Rac1 meets ubiquitin: New insights in Rac1 signalling
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Chapter 7

Summary and concluding remarks
The Rho-like small GTPases are members of the superfamily of Ras GTPases and are key regulators of the cellular cytoskeleton. The subfamily of RhoGTPases contains 22 members which show very high sequence homology, yet show unique biological effects (Didsbury et al., 1989; Hall, 1990; Kozma et al., 1997; Ridley et al., 1992; Ridley and Hall, 1992). The aim of this thesis was to gain more insight in the complex regulation and signalling of Rac1, one of the best studied Rho-like GTPases. It has been almost two decades since Rac1 was recognized as a crucial regulator of the actin cytoskeleton (Bosco et al., 2009; Didsbury et al., 1989; Ridley et al., 1992; Ridley and Hall, 1992). Accordingly, Rac1 was rapidly established to be a key regulator for various cellular processes, including cell polarization and migration, cell division, cell-cell and cell-matrix adhesion to the extracellular matrix as well as to other cells (Abo et al., 1991; Arulanandam et al., 2009; Bishop and Hall, 2000; Bosco et al., 2009; Didsbury et al., 1989; Hall, 1990; Ridley et al., 1992; Ridley and Hall, 1992). As a result, Rac1 signalling controls various physiological processes, such as differentiation, morphogenesis and immune responses. Finally, Rac1 signalling is implicated in development and metastasis of cancer (Kamai et al., 2010; Qi et al., 2009; Qiu et al., 1995). Thus, a better understanding of the regulatory pathways controlling Rac1 activation and downstream signalling is important.

Most small RhoGTPases, such as Rac1, are regulated by a series of regulatory proteins including Guanine nucleotide Exchange Factors (GEFs), GTPase-activating proteins (GAPs) and Guanine nucleotide Dissociation Inhibitors (GDIs) (Hall, 1990). GEFs and GAPs regulate the cycling of the GTPases between the active (GTP-bound) and inactive (GDP-bound) state, whereas the GDI is a cytosolic chaperone that binds inactive RhoGTPases (Bishop and Hall, 2000; Bosco et al., 2009; Rossman et al., 2005; van Aelst and D'Souza-Schorey, 1997). Interestingly, although the GDP-bound form of RhoGTPases is generally considered to be inactive, GDP-bound RhoGTPases have, in contrast to the current dogma, been shown to exert signaling functions (Grizot et al., 2001; Illenberger et al., 1998; Neel et al., 2007). This indicates that GDP-bound RhoGTPases are signalling competent,
which infers that there must also be additional pathways to limit this mode of RhoGTPase-dependent signaling. An increasing number of studies (including from our group, chapter 4 (Nethe et al., 2010)) have identified ubiquitylation as an important means to regulate RhoGTPase signalling (Boyer et al., 2006; Chen et al., 2009; Doye et al., 2002; Ho et al., 2008; Lanning et al., 2004; Lerm et al., 1999b; Lerm et al., 1999a; Lerm et al., 2002; Nethe et al., 2010; Perez-Sala et al., 2009; Pop et al., 2004; Rolli-Derkinderen et al., 2005; Sahai et al., 2007; Visvikis et al., 2008; Wilkins et al., 2004; Zhang et al., 2004). Ubiquitylation comprises a post-translational modification during which ubiquitin, a 76 amino acid protein, is covalently conjugated to lysine residues within target proteins. Whereas poly-ubiquitylation is established as a key signal for proteasome-mediated degradation, mono-ubiquitylation controls protein targeting and internalisation as well as protein-protein interactions (Acconcia et al., 2009). Although information on ubiquitylation of RhoGTPases is limited, this type of post-translational processing could represent an important extension of the canonical models describing the regulation of RhoGTPases. This topic has been reviewed in Chapter 2 of this thesis.

As mentioned above, Rho-like GTPases show very high sequence homology. An exception to this rule is the so-called hypervariable region in the C-terminonal region of these GTPase, just upstream of terminal lipid anchor. Previously, we identified a series of regulatory proteins that associate selectively to the hypervariable C-terminus of the Rac1 GTPase. These include the Rac1 GEF β-PIX, which recruits Rac1 towards Focal Adhesions (FAs) (ten Klooster et al., 2006), the lipid kinase PIP-5-Kinase (Hennik 2003, Halstead 2010) and the nuclear proto-oncogene SET/I2PP2A. In Chapter 3 we describe the identification of the membrane-associated adapter protein caveolin-1 (Cav1) as a Rac1 interactor. The interaction of Rac1 with Cav1 had been previously suggested but was never mapped (del Pozo et al., 2004; Grande-Garcia et al., 2007), and in this chapter we describe that this association requires the C-terminal domain of Rac1 and the scaffolding domain of Cav1. In addition, the interaction of Rac1 with Cav1 was found to be driven by the activation of Rac1, and activation of Rac1 significantly promoted a pool of Cav1 to co-localise with Rac1 at Focal Adhesions (FAs). In agreement with
this finding are previous suggestions of Cav1 associating to integrins (Wary et al., 1998). Moreover, cell adhesion stimulated both the association of Rac1 with Cav1 as well as the recruitment of Cav1 towards FAs, suggesting that Rac1 and integrins cooperate in the targeting of Cav1 to FAs. In the FAs, Cav1 might functions in integrin- and Rac1-mediated signalling by regulating the internalization of specific membrane domains (del Pozo et al., 2002; del Pozo et al., 2004; Grande-Garcia et al., 2007).

Several studies have suggested that Cav1 tyrosine phosphorylation drives Cav1 targeting to FAs (del Pozo et al., 2005; Goetz et al., 2008; Grande-Garcia et al., 2007; Nethe et al., 2010). However, these studies were questioned when it was shown that the commonly used mouse antibody to tyrosine-phosphorylated Cav1 cross-reacts with tyrosine-phosphorylated paxillin, which also resides in FAs (Hill et al., 2007). In Chapter 4, the issue of FA localization of Cav1 was studied in more detail, which, based on these and recent findings in the literature, resulted in the presentation of an alternative model. This model proposes that Src kinase-driven phosphorylation of Cav1 at Tyr14 stimulates the translocation of Cav1 from cholesterol-enriched membrane patches towards cholesterol-poor regions, such as FAs (Swaney et al., 2006). Dephosphorylation of Cav1, by an as yet unidentified tyrosine phosphatase, would subsequently destabilize Cav1 at FAs, thereby leading to the internalization of Cav1 and Cav1-associated FA components.

Cav1 targeting to FAs has been suggested to promote FA turnover as well as to silence Rac1 signalling by stimulating Rac1 internalization (del Pozo et al., 2002; del Pozo et al., 2004; Goetz et al., 2008; Grande-Garcia et al., 2007). Consequently, removal of Rac1 from the cellular periphery is assumed to drive its inactivation, as cytosolic Rac1 is predominantly found in its inactivate, GDP-bound form. In Chapter 5 the underlying mechanisms by which Cav1 drives Rac1 inactivation was studied in more detail. To confirm earlier suggestions that Cav1 is a negative regulator of Rac1, Rac1 activity and signalling was examined in Cav1-deficient cells. Depletion of Cav1 by siRNA and shRNA led to an increase in the activation of Rac1, thereby enhancing cell spreading. Unexpectedly however, depletion of Cav1 also increased Rac1 protein expression levels. Further analysis
showed that Cav1 regulates Rac1 expression levels by promoting the poly-ubiquitylation of Rac1. Because loss of Cav1 does not impair Rac1 mono-ubiquitylation, this step might occur at the plasma membrane, independent of Cav1-dependent events. Interestingly, expression of an N-terminally linked ubiquitin-Rac1 fusion, used to mimic mono-ubiquitylated Rac1, showed that such a protein localized prominently to endosomal structures, rather than to the plasma membrane (described in Chapter 2). In addition, a K147R mutant of activated Rac1, which cannot be ubiquitylated, shows an enhanced accumulation at the plasma membrane. Since activated Rac1 colocalises with Cav1 at FAs, and Cav1 can drive Rac1 internalization, it is tempting to speculate that (mono)-ubiquitylation of Rac1 drives its internalisation in a Cav1-dependent fashion.

Several E3 ubiquitin ligases were previously identified as binding partners of Rac1, such as the RING-finger ubiquitin E3-ligases POSH (Plenty Of SH3s) (Goetz et al., 2008; Grande-Garcia et al., 2007; Kim et al., 2006; Visvikis et al., 2008), Cbl (Cas-Br-M (murine) ecotropic retroviral transforming sequence) (Sattler et al., 2002; Schmidt et al., 2006; Teckchandani et al., 2005) and the SCF(βTrCP)-E3 ligase complex (Boyer et al., 2004; Senadheera et al., 2001). However, none of these were found to target Rac1, leaving the Rac1 ubiquitin ligase(s) to be identified.

As a result of the above findings, our discovery that the HECT (Homologous to the E6-AP Carboxyl Terminus) E3 ligase Nedd4-1 associates with the Rac1 C-terminus, as described in chapter 6, was of great interest. Nedd4-1, initially found to be down-regulated during neuronal development, is a ubiquitin E3 ligase that is highly conserved from yeast to human (Kumar et al., 1997). Nedd4-1 is ubiquitously expressed and essential for development, since depletion of Nedd4-1 causes perinatal lethality. However, the mechanisms underlying this phenotype remain elusive (Fouladkou et al., 2010; Liu et al., 2009).

Despite its association to Rac1, we found no evidence for Nedd4-1 being a E3 ubiquitin ligase for Rac1. Its pronounced co-localization with active Rac1 at cell-cell junctions, however, prompted us to further examine its potential role in the regulation of intercellular contacts. Other than the finding that Nedd4-1 binds connexion-43, a GAP junctional protein (Leykauf et al., 2006), its contribution to
the regulation of epithelial cell junctions has received little attention. We found that lentiviral-based depletion of Nedd4-1 notably affects junctional integrity of epithelial monolayers. Similarly, ECIS-based, real-time analysis of transepithelial resistance indicated that cell-cell contact maturation is hampered upon depletion of Nedd4-1. These data thus suggest that Nedd4-1 promotes the maturation of cellular junctions, as further confirmed by the increased maturation of cell-cell junctions following overexpression of Nedd4-1. Interestingly, in the course of these studies, examining effects on the cell cytoskeleton, we found that Nedd4-1-expressing cells showed a decrease in the levels of acetylated microtubules (MTs). Posttranslational modification of MTs by acetylation is a highly conserved process comprising conjugation of an acetyl moiety on the luminal site of α-tubulin at Lys 40 (Verhey and Gaertig, 2007). MT acetylation reduces their turnover, and increases MT stability in response to MT-destabilising pharmaceutical compounds such as nocodazole (Verhey and Gaertig, 2007). It is well established that MTs contribute to the formation of intercellular junctions, by facilitating transport of junctional proteins such as N-cadherin and connexin-43 towards the cell-cell border, enhancing the maturation of cell-cell contacts (Mary et al., 2002; Shaw et al., 2007; Teng et al., 2005).

Acetylated MTs, in comparison to non-acetylated MTs, show different binding affinities towards MT-linked motor proteins and show altered directionality of motor protein-mediated vesicular traffick (Reed et al., 2006). As a result, MT acetylation could well interfere with MT-driven maturation of cell junctions. We therefore hypothesised that Nedd4-1-mediated downregulation of MT acetylation promotes cell-cell junction maturation. To test this hypothesis, candidate proteins known to affect MT acetylation were examined for encoding a PPXY or RXXQE motif, previously shown to be required for subsequent ubiquitylation by Nedd4-1 (Persaud et al., 2009). This search identified dishevelled-1 (DVL-1) as a potentially new substrate for Nedd4-1. Binding of the WW-region of Nedd4-1 to the DEP-C-terminal portion of DVL-1 was found to drive DVL-1 ubiquitylation on two conserved lysine residues situated between DVL-1 DIX and PDZ domains. Conversely, depletion of Nedd4-1 increased protein levels of endogenous DVL-1 as
well as the acetylation of MTs, confirming that Nedd4-1 controls MT acetylation by regulating DVL-1 protein levels. To confirm that MT acetylation impairs junction maturation, cells were treated with the pharmaceutical inhibitor TrichostatinA (TSA) to inhibit histone deacyetylase (HDAC) activity. HDACs are known to regulate histone acetylation, but were more recently also found to deacetylate MTs (Zhang et al., 2003). TSA-based induction of MT acetylation, like we showed following the downregulation of Nedd4-1, impairs junction maturation as measured by ECIS and by live-cell imaging. Moreover, the fast response of junction reformation after treating cells with TSA, abrogated the involvement of TSA-driven alteration of gene expression, but implicated Ac-MTs to impair ECJ maturation. In summary, we propose Nedd4-1 to alter junction stability by controlling the expression levels of DVL-1 which regulates MT acetylation.

Rac1 has been well established to regulate the formation and maturation of cell junctions, by coordinating WAVE-mediated activation of the ARP2/3 complex, which stimulates formation of F-actin-based cellular protrusions (Harris and Tepass, 2010). In addition, since active Rac1 bound and stimulated Nedd4-1-mediated proteasomal degradation of DVL-1, we propose that Rac1 also stimulates formation of cell junctions by coordinating the Nedd4-1-DVL-1 axis to control MT acetylation. HDAC-6 has been identified to control MT deacetylation (Valenzuela-Fernandez et al., 2008), and its activity is inhibited by the deubiquitylating enzyme CYLD (Wickstrom et al., 2010). Interestingly, CYLD also to deubiquitylates DVL-1, thereby altering DVL-1 signalling (Tauriello et al., 2010). These findings therefore indicate that CYLD and HDAC-6 could coordinate the Rac1-Nedd4-1-DVL-1 axis. As a result, these proteins are obvious candidates for future studies aimed at the underlying mechanisms concerning the regulation of MT acetylation and its role in junctional integrity.

At the time of writing this chapter, a Pubmed search for Rac1 revealed 3769 publications. Yet, we are only just beginning to understand and appreciate the complexity of Rac1 regulation and signalling. This thesis aims to provide new insights regarding the molecular basis of the Rac1 localization, downstream effects and inactivation. Identification of the relevant E3 ubiquitin ligases that target Rac1
and thereby its signalling capacity and further analysis of the Rac1-Nedd4-1-DVL-1 axis as a novel pathway regulating cell-cell junctions will be obvious and exciting goals for future research.

References


