Review

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Mechanistic insights on spatiotemporal control of Ras-signaling

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Abstract: Proteins of the Ras-family are guanine nucleotide binding proteins (GNBPs) involved in a variety of fundamental cellular processes, including cell proliferation, cell differentiation, cytoskeleton dynamics, vesicular processes and intracellular transport. A dysregulation of Ras-signaling has been found to be causative for the development of diseases, such as diverse cancer types, RASopathies, neurodegenerative diseases and ciliopathies. Ras-proteins cycle between a GTP-bound on-state and a GDP-bound off-state. Ras-proteins show low intrinsic rates for nucleotide exchange and nucleotide hydrolysis. They need guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) to accelerate both functions in order to act as true molecular switches in the physiological context. Ras-proteins and their regulators/effectors are targets of post-translational modifications (PTMs) such as phosphorylation, ac(et)ylation, lipidation and ubiquitination. These PTMs regulate their activity, subcellular localization and turnover. In a biological perspective, PTMs are essential components for cellular signaling cascades and for molecular pattern formation. Bacterial pathogens use PTMs of Ras-proteins to allow efficient infection processes. Besides, modifications of Ras-proteins were shown to be of therapeutic potential in oncogenic variants such as Ras G12C. In this review, we summarize current knowledge on Ras-signaling, while emphasizing PTMs as dynamic signaling hubs for its precise spatiotemporal control.

Keywords: Ras; guanine nucleotide binding proteins; post-translational modification; acetylation

1 Introduction

Cells must communicate with their environment to adjust processes to changing conditions. To this end, signals are sensed by cell surface receptors including G-protein coupled receptors (GPCRs), integrins, and receptor tyrosine kinases (RTKs) (Bahar et al. 2023; Sinkala et al. 2021). Receptors are activated by binding extracellular ligands such as growth-factors and this binding energy is translated into conformational changes, which are transferred from the extracellular into the intracellular space (Bahar et al. 2023; Sinkala et al. 2021). Activation of the receptors can result in the activation of a signal transduction cascade, involving GTP-binding proteins, including heterotrimeric G-proteins or small guanine nucleotide binding proteins (GNBPs) of the Ras-family (Sinkala et al. 2021). From an evolutionary view, Ras-proteins belong to the superfamily of P-loop containing nucleoside triphosphate hydrolases (P-loop NTPases) and within this superfamily to the family of G-proteins (Figure 1A) (Chandonia et al. 2022; Fox et al. 2014). G-proteins also encompass translation elongation and initiation factors such as eukaryotic eEF2, eEF-1α or prokaryotic EF-G, EF-Tu, dynamin-related large G-proteins, the Gα-subunit of heterotrimeric G-proteins, signal-recognition particle (SRP) and its receptor (SR), septins and other proteins such as the bacterial cell division GTPase MinD, ribosomal assembly GTPases EngA/B/C and the tRNA modification enzyme TrmE (MnmE) (Chandonia et al. 2022; Fox et al. 2014; Leipe et al. 2002, 2003; Saraste et al. 1990; Walker et al. 1982). Today, more than 150 members are reported to belong to the Ras-family (Colicelli 2004). These are classified in five major subfamilies, i.e., the Ras (Rat sarcoma) subfamily involved in cell proliferation and differentiation, the Rho (Ras-homologous) subfamily involved in regulation of the actin cytoskeleton, the Arf/Arl (ADP-ribosylation factor/Arf-like) subfamily involved in vesicular trafficking and ciliary processes, the Rab (Ras gene from rat brain) subfamily involved in intracellular membrane trafficking and the Ran (Ras-like nuclear)

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subfamily involved in nucleo-cytosolic transport processes (Figure 1A) (Barbacid 1987; Colicelli 2004; Der et al. 1986; Kahn and Gilman 1984; Karnoub and Weinberg 2008; Malumbres and Barbacid 2003; Seeburg et al. 1984). Members of the different sub-families bind to different effectors, and even the Ras-proteins within the subfamilies target a different set of effectors although their sequences and structures are very similar. We previously studied the interaction of the Rho-proteins RhoA, Rac1 and Cdc42 with different isoforms of the actin-polymerization regulator mDia. The interactions suggest that during evolution, only a few amino acid exchanges are needed to determine the specificity of Ras-proteins for the interaction with their effectors (Lammers et al. 2005, 2008; Rose et al. 2005). The founding member Ras was initially identified as the product of a virus-encoded oncogene (v-*ras*) eliciting formation of sarcoma in infected rats (Cox and Der 2010; Harvey 1964; Kirsten and Mayer 1967; McCormick 2022; Scolnick et al. 1973, 1979; Scolnick and Parks 1974; Shih et al. 1979; Wittinghofer and Nassar 1996). For a full historical outline of discoveries in the Ras research field see other excellent reviews (Cox and Der 2010; McCormick 2022). Later, Ras encoding genes were also identified in humans and Ras was shown to activate different signaling pathways (Figure 1B) (Ellis et al. 1981; Langbeheim et al. 1980). Initial studies focused on three isoforms: H-Ras (Harvey-Ras), K-Ras4A/K-Ras4B (Kirsten-Ras), and N-Ras (Neuroblastoma-Ras) (Shih et al. 1981). They are proto-oncogenes, converted to oncogenes by mutation. Ras is found to be mutated in about 30 % of all human cancers, and even up to 90 % of pancreatic cancers, showing its key role in cancer development (Prior et al. 2012). Mutations predominantly occur at key residues like Gly12 or Gly13 as well as Gln61 (Prior et al. 2012), which render Ras constitutively active. The different Ras-isoforms, i.e., H-Ras (bladder cancer), K-Ras (colorectal/pancreatic cancer), and N-Ras (neuroblastoma, melanoma), are found to be mutated in different prevalence in human tumors. Mutated Ras contributes to different stages in cancer development, i.e., initiation, progression and metastasis. Ras-signaling is regulated on diverse layers ensuring a precise coordination within cells in space and time (Hancock 2003; Mor and Philips 2006; Omerovic et al. 2007; Plowman and Hancock 2005; Rocks et al. 2006). One layer is at the transcriptional level by transcription regulators controlling expression of genes encoding for Ras-proteins, their specific regulators and effectors. Another layer of regulation exists at the post-transcriptional level in form of regulatory RNAs including long non-coding RNAs (lncRNAs) (Rotblat et al. 2011; Saliani et al. 2022). Finally, Ras-signaling is regulated by other proteins and a diverse set of post-translational modifications (PTMs), including lipidation, phosphorylation, *S*-nitrosylation, *S*-oxygenation, ac(et)ylation, ubiquitination/SUMOylation and proteolytic processing (Abdrabou and Wang 2018; Ahearn et al. 2018; Campbell and Philips 2021; Dharmaiah et al. 2025; Evans et al. 1991; Navarro-Lerida et al. 2021; Osaka et al. 2021; Walton et al. 2020). Several bacterial pathogens directly target proteins of the Ras-family to achieve an efficient infection process. This review highlights the current state-of-the-art of Ras-signaling regulation by GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins) and solubilization factors. Moreover, it emphasizes the role of PTMs for Ras-signaling, their importance for intracellular molecular pattern formation and subsequent spatiotemporal coordination of Ras-signaling. We describe examples of how Ras-signaling is targeted by bacterial pathogens for efficient infection. We illustrate recent methodological advances to study PTMs involved in Ras-signaling and summarize novel therapeutic developments aiming to inhibit oncogenic Ras.

2 The G-domain as the core-structure of the molecular switch

The first correct structure of H-Ras, bound to the non-hydrolyzable GTP-analog GppNHp, revealed structural similarities with the so-called guanine nucleotide binding domain (G-domain) of *Escherichia coli* EF-Tu, composed of an α/β-fold with a central β-sheet containing six β-strands, five of which were in parallel (β1, β3, β4, β5, β6) and one in antiparallel orientation (β2), surrounded by five α-helices (Figure 1C) (Lacour et al. 1985; Pai et al. 1989; Wittinghofer and Pai 1991). Members of the Ras-family show variations in this structural G-domain fold: Rho-proteins contain an additional α-helix connecting α4 and β5, Ran has an extended C-terminus containing acidic residues, and Arf/Arl-proteins have an additional N-terminal amphipathic α-helix important for membrane binding (Kahn and Gilman 1984; Scheffzek et al. 1995; Wei et al. 1997). Ras-proteins are molecular switches which cycle between an inactive GDP-bound and an active GTP-bound conformation (Figure 1D) (Gasper and Wittinghofer 2019; Vetter and Wittinghofer 2001; Wennerberg et al. 2005). As also known for other proteins and enzymes, important functional regions of Ras-proteins are located in loop regions connecting the secondary structure elements of the G-domain (Figure 2A). In the GTP-bound conformation, the two switch regions, switch I (G2 motif; Ras: xTx; T: Thr35) and switch II (G3 motif; Ras: DxxG; D: Asp57, G: Gly60), are adopting a stable conformation by contacting the γ-phosphate (Vetter and Wittinghofer 2001). The GDP-bound state is characterized by more flexible switch-regions with increased conformational dynamics. This is known as “loaded-spring mechanism” (Figure 2B).

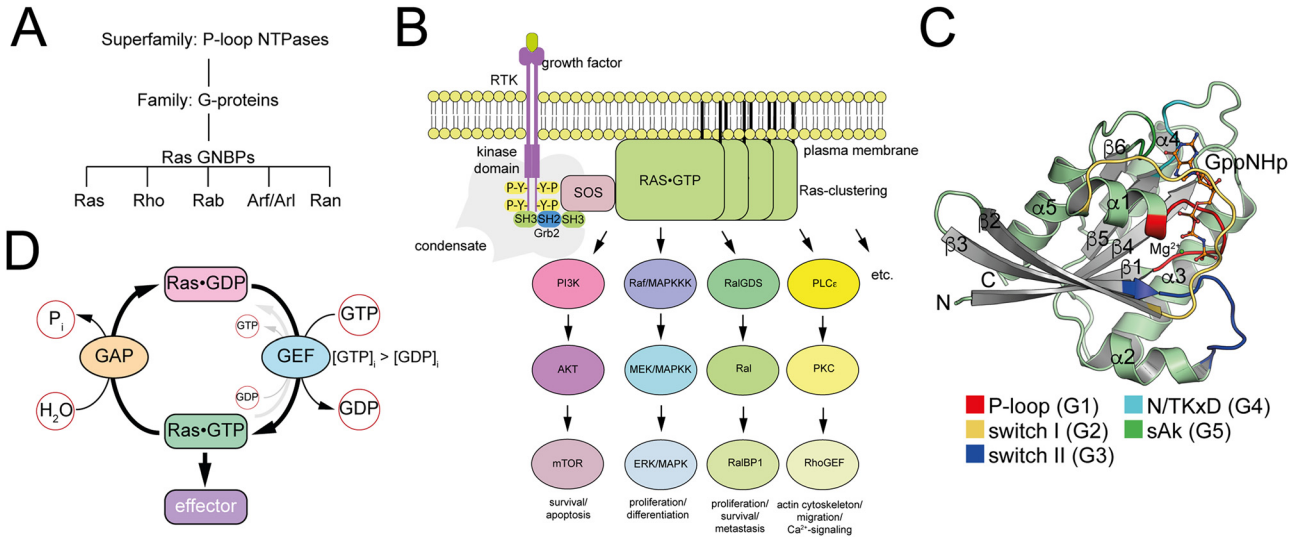

Figure 1: Ras-proteins are molecular switches regulating fundamental cellular functions. (A) Ras-proteins belong to the superfamily of P-loop NTPases and to the family of G-proteins. They can be subdivided into five main groups, i.e., Ras, Rho, Rab, Arf/Arl and Ran. (B) Ras regulates fundamental cellular processes such as cell proliferation, cell differentiation, cell migration and the actin cytoskeleton. It is activated at the plasma membrane (PM) by the GEF SOS, which is recruited to the PM upon stimulation of transmembrane receptors by binding to ligands such as growth factors (temporal control). Receptors include receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR) or platelet derived growth factor receptor (PDGFR). Ras itself is also recruited to the PM by lipidation of C-terminal residues and/or a poly-basic region (spatial control). (C) Ras-proteins are structurally composed of the G-domain, an α/β-fold consisting of a central six-stranded β-sheet surrounded by five α-helices. The structure shows that in the G-domain of H-Ras·GppNHp (PDB: 5P21) the β-sheet is parallel for β-strands β1-β3-β4-β5-β6 while β2 is oriented antiparallel to β3. Five conserved sequence motifs (G1-G5) are important for binding GTP/GDP, for binding of Mg^{2+} needed to establish high-affinity for nucleotides, and for the switch function. In the GTP-bound state, switch I and switch II adopt a stable conformation enabling binding to effector proteins. Regulators such as GEFs, GAPs and GDIs also bind to this side. (D) The functional cycle of Ras-proteins. Ras-proteins are molecular switches that can change from a GDP-bound inactive state, to a GTP-bound active state. As the intrinsic rates for nucleotide dissociation and nucleotide hydrolysis are very low, GEFs and GAPs are needed to complete the cycle by accelerating the rates by several orders of magnitude. GEFs stabilize a nucleotide free conformation, meaning in principle the reaction is reversible. Ras is predominantly charged with GTP due to the higher intracellular concentration of GTP compared to GDP. Effector proteins are defined as molecules that bind only to the GTP-bound state.

Effector proteins discriminate between the GDP- and GTP-bound conformation and specifically target the GTP-bound state of the Ras-protein. The P-loop (G1-motif; Walker A motif) contains the conserved consensus sequence GxxxxGKS/T (Ras: 10-GxxxxGKS-17) and constitutes the sequence motif most often found in P-loop NTPases. It is needed for high-affinity nucleotide binding and nucleotide hydrolysis (Figure 2C) (Saraste et al. 1990; Walker et al. 1982). Ras-proteins need a Mg^{2+}-ion for high-affinity nucleotide binding (Figure 2D). Based on structural considerations, nucleotide binding proteins can be subdivided into two classes: The first class comprises small GNBPs, myosin and kinesin, and the second class contains ASCE proteins (additional β-strand between the P-loop and the strand containing a catalytic Glu/E), including ABC (ATP-binding cassette) proteins (Leipe et al. 2002; Wittinghofer 2016). Notably, the preference for guanine nucleotides is established by the G4-motif (N/TKxD; Ras: 116-NKxD-119) and G5-motif (sAk; Ras: 145-SAK-147) contacting the guanine base (Figure 2C) (Vetter and Wittinghofer 2001). In fact, D119 of the G4-motif contacts the exocyclic N^2-amino-group of the guanine base and is mostly responsible for nucleotide specificity. Mutation D119N in Ras was shown to alter the specificity from guanine to xanthine (Zhong et al. 1995). Most Ras-proteins show equally strong binding to GDP and GTP, with high affinity in the nanomolar to picomolar range, while ATP/ADP and GMP binding is more than six-orders of magnitude lower and estimated to be in the millimolar range (John et al. 1990). This suggests that, during evolution, proteins involved in signaling were often selected for using guanine nucleotides rather than adenine nucleotides that are predominantly used in metabolic processes (Greiner and Glonek 2021). Along that line, the GTP:GDP ratio is regarded as a direct indicator for the state of cellular signal transduction (Haga and Ridley 2016). Cells often retain a high intracellular ATP concentration in the millimolar range while ADP concentration is more than one-order of magnitude lower (Traut 1994). GTP and GDP concentrations were shown to be in the micromolar range (GTP: ~300 µM; GDP: ~36 µM) and therefore at least tenfold lower compared to ATP and ADP, respectively (Boissan et al. 2014; Michailova and McCulloch 2001; Traut 1994). Although they might vary slightly depending on the cellular type and metabolic state, the tight binding of Ras towards GTP and GDP suggests these cellular

concentrations are sufficient to saturate Ras-proteins. GEFs lower the nucleotide affinity by several orders of magnitude by stabilizing a nucleotide-free conformation of the Ras-protein. To this end, establishment of a Ras-like molecular switch likely co-evolved with the cellular GTP/GDP concentrations. A similar system would not be possible with the existing cellular ATP/ADP concentrations, as these would result in different equilibria compared to GTP/GDP. For example, GEFs might not be able to displace the nucleotide to generate a nucleotide-free state of the Ras-protein-GEF complex. Ras-proteins, effectors and regulators are targeted by multiple co-translational and post-translational modifications that also modulate their functions, affecting their subcellular localization, stability and activities. These are described in the following sections.

3 Proteins involved in Ras-signaling undergo co-translational processing

Per definition, PTMs cover all measures to modify proteins following translation and folding. This contrasts with modifications occurring co-translationally, i.e., on the nascent polypeptide chain, which include removing the formyl group of initiator amino acid formyl-methionine by peptide deformylase (PDF), removing the resulting methionine by aminopeptidase (MetAP), and often acetylation of the new N-terminus of proteins by a family of N-terminal acetyltransferases (NATs), which use acetyl-CoA as donor molecule for the acetylation of the *N*-(α)-amino group (Aksnes et al. 2019). The nascent polypeptide-associated complex (NAC) was recently shown to recruit MetAP to the ribosomal exit tunnel to cleave-off the N-terminal methionine in cytosolic proteins (Gamerdinger et al. 2023). For K-Ras, N-terminal acetylation was shown to affect the conformation of the protein by interaction of the *N*-(α)-acetyl group with the central β-sheet modulating stability of the N-terminal region and switch I (Dharmaiah et al. 2019). If, after deformylation and removal of the initiator Met, a Gly is exposed within the consensus sequence Gly-x-x-x-(Ser/Thr/Cys) (x: varying amino acid), the α-amino group at the Gly can be co-translationally *N*-myristoylated by myristoyl-CoA:protein-*N*-myristoyltransferase (Gamerdinger et al. 2025). This was described for proteins of the Arf/Arl subfamily of the Ras-family (Arl1: Met-Gly-Gly-Phe-Phe-Ser; Arf1: Met-Gly-Asn-Ile-Phe; Arf2/4: Met-Gly-Leu-Thr-Ile-Ser). While Arf-proteins were identified to be *N*-myristoylated, only some Arl-proteins, including Arl1, were found to carry this N-terminal modification (Donaldson and Jackson 2011). *N*-myristoylation or *N*-acetylation was shown to be necessary for binding of Arf/Arl-proteins to cellular membranes, including Golgi and endomembranes (Behnia et al. 2004; Setty et al. 2004). Arl8a and Arl8b were reported to be acetylated at the N-terminal methionine, regulating their localization within lysosomes, where they might be involved in regulating lysosomal transport processes (Hofmann and Munro 2006). Recent reports also suggest N-terminal acetylation and N-terminal myristoylation can occur post-translationally, however, if this is also realized for Ras-family GNBPs or regulators thereof needs further investigations (Aksnes et al. 2019; Chen and Kashina 2021; Matsumoto et al. 2023; Varland et al. 2015). Further co-translational modifications are *N*-glycosylation of proteins at Asn side chains present in Asn-x-Ser/Thr motifs upon import of the growing polypeptide chain into the lumen of the endoplasmic reticulum (ER). H-Ras was reported to alter *N*-linked glycosylation of surface exposed proteins affecting the invasive potential occurring before morphological transformation in cancer cell development (Bolscher et al. 1988). Apart from these modifications, proteins undergo co-translational folding which is supported by binding to chaperones such as trigger factor (TF) in *E. coli* or RAC/NAC in eukaryotes and heat shock proteins [Hsp60 (GroEL)/Hsp70 (DnaK), etc.] (Deuerling et al. 2019; Preissler and Deuerling 2012). This is accompanied by the action of protein disulfide isomerase (PDI) catalyzing thiol-disulfide-exchange reactions, ensuring the correct formation of disulfide bonds, and peptidyl-prolyl-*cis*/*trans*-isomerase (PPI) catalyzing conversion of peptide bonds preceding proline side chains from *cis* to *trans* and *vice versa* (Gothel and Marahiel 1999; Hirayama et al. 2021). Both reactions can be rate-limiting steps in protein folding. Moreover, the peptidyl-prolyl-*cis*/*trans*-isomerase Pin1 is important in cellular signaling by catalyzing the *cis*/*trans*-isomerization of peptide-bonds preceding proline side chains on Ser/Thr-Pro-motifs, in which the Ser/Thr is phosphorylated (Liou et al. 2011). Pin1 was suggested to directly bind to H-Ras affecting the aggressiveness of breast cancer cells (Saeidi et al. 2020). Besides, quality control mechanisms acting co-translationally ensure the quality of newly synthesized proteins. One of such mechanism is degradation of proteins which are marked by co-translational *N*-(α)-acetylation via the *N*-end rule pathway (Nguyen et al. 2018; Varshavsky 2011). In this pathway, proteins are recognized by their N-termini, called *N*-degrons, by ubiquitin E3 ligases (Bachmair et al. 1986; Varshavsky 2008; Varshavsky 2011). Ubiquitination results in the proteolytic degradation via the proteasome (Varshavsky 2008, 2011). G-protein signaling was reported to be regulated by N-terminal acetylation and the *N*-end rule pathway (Park et al. 2015). Arl3 is protected against degradation via the *N*-end rule pathway by acetylation of the N-terminal methionine (Kim et al. 2014). Next to the co-translational processes described here, proteins involved in Ras-signaling also undergo various PTMs.

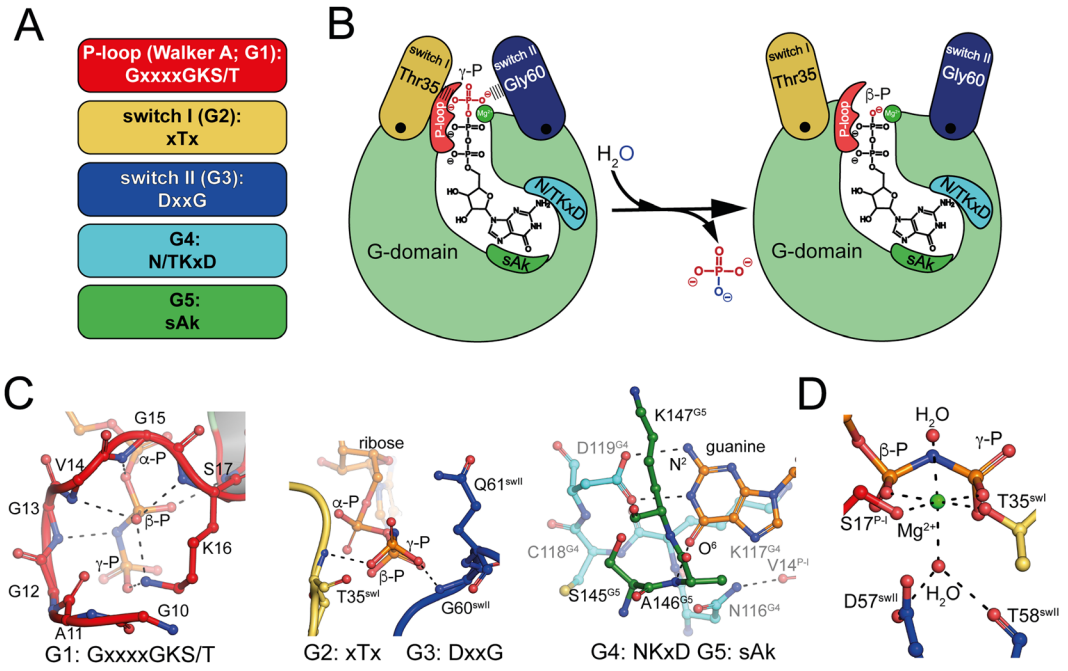

Figure 2: Conserved sequence motifs in Ras-proteins needed for the switch function. (A) Ras-proteins contain five conserved sequence motifs (G1-G5). The P-loop (phosphate-binding loop; G1) is needed for GTP/GDP-binding, switch I (G2) and switch II (G3) are needed for the switch function, G4 and G5 are contacting the guanine base. x: any amino acid; S/T: either Ser or Thr; N/T: either Asn or Thr; lower case: less strictly conserved. (B) Ras-proteins act as molecular switches using the "loaded-spring-mechanism" to switch between the GTP-loaded "on"-state and the GDP-loaded "off"-state. In this mechanism Ras is regarded as a loaded spring in the GTP-bound state by Thr35 of switch I (G2) and G60 of switch II (G3) contacting the γ-phosphate of GTP. Ras-proteins can intrinsically hydrolyze GTP, however, the hydrolysis rate is not sufficient to establish a molecular switch under physiological conditions. GAPs accelerate the hydrolysis rate by several orders of magnitude. Upon GTP-hydrolysis and dissociation of the P_i (or $H_2PO_4^-$) to form GDP, the loaded-spring relaxes and the switch regions become flexible. (C) The P-loop, switch I, switch II and the N/TKxD and sAk-motifs in Ras are needed for nucleotide binding and the molecular switch function. Left panel: The P-loop (G1; GxxxxGKS/T) creates a polyanion hole by binding the nucleotide's β- and γ-phosphates. The main chain amide groups contact the negatively charged phosphates by wrapping around them. This tight packing is only possible to the Gly-rich sequence of the P-loop. The side chain of Lys16 neutralizes the negative charges at the β- and γ-phosphates. Middle panel: Thr35 of switch I (G2) and Gly60 of switch II (G3) form main chain interactions with the γ-phosphate of GTP thereby stabilizing a conformation of the switches that creates a binding surface for effector proteins. In the GDP-bound state these interactions are abolished resulting in a higher flexibility of the switch-regions that does not allow binding to effector proteins. Right panel: the motifs G4 (N/TKxD) and G5 (sAk) mediate nucleotide binding by contacting the base with Lys117 of G4, forming a stacking interaction with the aromatic base as well as Asp119 of G4 and forming an interaction with the exocyclic N^2-amino group of the guanine base. Moreover, Ala146 of G5 contacts the exocyclic O^6-carbonyl with the main chain. Besides creating binding affinity, the latter two are also important for establishing specificity towards guanine nucleotides. (D) All Ras-proteins use Mg^{2+} for high-affinity nucleotide binding. The GTP/GDP is bound in the nanomolar to picomolar range. The Mg^{2+}-ion is hexacoordinated establishing an octahedral geometry. The β- and γ-phosphates of the GTP bind the Mg^{2+} in a bidentate fashion, and the square plane is completed by the side chains of Ser17 (P-loop) and Thr35 (switch I). Two water molecules complete the octahedral geometry by occupying the apical positions of the square bipyramide.

4 Post-translational modifications enable a dynamic regulation of Ras-signaling

While the human genome contains approximately 20.000 protein-encoding genes, estimations suggest that each cell contains one million protein variants (Omenn et al. 2016). The variety of the proteome is substantially increased by processes such as alternative splicing and PTMs (Stepankiw et al. 2023; Tress et al. 2017; Tress et al. 2007). Following translation and folding, proteins can undergo a variety of PTMs, including chemical modifications such as phosphorylation at Ser/Thr and Tyr side chains, ac(et)ylation of the epsilon amino group of lysine side chains and of the alpha amino group at the proteins' N-terminus, nitrosylation and lipidation, in the form of isoprenylation and/or fatty acyl modifications, ac(et)ylation of cysteine side chains, and glycosylation of Ser/Thr or Asn side chains (Keenan et al. 2021; Suskiewicz 2024). Next to these modifications, proteins can be modified by attaching other proteins by forming isopeptide bonds between the substrates' lysine side chains and the carboxyl group of the C-terminal Gly in ubiquitin, SUMO or other ubiquitin-like proteins (ULPs), such as

NEDD8, ISG15 or FAD10 (Dikic and Schulman 2023; Suskiewicz 2024). Additionally, proteins are often modified by proteolytic processing (Aepfelbacher et al. 2003; Lopez-Otin and Bond 2008; Park and Park 2024; Rogers and Overall 2013). Proteins of the Ras-family, their regulators and effectors are all targeted by these PTMs (Ahearn et al. 2011a; Campbell and Philips 2021; Shi et al. 2025). PTMs of Ras-family proteins regulate protein function by various mechanisms. They influence the subcellular localization of Ras-proteins, impact protein-protein interactions, act as allosteric regulators for protein function, mediate protein turnover and furthermore allow the modulation and integration of signals by PTM-crosstalk (Abdrabou and Wang 2018; Ahearn et al. 2011a; Campbell and Philips 2021; Shi et al. 2025). Moreover, regulation of protein function by PTMs allows dynamic modulation and coordination of signaling pathways depending on the cellular state, without the need to degrade and resynthesize proteins. In the following sections we will describe how Ras-proteins are regulated by GEFs, GAPs and GDIs/GDI-like solubilizing factors with an emphasis on how PTMs modulate their activity.

5 Guanine-nucleotide exchange factors ensure activation of Ras-proteins

The intrinsic rates for nucleotide dissociation (for H-Ras: k_{diss} of $2 \times 10^{-5}\,s^{-1}$) and nucleotide hydrolysis (for H-Ras: k_{cat} of $1.2 \times 10^{-4}\,s^{-1}$) of Ras-proteins are too low to constitute a molecular switch in a physiological context (Gideon et al. 1992; Lenzen et al. 1998). Notably, the exact values vary for each individual GNBP. There are GNBPs with higher off-rates for nucleotide dissociation and higher or lower catalytic rates for GTP-hydrolysis. To function as molecular switch, the Ras-protein must be quickly activated and inactivated to ensure a fast signaling response and avoid inappropriate physiological outcomes, such as an extended cell proliferation in case of activated Ras or actin dynamics in case of activated Rho. Therefore, although Ras-proteins accelerate nucleotide hydrolysis by several orders of magnitude compared to hydrolysis in solution, in the physiological context the term small GTPase is misleading. It is more appropriate to use the term guanine nucleotide binding proteins to define members of the Ras-family. Binding of extracellular ligands such as growth factors to the extracellular domains of transmembrane receptors often results in activation of GEFs, which activate Ras-proteins by accelerating nucleotide dissociation by more than five orders of magnitude (for H-Ras-Cdc25: intrinsic k_{diss} of $2 \times 10^{-5}\,s^{-1}$; GEF-catalyzed k_{diss} of $3.9\,s^{-1}$), resulting in exchange of the bound GDP to GTP and allowing Ras-proteins to function as molecular switches in a physiological context (Figure 1B and D) (Lenzen et al. 1998). GEFs are only specific for their specific Ras subfamily, i.e., they are structurally diverse (Figure 3A). However, they are functionally conserved and use similar strategies to charge their Ras-protein with GTP. This mechanism is known as the “push-and-pull mechanism” and includes multiple steps (Figure 3B). Initially, the GEF forms a low-affinity ternary complex with the GDP-bound Ras-protein (Figure 3C). Upon an isomerization step the low-affinity complex is transferred into a high-affinity ternary GEF·Ras·GDP complex with a lowered nucleotide affinity. Finally, the nucleotide is dissociated, and a binary GEF·Ras-protein complex is formed (Figure 3C). GEFs lower the nucleotide affinity by pushing switch I and pulling switch II out of its original position. They insert residues into the nucleotide-binding region, thereby re-orienting the P-loop (G1-motif) and resulting in the dissociation of the bound Mg^{2+} ion that is needed for high-affinity nucleotide binding (Figure 3B) (Vetter and Wittinghofer 2001). Finally, the GEF stabilizes a nucleotide-free conformation of the Ras-protein, which can be recharged with nucleotide. As both nucleotides bind with a similar and high nanomolar to picomolar affinity, the approximately tenfold higher intracellular concentration of GTP over GDP explains the predominant binding of GTP to the Ras-protein and adoption of its active conformation (Figure 1D) (Feuerstein et al. 1987; Ford et al. 2009; Goody and Hofmann-Goody 2002; Traut 1994). Many reported GEFs bind to the nucleotide-bound GNBP with a moderate affinity in the high nanomolar to micromolar range, while the affinity to the nucleotide-free GNBP is often higher. It is still not sufficiently understood how Ras-protein·GEF-complexes dissociate following nucleotide-exchange (Bos et al. 2007; Cherfils and Zeghouf 2013). This might include competition of GEFs and effector proteins for binding GTP-loaded Ras-proteins, similar as described for Ras-binding to a full repertoire of different effectors (Kiel et al. 2021). Regulators and effectors often use a similar binding site on the Ras-protein, covering the nucleotide-binding area including switch I and switch II but effectors often bind with a higher affinity (Bos et al. 2007). Moreover, PTMs might lower the Ras-protein-GEF affinity (Figure 3C) (Cherfils and Zeghouf 2013; Kiel et al. 2021). This needs further investigation. Many GEFs are multidomain proteins with accessory domains which are needed for their subcellular localization and for regulation of their activity. They are often regulated by an autoinhibitory mechanism mediated by additional domains N- or C-terminal from the catalytic GEF-domain (Bos et al. 2007). Binding of the second messenger cAMP to the cyclic nucleotide binding (CNB)-domains in the Rap1/

2GEFs EPAC1/2 (exchange protein activated by cAMP) was reported to result in its activation by converting it from an autoinhibited state into the active conformation, capable of binding Rap·GDP (de Rooij et al. 1998; Rehmann et al. 2006; Rehmann et al. 2003). PTMs were reported for GEFs of all sub-families of the Ras-family, regulating their GEF-activity, their subcellular localization and their turnover (Figure 3C) (Magliozzi et al. 2018; Morohashi et al. 2010). The following examples will indicate the functional diversity of GEF-phosphorylation. A sequential multi-step phosphorylation of the RhoGEF Vav on several Tyr side chains in the acidic region N-terminal of the catalytic dibble homology (DH) domain by Src- and Syk-family kinases was shown to result in a gain-of-function, stimulating its GEF activity by inducing a conformational change allowing RhoA access to the catalytic DH domain (Cherfils and Zeghouf 2013; Patel and Karginov 2014). The RhoA GEF GEF-H1 was shown to be regulated by phosphorylation catalyzed by diverse kinases such as PAK-1 and ERK1/2 on several Ser and Thr side chains, resulting either in inhibition or activation of its GEF-activity (Fujishiro et al. 2008; von Thun et al. 2013; Yamahashi et al. 2011). Phosphorylation of GEF-H1 by PAK-1 regulates its activity and generates a binding site for recruitment and relocation of 14-3-3 proteins to microtubles (Patel and Karginov 2014; Zenke et al. 2004). For the DH-PH RhoGEF ARHGEF3, phosphorylation by protein kinase C (PKC) within the pleckstrin homology (PH)-domain was shown to inhibit GEF activity by an allosteric mechanism resulting in reduction of actin stress fiber formation in cells (Moreno et al. 2025). The activity of the RasGEF son of sevenless 1 (SOS1) was reported to be suppressed by ERK-catalyzed phosphorylation at multiple Ser residues upon stimulation of cells by EGF, constituting a negative feedback regulatory loop (Kamioka et al. 2010). Aside from phosphorylation, GEFs were also reported to be targeted by lysine acetylation. Acetylation of the RhoGEF Net1A on lysine side chains within a nuclear localisation sequence (NLS) resulted in its cytosolic accumulation and inhibition of RhoA activation, as shown by treatment of cells with deacetylase inhibitors (Song et al. 2015). Furthermore, the E3 ligase Smurf1 was shown to ubiquitinate the Cdc42 GEF h-PEM-1 resulting in its proteasomal degradation (Yamaguchi et al. 2008). These few examples demonstrate the importance of PTMs in regulating fundamental characteristics of GEF functions, i.e., GEF activity, subcellular localization and turnover. Aside from those reported single PTMs, GEFs are targeted by many more PTMs, as can be accessed from the PhosphoSitePlus database (https://www.phosphosite.org/). Future studies will reveal their role in the regulation of GEF functions. Several GEFs were reported to be dysregulated in human cancers affecting different aspects in RASopathies and/or tumorigenesis (Vigil et al. 2010). Germline gain-of-function mutations resulting in activation of SOS1 were shown to contribute to the development of Noonan syndrome (Roberts et al. 2007; Vigil et al. 2010). The RhoGEF-encoding gene *vav1* was shown to be upregulated due to demethylation within the promoter region resulting in Rac1 activation in pancreatic cancer cells. A chromosome translocation was reported for ARHGEF12 in cells and in one patient with acute myelogenous leukemia (Kourlas et al. 2000). Another example of a chromosome translocation is found upon formation of the Philadelphia chromosome in 90 % of chronic myeloid leukemia (CML) patients by which a BCR-Abl1 fusion protein is generated. BCR contains a RhoGAP and RhoGEF domain and Abl1 is a tyrosine kinase. In BCR-Abl1 fusion protein, the RhoGEF domain is still functional and its activity results in activation of RhoA, contributing to oncogenesis as also does the constitutively active Abl1 kinase (Sahay et al. 2008; Vigil et al. 2010). The completion of the cycle between GTP- and GDP-bound states is necessary for Ras-proteins to function as a molecular switch of physiological relevance, which requires an increased GTP hydrolysis.

6 GTPase-activating proteins are essential components for Ras-inactivation

The signal of the Ras-protein is terminated by GTPase-activating proteins (GAPs) accelerating hydrolysis of the bound GTP to GDP, thereby bringing the Ras-protein into its inactive conformation. Similar as described for GEF-catalyzed nucleotide dissociation, these GAPs are needed to increase the slow intrinsic rates for nucleotide hydrolysis for Ras-proteins (for H-Ras: k_{cat} of $1.2 \times 10^{-4}\ s^{-1}$) to constitute a physiologically significant switch (Figure 1D) (Lenzen et al. 1998). In fact, GAPs accelerate nucleotide hydrolysis by up to five orders of magnitude (for H-Ras-p21-GAP k_{cat} of $19\ s^{-1}$) (Bos et al. 2007; Gideon et al. 1992). In the context of signaling, GAPs make the Ras-system switchable and regulatable by external sources, including activated transmembrane receptors (Bos et al. 2007). The different Ras-subfamilies are regulated by their own GAPs, which are often specific for certain Ras-proteins within the subfamilies (Bos et al. 2007). However, members of the GAP1 family contain GAPs active towards Ras and Rap, both belonging to the Ras-subfamily of the Ras-family (Kupzig et al. 2006). While these GAPs are structurally different, they are functionally conserved using similar although not identical strategies to accelerate nucleotide hydrolysis (Bos et al. 2007). Much knowledge on the mechanism underlying GAP-catalyzed nucleotide

hydrolysis on Ras-proteins was obtained by structural analysis using aluminum fluoride, i.e., AlF_3 or AlF_4^-, which is often used to study the mechanisms underlying phosphoryl group transfer reactions (Figure 4A) (Gremer et al. 2008; Ismail et al. 2010; Mittal et al. 1996; Rittinger et al. 1997a; Scheffzek et al. 1997; Scrima et al. 2008a). Several crystal structures of proteins involved in phosphoryl transfer reactions using AlF_4^- to mimic the transition state were solved. Surprisingly, in some protein structures, AlF_4^- was bound to the protein while AlF_3 is the actual mimic for transfer of a phosphoryl group. However, it could be shown that the pH of the crystallization condition affects the coordination of aluminum from AlF_3 to AlF_4^- (Schlichting and Reinstein 1999). Initial studies with heterotrimeric G-proteins suggested that AlF_4^- in presence of GDP occupies the position of the γ-phosphate, as aluminum and fluoride are similar to phosphor and oxygen, thereby acting as mimic for the ground state (Bigay et al. 1985). Later, structural studies showed that AlF_4^- in fact mimics a pentavalent transition state with a trigonal bipyramidal geometry rather than the ground state of phosphoryl group transfer. AlF_4^- showed an octahedral geometry, which included the nucleophile H_2O and the GDP leaving group, with AlF_4^- being in a square-planar geometry (Coleman et al. 1994; Prive et al. 1992; Tesmer et al. 1997). Studies on Ras showed that it binds aluminum fluoride only in presence of the RasGAP. Notably, in the structures of Arl3·RP2 and RhoA·p50RhoGAP, AlF_4^- was in a square-planar geometry resulting in an octahedrally coordinated transition state as observed in heterotrimeric G-proteins, also mimicking the pentavalent trigonal bipyramidal transition state consisting of H_2O as nucleophile attacking the γ-phosphate and GDP as leaving group (Graham et al. 2002; Grigorenko et al. 2005; Rittinger et al. 1997b; Schlichting and Reinstein 1999; Veltel et al. 2008a). The structures of Ras·RasGAP as well as Arf·ArfGAP crystallized in presence of aluminum fluoride show that in this case AlF_3 is bound mimicking a trigonal bipyramidal transition state (Figure 4A) (Ismail et al. 2010; Scheffzek et al. 1997). Recent reports raised the possibility that the AlF_3 might in fact be MgF_3^- as Mg^{2+} was also present in all crystallization conditions (Grigorenko et al. 2005). AlF_3 and MgF_3^- share a highly similar atomic scattering factor and a geometry capable of forming a trigonal bipyramidal transition state (Graham et al. 2002; Mishra and Lambright 2016). These structural data show that GAPs for proteins of the Ras-, Rho-, Arf/Arl/Sar1- and Rab-subfamilies use a positively-charged arginine finger *in trans*, while it is supplied *in cis* in the Gα subunit of heterotrimeric G-proteins, inserted into the nucleotide binding area, neutralizing negative-charges occurring in the transition state of catalysis (Figure 4A) (Dumas et al. 1999; Gremer et al. 2008; Rittinger et al. 1997a; Scheffzek et al. 1997; Scheffzek et al. 1998b). Further catalytic strategies were developed during evolution to achieve efficient nucleotide hydrolysis. This includes the nucleotide-dependent dimerization of GADs (GTPase activated by dimerization), in which catalytically important residues for nucleotide hydrolysis are oriented upon dimerization (Ash et al. 2012; Gasper et al. 2009). Additionally, cation-dependent G-proteins use monovalent cations including potassium and/or sodium for efficient nucleotide hydrolysis (Ash et al. 2012; Gasper et al. 2009). The Ras-related GTP-binding protein MnmE from *Thermotoga maritima* is conserved in all three domains of life and is involved in modification of tRNAs using a positively-charged potassium ion to induce dimerization and to stimulate nucleotide hydrolysis functionally similar to the Arg-finger (Meyer et al. 2008; Scrima and Wittinghofer 2006).

7 GAPs are regulated by a variety of PTMs

As described for GEFs, GAPs are also regulated by a multitude of diverse PTMs. PTMs regulate activation of GAPs, their subcellular localization and the protein turnover. We describe a few prominent examples for each of these outcomes. It was reported that RheBGAP hamartin·tuberin-complex (TSC1·TSC2; TSC: tuberous sclerosis complex) translocates to the lysosomes in a universal response to cellular stress, resulting in inactivation of RheB and the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) activity (Demetriades et al. 2016). Phosphorylation of TSC2 at S939 by AKT kinase creates a binding site for a 14-3-3 protein and results in its translocation from the lysosomal membrane to the cytosol. In the cytosol, it is not available to inactivate RheB, which in turn results in the activation of mTORC1 (Cai et al. 2006; Manning et al. 2002). Another example is the phosphorylation of a GAP, which can exert a dominant-negative effect: RasGAP was shown to be recruited to diverse receptor tyrosine kinases (RTKs) via its SH2-domain (Schlesinger et al. 1999). Platelet-derived growth factor receptor (PDGFR) is a RTK reported to tyrosine-phosphorylate RasGAP. This creates a binding site for the SH2-domain of Src kinase, leading to the formation of a RasGAP·Src-complex. This results in inhibition of Src kinase activity as shown by the impaired phosphorylation of the Src substrate phospholipase Cγ (PLCγ) (Schlesinger et al. 1999). Aside from these PTMs, RasGAP has been shown to be proteolytically processed by caspases during apoptosis resulting in an increased GAP-activity (Bartling et al. 2004; Wen et al. 1998; Yang et al. 2004; Yang and Widmann 2001). RanGAP1 was the first identified protein modified with SUMO1

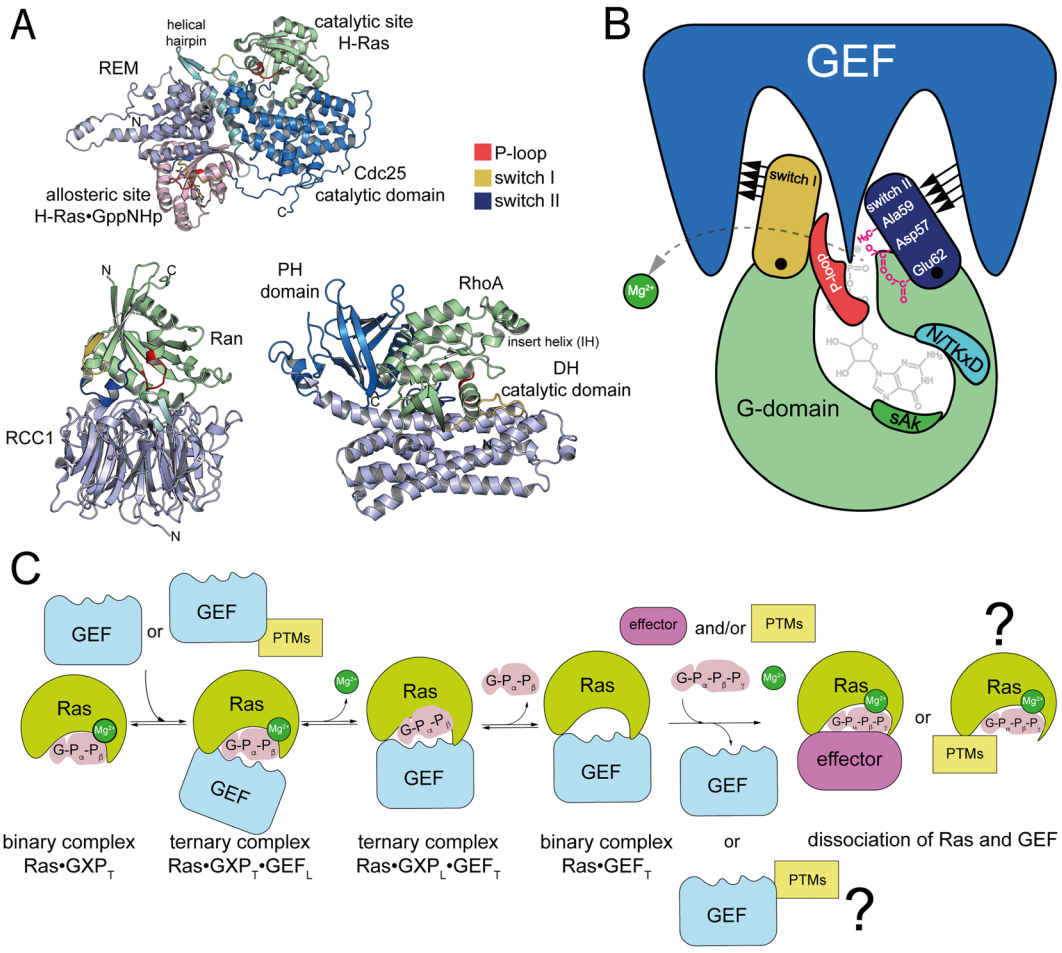

Figure 3: Guanine nucleotide exchange factors are structurally diverse but functionally conserved. (A) Crystal structures for the RasGEF SOS1 in complex with H-Ras (PDB: 1NVV), the RanGEF RCC1 (regulator of chromosome condensation) in complex with Ran (PDB: 1I2M) and the RhoGEF LARG (leukemia-associated RhoGEF) in complex with RhoA (PDB: 1X86). All GEFs bind the Ras-protein at the switch regions. The GEFs are specific for proteins of each Ras-subfamily. Although GEFs are structurally diverse, they use similar catalytic strategies to accelerate nucleotide dissociation by five-orders of magnitude. The catalytic fragment of SOS consisting of the regulatory REM (Ras-exchange motif) and catalytic Cdc25 domain is shown in complex with H-Ras·GppNHp bound to the distal, allosteric site and nucleotide-free H-Ras bound to the catalytic site. It was shown that binding of Ras to the allosteric site stimulates GEF-activity of SOS towards Ras. The crystal structure of the RanGEF RCC1 in complex with nucleotide-free Ran shows RCC1 is an all-β-protein composed of a seven-bladed β-propeller. In contrast, the crystal structure of the LARG dibble-homology (DH)-pleckstrin homology (PH)-tandem domain in complex with nucleotide-free RhoA reveals RhoA makes major contacts to the all-helical catalytic DH-domain, while the PH-domain was implicated in phospholipid-binding (Kristelly et al. 2004; Margarit et al. 2003; Renault et al. 2001). (B) "Push-and-pull-mechanism" used by GEFs to accelerate nucleotide dissociation on Ras-proteins. GEFs insert residues into the nucleotide binding region of the Ras-protein thereby resulting in displacement of the P-loop and dissociation of the Mg^{2+}-ion. This is achieved by pulling switch I and by pushing switch II into the nucleotide binding region bringing Ala53 and negatively charged residues such as Asp57 or the conserved Glu62 into the phosphate binding region. Overall, GEF-binding stabilizes a nucleotide-free conformation of the Ras-protein ready to be re-loaded with GTP as the intracellular concentration of GTP is approximately tenfold higher compared to GDP. (C) GEFs use a multiple step mechanism to catalyze nucleotide dissociation of either GTP or GDP (GXP). Initially, the GEF binds to the binary Ras·GDP complex in which the nucleotide is tightly bound (T: tightly bound). PTMs were reported to activate/inactivate GEF activity. A ternary GEF·Ras·GDP-complex is formed in which the GEF is loosely bound (L: loosely bound). The GEF-protein makes a second contact lowering the affinity of the bound nucleotide. The phosphate-groups are displaced first, the nucleoside moiety at the end. Finally, the nucleotide is displaced and a binary Ras·GEF-complex is formed in which the GEF binds tightly. In principle these steps are reversible. The complex of nucleotide-free Ras-protein·GEF is reloaded with GTP and dissociated by unknown mechanisms. This might include variations in the cellular GTP-concentration, effector binding competing with the GEF for binding to the Ras-proteins or by PTMs lowering the affinity between the Ras-protein and GEF.

(Mahajan et al. 1997; Matunis et al. 1996). SUMOylation of RanGAP at Lys524 targets it to the cytosolic site of the nuclear pore complex (Matunis et al. 1998). PTMs of Ras-proteins themselves also affect their regulation by GAPs. Ubiquitination of N-/K-Ras on Lys128 was shown to increase the binding affinity towards the GAPs NF1 and RASA1, thereby

also promoting GAP-catalyzed nucleotide hydrolysis on Ras. Lys128-ubiquitination restricted tumor growth by restricting RAL/TBK1 signaling (Magits et al. 2024). Lys128 was also shown to be targeted by lysine acetylation, thereby blocking it for ubiquitination, suggesting a PTM-crosstalk (Knyphausen et al. 2016; Magits et al. 2024). Along that line, phosphorylation of Ras on Tyr32 by Src kinase was reported to strengthen the interaction with RasGAP (Bunda et al. 2014; Kano et al. 2019). Time-resolved crystallography recently showed that Tyr32 can adopt various conformations in the GTP-bound state, accompanying switch I alternating between open- and closed-states (Lin et al. 2025). In the latter, it forms a hydrogen bond to the γ-phosphate of the bound GTP (Lin et al. 2025; Rudack et al. 2015). In the open conformation, the Tyr32 side chain is more flexible and is therefore accessible for phosphorylation by various kinases (Bunda et al. 2014; Kano et al. 2019). Src kinase was reported to bind to the GTP-bound state of Ras resulting in phosphorylation of Ras at Tyr32. This reduces binding to effectors such as Raf-kinase while the binding to RasGAP is increased. Effectively, Tyr32-phosphorylation promotes inactivation of Ras-signaling by interfering with effector binding and by improving GAP-catalyzed GTP-hydrolysis (Bunda et al. 2014). A more recent report suggested that simultaneous phosphorylation of Tyr32 and Tyr64 by Src affects the conformation of switch I and switch II, abolishing interaction with Raf kinase. The double phosphorylation attenuated GAP-catalyzed nucleotide hydrolysis and GEF-catalyzed nucleotide exchange. Intrinsic exchange rates were also increased, which resulted in more GTP-loaded protein, albeit incapable to bind to effectors (Kano et al. 2019). The phosphorylation of Ras at Tyr32 and Tyr64 can be reversed by the phosphotyrosine phosphatase (PTP) SHP2 and inhibition of SHP2 terminates Ras-mediated signal transduction (Kano et al. 2019). In another example, phosphorylation of a RacGAP by Aurora B kinase was reported to switch its activity towards RhoA (Minoshima et al. 2003). The next section highlights mechanisms used by GAPs to accelerate nucleotide hydrolysis.

8 Mechanistic strategies employed by Ras-proteins to achieve efficient nucleotide hydrolysis

The Arg-finger of the GAP positions Gln61 of Ras (Gln63 in RhoA; Gln71 in Arl3) by a main chain interaction (in Arl3 by a side chain interaction). In turn, Gln61 orients the catalytic water molecule for nucleophilic attack on the γ-phosphate of GTP and it stabilizes the γ-phosphate in the transition state during catalysis (Figure 4A). Notably, although earlier studies proposed a general base catalytic mechanism (with Gln61 as the general base) or a solvent-assisted catalytic mechanism (2-water mechanism; with a second water molecule involved in catalysis), later studies suggested that the γ-phosphate itself, with a pK_a of 6.5 in solution, would activate the catalytic water molecule by deprotonation in a substrate-assisted catalytic mechanism (1-water mechanism) (Berta et al. 2020; Schweins et al. 1995). The intrinsic nucleotide hydrolysis of Ras and other GTP-binding proteins was initially suggested to use a mechanism based on substrate-assisted catalysis (Pasqualato and Cherfils 2005; Schweins et al. 1995). A more recent report conducting empirical valence bond calculations suggested a solvent-assisted pathway to be energetically preferred over the substrate-assisted pathway for intrinsic and GAP-catalyzed GTP-hydrolysis used by diverse GTP-binding proteins, including small GNBPs of the Ras-family and heterotrimeric G-proteins (Figure 4B) (Calixto et al. 2019). In this mechanism, the water attacks the γ-phosphate and the rate-limiting transition state is formed before its deprotonation (Figure 4B) (Calixto et al. 2019). Further mechanistic studies initially suggested that the cleavage, not an isomerization step, i.e., a conformational change, occurring upon binding of the GAP to the Ras-protein, is rate-limiting in GAP-catalyzed nucleotide hydrolysis on Ras (Figure 4C) (Wittinghofer et al. 1997). This was strongly supported by the notion that Ras-proteins on their own are inefficient enzymes: GAP-binding to the Ras-protein induces a conformational change resulting in proper orientation of the inherent catalytic machinery needed for nucleotide hydrolysis. However, the supply of the Arg-finger by the GAP *in trans* is essential for accelerating nucleotide hydrolysis to the observed rates. More recent Fourier-transform infrared (FTIR) spectroscopy data show that the Arg-finger is located in the GTP-binding pocket and neutralizes negative charges at the GTP only in the transition state of hydrolysis and not in the ground-state. It was suggested that placement of the Arg-finger into the active site follows the conformational change of switch I to adopt an open conformation. This is driven by a gain in entropy due to a release of ordered-water molecules from the GTP-binding site upon Ras·RasGAP complex formation (Kotting et al. 2008). Following the movement of the Arg into the catalytic site, the bond cleavage of GTP occurs fast and a protein bound GDP·$H_2PO_4^-$ intermediate is formed. Further FTIR-spectroscopy and empirical valence bond analyses experiments suggest the cleavage to $H_2PO_4^-$ and GDP as final products in GAP-catalyzed GTP-hydrolysis on Ras. Ras returns to the ground state after switch I adopts its closed conformation, releasing $H_2PO_4^-$, accompanied by a conformational change of the Arg pointing into bulk solvent (Kotting et al. 2008). These data suggest that GAP-catalyzed

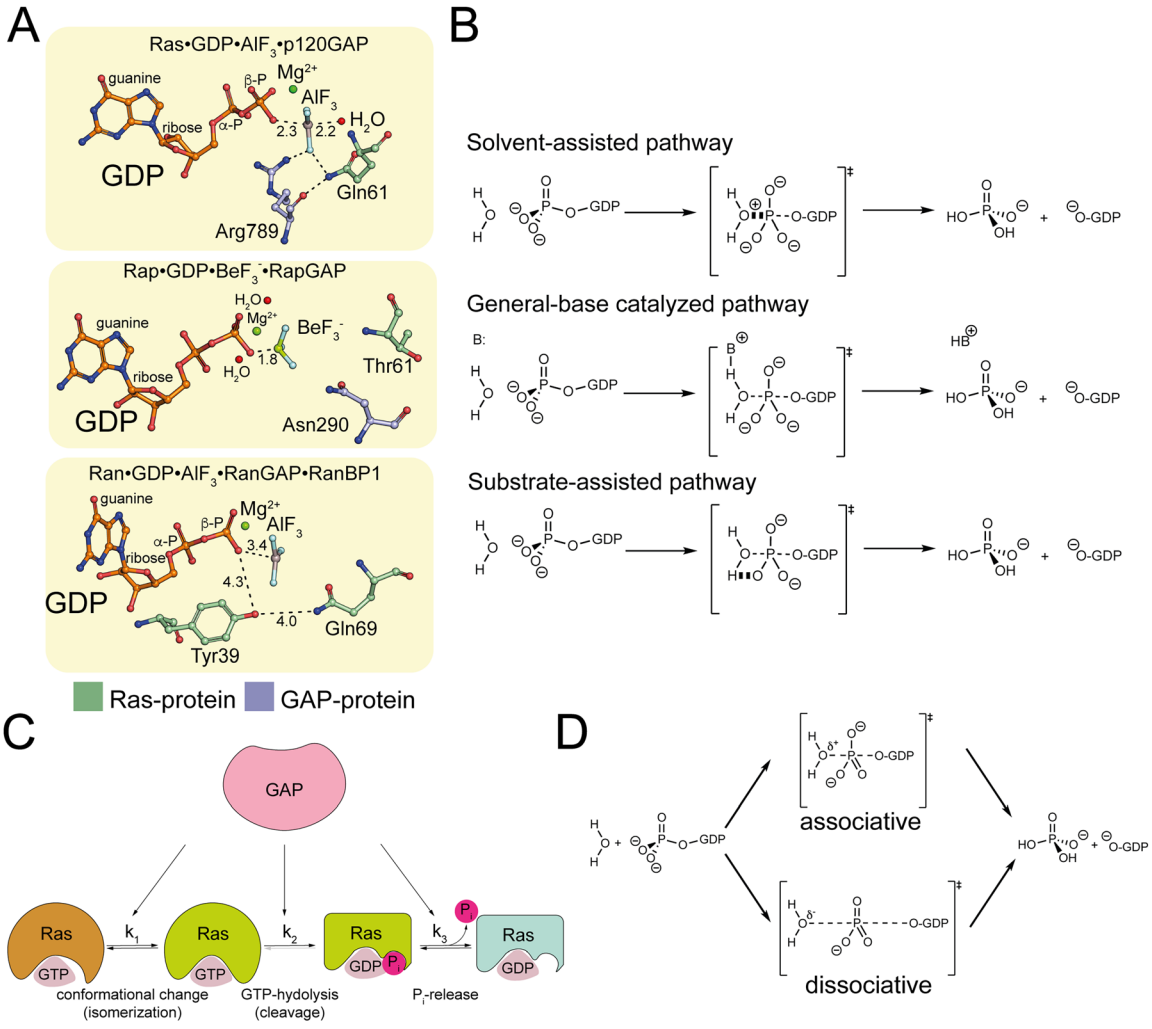

Figure 4: GTPase-activating proteins for Ras-family members are structurally different but use similar catalytic strategies to accelerate GTP-hydrolysis. (A) Closeup of the crystal structures of Ras-protein·GAP-complexes. Upper panel: Structure of human p120GAP in complex with Ras·GDP·AlF_3 (PDB: 1WQ1). The AlF_3 adopts a trigonal planar geometry mimicking the trigonal bipyramidal transition state observed in GAP-catalyzed nucleotide hydrolysis. The Arg-finger (Arg789) of p120GAP inserts *in trans* into the nucleotide-binding region of Ras stabilizing the transition state by neutralizing the negative charge developing upon formation of the transition state of catalysis. Arg789 also contacts the side chain of Gln61 thereby orienting it to position the catalytic water molecule for nucleophilic attack on the γ-phosphate. Gln61 also stabilizes the transition state as shown by the interaction towards AlF_3 (Scheffzek et al. 1997). This mechanism is also conserved in RhoGAPs, Arf/Arl/SAR1GAPs and RabGAPs. Middle panel: RanGAP accelerates GTP-hydrolysis on Ran without an Arg-finger (PDB: 1K5G). Instead, the Gln69 is positioned by Tyr39 *in cis* and Tyr39 also contacts the β-phosphate, although the distances are quite large for a direct interaction. As the distance between the oxygen of the GDP β-phosphate and the AlF_3 is 3.4 Å and as no Arg-finger is involved in the GAP-catalyzed nucleotide hydrolysis, a more dissociative mechanism is likely. Lower panel: RapGAP uses an Asn-thumb to accelerate GTP-hydrolysis on Rap (PDB: 3BRW). Gln61 in Ras is replaced by Thr61 in Rap. This Thr61 was shown to be involved in RapGAP binding rather than in catalysis. The RapGAP inserts an Asn-thumb *in trans* into the nucleotide binding region in Rap functionally replacing the RasGAP Arg-finger. The structure was determined with BeF_3^- modelled as tetrahedrally coordinated as mimic of the γ-phosphate of GTP in the ground state. As the Rap·RapGAP system also does not depend on a positively charged Arg-finger for catalysis, a dissociative mechanism was postulated (Scheffzek et al. 1997; Scrima et al. 2008b; Seewald et al. 2002). (B) Different mechanisms for catalysis were suggested that activate the nucleophilic water molecule. In the solvent-assisted mechanism the water molecule attacks the γ-phosphate to form the transition state before its deprotonation, in the general-base catalyzed pathway a base deprotonates the water before nucleophilic attack and in the substrate-assisted pathway, the γ-phosphate itself acts as base to activate the water molecule. While a substrate-assisted pathway was regarded as the mechanism used by Ras-proteins to achieve efficient GTP-hydrolysis, novel data suggest that the solvent-assisted mechanism is realized (Calixto et al. 2019). (C) GAP-catalyzed nucleotide hydrolysis proceeds through multiple steps. Binding of GAP to the Ras-protein induces a conformational change (isomerization) inserting the Arg-finger (in Ras/Rho) and positioning the intrinsic catalytic machinery of the Ras-protein (induced fit). This is followed by the nucleotide hydrolysis step in which the cleavage of the GTP occurs. A protein-bound $H_2PO_4^-$-species is formed, which is finally released into bulk solvent and converted to P_i. For Ras-, Ran- and Rap-proteins the step including the release of $H_2PO_4^-$ was suggested to be rate-limiting, while for Rho- and Rab-proteins the cleavage step is regarded as rate-limiting. (D) The formation of the transition state can occur by different mechanisms. In an associative mechanism bond formation precedes cleavage and departure of the leaving group. In contrast, in a fully dissociative

nucleotide hydrolysis is reversible and, in analogy to other processes dependent on nucleotide hydrolysis such as actin filament disassembly, $H_2PO_4^-/P_i$-release (P_i: inorganic phosphate) contributes to the rate limiting step (Figure 4C) (Kotting et al. 2006).

Notably, for Ran·RanGAP and Rap·RapGAP, in which both GAPs do not contain an Arg-finger, the $H_2PO_4^-/P_i$-release was also shown to be rate-limiting, while for Rab1b and RhoA, whose GAPs do contain an Arg-finger, bond breakage is rate-limiting in GAP-catalyzed nucleotide hydrolysis (Figure 4C) (Allin et al. 2001; Brucker et al. 2010; Chakrabarti et al. 2004; Kotting et al. 2006; Seewald et al. 2002; Wittinghofer et al. 1997). These data show that although GAP-catalyzed nucleotide hydrolysis is present for most Ras-proteins, the detailed underlying mechanisms might vary slightly. While hydrolysis of GTP in water follows a dissociative mechanism, it is not completely resolved which strategies Ras-proteins apply for intrinsic GTP-hydrolysis (Florián and Warshel 1998; Maegley et al. 1996; Wittinghofer 2006). Along that line, there was debate concerning the nature of the transition state and the precise mechanism of intrinsic, as well as GAP-catalyzed, nucleotide hydrolysis. Three alternative mechanisms were debated: (1) A fully dissociative mechanism in which bond cleavage and departure of the leaving group GDP precede bond formation of the γ-phosphate with the attacking water molecule, (2) a fully associative mechanism in which bond formation precedes cleavage and departure of the leaving group and (3) an S_N2-like mechanism in which formation and cleavage of the bonds occur in a concerted way (Figure 4D). This concerted S_N2-mechanism can have a more associative or dissociative nature. Overall, for GAP-catalyzed nucleotide hydrolysis on Ras-, Rab-, Arf/Arl- and Rho-proteins, data including X-ray crystallography, FTIR difference-spectroscopy and quantum mechanical (QM)/molecular mechanical (MM) calculations suggest an associative mechanism, as this is characterized by a pronounced negatively-charged transition state, explaining the need for the positively-charged Arg-finger in catalysis (Figure 4D) (Ismail et al. 2010; Kotting et al. 2006). In contrast, in RanGAP or RapGAP, which do not use an Arg-finger to accelerate nucleotide hydrolysis, the reaction mechanisms might involve more dissociative transition states with a metaphosphate-like configuration (Figure 4D) (Brinkmann et al. 2002; Daumke et al. 2004; Scrima et al. 2008b; Seewald et al. 2002). In Rap·RapGAP an Asn residue is supplied by RapGAP *in trans*, which takes over the function of the Gln61 in Ras/Gln63 in RhoA in orienting the catalytic water for catalysis (Figure 4A). Ran Tyr39 (Tyr32 in Ras; Tyr34 in RhoA) in the Ran·GppNHp·RanGAP complex was shown to directly contact the γ-phosphate and Gln69 in Ran (Gln61 in Ras) *in cis* to position the water for catalysis (Figure 4A). In complexes of Ras·RasGAP and Rho·RhoGAP, the corresponding Tyr is in an open conformation, allowing the Arg-finger of the GAP to insert into the nucleotide binding site. In contrast, in Ran·RanGAP and Rap·RapGAP this Tyr side chain (Ran: Tyr39; Rap1B: Tyr32) is in a closed conformation, sterically hindering the insertion of a potential Arg-finger (RanGAP: Arg170; RapGAP: Arg286) into the nucleotide binding side. For Rab·RabGAP, a dual-finger mechanism was suggested to be used for accelerating nucleotide hydrolysis (Pan et al. 2006). The structure of the RabGAP Gyp1p in complex with Rab33·GDP and AlF_3 revealed that RabGAP inserts an Arg-finger and a Gln-finger (Gyp1p: Arg343, Gln378) *in trans* into the nucleotide binding pocket upon binding to Rab. This Gln supplied *in trans* functionally resembles Gln61 in Ras (Q92 in Rab33), i.e., it is important to bind the γ-phosphate and to position the catalytic water for nucleophilic attack of the γ-phosphate (Pan et al. 2006). As both residues were conserved in most RabGAPs this mechanism is considered to be conserved in Rab·RabGAP. Overall, the data on the mechanistic details of GTP-hydrolysis in solution, intrinsic hydrolysis and GAP-catalyzed hydrolysis should be characterized for each individual system. Although for many Ras-proteins GEFs and GAPs were described, for some proteins such as RIT1 no GEFs nor GAPs were identified so far, although their slow intrinsic rates observed for nucleotide dissociation and nucleotide hydrolysis would hint at their existence. For RheB, a GAP was identified but no GEF was discovered so far. Future studies will show whether these Ras-proteins are regulated by GAPs and GEFs. Several diseases are caused by a dysfunction of nucleotide exchange or nucleotide hydrolysis on Ras-proteins, as described for Ras in the subsequent section.

mechanism the bond cleavage and departure of the leaving group, GDP would precede bond formation. Another mechanism is a concerted S_N2-like mechanism (not shown). Structure-function analyses suggest GTP-hydrolysis in water follows a dissociative mechanism, while Ras-, Rho-, Rab- and ArfArl-proteins were suggested to use an associative mechanism for GAP-catalyzed nucleotide hydrolysis with a pronounced negative charge emerging in the transition state that is neutralized by the Arg-finger. In contrast, for RanGap and RapGap, which do not use an Arg-finger, a more dissociative mechanism might be realized. As the electronic configurations vary in the different systems, the mechanisms might be diverse and must be inspected for each individual case.

9 Ras is a proto-oncogene product mutated in many human tumors and RASopathies

The *ras*-gene is an proto-oncogene converted to an oncogene in many human cancers caused by somatic mutations (Rosell and Karachaliou 2016). Mutated K-Ras can be found in about 75 % of all human cancers, followed by mutated N-Ras (17 %) and mutated H-Ras (7 %) (Prior et al. 2020). Mutated K-Ras is the most prevalent Ras-isoform mutated in lung cancer (35 %), pancreatic cancer (90 %) and colorectal cancer (45 %) (Herdeis et al. 2021; Li et al. 2025; Rosell and Karachaliou 2016). Moreover, mutations in Ras contribute to a number of developmental disorders known as RASopathies, which occur with a prevalence of 1:1,000 in newborns and are caused by germline mutations in genes encoding for components or regulators of the Ras/MAPK pathway (Rauen 2013). The Ras/MAPK pathway regulates fundamental cellular processes including cell proliferation, cell differentiation and cellular senescence. It is therefore not surprising that mutations in genes encoding for major players in this pathway have severe consequences on human health. The different RASopathies cause unique clinical symptoms, yet share multiple overlapping phenotypes. In neurofibromatosis type 1 (NF type 1), Noonan syndrome (NS), capillary malformation-arteriovenous malformation syndrome (CM-AVM), Costello syndrome (CS), cardio-facio-cutaneous syndrome (CFC), and Legius syndrome (LS) (Rauen 2013), many patients are characterized by craniofacial dysmorphology, dysfunctions of the heart, skin and muscles, neurocognitive impairment and a higher risk to develop cancer (Rauen 2013). NS and CS are amongst others caused by activating mutations in genes encoding for different Ras-isoforms (NS: K-Ras, N-Ras; CS: H-Ras). The mutations often occur on different positions in the Ras-isoforms, and the majority result in activation of the MAPK pathway, which for NS or CS, however, is less severe compared to the activation by oncogenic Ras in cancer (Aoki et al. 2005; Cirstea et al. 2010; Rauen 2007; Schubbert et al. 2007; Schubbert et al. 2006). Additionally, NS is based on mutations in several other genes encoding for proteins involved in the MAPK pathway, including the RasGEF SOS1, the phosphatase SHP2 and kinase CRAF (Rauen 2013). Interestingly, in CS, H-Ras is mostly mutated at positions Gly12 and Gly13, which are also most frequently mutated in oncogenic Ras (Bos 1989). In Ras-driven tumors, three residues were found mutated in 97 % of all cases, i.e., Gly12 and Gly13 in the P-loop (10-GxGGxGKS-17) and Gln61 in switch II (57-DxxGQ-61) (Barbacid 1987; Der et al. 1986; Seeburg et al. 1984). The oncogenic mutants Ras G12V and Ras Q61L are both constitutively active and incapable of intrinsic as well as GAP-catalyzed nucleotide hydrolysis. Mutation of Gly12 in the P-loop (G1 motif) to any other residue (G12X; mostly G12V) abolishes GAP-catalyzed nucleotide hydrolysis as it sterically interferes with insertion of the Arg-finger and the correct orientation of Gln61. Mutation of Gly12 to any other residue, except to Pro, lowers the pK_a-value of the γ-phosphate of GTP, thereby impairing the reactivity for nucleophilic attack of the water molecule (Schweins et al. 1995). The mutant Ras G12P has an increased intrinsic nucleotide hydrolysis rate compared to Ras wild-type and other mutations of Gly12, explaining why Ras G12P is not oncogenic despite also being inactive in GAP-catalyzed nucleotide hydrolysis (Schweins et al. 1995). The side chain of Gln61 is directly orienting and positioning the active site water exerting the nucleophilic attack on the γ-phosphate. Mutation of Gln61 (Q61X; mostly mutated to Leu, i.e., Q61L) renders Ras inactive in hydrolyzing GTP to GDP and P_i. Both mutations, i.e., G12X and Q61X, although inactivating GTP hydrolysis, activate Ras signaling by allowing effector binding. Mutations in several genes encoding for GAPs were shown to contribute to severe diseases (Vigil et al. 2010). The RasGAP neurofibromatosis type 1 (NF1) binds stronger to Ras compared to other GAPs such as p120-GAP (Bollag and McCormick 1991). Several point mutations were identified in the gene encoding for NF1. Mutation of the Arg-finger in NF1 contributes to the development of NF type 1, an autosomal-dominant disease resulting in the development of benign tumors in the skin and nerve tissues (Scheffzek et al. 1998a). Along that line, mutation of the Arg-finger was also identified in the Arl3GAP RP2, which is correlated with the development of an X-linked retinopathy called retinitis pigmentosa, resulting in the decline of photoreceptor cells in the retina with symptoms starting with night blindness, impaired peripheral vision, i.e., tunnel vision, and continuing with progression of vision loss (Kuhnel et al. 2006; Veltel et al. 2008a,b; Veltel and Wittinghofer 2009). In tuberous sclerosis the tuberous sclerosis complex TSC1·TSC2, with TSC2 encompassing a GAP-domain for the small GNBP RheB, was shown to be mutated. RheB plays a role in the activation of mTOR-kinase and mTORC1, involved in regulation of protein synthesis, cell proliferation and cell growth (He et al. 2025). Several described mutations within TSC1·TSC2 result in constitutive activation of RheB and, as a consequence, stimulation of the mTOR pathway, resulting in formation of benign tumors (hamartomas) in diverse organs, including skin, heart, brain and kidney (Kwiatkowski and Manning 2005). The TSC1·TSC2-complex was structurally characterized, indicating TSC1 dimerization with a coiled-coil region and positioning of TSC2. The GAP-mechanism exerted by TSC2 is similar as described for RapGAP, i.e., TSC2

inserts an Asn-thumb (Asn1643) rather than an Arg-finger *in trans*, thereby functionally replacing the Gln61 in Ras involved in stabilizing the γ-phosphate in the transition state and positioning the catalytic water molecule. Correct functions of Ras-proteins also require spatial and temporal control of localization. This includes their translocation from the cytosol to specific endomembranes, which is controlled by different types of lipidation.

10 Ras-proteins are post-translationally lipidated for membrane targeting

Proteins of several subfamilies of the Ras-family, including Ras, Rho, Rab and Arf/Arl, are recruited to different cellular membranes. While Ras- and Rho-subfamily proteins were shown to be enriched at the plasma membrane, Rab- and Arf/Arl-proteins are enriched at endomembranes (Figure 5A). It is still a matter of investigation how an enrichment of certain Ras-proteins at cellular membranes is achieved, considering the huge membrane surface area constituted by the endomembrane system, which should entropically be occupied by proteins if the migration of proteins constituted a purely passive mechanism. The directed transport is mediated in part by lipidation at the proteins' C-terminus. While the G-domain of proteins of the Ras-family is structurally highly conserved and also shows a high degree of sequence similarity, the region located C-terminally shows a high degree of variation and is hence called the hypervariable region (HVR) (Figure 5A). This HVR contains the C-terminal isoprenylation sequence, the so-called CaaX-box (C: Cys; a: aliphatic side chain; X: varying side chain), in which a C15-farnesyl- or a C20-gernalygeranyl-moiety is covalently attached to the CaaX-box Cys, forming an irreversible thioether linkage (Figure 5A). To further increase affinity towards membranes, a second specific interaction site is necessary for physiological function. This can either be a palmitoylation resulting in formation of a reversible thioester bond at other Cys side chains adjacent to the CaaX-box, as found in K-Ras4A, H-Ras and N-Ras, or a highly positively-charged poly-basic region (PBR) as observed in K-Ras4B, RhoA-C, Cdc42, Rac1 or RIT1/2, mediating interactions with negatively-charged phospholipids in the plasma membrane (Figure 5A) (Hancock et al. 1990; Omerovic et al. 2007). For Rac1, but not for RhoA, the PBR was also reported to serve as a nuclear localization sequence (NLS), important for nuclear localization of armadillo repeat proteins such as SmgGDS or p120 catenin (Lanning et al. 2003). Some proteins of the Arf/Arl subfamily are *N*-myristoylated at the α-amino group of the Gly exposed at the N-terminus after removal of the N-terminal Met. Other Arf/Arl-proteins are not *N*-myristoylated but the presence of an N-terminal amphipathic α-helix, exposed by structural rearrangements including a β-sheet register shift when going from the GDP-bound into the GTP-bound state, is sufficient for membrane binding (Hillig et al. 2000). The only subfamily not targeted to membranes is Ran, which regulates the directed transport of proteins and RNAs between nucleus and cytosol (Scheffzek et al. 1995). Isoprenylation of Ras-proteins is catalyzed by cytosolic (protein-)isoprenyltransferases (Marchwicka et al. 2022). The human genome encodes for a total of four isoprenyltransferases, i.e., farnesyltransferase (FTase) and three geranylgeranyltransferases (GGTase I-III), using the C15-isoprenoid farnesylpyrophosphat (FPP) or the C20-isoprenoid geranylgeranylpyrophosphate (GGPP) originating from the mevalonate pathway as activated isoprenyl-groups for isoprenylation (Liang et al. 2002). These are heterodimeric enzymes containing one out of four α-subunits and one out of three β-subunits encoded in the human genome (Marchwicka et al. 2022). Studies concerning substrate specificity of isoprenyltransferases showed that proteins, which contain a CaaX-box where X is Ser/Ala/Gln/His/Cys, are substrates for FTase (including H-Ras, K-Ras, N-Ras,Ras2, Rap2, pre-Lamin A, LaminB, RhoB, RhoE, and Rheb) while Leu or Val at this position define substrates for GGTase I (including most Rho-proteins; RhoA, RhoB, RhoC, Rac1, Rac2, Cdc42 as well as Rab8, Rab11, Rab13, RalA and Rap1B) (Fres et al. 2010; Zhao et al. 2020). FTase and GGTase I share the same α-subunit but differ in their β-subunits, determining substrate specificity. Substrates for both FTase and GGTase I contain Thr/Ile/Phe/Met as C-terminal residue in the CaaX-box motif (Liang et al. 2002). Rab-proteins contain a XXCC-, CCXX-, XCXC- or CCXXX-motif (C: Cys; X: varying residue), replacing the CaaX-box, which is targeted for geranylgeranylation at both Cys side chains by GGTase II (Figure 5A) (Pereira-Leal et al. 2001). GGTase III was the last identified isoprenyltransferase, shown to be composed of the α-subunit PTAR1 and the β-subunit of Rab GGTase II. The first characterized substrate of GGTase III was the E3 ubiquitin ligase FBXL2 (Kuchay et al. 2019). Geranylgeranylation of FBXL2 at the C-terminal CaaX-box was shown to mediate membrane targeting. Interestingly, FBXL2 is not isoprenylated by GGTase I, although it contains a prototypical GGTase CaaX-box motif (CVLL) (Kuchay et al. 2019). Although rules for substrate specificity were reported to be dependent on the exact sequences at the isoprenylation site like in the case of the CaaX-box, several studies showed examples of cross-reactivity. These examples indicate that apart from the primary structure, the substrate's tertiary structure also contributes to determination of substrate specificity. Substrate specificity of GGTase III for FBXL2 is conferred by extensive interactions of a leucine-rich repeat (LRR)-domain

of FBXL2 and the α-subunit of GGTase III. For GGTase II, it is known that an accessory protein, i.e., Rab escort protein (REP), is needed to confer substrate specificity for Rab proteins (Pereira-Leal et al. 2001). The *S*-prenylation of Ras-proteins results in localization of the proteins to the endoplasmic reticulum (Figure 5B). It was shown that isoprenylated Ras-proteins are localized at the cytosolic site of the ER, which also contains the enzymes for further post-translational processing, namely the integral membrane-spanning ER endoproteases Ras and α-factor converting enzyme (Rce1) as well as Ste24/Afc1 (α-factor converting enzyme), resulting in proteolytic cleavage of the C-terminal aaX residues of the CaaX-box (Figure 5C). Additionally, the ER contains the isoprenylcysteine carboxyl methyltransferase (ICMT) using S-adenosyl-methionine as methyl-group donor molecule to methylate and thereby neutralize the negative charge at the C-terminal carboxylate (Dai et al. 1998). Following isoprenylation and further post-translational processing at the ER, palmitoylation of Ras-proteins is catalyzed by the aspartate-histidine-histidine-cysteine (DHHC) palmitoyl transferases localized at the Golgi apparatus (Figure 5B and D) (Philippe and Jenkins 2019). Thioesterases are localized in the cytosol, at the nuclear membrane, at the plasma membrane and at the ER. APT1 (human: LYPLA1), APT2 (human: LYPLA2) (APT: acyl-protein thioesterase) or ABDH17s (isoforms: ABDH17A/B/C) are Ser-hydrolases with a typical Ser-His-Asp catalytic triad that belong to the α/β-hydrolase superfamily and were reported to be capable of depalmitoylating the Gα-subunit of heterotrimeric G-proteins and certain Ras-proteins (Lin and Conibear 2015). While APT1 was shown to depalmitoylate H-Ras, N-Ras was reported to be depalmitoylated by ABDH17s, which are themselves N-terminally palmitoylated, supporting their localization at the plasma membrane (Lin and Conibear 2015; Martin and Cravatt 2009). Palmitoylation of H- and N-Ras at the Golgi apparatus was shown to increase affinity of Ras to the Golgi membrane. The directionality of the secretory pathway ensures the directed vesicular transport of H-Ras and N-Ras from the Golgi to the plasma membrane (Figure 5B) (Rocks et al. 2006). Depalmitoylation is subsequently catalyzed by thioesterases, lowering membrane affinity. Proteins only modified by isoprenylation have a much lower affinity to membranes compared to transmembrane proteins (Figure 5B). Entropically, this would result in distribution of Ras-proteins in the extended cellular endomembrane system if the thioesterases were not localized to almost all cellular membranes. Overall, this creates directed transport cycles of H- and N-Ras accumulating at the Golgi by palmitoylation of the farnesylated Ras-proteins, vesicular transport to the plasma membrane and depalmitoyaltion (Rocks et al. 2006, 2010). The different Ras-proteins are enriched at the plasma membrane, albeit using slightly different underlying mechanisms, driven by GDI-like solubilization factors counteracting the entropy-driven distribution of Ras-proteins over all cellular membranes (Chandra et al. 2011). In the next section, we describe the functional details of these solubilization factors.

11 RhoGDIs, RabGDIs and PDE6δ are solubilization factors for lipidated Ras-proteins

Many Ras-proteins are bound to cellular membranes by lipid modifications (Chen et al. 2018; Erwin et al. 2017). These lipidations increase their hydrophobicity and impair their solubility in the cytosol (Figure 6A) (Chen et al. 2018). Therefore, besides regulation by GEFs and GAPs, guanine nucleotide dissociation inhibitors (GDIs) also regulate proteins of the Rho-subfamily and Rab-subfamily. The name derives from the observation that GDI-binding of the GNBP impairs GEF-catalyzed nucleotide exchange. GDIs are able to bind to lipidated GNBPs, extracting them from the membrane to form a cytosolic pool ready to be reactivated by membrane binding and GEF-catalyzed nucleotide exchange (Dransart et al. 2005). GDIs are structurally composed of a C-terminal immunoglobulin (IG)-domain forming a β-sandwich fold, thereby forming a hydrophobic cavity capable of binding the hydrophobic and isoprenylated C-terminus of the GNBP (Figure 6B) (Hoffman et al. 2000; Kuhlmann et al. 2016a; Longenecker et al. 1999; Rak et al. 2003; Tnimov et al. 2012). Moreover, they contain an N-terminal intrinsically unfolded region, which adopts a HTH (helix-turn-helix)-motif upon binding to the Rho-/Rab-protein contacting their nucleotide binding regions (Figure 6B) (Hoffman et al. 2000; Kuhlmann et al. 2016a; Longenecker et al. 1999; Tnimov et al. 2012). Mechanistic studies showed that GDIs bind much stronger to the GDP-bound Rho-/Rab-proteins compared to the GTP-bound forms. For RhoGDIs an almost three orders of magnitude higher affinity to the GDP-bound state of the GNBPs was described compared to the GTP-bound state (RhoA-GG·GDP·RhoGDIα K_D: 5 pM; RhoA-GG·GTP·RhoGDIα K_D: 3 nM, GG: geranylgeranyl) (Tnimov et al. 2012). Thereby, GDIs are able to sense the nucleotide-loading state of the GNBP to directly connect the GTP/GDP cycle to a cytosol/membrane cycle. As the affinity of the GDIs towards the GDP-loaded Rho-/Rab-proteins is in the low nanomolar to picomolar range, additional GDI-displacement factors (GDFs) were suggested to be necessary to dissociate the complexes, making them accessible for reactivation by GEF-catalyzed nucleotide exchange (Sivars et al. 2003; Yamashita and Tohyama 2003). Proteins of the ezrin/radixin/moesin (ERM)-

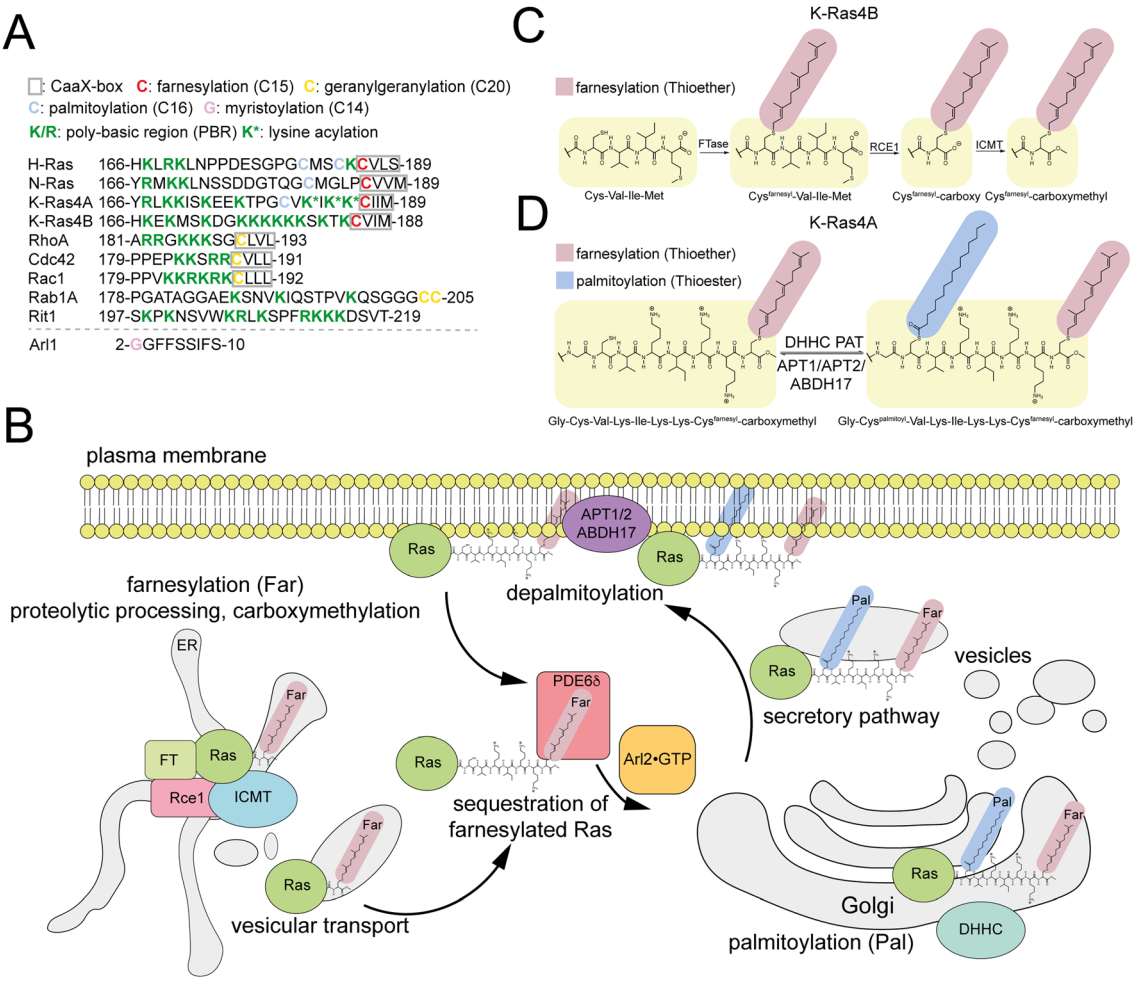

Figure 5: The hypervariable region of Ras-proteins is important for their subcellular localization. (A) The C-terminal hypervariable region (HVR) is the most diverse sequence area in Ras-proteins. The region is intrinsically unfolded. Depending on the Ras-protein it contains the C-terminal CaaX-box for isoprenylation, further Cys-side chains for lipidation and/or a poly-basic region (PBR) for membrane binding as well as lysine side chains that can be acylated. Asterisks indicate putative lysine acylation sites. (B) Ras-proteins are distributed in the cell due to post-translational modification by lipidation. The Ras-proteins are isoprenylated, either farnesylated or geranylgeranylated, at the endoplasmic reticulum (ER) at a Cys within the C-terminal CaaX-box (C: Cys; a: aliphatic side chain; X: any side chain). Subsequently, the enzymes Rce1 and ICMT catalyze the proteolyical processing and the carboxymethylation. The Ras-proteins are further distributed from the ER to the Golgi apparatus through the directionality of the secretory pathway and/or by the G-protein solubilization factor (GSF) PDE6δ, which binds to the isoprenylated Ras-proteins. Binding of Arl2·GTP to the PDE6δ-cargo-complexes releases the farnesylated Ras-protein at perinuclear membranes (not shown) and at the Golgi. Thereby, farnesylated Ras accumulates at the Golgi, where it is palmitoylated by DHHC-palmitoyltransferases at Cys-residues in the HVR N-terminally from the CaaX-box. This increases the affinity towards the Golgi-membrane. Fully modified Ras is subsequently transported by vesicular transport to the plasma membrane, where it initiates signal transduction. Thioesterases, i.e., APT1/2 and/or ABHD17, depalmitoylate Ras-proteins at the plasma membrane lowering their affinity to the membrane. GDFs as PDE6δ can bind and solubilize the isoprenylated Ras-proteins, ensuring that entropically not all isoprenylated Ras is distributed in the endomembrane system. (C) Enzymatic cascade resulting in isoprenylation of Ras-proteins at the ER-membrane. Initially, Ras-proteins are farnesylated or geranylgeranylated by farnesyltransferase or geranylgeranyltransferases at the C-terminal CaaX-box Cys side chain forming an irreversible thioether bond. Subsequently, Rce1 proteolytically processes the CaaX-box removing the three C-terminal residues before ICMT carboxymethylates the α-carboxylate thereby neutralizing the negative charge, which would interfere with membrane binding.

family and the neurotrophin receptor (p75NTR) were suggested to fulfill this role for the complex of Rho and RhoGDIα (Ivetic and Ridley 2004; Yamashita and Tohyama 2003). Notably, for other proteins of the Ras-family, including Ras-, RheB- and Arl/Arf-proteins, the delta subunit of type 6 phoshodiesterase (PDE6δ) was reported to act as a carrier for the lipidated GNBPs (Muratcioglu et al. 2017). Mechanisms for transport processes of lipidated GNBP cargo mediated by PDE6δ and cargo release at the intracellular destination were reported (Figure 6C). In contrast to GDIs, PDE6δ and the related proteins UNC119a/UNC119b lack the N-terminal region needed to sense the nucleotide-loading state. Thus,

transport by PDE6δ is suggested to be independent of nucleotide-loading of the GNBP, meaning the GTP/GDP-cycle is not coupled to membrane/cytosolic localization. The interplay of isoprenylated RhoA with RhoGDIα was studied. The binding of isoprenylated RhoA to the plasma membrane was suggested to strongly reduce the affinity of RhoGDIα to RhoA by almost three orders of magnitude (Kuhlmann et al. 2016a; Tnimov et al. 2012). Therefore, a mechanism was postulated according to which a low affinity complex is formed between membrane-bound RhoA and RhoGDIα, which is transformed into a high-affinity complex upon binding of the isoprenyl-moiety into the hydrophobic cavity within the IG domain. This gain in affinity is suggested to be the driving force for the membrane extraction of isoprenylated RhoA (Goody et al. 2005). Binding to GDP-loaded RhoA being almost three orders of magnitude tighter compared to GTP-loaded RhoA ensures that GDP-bound RhoA is predominantly extracted from the plasma membrane. The N-terminal helix-turn-helix motif of RhoGDIα is important for the interaction with RhoA. It contacts the nucleotide binding region of RhoA, thereby sensing the nucleotide loading state. Moreover, the N-terminal region preceding the helix-turn-helix motif is highly negatively charged by the prevalence of Asp and Glu side chains, which contribute to RhoA binding by forming electrostatic interactions with the C-terminal PBR in RhoA (Dransart et al. 2005). Upon contacting the plasma membrane, the negatively-charged phospholipids compete with RhoGDIα for binding to RhoA. Lowering the affinity of RhoGDIα to isoprenylated RhoA leads to an increase of propensity for a flip of the geranylgeranyl-moiety from the hydrophobic IG domain into the plasma membrane. This demonstrates that electrostatics are important for the interplay of RhoA, RhoGDIα and negatively-charged phospholipids in the plasma membrane. This suggests that lipid composition and the amount of negatively charged phospholipids, such as phosphatidylserine and PIP_2, might affect the efficiency of releasing isoprenylated RhoA from the RhoGDIα·RhoA-GG complex and binding or extracting isoprenylated RhoA to or from the membrane (Dransart et al. 2005). The mechanisms reported for membrane targeting and extraction are similar to the interaction of RabGDI and Rab-proteins. However, RabGDI was shown to bind double-isoprenylated Rab-proteins only in their GDP-bound state, while RhoGDI only binds mono-isoprenylated Rho-proteins with higher affinity in their GDP-bound state, but also with lower affinity in their GTP-bound state (Calero et al. 2003; Ignatev et al. 2008). Similar as observed for RhoGDI, an allosteric mechanism was reported for RabGDI, in which Rab binding results in opening of the hydrophobic prenyl-binding pocket in the IG domain to accommodate the hydrophobic isoprenyl-group (Grizot et al. 2001; Hoffman et al. 2000; Ignatev et al. 2008; Keep et al. 1997; Longenecker et al. 1999; Scheffzek et al. 2000; Tnimov et al. 2012). The interplay of GEFs and GAPs, the continuous cycling between activation and inactivation of Ras-proteins, their subcellular localization and the transport of Ras-proteins between different subcellular membranes creates cellular gradients that are essential to drive directed cellular processes.

12 Formation of cellular gradients by the interplay of GEFs, GAPs and shuttling of small GBNPs

Cellular patterns in Ras-signaling are established in a context-dependent manner by the spatiotemporal control of GEF and GAP activation, as has been studied systemically for Rho-dependent signaling (Muller et al. 2020). Similarly, differently localized regulators ensure the formation of a cellular Ran-gradient, which is essential for a targeted shuttling of proteins and other molecules between cytosol and nucleus (Figure 7A) (Ohtsubo et al. 1987). The RanGEF RCC1 is located in the nucleus and attached to chromatin, whereas RanGAP is located at the cytosolic side of nuclear pores (Görlich 1998; Macara 2001). The gradient is formed not only in interphase cells but also in mitotic cells, regulating processes such as chromosome condensation, mitotic spindle formation and nuclear envelope formation (Kalab et al. 1999; Matchett et al. 2014; Melchior 2001). A similar mechanism underlying a gradient of small GNBPs of the Arf/Arl subfamily was recently also described to regulate the transport of proteins to and from primary cilia and immune synapses (Figure 7A) (Powell et al. 2021). While Arl2 is a cytosolic protein involved in processes including tubulin folding, Arl3 was shown to be enriched in the cilia. The Arl3GAP retinitis pigmentosa 2 gene product (RP2) is located to the ciliary base and is able to accelerate GTP-hydrolysis on Arl3 but not Arl2, while Arl13B was shown to act as a GEF for Arl3 in the primary cilia. This ensures that activated, GTP-bound Arl is present in the lumen of the cilia while inactivated, GDP-bound Arl is found in the cytosol, thereby creating a gradient (Figure 7A) (Stephen and Ismail 2016). More recently, Arl13B was shown to be a poor GEF for Arl3 at physiological ratios of GTP:GDP. However, GEF activity can be strongly improved by binding of the co-GEF BART co-localizing with Arl13B in the primary cilium (ElMaghloob et al. 2021). *N*-myristoylated proteins, including protein kinase A (PKA), the tyrosine kinases Src and Lck, deubiquitinating enzymes, α-subunit of transducin, GNAT1, the renal protein nephrocystin-3 (NPHP3) and the formin protein

FMNL1, bind to the GDI-like solubilization factors (GSFs) UNC119a and/or UNC119b (Erwin et al. 2018; Ismail et al. 2012; Lee et al. 2013; Sanchez-Bellver et al. 2022; Sinha et al. 2013; Yelland et al. 2021; Zhang et al. 2011). This allows solubilization of *N*-myristoylated proteins to build a cytosolic pool, which allows sequestering of *N*-myristoylated proteins to cellular membranes. GTP-bound Arl2 and Arl3 are able to release the *N*-myristoylated cargo-proteins from their complexes with UNC119a and/or UNC119b. Structure-function analyses revealed that the proteins bound and solubilized by UNC119 proteins can be classified in low affinity cargoes and high affinity cargoes (Yelland et al. 2021). While both Arl2 and Arl3 are capable of releasing low affinity cargoes, only Arl3 also displaces high affinity cargoes. The GEF for Arl3 Arl13B is localized to the primary cilium and the immune synapse (Ismail et al. 2012; Stephen et al. 2018; Yelland et al. 2021). So far, no GEF was identified for Arl2, maybe due to its fast intrinsic nucleotide exchange rate (Bowzard et al. 2007; Nithianantham et al. 2015). Knock-out of UNC119b had stronger impacts on the transport of *N*-myristoylated cargoes into primary cilium compared to UNC119a, suggesting that UNC119b is an important GSF in primary cilia (Wright et al. 2011). Therefore, it was concluded that Arl3 is activated in these compartments, resulting in a release of high affinity cargoes from UNC119, while Arl2 is releasing cargoes outside of these compartments. Aside from the GSFs UNC119a and UNC119b, GTP-bound Arl2 and Arl3 also bind PDE6δ, that was shown to be a GSF for farnesylated cargo proteins including Ras-proteins, i.e., RheB, K-Ras, N-Ras and H-Ras. In analogy to UNC119, binding of active, GTP-bound Arl2 or Arl3 to PDE6δ results in release of the isoprenylated cargo proteins (Ismail et al. 2011). Studies show that PDE6δ plays a key role in localization of these Ras-proteins to the plasma membrane by spatial cycles of solubilization, trapping and vesicular transport (Chandra et al. 2011; Schmick et al. 2014). As stated above, palmitoylated H-Ras and N-Ras are predominantly localized to the plasma membrane and the Golgi. Upon depalmitoylation, PDE6δ is able to redistribute the Ras-proteins to the cytosol. It was shown that the capability of PDE6δ to bind farnesylated Ras-proteins depends on their palmitoylation state. While monopalmitoylated H-Ras was still efficiently solubilized by PDE6δ, Ras-proteins such as Rap2a and Rap2c, that were double palmitoylated at two C-terminal Cys side chains additionally to the farnesylation in the CaaX-box Cys, were not solubilized (Chandra et al. 2011). Along that line, PDE6δ was unable to solubilize geranylgeranylated Ras-proteins. This demonstrates that PDE6δ is a chaperone solubilizing depalmitoylated and farnesylated Ras-proteins and effectively trapping depalmitoylated Ras, i.e., N-Ras, H-Ras and K-Ras4A, at the Golgi, where re-palmitoylation and redistribution to the plasma membrane is initiated. Interestingly, for H-Ras, another layer of regulation was reported, i.e., the *cis*/*trans* isomerization of the peptide bond preceding the proline side chain directly preceding a C-terminal palmitoylation site, acting as molecular timer for its depalmitoylation (Ahearn et al. 2011b; Saeidi et al. 2020). K-Ras4B is farnesylated at the CaaX-box Cys and carries a positively charged PBR in the region preceding the CaaX-box, ensuring targeting to negatively charged plasma membrane containing phospholipids such as phosphatidylserine and PIP_2 (Plowman and Hancock 2005). However, considering the total surface area of cellular endomembranes, this is not sufficient to explain the accumulation of K-Ras4B at the plasma membrane. It was shown that farnesylated K-Ras4B is constantly sequestered from endomembranes by PDE6δ and subsequently released at perinuclear membranes by binding to GTP-loaded Arl2 (Schmick et al. 2014). K-Ras4B is trapped by electrostatic interactions at recycling endosomes from which K-Ras4B is transported by vesicular trafficking, resulting in enrichment of K-Ras4B at the plasma membrane (Schmick et al. 2014). These processes have important functions for intracellular signaling, counteracting the entropy-driven sequestration of Ras-proteins over all cellular membranes (Chandra et al. 2011; Ismail et al. 2011) Structurally, UNC119a, UNC119b and PDE6δ are highly similar, consisting of an IG domain forming a cavity that can accommodate the hydrophobic myristoyl- or farnesyl-moiety (Ismail et al. 2012). However, the GSFs differ in their mechanism described for cargo-release upon binding to GTP-loaded Arl2 or Arl3. While binding of Arl3 to UNC119b was reported to result in a widening of the hydrophobic pocket, impairing binding to the *N*-myristoylated cargo, binding of active Arl3 to PDE6δ results in closure of the hydrophobic pocket, thereby displacing the farnesylated cargoes (Ismail et al. 2012; Ismail et al. 2011). Besides lipidation of Ras-proteins by formation of thioethers and/or thioesters on Cys side chains Ras-proteins can be acylated on primary amino groups of lysine side chains or on the Ras-protein's N-terminus (Figures 5C, D and 6A).

13 Lysine ac(et)ylation allows the adjustment of Ras-signaling to the cellular metabolic state

The three different types of lipid modifications require distinct sets of enzymes. While isoprenylation by thioether formation at the CaaX-box Cys is irreversible, thioester formation by palmitoylation at Cys residues is reversible by the action of thioesterases (Figures 5 and 6). Moreover, while

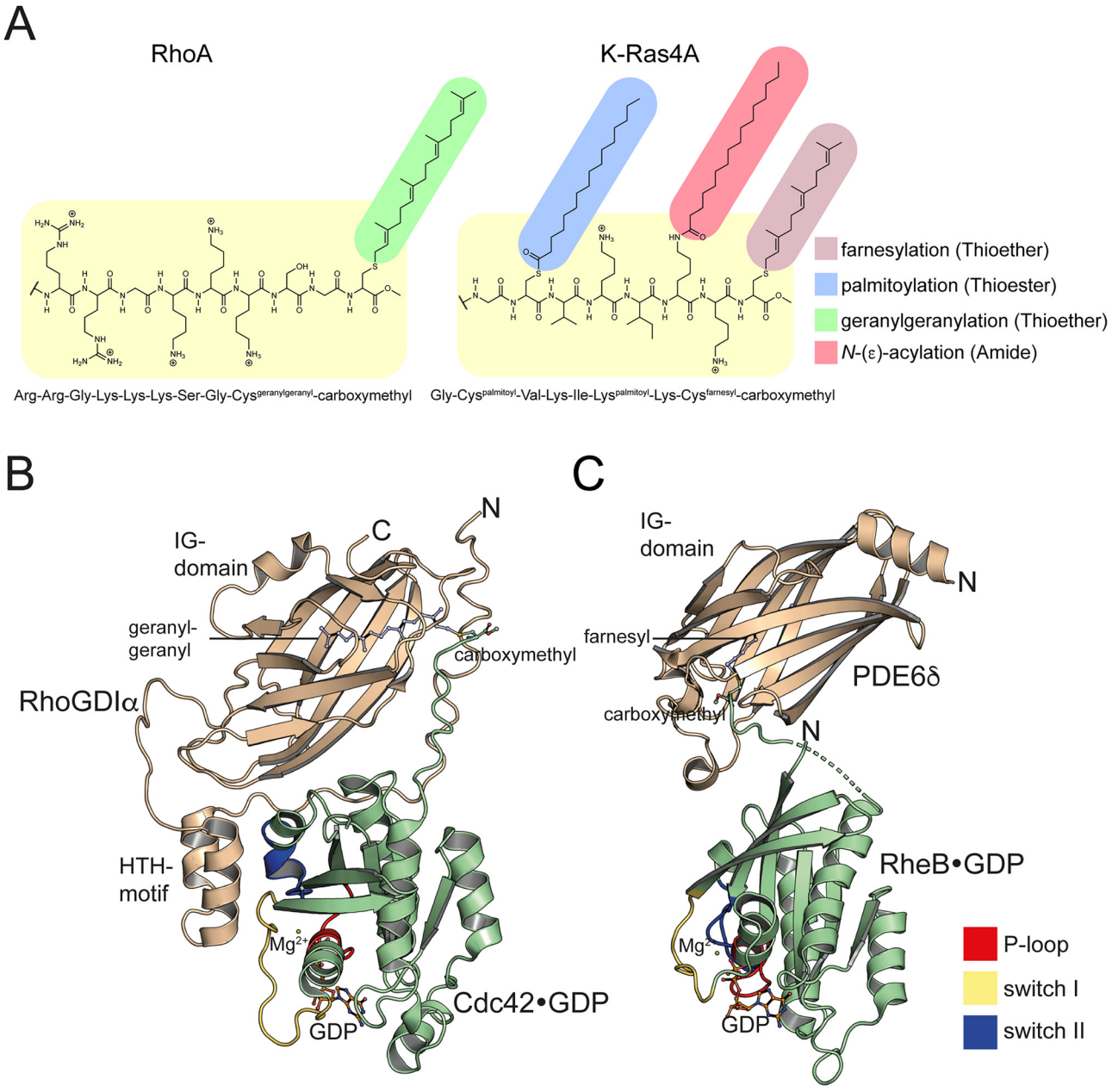

Figure 6: G-protein solubilization factors such as RhoGDI and PDE6δ are important factors for spatiotemporal regulation of Ras-signaling. (A) Ras-proteins can be post-translationally lipidated by multiple PTMs as exemplified on RhoA and K-Ras4A. Rho-proteins are geranylgeranylated at the CaaX-box Cys side chain forming an irreversible thioether. Next to this isoprenylation Ras-proteins need a second entity for membrane binding. This can be an extended PBR as realized amongst others in Rho-proteins or K-Ras4B or a second lipidation on Cys side chains or Lys side chains in the HVR. Rho-proteins have a highly positively charged Lys- and Arg-rich HVR, the PBR, involved in binding to negatively charged phospholipids. Moreover, this region is important for the interaction with the negatively charged N-terminal region of RhoGDI. This electrostatic network is needed for membrane delivery and extraction. K-Ras4A does not contain an extended PBR. However, it is palmitoylated by formation of a reversible thioester bond on a Cys side chain in the HVR and it can furthermore be lysine acylated by formation of a reversible amid-bond. (B) G-protein solubilization factors, such as RhoGDI and PDE6δ, use a hydrophobic cavity in an immunoglobulin-domain (ID) to accommodate the hydrophobic prenyl-group of Ras-proteins. Shown are the structures of RhoGDIα in complex with geranlygeranylated RhoA (PDSB: 1CC0) and PDE6δ in complex with farnesylated RheB (PDB: 3T5G). Both structures are highly similar in the IG-domain forming a two-layered β-sandwich with a hydrophobic cavity in between these β-sheets. In contrast to PDE6δ, RhoGDIs and RabGDIs (not shown) have an extended N-terminal region forming a helix-turn-helix (HTH)-motif contacting the switch regions of Ras-proteins. This allows sensing of the nucleotide-loading state of the Ras-protein and as a consequence to couple the GTP/GDP-cycle with a cytosol/membrane-cycle. While RhoGDIs and RabGDIs are in principle able to bind to farnesylated or gernalygeranylated Ras-proteins, PDE6δ only binds to farnesylated proteins. Most contacts to PDE6δ are established to the farnesyl-moiety, while only few interactions were described for some proteins with side chains directly flanking the farnesylation site.

acylation of lysine side chains is reversible by deacylases, including NAD^+-dependent sirtuins and classical Zn^{2+}-dependent histone deac(et)ylases (HDACs), the acylation at the N-terminus is irreversible (Blasl et al. 2021; Lammers 2021). As explained above, lysine acylation is a modification recently discovered on Ras-proteins, present on lysine side chains of the PBR in the C-terminal HVR or in other regions of the proteins modulating Ras-signaling, interfering with diverse processes including membrane binding, with consequences on cell migration, or transforming activity (Jing et al. 2017; Spiegelman et al. 2019a,b; Zhang et al. 2017b; Zhou et al. 2017a). This modification was shown to modulate Ras-membrane binding. In R-Ras, Rac1, K-Ras4A, RalB and H-Ras lysine side chains in the HVR preceding the CaaX-box were identified to be *N*-myristoylated or *N*-palmitoylated (Jing et al. 2017; Spiegelman et al. 2019a,b; Zhang et al. 2017b; Zhou et al. 2017a). Studies suggested that sirtuins (SIRTs) are capable to deacylate Ras-proteins (Figure 6A). It was discovered that SIRT6 is able to deacylate the Ras-related protein R-Ras and SIRT2 is able to deacylate the proteins K-Ras4A, RalB and H-Ras (Jing et al. 2017; Spiegelman et al. 2019b; Zhang et al. 2017b). Sirtuins are NAD^+-dependent enzymes, i.e., they use NAD^+ as stoichiometric co-substrate for the deacylation (Blasl et al. 2021). This relates the acylation state of proteins to the metabolic state of the cell, which means that at high cellular NAD^+ concentrations, the acylation stoichiometry might be lower compared to conditions of low cellular NAD^+ concentrations. There are still open questions on which enzymes are responsible for the acylation of Ras-proteins and on the actual acylation mechanism. Although lysine acyltransferases were reported to be capable of transferring palmitoyl-groups using palmitoyl-CoA as a donor-molecule for acylation, it has not been tested whether acyltransferases are capable of enzymatically acylating proteins of the Ras-family on lysine side chains in their HVR. One study suggests a mechanism including an S-N-acyl-transfer of a palmitoyl group forming a thioester with a Cys-side chain to the nucleophilic ε-amino group of lysine side chains (Komaniecki and Lin 2021). There are known bacterial enzymes that are capable to fatty acylate, i.e., *N*-palmitoylate or *N*-myristoylate, lysine side chains (Liu et al. 2018; Zhou et al. 2017a). The huge progress in quantitative mass spectrometry enabled the identification of thousands of acetylation and further acylation sites in the proteome of organisms of all domains of life (Choudhary et al. 2009; Lundby et al. 2012). These studies revealed that almost all proteins of the Ras-family, including K-Ras4A, Cdc42, Ran, their regulators and effectors, are targeted by lysine ac(et)ylation (Choudhary et al. 2009; Lundby et al. 2012). Our laboratory contributed to understanding how lysine acetylation regulates Ras-signaling. For the small GNBP Ran, we showed that lysine acetylation regulates all fundamental properties of Ran-function, i.e., GAP-catalyzed nucleotide hydrolysis, GEF-stimulated nucleotide dissociation, Ran-subcellular localization by interfering with nuclear transport factor 2 (NTF2) binding and the interaction with import and export receptors (de Boor et al. 2015). Our data revealed that the deacetylases SIRT1-3 are capable of deacetylating Ran and that diverse lysine acetyltransferases are capable of acetylating Ran at different lysine side chains (de Boor et al. 2015). A study suggested that K-Ras4B oncogenicity is regulated by acetylation of Lys104, counteracting SOS-catalyzed nucleotide exchange. The same laboratory further showed by indirect assays that the Ras-acetylation level is modulated by the deacetylases SIRT2 and HDAC6 (Yang et al. 2013). While these studies were performed on a Lys-to-Gln mutant, K-Ras4B K104Q, as mimic for lysine acetylation, we analyzed the impact of lysine acetylation by using site-specifically acetylated K-Ras4B protein prepared by using the genetic code expansion concept (GCEC) (Knyphausen et al. 2016). Our data revealed that Lys104-acetylation neither affected SOS-catalyzed nucleotide exchange on wildtype K-Ras4B nor on oncogenic K-Ras4B G12V. Moreover, Lys104-acetylated K-Ras4B was no substrate for SIRT2 or HDAC6, suggesting the impact reported for these enzymes on the K-Ras4B acetylation state is indirect (Knyphausen et al. 2016). We identified further acetylation sites in K-Ras4B, which are enzymatically regulated by lysine acetyltransferases (Knyphausen et al. 2016). These include Lys147 within the sAk/G5-motif, Lys101, Lys104 and Lys128 in the region of α3/α4. This region was previously suggested to be a potential interface for K-Ras4B dimer formation (Muratcioglu et al. 2015). We were not able to reproduce K-Ras4B dimerization (Knyphausen et al. 2016), which is in agreement with recent discussions suggesting that Ras forms nanocluster oligomers at the plasma membrane via its G-domain rather than real dimers (Figure 1B) (Simanshu et al. 2023). Lys101, Lys104, Lys128 and Lys147 in K-Ras4B were also reported to be ubiquitinated and/or SUMOylated (Heidelberger et al. 2018; Hendriks et al. 2017; Steger et al. 2021). Monoubiquitination of K-Ras4B at Lys147 impairs RasGAP-catalyzed nucleotide hydrolysis, thereby indirectly stabilizing the GTP-bound active state and activating downstream effectors (Knyphausen et al. 2016; Sasaki et al. 2011). In contrast, monoubiquitination of K- and N-Ras at Lys128 improves binding to RapGAP, switching-off signaling by acceleration of GTP-hydrolysis (Magits et al. 2024). These data suggest that a crosstalk of acetylation and ubiquitination might allow a regulation of Ras-function and Ras-signaling (Knyphausen et al. 2016). Along that line, a recent report showed that the Ras-proteins K-Ras, M-Ras and RIT1 are targeted by ubiquitination for proteasomal degradation in their GDP-bound form via the protein leucine zipper-like transcription regulator 1 (LZTR1), acting as substrate adapter for the Cullin-3 RING E3 ubiquitin ligase (Dharmaiah et al. 2025). We showed that the RhoA-C regulator RhoGDIα can

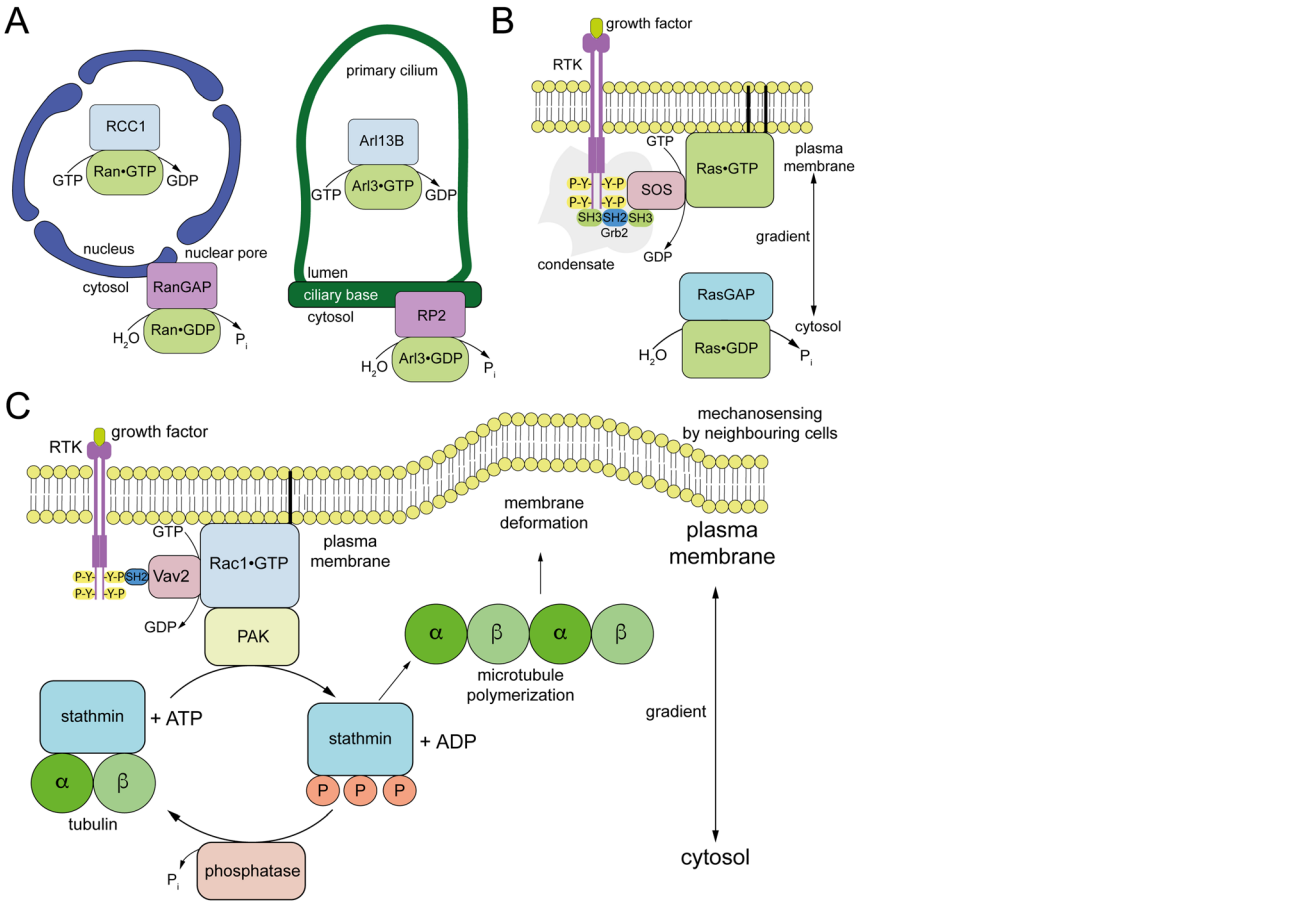

Figure 7: Molecular pattern formation by establishing subcellular gradients in Ras-signaling. (A) The distinct localization of GEFs and GAPs in different subcellular sites allows the formation of subcellular gradients of active/inactive Ras-proteins that drive cellular processes. As shown for Ran, the RanGEF RCC1 is localized in the nucleus bound to chromatin, while the RanGAP is localized on the cytosolic side of nuclear pores. This creates a gradient of GTP-bound Ran in the nucleus and GDP-bound Ran in the cytosol, allowing a directed shuttling of proteins to/from the nucleus/cytosol. Similarly, the Arl3-GAP RP2 is localized at the ciliary base and the Arl3GEF Arl13B within the lumen of primary cilia. This creates a gradient of active Arl3 in the cilia and inactive Arl3 in the cytosol, allowing a directed transport and release of lipidated cargo within the primary cilia. (B) Condensate formation allows the establishment of subcellular gradients in membraneless compartments as realized at the RTK. The phosphorylated RTK and Grb2 form a condensate that drives signal transduction. A further characteristic of cellular gradient formation is the localization of proteins involved in signal transduction at cellular membranes resulting in a strong increase in their effective concentrations. Localization of the RasGEF SOS to the plasma membrane increases its local concentration resulting in activation of membrane bound Ras. Again, the different localization of GEF and GAP allows the establishment of a cellular gradient of active and inactive Ras, enabling a directed activation of Ras-signaling. (C) Molecular pattern formation establishes recursive communication systems with neighboring cells. An example of molecular pattern formation that establishes a system to communicate with other cells is realized by activation of membrane-bound Rac1 through recruitment of its GEF Vav2 to the plasma membrane. Rac1 recruits the kinase PAK1 and activates it locally at the plasma membrane. PAK1 is able to phosphorylate stathmin at multiple sites resulting in release of bound α/β-tubulin heterodimers. The increase of the local concentration of α/β-tubulin drives polymerization of microtubles at the plasma membrane resulting in its deformation. This can be sensed by neighboring cells (inside-out signaling; mechanosensing) that can in turn communicate back (outside-in signaling).

also be regulated by lysine acetylation (Kuhlmann et al. 2016a; Kuhlmann et al. 2016b). RhoGDIα is a solubilization factor of geranylgeranylated Rho-proteins involved in membrane extraction of GDP-loaded Rho-proteins, forming a cytosolic pool of inactive Rho ready to be re-activated by GEF-catalyzed nucleotide exchange. However, as the affinity towards GDP-loaded, isoprenylated RhoA is reported to be in low nanomolar to picomolar range, a GEF might not be able to directly displace the bound RhoGDIα from RhoA for subsequent reactivation (Kuhlmann et al. 2016a,b; Tnimov et al. 2012). To this end, GDI-displacement factors (GDFs) were reported to dissociate the complexes of Rho and Rab-proteins from their GDIs (DiracSvejstrup et al. 1997). For Rho·RhoGDI complexes, proteins of the ERM-family were reported to act as GDFs (Dransart et al. 2005; Ivetic and Ridley 2004). Our data on lysine acetylation of RhoGDIα show that post-translational acetylation of lysine side chains can also act as a GDF. Acetylation of Lys52, in the N-terminal region of

RhoGDIα contacting the nucleotide binding region of RhoA, and of Lys178, in the IG-domain, strongly lowers the affinity of isoprenylated RhoA towards RhoGDIα (Kuhlmann et al. 2016a; Kuhlmann et al. 2016b). Besides acting as GDF, the reduction of affinity of RhoGDIα towards the isoprenylated RhoA-protein by acetylation can also interfere with its capacity to extract the membrane-bound RhoA from the membrane (Kuhlmann et al. 2016a; Kuhlmann et al. 2016b). Lys52 is located in the N-terminal helix-turn-helix motif of RhoGDIα contacting the nucleotide-binding region in the RhoA-bound form, but it is intrinsically unstructured in the unbound form. As acetylation of Lys52 reduces the affinity towards isoprenylated RhoA by four-orders of magnitude, these data also support reports suggesting that the N-terminus of RhoGDIα communicates with the IG-domain forming the hydrophobic isoprenyl-binding cavity (Goody et al. 2005; Kuhlmann et al. 2016a,b). For acetylation of RhoGDIα at Lys178, we observed a widening of the IG-domain, explaining mechanistically how the affinity towards isoprenylated RhoA is reduced, supporting data reported for the solubilizing factor UNC119b (Ismail et al. 2012; Kuhlmann et al. 2016a). One acetylation site in RhoGDIα, i.e., Lys138 in the IG-domain, was shown to also be targeted by SUMOylation, increasing the affinity towards RhoA (Yu et al. 2012). This is an example for a PTM crosstalk in which acetylation blocks subsequent SUMOylation and *vice versa*. Finally, complex formation between Rho-proteins and RhoGDIα was shown to be important to protect Rho-proteins from being targeted by ubiquitination for proteasomal degradation (Boulter et al. 2010; Cox and Der 1992). In another example for PTM-crosstalk, acetylation of RhoGDIα also indirectly modulates RhoA turnover. Cdc42 is phosphorylated by Src kinase on Tyr64, resulting in an enhanced complex formation with RhoGDIα (Tu et al. 2003). This might be important to shuttle Cdc42 to specific subcellular sites. Moreover, Src also phosphorylates and activates the Cdc42 GEF Vav2, stimulating its activity (Tu et al. 2003). Similarly, phosphorylation of RhoA on Ser188 in the C-terminus by cAMP-dependent protein kinase A (PKA) or cGMP-dependent protein kinase G (PKG) also increases the interaction with RhoGDIα, resulting in impaired RhoA-signaling in cells (Ellerbroek et al. 2003). Structural data on the complexes RhoA-GG/F·RhoGDIα or Cdc42-GG·RhoGDIα (GG: geranylgeranyl; F: farnesyl) show that the highly negatively charged N-terminus of RhoGDIα binds to the positively-charged region of the Rho-protein. The PBR is important for membrane binding of the Rho-protein. The negatively charged phospholipids in the plasma membrane compete with the negatively charged N-terminal patch in RhoGDIα for binding to the PBR in RhoA. Phosphorylation of a residue in the PBR introduces a negative charge that might impair RhoA plasma membrane binding and indirectly stabilizes the RhoA-GG·RhoGDIα-complex. These few examples show how important PTMs including post-translational lysine acylation are for Ras-signaling. As lysine acyltransferases use acyl-CoA as the donor molecule for the acylation of lysine side chains or the α-amino group of proteins, and sirtuins use NAD^+ as stoichiometric co-substrate for the deacylation, the regulation of Ras-signaling can be tightly adjusted to the cellular metabolic state (Blasl et al. 2021; Lammers 2021). Thousands of acylation sites were identified in proteins of the Ras-family as well as in their regulators and effectors and all these await their functional characterization (https://www.phosphosite.org/) (Choudhary et al. 2009, 2014; Hornbeck et al. 2015; Lundby et al. 2012). In general, PTMs contribute to the adaptation of the cells to its environment through directed distribution of proteins.

14 PTMs are fundamental for Ras-driven molecular pattern formation in cells

In a more global and systemic view, PTMs in Ras-signaling are essential for transfer of information from the cellular environment into the intracellular cytosol. In these processes, a signal or stimulus, i.e., a growth factor or morphogen, is sensed by cell surface receptors such as receptor tyrosine kinases (RTKs) like platelet-derived growth factor receptor (PDGFR) or epidermal growth factor receptor (EGFR). The signal is then transmitted into the cell, amplified and further processed (Bahar et al. 2023; Prajapati et al. 2025; Sinkala et al. 2021). Notably, recent data suggest that Ras-signaling is also strongly modulated by formation of biomolecular condensates at the plasma membrane (Strzyz 2019). These condensates are membraneless subcompartments in cells that form upon liquid-liquid phase separation (LLPS) by intermolecular interactions (Figure 7B) (Banani et al. 2017). Often, intrinsically disordered proteins or intrinsically disordered regions (IDRs) within proteins contribute to formation of condensates. Recent reports describe a biomolecular condensate at the plasma membrane formed by contribution of the C-terminal IDR of EGFR, which contains the Tyr-phosphorylation sites needed to recruit the adaptor protein Grb2 (Lin et al. 2022). This condensate was shown to increase the concentration and the membrane-dwell time of the GEF SOS, thereby establishing a system of kinetic proofreading and modulating Ras-activation by SOS (Huang et al. 2019). Similarly, biomolecular condensate formation was shown to affect regulation of actin polymerization (Case et al. 2019). This includes the recruitment of GEFs activating small GNBPs, which in turn activate downstream signal transduction processes including phosphorylation cascades or activation of actin-nucleation factors by Rho-proteins. This finally results in

cellular responses, such as modulation of cellular gene expression programs or formation of F-actin polymers by the formin protein mDia1 (Lammers et al. 2005, 2008; Rose et al. 2005). This demonstrates how essential PTMs are for cells to translate their perception of the environment into a cellular response, manifested in outcomes including change in morphology, change in metabolism, alteration of cellular migration, differentiation and proliferation. PTMs are dynamically regulated by enzymes, including so-called writers that specifically modify proteins, such as kinases, ac(et)yltransferases or E3 ubiquitin ligases, and erasers that can remove these PTMs, like phosphatases, deac(et)ylases/esterases, deubiquitinases/ubiquitin-like specific proteases (USPs) (Suskiewicz 2024). The dynamic nature of PTMs and the inherent property to adjust the activity of writers and erasers to the perceived cellular inner state and to the outside environment establishes a recursive communication system, in which the outside affects the inside and *vice versa* (Koseska and Bastiaens 2017). For the functionality of these recursive interaction systems, reader domains in proteins allow targeting of the dynamic PTMs and establishment of a system described by the concept of dimensionality reduction (Koseska and Bastiaens 2017). This concept explains the reduction of a three-dimensional space in the cytosol allowing free diffusion to a two-dimensional space at the cellular membrane, in which new equilibria are established by locally concentrating the enzymes (Adam 1968). PTMs help in shifting equilibria, increasing the affinity as well as the residence time of proteins to the post-translationally modified target protein with consequences on local concentrations of proteins (Koseska and Bastiaens 2017). As examples, protein domains including Src-homology 2 (SH2)-domains and phosphotyrosine binding (PTB)-domains allow binding to phosphotyrosine (pTyr) side chains, such as created upon activation of RTKs, SH3-domains enable recruitment to proline-rich sequences, 14-3-3 domains are able to bind to phosphoserine (pSer) or phosphothreonine (pThr) and bromodomains (BRDs) as well as YEATS (Yaf9, ENL, Af9, Taf14, Sas5)-domains target ac(et)ylated lysine side chains. In Ras-signaling, GEFs are recruited directly by adaptor proteins such as Grb2 via their SH2-domains to phosphorylated RTK-receptors and the RasGEF SOS is recruited via the Grb2 SH3-domain to the site of receptor activation at the plasma membrane (Figures 1B and 7B). An impressive example for this recursive communication is highlighted in the regulation of microtuble dynamics by formation of stathmin-tubulin interaction gradients driven by cycles of stathmin phosphorylation and dephosphorylation ultimately resulting in deformation of the cellular morphology, which is sensed by neighboring cells (Figure 7C) (Gavriljuk et al. 2021; Niethammer et al. 2004; Zeitz and Kierfeld 2014). This example also illustrates further important basic concepts of cellular signaling, which are manifested in the establishment of morphogenesis through Ras-signaling, and how this depends on PTMs. To generate ordered cellular structures, i.e., a system that is held out-of-equilibrium, cells have to counteract the gain in entropy by investing energy (Koseska and Bastiaens 2017; Niethammer et al. 2004). Ras-signaling drives cellular morphogenesis as it combines several features essential for these processes. Many Ras-dependent processes are initiated from cellular membranes in an example of the reduction of dimensionality (Adam 1968). This is possible as activation of the signal cascade and activation of Ras-proteins occur at cellular membranes and are precisely regulated in time and space, as described above for activation of the MAPK pathway by Ras (Figure 1B). This precise spatial restriction of Ras-activation at cellular membranes results in formation of molecular gradients of activated Ras-proteins, and in turn of activated effector proteins. In this review, we put a special focus on how PTMs contribute to formation of molecular patterns in the context of signaling. Ras-signaling does also include gradients of GTP-bound active and GDP-bound inactive Ran or Arf/Arl-proteins, which are established by the defined cellular localization of the GEFs or GAPs, as described above (Cavazza and Vernos 2015; Dasso 2002; Gotthardt et al. 2015; Watzlich et al. 2013). Activation of Ras-signaling is achieved by an activation of a cell surface receptor after perception of an environmental signal such as binding of a growth factor, often resulting in recruitment of GEFs to the plasma membrane to the activated receptors, either indirectly by recruitment of adaptor proteins, such as Grb2, or by direct binding of GEFs to those activated receptors, such as Vav2 binding via their SH2-domains to pTyr residues of the activated RTK (Figures 1B and 7B). By translocation of the GEF to the plasma membrane after activation of the receptor, the local effective concentration of the GEF is increased by one-order of magnitude (Grecco et al. 2011b; Leonard et al. 2023). This leads to a dominating GEF-catalyzed exchange of the Ras-protein bound GDP to GTP and therefore to a local activation of the Ras-protein. The localization of SOS to the plasma membrane substantially increases the turnover rate for nucleotide exchange (Gureasko et al. 2008; Huang et al. 2024; Iversen et al. 2014). This shift of Ras-activation to the membrane locally alters the balance of the GTP/GDP-cycle established by GEFs and GAPs towards the GTP-bound active state, finally resulting in local increase of activated Ras-protein at the membrane. Importantly, Ras-proteins would not be functional as molecular switches at a physiological scale without the action of GAPs. Only through GAPs can the signal be switched-off and thereby completed cycles of precisely coordinated Ras-activation and -inactivation can be established. Again, this is efficient as many Ras-proteins are often targeted to the membrane due to its lipidation or the presence of a PBR interacting with negatively-charged phospholipids such as phosphatidylserine (Clarke 2023).

For K-Ras4B and other GTP-binding proteins, it was recently shown that the PBR is not primarily important for an electrostatic interaction of the protein to the membrane but is rather important for lipid recognition by adopting different conformations (Clarke 2023). This supports nanoclustering of K-Ras4B and the segregation of K-Ras4B and H-Ras (Zhou et al. 2014, 2017b). Lipidated Ras was described to be predominantly enriched in plasma membrane microdomains (Plowman and Hancock 2005; Prior et al. 2003; Truong-Quang and Lenne 2014). For H-Ras, it was shown that membrane orientation depends on its nucleotide loading state (Abankwa et al. 2008, 2010; Gorfe et al. 2007). The effectors Raf-kinase, MEK and ERK were found to localize to membrane microdomains upon activation of the MAPK pathway (Harding et al. 2005). The majority of the cellular K-Ras pool was predominantly found to localize to non-raft plasma membrane independent of its nucleotide loading state. H-Ras, in its GDP-bound state, was identified to be present at half of its concentration at lipid rafts, but H-Ras in its GTP-bound state was completely absent from rafts. Overall, different Ras-isoforms were shown to localize to different plasma membrane microdomains. This might be due to the different lipidation of H-Ras and K-Ras, K-Ras4A and K-Ras4B, by palmitoylation, farnesylation and/or by presence of a PBR in the HVR (Prior and Hancock 2012). The enrichment of lipidated Ras at the sites of GEF-catalyzed activation at the membranes constitutes a self-amplifying system, based on Ras-clustering (Figures 1B, 5 and 6). Notably, several studies suggest that different Ras-isoforms can form dimers using different surfaces of the G-domain. The idea of Ras forming dimers via their G-domain was sparked by the observation that Raf-kinase also forms obligate dimers, suggesting that only Ras dimers would be able to fully activate Raf (Muratcioglu et al. 2015; Nan et al. 2015; Philips and Der 2015; Simanshu et al. 2023). However, the dimerization of Ras could not be experimentally shown in solution, though recent data and discussions suggest that Ras does not form dimers via association of their G-domains. Instead, the C-terminal lipid modification results in clustering of two or more Ras molecules in membrane microdomains (Janosi et al. 2012; Knyphausen et al. 2016; Mu et al. 2022; Simanshu et al. 2023; Zhou et al. 2024). Differences in the C-terminal lipidation and the sequence in the HVR in Ras-proteins explain the different localization in the plasma membrane and endomembrane microdomains (Chavan et al. 2015; Jang et al. 2015; Plowman and Hancock 2005; Plowman et al. 2005; Prior et al. 2003; Truong-Quang and Lenne 2014). Rising of the local concentration of activated Ras leads to a stimulation of the MAPK pathway. This accumulation of activated Ras leads to a clustering of effector proteins, such as the kinase PAK that results in a strong increase of the phosphorylated substrates at this site. This is another example of dimensionality reduction, which was reported to be able to increase the affinity between Ras and its effectors by more than 100-fold (Adam 1968; Kholodenko et al. 2000). Overall, this concept shows how signals are locally strongly amplified in the cell, resulting in the establishment of molecular patterns within the cell. Dynamic PTMs are the basis for those processes at the molecular level as they allow to modulate the spatial organization of the proteins involved in the processes and their effective local concentrations (Huang et al. 2024; Leonard et al. 2023). Often these systems are self-organizing and depend on the affinity and accumulation of receptors and Ras-proteins in areas of cellular membranes of certain lipid compositions. Along that line, it was shown that isoprenylated Ras predominantly exists at the ER membrane, while palmitoylation at the Golgi results in accumulation at the plasma membrane (Prior and Hancock 2012). These processes can be manipulated by pathogens using virulence factor to promote infection.

15 Bacterial pathogens hijack host cells by targeting Ras-signaling by PTMs

Gram-negative bacterial pathogens use sophisticated megadalton systems, including the type-I-secretion system (T1SS), type-III-secretion system (T3SS) and type-IV-secretion system (T4SS) to inject a plethora of virulence factors into host cells supporting an efficient infection process. Alternatively, exotoxins are taken up through endocytosis by host cells. These virulence factors manipulate the host cell to allow the cellular uptake of pathogens to enable proliferation, differentiation and survival in the host cell by counteracting anti-infective strategies, as well as to facilitate the propagation and release at the end of infection. Several bacterial virulence factors were characterized structurally and functionally at the mechanistic level up to atomic resolution. Many target GNBPs, exerting either gain-of-function effects by activation of G-protein signaling or loss-of-function effects by its inactivation. Mechanistically, the virulence factors often act by post-translationally modifying G-proteins resulting either in modulation of effector binding, GEF-catalyzed nucleotide exchange, GAP-catalyzed nucleotide hydrolysis, or solubilization by GDIs. As one of the first concisely characterized virulence factors affecting a G-protein, the enterotoxin cholera toxin was shown to directly modify the stimulatory Gα-subunit, $G\alpha_s$, of a heterotrimeric G-protein (Bourne 1990; Moss et al. 1979; Spangler 1992). Cholera toxin was shown to have an ADP-ribosyltransferase

activity transferring an ADP-ribosyl-group to the Arg-finger (R201) supplied *in cis* in $G\alpha_s$ (Beckner and Blecher 1981; Chang and Bourne 1989; O'Neal et al. 2005; Spangler 1992). Consequently, $G\alpha_s$ exists permanently in the GTP-bound active state, resulting in constitutive activation of a signal transduction cascade. Besides, *Salmonella typhimurium* uses various virulence factors encoded in pathogenicity islands integrated into the bacterial genome, directly manipulating Rho-signaling. One of these factors is *Salmonella* outer protein E (SopE), which is a T3SS effector that has GEF activity towards Cdc42 and Rac1, contributing to the reorganization of the actin-cytoskeleton (Buchwald et al. 2002). In analogy, *Shigella flexneri* uses the T3SS to inject the WxxxE-type virulence factors IpgB1 and IpgB2, acting as GEFs towards Cdc42/Rac1 and RhoA, respectively, to modulate the actin cytoskeleton for efficient cellular invasion by inducing membrane-ruffles (Hachani et al. 2008; Klink et al. 2010; Weddle et al. 2022). These bacterial GEFs are structurally unrelated to the eukaryotic counterparts, suggesting a development by convergent evolution. The *Shigella* T3SS effector IpaJ is a Cys-protease that acts as a demyristoylase, cleaving *N*-myristoylated proteins, including human Arf1, between Gly2 and Asn3, resulting in inhibition of the secretory pathway by a pathogen (Burnaevskiy et al. 2013). Various other bacterial species use virulence factors that manipulate Ras-signaling by PTMs. The *Vibrio cholerae* multidomain, multifunctional autoprocessing repeats-in-toxin (MARTX) toxins contain a lysine N-(ε)-fatty acyltransferase activity using palmitoyl-CoA as substrate within their RhoGTPase inactivation domain (RID), resulting in acylation of RhoGNBPs in the lysine-rich PBR (Zhou et al. 2017a). Defective Rho-signaling manifested in disruption of the host cell actin cytoskeleton (Zhou et al. 2017a). *Yersinia enterolytica* or *Yersinia pestis* use the T3SS to inject the protease YopT into host cells (Iriarte and Cornelis 1998; Schmidt 2011; Shao et al. 2002). According to the MEROPS-database, YopT is a Cys-protease that belongs to the CA-clan. YopT efficiently cleaves RhoA directly N-terminally of the CaaX-box Cys only when RhoA is geranylgeranylated and proteolytically processed by Rce1, while carboxymethylation is not needed (Schmidt 2011). Thereby, RhoA is released from the membrane and forms complexes with RhoGDIα resulting in disruption of the actin cytoskeleton in the host cell, priming the formation of phagocytic cups (Aepfelbacher et al. 2003; Fueller et al. 2006). The CE-clan protease related *Yersinia pseudotuberculosis* virulence factor YopJ has an acetyltransferase (AcT) activity stimulated by inositol hexakisphosphate (IP_6) in the host cell, inhibiting MAPK signaling and the NFκB pathway by acetylating the kinases MEK2, MEK6 and IKK within the activation segment (Mittal et al. 2006; Mittal et al. 2010). Notably, the AcT can acetylate Ser and Thr side chains as well as Lys side chains (Mittal et al. 2006, 2010). Other members of this family of virulence factors act as deubiquitinases (DUB), deSUMOylases or even have a dual activity as AcT and DUB/deSUMOylases (Boll et al. 2023; Pruneda et al. 2018; Schmöker et al. 2025). *Yersinia* also uses the virulence factor *Yersinia* protein kinase A (YpkA) that has a dual activity, showing structural similarities to eukaryotic Ser/Thr-kinases and a domain, which functionally mimics a RhoGDI (Barz et al. 2000; Prehna et al. 2006). It was shown that YpkA has an autophosphorylation activity and its binding to Rac1/RhoA inhibits GEF-catalyzed nucleotide exchange (Barz et al. 2000; Prehna et al. 2006). In cell culture, both the kinase domain and the GDI-domain are needed to induce cytoskeletal disruption (Prehna et al. 2006). A mutant *Y. pseudotuberculosis* strain lacking the GDI-domain of YpkA showed impaired virulence in a mouse infection assay (Prehna et al. 2006). The *E. coli* single-chain exotoxin cytotoxic necrotizing factor (CNF1) has a deamidase activity resulting in constitutive activation of RhoA, Cdc42 and Rac1 by deamidation of Gln63 in RhoA or Gln61 in Cdc42/Rac1 rendering them incapable of hydrolyzing the bound GTP (Figure 8A) (Chaoprasid and Dersch 2021; Schmidt et al. 1997). This results in reorganization of the actin cytoskeleton with formation of thickened actin fibers, membrane ruffles, microspikes and filopodia. Moreover, CNF1 causes a polynucleation phenotype in human epithelial cells (Chaoprasid and Dersch 2021). The *Clostridium difficile* C3 exoenzyme has an ADP-ribosyltransferase activity that ADP-ribosylates RhoA, -B- and -C at Asn41 near switch I resulting in inhibition of Rho-signaling (Figure 8A). While the interaction with various effectors was shown to be unaffected by this modification, the Rho-protein is not accessible for GEF-catalyzed nucleotide exchange (Sehr et al. 1998). Other *Chlostridium difficile* enterotoxins are *C. difficile* toxin A/B (TcdA and TcdB), which are taken up by the host cell by receptor-mediated endocytosis, monoglycosylate Thr37 or Thr35 in switch I of RhoA or Cdc42/Rac1, respectively, resulting in their inactivation (Voth and Ballard 2005). *Vibrio parahaemolyticus* inhibits Rho-signaling by AMPylation of RhoA, Cdc42 and Rac1 at Thr37 or Thr35 by the T3SS effector VopS (Figure 8A) (Yarbrough et al. 2009). Thr37-/Thr35-AMPylation by VopS prevents the interaction of Rho-proteins with effector proteins, thereby inhibiting actin assembly in infected cells (Yarbrough et al. 2009). *Legionella pneumophila* injects several hundreds of virulence factors into host cells using the Dot/Icm T4SS, manipulating various cellular processes in the host cells. SidM/DrrA is a bifunctional effector used to recruit Rab1 from the cytosol to the *Legionella*-containing vacuole (LCV) and acts as GEF for Rab1 resulting in loading of Rab1 with GTP, leading to its dissociation from RabGDI (Schoebel et al. 2009). Furthermore, SidM

has an adenylyl-transferase activity that adenylates Rab1 in its GTP-bound conformation within switch II, impairing GAP-catalyzed GTP-hydrolysis (Muller et al. 2010; Murata et al. 2006; Schoebel et al. 2009; Yu et al. 2013). Notably, the *L. pneumophila* effector SidD acts as a deAMPylase that can revert Rab1 AMPylation catalyzed by SidM/DrrA, thereby establishing a system of temporal control of Rab1 activity by cycles of AMPylation and deAMPylation during different phases of infection (Tan and Luo 2011). The *L. pneumophila* effector LepB has GAP-activity towards Rab1, accelerating nucleotide hydrolysis by rearrangement of the Rab1 Q70, thereby acting as *cis*-Gln (Yu et al. 2013). These examples show that during evolution, bacterial pathogens developed various strategies using PTMs to manipulate cellular processes in the host cells targeting molecular switches of the Ras-family, their regulators or effectors. Obtaining profound biochemical and biophysical knowledge on protein-protein interactions, protein-lipid interactions and how their interplay is affected by PTMs is critical not only to understand infections but also normal and dysregulated Ras-signaling. This includes the exact understanding of equilibrium dissociation constants (K_D) and the exact knowledge on the dynamics of the interactions, including their association rate constants, k_{ass}, and dissociation rate constants, k_{diss}, in order to be able to describe and assess these fundamental processes in pattern formation in cells.

16 Methodological advances to study PTMs in Ras-signaling

The last years have seen tremendous methodological progress in studying growth factor signaling and Ras-signaling. This includes the development of advanced single-cell proteomics and high-resolution microscopy, including lifetime imaging techniques such as fluorescence lifetime imaging (FLIM), fluorescence resonance energy transfer (FRET) and the combination of FLIM and FRET. These enable the study of protein function, subcellular localization and the dynamics of protein-protein network with high level of spatial and temporal resolution, even at single-molecule level (Grecco et al. 2010, 2011a; Mund et al. 2022; Peyker et al. 2005; Torrado et al. 2024; Verveer et al. 2006a,b). In this section, we will mainly focus on technologies that enable the study of the impact of PTMs in Ras-signaling. Several methods were developed to isoprenylate Ras-proteins for *in vitro* studies. This encompasses co-expression of Ras-proteins with farnesyltransferases or geranylgeranyltransferases *in vivo* and subsequent purification of the isoprenylated proteins (Fres et al. 2010; Schaber et al. 1990). In an alternative approach, the recombinantly produced and purified Ras-protein can be isoprenylated *in vitro* using purified prenyltransferases and farnesylpyrophosphate or geranylgeranylpyrophosphate as substrates for the isoprenylation (Kuhlmann et al. 2016a; Schaber et al. 1990). Production of CaaX-box containing Ras-proteins in a eukaryotic expression system such as the baculoviral expression in Sf9 cells does also result in full C-terminal processing as described for Ras and Rap-proteins (Porfiri et al. 1995). Further strategies to prepare fully processed and lipidated Ras-proteins include semisynthetic approaches, in which a protein is recombinantly produced, purified and subsequently ligated to a lipidated peptide (Brunsveld et al. 2006; Hanna et al. 2022). To this end, C-terminally truncated Ras-proteins can be chemoselectively modified by lipidated peptides, generated by solid-phase synthesis (Bader et al. 2000; Brunsveld et al. 2006). Moreover, protein ligation using the intein-technology has also been applied to obtain C-terminally lipidated Ras-proteins (Alexandrov et al. 2002; Brunsveld et al. 2006). In a chemoenzymatic semisynthetic strategy, the enzyme sortase (SrtA) can be used to facilitate linkage of a protein carrying a SrtA recognition motif to a modified peptide (Hanna et al. 2022; Tschirpke et al. 2024). Workflows were also reported that allow the preparation of *N*-myristoylated Arl-proteins by co-production of Arl with human N-terminal myristoyltransferase (Van Valkenburgh and Kahn 2002). Progress in synthetic biology allows the site-specific incorporation of various non-canonical, unnatural amino acids (UAAs) into proteins following the genetic code expansion concept (GCEC). These systems are based on synthetically evolved aminoacyl-tRNA-synthetases/$tNRA_{CUA}$-pairs that fulfill the rules of orthogonality. This means that there is no cross-reactivity with the host cells' translational machinery. The aminoacyl-tRNA-synthetase charges the cognate $tRNA_{CUA}$, with CUA decoding the amber stop codon, with the desired amino acid. One of the most often used synthetic biological systems is the pyrrolysyl-tRNA-synthetase (PylRS)/$tRNA_{CUA}$ (PylT)-pair from *Methanosarcina* species. Pyrrolysine is known as the 22nd amino acid and is incorporated by methanogenic archaea into enzymes involved in methane biosynthesis. Studies were done in all model organisms showing that this system can be applied in eukaryotes and prokaryotes (Bianco et al. 2012; Davis et al. 2021; Gautier et al. 2010; Greiss and Chin 2011; Lammers et al. 2010; Neumann et al. 2008, 2009). PylRS was shown to have a substrate binding cavity capable of accommodating a variety of amino acid side chains. It has a high degree of substrate promiscuity and it does not have any proof-reading activity (Fekner and Chan 2011). PylRS/PylT is applied to incorporate more than 100 UAAs as response to an amber stop codon into proteins. A PylRS/PylT-pair system derived from

Methanosarcina barkeri was developed to allow the incorporation of acetyl-L-lysine into proteins (Lammers et al. 2010; Neumann et al. 2009; Neumann et al. 2008). Notably, the synthetic evolution of PylRS-variants with altered substrate specificity requires the development of a sophisticated selection strategy. Novel approaches applying machine learning were developed for the evolution of PylRS-variants with improved incorporation efficiencies for diverse non-canonical amino acids (Zhang et al. 2025b). We applied this GCEC to produce site-specifically and quantitatively acetylated proteins suitable for biochemical and biophysical studies, including structural studies using X-ray crystallography (de Boor et al. 2015; Kuhlmann et al. 2016a,b; Lammers 2018; Lammers et al. 2010). This resulted in the determination of the first crystal structure of a site-specifically lysine-acetylated protein, the peptidyl-prolyl-*cis*/*trans*-isomerase Cyclophilin A (CypA) acetylated at Lys125, and of a protein complex, CypA AcK125·HIV-1-capsid (Lammers et al. 2010). Subsequently, we also determined the first crystal structure of a double-acetylated protein, RhoGDIα acetylated at Lys127 and Lys141 (Kuhlmann et al. 2016b). Our data on the impact of lysine acetylation on Ras-proteins and their regulators suggest that this PTM is important to regulate fundamental processes in Ras-signaling and to adjust them to the cellular metabolic state. Incorporation of photo-caged lysine was possible in nuclear localization sequences (NLS) in proteins, to stimulate their nuclear localization, and into kinases of the MAPK pathways using synthetically evolved variants of PylRS (Gautier et al. 2010, 2011). This enables the activation of kinase signaling in living mammalian cells to obtain further insight into the kinetics of nuclear transport or of individual steps in kinase signaling cascades, as well as to dissect outcomes of activation of individual kinases (Gautier et al. 2010, 2011). Similar systems might be interesting to apply to Ras-proteins and their regulators. Another breakthrough in synthetic biology was the use of the GCEC system to allow the incorporation of phosphoserine, phosphothreonine, phosphotyrosine and of their non-hydrolyzable analogs into proteins (Luo et al. 2017; Park et al. 2011; Rogerson et al. 2015; Zhang et al. 2017a). This enabled the recombinant production and purification of site-specifically phosphorylated kinases from *E. coli* and to study kinase signaling, including MAPK signaling, in mammalian cells (Beránek et al. 2018; Luo et al. 2017; Neumann 2018; Park et al. 2011; Rogerson et al. 2015; Zhang et al. 2017a).

The last years were characterized by massive progress in protein structure prediction and protein design (Jumper et al. 2021; Krishna et al. 2024; Lisanza et al. 2025). Protein structure prediction is now highly accurate, although protein complexes and intrinsically unfolded regions are still difficult to predict in several cases. The releases of AlphaFold3 and RoseTTAFold All-Atom also allow the prediction of the binding of ligands, including small molecule ligands, and of PTMs (Abramson et al. 2024; Krishna et al. 2024). Miniprotein binders were selected from a naïve library that bind with exceptional picomolar affinity towards K-Ras. These miniproteins were shown to bind as dimers to the effector binding region including switch I and switch II, stabilizing K-Ras in an open conformation. In this open conformation, an extended pocket is generated that might be used to develop novel small molecules targeting this site (McGee et al. 2018). Besides these approaches, the *de novo* design of Ras-isoform specific binders is also ongoing (Zhang et al. 2025a). The possibility to design protein binders with high selectivity and potency might also have a huge impact on basic science. Studying Ras-signaling will also play an important role for the development of novel therapeutics to tackle mutations in Ras-signaling.

17 Novel therapeutic avenues are promising to tackle oncogenic Ras and RASopathies

Several therapeutic attempts aimed at interfering with PTMs of Ras-proteins. Targeting the subcellular localization of oncogenic Ras was regarded initially a promising strategy. This led to the development of small molecule farnesyltransferase inhibitors (FTIs), i.e., tipifarnib and lonafarnib, that entered clinical trials (Wittinghofer 2013, 2014). However, both compounds failed clinical trials, most likely due to severe and unwanted side-effects and because impaired farnesylation can be compensated functionally in N-Ras and K-Ras by geranylgeranylation (Berndt et al. 2011; Berndt and Sebti 2011; Schaffner-Reckinger et al. 2025). Both compounds were later shown to be valuable for treatment of progeria and cancer caused by H-Ras mutations (Ho et al. 2021; Schaffner-Reckinger et al. 2025; Suzuki et al. 2023). The development of dual-inhibitors targeting both farnesyltransferase and GGTase I was not successful due to their massive side effects (Lobell et al. 2001). Along that line, inhibitors that approach proteolytic processing of isoprenylated Ras, such as salirasib inhibiting carboxymethylation catalyzed by the enzyme ICMT, did show considerable off-target effects and are therefore not suitable for development of a therapeutic (Berndt et al. 2011). Another molecular target is the palmitoylation/repalmitoylation cycle of Ras affecting the localization of H-Ras, N-Ras and K-Ras4A. While H-Ras and N-Ras are recruited to the Golgi membrane to become palmitoylated and further transported to the plasma membrane by vesicular transport,

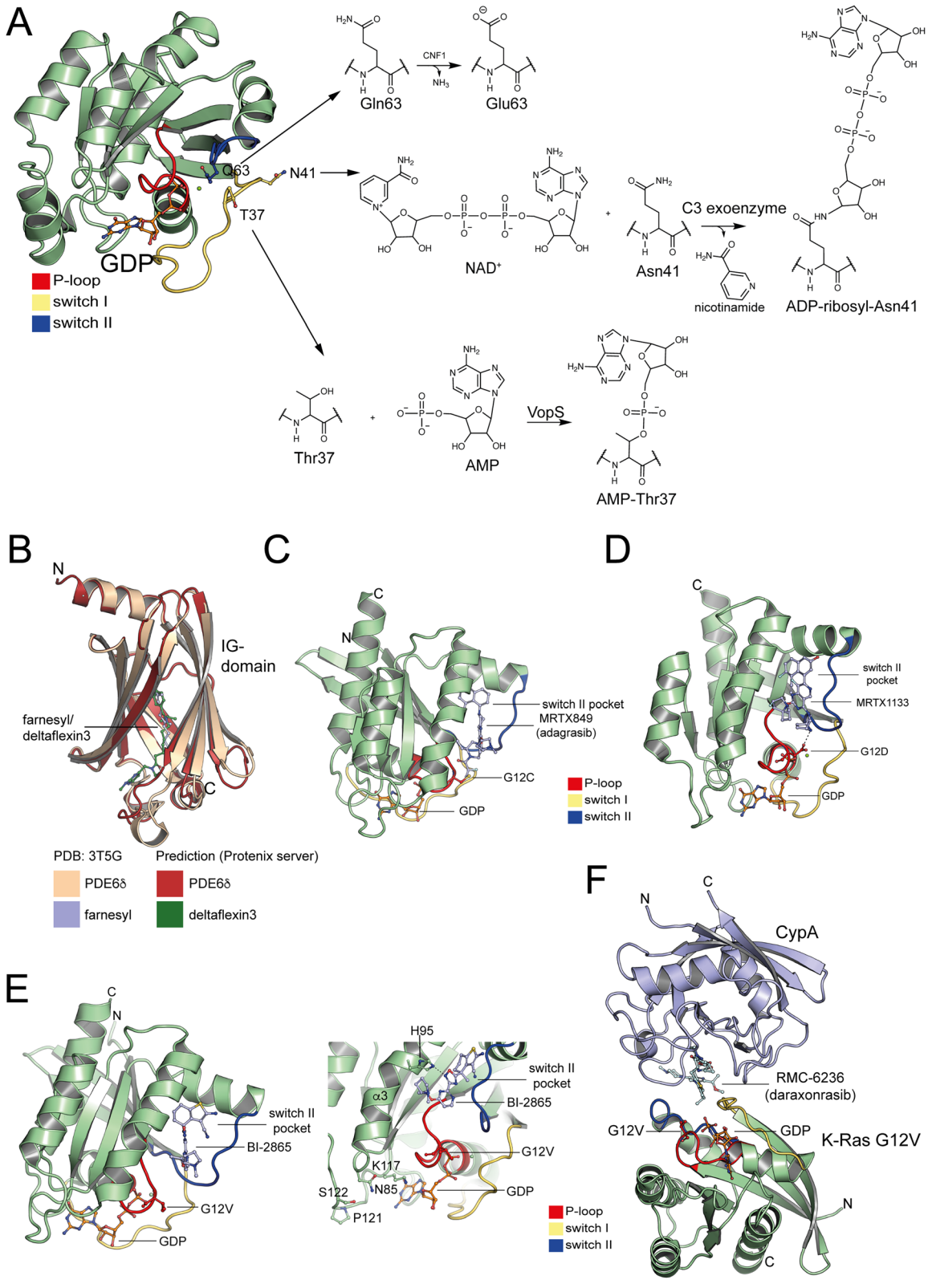

Figure 8: Targeting Ras-proteins by virulence factors and novel therapeutic advances to tackle mutant Ras. (A) Bacterial pathogens use a plethora of virulence factors that use PTM of Ras-proteins and their regulators to allow an efficient infection process. Shown is the structure of RhoA (PDB: 1FTN) and selected sites that were post-translationally modified by bacterial pathogens. The *E. coli* exotoxin CNF1 (cytotoxic necrotizing factor) has a deamidase activity resulting in constitutive activation of RhoA, Cdc42 and Rac1 by deamidation of Gln63 in RhoA/Gln61 in Cdc42/Rac1. The *Clostridium difficile* C3

K-Ras4A is found at the mitochondrial membranes upon depalmitoylation, activating hexokinase I. Small molecule compounds, such as palmostatin B, were developed to interfere with Ras localization, inhibiting the palmitoyltransferases APT1 (ML348)/APT2 (ML349) or ABHD17, affecting trafficking of N-Ras and H-Ras. These studies suggest that inhibition of ABHD17 is particularly important for inhibiting growth of cancer cells that depend on N-Ras (palmostatin B, ML348). Other studied molecular targets for the development of therapeutics are Ras solubilization factors, including PDE6δ that binds to isoprenylated proteins, thereby not differentiating between the GDP- and GTP-bound states. Palmitoylation of isoprenylated Ras-proteins was shown to abolish the interaction with PDE6δ, suggesting that K-Ras4B is the main Ras-protein that can be affected by PDE6δ-inhibitors. Some promising compounds selectively binding to the hydrophobic pocket in PDE6δ were developed, however, these showed significant side-effects and were poorly soluble. Another compound, deltaflexin3, showed less off-target effects and improved solubility, however, it also resulted in only weak activation of the MAPK pathway (Figure 8B). During treatment, deltaflexin3 is suggested to be used in a combination therapy with sildenafil, an inhibitor of PKG2, which phosphorylates K-Ras4B at S181, lowering the affinity towards PDE6δ.

Despite many attempts to directly therapeutically tackle oncogenic Ras, i.e., Ras G12X, G13X or Q61X, it was long considered to be undruggable, for several reasons. Firstly, Ras-proteins bind GDP and GTP with high nanomolar/picomolar affinity, making it difficult to develop potent molecules that are able to compete with nucleotide binding. The cellular concentrations of GDP and GTP in the micromolar range suggest that Ras-proteins are saturated with either GDP or GTP. Secondly, apart from the nucleotide binding pocket, there are no obvious solvent-accessible pockets suitable to be targeted by small molecules. Thirdly, the development of selective compounds discriminating between different Ras-proteins is demanding as the G-domain of Ras is structurally highly conserved. Fourthly, there is a broad spectrum of known mutations in switch I or switch II that render Ras constitutively active, with G12X, G13X and Q61X being the most prevalent without substantially altering its structure. This makes it really challenging to develop small molecules specifically targeting the individual oncogenic variants. Recent data suggest that the wildtype Ras-signaling has to be simultaneously targeted therapeutically alongside the mutated Ras in cancer, in a combination therapy, as both contribute to disease development (Sealover et al. 2025).

Recently, there has been tremendous success in the development of small molecules targeting specific K-Ras mutations, including the P-loop G12C, one of the most prevalent mutations frequently found in lung cancer (Li et al. 2025; Ou et al. 2022; Rosell et al. 2024; Rosell and Karachaliou 2016; Skoulidis et al. 2021). Covalent inhibitors such as sotorasib and adagrasib were developed and entered clinical trials (Figure 8C). They specifically target the K-Ras mutant G12C by forming a thioester linkage and trapping K-Ras in the inactive conformation by binding to the switch II pocket (Ou et al. 2022; Skoulidis et al. 2021). Further potent non-covalent K-Ras inhibitors were developed, such as the compound MRTX1133 targeting K-Ras G12D in its GDP- and GTP-bound state, so-called Ras-on inhibitors (Figure 8D) (Wang et al. 2022). MRTX1133 has picomolar affinity to K-Ras G12D binding to the switch II pocket (Wang et al. 2022). While these inhibitors are a major step towards treatment of Ras-driven cancers, they are only targeting individual mutants. However, progress was made in the development of non-covalent inhibitors that also target the switch II pocket selectively and potently target several variants of oncogenic K-Ras, i.e., pan-K-Ras inhibitors such as BI-2865 (Kim et al. 2023) BI-2865 targets both, the GTP-bound and GDP-bound state with higher potency towards the latter (Figure 8E) (Kim et al. 2023). Structural studies show that selectivity towards K-Ras is created by residues that evolutionary diverged in K-Ras, H-Ras and N-Ras. His95 in K-Ras forms a direct interaction with BI-2865 (H-Ras: Gln95; N-Ras: Leu95). Moreover, differences between K-Ras, H-Ras and N-Ras are at positions Pro121 and Ser122 in K-Ras, which might contribute allosterically to the inhibitory potency of BI-2865 (Figure 8E) (Kim

exoenzyme has ADP-ribosyltransferase activity modifying RhoA, -B- and -C at Asn41 near switch I, resulting in inhibition of Rho-signaling. *Vibrio parahaemolyticus* adenylates RhoA, Cdc42 and Rac1 at Thr37 or Thr35 by the effector VopS. Thr37-/Thr35-AMPylation by VopS abolishes the interaction of Rho-proteins with effector proteins. (B) Small molecule inhibitors were developed directly targeting the G-protein solubilization factor PDE6δ. Shown is a superposition of the crystal structure of PDE6δ in complex with a farnesyl-moiety (PDB: 3T5G) with a predicted structure of PDE6δ in complex with deltaflexin3. The prediction was done with the Protenix server (pTM: 0.96; ipTM: 0.91). the superposition shows that deltaflexin3 uses the same binding site in the hydrophobic cavity as the farnesyl-moiety. (C) The K-Ras G12C specific inhibitor MRTX849 binds covalently to the Cys12 and occupied the switch II pocket (PDB: 6UT0). (D) MRTX133 is a small molecule non-covalently targeting K-Ras G12D. The specificity toward the G12D mutant is created by formation of a hydrogen bond of Asp12 with the MRTX133 molecule. MRTX133 occupies the switch II pocket. (E) The small molecule BI-2865 is a compound targeting multiple K-Ras mutants. It is selective towards K-Ras as binding is mediated by His95, which is divergent in H-Ras and N-Ras. (F) The molecular glue RMC-6236 is a non-covalent inhibitor that allows formation of a tri-complex between K-Ras and CypA (cyclophilin A). This blocks interaction with effector proteins. RMC-6236 is able to target various Ras-mutants and is active towards K-Ras, H-Ras and N-Ras.

et al. 2023). Another strategy focusses on the development of so-called molecular glues to target oncogenic Ras (Holderfield et al. 2024; Schulze et al. 2023). Several reports describe the usage of molecular glues to target Ras-signaling by recruiting the peptidyl-prolyl-*cis*/*trans*-isomerase CypA, belonging to the immunophilins, as also FKBP12, which is known to be involved in mTOR signaling (Harikishore and Yoon 2015; Holderfield et al. 2024; Schulze et al. 2023). CypA binds to cyclic peptides, such as cyclosporin A or sanglifehrin A, forming a composite surface interacting with cellular effectors like calcineurin phosphatase, which has an immune suppressive effect (Jin and Harrison 2002). These cyclic molecular glues encompass a CypA binding motif and a Ras-binding motif, enabling the formation of ternary complexes between both proteins. In contrast to other small-molecule inhibitors, these molecular glues, including RMC-6236, are non-covalent Ras-on inhibitors, however, they target multiple oncogenic Ras-variants as well as Ras-wildtype and different Ras-isoforms (Figure 8F) (Cregg et al. 2025). Overall, formation of these tri-complexes results in blockage of effector recruitment as CypA occupies the effector binding site on Ras (Holderfield et al. 2024; Schulze et al. 2023). Notably, our research showed that CypA is targeted by lysine acetylation, impairing its catalytic activity and interaction with cyclosporin A, suggesting that the metabolic state of cells targeted by these molecular glues might interfere with their therapeutic potency (Lammers et al. 2010). Along this line, therapeutic strategies might also include targeting the interaction sites of Ras effectors and/or regulators. Next to the development of protein binders or small-molecule compounds binding the effector to block interaction with Ras, as described for PI3 kinase, it was recently shown that interfacial compounds or proteins can also stabilize the complex between Ras and effector or regulator (Klebba et al. 2025). An example for the latter is the fungal toxin brefeldin A, which binds the complex between Arf1 and the Sec7 GEF-domain, stabilizing a GDP-bound conformation that represents the pre-catalysis state before nucleotide dissociation (Mossessova et al. 2003). Based on available crystal structures on Ras-proteins, small-molecule PROTACs (proteolysis targeting chimeras) were developed as heterobifunctional degraders recruiting E3 ubiquitin ligases, such as van Hippel-Lindau (VL) or Cereblon, to mutated K-Ras (Bond et al. 2020; Inano et al. 2025; Zeng et al. 2020). The recent progress in protein design also resulted in the development of the first isoform-specific and potent Ras-binders that might also have therapeutic applications (Zhang et al. 2025a). These isoform-specific binders might also be used for therapeutic strategies using bioPROTACs, such as recently described for tripartite motif-containing protein 21 (TRIM21) and its applications with Trim-Away, and other E3 ligases (Clift et al. 2018; Clift et al. 2019). TRIM21 is a protein encompassing an N-terminal RING E3 ligase domain, followed by a coiled-coil domain needed for dimerization and a C-terminal PRY-SPRY domain for binding to antibodies' constant Fc-region. Several reports described that replacement of the C-terminal PRY-SPRY-domain in TRIM21 by any type of recruitment domain can be used to target a protein for proteolysis in the proteasome. Future studies will reveal whether this can be extended to also target oncogenic variants of Ras, effectors and/or regulators thereof for therapeutic interventions.

18 Conclusions

Research on the Ras-family has seen tremendous progress in the last decades due to developments in structural biology, systemic biology, synthetic biology and computational approaches including protein structure prediction and protein design. Further studies on formation of condensates by liquid-liquid phase separation will show their impact on Ras-signaling and on the potency of therapeutic compounds. Here, we showed that PTMs are essential components for Ras-signaling. They modulate the activities of GEFs, GAPs and solubilization factors, they modulate interactions of Ras-proteins with other proteins as well as lipids, they regulate protein turnover and they affect protein conformations by acting as allosteric regulators. Moreover, PTMs are mainly responsible for molecular pattern formation and formation of molecular gradients in Ras-signaling, necessary to establish a directionality in signal transduction. To this end, PTMs are essential for recruitment of proteins to spatially organize Ras-signaling and to temporally organize it by establishing dynamic cycles of modifications, exemplified by kinases-phosphatases or acyltransferases-deacylases/thioesterases. Ras-signaling is targeted by a plethora of bacterial virulence factors, many of which also post-translationally modify Ras-proteins to achieve an efficient infection. While long regarded as almost undruggable, recent examples show a huge success in the development of small molecules and protein binders with a high degree of potency and selectivity towards specific Ras-isoforms suitable for therapeutic interventions. Future studies including novel tools of artificial intelligence and protein design, development of PROTACs and of molecular glues open exciting avenues to further understand Ras physiology, pathophysiology and to develop strategies to fight Ras-driven cancers and RASopathies.

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Use of Large Language Models, AI and Machine Learning Tools: The protein structure prediction server Protenix was used to predict the complex structure of PDE6δ and the drug deltaflexin3 (https://protenix-server.com).

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