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To my family

ABSTRACT

Proteins of the Ras family are central regulators of crucial cellular processes, such as proliferation, differentiation and apoptosis. Their importance is emphasized in cancer, in which the isoforms H-ras, N-ras and K-ras are misregulated by mutations in approximately 20 – 30 % of cases. Thus, they represent major cancer oncogenes and one of the most important targets for cancer drug development. Ras proteins are small GTPases, which cycle between the GTP-bound active and GDP-bound inactive state. Despite the tremendous research conducted in the last three decades, many fundamental properties of Ras proteins remain poorly understood. For instance, although new concepts have recently emerged, the understanding of Ras behavior in its native environment, the membrane, is still largely missing. On the membrane Ras organizes into nanoscale clusters, also called nanoclusters. They differ between isoforms, but also between activation states of Ras. It is considered that nanoclusters represent the basic Ras signaling units. Recently, it was demonstrated that on the membrane Ras adopts distinct conformations, the so-called orientations, which are dependent on the Ras activations state. The membrane-orientation of H-ras is stabilized by the helix α4 and the C-terminal hypervariable region (hvr). The novel switch III region was proposed to be involved in mediating the change between different H-ras orientations. When the regions involved in this mechanism are mutated, H-ras activity is changed by an unknown mechanism.

This thesis has explained the connection between the change of Ras orientation on the membrane and Ras activity. We demonstrated that H-ras orientation mutants exhibit altered diffusion properties on the membrane, which reflect the changes in their nanoclustering. The altered nanoclustering consequently rules the activity of the mutants. Moreover, we demonstrated that specific cancer-related mutations, affecting the switch III region of different Ras isoforms, exhibit increased nanoclustering, which consequently leads to stronger Ras signaling and tumorigenicity. Thus, we have discovered nanoclustering increase as a novel mechanism of Ras activity modulation in cancer.

The molecular architecture of complexes formed on the membrane upon Ras activation is another poorly understood property of Ras. The following work has provided novel details on the regulation of Ras nanoclustering by a known H-ras-GTP nanoclustering stabilizer galectin-1 (Gal-1). Our study demonstrated that Gal-1 is not able to bind Ras directly, as it was previously proposed. Instead, its effect on H-ras-GTP nanoclustering is indirect, through binding of the effector proteins.

Collectively, our findings represent valuable novel insights in the behavior of Ras, which will help the future research to eventually develop new strategies to successfully target Ras in cancer.

SAMMANFATTNING (Swedish abstract)

Proteinerna i Ras-familjen är centrala reglerare av väsentliga cellulära processer som cellproliferering, -differentiering och apoptos. Ras proteinernas betydelse är betonad i cancer, var regleringen av H-ras, N-ras och K-ras är störd på grund är mutationer i ca 20-30% av alla cancer. Således är Ras proteinerna några av de mest betydelsefulla oncogenerna och viktigaste målen för utvecklingen av nya botemedel mot cancer. Ras proteinerna är små GTPaser som växlar mellan den GTP-bundna aktiva formen och GDP-bunda inaktiva formen. Trots de enorma forskningsinsatser som gjorts under de tre senaste årtiondena har många fundamentala egenskaper hos Ras förblivit gåtfulla. Som ett exempel, även om nya koncept har vuxit fram under de senaste åren, saknas fortfarande en stor del information om Ras beteende i sin naturliga miljö, d.v.s. cellmembranet. I membranet är Ras organiserat i så kallade nanoklusters. Dessa nanoklusters skiljer sig mellan de olika isoformerna, men också mellan de olika aktiveringstillstånden. Man anser att nanoklustren är de grundläggande enheterna för Ras signalering. Nyligen påvisades det att Ras antar olika konformation (orientering) i membranet beroende på Ras aktiveringstillstånd. H-ras orientering i membranen stabiliseras av helix α4 och den C-terminala hypervariabla regionen (hvr). Den nyupptäckta switch III-regionen föreslogs vara involverad i förändringen av de olika H-ras orienteringarna. Då regionerna som är involverade i denna mekanism muteras förändras H-ras aktiviteten genom en okänd mekanism.

I denna avhandling förklaras sambandet mellan förändringen av Ras orientering i membranet och Ras-aktiviteten. Vi visar att H-ras orienteringsmutanter uppvisar en förändring i hur de diffunderar i membranet, som speglar förändringarna i bildandet av nanoklusters. Denna förändring i bildande av nanoklusters styr följaktligen mutanternas aktivitet. Dessutom visade vi att specifika cancerrelaterade mutationer som påverkar switch III-regionen i olika Ras isoformer ökar mängden Ras i nanoklusters, vilket leder till starkare Ras-signalering och ökad tumorigenicitet. Således har vi upptäckt att ökningen av nanoklusters är en ny mekanism som modulerar Ras-aktiviteten i cancer.

Den molekylära arkitekturen hos komplex som bildas på membranet då Ras aktiveras är en annan dåligt förstådd egenskap hos Ras. Denna avhandling har bidragit till ökad förståelse om hur galektin-1 (Gal-1), som stabiliserar H-ras GTP-nanoklusters, reglerar Ras-nanoklusters. Vår studie visade att Gal-1 inte kan binda direkt till Ras, som tidigare föreslagits, utan att effekten på H-tas GTP nanoklusters sker indirekt, genom bindningen till effektorproteiner.

Sammantaget ger resultaten i denna avhandling värdefulla nya insikter om hur Ras regleras. Dessa kommer att hjälpa framtida forskning att så småningom utveckla nya strategier för att framgångsrikt kunna behandla Ras i cancer.

TABLE OF CONTENTS

ABBREVIATIONS

APT	Acyl protein thioesterase
Arf	ADP ribosylation factor
ATP	Adenosine-5'-triphosphate
BHK	Baby hamster kidney
CBR	Carbohydrate binding region
CFCS	Cardiofaciocutaneous syndrome
CM-AMS	Capillary malformation–arteriovenous malformation syndrome
COSMIC	Catalogue of somatic mutations in cancer
CR	Conserved region
CRD	Cysteine rich domain
CS	Costello syndrome
DMEM	Dulbecco's modified Eagle medium
DMPC	1,2-dimyristoylglycero-3 phosphocoline
DNA	Deoxyribonucleic acid
EBNA	Epstein–Barr nuclear antigen
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FCS	Fluorescence correlation spectroscopy
FLIM	Fluorescence lifetime microscopy
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
FTase	Farnesyl transferase
GA	Golgi apparatus
Gal	Galectin
GAP	GTPase-activating protein
GDI	GDP dissociation inhibitors
GEF	Guanine nucleotide exchange factor
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine-tri-phosphatase
HEK	Human embryonic kidney
hvr	Hypervariable region
Icmt	Isoprenyl cysteine transferase
IQGAP	IQ motif containing GTPase activating protein
KSR	Kinase suppressor of Ras
MAPK	Mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
mCFP	Monomeric cyan fluorescent protein
mCit	Monomeric citrine

GDP	Guanine-5'-diphosphate
MD	Molecular dynamics
MEK	Mitogen/extracellular signal-regulated kinase
mGFP	Monomeric green fluorescent protein
MP1	MEK partner 1
mRFP	Monomeric red fluorescent protein
NF1	Neurofibromatosis type 1
NGF	Nerve growth factor
	Nuclear magnetic resonance
NS	Noonan syndrome
NSML	Noonan syndrome with multiple lentigines
PA	Phosphatidic acid
PAT	Palmitoyl acyltransferase
PBR	Polybasic region
PC12	Rat adrenal pheochromocytoma cells
PDEδ	Delta subunit of the phosphodiesterase 6
PE	Posphatidylethanolamine
PH	Pleckstrin homology
PHB	Prohibitin
PI	Phosphatidylinositol
PIP2	Phosphatydyl-inositol-2-phosphate
PIP3	Phosphatidyl-inositol-3-phosphate
PS	Phosphatidylserine
Rab	Ras-related proteins in brain
Raf	Rapidly accelerated fibrosarcoma
Ral	Ras-related
Ran	Ras-like nuclear protein
Ras	Rat sarcoma (protein)
RBD	Ras binding domain
Rce1	Ras-converting enzyme 1
Rho	Ras homologous
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SH	Src homology
Shoc2	Soc-2 suppressor of clear homolog
SOS	Son of sevenless
SPT	Single particle tracking
STED	Stimulated emission depletion
wt	Wild type

Amino acids

Ala	Alanine (A)	**Leu**	Leucine (L)
Arg	Arginine (R)	**Lys**	Lysine (K)
Asn	Asparagine (N)	**Met**	Methionine (M)
Asp	Aspartate (D)	**Phe**	Phenylalanine (F)
Cys	Cysteine (C)	**Pro**	Proline (P)
Gly	Glycine (G)	**Ser**	Serine (S)
Glu	Glutamate (E)	**Thr**	Threonine (T)
Gln	Glutamine (Q)	**Trp**	Tryptophane (W)
His	Histidine (H)	**Tyr**	Tyrosine (Y)
Ile	Isoleucine (I)	**Val**	Valine (V)

LIST OF ORIGINAL PUBLICATIONS

Bellow is the list of publications and manuscripts used in the thesis. They are referred to in the text by their Roman numerals.

I. Guzmán, C.*, **Šolman, M.***, Ligabue, A., Blaževitš, O., Andrade, D.M., Reymond, L., Eggeling, C., and Abankwa, D. (2014). The efficacy of Raf kinase recruitment to the GTPase H-ras depends on H-ras membrane conformer-specific nanoclustering. J. Biol. Chem. *289*, 9519–9533.

II. **Šolman, M.**, Ligabue, A., Blaževitš, O., Jaiswal, A., Zhou, Y., Liang, H., Lectez, B., Kopra, K., Guzmán, C., Härmä, H., Hancock, J.F., Aittokallio, T., and Abankwa, D. (2015). Specific cancer associated mutations in the switch III-region of Ras increase tumorigenicity by nanocluster augmentation. Elife *4*.

III. Blaževitš, O.*, Mideksa, Y.G.*, **Šolman, M.**, Ligabue, A., Ariotti, N., Nakhaeizadeh, H., Fansa, E.K., Papageorgiou, A.C., Wittinghofer, A., Ahmadian, M.R., and Abankwa, D. Galectin-1 dimers can scaffold Raf-effectors to increase H-ras nanoclustering. *Manuscript under revision in Scientific Reports.*

* annotates equal contribution.

INTRODUCTION

The cellular genomes are constantly subject to modifications by mutations. Mutations in specific genes can drastically change the behavior of cells and lead to acquisition of cancerous properties, such as uncontrolled proliferation and evasion of cell death. These abnormal cells are no longer responsive to the surrounding signals and behave autonomously, with the only goal to reproduce themselves.

Ras proteins are the central regulators of cellular fates. They function as signaling nodes, which respond to external stimuli and convey the signal to multiple downstream signaling pathways, in order to stimulate a certain cellular response, such as proliferation, differentiation or cell death.

Having such a critical role makes Ras highly vulnerable. Mutations can leave Ras constitutively active, ignoring the external stimuli. Its hyperactivation in turn leads to the continuous stimulation of the downstream signaling pathways and consequently to the acquisition of cancer cell properties. Mutations in the isoforms H-ras, N-ras and K-ras are present in 20-30 % of cancers, making them one of the most important oncogenes. Ras mutations are also involved in distinct developmental diseases. The significance of Ras was recognized early after its discovery in the 1980s; since then it represents one of the most important targets for cancer-drug development. Despite tremendous efforts, successful inhibition of Ras was not yet accomplished.

The function of Ras depends on its attachment and primary localization to the plasma membrane. Only in recent years, a colossal development of advanced biophysical and microscopy techniques sheds light on the Ras nanoscale behavior in its native membrane environment. Novel exciting properties were unveiled, giving hope for better understanding of Ras and finding new avenues for its targeting. It was demonstrated that on the membrane Ras organizes into dimer and oligomers, the so-called nanoclusters; they are dependent on the isoform and the activation state of the protein. Nanoclusters are considered to be the basic signaling units of Ras, enabling a high-fidelity signal transmission. Nanoclustering can be additionally regulated by several proteins. One of them is Galectin-1 (Gal-1), which enhances H-ras nanoclustering. Another newly discovered Ras feature is its ability to adopt distinct conformations on the membrane. Conformers are dependent on the Ras activation state and they differ between distinct isoforms. This thesis is largely focused on depicting the additional molecular features of these novel properties of Ras and their significance in (patho)physiological conditions. In addition, the last part of the thesis is focused on the detailed mechanistic characterization by which Gal-1 regulates Ras nanoclustering.

REVIEW OF THE LITERATURE

1. The biology of Ras

1.1. Ras superfamily

K-ras, N-ras and H-ras are members of the Ras (Ras sarcoma) protein family which, together with Rho (Ras homologous), Rab (Ras-like proteins in brain), Arf (ADP-ribosyation factor) and Ran (Ras-like nuclear) families and a few unclassified members, constitute the large Ras superfamily (Goitre et al., 2014; Wennerberg et al., 2005). Ras superfamily has a total of 164 known members in humans. Although proteins from the Ras superfamily share similar molecular mechanisms, they are involved in tremendously versatile processes. Grouping into different families within the superfamily is based on the sequence homology and the function of the proteins (Rojas et al., 2012).

Their ability to bind GTP or GDP classifies them as guanine-nucleotide binding proteins (G proteins). G proteins are divided into two groups: large heterotrimeric G-proteins built from α, β and γ subunit and monomeric small GTPases, homologous to α subunit of heterotrimeric G proteins (Cabrera-Vera et al., 2003). Proteins from the Ras superfamily are small GTPases. They share a set of conserved nucleotide binding motifs and they all function as molecular switches, which cycle between the active, GTP-bound conformation, and inactive, GDP-bound conformation (Fig. 1). When in the active form, they can bind their effector proteins (Tetlow and Tamanoi, 2013). Since small GTPases bind guanine-nucleotides with high affinity and have very low intrinsic hydrolase activity, additional proteins are required to facilitate the GTP/GDP cycling. Guanine nucleotide exchange factors (GEFs) activate small GTPases by catalyzing the release of GDP, so GTP, which exists in much higher cellular concentration than GDP, could bind (Cherfils and Zeghouf, 2013). GTPase activating proteins (GAPs) catalyze the GTP hydrolysis to inactivate the protein (Vigil et al., 2010). Most small GTPases are membrane-associated proteins. Membrane anchorage is achieved through the post-translational attachment of the lipid group, such as geranylgeranyl, farnesyl and myristoyl groups, which defines their cellular localization (Vidal, 2010).

Rho GTPases are mainly involved in the organization of actin cytoskeleton, which orchestrates movements of the cell, such as migration, phagocytosis, cell division, cell polarity and endocytosis. There are 22 members of the Rho family, of which RhoA, Rac1 and Cdc42 are the best characterized (Hall, 2012). Rab is the largest family of small GTPases, which accounts for 65 members. Rab proteins are involved in regulation of different steps required for vesicular trafficking, including the choice of

the vesicular cargo, budding of the vesicle, transport of the vesicle to its destination and uncoating, tethering and fusion with the destination membrane (Zhen and Stenmark, 2015). Beside regulations by GEF and GAP proteins, Rho and Rab proteins are also regulated by guanosine nucleotide dissociation inhibitors (GDIs) (Cherfils and Zeghouf, 2013). They bind to their GDP–bound form, and prevent the nucleotide exchange and their membrane localization. The Arf family contains more than 30 members, which are similarly to Rab proteins, involved in vesicular trafficking (D'Souza-Schorey and Chavrier, 2006). The Ran family has only one member in humans, which is involved in regulation of protein shuttling between nucleus and cytoplasm (Dasso, 2002).

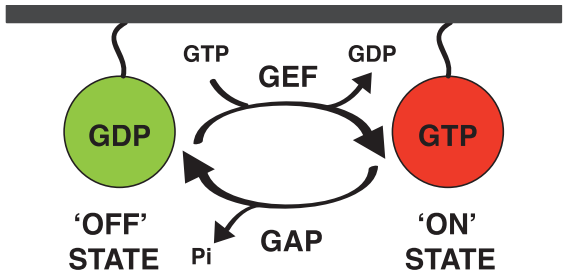


Figure 1. The activation cycle of Ras superfamily of small GTPases.
Proteins from the Ras superfamily are membrane-anchored (the membrane is represented by a thick grey line). They function as molecular switches, cycling between the GDP-bound inactive (green) and GTP-bound active form (red). The GEF and GAP proteins catalyze the nucleotide exchange and hydrolysis, respectively.

1.2. Ras family

The Ras family counts 36 genes in humans, which encode 39 proteins (Rojas et al., 2012). Because of their implication in cancer, the isoforms K-ras, N-ras and H-ras are the most extensively studied. My thesis is focused on these Ras isoforms and further on, if not specified, Ras will refer to any of them. They regulate a plethora of crucial signaling pathways, such as cell proliferation, survival, cell growth and differentiation. Thus, the misregulation of Ras proteins can lead to the development of diseases, such as cancer (Fernández-Medarde and Santos, 2011). H-ras and K-ras4A proteins were the first described members of the whole Ras superfamily. The discovery of *RAS* genes dates back to the 1960s when the cancer research field was focused on tumorigenic viruses, which were considered to be the main cause of cancer. Two retroviruses isolated from murine sarcoma, Kirsten and Harvey murine sarcoma viruses were able to transform fibroblasts, so that they acquire cancerous

properties (HARVEY, 1964; Kirsten and Mayer, 1967). It was later demonstrated that the transforming viral genes exist also in normal, not virally-infected cells, where they encode for proteins 21 kDa in size, termed H-ras (also Ha-Ras) and K-ras (also Ki-Ras) (Ellis et al., 1981). The name Ras reflects that it was originally isolated from the rat sarcoma. In the 1980s, it was shown that many cancers carry mutations in *KRAS*, *HRAS* and the third oncogenic isoform *NRAS* (Shimizu et al., 1983), which established Ras as one of the most prominent drug targets (Cox and Der, 2010). Nevertheless, no efficient way of Ras inhibition exists so far, despite intensive research in the last 30 years (Cox et al., 2014).

The *KRAS, NRAS* and *HRAS* genes are localized on the chromosomes 12, 1 and 11, respectively, and consist of 4 exons interrupted by 3 introns (Hall et al., 1983; McBride et al., 1982; O'Brien et al., 1983). These three genes encode for 4 proteins, H-ras, N-ras, K-ras4A and K-ras4B. The latter two are products of differently spliced alternative exon 4 in the *KRAS* gene (Barbacid, 1987). Ras proteins are approximately 21 kDa in size and consist of 189 (H-ras, N-ras and K-ras4A) or 188 (K-ras4B) amino acids. The first 165 residues are almost completely identical among the different isoforms. These residues comprise the so-called G domain to which nucleotides and effector proteins bind (Wittinghofer and Vetter, 2011) (Fig. 2). The structure of the G-domain will be discussed in more detail in the following section. The remaining amino acids, on the C-terminus, build the hypervariable region (hvr) that shares only 15 % of similarity among isoforms. The hvr serves Ras to establish contacts with the cellular membranes (Prior and Hancock, 2011), through its post-translationally added lipid anchors, which will be discussed in detail in the next chapter.

Although Ras proteins are highly similar in terms of sequence and share a common set of regulator and effector proteins, it is now clear that they are isoform specific (Castellano and Santos, 2011). For instance, distinct Ras isoforms show different preference for specific effectors and consequently trigger different transcriptional profiles (Yan et al., 1998; Zuber et al., 2000). Their expression levels vary in different tissues (Furth et al., 1987; Leon et al., 1987) and the frequency of mutations in different isoforms varies depending on the cancer type (Prior et al., 2012). Furthermore, they differ in the subcellular localization and trafficking routes (Omerovic and Prior, 2009; Rocks et al., 2006). How this specificity is achieved remains one of the most exciting questions of Ras biology today.

1.3. The structure of Ras

Ras proteins are bound to the membrane through the C-terminal hvr (Fig. 2). As for many other membrane proteins, structural studies of the full-length Ras proteins remain challenging. However, the structure of the soluble G-domain was studied in detail using X-ray crystallography, nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR) and computational simulations. Currently >550 entries are available in the protein data bank, giving the structures of the Ras G-domains in nucleotide free, GTP-, GDP-, various effector-, GEF- and GAP-bound states.

1.3.1. The G-domain structure

The G-domain is built of 6 α-helices and 5 β-sheets interconnected with loops. Residues required for the nucleotide- and effector- binding are well defined (Fig. 2). Binding of the nucleotide is accomplished through several nucleotide-binding motifs, also called G-boxes, which are highly conserved among members of the Ras superfamily (Wennerberg et al., 2005). The GXXXXGKS/T motif, X denotes any nucleotide, also called the P-loop (G1 box), the residue T35 (G2 box) and the DXXGQ/H/T motif (G3 box) make contacts with the phosphates and Mg^{2+}, which is required for nucleotide binding and also GTP hydrolysis. The motifs N/TKXD (G4 box) and SAK (G5 box) contact the nucleotide base, the latter giving the largest contribution to the guanine base selectivity (Vetter and Wittinghofer, 2001; Wittinghofer and Vetter, 2011). Major conformational rearrangements of the G-domain happen in the well-defined switch I (residues 32-38) and switch II (residues 59-67) motifs during the cycling of Ras between the active and inactive state (Milburn et al., 1990; Schlichting et al., 1990). The trigger for these structural changes is described with the so-called loaded-spring model, which is highly conserved among the members of the Ras superfamily (Wittinghofer and Vetter, 2011). In the GTP-bound state residues Thr35 and Gly60 establish hydrogen bonds with the γ-phosphate group of the nucleotide that keep the switch motifs in a GTP-bound conformation. The γ- phosphate group is lost with the GTP-hydrolysis, this loss causes the relaxation of switch motifs into a GDP-bound conformation (Vetter and Wittinghofer, 2001). The G-domain can be divided into two lobes, the N-terminal effector lobe (residues 1-86) and the C-terminal allosteric lobe (residues 87-171) (Fig. 2) (Gorfe et al., 2008). The effector lobe is completely conserved among the Ras isoforms and it contains the switch regions and binding sites for the effectors and nucleotides. The allosteric lobe is not completely conserved among the different Ras isoforms. Together with hvr, it establishes the contacts with the membrane, which will be discussed in detail in the next chapter. The allosteric and effector lobes are connected with different communication routes, some of which are isoform specific

(Gorfe, 2010; Gorfe et al., 2008; Parker and Mattos, 2015). These communication routes can serve to sense the nucleotide state of the effector lobe and translate it into a specific membrane binding of the allosteric lobe. One of such communication routes in H-ras is the recently described switch III region (Abankwa et al., 2008; Gorfe et al., 2008). It is built of hydrogen bonds between the residues R161, R164 of the helix α5 in the allosteric lobe and D47, E49 of the β2-β3-loop in the effector lobe. These hydrogen bonds are dependent on the bound nucleotide and they guide the conformation switch of H-ras relative to the plasma membrane (discussed in detail in the following chapter).

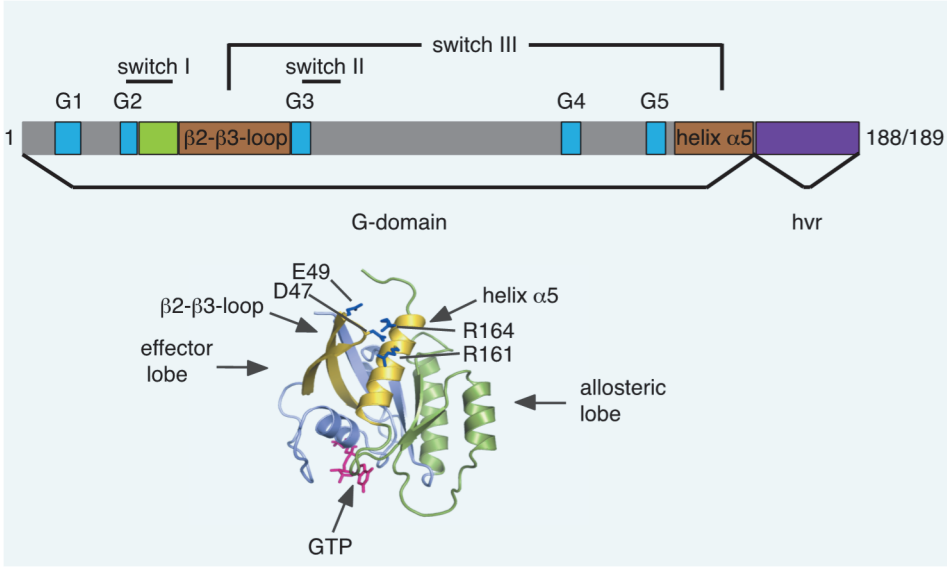


Figure 2. The structural features of the G-domain of Ras.
Ras is built of the globular G-domain and the unstructured hypervariable region (hvr) on its C-terminus (above, highlighted in violet). The nucleotide binding motifs (G1-G5 boxes) are highlighted in blue, while the effector-binding site in green. The elements of the novel switch III region are highlighted in brown. The structure of the soluble G-domain is well characterized (bellow). The G-domain can be split into the N-terminal effector lobe (residues 1-86, labeled in violet), through which the contacts with the nucleotide (labeled in pink) and effectors are established, and the C-terminal allosteric lobe (residues 87-171, labeled in green), which is involved in establishing the contacts with the membrane. In yellow are highlighted the β2-β3-loop in the effector lobe and the helix α5 in the allosteric lobe. They constitute the novel switch III region, which is implicated in the novel orientation mechanism of H-ras on the membrane, presumably by guiding the reorientation. This is established through the rearrangements of the hydrogen bonds between the residues D47, E49, R161 and R164 indicated in blue.

1.4. Ras signaling

Ras can be activated in response to distinct extracellular stimuli, such as hormones, growth factors and cytokines. For instance, binding of a growth factor to the extracellular domain of the receptor tyrosine kinases leads to its activation characterized by phosphorylation of numerous tyrosines in its cytoplasmic tail. Phosphorylated tyrosines serve as docking sites for Src homology 2 (SH2) domains of different proteins and recruit them to the plasma membrane. Among them are also Grb2 and Shc proteins (Li et al., 1993; Pelicci et al., 1992). They are adaptor proteins, without any enzymatic activity (Fig. 3). Besides the SH2 domain, Grb2 also contains two SH3 domains, which bind and recruit RasGEF SOS1 to the plasma membrane. When recruited to the plasma membrane, SOS1 can, in turn, activate Ras. (McKay and Morrison, 2007).

Once activated, Ras can initiate distinct downstream signaling cascades through binding of its effector proteins. Effectors usually contain a Ras binding domain (RBD) with a characteristic ubiquitin fold (Herrmann, 2003; Kiel and Serrano, 2006). At least 10 distinct signaling pathways are regulated by Ras, leading to various biological effects (Rajalingam et al., 2007). The three best characterized pathways are Raf, PI3K and RalGEF pathways (Fig. 3). The Raf (rapidly accelerated fibrosarcoma) pathway was the first described Ras signaling pathway. Its major biological effect is the stimulation of cell proliferation, but it is also involved in regulation of other, rather divergent processes, such as the cell differentiation, survival, senescence, apoptosis and migration (Lu and Xu, 2006; Roskoski, 2012). The Raf signaling pathway will be discussed in detail in the following section.

In the PI3K pathway, Ras recruits phosphoinositide-3-kinase (PI3K) for activation at the plasma membrane. Active PI3K then, in turn, generates phosphatidyl-inositol-3-phosphate (PIP3) on the plasma membrane by phosphorylating phosphatydyl-inositol-2-phosphate (PIP2). PIP3 serves as a docking site for the proteins containing pleckstrin-homology domain (PH domain). Among them is the tyrosine kinase Akt that upon activation phosphorylates its target proteins. Those proteins have important roles in regulating cell growth, proliferation and survival (Castellano and Downward, 2011).

In the third pathway, Ras induces activation of RalGEF (Ras-related protein GEF) proteins, which in turn activate RalA and RalB. They belong to the Ras family and regulate distinct processes, such as endocytosis, cell migration and gene expression (Neel et al., 2011).

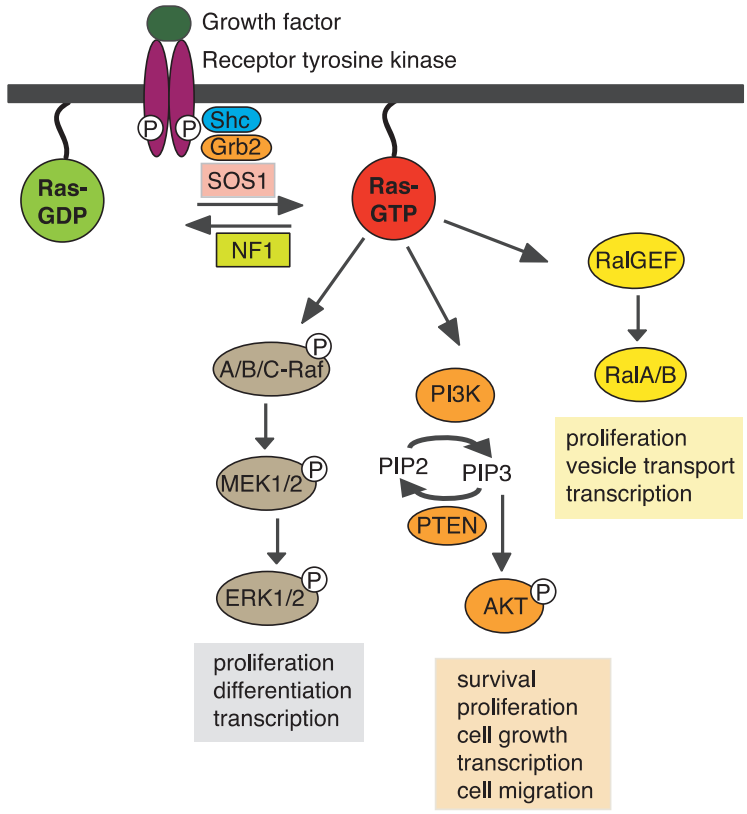


Figure 3: A simplified overview of the major Ras signaling pathways
Ras gets activated in response to distinct stimuli. For instance, binding of a growth factor to a receptor tyrosine kinase induces the phosphorylation of their cytoplasmic tails. Phosphorylated sites serve as a docking sites for adaptor proteins Shc and Grb2. Grb2 then binds and recruits Raf-GEFs, such as SOS1, which activate Ras by catalyzing the GDP-GTP exchange. Activated Ras activates several signaling pathways, of which the major ones are Raf, PI3K and Ral-GEF signalling pathways. In the Raf signaling pathway, activated Ras binds and recruits Raf kinases (A-Raf, B-Raf and C-Raf) to the membrane, where they become active after a series of phosphorylation events and dimerization. Active Raf phosphorylates and activates MEK kinases (MEK1 and MEK2), which in turn phosphorylate and activate ERK kinases (ERK1 and ERK2). ERK phosphorylates a plethora target proteins and initiates events, such as cell proliferation or differentiaion. Active Ras can also activate PI3K, which then converts membrane lipid PIP2 to PIP3. The kinase Akt binds PIP3 through its PH domain. That leads to Akt activation and phosphorylation of its target proteins and stimulation of cell growth, survival, cell migration and proliferation. Another well characterized Ras signaling pathway is Ral-GEF, which activates small GTPases RalA and RalB, which are involved in vesicular transport and endocytosis.

1.5. Raf signaling pathway

The Raf signaling pathway belongs to the group of 6 evolutionary conserved, mitogen-activated protein kinase (MAPK) signaling cascades, together with ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase (JNK) and the p38 pathways (Dhillon et al., 2007). The basic components of MAPK signaling cascades are three dual-specificity kinases, which can phosphorylate both serines/threonines and tyrosines of their downstream targets. They are called MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAPK. MAPKKK phosphorylates and activates MAPKK, which in turn phosphorylates and activates MAPK (Chen et al., 2001).

In the Raf pathway, Raf is the first kinase of the cascade, MAPKKK. There are three Raf isoforms in vertebrates, A-Raf, B-Raf and C-Raf (Cseh et al., 2014). The active Ras-GTP recruits them for activation at the plasma membrane. Raf proteins then activate the second kinases of the cascade, mitogen/extracellular signal-regulated kinase MEK1 and MEK2 (MAPKK), by phosphorylating two serine residues of their activation loop. Active MEKs in turn dually phosphorylate the last kinases of the cascade, extracellular signal-regulated kinases ERK1 and ERK2 (MAPK) (Plotnikov et al., 2011). Ultimately, activated ERK proteins phosphorylate their targets in the cytoplasm and nucleus, where they translocate from the cytoplasm after activation. ERK has many various targets, such as transcription factors, protein kinases and phosphatases (Roskoski, 2012).

The biological effects of ERK1/2 signaling vary depending on the stimulus. For instance, stimulation with EGF induces transient activation of ERK signaling, resulting in cell proliferation of rat adrenal pheochromocytoma neuronal cells (PC12), while stimulation with nerve growth factor (NGF) induces prolonged ERK activity, resulting in cell differentiation (Vaudry et al., 2002; York et al., 1998)

To accomplish the specificity of signaling responses, a precise spatiotemporal regulation exists. It is governed by various mechanisms, which include feedback loops, mediated either directly by ERK or by distinct phosphatases; cross-talks with other signaling pathways and formations of protein complexes (Kholodenko et al., 2010; Udell et al., 2011; Whitmarsh, 2007). Scaffolding proteins have an important contribution for the signaling specificity (Brown and Sacks, 2009; Vetterkind et al., 2013). They bind various components of the cascade, bring them together and regulate the kinetics of the signal transduction, by changing the duration and intensity of the signal. They also regulate the localization of the signaling pathway, cross-talk with other pathways and feedback loops. Among the ever-expanding scaffolds of the Ras-Raf pathway are kinase suppressor of Ras (KSR) (Zhang et al., 2013), MEK partner 1 (MP1) (Teis et al., 2002), prohibitin (PHB) (Rajalingam and Rudel, 2005), Shock2/Sur8 (soc-2 suppressor of clear homolog) (Matsunaga-

Udagawa et al., 2010) and IQ motif containing GTPase activating protein 1 (IQGAP1) (Roy et al., 2005).

The KSR proteins are the most extensively studied Ras-Raf signaling pathway scaffolds, which potentiate the pathway signaling (McKay et al., 2009). KSR proteins are homologues of Raf, with which they share a high sequence identity (Therrien et al., 1995). They are considered to be pseudokinases (Michaud et al., 1997; Roy et al., 2002; Stewart et al., 1999), although their kinase activity was demonstrated in several studies (Zhang et al., 2013). Upon the Ras activation KSR proteins translocate to the plasma membrane. Initial studies identified their binding to all three kinases of the Raf pathway, and it was proposed that they act as scaffolds, which enhance the Ras-Raf pathway signaling by bridging the components of the pathway (Nguyen et al., 2002; Roy et al., 2002; Stewart et al., 1999). However, the latest research demonstrated that instead of serving as a passive scaffold, KSR form side-to-side homo- and heterodimers with Raf proteins (Brennan et al., 2011; McKay et al., 2011; Rajakulendran et al., 2009). When dimerized with a Raf protein they induce their activity in an allosteric way. They use a similar structural mechanism as the Raf dimers described in the section 1.6.1.

1.6. Raf proteins

Vertebrates have three isoforms of Raf, A-Raf, B-Raf and C-Raf, while the lower organisms have only one Raf isoform to which B-Raf is the most similar (Desideri et al., 2015). The most intensively studied isoform is C-Raf, which was also the first one discovered (Beck et al., 1987). The focus switched to B-Raf after the observation that, unlike other Raf isoforms, it is often mutated in cancer, such as 40% of melanomas. The most frequent mutation, present in more than 80% of cancers with B-Raf mutation, is B-Raf-V600E (Davies et al., 2002). Although Raf isoforms share the same activators and effectors, they exhibit biological differences. It is considered that B-Raf is the prototypical Raf which activates only MEK, while the other isoforms evolved to perform additional functions (Desideri et al., 2015; Murakami and Morrison, 2001). For instance, C-Raf is considered to be important for the Rho signaling (Ehrenreiter et al., 2005). The function of A-Raf still remains unresolved. Compared to the other isoforms, B-Raf has the strongest ability to bind upstream Ras and to activate downstream MEK (Desideri et al., 2015).

Raf proteins are 55-75 kDa in size. They share three conserved regions (CR1-CR3). CR1 contains the RBD and the cysteine-rich domain (CRD) (Ghosh et al., 1994; Nassar et al., 1995) important for the recruitment and binding to the active Ras at the plasma membrane, CR2 contains phosphorylation sites required for the inhibition of Raf, while the CR3 contains the activation segment and the kinase domain (Chong et al., 2001; Dhillon et al., 2002; Tran et al., 2005). According to the

function, Raf structure can be divided into the N-terminal regulatory domain and C-terminal kinase domain. When inactive, Raf resides in the cytoplasm where its regulatory domain autoinhibits the kinase domain (Chong and Guan, 2003; Cutler et al., 1998). It is considered that the autoinhibitory state is further stabilized by the protein 14-3-3 which binds to the phosphorylated residues in both regulatory and kinase domain (S259, S621 residues in C-Raf) (Dhillon et al., 2002; Dumaz and Marais, 2003).

1.6.1. The activation of Raf kinases

The activated Ras recruits Raf to the plasma membrane. Here, the RBD of Raf contacts the effector binding sites of Ras, (Fabian et al., 1994; Nassar et al., 1995) while CRD contacts the C-terminal farnesyl moiety of Ras (Luo et al., 1997; Winkler et al., 1998). CRD also associates with the membrane lipid phosphatidylserine (PS) and is required for proper translocation of Raf to the plasma membrane (Bondeva et al., 2002; Ghosh et al., 1996; Roy et al., 1997). The binding of Raf to Ras induces a conformational change, which in turn activates a series of dephosphorylation, phosphorylation events and protein interactions which ultimately lead to the activation of the kinase domain (Lavoie and Therrien, 2015). Dephosphorylation of S259 leads to the release of the p14-3-3 from the regulatory domain (Jaumot and Hancock, 2001; Ory et al., 2003). For the activation, Raf then needs to be dimerized (Freeman et al., 2013; Rushworth et al., 2006; Weber et al., 2001). Raf isoforms can form homo- and heterodimers with each other, but also with KSR protein, each of them having different ability to further activate MEK. The most active is B/C-Raf heterodimer (Rushworth et al., 2006). Although dimerization was already described in 1996 (Farrar et al., 1996; Luo et al., 1996), it has been intensively studied only recently, when its physiological relevance was recognized. High incidence of B-Raf-V600E mutation in tumors led to the development of inhibitors, such as Vemurafenib, which bind B-Raf ATP-binding pocket and block the kinase activity. Although they inhibited tumors carrying B-Raf-V600E with high efficiency, they led to the paradoxical development of tumors in which wild-type B-Raf and mutated Ras were present (Carnahan et al., 2010; Freeman et al., 2013; Lavoie et al., 2013; Poulikakos et al., 2010). Other studies showed that mutations, which paradoxically impair the function of the B-Raf catalytic domain exhibit stronger downstream signaling (Garnett et al., 2005; Heidorn et al., 2010; Wan et al., 2004). In both cases Raf dimerization was augmented, either by inhibitors or by a mutation. Crystal structure studies revealed that dimerization occurs through the side-to-side interface of the kinase domains. Dimerization alosterically induces the conformational change in the catalytic cleft of the kinase domain promoting its active conformation (Lavoie et al., 2013; Thevakumaran et al., 2015). It was shown that Ras promotes dimerization of Raf. This could be explained by the fact that Ras forms dimers and oligomers

(nanoclusters) on the membrane (explained in detail in the following chapter) (Cho et al., 2012a; Poulikakos et al., 2010; Weber et al., 2001). The p14-3-3 was also shown to promote Raf dimerization, presumably through simultaneous binding of the phosphorylated residues in the regulatory domain in both kinases (Rushworth et al., 2006; Weber et al., 2001).

Beside dimerization, phosphorylation of distinct residues throughout the Raf sequence are involved in the promotion of kinase activity. They involve the phosphorylation sites in the activation segment; the N-region which consists of four amino-acids in the C-terminal end of the regulatory domain and phosphorylation site required for the binding of p14-3-3 in the kinase domain (Lavoie and Therrien, 2015). In most cases, the exact mechanism for kinase activity promotion is not completely clear.

2. Organization of Ras on the membranes

2.1. Cellular membranes

Beside their role of providing a physical barrier for the cell and cellular organelles, biological membranes have a key role to enable communication of the cell with the surrounding. Singer and Nicholson introduced the fluid mosaic model in 1972, which describes biological membranes as fluid lipid bilayer in which membrane proteins are incorported (Singer and Nicolson, 1972). According to the model, membrane proteins and lipids have random distribution and free lateral diffusion in the bilayer. We now know that cellular membranes are much more complex**.** Lipid bilayers contain more than 1000 different lipid species which are grouped into three major groups: phospholipids, sphingolipids and cholesterol (van Meer et al., 2008). Membrane lipids are amphipathic, thus having the hydrophilic head and hydrophobic lipid group. The hydrophilic part orients to the polar aqueous environment, while the hydrophobic part towards the middle of the bilayer (van Meer and de Kroon, 2011).

The lipid composition varies between membranes of different organelles, but also between outer and inner leaflets of the bilayer. For instance, PS, phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) are enriched in the inner leaflet of plasma membrane, while sphingolipids localize to the outer leaflet of the plasma membrane (van Meer, 2011).

Both lipids and proteins are not homogenously distributed, but rather segregate into proteo-lipid domains. Although many different membrane domains may exist, most of the research was focused on the so called lipid rafts. Lipids rafts were first defined as immobile liquid ordered domains of 100-500 nm in size, enriched in cholesterol and saturated sphinoglipids. They serve as sites for segregation of specific proteins (Simons and Ikonen, 1997; Simons and van Meer, 1988). This initial lipid raft concept has been controversial, because it is based on a debatable experimental evidence (Hancock, 2006; Munro, 2003; Shaw, 2006). For instance, the phase separation into liquid ordered and liquid disordered domains, observed in the simple model membranes (Ahmed et al., 1997), were not confirmed in the membranes of living cells (Maxfield and Mayor, 1997). Also, the biochemical methods used for the isolation of detergent resistant membranes (Brown and Rose, 1992), which are considered to reflect lipid rafts, are very crude and could lead to formation of membrane structures which do not reflect the constitution of physiological membrane (Foster et al., 2003; Heerklotz, 2002). Furthermore, the results obtained by the method of cholesterol depletion, using for instance cyclodextrin treatment, are also difficult to interpret, since these treatments can have additional effects on the

cell, such as change in permeability and fluidity of the membrane (Kurzchalia and Ward, 2003; Ohvo-Rekilä et al., 2002).

In recent years, lipid raft theory has shifted, due to the development of more sensitive super-resolution microscopy and other biophysical techniques. They are now called membrane rafts which are considered to be rather small (10-100 nm) and dynamic, and their formation is governed by complex interactions between proteins and lipids (Pike, 2006; Simons and Gerl, 2010).

2.2. The spatial dynamics of Ras in the cell

2.2.1. Binding of Ras to the membrane

Ras proteins establish contacts with the membrane through their C-terminal hvr. The membrane attachment and localization is required for their function. They are primarily localized to the plasma membrane, although they can also localize to different endomembrane systems, such as endoplasmatic reticulum (ER), golgi apparatus (GA), endosomes and mitochondria (Prior and Hancock, 2011). The membrane attachment varies between the isoforms. All the Ras isoforms share a unique motif on their C-terminus, called the CAAX box, whose cysteine becomes farnesylated (Hancock et al., 1989). The CAAX motif contains cysteine (C), followed by two aliphatic (AA) and any (X) amino acid. Once the protein is synthesized in the cytoplasm, the CAAX box becomes modified in a series of three reactions, as reviewed in (Wright and Philips, 2006) (Fig. 4). First, a farnesyl moiety is attached to the cysteine via a thioether bond by a farnesyl protein transferase (FTase). In the next two steps, which occur on the ER, the AAX motif is first cleaved by the Ras-converting enzyme-1 (Rce1). After the AAX motif cleavage, the isoprenyl cysteine transferase (Icmt) catalyzes the carboxymethylation of the farnesylated cysteine. The membrane binding achieved by farnesylation is further enhanced by additional membrane binding motifs in their hvr region (Hancock et al., 1989; 1990).

H-ras, N-ras and K-ras4A are targeted to the GA, where they become palmitoylated by the enzymes palmitoyl acyltransferases (PATs) (Swarthout et al., 2005) (Fig. 4). PATs predominantly localize to the membranes of GA where they catalyze the attachment of the palmitate moiety to the cysteine via a thioester bond (Rocks et al., 2010). H-ras is di-palmitoylated on the residues Cys181 and Cys184, while N-ras and K-ras4A are mono-palmitoylated on the residues Cys181 and Cys180, respectively (Hancock et al., 1989). Palmitoylated isoforms then traffic unidirectionally from the GA to the plasma membrane in an exocytic vesicular fashion, presumably via the recycling endosomes (Apolloni et al., 2000; Choy et al., 1999). Both N-ras and K-ras4A, besides being palmitoylated, also contain charged polybasic regions (PBRs). In the case of N-ras the PBR is located upstream of hvr, between residues 166-171, while K-ras4A has two PBRs, PBR1 contains residues 167-

170 and PBR2 residues 182-185 (Laude and Prior, 2008; Tsai et al., 2015). PBRs of both N-ras and K-ras4A are required for their proper localization.

On the other hand, K-ras4B is not palmitoylated, but instead its hvr contains a hexalysine PBR (Hancock et al., 1990) (Fig. 4). PBR interacts electrostatically with the negatively charged lipids in the membrane, which are abundant in the plasma membrane. Abundance of the negatively charged lipids results in the predominant localization of K-ras in the plasma membrane to where it travels directly from the ER (Apolloni et al., 2000; Okeley and Gelb, 2004).

2.2.2. Ras trafficking

Ras proteins are highly dynamic and they are constantly moving inside the cell, between different membrane compartments. Unlike farnesylation, both electrostatic interactions and palmitoylation are reversible processes, and their reversibility enables Ras proteins to traffic between the different compartments. Despite their constant movement, they have a very determinate localization, predominantly to the plasma membrane. The knowledge on the spatial organization of Ras is increasingly growing in the recent years, with many new factors which affect their localization and trafficking, such as enzymes, posttranslational modifications or chaperons, being constantly revealed (Prior and Hancock, 2011; Schmick et al., 2015).

Palmitoylation of H-ras, N-ras and K-ras4A determines their stable association with the membrane by a so-called mechanism of kinetic trapping (Schmick et al., 2015; Schroeder et al., 1997). After some time, they become de-palmitoylated, and rapidly translocated to the GA in a non-vesicular fashion (Goodwin et al., 2005; Rocks et al., 2005). On the GA they are available for the next round of palmitoylation and recycling to the plasma membrane (Rocks et al., 2010). So far, it was shown that H-ras and N-ras can be de-palmitoylated by the acyl protein thioesterase (APT1 and APT2), which catalyze the thioester bond hydrolysis (Dekker et al., 2010; Rusch et al., 2011). The prolyl isomerase FKBP12 also affects the depalmitoylation of H-ras (Ahearn et al., 2011). The FKBP12 isomerizes the bond Gly^{178}-Pro^{179} in H-ras, which in turn facilitates the depalmitoylation by APTs. Since the electrostatic interactions are relatively weak, K-ras4B is less stably bound and has a short residency time on the membrane (Silvius et al., 2006).

Due to the depalmitoylation and/or destabilization of electrostatic interactions, but also after initial farnesylation after its synthesis, the hydrophobic prenylated Ras species are found in the unfavorable hydrophilic cytoplasm. In the recent years it was shown that the movement of the free prenylated Ras species in the cytoplasm can be supported by certain chaperons, which solubilize them, but also enable their delivery to specific locations (Fig. 4). The delta subunit of the phosphodiesterase 6 (PDEδ) was found to bind to various proteins including H-ras, N-ras and K-ras4B, independent on their activation state (Nancy et al., 2002). PDEδ is called the GDI-

like factor because it resembles the structure of the Rho-GDI-factors which solubilize members of the Rho family of small GTPases (Hanzal-Bayer et al., 2002). It contains a hydrophobic pocket which can accommodate the farnesyl moiety of prenylated and de-palmitoylated proteins (Chandra et al., 2012), and solubilize them. PDEδ can also bind the Arl2, a member of the Arf family of small GTPases, which is not prenylated (Hanzal-Bayer et al., 2002; Nancy et al., 2002). PDEδ binds to the Arl2 in the perinuclear space inducing the discharge of the Ras protein bound to PDEδ (Schmick et al., 2014). The perinuclear space is composed of different endomembranes, such as GA and endosomal membrane. Once discharged from the PDEδ, H-ras and N-ras can bind to the membranes of GA, where they can become palmitoylated by GA-localized PATs and get kinetically trapped. On the other hand, K-ras4B binds to recycling endosome, since its membranes are enriched in negatively charged lipids, from where it can travel back to the plasma membrane (Schmick et al., 2014). Therefore, the PDEδ represents a Ras chaperone which, together with Arl2, is involved in regulation of the Ras spatial organization (Schmick et al., 2015). Another two chaperons, which are the splice variants of the gene SmgGDS, called SmgGDS-607 and SmgGDS-558, can bind the small GTPases which contain the PBR region, such as K-ras4B. SmgGDS-607 binds to the non-prenylated proteins and is involved in the regulation of their prenylation after synthesis, while SmgGDS-558 binds to the prenylated proteins and is involved in the regulation of their localization (Berg et al., 2010; Schuld et al., 2014). Ras localization can also be regulated by phosphorylation and ubiquitination. K-ras4B can be phosphorylated by protein kinase C on the residue Cys181, which leads to weakening of the electrostatic interaction of K-ras4B with the plasma membrane and its subsequent relocalization to the endomembranes, for instance ER, GA and mitochondria. On the mitochondria it binds to the pro-apoptotic protein BclXL and induces apoptosis (Quatela et al., 2008). However, other studies have shown that the relocalization to endomembranes is only partial, and the phosphorylated K-ras-GTP is found in high abundance on the plasma membrane where it is segregated into distinct plasma membrane domains and its signaling is increased (Alvarez-Moya et al., 2011; Barceló et al., 2013; 2014). Phosphorylation is inhibited by calmodulin, which binds to the PBR of K-ras (Alvarez-Moya et al., 2010; 2011). Once the interaction of calmodulin and K-ras is inhibited by the calmodulin kinase II calmodulin-binding domain phosphorylation can occur (Villalonga et al., 2001).

On the other hand, ubiquitination can modulate localization of H-ras and N-ras, but not K-ras4B. Ubiquitination of the Lys 63 is catalyzed by Rabex-5 and it stimulates internalization of H-ras and N-ras from the plasma membrane to the early endosomes and decreases the Ras-Raf pathway signaling (Jura and Bar-Sagi, 2006; Rodriguez-Viciana and McCormick, 2006) .

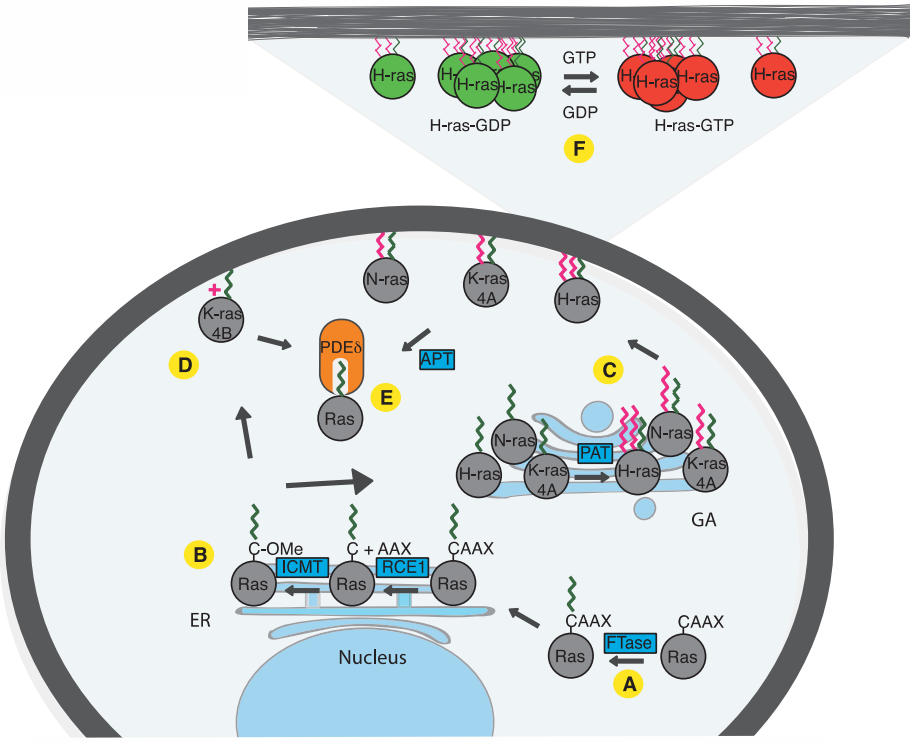


Figure 4. Ras cellular trafficking and nanoclustering on the plasma membrane.
After the synthesis of Ras proteins in the cytoplasm, FTase catalyzes the attachment of the farnesyl moiety to the cysteine on their N-terminal CAAX box (**A**). This induces the translocation of Ras to ER, where the CAAX box becomes further modified by the enzymes Rce1 and Icmt (**B**). From ER, the isoforms H-ras, N-ras and K-ras4A travel to the GA. Here PAT enzymes catalyze the mono-palmitoylation of N-ras and K-ras4A, and di-pamitoylation of H-ras (**C**). From the GA these three isoforms travel to the plasma membrane in a vesicular fashion. K-ras4B travels to the plasma membrane directly to the ER (**D**). It is not palmitoylated, but contains a polybasic hexalysine patch, which establishes electrostatic interactions with the lipids in the plasma membrane. After the depalmitoylation or destabilization of the electrostatic interactions farnesylated isoforms in the cytoplasm can be solubilized by distinct chaperons, such as PDEδ (**E**). On the plasma membrane, Ras proteins organize into nanoclusters, which are distinct between the isoforms and dependent on their activation state (**F**).

2.3. Nanoscale organization of Ras proteins on the membrane

In recent years, development of different techniques enabled looking at the nanoscale organization of proteins at the plasma membrane. These observations led to the recognition of two novel phenomena in behavior of Ras proteins, both of them isoform- and nucleotide state- dependent: 1. they exhibit distinct conformations in regard to the membrane and 2. they laterally segregate and form dimers and larger oligomers, termed nanoclusters (Zhou and Hancock, 2015).

2.3.1. The orientation of Ras on the membrane

Ras proteins can adopt specific guanine-nucleotide dependent conformational states on the membrane. This phenomenon was first identified using the molecular dynamics (MD) simulations of the full length H-ras anchored to the 1,2-dimyristoylglycero-3-phosphocholine (DMPC) bilayer (Gorfe et al., 2007). It was shown that on the membrane H-ras adopts two different conformation states, also called orientations (Fig. 5). They exist in an equilibrium, which is shifted with the binding of the nucleotide. The orientation predominant in the H-ras-GDP form is stabilized through interaction of the hvr with the membrane. The basic residues R169 and K170 are the major contacts, which form hydrogen bonds with the phosphate groups of the membrane. The stabilization of the orientation predominant in the H-ras-GTP form is established through the helix α4, with the residues R128 and R135 forming the major contacts with the membrane.

Our group has shown that mutating the critical orientation stabilizing residues to neutral alanines changes the activity of H-ras-GTP, presumably through changing its orientation (Abankwa et al., 2008; 2010). Mutations in the hvr residues, which stabilize the H-ras-GDP conformation increase, while the mutations in the helix a4 residues, which stabilize the H-ras-GTP orientation decrease the activity of H-rasG12V. Thus, the mutant H-rasG12V-R169A,K170A has increased recruitment of the Ras binding domain (RBD) of the effector C-Raf or PI3K. Increase in the activity of the mutant is reflected on the stronger Raf pathway signaling, measured by PC12 cells differentiation assay. On contrary, the mutant H-rasG12V-R128A,R135A displays reduced RBD-recruitment and PC12-cell differentiation.

In addition to the orientation-stabilizing regions, MD simulations identified orientation-dependent rearrangements within helix α5 and β2-β3-loop, where residues D47, E49, R161, R164 form distinct salt bridge networks (Abankwa et al., 2008). It was proposed that helix α5 and β2-β3-loop function as a novel switch region, termed switch III, which guides the reorientation of H-ras on the membrane upon GTP loading or hydrolysis (Abankwa et al., 2008). Similar to the mutations in the orientation-stabilizing residues, certain mutations in the switch III residues altered the signaling strength of the H-rasG12V, which was measured by PC12 cell differentiation assay. The mutant H-rasG12V-D47A,E49A increased, while the

mutant H-rasG12V-R161A decreased the PC12 cell differentiation (Abankwa et al., 2008).

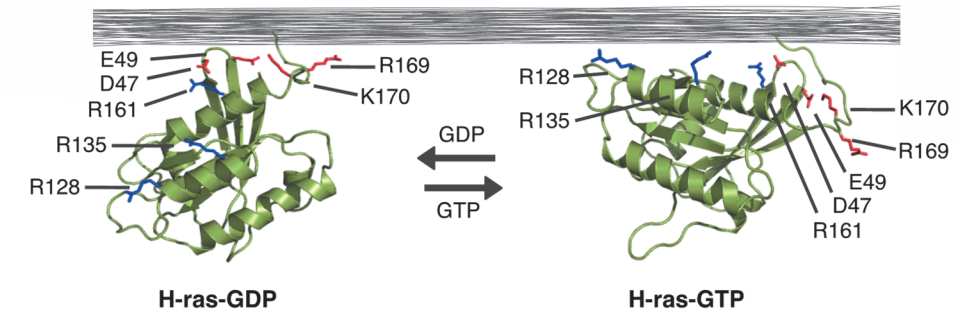


Figure 5. H-ras membrane-orientation mechanism.
The figure is modified from (Gorfe et al., 2007). It represents the two nucleotide dependent orientations of the membrane bound H-ras derived from the MD simulations. The residues R169 and K170 in the hvr stabilize the H-ras-GDP orientation, while the R128 and R135 in the helix α4 stabilize the H-ras-GTP orientation. It is suggested that the switch III region, composed of helix α5 (critical residues D47 and E49) and β2-β3-loop (critical residue R161 is highlighted) guides the reorientation (Abankwa et al., 2008). Residues highlighted in blue represent the decreased H-rasG12V activity when they are mutated to alanines, while the ones highlighted in red represent that the H-rasG12V activity is increased once the mutations are introduced.

It was demonstrated that other two isoforms, K-ras and N-ras also adopts isoform-specific and nucleotide-dependent orientation states on the plasma membrane. MD simulations showed that contrary to H-ras, the K-ras-GDP conformation is stabilized by the helix α4, while the K-ras-GTP conformation is stabilized by the hvr (Abankwa et al., 2010). Thus, the mutant K-rasG12V-R169A,K170A displays decreased, while the mutant K-rasG12V-R128A,R135A increased RBD recruitment. When introduced into the N-rasG12V, these mutations increase the RBD recruitment in both cases, indicating yet another orientation mechanism (Abankwa et al., 2010). Thus, effector proteins distinguish between different Ras orientations on the membrane and lead to distinct signaling outputs. However, it remains unknown how can orientation on the membrane affect Ras activity.

Recent studies provided more evidence supporting the existence of different orientation of Ras on the membrane (Kapoor et al., 2012a; 2012b; Mazhab-Jafari et al., 2015). Spectroscopic studies of the full length K-ras and N-ras demonstrated that N-ras adopts two conformation states similarly as molecular dynamics simulations

suggested for H-ras (Kapoor et al., 2012a). K-ras-GDP also adopts a stable conformation on the membrane, in which helices are parallel to the membrane, while K-ras-GTP is more flexible, and does not adopt any preferred conformation (Kapoor et al., 2012b). More recently, NMR was used to study conformations of K-ras tethered to the nanodisc membrane (Mazhab-Jafari et al., 2015). K-ras adopted two distinct orientations, depending on the bound nucleotide. Similar to the spectroscopic study(Kapoor et al., 2012b), in the K-ras-GDP orientation most of the helices are parallel to the membrane. In the GTP-bound state, K-ras displays a conformation in which the effector binding part is occluded. Furthermore, they determined the structures of K-ras-GTP bound to the RBD of A-Raf and Ral-GDS. The effector binding requires the reorientation of the occluded state. Upon the formation of the complex with the effector, the occluded conformation is released. They also revealed that Noonan syndrome related mutations K5N and D153V disturb the occluded orientation of the K-ras, which then directly increases the binding of the effector (described in details in the following chapter).

2.3.2. Ras nanoclustering

In recent years, it was realized that many membrane proteins do not have random distribution on the membrane, but rather form clusters. For instance, GPI-anchored proteins are various proteins which are tethered with their glycosylphosphatidylinositol anchor to the outer leaflet of the plasma membrane. Here, 20-40 % of them segregate into nanoclusters smaller than 5 nm, which consist of at most 4 proteins (Mayor and Riezman, 2004; Sengupta et al., 2011; Sharma et al., 2004; van Zanten et al., 2010). They are dependent on cholesterol and sphingomyeline, thus they have been used as the lipid raft markers (Mayor and Riezman, 2004).

Clustering was also identified for Ras proteins, and it is termed nanoclustering, due to cluster's nano-dimension of only ≈20 nm in diameter (Plowman et al., 2005). The phenomenom of Ras nanoclustering is studied by different methodological approaches, such as electron microscopy (EM), single particle tracking (SPT), fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer measured by fluorescence lifetime microscopy (FLIM-FRET) (Hancock, 2003; Murakoshi et al., 2004; Plowman et al., 2008; Prior et al., 2003). Only a fraction of 40 % Ras proteins is found in nanoclusters, while others exist as monomers (Plowman et al., 2005; Prior et al., 2003) (Fig. 4). Nanoclusters are built from approximately 6-8 Ras molecules (Plowman et al., 2005). Their lifetime is only 0.1-1 s (Hancock and Parton, 2005; Hibino et al., 2003; Murakoshi et al., 2004), making them very dynamic entities which are being constantly assembled and disassembled**.** Nanoclustering is essential for Ras signal transduction, since with the decrease in Ras nanoclustering, the signaling output is also decreased. In fact, it was

demonstrated that effectors are binding to nanoclusters only, thus they serve as platforms to which effectors are recruited for their activation (Tian et al., 2007). Furthermore, nanoclusters establish an analog-digital-analog circuit, to enable a high precision signal transmission across the plasma membrane (Harding and Hancock, 2008b). Signaling stimulant, such as EGF, operates in an analogous way. Depending on the amount of EGF, certain amount of active Ras nanoclusters is formed. Nanoclusters then generate signal in a digital fashion, always producing the maximal signaling output during their lifetime. The final pERK signaling output is again analogous, being a sum of all the digital signals emitted from the nanocluster. In this way it is ensured that the EGF signaling input precisely matches the pERK signaling output (Harding and Hancock, 2008b; Kholodenko et al., 2010; Tian et al., 2007).

The segregation of Ras into nanoclusters is determined by the complex interactions of Ras protein with the membrane. Ras interacts with the membrane through its lipid anchors and the linker domain in the hypervariable region, but also through specific residues in the G-domain, which differ with the nucleotide-dependent Ras orientation (explained in detail in the previous section). Since the different Ras isoforms establish distinct interactions with the membrane, nanoclustering is isoform-specific, meaning that H-ras, N-ras and K-ras will form distinct nanoclusters (Hancock and Parton, 2005; Plowman et al., 2005). Also, for the same isoform, nanoclusters are different depending on the activation state of the protein, e.g. H-ras-GTP and H-ras-GDP nanoclusters are distinct (Janosi et al., 2012; Prior et al., 2001; 2003).

Since Ras proteins interact differently with the membrane lipids, each nanocluster type has a specific lipid composition, which is only beginning to be understood. The early studies clearly demonstrated the dependency of nanoclustering on the cholesterol composition. Nanoclustering of the minimal anchor of H-ras, termed tH, and H-ras-GDP is dependent on cholesterol. Upon activation H-ras-GTP segregates into cholesterol-independent nanoclusters (Niv et al., 2002; Plowman et al., 2005; Prior et al., 2001; 2003). In the case of K-ras, the minimal anchor of K-ras, termed tK, K-ras-GDP and K-ras-GTP all form nanoclusters which are independent on cholesterol (Niv et al., 2002; Plowman et al., 2005). tN and N-ras-GDP nanoclusters are also cholesterol-independent, while the N-ras-GTP nanoclusters are cholesterol-dependent (Plowman et al., 2005). Nanoclustering of K-ras4A has not yet been studied. Recent study has demonstrated that different nanoclusters have distinct composition of anionic lipids, such as PS, PA, PIP2, PIP3, phosphatidylinositol 3-phosphate (PI3P), and phosphatidylinositol 4-phosphate (PI4P) (Zhou et al., 2014). It is not yet clear what is the importance of each of these lipids for nanoclustering. It was demonstrated that PS is a crucial component of K-ras nanoclusters. Changing the level of PS or its distribution in the plasma membrane drastically affects nanoclustering of both tK and

K-ras-GTP, while tH and H-ras-GTP nanoclustering is not affected (Zhou et al., 2014). For example, lowering the PS levels leads to decreased K-ras nanoclustering and also to the removal of K-ras from the plasma membrane (Cho et al., 2013; van der Hoeven et al., 2013; Zhou and Hancock, 2015). It seems that lipid distribution is very important for the segregation of isoforms into distinct nanoclusters. For instance, if the concentrations of cholesterol and PS fall outside the optimal concentration, the heterotypic clustering between K-rasG12V and H-rasG12V or H-ras(wt) and H-rasG12V occurs (Ariotti et al., 2014; Zhou et al., 2012).

However, despite the accumulated evidence for a non-random Ras distribution on the membrane, the evidence demonstrating the biological significance of Ras nanoclustering in the (patho)physiological conditions is still missing.

2.3.3. Ras dimerization

In recent years distinct studies have reported the existence of Ras dimers, rather than nanoclusters. The dimerization of full length N-ras bound to the POPC membrane was demonstrated using computational simulations (Güldenhaupt et al., 2012). In the dimers N-ras establishes an orienatation perpendicular to the membrane, which correlates with the orientation they observed by FTIR spectroscopy. It was proposed that the dimerization interface is established through the helix α4, helix α5 and β2-β3-loop. Another study demonstrated the existence of H-ras dimers on the artificial membranes using a combination of time-resolved fluorescence spectroscopy and microscopy (Lin et al., 2014). They demonstrated that dimerization is a general property of the membrane-bound H-ras. They identified the switch II region to be crucial for the dimer formation, presumably as a part of the dimer interface or through allosteric coupling to it. Dimerization of the G-domain of K-ras in solution was demonstrated by a recent study (Muratcioglu et al., 2015). The two most populated dimeric interfaces determined by NMR are the α interface involving helices α3 and α4, and the β interface involving helix α1, β2 and β3 sheets. A recent study has demonstrated the existence of K-ras dimers also in cells by employing a quantitative photoactivated localization microscopy (PALM) and it was demonstrated that Ras dimers, rather than oligomers, represent the Ras signaling units (Nan et al., 2015). These results gave rise to a new controversy in the field, which urges further studies to better describe the Ras complexes formed on the membrane.

2.4. Modulators of Ras nanoclustering

So far, different mechanisms have been recognized, which can modulate the level and stability of nanoclusters and, as a direct consequence, the signaling output. For instance, proteins Gal-1 and Gal-3 increase the H-ras-GTP and K-ras-GTP nanoclustering, respectively (Belanis et al., 2008; Shalom-Feuerstein et al., 2008). Nucleophosmin and nucleolin bind to membrane bound K-ras, regardless of the nucleotide bound, enhance its levels on the membrane, and in the case of nucleophosmin also increase its nanoclustering (Inder et al., 2010; 2009). All four proteins are often overexpressed in cancer, underscoring the importance of Ras signaling pathways strength for cancer development. Recently, it was shown that H-ras-G12V can negatively regulate K-rasG12V nanoclustering, in a phenomenon termed spatial cross-talk (Zhou et al., 2014). This occurs because both H-rasG12V and K-rasG12V sequester PS in their nanoclusters. Thus, increase in the H-rasG12V nanoclusters reduces the PS pool available for the K-rasG12V nanoclusters and K-rasG12V nanoclustering is reduced. Both actin cytoskeleton and caveolae also sequester the PS on the plasma membrane (Zhou et al., 2014). Consequently, decrease in caveolae increase (Ariotti et al., 2014), while decrease in actin decrease K-ras-GTP nanoclustering (Plowman et al., 2005). As described in the section 2.2.2., the phosphorylation of Cys181 by protein kinase C segregates K-ras-GTP into distinct plasma membrane domains and enhances signaling output (Barceló et al., 2013; 2014).

Ras nanoclusters serve as the sites of effector recruitment and activation, thus their perturbation has been recognized as a prominent drug target (Cho and Hancock, 2013). Since the lipid environment is crucial for Ras nanoclustering, changing it could lead to the defective nanoclustering. For instance, it was shown that fendiline and staurosporine induce relocalization of K-ras to the endomembranes and decrease its nanoclustering on the plasma membrane. They do that by altering the PS trafficking to the plasma membrane (Cho et al., 2013; 2012b; van der Hoeven et al., 2013). Indomethacine, a non-steroidal anti-inflammatory drug, increases and stabilizes the cholesterol-dependent nanoclustering of tH, H-ras-GDP and N-ras-GTP. At the same time, N-ras-GDP and H-ras-GTP lateral segregation into the cholesterol-independent nanoclusters is hindered, resulting in the formation of mixed, heterotypic, nanoclusters. The homotypic K-ras nanoclustering is not affected, but the heterotypic aggregates with the tH cholesterol-dependent nanoclusters form, in which the actual mixing does not occur. (Zhou et al., 2010). Another membrane intercalators, bile acids, interact specifically with disordered domain and affect its organization. Consequently, tK and K-rasG12V, but not H-rasG12V and tH, nanoclustering and its downstream signaling is increased (Zhou et al., 2013). Interestingly, nanoclustering of N-ras and K-ras is also altered by

inhibitors of B-Raf-V600E, which were previously shown to induce dimerization of B-Raf with B-Raf or C-Raf. The formed Raf dimers crosslink the Ras molecules and consequently increase the probability of the nanoclusters formation (Cho et al., 2012a).

2.5. Galectin-1 is a regulator of H-ras nanoclustering

Gal-1 is a 14.5 kDa protein, which belongs to the family of galectins. There is 15 known mammalian galectins and they all share a highly conserved carbohydrate binding region (CBR), which can bind β-galactosides. Galectins can be segregated into three groups, depending on their CBR, proto-type galectins have one CBR (Gal-1, -2, -5, -7, -10, -11, -13, -14 and -15), tandem-repeat-type galectins have two CBRs connected with a linker domain (Gal-4, -8, -9 and -12) and chimeric-type have one CBR which is fused to a non-lectin domain (Gal-3)(Camby et al., 2006). The structure of Gal-1 consists of two antiparallel β-sheets. Gal-1 can exist as a monomer or a dimer (Bourne et al., 1994; López-Lucendo et al., 2004). It is localized in the nucleus and cytoplasm of the cell from where it can get secreted to the extracellular space, through an unconventional mechanism, which is dependent on the interaction with glycosylated cell surface receptors (Seelenmeyer et al., 2008).

Gal-1 is upregulated in many tumor cells and the surrounding stroma and its upregulation is usually related to more progressive and invasive stages of cancer (Ebrahim et al., 2014; Satelli et al., 2008). Rather than having a unique role, this multifaceted protein is involved in many processes of the tumor development: cell transformation, radio- and chemoresistance through intracellular binding of activated H-ras, tumor growth, angiogenesis, cell adhesion, metastasis and immunosuppression through extracellular binding of different N- or O-glycosylated proteins (Astorgues-Xerri et al., 2014; Ebrahim et al., 2014; Ito et al., 2012).

Gal-1 is a known modulator of H-ras signaling. It was identified as an interacting partner of farnesylated Ras using the biochemical crosslinking and co-immunoprecipitation with the pan-Ras or Gal-1 antibody (Elad-Sfadia et al., 2002; Paz et al., 2001). It was shown that it binds preferentially to H-ras and more weakly to K-ras, but not to N-ras. Gal-1 can also recognize the activation state of Ras, binding strongest to H-ras-GTP. Computational structural analysis of the Gal-1 structure superimposed with the Rho-GDI structure suggested that Gal-1 could also, similarly to Rho-GDI, have a hydrophobic cavity which could accommodate farnesyl chain of H-ras (Rotblat et al., 2004). Mutation L11A in the hydrophobic cavity increases the fraction of H-ras-GTP in the cytoplasm, although this mutant has the same binding to H-ras-GTP as the wild-type Gal-1. It was also proposed that Gal-1 acts as a chaperone for de-palmitoylated H-ras-GTP, which delivers it to the GA (Belanis et al., 2008; Paz et al., 2001).

Overexpression of Gal-1 was found to affect the H-ras signaling output by simultaneously increasing and prolonging the MAPK-pathway signaling, while decreasing the PI3K-pathway signaling (Elad-Sfadia et al., 2002). Increased MAPK signaling and also cell transformation is due to its ability to increase and stabilize the H-ras-GTP nanoclustering (Belanis et al., 2008; Prior et al., 2003; Rotblat et al., 2010) (Fig. 6). Gal-1 can also distinguish between the specific orientations of H-ras on the membrane, interacting more with the GTP-like orientation (Abankwa et al., 2010).

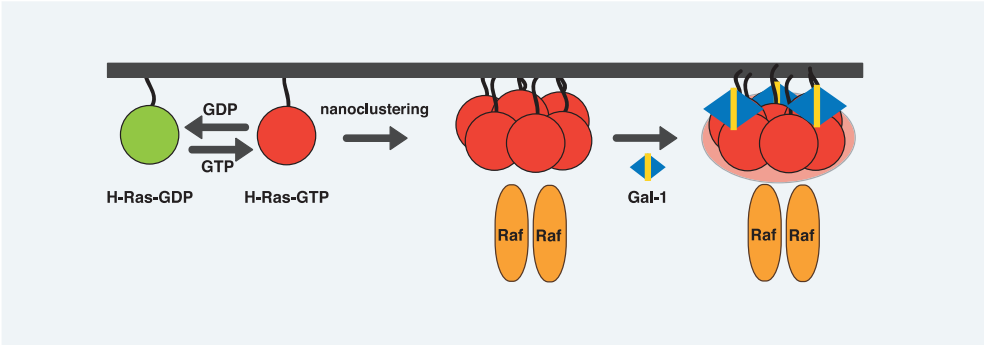


Figure 6. The current model of H-ras-GTP nanoclustering regulation by Gal-1.
After H-ras activation H-ras-GTP forms nanoclusters, which serve as recruitment and activation sites for Raf effectors. Gal-1 binds to the farnesyl moiety of H-ras-GTP and stabilizes the nanoclusters. Increased H-ras-GTP nanoclustering results in stronger downstream signaling. The scheme is modified from (Belanis et al., 2008).

3. Ras in diseases

Misregulation of Ras often leads to pathogenesis. Shortly after its discovery it was demonstrated that somatic mutations in Ras genes are frequently present in tumors, where they are one of the most important drivers of the oncogenesis (Pylayeva-Gupta et al., 2011). More recently it was discovered that Ras can also be mutated in the germline, where it gives rise to various developmental diseases which belong to the RASopathies group of diseases (Fernández-Medarde and Santos, 2011).

3.1. Ras in cancer

Tumor development is a long process during which normal cells accumulate several somatic mutations in their DNA (Stratton et al., 2009). Some of these mutations provide cells with advantages compared to normal cells, such as uncontrolled proliferation, anchorage-independence and immortality, which lead to the outgrowth of the cell with the mutation (Hanahan and Weinberg, 2011). Somatic mutations can be deletions, insertions, substitution of nucleotide bases, chromosomal rearrangements and gene amplifications or deletions. A cancer cell usually carries many mutations, which can be classified either as driver mutations or passenger mutations (Greenman et al., 2007). Driver mutations contribute to development of cancerous properties and are considered to be required for the oncogenesis. Passenger mutations are present in the genome, but do not seem to have an impact on the cancer development. It was recently proposed that there should be a third class of mutations, the so-called latent drivers. They do not seem important for the cancer development, but could play an important role for providing cancer cells with specific advantages in certain situations, such as the acquisition of the drug resistance (Nussinov and Tsai, 2015).

Cancer mutations are deposited into different databases, such as Catalogue of Somatic Mutations in Cancer (COSMIC) database, which is considered to be the most thorough (Forbes et al., 2011). According to the COSMIC database, Ras is mutated in 27% of cancers. The frequency of mutations is not the same for different isoforms. K-ras mutations occur in 19% of tumors, which makes it the most frequently mutated oncogene. N-ras mutations are found in 5% and H-ras in only 2.6% of mutations. Different isoforms are also not equally mutated among different cancer types (Prior et al., 2012; Pylayeva-Gupta et al., 2011), except in the thyroid cancer where mutations of all three isoforms are equally distributed. K-ras is the preferentially mutated Ras isoform in pancreatic cancer (>60% of incidence), lung cancer (>30% of incidences) and colorectal cancer (>40% of incidence); N-ras mutations in skin cancer (>20% of incidence) and hematopoietic cancers (>15%); and H-ras in bladder cancer (>10%) (Pylayeva-Gupta et al., 2011).

3.1.1. Ras hot-spot mutations

More than 99% of Ras mutations are substitutions in residues G12, G13 and Q61 (Fig. 7). Therefore, they are called hot-spot mutations. Although mutations in all three hot-spot residues affect Ras proteins in a similar manner, they are differently distributed among distinct isoforms. K-ras is preferentially mutated in G12 residue (90% of K-ras mutations), N-ras in Q61 (60% of N-ras mutations) and H-ras equally in both G12 and Q61 (40%/50% respectively) (Prior et al., 2012).

Ras hot-spot mutations lead to impaired Ras hydrolysis, leaving the mutants constitutively active. In order to catalyze the GTP-hydrolysis GAP proteins insert the, so-called Arg finger into the hydrolysis site of Ras, where it serves to neutralize the negatively charged γ-phosphate after the hydrolysis reaction. GAPs also help to position the Q61 residue into the hydrolysis site. Q61 residue in the switch II is important for the activation of the water molecule required for the nucleophilic attack during the hydrolysis reaction (Scheffzek et al., 1997). The residue G12 is in the van der Waals proximity to the Arg finger of GAP. Mutations in G12 lead to sterical clashes, which prohibit the positioning of Arg finger in the hydrolysis site. Thus, mutations in the G12 residue of Ras lead to its insensitivity to GAP-mediated GTP hydrolysis, although they can bind to GAP with the same affinity as wild-type Ras (Scheffzek et al., 1997; Wittinghofer and Vetter, 2011). The structural effects of the mutations in G13 residue has not been studied in detail, but because of their proximity to hydrolysis site it is considered that they would also, similar to mutations in G12, lead to sterical clashes which would disable the formation of the transition state with Arg finger of GAP proteins. In the case of G12, any substitution, except G12P, leads to insensitivity to GAP, while in the case of G13 only substitutions to larger amino-acids lead to such effects. Residue Q61 gets positioned into the hydrolysis site after GAP binding, and here it establishes contacts with the Arg finger of GAP and with the water molecule needed for the nucleophilic attack. Thus, mutations in Q61 also lead to GAP insensitivity, since the transition state can not be achieved (Scheffzek et al., 1997). It was also proposed that hot spot mutations affect the mechanism of Raf-dependent allosteric intrinsic hydrolysis (Buhrman et al., 2010; 2011). In this mechanism the Y32 residue, similar to the Arg finger of GAP, neutralizes negative charge. Binding of a ligand, most probably a membrane component, to the allosteric lobe of Ras when it is bound to Raf induces a conformational change in the switch II, positioning Q61 into the optimal position for the hydrolysis reaction. Hot-spot mutations disable the proper positioning of the bridging water molecule which is crucial for the stabilization of negative charge during the formation of transition state (Parker and Mattos, 2015; Prior et al., 2012).

3.1.2. Noncanonical Ras mutations

Beside the hot-spot mutations, there are a number of noncanonical Ras mutations, which can be found throughout the whole Ras sequence (Fig. 7). So far, due to their infrequency, they have been rarely studied.

For most of the noncanonical mutations the mechanism of Ras activation has not been demonstrated. Some mutations affect the regions involved in nucleotide binding or switch I and II regions. Mutations in few of these residues have been analyzed (V14I, Q22K, P34R, G60R, K117R, A146T, F28L) and it was confirmed that they activate the protein by either impairing GTP hydrolysis or increasing nucleotide exchange rate (described in detail in the following section) (Cirstea et al., 2013; 2010; Denayer et al., 2012; Gremer et al., 2011; Janakiraman et al., 2010; Reinstein et al., 1991). Several mutations affect the regions involved in the mechanism of Ras orientation on the membrane (Abankwa et al., 2008; 2010). They overlap with the regions that either stabilize specific Ras conformation on the membrane, helix α4 and hvr, or regions involved in the novel switch III mechanism of reorientation, β2-β3-loop and helix α5. Interestingly, GTP hydrolysis and nucleotide exchange rate was not altered by mutations in switch III region, N-ras-T50I and K-ras-D153K (Cirstea et al., 2010; Gremer et al., 2011). Therefore, it could be that they affect the orientation of Ras on the membrane, which then alters the activity of the protein, as it was previously shown for the MD simulation derived orientation-switch III mutants (Abankwa et al., 2008; 2010), and proposed for the N-ras-T50I mutant (Cirstea et al., 2010). Understanding the mechanisms of these mutations could provide clues for novel ways of targeting Ras. Furthermore, it is unclear what is the tumorigenic potential of noncanonical mutations. Most of these mutations activate Ras only mildly, and have weak transforming ability, or no ability at all (mutant K-ras-R164Q) (Smith et al., 2010).

However, a recent study demonstrated that the effect of otherwise mild noncanonical H-ras-F28L mutation has a strong activation profile if it co-occurs with the loss in the GEF protein NF1 (Stites et al., 2015). They also showed that there is a higher rate of co-occurrence between noncanonical Ras mutations and either GAP or GEF mutations.

Specific noncanonical K-ras mutations were recently demonstrated to be important in colorectal cancer, where the mutational status of K-ras is used as an indicator of resistance to treatment with epidermal growth factor receptor (EGFR) inhibitors. Most of the clinical methods used to detect Ras mutations in tumors concentrates on detecting the hot-spot mutations (Franklin et al., 2010). In 15-27 % of the tumors, which were classified as K-ras(wt) did not respond to the EGFR treatment. Sequencing of the whole *KRAS* gene demonstrated that these tumors were not K-ras(wt), but rather carried a noncanonical K-ras mutations, most commonly A146T, but also L19F, L117N and R164Q (Douillard et al., 2013; Parsons and Myers, 2013; Smith et al., 2010).

3.2. Ras misregulation in developmental diseases

RASopathies are a group of developmental diseases characterized by the aberrant Ras/MAPK pathway signaling due to germline mutations in certain genes of the pathway. Diseases which belong to this group are neurofibromatosis type 1 (NF1), Legius syndrome, Capillary Malformation–Arteriovenous Malformation Syndrome (CM-AMS), Noonan syndrome (NS), Noonan syndrome with multiple lentigines (NSML, also called Leopard syndrome), Costello syndrome (CS) and cardiofaciocutaneous syndrome (CFCS) (Rauen, 2013).

Mutations in *RAS* genes are found in CS, NS and CFCS (Fig. 7). Patients with these syndromes have many overlapping symptoms, such as craniofacial dysmorphism, cutaneous anomalies, cardiac and musculoskeletal abnormalities and delayed neurocognitive development, which is more strongly affected in the CS and CFCS (Schubbert et al., 2007). They also have a higher risk of developing cancer, with the highest risk for CS patients (Kratz et al., 2011). Syndromes can be distinguished according to the more specific symptoms and mutated genes. Most of the Ras mutations detected in these syndromes hyperactivate it only mildly, leading to a mild increase in downstream signaling, which makes these mutants tolerable during development.

3.2.1. Ras mutations in Costello syndrome

The CS is one of the most infrequent RASophaty, affecting only 200 to 300 people worldwide. It is the first syndrome in which a germline Ras mutation was detected, in 2005 (Aoki et al., 2005). CS patients carry a mutation in *HRAS* gene (Fig. 7), in most cases affecting the same amino-acid residues (G12, G13, Q61) as in cancer, but most frequently leading to substitutions which occur very rarely in cancer (Rauen, 2007). Mutational range in CS is very narrow, resulting in a uniform phenotype among patients. Most frequent mutations are G12S (80% of cases) and G12A (10% of cases) (Tidyman and Rauen, 2008). The most common G12S and G12A mutations have much weaker transforming potential and stronger GAP-catalyzed GTPase activity compared to the G12V mutation (Prior et al., 2012). This is also demonstrated by a more severe phenotype exhibited by the CS patient with the G12V mutation (Viosca et al., 2009). Mutations occurring at the residues other than G12 and G13, also result in a distinct CS phenotype, usually of milder nature (mutations T58I, K117R, A146T, A146V) or with predominant musculoskeletal abnormalities (mutations Q22K and E63K) (Gripp and Lin, 2012; McCormick et al., 2013). Mutations in G12, G13 and Q22 residues affect the GAP-catalyzed GTP-hydrolysis, while the other mutations can increases nucleotide exchange rate (K117R) (Denayer et al., 2008; Janakiraman et al., 2010), at the same time decrease GAP sensitivity and effector binding (E37dup) (Gremer et al., 2010). Mutations in Q22K, A146T/V and T58I have been studied in K-ras background, where they affect GAP-catalyzed GTP-hydrolysis, nucleotide exchange rate or have no effect on the biochemical properties of the protein, respectively (Gremer et al., 2011; Janakiraman et al., 2010). The mechanism of the mutation E63K has not yet been determined.

3.2.2. Ras mutations in Noonan syndrome and Cardiofaciocutaneous syndrome

The NS is a more common syndrome, affecting 1:2000-3000 individuals, while the CFCS is, similar to CS, very rare (Tidyman and Rauen, 2008). In NS *PTPN11* (50% of cases) or *SOS1* (15% of cases) are most frequently mutated genes, but also mutations in other genes, such as *KRAS*, *NRAS*, *SHOC2*, *CBL*, *RAF1* and *BRAF* were reported. Most frequently mutated genes in CFCS are *BRAF* (75% of cases), *MAPK1* and *MAPK2* (25% of cases) (Rauen, 2013).

K-ras mutations occur in <2% of NS patients and only a few CFCS patients (Fernández-Medarde and Santos, 2011). Role of K-ras mutations in CFCS is controversial because it is difficult to give a clear diagnosis since patients exhibit overlapping symptoms of both NS and CFCS (Schubbert et al., 2007). So far, 19 distinct mutations were detected (Fig. 7). Unlike CS mutations in H-ras, NS and CFCS mutations in K-ras are spread throughout its coding sequence, affecting residues, which are almost never affected in cancer. This is reflected on a very broad spectrum of phenotypes exhibited by patients. Mutations lead to a mild hyperactivation of K-ras, never as severe as in cancer (Cirstea et al., 2013; Gremer et al., 2011). They hyperactivate Ras by either increasing its intrinsic or GEF-stimulated nucleotide exchange rate (V14I), impairing its sensitivity to GAP-stimulated GTP-hydrolysis (Q22R, Q22K), or affecting both properties (Q22E, F156L). Some mutations strongly impair K-ras sensitivity to GAP-stimulated GTP-hydrolysis, but at the same time decrease its effector binding (P34L/R/Q, G60R and K147E, latter two also exhibit increased nucleotide exchange rate), resulting in a mild activation of K-ras. Some mutations have no effect on these biochemical properties (K5E/N, T58I, Y71H, D153V) (Cirstea et al., 2013; Gremer et al., 2011; Janakiraman et al., 2010), while for other mutations (V152G (Carta et al., 2006), N116S (Razzaque et al., 2012), I36M, F156I (Zenker et al., 2007), M72L (Brasil et al., 2012)) such an analysis has not yet been performed. Structural analysis of mutations D153V and V152G in helix α5 based on the H-ras structure proposed that these mutations might increase the nucleotide exchange rate (Carta et al., 2006). Biochemical analysis of the mutant D153V showed that this is not the case (Gremer et al., 2011; Schubbert et al., 2007). Recently, a NMR study of the membrane bound D153V and K5N mutants demonstrated that these mutations could affect the conformation of K-ras on the membrane, making it more assessible to the effectors by exposing the effector binding sites (Mazhab-Jafari et al., 2015).

N-ras mutations were detected only in NS, where they occur rarely, in less than 2% of the cases. So far, only four mutations, I24N (Denayer et al., 2012; Kraoua et al., 2012), P34R (Denayer et al., 2012), T50I and G60E (Cirstea et al., 2010), were detected which are, as K-ras germline mutations, dispersed throughout the coding sequence. G60E and P34R activates N-ras by affecting its nucleotide exchange rate,

rendering it insensitive to GAP and decreasing its effector binding (Cirstea et al., 2010; Gremer et al., 2011). The mutation T50I did not affect the biochemical properties of N-ras. Instead, it was suggested that T50I could alter the conformation of N-ras on the membrane (Cirstea et al., 2010). The T50 residue is in the β2-β3-loop of the novel switch III region, which was shown to be important for the establishment of H-ras conformations on the membrane (Abankwa et al., 2008). The mechanism by which mutations I24N activate N-ras has not yet been analyzed. It was demonstrated recently that mutations I24N, G60E and T50I affect zebra-fish embryo development, in a way that resembles their effect on the patients with NS. T50I mutation exhibited a very mild effect (Runtuwene et al., 2011).

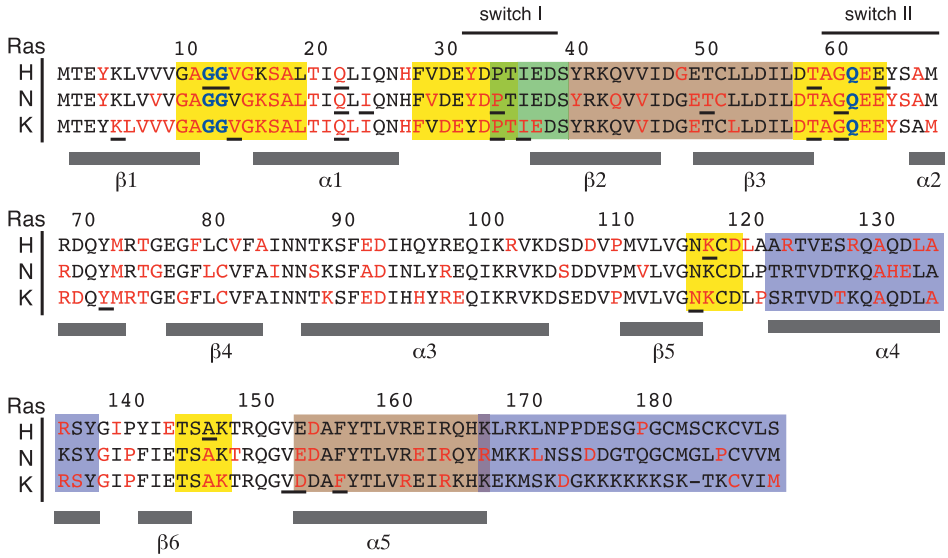


Figure 7. Amino-acid residues affected by cancer- and developmental diseases-related mutations.

This figure represents the overview of the mutated amino-acid residues in the H-, N- and K-ras isoforms. More than 99.2 % of the cancer-related mutations occur in the hot-spot residues, highlighted in bold. Other cancer-related mutations (noncanonical mutations) affect the residues highlighted in red. Residues that were found to be mutated in developmental diseases are underlined. The yellow background indicates the residues involved in nucleotide binding, while the green background indicates the residues involved in binding of the effectors. Blue background indicates the orientation-stabilizing helix α4 and the C-terminal hvr region, while the switch III region is highlighted with a brown background. The switch I and switch II regions are annotated above the sequence, while the motifs of the secondary structure, alpha helices (α) and beta sheets (β) are annotated bellow the sequence.

AIMS OF THE STUDY

When we began this study, nucleotide dependent Ras orientation on the membrane was a newly described phenomenon. Our laboratory has demonstrated that the mutations in the critical residues of the orientation-switch III regions affect the activity of H-ras. Currently, the known mechanisms by which Ras mutations can affect Ras activity is by affecting its intrinsic or GEF-catalyzed nucleotide exchange rate, its intrinsic or GAP-catalyzed GTP hydrolysis or its affinity towards the effector. These defects are easily detectable by common biochemical techniques. However, the orientation-switch III regions of Ras are not known to be involved in the effector binding and nucleotide exchange. Therefore, we aimed to provide further molecular mechanistic details on the novel orientation-switch III mechanism, with the ultimate goal to untangle the connection between the orientation of Ras on the membrane and its activity.

Next we aimed to understand the biological relevance of the orientation-switch III mechanism. It was recently demonstrated that particular mutants of Ras, associated with the developmental diseases, display unaffected biochemical properties, but still increase the Ras-dependent signaling. Interestingly, some of these mutations were affecting the orientation stabilizing- and switch III- regions. Moreover, a number of mutations in the orientation stabilizing- and switch III-region have been identified in cancer. Their mechanism of Ras activation and role in pathogenesis remain completely unknown.

Thus, as the main goal of this thesis we comprehensively analyzed several cancer- and developmental disease-associated mutations in the switch III region of Ras. The goal was to answer the following questions: Can these mutations activate Ras and induce aberrant downstream signaling? What is the mechanism by which they activate it? Can these cancer-related switch III mutations induce the tumorigenic properties of the cells?

Finally we aimed to better characterize the interaction between H-ras-GTP and its nanoclustering scaffold protein Gal-1. The existing model proposed that Gal-1 binds to the farnesylated H-ras-GTP and enhances the level and stability of the H-ras-GTP nanoclusters, which then increase the downstream Raf pathway signaling. However, sufficient evidence describing the direct interaction of H-ras-GTP and Gal-1 and the complex formed on the membrane is still missing. Puzzled by the recent study, in which the direct interaction between the farnesylated H-ras peptide and Gal-1 was not observed, we reassessed the binding of the Gal-1 and H-ras and the overall mechanism by which Gal-1 regulates the signaling of H-ras.

MATERIALS AND METHODS

1. Cell culture (I, II, III)

Baby hamster kidney (BHK) cells were used in publication I, II and III, Gal-1-knockout mouse embryonic-fibroblasts (Gal-$1^{-/-}$ MEFs) in I, rat adrenal pheochromocytoma (PC12) and NIH/3T3 cells in II, human embryonic kidney (HEK) 293-EBNA cells in III.

BHK, Gal-$1^{-/-}$ MEF, HEK-293-EBNA and NIH/3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/mL). In the case of NIH/3T3 cells FBS was not heat inactivated. PC12 cells were grown on plates coated with 50 μg/ml of rat tail collagen I (Gibco). Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5 % FBS, 10 % horse serum, L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/mL) was used to culture them. All cell lines were passaged every 2-3 days, when they reached a confluency of 80 %.

2. DNA constructs and molecular cloning (I, II, III)

The following plasmids were previously described:
pmGFP-H-rasG12V, pmRFP-C-Raf-RBD, pcDNA3-antisense-Gal1, pcDNA3-Gal-1, pmRFP-Gal-1 (used in I, II, III)
pmGFP-H-rasG12V-R128A,R135A, pmGFP-H-rasG12V-R169A,K170A (used in I, II)
pGEX-4T1-huNF1-333, pGEX-2T-huCRAF-RBD, pmGFP-H-rasG12V-D47A,E49A, pmGFP-tH, pmCFP-K-rasG12V, pmCit-N-rasG12V (used in II)
pmRFP-PI3K-αRBD, pmRFP-C-Raf-CRD-RBD, pEGFP-A-Raf, pEGFP-B-Raf, pEGFP-C-Raf (used in III)

The following plasmids were generated using molecular cloning as described in the original publications (I-III):
pQE-A1-H-ras(wt), pQE-A1(used in I, II, III)
pQE-A1-C-Raf-RBD (used in I, III)
pmCherry-H-rasG12V, pmGFP-K-rasG12V, pmCherry-K-rasG12V, pmGFP-N-rasG12V, pmCherry-N-rasG12V, pmGFP-N-rasG12V, pmCherry-N-rasG12V (used in II, III)
pSNAP-H-rasG12V, pSNAP-H-rasG12V-R128A,R135A, pSNAP-H-rasG12V-R169A,K170A, pQE-A1-H-ras-R128A,R135A, pQE-A1-H-ras-R169A,K170A (used in I)

pmCFP-H-rasG12V, pmCFP-N-rasG12V, pQE-A1-H-ras-D47A,E49A, pQE-A1-H-ras-G48R, pmCherry-H-rasG12V-G48R,D92N, pmCherry-H-ras-G48R,D92N, pmCherry-H-rasG12V-G48R, pmCherry-N-rasG12V-C51Y, pmCherry-N-rasG12V-E49K, pmCherry-N-rasG12V-T50I, pmGFP-N-rasG12V-C51Y, pmGFP-N-rasG12V-E49K, pmGFP-N-rasG12V-T50I, pmCherry-K-rasG12V-V152G, pmGFP-K-rasG12V-V152G, pmCherry-K-rasG12V-R164Q, pmGFP-K-rasG12V-R164Q (used in II)
pmCit-Gal-3, pmCit-Gal-1, pQE-A1-Gal-1, pcDNA3-N-Gal-1 (used in III)

The following plasmids were generated using the site-directed mutagenesis (GenScript Inc.):
pmGFP-H-ras(wt) (used in II, III)
pmGFP-H-rasG12V-R128A,R135A,D47A,E49A, pmCFP-K-rasG12V-R164Q, pmGFP-H-ras-G48R, pmGFP-H-ras-R161A,R169A, K170A, pmGFP-H-ras-R128A, R135A, G48R, pmCFP-K-ras(wt), pQE-A1-H-ras-G48R, D92N, pmCit-N-ras(wt), pmGFP-H-rasG12V-G48R, pmCherry-H-rasG12V-G48R,D92N, pmCit-N-ras-C51Y, pmCit-N-ras-E49K, pmCit-N-ras-T50I, pmCit-N-ras-G12V-C51Y, pmCit-N-ras-G12V-E49K, pmCit-N-ras-G12V-T50I, pmCFP-K-ras-V152G, pmCFP-K-ras-R164Q, pmCFP-K-rasG12V-V152G (used in II)
pmGFP-H-rasG12V-D38A, pmRFP-Gal-1-C3S,L5Q,V6D,A7S (pmRFP-N-Gal-1), pmRFP-Gal-1-V6D, pmRFP-Gal-1-S63A, pmRFPGal-1-S63A,D65A, pmRFP-C-Raf-RBD-D113A,D117A, pmRFP-C-Raf-RBD-D117A, pmRFP- C-Raf-RBD-K109A, W114A, T116A, L121A, E124A, L126A (6A), pmRFP-C-Raf-RBD- K109A, W114A, T116A (N3A), pmRFP-C-Raf-RBD- W114, T116A, L121A (C3A) (used in III)

3. Microscopy methods

3.1. Spot variation STED-FCS (I)

Stimulated emission depletion (STED) microscopy uses the non-linear fluorescence processes to selectively excite fluorophores in the sample and increase the resolution (Müller et al., 2012). In STED microscopy fluorescent molecules are excited by a laser as in confocal microscopy. Before the emission happens, another laser, the so-called depletion laser, de-excitates the molecules by stimulated depletion in a donut shaped focus and only molecules from the center of the donut can emit. Depletion laser has to be perfectly aligned with the excitation laser. The size of the donut, and as a consequence the final resolution, depends on the intensity of the depletion laser and can go to values lower than 20 nm. The fluorescence correlation spectroscopy (FCS)

is used to determine the apparent diffusion coefficient in the observation spot (D) (Billaudeau et al., 2013; He and Marguet, 2011). The combined spot variation STED-FCS enables the measurements of apparent diffusion coefficient by FCS in the observations spots, which size is varied by STED (Eggeling et al., 2009; Mueller et al., 2013). The STED-FCS experiments were performed in the BHK cells kept in HDMEM at 23 °C and transiently expressing the plasmids pSNAP-H-rasG12V, pSNAP-H-rasG12V-R128A,R135A, pSNAP-H-rasG12V-R169A,K170A. The SNAP tag is a mutant of the DNA repair protein O6-alkylguanine-DNA alkyl-transferase (AGT), which binds covalently the derivatives of benzylguanine (Keppler et al., 2004). Here the benzylguanine derivative with the attached SiR fluorophore (Lukinavičius et al., 2013) was used to label the SNAP-tagged orientation mutants. The FCS measurements were acquired for 10 s in the STED observation spots of different sizes (150 nm – 250 nm) at the lower plasma membrane of cells. The apparent diffusion coefficient (D) was calculated in at least 7 distinct spots of the same size per sample. Determined averages of the apparent diffusion coefficients were plotted against the observation spot size (d). The diameter of the observation area $d(P_{STED})$ formed by a certain STED power P_{STED} was determined by measuring the diffusion times of the lipids labeled with Atto647N dye in the DOPC supported lipid bilayers (SLBs), the ration of labeled to unlabeled lipids in the bilayer was 1:10000. These lipids exhibit a free diffusion in the bilayer and their apparent diffusion coefficient is the same no matter of the spot size, while their average transient time through the observation spot (t_D) scales proportionally with the size of the observation area. Therefore, knowing the size of the confocal observation area $d(P_{STED}=0)$, which was determined by fluorescent beads to be ≈ 240 nm, the sizes of the STED observation spots can be calculated using the following formula:

$$\frac{d(P_{STED}=0)}{d(P_{STED})} = \sqrt{\frac{t_D(P_{STED}=0)}{t_D(P_{STED})}}$$

3.2. Fluorescence recovery after photobleaching (FRAP) (I)

FRAP was used to measure the immobile fraction of H-ras-orientation mutants, which correlates with the nanoclustered fraction of mutants on the membrane (Guzmán et al., 2014b). BHK cells were seeded on coverslips, on a 6-well plate at a density of 200'000 cells/well. After 24 h they were transfected with the mGFP-tagged H-rasG12V mutants using the JetPRIME transfection reagent (Polyplus transfection). 24 h post-transfection the culture medium was exchanged for Ringer's buffer (10 mM HEPES, 10 mM glucose, 2 mM $NaH_2PO_4 \bullet H_2O$, 1 mM $MgCl_2$, $6H_2O$, 2 mM $CaCl_2$, 5 mM KCl, 155 mM NaCl, pH 7.2) at 22 °C. FRAP data was obtained using the FRAP wizard on the Leica TCS microscope in the confocal mode

(excitation at 488 nm, detection at 497-568 nm). The frames were taken every 1.51 seconds. The first 5 frames were taken at 10 % of the laser intensity (pre-bleaching). In the next 20 frames a selected squared region of 2.5 μm interest (ROI) was bleached at the maximal laser intensity to 40 – 60 % of initial fluorescence intensity. The following 75 frames were taken at 10 % of laser intensity, in order to follow the recovery of the fluorescence in the ROI. Image J 1.47g software (National Institutes of Health) was used to quantify the fluorescence signal and determine the recovery curves, which were initially normalized to the fluorescence intensity from the prebleaching frames. These normalized curves were fitted in the IgorPRO software (WaveMetrics) using the formula described in (Feder et al., 1996). The immobile fractions were then determined using the following formula in which Q represents the immobile fraction, F_∞ the normalized intensity after an infinite time and F_0 the normalized intensity at the time 0 after bleaching:

$$Q = (1 - F_\infty)/(1 - F_0)$$

Three biological repeats were performed with more than 10 cells imaged for each sample.

3.3. Wide-field FLIM-FRET measurements (I, II, III)

Cellular fluorescence resonance energy transfer (FRET) measured by wide-field fluorescence lifetime microscopy (FLIM) experiments were performed in all three publications, while in III they were also performed in solution. The FRET phenomenon occurs between two fluorescent molecules (donor and acceptor) if the emission spectrum of the donor overlaps significantly with the excitation spectrum of the acceptor, the dipoles of the two fluorophores are in the favorable orientation and the distance between them is less than 10 nm (Jares-Erijman and Jovin, 2006). The fraction of the transferred energy, FRET efficiency (E), is defined by the following formula, in which R represents the distance between the fluorophores and R_0 represents the Förster radius:

$$E = \frac{1}{1 + (R/R_0)^6}$$

There are two groups of techniques for FRET detection- intensity based techniques and fluorescence lifetime based techniques. Fluorescence lifetime is defined as the time an electron resides in the excited state before returning to the ground state while emitting a photon. The intensity based techniques have several disadvantages, such as susceptibility to photobleaching, signal cross-contamination and the dependency on the concentrations of the fluorophore (Berney and Danuser, 2003). On the other hand fluorescence lifetime techniques are less dependent on these influences (Sun et al., 2011).

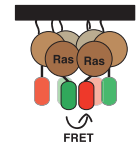
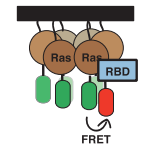
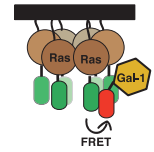
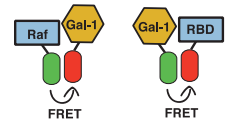
The fluorescence lifetime of a fluorophore depends primarily on the environmental factors, such as FRET, which decreases the donor's lifetime. Thus, FLIM-FRET was used to report on the close proximities between two proteins tagged with the donor and acceptor fluorescent tags.

BHK (used in I, II and III) or HEK-EBNA (used in III) cells grown on coverslips were transiently transfected with the indicated fluorescently tagged constructs as described in Table 1 using Fugene 6 (Promega Biotech AB) or JetPRIME (Polyplus transfection) transfection reagent, respectively. After 24 h or 48 h cells were fixed for 20 min with 4 % paraformaldehyde (PFA), washed with phosphate buffer saline (PBS) and mounted using Mowiol 4-88 (Sigma-Aldrich). In III wide-field FLIM-FRET was also used to determine the interactions Gal-1 and H-ras-GTP in solution. Purified DY-547-tagged C-Raf-RBD and Gal-1 were incubated with mGFP-H-rasG12V derived from cellular lysates; or purified Atto-488 tagged H-ras-GTPγS with mRFP-Gal1 or mRFP-RBD derived from cellular lysates.

Imaging was performed using the inverted microscope Zeiss AXIO Observer D1. As a light source the sinusoidally modulated 3W, 497 nm LED lamp at 40 MHz under epi-illumination was used and cells were imaged using the 63X, NA 1.4 oil objective. To monitor the mGFP fluorescence the GFP filter set was used (excitation: BP 470/40, beam splitter FT 495, emission: BP 525/50). The lifetimes of the mGFP were calculated using a fluorescence lifetime imaging attachment (Lambert Instruments). Three biological repeats were performed and fluorescence lifetimes were determined from at least 40 cells. From the determined lifetimes the apparent FRET efficiency (E_{app}) was calculated according to the following formula, in which the τ_D represents the measured fluorescence lifetime of the donor molecule (mGFP), while the τ_{DA} represents the measured fluorescence lifetime of the donor-acceptor pair.

$$E_{app} = \left(1 - \frac{\tau_{DA}}{\tau_D}\right) * 100$$

Table 1. Different cellular wide-field FLIM-FRET experiments performed in this thesis. The column on the left provides the title of distinct FLIM-FRET experiments with a short description where it was used and how it was performed. The column on the right provides the schematic representations of each FLIM-FRET experiments.

Nanoclustering Used in II and III. Cells were transiently co-transfected with mGFP- and mCherry-tagged Ras constructs.	
Ras binding domain (RBD)-recruitment Used in I, II and III. Cells were transiently co-transfected with the mGFP-tagged Ras constructs and mRFP-tagged C-Raf-RBD.	
Ras / Gal-1 complexation Used in II and III. Cells were transiently co-transfected with mGFP-tagged Ras and mRFP-tagged Gal-1 constructs.	
Raf (RBD) / Gal-1 complexation Used in III. Cells were transiently co-transfected with mGFP-Raf isoforms and mRFP-Gal-1; or mCit-Gal-1 and mRFP-RBD constructs.	

4. PC12 cell differentiation assay (II)

PC12 cell differentiation assay was used in publication II to report on signaling strength of different cancer-derived mutants in switch III region of N-ras and K-ras, similar to (Gorfe et al., 2007). Cells were seeded on the 4 well Lab-TekTM II Chambered Coverglass (Termo Fischer Scientific Nunc) coated with 0.1 % of rat tail collagen I (Termo Fischer Scientific Gibco) in 30 % ethanol, at the density of 10^4 cells/well. After 24 h cells were transfected with the pmGFP-tagged Ras mutants or the control plasmid pmGFP-tH using the JetPRIME transfection reagent (Polyplus-transfection). After 2 days cells were fixed with 4 % PFA and mGFP-positive cells were imaged using the confocal microscope Zeiss LSM 510 META, 40x, NA 1.4 oil immersion objective. Neurite lengths in at least 25 cells per mutant from three biological repeats were determined using "NeuronJ" plug in for ImageJ software (Meijering et al., 2004).

5. Tumorigenicity assays in NIH/3T3 cells (II)

NIH/3T3 mouse fibroblast cell proliferation, transformation and anchorage-independent growth are well established and widely used assays for determination of Ras oncogenicity. These assays were used to determine the oncogenic potential of switch III mutants N-rasG12V-C51Y and K-rasG12V-R164Q, compared to N-rasG12V and K-rasG12V respectively.

First, to generate cells stably expressing indicated Ras mutants or GFP control only, cells were transduced with lentiviral particles containing HA-tagged Ras mutants under CMV promoter and GFP-Puromycin marker under RSV promoter (AMS Biotechnology). 72 h post-infection transduced cells were selected by 1 μg/ml puromycin treatment for the next 72 h. The pool of transduced cells was then expanded in the presence of 0.5 μg/ml of puromycin and used in the proliferation assay, transformation assay and anchorage independent growth assay.

Proliferation assay

Cells were seeded on a 96-well plate in a density of 500 cells/well. At intervals of 0 h, 24 h, 48 h and 72 h, 15 μl of Alamar Blue (Termo Fisher Scientific Invitrogen) was added to each well. After three hours fuorescence intensity at excitation of 570 nm and emission of 590 nm was measured using Synergy H1 Hybrid Reader (BioTek). Alamar blue penetrates the living cells, thus fluorescence intensity reports on the cell amount. For each sample experiment was performed in a hexaduplicate. The average of the measured fluorescence intensity was determined and normalized to the 0 h time point. Three biological repeats were performed and averages for each time point were calculated.

Transformation assay

1'500 cells were seeded on a 6 well plate in a triplicate. After 7 days cells were fixed with 4 % PFA for 20 min, stained using 0.5 % crystal violet in 10 % EtOH for 15 min and washed several times with PBS until excess stain was removed. Air-dried plates

were scanned and average colony area percentage was determined using "ColonyArea" plug in for ImageJ software (Guzmán et al., 2014a). Three biological repeats were performed and average was determined.

Anchorage independent growth

6-well plates were first coated with the bottom layer containing 1.2 % agarose and growth media, on top of which the top layer containing 50'000 cells, 0.5 % agarose and growth media was plated. After drying, the growth media supplemented with 0.5 µg/ml of puromycin was added on top of the layers. Cells were fixed after 10 days of growth using methanol/acetone (1:1). Formed colonies were imaged using the Zeiss SteREO Lumar V12 stereomicroscope.

Quantification was done in the ImageJ software. In order to analyze the area percentage covered with the colonies, the function 'Analyze Particles' was used; a filter for particles smaller than 500 μm^2 was applied. Averages from three biological repeats were calculated.

6. Other methods (I, II, III)

The detailed descriptions of each listed biochemical assays can be found in the original publications (I-III).

METHODS	
Protein expression, purification and fluorescence labeling	I, II, III
Fluorescence anisotropy assay	I, II, III
GAP-dependent GTP-hydrolysis assay	II
SOS-dependent nucleotide exchange assay	II
GST pull-down assay	III
in vitro FRET assay	III
Western blotting (WB)	I, II, III
Confocal FLIM-FRET	I
Co-localization analysis	II
Electron microscopy	II, III
Confocal FLIM-FRET	I
Co-localization analysis	II
Electron microscopy	II, III
Statistical analysis	I, II, III
Bioinformatics analysis	II
Computational modeling	I, III

7. Antibodies used in WB (I, II, III)

List of antibodies	
β-actin (A1978, Sigma-Aldrich)	I, II, III
GAPDH (SAB1405848, Sigma-Aldrich)	I
galectin-1 (sc-28248, Santa Cruz Biotechnology)	I
pC-Raf (Ser338) (9427S, Cell Signaling)	II
pMEK (9154S, Cell Signaling)	II
pERK (4695S, Cell Signaling)	II
C-Raf (9442, Cell Signaling)	II
MEK1/2 (9126, Cell Signaling)	II
pMEK1/2 (9121, Cell Signaling)	II
pERK1/2 (9101, Cell Signaling)	II
ERK1/2 (9102, Cell Signaling)	II, III
AKT (9272, Cell Signaling)	II
pERK (9106, Cell Signaling)	III
pAKT1 (MAB7419, R&D Systems)	II
GFP (3999-100, BioVision)	II

RESULTS AND DISCUSSION

1. Coupling between the orientation-switch III mechanism and Ras activity

The orientation of H-ras on the membrane is stabilized either by helix α4 in the GTP-bound state or by hvr in the GDP-bound state (Gorfe et al., 2007). Our group has recently demonstrated that mutations introduced in the residues, which are critical for the stabilization of certain orientation, change the activity of H-rasG12V (Abankwa et al., 2008; 2010). The mutant H-rasG12V-R169A,K170A is more active, while the mutant H-rasG12V-R128A,R135A is less active. The switch III region, composed of β2-β3-loop and helix α5 is also suggested to be implicated in the orientation mechanism of H-ras on the membrane, presumably by guiding the reorientation. This prediction is based on the MD simulations, which showed perturbation in the salt-bridge network formed by residues D47, E49, R164 and R161 during the nucleotide dependent conformational change of the membrane-bound H-ras (Abankwa et al., 2008; Gorfe et al., 2007). The mutations D47A,E49A or R161A increased or decreased the activity of H-rasG12V, respectively (Abankwa et al., 2008). The first goal of this thesis is to understand the mechanism by which the change in orientation introduced by mutations in the orientation stabilizing- and switch III- regions affects the observed change in H-ras activity. The mutations studied in the publication I and II are sumarized in the Figure 8.

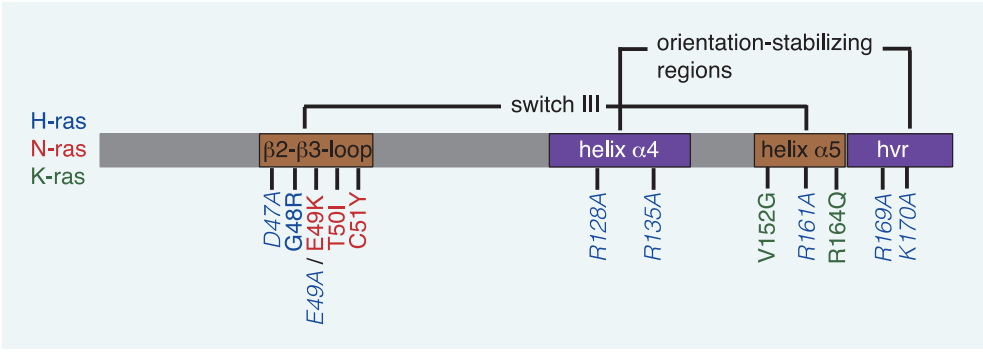


Figure 8. Mutations studied in the publication I and II
This figure summarizes all the orientation-switch III mutations studied in the the publication I and II. Highlighted in brown are the switch III elements β2-β3-loop and helix α5, while highlighted in violet are orientation stabilizing regions helix α4 and hvr. Mutations labeled in blue were introduced in H-ras, in red in N-ras and in green in K-ras. Annotated in italic are the H-ras mutations in residues derived from the MD simulations (Gorfe et al., 2007)

Of note, here and in the following sections, in the experiments where we followed the biochemical properties of the mutants, such as guanine nucleotide exchange and Ras binding domain (RBD) binding of purified proteins, mutations were introduced in the wild-type background of the parent Ras construct. On the contrary, in the cellular nanoclustering and activity assays, we wanted to ensure that we follow the effect of orientation-switch III mutations on the active, GTP-bound form of Ras. Therefore, if not stated differently, mutations were introduced to the G12V background of the original Ras proteins.

1.1. H-ras orientation mutants display differences in effector binding only when they are bound to the membrane

To see if the mutations directly affect the affinity of H-ras to its effectors, we addressed the binding of H-ras orientation mutants to the Ras binding domain (RBD) of the C-Raf effector (C-Raf-RBD) in the absence of the mutant's attachment to the membrane.

The fluorescence anisotropy assay was used to measure the binding affinities of purified C-Raf-RBD to the H-ras-R169A,K170A and H-ras-R128A,R135A orientation mutants in solution. Their binding was identical to the one of H-ras(wt) and C-Raf-RBD (Fig. 1b, I), indicating that the affinity to RBD in solution is not affected with the introduction of orientation mutations.

Binding of the C-Raf-RBD to soluble Ras was also followed in BHK cells by Förster resonance energy transfer (FRET) measured by fluorescence lifetime imaging microscopy (FLIM), after the treatment of cells with 5 μM compactin. Compactin is an inhibitor of HMG-CoA reductase, which is an enzyme required for the production of isoprenoid chains needed for Ras prenylation and membrane binding. Thus, treatment with compactin induces the re-localization of Ras from the membrane to the cytoplasm (Köhnke et al., 2012; Nguyen et al., 2009).

Confocal FLIM-FRET images demonstrate the re-localization of the mutants to the cytoplasm after the compactin treatment, but also show the decrease in the C-Raf-RBD-recruitment FRET (Fig. 2A, I). To quantify the changes in FRET after the compactin treatment observed by confocal FLIM-FRET measurements, we next measured the C-Raf-RBD recruitment using the wide-field FLIM-FRET. It was demonstrated that after compactin treatment C-Raf-RBD recruitment is the same for both orientation mutants H-rasG12V-R169A,K170A, H-rasG12V-R128A,R135A and the control H-rasG12V (Fig. 2B, I).

These results demonstrate that the orientation mutations do not affect binding of H-ras to the effector C-Raf-RBD in solution. Instead, the differences in the effector recruitment are accomplished by an unknown mechanism only when the proteins are membrane bound.

1.2. H-ras orientation mutants display distinct Gal-1 level dependent nanoclustering responses

On the membrane, Ras proteins have a non-random nanoscale organization. They organize into nanoclusters, which are isoform- and nucleotide- specific (Zhou and Hancock, 2015). Nanoclusters serve as signaling platforms to which effectors are recruited and they enable the high-fidelity signal transduction by acting as analog-digital-analog switches (Harding and Hancock, 2008a; Tian et al., 2010). The extent of nanoclustering defines the signaling output: more nanoclusters are related to stronger signaling output.

Gal-1 is a well-known scaffold of the H-ras-GTP nanoclusters, which increases the amount of H-ras-GTP nanoclusters and their stability (Belanis et al., 2008; Prior et al., 2003; Rotblat et al., 2010). Previous work has shown that Gal-1 can distinctly complexate with the H-ras orientation mutants(Abankwa et al., 2010). Similar to their C-Raf-RBD-recruitment and activity, the mutant H-rasG12V-R169A,K170A has stronger and H-rasG12V-R128A,R135A weaker complexation to Gal-1.

Therefore, we hypothesized that differential complexation of Gal-1 and H-ras orientation mutants could cause distinct nanoclustering responses, which would ultimately give rise to distinct effector recruitment.

To address our hypothesis we followed the nanoclustering abilities and effector recruitment of H-ras orientation mutants on the plasma membrane using sensitive fluorescence microscopy techniques stimulated emission depletion combined with fluorescence correlation spectroscopy (STED-FCS), fluorescence recovery after photobleaching (FRAP) and fluorescence lifetime microscopy combined with Förster resonance energy transfer (FLIM-FRET). STED-FCS is used to capture the occurrence of the nanoclusters, FRAP measures the established nanoclusters and FLIM-FRET was used to measure the C-Raf-RBD recruitment.

The experiments were performed in BHK cells with Gal-1 level variation, either by depleting endogenous Gal-1 using antisense-Gal-1 construct, or by overexpressing unlabeled or mRFP-tagged Gal-1. The endogenous concentration of Gal-1 in BHK cells was ~ 10.0 ± 0.9 μM (Fig. 4B, I). We determined it from the standard curve of purified Gal-1. Overexpression of mRFP-Gal-1 increased Gal-1 levels by 5 folds, while the overexpression of untagged Gal-1 increased it by 2 folds (Fig. 4F and 4G, I). The antisense-Gal-1 depleted the endogenous levels by approximately 50 % (Fig. 4D, I).

First we used STED-FCS, which enables us to follow the diffusion properties of the plasma membrane bound H-ras orientation mutants on a nanoscale (Eggeling et al., 2009; He and Marguet, 2011).The super-resolution microscopy STED was used to vary the size of the observation spot, on a scale bellow the diffraction limit (120-240 nm). Using FCS the apparent diffusion coefficients (*D*) of H-ras orientation

mutants were measured in the observation spot of varying sizes (*d*) (Billaudeau et al., 2013; He and Marguet, 2011). The change of the apparent diffusion coefficient with the variation of the observation spot size was used to distinguish the free from the anomalous diffusion (Eggeling et al., 2009; Mueller et al., 2013). Free diffusion is characterized by the constant apparent diffusion coefficient (*D*), while in the case of anomalous diffusion the coefficient changes with the variation in the size of the observation spot (*d*). The decrease in the apparent diffusion coefficient with the decrease of the observation spot size indicates hindered diffusion, e.g. nanoclustering. The slopes of the determined *D(d)* curves report on the extent of hindered diffusion.

Our results demonstrate that the extent of the Gal-1 level dependent hindered diffusion varies between the H-ras orientation mutants (Fig. 3C, I). In low levels of Gal-1, mutants have diffusion properties similar to free diffusion and the mutant H-rasG12V-R169A,K170A shows the strongest hindered diffusion. Already in the endogenous levels of Gal-1 the mutant H-rasG12V-R169A,K170A achieves its maximal hindered diffusion, which does not elevate further with the Gal-1 overexpression. For the mutant H-rasG12V-R128A,R135A the increase in hindered diffusion is much weaker, and it elevates only slightly with Gal-1 overexpression.

Next, we monitored the fraction of the established nanoclusters of the H-ras orientation mutants with the variation of Gal-1 levels using FRAP. We determined the immobile fraction, which corresponds to the immobilized Ras in nanoclusters (Guzmán et al., 2014b; Rotblat et al., 2010). These mutants exhibited distinct nanoclustering responses depending on the Gal-1 dose, similar to what was observed by STED-FCS experiments (Fig. 5C, I). At endogenous levels of Gal-1 the mutant H-rasG12V-R169A,K170A had a significantly higher nanoclustering level than the mutant H-rasG12V-R128A,R135A. When Gal-1 was depleted the nanoclustering levels of both mutants were at a similar level. With the Gal-1 overexpression the nanoclustering level of both mutants reached a plateau at approximately 60%.

Thus, our results demonstrate that the conformational changes induced by orientation mutations, change the nanoscale behavior of H-ras at the membrane. This affects both the assembly of nanoclusters (measured by STED-FCS) and the fraction of established nanoclusters (measured by FRAP) with the change in the Gal-1 level.

1.3. Distinct nanoclustering responses govern the distinct activity of H-ras orientation mutants

Since nanoclusters serve as the sites of Ras signaling, we assumed that the observed differences in Gal-1 dose dependent nanoclustering of H-ras orientation mutants could directly govern the differences in the Gal-1 dependent effector recruitment.

Therefore, we monitored the recruitment of C-Raf-RBD to the H-ras orientation mutants with the varying levels of Gal-1. Similar to what we observed in STED-FCS and FRAP nanoclustering experiments, orientation mutants exhibited differences in the C-Raf-RBD-recruitment with the Gal-1 level variation (Fig. 6C, I).

In the endogenous levels of Gal-1, the mutant H-rasG12V-R169A,K170A had much stronger activity than H-rasG12V-R128A,R135A, as reported previously (Abankwa et al., 2010). Compared to the control H-rasG12V, the mutant H-rasG12V-R128A,R135A had similar, but not significantly lower activity as it was observed previously (Abankwa et al., 2010). With the Gal-1 overexpression, effector recruitment was further increased for both mutants, while Gal-1 depletion decreased the C-Raf-RBD recruitment to the same level for both mutants.

Our results demonstrate that the Gal-1 level dependent C-Raf-RBD-recruitment response differs for the two H-ras orientation mutants. If the differences in the nanoclustering fraction govern the differences in their C-Raf-RBD-recruitment we would expect a strict correlation between the C-Raf-RBD-recruitment response and the nanoclustering response measured by FRAP. However, this was not the case. Therefore, we developed a computational model in order to explain the observed discrepancy. Our model demonstrates that although there is a correlation between the nanoclustered fraction and the C-Raf-RBD-recruitment, the correlation is not strict, because the C-Raf-RBD-recruitment to the nanoclusters depends not only on the fraction of established nanoclusters, measured by FRAP, but also on the lifetime of nanoclusters. Nanocluster lifetime, in turn, depends on the differential binding of Gal-1 to different orientation mutants.

Our results demonstrate that H-ras orientation mutants display different activity due to their distinct nanoclustering abilities, which are dependent on the distinct complexation with the H-ras-GTP nanocluster stability modulator Gal-1.

1.4. Changes in nanoclustering also govern the activity of the mutant in switch III region identified with computational modeling

Our next goal was to better characterize the novel switch III region of H-ras, recently described by our laboratory (Abankwa et al., 2008).

In order to confirm the coupling of the switch III region to the H-ras membrane orientation mechanism, we combined orientation and switch III mutations with the opposite activities and analyzed their C-Raf-RBD recruitment FLIM-FRET in BHK cells (Fig. 1C, II). For both combined mutants H-rasG12V-R161A,R169A,K170A and H-rasG12V-D47A,E49A,R128A,R135A, the effect of the activating mutations (R169A,K170A or D47A,E49A) on the C-Raf-RBD recruitment was normalized by the decreasing/neutralizing mutations (R161A or R128A,R135A) to the C-Raf-RBD recruitment of the control H-rasG12V. These results indicate that the switch III

region and the orientation-stabilizing regions hvr and helix α4 allosterically connect to each other and represent the crucial elements required for the nucleotide-dependent orientation of H-ras on the membrane.

Based on these observations, we postulated that the observed increased activity of the H-ras-G12V-D47A,E49A mutant could be, similarly to orientation mutants H-rasG12V-R169A,K170A, a consequence of increased Gal-1 dose dependent nanoclustering.

Therefore, we monitored the nanoclustering abilities of this mutant. We used electron microscopy on the plasma membrane sheets and nanoclustering FLIM-FRET with Gal-1 level variation in BHK cells transiently expressing fluorescently tagged H-ras mutants (Fig. 2A and 2C, II). The mutations D47A,E49A induced strong increase in the nanoclustering in all Gal-1 levels compared to the control H-rasG12V. The increased nanoclustering was, in turn, followed by an increase in the activity of the mutant measured by C-Raf-RBD-recruitment FLIM-FRET (Fig. 2E, II), which was then reflected as stronger signaling in the Raf signaling pathway demonstrated by higher levels of the pC-Raf, pMEK1/2 and pERK1/2 (Fig. 2F, II).

Similarly as for the orientation mutants, we monitored if the D47A,E49A mutations in the switch III affect the basic biochemical properties of H-ras. Besides measuring the binding of mutants to C-Raf-RBD in solution by fluorescence anisotropy (Fig. 2G, II), which remained unaltered, we also assessed its sensitivity to GAP-mediated GTP-hydrolysis and SOS-dependent nucleotide exchange (Fig. 2H, Supplementary file 1, II). While the mutant remained sensitive to GAP, there was a slight decrease in the SOS-catalyzed GTP-dissociation rate, which were then reflected on the significant increase in the fraction of active Ras after the stimulation with EGF (Fig. 2-figure supplement 1, II). Therefore, the mutations D47A,E49A in the switch III region of H-ras increase its activity by strongly augmenting its Gal-1 dose dependent nanoclustering, but also by slightly increasing its SOS-dependent nucleotide exchange.

In conclusion, our results provide further proof that the switch III regions are involved in the regulation of the H-ras orientation mechanism on the membrane. We also demonstrate that the activity of the switch III mutant H-rasG12V-D47A,E49A is increased due to the increase in Gal-1 dose dependent nanoclustering.

2. Pathophysiological role of the novel switch III region

As described in the chapter 3 of the literature review, the defects in Ras mediated signaling have strong impact on the pathogenesis, both in cancer and developmental diseases. The main focus of my thesis was to explore whether the novel mechanism of Ras activity modulation is exploited in disease.

Several mutations of Ras in the orientation stabilizing- and switch III- regions have been described. However it remains completely unknown whether they activate Ras and what is the mechanism behind it. Furthermore, the ability of these mutations to promote the tumorigenic potential of Ras has not yet been investigated. Thus, we hypothesized that these mutations activate Ras by augmenting its nanoclustering. We broadened our focus on the K-ras and N-ras isoforms as well, since they also exhibit isoform-specific and nucleotide-dependent conformations on the membrane, as described in section 2.4.1. Current evidence suggests that similar regions as for H-ras could be of importance for the membrane conformation of N-ras and K-ras as well (Abankwa et al., 2010; Kapoor et al., 2012a; 2012b; Mazhab-Jafari et al., 2015). Furthermore, N-ras and K-ras are, compared to H-ras, more severe oncogenes, as they are more frequently mutated in cancer (Prior et al., 2012).

2.1. Orientation stabilizing- and switch III- regions of Ras are mutated in cancer

To understand the pathophysiological relevance of orientation-switch III mechanism, we first searched for the mutations affecting helix α4, hvr and switch III region of H-, N- and K-ras from 140'000 cancer sample entries from different databases (COSMIC, cBioPortal and ICGC) (Fig. 3, Supplementary file 2, II). Analysis showed that mutations in orientation-switch III occur rarely, with only 15 cases of H-ras, 20 cases of N-ras and 28 cases of K-ras mutations reported so far. For H-ras mutations occur most frequently in helix α4, for N-ras in β2-β3-loop and for K-ras in helix α5. Due to the low number of cases it is difficult to conclude if there is any preference for a certain cancer type, however mutations were most frequently associated with the colorectal cancer.

Seven Ras mutants in the switch III region were selected and a comprehensive analysis of their biochemical properties, nanoclustering, downstream signaling and tumorigenicity was performed (Fig. 8). Five mutants were detected in the cancer patients: H-ras-G48R, N-ras-E49K, N-ras-C51Y and K-ras-R164Q (Table 2). Mutation H-ras-G48R co-occurred with D92N mutation in the original tumor sample (Simi et al., 2011). Therefore H-ras-G48R mutation was analyzed in the presence and absence of the second, D92N mutation.

We also analyzed two mutations found in Noonan syndrome patients: N-ras-T50I and K-ras-V152G, whose mechanism of Ras activation remains unclear (Carta et al., 2006; Cirstea et al., 2010).

Table 2: Summary of the studied cancer-related switch III mutations.
The table provides the basic information on the mutants used in this study. The column *Mutation* defines the mutated isoform and mutated residues. The column *Region* reports on the part of the switch III in which mutation was found and the column *Cases* on the number of cases the mutation was reported in. The column *Disease* specifies the cancer or developmental disease type in which mutations were found, with the number in bracket specifying the number of cases for a particular disease type.

Mutation		Region	Cases	Disease
H-ras	G48R,(D92N)	β2-β3-loop	1	Skin cancer
N-ras	T50I	β2-β3-loop	3	Skin cancer (2) and Noonan syndrome (1)
	E49K	β2-β3-loop	1	Colorectal cancer
	C51Y	β2-β3-loop	1	Hematopoietic cancer
K-ras	R164Q	helix α5	6	Colorectal (5) and endometrial (1) cancers
	V152G	helix α5	1	Noonan syndrome

2.2. Biochemical properties and cellular localization of disease-related switch III mutants remain intact

To understand if the mutations in the switch III region affect basic biochemical properties of Ras, which could lead to activation of Ras by a classical mechanism, we analyzed SOS-mediated nucleotide exchange, GAP-mediated GTP-hydrolysis, binding of C-Raf-RBD in solution and downstream signaling under serum starvation.

The SOS-dependent nucleotide exchange was addressed in the case of mutants H-ras-G48R and H-ras-G48R,D92N using the QRET technique(Kopra et al., 2014) (Supplementary file 1, II). Mutant H-ras-G48R exhibited unaltered SOS-dependent nucleotide exchange, while the addition of the second D92N mutation slightly increased it. The increased nucleotide exchange was reflected on the increased fraction of GTP-loaded Ras, which we observed after the stimulation with EGF (Fig. 4F, II).

The GAP-mediated GTP-hydrolysis was monitored for all mutants and remained the same as for their wild-type Ras parent constructs in all cases (Fig 4F, 5E and 6E, II).

Also the binding of the effector C-Raf-RBD in solution was not affected by any of the mutations in the switch III region. To address it, fluorescence anisotropy assay with purified proteins was used in the case of H-ras mutants (Fig. 4E, II), while in the case of N-ras and K-ras mutants the cellular FLIM-FRET, after compactin treatment, was used (Fig. 5-figure supplement 1B, Fig. 6-figure supplement 1B, II). As observed for the

H-ras orientation mutants in the previous study, both assays provide us with the comparable information on protein binding in solution (Guzmán et al., 2014c).

The downstream signaling under serum starvation of the N-ras and K-ras mutants was also addressed, by monitoring the levels of pMEK, pERK and pAKT. Under serum starvation all analyzed mutants showed the same signaling strength as their parent wild type construct (Fig. 5-figure supplement 1D, Fig. 6-figure supplement 1D, II).

Finally, we determined whether switch III mutations affect sub-cellular localization of the proteins. We measured the Manders co-localization coefficient (*R*) from the confocal images of BHK cells co-expressing the mCherry-tagged RasG12V mutant proteins and their mCFP-tagged parent constructs. None of the monitored switch III mutations affected the localization of the protein (Fig. 4A, 5A and 6A, II).

Our results demonstrate that the basic biochemical properties and the sub-cellular localization of the switch III mutants remain unaltered. Thus, these mutations activate Ras in a completely unknown alternative way. Based on our previous results, we hypothesized that similarly to the orientation-switch III mutants identified with computational modeling, disease-related switch III mutants activate Ras by increasing its nanoclustering.

2.3. Specific cancer-derived switch III mutants exhibit increased nanoclustering

Nanoclustering was monitored using electron microscopy on plasma membrane sheets and FLIM-FRET in BHK cells transiently expressing fluorescently tagged Ras mutants. For H-ras mutants, Gal-1 level modulation was done in FLIM-FRET experiments, similar as in the previous study with the orientation-switch III mutants (Guzmán et al., 2014c) identified by MD simulations (Abankwa et al., 2008; Gorfe et al., 2007). Complexation with Gal-1 was not affected by K- and N-ras switch III mutations. Therefore, in the case of these mutants nanoclustering was measured only in the endogenous Gal-1 levels (Fig. 5-figure supplement 1A, Fig. 6-figure supplement 1A, II).

All cancer-related mutants, except the N-ras-T50I mutant, show a significant increase in nanoclustering, both in electron microscopy and FLIM-FRET experiments (Fig. 4B,C, 5B,C and 6B,C, II). Based on these results we propose that these mutations affect the conformation of the protein on the membrane, which is then coupled to its nanoclustering, similar to computational modeling-identified orientation-switch III H-ras mutants (Guzmán et al., 2014c). On the other hand, nanoclustering of Noonan-syndrome related mutants is not affected (Fig. 5B,C and 6B,C, II).

At this point, we still lack the precise structural details of the conformational changes induced by switch III mutations, but also the explanation how they change the nanoclustering response. We propose that the G48R mutation in H-ras could affect the conformation of H-ras in a similar way as it was proposed for mutations in its neighboring residues, D47A and E49A, by stabilizing the active conformation of H-ras.

Recently accumulated data provided more structural details on orientation mechanism of isoforms N- and K-ras on the artificial membranes or by MD simulations (Abankwa et al., 2010; Kapoor et al., 2012a; 2012b; Mazhab-Jafari et al., 2015). The study from Mazhab-Jafari *et al*. provided the first structural evidence that Noonan syndrome/CFCS- derived noncanonical mutations K5N and D153V induce a conformational change in K-ras (Mazhab-Jafari et al., 2015). They also demonstrated that the conformational change directly affects the effector binding. However, these single-molecule studies provide no insight on the consequence of distinct membrane orientations on the formation of higher order Ras complexes, dimers and nanoclusters, which are considered to be the Ras signaling units. Change in the orientation could directly influence the binding of Ras molecules upon formation of dimers and nanoclusters. This was demonstrated in a recent study, in which they show that, depending on the conformation state, specific interfaces between the Ras proteins are preferred (Li and Gorfe, 2013). In the recent work from Muratcioglu *et al*., the structural details of K-ras-GTP dimerization were determined and a β-interface was suggested as the predominant interface (Muratcioglu et al., 2015).

Thus, we propose that a conformational change induced by the switch III mutants could directly affect the binding of Ras proteins and affect their complexation on the membrane and formation of dimers and nanoclusters. Our observations could be better understood if the structural change of the membrane bound Ras mutants and its effect on the nanoscale distribution would be measured. We believe that the development of the precise high-resolution methodology could overcome this highly challenging task.

2.4. Specific switch III mutations affect the activity and tumorigenic potential of Ras

Our next goal was to assess if the observed increase in nanoclustering has any effect on the activity and the tumorigenic potential of the mutants in the switch III region of Ras.

First the C-Raf-RBD recruitment was measured in BHK cells using FLIM-FRET and in the case of H-ras mutants with the Gal-1 level modulation. Nanoclustering response measured by FLIM-FRET and C-Raf-RBD recruitment were correlated. All

cancer-related mutants in switch III region, except the N-ras-T50I mutant, exhibited increased effector recruitment, while for the developmental disease-related mutants it was unchanged (Fig. 4D, 5D and 6D, II).

We performed a more detailed analysis of signaling impact of N-ras and K-ras mutants, by following the phosphorylation of MEK1/2 and ERK1/2 in the MAPK-pathway and AKT in PI3K-pathway using western blotting from BHK cells transiently transfected with the switch III mutants and stimulated with EGF. The N-ras and K-ras switch III mutants displayed only a mild increase of the phosphorylation of ERK1/2 and MEK1/2, while the pAKT levels were not affected (Fig. 5-figure supplement 1D, Fig. 6-figure supplement 1D, II). Noonan syndrome-related mutant K-ras-V152G did not have any effect on these signaling pathways. Such an effect is consistent with the previously observed mild increase in Ras activity for different Noonan/CFC- syndrome related mutants (Cirstea et al., 2010; Gremer et al., 2011).

We also used the PC12 cell differentiation assay, as a more sensitive readout for the biological activity of the mutants. PC12 cells have the ability to undergo differentiation into sympathetic neurons when Ras oncogenes are expressed (Qui and Green, 1992). The length of developed neurites reports on the strength of signaling through MAPK-pathway (Cowley et al., 1994; Vaudry et al., 2002). A stronger PC12-cell differentiation was observed for the N-ras and K-ras switch III mutants that displayed stronger nanoclustering and C-Raf-RBD-recruitment (Fig. 5F and 6F, II). The strongest effect on N-ras was observed for C51Y mutation, while for K-ras with the R164Q mutation, which is the most frequent switch III mutation, found in 6 cancer patients.

The two most active mutants were then tested for their tumorigenic potential in NIH/3T3 mouse fibroblasts. The NIH/3T3 proliferation, transformation and anchorage-independent growth are the most commonly used assays for determination of the tumorigenic potential of Ras mutants (Clark et al., 1995). The anchorage independent growth measured by colony formation in soft agar can usually be correlated with the tumorigenic potential *in vivo* (Shin et al., 1975).

We established NIH/3T3 cells stably expressing the N-rasG12V-C51Y, K-rasG12V-R164Q and their parent constructs. Both mutants increased the cell growth rate, transforming ability and anchorage independent growth, demonstrating that these mutants have increased tumorigenic potential (Fig. 5G-I, Fig. 6G-I, II). Another group has recently examined the RNA transcription profile and transforming potential of the K-ras-R164Q mutant in NIH/3T3 cells. They introduced the mutation into the wild-type K-ras and used transient transfection. Although they observed differences in the RNA transcription profile, the

transforming potential was not changed compared to K-ras(wt), suggesting that this mutation activates Ras only mildly (Smith et al., 2010).

2.5. Increased nanoclustering is a novel mechanism for Ras activation in cancer

Our results revealed a novel mechanism for Ras activation by specific cancer-related mutations in the switch III region. Contrary to known mechanisms, specific mutations in the switch III region do not affect the biochemical properties of the protein, but instead increase its nanoclustering (Fig. 9). Enhanced nanoclustering then directly governs the increase in effector recruitment, signaling output and the tumorigenic potential of the mutant.

Our study also represents the first example of (patho)physiological relevance of nanoclustering. We showed that nanoclustering phenomenon can be exploited in disease, where the change in nanoclustering ability can lead to Ras hyperactivation. Our findings suggest that decrease in Ras nanoclustering abolishes Ras activity. Thus, development of inhibitors that block Ras nanoclustering represents an exciting future challenge.

In contrast to the classical mechanisms of Ras activation, such as GEF activation, which increase Ras activity even more than 50,000 fold, nanoclustering augmentation increases it rather weakly, by only 2-3 fold. Such a weak activation could be the reason why these mutations appear so infrequently.

We have no insight in the importance of these mutations for the tumor development. Based on their low activity we can speculate that these mutation would act as latent drivers, rather than driver or passenger mutations, as it was proposed previously for the mutant K-ras-R164Q (Nussinov and Tsai, 2015). Latent drivers are a recently proposed class of mutations found in cancer, which do not have any effect on the tumor development. That would classify them as passenger mutations. However, latent drivers play a key role in specific situations, such as development of drug resistance, when they provide specific advantages for the cancer cells. It could also be that the effect of switch III mutations depends on the presence of mutations in some other genes, as it was recently demonstrated for the noncanonical Ras mutation F28L, which activation profile depends on the presence of the GEF protein NF1 (Stites et al., 2015). Otherwise mildly activating mutant increases its activation profile drastically when the loss of NF1 co-occurs.

The mutants K-ras-V152G and N-ras-T50I have intact biochemical properties, but also display unaltered nanoclustering. Thus, we still lack the mechanism of their activation. Both mutants were found in Noonan syndrome, while the N-ras-T50I was also found in cancer. Presumably, these mutations could lead to such a mild activation of Ras that the effects were beyond the detection limit. Such a mild

activation could still be sufficient to lead to development of disorders such as Noonan syndrome. For instance, it was reported previously that although N-ras-T50I activates N-ras weakly, its expression leads to small, but significant defects in the development of the zebra-fish (Runtuwene et al., 2011).

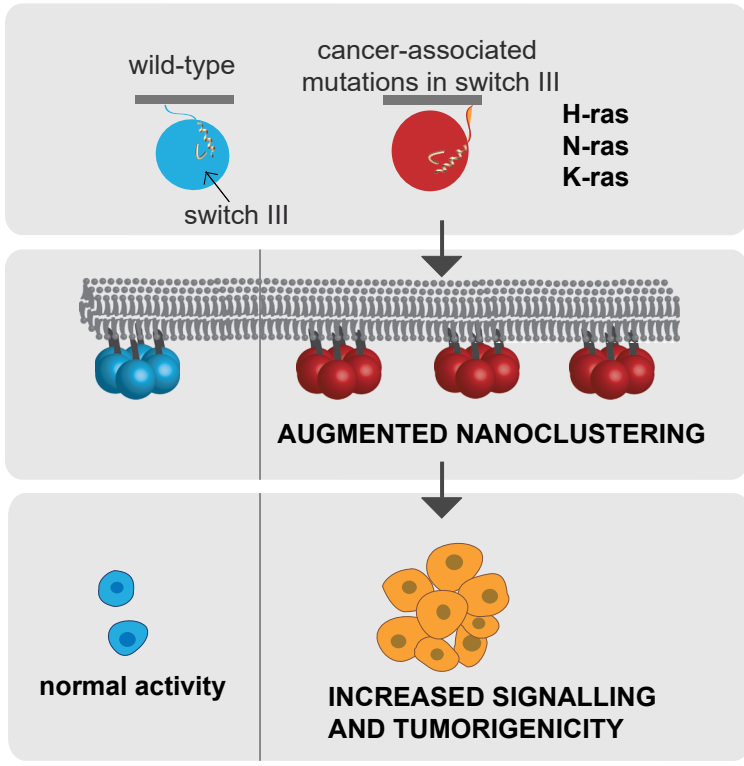


Figure 9. A novel mechanism of Ras activation in cancer by increasing its nanoclustering.
Specific cancer-related mutations in the switch III region of Ras increase the nanoclustering of Ras, presumably as a consequence of the change in its orientation on the membrane, governed by the orientation-switch III mechanism. Increased nanoclustering is then a direct cause for the increase in activity and the tumorigenic potential of the Ras switch III mutants.

3. Novel mechanistic insights into the Gal-1 regulation of H-ras-GTP nanoclustering

The emphasis of the last part of this thesis was put on the Gal-1 protein and the mechanism by which it accomplishes its effect on the H-ras-GTP nanoclustering. According to the current model (Fig. 6), Gal-1 interacts directly with the H-ras-GTP on the plasma membrane, by binding to its farnesyl moiety. Binding of Gal-1 to H-ras-GTP leads to the stabilization and longer lifetime of H-ras-GTP nanoclusters (Belanis et al., 2008; Rotblat et al., 2010). Consequently, its downstream effector recruitment and signaling through the Ras-MAPK pathway are increased (Elad-Sfadia et al., 2002). So far, the interaction of Gal-1 and H-ras-GTP was observed by co-immunoprecipitation experiments (Elad-Sfadia et al., 2002; Paz et al., 2001). The identification of a hydrophobic pocket in Gal-1, which could accommodate the farnesyl moiety of Ras is based on the computational modeling, where the structure of Gal-1 was superimposed on the Rho-GDI structure (Rotblat et al., 2004). The direct binding of Gal-1 and the analogues of farnesyl chain, farnesyl-diphosphate or farnesol was recently demonstrated by saturation transfer difference NMR. This method monitors the chemical shift in the signal of the ligand (farnesyl analogues) upon the addition of the protein (Gal-1) (Yu et al., 2015). However, the proof of a direct interaction between Gal-1 and full length farnesylated Ras is still missing. A recent study displays that neither Gal-1, nor Gal-3 bind to the farnesylated H-ras and K-ras peptides (Mejuch et al., 2015). The interaction was measured using the fluorescence anisotropy. Moreover, in the same study no binding of the Gal-3 to the full length farnesylated K-ras was observed. Gal-3 was previously proposed to have a similar farnesyl-binding pocket as Gal-1 (Elad-Sfadia et al., 2004). Interestingly, in one of our experiments we followed the Gal-1 complexation with H-rasG12V using FLIM-FRET in BHK cells treated with compactin (SI Fig. 1A, III). Despite the compactin treatment, which leads to the loss of Ras farnesylation, we were still able to detect the complexation between Gal-1 and H-rasG12V.

These opposing results suggested that Gal-1 is able to bind to the non-farnesylated H-rasG12V and prompted us to re-assess the existing model of H-ras-GTP and Gal-1 interaction.

3.1. There is no direct interaction between Gal-1 and H-ras-GTP

The interaction between Gal-1 and H-ras-GTP was addressed *in vitro* using purified proteins. This enabled us to directly look at the interaction using a two-component system where we avoid the possibility of an indirect interaction, which is one of the drawbacks of the co-immunoprecipitation experiments.

First we looked at the interaction of purified Gal-1 with the fluorescently labeled farnesylated peptide derived from the protein Rheb, which is another member of the Ras family (Fig.1A and 1B, III). The fluorescence polarization was followed with the increasing concentration of either Gal-1 or a positive control the protein PDEδ, which binds to the farnesyl moiety of prenylated and depalmitoylated proteins of the Ras family (Nancy et al., 2002). Although binding of the farnesylated peptide to PDEδ was detected, no binding to Gal-1 was observed. To further validate that the complexation of Gal-1 and H-ras-GTP is not dependent on the H-ras farnesylation, we used a H-rasG12V-C186S mutant, which can not be farnesylated. This mutant displayed a similar complexation with Gal-1 as the control H-rasG12V measured by FLIM-FRET in BHK cells (unpublished data).

Besides monitoring the binding to its farnesyl moiety, binding of Gal-1 to the G-domain of H-ras-GTP was also monitored by FRET using purified Gal-1 and H-Ras, which were labeled with DY-547 and Atto-488 on their N-terminally located A1 tag (Guzmán et al., 2014c), respectively (Fig. 1C, III). The H-ras was loaded with GTPγS, which is a nonhydrolyzable analog of GTP. Interaction of H-ras-GTPγS and C-Raf-RBD was used as a positive control. We could not observe any binding of Gal-1 to the G-domain of H-ras-GTP.

Since additional post-translational modifications of Gal-1 or H-ras may be required for their interaction, the binding of the proteins derived from the cellular extracts was followed by FLIM-FRET (Fig. 1D, III). We incubated the purified and fluorescently labeled Dy547-Gal-1 or Atto-488-H-ras-GTPγS proteins with the extracts of cells overexpressing mGFP-H-rasG12V or mRFP-Gal-1, respectively. Binding of purified Atto-488-H-ras-GTPγS to the mRFP-C-Raf-RBD from the cellular lysates was used as a positive control. Even when Gal-1 or H-rasG12V were derived from the cellular lysates, no interaction between them was observed.

Our results show a discrepancy with the existing model and suggest that there is no direct binding of Gal-1 to H-ras-GTP, neither to its farnesyl chain nor the G-domain. These results led us to consider that the Gal-1 and H-ras-GTP interaction and nanoclustering regulation could be achieved in an indirect way, presumably through binding of another protein.

3.2. Gal-1 indirectly associates with H-ras-GTP, through binding to the Raf effector proteins

We hypothesized that Gal-1 could regulate H-ras-GTP nanoclustering through the binding to the effector proteins. This hypothesis arose from the experiments in which we tested the C-Raf-RBD-recruitment and nanoclustering of the H-rasG12V-D38A mutant. D38A mutation inhibits the interaction of Ras with its three major effectors: RalGEF, Raf and PI3K (Herrmann et al., 1995). The introduction of D38A mutation

to H-rasG12V abolished the C-Raf-RBD recruitment, but also the recruitment of Gal-1 measured by FLIM-FRET in HEK-EBNA cells (Fig. 2B, III). Furthermore, nanoclustering of this mutant followed by electron microscopy is reduced to similar extent as nanoclustering of H-rasG12V when Gal-1 is depleted (Fig. 2A, III).

Gal-1 is known to preferentially increase the Raf pathway signaling, while the PI3K pathway signaling is decreased simultaneously (Elad-Sfadia et al., 2002). Thus, we considered Raf kinases as the most plausible interacting partners of Gal-1. FLIM-FRET binding experiments of Gal-1 and A-Raf, B-Raf or C-Raf performed in HEK-EBNA cells, showed that Gal-1 binds all three Raf isoforms, with the strongest binding to B-Raf (Fig. 2C and 2D, III). We also looked at how siRNA silencing of each Raf isoform affects the H-rasG12V nanoclustering when Gal-1 is overexpressed (SI Fig. 2A, III). The silencing of A-Raf isoform decreased Gal-1 dependent and independent H-rasG12V nanoclustering, while the silencing of the B- and C-Raf has no effect. Why this effect is observed only for A-Raf isoform is an important question to follow in the future, which could provide insights in both Ras and Raf isoform specificity.

Next, we identified the minimal domain of Raf required for the binding with Gal-1. We observed strong FRET between Gal-1 and C-Raf-RBD, which did not increase with the addition of the CRD fragment of C-Raf (Fig 2D and 2E, III). This result indicated that RBD is the minimal Gal-1 binding domain. Since RBD is structurally similar among Ras effectors, we also tested if we can observe any FRET between Gal-1 and the RBD of PI3Kα effector protein. Although much weaker, we could still observe FRET by the FLIM-FRET experiments in the BHK cells. The interaction of C-Raf-RBD and Gal-1 was also confirmed using the co-immunoprecipitation assay in which GST-Gal-1 was able to pull-down the purified C-Raf-RBD and GST-C-Raf-RBD to pull-down the purified Gal-1 (Fig. 2F and SI Fig. 2C, III). Finally, we validated this interaction by *in vitro* FRET experiments using purified Gal-1 and H-Ras-GTPγS, labeled with DY-547 and Atto-488 on their N-terminally located A1 tag, respectively. From these experiments we determined a dissociation constant of Kd= 106 ± 40 nM for Gal-1 and C-Raf-RBD binding (Fig. 2G, III).

These results show that rather than binding to H-ras-GTP directly, Gal-1 binds to the RBD domain of the effector proteins and indirectly regulates H-ras-GTP nanoclustering.

3.3. Dimeric Gal-1 regulates H-rasG12V and K-rasG12V nanoclustering

To better characterize the emerging complex of Ras, Raf and Gal-1 on the membrane, we questioned whether Gal-1 exhibits its effect on the nanoclustering in a monomeric or dimeric form. Previous studies showed that Gal-1 starts forming dimers at the ≈7 μM concentration (Cho and Cummings, 1995).

We used previously reported dimerization-deficient mutant of Gal-1, named N-Gal-1, which contains four mutations at its N-terminus (Gal-1-C3S,L5Q,V6D,A7S, Kd ≈ 250 μM) (Cho and Cummings, 1996). First we confirmed its dimerization-deficiency, by looking at its dimerization using FLIM-FRET in HEK-EBNA cells (SI Fig. 4A, III). We then followed whether the abrogated dimerization has any effect on the binding of Gal-1 to C-Raf-RBD. The Gal-1 dimerization-deficiency did not reduce its binding to C-Raf-RBD, but instead it led to a significantly stronger interaction (SI Fig. 4B, III).

On the other hand, H-rasG12V nanoclustering-FLIM-FRET increase, induced by the Gal-1(wt) overexpression, was completely lost when N-Gal-1 was overexpressed (Fig. 4A, III). Consequently, N-Gal-1 did not increase the C-Raf-RBD-recruitment-FLIM-FRET (Fig. 4B, III). Finally, we followed the pERK1/2 levels in HEK-EBNA cells transiently expressing H-ras(wt) at different time intervals (0-30 min) after the stimulation with EGF, in order to determine the downstream Raf pathway signaling strength. Contrary to the increased and sustained pERK level observed after the Gal-1 overexpression, N-Gal-1 overexpression did not affect it (Fig. 4C, III).

Next we followed the effect of Gal-1 on the nanoclustering of other two isoforms, N-rasG12V and K-rasG12V measuring the nanoclustering-FLIM-FRET with the Gal-1 level modulation in BHK cells. The Gal-1 complexation FLIM-FRET experiments indicated that both N-rasG12V and K-rasG12V form complexes with Gal-1, which are sustained after the treatment with compactin (SI Fig. 4C, III). This is another discrepancy with previous studies where it was shown using co-immunoprecipitation that Gal-1 binds to H-ras-GTP and weakly to K-ras-GTP, while no interaction was detected for N-rasG12V (Paz et al., 2001). The N-rasG12V nanoclustering remains unchanged, despite the Gal-1 level modulation (Fig. 4D, III). In contrary, K-rasG12V nanoclustering is altered with the Gal-1 level modulation, in an opposite fashion to the H-rasG12V. The increase in Gal-1 levels leads to a decrease in nanoclustering, while the Gal-1 depletion leads to its increase. Gal-1 in the dimeric form was once again required for the observed effect on the K-rasG12V nanoclustering, which was demonstrated by our nanoclustering-FLIM-FRET experiments in HEK-EBNA cells, where we observed a smaller effect on the K-rasG12V nanoclustering when N-Gal-1 was used (Fig. 4E, III).

Our results suggest that an intact dimer interface is required for increase in the H-rasG12V nanoclustering, effector recruitment and downstream signaling. We also show that Gal-1 overexpression has a negative impact on K-rasG12V, while it has no effect on the N-rasG12V nanoclustering. The observed effect of Gal-1 on the K-rasG12V nanoclustering is again dependent on the Gal-1 dimerization.

3.4. Galectin-1 might be a scaffold of Raf dimers

In contrast to the current model, our results suggest that Gal-1 regulates the H-ras-GTP nanoclustering indirectly by binding to the Raf effectors, rather than binding directly to the H-ras-GTP (Fig. 10).

In recent years it became evident that Raf proteins require dimerization for their activation after the binding to the active Ras. Raf dimers can also modulate the nanoscale distribution of Ras on the membrane. It was recently demonstrated that paradoxical inhibitors of B-RafV600E mutant, which induce the wild-type B-Raf / B-Raf homodimerization and B-Raf / C-Raf heterodimerization lead to an increase of K- and N-ras nanoclustering (Cho et al., 2012a). The same effect was observed for the tandem construct in which the B-Raf-CRD-RBD and C-Raf-CRD-RBD fragments were fused together, in order to mimic the constitutive B-Raf / C-Raf dimer. Based on our finding that Gal-1 requires dimerization to be able to affect H-ras-GTP and K-ras-GTP nanoclustering, we propose that upon Ras activation dimeric Gal-1 binds and stabilizes the dimers of Raf, which in turn leads to the increase in the H-ras-GTP nanoclustering.

Our study provides novel insights on the three-component complex formed by Ras-GTP, Raf and Gal-1 on the plasma membrane, but it also leaves many open questions for the future research. For instance, the formal proof demonstrating the effect of Gal-1 on Raf dimerization is an essential requirement to validate our model. If Gal-1 dimers bind to the Raf dimers, does it bind preferentially to a specific Raf homo- or heterodimer?

Furthermore, the biological function and significance of such interaction remains unclear. Our model suggests that Gal-1 could serve to project the H-ras signaling to the Raf pathway specifically, through binding and stabilization of the Raf dimers. A positive effect of Gal-1 on the Raf pathway signaling, at the expense of the PI3K pathway signaling was previously reported (Elad-Sfadia et al., 2002). However, we cannot rule out the possibility that an interaction between Gal-1 and PI3Kα exists. Although much smaller than in the case of C-Raf-RBD, we still observed significant FRET between Gal-1 and PI3Kα-RBD. It would be important to inspect if Gal-1 interacts with the full length PI3Kα, or any other Ras effector.

We observed complexation of Gal-1 with all three Ras isoforms, but the effect on their nanoclustering was drastically distinct. It is unclear how Gal-1 negatively affects the K-rasG12V nanoclustering. There might be some additional proteins involved in the signaling complexes formed on the membrane upon Ras activation, which bind to a specific Ras isoform. The decrease in K-rasG12V nanoclustering could also be a direct consequence of the recently described spatial cross-talk effect, according to which increased H-rasG12V nanoclustering induces a decrease in the K-rasG12V nanoclustering (Zhou et al., 2014).

Our results also suggest that we should revisit established conclusions from the publication 1 (described in the section 1 of the results and discussion), which were based on the previous model, according to which Gal-1 can directly bind to H-rasG12V. There we proposed that H-ras orientation mutants have different nanoclustering responses, which are dependent on their intrinsic ability to interact with other Ras proteins and on their differential ability to complexate with Gal-1. These two properties regulate the amount and stability of the H-rasG12V nanoclusters, upon which depends the effector recruitment. Based on the latest results, we propose a different interpretation of the observed results. We suggest that the different nanoclustering abilities of H-ras orientation mutants originate exclusively from their different abilities to bind to each other. In fact, small differences in extend of the hindered diffusion at low levels of Gal-1 measured by STED-FCS (Fig. 3c, I) are observed between the orientation mutants. The H-rasG12V-R169A,K170A has stronger ability to incorporate into nanoclusters than the mutant H-rasG12V-R128A,R135A. The small increase in nanoclustering could then lead to a small increase in the effector recruitment. In the higher doses of Gal-1 the differences in the nanoclustering and RBD recruitment are more prominent. This can be assigned to the effect of Gal-1 on the stability of nanoclusters, which could then magnify the differences in nanoclustering of the mutants and in turn increase differences in their effector recruitment. However, the differences between the nanoclustering of the mutants at low doses of Gal-1 are not observable by FRAP (Fig. 5C, I). Using STED-FCS the hindered diffusion of moving Ras molecules was followed. The size of the observation spot is small (<250 nm), so that relatively small number of molecules is followed. FCS detects the events with the time resolution as low as 1.56 ns. Thus, the small differences in the diffusion properties are possible to observe. On the other hand, FRAP was used to determine the immobile fraction, which reports on the amount of immobilized nanoclusters in the observation region ROI. Due to the large size of the ROI (2.5 μm^2), the diffusion properties of a large number of molecules was followed. Also, the time between frames was 1.514 s. This timescale is longer than the lifetime of the nanoclusters (0.1-1 s), which makes the small differences in nanoclustering difficult to observe.

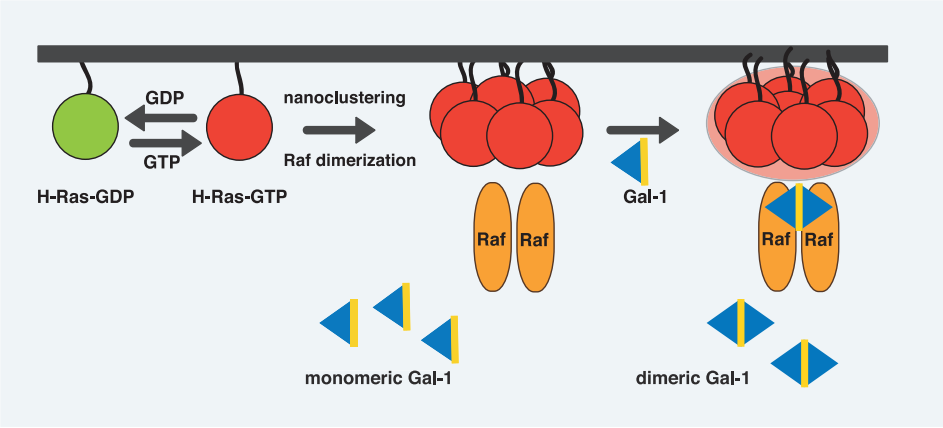


Figure 10: A novel model of H-ras-GTP nanoclustering regulation by Gal-1.
After its activation, H-ras-GTP forms active nanoclusters, which serve as sites to which Raf is recruited. Next, Raf forms dimers to become activated. Gal-1 can exist in a monomeric and dimeric form, the latter formed when cellular Gal-1 concentration is elevated. We propose that Gal-1 dimers bind to the Raf dimers and stabilize them, indirectly increasing the stability of H-ras-GTP nanoclusters.

CONCLUDING REMARKS

This thesis was started with the aim to better characterize the novel orientation-switch III mechanism in order to understand the connection between the H-ras membrane orientation and its activity. Contrary to the known mechanisms for Ras activity modulation, orientation-switch III mutations have no effect on the H-ras nucleotide exchange or the effector binding. By employing advanced fluorescence microscopy techniques we demonstrated that H-ras orientation mutants differ in their abilities to organize into nanoclusters on the plasma membrane. Their distinct nanoclustering then governs the activities of the mutants. Our results unveiled altered nanoclustering as a novel mechanism for the regulation of H-ras activity.

The next step of the thesis demonstrated that this novel mechanism of Ras activity regulation by nanoclustering alteration is exploited in cancer. Intriguingly, several noncanonical mutations in the switch III region of distinct Ras isoforms have been found in cancer, but due to their infrequency their characterization has been largely ignored. Our study demonstrates that specific noncanonical cancer-related mutations in switch III increase nanoclustering of Ras, while leaving the protein biochemically intact. These mutants also exhibit an increase in downstream signaling and increased tumorigenicity (Fig. 9). Compared to the Ras hot-spot mutations, switch III mutations hyperactivate Ras rather mildly. The mild activation can explain the low frequency of these mutations in cancer. The future challenge would be to provide the precise structural insights of the influence of these mutations on the membrane orientation of the protein and the effect of the rendered orientation for the formation of higher order Ras complexes.

The last step of the thesis provided intriguing novel details on the molecular regulation of Ras nanoclustering by the scaffold Gal-1. We challenged the existing model and demonstrated that Gal-1 is not able to directly bind to the H-ras-GTP, regardless of the H-ras farnesylation state. Instead, the interaction with the downstream effector Raf was observed. The interaction of Gal-1 with Raf was independent on the level of Gal-1 dimerization. However, the effect of Gal-1 on the H-ras-GTP was observed only when Gal-1 is in the dimeric form. Although the precise molecular details of the mechanism are still missing, we propose a novel model, where we suggest that Gal-1 binds to Raf dimers and stabilizes them. Raf dimers, in turn, increase the H-ras nanoclustering and its downstream signaling (Fig. 10).

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Turku, February 2016
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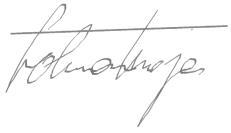

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Nanoclustering augmentation - a novel mechanism of Ras activation in cancer

This thesis describes a novel mechanism for the regulation of the activity of Ras proteins, one of the most frequently mutated genes in cancer. We demonstrated that Ras conformers on the membrane have distinct activities due to their distinct abilities to form nanoscale oligomers, the so called nanoclusters, which serve as the Ras signalling units. As the major finding we demonstrated that this mechanism of Ras activity modulation is exploited in cancer, where specific mutations affecting the novel switch III region of Ras activate it by increasing its nanoclustering. The switch III region of Ras is involved in guiding the reorientation of Ras conformers on the membrane.

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