Review

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The Ras switch in structural and historical perspective

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Abstract: Since its discovery as an oncogene more than 40 years ago, Ras has been and still is in the focus of many academic and pharmaceutical labs around the world. A huge amount of work has accumulated on its biology. However, many questions about the role of the different Ras isoforms in health and disease still exist and a full understanding will require more intensive work in the future. Here we try to survey some of the structural findings in a historical perspective and how it has influenced our understanding of structure-function and mechanistic relationships of Ras and its interactions. The structures show that Ras is a stable molecular machine that uses the dynamics of its switch regions for the interaction with all regulators and effectors. This conformational flexibility has been used to create small molecule drug candidates against this important oncoprotein.

Keywords: effector; GAP; GEF; Ras; switch regions.

Introduction: the structure of Ras

Since the first structure of Ras was presented in 1988/89 a lot of structural information has been accumulated. This has led to ~250 different structural records in the Protein Data Base (PDB), which are summarized in Table 1. They constitute an overwhelming source of structural and mechanistic information. Here we present a short history of milestones along the way which is not meant to be comprehensive but rather tries to highlight the ups and downs along the path to the now familiar picture of a small G protein which functions as a molecular switch, the activity of which is regulated by GEFs and GAPs. Structural findings on Ras have substantially influenced the biology of Ras but also those of other small and large G proteins. The history of these findings is presented by the timeline diagram in Figure 1. The complex biology of Ras has been summarized previously in a plethora of reviews focusing on different aspects of the Ras protein(s) (see Malumbres and Barbacid, 2003; Cox and Der, 2010; Ahearn et al., 2011; Shimanshu et al., 2017).

The history of the Ras structure started when Gay and Walker, by comparing the first 37 residues of the H-Ras protein, identified a high homology with the β-subunit of the mitochondrial and bacterial ATP synthases (Gay and Walker, 1983). This region of the protein contains what has been described as the Walker A motif present in various nucleotide binding proteins and is now more commonly called the P-loop, with the conserved sequence motif GxxxxGKS/T (Saraste et al., 1990). As residues in ATP synthase, myosin and nucleotide kinases were believed to be involved in nucleotide binding, various authors suggested that the Ras protein may also bind nucleotides (Walker et al., 1982; Wirenga and Hol, 1983). Later, it has been established that Ras proteins bind guanine nucleotides and seem to have no affinity to ADP or ATP. Leberman and Egner (1984) compared the sequence of various GTP-binding proteins and identified a remarkably extensive homology, in particular between Ras and the protein synthesis elongation factor EF-Tu. Correspondingly, structural models of Ras based on the preliminary structures of EF-Tu were described (Jurnak, 1985; McCormick et al., 1985).

The first (correct) three-dimensional structure then showed that the Ras protein indeed has the same fold as one of the domains of EF-Tu (Pai et al., 1989) and is not, as proposed earlier, different from EF-Tu (deVos et al., 1988). Almost all these and later structural studies used a truncated version of H-Ras (residues 1-166, or occasionally 1-171), and much later of other Ras isoforms, as it was found that recombinant full-length protein was proteolysed at the C-terminal end suggesting that these residues were flexible. A nuclear magnetic resonance (NMR) study of full-length Ras showed that residues beyond 172 could not be assigned because they are conformationally averaged, confirming earlier observations on the flexibility of the

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Table 1: Released structures of H-, N- and K-Ras.

Ras type	Small mol. ligand	Protein complex	Nucleotide	# Strc	PDB codes
H-Ras *Diff. mut.*		Effectors	Diff.	11	6amb, 1k8r, 4k81, 3ddc, 1he8, 2c5i, 1lfd, 3kud, 6axg, 4g3x, 4g0n
H-Ras *Diff. mut.*		SOS		6	1bkd, 1nnv, 1nvw, 1nvu, 1nvx, 1xd2
H-Ras *Diff. mut.*	Different compounds	SOS		35	4uru, 4urw, 4urx, 4us1, 4us2, 4us0, 4ury, 4urv, 4urz, 4nyi, 4nyj, 4nym, 5wfo, 5wfq, 5wfr, 5wfp, 6bvj, 6bvk, 6bvm, 6bvl, 6bvi, 6cuo, 6cup, 6cur, 6d5j, 6d5h, 6d5g, 6d5e, 6d59, 6d56, 6d5l, 6d55, 6d5v, 6d5m, 6d5w
H-Ras		P120GAP (RASA1)	GDP-AlF3		1wq1
H-Ras	YCN (Cyclen)		GNP		3l8y
H-Ras *T35S*	KOB (Kobe2601, 2-(2,4-dinitrophenyl)-N-(4-fluorophenyl) hydrazinecarbothioamide)		GNP		2lwi
H-Ras *T35S*	KBFM123 (3-oxidanyl-~{N}-[[(2~{R})-oxolan-2-yl]methyl] naphthalene-2-carboxamide		GNP		5zc6
H-Ras *Diff. mut.*	Soaking experiments		GNP	16	3rsl, 3rso, 3rs0, 3rs2, 3rs3, 3rs4, 3rs5, 3v4f, 4dlz, 4dly, 4dlw, 4dlv, 4dlu, 4dlt, 4dls, 4dlr
H-Ras *M72C*	Covalent inhibitors		GNP, GDP	3	5vbe, 5vbz, 5vbm
H-Ras *wt*	Monobody (NS1)		GDP		5e95
H-Ras *Wt G12P*	–	–	GCP	4	121p, 1jah, 1jai, 6q21
H-Ras *Diff. mut.*	–	–	GDP	16	1aa9, 1crp, 1crq, 1crr, 1ioz, 1lf5, 1pll, 1q21, 1zvq, 2cld, 2q21, 2quz, 2x1v, 3lo5, 4l9s, 4q21
H-Ras *Diff. mut.*	–	–	GNP	44	1lf0, 221p, 2lcf, 2rga, 2rgb, 2rgc, 2rgd, 2rge, 2rgg, 3i3s, 3k8y, 3k9l, 3k9n, 3kkm, 3kkn, 3l8z, 3lbh, 3lbi, 3lbn, 3oiu, 3oiv, 3oiw, 3rry, 3rrz, 3rs7, 3tgp, 421p, 4efl, 4efm, 4efn, 4l9w, 4rsg, 4xvq, 4xvr, 5b2z, 5b30, 5p21, 5wdo, 5wdp, 5wdq, 5x9s, 621p, 721p, 821p
H-Ras *Diff. mut.*	–	–	GTP	3	1plk, 1qra, 521p
H-Ras *wt*	–	–	mant-dGNP		1gnp
H-Ras *wt*	–	–	Nitrophenyl-Caged-GTP	3	1gnr, 1gnq, 1plj
H-Ras *wt, G12V*	–	–	DABP-GNP	2	1clu, 1rvd
K-Ras *wt*		Effector: A-Raf (RBD)	GNP	1	2mse
K-Ras *wt*	–	PDEdelta	GDP	2	5tar, 5tb5
K-Ras *T35S*			Nt-free (NMR)	2	2n42, 2n46
K-Ras *wt*		Darpins	GDP, GSP	4	5mlb, 5o2s, 5o2t, 5mla

Table 1 (continued)

Ras type	Small mol. ligand	Protein complex	Nucleotide	# Strc	PDB codes
K-Ras *G12D*	Cyclic inhibitory peptide Krpep-2d	–	GDP		5xco
K-Ras *G12C*	BOQ (ethyl	SOS (487aa)	Nt-free		6epp
C118S D126E	2-(aminomethyl)-5-~{tert}-butyl-				
T127S K128R	furan-3-carboxylate)				
	BPW (3-(4-chlorophenyl)propan-1-amine)	SOS (487aa)	Nt-free		6epo
	BQ2 (1-(3,4-dihydro-1~{H}-isoquinolin-2-yl)-2-oxidanyl-ethanone)	SOS (487aa)	Nt-free		6epn
	BQ5 ((1-phenyl-5,6-dihydro-4~{H}-cyclopenta[c]pyrazol-3-yl)methanamine)	SOS (487aa)	Nt-free		6epm
	none	SOS (487aa)	Nt-free		6epl
K-Ras *G12C*	8ZG (Quinazoline)		GDP		5v71
K-Ras *G12C*	91D (Quinazoline)		GDP		5v9l
K-Ras *G12C*	91G (Quinazoline)		GDP		5v9o
K-Ras *G12D*	9LI (2-(4,6-dichloro-2-methyl-1H-indol-3-yl)ethanamine)		GCP		4dst
K-Ras *wt*	9R5 ([(2~{R})-6-chloranyl-2,3-dihydro-1,4-benzodioxin-2-yl]methanamine)		GNP	2	5ocg, 5oct
K-Ras *wt*	9RK (~{N}-[[(3~{R})-2,3-dihydro-1,4-benzodioxin-3-yl]methyl]furan-2-carboxamide)		GNP		5oco
K-Ras *wt*	Benzamidine		GSP		4dso
K-Ras *Q61H*	CVK (4-(2,3-dihydro-1,4-benzodioxin-5-yl)-~{N}-[3-[(dimethylamino)methyl]phenyl]-2-methoxy-aniline)		GNP		6f76
K-Ras *Q61H*	D2Z (2-[4-[[(3~{R})-2,3-dihydro-1,4-benzodioxin-3-yl]methylcarbamoyl]phenoxy]ethyl-dimethyl-azanium)		GNP		6fa1
K-Ras *Q61H*	D2W (4-[2-(dimethylamino)ethoxy]-~{N}-[[(3~{R})-5-(6-methoxypyridin-2-yl)-2,3-dihydro-1,4-benzodioxin-3-yl]methyl]benzamide)		GNP		6fa2
K-Ras *Q61H*	D1Z (~{N}-[[(3~{R})-5-[5-[[3-[(dimethylamino)methyl]phenyl]amino]-6-methoxy-pyridin-2-yl]-2,3-dihydro-1,4-benzodioxin-3-yl]methyl]oxane-4-carboxamide)		GNP		6fa3
K-Ras *Q61H*	D1W (6-(2,3-dihydro-1,4-benzodioxin-5-yl)-~{N}-[4-[(dimethylamino)methyl]phenyl]-2-methoxy-pyridin-3-amine)		GNP		6fa4
K-Ras *Q61H*	F8T ([4-[[4-(3-methoxyphenyl)phenyl]amino]phenyl]methyl-dimethyl-azanium) (CH-1))		GNP		6gqw
K-Ras *Q61H*	F8N (~{N}-(3-imidazol-1-ylpropyl)-4-[[3-(3-methoxyphenyl)phenyl]methyl]oxane-4-carboxamide)		GNP		6gqt
K-Ras *Q61H*	F8K ([4-[[2-methoxy-4-(3-methoxyphenyl)phenyl]amino]phenyl]methyl-dimethyl-azanium) (CH-2))		GNP		6gqx

Table 1 (continued)

Ras type	Small mol. ligand	Protein complex	Nucleotide	# Strc	PDB codes
K-Ras *Q61H*	F6E ((6 ~ {S})-1-(1 ~ {H}-imidazol-4-ylcarbonyl)-6-[(4-phenylphenyl) methyl]-4-propyl-1,4-diazepan-5-one) (Ppin-1))		GNP		6gom
K-Ras *Q61H*	F8Q ([4-[[3-fluoranyl-2-methoxy-4-(3-methoxyphenyl)phenyl]amino] phenyl]methyl-dimethyl-azanium)		GNP		6gqy
K-Ras *C118S*	0QX (2-(1H-indol-3-ylmethyl)-1H-imidazo[4,5-c]pyridine)		GDP		4epv
K-Ras *C118S*	0QV ((4-hydroxypiperidin-1-yl) (1H-indol-3-yl)methanethione)		GDP		4epw
K-Ras *C118S*	0QW ((2-hydroxyphenyl)(pyrrolidin-1-yl)methanethione)		GDP		4ept
K-Ras *C118S G12V*	0QR (N-(6-aminopyridin-2-yl)-4-fluorobenzenesulfonamide)		GDP		4epx
K-Ras *Diff. mut.*	Different compounds	Nanodiscs	Diff.	5	2msc, 2msd, 6cch, 6cc9, 6ccx
K-Ras *Diff. mut.*	–	–	GCP	2	4dsn
K-Ras *wt*	–	–	GCP + GDP	1	5uk9
K-Ras *Diff. mut.*	–	–	GDP	16	2msc, 4dsu, 4l8g, 4l9s, 4ldj, 4lpk, 4lrw, 4obe, 4ql3, 4tq9, 4tqa, 4wa7, 5uqw, 5us4, 6asa, 6ase
K-Ras *Diff. mut.*	–	–	GNP	7	3gft, 4l9w, 5usj, 6god, 6goe, 6gof, 6gog
K-Ras *G12C*	Covalent inhibitors	–	Y9Z (SML-8-73-1)		4nmm
K-Ras *G12C C51S C80L C118S*	Covalent inhibitors		GDP	12	4luc, 4lyj, 4m22, 4m21, 4m1y, 4m1w, 4m1t, 4m1s, 4m1o, 4lyh, 4lyf, 4lv6
K-Ras *G12C C51S C80L C118S*	ARS-853		GDP		5f2e
K-Ras	–	R11.1.6	GNP	2	5ufe, 5ufq
K-Ras *G12V*	–	Miniprotein	Diff.	3	5wha, 5whb, 5wlb
N-Ras *wt*	–	–	GNP	1	5uhv

Figure 1: Timeline of important discoveries relating to the structure of Ras.

C-terminal end (Thapar et al., 2004). H-Ras (1-166) ('Ras' from now on, unless other forms were used) was crystallized in the GTP-bound form using GppNHp as the slowly hydrolyzing triphosphate analogue. It showed the typical α,β-fold of nucleotide binding proteins with six β-strands and five α-helices as shown in Figure 2 (see also the video in the online Supplementary Material). This arrangement of secondary structure elements is now commonly called the G domain (the Ras domain by some) and is basically conserved in all proteins of the Ras superfamily, in protein synthesis factors, in the α-subunit of heterotrimeric G proteins and in many other GTP-binding proteins such as MnmE, a protein involved in tRNA modification (Scrima and Wittinghofer, 2006). It is modified by insertions and additions or is part of multi-domain proteins as described before (Vetter and Wittinghofer, 2001).

The β,γ-phosphates are trapped in the P-loop such that the main chain hydrogens and the conserved lysine point towards the negative charges, an arrangement which has been called 'giant anion hole' during the structural analysis of adenylate kinase (Dreusicke and Schulz, 1986). Negative charge is further neutralized by the Mg^{2+} ion which forms a β,γ-bidentate complex with oxygen atoms from the β- and γ-phosphates. The guanine base is contacted by the highly conserved NKxD motif and the coordination explains the high specificity of the protein for guanine nucleotides. The affinity for ADP/ATP has been estimated to be in the order of millimolar, at least a million-fold weaker than the affinity for GDP/GTP, while GMP binding is equally weak (John et al., 1990). For a detailed description of structural features see the iBiology seminar (https://www.youtube.com/watch?v=AVKZtfQ-Nmg).

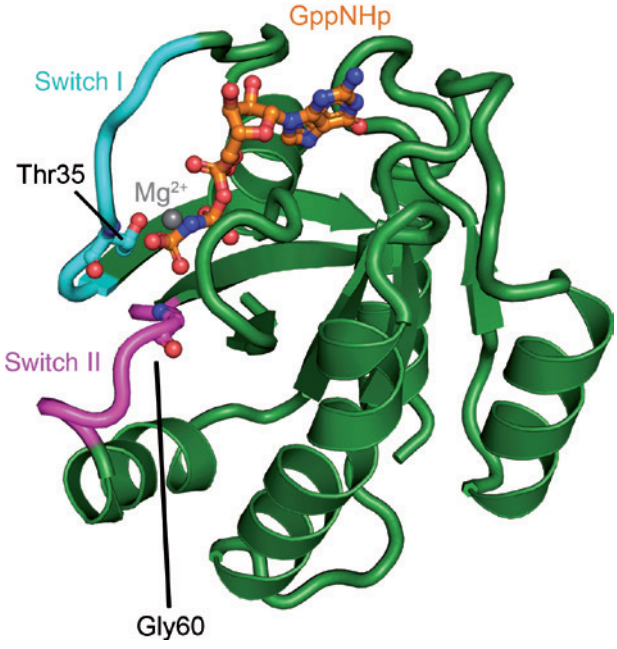

Figure 2: Ribbon diagram/cartoon of the structure of Ras in the triphosphate conformation.
Switch I is in cyan, switch II in magenta, with details of the anchoring residues Thr35 (switch I) and Gly60 (switch II). GppNHp (orange) is shown in atomic detail.

Although much of the mechanistic structural work has been done on H-Ras, K-Ras is by far the most important oncogene and accordingly a large number of structures have been solved in the course of drug development efforts (see below). The four isoforms (including the two splice variants of K-Ras) are identical in the first 86 residues comprising one side of the molecule which has been called the effector lobe as it interacts with GAP, GEF and effectors. The opposite half (the allosteric lobe), residues 87-166, is more diverse and shows a small number of differences. The C-terminal ends are more divergent, as the name hypervariable implies. Some biochemical properties are slightly different between the isoforms, i.e. the intrinsic GTPase, which may potentially be significant (Johnson et al., 2017). However, one has to consider the fact that most likely the switch-off in cells is mediated by the interaction with GAP. The structure of N-Ras in the triphosphate form has been solved (Johnson et al., 2017). The basic fold is obviously identical and only shows differences in the switch II region and in the remote part away from the active site. It is reasonable to assume that the differences in sequence influence the local conformation and the dynamics of the proteins, as shown by molecular dynamics simulations (Grant et al., 2009; Kapoor and Travesset, 2015). It is to remember however that the switch regions are highly dynamic and that the details of their conformation in crystal structures are influenced by the crystal packing.

Over the years of Ras history there have been conflicting reports on Ras being a dimer (see review by Shimanshu et al., 2017). Such reports were fueled by models, whereby the dimeric Raf would need to be activated by a dimeric Ras, or by the consistent observation that wildtype Ras is a suppressor of oncogenic Ras. *In vitro* measurements of the Ras G domain dimerization may have been deceived by the high concentrations used in some of these experiments such as NMR (Muratcioglu et al., 2015) or FTIR (Gueldenhaupt et al., 2012). A possible dimerization of the G domain should have been observed in the high concentration of a crystal, but different crystals of Ras show different crystal packings (i.e. compare PDB structures 1ctq and 3l8z). The most convincing report on the issue has shown that at least *in vitro* the fully modified full-length Ras is a monomer under a variety of concentrations in supported lipid bilayer membranes of various

compositions (Chung et al., 2017). This does not exclude that *in vivo* Ras may dimerize in the presence of other factors, as it has been found in higher order nanoclusters containing several molecules of the protein (reviewed by Zhou and Hancock, 2015).

The switch motifs of Ras

The early structural findings on EF-Tu (Jurnak, 1985; la Cour et al., 1985) and the high resolution structure of Ras (Milburn et al., 1990; Pai et al., 1990) together with the sequence homology of rapidly appearing new Ras-like genes such as *Rho* (Madaule et al., 1987), *Ypt/Rab1* (Gallwitz et al., 1983) and different genes coding for Gα proteins discovered earlier induced Bourne et al. to write two seminal reviews and to define the GTPase Switch Superfamily (Bourne et al., 1990, 1991). They defined five sequence motifs called G1 to G5 where G1 is the Walker A motif or P loop and G4 the NKxD motif. G2 and G3 are the major motifs involved in the switch function, and are parts of switch I and II (see Vetter and Wittinghofer, 2001; iBiology: https://www.youtube.com/watch?v=AVKZtfQ-Nmg).

One of the major questions concerning the function of Ras or any other G protein is the structural basis of the GDP-GTP transition from an inactive to an active conformation. The first structural demonstration of the switch

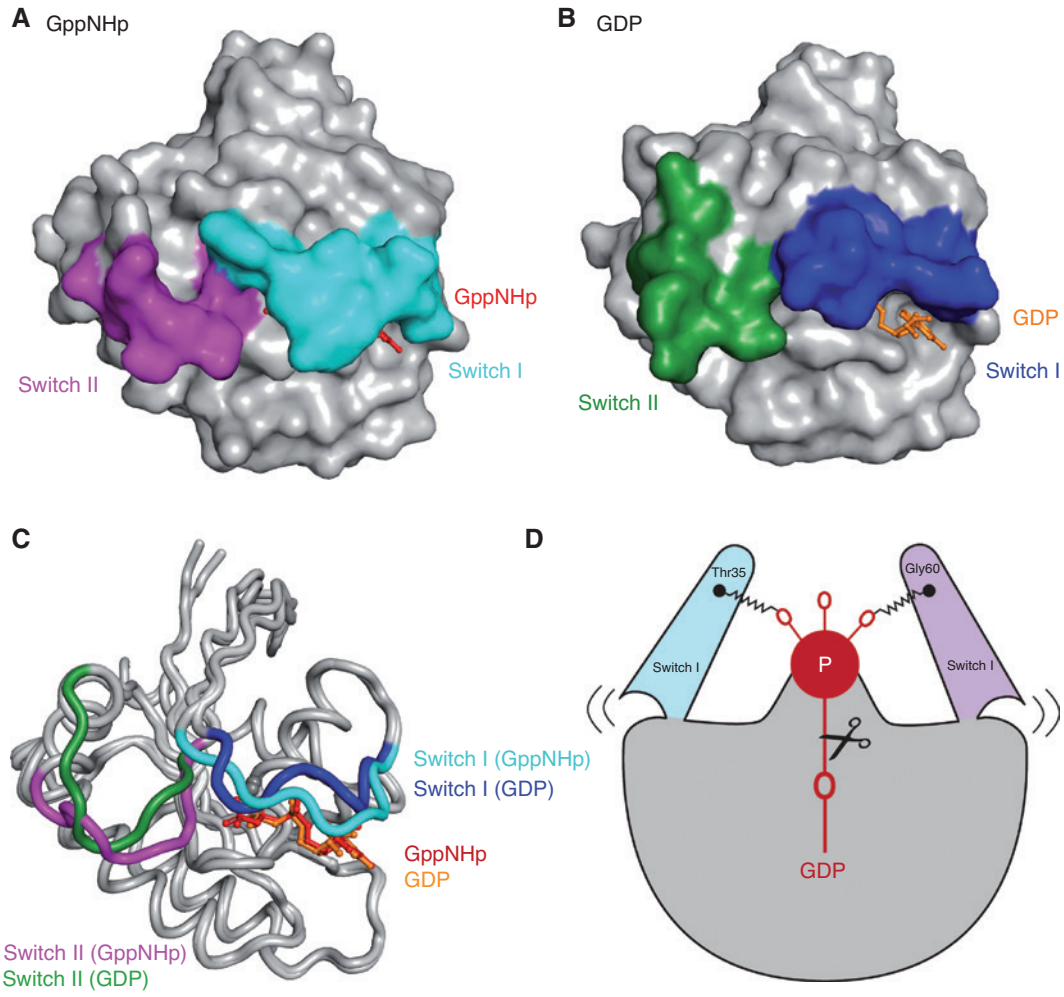

Figure 3: The conformational change.
Surface representation of H-Ras in GppNHp-bound state (A), showing switch I in blue and switch II in magenta with GppNHp shown in red (pdb 5p21). (B) GDP bound state, with switch I in blue, switch II in green and GDP in orange (pdb 4q21). (C) Worm plot of the conformational change with colors of the switches as in A, B. The overall Ras structure is similar with low r.m.s.d., while the switch regions show profound differences. (D) Schematic model of the switch mechanism. Ras acts as loaded spring, that releases switch I and II in different conformations, once the gamma-phosphate is hydrolyzed, adapted from Vetter and Wittinghofer (2001). The conformational switch is shown as a video in the online Supplementary Material.

reaction of Ras, and the definition of the switch regions, was published by Milburn et al. (1990), shortly after the Ras·GppNHp structure had been published. They showed that the basic fold and most of the structural elements were identical and that only two regions of the protein change their conformation. These regions of no more than ten residues (size depending on crystal context) were called switch I and switch II by the authors (Figure 3A–C) (for a video of the conformational change, see the online Supplementary Material). The underlying mechanism is based on the interaction of Thr35 in switch I (called G2 by Bourne et al.) and Gly60 in switch II (G3 motif). These residues are totally conserved in G binding proteins and make main chain hydrogen bonds to the γ-phosphate (Figure 3D). These interactions are lost upon GTP hydrolysis allowing these regions to adopt a different conformation. This structural change that has been called the loaded spring mechanism (Figure 3D) (Vetter and Wittinghofer, 2001). The dynamic behavior of the G domain fold was demonstrated in the first NMR structure of Ras in the triphosphate form which showed a pronounced polysterism (Ito et al., 1997). This is confirmed by comparing many different structures, which show a greater flexibility in the GDP-bound form as compared to the GTP-bound structure (Vetter and Wittinghofer, 2001).

A direct demonstration of the conformational change occurring in a crystal was achieved by using a crystal that contained caged-GTP bound to Ras (which has the cage group on the γ-phosphate and is non-hydrolysable) rather than GTP or a slowly hydrolyzing GTP analogue. After photolytic cleavage of the cage group the resulting GTP was allowed to hydrolyze to GDP, allowing the comparison between the two states. The conformational states in switch I could be followed by using the Laue technique of multi-wavelength crystallography (Schlichting et al., 1990). As crystal quality was not optimal, various regions of the protein were not well resolved. A much more detailed and higher resolution study of the reaction in the crystal was later performed by Scheidig et al. (1999). One of the intriguing outcomes of these studies was the detailed analysis of the pre-hydrolysis GTP-bound state of Ras, which showed that the non-hydrolyzable (or rather slowly hydrolyzing) GppNHp is a good mimic of GTP in the active site of Ras, at least as far as the position of the phosphates is concerned.

Mutants of Ras

Ras is most likely the most heavily mutated protein in biochemical history. In fact, in a recent mega-project of saturation mutagenesis every residue has been mutated to every other possible amino acids (Bandaru et al., 2017). This study shows that in the context of its signaling machinery, Ras is sensitive to mutational alteration across the sequence space. Mutational hotspots which lead to activation are similar to those that have been observed in the context of human diseases.

The most interesting aspect of Ras biology is it being the most frequently mutated oncogene (see Prior et al., 2012, for a complete list of mutations). The most commonly mutated residues found in cancer are Gly12, Gln61 and Gly13 (in that order of frequency) and a few others much less frequent ones. It has in fact been shown that every mutation of Gly12 (except Pro) (Seeburg et al., 1984) and every mutation of Gln61 (Der et al., 1986) are transforming. The most obvious defect is the greatly reduced GAP mediated GTP hydrolysis, although the extent of reduced activity is possibly different for each mutant and may lead to differences in the extent by which these mutants occur in the GTP-bound state inside the cell. A few of the oncogenic mutations have been investigated structurally but no significant change could be demonstrated. The P-loop, in the presence of G nucleotides seems to be a very stable structural motif and mutations of Gly12 have no significant impact, while Gln61 and switch II are highly mobile both in the wildtype and the mutant situation (Krengel et al., 1990; Ito et al., 1997).

It is counterintuitive that the two residues that contact the γ-phosphate, Thr35 and Gly60 in Ras, are almost totally conserved in many G domain proteins, even though their interactions are mediated by main chain hydrogen bonds. This finding has stimulated a number of mutational studies. The most conservative change is the mutation of Thr35 to Ser. This should in principle allow the interaction of the hydroxyl side chain to interact with the Mg^{2+} ion and the main chain nitrogen to make a H-bond to the γ-phosphate, as in wildtype Ras. However, the properties of Ras(T35S) are remarkably different from wildtype (Spoerner et al., 2001). The switch regions become highly dynamic and the affinity towards effectors such as Raf-RBD is strongly reduced. It is even more reduced when Thr35 is replaced by Ala, which would be unable to make an interaction with Mg^{2+}. These results suggest that the methyl group of Thr35 significantly stabilizes the switch I region.

The mutation of the equally well (or totally) conserved Gly60 at the N-terminal end of switch II is also very sensitive to mutation. Even the sterically most conservative G60A mutation alters the properties of Ras (and other G proteins) dramatically. While the nucleotide binding ability is not compromised significantly, the GTPase is

greatly reduced. The biological activity of oncogenic v-Ras is severely compromised by the G60A mutation suggesting that it acts as a dominant negative form of the protein (Hwang et al., 1996). The structure of the G60A mutant (Ford et al., 2005) shows a remarkable conformational change in the structure of the switch regions, which resemble the conformation of Ras in complex with the exchange factor SOS. As SOS binds to Ras(G60A) but does not catalyze nucleotide exchange, the authors postulate that the two proteins form an unproductive tight complex that prevents SOS from activating normal Ras. In any case a methyl group on residue 60 severely destabilizes the switch regions, even though Gly60 shows a normal phi-psi angle in the Ramachandran plot, at least in the GTP-bound conformation.

It has been shown by ^{31}P-NMR that Ras·GppNHp shows (at least) two conformational states, state 1 and state 2, where two peaks are observed for the beta and gamma-phosphates (originally wrongly assigned to the α- and β-phosphates) (Geyer et al., 1996; Spoerner et al., 2001). State 2 is believed to be in the well-defined Raf effector-bound conformation (discussed below), whereas state 1 could originally not be defined structurally. The T35S and T35A mutant ^{31}P-NMR spectra show only state 1, and the X-ray crystallographic structure shows a completely undefined electron density for switch I, indicating a highly flexible conformation (Spoerner et al., 2001). A later structure obtained from a different space group showed Ras(T35S) to occur in two different conformations (Shima et al., 2010) and other mutants also crystallized in state 1, confirming the NMR data. In both conformations, Ser35 is no longer coordinated to the Mg ion and Gly60 contacts the γ-phosphate in only one of them. Finally, using crystals of Ras(T35S) as seeds, Muraoka et al. (2012) were able to get crystals of wt Ras in state 1: The structure showed loss of both Thr35 and Gly60 interactions, and the open conformations created two new pockets apparently suitable for drug targeting the inactive state (see below). Similar findings have also been reported for other Ras-like proteins such as M-Ras (Shima et al., 2010). This underscores the important role of the methyl group of Thr35 for stability of the active conformation of switch I and interaction with effector. Although conclusive structural explanations for the conservation are not available, these studies show that the dynamic properties of the switch regions are controlled by the two main chain interactions, and are severely perturbed by the absence (T35S) or the presence (G60A) of a methyl group.

Apart from the somatic mutations found in cancers, germline mutations have been found in all three Ras isoforms. They lead to developmental diseases such as Noonan or Costello syndromes. They are summarized as Rasopathies and include mutations in almost all components of the Ras-Map kinase pathway. Mutations in H-Ras, found in Costello syndrome, affect residues that are also mutated in cancer (Aoki et al., 2005), whereas the mutations in Noonan syndrome are found in K-Ras and target residues differ from those found in cancer (Schubbert et al., 2006). Noonan mutations are distributed across the K-Ras sequence and include alterations such as V14I, Q22E, P34L, T58I, G60R, E153V and F156L, to name just a few. It appears that oncogenic mutations in H-Ras are tolerated during human development to a postnatal stage, but not in K-Ras, confirming the knockout studies of the three Ras genes in mice. Not much structural information is available for the K-Ras mutants, but the biochemical analysis shows that they affect different aspects of Ras function and lead to increased nucleotide release or (somewhat) decreased GAP-mediated GTP hydrolysis, in combination with reduced affinity to effectors (Gremer et al., 2011).

Interaction with RasGAPs

The GTPase reaction of Ras and of many other small and large G proteins (for review on regulatory proteins see Bos et al., 2007; Cherfils and Zeghouf, 2013; Shimanshu et al., 2017) is very slow, in the order of 10^{-4} s^{-1}. By analyzing the amount of GDP- vs. GTP-bound Ras, Trahey and McCormick (1987) showed very elegantly that the GTPase *in vivo* should be faster than what is measured *in vitro* with pure protein, leading them to postulate a GTPase activating Protein (GAP). Shortly thereafter such a protein, later called p120-GAP and in a revised nomenclature RASA1, was isolated and defined molecularly (Trahey et al., 1988; Vogel et al., 1988). This protein does accelerate the GTPase of normal, but not of an oncogenic version of Ras (under the conditions used). As shown later, the acceleration is in fact 10^5-fold (Gideon et al., 1992). The causal gene for the benign tumor neurofibromatosis type 1 (*NF1*) was shown to code for a very large protein (2818 residues) with even today mostly unknown functional domains. Surprisingly (at the time), it contains a domain of 333 residues highly homologous to the catalytic domain of similar length in p120GAP. Today more than ten proteins with a RasGAP domain (called GRD: GAP-related domain) have been identified, some of which are specific for Ras, while others show very interesting dual specificity for Ras and Rap (see e.g. Sot et al., 2010).

A large number of papers on the mechanism of GTP hydrolysis using both experimental and theoretical approaches have been published over the last 30 years (Scheffzek and Ahmadian, 2005). The reaction induces

a reversal of stereochemistry at the γ-phosphate (Feuerstein et al., 1989). While ATP or GTP hydrolysis in water follows a dissociative mechanism, this is much less clear and highly disputed for the intrinsic Ras-mediated reaction, where one cannot introduce other leaving groups or use other nucleophiles without blocking the reaction (for parts of this discussion, see Maegley et al., 1996; Florian and Warshel, 1998; Wittinghofer, 2006). There is a water molecule close to the γ-phosphate in most structures which could serve as the nucleophile. How it might be activated by a second water molecule or by substrate-assisted catalysis, and how the transition state is stabilized remains to be agreed upon (for a recent contribution, which favors solvent-assisted catalysis, see Calixto et al., 2019). The structure and various other evidences suggested however that Gln61, being close to the active site could be involved in the reaction although not as a general base as its pKa value is very low (actually negative). In any case it appears that the GAP-mediated reaction might be the one to scrutinize as the physiologically more relevant reaction.

The biochemistry of the GAP-mediated reaction showed that the GTPase is stimulated 10^5-fold by GAP (Gideon et al., 1992) and that a rate-limiting step observed by fluorescence using mant-GTP is stimulated by GAP. Whether this was a conformational step preceding the cleavage, the chemical step or something else could not be resolved by this technique (Neal et al., 1990; Rensland et al., 1991). Mutational analysis of conserved residues and NF1 patient mutations suggested various residues to be important for the reaction. Previous investigations on phosphoryl transfer reactions had shown that arginine residues might be important. In adenylate kinase, which is one of the fastest P-transfer enzymes known (turnover is around 1000 s^{-1}), the movement of two Arg residues into the active site is the catalytic step (Müller et al., 1996). In Gα proteins an arginine in switch I is ADP-ribosylated by cholera toxin, and this inactivates the GTPase reaction. From the conserved arginines in the GAP domain of p120-GAP or NF1, one was indeed found to severely affect catalysis without much effect on binding. A step forward in mechanistic understanding was the finding that aluminum fluoride (AlFx) can bind to Ras·GDP in the presence of stoichiometric amounts of the GAP domain (Mittal et al., 1996). AlFx complexes have been found to be located in the active site of many P-transfer enzymes including Gα proteins, where they mimic the transition state of the reaction (in the presence of ADP/GDP). Mutating the critical Arg residue in GAP or using the oncogenic mutants of Ras

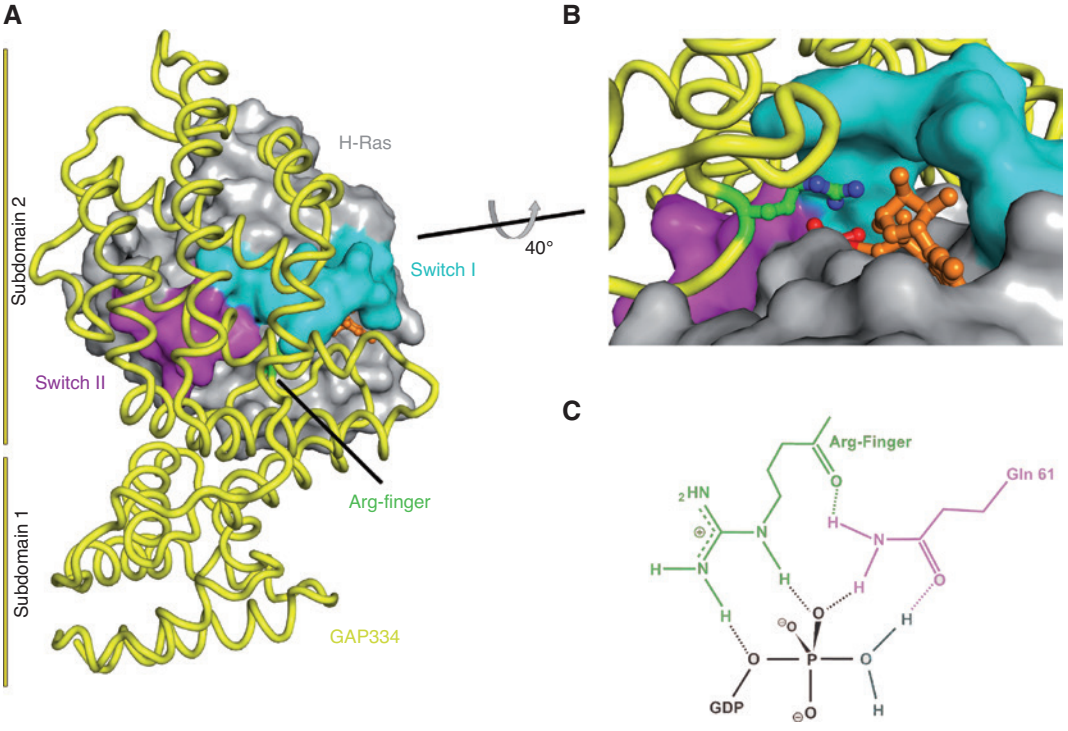

Figure 4: The GAP interaction.
(A) Ras (gray) surface showing switch I in cyan and switch II in magenta as above. The GAP domain of p120GAP is shown as yellow ribbon with the Arg-finger (green) pointing into the active site of Ras (pdb 1wq1). (B) Blow-up of the active site. (C) Model of the transition state deduced from the structure, where the attacking water is stabilized by Gln61 of Ras (purple). The arginine finger of GAP (green) stabilizes the position of Gln61 and neutralizes negative charges.

blocks the formation of the Ras·GDP·AlFx·GAP complex. All of this showed that Ras is an incomplete enzyme, which requires the presence of GAP and that an Arg residue of GAP, later called the arginine finger, is required for catalysis (Ahmadian et al., 1997) (Figure 4).

The structure of the GAP domain (GAP-334) of p120-GAP and later of NF1 (NF1-333) showed an all-helical protein consisting of two subdomains and explained some of the features of previous mutational studies, but did not reveal the mechanism of GTP hydrolysis (Scheffzek et al., 1996, 1998). The structure of the complex of RasGAP (using GAP334) with Ras·GDP and AlFx revealed the mechanistic aspects of the interaction (Scheffzek et al., 1997). It showed how Ras interacts with GAP via the switch regions forming an extended interface, very surprisingly considering the low affinity between GAP-334 and Ras (in the 10–20 μM range) (Figure 4A). Gln61, which was highly flexible in the structure of Ras alone (high B-factor) was stably fixed close to the nucleophilic water and the mimic of the γ-phosphate. Its position in turn is stabilized by the arginine finger (Figure 4B), which also interacts with negative charges of the phosphates. AlFx forms a trigonal plane, which was originally interpreted as neutral AlF_3, but considering recent results on fluorometallates and the affinity of the P-loop for negatives charges, it is more likely an MgF_3^- (Jin et al., 2017). The structure suggests that Arg stabilizes the transition state by neutralizing negative charges and thus makes an associative mechanism with a tighter transition state more likely, although this is not universally accepted from biochemical studies (see Nixon et al., 1995; Du et al., 2004). It also shows that Gln61 has a direct role in catalysis by stabilizing the position of the nucleophilic water relative to the γ-phosphate (Figure 4C).

Time-resolved Fourier transform infrared (FTIR) spectrocopy studies later showed that for the GAP-stimulated reaction, the reorientation of the Arg finger determines the rate of the cleavage reaction to produce Ras·GDP·Pi·GAP. This state is capable for the reverse reaction to produce GTP. Finally, the release of Pi to the Ras·GDP state is the rate-limiting step of catalysis (Kötting et al., 2006). Biochemical studies show that the arginine finger mutant binds to the Ras·GppNHp state with wildtype affinity but does not form the transition state mimic with AlFx (Mittal et al., 1996; Gremer et al., 2008). These findings are supported by two structures from the Rho-RhoGAP system which appeared simultaneously with the Ras-RasGAP structure, where both the ground state Rho-GppNHp complex and the transition state GDP-AlFx complexes were solved. They showed that the catalytic Arg is oriented towards the active site only in the transition state (Rittinger et al., 1997a,b).

The most important results of the structure of the Ras-RasGAP complex was to show that the steric requirements of the active site in the transition state do not allow a residue change from glycine to any other amino acid. Even a Gly12-Ala mutation, the sterically smallest possible replacement of the Gly12 position, and even more drastic oncogenic mutants such as G12V, G12D, G12C, G12R would interfere with the position of atoms in the active site in the transition (but not the ground) state (Scheffzek et al., 1997). As Gln61 directly participates in the chemistry of the reaction, it cannot be replaced by any other residue, and even Asn61 would not be able to position its carboxamide side chain properly, relative to the nucleophilic water. This also explains the findings that the oncogenic mutants bind to GAP with at least wildtype affinity (Gln 61 mutants bind even better) but do not allow catalysis to happen, as the arginine residue moves into the active site only in the transition state (Scheffzek et al., 1997; Gremer et al., 2008).

Interaction with Ras GEFs

The sub nanomolar affinity of guanine nucleotides to Ras (Neal et al., 1988; John et al., 1990) suggested that, in analogy to heterotrimeric G proteins and EF-Tu, there should be an exchange factor, which increases the dissociation rate. Such factors, which were originally called GNRPs (for guanine nucleotide releasing proteins) are now called GEF (for guanine nucleotide exchange factors) and have been identified for almost all small G proteins of the Ras superfamily (for reviews see Bos et al., 2007; Cherfils and Zeghouf, 2013). Genetic studies in yeast *Saccharomyces cerevisiae* suggested that the gene *CDC25* is upstream of the Ras pathway and it was shown that the C-terminal fragment indeed functions as a Ras GEF (Crechet et al., 1990). A large number of mammalian proteins were identified, which contain what is now called the Cdc25 homology domain that act as GEFs for the proteins of the Ras subfamily such as Ras, Ral, R-Ras and Rap1/2.

The most well-known Ras-specific GEFs, Ras GEFs, are $Cdc25^{Mm}$ (Martegani et al., 1992); more commonly called Ras-GRF1 (Shou et al., 1992) and SOS1 – named after the son-of-sevenless gene in the sevenless eye development pathway of *Drosophila* (Chardin et al., 1993). These and other Ras GEFs contain a number of domains, one of which is the Cdc25 domain which was shown to be active on Ras *in vitro* (e.g. Mistou et al., 1992). A detailed kinetic study using various mant-labeled nucleotides showed that the mechanism of exchange involves the formation of

a transient ternary Ras·GXP·GEF complex (Lenzen et al., 1998) and the transient but stable (in the absence of nucleotide) Ras·GEF complex as proposed earlier by various authors (Figure 5A). Such a ternary complex was demonstrated spectroscopically for the Ran·RCC1 system (Klebe et al., 1995). For Ran and Ras, and for most other small G proteins, the ternary complex is unstable and reduces the affinity of nucleotide by various orders of magnitude, just as the rate of dissociation of nucleotide is increased by a similar order of magnitude (Klebe et al., 1995). The data also suggested a model whereby Ras and Cdc25 form a ternary complex, which isomerizes from a tightly bound nucleotide to a weakly bound form, from which the nucleotide is released to form a tight binary complex. In the reverse reaction, Ras, via association and isomerization, returns to a state with tightly bound nucleotide. The maximum rate of release is 10^5-fold faster with $Cdc25^{Mm}$, but much slower for SOS-cat (see below). It should be stressed that Ras GEFs (and most other, if not all other GEFs) are merely catalysts, which work in any direction, and this is dictated by the relative affinities of the competing nucleotides and their relative concentration (Guo et al., 2005).

The structure and mechanism of action of SOS was published in a series of papers from the groups of Kuriyan and Bar-Sagi. The first structure was from a stable nucleotide-free complex between a ~500 residue C-terminal fragment and H-Ras(1-166) (Boriack-Sjodin et al., 1998) (Figure 5B). The active fragment, called SOS-cat, is an all-helical protein, which severely distorts the phosphate region of the nucleotide binding site. A helical hairpin is responsible for opening the switch I region and positioning switch II residues such that they distort the active site. One of these is Ala59, which now occupies the position of the Mg^{2+} ion and this effect alone would increase the rate of release around 10^3-fold (for a video featuring details

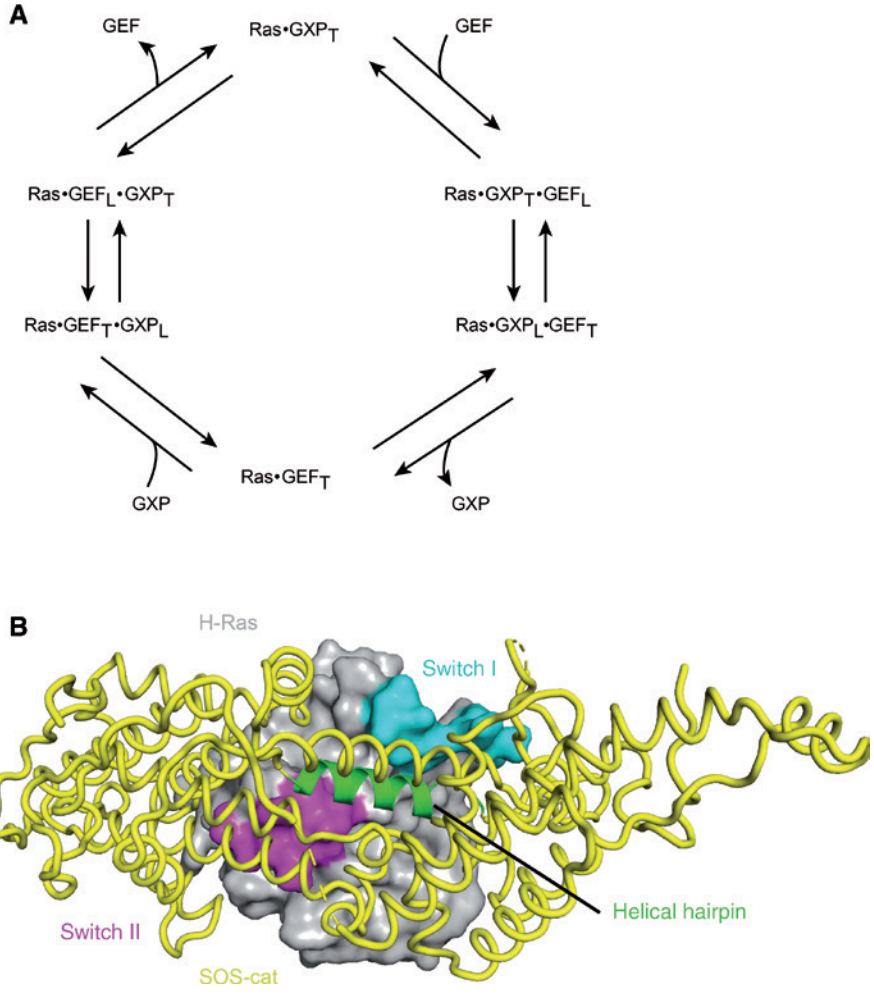

Figure 5: The GEF interaction.
(A) Kinetic model of the GEF mechanism, showing the minimum number of transient intermediates. The reaction involves conformational changes between tight (T) or loose (L) binding states of either nucleotide or GEF. (B) The structure of the Ras·SOS-cat complex, Ras coloring and orientation as above, with SOS-cat in yellow and its catalytic helix in green (pdb 1nvw).

of the GEF reaction, see the online Supplementary Material). The second important interaction is to move Glu62 from switch II into a position to interact with the lysine residue of the P-loop, which thus stabilizes the nucleotide free form of the complex. It turned out that Glu62 in many other small G proteins is conserved and functions to mediate an interaction with the P-loop lysine in their complex with cognate GEF (Gasper et al., 2008). The overall effect of SOS is to disrupt the interactions of the phosphate but not the base binding region, which suggests a mechanism for nucleotides being released in a phosphate-first/base-last mechanism and its reversal in the binding reaction. The data also show that the intrinsic (albeit slow) exchange reaction of Ras and other small G proteins might also use residues of switch II such as Ala59 and Glu62 to stabilize the nucleotide-free intermediate, and that GEFs just accelerate this mechanism.

A surprising finding came when Margarit et al. investigated the interaction of a Ras mutant (A59G) with SOS-cat (Margarit et al., 2003). It had been shown earlier that many GEFs for the Ras subfamily contain in addition to the Cdc25 domain a domain/region of ~200 residues called Ras exchange motif (REM). This REM domain/motif was shown to bind a second molecule of Ras in a site distal to where the nucleotide free Ras is bound. This second site contains Ras in the GTP-bound form and such a complex can be demonstrated also for wt Ras, both in the crystal and in solution. The second binding site behaves like an effector binding site in that it seems to specifically require Ras in the GTP-bound conformation. The authors show by measuring the kinetics of nucleotide exchange that SOS operates by feedback activation whereby the result of the exchange reaction, Ras·GTP, is an activator of GEF.

The N-terminal to the SOS-cat region is a DH-PH tandem unit required for activation of a Rho protein. Structural analysis of a DH-PH-cat structure of SOS shows that the DH-PH unit blocks the allosteric site and would thus not allow for high GEF activity (Sondermann et al., 2004). Hence, full activity of SOS requires further interaction with the membrane for proper localization of the lipophilic C-terminal ends of both Ras molecules. The autoinhibitory role of the DH-PH tandem is relieved by features of the membrane such as the concentration of PIP2 and the density of Ras molecules (Gureasko et al., 2008).

Ras-GRP (for guanine nucleotide releasing protein) is, other than SOS, a tissue specific GEF for Ras, which is important for the development of white blood cells. It consists of an REM and CDC25 domain in addition to an EF hand and a C1 domain. Iwig et al. (2013) report on a structure, which shows that this protein is in a dimeric auto-inhibited conformation. It is suggested that it may require both Ca^{2+} and diacylglycerol binding for full activation.

Interaction with effectors

After the discovery of GAP it was proposed that GAP might also be an effector (McCormick, 1990). This was in part caused by the longtime failure to identify a true effector. Part of the problem was due to pulldown experiments using the Y-259 antibody, which does in fact inhibit effector binding. The first effector to be identified by several groups in 1993 (see reviews by Malumbres and Barbacid, 2003; Cox and Der, 2010) was the protein kinase c-Raf which had already previously been assumed to be involved in the Ras pathway. Deletion constructs quickly identified a region in the molecule of around 80 resides as the Ras binding domain (RBD). The first structure of this domain in complex with Rap1 (which happened to form better crystals than Ras) was solved soon thereafter (Nassar et al., 1995) Rap1 is a close homologue of Ras and binds to the RBD. Structure guided mutations introduced two residues, which converted Rap1 to 'Raps', a protein which in terms of effector binding looks and behaves like Ras (in terms of affinity) (Nassar et al., 1996). The structure showed that RBD forms a β-sandwich ubiquitin-like-fold (Figure 6). The complex uses a number of mostly negatively charged residues from switch I for the interaction with positive ones from RBD. Interaction with a switch region would, as anticipated, explain the finding that the binding is 1000-fold tighter for the GTP- than the GDP-bound form of Ras (Herrmann et al., 1995). The two proteins form a continuous β-sheet in the interface by using B1-B2 from RBD and β2-β3 from Ras.

Raf kinase contains, in addition to the kinase domain, a conserved region, which is a cysteine-rich domain (CRD). It has been shown that it also binds to Ras and is required for activation of the kinase. *In vitro* the affinity of CRD to Ras is very low (approximately 200 μM) but is increased ten-fold when Ras is farnesylated (Thapar et al., 2004). The NMR structure of the CRD was solved by Mott et al. (1996). To delineate the second binding site, full-length Ras (a mixture of 1-189 and 1-185 proteins) farnesylated on Cys185 was investigated by NMR and titrated with RafCRD (135-186, as GST-fusion). The interaction was followed by ^{1}H-^{15}N HSQC chemical shift data and showed perturbation on residues N-terminal to switch I, such as Asn26 and Glu30 (Thapar et al., 2004). These changes were not observed using non-farnesylated Ras supporting the notion that farnesylation is important for the interaction.

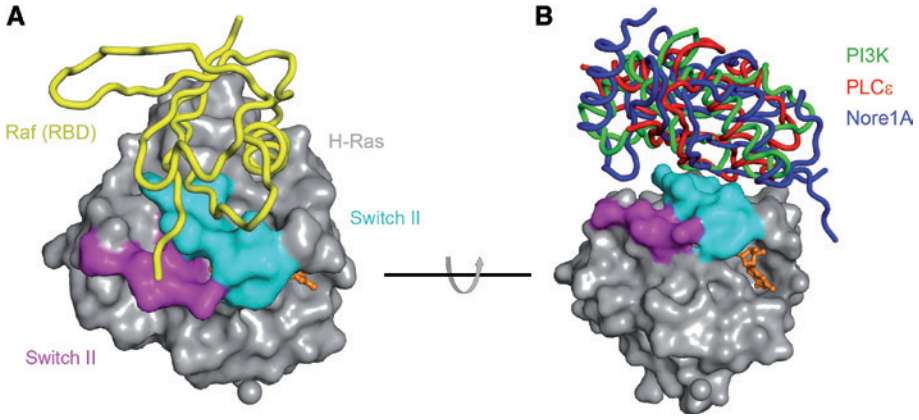

Figure 6: Effector interaction.
(A) Ras in complex with RafRBD, in yellow ribbon, Ras coloring and orientation as above (pdb 4g0n). (B) Overlay of Ras binding domains (RBD) and Ras association domains (RA) of some of the effectors which have been structurally analyzed up to now. Binding along switch I is similar for all (pdb 1he8, 2c5l, 3ddc).

Unfortunately, full-length C-Raf1 or any other Raf isoform could not be expressed and fragments containing RBD and CRD could not, in case expression worked, be investigated structurally, either in the presence or absence of Ras (see below). This leads to the conclusion that RBD and CRD are required for activation but the exact mechanism of the role of Ras in the complex activation of the kinase beyond just targeting it to the membrane is still missing. For a general review on effector complexes and what we have learnt from structural studies, see Mott and Owen (2015).

The structure of a complex of Ras itself with RafRBD was published much later (Fetics et al., 2015) (Figure 6A). It was intended to show the interaction of Ras with the RBD plus CRD domains which were contained in the construct. However, only the RBD domain of Raf could be traced while the CRD was unordered, which may indicate that this interaction is indeed very week and/or may require the full Raf protein and/or farnesylation of Ras, as suggested earlier (Thapar et al., 2004). In any case, the structure was very similar to the structure of the Raps complex [root mean square deviation (r.m.s.d.) 0.93 Å]. As switch II is not in direct contact with RBD (but is supposedly in contact with CRD) the significant sequence difference in switch II between Ras and Rap turned out not to influence earlier conclusions concerning the interaction. The Mattos group also solved the structure of the complex with the oncogenic Ras(Q61L) mutant. There are small perturbations of switch II close to the active site, which are presented as to have an effect on the hydrolytic activity, such as the nucleophilic water which is not seen at this low resolution structure (3.3 Å). However, in the absence of other arguments it appears that the absence of the catalytic Gln is the major obstacle for a normal intrinsic GTP hydrolysis.

It should be pointed out that, while the basis of the molecular switches is to interact with effectors in the GTP-bound state, this does not mean that an interaction with the GDP-bound form is unallowed *per se*. By introducing structure-guided mutations (more positive charges) into Raf-RBD, Filchtinski et al. (2010) were able to make a stable complex between Ras·GDP and Raf-RBD. The three-dimensional structure showed a similar interaction pattern as compared to the GTP-bound complex.

A large number of RBDs have been identified in addition to RA (Ras association) domains, a subset of potential Ras effector molecules with a similar signature (Figure 6B). They bind to Ras with affinities ranging from submicromolar to 10–20 μM affinity, and some of these may in fact be effectors of Rap1 or other Ras-like small G proteins (Wohlgemuth et al., 2005). Besides Raf, the most prominent effector proteins are the PI3 kinases. A structure was solved between PI3Kγ and Ras·GppNHp (Pacold et al., 2000). Here, Ras uses both switch 1 and 2 to bind to the RBD and other parts of the molecule to activate the kinase activity. Mutagenesis shows that both regions of Ras are required for binding and/or activation. The PI3K data are thus the showcase for the role of Ras and its interaction with effectors, which involves the dual role of membrane recruitment and allosteric activation and might well be applicable to Raf kinase and byr2.

PLCε is another large multi-domain protein, which is partially regulated by Ras. It is unique (besides Afadin) in that it contains two RA domains and the NMR structure analysis shows that they have a regular ubiquitin fold very similar to Raf-RBD. However only RA2 binds Ras (with measurable affinity) and the structure of the complex (by X-ray) shows features more similar to the PI3K interaction in that it contacts both switch I and II (Bunney et al., 2006).

Ral-GDS is a GEF for the Ras-like protein Ral. It has an RBD and the structures with a tighter binding Ras(E31K) mutant (Huang et al., 1998) and with native Ras (Vetter et al., 1999) were solved. They showed the ubiquitin fold of the RBD and a similar inter-β-sheet interaction between the two proteins. However, the details of the interaction and the residues involved were remarkably different from the Ras·RafRBD interaction. Similar findings were made with the RBD of Byr2, the kinase from *Schizosaccharomyces pombe* which is homologous to Raf and acts downstream of Ras in the *S. pombe* Map kinase cascade (Scheffzek et al., 2001). A novel feature of this complex is that a C-terminal helical extension of byr2-RBD also seems to make contact to Ras.

Other structures are available showing complexes of Ras with RBD/RAs whose role in biological signal transduction are less clear. NORE1A (Novel Ras Effector), a splice variant of RasSSF5 is a member of the RASSF (Ras association) family of proteins which are believed to act as negative regulators of growth and/or tumor formation. The structure of the complex shows that the RBD is a much larger protein as it has an insertion between β1 and β2 of the regular ubiquitin fold and an N-terminal extension (Stieglitz et al., 2008). The complex shows, in addition to the inter-β-sheet, the typical polar interaction between Ras and NORE1A. Afadin/AF6, a very large multidomain protein, contains two RA domains at the N-terminus which in acute myeloid leukemia (AML) is fused to the *MLL* gene. The structure of the first RA domain showed that it is similar to the NORE1A RA domain it that it contains an N-terminal helix in addition to the core ubiquitin fold by which it makes contact to switch II. This helix increases the affinity between Ras and the core RA domain from 18 to 4 μM, well in the range of typical Ras effectors (Smith et al., 2017). Sequence alignment using more than 50 RA/RBDs identifies such a helix only in the RASSF1-6 family and in AF6.

Grb14 (growth factor receptor binding) is a member of the large family of adaptor proteins. Grb7-10-14 contain in addition to the SH2 and PH domain an RA domain. The structure of Grb14 with Ras shows that the RA and PH domains form a structural tandem. Only the RA interacts with Ras and forms the classical interaction surface, again very similar to previous complexes (Qamra and Hubbard, 2013).

Interaction with PDEδ

By solving the structure of the delta subunit of phosphodiesterase 6 (PDEδ from now), it was shown that its structure was similar to that of RhoGDI, the chaperone/transport factor for the prenylated Rho proteins (Hanzal-Bayer et al., 2002). What this suggested was verified by showing that PDEδ does indeed bind prenylated proteins like Ras or RheB, and that this was mutually exclusive with the binding of Arl2/3·GTP, factors which mediate the unloading of the prenylated proteins (Ismail et al., 2011). Structural studies with C-terminal peptides from Ras and RheB and fully modified full-length RheB showed the binding pocket to be capable of binding the prenylated C-termini of various peptides or proteins. The structure with fully modified (farnesylated and methylated) K-Ras4B supported this observation and showed for the first time the structure of Ras beyond the G-domain (Dharmaiah et al., 2016) (Figure 7). Surprisingly, residues 166-180 have a stable secondary structure and extended helix α5. The last four residues are inserted into the PDEδ hydrophobic pocket. The interaction with Ras is relevant for the spatial organization of Ras because knock-down of PDEδ randomizes Ras localization to all membranes and severely impedes Ras signaling (Chandra et al., 2012; Schmick et al., 2014).

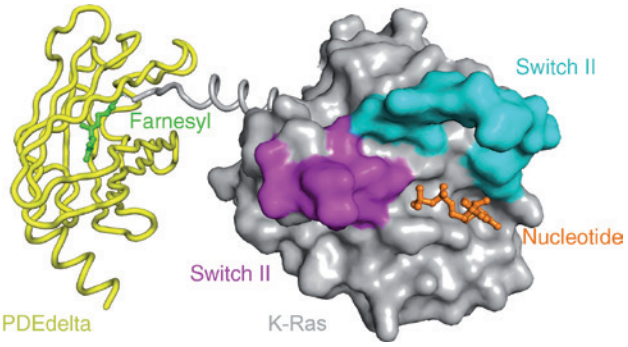

Figure 7: The complex between PDEδ and fully modified K-Ras4B. Ras coloring and orientation as above, showing PDEδ in yellow that harbors the farnesylated (green) C-terminus of Ras (pdb 5tar).

Efforts towards Ras drugs

Being such an important oncogene, it is obvious that numerous attempts have been made to target Ras and the Ras pathway leading to proliferation. This has been summarized in many reviews (see e.g. Cox et al., 2014; Spiegel et al., 2014; McCormick, 2015). We focus here on approaches to target Ras directly, or its interface with effectors or regulators (SOS), using structure-guided approaches, rather than components of the Ras pathway, such as Raf or MEK. The compounds are summarized in Table 1 and illustration of some of the binding sites are

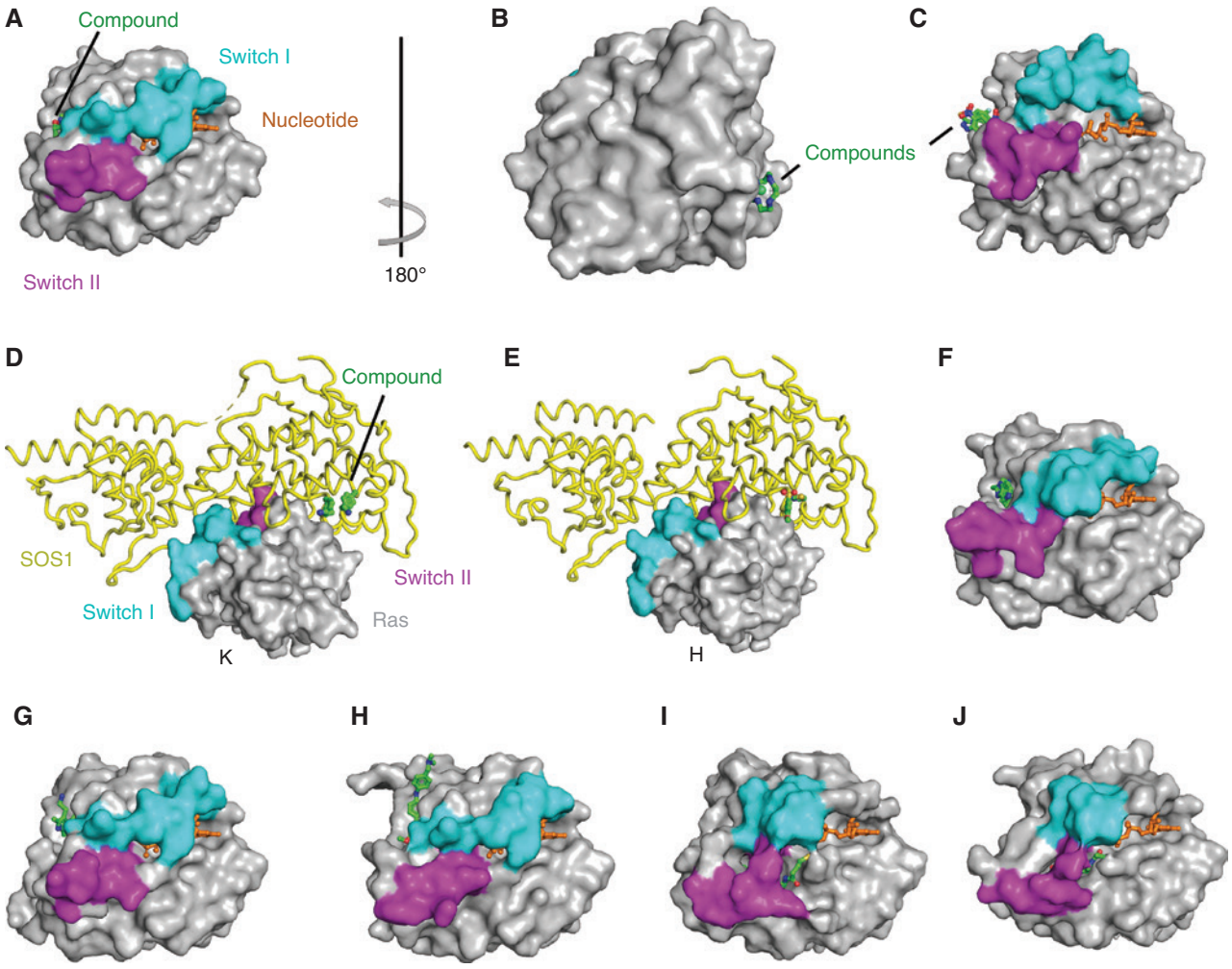

Figure 8: Ras and small molecules.
Ras coloring as above, small molecule compounds shown in green. (A) K-Ras·9R5, Quevedo et al., 2018 (pdb 5ocg). (B) H-Ras·Cyclen rotated 180° in comparison to the other orientations. Rosnizeck (pdb 3l8y). (C) H-Ras·Kobe2601, Shima et al., 2013 (pdb 2lwi). (D) K-Ras·SOS·BPW, Hillig et al., 2019 (pdb 6epo). (E) H-Ras·SOS·6W2, Winter et al., 2015 (pdb 4uru). (F) K-Ras·0QX, Sun et al., 2012 (pdb 4epv). (G) K-Ras·9LI, Maurer et al., 2012 (pdb 4dst). (H) K-Ras·F8T, Cruz-Migoni et al., 2019 (pdb 6gqw). (I) K-Ras·20G, Ostrem et al., 2013 (pdb 4luc). (J) K-Ras·ARS-853, Patricelli et al., 2016 (pdb 5f2e).

shown in Figure 8. The story of drugging Ras seems to follow a wave function with periods of intense efforts and times when Ras is considered undruggable. The first wave focused on the development of inhibitors of Ras farnesylation. While biochemically potent nanomolar inhibitors were developed, many problems were encountered in clinical trials. In the end these inhibitors may be effective against tumors with mutations in H-Ras.

Another effort focused on developing compounds that would bind into the GTP binding pocket, in analogy to those hitting the ATP binding site of protein kinases. These effects proved fruitless after it was realized, somewhat late by some, that the affinity of G nucleotides is in the subnanomolar to picomolar range. The idea of G nucleotide analogues has been picked up lately by the development of GDP/GTP derivatives, which form covalent adducts. Some of these have used the β-phosphate of GDP for modification resulting in derivatives with drastically reduced affinity (Lim et al., 2014; Xiong et al., 2017). It has been pointed out that, apart from the problem with intracellular delivery, covalent GDP/GTP derivatives will only work when they have similar affinity as the native nucleotide and can be efficiently exchanged by Ras GEFs (Muller et al., 2017). It is suggested that the 2′ and 3′ ribose position of the nucleotide would be a good position to introduce chemical warheads for covalent labeling (Wiegandt et al., 2015).

That the Ras·effector interaction might be a difficult target for small molecules as was indicated by the structural analysis of the Ras·RafRBD complex and confirmed

by other such complexes which showed a rather flat interface with no good druggable pockets (Nassar et al., 1996; Fetics et al., 2015). Not surprisingly those early efforts in many labs lead to compounds whose affinity never improved much below the two-digit micromolar number. That the interface can be inhibited was shown much earlier by the Y-259 antibody. The idea was revitalized by screening for monobodies that bind with nanomolar affinity to Ras. The three-dimensional structure showed binding to a site on H- or K-Ras which is opposite the switch regions and not involved in effector binding. The monobody potently inhibits Ras-mediated signaling (Spencer-Smith et al., 2017). Whether such biologicals will ever be stable and suitable for the clinic will have to be worked out. In a related strategy an inhibitory cellular antibody fragment was used as a competitor in a screen for small molecules that bind to Ras. The initial hits were optimized by structure-based design and created compounds (called Abd = antibody derived) that bind to Ras with some potency and inhibit effector binding (Bery et al., 2018). Further structure-guided improvement produced a compound Abd-7, which had a remarkably high affinity for Ras (50 nM) and overlapped with the effector binding site. Unfortunately, the inhibitory IC_{50} in cells was much lower (8–10 μM), a feature not unusual for small molecules (Quevedo et al., 2018) (Figure 8A).

A different approach was taken by Rosnizeck et al., 2010 who relied on the finding that Ras is in two conformational states and only one of which binds to an effector. By NMR screening they found a Cu^{2+}cyclen (Figure 8B), which shifts the protein to state 1. Unfortunately, the affinity is too low to serve as a lead compound. A similar strategy was followed by Shima et al. (2013) who used a new pocket on the state 1 conformation of Ras to find a compound by an *in silico* screen inhibiting Ras·Raf interaction with micromolar affinity (Figure 8C), a remarkable progress compared to other such efforts (see below).

The Ras interaction with the most important Ras GEF SOS was also considered for drug development although it is not clear if and how much oncogenic Ras requires interaction with and activation by SOS, and if there are differences between the various mutants (Figure 8D,E). Stapled peptides mimicking the catalytic SOS helix were developed, which had up to nanomolar affinities but only low cellular activity (Patgiri et al., 2011; Leshchiner et al., 2015). A recent approach to target Ras·SOS interaction identified a small molecule, which required both Ras and SOS for binding although its binding site was exclusively on SOS itself (Hillig et al., 2019). This compound was further developed by combining fragment-based compounds with HTS derived chemistry and ended up being an efficient two-digit nanomolar compound which acted on SOS, and inhibited activation of wild-type Ras. It had however a much smaller effect on mutant Ras(G12C), which seems to indicate that the latter does not require a GEF for activation. Erk activity could be fully suppressed by a combination of this compound with ARS-853 (see below). Another such approach was followed by Winter et al. (2015). Considering that most malignancies have an activated Ras-Raf-MEK-Erk pathway (even without a Ras mutation) it is not obvious why activators of the Ras pathway would be good lead compounds for drug development. However, a number of potent submicromolar activators of SOS have been developed, which increase Ras·GTP levels and have an effect on Erk signaling believed to be induced by negative feedback (see e.g. Hodges et al., 2018).

Considering the role of PDEδ for the spatial organization and signaling of Ras, it is not surprising that PDEδ, with its large hydrophobic pocket, was considered a target for anti-Ras drugs. By knocking out PDEδ all Ras isoforms become indeed mis-localized. A number of medium to high affinity compounds for the PDEδ pocket were developed, which achieved a similar effect in cells. However, the compounds proved only effective in a small window of concentrations such that the compounds would need to be improved for targeting Ras in disease (Zimmermann et al., 2013; Papke et al., 2016). The negative effect was most likely due to PDEδ being a general prenyl binding protein, PrBP (Zhang et al., 2012), with affinities in the micro- to nanomolar range for different target molecules (Fansa et al., 2016).

An intensive effort has been ongoing to target Ras directly by small molecules, preferably K-Ras. The development was done by fragment-based screening using NMR or crystallography. The development of such compounds involved a lot of sophisticated structural and chemical efforts (Maurer et al., 2012; Sun et al., 2012; Matsumoto et al., 2018; Cruz-Migoni et al., 2019). Such compounds ended up binding in a similar pocket (Figure 8F–H), which is formed between switch I and II. This pocket had not been observed in the structural analysis of Ras·GDP or Ras·GppNHp. While this seemed surprising to some, it is necessary to remember that switch regions are highly flexible, in particular in the GDP-bound form, and can be assumed to adopt a number of conformation states, which can be visualized by high-pressure NMR (Kalbitzer et al., 2013). One of these is thus stabilized here by small molecules. Not surprisingly those compounds, when tested, would bind to all four isoforms of Ras and were more or less unselective for wildtype and mutant Ras proteins. Unfortunately, such compounds were only active in the

two-digit micromolar range and were put aside as possible drug target candidates.

The most innovative and most promising approach to target a specific oncogenic Ras mutant has been introduced by the group of Kevan Shokat (Ostrem et al., 2013). Using a structure-guided approach they developed inhibitors that bind covalently to the reactive Cys of Ras(G12C) without touching other cysteines (Figure 8I). G12C is a frequent K-Ras mutation in certain cancers (i.e. lung cancer) and the finding was exciting news for the community. They identified a new pocket near switch II (SII-P), which was not visible before in Ras structures. This original finding has stimulated an intense effort in many academic and pharmaceutical laboratories and has led to a series of efficient compounds of the ARS (Patricelli et al., 2016) (Figure 8J) or SML series (Lim et al., 2014) and others (Lito et al., 2016). These compounds bind and stabilize the GDP-bound form of Ras and thus inhibit interaction with effectors. Surprisingly, it was demonstrated that the G12C mutant is not, as assumed, in a static GTP-bound state but rather dynamically switches between the two states and responds to Ras regulators. Compounds such as ARS853 are active in cells in a low micromolar range. They are really hot Ras drug candidates and some of these are in clinical trials. The Ras-drugging wave has thus reached a new stable peak and promises to finally deliver drugs for the undruggable Ras, albeit thus far only for a specific oncogenic mutant.

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