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The E3 ubiquitin ligase HACE1 : characterization of its regulation by phosphorylation and demonstration of its role in cellular cohesion

María Isabel Acosta-López

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Unité de recherche : Inserm 1065- C3M

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Mention : Interactions moléculaires et cellulaires

Défendue publiquement par
Maria Isabel ACOSTA-LOPEZ

The E3 ubiquitin ligase HACE1: Characterization of its regulation by phosphorylation and demonstration of its role in cellular cohesion.

HACE1 E3 ubiquitine ligase : Caractérisation de sa régulation par phosphorylation et mise en évidence de son rôle dans la cohésion cellulaire.

*Dirigée par Emmanuel Lemichez, Directeur de Recherche, C3M
et co-dirigée par Orane Visvikis, Chargé de Recherche, C3M*

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Devant le Jury de soutenance composé de :

Dr. Anne DEBANT	<i>Directeur de Recherche</i>	<i>Examinatrice et Présidente du Jury</i>
Dr Serge ROCHE	<i>Directeur de Recherche</i>	<i>Rapporteur</i>
Dr. René-Marc MEGE	<i>Directeur de Recherche</i>	<i>Rapporteur</i>
Dr Emmanuel LEMICHEZ	<i>Directeur de Recherche</i>	<i>Directeur de thèse</i>
Dr Orane VISVIKIS	<i>Chargé de Recherche</i>	<i>Co-Directrice de thèse</i>

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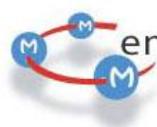
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List of Abbreviations

AJ: Adherens Junctions
AMP-Ub: Ubiquitin adenylyate
CISK: Cytokine-independent survival kinase
CNF1: Cytotoxic necrotizing factor 1
DUBs: Deubiquitinating enzymes
EGF: Epidermal growth factor
EMT: Epithelial-mesenchymal transition
ENaC: Epithelial sodium channels
GAP: GTPase Activating Protein
GDF: GDI Displacement Factor
GDP: Guanosine Di-Phosphate
GEF: Guanin nucleotide Exchange Factor
GTP: Guanosine Tri-Phosphate
HACE1: HECT-domain and Ankyrin-repeat containing E3 ubiquitin protein
ligase 1
HECT: Homologous to E6AP C-terminus
HGF: Hepatocyte growth factor
HRG: Heregulin
IFN: Interferon
IKK β : IkappaB kinase beta/ Nuclear Factor NF-Kappa-B Inhibitor Kinase Beta
JNK: c-Jun N-terminal kinase
LIMK: LIM-motif containing kinase
MAPK: mitogen-activated protein kinase
MET: Mesenchymal-epithelial transition
MLC: Myosin light chain
MLCP: MLC phosphatase
NADPH: nicotinamide adenine dinucleotide phosphate
Nedd8: Neural precursor cell-expressed, developmentally downregulated 8
NF-kB: Nuclear Factor kappa B.
NRF2: nuclear factor erythroid 2-related factor 2

PAK: p21-activated kinase
PDGF: Platelet-derived growth factor
PKA: Protein kinase A
PTM: Post-translational modifications
Rac: Ras-related C3 botulinum toxin substrate
RBR: ring between ring
Rho: Ras homologue
RhoGDI: Rho GDP Dissociation Inhibitor
RING: Really interesting New gene
ROS: Reactive Oxygen Species
SGK1: serum and glucocorticoid related kinase 1
Smurf: Smad ubiquitination-related factor
SRF: Serum-response factor
 β AR: β -Adrenergic receptor
SUMO: small ubiquitin-related modifier
Syn5: Syntaxin5
TGF β : transforming growth factor beta
TNF: Tumor necrosis factor
TNFR1: TNF receptor 1
TRAF: TNFR associated factor
UBD: Ubiquitin binding domain
UBEx: Ubiquitin binding exosite
Ubls: Ubiquitin-like modifiers
UBP: ubiquitin binding protein
UBS: ubiquitin binding site
UPS: Ubiquitin Proteasome System
WASP: Wiskott-Aldrich syndrome protein
WAVE: WAVE-family verprolin homologous protein

General Introduction

The post-translational modification of proteins by ubiquitination serves multiple cellular purposes. It modulates protein-protein interactions along diverse signaling pathways and targets proteins for degradation by the proteasome, thereby contributing to protein homeostasis and to the temporal dynamics of signaling networks. In 2002, our team demonstrated that small intracellular signaling proteins of the Rho GTPase family can be ubiquitinated and addressed to the ubiquitin proteasome system. The members of the Rho GTPase family are present in all eukaryotes and act as molecular switches best known to regulate cytoskeletal dynamics. In addition, they take part in the regulation of gene expression, cell cycle progression, as well as in the cellular response to pathogenic agents. Through the study of the regulation of Rho GTPase activity by ubiquitination, our team identified the first E3 ubiquitin ligase that directly ubiquitinates and promotes the proteasomal degradation of Rac1, the founding member of the Rho GTPase family. This E3 ligase is HACE1, a protein first found epigenetically silenced in sporadic Wilms' tumor and then repeatedly found to be downregulated in numerous human diseases, including cancer, neurodegenerative diseases and developmental conditions. Despite the important role of HACE1 in the maintenance of cell homeostasis, nothing is known about the post-translational regulation of its activity. In this work, we aim to contribute to this knowledge gap by studying how HACE1 is regulated by phosphorylation and, in parallel, explore the role of HACE1 in the regulation of intercellular adhesion, a cellular process that heavily depends on Rho GTPase signaling.

The first three chapters of this thesis comprises the introduction to my work. Chapter 1. aims to give a general view of the Ubiquitin field with particular emphasis on the characteristics and regulatory mechanisms of the E3 ubiquitin ligase family; Chapter 2. is dedicated to the Rho GTPases, their mechanisms of action, cellular functions and implications in human disease; and Chapter 3. gives a more detailed description of the current knowledge of HACE1.

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Ubiquitination is a dynamic and complex type of post-translational modification implicated in nearly all aspects of eukaryotic biology. Ubiquitin is a 8.5kDa protein that was first isolated from bovine thymus and was associated with the induction of lymphocyte differentiation (Goldstein et al. 1975). This new small protein was believed to be ubiquitously expressed (hence its name) from bacteria and yeast to animals and higher

plants. Moreover, the 76 amino acid long sequence of ubiquitin was found to be remarkably conserved among eukaryotes (Goldstein et al. 1975), which suggested a fundamental role. A role so vital that it imposed incredibly tight evolutionary constraints on ubiquitin's structure. The first observation of covalently attached ubiquitin was made by H. Busch and collaborators: they described a protein, which they called "A24", that curiously had one C-terminus and two N-termini (Goldknopf and Busch 1977). This particular protein was later found to be the histone H2A bound to ubiquitin by an isopeptide bond, which made H2A the first substrate of the ubiquitin pathway to be found (Hunt and Dayhoff 1977). However, it wasn't until some years later, between 1978 and 1983 that the teams of A. Herschko and I. Rose discovered the central role of ubiquitin in non-lysosomal, ATP-dependent intracellular protein degradation (Ciechanover et al. 1978; Wilkinson et al. 1980). They isolated and described the enzymatic cascade composed of three enzymes (E1, E2, and E3) necessary for the activation and covalent attachment of ubiquitin onto protein substrates (Ciechanover et al. 1981; Hershko et al. 1981; Hershko et al. 1983), a work that granted them the Nobel prize in chemistry in 2004. Since then, it has been shown that the ubiquitin system is not only involved in the degradation of proteins by the proteasome (Finley 2009) but also in non-proteolytic processes such as membrane trafficking (Hicke and Dunn 2003), DNA repair (Jentsch et al. 1987; Cohn et al. 2007; Stewart et al. 2009) and chromatin dynamics (Wright et al. 2012). In this chapter, I will briefly describe the properties and mechanisms of action of the proteins involved in ubiquitination, explore the variety of signals ubiquitination can transduce, and finally focus on one of the key components of the ubiquitin system: the E3 ubiquitin protein ligases.

1.1. The Ubiquitin system.

The parallel between cell signaling and language is one often drawn, and with good reason. Both are communication systems that encode and relay precise signals that elicit specific actions. Within this allegory, one can say that the ubiquitin system is composed of words based on a common root (ubiquitin). These words form sentences that are written down

by the ubiquitin conjugating system (E1, E2, E3), are read by ubiquitin binding proteins (UBPs), and are erased by deubiquitinating enzymes (DUBs).

1.1.1. Ubiquitin

Ubiquitin is the founding and best-studied member of a family of ubiquitin-like modifiers (Ubls) encompassing nearly 20 proteins in eukaryotes that share 9-58% homology with ubiquitin. Notable examples of Ubls are SUMO (small ubiquitin-related modifier) and Nedd8 (Neural precursor cell-expressed, developmentally downregulated 8) (van der Veen and Ploegh 2012). In humans, ubiquitin precursors are encoded by four genes: UbB and UbC, which encode poly-ubiquitin peptides of three and nine units, respectively; and UBA52 and RPS27A/UBA80, which encode ribosomal subunits fused to the C-terminus of a ubiquitin monomer. Following translation, free ubiquitin is generated by the cleavage of the gene products by DUB proteases (Monia et al. 1989).

Much of the communication potential of the ubiquitin system is based on the unique properties of ubiquitin itself. Ubiquitin is a highly stable protein of 8.5kDa and 76 amino acids with a chain ball-like structure (Vijay-Kumar et al. 1987) whose surface presents several recognition patches that have been implicated in binding to E3 ubiquitin protein ligases, UBPs and DUBs (Dikic et al. 2009; Kamadurai et al. 2009; Cui et al. 2010; Ye et al. 2011) (**Fig. 1.1.C**). Ubiquitin is covalently conjugated onto substrate proteins by an isopeptide bond between the C-terminal glycine 76 of ubiquitin and usually the side chain amine of a lysine residue in the substrate protein (**Fig. 1.1B**). This reaction can lead to monoubiquitination or multi-monoubiquitination of a substrate protein. Moreover, ubiquitin itself disposes of seven lysines and its first methionine that can act as ubiquitin linking sites and enable the formation of ubiquitin chains of different topologies (**Fig. 1.1A**). The particular conformation of the chains is known to expose or restrict access to ubiquitin recognition patches, determining the type of ubiquitin-binding proteins that can be recruited and thus affecting the induced downstream signaling response (Komander and Rape 2012).

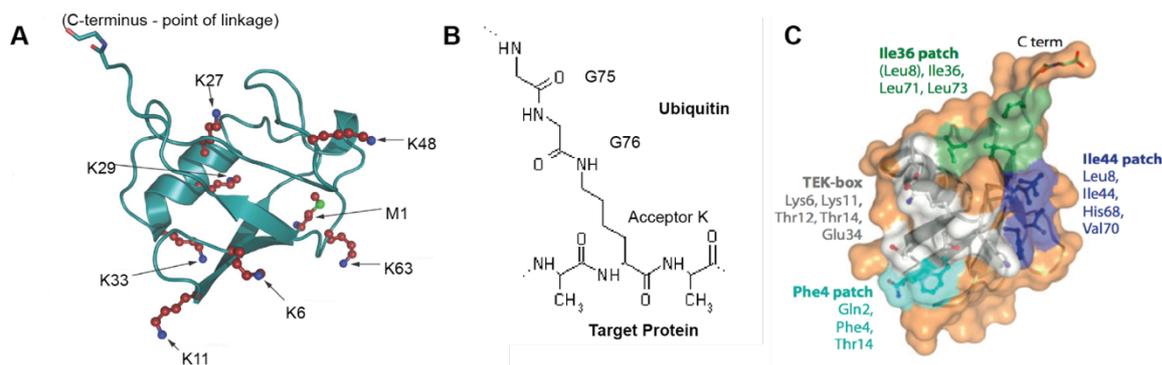


Figure 1.1. Ubiquitin structure characteristics. **A.** Structure of ubiquitin indicating that all its linking residues: seven lysine residues (red, with blue nitrogen atoms) and a methionine (with a green sulfur atom). The lysines are located on different surfaces of the molecule; M1 is the linkage point in linear chains, and is spatially close to K63. The C-terminal G75-G76 motif involved in isopeptide bond formation is indicated (red oxygen atoms, blue nitrogen atoms). **B.** Representation of the isopeptide bond between ubiquitin and a target protein. **C.** Representation of ubiquitin's surface indicating four recognition patches in different colors. The name of each patch and the residues that form part of it are indicated in the same color as the patch's surface. Adapted from (Komander 2009) and (Komander and Rape 2012)

1.1.2. The writers: The enzymatic cascade of ubiquitin conjugation

The writer system that leads to ubiquitin chain formation and conjugation onto substrates is composed of three types of enzymes that work in a hierarchical fashion (Hershko et al. 1983): E1s (ubiquitin-activating enzymes), E2s (ubiquitin-conjugating enzymes), and E3s (ubiquitin protein ligase enzymes). E1 enzymes begin the cascade by using the hydrolysis of ATP to catalyze the formation of a phosphodiester bond between the C-terminus of ubiquitin and the phosphate group of AMP, producing ubiquitin adenylate (AMP-Ub). Next, the sulfhydryl group of the E2 active cysteine attacks the AMP-Ub, forming a high-energy thioester bond with ubiquitin's C-terminus and displacing AMP. The E1 then catalyzes for a second time the adenylation of a ubiquitin monomer and forms a non-covalent complex with it in its adenylation domain. The double-ubiquitin-loaded E1 can then facilitate the transfer of the thioester-bound ubiquitin from E1 to an active cysteine in E2 (Haas et al. 1982; Haas et al. 1983; Schulman and Harper 2009; Schäfer et al. 2014). Subsequently, the ubiquitin-charged E2 can bind to E3 enzymes that lack an active cysteine (RING family) or that possess an active cysteine (HECT and RBR families); in the first case the E2 transfers the charged ubiquitin directly to a substrate bound to the

E3, and in the second case the E2 transfers the ubiquitin to the active cysteine of an E3, which then conjugates the ubiquitin onto a substrate (Deshaies and Joazeiro 2009; Stewart et al. 2016) (**Fig. 1.2**).

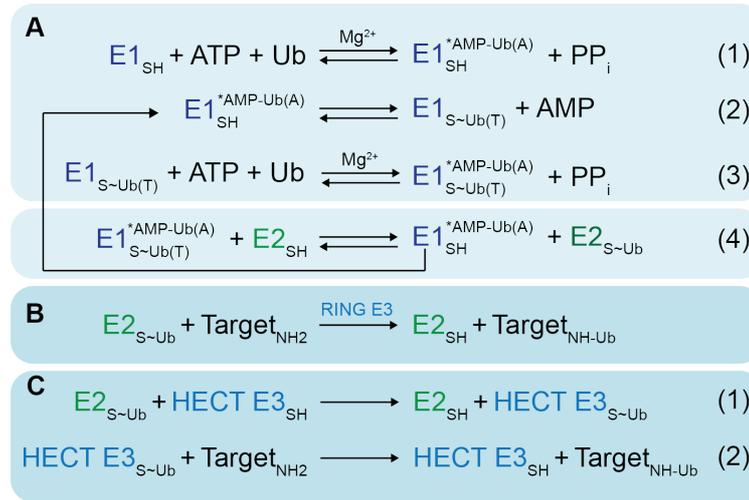


Figure 1.2. Generalized enzymatic mechanisms of ubiquitin (Ub) transfer between enzymes and ultimately to a target. * refers to a noncovalent complex, ~ refers to a high-energy thioester bond, - refers to a covalent bond (phosphodiester in AMP-Ub or isopeptide in Target-Ub). **A.** Initial steps catalyzed by E1. (1) E1 binds Mg^{2+} , ATP and a Ub, and catalyzes the acyl-adenylation of the Ub's C-terminus. (2) E1 catalytic cysteine attacks the Ub~AMP intermediate, to form the covalent thioester-linked E1~Ub intermediate. (3) E1 then adenylates a 2nd Ub molecule, such that E1 binds 2 Ub molecules: Ub(T) is thioester-linked to E1's catalytic cysteine; Ub(A) is associated noncovalently at the adenylation site. (4) Doubly-Ub-loaded E1 binds an E2 and Ub(T) is transferred from the E1 to the E2 catalytic cysteine. **B.** RING E3s enhance Ub transfer from E2 to a target. **C.** HECT and RBR E3s (represented by the HECT family in this figure) contain a catalytic cysteine, and (1) form a covalent thioester intermediate with a Ub prior to Ub ligation to a target lysine (2). Adapted from (Schulman 2011)

The human genome encodes 2 E1s, 37 E2s and more than 600 E3s (Li et al. 2008; Komander 2009), making the E3 enzymes the most diverse and evolutionarily refined actors of the cascade. E3 enzymes regulate target specificity and, together with E2 enzymes, direct bond formation.

E3 enzymes are classified in three categories based on the structure of their E2-binding domain and on their ubiquitin transfer mechanism (**Fig. 1.3**): (i) The Really Interesting

New Gene (RING) family catalyzes the single-step transfer of ubiquitin from the E2 to the substrate, while (ii) the Homologous to E6AP C-Terminus (HECT) and (ii) the RING-Between-RING (RBR) families ubiquitinate substrates in a two-step reaction where ubiquitin is first transferred from the E2 to an active cysteine within the E3 catalytic domain and then from the E3 to the substrate (Huibregtse et al. 1995; Deshaies and Joazeiro 2009; Smit and Sixma 2014). I will further expand on the mechanisms of action of these three E3 families in the section 1.2.

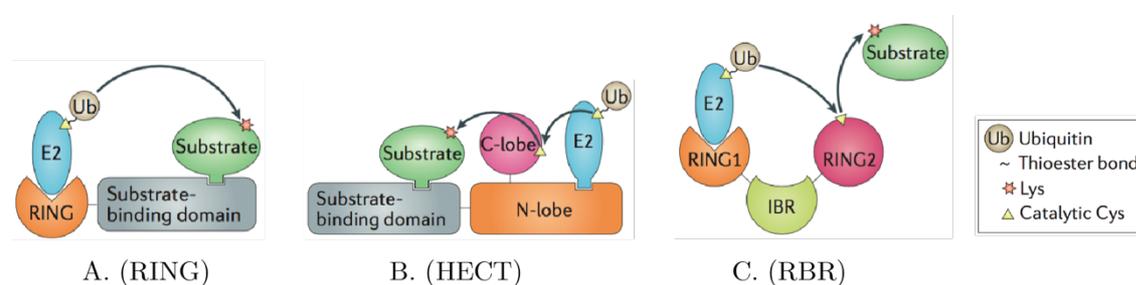


Figure 1.3. Classes of E3 ubiquitin ligases. Simplified representation of domain structure and the reactions catalyzed by three classes of E3 ubiquitin ligases. **A.** Schematic of Really Interesting New Gene (RING) E3-mediated catalysis. The RING domain binds E2~ubiquitin (~ indicates a thioester bond) and a substrate-binding domain recruits the substrate. Ubiquitin is transferred directly from the catalytic cysteine of E2 to a substrate lysine. **B.** Homologous to E6AP Carboxyl Terminus (HECT) E3 catalysis. The N-lobe of the HECT domain binds E2~ubiquitin and a substrate-binding domain recruits the substrate. Ubiquitin is transferred from E2 to the catalytic cysteine of the C-lobe of the HECT domain and subsequently to a substrate lysine. **C.** RING-between-RING (RBR) E3 catalysis. the RING1 domain binds E2~ubiquitin and ubiquitin is transferred from E2 to the catalytic cysteine of RING2 and then to a substrate lysine. Adapted from (Buetow and Huang 2016).

In addition to the previously described types of E3 ligases, which are present in eukaryotes; it has been shown that despite having no intrinsic ubiquitination machinery, pathogenic bacteria encode bacterial E3 ligases (BELs) that have the capacity to manipulate the host ubiquitin system during infection (reviewed in (Ashida and Sasakawa 2016) and (Ashida et al. 2014)). Some of these BELs structurally and functionally mimic host HECT-type and RING-type E3s (Maculins et al. 2016; Ashida and Sasakawa 2016). However, a third class of BELs called Novel E3 ligases (NELs) do not share any structural similarity with

any eukaryotic E3 ligases (Quezada et al. 2009). They are characterized by the presence of a C-terminus NEL catalytic domain and N-terminal Leucine-Rich Repeat (LRR) domains which are required for substrate binding and also inhibit the NEL domain in absence of a substrate (Chou et al. 2012).

1.1.3. The ubiquitin code: Signaling implications of ubiquitination

Ubiquitin can be singularly conjugated or attached to substrates as chains with different conformations determined by the residue that links one ubiquitin to the next. This can be seen as words (ubiquitin) that associate and form different sentences (ubiquitin chains), and constitutes the base of a system that D. Komander and M. Rape have called the “ubiquitin code” (Komander and Rape 2012). The panoply of ubiquitination chain types attached to substrates by ubiquitin conjugating enzymes can be recognized and disassembled by specific DUB enzymes, contributing to the dynamic regulation of the code (Mevisen and Komander 2017). To interpret the ubiquitin code, cells count on an array of UBPs with a variety of ubiquitin binding domains (UBDs) that specifically recognize specific types of ubiquitin chains and can then mediate particular cellular responses (Husnjak and Dikic 2012) (**Fig. 1.4A**).

It has been estimated that the majority of ubiquitin in mammalian cell lines is conjugated as a single ubiquitin (>60%), most likely due to monoubiquitination of histone H2A, which is one of the most abundant cellular components (Clague et al. 2015). Monoubiquitination of substrates is typically associated with alterations of intra- or intermolecular interactions that in turn affect their localization, complex formation or activity. After monoubiquitination, ubiquitin chains with K48- and K63- linkages are the second most abundant types of chains. K48-chains are mainly involved in the targeting of proteins for proteasomal degradation. While K63-chains often act as secondary messengers and scaffolds, allowing the formation of rapid and reversible signaling complexes involved in processes such as the activation of the Nuclear Factor kappa B (NF- κ B) transcription factor, DNA repair, innate immune responses, clearance of damaged mitochondria, and protein sorting (Komander and Rape 2012).

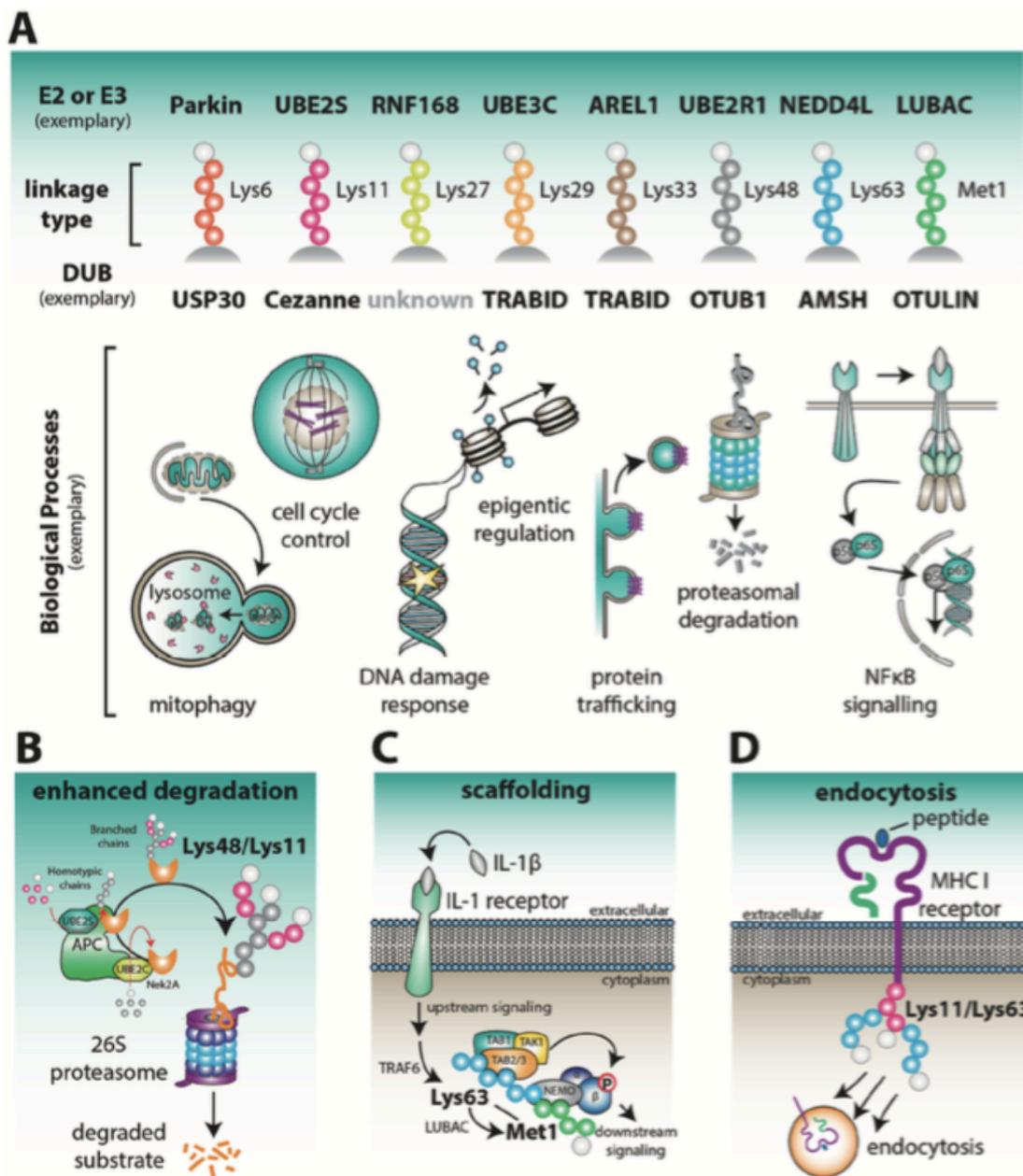


Figure 1.4 Physiological roles associated with individual chain types. **A.** Examples of E2 or E3 enzymes that assemble and DUBs that disassemble ubiquitin chains with linkage preferences is indicated. Below, illustrations show some of the biological processes that determined ubiquitin linkage types have been associated with. **B.** APC/C is active during early mitosis and modifies cell cycle regulators such as Nek2A with Lys48/Lys11-linked branched polyubiquitin. In this process, UBE2C first assembles short chains on the substrates, and these are then elongated on each ubiquitin by Lys11-linked polymers. Lys48/Lys11 branched chains enhance proteasomal degradation. **C.** Mixed or branched Lys63/Met1-linked chains serve as protein scaffolds at immune receptors, such as IL-1 receptors, to promote NF-κB signaling. **D.** A viral E3 ligase initiates endocytic internalization of the MHC class I receptor through the attachment of mixed or branched Lys11/Lys63-linked ubiquitin chains. Taken from (Swatek and Komander 2016).

Proteomic studies have revealed that chains linked by the remaining 5 lysines (K6-, K11-, K27-, K29-, K33-) and the first methionine (M1-) of ubiquitin also exist in cells but in much smaller proportions and are referred to as "atypical chains". In human cells, conjugated K11-linked chains increase in abundance during mitosis and early G1 phase and have, accordingly, been found to target cell cycle regulators for degradation by the proteasome, which is reminiscent of the function of K48-linked chains. K11 chains are produced by specific E3 enzymes like APC/C (anaphase promoted complex) in partnership with the E2 Ube2S. M1-linked chains are quickly synthesized following activation of inflammatory signaling cascades and are recognized by UBPs with linkage-specific UBDs, such as the ubiquitin binding in ABIN and NEMO (UBAN) domain (Yau and Rape 2016). Like K11 chains, M1-chains are assembled by particular E3 enzymes, like the Linear Ubiquitin Chain Assembly Complex (LUBAC), which play pivotal roles in immune signaling and NF- κ B activation (Yau and Rape 2016). The four other types of lysine linkages of ubiquitin are much less characterized and seem to be assembled by E3 ligases that have mixed specificity. K6-linked chains are observed during the removal of damaged mitochondria from cells, K-27 chains seem to be involved in regulating DNA repair and autoimmunity, K29-chains are reported to have roles in proteasomal degradation, and K33-linked chains appear to regulate trafficking through the trans-Golgi network (Swatek and Komander 2016; Yau and Rape 2016).

So far, I have described homotypic chains, that is, chains where ubiquitin monomers are connected by a single type of linkage. However, heterotypic chains are also formed on substrates, and they can be either of mixed or branched nature. Mixed chains are composed of ubiquitin subunits connected to only one ubiquitin subunit at a time by various lysines (or M1), while branched chains have ubiquitin subunits conjugated to more than one lysine or the M1 residue at a time. These kinds of chains are proving to be functionally diverse, participating in numerous signaling cascades due to their ability to attract several UBPs in specific combinations and thus elicit unique reactions as exemplified in figure 1.4B-D (Swatek and Komander 2016).

In addition to the abundance of linkage possibilities, the complexity of this code has

increased enormously by recent discoveries showing that ubiquitin can be subjected to phosphorylation, acetylation, and modification by Ubls (like SUMO and Nedd8), adding another layer of regulation and of interaction with other PTM systems (Cui et al. 2010; Herhaus and Dikic 2015; Swatek and Komander 2016; Yau and Rape 2016).

Additionally, pathogenic bacteria strategically modify ubiquitin and Ubls to interfere with the host ubiquitination system and achieve successful infection. For example, the bacterial effector Cif (cycle inhibiting factor) family encoded by *Burkholderia pseudomallei* and by enteropathogenic *Escherichia coli* can deamidate glutamine 40 of ubiquitin and Nedd8, thereby inhibiting ubiquitin chain extension and hampering the Nedd8-dependent activation of CRLs (Cullin-RING Ligases) (Cui et al. 2010). Similarly, arginine phosphorylation of ubiquitin induced by SdeA produced by *Legionella pneumophila* has been shown to impair host ubiquitin-dependent processes (Bhogaraju and Dikic 2016).

1.1.4. When ubiquitination goes awry: Implications in human diseases

Considering the widespread implication of ubiquitination in cell signaling, it is not surprising that the deregulation of the ubiquitin system contributes to the development and progression of several pathologies including cancer, neurodegenerative diseases, autoimmunity, metabolic and inflammatory disorders, infection and muscle dystrophies (reviewed in (Popovic et al. 2014)).

Disease-associated perturbations in the ubiquitin system may occur at multiple levels: i) at any point during the multi-step process of ubiquitin conjugation, commonly via mutation or deletion of E1, E2, E3 enzymes, or of the substrate itself, ii) during ubiquitin recognition (by de-regulation of UBPs), or iii) during de-ubiquitination (by de-regulation of DUBs) (Popovic et al. 2014; Groen and Gillingwater 2015). One of the best-known examples of an E3 ligase whose perturbation leads to a pathology is E6AP (also known as Ube3a). Genetic alterations on *E6AP* that result in loss of function are known to cause Angelman syndrome, a rare neurogenetic disorder characterized by severe mental retardation, speech impairment, ataxia, seizure and frequent bursts of laughter (among other symptoms) (Buiting et al. 2016). Moreover, several types of cancer and immune

pathologies present alterations in the canonical NF- κ B pathway, which heavily relies on the conjugation and recognition of different types of ubiquitin chains (as described in section 1.1.2). For instance, it has been shown that patients with inherited deficiency in HOIL-1 (an E3-ligase present in the LUBAC complex that regulates NF- κ B activation) suffer from chronic autoinflammation, muscular amylopectinosis and susceptibility to bacterial infections (Boisson et al. 2012). Another example is the DUB A20/TNFAIP3, which is considered a tumor suppressor due to its role in restraining exacerbated inflammation via the NF- κ B pathway. Concordantly, mutations in A20/TNFAIP3 that decrease its expression or compromise its activity are commonly found in patients with lymphomas as well as in patients suffering from inflammatory conditions including rheumatoid arthritis, psoriasis, systemic lupus erythematosus, celiac disease, Crohn's disease and diabetes (Hymowitz and Wertz 2010; Ma and Malynn 2012). Also associated with the NF- κ B pathway is the UBP and autophagy adaptor Sequestosome 1 (SQSTM1, also known as p62). A mutation near the UBD of SQSTM1/p62 has been shown to cause Paget disease of bone, a common and chronic skeletal disorder (Laurin et al. 2002). In addition to inflammation, one of the key ubiquitin-dependent processes deregulated in cancer is genomic instability. For instance, FANCL, an E3 ligase whose activity is necessary for the correct localization of DNA repair factors (Garcia-Higuera et al. 2001), has been found to be mutated in hereditary ovarian and breast cancer as well as in Fanconi anemia, a rare cancer-prone genetic disease characterized by chromosomal instability (Peng et al. 2007; Xie et al. 2010).

Due to the increasingly recognized implication of the ubiquitin system in disease, great efforts have been made towards the development of inhibitors and agonists of the enzymes of the ubiquitin system (E1, E2, E3 and DUBs) for therapeutic applications (Huang and Dixit 2016). More about this topic, focusing on regulatory strategies of HECT E3 ligases, can be found in section 1.3.

1.2. E3 ubiquitin ligases: focus on the HECT family.

As seen briefly in section 1.1.2, the E3 ubiquitin ligases (E3s) are the main contributors of specificity in the ubiquitin conjugation system. In accordance with this role there is a great variety of these enzymes (>600 in humans), which are classified in three types: RING, RBR, and HECT. In this section I will expand on the characteristics of each family of E3s and their mechanisms of action with a special emphasis, at the end of the section, on the HECT family.

1.2.1. RING E3 ligases.

The great majority of E3 ligases belong to the RING family. Bioinformatic analyses estimate that there are around 600 members in humans (Li et al. 2008). Example members of this family are c-Cbl, which is essential for ubiquitination and lysosomal degradation of the epidermal growth factor receptor (EGFR) (Levkowitz et al. 1999), and APC/C, which promotes ubiquitination and proteasomal degradation of anaphase inhibitors, ensuring timely chromatid separation and mitotic exit (Craney et al. 2016).

RING E3s are characterized by a catalytic RING domain that requires the coordination of two zinc ions to fold correctly, or a U-box domain, which closely resembles RING domains in structure but does not coordinate zinc ions (Deshaies and Joazeiro 2009). RING E3 ligases are very diverse and can be active as monomers, homodimers, heterodimers or as part of large multi-subunit complexes (as reviewed in (Buetow and Huang 2016)). Examples of these large complexes are APC/C (Anaphase promoting complex/cyclosome) (Chang and Barford 2014) and the E3 Cullin-RING ligases (CRL), a large family of multi-subunit RING E3s (Petroski and Deshaies 2005a; Lydeard et al. 2013). The prototypical CRL is the Skip/Cullin/F-box complex (SCF).

RING ligases simultaneously bind the substrate protein and the ubiquitin-loaded E2, and mediate the direct transfer of ubiquitin from E3 to substrate. It has been observed that the RING-mediated approximation of E2~Ub and Substrate is not sufficient to reach optimal transfer rates (Seol et al. 1999; Petroski and Deshaies 2005b; Saha and Deshaies

2008) and that the nature of the E2-RING interaction is important since not all E2-RING pairings lead to substrate ubiquitination (Brzovic et al. 2003; Ozkan et al. 2005). In 2012, the crystal structures of RNF4 and BIRC bound to E2~Ub were elucidated (Dou et al. 2012; Plechanovová et al. 2012) and they revealed that RING E3s are more than scaffolds: they prime ubiquitin for transfer by stabilizing the highly dynamic E2~Ub into a closed conformation that renders it more reactive towards transfer (Page et al. 2012; Dou et al. 2012; Pruneda et al. 2012; Soss et al. 2013). This mechanism has been recently expanded from homodimeric RINGs to monomeric RINGs (Dou et al. 2013; Buetow et al. 2015; Branigan et al. 2015), indicating that the mechanism might be universal to many other RING E3-E2 pairs.

After the first ubiquitin is transferred to a substrate, the formation of polyubiquitin chains often ensues. RING-E3 ligases can catalyze chain elongation much faster than chain initiation and in cooperation with a single E2 enzyme. For example, the E2 Cdc34 (also known as UbcH3 or UBE2R1) is 5 to 30 times faster at chain elongation than at initiation and is specific of Lys48 (Petroski and Deshaies 2005b). In other cases, chain initiation and elongation can be carried out by separate E2s. An example of this is APC/C-mediated polyubiquitination: the E2 UbcX (also known as UbcH10 or UBE2C) has a preference for monoubiquitination or short ubiquitin chains, whereas the E2 Ubc4 (also known as UbcH5 or UBE2D) usually assembles long polyubiquitin chains (Yu et al. 1996), the authors of this study proposed that they operate sequentially.

In RING E3- catalyzed ubiquitination, chain linkage specificity (chain topology) is thought to be determined by the E2 enzyme cooperating with the RING E3 ligase (Chen and Pickart 1990; Haas et al. 1991; Hofmann and Pickart 1999).

1.2.2. RBR E3 ligases.

The RBR E3 ligases are viewed as hybrids between RINGs and HECTs and were only recently defined as a distinct type of E3 ubiquitin ligases (Wenzel et al. 2011). They are characterized by a catalytic domain composed of two RING fingers (RING1 and RING2) and a central in-between RING (IBR) zinc-binding domain. The RING1 domain binds to

the loaded E2 and the RING2 domain contains an active cysteine residue capable of forming a reversible thioester intermediate with ubiquitin. Besides the common RING1-IBR-RING2 motif, RBR family members possess other domains, which gives diversity to the family (Wenzel et al. 2011; Stieglitz et al. 2012; Smit et al. 2012; Spratt et al. 2014).

There are 14 RBRs encoded in the human genome, and the three best-characterized are PARKIN, commonly mutated in Parkinson disease; HOIP (HOIL-1L interacting protein), the central E3 subunit of LUBAC (linear ubiquitin chain assembly complex) in NF- κ B signaling; and HHARI (human homologue of Ariadne) (Kitada et al. 1998; Kirisako et al. 2006; Ikeda et al. 2011).

RBR proteins are commonly found in auto-inhibited conformations that are not competent for ubiquitin transfer. Release from the auto-inhibited state occurs once RBRs bind to E2~Ub, as observed by Lechtenberg and colleagues in a study where they solved the structure of the fully active human HOIP in complex with an E2~Ub (Lechtenberg et al. 2016). Moreover, they observed that contrary to RINGs, and similar to HECTs (see section below), HOIP stabilizes E2~Ub in an extended conformation where the E2~thioester bond is juxtaposed with the RING2 active site cysteine and is optimal for transfer. This is in agreement with the fact that RBR's RING1 finger alone cannot promote ubiquitin transfer (Wenzel et al. 2011), as it does not activate E2~Ub like a canonical RING domain (in a closed conformation).

Little is known about how chain type is determined by RBRs. So far, the only well studied case is HOIP, where M1-chain specificity has been shown to depend on the presence of a linear ubiquitin chain-determining domain in HOIP (Smit et al. 2012; Riley et al. 2013).

1.2.3. HECT E3 ligases.

Members of this family of E3 ligases were among the first E3 enzymes to be cloned, and are the best functionally characterized among the thioester-forming E3s. The ubiquitin ligase function of the HECT family of proteins was first observed through studies of the degradation of the p53 tumor suppressor in cells infected by oncogenic forms of human

papillomavirus (HPVs) (Scheffner et al. 1990). Biochemical studies revealed that p53 degradation depended on the HPV E6 gene and a host protein named E6-AP (E6-associated protein), and that the complex of these proteins functioned as a p53-specific E3 {Scheffner:1993ur}. Further studies determined that E6-AP contained a conserved region of about 350 amino acids towards its C-terminus (Huibregtse et al. 1995) and that within this region, called the homologous to E6-AP C-terminus (HECT) domain, was a highly conserved cysteine located around 35 residues upstream of the C-terminus that is required for E6-AP activity (Scheffner et al. 1995).

Most HECTs arose before the emergence of animals or very early in metazoan evolution (Marín 2010), and the human genome encodes 28 HECT E3 ligases (Rotin et al. 2009). All members of the HECT family are characterized by a HECT domain located at their C-terminus and most also contain a variety of protein-protein or protein-lipid interaction domains towards their N-terminus (Rotin et al. 2009). Based on their N-terminal domain architecture, the 28 human HECT E3s are commonly divided into three sub-families (**Fig. 1.5**): (i) the Nedd4 family has 9 members in humans and is characterized by C2 and WW domains that allow them to bind phospholipids and PY motifs in substrate proteins. This is the best-studied family, most of what is known about structure and enzymatic mechanisms of HECT E3s come from studies done with NEDD4 E3 ligases; (ii) the HERC family, with 6 human members, contain regulator of chromosome condensation 1 (RCC1)-like domains (RLDs) that are not well described. This family can be divided in two groups, the large HERCs (>500 kDa) and the small HERCs (around 100kDa), which have a single RLD; (iii) the “other” family, with 13 human members, comprises HECTs which contain protein-protein interaction domains different from the previously mentioned

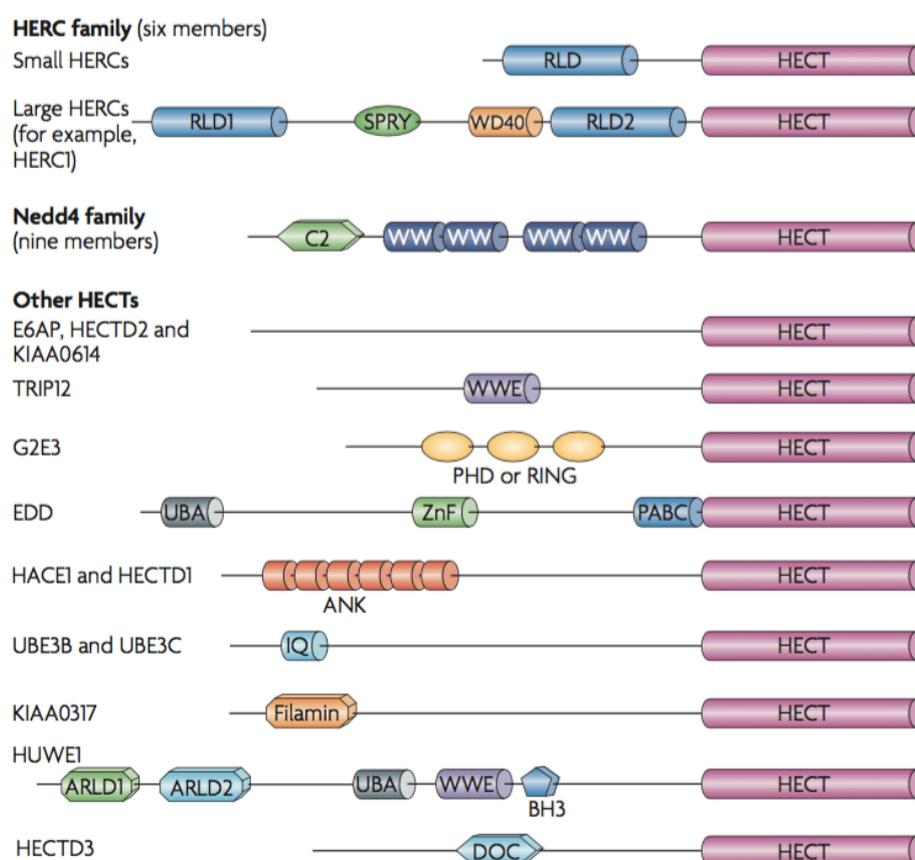


Figure 1.5. The mammalian HECT E3 ligases. The 28 human HECT E3 ligases are often grouped into three families. Two of these, the Nedd4 family and the HERC family, can be clearly distinguished by their domain architecture. The HERC family members, which contain one or more regulator of chromosome condensation 1 (RCC1)-like domains (RLDs), can be divided into two groups — the small HERCs that carry a single RLD and the large HERCs that contain more than one RLD and additional domains, such as SPRY and WD40. Nedd4 family members are characterized by a unique domain architecture, with all members containing an N-terminal C2 domain and two to four WW domains. The remaining HECT proteins contain a myriad of domains (as shown). From (Rotin et al. 2009).

The details of the enzymatic mechanism by which HECT E3s catalyze the transfer of ubiquitin onto a substrate have proven to be elusive for a long time. Currently, this knowledge gap is rapidly closing thanks to the increasing abundance of structural information that captures the different stages of the two step-ubiquitin transfer reaction that HECTs catalyze (Buetow and Huang 2016; Zheng and Shabek 2017). The catalytic HECT domain has a bi-lobed structure, where the two lobes are connected by a flexible hinge loop. The N-terminal lobe (N-lobe) binds to E2~Ub and the C-terminal lobe (C-

lobe) contains the catalytic cysteine (Huang et al. 1999). The flexible hinge allows the two lobes to rotate, a characteristic that is necessary for ubiquitin transfer (Verdecia et al. 2003; Ogunjimi et al. 2005). Thanks to the elucidation of the structure of Nedd4-2 bound to the E2 UBE2D2 (UbcH5b) loaded with ubiquitin (Kamadurai et al. 2009), the current model of transthiolation is the following: Nedd4-2's N-lobe binds to E2~ub and upon rotation of the hinge, the C-lobe binds ubiquitin. Interestingly, this interaction stabilizes E2~Ub in an open conformation, contrary to the closed conformation induced by RING E3s. This arrangement brings together the two catalytic cysteine residues of E2 and E3 and promote the formation of the HECT E3~Ub intermediate. This mechanism is likely to be shared among other HECT E3s (Buetow and Huang 2016). Once the E2 leaves, the interaction between the Ub and the C-lobe remains the same, as evidenced in the crystal structure of Nedd4-1~Ub (Maspero et al. 2013). In the next step, however, Kamadurai and collaborators observed that the N-lobe of the HECT domain rotates almost 130° from its previous position (while bound to E2~ub or conjugated with Ub). This change juxtaposes the active cysteine bound to the Ub and the acceptor lysine of the substrate (in this case the yeast Nedd4 homolog Rsp5 and its target Sna3) (Kamadurai et al. 2013).

Not much is known about how HECT E3s catalyze chain elongation and determine linkage specificity. Kim and colleagues demonstrated that the linkage specificity of several HECT E3s depends on the particular sequence identity of the C-lobe of the ligase's HECT domain and is, contrary to RING E3s, independent of the identity of the cooperating E2 enzyme (Kim and Huibregtse 2009). Nedd4 E3s have a UBD (aka ubiquitin-binding exosite hereafter referred as UBEx) within the N-lobe of their HECT domain. It has been shown that the UBEx is critical for polyubiquitin chain formation (Ogunjimi et al. 2005; French et al. 2009; Kim et al. 2011; Maspero et al. 2011; Maspero et al. 2013). Indeed, it has been reported that inhibiting the binding of ubiquitin to the UBEx, either by mutation or by small chemical inhibitors, impairs polyubiquitin chain elongation but not E2-/E3-transthiolation nor the conjugation of the first ubiquitin to a substrate (Kim et al. 2011; Maspero et al. 2011; Kathman et al. 2015). On the other hand, Zheng and colleagues recently showed that occupation of the UBEx by ubiquitin variants in different members

of the Nedd4 family can influence many properties of the reaction both positively and negatively (Zhang et al. 2016).

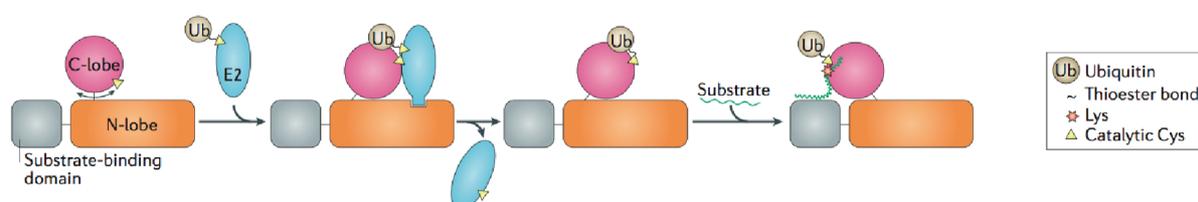


Figure 1.6. Schematic showing the HECT E3 catalytic cycle. In the absence of any binding partner, the C-lobe can rotate relative to the N-lobe thanks to the hinge loop. Upon encountering E2~ubiquitin, the N-lobe binds E2 and the C-lobe rotates to bind ubiquitin, thereby juxtaposing the catalytic cysteine residues from E2 and E3. Upon ubiquitin transfer onto the catalytic cysteine of the C-lobe, E2 is released. E3 binds the substrate through its substrate-binding domain and the C-lobe undergoes rotation to juxtapose the catalytic cysteine of E3 and a substrate lysine for ligation. The actual order in which HECT E3 recruits E2~ubiquitin and substrate is not yet known. From (Buetow and Huang 2016)

The number of HECT E3 ligases represent less than 5% of all the E3 ligases found in humans, yet they have been shown to have important physiological roles in many biological processes such as, fetal growth and development, regulation of DNA damage and replication, modulation of immune responses, among other processes. Consequently, their de-regulation leads to development of different pathologies (reviewed in (Scheffner and Kumar 2014) and (Rotin et al. 2009)). Therefore, it is important to understand how this family of E3 ligases is regulated.

1.3. Regulation of HECT E3s.

The activity of E3 ligases are tightly regulated on a variety of levels including cooperation with E2 enzyme(s), E3 processivity and substrate recognition. The regulation of these processes is usually mediated by structural rearrangements triggered by interaction with auxiliary factors or by post-translational modifications (PTMs) such as phosphorylation and ubiquitination (Ogunjimi et al. 2005; Wiesner et al. 2007; Rotin et al. 2009; Maspero et al. 2011). Currently, most of what is known about the regulation of HECT E3 ligase

activity comes from studies focusing on members of the Nedd4 subfamily. Therefore, this section will mainly discuss the literature involving the regulation of this subfamily of HECT ligases, then mention a couple of recent studies involving two HECT ligases outside of the Nedd4 subfamily, and finally go through some examples of regulation of HECT ligase activity by small chemical inhibitors.

1.3.1. Regulation of Nedd4 family ligases.

Nedd4 family ligases share a common domain structure and, at steady state, most of them favor inhibitory conformations that protects them and their targets from untimely ubiquitination (**Fig. 1.7**). Smurf2, Nedd4-1 (aka Nedd4), Nedd4-2 (aka Nedd4-L), and WWP2 are negatively regulated under basal conditions through intramolecular interactions involving the C2 and HECT domains (Wiesner et al. 2007; Mund and Pelham 2009; Wang et al. 2010a); (Bruce et al. 2008). In the case of Itch, auto-inhibitory conformation is mediated by its WW and HECT domains and is proposed to occur intramolecularly (Gallagher et al. 2006; Riling et al. 2015). Despite the high degree of homology between Smurf1 and Smurf2, the *cis* interaction between the C2 and the HECT domains that inactivates Smurf2 is not found in Smurf1 due to the shorter linker region between its HECT and C2 domains (Wiesner et al. 2007). Instead, it has been reported that the full-length Smurf1 forms homodimers through intermolecular contacts mapped to a fragment containing the C2 and the WW domains of one molecule and the HECT domain of the partner (Wan et al. 2011).

The details of how these closed conformations block enzymatic activity is not yet clear for all the ligases mentioned above but, to date, the best characterized one is Smurf2. As previously mentioned, Smurf2 auto-inhibition is governed by the interaction between the C2 and HECT domains; specifically, the C2 domain binds the N-lobe of the HECT domain and restricts movement of the C-lobe, which makes the active cysteine inaccessible for an incoming E2~Ub and thereby precluding transthiolation (Wiesner et al. 2007; Mari et al. 2014). Moreover, the C2 domain partially buries the UBEx, which is essential for E3 processivity (Mari et al. 2014). Another enzymatic step affected by the auto-inhibitory

conformation of Smurf2 is the recruitment and binding of the its associated E2 (UbcH7); it has been shown that Smurf2 interacts very weakly with UbcH7, and that binding to the adaptor protein SMAD7 (disrupting its intra-inhibitory interactions) is necessary for a functional interaction between Smurf2 and UbcH7 (Ogunjimi et al. 2005).

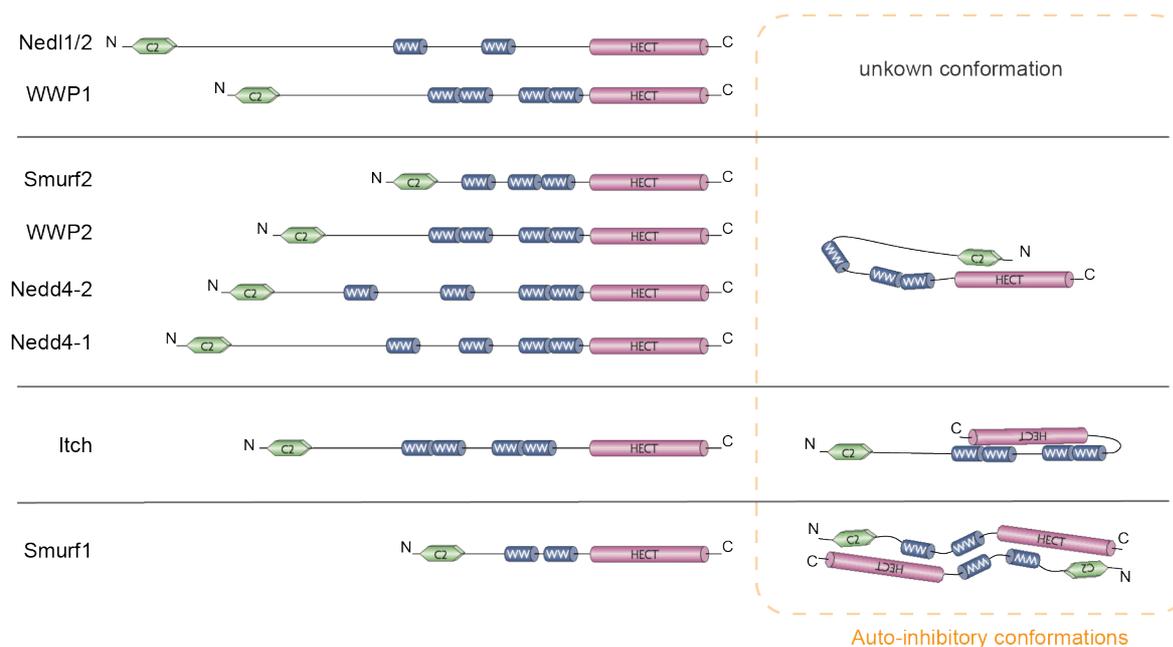


Figure 1.7. Domain structure of the nine members of the Nedd4 family and their auto-inhibitory conformations. Smurf2, WWP2, Nedd4-2 and Nedd4-1 form intra-molecular interactions between their C2 and HECT domains, as exemplified with Smurf2; Itch auto-inhibitory conformation is similar, but the HECT domain interacts with WW domains instead of the C2 domain. Smurf1 presents inhibitory interactions between the C2 domain, a WW domain and the HECT domain in *trans* instead of *cis*, forming an inactive homo-dimer.

Similar to Smurf2, nuclear magnetic resonance (NMR) and biochemical analysis have shown that in Nedd4-1 the C2 domain has the potential to regulate E3 activity by keeping the HECT domain in a low-activity state where its ability for transthiolation and non-covalent ubiquitin binding is impaired (Mari et al. 2014). Contrary to Smurf2, recent studies have shown that Itch can bind to E2s while in its auto-inhibitory conformation. However, the transfer of ubiquitin from E2 to the active cysteine of Itch is thwarted (Riling et al. 2015).

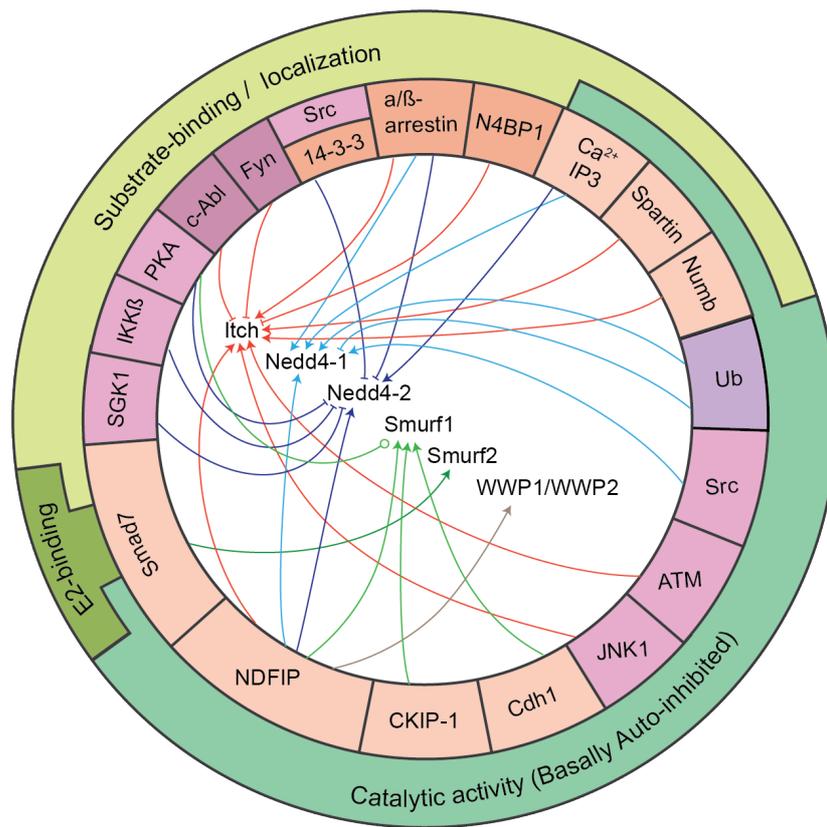


Figure 1.8. Mechanisms of regulation of Nedd4-family ligases. An increasing number of studies have shown that adaptors (orange) and PTMs such as ubiquitination and phosphorylation (kinases shown in mauve) can promote (lighter orange or mauve, and pointed arrow), inhibit (darker orange or mauve, and blunt arrow) or modulate (circle-ending line) one or more aspects of Nedd4-ligases activity; such as the ligase localization and interaction with substrates, its binding to E2 enzymes, and its intrinsic E3 catalytic activity. Details about how each adaptor and PTM modulates the different E3 ligases are discussed in the main text.

The last ten years have seen a boom in the number of studies exploring how the cell controls the activity of Nedd4 E3 ligases, and a multitude of adaptors and PTM events (mainly phosphorylation) have been shown to modulate one or more aspects of Nedd4-ligases activity. This includes the disruption or promotion of (i) their auto-inhibitory conformations, which affects their intrinsic catalytic activity; (ii) their cooperation with E2 enzymes; (iii) their cellular localization; and (iv) their substrate-affinity (**Fig. 1.8**). As shown in figure 1.8, some adaptors bind and regulate one or more E3 ligases by several mechanisms. Despite this, for practical purposes I will classify the PTMs and Adaptors into two groups: those which modulate the catalytic activity of the E3 ligase by affecting

their conformation, and those who modify the E3 ligase interaction with substrates by affecting its cellular localization and/or its affinity for its targets.

a. Release of auto-inhibition by adaptors

The Nedd4 family-interacting proteins (NDFIP) 1 and 2 have been described as activating adaptors targeting several Nedd-4 E3 ligases. Indeed, they promote the auto-ubiquitination of Itch, Nedd4-1, Nedd4-2, Smurf1, WWP1 and WWP2, as well as the ubiquitination of JunB, c-Jun and endophilin by Itch and Nedd4-1 (Mund and Pelham 2009). Mechanistically, it was shown that NDFIP1 binds multiple WW domains of Nedd4 ligases through its PY motifs and disrupts Nedd4 auto-inhibition (Riling et al. 2015). In contrast with NDFIP 1 and 2, most other adaptors reported to date activate only one or two E3 ligases.

Smurf2 is activated upon binding the adaptor protein SMAD7, whose expression is regulated by extracellular stimuli like the transforming growth factor beta (TGF β) (Kee and Huibregtse 2007). SMAD7 PY motifs interact with the WW domains of Smurf2 and the N-terminus of SMAD7 interacts with the HECT domain of Smurf2, causing the release of the C2 domain (Wiesner et al. 2007; Aragón et al. 2012) and enabling Smurf2-mediated transthiolation. In addition, Smad7 activates Smurf2 in two other ways: first, it facilitates the recruitment of the E2 (Ogunjimi et al. 2005) and second, it directs Smurf2 to the plasma membrane, where it mediates its interaction with several substrates (Kavsak et al. 2000; Di Guglielmo et al. 2003; Izzi and Attisano 2004).

Smurf1 has been shown to be regulated by two adaptor proteins: CKIP-1 (casein-kinase-2 interacting protein -1) and with Cdh1 (Lu et al. 2008; Wan et al. 2011). Wan and colleagues showed that CKIP-1 and Cdh1 bind to Smurf1 and activate it by disrupting the formation of inhibitory Smurf1 dimers, thereby promoting Smurf1 autoubiquitination and ubiquitination of RhoA, one of Smurf1's targets (Wan et al. 2011).

There are several examples of auxiliary proteins that activate Itch, notably Spartin and Numb. Spartin binds to Itch via its PY motifs and recruits it to lipid droplets. This

interaction increases Itch enzymatic activity at a specific cellular location and enables the ubiquitination of proteins present on the lipid droplets, such as adipophilin (Hooper et al. 2010). Di Marcotullio and colleagues showed that Numb activates Itch by disrupting inhibitory intramolecular interactions between its HECT and WW domains; moreover, Numb recruits G11 and mediates its interaction with Itch, leading to G11 ubiquitination and degradation (Di Marcotullio et al. 2011).

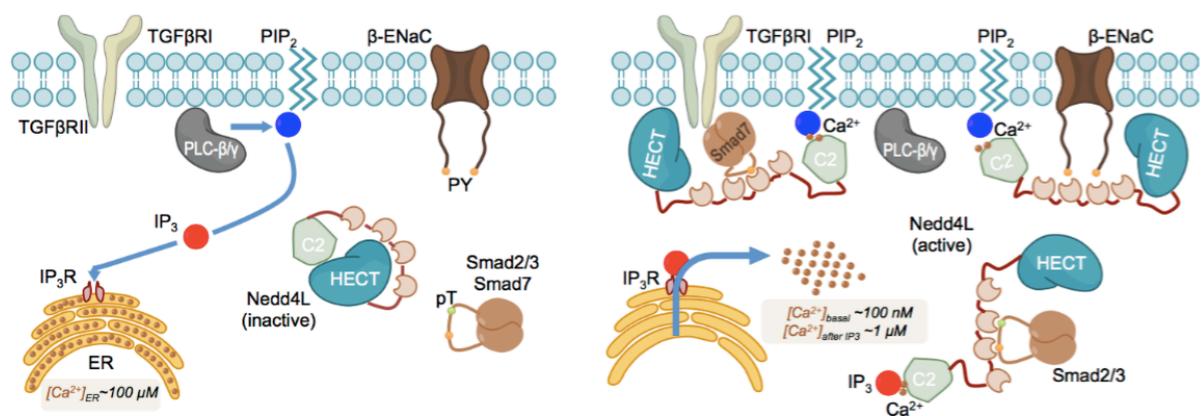


Figure 1.9. Activation and cellular localization of Nedd4-2 by IP₃ and Calcium. IP₃-induced Ca²⁺ delivery into the cytoplasm triggers the transition from the closed an inactive conformation of the ligase (**left panel**) to the active one (**right panel**). Once active, different WW domains in Nedd4-2 are able to recognize the ligase's cytoplasmic targets including Smad2/3 and Smad7. The ligase is also able to relocate to the plasma membrane, where it possibly anchors to the IP₃ head groups of the PIP₂ lipids and targets the membrane receptors, such as TGFβRI and β-ENaC. From: (Escobedo et al. 2014).

Small cellular messengers can also act as activating adaptors that release the auto-inhibition of Nedd4 ligases. Wang and colleagues demonstrated that calcium ions release the C2 domain-mediated auto-inhibition in both Nedd4-1 and Nedd4-2 by disrupting the binding of the C2 domain to the HECT domain (Wang et al. 2010a). More recently, a study using NMR revealed that Ca²⁺ and inositol 1,4,5-triphosphate (IP₃) bind to the C2 domain of Nedd4-2 using the same region that mediates the interaction with the HECT domain (Escobedo et al. 2014). Thus, the balance between the closed and open conformation of Nedd4-2 results from the competition between Ca²⁺, IP₃, and the HECT

domain to bind the C2 domain. Therefore, the activity of Nedd4-2 depends on the intracellular levels of Ca^{2+} and IP_3 . IP_3 is generated by hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2) and is found in the cytosol and the endoplasmatic reticulum, where it binds to its receptor. Both IP_3 and PIP_2 can bind to Nedd4-2 in the presence of Ca^{2+} , which enables Nedd4-2 to act either at the cytosol or at the membrane; where it can target specific substrates, including the cytosolic Smad7 (which makes Nedd4-2 an indirect regulator of Smurf2 activity) (**Fig. 1.9**) (Escobedo et al. 2014).

b. Modulation of auto-inhibition by PTM

Release of auto-inhibition by post-translational modifications has been less reported than activation via adaptors. Here, I will mention three cases where phosphorylation activates Nedd4 ligases and a recent study that shows that ubiquitination of Nedd4 and Rsp5 inhibits their activity in a proteasome-independent manner.

JNK1 kinase phosphorylates Itch at S199, S232, and T222. These modifications disrupt the auto-inhibited conformation of Itch mediated by its WW and HECT domains and therefore induces its activation (Gao et al. 2004; Gallagher et al. 2006). A second kinase, ATM, phosphorylates Itch in response to DNA damage and induces its activation (Santini et al. 2014). The authors of this study identify that phosphorylation of S161 is critical for ATM mediated activation of Itch and they propose that this modification disrupts Itch's intra-inhibitory interactions, as reported for JNK induced phosphorylation. Following the same trend, Persaud and colleagues have described that following activation of FGFR1 or EGFR, the effector tyrosine kinase c-Src is activated, which then phosphorylates Nedd4-1 on Y43 (C2 domain) and Y585 (HECT domain). They demonstrate that phosphorylation of Nedd4-1 disrupts the C2-HECT interaction and thereby relieves inhibition of the E3 ligase, which results in the ubiquitination and degradation of substrates such as FGFR1 (Persaud et al. 2014).

Earlier this year, Attali and colleagues have shown that ubiquitination also affects Nedd4 ligases conformation and activity. Specifically, they demonstrated that ubiquitination of a

flexible alpha-helix in the HECT domain of Nedd4-1 and Rsp5 (Nedd4-1 homologue in Yeast) induces the formation of an inactive homo-trimer (Attali et al. 2017). The described mechanism relies on both ubiquitination of specific lysine residues located in the alpha-helix and on the presence of a UBE_x within the HECT domain. Upon ubiquitination, the alpha helix rotates to approach its linked ubiquitin to the UBE_x. This structural change clears a region that is required for oligomerization (**Fig. 1.10**). How this change in conformation inactivates NEDD4-1 and RSP5 is still unclear.

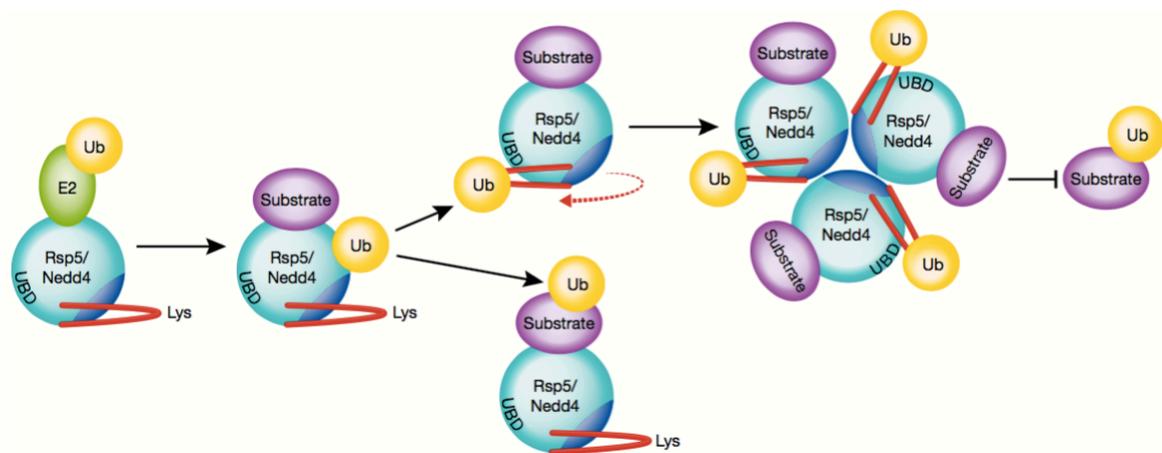


Figure 1.10. Schematic representation of how the Nedd4 family ubiquitin ligases self-regulate through auto-ubiquitination. Ubiquitin (Ub) is transferred from a ubiquitin-conjugating enzyme (E2) to either the Rsp5/Nedd4-1 active-site cysteine and then to E3-bound substrate, or onto a flexible α -helix on the E3. Upon auto-ubiquitination of the α -helix, the position of the helix rotates to interact with a conserved UBE_x (“UBD” in the figure) opening access to an oligomerization domain and trimerization of Nedd4-1. Self-association renders the E3 inactive. From (Hill and Kleiger 2017).

Up to this point I have described adaptors and PTMs that directly influence E3 catalytic activity, and some that in addition modulate the localization of the E3 ligase, thereby dictating their interaction with a set of substrate proteins. In the next two sections I will show some examples of PTMs and auxiliary proteins that regulate substrate recognition, reportedly without affecting the E3 catalytic activity.

c. Regulation of substrate interaction by adaptors.

The 14-3-3 family members bind targets that have been phosphorylated at specific serine or threonine residues and are key regulators of a wide variety of cell signaling pathways mediated by phosphorylation (Muslin et al. 1996). 14-3-3 proteins have been shown to associate to Nedd4-2 following its phosphorylation by SGK1 (Serum- and Glucocorticoid-related Kinase 1) and PKA (Protein Kinase A). This association with 14-3-3 proteins inhibits the interaction between Nedd4-2 and its substrate ENaC (epithelial sodium channel) leading to reduced ENaC ubiquitination and subsequent degradation (Ichimura et al. 2005; Bhalla et al. 2005; Nagaki et al. 2006; Chandran et al. 2011).

Similarly, Oberst and colleagues demonstrated that Nedd4-binding partner 1 (N4BP1) binds to the second WW domain of Itch and inhibits its interaction with several substrates (Jun, p73 and p63) by binding competition, thereby preventing their ubiquitination (Oberst et al. 2007).

Auxiliary proteins can also promote enzyme-substrate interactions. Members of the α - and β -Arrestin families have been shown to bind to the WW domains of Itch, Nedd4-1 and Nedd4-2 and mediate their association with β 2 adrenergic receptor, which leads to ubiquitination and recycling of the receptor (Shea et al. 2012; Han et al. 2013).

d. Regulation of substrate interaction by PTM.

Contrary to the activating effect of serine and threonine phosphorylation of Itch, tyrosine phosphorylation seems to negatively modulate the ability of Itch to selectively bind and ubiquitinate some of its targets, such as JunB and c-Jun. Gao and colleagues found that phosphorylation of a tyrosine within the PPXY motif of Itch by c-Abl inhibited its binding to c-Jun (Gao et al. 2006). Similarly, Fyn kinase phosphorylates Itch on Tyr-371, which inhibits its binding to JunB and therefore hampers JunB ubiquitination (Yang et al. 2006).

Numerous studies have shown that hormone-induced phosphorylation of Nedd4-2 by PKA, SGK and IKK β inhibits Nedd4-2 interaction with ENaC, inhibiting its ubiquitination (reviewed in (Snyder 2009)). For instance, SGK1 phosphorylates Nedd4-2 at S221, S327

and T245 and increases the surface abundance of all three ENaC subunits (α -, β - and γ -), while Nedd4-2 phosphorylation by PKA at S221 and S327 specifically increases the abundance of α -ENaC (Ismail et al. 2014). These studies suggest that the pattern of phosphorylation on Nedd4-2, modulated by different kinases, controls its association with the different subunits of ENaC. Similarly, it has been shown that Nedd4-2 phosphorylation by SGK1 downstream mTORC2 activation results in reduced Nedd4-2-JunB interaction and increased JunB stability (Heikamp et al. 2014). In another study, it has been demonstrated that following their TGF β -induced phosphorylation, SMAD2/3 interact with Nedd4-2 via its second WW domain (WW2), which results in their ubiquitination and degradation; and that SGK1 inhibits this interaction by phosphorylating two serine residues flanking Nedd4-2 WW2 domain (Gao et al. 2009).

Another remarkable case of modulation of substrate binding by phosphorylation was shown in a study by Cheng and colleagues. They find that PKA phosphorylates Smurf1 at Thr-306 and shifts Smurf1's affinity for its substrates. Namely, phosphorylation at Thr-306 on Smurf1 reduces its affinity for Par6 but increases it towards RhoA (Cheng et al. 2011).

Finally, we have seen that ubiquitination of Nedd4 ligases can lead to inhibition of their catalytic activity by trimerization (Attali et al. 2017). Additionally, Woelk and collaborators reported an example of self-ubiquitination-dependent recruitment of substrates: they observed that self-catalyzed monoubiquitination of Nedd4-1 serves to recruit EPS15, which is subsequently monoubiquitinated by Nedd4-1 (Woelk et al. 2006).

In summary, Nedd4 ligases are regulated by auto-inhibition, either by intra-molecular interactions or by inter-molecular interactions, forming steady state homodimers like Smurf1 or ubiquitination-triggered trimers like Nedd4-1 and Rsp5. With the exception of these trimers, the auto-inhibitory conformations are stable at basal levels and upon a stimulus (phosphorylation or binding of an adaptor protein) they can be disrupted. Each particular mechanism of activation disrupts the same interaction but in response to different upstream signals and, as seen before, they can additionally dictate the subcellular localization of E3s, thereby influencing their access to substrates. In other cases, the

adaptors or the modifications do not activate the ligase directly but solely modulate its affinity to different proteins, further fine-tuning the activity of Nedd4 E3 ligases.

1.3.2. Regulation of HECT ligases outside the Nedd4 family.

Very few studies about the regulation of HECT E3 ligases outside the Nedd4 family exist. The ones published pertain E6AP, the founding member of the HECT E3 ligases, and HUWE1, the giant of many names.

a. E6AP/Ube3A

As previously mentioned, E6AP was first described as the E3 ligase that worked in partnership with the E6 viral protein to induce p53 degradation in cells infected with HPV (Scheffner et al. 1990). A recent study elucidated the crystal structure of a E6/E6AP/p53 ternary complex and determined that the viral protein E6 behaves as an adaptor of E6AP that enables it to bind to p53 by inducing the formation of a p53 binding site. Interestingly, it has been shown that phosphorylation of E6AP by c-Abl inhibits its capacity to ubiquitinate p53 (Chan et al. 2013).

Besides regulating p53 during viral infection, E6AP also targets cellular proteins such as activity-regulated cytoskeleton-associated protein (Arc), RhoA-GEF Ephexin-5, and a human homologue of yeast Rad23 (HHR23A), a protein involved in DNA repair (Kumar et al. 1999; Kühnle et al. 2013). Incidentally, a recent study showed that phosphorylation of E6AP on Thr-485 by PKA downregulates E6AP activity by strengthening its affinity for itself and for HHR23A; and that disruption of this phosphorylation site (T485A) disables E6AP regulation by PKA and causes both excessive dendritic spine development in the brain and autism (Yi et al. 2015).

b. HUWE1

Huwe1, also known as Mule, ARF-BP1, UREB1, HectH9 and LASU1, is a 482kDa HECT E3 ligase implicated in numerous physiological processes (Scheffner and Kumar 2014) and one of its most prominent targets is the tumor suppressor p53. It has been shown that

HUWE1 activity towards p53 is inhibited when it binds p14ARF (Alternative ReadinG Frame in the CKN2A gene) (Chen et al. 2005). In a more recent study, it was determined that p14ARF also inhibits HUWE1's ability to ubiquitinate Miz1, a transcription factor that suppresses TNF-induced cell death (Lee et al. 2015). Moreover, this study uncovered that TNF activates HUWE1 by inducing its Syk (Spleen Tyrosine Kinase)-mediated tyrosine phosphorylation, which in turn disrupts HUWE1 interaction with its inhibitor p14ARF. Together these studies show that HUWE1 activity can be inhibited by the adaptor protein p14ARF and that their interaction can be modulated by phosphorylation. However, the molecular mechanism by which p14ARF inhibits HUWE1 activity is not clear.

Sander and colleagues published this year a structural study of an isolated C-terminal fragment of HUWE1 (3951-4374), which comprised the HECT domain plus a 42-residue extension. This work showed that HUWE1's HECT domain can form an asymmetric dimer in which one subunit adopts a rigid auto-inhibited conformation; in addition, they identified an "activating region" located around 50 residues upstream the initially analyzed C-terminal fragment that could interact in *cis* with the dimerization region and thus disrupt the formation of the inhibitory dimer. The authors measured the strength of these inter- and intra-molecular interactions and found that they were quite weak and very similar, indicating that the dimeric and monomeric forms of HUWE1 are in a dynamic equilibrium. Interestingly, they found that the HUWE1 inhibitor p14ARF binds to the activating region of HUWE1 and shifts its conformational equilibrium towards the inactive dimer (**Fig. 1.11**) (Sander et al. 2017). This work provided an initial structural framework of HUWE1 activity, however more biochemical and structural studies are needed to identify which step of the catalytic cycle is hampered by the dimeric conformation of HUWE1 and which mechanisms mediate its activation.



Figure 1.11. Model of the conformational regulation of HUWE1 and the proposed mechanism of its inhibition by p14ARF. The catalytic activity of HUWE1 is regulated conformationally by the balance of inter- and intramolecular interactions. The “thumb” and “pointer” helices adjacent to the catalytic HECT domain can mediate the dimerization of HUWE1. The dimer locks the position of the C-lobe, buries the C-terminal tail (magenta), and occludes a putative ubiquitin binding site on the C-lobe of one subunit, hence representing an auto-inhibited state. Alternatively, the dimerization region of HUWE1 can associate with the activation segment in cis, which precludes dimer formation. The activation segment and the dimerization region are separated by a 55- residue flexible linker, which allows the re-positioning of the activation segment. In the monomeric state of HUWE1, the C-lobe is mobile with respect to the N-lobe (arrow), and the C-terminal tail may anchor the C-lobe on the N-lobe or interact with substrates, as required for catalytic activity. The activation segment of HUWE1 presents a major interaction site for a physiological inhibitor of HUWE1, p14ARF. It is possible that the binding of p14ARF to the activation segment releases the dimerization region from its intramolecular engagement, thus shifting the conformational equilibrium of HUWE1 toward the auto- inhibited, dimeric state. From (Sander et al. 2017).

1.3.3. Modulation of the activity of HECT E3s by small chemical compounds.

The therapeutic potential of targeting proteasome-mediated degradation was successfully demonstrated in 2003 with the FDA approval of the reversible proteasome inhibitor Bortezomib (Velcade/PS-341) for the treatment of relapsed or refractory multiple myeloma (Adams 2002; Roccaro et al. 2006) and later by Carfilzomib (Krypolis), an irreversible second generation proteasome inhibitor (Khan and Stewart 2011). Despite their clinical success, these inhibitors have been associated with cutaneous and cardiac side effects in the case of Bortezomib (Nowis et al. 2010), and with hematological complications and minor side effects like fatigue, nausea and gastrointestinal upset in the case of Carfilzomib (Siegel et al. 2012; Kim and Crews 2013).

As components of the ubiquitin system, E3 ubiquitin ligases are involved in the regulation of virtually all physiological processes. Additionally, they are a diverse and highly specific group of enzymes whose activity is subjected to strict regulation. These characteristics make E3 ligases attractive targets for drug development with the potential to minimize the side effects observed in classical ubiquitin-proteasome inhibitors (Wilkie and Davies 2012). This is reflected in the recent interest in considering E3 ligases as therapeutic targets against cancer (Liu et al. 2015) and inflammatory diseases such as asthma, atherosclerosis and obesity (Goru et al. 2016).

In the particular case of HECT E3 ligases, the discovery of small molecule and peptide inhibitors has recently been reported. Mund and colleagues screened a library of bicyclic peptides for compounds that targeted the E2 binding site of the HECT domain and found candidates that inhibited Smurf2, Nedd4-1, HUWE1 and WWP1 activity *in vitro*. Among these peptide inhibitor candidates, one retains its inhibitory activity *in vivo*; however, they find that this compound does not impair E2 binding but instead it induces a conformational change that results in the oxidation of the E3 catalytic cysteine, impairing transthiolation (Mund et al. 2014).

In other cases, the search for chemical inhibitors leads to the discovery of fundamental properties of HECT ligases. For instance, Kathman and collaborators were looking for covalent modifiers of two Nedd4-1 cysteine residues: the catalytic cysteine (C876) and a cysteine important for elongation (C627); they identified a small inhibitor that weakened the processivity of Nedd4-1 and made it efficiently antagonized by the DUB USP8. By studying the mechanism of action of this molecule they demonstrated that Nedd4-1 is a processive enzyme, meaning that it transfers several ubiquitin monomers to the substrate in one round of binding. Subsequently, once bound to their candidate molecule it turns into a distributive enzyme, i.e. it needs to release and bind its substrate every time it attaches an ubiquitin (Kathman et al. 2015).

Recently, our team participated in the development of variants of ubiquitin (UbV) designed to strongly bind 20 HECT ubiquitin ligases with the purpose to inhibit them.

The approach was based on the fact that all HECT ligases bind to ubiquitin, either through a thioester bond with their active cysteine or, as seen in Nedd4-like ligases, through their UBE_x. Interestingly, we observed that not all UbV inhibited all HECT ligases, some also increased their activity, or changed the type of ubiquitin chains that they generated. Therefore, this study generated a fantastic set of tools to study the regulatory mechanisms of HECT E3 ligases and to evaluate their roles on various cellular processes (Zhang et al. 2016).

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2.1. The Rho GTPase family

The family of Rho GTPases is part of the superfamily of Ras-related small GTPases, a group of small G proteins in which most members have an intrinsic Mg²⁺-dependent

GTPase activity involving the binding to GTP and the catalysis of its hydrolysis into GDP (Takai et al. 2001). Rho GTPases are distinguished from other Ras-like GTPases by the presence of an insert region, which is involved in the recognition of binding partners (effectors and regulators) (Freeman et al. 1996). The first Rho GTPase gene was described three decades ago (Madaule and Axel 1985) and since then, members of the family have been found in all eukaryotic cells, among which they share a high degree of conservation (Boureau et al. 2007). In humans, the Rho GTPase family consists of 20 members, 12 of which are considered “classical” and can catalyze the hydrolysis of GTP, and 8 of which are considered “atypical” and are predominantly bound to GTP either due to key amino acid substitutions that hamper their GTPase activity, or to an increased nucleotide exchange rate (like Wrch1) (**Fig. 2.1**) (Vega and Ridley 2008; Heasman and Ridley 2008). Among the classical Rho GTPases, the most studied and well understood members are RhoA, Rac1 and Cdc42 (Vega and Ridley 2008).

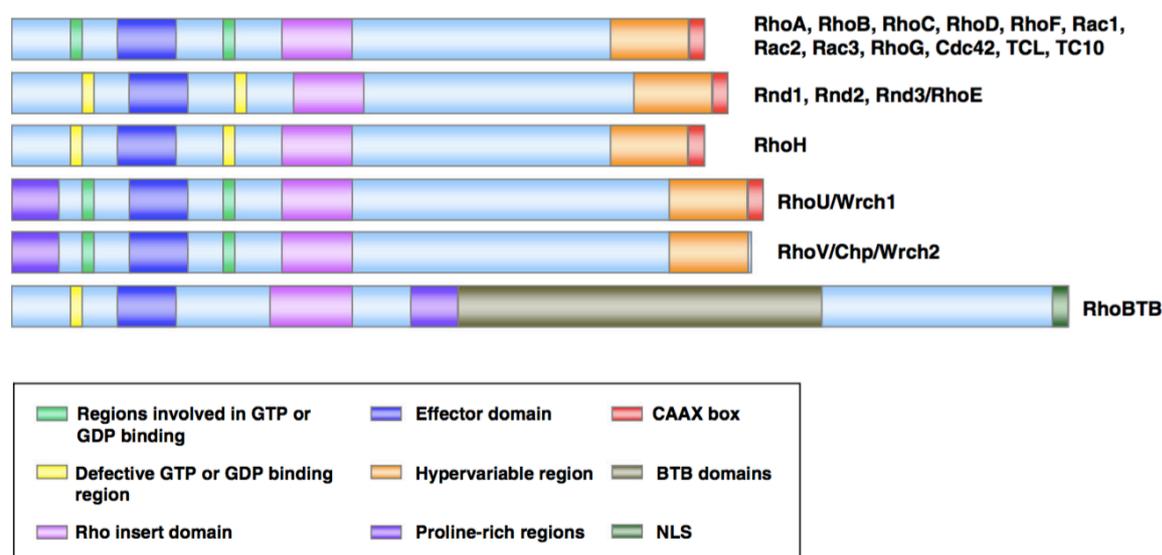


Figure 2.1. Domain architecture of the Rho GTPases. RhoA, RhoB, RhoC, Rac1, Rac2, Rac3, RhoG, RhoD, RhoF, Cdc42, TCL and TC10 have a similar basic protein structure. Rnd1, Rnd2, Rnd3/RhoE and RhoH are considered atypical Rho GTPases that have modifications in the GTP/GDP binding region that make them lack GTPase activity. Wrch1 and Wrch2 are characterized by the presence of an N-terminal proline-rich region. RhoBTB1 and 2 have the most divergent protein organizations with two characteristic BTB domains NLS, nuclear localization sequence. From (Vega and Ridley 2008)

Like the majority of Ras-like GTPases, most Rho-family GTPases work as molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state that can interact with effector proteins to relay signals through different pathways. They are best known as master regulators of cytoskeletal dynamics. Therefore, they are critical regulators of cell adhesion, migration and vesicle trafficking. In addition, they take part in the regulation of gene expression, cell cycle progression, as well as in the cellular response to pathogenic agents (Jaffe and Hall 2005; Bustelo et al. 2007; Lemichez and Aktories 2013; Hodge and Ridley 2016).

2.2. Regulation of the activity of Rho GTPases.

2.2.1. Structural basis of Rho GTPase activity.

The signal transducer functionality of Rho GTPases comes from the fact that their GDP-bound (inactive) and GTP-bound (active) states have different affinities for regulators and effectors. This change in affinity results from a nucleotide-dependent conformational rearrangement that occurs in two localized regions appropriately called switch I and II (**Fig. 2.2A-B**) (Vetter and Wittinghofer 2001; Hakoshima et al. 2003; Dvorsky and Ahmadian 2004). These two regions, together with the five conserved G-box regions, constitute the nucleotide binding pocket and also contain the Mg^{2+} binding site, which is essential for GTPase activity (**Fig. 2.2A-B**). In the active state, the γ -phosphate of the bound GTP forms two hydrogen bonds between the side-chain oxygen of a conserved threonine in the switch I region and the main-chain oxygen of a conserved glycine in the switch II region, forming a stable closed conformation. These interactions are unlatched upon loss of the γ -phosphate during GTP hydrolysis, which relaxes the switch I and II regions giving them great conformational variability. This rearrangement process is referred to as the “loaded-spring mechanism” and is the basic principle for the signal transducing capabilities of Rho GTPases (**Fig. 2.2C**) (Vetter and Wittinghofer 2001).

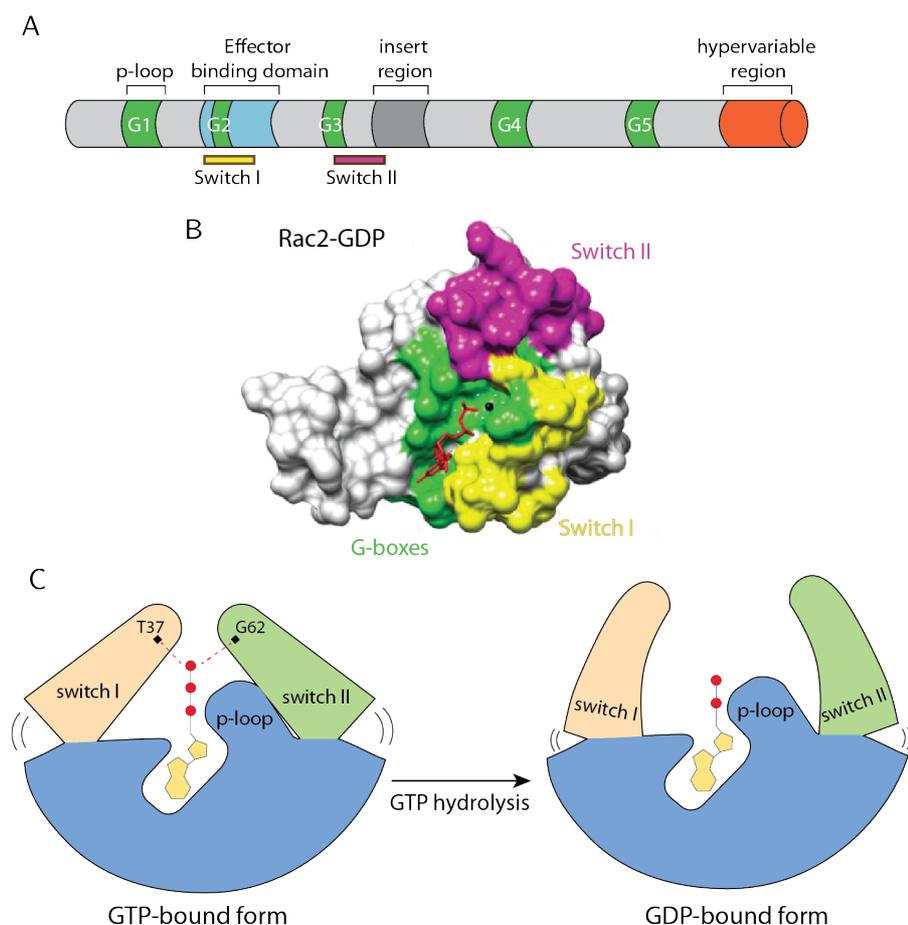


Figure 2.2. RhoGTPase activity. **A.** Schematic representation of the general domain structure of Rho GTPases. The highly conserved G domain of Rho-GTPases is characterized by five G-box motifs responsible for nucleotide binding. The P-loop (phosphate-binding loop), also known as the G1 domain, is a conserved motif that is responsible for binding to the γ -phosphate of the guanine nucleotide. The hypervariable domain at the C-terminus gives Rho GTPases specificity since it shows the highest level of variability between Rho proteins, and is a key region for posttranslational modifications, notably lipidation, which mediates the membrane targeting of RhoGTPases. **B.** The Rac2-GDP (PDB ID: 2W2T) three-dimensional structure with G boxes, switch I and switch II regions indicated (using the same color scheme as in A) GDP is shown in red and Mg^{2+} as a black sphere. **C.** Rho GTPase's conformational changes upon GTP hydrolysis. the switch I and II domains bind to the γ -phosphate via the main chain NH groups of the conserved Thr and Gly residues (T37 and G62 in RhoA). Release of the γ -phosphate after GTP hydrolysis allows the switch regions to relax into a different conformation. This switch is termed the “loaded-spring mechanism”. Modified from (Weirich et al. 2008) and (Olson 2016).

2.2.2. Regulation of the GDP-GTP cycle

Due to the high binding affinity of GTPases for both GDP and GTP and the slow rate of intrinsic GTP hydrolysis, the GTPase cycle is controlled by GEFs (guanine nucleotide

exchange factors) and GAPs (GTPase-activating proteins). GEFs promote the activation of Rho GTPases by facilitating the exchange of GDP by GTP once the GEFs themselves are activated by upstream stimuli. GEFs preferentially interact with GDP-bound Rho GTPases through their switch I and II regions and insert residues close to or into the P-loop and the Mg^{2+} binding area (**Fig. 2.2A-B**), which creates structural changes that impair the binding of phosphates and the metal ion and drastically decreases the affinity for nucleotides leading to GDP release. This results in a nucleotide-free GTPase that quickly associates with GTP more often than with GDP due to the 100-fold higher concentration of GTP in cells (Vetter and Wittinghofer 2001; Hakoshima et al. 2003). On the other hand, GAPs accelerate the inactivation of Rho GTPases by stimulating their intrinsic GTPase activity by up to 10^5 times; they do so by binding both the switch I and II regions and by inserting a highly-conserved arginine containing “finger” into the Rho GTPase active site, which stabilizes the catalytic glutamine residue into a position optimal for GTP hydrolysis (Moon and Zheng 2003; Hakoshima et al. 2003). Over 80 GEFs and more than 70 GAPs have been reported in mammals, greatly surpassing the number of Rho GTPase family members, which indicates that the regulation of Rho GTPase activity is highly complex and has the potential to be incredibly precise (Hall 2012).

In addition to the regulation of the GTP/GDP cycle by GAPs and GEFs, the equilibrium of the cycle depends on (i) the intrinsic affinity of each Rho GTPase for GDP versus GTP, (ii) on the cellular levels of GTP, and (iii) on the availability of effectors, which bind to the GTP-bound form of Rho GTPases and thus shifts the equilibrium towards the GTP-bound state (Vetter and Wittinghofer 2001).

Another set of proteins that impact the equilibrium of the GTPase cycle and the cellular localization of Rho GTPases is the family of RhoGDIs (GDP dissociation inhibitors). As their name indicates, RhoGDIs bind preferentially the GDP-bound form of some GTPases and prevent their dissociation from GDP, thus keeping them in their inactive form. RhoGDIs also spatially regulate RhoGTPases by sequestering them in the cytosol, preventing them from localizing to membranes and being activated by GEFs. RhoGDIs maintain Rho GTPases as soluble cytosolic proteins by forming high-affinity complexes in

which the isoprenoid membrane-targeting moiety present at the C-terminus of the Rho GTPases is shielded from the aqueous cytosolic environment (DerMardirossian and Bokoch 2005). In addition to the negative effect that RhoGDIs have on the re-activation of Rho GTPases, it has been reported that RhoGDI-bound GTPases are protected from proteasomal degradation, and thus RhoGDI proteins also have a positive effect on their stability and contribute to the regulation of Rho GTPases protein levels (Boulter et al. 2010). Rho GTPases can escape or prevent binding with RhoGDIs through post-translational modifications of the GTPase or of the GDI, such as phosphorylation and s-palmitoylation (Hodge and Ridley 2016).

2.2.3. Additional regulatory mechanisms

Mechanisms other than cycling (termed “unconventional”) also regulate Rho GTPase signaling (summarized in **Fig. 2.3**). For example, Rho GTPases can be regulated at the level of gene expression or at the translational level by micro RNAs. Additionally, Rho GTPase activity can be modulated at the protein level by post-translational modifications such as lipidation, which occurs mainly at their C-terminus and is essential for membrane localization and necessary for activation. Some Rho GTPases can be phosphorylated, which modulates their interaction with regulators and effectors; and sumoylated, which regulates their activity. Moreover, Rho GTPases can be ubiquitinated and their protein levels can be regulated by the ubiquitin-proteasome system (Hodge and Ridley 2016). This last topic will be further developed in Chapter 3.

Together, both classical (GEFs, GAPs and RhoGDIs) and unconventional regulatory mechanisms precisely regulate the spatiotemporal activation of Rho GTPases and their interaction with available downstream effectors, which determines the outcome of GTPase-specific cellular responses (Pertz 2010; Hodge and Ridley 2016).

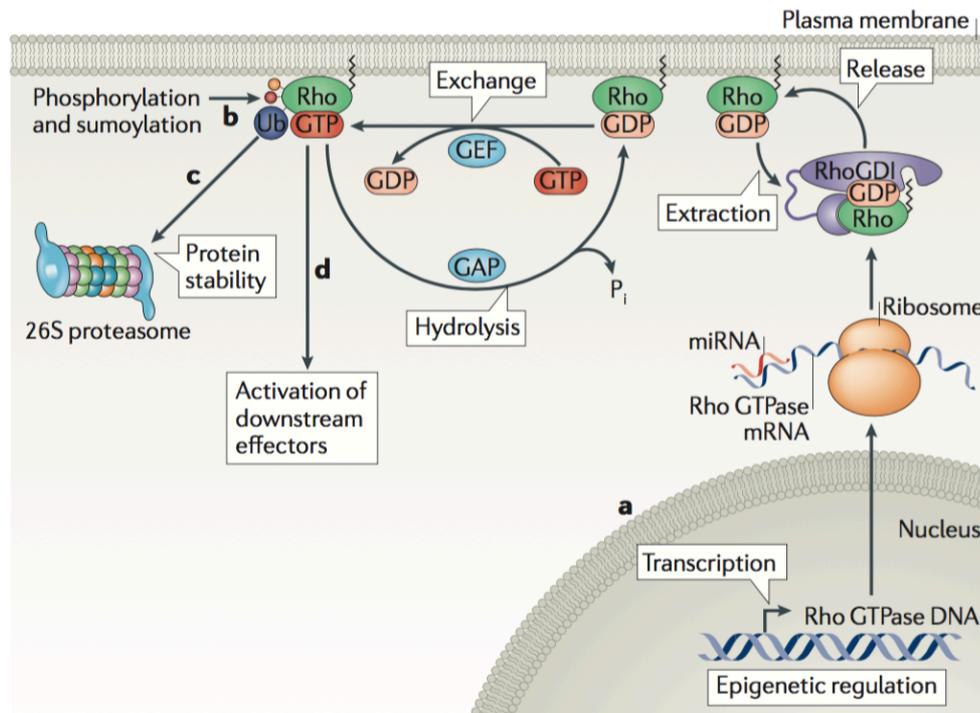


Figure 2.3. Overview of Rho GTPase regulation. The route from Rho GTPase protein expression to effector protein activation is tightly regulated. GEFs, GAPs and GDIs constitute the canonical regulators of the GTPase cycle. GEFs activate Rho GTPases by catalyzing the exchange of GDP for GTP, whereas GAPs stimulate the intrinsic GTPase activity of Rho GTPases and inactivate them. GDIs extract prenylated Rho GTPases from the membrane by binding the isoprenoid moiety and sequester them away in the cytoplasmic compartment. Unconventional mechanisms and their importance in the regulation of Rho GTPases are becoming more apparent. **A.** Rho GTPase expression can be controlled at the transcriptional level by epigenetics and at the translational level by the action of micro RNAs (miRNAs). **B.** Post-translational covalent modifications of Rho GTPases, including phosphorylation and sumoylation, can result in the activation or inactivation of Rho GTPases, depending on the cellular context. **C.** Protein levels of Rho GTPases can be efficiently regulated by the ubiquitin–proteasome system. **D.** The combination of classical and unconventional regulatory mechanisms ensures the appropriate spatiotemporal activation of the Rho GTPases during various cellular processes. From (Hodge and Ridley 2016)

2.3. Molecular and cellular aspects of Rho GTPase signaling

2.3.1. From extracellular stimuli to effector proteins: Focus on PAK

There are many signaling pathways that lead to the activation of Rho GTPases, including those initiated by physical stimuli (mechanical stress or cell–cell and cell–substrate

adhesion) and chemical factors (phospholipids, growth factors and cytokines). These initial signals are transduced by cell–cell or cell–extracellular matrix adhesion receptors, like cadherins and integrins; and G-protein-coupled receptors; growth factor receptors and cytokine receptors. Upon activation, GTP-bound Rho GTPases interact with a wide spectrum of effector proteins to regulate various cellular pathways, such as actin cytoskeleton reorganization, cell motility, cell growth, membrane trafficking, apoptosis, and transcription (**Fig. 2.4**) (Buchsbbaum 2007; Citi et al. 2011).

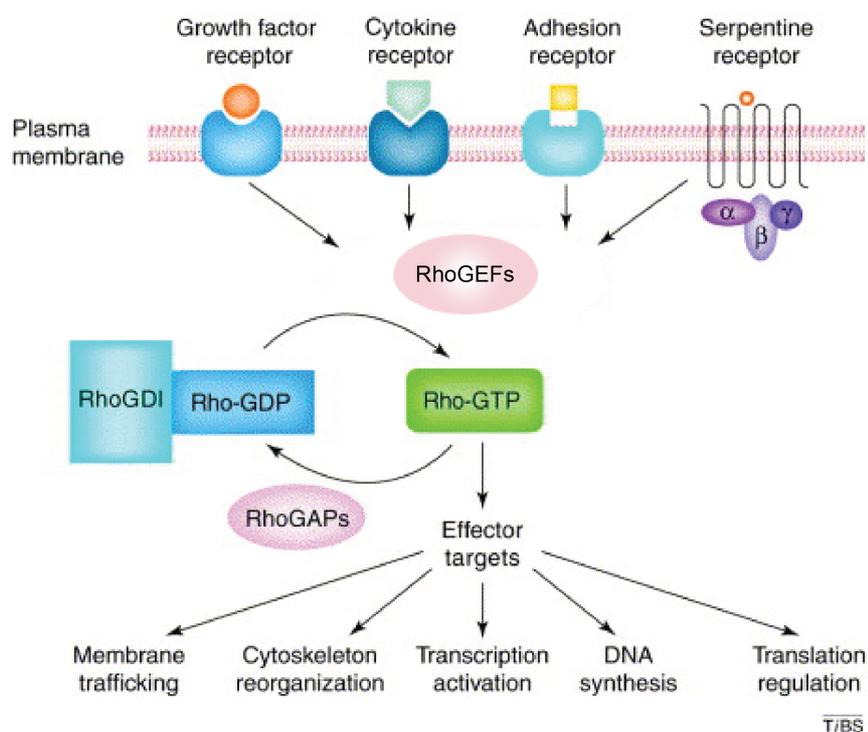


Figure 2.4. Signaling of Rho GTPases. In mammalian cells, stimulation of a variety of cell surface receptors leads to the activation of specific GEFs, which in turn catalyze the exchange of bound GDP for GTP on specific Rho GTPases, resulting in their activation. The receptor-mediated signals might also affect the biochemical activity of two classes of negative regulators of Rho GTPases: RhoGAPs and RhoGDIs. Modulation of these two factors and of GEFs, regulate the dynamics of the Rho GTPase cycle. In the GTP-bound state, Rho proteins can then interact with multiple effector targets, leading to diverse cellular responses. Adapted from (Zheng 2001)

More than 100 Rho GTPase effectors have been identified (Hall 2012), some of these are scaffold proteins and others are enzymes, such as kinases, phosphatases and phospholipases.

These effectors associate with GTP-bound Rho GTPases through interacting regions that commonly overlap with the switch I and II domains, which grants them specificity for the GTP-bound state. Despite the high amino acid sequence and structural homology between the members of the Rho GTPase family, they interact with and activate distinct targets and have few common effectors (Bishop and Hall 2000; Bustelo et al. 2007). Once activated, these effectors propagate signals through numerous signaling networks that culminate in the modulation of the cellular processes mentioned in the previous paragraph and also create feedback loops that in turn regulate the activity of Rho GTPases. Indeed, Kim and colleagues recently built a model of the signaling network around Rac1, RhoA and Cdc42 triggered by the epidermal growth factor (EGF) and found that it was a highly connected web of proteins that comprised 121 feedback loops, which the authors proposed to be vital for the dynamic regulation of these three Rho GTPases during cell migration (Kim et al. 2015).

The PAK family of effectors

The family of p21-activated kinases (PAKs) were the first Rho GTPase targets to be identified back in 1994 by Edward Manser and colleagues while searching for new GAPs for Rac1 and Cdc42 (Manser et al. 1994). PAKs are a group of serine/threonine kinases that share an N-terminal Cdc42/Rac interaction/binding (CRIB) motif that allows them to bind to active Rac1 and/or Cdc42. The CRIB domain overlaps with an autoinhibitory domain (AID) that, in the absence of Rac1 or Cdc42, mediates interactions that keep PAKs in an inactive conformation (**Fig. 2.5A-B**). In humans, the PAK family consists of six members classified in two groups according to their structural differences: Pak1-3 are in group I, while Pak4-6 are in group II. The two groups have different mechanisms of activation. Notably Group I PAKs can be activated by Rac1 and Cdc42 and require auto-phosphorylation of a conserved residue within the kinase domain (**Fig. 2.5B**); while Group II PAKs have higher basal activities, do not require auto-phosphorylation, and are activated only by Cdc42 (Zhao and Manser 2012).

Group I members are the most studied among the PAKs. They share between 92 to 95% homology in the kinase domain, which accounts for similarities in phosphorylation targets found *in vitro*. However, isoform-specific responses *in vivo* are evident and are believed to be due to differential tissue or cellular localization since PAK2 is thought to be ubiquitously expressed, whereas PAK1 and PAK3 expression is more restricted. PAK1 is prominently expressed in mammary gland, muscle, spleen, liver and brain; while PAK3 has only been detected in the brain (King et al. 2014).

Group I PAKs are implicated in the regulation of numerous cellular functions via their great number of targets (Bokoch 2003; Arias-Romero and Chernoff 2008; Chan and Manser 2012). Figure 2.5C illustrates this for PAK1, the best-studied member of group I PAKs. Through its targets, PAK1 is able to exert pro-survival and anti-apoptotic functions, regulate cytoskeletal dynamics, cell cycle progression, gene expression, immune functions and host-pathogen responses (Chiang and Jin 2014).

Considering that PAKs promote cell cycle progression, protect cells from apoptosis and promote migration and invasion; it is no wonder that PAK genes are found to be frequently amplified (PAK1 and PAK4) or mutated (PAK5) in human cancers, and have been linked to cancer progression (Kelly and Chernoff 2012; Kumar and Li 2016). Moreover, PAK genes seem to play an important role during tissue development; and deregulation of PAK expression is associated with brain disorders like non-syndromic X-linked mental retardations and Alzheimer's disease (Chan and Manser 2012; Kelly and Chernoff 2012).

Many (but not all) of these processes are dependent on Rho GTPase mediated activation of PAKs. In the next three sections, the cellular roles of Rho GTPases mediated by different effectors will be discussed, focusing on cytoskeleton remodeling and on intercellular adhesion.

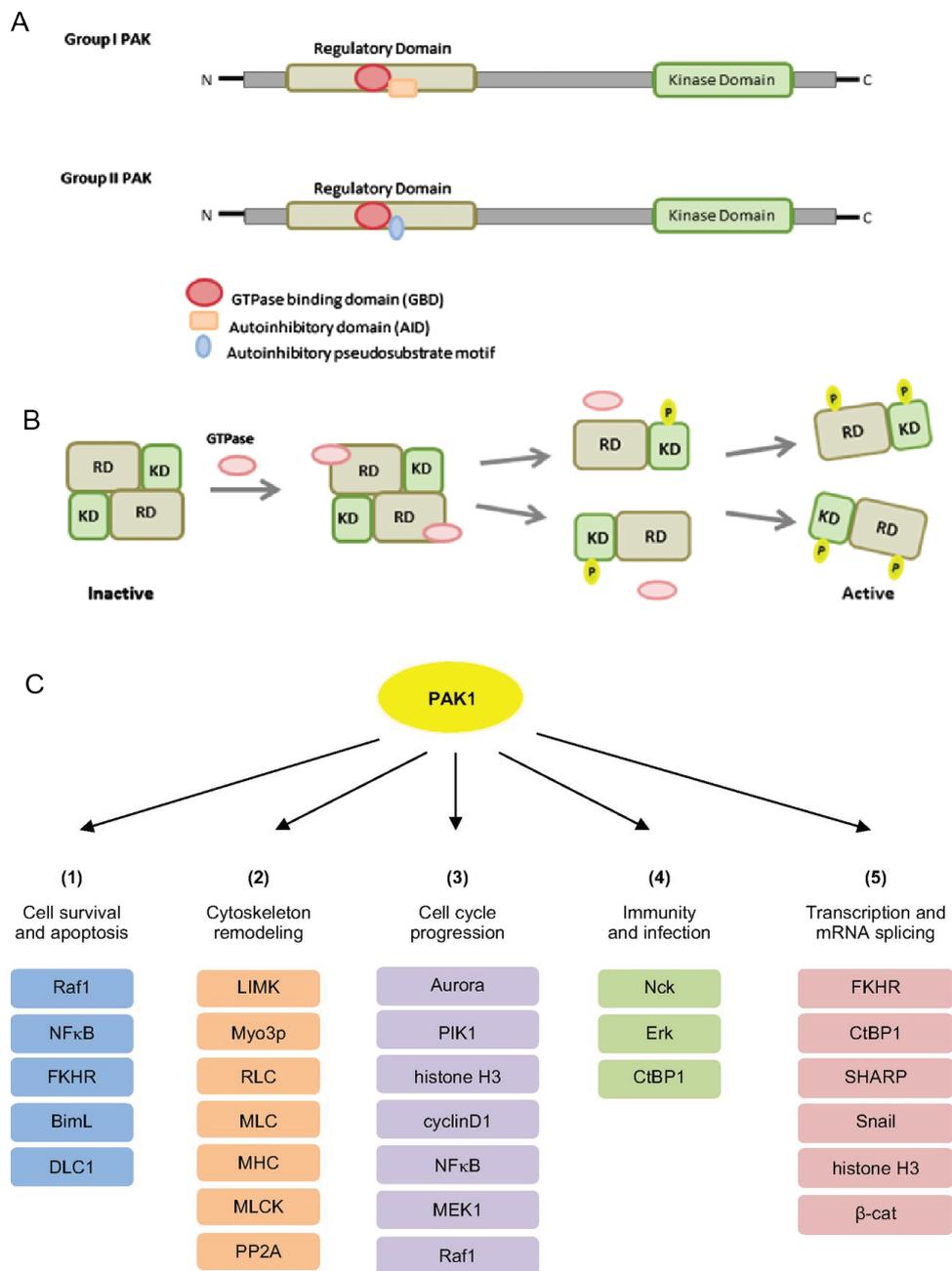


Figure 2.5. Overview of PAK structure, activation and targets. **A.** Simplified domain organization of group I and Group II PAKs. **B.** Mechanism of group I PAK activation. The N-terminal autoinhibitory domain (AID) keeps PAK1 as a dimer in an auto-inhibited state. The AID partially overlaps with the GTPase binding domain. GTP-bound Rho GTPases (Cdc42/Rac1) release PAK1 from its auto-inhibitory conformation, allowing its auto-phosphorylation (Thr-423). Phosphorylation at Thr-423 is critical for PAK1 activity. Subsequently, additional residues are phosphorylated at N-terminus, blocking auto-inhibition. **C.** Non-exhaustive list of PAK1 targets, linking PAK signaling to (1) cell survival and apoptosis, (2) cytoskeleton remodeling, (3) cell cycle progression, (4) immunity and infection, and (5) gene transcription and mRNA splicing. Adapted from (Dammann et al. 2014) and (Chiang and Jin 2014).

2.3.2. General view of the cellular roles of Rho GTPases

Via their panoply of effectors, Rho GTPases are involved in three critical processes: (i) actin cytoskeleton reorganization, (ii) the regulation of gene expression and (iii) the generation of ROS (reactive oxygen species). This, in turn, implicates Rho GTPases in the regulation of cell morphology and motility, cell growth, membrane trafficking, apoptosis, and inflammation (Jaffe and Hall 2005; Hall 2012).

Back in 1991, the first reported activity of a Rho GTPase family member was for Rac acting as an allosteric regulator of the phagocytic NOX (NADPH oxidase) enzyme complex, which transports electrons across membranes and generates superoxide anions from molecular oxygen (Abo et al. 1991). Later studies found that the subunits of the NADPH oxidase complexes p67phox (Segal and Hall 1994), and its homolog NOXA1 (Ueyama et al. 2006; Cheng et al. 2006) directly interact with Rac1 and require this interaction to localize to the membrane and integrate in the functional NADPH oxidase complex. This, along with other mechanisms, involves Rho GTPases in the regulation of redox balance with implications in DNA damage and inflammation, the latter via the activation of NF- κ B (Hobbs et al. 2014).

Rac1, RhoA and Cdc42 can promote the activation of the NF- κ B transcription factor (Perona et al. 1997; Cammarano and Minden 2001). Crosstalk with the NF- κ B pathway is involved in cytokine production, inflammation, cell cycle progression, cell adhesion, ROS production, and metabolism (Tong and Tergaonkar 2014). Moreover, other pathways associated with the regulation of gene transcription are controlled by Rho GTPases. For instance, Rho indirectly activates the transcription factor SRF (serum-response factor) through its effects on actin, while Rac and Cdc42 activate the JNK (c-Jun N-terminal kinase) and p38 MAPK (mitogen-activated protein kinase) pathways through targets such as the mixed lineage kinases (Gallo and Johnson 2002; Miralles et al. 2003).

In addition to their ability to control gene expression and ROS generation, the Rho GTPases are central regulators of cytoskeletal dynamics, an aspect that will be discussed in the next section in more detail. Indeed, most of the cellular functions of the Rho

GTPases stem from their ability to remodel the cytoskeleton. By doing so, they are involved in the control of cell shape, cell migration and chemotactic responses, axonal guidance and dendrite outgrowth in neurons, endocytosis and vesicle trafficking (Hall 2012).

One of the cellular aspects that is affected by all the three central processes controlled by Rho GTPases (Redox balance, gene expression, cytoskeleton remodeling) is cell proliferation (Vega and Ridley 2008). The dynamics of the actin cytoskeleton regulated by Rho GTPases are critical during cell cycle progression and mitosis: Rho GTPases and their effectors are involved in cell rounding at mitosis onset (Bakal et al. 2005), in chromosome alignment (Yasuda et al. 2004) and are required for contraction of the actomyosin ring that separates daughter cells at the end of mitosis (Madaule et al. 1998; Kamijo et al. 2006; Miller and Bement 2009). Rho GTPases have also been shown to regulate cell cycle entry and cell cycle progression, in particular by regulating the expression of a number of genes involved in G₁/S transition, notably, CyclinD1 and p21^{waf1} (Villalonga and Ridley 2006).

2.3.3. Role of Rho GTPases in cytoskeleton reorganization

a. A historical perspective on the roles of Rho GTPases in cytoskeletal dynamics.

The first direct evidence of the involvement of Rho GTPases in the regulation of cytoskeletal dynamics came from two papers in 1992 (Ridley and Hall 1992; Ridley et al. 1992). These seminal works reported that microinjection of recombinant constitutively active mutants of Rac induced the dramatic formation of lamellipodia (large, flattened and ruffling protrusions) by regulating actin polymerization, while active mutants of Rho induced the formation of stress fibers and adhesion structures. Shortly after, it was found that activation of Cdc42 induced the formation of protrusive actin-rich filopodia (**Fig 2.6A**). Thus, for a long time the consensus in the field was that Rho, Rac and Cdc42 regulated three separate signal transduction pathways that linked plasma membrane receptors to the assembly of distinct structures of actin filaments (Etienne-Manneville and Hall 2002), which led to the following general view: In polarized cells, Rac1 and Cdc42 would only be active at the migration front where they would promote the formation of cell protrusions,

whereas RhoA would only be active at the back where it would control cell contraction. However, recent studies taking advantage of imaging techniques that allow the visualization of the time and location of Rho GTPase activation have shown that this model is too simple (reviewed in (Pertz 2010; Spiering and Hodgson 2011)). For instance, all three GTPases have been shown to be activated at the front of migrating cells, where RhoA has a role in the initial events of protrusion, whereas Rac1 and Cdc42 are involved in reinforcement and stabilization of newly expanded protrusions in randomly migrating fibroblasts (Nalbant et al. 2004; Shen et al. 2006; Machacek et al. 2009; Martin et al. 2016). Moreover, the nature of the spatiotemporal activation pattern seems to depend on the cellular context and the triggering factors, like the platelet-derived growth factor (PDGF) and the epidermal growth factor (EGF) (Bravo-Cordero et al. 2013).

Although the spatiotemporal coordination of the activity of Rho GTPases in morphogenic events like cell migration, axonal guidance, endocytosis and vesicle trafficking is still poorly characterized due to technical difficulties (Fritz and Pertz 2016); the various upstream and downstream pathways around Rho, Rac and Cdc42 and the biochemical processes that lead to actin fiber remodeling are better understood.

b. Rho GTPases effectors involved cytoskeleton reorganization.

Among the plethora of effectors of Rho, Rac and Cdc42 there are kinases and nucleation-promoting factors that are involved in the regulation of the cytoskeleton (**Fig. 2.6B**). For instance, different mDia isoforms can be activated upon binding active Rho, Rac or Cdc42 (Lammers et al. 2008). mDias are part of the Formin family of proteins and promote the formation of unbranched actin filaments. Cdc42 and Rac can also promote actin polymerization by activating WASP (Wiskott-Aldrich syndrome protein) and WAVE (Wiskott-Aldrich syndrome protein-family verprolin homologous protein), respectively. Active WASP and WAVE then promote the branching of actin filaments through the activation of the Arp2/3 complex (Symons et al. 1996; Kolluri et al. 1996; Miki et al. 2000; Millard et al. 2004). Furthermore, PAKs activated downstream of Rac and Cdc42, mediate the phosphorylation of LIM-motif containing kinase (LIMK), which in turn phosphorylates

and inhibits the actin filament-severing protein cofilin (Edwards et al. 1999). By severing actin filaments, cofilin creates free barbed and pointed ends which become available for polymerization or de-polymerization, thereby promoting actin-filament turnover (Maciver et al. 1998).

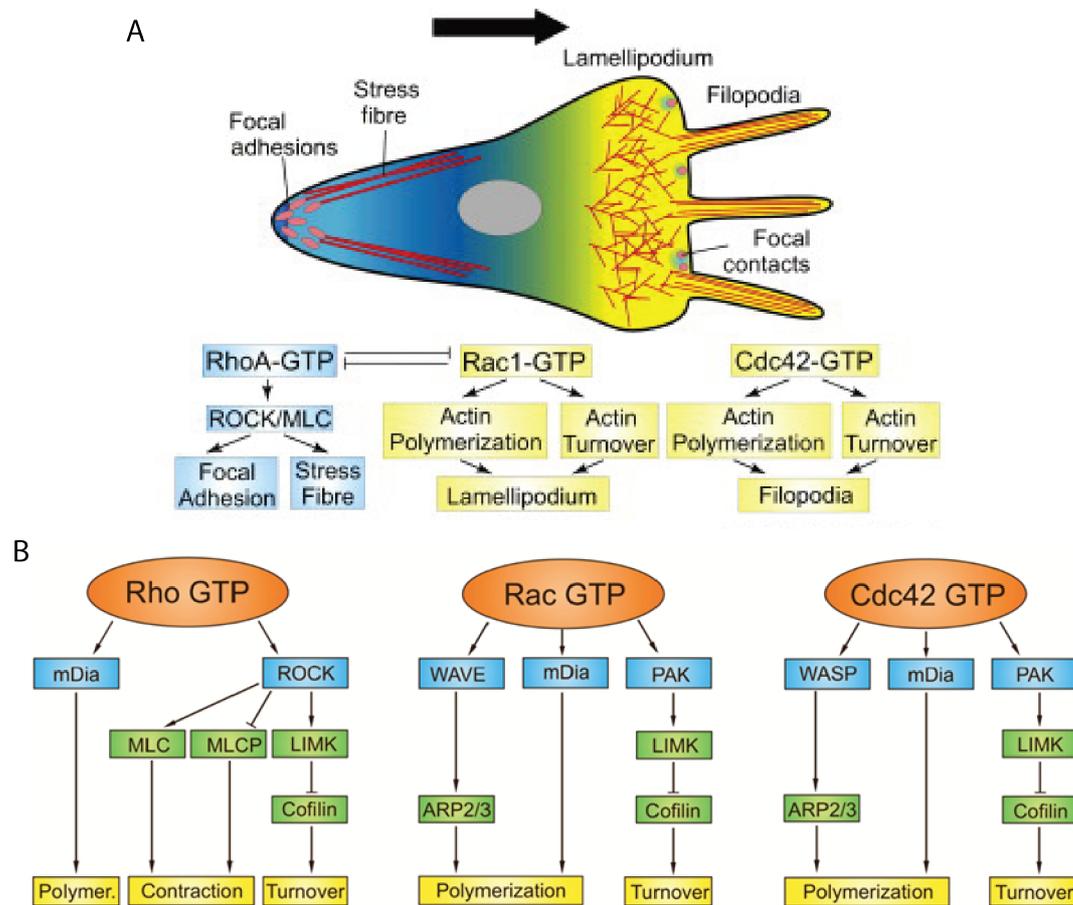


Figure 2.6. Rho, Rac and Cdc42 in cytoskeleton dynamics. **A.** Control of cell protrusion during migration; Rho mainly activates the formation of stress fibers and focal adhesions, Rac activates the formation of lamellipodia and membrane ruffles, and Cdc42 activates the formation of filopodia. **B.** Downstream effectors of Rho, Rac and Cdc42 involved in the generation of actin cytoskeletal structures. Adapted from (Mayor and Carmona-Fontaine 2010) and (Dráber et al. 2012).

Besides modulating the growth of actin filaments, Rho can promote myosin-actin interactions through ROCK, a kinase that phosphorylates a number of actin cytoskeleton regulators. Primarily it phosphorylates and inactivates myosin light chain (MLC) phosphatase (MLCP), thus increasing the levels of phosphorylated MLC (Swärd et al. 2000); although, other studies indicate that ROCK may also phosphorylate MLC directly

(Amano et al. 1996; Totsukawa et al. 2000). This increase of phosphorylated MLC contributes to actin filament contractility (Zaidel-Bar et al. 2015). Like PAK, ROCK has also been described to phosphorylate LIMK, thus promoting actin polymerization (Ohashi et al. 2000; Sumi et al. 2001; Katoh et al. 2001).

2.3.4. Role of Rho GTPases in adherens junctions

Intercellular adhesion is one of the cellular processes that heavily depends on the actin remodeling activity of Rho GTPases. It provides tissues with mechanical and functional integrity and constitutes barriers between distinct body compartments. In vertebrates, cell-cell adhesion is carried out by three specialized complexes: tight junctions (TJ), adherens junctions (AJ), and desmosomes (Gumbiner 1996). Although it is known that Rho GTPase signaling cross talks with all three adhesion complexes (Spindler and Waschke 2011; Citi et al. 2014; Komarova et al. 2017; Arnold et al. 2017), here I will focus on their role in AJ regulation.

a. The dynamic structure of adherens junctions

AJs are present in epithelial, endothelial and non-epithelial cells, such as cardiac myocytes, fibroblasts, and neurons. This type of junction is characterized by the presence of a transmembranal protein, which can be a member of the nectin family or of the classical cadherin family, which includes E-cadherin (expressed in epithelial cells), VE-cadherin (expressed in endothelial cells), and N-cadherin (expressed in neural and mesenchymal tissues). The extracellular domain (ectodomain) of cadherins can form calcium-dependent homotypic adhesive contacts with neighboring cells; while the intracellular domain of cadherins behaves like a scaffold for cytoplasmic proteins, such as β -catenin, α -catenin, p120-catenin, among many others (**Fig. 2.7**) (Ratheesh and Yap 2012; Mège and Ishiyama 2017).

AJs are connected to the actin cytoskeleton and their functionality depends on actin cytoskeleton dynamics (Ratheesh and Yap 2012; Mège and Ishiyama 2017). Cadherins are indirectly linked to F-actin via catenin proteins in a tension-dependent manner. Specifically, cadherins cytoplasmic tail binds to β -catenin, which in turn binds to α -catenin, a protein

that can bind simultaneously to the cadherin/catenin complex and to F-actin only under actomyosin-generated force. Indeed, recent studies have shown that α -catenin undergoes conformational changes upon being subjected to mechanical tension, thereby facilitating its binding to F-actin and the recruitment of Vinculin, an actin-binding protein, which further reinforces cell-cell adhesion and the linkage to F-actin under mechanical force (Yonemura et al. 2010; le Duc et al. 2010; Yao et al. 2014; Buckley et al. 2014).

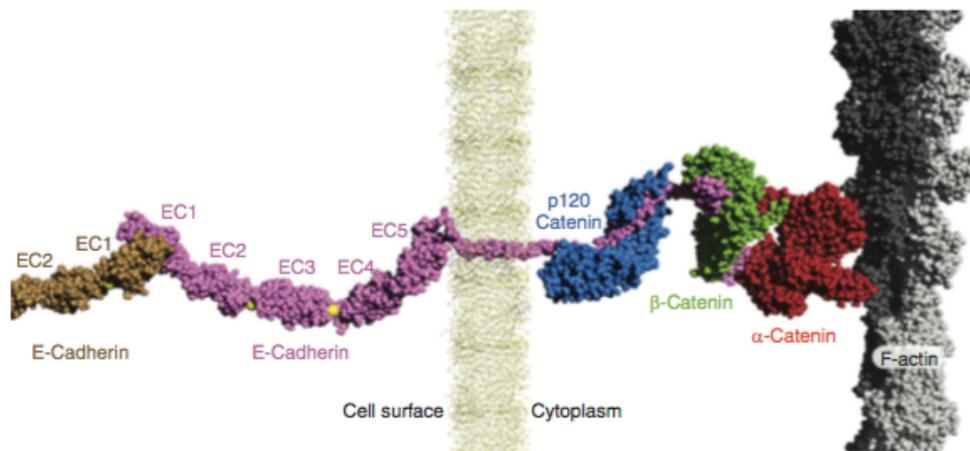


Figure 2.7. Structural model of the core E-cadherin/catenin cell adhesion complex. Ectodomains of E-cadherin cell adhesion receptors (orange and pink) from adjoining cells engage in Ca^{2+} -dependent extracellular strand-swap trans interaction. The cytoplasmic region of E-cadherin binds directly to β - and p120-catenins, and indirectly to α -catenin through β -catenin. α -catenin binds directly to F-actin or indirectly by associating with other F-actin-binding proteins, such as vinculin (not shown here).

The association of the cadherin/catenin complex to the actin cytoskeleton and actomyosin generated force are essential for the transition from nascent adhesions to the more stable mature junctions (Hansen et al. 2013; Buckley et al. 2014; Chen et al. 2015). While force on cadherin-based cell-cell junctions is necessary for their stabilization and proper function, excessive actomyosin-generated pulling force can lead to junction disassembly (reviewed in (Gomez et al. 2011)).

The modulation of intercellular junction integrity occurs physiologically during cell division, cell extrusion, and wound healing, which take place both during tissue development and

in adult tissues (Baum and Georgiou 2011). Notably, loss of adherens junctions occurs during epithelial-to-mesenchymal transition (EMT), a key feature of embryogenesis and also one of the stepping stones to tumor metastasis. During EMT, epithelial cells lose their polarity as well as their cellular junctions, commonly due to the transcriptional repression of E-cadherin expression (Lamouille et al. 2014).

The dynamic nature of cell-cell junctions is evident during their assembly, maturation, and maintenance, and is linked to the remodeling of the cytoskeleton, which is substantially orchestrated by the careful control of Rho GTPases (Menke and Giehl 2012; Citi et al. 2014; van Buul and Timmerman 2016). In the context of AJ regulation, RhoA, Rac1 and Cdc42 are, so far, the best-characterized Rho GTPases.

b. Rho GTPases in epithelial AJ Assembly and Maturation

In epithelial cells, *de-novo* cell-cell junction formation occurs after migrating cells make contact with one another by waves of protruding and retracting lamellipodia (Yonemura et al. 1995). Yamada and colleagues performed a study on the localization of active RhoA and Rac1 during junction formation using high resolution live-cell imaging with a FRET-based biosensor; they demonstrated that Rac1 activity is high at the periphery of contacting membranes and triggers the initiation of cell-cell adhesion, while active RhoA was observed at the contact edges at later time points, and was required to drive the expansion and completion of the epithelial cell-cell junction (Yamada and Nelson 2007). Indeed, it has been shown that following engagement of E-cadherin at primordial junctions, Rac1 and Cdc42 promote the polymerization of branched actin filaments in lamellipodia and filopodia (in the case of Rac1, through activation of the Arp2/3 complex by WAVE2), which extends the interface between the two adjacent cells and stimulates cadherin ligation (Vasioukhin et al. 2000; Verma et al. 2004; Yamazaki et al. 2007; Samarin and Nusrat 2009). As junctions mature, RhoA plays a fundamental role through mDia and ROCK to convert the branched actin network into contractile actin filaments (Citi et al. 2014; Arnold et al. 2017).

c. Rho GTPases in epithelial AJ integrity and maintenance

In addition to their role in epithelial junction assembly and maturation, the activity of Rho GTPases is required once junctions are mature, and play a role in the maintenance of homeostatic junction architecture and tension (Citi et al. 2014). Most studies report a positive effect of Rac1 and Cdc42 activity in the stability of mature AJs (reviewed in (Mack and Georgiou 2014)). For instance, it has been reported that Rac1- and Cdc42-dependent Arp2/3 activity is required to maintain mature AJs (Brieher and Yap 2013). Also, it has been shown that active Cdc42 and Rac1 bind to their effector IQGAP, which impedes IQGAP inhibitory interaction with β -catenin, thereby stabilizing AJs (Kuroda et al. 1998; Fukata et al. 1999). Moreover, Tiam1-Rac1 activity seems to restore AJs and an epithelial morphology in several cell types (Hordijk et al. 1997; Malliri et al. 2004), and it has been shown that Tiam1 degradation at AJs is required for Src- induced AJ disassembly (Woodcock et al. 2009). On the other hand, several studies have shown that increased Rac signaling can disrupt AJs under particular physiological situations (Mack and Georgiou 2014). For instance, during HGF (Hepatocyte growth factor)-induced cell scattering (Potempa and Ridley 1998; Shintani et al. 2006) and during tumorigenesis (Menke and Giehl 2012). Interestingly, the loss of cell-cell junctions following an increase of Rac1 activity is often related to the development of EMT (Yagi et al. 2007; Zhou et al. 2016).

Concerning RhoA, limitation of its activity at AJs has been suggested to be important for their stabilization since excessive RhoA activation leads to the strong generation of actomyosin pulling force and the consequential disruption of AJs (Sahai and Marshall 2002; Chang et al. 2006; Holeiter et al. 2012; Lee et al. 2016). Therefore, RhoA activity must be tightly controlled and is downregulated partly by the p120-dependent recruitment of p190RhoGAP to AJ, and by the antagonism between RhoA and Rac1 (Wildenberg et al. 2006; Ratheesh et al. 2012). Conversely, RhoA signaling can also help stabilize AJs; it has been shown that RhoA helps to maintain AJs via Dia1 (Sahai and Marshall 2002) and non-muscle myosin II (Shewan et al. 2005; Smutny et al. 2010). Moreover, the Rho GEF TEM4 has been found to regulate AJ integrity by associating with the cadherin-catenin complex (Ngok et al. 2013).

2.4. Rho GTPases and pathology.

The essential role of Rho GTPases is evidenced by the fatal fate of mice whose Rac1 or Cdc42 genes have been deleted (they die in the early stages of embryonal development) (Sugihara et al. 1998). Given the central role of Rho GTPases in cellular homeostasis, it is not surprising that their dysregulation has been found related to the development of an array of pathological phenotypes, such as immunodeficiency syndromes, neurological diseases, cancer, and bacterial infections (Boettner and Van Aelst 2002; DeGeer and Lamarche-Vane 2013; Lemichez and Aktories 2013; Orgaz et al. 2014; Bai et al. 2015).

2.4.1. Rho GTPases in neurological diseases

Given the dynamic nature of the neuronal cytoskeleton, precise spatial and temporal regulation of Rho family GTPases has been shown to be indispensable in nerve cell function, from neuronal specification and polarization to axon guidance, survival and nerve growth (Govek et al. 2005; Stankiewicz and Linseman 2014). Accordingly, an increasing number of studies suggest that de-regulation of Rho GTPase signaling is associated with a number of neuropsychiatric and neurodegenerative diseases like Amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease, Parkinson's disease, glaucoma, and Charcot-Marie-tooth disease (reviewed in (DeGeer and Lamarche-Vane 2013)); and efforts are being made to understand the role of Rho GEFs and GAPs in such diseases in order to identify new specific therapeutic targets (Bai et al. 2015).

2.4.2. Rho GTPases and cancer

a. Aberrant regulation of Rho-GTPase activity in cancer

Rho GTPase signaling is deregulated in cancer by a wide range of mechanisms, some of which target their regulators. For instance, GEFs are commonly overexpressed in various cancer types, and negative regulators of Rho GTPases such as Rho GAPs and Rho GDIs have been shown to act as tumor suppressors and are frequently lost in human cancers (Vigil et al. 2010; Barrio-Real and Kazanietz 2012). In contrast, other mechanisms target the Rho GTPases directly. For example, Rho GTPases can be overexpressed in human

cancers and are associated with aggressiveness and poor patient survival (with the curious exception of RhoB, which seems to act as a tumor suppressor) (Orgaz et al. 2014; Ji et al. 2015); additionally, Rac1b, a constitutively active splice variant of Rac1, has been reported in colorectal (Jordan et al. 1999), breast (Schnelzer et al. 2000), lung (Liu et al. 2012), thyroid (Silva et al. 2013), and pancreatic (Mehner et al. 2014) cancers.

While early studies identified recurrent chromosomal alterations involving the RhoH-encoding gene in patients with hematopoietic malignancies (Preudhomme et al. 2000; Pasqualucci et al. 2001), missense mutations within Rho GTPases were thought to be rare in cancer. It was only with the advent of large scale sequencing that sporadic mutations in all 20 Rho GTPases were identified in a large variety of cancers (Olson 2016). Most mutations have been reported in Rac1 and RhoA. Indeed, a whole-exome sequencing study performed in melanoma samples revealed that 5% of them were found to harbor missense mutations in the Rac1 gene, making Rac1 the third most highly mutated gene in melanoma after BRaf and NRas (Hodis et al. 2012).

One of the most common mutations of Rac1 in sun-exposed melanomas is the P29S substitution. P29S is a gain-of-function mutation that still allows Rac1 to hydrolyze GTP (contrary to the GTPase-dead constitutively active Q61L mutation). Structural analysis of this mutant indicates that the P29S substitution alters switch 1 conformation, destabilizes the GDP-bound state, and stabilizes the GTP-bound form of Rac1, which agrees with biochemical assays showing that Rac1(P29S) binds to a greater extent to its effectors compared to Rac1(WT) (Hodis et al. 2012; Krauthammer et al. 2012). P29 seems to be a hotspot for Rac1 mutations and, in addition to melanoma, it has been detected in cases of head and neck squamous cell carcinoma (Stransky et al. 2011), as well as in breast cancer cell lines (Kawazu et al. 2013). Additional activating Rac1 mutations have been identified in various cancer cell lines (N92I and C157Y), each of which were found to increase spontaneous GDP release to allow rapid GDP/GTP cycling that increases signal output (Kawazu et al. 2013).

b. Rho GTPases in cancer cell biology

In accordance to their central role in cell signaling, deregulation of Rho GTPases has been shown to be involved in all stages of cancer progression and is linked to many of the “hallmarks of cancer”, including oncogenic transformation, cell survival, tumor metabolism as well as metastasis (reviewed in (Orgaz et al. 2014) and (Vega and Ridley 2008)).

The deregulation of Rho GTPases is required for Ras GTPase-mediated oncogenesis and also for aberrant growth induced by other oncoproteins. Additionally, Rho GTPases have been associated with either promotion or inhibition of tumor suppressors, which seems to depend on cell type, and stage of tumor progression (Orgaz et al. 2014). For instance, overexpression of the tumor suppressor Merlin blocks Rac1-induced transformation, since Merlin deficiency enhances Rac1 activity and aberrant cell growth. Conversely, Rac1 has been shown to promote Merlin inactivation by inducing its phosphorylation (Shaw et al. 2001; Sherman and Gutmann 2001).

In addition to their capacity to modulate oncogenes and tumor suppressors, it has been shown that Cdc42, Rac1 and RhoC contribute to cell transformation by altering and enhancing the glutamine metabolism used by cancer cells to sustain their exacerbated proliferation rates (Wang et al. 2010b). Moreover, Rho GTPases have been reported to promote the formation of new blood vessels by promoting the production and secretion of angiogenic factors (Bryan and D'Amore 2007). Finally, Rho GTPase activity has been shown to contribute to the production of proinflammatory cytokines and the development of chronic inflammation, which is involved in cancer initiation and progression. (Vega and Ridley 2008).

2.4.3. Rho GTPases and bacterial infection.

Rho GTPases are involved in the host immune defenses in response to bacterial pathogens (Stuart et al. 2013) and, in accordance, many pathogens produce virulence factors, which exploit and/or impair diverse aspects of Rho protein activity and signaling. The molecular mechanisms of action of these effectors are diverse (Aktories 2011); some mimic the activity of Rho GTPase regulators (GEFs, GAPs, GDI) while others directly target the GTPase.

The effectors that directly targets Rho GTPases possesses enzymatic activities, which allows them to modify key functional residues of Rho GTPases by catalyzing chemical modifications, such as glucosylation, adenylation and deamidation (Visvikis et al. 2010; Lemichez and Aktories 2013). An example of this type of effector that has been studied and used extensively by our research group is the Cytotoxic necrotizing factor 1 (CNF1) toxin, a virulence factor produced by some pathogenic strains of *Escherichia coli* that has the capacity to translocate its catalytic domain into the host cell cytosol (Lemichez et al. 1997; Knust et al. 2009), where it binds members of the Rho GTPase family and deamidates a glutamine residue that is essential for GTPase activity into a glutamate residue (Q63 in RhoA or Q61 in Rac1 and Cdc42), thereby locking the Rho-family protein in the active GTP-bound state (Flatau et al. 1997; Schmidt et al. 1997). Further examples of these factors and their effect on the Rho GTPase cycle are shown in figure 2.8.

The modulation of Rho GTPase signaling by pathogens has been reported to disrupt host epithelial/endothelial barriers, hinder immune cell phagocytic functions, facilitate the invasion of epithelial cells by pathogenic bacteria and enable them to replicate, form intracellular reservoirs and disseminate through different tissues (Lemichez and Aktories 2013).

Moreover, toxins that modulate Rho GTPase activity have been remarkably useful to understand the diverse cellular roles of Rho GTPases and to reveal novel cellular regulatory mechanisms of these GTPases. It was, after all, through the study of the effect of the CNF1 toxin in Rho GTPases that their regulation by the ubiquitin and proteasome system was first demonstrated (Lerm et al. 2002; Doye et al. 2002).

In addition to their usefulness in basic research, several studies have indicated that toxins targeting Rho proteins have a great potential for medical applications applied to pathologies where the function of Rho GTPases is known to play a key role, such as neurological diseases (Lemichez and Aktories 2013). For instance, studies from our team have indicated that the CNF1 toxin is a potential mucosal immunoadjuvant for

prophylactic or therapeutic vaccines against intracellular pathogens (Munro et al. 2005; Michel et al. 2016).

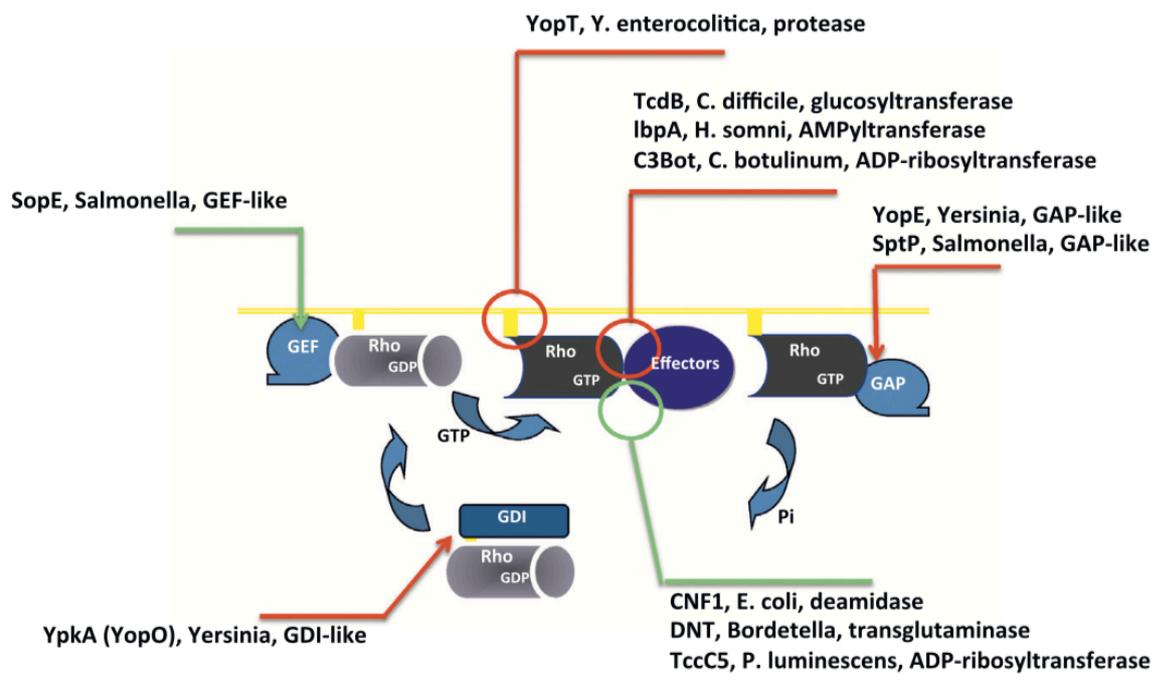


Figure 2.8. Selected examples of bacterial virulence factors targeting various stages of Rho protein regulation. Bacterial factors target all of the key steps of GTPase regulation for activation (green) or inactivation (orange). Modulation of Rho activity can occur by mimicry of cellular GEF, GAP or GDI factors (e.g., SopE, YopE, YpkA, respectively). Other strategies consist of catalyzing direct post-translational modifications (PTM) on Rho. These PTMs include activating the key glutamine residue of the switch I domain (Q63 for RhoA) by ADP-ribosylation (TccC5), transglutamination (DNT) or deamidation (CNF1, converting Q63 into E63 in RhoA). Key residues of the switch II domain are also subjected to inactivating PTMs, e.g., ADP-ribosylation (C3bot, N41 of RhoA, B, C members), AMPylation (LbpA, Y34 for RhoA) or the addition of sugar groups (TcdA/B, T37 for RhoA). Taken from (Lemichez and Aktories 2013)

Ubiquitination of Rho GTPases: Rac1 and its E3 ligase HACE1

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e. Cell death (apoptosis and necroptosis)	81

3.1. Regulation of Rho GTPases by ubiquitination

The first demonstration that Rho GTPases were ubiquitinated and addressed to the ubiquitin proteasome system (UPS) came from our lab in 2002, from the study of the Rho GTPase-activating effect of the CNF1 toxin (Doye et al. 2002). In this work, we showed that despite the constitutive activation of Rac1, Cdc42 and RhoA by CNF1, the active level of these GTPases did not remain constant but reached a peak at 4 to 6 hours after CNF1 intoxication and then rapidly diminished. This compensatory cellular response was found to be mediated by ubiquitination and proteasomal degradation. Since then, it has been reported that the ubiquitination of Rho GTPases can be modulated by its regulatory proteins. For instance, increased activity of Rho GEFs have been shown to promote the

ubiquitination of members of the Rho family (Doye et al. 2002), and it has been found that RhoGDI-bound GTPases are protected from proteasomal degradation (Boulter et al. 2010). These findings show that regulation of Rho GTPases by ubiquitination is closely interweaved with the classical cellular mechanisms that regulate Rho GTPase signaling.

Currently, 15 of the 20 Rho family members existing in humans are reported to be ubiquitinated in the PhosphoSitePlus resource (Hornbeck et al. 2015), indicating that ubiquitination is a common mechanism of regulation. However, only the ubiquitination of RhoA and Rac1 are currently characterized in detail. In this section I will briefly describe what is known about RhoA ubiquitination and then focus on Rac1 ubiquitination.

3.1.1. Ubiquitination of RhoA

The ubiquitination of RhoA is the best described among the Rho family. Several laboratories (ours included) have shown that RhoA is ubiquitinated by the HECT-type E3 ligase Smurf1. Moreover, recent studies have suggested that some E3 Cullin-RING ligases (CRL) are also able to induce RhoA ubiquitination, namely, $CUL3^{BACURD}$, SCF^{FBXL19} , and SCF^{Fwb7} (Hodge and Ridley 2016).

SMURF1 (SMAD-specific E3 ubiquitin protein ligase 1) was the first E3 ubiquitin ligase identified to target a member of the RhoGTPases (Wang et al. 2003). SMURF1 is a Nedd4-like E3 ubiquitin ligase involved in the regulation of TGF- β signaling. This E3 ubiquitin ligase is able to bind RhoA in a guanine nucleotide-independent manner (Ozdamar et al. 2005). However, whether Smurf1 ubiquitinates all forms of RhoA or whether it is specific to the GDP- or GTP- bound state is still a controversial issue (Wang et al. 2003; Ozdamar et al. 2005; Boyer et al. 2006). The Rac1 and Cdc42 effector complex PAR6/ PKC ζ recruits SMURF1 to the leading edge of migrating cells, where it ubiquitinates RhoA at lysines 6 and 7, which suggests a mechanism for the site-specific degradation of RhoA (Wang et al. 2003; Ozdamar et al. 2005). This degradation inhibits the RhoA-mediated formation of stress fibers at the leading edge, giving way to Rac-driven protrusion of lamellipodia. Also, it has been shown that upregulation of SMURF1 expression reduces the levels of RhoA and stimulates cancer cell migration, invasion and metastasis (Yu et al. 2015), but whether

this reduction occurs at a specific location or is general is still unclear. Interestingly, phosphorylation of RhoA has been shown to modulate its SMURF1-dependent ubiquitination. During axon extension, RhoA phosphorylation promotes its ubiquitination by SMURF1 (Cheng et al. 2011; Deglincerti et al. 2015), while our team has shown that phosphorylation of RhoA on Ser-188 promotes its binding to RhoGDI and protects RhoA from proteasomal degradation in vascular smooth muscle cells (Rolli-Derkinderen et al. 2005).

Out of the three CRL ligases that have been reported to ubiquitinate RhoA, the BTB/POZ domain-containing adaptor for CUL3-mediated RhoA degradation (BACURD)-CUL3-RING ubiquitin ligase complex (CUL3^{BACURD}) is the best-supported. This CRL from the C3RL subfamily is composed of the BACURD adaptor protein, the Cullin3 scaffold and the Rbx E2-binding protein. BACURD binds Cullin3 through its BTB and interacts with RhoA through its POZ domain to selectively ubiquitinate RhoA (Chen et al. 2009). Depletion of CUL3 or BACURD has been shown to stimulate formation of actin stress fibers by increasing RhoA levels (Chen et al. 2009; Ibeawuchi et al. 2015). The two other CRL ligases that have been reported to mediate RhoA ubiquitination are: (i) The SCF ligase coupled with the F-box protein FBXL19 (SCF^{FBXL19}), which has been shown to induce the ubiquitination of RhoA on Lys-135 in lung cells following ERK2-mediated phosphorylation of RhoA (Wei et al. 2013); and (ii) the SCF ligase coupled with the F-box protein FBW7 (SCF^{FBW7}), which has been shown to regulate the ubiquitination and proteasomal degradation of RhoA in gastric cancer cells (Li et al. 2016).

3.1.2. Ubiquitination of Rac1.

The degradation of Rac1 by the UPS has been consistently reported to occur after Rac1 sustained activation. It can be mediated either by bacterial effectors, such as the CNF1 toxin from *E. coli* and the dermonecrotic toxin (DNT) from *Bordetella* (Doye et al. 2002; Munro et al. 2005); by physiological factors, such as hepatocyte growth factor (HGF) (Lynch et al. 2006); by the sustained presence of Dbl-exchange factors; or by Rac1 point mutations (Q61L, G12V) (Doye et al. 2002). We have shown that the ubiquitination of

Rac1 activated by the Q61L point mutation mainly involves its Lys-147 (Visvikis et al. 2008). In 2011, our team identified the first E3-ubiquitin ligase that directly targets Rac1 for degradation: the HECT domain E3 ubiquitin ligase HACE1 (Torrino et al. 2011). In addition to HACE1, three ligases of the RING family have been reported to mediate Rac1 ubiquitination: XIAP, cIAP1 and the SCF^{FBXL19} complex.

In a seminal study, our team found that the HECT-domain and Ankyrin-repeat containing E3 ubiquitin protein ligase 1 (HACE1) binds to and ubiquitinates specifically GTP-loaded Rac1. This binding and subsequent targeting is induced by CNF1-intoxication, activation of the GTPase by point mutations or by expression of the GEF-domain of Dbl (Torrino et al. 2011). Since then, other groups have confirmed and expanded these observations and identified lys-147 as the residue of Rac1 that is ubiquitinated by HACE1 (Castillo-Lluva et al. 2012; Daugaard et al. 2013; Goka and Lippman 2015). The details and implications of HACE1-mediated regulation of Rac1, as well as other properties of this E3 ubiquitin ligase will be developed in section 3.2.

Oberoi and colleagues have reported that the inhibitor of apoptosis (IAP) proteins X-linked IAP (XIAP) and cellular IAP1 (cIAP1) bind to GTP- and GDP-bound Rac1 and promote its polyubiquitination at Lys-147, leading to Rac1 proteasomal degradation (Oberoi et al. 2012; Oberoi-Khanuja and Rajalingam 2012). They observed that depletion of cIAP1 and XIAP resulted in hyper-activation of Rac1, leading to an elongated morphology and enhanced cell migration in both normal and tumor cells (Oberoi et al. 2012). However, other groups have described instead a reduction of cancer cell migration following IAPs downregulation (reviewed by (Orme et al. 2012)) questioning the results found by Oberoi et al. More recently, it has been suggested that SCF^{FBXL19} targets Rac1 and Rac3 for ubiquitination, in addition to the previously reported RhoA, on Lys-66 (Zhao et al. 2013; Dong et al. 2014). SCF^{FBXL19} targets Rac1 in a guanine nucleotide-independent manner and requires phosphorylation of Rac1 on Ser-71 by AKT (Zhao et al. 2013).

3.2. HACE1 E3 ubiquitin ligase

3.2.1 Brief historical context and association with human diseases

The *Hace1* gene was first identified in 2004 by the group of Dr. Sorensen just ~50kb downstream of a translocation breakpoint in the chromosome 6q21 present in a patient with Wilms' tumor, a pediatric renal tumor (Anglesio et al. 2004). In this seminal paper, HACE1 was shown to be a functionally active E3 protein ligase with a catalytic HECT domain at its C-terminus and a series of Ankyrin repeats at its N-terminus (initially thought to be six but more recently described to be seven (Andrio et al. 2017)), that mediate protein-protein interactions (Li et al. 2006).



Figure 3.1. Representation of HACE1 domain organization. HACE1 is a 909 residue-E3 ubiquitin ligase with 7 N-terminal Ankyrin repeats, thought to mediate protein-protein interactions, and a C-terminal HECT catalytic domain. The position of the catalytic cysteine (*Cys876) within the HECT domain is indicated.

HACE1 is widely expressed in human tissues, including mature and fetal kidney, skeletal muscle, liver and ovaries. It is relatively highly expressed in the brain, placenta, thymus, prostate and peripheral blood leukocytes (Anglesio et al. 2004; Zhang et al. 2007).

HACE1 was initially classified as a tumor suppressor gene by the team of Dr. Sorensen. They pointed out that the *hace1* gene locus is within a region previously described as a hotspot of deletions or loss of heterozygosity in multiple human tumors. In accordance with this, they observed that HACE1 mRNA expression was very often repressed in primary Wilms' tumors (Anglesio et al. 2004). This work was later expanded in collaboration with the team of Dr. Penninger, where they show that HACE1 is not only repressed in Wilms' tumors but also in several other human cancers, including mammary, hepatic, gastric, ovarian and prostate cancers (Zhang et al. 2007). Independent studies

have genetically linked reduced *hace1* expression to various types of cancer, including neuroblastoma, lung, stomach, mammary and liver cancer (Hibi et al. 2008; Sakata et al. 2009; Diskin et al. 2012; Liu et al. 2014; Goka and Lippman 2015)

In agreement with the genetic association studies in humans, *hace1*-KO mice generated by the team of Dr. Sorensen show a tendency to spontaneously form late-onset tumors originated from all of the three germ layers, resulting in the development of different carcinomas, lymphomas and sarcomas (Zhang et al. 2007). Their work has also shown that loss of HACE1 makes mice highly susceptible to environmental stress (like exposure to DNA alkylating agents and gamma irradiation) and secondary genetic insults, such as loss of another tumor suppressor like p53. Under this kind of environmental and genetic stress, *hace1*-KO mice present a significantly worse phenotype compared to control mice: they develop more aggressive tumors and also present more types of malignancies than the ones that normally arise in HACE1-expressing mice (Zhang et al. 2007).

In addition to generating and characterizing their murine model, Zhang and colleagues showed that HACE1 expression in human cancer cells suppresses cellular proliferation and hinders their ability to form tumors *in vivo*. These authors showed that HACE1 controls cellular proliferation by reducing CyclinD1 protein stability in an E3 ligase-dependent manner. However, they did not find any evidence that HACE1 targets CyclinD1. It was not until 2011, when our group identified GTP-bound Rac1 as the first target of HACE1 (Torrino et al. 2011), that the signaling mechanisms of HACE1 tumor suppressor activity were further elucidated and a connection between Rac1, HACE1 and the control of cyclin-D1 stability was drawn. As shown in Chapter 2, Rac1 is an essential protein whose deregulation is known to play a role in the development of cancer. Accordingly, subsequent studies have found that HACE1's role in repressing cell migration in response to HGF depends on Rac1 ubiquitination (Castillo-Lluya et al. 2012). Moreover, HACE1 has been shown to repress cellular migration, invasion and proliferation in human mammary epithelial cells and hinders breast cancer progression in HER2/Neu expressing cells by regulating cellular levels of GTP-Rac1 (Goka and Lippman 2015).

hace1-KO mice generated by Zhang and colleagues were born at the expected time and were fertile. They looked normal, showed no signs of developmental problems and were able to reach old age (2 years) despite their propensity to develop late-onset tumors (Zhang et al. 2007). However, in the last couple of years, there have been studies associating the genetic alterations in *hace1* with developmental diseases, both in the animal model *Xenopus laevis* (Iimura et al. 2016) and in humans (Akawi et al. 2015; Hollstein et al. 2015). These last two studies in humans identified putative loss-of-function variants in HACE1, including biallelic missense mutations, and frameshift mutations that were predicted to result in the truncation of HACE1 before or within its HECT domain, or to perturb the HECT domain folding. These mutations were associated with developmental autosomal recessive disorders in individuals from different countries. The affected individuals presented a series of common symptoms: intellectual disability, brain atrophy, abnormal gait and ocular abnormalities. The authors of these studies hypothesized that HACE1's role during development might be mediated by the deregulation of Rac1 activity, since it is known that Rac1 plays a role in developmental processes, such as photoreceptor morphogenesis and cerebellar development. This hypothesis is congruent with the fact that knockdown of Rac1 partially rescues the defects in early embryonic development observed in *Xenopus laevis* lacking HACE1, suggesting that indeed, HACE1's role in development might be mediated by modulation of Rac1 activity (Iimura et al. 2016).

Collectively, these studies indicate that HACE1 plays an important role in maintaining cell and organism homeostasis, as evidenced by its tumor suppressor activity and role in early development. Whether HACE1's ability to regulate Rac1 activity is the only molecular mechanism mediating such diverse effects, or whether there is a contributing role of other known or yet uncharacterized targets and partners of HACE1 is currently a subject of much interest.

3.2.2. Targets of HACE1: Rac1 and beyond

Our team identified the GTP-bound form of Rac1 as the first target of the catalytic activity of HACE1, and since then seven other targets have been proposed, expanding HACE1's

role in cell signaling and shedding light on its remarkable capability to conjugate different kinds of ubiquitin chains in a substrate-specific manner. In this section I will go briefly through these targets starting with the most recently identified, so that the end of the section will be dedicated to the earliest and most-studied target of HACE1: Rac1.

A recent study has suggested that HACE1 directly mediates K63-linked ubiquitination of TRAF2 *in vivo* and *in vitro*. Since TRAF2 is a RING E3 ubiquitin ligase that plays a role in the induction of NF- κ B activation and in the inhibition of necroptosis downstream the tumor necrosis factor receptor 1 (TNFR1), the authors suggested that HACE1 is a critical regulator of TNFR1-mediated cell fate (Tortola et al. 2016a). The same year, Huang and colleagues demonstrated that HACE1 monoubiquitinates Syntaxin5 (Syn5), a SNARE protein involved in Golgi reassembly, at Lys-270. The authors showed that ubiquitinated Syn5 has a lower affinity for HACE1 than non-modified Syn5, which gives a clue as to why HACE1 transfers a single ubiquitin molecule to Syn5. Interestingly, the monoubiquitination of Syn5 by HACE1 occurs early during mitosis, and is reversed by the action of the DUB VCIP135 during the late stages of mitosis. This gives an insight into a temporal and/or spatial modulation of HACE1 activity (Huang et al. 2016).

Another recently described target of HACE1 is the Y-box-binding protein 1 (YB-1). YB-1 is a DNA- and RNA-binding protein involved in the regulation of gene transcription, mRNA stability, protein translation, splicing and DNA repair. Moreover, there is evidence that YB-1 is secreted, but it is unclear through which mechanisms and for what purpose this happens. Studying this aspect, Palicharla and Maddika have found that HACE1 modifies YB-1 with K27-linked polyubiquitin, giving YB-1 a new surface of interaction that is necessary for its association with the ESCRT-1 complex and its secretion through the multivesicular bodies pathway (Palicharla and Maddika 2015).

In 2014, four more targets for HACE1 were proposed. Three of them are members of the Rab GTPase family and were identified in the same study published by Lachance and colleagues. This study suggested that the β 2-Adrenergic receptor (β 2AR) mediates HACE1 interaction with Rab11a, Rab6a, and Rab8a and facilitates their ubiquitination (Lachance

et al. 2014). The fourth target has been reported by Liu and colleagues in a study that showed that HACE1 binds and targets the autophagy receptor Optineurin (OPTN), a protein genetically implicated in glaucoma, amyotrophic lateral sclerosis (ALS) and Paget disease. In this study, the authors showed that HACE1 binds to OPTN through its N-terminal Ankyrin repeats and conjugates K27- and K48-linked ubiquitin chains on OPTN's Lys-193 in cells (and on 12 other lysines *in vitro*). This ubiquitination promotes the interaction between OPTN and p62 leading to an accelerated autophagic flux, which in turn lowers cellular ROS levels and mitigates oxidative DNA damage (Liu et al. 2014). A study currently under review from our team confirmed that HACE1 and OPTN interact. However, though this interaction seems to be important for HACE1 function towards Rac1, we did not observe that OPTN ubiquitination was dependent on HACE1 activity (Hamaoui et al, under review).

As discussed in the previous section, Rac1 in its GTP-bound form is to date the best described target of HACE1 (Torrino et al. 2011; Castillo-Lluva et al. 2012; Daugaard et al. 2013; Goka and Lippman 2015). HACE1 targets Rac1 after it is activated in response to several stimuli such as CNF1-intoxication, Rac1 point mutations (Q61L, Q61E or G12V), the expression of the GEF-domain of Dbl (Torrino et al. 2011), or by stimulation with growth factors that are known to activate Rac1 like: HGF (Hepatocyte growth factor) (Castillo-Lluva et al. 2012), EGF (Epidermal growth factor) or HRG (Heregulin) (Goka and Lippman 2015). Altogether, these findings demonstrate that the regulation of Rac1 by HACE1 is a general and constitutive mechanism to modulate the levels of active Rac1 in the cell.

HACE1 has been shown to ubiquitinate Rac1 at the Lys-147 *in vitro* and in cells in response to HGF (Castillo-Lluva et al. 2012) and EGF (Goka and Lippman 2015).

We have seen before that HACE1 is able to conjugate on its substrates a single ubiquitin peptide or different types of ubiquitin chains (K63-, K48-, and K27-linked chains). However, the type of ubiquitin chains that HACE1 attaches to Rac1 has not been completely elucidated. It is known that the expression of a K48R-ubiquitin mutant partially dampens

the polyubiquitination profile of active Rac1 (Q61L mutant) above the size of 72kDa in control and HACE1 expressing cells (Torrino et al. 2011). This indicates that HACE1 does transfer K48-linked ubiquitin chains (with 6 or more ubiquitin moieties) to Rac1. However, it seems it is not the only type of chain that HACE1 can catalyze on Rac1 and further studies are necessary to dissect which other types of ubiquitin chains can be crosslinked on Rac1 and what their impact is on Rac1-dependent signaling.

A characteristic that sets apart HACE1 from other E3 ubiquitin ligases that target Rac1 is that it preferentially binds and targets GTP-bound Rac1. A recent study from our group showed structural aspects of HACE1 that regulate this specificity (Andrio et al. 2017). This work established that a specific surface encompassing amino acids located in Ankyrin repeats 5 to 7 of HACE1 is critical for its binding to Rac1 (**Fig. 3.2**). Moreover, it demonstrated that the MID region cooperates with the Ankyrin domain in HACE1 to confer specificity of association to the active form of Rac1. Finally, through the study of the effect of mutants of these critical residues in HACE1 on Rac1 ubiquitination (some of which are listed in the Catalogue of Somatic Mutations in Cancer), we also demonstrated that the efficiency of association of HACE1 with Rac1 correlates with the efficiency of Rac1 ubiquitination. All of which suggests that HACE1's capacity to target specific substrates can be modulated at the protein level and that it is a mechanism, apart from genetic inactivation, by which cancer cells can modulate HACE1 function.

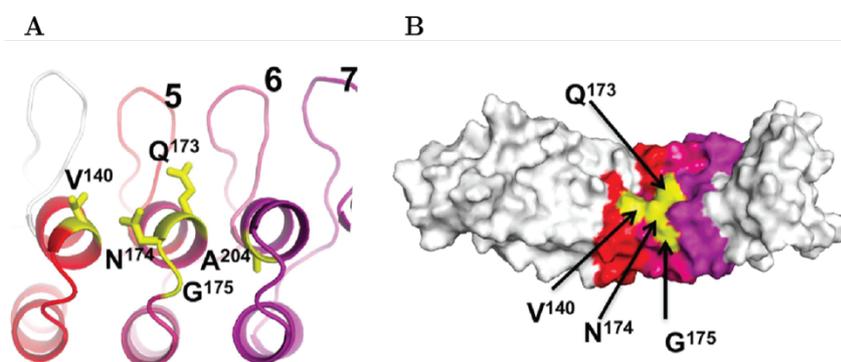


Figure 3.2. 3D model of HACE1 Ankyrin repeats important for its interaction with Rac1. **A.** Ribbon diagram of HACE1 Ankyrin repeats 5, 6 and 7 depicting surface-located amino acids V140, Q173, N174, G175. The alanine in position 204 is located on the internal part of the helix. **B.** Surface representation of the same Ankyrin repeats in HACE1, depicting the cluster of residues controlling

Rac1 binding and ubiquitination (yellow) at the surface of the repeats 5, 6 and 7. Taken from (Andrio et al. 2017)

As seen in the previous section, HACE1 targets proteins that are located at different subcellular sites, suggesting that HACE1 activity is spatially regulated. However, evidence of HACE1 intra-cellular localization is rather sparse. Endogenous HACE1 has been shown to co-localize with Grasp65 and other Golgi markers by immunofluorescence and cellular fractionation (Tang et al. 2011). These authors found that exogenously expressed HACE1 wild type, but not the catalytic inactive mutant C876S, still co-localizes with Golgi markers but it is also found (in big quantities) spread in the cytosol. This observation is in accordance with other studies that have detected exogenously expressed HACE1 mainly in the cytosol and in the perinuclear region (Anglesio et al. 2004; Torrino et al. 2011). In conditions where HACE1 is over-expressed and Rac1 activation is induced by point mutations or stimulation with HGF, HACE1 is still mainly cytosolic but a small part co-localizes with Rac1 at the cell periphery and in membrane ruffles (Torrino et al. 2011; Castillo-Lluva et al. 2012). Contrary to HACE1 localization in the Golgi, co-localization of active Rac1 with HACE1 at the cell membrane does not require HACE1 catalytic activity. Nevertheless, cellular fractionation assays have shown that overexpression of HACE1 exclusively promotes the degradation of the fraction of Rac1 located at membranes (Castillo-Lluva et al. 2012), and it has been shown that endogenous Rac1 ubiquitination by exogenously expressed HACE1 requires the presence of NOXA1, a component of the transmembranal holoenzyme NADPH oxidase (Daugaard et al. 2013).

Altogether, these observations indicate that HACE1 exerts some of its function as an E3 ubiquitin ligase at cellular membranes: either at the cell periphery, where it ubiquitinates Rac1 and targets it for degradation; or at the Golgi, where it targets Syn5. How exactly HACE1 is recruited to its sites of action, and which cellular contexts determine its localization and target recognition remain aspects of great interest.

3.2.3. HACE1: a dual function protein involved in numerous cellular processes

It has been shown that HACE1 targets Rac1 and at least seven other proteins that are involved in a wide array of cellular mechanisms. Consequently, most of the cellular

processes in which HACE1 has been implicated require its catalytic activity. However, HACE1 has also been shown to play a role in the regulation of autophagy, anti-oxidative stress and anti-viral response in a way that is independent of its catalytic activity. The precise mechanisms by which HACE1 mediates these functions are still largely unknown (Zhang et al. 2014; Rotblat et al. 2014; Mao et al. 2016). This dichotomy makes HACE1 a dual function protein that is capable of modulating signaling events by two independent mechanisms that differ in their requirement for E3 ligase activity (summarized in **Table 3.1**). In this section, I will expand on some of the better studied cellular events where HACE1 plays a regulatory role.

a. Membrane organization and protein trafficking

In 2011, the teams of Dr. Wang and Dr. Zerial published an elegant study where they demonstrate that HACE1 catalytic activity is necessary for Golgi membrane fusion after mitosis (Tang and Wang 2013). They identify HACE1 as a binding partner of Rab-GTPases, which are important membrane organizers (Zerial and McBride 2001). Specifically, they show that HACE1 binds to the GTP-bound form Rab1, Rab4 and Rab11 (but not Rab2, 5, or 6); and through its interaction with GTP-Rab1, HACE1 is recruited to the Golgi and promotes Golgi stability. Mechanistically, they found that the presence of catalytically active HACE1 plays a role during mitotic disassembly of the Golgi membrane that is essential for Golgi reassembly after mitosis. In a follow up study, Dr. Wang's group demonstrated that HACE1 regulates this process by monoubiquitinating Syntaxin5 at Lys-270 specifically during early mitosis, as illustrated in figure 3.3 (Huang et al. 2016).

The authors of this study have suggested that HACE1's role as a tumor suppressor and regulator of cell proliferation might be connected to the role of HACE1 in Golgi biogenesis during the cell cycle. In the absence of HACE1, Golgi structure and therefore function is disrupted, which may directly affect cell growth and proliferation and impair accuracy of protein glycosylation and sorting, including cell adhesion molecules on the cell surface that are known to contribute to metastasis.

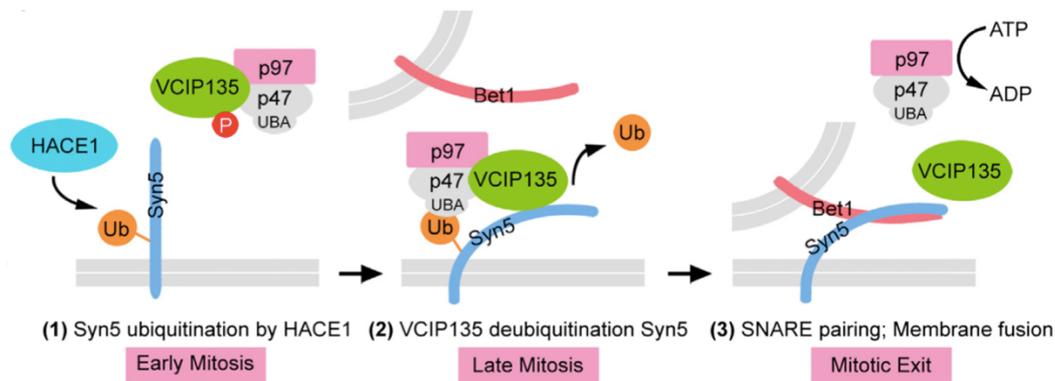


Figure 3.3. Representation of the model for the role of Syn5 ubiquitination in p97/p47-mediated post-mitotic Golgi membrane fusion. (1) In early mitosis, Syn5 is monoubiquitinated by HACE1 on the Golgi membranes, while the DUB VCIP135 is inactivated by mitotic phosphorylation. Syn5 recruits the p97/p47 complex and VCIP135 to the Golgi membranes through the interaction between the ubiquitin moiety on Syn5 and the UBA domain of p47. (2) In late mitosis, ubiquitin on Syn5 is removed by VCIP135 that is reactivated by dephosphorylation, (3) enabling Syn5-Bet1 SNARE complex formation and thus membrane fusion by p97 at mitotic exit. Taken from (Huang et al. 2016)

HACE1 association to Rab1, 4, and 11 indicates that HACE1 might have functions in protein trafficking. Indeed, HACE1 has been implicated in protein transport: HACE1 promotes secretion of YB-1 by conjugating K27-linked ubiquitin chains onto it (Palicharla and Maddika 2015), and regulates the recycling of the β 2-adrenergic Receptor (β 2AR) through a Rab11a-dependent mechanism (Lachance et al. 2014). This study from Lachance and colleagues showed that β 2AR mediates HACE1 interaction with Rab11a, Rab6a and Rab8a and facilitates their ubiquitination. They propose that HACE1 mediates Rab11a ubiquitination at Lys-145 in a way that does not affect its stability but rather leads to its activation (necessary for β 2AR recycling), and thus they introduce the idea that a cargo protein (like β 2AR) can regulate its own trafficking by inducing ubiquitination and activation of Rab GTPases.

b. Cell migration

Several studies support a repressive role of HACE1 on cell migration. Castillo-Lluva and collaborators have shown that HACE1 depletion in MEFs enhances cell migration independently of growth factor stimulation, and increases the accumulation of actin and Rac1 at the leading edge of cells (Castillo-Lluva et al. 2012). In this study, the authors

show that this highly motile phenotype depends on the absence of HACE1-mediated Rac1 ubiquitination, since this phenotype can be reversed by knocking down Rac1; and in cells expressing HACE1, the turnover of Rac1 at the leading edge of cells seems to be regulated by its ubiquitination. In agreement with these findings, another study has shown that HACE1 represses cellular migration and invasion in human mammary epithelial cells (MCF12A) in a Rac1 dependent manner (Goka and Lippman 2015). Moreover, a recent study focusing on the development and characterization of ubiquitin variant probes (UbV) that modulate HECT E3 ligase activity reported that among 13 HECT-ligases, the modulation of HACE1 activity had the most impact on cell migration. In line with the first two studies, this paper showed that inhibition of HACE1 greatly promoted cell migration (Zhang et al. 2016).

c. Cell growth and redox balance

As mentioned previously, lack of expression of active HACE1 is correlated with the aberrant stabilization of CyclinD1 protein levels and with increased cell proliferation due to the bypass of the G0/G1 arrest (Zhang et al. 2007; Goka and Lippman 2015). In a later study, the group of Dr. Sorensen in collaboration with our team found that HACE1 control of CyclinD1 levels is mediated by the inhibition of Rac1-dependent NADPH oxidase activity, which is required by the AP-1 transcription factor to induce CyclinD1 gene expression (Daugaard et al. 2013).

The mechanism proposed by this last study is illustrated in figure 3.4. Loss of HACE1 leads to chronic high ROS (Reactive Oxygen Species) levels in mammalian cells and in zebrafish, this imbalance is due to an overproduction of ROS at the Rac1-dependant NADPH oxidase complexes and not at the mitochondria. This study demonstrated that HACE1 targets Rac1 for degradation when it is localized to the NADPH oxidase holoenzyme, thus limiting *de novo* generation of ROS. In this way, HACE1 protects the cells against ROS induced DNA damage and increases cell proliferation. In accordance with this, another study reported that re-expression of HACE1 and Optineurin (an

autophagy receptor and HACE1 target) in a human lung cancer cell line synergistically reduced ROS production and DNA damage (Liu et al. 2014).

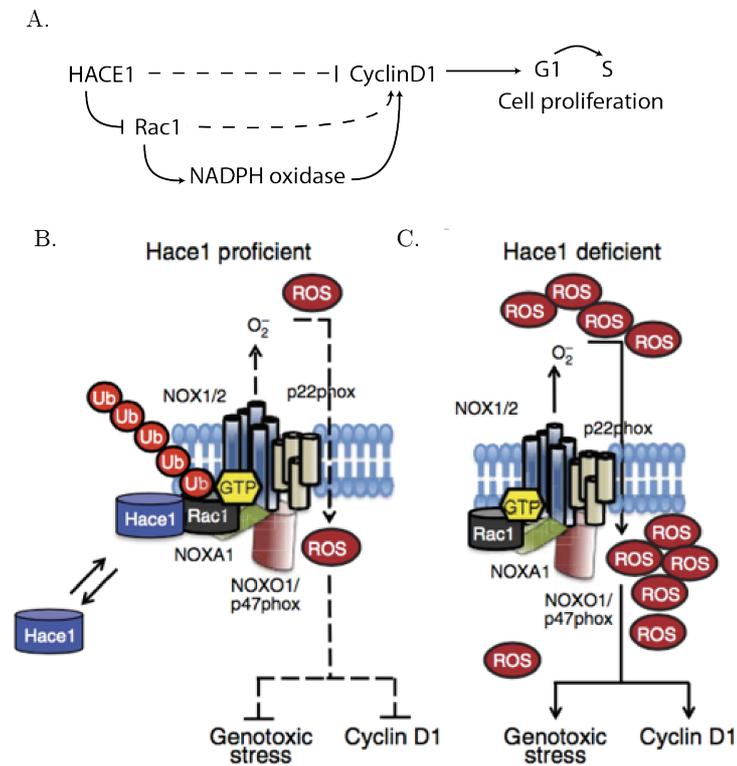


Figure 3.4. HACE1 controls Rac1-dependent NADPH oxidases. **A.** Simple arrow scheme showing the way that HACE1 controls cell proliferation by indirectly inhibiting CyclinD1 levels, through its control of Rac1 activity. **B.** HACE1 targets NADPH oxidase-bound Rac1 to regulate ROS production, cyclin D1 expression and DNA damage susceptibility. **C.** Loss of HACE1 hyperactivates NADPH oxidase activity, this increases ROS generation, cyclin D1 expression and ROS-induced DNA damage. Adapted from (Daugaard et al. 2013)

In addition to repressing ROS production, HACE1 has been found to indirectly promote the activity of the nuclear factor erythroid 2-related factor 2 (NRF2), a master regulator of the anti-oxidative stress response. HACE1 is essential for optimal activation of the NRF2 response under oxidative stress conditions by promoting NRF2 protein synthesis, stabilization, and nuclear localization. This function is independent of HACE1's catalytic activity but it requires the presence of its Ankyrin repeats and HECT domain. Interestingly,

it also seems that *hace1* itself is an oxidative stress response gene, since mRNA levels of HACE1 increase when cells are under acute oxidative stress (treated with H₂O₂ or arsenate) (Rotblat et al. 2014). This latter observation is in line with a previous study that identifies HACE1 as a target gene of the NRF2 transcription factor (Malhotra et al. 2010). Collectively, these studies demonstrate the central role of HACE1 in the control of cellular redox balance, where it inhibits ROS generation in an E3 ligase-dependent way and promotes the anti-oxidative stress response in an E3 ligase-independent way (**Fig. 3.5**).

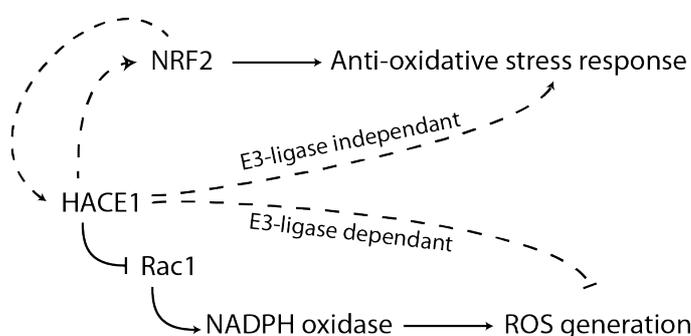


Figure 3.5. HACE1 is a central player in the control of cellular redox balance. Simplified scheme indicating the two complementary pathways that involve HACE1 in the control of oxidative stress. (**lower branch**) HACE1 targets Rac1 for degradation when located at the NADPH oxidase holoenzyme, thus inhibiting its activity and stopping ROS generation. (**Upper branch**) HACE1 indirectly promotes the activity of the NRF2 transcription factor, a master regulator of the anti-oxidative stress response, which in turn promotes HACE1 expression in response to severe oxidative stress.

d. Autophagy

HACE1 is involved in maintaining protein homeostasis (proteostasis) in response to stress by two mechanisms: 1) HACE1 can ubiquitinate and address its targets to UPS, and 2) HACE1 can increase protein degradation by promoting autophagic flux.

There are two studies that link HACE1 to autophagy. The first one starts from the observation that HACE1 expression is upregulated in human heart failure, and studies the

role of HACE1 in mice hearts under hemodynamic stress. They find that *hace1*-KO mice have frailer hearts and their cardiomyocytes display an accumulation of LC3, p62 and ubiquitinated proteins, which indicates impaired autophagy. Indeed, they find that HACE1 is required for efficient clearance of protein aggregates in an E3 ligase-independent manner. Interestingly, loss of HACE1 only impairs autophagy in cardiomyocytes under hemodynamic stress, not at basal conditions. The authors suggest that in absence of stress, redundant pathways can control proteostasis without requiring HACE1. However, during stress, the cell cannot cope without HACE1, perhaps because the redundant systems are either impaired or inadequate (Zhang et al. 2014).

In the second study, HACE1 is shown to ubiquitinate the autophagy receptor Optineurin at Lys-193, promoting its interaction with p62/SQSTM1 to form an autophagy receptor complex, accelerating autophagic flux in HEK293, MEFs and lung cancer cells. Different from the previous study, they show that HACE1 E3 ubiquitin ligase activity is essential for protein degradation by autophagy (Liu et al. 2014).

e. Cell death (apoptosis and necroptosis)

Recently, HACE1 has been shown to control tumor necrosis factor (TNF)-elicited cell fate decisions and to exert tumor suppressor and anti-inflammatory activities downstream of the TNF receptor 1 (TNFR1) (Tortola et al. 2016a). Tortola and colleagues observed that *hace1*-KO mice present dampened TNF-stimulated NF- κ B activation, reduced TNFR1-NF- κ B-dependent pathogen clearance, and impaired TNF-induced apoptosis. However, loss of HACE1 did not hamper TNFR1-mediated necroptosis via RIP1 and RIP3 kinases. This indicated that HACE1 acts as a gatekeeper of apoptotic vs necroptotic cell fate. The authors suggest that this new role of HACE1 is mediated by its ability to conjugate K63-linked ubiquitin chains on TRAF2 (**Fig. 3.6.**). Moreover, it was shown in that same study that loss of HACE1 predisposed mice to colonic inflammation and carcinogenesis *in vivo*, which is in line with a previous genetic study that draws a link between HACE1 deficiency and celiac disease (Einarsdottir et al. 2011; Tortola et al. 2016a). Tortola and colleagues observed that this phenotype was alleviated by the knock-out of RIP3 kinase or of TNFR1.

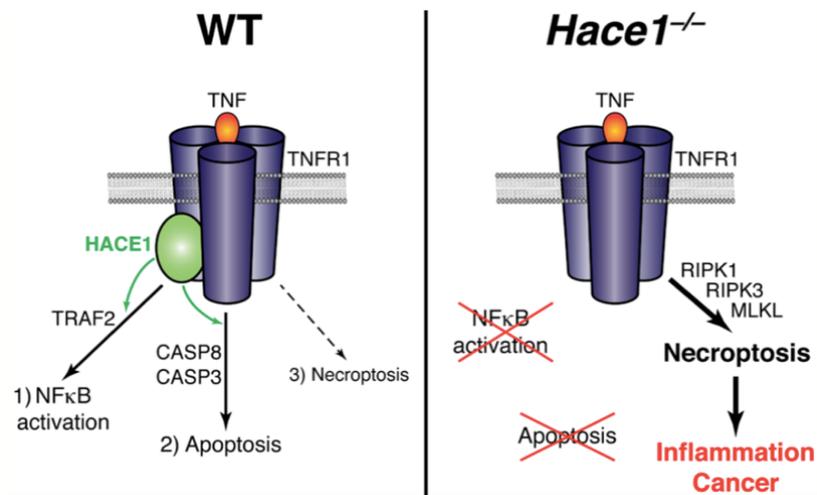


Figure 3.6. HACE1 controls cell fate after TNFR1 activation. Upon stimulation with TNF, HACE1 mediates the induction of NF-κB activation and apoptosis, which keeps a balance between the possible cell fates downstream of TNFR1 (left panel). In the absence of HACE1, NF-κB activation and apoptosis induction downstream of TNFR1 are impaired and cells are predisposed to necroptotic death, which promotes intestinal inflammation and carcinogenesis (right panel). Taken from (Tortola et al. 2016b)

Process	Notes on Mechanism	Reference
<i>E3-ubiquitin ligase- Dependent</i>		
Post-mitotic Golgi reassembly	Mono Ub of Syn5	(Tang et al. 2011) (Huang et al. 2016)
β 2AR recycling	Ub of Rab11a (mono- or poly- Ub that does not lead to protein degradation)	(Lachance et al. 2014)
Yb-1 secretion	K27 polyub of YB-1	(Palicharla and Maddika 2015)
Inhibition of cell migration	Ub of Rac1 \rightarrow degradation	(Castillo-Lluva et al. 2012; Goka and Lippman 2015)
Inhibition of ROS generation	Ub of Rac1 \rightarrow degradation K27 and K48 Ub of OPTN	(Castillo-Lluva et al. 2012) (Liu et al. 2014)
Induction of autophagy	K27 and K48 Ub of OPTN	(Liu et al. 2014)
Control of cell shape	Ub of Rac1 \rightarrow degradation	(Torrino et al. 2011)
Control of cell death	K63 Ub of TRAF2	(Tortola et al. 2016a)
Cell cycle: Induction of G0/G1 arrest	Ub of Rac1 \rightarrow degradation	(Zhang et al. 2007; Daugaard et al. 2013)
<i>E3-ubiquitin ligase- Independent</i>		
Anti-oxidative stress response: promotion of NRF2 activity	Via protein-protein interactions requiring HACE1 ANK repeats and HECT domain	(Rotblat et al. 2014)
Autophagy in the heart	-	(Zhang et al. 2014)
Inhibition of virus triggered type I IFN signaling	Via interaction with TRAF3	(Mao et al. 2016)
Regulation of RAR β transcriptional activity	Via interaction with RAR β 1, 2, and 3	(Zhao et al. 2009)

Table 3.1. Cellular processes regulated by HACE1. The processes are classified according to their requirement of HACE1's E3 ubiquitin ligase activity. (Ub) stands in short for "ubiquitination" and (\rightarrow) stands for "leading to".

Thesis objectives

Since its discovery, the downregulation of HACE1 expression has been associated with numerous human diseases, including cancer, neurodegenerative diseases and developmental conditions (Anglesio et al. 2004; Zhang et al. 2007; Goka and Lippman 2015; Akawi et al. 2015; Hollstein et al. 2015). Several mechanisms responsible for this inhibition are reported in the literature: there are examples of methylation of the *hace1* gene or its upstream regulatory regions (Anglesio et al. 2004; Zhang et al. 2007; Hibi et al. 2008; Sakata et al. 2009; Küçük et al. 2013; Gao et al. 2016a), chromatin modifications (Bouzelfen et al. 2016), deletion of the gene (Stewénus et al. 2008), and chromosomal translocations that truncate *hace1* (Slade et al. 2010).

In contrast to the large amount of data concerning the regulation of HACE1 gene expression, little is known about the mechanisms that modulate its catalytic activity and/or substrate recognition at the protein level. Considering the number of cellular mechanisms that require the normal activity of HACE1, it is of great importance to understand how HACE1 E3 ubiquitin ligase activity is modulated. Not only to better understand the processes it controls but also to eventually manipulate its activity for therapeutic purposes. As I have described in Chapter 1.4, other HECT domain ubiquitin ligases are regulated by structural rearrangements triggered by PTM. **Therefore, this work aims to study how HACE1 is regulated by PTMs, and more specifically by phosphorylation.**

In parallel to this work, our team screened for new HACE1 interactors using the yeast two hybrid system in order to identify potential new targets or regulatory adaptors of HACE1. Interestingly, two of the novel binding proteins candidates identified, α -catenin and E-cadherin, are core components of epithelial adherens junctions. **Hence, another aim of this study is to investigate whether and how HACE1 is involved in the regulation of epithelial adherens junctions.**

Results and Discussion

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**4.1. Research Article -imminent submission-:
Group-I PAKs mediate the phosphorylation of HACE1 on serine 385
in order to regulate its oligomerization state and Rac1 ubiquitination**

Group-I PAKs mediate the phosphorylation of HACE1 at serine 385 to regulate its oligomerization state and Rac1 ubiquitination

Maria I. Acosta¹, Serge Urbach², Anne Doye¹, Yuen-Wai Ng⁴, Jérôme Boudeau³, Amel Mettouchi¹, Anne Debant³, Edward Manser⁴, Orane Visvikis^{1,5}, Emmanuel Lemichez^{1,5}

C3M, Inserm, Equipe labellisée Ligue contre le Cancer, University of Côte d'Azur, Nice F-06204, France.

FPP at IGF, CNRS, Inserm, University of Montpellier, Montpellier F-34094, France

CRBM, CNRS, University of Montpellier, Montpellier F-34293, France.

sGSK Group, IMCB, Singapore 138673, Singapore.

Equal contribution

Corresponding author emmanuel.lemichez@inserm.fr

Abstract

The regulation of Rac1 by HACE1-mediated ubiquitination and proteasomal degradation is emerging as an essential element in the maintenance of cell homeostasis. However, how the E3 ubiquitin ligase activity of HACE1 is regulated remains undetermined. Here, we have addressed this question by using a proteomics approach to identify dynamically regulated phospho-residues of HACE1 in response to Rho GTPase activation. We report that serine 385, which is located in the middle region of HACE1, is phosphorylated in response to Rac1/Cdc42 activation and that group-I PAK kinases phosphorylate serine 385 *in vitro* and *in vivo*. Mechanistically, we define that the phospho-mimetic mutant HACE1(S385E), as opposed to HACE1(S385A), displays a lower capacity to ubiquitinate Rac1 in cells while maintaining its activity toward Rac1 *in vitro*. We also found that phosphorylation of serine 385 plays a pivotal role in controlling the HACE1 oligomerization state. Together, our work identifies, for the first time, a phospho-mediated regulation of HACE1 activity that is under the control of group-I PAKs.

Introduction

E3 ubiquitin ligases (E3s) are critical gatekeepers of cell homeostasis ¹. While we have begun to appreciate their structure and the diversity of their targets, we fall short on knowledge about their general integration in cell signaling. One essential question in particular is to better understand the cross-talk between kinases and ubiquitin ligases ². This is particularly true for HACE1, which is a critical regulator of active Rac1 flux for which we still lack identified regulators ³.

Rac1, together with Cdc42 and RhoA, are the most extensively studied members of the Rho GTPase family, which are intracellular signaling proteins that control a variety of cellular processes, such as actin remodeling and transcription ⁴. Rho GTPases act as molecular switches that cycle between an inactive form bound to GDP and an active form bound to GTP. In response to various environmental stimuli, inactive Rho proteins are charged with GTP, which induces conformational changes allowing Rho proteins to bind to effector proteins. These effectors, in turn, either relay or directly execute cellular responses driven by the Rho-activating environmental stimuli. More than 100 effector proteins of Rho GTPases have been described; some correspond to scaffold proteins, while others harbor enzymatic activities, notably kinases ⁴. The first identified and best characterized effectors activated by Rac1 are the family of P-21 Activated serine/threonine Kinases (PAKs) ⁵. In mammals, the PAK family consists of six members classified into two groups. Rac1 and Cdc42 activate group-I, which comprises PAK1, PAK2 and PAK3. Group-II, which comprises PAK4, PAK5 and PAK6, are only regulated by Cdc42 ⁶. Group-I PAKs are highly homologous but show different profiles of tissue expression. While PAK2 is found in virtually all tissues, PAK1 and PAK3 display a more restricted expression patterns; PAK1 is expressed in the mammary gland, muscle, spleen and brain tissues, while PAK3 expression is restricted to the brain ⁷. Genetics studies have established that PAKs play a significant role in tissue development and that PAK expression deregulation is linked to cancer progression ^{8,9}.

The regulated activity of Rac1 allows the integration of the many signals involved in the maintenance of cell homeostasis and cellular dynamics. The GDP/GTP cycle is controlled by the following regulatory proteins: (i) Guanine nucleotide Exchange Factors (GEFs), which facilitate the exchange of GDP and GTP; (ii) GTPase activating Proteins (GAPs), which increase the intrinsic rate of GTP hydrolysis; and (iii) RhoGDIs, which sequester Rho proteins in the cytosol. Additionally, the control of active Rac1 flux by the E3 ubiquitin ligase HACE1 is emerging as an important aspect of Rac1 signaling³. We and others have shown that HACE1 ubiquitinates Rac1 once it is activated, either by using point mutants (Q61L, Q61E, and G12V), by over-expressing the GEF domain of Dbl, or in response to growth factors such as Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF) and Heregulin (HRG)^{3,10,11}. The regulation of Rac1 by ubiquitination was first revealed in cells intoxicated by Cytotoxic Necrotizing Factor 1 (CNF1)¹², which is produced by pathogenic strains of *E. coli* from phylogenetic group B2. After endocytosis into the host cells, the toxin CNF1 translocates its catalytic domain into the cytosol, where it deamidates RhoA glutamine residue Q63 (Q61 in Rac1 and Cdc42) into a glutamic acid^{13,14}. Because this residue is essential for the GTP hydrolysis, its deamidation by CNF1 impairs the GTPase activity of Rho proteins and locks them in a GTP-bound state. As a consequence of its permanent activation, Rac1 gets ubiquitinated by HACE1 and is subsequently targeted by the 26S proteasome degradation machinery^{3,12}.

HACE1 is an important tumor suppressor whose expression is lost in a variety of human cancers, including Wilm's tumor, B-cell lymphoma, and colorectal, gastric and breast cancers^{11,15-19}. The repression of HACE1 expression has been shown to be a consequence of epigenetic silencing or chromosomal alterations^{6,15-19}. A major demonstration of HACE1 tumor suppressor activity came a decade ago from the observation that *hace1* KO mice develop spontaneous late-onset cancers from the three germ-layers¹⁶. Since then, several studies have converged to the idea that HACE1 is a guardian of cell homeostasis by controlling ROS levels and the autophagy of protein aggregates²⁰⁻²². Moreover, HACE1 also controls cell growth, migration and invasion, which are key features of cancer

progression^{10,11}. Interestingly, the deregulation of Rac1 ubiquitination due to the loss of HACE1 contributes to higher NADPH-dependent ROS production, which leads to DNA damage and cell hyper-proliferation²⁰. Additionally, a recent study has revealed that HACE1-induced ubiquitination of Rac1 in mammary gland epithelial cells (MCF12A) plays a major protective role against HER2/Neu-mediated breast tumorigenesis¹¹. More recently, several cancer-associated missense mutations in the *hace1* gene that inhibit Rac1 ubiquitination and impair cell growth have been identified, indicating that HACE1 activity can be altered in cancer²³.

Despite its importance in cell homeostasis, nothing is known about HACE1 regulation at the post-translational level. HACE1 possesses an N-terminal ankyrin-repeat domain (ANK) and a C-terminal catalytic HECT domain. The ANK and HECT domains are separated by a Middle region (MID) that does not harbor any structural homology with other known domains. Several phospho-proteomics studies have identified residues in HACE1 that are phosphorylated²⁴. Nevertheless, the context of these post-translational modifications and their consequences on the activity of this essential regulator remain to be defined. Here, we show the essential roles of group-I PAK kinases on the phospho-regulation of HACE1 E3 ubiquitin ligase activity and its oligomerization state.

Results

CNF1 increases HACE1 Ser-385 phosphorylation

To explore the possible cross-talk between Rho GTPases and HACE1, we conducted a study aimed at characterizing the phosphorylation status of HACE1 upon activation of Rho GTPases. We undertook an unbiased proteomics approach to identify HACE1 amino acid residues that were differentially phosphorylated in cells treated with the Rho-activating toxin CNF1. This was performed by tandem mass spectrometry (MS/MS) comparative analysis of trypsin-digested immuno-purified HA-HACE1 wild-type (WT) from primary Human Umbilical Vein Endothelial Cells (HUVECs) that were either

untreated or treated with CNF1 for 24 hours. Following MS/MS analysis, we performed a database search using phosphorylation as a variable modification. This allowed for the identification of, among others, one peptide with a single phosphorylated residue corresponding to serine 385 (Ser-385) located in the MID region (**Fig. 1a**). Quantification of the corresponding phosphorylated peptide ion signal from the extracted ion chromatograms indicated that CNF1 treatment induces a strong increase in Ser-385 phosphorylation (**Fig. 1b and Supplementary Figure S1a**). The same results were observed with two other Ser-385-containing peptides resulting from incomplete trypsin digestion (**Supplementary Figure S1b-c**). We thus conclude that CNF1 induces an increase in HACE1 phosphorylation at Ser-385.

To validate these results and to study the phospho-modulation of HACE1 on Ser-385, we generated a Ser-385 phospho-specific polyclonal antibody (referred to as pS385) and measured the levels of phosphorylated HACE1 by immunoblot assay. In agreement with the proteomics analysis, we found that CNF1 treatment of HUVECs expressing HACE1 leads to a significant increase in Ser-385 phosphorylation levels compared to control cells (**Fig. 1c-d**). To ascertain the specificity of the pS385 antibody towards phosphorylated HACE1, protein extracts were incubated with λ -phosphatase. As shown in **Figure 1c**, signals detected using the pS385 antibody disappeared after λ -phosphatase treatment, indicating that the pS385 antibody specifically recognizes the phosphorylated form of HACE1. Additionally, we found that the pS385 antibody detects HACE1(WT) but not the phospho-resistant mutant HACE1(S385A), indicating that the pS385 antibody specifically recognizes the phosphorylated Ser-385 in this context (**Fig. 1e**). The kinetics of HACE1 Ser-385 phosphorylation in CNF1-treated cells showed an increase in phosphorylation of up to 24 hours (**Fig. 1f**). Even though endogenous HACE1 is expressed at very low levels in HUVECs, we detected the increase in endogenous HACE1 phosphorylation on Ser-385 in CNF1-treated cells (**Fig. 1g**). Collectively, our data establish that HACE1 phosphorylation at Ser-385 is more abundant in cells treated with CNF1 than in control cells.

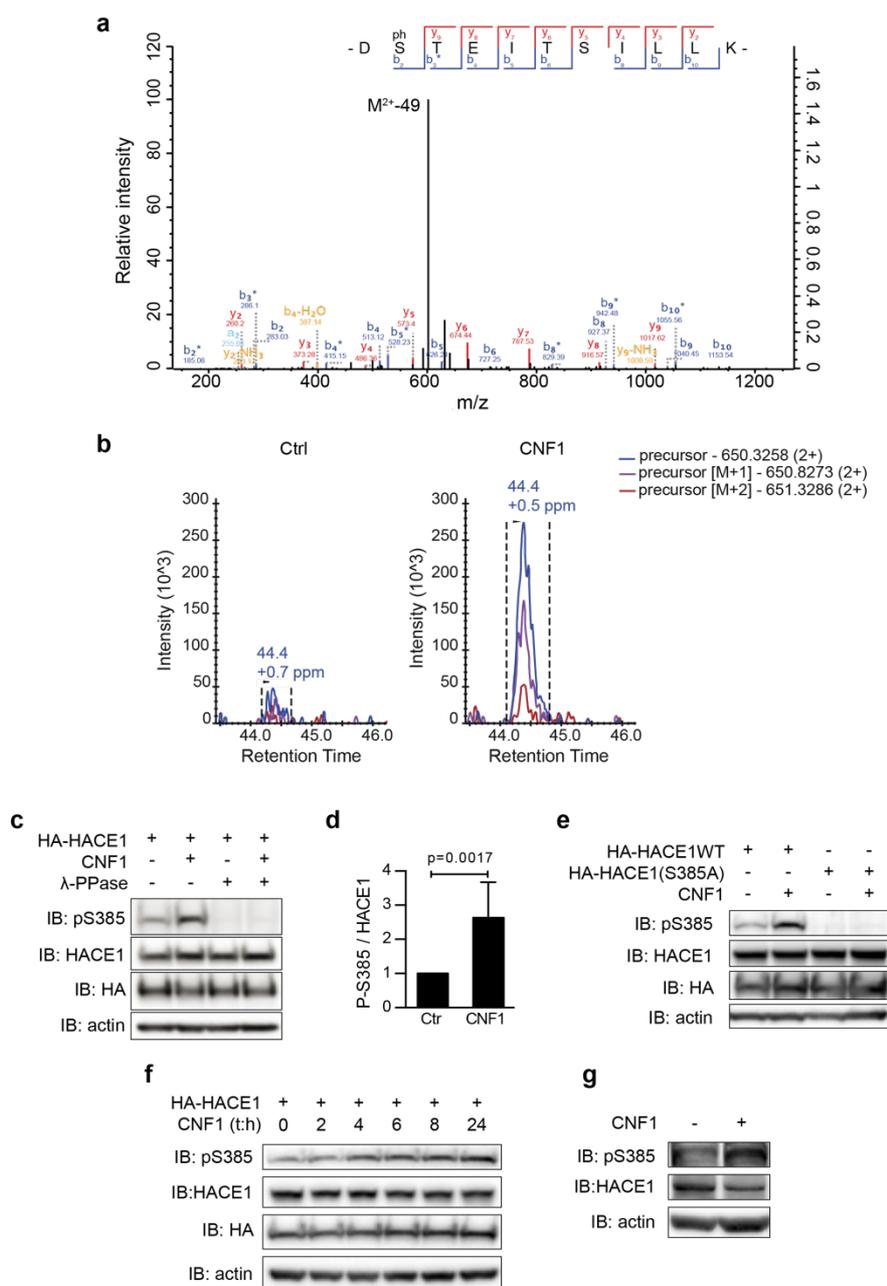


Figure 1: CNF1 increases phosphorylation of HACE1 on Ser-385. **a.** Fragmentation spectra of the DS(p)TEITSILK(+2) peptide showing that Ser-385 is phosphorylated. **b.** Extracted signal (precursor monoisotopic peak at +1 and +2) for the DS(p)TEITSILK(+2) peptide obtained with Skyline. **c.** Protein lysates from HUVECs transfected with HA-HACE1(WT) and treated with CNF1 at 10^{-9} M for 24 hours were treated or not with λ -phosphatase (λ -PPase) and analyzed by immunoblot (IB) using the indicated antibodies. IB: actin is used as a loading control. **d.** Graph showing levels of P-HACE1 relative to HACE1 total protein levels quantified by densitometry from the IB analysis. Data correspond to the mean \pm SD of > 3 biological replicates. p value was determined by one-sample t-test. **e.** Protein lysates from HUVECs transfected with HA-HACE1(WT) or HA-HACE1(S385A), treated with CNF1 at 10^{-9} M for 24 hours and analyzed by IB. **f.** Protein lysates from HUVECs transfected with HA-HACE1(WT), treated with CNF1 at 10^{-9} M for the indicated times, and analyzed by IB. **g.** Protein lysates from HUVECs treated with CNF1 at 10^{-9} M for 24 hours.

Rac1/Cdc42 controls HACE1 Ser-385 phosphorylation

Because CNF1 triggers the constitutive activation of several Rho GTPases^{13,14}, we assessed whether HACE1 phosphorylation in CNF1-treated cells is mediated by Rho GTPase activation. Using the pS385 antibody, we first analyzed the levels of HACE1 phosphorylation on Ser-385 in HUVECs co-expressing active forms of Rac1, Cdc42 and RhoA. The expression of both Rac1(Q61L) and Cdc42(Q61L) induced higher levels of Ser-385 phosphorylation compared to control conditions (**Fig. 2a-b**). Conversely, the expression of RhoA(Q63L) had no effect (**Fig. 2a-b**). The expression of Rac1(Q61L) also induced a clear increase in endogenous HACE1 phosphorylation on Ser-385 (**Fig. 2c**). In good agreement with previous results, over-expression of the dominant negative forms Rac1(T17N) or Cdc42(T17N) hindered the phosphorylation of Ser-385 triggered by CNF1 (**Fig. 2d-e**). Altogether, these results show that CNF1 treatment promotes the phosphorylation of HACE1 on Ser-385 in a Rac1- or Cdc42-dependent manner in HUVECs.

We then sought to determine whether this phosphorylation of HACE1 is specific to endothelial cells or whether it can be observed in other cell types. We analyzed the phosphorylation of HACE1 in mammary gland epithelial MCF12A cells, a cell type in which the HACE1/Rac1 signaling axis is functional¹¹. Interestingly, we found that CNF1 treatment or the expression of Rac1(Q61L) also greatly increased the phosphorylation of Ser-385 in MCF12A cells (**Supplementary Figure S1d-e**). This result shows that the induction of HACE1 phosphorylation on Ser-385 is not restricted to endothelial cells and might be a broader mechanism of HACE1 regulation. Altogether, these results show that CNF1 and Rac1 induce HACE1 phosphorylation on Ser-385 in various cell types.

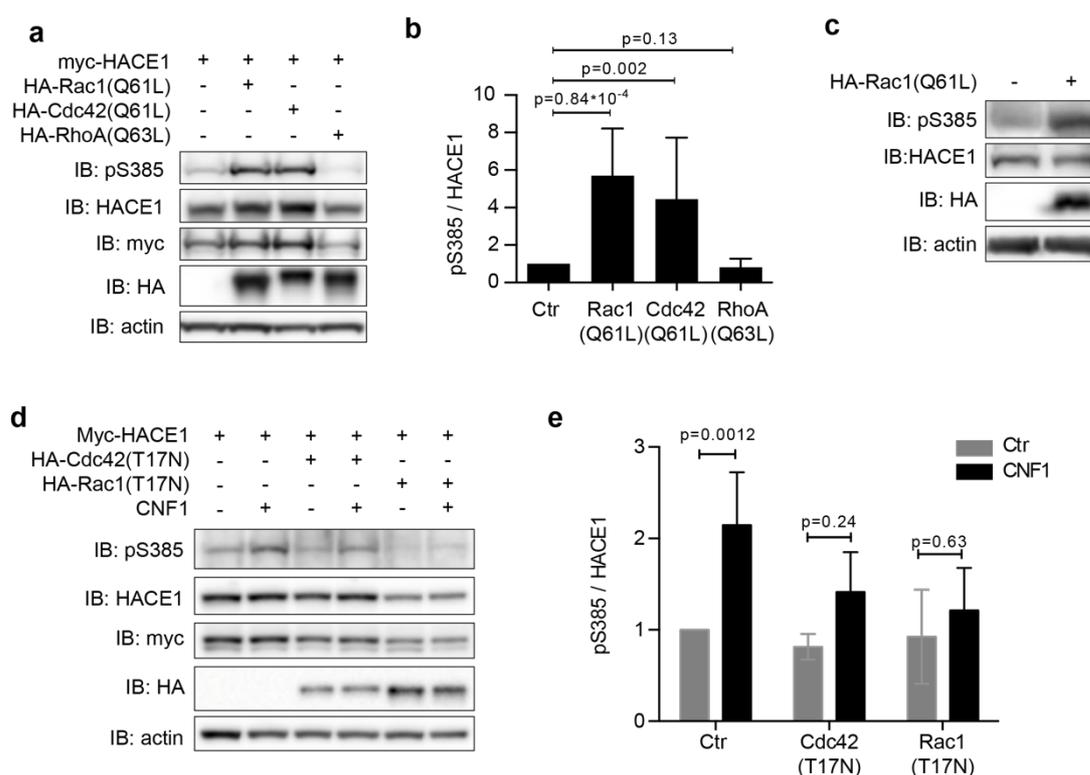


Figure 2: Activation of Rac1 or Cdc42 mediates the phosphorylation of HACE1 at Ser-385.

a. Protein lysates from HUVECs transfected with myc-HACE1(WT) together with HA-tagged active mutant of Rho-GTPases were analyzed by immunoblot (IB) using the indicated antibodies. IB: actin is used as a loading control. **b.** Graph showing levels of P-HACE1 relative to HACE1 total protein levels quantified by densitometry from the IB analysis described in A. Data correspond to the mean \pm SD of 3 biological replicates. p values were determined by unpaired two-sample t-test. **c.** Protein lysates from HUVECs transfected with HA-Rac1(Q61L) and analyzed as in (a). **d.** Protein lysates from HUVECs transfected with HA-HACE1(WT) alone or together with dominant negative myc-Rac1(T17N) or myc-Cdc42(T17N), treated with CNF1 at 10^{-9} M for 24 hours and analyzed as in (a). **e.** Graphs showing levels of P-HACE1 relative to HACE1 total protein levels quantified by densitometry from the IB analyses described in (d). Data correspond to the mean \pm SD of 3 biological replicates. p values were determined by unpaired two-sample t-test.

Group-I p-21 activated kinases phosphorylate HACE1 on Ser-385

We then sought to determine the kinases responsible for the phosphorylation of HACE1 on Ser-385. Two independent phospho-proteomics screens performed in HEK293 cells have previously identified the phosphorylation of Ser-385 in HACE1 but found that it is independent of Protein Kinase D1 (PKD1) or mammalian Target of Rapamycin (mTOR)

activity^{25,26}. Accordingly, inhibition of mTOR using rapamycin or Torin1 had no impact on the level of Ser-385 phosphorylation in CNF1-treated cells (**Supplementary Figure S2**). Moreover, we found equal levels of Ser-385 phosphorylation in PKD1-depleted cells (data not shown). To narrow down the potential kinases responsible for Ser-385 phosphorylation, we screened consensus kinase recognition motifs in the sequence encompassing Ser-385 using the prediction tool NetPhorest²⁷. As depicted in **Supplementary Figure S3a**, our analysis identified a site of recognition by PAKs. Together with our finding that both Rac1 and Cdc42, but not RhoA, induce the phosphorylation of HACE1 on Ser-385, this *in silico* prediction strongly suggested a role for group-I PAKs in HACE1 phosphorylation.

To assess whether group-I PAKs directly target HACE1, we performed *in vitro* kinase assays with recombinant PAK1 and HACE1. As shown in **Figure 3a**, we found that HACE1(WT) gets phosphorylated in the presence of PAK1. This revealed that HACE1 is a direct substrate of PAK1. We then analyzed the role of group-I PAK kinases in HUVECs and MCF12A cells. Because PAK3 expression is restricted to the brain⁵, we focused on PAK1, which is notably expressed in mammary glands, and on PAK2, which is ubiquitously expressed. We first verified PAK1 and PAK2 expression in HUVECs and MCF12A cells using specific anti-PAK1 and anti-PAK2 antibodies. This established that PAK2 is expressed both in HUVECs and MCF12A cells, while PAK1 is only expressed in MCF12A cells (**Fig. 3b**). Using three independent siRNAs specifically targeting PAK2, we found that depletion of PAK2 markedly reduced Rac1(Q61L)-induced phosphorylation of Ser-385 in HUVECs and MCF12A cells (**Fig. 3c-d**). This result strongly indicates that PAK2 acts downstream of Rac1 to induce HACE1 phosphorylation. Additionally, depletion of PAK2 also reduced the CNF1- and Cdc42(Q61L)-mediated phosphorylation of Ser-385 (**Supplementary Figure S3b-c**). Because PAK1 is expressed in MCF12A cells, we assessed its role on HACE1 phosphorylation in these cells and found that silencing of PAK1 reduced Rac1(Q61L)-induced phosphorylation of HACE1 (**Fig. 3e**). Together, these data establish that the PAK1 and PAK2 kinases both regulate the phosphorylation levels of HACE1 on Ser-385. Consistently, we found that treatment of MCF12A cells with the group-I PAK inhibitor FRAX597 suppresses HACE1 phosphorylation induced by Rac1(Q61L) and

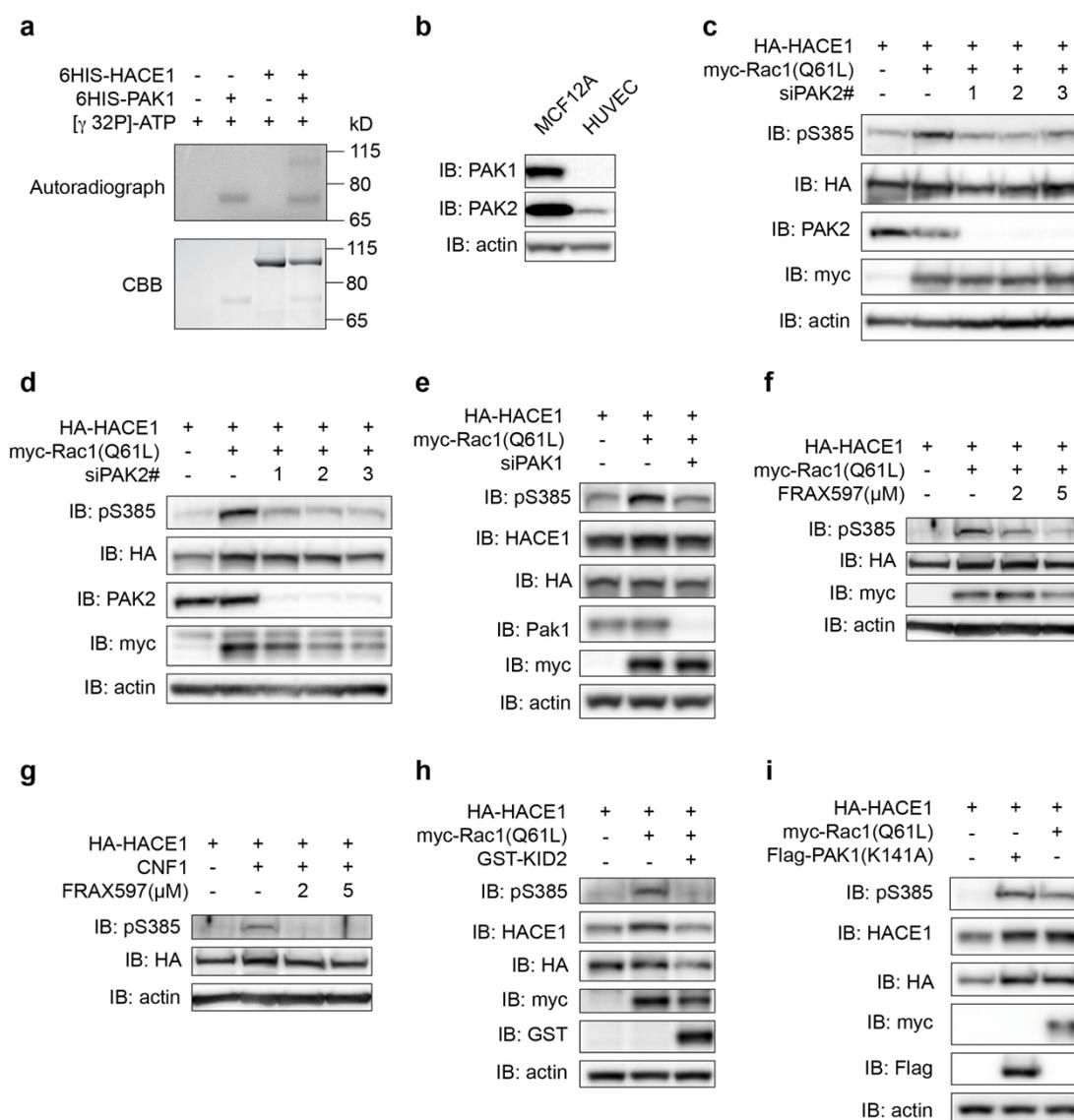


Figure 3: Group-I PAKs induce direct phosphorylation of HACE1.

a. *In vitro* [γ ³²P]-ATP kinase assay using recombinant 6His-HACE1 and recombinant 6His-PAK1 analyzed by autoradiography and Coomassie Brilliant Blue (CBB) staining. **b.** MCF12A and HUVEC protein lysates analyzed by immunoblot (IB) using anti-PAK1 and anti-PAK2 antibodies. IB: actin is used as a loading control. **c-e.** Protein lysates from (c) HUVECs and (d-e) MCF12A cells transfected with siRNAs targeting (c-d) PAK2 or (e) PAK1 and plasmid expressing HA-HACE1 and myc-Rac1(Q61L) and analyzed by IB using the indicated antibodies. **f-g.** Protein lysates from MCF12A cells transfected with HA-HACE1, either (g) intoxicated with CNF1 for 16 hours or (f) co-transfected with myc-Rac1(Q61L), and treated with FRAX597 at the indicated concentration for 16 hours before IB analysis. **h-i.** Protein lysates from MCF12A cells transfected with HA-HACE1, myc-Rac1(Q61L) and GST-KID2 or Flag-PAK1(K141A) and analyzed by IB.

CNF1 (**Fig. 3f-g**). Similarly, expression of the dominant negative Kinase Inhibitory Domain of PAK2 (KID2) reduces the levels of Ser-385 phosphorylation induced by Rac1(Q61L) (**Fig. 3h**). Finally, we found that expression of a constitutively active PAK1(K141A), which is mutated in the auto-inhibited domain, could promote HACE1 phosphorylation on Ser-385. Thus, PAK1 activation is sufficient to induce HACE1 phosphorylation of Ser-385 in MCF12A cells (**Fig. 3i**). Altogether, these results indicate an essential role for group-I PAK kinases in regulating HACE1 phosphorylation at Ser-385 downstream of Rac1 in a variety of cell types.

Phospho-mimetic mutation S385E hampers HACE1 ability to ubiquitinate Rac1 in cells

To determine whether Ser-385 phosphorylation could modulate the capacity of HACE1 to ubiquitinate Rac1, we used an assay based on the purification of cellular proteins cross-linked to 6His-tagged ubiquitin²⁸. We found that the phospho-resistant mutant HACE1(S385A) induces Rac1(Q61L) ubiquitination as efficiently as HACE1(WT) (**Fig. 4a**). This indicates that phosphorylation of Ser-385 is not required for the activity of HACE1 on Rac1. Conversely, we found that the phospho-mimetic mutant HACE1(S385E) displays a reduced capacity to ubiquitinate Rac1(Q61L). This strongly suggests that the phosphorylation of Ser-385 down-regulates HACE1 activity on Rac1 (**Fig. 4a**).

To decipher how phosphorylation of Ser-385 interferes with Rac1 ubiquitination, we analyzed the properties of the association between HACE1(S385E) and Rac1, as well as the intrinsic catalytic activity of HACE1(S385E). Using a co-immunoprecipitation (co-IP) assay, we found that HACE1(S385A) and HACE1(S385E) interact with Rac1(Q61L) to the same extent as HACE1(WT) (**Fig. 4b**). This suggests that the HACE1-Rac1 association occurs regardless of the phosphorylation status of HACE1 on Ser-385. We next sought to determine whether phosphorylation on Ser-385 down-regulates the intrinsic catalytic activity of HACE1. A classical way to assess the catalytic activity of an E3 ligase is to measure its auto-ubiquitination levels. However, we could not detect the specific auto-ubiquitination of HACE1 (**Supplementary Figure S4**), indicating that HACE1 does not

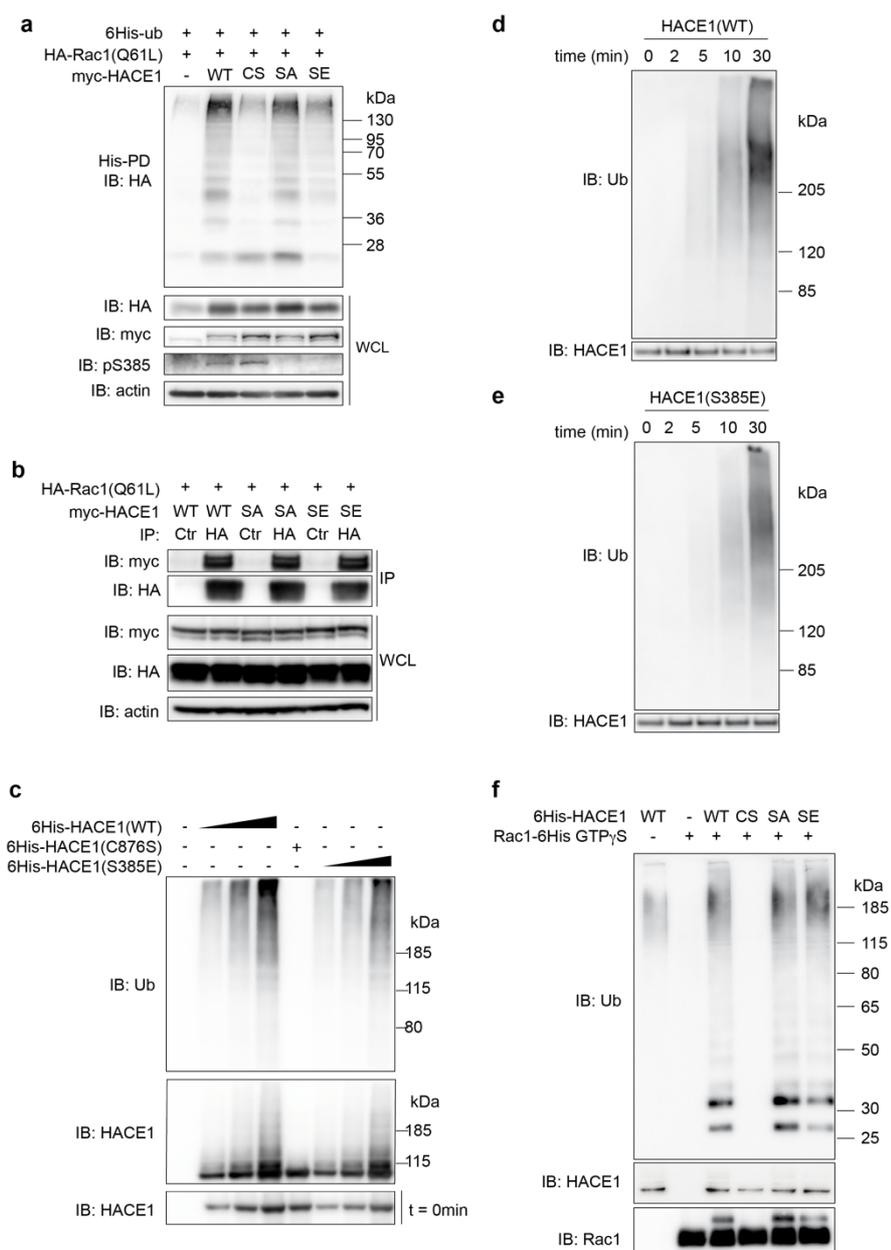


Figure 4: HACE1(S385E) phospho-mimetic blocks Rac1 ubiquitination in cells.

a. Protein lysates from MCF12A cells transfected with the indicated plasmids were subjected to His pull-down (His-PD) prior to immunoblot analysis (IB). Whole cell lysate (WCL) IB analysis showing total protein expression. **b.** Protein lysates from MCF12A cells transfected with the indicated plasmids were subjected to immunoprecipitation (IP) using Ctrl or HA antibodies prior to immunoblot analysis (IB). Whole cell lysate (WCL) IB analysis shows total protein expression. **c.** *In vitro* ubiquitination assay using recombinant 6His-tagged HACE1(WT), catalytic inactive mutant C876S (CS) and HACE1(S385E) (SE) analyzed 30 min post-reaction by immunoblot using the indicated antibodies. IB at t=0 min shows the input protein levels. **d-e.** *In vitro* ubiquitination assay using HACE1(WT) and HACE1(S385E) (SE) analyzed by IB at the indicated time points. **f.** *In vitro* ubiquitination assay using recombinant 6His-tagged HACE1(WT), catalytic inactive mutant HACE1(C876S) (CS), HACE1(S385A) (SA), HACE1(S385E) (SE) and Rac1 loaded with GTPγS and analyzed 30 min post-reaction by immunoblot using the indicated antibodies.

induce its own ubiquitination in MCF12A cells, which prevented us from using this approach. To overcome this technical obstacle, we performed *in vitro* ubiquitination experiments using purified recombinant 6His-tagged HACE1(WT) and mutants. With this assay, we could detect auto-ubiquitination of HACE1, as evidenced by the ubiquitination of HACE1(WT) and the absence of ubiquitination of the catalytic inactive mutant HACE1(C876S) (**Fig. 4c**). This indicated that the *in vitro* ubiquitination assay is a reliable method to examine HACE1 catalytic activity. Measuring HACE1 auto-ubiquitination using this assay showed no drastic decrease in HACE1(S385E) activity compared to HACE1(WT). Indeed, we measured a proportional increase in self-ubiquitination with both forms of HACE1 by using increasing amounts of HACE1(WT) and HACE1(S385E) (**Fig. 4c**). We also found that HACE1(WT) and HACE1(S385E) display similar auto-ubiquitination kinetics (**Fig. 4d-e**). Taken collectively, these results indicate that the phospho-mimetic mutation S385E does not alter the intrinsic catalytic activity of HACE1 *in vitro*. In good agreement with our findings that HACE1(S385E) retains its catalytic activity, we also determined that HACE1(S385E) induces the ubiquitination of recombinant Rac1 loaded with GTP γ S *in vitro* (**Fig. 4f**). Altogether, these results established the paradoxical impact of the S385E mutation on HACE1 activity measured *in vitro* versus *in vivo*. Indeed, we found that HACE1(S385E) is catalytically active on Rac1 *in vitro* (**Fig. 4f**), though it displays low activity in cells (**Fig. 4a**), suggesting the combined involvement of Ser-385 phosphorylation and an inhibitory cellular factor that has yet to be identified to down-modulate HACE1 activity.

Phosphorylation of Ser-385 modulates HACE1 homo-oligomerization

It has previously been shown that several HECT-E3 ligases are regulated by phospho-dependent intra- or inter-molecular interactions^{29,30}. To determine whether HACE1 undergoes such regulation in cells, we first performed co-IP experiments using a combination of epitope-tagged HACE1 constructs. We found that HA-HACE1(WT) binds to myc-HACE1(WT) (**Fig. 5a**), which demonstrates that HACE1 is capable of forming

homophilic intermolecular interactions. This result indicates that HACE1 can form oligomers. We next performed size exclusion chromatography (SEC) using the recombinant 6His-HACE1. By comparing the elution profiles of 6His-HACE1 with molecular weight markers, we found that 6His-HACE1 is efficiently eluted at an apparent molecular weight of ≥ 200 KDa (**Fig. 5b**). With a theoretical molecular weight of monomeric 6His-HACE1 of 106 KDa, this SEC result indicates that HACE1 can directly form homo-oligomers of at least two proteins and that the homophilic interactions observed by co-IP are likely direct. To narrow down the HACE1 domains that are engaged in the homophilic interactions, we performed co-IP experiments using different tagged versions of full length (FL) HACE1 or deletion mutants as depicted in **Supplementary Figure S5a**. As shown in **Supplementary Figure S5b**, we confirmed that HACE1 FL binds to HACE1 FL. We also detected an efficient interaction between HACE1 FL and HACE1 ANK+MID (**Supplementary Figure S5b**). A weaker binding of HACE1 FL with the HECT domain alone was also detected. Additionally, another series of co-IPs showed that HACE1 ANK+MID binds most efficiently to the HECT domain alone (**Supplementary Figure S5c-d**). Altogether, these results indicate that HACE1 can form homo-oligomers that most likely involve interactions between the ANK + MID region and the HECT domain.

We next sought to determine whether phosphorylation of Ser-385 can interfere with HACE1 homo-oligomerization. We found that HA-HACE1 binds to myc-HACE1 to the same extent as HA-HACE1(S385A) with myc-HACE1(S385A) (**Fig. 5d**). This suggests that HACE1 homo-oligomerization occurs in the absence of Ser-385 phosphorylation. Interestingly, we found that HACE1(S385E) displays higher homophilic interaction levels than HACE1(WT) or HACE1(S385A), suggesting that phosphorylation modifies the state of HACE1 homo-oligomerization (**Fig. 5d**). In accordance with this result, we found that overexpression of active PAK1(K141A) greatly increases HACE1 homophilic interaction levels (**Fig. 5e**). This is specific to Ser-385 phosphorylation as PAK1(K141A) overexpression did not modify the extent of HACE1(S385A) homophilic interactions (**Fig. 5e**). Altogether, these results indicate that the phosphorylation at Ser-385 modulates HACE1 homo-oligomerization properties.

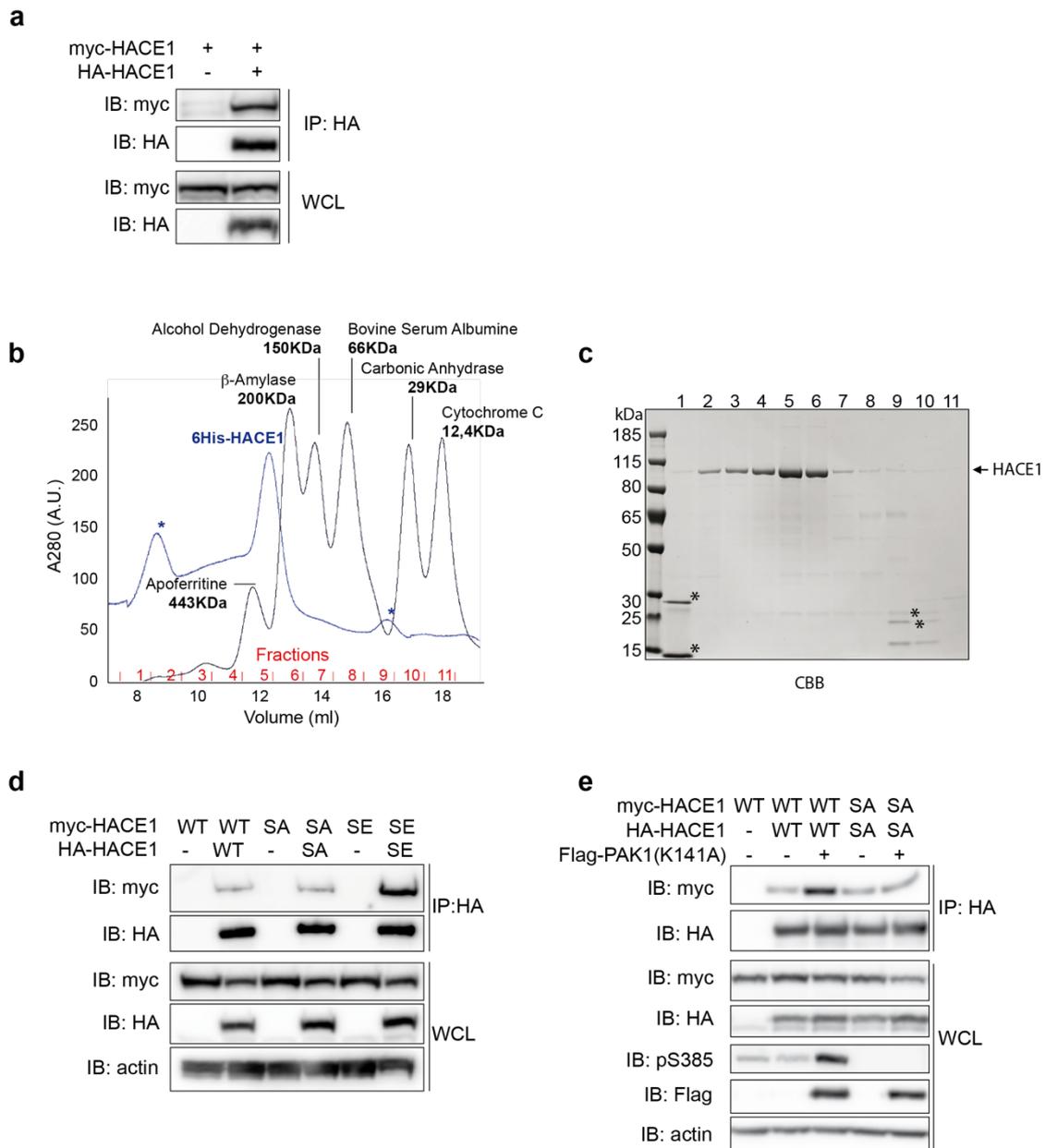


Figure 5: Ser-385 phosphorylation modulates HACE1 oligomerization.

a, d-e. Protein lysates from MCF12A cells transfected with the indicated plasmids were subjected to immunoprecipitation (IP) using HA or Flag antibodies prior to immunoblot analysis (IB). Whole cell lysate (WCL) IB analysis shows the total protein expression. SA is S385A and SE is S385E. **b.** Superposition of the size exclusion chromatograms from recombinant 6His-HACE1(WT) and the molecular weight markers suggests that HACE1 oligomerizes as a dimer or a trimer. Absorbance at 280 nm is expressed in arbitrary units (A.U.). *: non-specific picks. **c.** Elution fractions from 6His-HACE1 SEC analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (CBB). *: non-specific contaminants of 6His-HACE1 purification fractions corresponding to the non-specific picks seen in (a).

Discussion

Using an unbiased proteomics approach, we have identified HACE1 Ser-385 as a pivotal amino acid residue that is phosphorylated in response to Rho GTPase activation by the CNF1 toxin. The GTPases Rac1 and Cdc42 are not only required for Ser-385 phosphorylation downstream of CNF1, but either Rac1 or Cdc42 activation is sufficient to induce the phosphorylation of Ser-385. Our data points to group-I PAKs as being responsible for the phosphorylation of HACE1 downstream of Rac1. Finally, we have shown that the phosphorylation of Ser-385 is critical for the modulation of HACE1's capacity to ubiquitinate Rac1 and its oligomerization properties. This establishes the phospho-regulation of HACE1 as a critical modulator of Rac1 signaling.

Interestingly, the phospho-mimetic mutant HACE1(S385E) has a reduced capacity to ubiquitinate GTP-bound Rac1 in cells. This is, to our knowledge, the first description of a post-translational regulation of HACE1. Our data support a model where Rac1 down-regulates the activity of its own E3 ubiquitin ligase. Considering the essential role of Rac1, such a physiological regulation of HACE1 would ensure the adequate control of the levels of Rac1 activation to maintain proper cell homeostasis. Conversely, one could hypothesize that excessive phospho-inhibition of HACE1 would lead to a pathological level of Rac1 signaling activation. Consistently, it has already been established that enhanced Rac1 signaling, due to loss of *hace1* expression, promotes (i) excessive ROS production which leads to DNA damage and cell hyper-proliferation and (ii) breast cancer progression^{11,20}. In line with this, we have found that over-expression of an active form of PAK1 induces high levels of HACE1 Ser-385 phosphorylation. Interestingly, group-I PAKs are over-expressed or hyper-activated in a wide variety of cancers, including breast, lung, kidney, colorectal or gastric cancers, which could lead to excessive HACE1 phospho-inhibition^{9,31}. Therefore, in future studies, measurements of the phosphorylation levels of HACE1 Ser-385 in cancer samples might be a good readout of the deregulation of Rac1 signaling mediated by PAK. Interestingly, PAK also promotes Rac1 activation by binding to the Rac1 exchange factor PAK-interacting exchange factor (PIX) and by mediating the

phospho-inhibition of RhoGDI^{32,33}. Therefore, our results position PAK at the center of a coordinated pathway between the canonical system of Rac1 activation via a GDP/GTP switch and the system that controls Rac1 activation by proteasome-mediated degradation.

How can phosphorylation of Ser-385 reduce the capacity of HACE1 to ubiquitinate Rac1? Phosphorylation sites can serve as recognition sites for adaptor proteins such as the 14-3-3 family of proteins³⁴. Interestingly, phosphorylation of the E3-HECT Nedd4-2 has been shown to trigger its association with 14-3-3 proteins, which prevents Nedd4-2 from binding to its target — the epithelial Na⁺ channel (ENaC)³⁵. Here, we failed to detect a specific association between the Ser-385-phosphorylated form of HACE1 with 14-3-3 proteins (**Supplementary Figure S6**). Accordingly, although we have found that the HACE1(S385E) mutant is less able to ubiquitinate active Rac1, it is known that glutamic acid phospho-mimetic residues do not bind 14-3-3 adaptor proteins because they do not fit into their binding pockets³⁶. Thus, the hypothesis of HACE1 phospho-inhibition by 14-3-3 proteins seems unlikely. An alternative hypothesis is that the phosphorylation of HACE1 might directly affect its binding to Rac1. Indeed, this is the case for the HECT E3 ligase Smurf1 whose phosphorylation modulates its own affinity for its substrates Par6 and RhoA³⁷. However, we found that the HACE1(S385E) phospho-mimetic mutant binds to Rac1 as efficiently as HACE1(WT). Therefore, we hypothesized that the phosphorylation of Ser-385 might regulate HACE1 catalytic activity. Indeed, such a regulation has been previously reported for Itch/AIP4 and Nedd4-1, whose activities are regulated upon JNK- and Src-dependent phosphorylation, respectively^{29,30}. However, the absence of HACE1 auto-ubiquitination activity in our cellular model precluded us from testing this possibility. Nevertheless, *in vitro* ubiquitination experiments indicate that HACE1(S385E) is functional, as it can ubiquitinate itself and Rac1. This important result allowed us to discard the possibility that the lowered HACE1(S385E) activity measured in cells is due to intrinsic alterations in the protein. The paradoxical differences between HACE1(S385E) activities *in vitro* and *in vivo* suggest that, in addition to Ser-385 phosphorylation, a cellular adaptor mediates HACE1 inhibition. Such regulation requiring both the phosphorylation of the HECT E3 ligase and an adaptor protein has been recently described

for HUWE1 (aka MULE, ARF-BP1)³⁸. Indeed, although it works in the opposite direction, it was shown that phosphorylation of HUWE1 disrupts the interaction with its inhibitory co-factor p14-ARF and ultimately activates the E3 ligase³⁸. A similar scenario involving the phospho-dependent binding of a regulatory adaptor to HACE1 is likely to occur here. One could hypothesize that an activator can bind the non-phosphorylated form of HACE1 to trigger its activity or, conversely, that an inhibitor binds the phospho-form of HACE1 to abrogate its activity. Therefore, our work opens the way for the search for regulatory adaptors whose binding is modulated by HACE1 phosphorylation at Ser-385.

In most cases, the regulation of HECT E3 ligases involving phosphorylation and/or adaptors is the consequence of structural modifications. This has been particularly well-described in the Nedd4 family, which undergoes inhibitory intra- or intermolecular interactions between their C-terminal HECT domain and C2 or WW N-terminal domains^{39,40}. These interactions, whether they induce a closed monomeric conformation or the formation of an inactive homodimer, are relieved by phosphorylation or binding to an adaptor protein^{29,30,41-45}. Similarly, it has recently been proposed that the HUWE1 E3 ligase can adopt an inhibitory asymmetric homodimeric conformation that leads to its inhibition and that binding of p14-ARF shifts the dynamic conformational equilibrium of HUWE1 toward the inhibitory dimer⁴⁶. Our co-IP data indicate that HACE1 forms homophilic interactions that involve the binding of the HECT domain to the ANK+MID region. Interestingly, the HECT domain does not bind to either the ANK domain or the MID region alone. This suggests that either the HECT domain binds a motif intersecting the ANK and MID regions or that the ANK+MID region adopts a particular conformation that enables its binding to the HECT domain. In line with this second hypothesis, we have recently demonstrated that the cooperation of ANK and MID regions are important for the efficient binding to the active form of Rac1²³. Here, we found that recombinant HACE1 can form a homo-oligomer *in vitro* by SEC analysis. This indicates that homophilic HACE1 interactions found in cells are likely direct. Moreover, our SEC data also indicates that HACE1 homo-oligomers correspond to dimers or trimers. The formation of trimers of HECT E3 ligases has been reported for Ube3A/E6AP, which promotes full activity of the

ligase⁴⁷. Interestingly, an independent study has shown that phosphorylation of threonine 485 inhibits Ube3A/E6AP catalytic activity⁴⁸. Whether this phosphorylation alters Ube3A/E6AP trimerization has not been explored. However, mutation of this specific threonine is associated with autism, demonstrating the importance of this phosphoregulation in physiology. A recent study has shed light on the importance of post-translational modifications in the trimerization of several Nedd4 proteins⁴⁹. It was shown that the formation of Nedd4 trimers is promoted by ubiquitination of their HECT domains, which leads to their inactivation⁴⁹. Our data indicates that homodimerization or homotrimerization of HACE1 occurs in the absence of Ser-385 phosphorylation as the phospho-null mutant HACE1(S385A) and HACE1(WT) display similar levels of homophilic interactions. Interestingly, our results suggest that phosphorylation of Ser-385 modifies HACE1 oligomerization properties. Indeed, we found higher levels of homophilic HACE1(S385E) interactions compared with HACE1(WT). Similarly, specific phosphorylation of Ser-385 induced by PAK1(K141A) increases homophilic HACE1(WT) interactions. This increase in homophilic phospho-HACE1 interactions may thus reflect an increase in the stability of the oligomer or an increase in the extent of oligomerization, i.e., number of units per oligomer. Moreover, our data indicate that an adaptor is likely required to account for the decrease in HACE1(S385E) activity in cells. Thus, one could hypothesize that an adaptor binds to the phospho-oligomer to mediate HACE1 inhibition. An alternative hypothesis would be that an adaptor binds to HACE1 once it is phosphorylated on Ser-385, which in turn modifies the properties of HACE1 to oligomerize into an inactive complex. Interestingly, we found that Ser-385 is located within an intrinsically disordered region (IDR)⁵⁰ (**Supplementary Figure S7**). These IDRs, which lack stable tertiary structures, are phosphorylation hotspots⁵¹; thanks to their flexibility, IDRs allow easy access and recognition of their phosphorylated residue to binding surfaces⁵². Further work remains to be completed to determine how phosphorylation of Ser-385 modifies HACE1 oligomerization properties. In conclusion, we have uncovered a pivotal role for Ser-385 in the regulation of HACE1 that sets the basis for deciphering the relationship between the structure and activity of HACE1.

Methods

Plasmid constructs, primers, siRNAs, antibodies and reagents

All plasmids used in this study are listed in Supplementary table S1. Site-directed mutagenesis was performed using the primers listed in Supplementary table S2 and the QuickChange Lighting (Agilent) and QuickChange II (Agilent) kits for HACE1 and PAK1 plasmids, respectively. The pSY5M-6His-PAK1 plasmid was obtained by subcloning the rat PAK1 sequence⁵³ into the pET21d+ vector. pXJ-GST-KID2 was obtained by subcloning the rat PAK2 kinase inhibitory domain (85-144 aa) into the pXJ40-GST vector using the XhoI/KpnI restriction sites. All the plasmid sequences were verified by sequencing. SMART-pool siRNA mixes against PAK1 (#5058) and PAK2 (#5062) were acquired from GE Dharmacon. The silencer select siRNAs against PAK2 were labeled as siPAK2#1 (s10022), siPAK2#2 (s10023), and siPAK2#3 (s10024) and were purchased from Ambion® ThermoFisher Scientific. All the antibodies used in this study are listed in Supplementary Table 3. The rabbit anti-HACE1pS385 (pS385) antibody was raised against the phospho-peptide sequence KNKRD[pS]TEITS and purified by positive and negative affinity purification using the phosphorylated and unphosphorylated peptide sequences, respectively (Perbio Science France SAS). The CNF1 toxin was purified as described in Doye et al., 2006. The PAK1 kinase inhibitor FRAX597 (Selleckchem) was used at 2 or 5 μ M for 16 hours and the mTOR inhibitors Torin1 (Tocris) and Rapamycin (Sigma-Aldrich) were used at 0.1 μ M and 0.1 nM respectively, for 4 hours.

Cell culture, transfection and lysis

HUVECs were obtained from PromoCell and maintained in human endothelial SFM medium (Gibco™) supplemented with 20% fetal bovine serum (Gibco™), 20 ng/ml FGF-2, 10 ng/ml EGF (Peprotech), 1 μ g/ml heparin (Sigma-Aldrich), and 1% penicillin-streptomycin (Gibco™). MCF-12A mammary gland epithelial cells (CRL-10782, ATCC) were maintained in DMEM/F12 (Gibco™) supplemented with 7.5% horse

serum (Biowest), 20 ng/ml recombinant human EGF (Peprotech), 10 µg/ml human recombinant insulin (Gibco™), 0.5 µg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), and 1% penicillin-streptomycin (Gibco™). Human embryonic kidney (HEK293) cells were maintained in DMEM (Gibco™) supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin (Gibco™).

Plasmid DNAs and siRNAs were transfected into MCF12A cells using Lipofectamine LTX and Lipofectamine RNAiMAX, respectively, according to manufacturer's procedures (Invitrogen). For DNA transfection, cells were seeded at 70% confluence 16-24 hours before DNA transfection. For DNA and siRNA co-transfection, 1.3×10^6 cells were directly reverse transfected with a 150 nM final concentration of siRNA in a 12-well plate, incubated for 48 hours, transfected with plasmid DNA and incubated an additional 24 hours before lysis. HUVECs were transfected with plasmid DNA by electroporation or using the PolyMag reagent (OZ Biosciences) as described previously (Doye et al., 2006) 24 hours before lysis unless otherwise stated. HUVECs were transfected with siRNAs using the PolyMag reagent at a final concentration of 50 mM and incubated for 72 hours before lysis. For DNA and siRNA co-transfection, cells were first transfected with siRNA using PolyMag, incubated for 48 hours, transfected with plasmid DNA using PolyMag and incubated an additional 24 hours before lysis. HEK293 cells were transfected with Lipofectamine 2000 according to manufacturer's procedure (Invitrogen).

For the analysis of the total protein levels analyzed by immunoblot, cells were lysed in 1X RIPA buffer (Bio Basic) supplemented with protease and phosphatase inhibitors (Pierce) and analyzed by SDS-PAGE and immunoblot.

Immunoblot analysis

Protein lysates were resolved using NuPAGE 3-8% Tris Acetate, 4-12% Bis-Tris pre-cast gels (Invitrogen) or 8%, 10% or 12% Tris Glycine SDS-PAGE gels. Separated proteins were transferred onto Immobilon-PVDF membranes (Millipore) using a semi-dry method

(Trans-blot[®] Turbo[™] transfer, Biorad) or by overnight transfer in carbonate buffer (1.25 mM NaHCO₃, 0.37 mM Na₂CO₃, and 20% v/v ethanol, pH 9.9). Membranes were probed with the indicated primary and secondary antibodies (Supplementary table S3), and then incubated with the Immobilon Western Chemiluminescent HRP substrate (Millipore). The emitted chemiluminescent signals were detected with a Syngene Pxi4 imaging system. When appropriate, immunoblot signals were quantified by densitometry using the Image Studio 3.1.4 software.

Tandem mass spectrometry analysis

HUVECs transfected with HA-HACE1 expressing plasmid were left untreated or were treated with CNF1 at 10⁻⁹ M for 24 hours. Following lysis and an immunoprecipitation assay, immuno-purified HA-HACE1 from both conditions were separated on an SDS-PAGE gel, and trypsin-digested samples obtained from the HA-HACE1 cut gel slices were analyzed as described previously⁵⁴. Briefly, proteins were digested in-gel using trypsin (Gold, Promega). The generated peptides were analyzed online using an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate 3000 HPLC (Dionex, Thermo Fisher Scientific). Desalting and pre-concentration of the samples was performed online on a Pepmap[®] pre-column (0.3 mm×10 mm, Dionex). A gradient consisting of 0–40% B in A for 60 min, followed by 80% B and 20% A for 15 min (A was 0.1% formic acid with 2% acetonitrile in water and B was 0.1% formic acid in acetonitrile) at 300 nl/min was used to elute peptides from the capillary reverse-phase column (0.075 mm×150 mm, Pepmap[®], Dionex). Eluted peptides were electrosprayed online at a voltage of 1.9 kV into an LTQ Orbitrap Elite mass spectrometer. A single full-scan mass spectrum cycle (400–2000 *m/z*) at a resolution of 120,000 (at 400 *m/z*) in the orbitrap, followed by twenty data-dependent MS/MS spectra were repeated continuously throughout the nanoLC separation. All the MS/MS spectra (acquired using the linear trap quadrupole) were recorded using a normalized collision energy (33%, activation Q of 0.25 and activation time of 10 ms).

Spectral data were analyzed using the MaxQuant software⁵⁵ with standard settings and the following variable modifications: (1) Acetyl (Protein N-term), (2) Oxidation (M), and (3) Phosphorylation (STY). For quantification, signal extraction of the identified peptides was performed using Skyline⁵⁶.

Co-Immunoprecipitation and His-Ub pull-down

For co-immunoprecipitation, MCF12A cells were scraped 20 hours post-transfection in 1 ml of immunoprecipitation buffer (SLB) (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton-X100, and 0.27 M sucrose) supplemented with protease and phosphatase inhibitors (Pierce) and lysed by a freeze-thaw cycle in liquid nitrogen. Cleared lysates were incubated with 30 μ l of Ezview Red Anti-HA or anti-Flag Affinity gel (Sigma-Aldrich) for 2 hours at 4 °C. Beads were washed at least twice with 1 ml of SLB and resuspended in 30 μ l of 1X LDS buffer with 50 mM DTT. For the His-Ub pull-down experiments, MCF12A cells were lysed 7 hours post-transfection in ULB (8 M Urea, 20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 10 mM imidazole, and 0.1% Triton X-100). The proteins that covalently bound to 6His-tagged ubiquitin were pulled-down by incubating 95% of the cleared lysate with 30 μ l cobalt-chelated resin (Clontech), which was previously blocked in ULB+5% bovine serum albumin (RIA grade, Sigma) for 1 hour. After lysate incubation, the beads were washed four times in ULB and resuspended in one volume of Laemmli's buffer.

***In vitro* kinase assays**

Purified PAK1 (2 ng/ μ l) was incubated with recombinant purified HACE1 (40 ng/ μ l) and 30 μ M ATP (10 μ Ci of [γ 32P]-ATP) in kinase buffer (25 mM HEPES pH 7.3, 0.02% Triton X-100, 25 mM NaCl, 5 mM β -glycerophosphate, 2.5 mM NaF, 5 mM MgCl₂, and 0.1 mM MnCl₂) at 30 °C for 30 min in a final volume of 50 μ l. Samples were analyzed by immunoblot followed by autoradiography and Coomassie blue staining.

Purification of recombinant proteins by IMAC

E. coli BL21 strains transformed with pET-28a plasmids encoding 6His-HACE1 (WT) and mutants were grown for 24 hours at 37°C in 1 L of Luria-Bertani (LB) Broth with kanamycin 50 µg/ml. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 100 µM and the culture was grown for another 8 hours at 30°C. Bacteria were harvested by centrifugation and lysed using a French Press in 20 ml of Buffer A (Tris-HCl pH 7.5 and 200 mM NaCl) with 1 mM phenylmethylsulfonyl fluoride (PMSF). Cleared lysate was subjected to Immobilized Metal Affinity Chromatography (IMAC) using a Chelating Sepharose Fast Flow column (GE healthcare) charged with NiSO₄ in an AKTA system with the UNICORN software (GE healthcare). Elution was performed with Buffer A containing increasing concentrations of imidazole. The approximately 15 ml fraction eluted with 250 mM imidazole that contained most of the 6His-HACE1 was dialyzed overnight in 25 mM Tris-HCl with 125 mM NaCl and concentrated the next morning to 1 µg/µl using an Amicon Ultra-15 50K (Millipore).

E. coli BL21 strains transformed with pSY5M plasmids encoding 6His-PAK1 were grown overnight at 30°C in 50 ml of LB with 50 µg/ml chloramphenicol and 50 µg/ml ampicillin and incubated overnight (ON) at 30°C. The next morning, 200 ml of LB with antibiotics was inoculated with 20 ml of the overnight culture. When $0.6 < OD_{600} < 1$, IPTG was added to a final concentration of 500 µM and the culture was grown another 4 hours at room temperature with shaking. The bacteria were harvested by centrifugation and lysed by sonication in 10 ml of cold bacterial lysis buffer (50 mM Tris [pH 8.0], 0.5% Triton-X100, 5 mM MgCl₂, 20 mM imidazole, 1 mg/ml lysozyme, 5 mM DTT, 0.5 mM PMSF and 1X Protease Inhibitors Cocktail (Roche)). The cleared lysate was incubated with 250 µl of Ni-NTA-agarose slurry and roll at 4°C for 2 hours. The 6His-Pak1 bound to the Ni-NTA-agarose beads was transferred into a 10-ml disposable column, washed and eluted in 5x1 ml of elution buffer (50 mM Tris [pH 8.0], 0.5% Triton-X100, 5 mM MgCl₂, 250 mM imidazole and 5% glycerol). Fractions with >1 mg/ml of 6His-PAK1 were pooled, aliquoted and snap-frozen in liquid nitrogen.

Size-exclusion chromatography

Size Exclusion Chromatography (SEC) was performed using 500 μ l of IMAC-purified 6His-HACE1 (WT) at 1 μ g/ μ l using a Superdex 200 Increase 10/300 GL with an AKTA system and the UNICORN software program. A mix of *gel filtration marker kit for protein molecular weights 12,000- 200,000 Da* (Sigma-Aldrich) with *apoferritin from equine spleen* (Sigma-Aldrich) was used as the control.

***In vitro* ubiquitination assay**

HACE1 auto-ubiquitination and HACE1-induced ubiquitination of Rac1 was performed in a 40 μ l final volume containing 20 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 5 mM ATP, and 1 mM DTT using 250 ng of recombinant human 6His-Ube1 (RD system), 500 ng of recombinant human UbcH7 (RD system), 1 μ g of ubiquitin (RD system), 100 ng to 1 μ g of 6His-HACE1 and 1 μ g of Rac1-6His that was previously loaded with GTP- γ S. Reactions were incubated at 37°C for 30 min unless otherwise indicated and were stopped by the addition of 1X LDS and 1X reducing agent (Invitrogen).

Lambda phosphatase treatment

One thousand units of λ -Protein Phosphatase (Sigma-Aldrich) was used to dephosphorylate 50 μ g of proteins from whole cell lysates. Samples were incubated for 40 min at 30°C, and the reactions were stopped by the addition of 1X LDS and 1X Reducing Agent (ThermoFisher Scientific).

Bioinformatics analysis

Prediction of IDRs was performed using the online tool PONDR predictor with the VL-XL, XL1-XT and CaN-XT algorithms <http://www.pondr.com/>⁵⁰. The PAK consensus

kinase sequence was obtained using the online tool NetPhorest <http://www.netphorest.info/index.shtml>²⁷.

Statistical analysis

The data were analyzed with the statistical software Graphpad Prism 6.0f. Statistical significance was evaluated by one- or two-sample unpaired t-tests.

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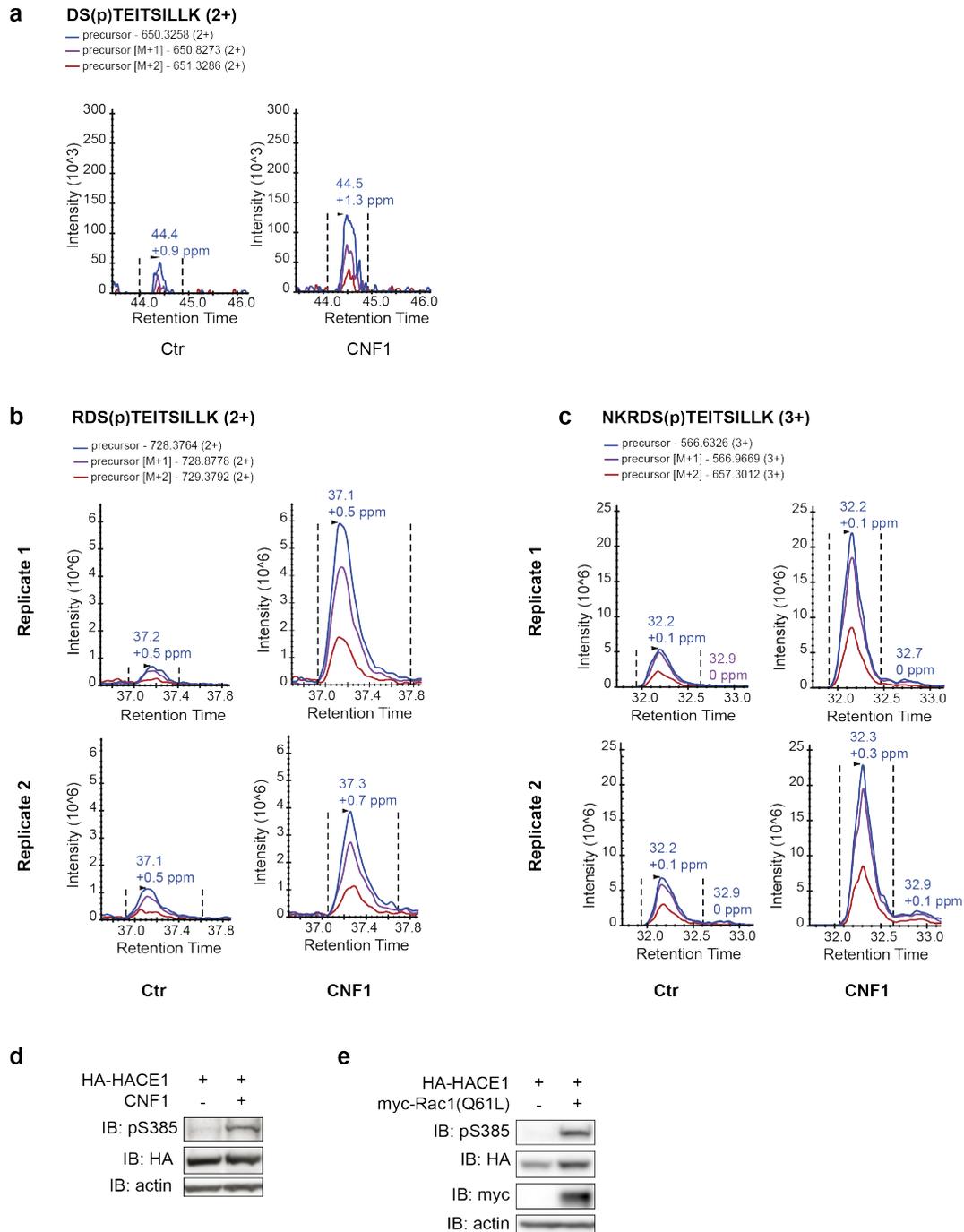
Author contributions

Conception or design of the work: E.L., O.V., E.M., J.B, A.M. Data collection: M.I.A., O.V., A. Do., S.U., Y.W.N. Analysis and Interpretation of the data: M.I.A., O.V., E.L., S.U., J.B., Y.W.N., E.M. Drafting the article: O.V., E.L., M.I.A., S.U. Critical revision of the article: S.U., J.B., A.M., A. De, E.M., Y.W.N. Approved manuscripts: M.I.A., A. Do., S.U., Y.W.N., J.B., A.M., A. De., E.M., O.V., E.L.

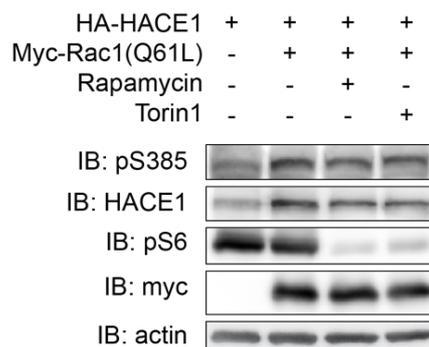
Competing Interests

The authors declare no competing financial interests.

Supplementary Information

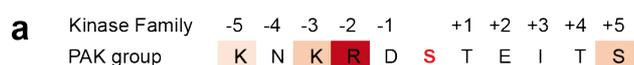
**Supplementary Figure S1: CNF1 and Rac1 induce phosphorylation of Ser-385.**

a. Extracted signal (precursor monoisotopic peak at +1 and +2) for the DS(p)TEITSILLK(+2) peptide in CNF1 and control conditions from a second biological replicate. **b-c.** Extracted signal (precursor monoisotopic peak at +1 and +2) for the (b) RDS(p)TEITSILLK(+2) and (c) NKRDS(p)TEITSILLK(+3) peptides in CNF1 and control conditions from two biological replicates. **d-e.** Protein lysates from MCF12A cells transfected with HA-HACE1(WT) and (d) treated with CNF1 for 24 hours or (e) co-transfected with myc-Rac1(Q61L) and analyzed by immunoblot (IB) using the indicated antibodies. IB: actin is used as a loading control.

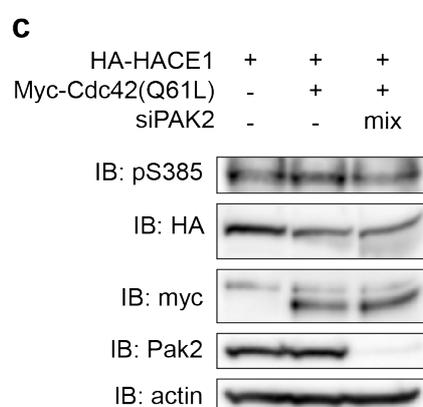
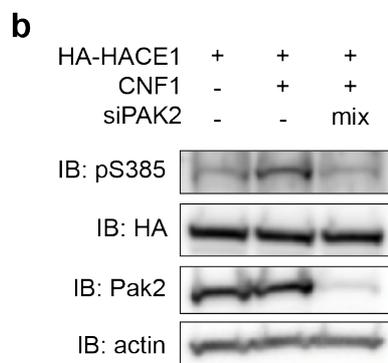


Supplementary Figure S2: Phosphorylation of HACE1 S385 is independent of mTOR signaling.

HUVECs transfected with the indicated plasmids were treated with rapamycin (0.1 μ M) or Torin1 (0.1 M) for 4 hours prior to lysis and analysis by immunoblot (IB) using the indicated antibodies. IB: actin is used as a loading control and pS6 is used as a control for mTOR activation.

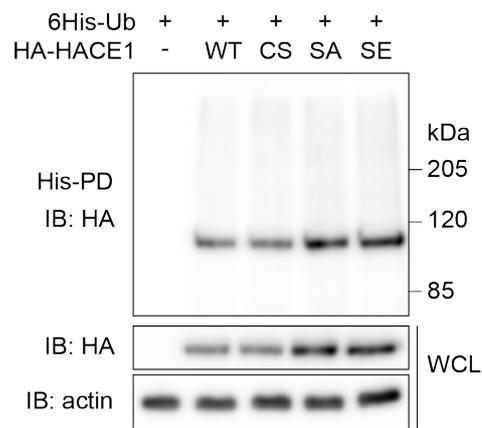


Consensus level



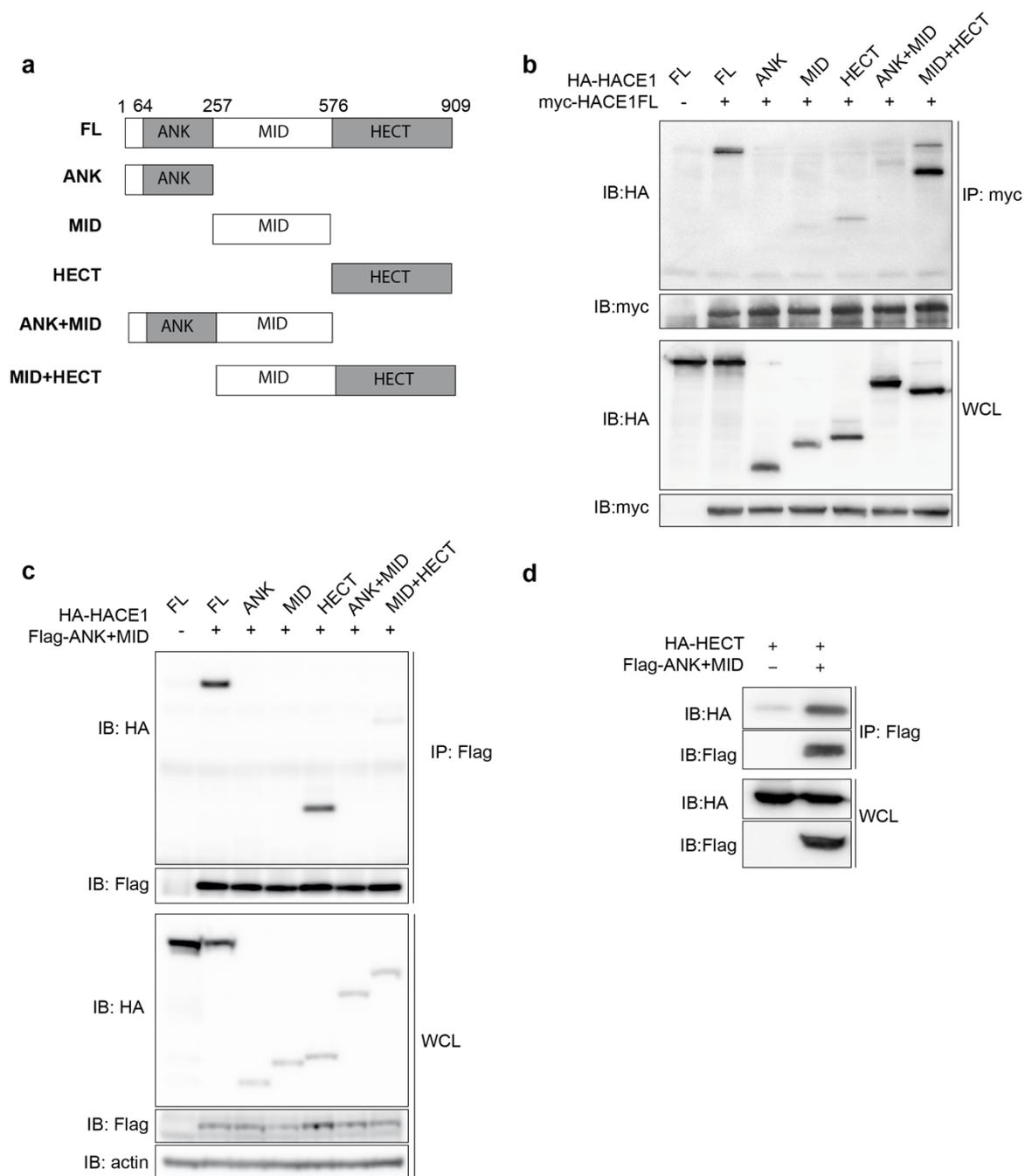
Supplementary Figure S3: Group I PAKs induce the direct phosphorylation of HACE1.

a. NetPhorest software analysis of the sequence surrounding Ser-385 identified a conserved PAK consensus motif. **b-c.** Protein lysates from MCF12A cells transfected with siRNA mix targeting PAK2 (**c**) with plasmids expressing HA-HACE1 and Cdc42(Q61L) or (**b**) intoxicated with CNF1 for 24 hours were analyzed by immunoblot (IB) using the indicated antibodies.



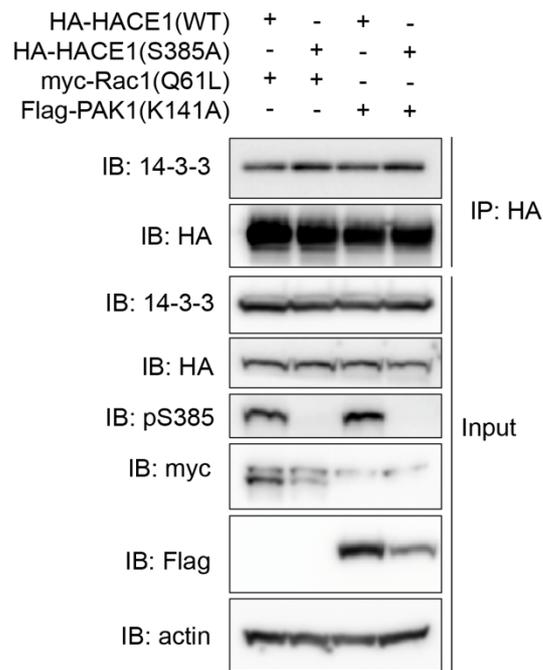
Supplementary Figure S4: HACE1 does not induce its own ubiquitination in MCF12A cells.

Protein lysates from MCF12A cells transfected with the indicated plasmids were subjected to His pull-down (His-PD) prior to immunoblot analysis (IB). Whole cell lysate (WCL) IB analysis showing total protein expression. CS is C876S, SA is S385A, and SE is S385E.



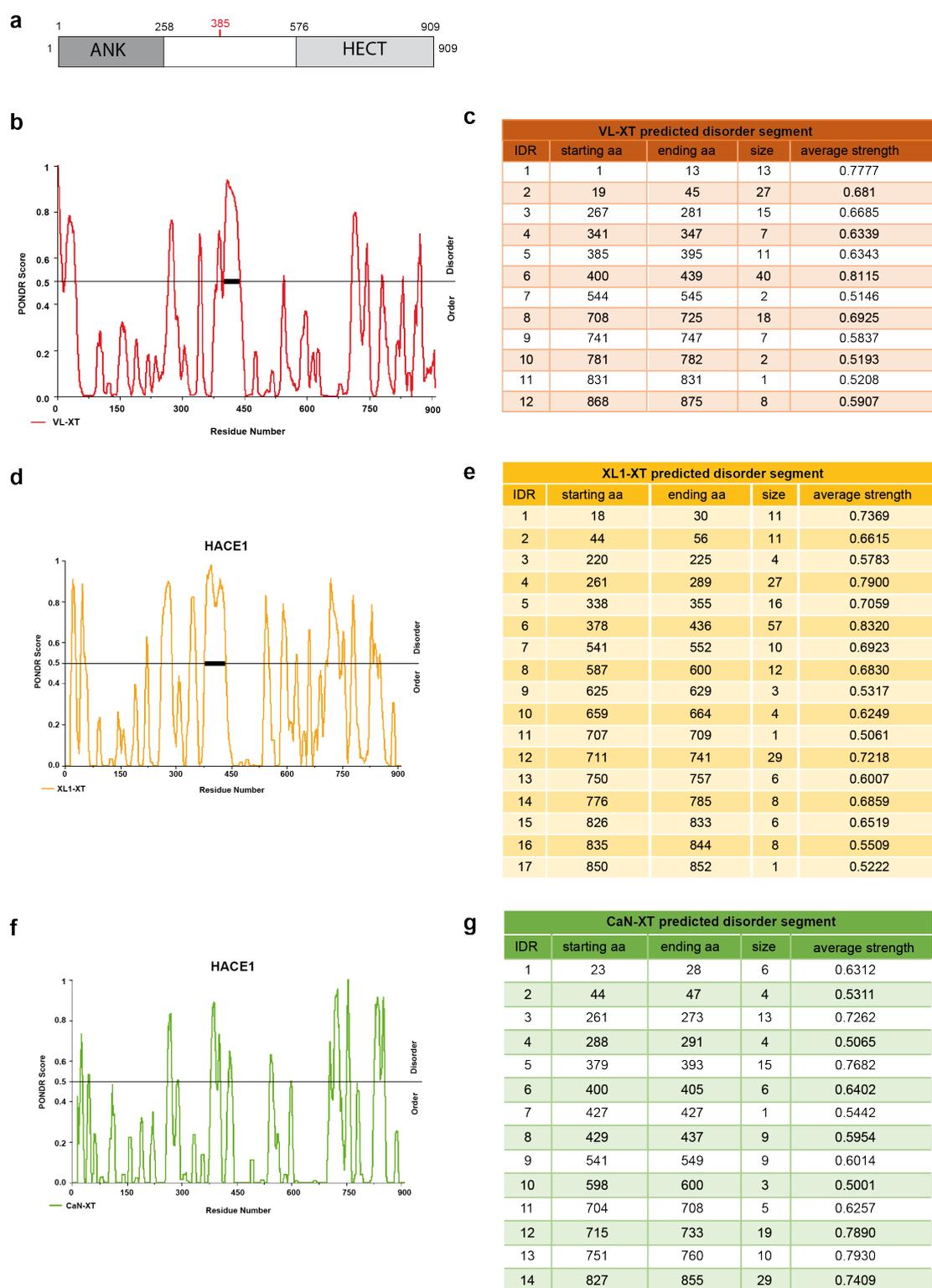
Supplementary Figure S5: *In vivo* homophilic interactions between HACE1 requires HECT binding to the ANK+MID region.

a. Schematic representation of the HACE1 protein indicating the position and size of the ANK and HECT domains as well as the MID region. The structures of the deletion mutants are also represented. **b-d.** Protein lysates from **(b)** HEK293 or **(c-d)** MCF12A cells transfected with the indicated plasmids were subjected to immunoprecipitation (IP) using myc or Flag antibodies prior to immunoblot analysis (IB). Whole cell lysate (WCL) IB analysis shows the total protein expression.



Supplementary Figure S6: Phosphorylation of Ser-385 does not promote a specific association between HACE1 and 14-3-3 proteins.

Protein lysates from MCF12A cells transfected with the indicated plasmids were subjected to immunoprecipitation (IP) using HA antibodies prior to immunoblot analysis (IB). Whole cell lysate (WCL) IB analysis shows the total protein expression.



Supplementary Figure S7: Ser-385 is located within an intrinsically disordered region.

a. Schematic representation of the HACE1 protein that shows Ser-385 within the MID region. **b-g.** HACE IDR prediction using the PONDRL® predictor with the **(b-c)** VT-XL, **(d-e)** XL1-XT and **(f-g)** CaN-XT algorithms. **(b, d, and f)** Graphs represent the PONDRL score relative to the residue position. Scores under and above 0.5 correspond to ordered and disordered regions, respectively. **(c, e, and g)** Tables listing the IDR domains found by the 3 algorithms.

Supplementary table S1: Plasmid List

#	Plasmid	Reference	Design
1	PKH3-HA3-HACE1(WT)	Andrio et al. 2017	
2	PKH3-HA3-HACE1(S385A)	This study	mutagenesis of plasmid #1 using primers #1-2
3	PKH3-HA3-HACE1(S385E)	This study	mutagenesis of plasmid #1 using primers #3-4
4	PKH3-HA3-HACE1(C876S)	Torrino et al., 2017	
5	pXJ40-HA-Rac1(Q61L)	Doye et al., 2002	
6	pKH3-HA3-HA-Cdc42(Q61L)	Doye et la., 2006	
7	pKH3-HA3-HA-RhoA(L63)	Doye et la., 2006	
8	pRK5-myc-HACE1(WT)	Andrio et al., 2017	
9	pRK5-myc-HACE1(S385A)	This study	mutagenesis of plasmid #8 using primers #1-2
10	pRK5-myc-HACE1(S385E)	This study	mutagenesis of plasmid #8 using primers #3-4
11	pXJ40-HA -Rac1(T17N)	Doye et el., 2002	
12	pKH3-HA3-Cdc42(T17N)	This study	mutagenesis of pKH3-HA3-Cdc42WT (Doye et al., 2006) using primers #7-6
13	pRK5-myc-Rac1(Q61L)	Visvikis et al., 2008	
14	6His-HACE1(WT)	Torrino et al., 2011	
15	6His-HACE1(C876S)	This study	mutagenesis of plasmid #14 using primers #5-6
16	6His-HACE1(S385A)	This study	mutagenesis of plasmid #14 using primers #1-2
17	6His-HACE1(S385E)	This study	mutagenesis of plasmid #14 using primers #3-4
18	pSY5M-6His-PAK1	This study	See methods
19	pXJ-Flag-PAK1(K141A)	This study	Mutagenesis of plasmid pXJ-Flag-PAK1 (Ng et al., 2010) using primers #7-8
20	pXJ40-GST-KID2 (85-144)	This study	See methods
21	pRGB4-6His-Ub	Doye et al., 2006	
22	pKH3-HA3-ANK (1-257aa)	Andrio et al, 2017	
23	pKH3-HA3-MID (258-571aa)	Andrio et al, 2017	

25	pKH3-HA3-HECT (574-909aa)	Andrio et al, 2017
26	pKH3-HA3-ANK+MID (1-571)	Andrio et al, 2017
24	pKH3-HA3-MID+HECT (258-909aa)	Andrio et al, 2017
25	pCMV-Tag2B-Flag-ANK+MID (1-574a)	Andrio et al, 2017

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Supplementary table S2: Primer List

#	Primer name	Sequence
1	HACE1(S385A)-Fw	5'- GAA TTG ATG AAA AAC AAA AGA GAC GCA ACA GAG ATC ACT TCT ATT TTA C
2	HACE1(S385A)-Rev	5'- GTA AAA TAG AAG TGA TCT CTG TTG CGT CTC TTT TGT TTT TCA TCA ATT C
3	HACE1(S385E)-Fw	5'- CAC AGA ATT GAT GAA AAA CAA AAG AGA CGA AAC AGA GAT CAC TTC TAT TTT ACT GAA A
4	HACE1(S385E)-Rev	5'- TTT CAG TAA AAT AGA AGT GAT CTC TGT TTC GTC TCT TTT GTT TTT CAT CAA TTC TGT G
5	HACE1(C876S)-Fw	5'- CTT TTA CCA ACT TCA AGC ACA TCC ATC AAC ATG CTC AAG
6	HACE1(C876S)-Rev	5'- CTT GAG CAT GTT GAT GGA TGT GCT TGA AGT TGG TAA AAG
7	Cdc42(T17N)-Fw	5'- GGT GCT GTT GGT AAA AAC TGT CTC CTG ATA TCC TAC
8	Cdc42(T17N)-Rev	5'- GTA GGA TAT CAG GAG ACA GTT TTT ACC AAC AGC ACC
9	PAK1(K141A)-Fw	5'- GTC AGG CGT ACA TGA GTT TTA C
10	PAK1(K141A)-Rev	5'- GTA CGC CTG ACT ATT GGA G

Supplementary table S3: Antibody List

Antibody	Clone Number	Supplier
Rabbit monoclonal anti-HACE1	EPR7962	Abcam
Mouse monoclonal anti-HA.11	16B12	Biologend
Rat monoclonal anti-HA	3F10	Roche
Mouse monoclonal anti β -Actin	AC-74	Sigma-Aldrich
Mouse monoclonal anti-c-Myc	9E10	Biologend
Rabbit polyclonal anti-PAK1	N/A	CST
Rabbit polyclonal anti-PAK2	N/A	CST
Mouse monoclonal anti-GST	26H1	CST
Mouse monoclonal anti-Flag	M2	Sigma-Aldrich
Rabbit polyclonal anti-pS6(S235/236)	N/A	CST
Rabbit polyclonal anti-pS385	N/A	This study*
Polyclonal Swine anti-mouse immunoglobulin HRP	N/A	DAKO
Polyclonal Rabbit anti-rat immunoglobulin HRP	N/A	DAKO
Polyclonal Goat anti-rabbit immunoglobulin HRP	N/A	DAKO

*See: methods

4.2. Implication of HACE1 in epithelial cell-cell adhesion

Taking into account HACE1's important role in pathophysiology and the poor understanding of the regulation of HACE1's activity, our team performed a yeast two hybrid screen seeking to identify novel HACE1 interactors that might be targets or regulatory adaptors. We successfully identified partners of HACE1 like OPTN, an autophagy adaptor that we recently demonstrated to be a regulatory adaptor of HACE1 activity towards Rac1, with important implications in mechanotransduction (Hamaoui *et al.*, under review). Interestingly, several proteins implicated in cell-cell adhesion, including E-cadherin and α -catenin, were also identified as possible partners of HACE1; which made us wonder whether HACE1 has a role in the control of cell-cell junctions and in particular of adherens junctions.

HACE1 interacts with α -catenin

In order to validate the interaction of α -catenin and E-cadherin with HACE1 detected by the yeast two hybrid screen, we performed co-immunoprecipitation (Co-IP) experiments, pulling down HA-tagged HACE1 and probing the Co-IP fraction with antibodies against α -catenin or E-cadherin. These experiments were done in the human mammary epithelial cell line MCF12A, a commonly used epithelial model in which HACE1 activity towards Rac1 is regulated by phosphorylation (section 4.1) and in which Rac1 ubiquitination by HACE1 is crucial for controlling cell migration and proliferation (Goka and Lippman 2015), both of which involve cell-cell junctions remodeling. As shown in **figure 4.1**, we succeeded to detect an interaction between HACE1 and α -catenin in MCF12A cells. However, we could not detect an interaction with E-cadherin in all the experimental conditions tested (different detergents and salt concentrations in lysis and washing buffers, different order of pull-down). This negative result suggests that either the interaction found in the yeast two hybrid screen is a false-positive, or that the interaction is very faint and/or transient, and we did not find the optimal experimental conditions to detect it.

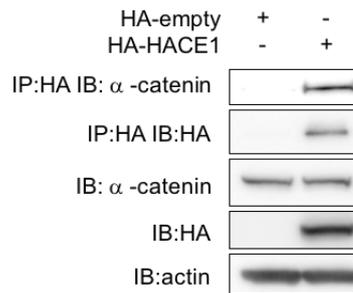


Figure 4.1. HACE1 interacts with α -catenin. Immunoprecipitation (IP) of HA-HACE1 in MCF12A cells showing that HACE1 interacts with endogenous α -catenin; correct expression of proteins of interest was verified by immunoblot (IB).

Despite the fact that we only confirmed the interaction of HACE1 with α -catenin and not with E-cadherin, this result still suggests that HACE1 might have a role in α -catenin signaling; notably in the regulation of cell-cell junctions.

HACE1 is required for epithelial integrity

To explore the possibility that HACE1 is involved in the regulation of intercellular adhesion, we generated cell lines that stably repressed the expression of HACE1 by transducing MCF12A cells with a lentiviral vector encoding shRNA targeting HACE1 (shHACE1), or control shRNA for control cell lines (shCtr). We validated the silencing of HACE1 in this cell line by western blot (**Fig. 4.2A**), which is reflected in the disrupted control of active Rac1 levels after CNF1 intoxication in shHACE1 cells (**Fig. 4.2B**). Remarkably, we observed that the cells expressing shHACE1 no longer had a conventional epithelial morphology but instead tended to be elongated, and they never formed homogenous “cobble stone-like” monolayers (**Fig. 4.3A**). To functionally evaluate the cohesion of the shHACE1 cell monolayer, we performed a monolayer permeability assay based on the measurement of the amount of Dextran-FITC that diffused through a cell monolayer seeded on a porous membrane sitting between two compartments. This assay allowed us to determine that cells lacking HACE1 formed significantly looser, more permeable monolayers compared to control cells (**Fig. 4.3B**), which is in agreement with our first observation of perturbed monolayer morphology in shHACE1 cells.

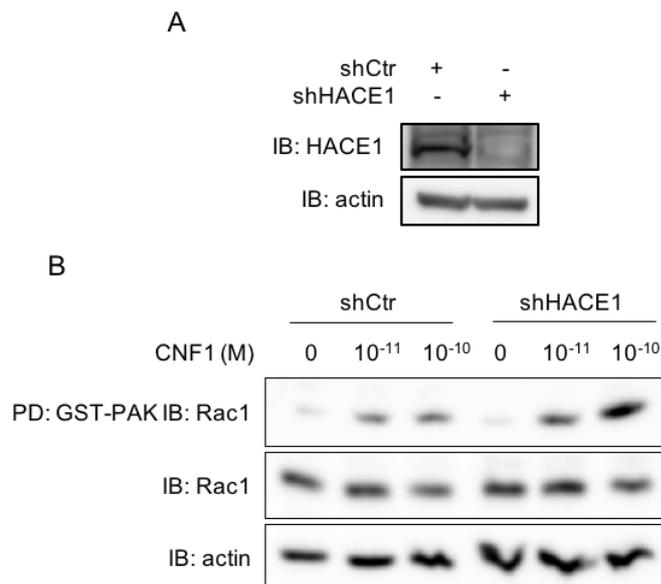


Figure 4.2. Generation of MCF12A shHACE1 cell lines. **A.** Immunoblot (IB) showing the successful knock down of HACE1 in shHACE1 cells. **B.** IB showing that in shHACE1 cells, CNF1 intoxication leads to increased Rac1 activation compared to shCtr cells. Active levels of Rac1 were isolated from cell lysates by co-precipitation with GST-PAK bound beads (PD). PD and total protein levels of Rac1 in the cell lysates were analyzed by IB.

HACE1 is required for AJ integrity

Considering that intercellular adhesion is vital to maintain epithelial polarity, morphology and tissue cohesion, we wondered whether loss of HACE1 disrupts epithelial adherens junctions (AJ). **Figures 4.3C** and **4.3D** show the great difference in morphology between the shCtr and the shHACE1 cells, the first readily forms organized and tightly packed monolayers while the second grows into a disorganized mesh of elongated cells. Specifically, **figure 4.3C** shows that shHACE1 cells do not form strong cell-cell cadherin-based junctions and, in general, shows very low signal levels for E-cadherin compared to control cells; while **figure 4.3D** shows that β -catenin, a direct cytosolic interactor of E-cadherin at AJ, is still expressed in shHACE1 cells but is not as strictly localized at the membrane as in control cells. In accordance with these results, the protein levels of E-cadherin and its associated catenins (α -, β -, and p120-catenin) are all downregulated in cells that repress HACE1 expression (**Fig. 4.3E**).

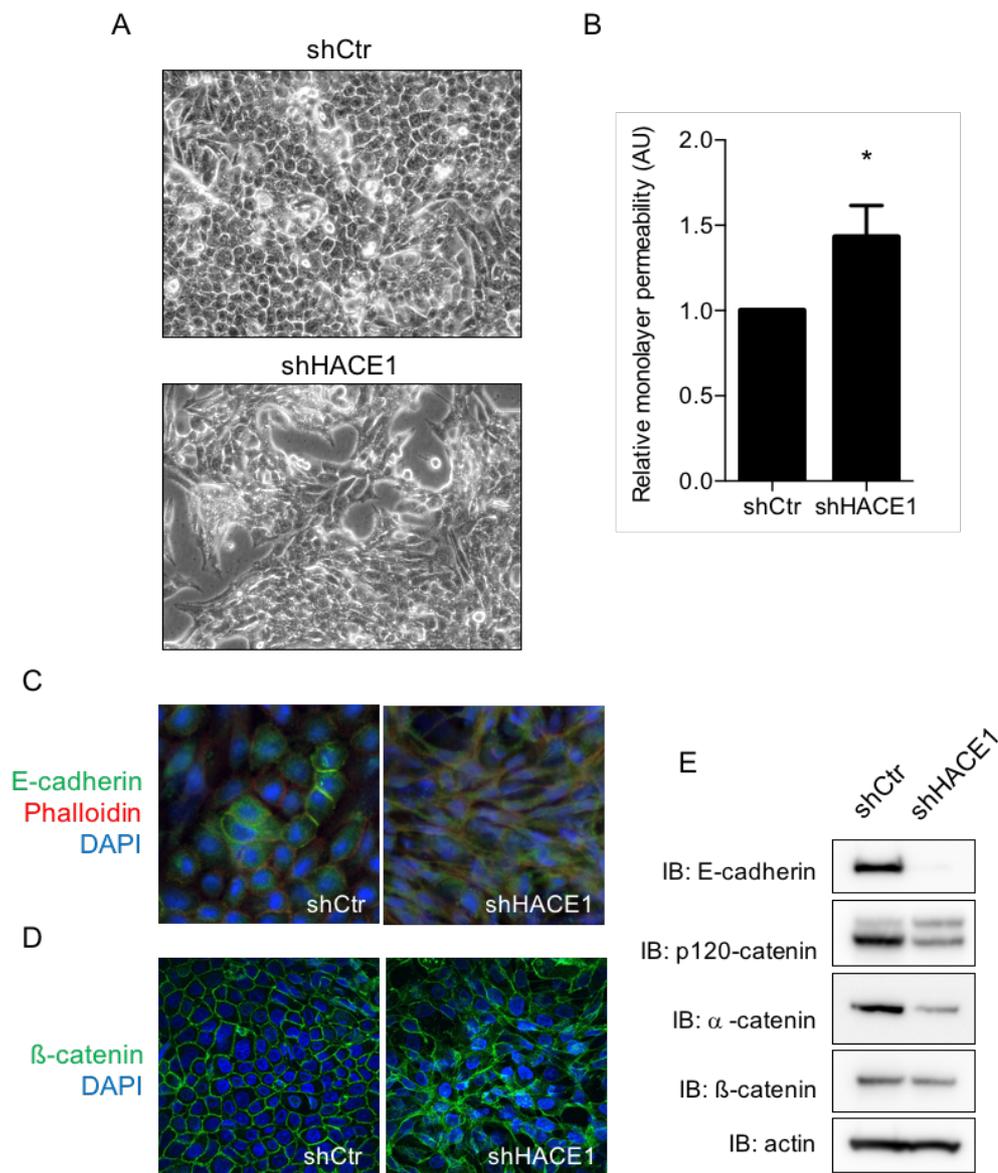


Figure 4.3. Loss of HACE1 disrupts the epithelial monolayer integrity. **A.** Bright field images of two MCF12A cell lines stably expressing either control shRNA (shCtr), or shRNA against HACE1 (shHACE1). **B.** Monolayer permeability to Dextran-FITC of shCtr and shHACE1 cells. Data correspond to mean \pm SD of 3 biological replicates. * $p < 0.05$ (one-sample t-test). **C-D.** IF of monolayers of shCtr and shHACE1 cells showing F-actin (phalloidin-TRITC: red), the nuclei (DAPI: Blue), and E-cadherin (C) or β -catenin (green) and the nuclei (DAPI: Blue) (D). **E.** IB showing that MCF12A shHACE1 cells present very low protein levels of E-cadherin and its associated catenins (α -catenin, β -catenin, and p120-catenin) compared with shCtr cells.

HACE1 prevents epithelial-mesenchymal transition of MCF12A cells

The down-regulation of epithelial cell-cell junction proteins along with the acquisition of a mesenchymal-like morphology are both characteristic events during epithelial-

mesenchymal transition (EMT), a differentiation program which epithelial cells undergo to transform into motile mesenchymal cells during physiological processes such as development and tissue repair, but also during cancer progression, where it contributes to metastasis (Kalluri and Weinberg 2009). Since HACE1 is widely reported as a tumor suppressor, and has been shown to be involved in the control of cell migration, proliferation and ROS production (Zhang et al. 2007; Castillo-Lluva et al. 2012; Daugaard et al. 2013; Goka and Lippman 2015), we hypothesized that HACE1 depletion may promote EMT.

To assess this hypothesis, we compared the expression levels of a set of conventional epithelial and mesenchymal markers and EMT-involved proteins (transcription factors and kinases) in control and shHACE1 cells (**Fig. 4.4A**). We observed once again that cell-cell junction proteins, which are epithelial markers, are expressed at very low levels in shHACE1 cells; whereas the mesenchymal markers Fibronectin, Cd44 and N-cadherin were up-regulated. However, not all the mesenchymal markers tested were expressed at higher levels in shHACE1 cells. For instance, SPARC and MMP14, which are both matrix remodeling and organizing proteins, were curiously downregulated. On the other hand, it is worth pointing out that shHACE1 cells have unequivocally undergone the cadherin switch, one of the hallmarks of EMT in which E-cadherin gets replaced by N-cadherin at junctions. This switch is known to lead to loss of cell polarity, weakened connections between epithelial cells and increased cell migration (Lamouille et al. 2014). We have also observed this event in an independent shHACE1 cell line expressing a different shRNA sequence (**Fig. 4.4B**). All together, these data indicate that the loss of HACE1 leads to the acquisition of an EMT-like phenotype.

HACE1 loss induces an EMT-promoting transcriptional program

EMT is a multifaceted and flexible program defined by known hallmarks. At the same time, EMT can be initiated and progress by different mechanisms depending on cell type and tissue context (Lamouille et al. 2014). One of the earliest hallmarks of EMT is the loss

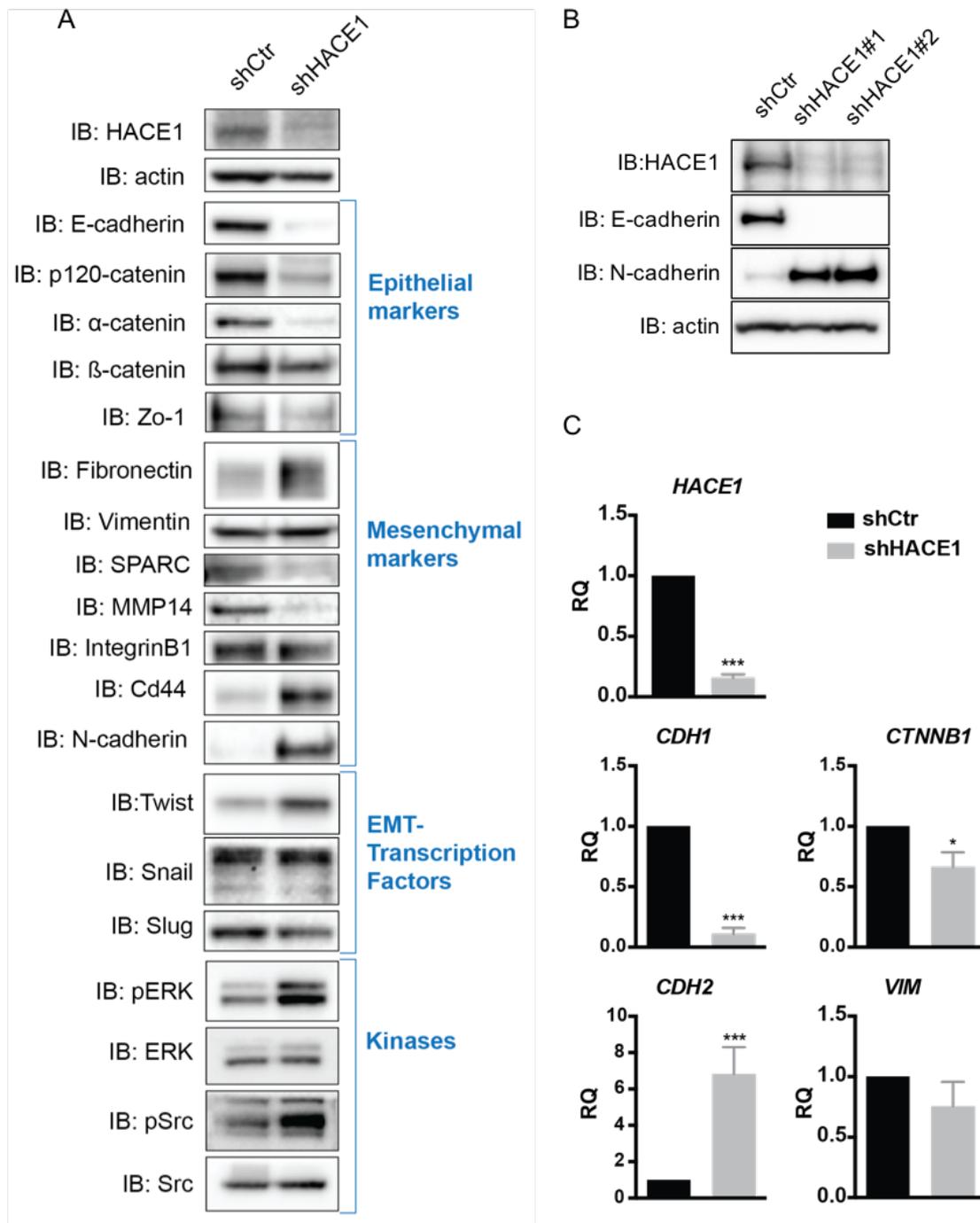


Figure 4.4. HACE1 loss results in the acquisition of an EMT-like signature. **A.** Immunoblot (IB) showing the expression profile of epithelial and mesenchymal markers, as well as EMT-promoting transcription factors in MCF12A cells shCtrl and shHACE1. **B.** The E-cadherin to N-cadherin switch occurs in two independent cell lines expressing different shRNA sequences against HACE1 (IB). **C.** mRNA levels of HACE1, E-cadherin (*CDH1*), β -catenin (*CTNNB1*), N-cadherin (*CDH2*) and Vimentin (*VIM*) in shCtrl and shHACE1 cells. Data correspond to mean \pm SD of 3 biological replicates. * $p < 0.05$, *** $p < 0.001$, blank: not significant (one-sample t-test).

of cadherin-based junctions, which can be disrupted at the protein level and/or at the transcriptional level by the induction of master transcription factors that promote the repression of epithelial genes like E-cadherin and the expression of mesenchymal genes. Among these EMT-promoting transcription factors are Snail, Slug, Twist and ZEB (Peinado et al. 2007). We tested whether the expression of some of these transcription factors was upregulated in shHACE1 cells, and found that Twist in particular was clearly more expressed in shHACE1 cells than in control cells (**Fig. 4.4A**). In agreement with this, we found that the mRNA levels of E-cadherin and β -catenin were downregulated, while the levels of N-cadherin were upregulated (**Fig. 4.4C**). All of which indicated that the EMT-like phenotype observed in shHACE1 cells was regulated at the transcriptional level.

The Mesenchymal-like phenotype of HACE1 depleted cells is not an immediate consequence of HACE1 loss.

Next, we wondered whether this phenotype was the direct result of the loss of HACE1 catalytic activity. Therefore, we attempted to evaluate the effect of the re-expression of HACE1 WT and of its catalytically deficient mutant HACE1(C876S) in shHACE1 cells. However, technical difficulties precluded us from using this approach. Namely, transient methods of transfection were very inefficient and stable transfection using retroviruses proved lethal to the shHACE1 cells. Due to this, we instead tested whether some of the EMT markers whose expression is modulated in shHACE1 cells were also regulated when HACE1 was silenced using siRNA for 72h in MCF12A cells, or when HACE1(WT) or its catalytically inactive mutant HACE1(C876S) was over-expressed for 24h. The results show that none of these short-term manipulations of HACE1 protein levels resulted in a change of expression of the EMT markers tested (**Fig. 4.5A, 4.5B**), with the exception of α -catenin, whose protein levels present a small decrease upon HACE1 silencing. These results show that the phenotype of shHACE1 cells is not an immediate consequence of the loss of HACE1, which led us to think that the loss of HACE1 is not directly affecting the stability or the expression of the analyzed EMT markers, but probably is contributing to other signaling pathways that in turn trigger the EMT program.

The EMT-promoting signaling network activated by HACE1 loss partially depends on extracellular signals and might be connected to α -catenin degradation.

We next addressed the question of which signaling pathways initially trigger the EMT program once HACE1 is lost? A multitude of signaling pathways are involved in the initiation and regulation of EMT. Among the more prominent ones are those triggered by TGF- β , and by growth factors through receptor tyrosine kinases (RTK), such as the EGF, HGF, FGF (fibroblast growth factor), and IGF1 (Insulin-like growth factor 1) pathways (Lamouille et al. 2014). Moreover, these pathways have been shown to crosstalk and cooperate extensively during EMT (Lindsey and Langhans 2015). All of this makes it challenging to determine which of these pathways might be modulated by the loss of HACE1. As a first general approach, we tested whether the EMT-like signature present in shHACE1 cells was mediated by extracellular factors by stimulating cell monolayers of shCtr and shHACE1 cells with either fully complemented media, or with media without serum and growth factors after 16h of starvation (**Fig. 4.5C**). We observed that the cadherin switch in shHACE1 cells is independent of extracellular factors. Interestingly, it has been shown that the engagement of transcriptional repressors at the E-cadherin gene promoter eventually leads to a more stable epigenetic silencing of the gene by histone modifications and subsequently by DNA hypermethylation (Yilmaz and Christofori 2009). Since we have shown that the transcription factor Twist is upregulated in shHACE1 cells and that E-cadherin and N-cadherin are regulated at the transcriptional levels, it is tempting to think that such an epigenetic silencing of the E-cadherin promoter may occur in shHACE1 cells.

On the other hand, the protein levels of Fibronectin and Twist, and the phosphorylation levels of ERK and Src seem to be sensitive to extracellular factors. Indeed, the kinases ERK and Src are known to be activated downstream of several RTKs and are involved in the regulation of EMT. Src is known to induce AJ disassembly (Woodcock et al. 2009) and EMT-related transcriptional changes through STAT3 and ERK activation (Gonzalez and Medici 2014); while ERK has been reported to phosphorylate and stabilize several EMT-

transcription factors, including Twist (Hong et al. 2011; Zhu et al. 2016), which might explain why Twist levels decrease partially upon growth factor starvation in shHACE1 cells. Regarding the dramatic decrease in Fibronectin expression upon growth factor deprivation, it might be a consequence of reduced Twist activity alone or in addition to other signaling pathways that are known to promote its expression like the Wnt/ β -catenin pathway (Gonzalez and Medici 2014). Altogether, these results indicate that the EMT signature characteristic of shHACE1 cells is in part dependent on exogenous stimuli.

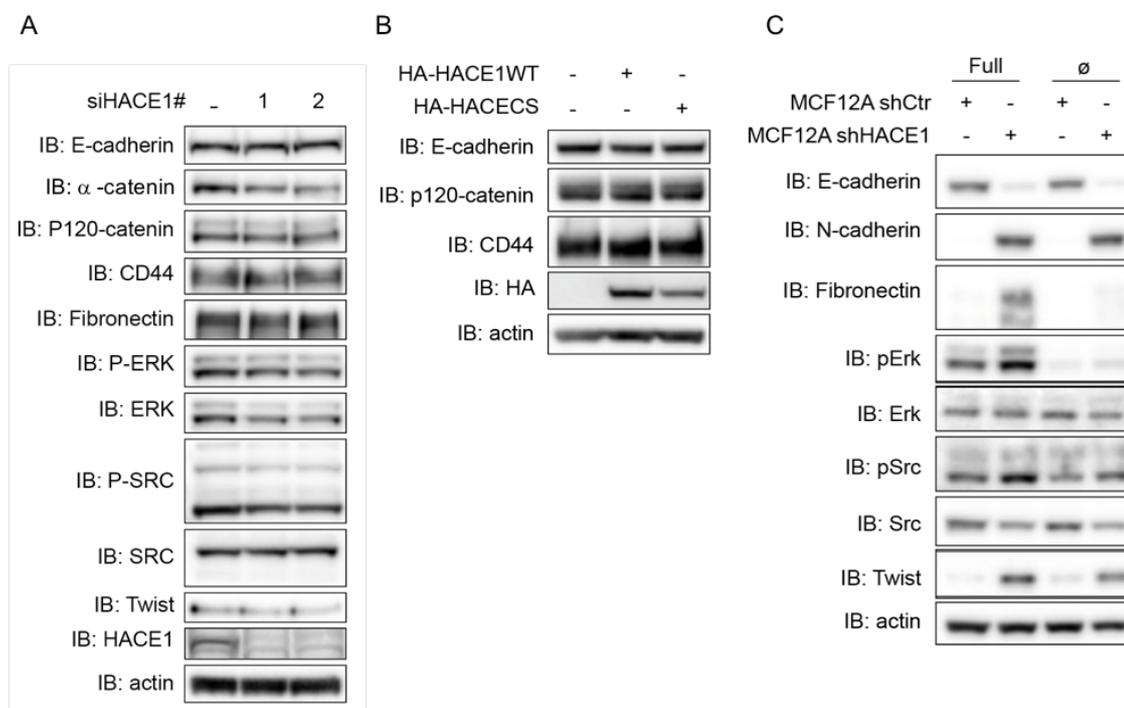


Figure 4.5. The EMT-like signature is not an immediate effect of HACE1 loss and is partially dependent on exogenous signals. **A.** Immunoblots (IBs) showing that downregulation of HACE1 using two different siRNA sequences, or **B.** over-expression of HACE1 WT or CS in MCF12A cells, does not change the expression of epithelial and mesenchymal markers. **C.** IB showing the levels of shHACE1-related EMT signature proteins in cells cultured in fully complemented medium (complete) or serum- and growth factors-free medium (\emptyset) for 6h after O/N starvation.

We have observed that α -catenin levels faintly, but consistently, decrease when HACE1 is silenced by siRNA for 72h in MCF12A cells (**Fig. 4.5A**) and they are almost completely lost in shHACE1 cells (**Figs. 4.3E, 4.4A**). Moreover, we have determined by yeast two hybrid screen and co-IP that α -catenin interacts with HACE1 (**Fig. 4.1**). This made us

wonder whether HACE1 could modulate α -catenin by catalyzing its ubiquitination. To test this, we performed ubiquitination assays in Chinese Hamster Ovary (CHO) cells over-expressing HA-tagged HACE1 and α -catenin (**Fig. 4.6**). Using this approach, we found no evidence that HACE1 induces α -catenin ubiquitination. Intriguingly, it has been suggested that α -catenin can be degraded by a proteasome-dependent and ubiquitin-independent mechanism that depends on α -catenin interaction with ARMc8 (armadillo-repeat-containing protein 8) (Hwang et al. 2005; Suzuki et al. 2008). This would be in agreement with our inability to detect ubiquitinated α -catenin, and opens the possibility that HACE1 might be involved in this ARMc8-dependent proteasomal degradation of α -catenin which should be evaluated in the future. Whichever the mechanism is that is reducing the levels of α -catenin could be involved in the EMT-like phenotype induced upon HACE loss. Indeed, α -catenin is considered a tumor suppressor whose loss leads to dysfunctional intercellular junctions that are not anchored to the actin cytoskeleton (Morton et al. 1993; Sun et al. 2014). Additionally, α -catenin has been shown to inhibit signaling pathways involved in EMT progression, such as the NF- κ B signaling in E-cadherin negative basal like breast cancer cells, and the Wnt/ β -catenin signaling in glioma and colon cancer cells (Sun et al. 2014). Interestingly, it has been shown that α -catenin proteasomal degradation is closely associated with the canonical Wnt/ β -catenin signaling (Hwang et al. 2005). More work is necessary to determine whether the downregulation of α -catenin is the trigger that leads to EMT in HACE1 depleted cells.

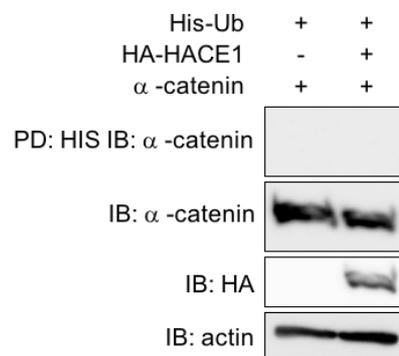


Figure 4.6. HACE1 does not ubiquitinate α -catenin. Purification of His-Ub conjugated proteins by histidine pull down in CHO cells over-expressing the indicated proteins shows that HACE1 over-expression does not induce detectable levels of α -catenin ubiquitination; correct expression of proteins of interest was verified by IB.

Conclusion

Collectively, our results draw a novel link between HACE1 and EMT. We have shown that loss of HACE1 results in the transcriptional regulation of adherens junctions through a mechanism that activates the Erk and Src kinases in response to exogenous factors and leads to the up-regulation of Twist protein levels, which is consistent with the repression of E-cadherin expression, and the upregulation of N-cadherin, Fibronectin and CD44. This study generates interesting questions about what molecular events drive this mechanism and how HACE1 is connected to them.

Discussion

a. HACE1, α -catenin and EMT.

We have shown here that HACE1 expression is required for α -catenin stability. However, although we showed that HACE1 binds to α -catenin, we could not demonstrate that HACE1 mediates its ubiquitination. Nonetheless, as discussed before, it is possible that the loss of α -catenin upon HACE1 depletion contributes to the development of the EMT-like phenotype. It would, therefore, be interesting to explore how HACE1 stabilizes α -catenin by studying the nature of their interaction, if there are any partners or scaffolds involved in it (such as ARMc8), and if they are regulated by HACE1 catalytic activity. More work remains to be done in order to assess this interesting aspect.

b. HACE1, YB-1 and EMT.

Considering the central role of HACE1 in cell homeostasis and the literature about the signaling pathways involved in EMT one can see various links that could direct further investigations on this subject. For example, it has been reported that HACE1 ubiquitinates the Y-box-binding protein 1 (YB-1), a DNA- and RNA-binding protein involved in the regulation of gene transcription, mRNA stability, protein translation, splicing and DNA repair (Palicharla and Maddika 2015). HACE1-mediated YB-1 ubiquitination is required for its secretion, which has been shown to protect cells from TGF- β triggered EMT in lung epithelial cells and in the breast cancer cell line MDA-MB 231 (Palicharla and Maddika

2015). Whether YB-1 is implicated in MCF12A EMT upon HACE1 depletion is an interesting possibility that should be addressed.

c. HACE1, Twist and EMT.

The Twist transcription factor is known to be regulated by ubiquitination, which has been reported to result in its lysosomal and proteasomal degradation. A recent study has shown that macro-autophagy deficiency, through p62 accumulation, stabilizes Twist by promoting the binding of p63 to Twist's polyubiquitinated form, which inhibits its degradation (Qiang et al. 2014). Interestingly, it has been shown that loss of HACE1 leads to impaired p62-mediated autophagy and therefore results in the accumulation of p62 (Zhang et al. 2014; Liu et al. 2014). It is therefore tempting to think that HACE1 might control Twist activity by moderating the levels of p62 and promoting Twist degradation.

Additionally, it has been shown that the stability of Twist is regulated by phosphorylation; JNK, ERK, and p38 MAPKs phosphorylate Twist at Ser68, which inhibits its ubiquitination and protects Twist from ubiquitin-mediated degradation and increases its activity (Hong et al. 2011). MAPKs are activated by several RTKs, notably by the EGFR family, which has been shown to promote Twist1 activity (Gonzalez and Medici 2014; Zhu et al. 2016). Considering that shHACE1 cells show elevated levels of phosphorylated Erk and Src, it would be interesting to test whether the inhibition of these kinases can destabilize Twist in shHACE1 cells. Further work would also be necessary to determine the particular signaling pathways leading to Erk and Src activation upon HACE1 depletion.

d. HACE1, Rac1 dependent signaling and EMT

Alternatively, it is possible that over-activation of Rac1 due to loss of HACE1 might play a role in the development of the EMT-phenotype. It has been demonstrated that HACE1, through its capacity to bridle Rac1 activity, represses cell migration, proliferation, and ROS production (Zhang et al. 2007; Castillo-Lluva et al. 2012; Daugaard et al. 2013; Goka and Lippman 2015). All of these three processes are correlated with AJ remodeling, so it is worth assessing whether Rac1 activity in shHACE1 cells is necessary for the development

of EMT. Furthermore, increased Rac1 activity and abnormally high ROS levels have been correlated with genomic instability and the induction of EMT via the activation of EMT-promoting transcription factors Snail (Radisky et al. 2005) and Zeb1 (Lam et al. 2013). Hence, it would be particularly interesting to evaluate whether the MCF12A shHACE1 cells present higher ROS levels than shCtr cells and whether normalizing cellular redox balance would counteract the effects of HACE1 depletion.

So far, I have discussed molecular actors that might contribute to the EMT-like phenotype acquired by cells after long-term (>72h) HACE1 depletion. It is important to consider that the proposed mechanisms might be the product of the genomic instability characteristic of HACE1 depleted cells (Zhang et al. 2007; Daugaard et al. 2013), and (considering the time-scale) that they likely accumulate through successive generations of cells. Such a situation would cloud the untangling of the particular HACE1-dependent signaling pathways responsible for the observed phenotype. Therefore, a cautious approach to continue the study of HACE1's role in EMT would entail the development of a cellular model that enables a more refined control of HACE1 expression; such as the tet-on or tet-off inducible expression system. Having a better temporal control of HACE1 expression (and an easy way to silence and re-express HACE1) would simplify future work on this subject.

e. HACE1, EMT and pathology

This work has identified a novel role of HACE1 in the regulation of epithelial AJ and drawn a link between HACE1 loss and the development of EMT-like phenotype. Intriguingly, several studies have associated HACE1 with diseases where a disruption of the cell-cell junctions of the intestinal epithelium is implicated, including Crohn's disease (Kenny et al. 2012), Celiac disease (Einarsdottir et al. 2011), colitis and colorectal cancer (Hibi et al. 2008; Tortola et al. 2016a). Furthermore, a recent study has suggested that EMT might play an important role in the development of Crohn's disease (Scharl et al. 2015). Together, these observations point towards a potential protective role of HACE1 in inflammatory intestinal diseases that would be interesting to explore.

EMT was first described as a developmental cellular process taking place during early embryonic development. Notably, during gastrulation, neural crest migration and heart morphogenesis (Thiery et al. 2009; Savagner 2015). Interestingly, HACE1 expression been proposed to be required for proper embryonic development (Iimura et al. 2016) and genetic alterations in *hace1* have been correlated to neurodevelopmental conditions in humans (Akawi et al. 2015; Hollstein et al. 2015). Since our results involve HACE1 in the regulation of EMT in mammary epithelial cells, it would be interesting to evaluate whether the regulation of HACE1 expression or activity is important for the rapid EMT and MET (mesenchymal-epithelial transition) cycles that occur during early embryonic development.

Lastly, EMT is heavily studied in the cancer field and is generally correlated with the malignancy and the metastatic capacity of a tumor (Gurzu et al. 2015). Incidentally, Hibi and colleagues reported that colorectal carcinomas with aberrant methylation of HACE1 tend to develop lymph node metastasis (Hibi et al. 2008), and a different study suggested that in hepatocellular carcinoma, low levels of HACE1 expression are correlated with tumor differentiation and vascular invasion (Gao et al. 2016b). Therefore, it would be interesting to study the role of HACE1 in the regulation of AJ and in EMT in the context of tumor metastasis.

General Discussion and Perspectives

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5.1. Phospho-regulation of HACE1 by Rac1/Cdc42 and group I PAKs.

5.1.1. Regulation of HACE1 by phosphorylation of Ser-385 *in vivo*: Is an adaptor at play?

The first part of the results of this thesis demonstrated that HACE1 phosphorylation at Ser-385 is induced by active Rac1 and Cdc42 through the activation of group I PAKs. Puzzlingly, we observed that the phosphomimetic mutant HACE1(S385E) reduced the levels of ubiquitination of Rac1 *in vivo* but not *in vitro*. As discussed in section 4.1, these results suggest that the phosphorylation at Ser-385 does not change the intrinsic catalytic activity of HACE1 and that the reaction of Rac1 ubiquitination by HACE1 might be modulated by a cellular factor. In accordance with this last hypothesis, we have observed that the degree of inhibition of Rac1 ubiquitination upon HACE1 Ser-385 phosphorylation

was cell dependent. Indeed, the clear inhibitory effect observed in MCF12A was also observed in Chinese Hamster Ovary (CHO) epithelial cells. However, the inhibitory effect of phosphorylation of HACE1 on Ser-385 was not as strong and less consistent in HUVEC cells (not shown). These observations reinforce our hypothesis of an adaptor modulating HACE1's ability to ubiquitinate Rac1, which would be cell-specific. It is worth noting that such an adaptor could be an activator that positively modulates the activity of the non-phosphorylated form of HACE1 or an inhibitor that negatively modulates the activity of the phosphorylated-form of HACE1. However, since we detect strong auto-ubiquitination of HACE1 *in vitro*, we think it is more likely that phosphorylation of HACE1 triggers its binding to an inhibitory adaptor.

To identify regulatory adaptors of HACE1, our first approach consisted in evaluating whether known HACE1-binding proteins interact differently with phosphorylated HACE1 by co-immunoprecipitation followed by western-blot analysis. We attempted to do this with α -catenin and E-cadherin, two novel interacting candidates identified by our yeast two hybrid screen that could potentially connect HACE1 phosphorylation to AJ regulation. By this approach, we could not identify an interaction between E-cadherin and HACE1 and, although we validated the interaction of HACE1 with α -catenin, we did not detect any difference in their interaction with phosphorylated HACE1. Even though these preliminary results are negative, it does not exhaust the possibilities of the approach since HACE1 has been reported to interact with other proteins as discussed in section 3.2.2. An alternative, more unbiased approach consists in analyzing by mass spectrometry the proteins co-immunoprecipitated either with HACE1(S385A) or HACE1(S385E). This proteomic approach is currently at the technical optimization stage in the laboratory. It would also be interesting to compare the identity of the proteins that co-immunoprecipitate with HACE1 in cells that have high and low levels of PAK-mediated phosphorylated Ser-385 (by over-expression of active Rac1(Q61L) or PAK1(K161A), or by CNF1 treatment). Since our results indicate that this adaptor might be present in MCF12A cells but not (or in lower quantities) in HUVEC cells, it would be useful to do this comparative analysis in both cell lines, to narrow down the prospective candidates.

To validate the functionality of the identified adaptors, their expression could be knocked-down (KD) by RNA interference (RNAi) and ubiquitination of Rac1(Q61L) levels in presence of HACE1(WT) and HACE1(S385E) will be measured in MCF12A cells. If the adaptor is an activator of non-phosphorylated HACE1, we expect that its KD reduces HACE1(WT) activity to the level of HACE1(S385E). Conversely, if the adaptor inhibits the phosphorylated form of HACE1, we expect that its KD restores HACE1(S385E)'s ability to ubiquitinate Rac1 up to HACE1(WT) level. The identification of adaptor proteins of HACE1 whose interaction is modulated by HACE1 phosphorylation would cast light on the cellular relevance of this new regulatory mechanism. It could place HACE1 within a signaling network and indicate its probable intracellular localization.

As discussed in section 1.3, there are about a dozen examples in the literature of adaptors/auxiliary proteins that modulate the function of HECT E3 ligases. While most of them pertain members of the Nedd4 family and only two regulate ligases outside of this family, all of them modulate the function of the HECT E3 ligase(s) by affecting one or more the following aspects: (i) interaction with E2 enzymes, (ii) E3 localization and/or substrate binding, and (iii) intrinsic catalytic activity. Therefore, it would be interesting to determine if HACE1's hypothetical adaptor affects some of these three aspects.

(i) Interaction with E2. HACE1's ability to interact with E2 enzymes *in vivo* after it is phosphorylated could be tested by evaluating whether the UbcH7 E2 enzyme (reported to work well with HACE1 (Anglesio et al. 2004; Torrino et al. 2011)) immunoprecipitates with HACE1(S385E) at a different rate than with HACE1(S385A). Alternatively, the answer to this question could be given by the previously proposed mass spectrometry (MS) analysis of HACE1-interacting proteins that depend on phosphorylation of Ser-385 if the change in interaction with the E2 is dramatic.

(ii) Substrate binding and localization. We have defined that HACE1(S385E) binds to activated Rac1 as well as with the non-phosphorylated HACE1(WT) and HACE1(S385A). However, this does not exclude that the phospho-regulation of Ser-385 could affect HACE1's binding to other targets. Thus, the MS analysis could reveal targets whose

interaction with HACE1 is modified by HACE1 phosphorylation. To more directly evaluate whether the target candidates, derived from the MS analysis or from the literature, are ubiquitinated more or less once HACE1 is phosphorylated, we could perform *in vivo* ubiquitination assays with them and with either HACE1(WT), HACE1(S385A) or HACE1(S385E). Regarding the possibility that HACE1 phosphorylation might affect its cellular localization, and consequently, its proximal targets, we did some preliminary immunofluorescence and cytosol-membrane fractionation experiments to compare the localization of HACE1(WT) against the S385A and S385E mutants. These experiments did not yield an obvious change in localization, which might be true but could also be a technical artifact caused by saturation of the system due to HACE1 over-expression. Further work would be necessary to develop techniques to determine the localization of endogenous HACE1 before and after it has been phosphorylated.

(iii) E3 catalytic activity. Subtle changes in the intrinsic catalytic activity of an E3 ligase are difficult to examine *in vivo*. Therefore, to evaluate the possible influence of a cellular adaptor on the enzymatic steps catalyzed by HACE1, *in vitro* assays need to be performed. This requires the prior identification of the adaptor, in order to include it in *in vitro* HACE1 auto-ubiquitination or Rac1 ubiquitination assays and evaluate whether the kinetics of the reaction changes. We would specifically evaluate the level of ubiquitin transfer from E2 to E3 (thioester bond formation) and the level of ubiquitin transfer from the catalytic cysteine to a target lysine residue in HACE1 (isopeptide bond formation).

Alternatively, we could consider that HACE1 phosphorylation on Ser-385 in *in vivo* inhibits HACE1 activity towards Rac1 through a cellular factor that does not bind to HACE1 (and thus would not be detectable by a co-IP approach). For instance, it has been shown that the activity of a DUB towards a specific substrate may be promoted by a change in the type of ubiquitin chain conjugated upon it or by a change in the mechanism of ubiquitin transfer from E3 to substrate. Indeed, it has been shown that upon binding to a chemical inhibitor Nedd4-1 changes its conjugating mechanism from processive (it transfers several ubiquitin monomers to the substrate in one round of binding) to distributive (it needs to release and bind its substrate every time it attaches a ubiquitin), and this makes the

substrate ubiquitination more efficiently reversed by the DUB USP8 (Kathman et al. 2015). This possibility can be evaluated by inhibiting DUB activity in cells using commercial chemical inhibitors or RNAi and testing whether the decreased ubiquitination of Rac1 by HACE1(S385E) is restored to HACE1(WT) levels.

5.1.2. How can the conformational change upon Ser-385 phosphorylation affect HACE1 function?

In addition to the lower activity towards Rac1 of the HACE1(S385E) mutant, another interesting result of the study is that phosphorylation of Ser-385 is correlated with an increased capacity to homo-oligomerize, suggesting that structural rearrangements occur upon HACE1 phosphorylation. As discussed before, many HECT E3 ligases (mostly of the Nedd4 family) activities are regulated by intra- or inter-molecular interactions. In light of this, one can wonder what the relationship is between the modulation of HACE1's function and oligomerization state upon its phosphorylation *in vivo*.

In order to assess this question, we would need to determine how the HACE1(S385E) oligomers are formed (what are the interaction regions) and be able to disrupt them *in vivo*. By blocking the formation of the HACE1(S385E) oligomers and subsequently assessing whether this disruption affects the activity of HACE1(S385E) towards Rac1, we could determine if this change in oligomerization state due to phosphorylation of Ser-385 affects HACE1 activity in cells. A first strategy to characterize the oligomers formed by HACE1(S385E) would be to map the region(s) of interaction in HACE1(S385E) oligomers by inter-domain co-immunoprecipitation assays in cells. One could propose, based on our inter-domain interaction assays for HACE1 (WT), that the oligomerization of HACE1 occurs between its HECT domain and a region overlapping the ANK domain and the MID region. We are currently collaborating with the laboratory of Jaqueline Cherfils in Paris to elucidate the crystal structure of HACE1, this might help to model and understand how phosphorylation of Ser-385 might affect its conformation.

In section 5.1.1, we have discussed how a cellular factor is most likely required for the phospho-regulation of HACE1 activity. Considering this, and if we find that the change in

oligomerization state upon Ser-385 phosphorylation is required for the modulation of HACE1 activity, we might then ask what the relationship is between the change in oligomerization and the cellular adaptor. Reducing our analysis to the possibility that the adaptor is an inhibitor, two scenarios come to mind: i) the phosphorylation of HACE1 at Ser-385 induces the binding to an adaptor, which then elicits the oligomerization change (**Fig. 5.1a**); or ii) the phosphorylation is inducing a change in the oligomerization state, that leads to the recruitment of an adaptor (**Fig. 5.1b**). An approach to discriminate between the two possibilities would be to compare the oligomerization states of HACE1(WT) and HACE1(S385E) *in vitro* using size exclusion chromatography (SEC). If their elution profile is the same, it would suggest that the higher order oligomerization of HACE1(S385E) observed in cells depends on a cellular factor. In that case, purification of the recombinant adaptor would be helpful to determine if, indeed, this adaptor shifts the SEC elution spectra of HACE1(S385E). Conversely, if HACE1(S385E) is eluted faster than the WT, it suggests that oligomerization of HACE1(S385E) occurs in absence of an adaptor. Additionally, the SEC analysis could be supported by crosslinking assays of recombinant purified HACE1(WT) and HACE1(S385E) followed by western-blot analysis to determine the proportion of HACE1(WT) and HACE1(S385E) that forms oligomers *in vitro* and the size of these oligomers in the absence or presence of the adaptor. Cellular assays could also be performed once the adaptor is known. For instance, we could evaluate the level of homo-oligomerization of HACE1(WT) and HACE1(S385E) by co-IP in cells where the expression of the adaptor has been KD by RNAi. If we find that HACE1(S385E) (or HACE1 in the presence of active PAK1) still homo-oligomerizes in a much stronger manner than the non-phosphorylated HACE1(WT) or HACE1(S385A), this would mean that the adaptor is not required for phospho-oligomer formation. Conversely, if the KD of the adaptor reduces the level of HACE1(S385E) oligomerization to the level of HACE1(WT), it indicates that this adaptor is necessary for HACE1(S385E) oligomerization.

Altogether, these analyses could shed light on the functional impact of HACE1's altered oligomerization upon Ser-385 phosphorylation and on the nature of this structural rearrangement. Specifically, the study of HACE1(S385E) would identify the interaction

regions, and determine whether the strong homophilic interaction of phosphorylated HACE1 is due to a gain in affinity (either interacting through the same or different regions than HACE1(WT)), or due to the formation of higher-order oligomers (trimers, tetramers, etc). In addition, we would have a better understanding of the mechanism by which the cellular adaptor influences HACE1's function and conformation upon Ser-385 phosphorylation.

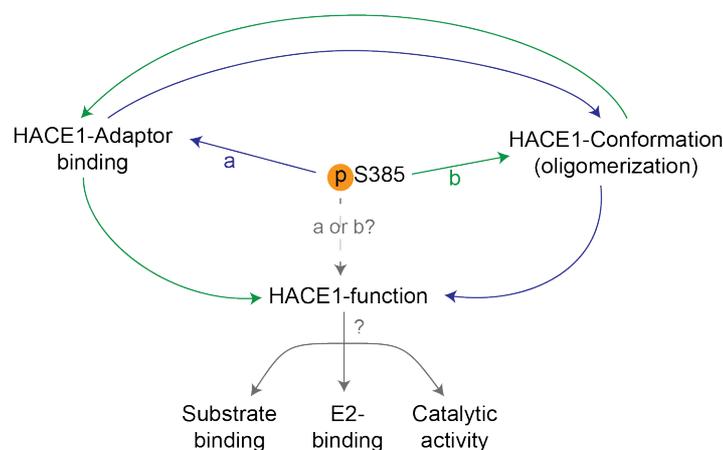


Figure 5.1. Representation of the possible relationships between p-HACE1S385, the binding to a cellular adaptor, the change in HACE1 oligomerization state and the modulation of HACE1's function.

5.1.3. PAK controls Rac1 ubiquitination by HACE1: signaling implications

The interplay between Rho GTPases, specially the antagonism between Rac1 and RhoA signaling, has been shown to occur in multiple contexts and by several mechanisms (Guilluy et al. 2011). Less known, however, is the crosstalk between Cdc42 and Rac1. To date, and to our knowledge, two mechanisms of Rac1 regulation by Cdc42 have been reported. The first one showed that Cdc42 limits Rac1 activity at the front of migrating cells through PAK-mediated recruitment and activation of the Rac-specific GEF β -Pix (Cau and Hall 2005), and the second study showed that in murine stem cells, loss of Cdc42 down regulated the levels of GTP-bound Rac1 without altering the total levels of Rac1 (Wu et al. 2007). In both cases, Cdc42 regulates Rac1 activation. Our study indicates that

there might be another mechanism of crosstalk between Cdc42 and Rac1 by demonstrating that group I PAKs (activated by both active Rac1 and Cdc42) control HACE1-mediated Rac1 ubiquitination. Therefore, our findings suggest that Cdc42, through PAK, could promote Rac1 activity by inhibiting its ubiquitin-mediated Rac1 degradation in addition to stimulating GEF-mediated Rac1 activation (schematized in figure **Fig. 5.2**). In the future, it would be interesting to determine where this regulation occurs; for instance, if it takes place in ruffles to allow sustained extension of Rac1-dependent lamellipodia.

We have shown that silencing PAK1 or PAK2 in MCF12A cells by siRNA inhibits the phosphorylation of HACE1 at S385 induced by Rac1, indicating that both kinases are required for Rac1-induced phosphorylation of HACE1 and that the presence of only one PAK isoform is not enough to reach full levels of phosphorylation. Interestingly, these results suggest that PAK1 and PAK2 might form active homo-dimers in MCF12A cells. To date, the formation of such a heterodimer has only been described for PAK1 and PAK3 in the brain, and has been shown to inhibit PAK activity (Combeau et al. 2012). Further studies on the relationship between PAK1 and PAK2 in mammary epithelial cells would be required to test this hypothesis.

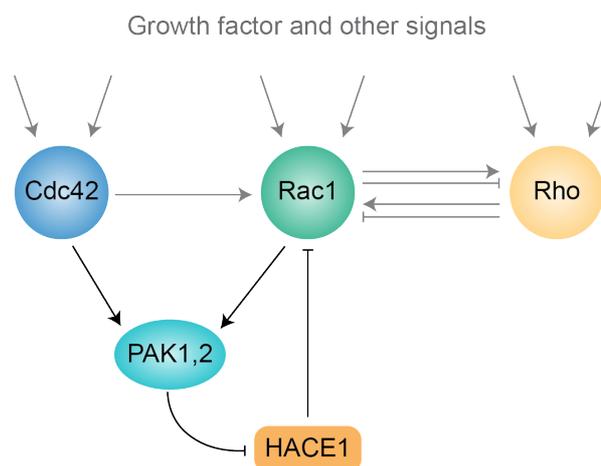


Figure 5.2. Crosstalk between Rho, Rac1 and Cdc42. The Cdc42-Rac1/PAK/HACE1 axis constitutes a novel crosstalk pathway between Cdc42 and Rac1 as well as a positive feedback loop for Rac1. Adapted from {Samuel2011}

5.2. HACE1 and epithelial cell-cell adhesion: keeping EMT at bay.

5.2.1. Does HACE1 have a role in the establishment and maintenance of AJ?

We have shown that long term HACE1 depletion in MCF12A starkly reduces the mRNA and protein levels of AJ core complex components (E-cadherin, p120-, α -, and β -catenin). This, coupled with the interaction between HACE1 and α -catenin and the small downregulation of α -catenin protein levels when HACE1 was depleted for 72h with siRNA, makes us wonder if HACE1 has a role in the short-timed regulation of epithelial AJs (establishment and maintenance). Furthermore, CNF1 has been shown to greatly induce uroepithelial cell motility (Doye et al. 2002) and our own preliminary results show that this is also the case in MCF12 cells (not shown). Additionally, PAK1 has been shown to disrupt E-cadherin based adhesions in keratinocytes (Lozano et al. 2008), and during embryonic development in drosophila (Pirraglia et al. 2010). We thus wonder whether the phosphorylation of HACE1 on Ser-385 induced by CNF1/Rac1-mediated PAK activation is involved in AJ regulation.

In order to determine the potential role of HACE1 in the establishment and maintenance of AJ, one interesting approach would be to generate a stable MCF12A cell line with an inducible (like the tet-on or tet-off system) shRNA that represses HACE1 expression in a controlled manner. With these cells, we could allow the formation of a mature epithelial monolayer before inducing HACE1 depletion. Assessing AJ integrity by evaluating monolayer permeability, E-cadherin protein levels, and E-cadherin localization in HACE1 depleted monolayers would indicate whether HACE1 is necessary for AJ maintenance.

In order to assess whether HACE1 is required during *de novo* AJ formation, we could repeat the setting described in the previous paragraph and disrupt E-cadherin cell-cell contacts in the mature monolayer by Ca^{2+} depletion and measure if junctions are re-established upon addition of Ca^{2+} in HACE1 depleted cells.

To evaluate whether phosphorylation on Ser-385 has a role in AJ formation and maintenance, we could attempt to repeat the previously suggested experiments using

transgenic MCF12A stable cells that bear an inducible shRNA against HACE1 as well as shRNA-resistant HACE1(WT), HACE1(S385E) or HACE1(S385A). This would allow us to induce the silencing of endogenous HACE1 and the simultaneous expression of shRNA insensitive mutants of HACE1, which would allow us to evaluate the capacity of HACE1 mutants to maintain (or not) AJ when endogenous HACE1 is depleted.

5.2.2. A parallel between HACE1 and PAK during EMT in cancer

At the time of its discovery, HACE1 was described as a tumor suppressor gene (Anglesio et al. 2004). Over time, this first designation has proven to be true for multiple types of human cancers, where loss of HACE1 is associated with tumorigenesis and cancer progression (Zhang et al. 2007; Hibi et al. 2008; Sakata et al. 2009; Diskin et al. 2012; Liu et al. 2014; Goka and Lippman 2015). HACE1 tumor suppressor activity has been correlated with its ability to control Rac1 hyper-activation (Castillo-Lluva et al. 2012; Daugaard et al. 2013; Goka and Lippman 2015) and in this work, we have defined that phosphorylation of HACE1 at Ser-385 by group I PAK leads to reduced ubiquitination of Rac1. Therefore, one could hypothesize that excessive PAK-mediated phospho-inhibition of HACE1 would lead to a pathological level of activation of Rac1 signaling.

In agreement with this hypothesis, PAK kinases are known to be at the center of signaling pathways required for oncogenesis and they are frequently over-expressed or hyper-activated in a wide variety of cancers, with PAK1 and PAK4 being the most commonly reported isoforms (Kumar and Li 2016). Interestingly, both the *pak* genes and *hace1* are located in chromosomal regions (11q13 and 6q21, respectively) that are hotspots of genomic alterations in human cancers (Bekri et al. 1997; Anglesio et al. 2004; Brown et al. 2008). Moreover, the cellular effects of PAKs hyper-activation and the consequences of HACE1 depletion are in some cases very similar, as listed in table 5.1. Interestingly, both HACE1 loss and PAK1 activation cooperates with HER2 overexpression in breast cancer cells to drive transformation and the acquisition of an invasive and migratory phenotype (Adam et al. 1998; Goka and Lippman 2015), indicating that HER2 over-activation signals through pathways repressed by HACE1 and promoted by PAK1 to favor cancer progression. However, not all HER2 positive breast carcinomas present HACE1 loss (Goka

and Lippman 2015), so it is possible that another mechanism of HACE1 inhibition might be involved. Taking this into account, it would be interesting to explore the expression and activity levels of PAK in HER2- and HACE1-positive breast carcinomas and whether PAK1 inhibits HACE1 by phosphorylating Ser-385 in this context.

Moreover, it has been shown that PAK1 contributes to the development of EMT during tumor metastasis by activating the master transcription factor Snail in breast cancer cells (Yang et al. 2005). Snail is then able to promote the expression of other transcription factors like Twist1 and Zeb1, and of mesenchymal markers such as Fibronectin and N-cadherin, while repressing the expression of various epithelial markers (Lamouille et al. 2014). Our findings implicate HACE1 loss in the development of EMT and, interestingly, Palicharla and colleagues proposed that ubiquitination of YB-1 by HACE1 protects cells from TNF- α induced EMT (Palicharla and Maddika 2015). Considering this, it would be interesting to explore whether the mesenchymal phenotype we observe after long-term HACE1 loss in MCF12A depends on PAK or Rac1/Cdc42 activity, and also, whether over-expression or hyper-activation of PAK promotes EMT in a HACE1-dependent manner. To assess this last question, an approach would be to generate cell lines stably expressing HACE1WT, HACE1(S385A), HACE1(S385E) or HACE1(C876S) mutant using CRISPR-cas9 knock-in on MCF12A cells and a HACE1 knock out cell line by CRISPR-cas9 (as a control). Then, we would compare if, over time, these cell lines lose their epithelial characteristics and acquire mesenchymal features by monitoring morphological changes, gain in motility and differential expression of EMT markers. We would expect that the knock out cell-line behaves like our previously studied shHACE1 cell lines. Comparison with the catalytic inactive HACE1(C876S) mutant would tell us if HACE1 E3 ligase activity is important for restraining EMT development, while the phospho-mimetic (HACE1(S385E)) and phospho-null (HACE1(S385A)) mutant would indicate whether phosphorylation of HACE1 on Ser-385, and probably PAK, play a role in EMT. Of course, the most robust way to assess the implication of HACE1 phosphorylation or catalytic activity in EMT development would be to attempt to reverse the mesenchymal phenotype of HACE1 depleted cells into an epithelial phenotype by over-expressing the

HACE1(S385A), HACE1(S385E) or HACE1(C876S) mutants. While we could take advantage of an inducible system (described in 5.2.1) to control the loss and re-expression of endogenous HACE1 and evaluate the impact on EMT, it seems that re-expressing a mutant of HACE1 in a previously HACE1 depleted cell could be challenging based on our experience. However, we cannot discard that our issues with this approach were unique of our lentiviral shRNA strategy and re-expression on a CRISPR-cas9 knock-out background would work better.

Phenotype	HACE1 depletion		PAK hyper-activity	
	Mechanism	Reference	Mechanism	Reference
→migration	— Ub and degradation of Rac1	(Castillo-Lluva et al. 2012; Goka and Lippman 2015)	TGF- β →AKT→PAK2 HER2, HRG →PI3K→PAK1	(Sato et al. 2013) (Adam et al. 1998) breast cancer
→invasion	— Ub and degradation of Rac1	(Goka and Lippman 2015) breast cancer	→MMP9 →Fibronectin expression	(Adam et al. 1998) breast cancer (Zhou et al. 2009; Jagadeeshan et al. 2015)
→ Proliferation	— G0/G1 arrest. (Rac1 → ROS → CyclinD1) →anchorage-independent growth (clonogenicity)	(Zhang et al. 2007; Daugaard et al. 2013; Liu et al. 2014; Goka and Lippman 2015)	→MAPK and MET signaling →anchorage independent growth (clonogenicity) →CyclinD1 expression → β -catenin	(Balasenthil et al. 2004; Tao et al. 2011; Shrestha et al. 2012) breast cancer (Zhu et al. 2012)
→ EMT-genetic program	— YB-1 secretion — TNF- α induced EMT	(Palicharla and Maddika 2015) (this thesis)	→Snail	(Yang et al. 2005) breast cancer

Table 5.1. Examples of common phenotypic effects of HACE1 depletion and PAK hyper-activity in cancer cells. → stands for “stimulation of”, and —| stands for “inhibition of”. Mechanisms presented in the case of HACE1 loss are all downstream of HACE1 since there are no known upstream regulators of HACE1. While for PAK, upstream activation pathways are sometimes indicated

Conclusions

It has been repeatedly shown that HACE1 plays an important role in the maintenance of cell homeostasis and that the downregulation of HACE1 expression by genetic and epigenetic mechanisms has been associated with numerous human diseases. However, the regulatory mechanisms of HACE1 activity at the post-translational level have not been studied.

My thesis work has established that HACE1 is subjected to regulation by PTM. We have demonstrated that HACE1 gets phosphorylated downstream of the Rac1-Cdc42/PAK signaling pathway, which results in the modulation of HACE1 ubiquitin ligase activity and induces changes in its oligomerization properties. Our results suggest that PAKs are not only effectors of active Rac1, but are also involved in a positive feedback loop that promotes Rac1 activity by restraining its HACE1-dependent and ubiquitin-mediated degradation.

In parallel, this work has identified a novel role of HACE1 in the regulation of epithelial adherens junctions and has drawn a link between HACE1 depletion and EMT in mammary epithelial cells. We have shown that the loss of HACE1 indirectly promotes the disruption of epithelial monolayer integrity and the acquisition of an EMT-like signature characterized by a strong E-cadherin to N-cadherin switch that is transcriptionally regulated. This is in line with the increased expression of Twist and the upregulation of the phosphorylation levels of the EMT-promoting kinases Erk and Src.

In the future, it would be interesting to define the cellular processes that are influenced by the phospho-regulation of HACE1, and to determine by which pathways HACE1 stabilizes adherens junctions and inhibits EMT.

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Collaborative work

During my PhD, I had the opportunity to participate in one of the on-going projects of the team which resulted in the publication of a research paper in the journal *Cytoskeleton* (included in the next page). In this study, we characterized the mechanical properties of the stress fibers induced by the *Bacillus anthracis* lethal toxin (LT) in HUVEC cells and determined their role in the disruption of adherens junctions observed in LT-treated cells. Additionally, we determined that the formation of LT-induced stress fibers is dependent on histone acetylation and on Rnd3/RhoE expression. Moreover, our results indicate that, in general, the HDAC/HAT histone acetylation machinery controls the organization of the actin cytoskeleton.

Working in this project allowed me to optimize experimental protocols to study the cohesion of cell monolayers (permeability assays) and visualize cell-cell junctions. Which was very useful to develop the second aim of my thesis that consisted in exploring the role of HACE1 in epithelial adherens junctions.



Contractile Actin Cables Induced by *Bacillus anthracis* Lethal Toxin Depend on the Histone Acetylation Machinery

Monica Rolando,^{1,2,3,4} Caroline Stefani,^{1,2,5} Anne Doye,^{1,2} Maria I. Acosta,^{1,2} Orane Visvikis,^{1,2} Hannah G. Yevick,⁶ Carmen Buchrieser,^{3,4} Amel Mettouchi,^{1,2} Patricia Bassereau,⁶ and Emmanuel Lemichez^{1,2*}

¹Microbial Toxins in Host–Pathogen Interactions, Equipe Labellisée La Ligue Contre Le Cancer, INSERM, U1065, Centre Méditerranéen De Médecine Moléculaire (C3M), 151 Route St Antoine de Ginestière, BP 2 3194, 06204 Nice Cedex, France

²UFR Médecine, IFR50, Faculté De Médecine, Université De Nice-Sophia Antipolis, Nice, France

³Biologie Des Bactéries Intracellulaires, Institut Pasteur, Paris, France

⁴UMR 3525, CNRS, Paris, France

⁵Immunology Program, Benaroya Research Institute at Virginia Mason, Seattle, WA 98101, USA

⁶Institut Curie—Centre de Recherche, Membrane and Cell Functions Group; CNRS UMR 168, Physico-Chimie Curie, Université Pierre et Marie Curie, 26 Rue d'ulm, Paris Cedex 05, 75248, France

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It remains a challenge to decode the molecular basis of the long-term actin cytoskeleton rearrangements that are governed by the reprogramming of gene expression. *Bacillus anthracis* lethal toxin (LT) inhibits mitogen-activated protein kinase (MAPK) signaling, thereby modulating gene expression, with major consequences for actin cytoskeleton organization and the loss of endothelial barrier function. Using a laser ablation approach, we characterized the contractile and tensile mechanical properties of LT-induced stress fibers. These actin cables resist pulling forces that are transmitted at cell–matrix interfaces and at cell–cell discontinuous adherens junctions. We report that treating the cells with trichostatin A (TSA), a broad range inhibitor of histone deacetylases (HDACs), or with MS-275, which targets HDAC1, 2 and 3, induces stress fibers. LT decreased the cellular levels of HDAC1, 2 and 3 and reduced the global HDAC activity in the nucleus. Both the LT and TSA treatments induced Rnd3 expression, which is required for the LT-mediated induction of actin stress fibers. Furthermore, we reveal that treating the LT-intoxicated cells with garcinol, an inhibitor of

histone acetyl-transferases (HATs), disrupts the stress fibers and limits the monolayer barrier dysfunctions. These data demonstrate the importance of modulating the flux of protein acetylation in order to control actin cytoskeleton organization and the endothelial cell monolayer barrier. © 2015 Wiley Periodicals, Inc.

Key Words: *Bacillus anthracis* toxin; HAT; HDAC; gene expression; actin cytoskeleton; vascular permeability

Introduction

Treatment with *Bacillus anthracis* lethal toxin (LT) provides a model system for monitoring the relationship between delayed actin cytoskeleton remodeling and gene expression [Raymond et al., 2009; Rolando et al., 2010; Trescos and Tournier, 2012]. Here, we first characterized the mechanical properties of actin cables in LT-treated cells, and we explored the relationship between the organization of the actin cytoskeleton and the enzymatic activity of both histone acetyl-transferases (HATs) and histone deacetylases (HDACs).

Numerous pathogens disrupt the endothelial barrier function of the host via targeting the organization of the actin cytoskeleton [Lemichez et al., 2010; Aktories et al., 2011]. For example, vascular leakage, edema, and hemorrhages are hallmarks of toxemia during systemic infection by the Gram-positive bacterium *Bacillus anthracis* [Abramova et al., 1993; Cui et al., 2004; Moayeri and Leppla, 2009]. *B. anthracis* synthesizes a three-component toxin; heptamers/octamers of the protective-antigen (PA₆₃) bind

Monica Rolando and Caroline Stefani contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

*Address correspondence to: Dr. Emmanuel Lemichez, INSERM U1065, C3M, 151 route Saint Antoine de Ginestière, BP 2 3194, 06204 Nice, Cedex 3, France. E-mail: lemichez@unice.fr
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to receptors on the host cell and associate with lethal factor (LF) to generate lethal toxin (LT for PA₆₃+LF) and/or with edema factor (EF) to form edema toxin (ET for PA₆₃+EF). The toxin complexes traffic through the endocytic pathway and are subsequently translocated across the endosomal membranes [Abrami et al., 2006]. Lethal factor is an endoprotease that cleaves the amino-terminal region of MAP kinases/extracellular signal-regulated kinase kinases (MEKs) and NLRP1, a key component of the inflammasome system [Duesbery et al., 1998; Moayeri et al., 2012; Chavarría-Smith and Vance, 2013]. Previous studies demonstrated that inhibition of MEKs by LT provokes a progressive and delayed induction of stress fibers during the first 24 h of endothelial cell intoxication, a phenomenon that is associated with the loss of the cortical network of actin filaments [Warfel et al., 2005; Rolando et al., 2010]. Moreover, the intercellular linear adherens junction (AJs) reorganize into discontinuous adherens junctions (DAJs) in LT-treated endothelial cells [Warfel et al., 2005; Rolando et al., 2010]. DAJs are composed of short actin cables orthogonal to cell–cell boundaries and are interconnected by VE-cadherin cell–cell adhesion molecules [Vestweber et al., 2009; Millan et al., 2010]. This reorganization of AJs results from a combination of a reduction of the cortical F-actin network and a reduction of the Rab11/Sec15 exocyst-regulated delivery of VE-cadherin [Guichard et al., 2010; Rolando et al., 2010].

The rapid reorganization of the actin cytoskeleton into stress fibers is primarily controlled by inducing the activity of the small GTPase RhoA, together with the interplay of actin-binding proteins [Heasman and Ridley, 2008; Tojkander et al., 2012]. RhoA, via its effector Rho kinase (ROCK), controls the ability of non-muscle myosin II (NMII) to bundle actin filaments into contractile cables by the phosphorylation-mediated activation of the NMII regulatory subunit (MLC). Other signaling pathways, which remain to be further elucidated, control the formation of actin cables and their mechanical properties in parallel to the RhoA/ROCK or MLCK pathways [Tojkander et al., 2012]. For example, in endothelial cells, the expression of the atypical GTPase Rnd3/RhoE promotes the formation of stress fibers [Chardin, 2006; Gottesbuhren et al., 2013].

Since its development, laser ablation nanosurgery has proven to be a powerful method for analyzing the mechanical properties of stress fibers [Strahs and Berns, 1979; Colombelli et al., 2005, 2009], thereby improving our knowledge on the mechanical interplay between actin structures and cell–cell or cell–extracellular matrix (ECM) contacts, as well as characterizing the diverse types of actin cables [Kumar et al., 2006; Chang et al., 2009; Tanner et al., 2010; le Duc et al., 2010]. For instance, the stress fibers regulated by ROCK or MLCK exhibit differences in their mechanical properties, indicating the existence of physical and functional heterogeneities in actin cables

[Kumar et al., 2006; Russell et al., 2009; Tanner et al., 2010].

In response to LT, endothelial cells progressively produce actin stress fibers [Warfel et al., 2005; Rolando et al., 2010, 2009]. Remarkably, this process occurs in the absence of detectable activation of the small GTPase RhoA and MLC phosphorylation, although inhibition of RhoA/ROCK disrupts the actin stress fibers that form in LT-treated cells [Rolando et al., 2010, 2009]. LT-treated cells experience massive transcriptional changes, indicating the importance of post-translational histone modifications in actin cytoskeleton reorganization [Raymond et al., 2009; Rolando et al., 2010]. Here, we sought to define the mechanical properties of the stress fibers that form in LT-treated cells and to characterize the interactions between the histone acetylation machinery, the formation of stress fibers and the integrity of the endothelial cell monolayer barrier.

Results

LT Promotes the Formation of Tensile and Stretched Stress Fibers

As previously reported, the cleavage of MAPK kinases (MEKs) by LT has profound consequences on the organization of the actin cytoskeleton in endothelial cells (Figs. 1A–1C) [Warfel et al., 2005; Rolando et al., 2010]. These effects can be visualized over time in Fig. 1A, in which all images of actin staining were acquired using control cell settings of signal acquisition (Fig. 1A). LT treatment induced a typical dense network of thick, parallel stress fibers, which filled the cells and was associated with the loss of the cortical F-actin network (Fig. 1A, LT 24 h). The evaluation of the percentage of cells displaying this typical dense network of thick stress fibers and loss of cortical actin, as defined in the inset image of Fig. 1B, established that the actin cytoskeleton reorganization progressively affected the entire cell population after 24 h of intoxication (Fig. 1B). Immunofluorescence analyses of the phospho-active form of MLC (p-MLC) revealed its association with thick stress fibers (Fig. 1D). Paxillin immunostaining showed that the majority of the actin cables displayed features of ventral stress fibers anchored at the extracellular matrix at both ends by focal adhesions (FAs) (Fig. 1E). Together, these findings indicated the contractile characteristics of the stress fibers in the LT-treated cells. We then determined the mechanical properties of this type of stress fiber. We performed our analyses on HUVECs expressing LifeAct-GFP to monitor the actin cytoskeleton dynamics after laser ablation. In untreated and LT-treated cells, we observed that both ends of the cables undergo a marked recoil along the axis of the cable in both directions immediately after cutting (Fig. 2A and Supporting Information Movies S1 and S2). Figure 2A also shows that the neighboring cables that were not targeted by the ablation remained stable. Note that the LifeAct-GFP signal

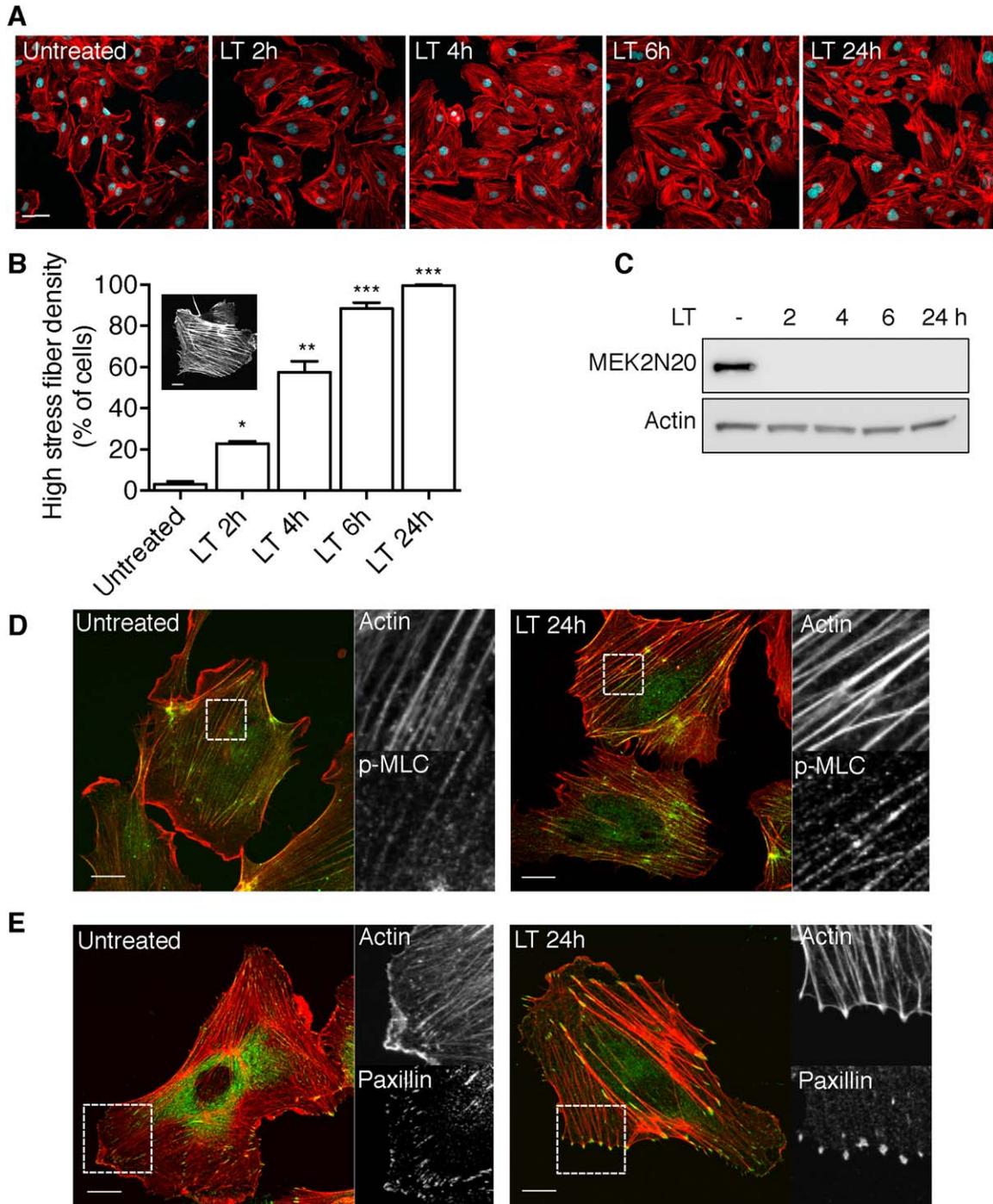


Fig. 1. Formation of stress fibers anchored to focal adhesions in endothelial cells treated with the lethal toxin (LT) of *B. anthracis*. (A–C) HUVECs were treated with LT (PA+LF, $3 + 1 \mu\text{g ml}^{-1}$) for the indicated periods of time (h). (A) F-actin was labeled with phalloidin-TRITC (red), and the nuclei were labeled with DAPI (cyan). Scale bar, $50 \mu\text{m}$. The images were taken using equivalent parameters for image acquisition to evaluate the differences in the stress fiber signal intensities. (B) Percentage of cells displaying an accumulation of thick stress fibers using the inset image as a model. The data represent the means \pm SEM, $n = 100$ cells per experiment from three independent experiments (ANOVA compared to the untreated condition: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (C) Immunoblot for the cleaved amino terminus of MEK2 (MEK2N20). Actin was used as a loading control. (D and E) Immunofluorescence analyses of the phalloidin-TRITC-labeled actin stress fibers (red) in untreated or LT-treated (PA+LF, $3 + 1 \mu\text{g ml}^{-1}$) HUVECs after 24 h. Scale bars, $10 \mu\text{m}$. Insets: details of the single channels are shown in gray. The active form of MLC was labeled with an anti-pMLC antibody (D, green), and the focal adhesions were labeled with an anti-paxillin antibody (E, green).

increased at the stress fiber extremities concomitant with their retraction (Fig. 2A, arrows). In parallel, we measured the distance of retraction between the split ends and the instantaneous velocity of retraction (Figs. 2B and 2C). This analysis revealed that at short time points (<4 s), the

retraction distance and the velocity of recoil of the extremities were higher in the LT-treated cells than in the controls (Figs. 2B and 2C). These data suggested the existence of higher tension in the stress fibers that form in the LT-treated cells. Subsequently, the retraction of the actin stress

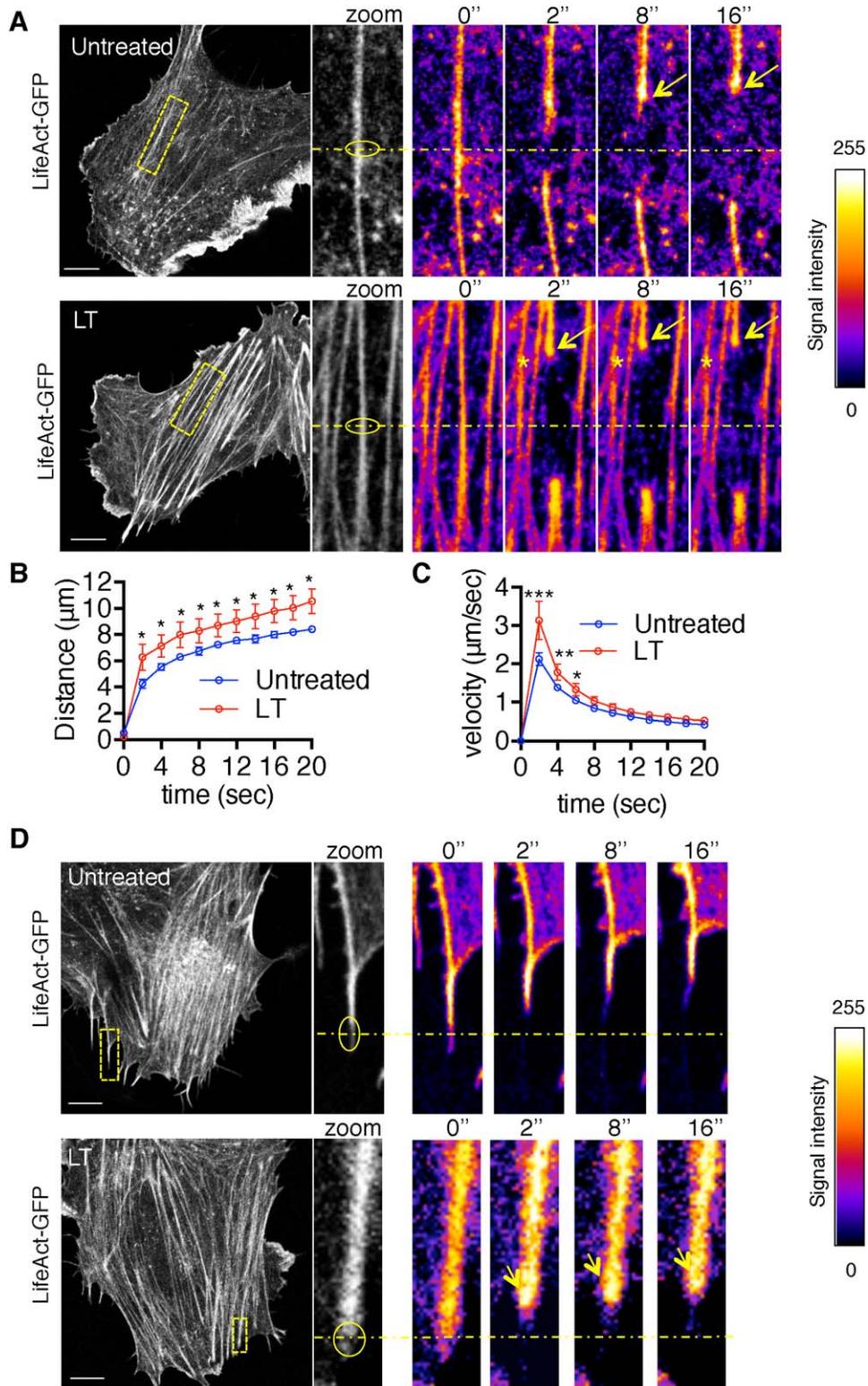


Fig. 2.

fibers exhibited similar kinetics in both cases. Next, we performed a series of ablations at the extremities of the stress fibers. We found that the stress fibers retracted away from the site of ablation (Fig. 2D and Supporting Information Movie S3 and S4). We also observed that the LifeAct-GFP signal increased at the ends of stress fibers concomitant with their retraction (Fig. 2D arrows).

We concluded that cells react to the LT treatment by inducing the formation of contractile stress fibers that are stretched by the traction forces originating from focal adhesions.

Mechanical Properties of the Stress Fibers at Discontinuous Adherens Junctions

We then characterized the mechanical properties of the actin cables connecting cells in monolayers treated with LT. In control monolayers, the major adherens junction molecule VE-cadherin is linearly arranged (Fig. 3A). However, after 24 h of LT treatment, we demonstrated that $95.8\% \pm 1.3\%$ of cells displayed discontinuous adherens junctions (DAJs) that were interspersed with intercellular gaps (Fig. 3A). We went on to determine whether the actin cables at the DAJs were pushing or pulling on each other. To answer this question, we selected adjacent endothelial cells expressing both LifeAct-mCherry and VE-cadherin-GFP and performed a series of local laser ablations (Figs. 3B and 3C, yellow circles). We first severed the actin cables connecting the cells at the level of the VE-cadherin-GFP signal (Fig. 3B and Supporting Information Movie S5). This type of ablation produced a retraction of both extremities toward each cell center and was also associated with a retraction of the edges of the adjacent cells. This observation suggested that the cells were pulling on each other. To further investigate this phenomenon, we performed sequential ablations at the rear of the DAJs in two neighboring cells (Fig. 3C and Supporting Information Movie S6). The first laser ablation at the rear of the actin cable produced a translocation of the VE-cadherin signal in the direction of the neighboring cell along the axis defined by the actin cable connecting the two cells. Ablation at the rear of the actin cable of the cell located on the right resulted in the movement of VE-cadherin signal in the opposite direction (Fig. 3C, yellow arrows).

Thus, the actin cables at DAJs equilibrate the tension forces emanating from neighboring cells to maintain the intercellular connections.

Treatment of Cells With TSA Promotes the Formation of Stress Fibers

Inhibition of MEKs with LT selectively prevents the phosphorylation of histone H3 at serine-10 and reprograms the cell transcriptome [Raymond et al., 2009; Rolando et al., 2010]. With the aim of counteracting the LT-induced actin reorganization and restoring the endothelial barrier function, we searched for chemical inhibitors known to interfere with gene expression and post-translational histone modifications and we examined their impact on the formation of stress fibers. Treating the human endothelial cells with Actinomycin-D (ActD), a polypeptide from *Streptomyces* that binds to DNA and prevents the elongation of transcripts, promoted the formation of a dense network of thin actin cables, which were less bundled than those induced by LT (Figs. 4A and 4B). We concluded that interfering with transcription changes the organization of the actin cytoskeleton. Histone acetyl-transferases (HATs) and deacetylases (HDACs) control the flux of histone acetylation, thereby modulating gene expression [Cheung et al., 2000]. This prompted us to test the effect of broad HAT and HDAC inhibitors on actin organization. HAT inhibitors (anacardic acid and garcinol) had no marked effect on the actin cytoskeleton in control cells, whereas trichostatin-A (TSA), a broad inhibitor of HDACs, produced ventral stress fibers similar to those induced by LT (Figs. 4A and 4B). Moreover, we observed paxillin accumulation at the ends of the stress fibers in the TSA-treated cells, indicating that these stress fibers are anchored to the FAs (Fig. 4C). Stress fibers result from the combined polymerization of actin filaments and their bundling into highly ordered cables. We next thoroughly compared the effects of the LT and TSA treatments on F-actin polymerization and bundling. To this end, we developed a semi-automated method of analysis, which is described in the Supporting Information. Briefly, we graphed the values of the phalloidin signal intensities (F-actin signal) along a line perpendicular to the actin fibers (Fig. 4D). From this graph, we obtained the integral of the signal intensity above the background. We then calculated a

Fig. 2. Mechanical characteristics of the stress fibers that form in the LT-treated cells. **(A)** Representative example of actin cable recoil after laser ablation in LifeAct-GFP-expressing untreated or LT-treated (PA+LF, $3 + 1 \mu\text{g}\cdot\text{ml}^{-1}$) HUVECs after 24 h. The laser ablation areas are depicted by a yellow circle on the zoomed images. The images at 0, 2, 8, and 16" were taken from Supporting Information Movies S1 and S2. The false colors reflect the intensity of the LifeAct-GFP signal (arbitrary units from 0 to 255). The yellow arrows indicate the LifeAct-GFP signal accumulation at the edge of actin cables after recoil. The yellow stars indicate the non-severed stress fibers. Scale bars, 10 μm . **(B and C)**. The graphs show the values for the distance of retraction (B) and velocities (C) between both extremities after different periods of retractions. The data are presented as the means \pm SEM, $n = 10$ fibers, and 1 fiber/cell from three independent experiments (unpaired Student's t test compared to the untreated condition: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). **(D)** Laser ablations performed at the end of the stress fibers in untreated or LT-treated (PA+LF, $3 + 1 \mu\text{g}\cdot\text{ml}^{-1}$) LifeAct-GFP-expressing HUVECs after 24 h. The images at 0, 2, 8, and 16" were taken from Supporting Information Movies S3 and S4. The false colors reflect the LifeAct-GFP signal intensity (arbitrary units from 0 to 255). The yellow arrows show the accumulation of the LifeAct-GFP signal at the edge of actin cables after recoil. Scale bars, 10 μm .

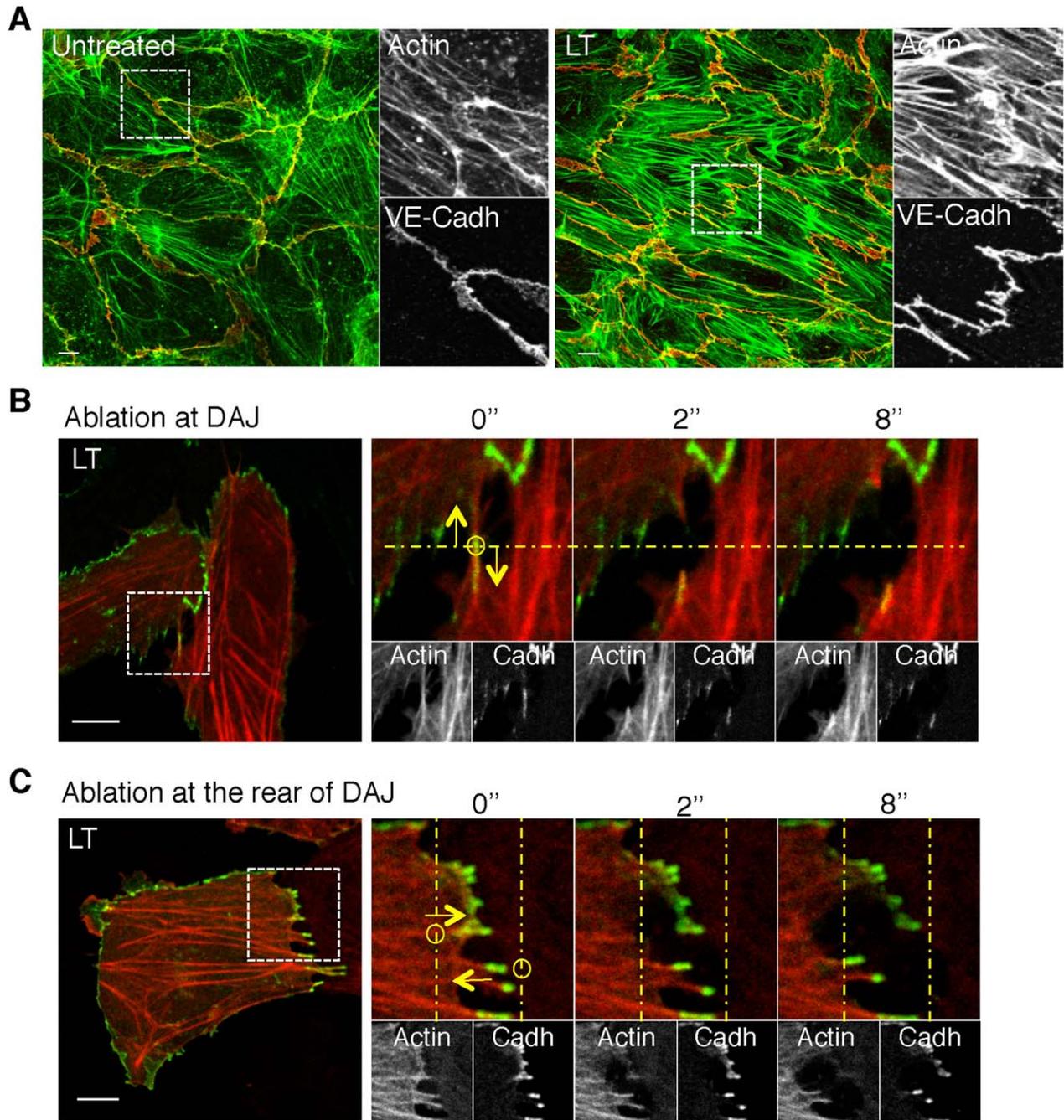


Fig. 3. Mechanical characteristics of the actin cables at DAJs in LT-treated endothelial cell monolayers. **(A)** Immunofluorescence analyses showing the reorganization of the actin cytoskeleton and the cell-cell junctions in endothelial cell monolayers treated with LT (PA+LF, $3 + 1 \mu\text{g ml}^{-1}$) for 24 h. F-actin was labeled with phalloidin-FITC (green), and the junctions were labeled with an anti-Cadherin-5 antibody (VE-Cadherin, red). The insets show the details for each individual channel in gray. Scale bars, 10 μm . **(B and C)** Zone of laser ablation at the discontinuous adherens junctions (DAJs) in LT-treated HUVECs expressing LifeAct-mCherry (red) and VE-Cadherin-GFP (green) at time 0'' are indicated by the yellow circles. The vertical bar serves as a reference location. The yellow arrows indicate the direction of movement of the VE-cadherin signal after ablation. The images were taken from the original Supporting Information Movies S5 and S6 at the indicated time points after cutting. Scale bar, 10 μm . **(B)** Example of an ablation performed at the level of VE-cadherin in a DAJ. **(C)** Two examples of ablations performed at the rear of the DAJ, first on the left (upper circle), followed by ablation on the right of the DAJs (lower circle). The yellow arrows indicate the direction of movement of the VE-cadherin-GFP (green) signal after ablation **(C)**. Insets: the details of the single channels for LifeAct-mCherry (Actin) or VE-cadherin-GFP (Cadh) are shown in gray.

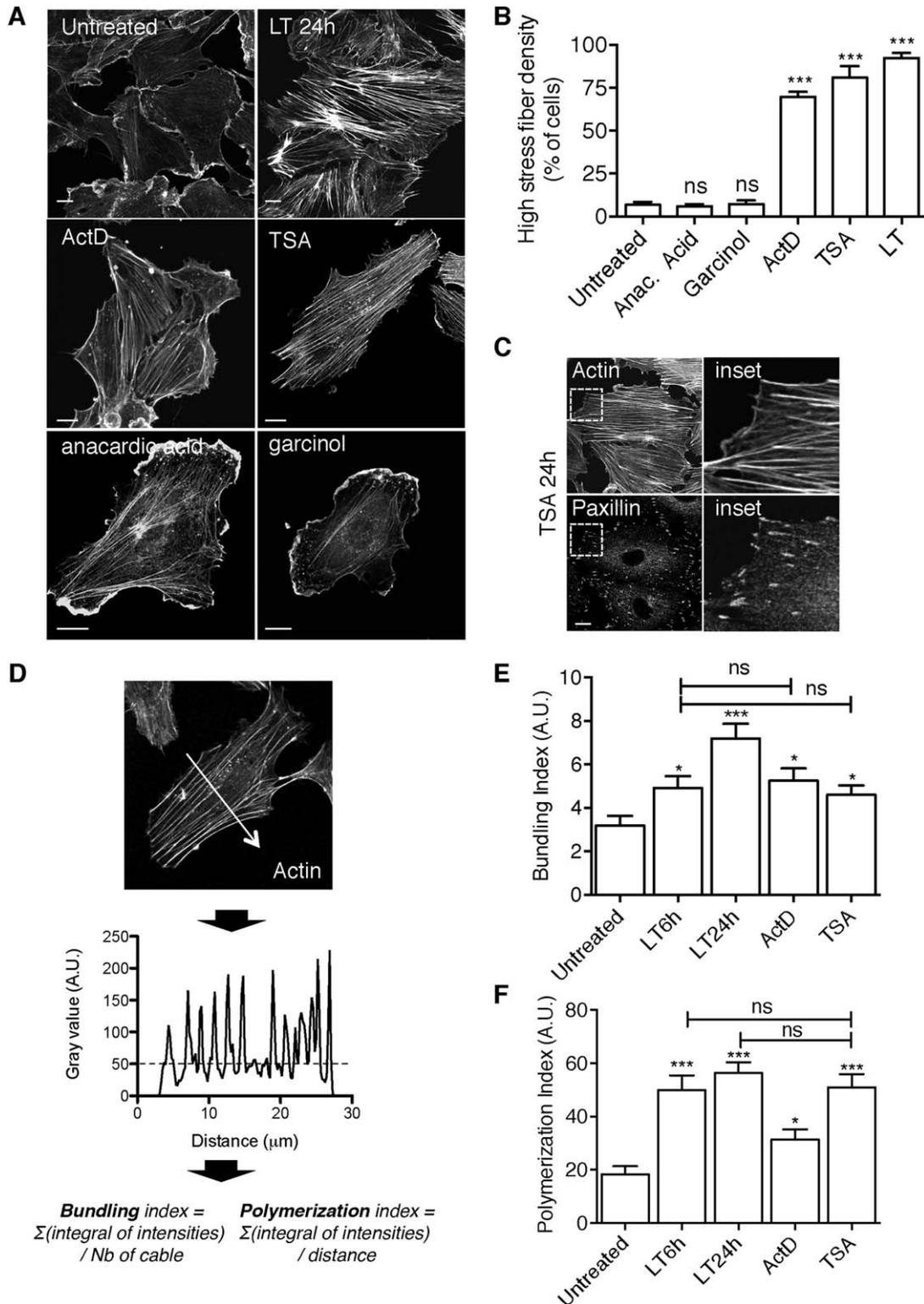


Fig. 4. Formation of stress fibers in TSA-treated cells. **(A)** Representative examples of HUVECs treated for 24 h with LT (PA+LF, $3 + 1 \mu\text{g ml}^{-1}$) or the following different inhibitors, as well as the untreated controls: Anacardic acid ($20 \mu\text{M}$), Garcinol ($25 \mu\text{M}$), Trichostatin-A (TSA, $50 \mu\text{M}$), or Actinomycin D (ActD, $1 \mu\text{g ml}^{-1}$ Supporting Information). F-actin was labeled with phalloidin-FITC (gray). Scale bar, $10 \mu\text{m}$. **(B)** The graph shows the percentage of cells displaying reorganization of actin cytoskeleton, as exemplified in (A). The data represent the mean values \pm SEM, $n = 100$ cells per experiment from three independent experiments (ANOVA compared to the Untreated condition: ns: non-significant, $***P < 0.001$). **(C)** Immunofluorescence analyses of the TSA-triggered actin stress fibers. Scale bar, $10 \mu\text{m}$. Insets: the details of the single channels are shown in gray. F-actin was labeled with phalloidin-TRITC (actin), and the focal adhesions were labeled with an anti-paxillin antibody (paxillin). Scale bar, $10 \mu\text{m}$. **(D)** Schema of the experimental methodology used in the text to determine the bundling and polymerization indexes. The dotted line corresponds to the mean value ($n = 3$ measurements per cell) of the background signal intensity measured between two cables. **(E and F)** Measures of F-actin polymerization and bundling in untreated cells or treated with LT, ActD ($1 \mu\text{g ml}^{-1}$) or TSA ($50 \mu\text{M}$) for 24 h. The data are represented as the mean values \pm SEM, $n = 15$ cells analyzed per experiment from three independent experiments (ANOVA compared to the untreated condition: ns: non-significant, $*P < 0.05$, $***P < 0.01$).

bundling index that corresponds to the sum of integrals divided by the number of cables in a cell and a polymerization index that corresponds to the sum of integrals divided by the total distance analyzed. The quantifications revealed that F-actin polymerization increased to a similar extent in both the TSA- and LT-treated cells (Figs. 4E and 4F),

whereas the F-actin bundling index was slightly higher in the LT-treated cells compared to the TSA-treated cells (Fig. 4E). We then screened chemical compounds targeting different HDAC members for their ability to induce stress fiber polymerization [Bolden et al., 2006]. Tubacin, a specific HDAC6 inhibitor, did not induce detectable changes

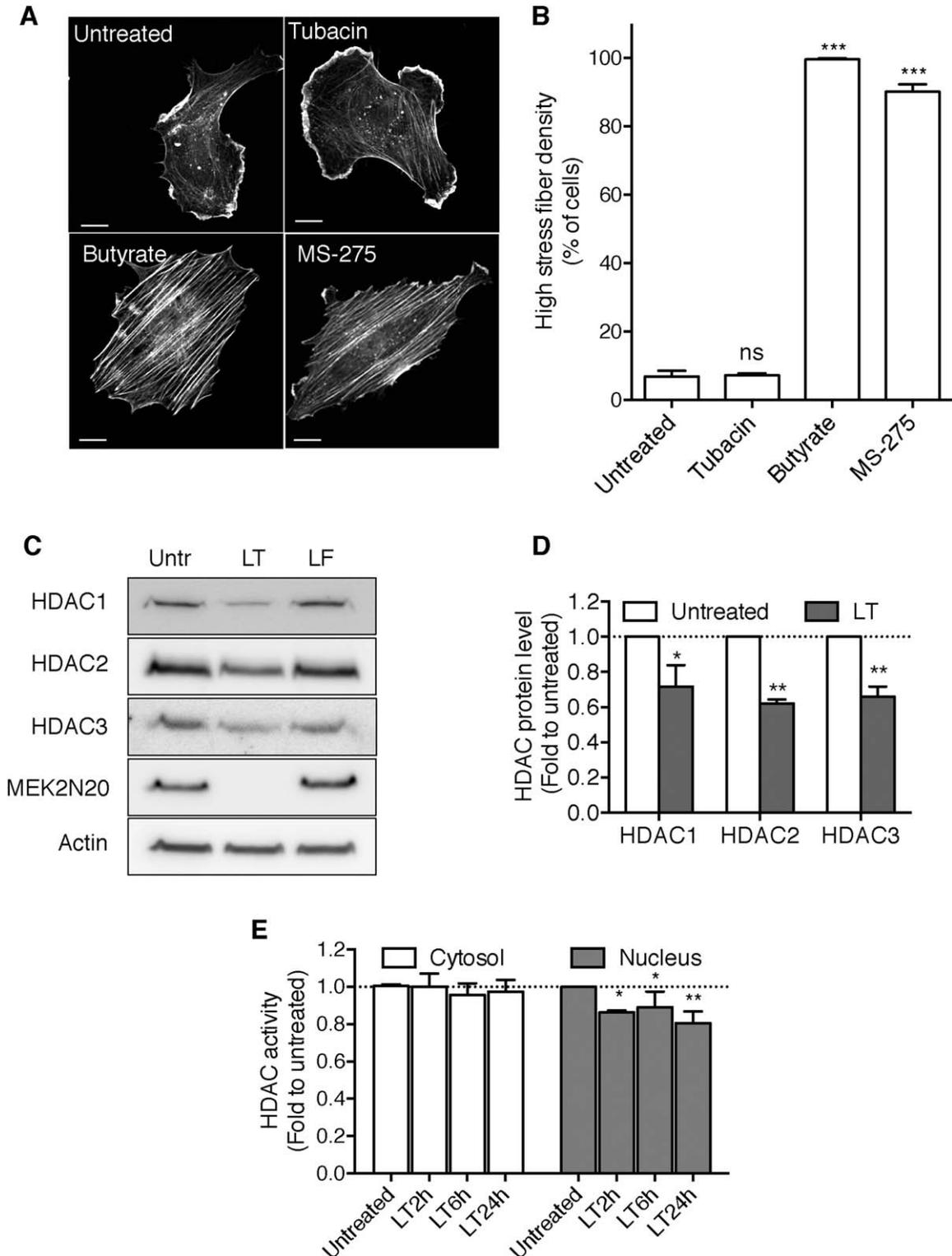


Fig. 5.

in the stress fibers (Figs. 5A and 5B) [Zhang et al., 2007]. However, both butyrate, which inhibits class I and IIa HDACs, and MS-275, which targets HDAC1, 2, and 3, produced a massive induction of stress fibers (Fig. 5A). Using the examples of actin reorganization shown in Fig. 5A as references, we estimated that the butyrate and MS-275 treatments affected a majority of the cells (Fig. 5B). Consistent with the above findings, we observed decreases in the protein levels of HDAC1, 2, and 3 in cells treated with LT for 24 h (Figs. 5C and 5D). Complementary to this approach, we evaluated the global HDAC activity in the cellular fractions using a colorimetric assay that reports the quantity of a deacetylated lysine substrate. No variation of the global HDAC activity was recorded in the cytosolic fractions of the LT-treated cells. In contrast, we measured a decrease of $12.5\% \pm 2.5\%$ in the global HDAC activity in the nuclear fractions of cells treated with LT for 24 h (Fig. 5E). Here, we link the LT-triggered cytotoxicity to actin cytoskeleton organization and to the activity of a subset of HDAC enzymes.

Induction of Rnd3 Expression in the LT- and TSA-treated Cells

We then assessed the level of RhoA activity in both the LT- and TSA-treated cells to better understand the molecular mechanisms leading to stress fiber induction in these conditions. Despite the formation of thick actin cables, we did not measure an increase in the level of active RhoA in either treatment conditions (Figs. 6A and 6B). Cells treated with the CNF1 toxin were included as a positive control for RhoA activation [Doye et al., 2002; Rolando et al., 2010]. We concluded that the massive increase in stress fiber formation observed in the LT- and TSA-treated cells occurs without a detectable increase in the level of active RhoA. Given that the expression of Rnd3 also promotes the formation of stress fibers, we hypothesized that the induction of this GTPase might favor the formation of stress fibers in these conditions. Figure 6C shows that both the LT and TSA treatments increased the Rnd3 mRNA levels (8.6-fold and 3.3-fold, respectively). Immunoblotting confirmed that the cells treated with LT or TSA displayed higher cellular levels of Rnd3 (Fig. 6D). Furthermore, we observed that a reduction of the Rnd3 level by RNAi impaired the

formation of actin stress fibers in the LT-treated cells (Fig. 6E). The quantification of the phenotypes shown in Fig. 6E established that the Rnd3 RNAi treatment inhibited the LT-induced effects on actin reorganization by twofold (Fig. 6F). The RNAi and toxin effects were verified and supported the idea that Rnd3 knockdown did not block LT proteolytic activity on MEKs (Fig. 6G). Additionally, we did not observe major changes in the cellular RhoB levels (Fig. 6G). Collectively, our results show that the accumulation of stress fibers in LT-treated cells requires the expression of the small GTPase Rnd3.

Garcinol Treatment Partially Reverses the LT-induced Actin Cytoskeleton Remodeling

The data described above prompted us to test the effect of inhibiting the flux of histone acetylation as a strategy for reducing the effects of LT on the actin cytoskeleton. HUVECs treated with the HAT inhibitor garcinol showed no significant changes in the organization of the actin cytoskeleton (Fig. 7A). Figure 7B shows the quantification of the inhibitory effect of garcinol on the LT-induced reorganization of the actin cytoskeleton using the phenotype of actin reorganization shown in Fig. 7A as examples. In contrast, we observed that treating HUVECs with both LT and garcinol partially reversed the toxin-induced actin cytoskeleton remodeling (Figs. 7A and 7B). In addition, we observed that treating the cells with garcinol limits the LT-triggered induction of Rnd3 (Fig. 7C). This result prompted us to evaluate the protective effect of garcinol on the toxin-induced reorganization of the intercellular junctions triggered. Figure 7D shows examples of the F-actin and VE-cadherin distributions at the intercellular junctions of endothelial cell monolayers treated with LT. In the LT-treated monolayers, we found that $95.75\% \pm 1.25\%$ of the cells displayed discontinuous adherens junctions (Fig. 7E). Cotreatment of the intoxicated cell monolayers with garcinol markedly reduced the formation of DAJs to $50\% \pm 13\%$ of the cell population (Figs. 7D and 7E). We next evaluated the effect of garcinol on the endothelial cell monolayer barrier function by measuring the diffusion of FITC-dextran across the monolayers. We found that LT reduced the monolayer barrier, an effect that was scaled down twofold upon treating monolayers with garcinol (Fig. 7F). Thus, we report here that the garcinol treatment reduced

Fig. 5. Relationship between the inhibition of HDAC isoforms and the formation of stress fibers. (A) Analyses of the actin cytoskeleton organization in HUVECs treated with Tubacin (10 μ M), Sodium butyrate (3 mM) or MS-275 (3 μ M) for 24 h and the untreated controls. F-actin was labeled with Phalloidin-FITC (gray). Scale bar, 10 μ m. (B) The graph shows the percentage of cells displaying reorganization of the actin cytoskeleton, as exemplified in (A). The data are presented as the means \pm SEM, $n = 100$ cells per experiment from three independent experiments. (C) Immunoblotting showing the level of HDAC1, 2 and 3 in response to LT (PA+LF, 3 + 1 μ g ml⁻¹) or LF alone (1 μ g ml⁻¹) after 24 h of treatment. Actin was used as a loading control, and the amino terminus of MEK2 (MEK2N20) was used as a control for toxin activity. (D) The graph shows the quantification of the HDAC protein levels normalized to the actin signal. The values are expressed as the fold changes relative to the untreated condition (means \pm SEM; $n = 4$, unpaired, one-sided Student's *t* test: * $P < 0.5$ and ** $P < 0.001$). (E) The graph shows the values of the total HDAC activities examined in the cytosolic and nuclear protein fractions, as described in the Materials and Methods section. The HUVECs were untreated or intoxicated with LT for the indicated periods of time (h) (means \pm SEM; $n = 3$, ANOVA compared to the untreated condition: * $P < 0.05$; ** $P < 0.01$).

the LT-induced cytoskeleton remodeling and partially restored the barrier function of the endothelial monolayers.

Discussion

It is important to delineate how the reprogramming of gene expression governs actin cytoskeleton reorganization. We

previously demonstrated that the lethal toxin (LT) of *B. anthracis* reprograms the cell transcriptome with major consequences for the organization of the actin cytoskeleton. Here, we report that the stress fibers that form in response to cellular intoxication with LT are tensile and under tension. Furthermore, we show that the actin cables at DAJs compensate for the disruptive tension generated between

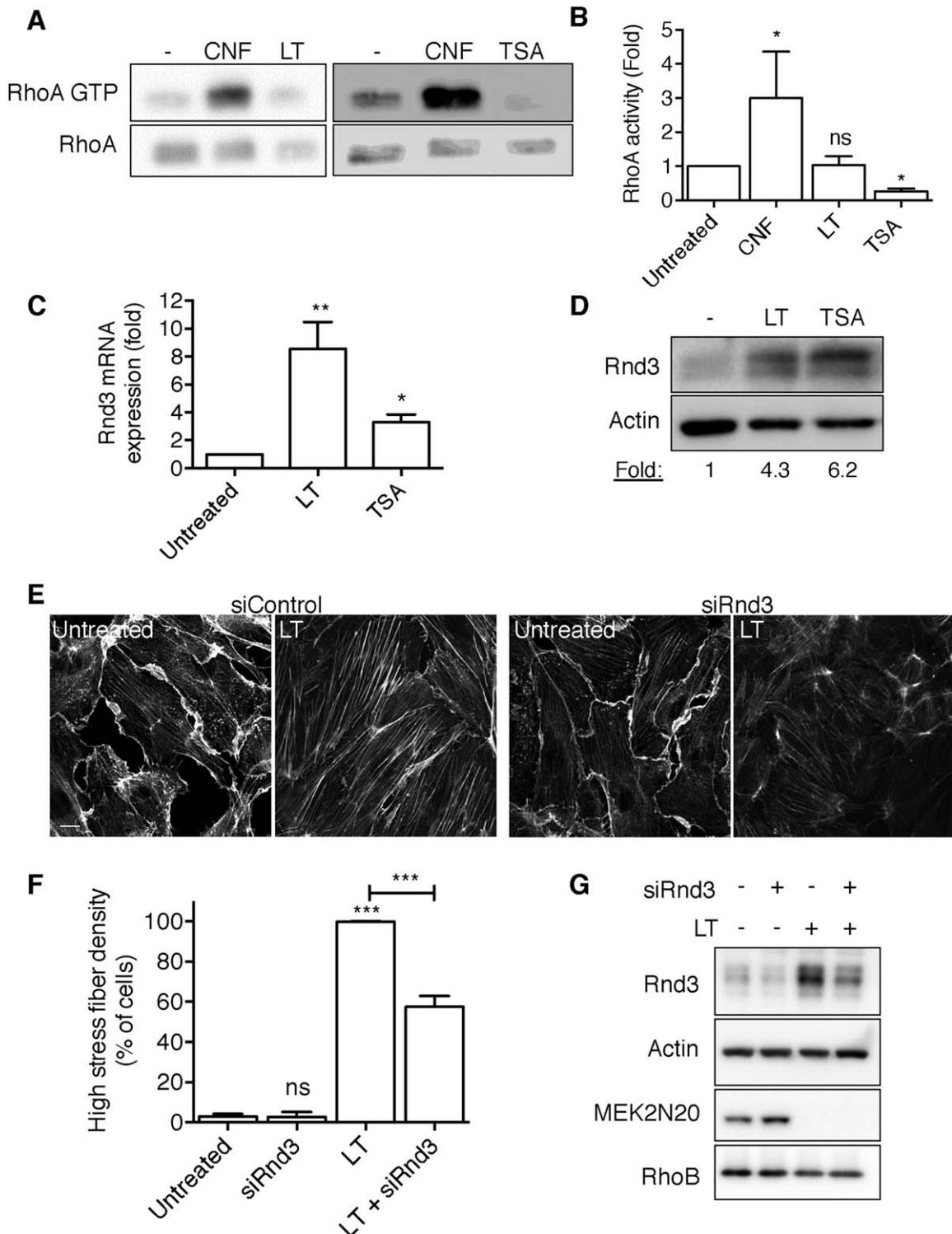


Fig. 6.

neighboring cells, thereby maintaining some cell–cell cohesion. LT treatment induces the formation of contractile actomyosin cables that are stretched by the traction forces originating from focal adhesions or cell–cell junctions. Importantly, we show that the flux of histone acetylation balanced by HAT/HDAC enzyme activities regulates the formation of stress fibers, and we implicate this regulation in the induction of actin stress fibers by LT. We also pinpoint the importance of Rnd3 expression in the induction of stress fibers by LT. Finally, we report that garcinol, an inhibitor of histone acetyl-transferases (HATs), reduces the cytotoxic effects of LT on the actin cytoskeleton and on endothelial cell monolayer barrier function.

The LT-treated cells display a dense network of thick actin cables. Severing the actin cables by means of laser ablation allowed us to characterize the mechanical properties of the stress fibers that form in the LT-intoxicated cells. This approach establishes their contractile and tensile characteristics. We unambiguously show that the stress fibers produced in the LT-intoxicated cells resist the contractile forces generated at the cell–matrix interface. A previous study reported different mechanical properties of thick stress fibers, specifically higher friction with the surrounding cytoskeleton [Kumar et al., 2006]. Here, our quantitative measurements show that the thick stress fibers display a faster retraction velocity in the LT-treated cells at a short time point that corresponds to the elastic regime. This finding suggests that these fibers are more tense, stiffer, or both, which is in good agreement with our observation that LT induced a thickening of the stress fibers. However, their viscous behavior after 4 s of recoil appears to be equivalent in the control cables. In accord with these findings, we found that the cables that form in the LT-treated cells exhibit a strong phospho-MLC signal, which suggested a high capacity of actomyosin contraction driven by myosin II. In the LT-treated monolayers, linear adherens junctions undergo a conversion into discontinuous adherens junctions. We also used laser ablation to characterize the actin cables at the DAJs. Here, we establish that the actin cables at DAJs resist the disruptive forces that tend to compromise

cell–cell cohesion in the monolayer. Based on these findings, a likely scenario is that actin cables form in response to treatment with LT to compensate for the disruptive forces induced by the toxin's actions.

The hijacking of post-translational histone modifications by microbial factors is emerging as an important aspect of host-pathogen interactions during infection [Hamon and Cossart, 2008]. Interestingly, we report here the importance of components of the histone acetylation machinery in the control of actin cytoskeleton organization. We also implicate the inhibition of HDACs in LT-induced actin cytoskeleton reorganization into actin cables. Note that contrary to LT, treatment with TSA does not trigger a decrease in the cellular phospho-ERK levels (data not shown). This result indicates that there are common features and differences between the modes of action of LT and TSA. As previously reported, we did not observe the activation of the RhoA/ROCK pathway in the LT-treated cells, although this signaling is required to maintain actin cables [Rolando et al., 2010]. Additional pathways involved in gene expression likely contribute to the promotion of actin cable bundling and contraction. Our previous data regarding LT indicated that the induction of stress fibers by the toxin involved the combined modulation of the expression of several genes, notably cortactin (CTTN), calponin-1 (CNN1), desmuslin (DMN), and raphilin-2 (RHPN2) [Rolando et al., 2010]. Here, we found that TSA modulates the expression of a majority of these genes (CTTN, DMN and RHPN2) in the same way as LT (data not shown). Moreover, we report here the induction of Rnd3, an atypical small Rho GTPase known to produce stress fibers in endothelial cells [Gottesbuhren et al., 2013], as a common consequence of treating the cells with LT or TSA. Together, our data indicate a sharp overlap between the effects of LT and TSA on actin cytoskeleton organization and the regulation of a set of actin regulators. The precise HDAC isoform(s) responsible for modulating the organization of the actin cytoskeleton remain to be identified; however, using MS-275, we pinpointed the role of HDAC 1, 2, and 3. Consistent with these findings, we also measured a decrease

Fig. 6. LT and TSA induce stress fiber formation via Rnd3 accumulation. (A and B) Measurement of active RhoA in cells treated with LT (PA+LF, $3 + 1 \mu\text{g ml}^{-1}$), TSA ($50 \mu\text{M}$) or the RhoA activating toxin CNF1 (positive control, 10^{-9} M , 4 h) for 24 h and the untreated controls. Immunoblotting for RhoA shows the levels of active RhoA (RhoAGTP) and total RhoA (RhoA). (B) The graph shows the values for active RhoAGTP normalized to the total amount of RhoA. The values are expressed as the fold change relative to the control (means \pm SEM; $n = 3$, ANOVA compared to the untreated condition: $*P < 0.05$). (C) Quantification of the Rnd3 mRNA level in untreated, LT-treated or TSA-treated cells after 24 h, as indicated. The values are expressed as the fold change relative to the control condition (means \pm SEM; $n = 3$, ANOVA compared to the untreated condition: $*P < 0.05$; $**P < 0.01$). (D) Immunoblotting showing the increase in the protein level of Rnd3 in the LT- or TSA-treated cells. Actin was used as the loading control, and the Rnd3 protein levels are expressed as the fold change relative to the control condition. One representative experiment of $n = 3$ experiments. (E–G) The cells were transfected with a control or Rnd3 siRNA and treated for 24 h with or without LT. (E) F-actin was labeled with Phalloidin-FITC (green). Scale bar, $10 \mu\text{m}$. (F) Percentage of cells displaying thick actin cables. As exemplified in (E) in the LT-treated condition. The data are represented as the means \pm SEM and compared to the untreated condition using an ANOVA: $***P < 0.001$, $n = 30$ to 50 cells analyzed per experiment from three independent experiments. (G) Immunoblots showing the Rnd3 RNAi-mediated knockdown as a control for the data presented in E, as well as the cellular levels of RhoB. The proteolytic activity of LT was analyzed by anti-MEK2N20 immunoblotting (MEK2N20). Actin was used as the loading control.

in the protein levels of HDAC1, 2, and 3 in the LT-intoxicated cells. Finally, we report that treating the endothelial cells with LT provokes a decrease in the global HDAC activity in the nuclear fractions. This result supports the hypothesis that histone deacetylases act at the level of specific gene promoters to control actin organization,

rather than a direct action of HDACs on cytosolic actin regulators. Our data reject the hypothesis that the effect of the toxin depends on HDAC6 activity; recent findings suggested a role of the cytoplasmic HDAC6 in controlling the association of cortactin with the actin filaments [Zhang et al., 2007; Kaluza et al., 2011]. Given that cortactin

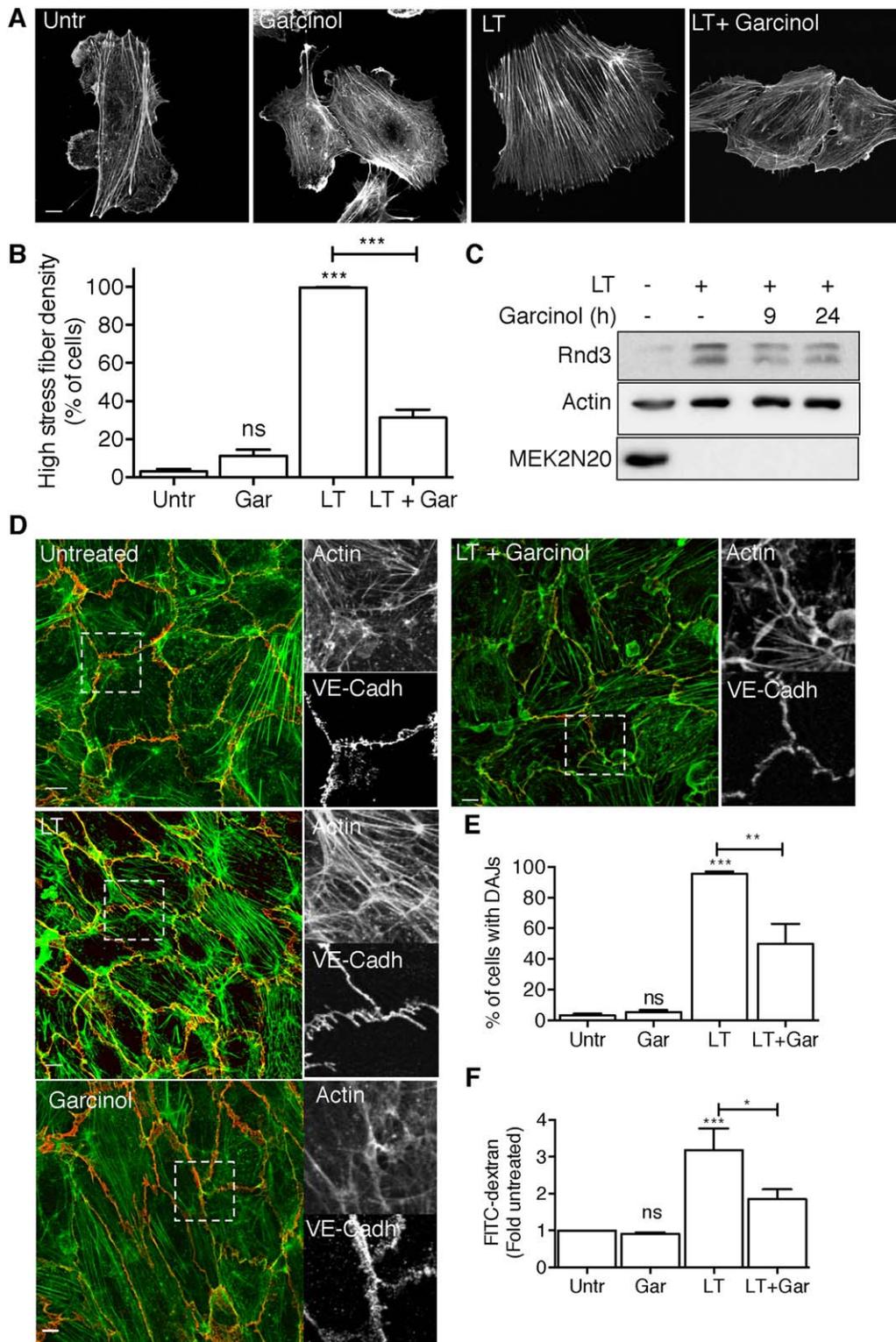


Fig. 7.

controls the assembly of the F-actin at the plasma membrane, its reduction by freeing a pool of actin molecules likely favors stress fiber assembly [Selbach and Backert, 2005]. The observation that the acetylation of cortactin by the cytoplasmic HDAC6 is likely not implicated in the LT-induced formation of stress fibers is also supported by the finding that tubacin had no detectable effect on actin polymerization. The effect of LT on nuclear HDACs does not correlate with a broad increase in histone acetylation, as observed using pan-acetyl histone H3 and H4 antibodies (not shown). These observations point to a possible action of the toxin on histone acetylation at the level of specific promoters. Clarifying this type of regulation is of interest, considering that little is known about the unconventional regulation of the actin cytoskeleton, notably by HDACs and HATs [Cheung et al., 2000].

Modulating the organization of the actin cytoskeleton is one strategy for controlling endothelial barrier function. The complete inhibition of RhoA or ROCK was previously assessed as a strategy for rescuing the alterations to the endothelial barrier following LT treatment. Unexpectedly, this strategy failed due to an increase in transcellular permeability driven by the opening of large transcellular tunnels [Rolando et al., 2009]. This prompted us to search for another strategy linked to the effects of LT on the cellular transcriptome. Histone acetylation is tightly controlled by the antagonistic activities of HDACs and HATs [Andrew and Bannister, 2011]. HDAC inhibition with TSA promotes the formation of DAJs and increases endothelial cell monolayer permeability (data not shown). Garcinol is a polyisoprenylated benzophenone derivative from the plant *Garcinia indica* that possess a potent inhibitory effect on the HATs p300 and PCAF and is a promising anti-cancer molecule *in vivo* [Liu et al., 2015]. Here, we report that garcinol treatment rescues the cytotoxicity of LT on HUVEC monolayers. We attributed this effect of garcinol to its ability to block the LT-induced actin cytoskeleton reorganization and to rescue the cohesion of cell–cell junctions. Note that the other HAT inhibitor, curcumin, also disrupted the actin cables produced by LT (data not shown). We report that treatment with garcinol partially

reversed the induction of Rnd3 by LT. These findings further point to the importance of Rnd3 in the LT-induced control of the actin cytoskeleton.

Collectively, our data reveal that the histone acetylation HDAC/HAT machinery controls the organization of the actin cytoskeleton.

Materials and Methods

Cell Culture Permeabilization Assays and Chemical Reagents

Endothelial HUVEC cell cultures (PromoCell, Heidelberg, Germany) and permeability assays were performed as previously described [Boyer et al., 2006]. Briefly, HUVEC monolayers were grown on gelatin-coated polyester filters (3- μm pore size; Greiner Bio-One) for 3 days and treated with 3 $\mu\text{g ml}^{-1}$ of PA and 1 $\mu\text{g ml}^{-1}$ of LF in supplemented SFM. The variations in the permeability of each monolayer were quantified after 24 h by measuring the amount of FITC-dextran 70 kDa (Invitrogen, Cergy Pontoise, France) that diffused across the monolayer (starting concentration: 0.5 mg ml^{-1}). The samples were collected from the lower chamber after 10 min. The levels of FITC-dextran were determined with a Fluoroscan Ascent (excitation: 485 nm; emission: 538 nm; Thermolab System). The HAT and HDAC inhibitors were purchased from ENZO Life Sciences and used at the indicated concentrations: garcinol (25 μM), Trichostatin A (TSA) (50 μM), tubacin (10 μM), MS-275 (3 μM), sodium butyrate (3 mM), anacardic acid (20 μM). Other biochemical reagents were purchased from Sigma Aldrich. The RNAi (Dharmacon) transfections were performed as previously described [Torrino et al., 2011].

Recombinant Toxin Production and Biochemical Measurements

The protective antigen (PA), lethal factor (LF) and CNF1 were purified as previously described [Doye et al., 2006; Rolando et al., 2010]. All proteins were applied onto an EndoTrap Red column, and the absence of endotoxin was assessed using the Limulus Amebocyte Lysate QCL-1000

Fig. 7. Protective effect of garcinol against LT-induced actin reorganization and endothelial cell monolayer permeability. (A–F) Endothelial cells were untreated (Untr) or treated with LT (PA+LF, 3 + 1 $\mu\text{g ml}^{-1}$), garcinol 25 μM (Gar) or a combination of LT and Garcinol (LT+Gar) for 24 h. (A) Representative images of the actin cytoskeleton organization. (B) The graph shows percentage of cells displaying thick actin cables, as exemplified in (A), due to the LT treatment (mean values \pm SEM; $n = 3$. ANOVA compared to the untreated condition: ns: non-significant, and $***P < 0.01$.) (C) Immunoblot showing the Rnd3 protein levels in the LT-treated cells or LT- and garcinol-treated (25 μM) cells after different periods of time (hours). Protein loading was verified by anti-actin immunoblotting (Actin). The LT activity was monitored by anti-MEK2N20 immunoblotting. (D) Representative images of the actin cytoskeleton in endothelial cell monolayers. F-actin was labeled with Phalloidin-FITC (Actin: green) and VE-Cadherin was labeled with an anti-Cadherin-5 antibody (VE-Cadh: red). The insets show the details for each single channel (gray). Scale bar, 10 μm . (E) The graph shows the quantification of the percentage of cells displaying DAJs with neighboring cells (mean values \pm SEM; $n = 3$. ANOVA compared to the untreated condition: ns for non-significant, $**P < 0.01$, $***P < 0.001$). (F) Measures of endothelial permeability: the values for the diffusion of FITC-dextran across the Transwell chamber are expressed in arbitrary units (A.U.). Each point corresponds to the mean values \pm SEM, $n = 3$ independent experiments; ANOVA compared to the untreated condition: ns for non-significant, $*P < 0.05$, $***P < 0.001$).

(Cambrex). The activity of the PA and LF proteins was routinely tested and resulted in 100% MEK2 cleavage after 2 h of HUVECs intoxication (PA+LF, 3 + 1 $\mu\text{g ml}^{-1}$) (see Fig. 1C as an example). The presence of active RhoA (RhoAGTP) in the cell lysates was determined by affinity chromatography, as previously described [Doye et al., 2006]. Cell fractionation was performed using the NE-PER TM Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's procedures (Life Technologies). The global HDAC activity was determined using the HDAC Colorimetric Assay Kit (BioVision). Fifty micrograms of the cytoplasmic and nuclear fractions were incubated with the acetylated-lysine substrate for 1 h at 37°C, followed by a 30-min incubation with the lysine developer. The absorbance of the deacetylated substrate reacting with the lysine developer was measured at 405 nm. For the immunoblots, the proteins were resolved on 12% SDS-PAGE gels using standard conditions and transferred to Immobilon-P PVDF membranes (Millipore). The antibodies used were as follows: an antibody directed toward the amino-terminal part of MEK2 (MEK2N20, Santa Cruz), anti-RhoA [clone 26C4] (BD Biosciences), anti-RhoE/Rnd3 [clone4] (Millipore), anti-RhoB (Santa Cruz), anti-HDAC1 [clone 2E10] (Millipore), anti-HDAC2 [clone 3F3] (Upstate), anti-HDAC3 [clone H99] (Santa Cruz), anti-beta-actin [clone AC74] (Sigma), anti-Histone H4ac (pan-acetyl), and anti-Histone H3ac (pan-acetyl) (Active-Motif). The primary antibodies were revealed using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (DAKO) followed by chemiluminescence using Immobilon Western (Millipore). The chemiluminescent signals were recorded on a FUJIFILM LAS-3000, and the data were quantified using the MultiGauge V3.0 software.

Immunofluorescence and Photoablation Experiments

Immunofluorescence studies were performed on cells fixed in 4% paraformaldehyde (Sigma). The actin cytoskeleton was labeled using 1 $\mu\text{g ml}^{-1}$ FITC- or TRITC-conjugated phalloidin (Sigma). The anti-pT18-pS19-MLC (Cell Signaling), anti-cadherin-5 (BD transduction), and anti-paxillin (BD transduction) antibodies were detected using Texas RED- or FITC-conjugated secondary antibodies (Invitrogen). The fluorescent signals were analyzed with an LSM510-Meta confocal microscope using a 63 \times or 25 \times magnification lens (Carl Zeiss). Each picture represents the projection of four serial confocal sections. We routinely set the conditions for signal acquisition based on the control cells prior to imaging the cells treated with LT or the different HDAC or HAT inhibitors to compare the stress fiber content and intensity. The laser ablation experiments were performed in HUVECs expressing LifeAct-mCherry (gift from P. Chavrier, Institut Curie, Paris) and LifeAct-GFP

(Ibidi) or VE-Cadherin-GFP [Boyer et al., 2006]. These experiments were performed with an Inverted Laser Scanning Confocal LSM710NLO microscope (Zeiss). The laser ablations were performed with a 2-photon-type laser scaled to 805 nm with a pulse width <100 fs (30 iterations \times acquisition every 2 s). The images were processed with ImageJ and QuickTime pro 7 software (Apple). The bundling and polymerization indexes were calculated using the ImageJ plugin described in the Supporting Information.

Statistical Analysis

The data were analyzed with the statistical software Prism 5.0b. Unless specified in the figure legend, the significance of the data was evaluated with a one-way ANOVA and Bonferroni's post hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

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Résumé

La protéine HACE1 est une enzyme de la famille des E3 ubiquitine ligase de type HECT qui joue un rôle clé dans la régulation de l'homéostasie cellulaire. Elle contrôle notamment l'activité de la petite GTPase Rac1 en catalysant l'ubiquitination de sa forme activée pour un ciblage au protéasome 26S. *Rac1* est un gène essentiel qui contrôle de nombreux processus cellulaires tels que l'adhérence, la migration et la prolifération. Aussi, la perte d'expression d'HACE1 dues à des altérations génétiques ou épigénétiques est associée à de nombreuses pathologies humaines tels que le cancer, des syndromes neurodégénératifs et des maladies développementales. Pourtant, malgré l'importance de HACE1 en physiopathologie, rien n'est connu à ce jour sur la régulation post-traductionnelle de son activité. Au cours de ce travail, nous avons montré que la serine 385 de HACE1 est phosphorylée par les kinase PAKs de groupe I, en réponse à l'activation de Rac1 et de Cdc42. Nous montrons que le mutant HACE1(S385E), qui mime la forme phosphorylée de HACE1, présente une activité réduite d'ubiquitination de Rac1. De plus, nous mettons en évidence un rôle centrale de la régulation de la Ser-385 par phosphorylation dans l'oligomérisation de HACE1, définissant ainsi les bases moléculaires de la relation entre structure et fonction de HACE1. En parallèle, nous avons déterminé que la perte d'expression d'HACE1 altère la cohésion des jonctions entre cellules épithéliales. Cet effet de dissociation s'apparente à une transition épithélio-mésenchymateuse (EMT) caractérisée par un échange d'expression de la E-cadhérine par la N-cadhérine régulé au niveau transcriptionnel. L'ensemble de ce travail a donc permis de mettre en évidence un mode inédit de régulation par phosphorylation de l'activité de HACE1 contrôlée par les kinases PAK du groupe I, ainsi qu'un rôle majeur de HACE1 dans la régulation de la cohésion cellulaire et l'EMT.

Abstract

The E3 ubiquitin ligase HACE1 is a key regulator of cellular homeostasis best-characterized for its ability to control the activity of the Rho GTPase Rac1. This GTPase is encoded by an essential gene whose product controls a wide array of cellular processes such as cell adhesion, migration and proliferation. Accordingly, the repression of HACE1 expression due to genetic and epigenetic alterations has been associated with numerous pathologies, including cancer, neurodegenerative and developmental diseases. However, nothing is known about the posttranslational regulation of HACE1 activity. Here, we unveiled that HACE1 gets phosphorylated at serine Ser-385 by Group-I Pak kinases in response to Rac1/Cdc42 activation. Mechanistically, we define that the phosphomimetic mutant HACE1(S385E) displays a lower capacity to ubiquitinate Rac1 in cells. In addition, our work attributes to the phosphorylation of Ser-385 a pivotal role in the state of HACE1 oligomerization, which sets the basis for deciphering the relationship between HACE1 structure and activity. In parallel, we have found that the loss of HACE1 expression leads to the disruption of epithelial monolayer cohesion characterized by disrupted of cell-cell junctions. Accordingly, we determined that loss of HACE1 results in the acquisition of epithelial-mesenchymal transition (EMT) features, including a transcriptionally regulated switch of expression between E-cadherin and N-cadherin. Altogether, this work reveals a phospho-mediated regulation of HACE1 activity that is under the control of Group I PAKs and implicates HACE1 in the balance between epithelium integrity versus EMT.