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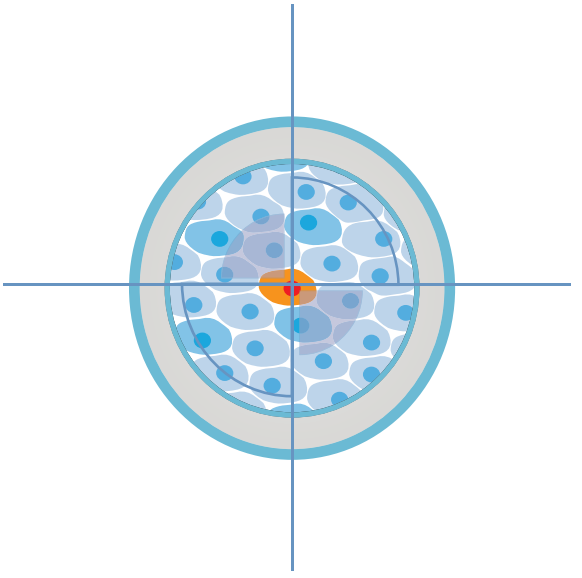
- new insights using a FRET-based chemical biology approach



Targeting oncogenic signaling proteins

- new insights using a FRET-based chemical biology approach

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To my family

ABSTRACT

Cancer affects more than 20 million people each year and this rate is increasing globally. The Ras/MAPK-pathway is one of the best-studied cancer signaling pathways. Ras proteins are mutated in almost 20% of all human cancers and despite numerous efforts, no effective therapy that specifically targets Ras is available to date. It is now well established that Ras proteins laterally segregate on the plasma membrane into transient nanoscale signaling complexes called nanoclusters. These Ras nanoclusters are essential for the high-fidelity signal transmission. Disruption of nanoclustering leads to reduction in Ras activity and signaling, therefore targeting nanoclusters opens up important new therapeutic possibilities in cancer. This work describes three different studies exploring the idea of membrane protein nanoclusters as novel anti-cancer drug targets. It is focused on the design and implementation of a simple, cell-based Förster Resonance Energy Transfer (FRET)-biosensor screening platform to identify compounds that affect Ras membrane organization and nanoclustering. Chemical libraries from different sources were tested and a number of potential hit molecules were validated on full-length oncogenic proteins using a combination of imaging, biochemical and transformation assays.

In the first study, a small chemical library was screened using H-ras derived FRET-biosensors. Surprisingly from this screen, commonly used protein synthesis inhibitors (PSIs) were found to specifically increase H-ras nanoclustering and downstream signalling in a H-ras dependent manner. Using a representative PSI, increase in H-ras activity was shown to induce cancer stem cell (CSC)-enriched mammosphere formation and tumor growth of breast cancer cells. Moreover, PSIs do not increase K-ras nanoclustering, making this screening approach suitable for identifying Ras isoform-specific inhibitors.

In the second study, a nanoncluster-directed screen using both H- and K-ras derived FRET biosensors identified CSC inhibitor salinomycin to specifically inhibit K-ras nanocluster organization and downstream signaling. A K-ras nanoclustering-associated gene signature was established that predicts the drug sensitivity of cancer cells to CSC inhibitors. Interestingly, almost 8% of patient tumor samples in the The Cancer Genome Atlas (TCGA) database had the above gene signature and were associated with a significantly higher mortality. From this mechanistic insight, an additional microbial metabolite screen on H- and K-ras biosensors identified ophiobolin A and conglobatin A to specifically affect K-ras nanoclustering and to act as potential breast CSC inhibitors.

In the third study, the Ras FRET-biosensor principle was used to investigate membrane anchorage and nanoclustering of myristoylated proteins such as heterotrimeric G-proteins, Yes- and Src-kinases. Furthermore, Yes-biosensor was validated to be a suitable platform for performing chemical and genetic screens to identify myristoylation inhibitors.

The results of this thesis demonstrate the potential of the Ras-derived FRET-biosensor platform to differentiate and identify Ras-isoform specfic inhibitors. The results also highlight that most of the inhibitors identified predominantly perturb Ras subcellular distribution and membrane organization through some novel and yet unknown mechanisms. The results give new insights into the role of Ras nanoclusters as promising new molecular targets in cancer and in stem cells.

Keywords: cancer stem cells, caveolin, drug screening, FRET, nanoclusters, Ras

SAMMANFATTNING (Swedish Abstract)

Cancer påverkar mera än 20 miljoner människor årligen och mängden människor som insjuknar i cancer ökar globalt. Ras/MAPK-signaleringsräckan är en av de mest studerade signaleringsräckorna i cancer. Ras proteinerna är muterade i nästan 20 % av alla humana cancer, men trots otaliga försök har man i nuläget inte lyckats hitta effektiva läkemedel som specifikt påverkar Ras. Det är nu etablerat att Ras proteiner återfinns i plasmamembranen i små tillfälliga signaleringskomplex som kallas nanoklusters. Dessa Ras nanoklusters är väsentliga för överföringen av information till de signaleringskaskader som är nedströms från Ras. En störning i bildandet av nanoklusters minskar Ras-aktiviteten och -signaleringen. Hämmare och aktiverare av nanokluster-bildningen kan således vara nya potentiella cancerläkemedel. Detta arbete beskriver tre olika studier var vi undersöker idén om rikta in anti-cancer läkemedelsbehandlingen mot olika membranproteiners nanoklusterbildning. Avhandlingen fokuserar på konstrueringen och implementeringen av en enkel cell-baserad FRET-biosensor metod för att screena för och identifiera läkemedel som påverkar Ras-proteinernas organisation i membranen och deras nanoklusters. Olika kemiska bibliotek testades, varefter ett antal potentiella molekyler validerades med hjälp av en kombination av mikroskopiering, biokemiska och biologiska analyser.

I den första studien screenades ett litet kemikaliebibliotek med hjälp av FRET-biosensorer som härletts från H-ras proteinet. Till vår överraskning upptäckte vi att proteinsyntesinhibitorer (PSI) specifikt ökar bildandet av H-ras nanoklusters och således även H-ras beroende nedströmssignalering. Vi kunde dessutom visa att den cycloheximid-inducerade ökningen i H-ras aktiviteten medförde att både bildandet av cancerstamcell (CSC)-mammosfärer och *in ovo* tumörtillväxten ökade. Nämnvärt är att PSI inte ökar mängden K-ras nanoklusters, vilket visar att denna screening-metod också är användbar för att identifiera isoform-specifika inhibitorer för Ras.

I den andra studien använde vi biosensorer som härletts från både H- och K-ras och vi upptäckte att CSC inhibitorn salinomycin specifikt inhibierar bildandet av K-ras nanoklusters och således dess nedströms signalering. Ut över detta upptäckte vi en specifik genisgnatur som predicerar hur känsliga cancercellerna är för CSC inhibitorer. Intressant nog återfanns denna gensignatur i cirka 8 % av tumörerna i The Cancer Genome Atlas (TCGA)-databasen och den korrelerade med en signifikant högre dödlighet. Genom att använda oss av denna gensignatur kunde vi identifiera ytterligare två ämnen, ophiobolin A och congoblatin A som hämmar tillväxten av CSCs. Dessa ämnen inhiberar specifik K-ras nanoklustering och verkar som potentiella bröst CSC-inhibitorer.

I den tredje studien använde vi oss av samma FRET-biosensor princip för att studera hur myristoylerade proteiner, t.ex. heterotrimeriska G-proteiner, Yes- och Src-kinaser, bildar nanokluster och hur de är förankrade i membranen. Vidare validerades Yes-biosensorerna som en passande platform för att utföra kemiska- och genetiska screener för att identifiera ämnen som inhiberar myristoylering.

Resultaten från denna avhandling visar tydligt att Ras-härledda FRET-biosensorer kan användas för att identifiera isoformspecifika Ras-inhibitorer. Resultaten påvisar även att de flesta nya inhibitorer som identifierades främst stör den subcellulära distributionen av Ras och dess organisering i membranen genom en fortfarande oupptäckt mekanism. Dessa resultat ger en djupare förståelse för Ras nanoklustrens roll som lovande nya molekylära mål i kampen mot cancer och cancerstamceller.

Nyckelord: cancerstamceller, caveolin, FRET, läkemedel screening, nanoklusters, Ras

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I. **Najumudeen, A.K.**, Posada, I., Lectez, B., Zhou, Y., Landor, S.J., Fallarero, A., Vuorela, P., Hancock, J.F., Abankwa, D. (2015) Phenotypic screening identifies protein synthesis inhibitors as H-ras-nanocluster increasing tumor growth inducers. Biochemistry. *54*, 7212–7221.

II. **Najumudeen, A.K.**, Jaiswal, A., Lectez, B., Oetken-Lindholm, C., Guzman, C., Siljamäki, E., Posada, I., Lacey, E., Aittokallio, T., Abankwa, D. Cancer stem cell drugs target K-ras signaling in a stemness context. *Manuscript under revision in Oncogene.*

III. **Najumudeen, A.K**.,* Köhnke, M.,* Šolman, M., Alexandrov, K., and Abankwa, D. (2013). Cellular FRET-biosensors to detect membrane targeting inhibitors of N-Myristoylated proteins. Plos ONE *8,* e66425.

ADDITIONAL PUBLICATIONS NOT INCLUDED IN THESIS

IV. Coxon, F.,* Joachimiak, L.,* **Najumudeen, A.K.,*** Breen, G., Gmach, J., Oetken-Lindholm, C., Way, R., Dunford, J., Abankwa, D., and Błażewska, K.M. (2014). Synthesis and characterization of novel phosphonocarboxylate inhibitors of RGGT. Eur. J. Med. Chem. *84*, 77-89.

V. **Najumudeen, A.K.**, Guzmán, C., Posada, I.M.D., and Abankwa, D. (2015). Rab-NANOPS: FRET biosensors for Rab membrane nanoclustering and prenylation detection in mammalian cells. Methods Mol. Biol. *1298*, 29-45.

* these authors contributed equally to the work

ABBREVIATIONS

Arl	Arf-like
APC	adenomatous polyposis coli
Arf	ADP ribosylation factors
AML	acute myeloid leukemia
ATCC	American type culture collection
ATP	adenosine triphosphate
BHK	Baby hamster kidney
CaM	calmodulin
CHX	cycloheximide
CRC	colorectal cancer
CSCs	cancer stem cells
Da	dalton
DMSO	dimethylsulfoxide
EGFR	epithelial growth factor receptor
EM	electron microscopy
ER	endoplasmatic reticulum
Erk	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FPP	farnesylpyrophosphate
FTase	farnesyl transferase
FTI	farnesyl transferase inhibitor
FITC	fluorescein isothiocyanate
FLIM	fluorescence lifetime imagining
FRAP	fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GGTase I	geranylgeranyl transferase I
GGTI	geranylgeranyl transferase inhibitor
GPI-AP	glycophosphatidylinositol – anchored proteins
GppNHp	5'-guanylyl imidodiphosphate
GDP	guanosine diphosphate
GTP	guanosine 5'-triphosphate
GTPase	guanosine triphosphatase
HEK	Human Embryonic Kidney
HTS	High Throughput Screening
MAPK	Mitogen Activated Protein Kinase
MDCK	Madin-Darby canine kidney
MEF	mouse embryonic fibroblasts
Met-AP2	methionine aminopeptidase 2
mTOR	mechanistic target of rapamycin
Myr	myristoylation
NANOPS	Nanoclustering and Prenylation Sensor
NANOMS	Nanoclustering and Myristoylation Sensor
NMT	N-myristoyltransferase
PAT	palmitoyltransferase
PBS	phosphate buffered saline
PDAC	pancreatic ductual adenocarcinoma
PDEδ	non catalytic δ-subunit of phosphodiesterase 6, prenyl binding protein PrBP/δ
ppErk	phosphorylated-Erk
PI3K	phosphoinositide 3-kinase
PM	plasma membrane
PSIs	protein synthesis inhibitors
PTEN	phosphatase and tensin homolog
Raf	rat-1 fibroblast kinase
Ras	rat sarcoma (protein)
Rab	Ras-like proteins in brain
RBD	Ras-binding domain

Rheb	ras homologue enriched in brain
Rho	Ras homologus
RT	room temperature
SDS	sodium dodecyl sulfate
S6K	ribosomal S6-kinase
siRNA	small interfering ribonucleic acid
SOS1	son of sevenless 1
TCGA	The Cancer Genome Atlas
TSC2	tuberous sclerosis complex 2/tuberin

Amino acids

Ala	alanine (A)
Arg	arginine (R)
Asn	asparagine (N)
Asp	aspartate (D)
Cys	cysteine (C)
Gly	glycine (G)
Glu	glutamate (E)
Gln	glutamine (Q)
His	histidine (H)
Ile	isoleucine (I)
Leu	leucine (L)
Lys	lysine (K)
Met	methionine (M)
Phe	phenylalanine (F)
Pro	proline (P)
Ser	serine (S)
Thr	threonine (T)
Trp	tryptophan (W)
Tyr	tyrosine (Y)
Val	valine (V)

INTRODUCTION

According to the World Health Organization around 15% of all deaths worldwide are caused by cancer. In 2012, 14.1 million new cancer cases and 8.2 million cancer deaths were registered worldwide. These numbers are expected to rise due to the changing lifestyles, growth and aging of the world population. This has gathered great attention and efforts from the academic and pharmaceutical industry to identify and develop anti-cancer agents. Cancer is defined as a group of diseases characterized by uncontrolled cell growth and spreading of cancer cells.

A number of strategies have been employed over the last century to treat cancer. They range from surgery, chemotherapy, radiation, hormone therapy, and immune therapy to targeted therapy, which employs drugs against specific molecules in cancer cells. However, success of these different therapeutic strategies varies widely.

The pioneers of molecular oncology identified a collection of genes that when mutated lead to uncontrolled cell proliferation and cancer. Many of these oncogenes and oncoproteins are now known to be drivers of human cancers. Several anti-cancer agents have been developed in the past few decades against human cancers. Cisplatin, a first-class of anti-cancer drugs, is a DNA-binding agent that induces cell death. Natural product-derived anti-cancer drugs such as paclitaxel (Taxol) that deregulate cell division, have potent anti-cancer properties. However, these drugs and their counterparts affect many cellular pathways and they are not specific for cancer cells, thus causing severe side effects. The identification of oncogenes and the molecular level understanding of their function have resulted in the identification of more specific anti-cancer drugs, such as the Abl kinase inhibitor imatinib (Gleevec) that is effective in treating chronic myeloid leukemia. In addition to small molecules, antibodies against kinase receptors have also been developed. For example, bevacizumab (Avastin), a humanized monoclonal antibody against the vascular endothelial growth factor A (VEGF-A) that targets angiogenesis has been in use for treating several cancers.

Ras proteins are an important class of anti-cancer targets that are frequently mutated in cancers. Yet, after thirty years of intense efforts to target these mutant proteins, no safe drugs have been developed successfully. However, these efforts have substantially increased our understanding of Ras function and regulation in normal and cancer cells. Today, we know that Ras forms nanoscale signaling domains called nanoclusters on the plasma membrane that are critical for Ras signaling.

This thesis summarizes efforts to identify chemical modulators of Ras membrane anchorage and nanoclustering, and postulates nanoclustering as a general characteristic of lipid-bound proteins.

REVIEW OF THE LITERATURE

1 Ras Superfamily of Small GTPases

Small guanosine triphosphatases (GTPases) are proteins that bind and hydrolyze guanosine triphosphate (GTP). The human Ras-superfamily of small GTPases consists of 160 proteins that are subdivided based on function and sequence similarities into five classical families consisting of 39 Ras proteins, 30 Arfs, 22 Rhos, 65 Rabs, 1 Ran and some 'unclassified' sequences (Figure 1). These families of proteins are involved in functions ranging from cellular signaling, proliferation, differentiation, cytoskeletal organization, vesicular trafficking to nuclear transport (Rojas et al., 2012).

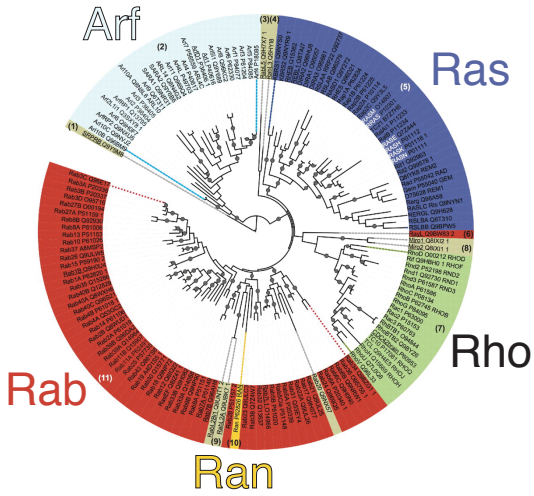

Figure 1. The Ras Superfamily of GTPases. Adapted from (Rojas et al., 2012)

Small GTPases act as molecular binary switches and they have the following characteristics that form a common biochemical paradigm for the whole superfamily: they cycle between an active GTP-bound 'On' and an inactive GDP-bound 'Off' state, they have high affinities for GDP and GTP, they all contain the G-domain that binds GTP or GDP, and they undergo large conformational changes following nucleotide binding (Wennerberg et al., 2005). The G-domain contains five conserved G box fingerprint motifs called G1-G5. Guanine nucleotide binding-induced structural changes happen in the 'switch' regions - switch I (Ras residues 30-38) and switch II (Ras residues 59-67). In short, activation of Ras proteins begins as the γ-phosphate of the GTP interacts with switch I (at threonine 35) and II (at glycine 60) pulling them close to the nucleotide into what is interpreted as the "loaded spring" mechanism (Vetter and Wittinghofer, 2001). This induces a conformational change to form an interaction surface that favors effector binding in

a GTP-dependent fashion. When the γ-phosphate is cleaved-off by the GTPase activity, it releases the "loaded spring" yielding the GDP-bound form. The GDP/GTP state of Ras is regulated by the guanine nucleotide exchange factors (GEFs) that turn "on" the molecular switch by promoting the dissociation of bound GDP in exchange for a new GTP (Figure 2). Since the cytosolic GTP concentration is much higher than GDP, GTP replaces GDP and changes Ras to an active state (Antonarakis and Van Aelst, 1998). Conversely, this active state of Ras is regulated by its very low intrinsic GTPase activity that is accelerated strongly by the GTPase-activating proteins (GAPs) that cause the hydrolysis of GTP to GDP and promote the formation of the inactive GDP-bound Ras. This cycling of Ras between the GDP and the GTP bound states is known as the GTPase cycle (Figure 2) (Vigil et al., 2010).

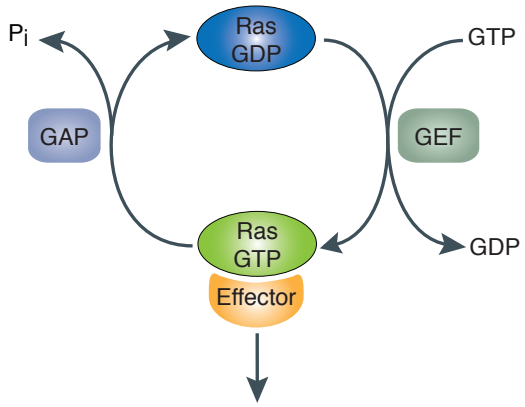

Figure 2. The GTPase cycle. Ras is a low-molecular weight guanine nucleotide binding protein that acts as a molecular switch cycling between a GDP- and GTP-bound state, which is regulated by the guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Inactive GDP-bound Ras is activated by GEFs that catalyze GTP binding of Ras. Consequently, GTP-bound active Ras interacts with, and activates, the downstream signaling effector proteins. The active state of Ras is regulated by its low intrinsic GTPase activity, which is accelerated strongly by GAPs leading to hydrolysis of GTP to GDP.

1.1 Ras as founding members of the Ras superfamily

Ras has the distinction of being the first oncogene to be identified in human cancers (Der et al., 1982; Parada et al., 1982; Santos et al., 1982). This observation came to fruition from the early discoveries of acutely transforming retroviruses that caused sarcomas in infected animals. This was followed by the discovery of the oncogenic Harvey murine sarcoma virus (Harvey, 1964) and Kirsten murine sarcoma virus (Kirsten and Mayer, 1967) and their ability to cause Rat Sarcomas (Ras). The viral genes were named *HRAS* and *KRAS* and their corresponding proteins came to be known as Ha-Ras or H-ras and Ki-Ras or K-ras. By 1980s, studies from Scolnick and colleagues made it clear that the *HRAS* and *KRAS* genes encode a membrane bound,

21 kDa protein that binds GDP and GTP. They also identified that the cellular equivalent of these viral genes had similar properties and their preferential GTP binding is key for transformation (Cox and Der, 2010). In 1982, three laboratories made the milestone discovery of identifying the transforming gene from human cancers to be homologues of the viral Harvey and Kristen-RAS genes (Der et al., 1982; Parada et al., 1982; Santos et al., 1982). This was followed by the identification of *NRAS* - named after the oncogene found in a human neuroblastoma cell line (Ireland, 1989). Today these Ras proteins are considered the founding members of the Ras superfamily of proteins. The Ras family has received much attention as it occupies a central role in many cellular signaling processes including cell growth, differentiation and apoptosis.

1.2 Ras isoforms have high sequence identity

After more than 30 years since the original finding, we now know that mammalian cells have three 'classical' RAS genes encoding four distinct clinically relevant isoforms of Ras – H-ras, K-ras4A and 4B (two splice variants of the K-ras gene) and N-ras. These four Ras isoforms are 188–189 amino acid long with an almost 82-90% overall sequence identity. The first 164 residues comprise the highly conserved G-domain, with the first 80 amino acids being identical and the next 85 amino acids display over 95% sequence identity (Figure 3). All the four Ras isoforms differ almost exclusively in the 25 amino acids of the carboxyl-terminal known as the hypervariable region (HVR) (Figure 3). Therefore, it is suggested that the functional differences between the Ras isoforms is due to the HVR. As discussed later, HVR contains the targeting information needed for Ras membrane anchorage that is essential for Ras function (Cox and Der, 2010).

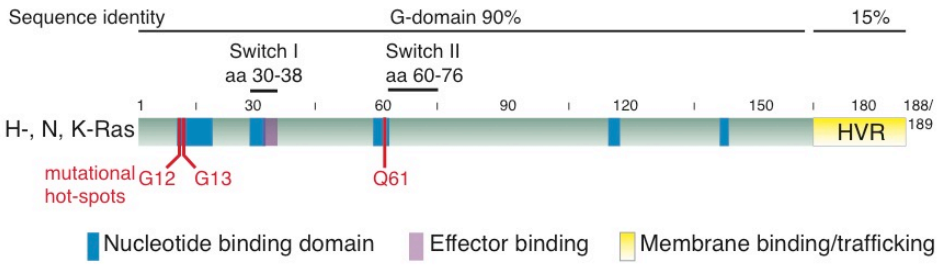

Figure 3. Ras overall domain structure. The four Ras isoforms (H-, N-, K-ras4A and 4B) that are 188-189 amino acid long share 82-90% overall sequence identity. The residues 1-164 comprise the G-domain that contains the nucleotide binding domains, the effector binding domain, and the switch regions. The nucleotide binding domains (blue) comprise the five conserved GTP-binding motifs. The effector-binding domain (purple) (residues 32-40) is involved in effector binding specificity. The switch I (residues 30-38) and II (residues 60-76) regions change conformation with GDP/GTP binding. The last 25 amino acids comprise the hypervariable region (HVR) that is essential for membrane binding. The key residues that are mutated in cancers are identical among the three Ras isoforms and are highlighted in red.

The *KRAS* gene encodes for two gene products that are generated by alternative splicing. These splice variants differ by the use of alternative fourth exons and are designated K-ras4A and 4B. The alternative fourth exons encode the C-terminal regions responsible for membrane targeting. K-ras4A can be palmitoylated and K-ras4B lacks the palmitoylation site. Recent data suggest that K-ras4A is expressed in several cancers including colorectal cancers (Tsai et al., 2015). However, K-ras4B is the ubiquitously expressed splice variant, and unless otherwise mentioned K-ras4B will hereafter be denoted as K-ras.

1.3 Ras activation is regulated by GEFs and GAPs

Ras proteins are activated by receptor tyrosine kinases (RTKs), G-protein coupled receptors or steroid hormones in response to various extracellular stimuli required for cell proliferation, growth, tissue development or repair. These cellular functions are tightly controlled via Ras proteins in normal cells and the deregulation of these key functions is a hallmark of cancer (Hanahan and Weinberg, 2011). In non-malignant cells, growth factor binding to the extracellular domain of the RTK activates the receptor, which initiates the recruitment of adaptor proteins, like Grb2 or Shc. This in turn leads to the recruitment of GEFs to the plasma membrane. Ras can be activated by one of the eight known GEFs, including the two SOS (Son of Sevenless) isoforms, two specific guanyl nucleotide releasing factors (RASGRF) and four RAS-specific guanine nucleotide-releasing proteins (RASGRP) (Vigil et al., 2010). These GEFs contain the CDC25 homology catalytic domain, which stimulates GDP release, and the N-terminal Ras exchange motif. SOS is one of the best-characterized GEFs; first identified to regulate *Drosophila melanogaster* eye development and later identified in humans (Rogge et al., 1991). The Grb2-SOS complex translocates via the SH2-domain close to the plasma membrane associated RTK and brings SOS in the vicinity of Ras. The Ras-SOS-binding stimulates the release of the bound nucleotide from Ras. Because the cytosolic GTP concentrations are ten-fold higher than GDP, GTP is rapidly exchanged for the previously utilized GDP, thus promoting the formation of active GTP-bound Ras (Vetter and Wittinghofer, 2001). This active Ras can bind with high affinity to the downstream effectors. The active GTP-bound Ras needs to be deactivated. As Ras has a very weak intrinsic GTPase activity, the deactivation is accelerated by the GAPs that can increase the rate of GTP hydrolysis by a factor of 10^5. Thus, GAPs, by acting as negative regulators of Ras, limit Ras signaling. In 1987, Trahey and McCormick characterized the first Ras-GAP, p120RasGAP (Trahey and McCormick, 1987). It was shown to bind Ras and increase the rate of GTP hydrolysis by 300 fold. This was followed by the identification of neurofibromin (NF1) as a RasGAP, from patients with type I neurofibromatosis, an inherited condition of benign brain tumors (Martin et al., 1990). Currently, there are seven known mammalian Ras-specific GAPs and they function through binding to the catalytic site of Ras and increasing the hydrolysis of Ras-GTP by inserting an 'Arg finger' into the active site (Ahmadian

et al., 1997; Vigil et al., 2010). This favors the nucleophilic attack of water on GTP and this catalysis is mediated by the conserved glutamine 61, as the GAP stabilises the switch II domain. However, oncogenic mutations on Ras, such as the mutations on Glycine 12, 13 and Glutamine 61 render Ras insensitive to GAP hydrolysis and thereby persistently lock Ras into a GTP-bound active state (Bos et al., 2007). Ras GEFs are deregulated in cancers through infrequent somatic mutations and increased gene expression leading to aberrant GEF activation. Though somatic mutations are rare, germline gain-of-function mutations in Ras GEF SOS are found in almost 13% of patients with Noonan syndrome, which is also associated to an increased risk to cancer (Roberts et al., 2006). However, these mutations are thought to be weakly activating and not potent enough to lead to cancer formation (Swanson et al., 2008). Ras GAPs stimulate the intrinsic GTPase activity and in general act as tumor suppressors. Ras GAP neurofibromin encoded by tumor suppressor *NF1* is found to have both germline and somatic mutations. Loss-of-function germline mutations in NF1 are found in neurofibromatosis type 1 patients that have an increased risk of developing cancers of the nervous system (Vigil et al., 2010).

1.4 Ras signal transduction

Active Ras transmits the signal downstream through interacting with proximal Ras effectors. There are more than ten reported Ras effectors that have a strong affinity for GTP-Ras, initiating downstream signaling cascades (Figure 4). The majority of the Ras effectors share the same structural feature known as the Ras-binding domain (RBD) that promotes interaction of the effectors with GTP-Ras. Although there are no obvious primary sequence similarity in the RBDs and RA (Ras-association) domains of the different effectors, they all exhibit the same topology of a ubiquitin superfold (Herrmann, 2003). This common structural fold of these otherwise diverse primary sequences accounts for the similar mode of interaction of different effectors with Ras (Repasky et al., 2004). These different Ras effectors determine the biological effects of Ras downstream signaling. The best-studied Ras effector pathways are the Raf and PI3K pathways.

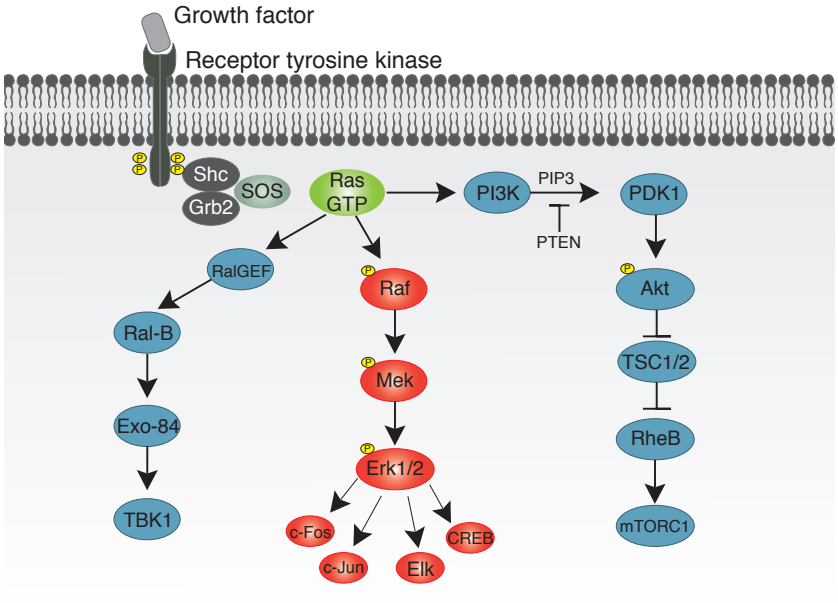

Figure 4. Simplified representation of selected Ras signaling pathways. In this highly simplified depiction of the Ras signal transduction pathway, growth factors activate receptor tyrosine kinases, which through a series of phosphorylations activate adaptor proteins and exchange factors that activate Ras. Active Ras binds and activates among others, the Raf/Mek/Erk, PI3K/PDK1/Akt and RalGEF/Ral/TBK1 pathways. Ras binds and recruits Raf to the plasma membrane, Raf phosphorylates Mek1/2 and Mek in turn activates Erk. Consequently, Erk activates a group of effector proteins and transcription factors that are involved in cell cycle regulation and proliferation. Furthermore, Ras activates PI3K that via PIP3 activates PDK1, which in turn phosphorylates and activates Akt that controls cell survival and growth via a number of key proteins. Ras also activates RalGEFs that in turn activate RalB, which activates TANK-binding kinase1 (TBK1).

The Raf-Mek-Erk pathway is a major part of the mitogen activated protein kinase (MAPK) signaling cascade. There are three Raf isoforms: A-Raf, B-Raf and C-Raf (Raf-1), and these Raf isoforms share sequence and domain identity. At the N-terminus they have an RBD followed by the cysteine-rich domain (CRD), and at the C-terminus a serine/threonine kinase domain. C-Raf serine/threonine kinase is the first and the best characterized Ras effector, identified originally from the retrovirus (3611-MSV) that caused rapidly accelerated fibrosarcomas (Lavoie and Therrien, 2015). Active Ras directly interacts with Raf through the RBD and relieves Raf auto-inhibition and initiates plasma membrane recruitment of Raf. This leads to additional phosphorylations that activate the catalytic region (Dickson et al., 1992; Koide et al., 1993; Lavoie and Therrien, 2015). Active Rafs proceed to phosphorylate and activate MAPK kinases/extracellular signal-regulated kinases (Mek1/2) (Kyriakis et al., 1992). Activated Mek phosphorylates and activates extracellular signal-regulated kinases (Erk1/2) (Gallego, 1992). Active Erk can phosphorylate cytosolic proteins, such as p90 S6 kinase, or it can translocate to the nucleus and

activate transcription factors such as Elk-1, c-Fos and c-Jun, which can regulate cell cycle or proliferation (Figure 4).

Phosphoinositide 3-kinase (PI3K) is the next best characterized Ras effector (Rodriguez-Viciana et al., 1994). There are three classes of PI3Ks, of which the class I PI3Ks are the best described. PI3Ks are heterodimeric lipid kinases consisting of one of three catalytic subunits (p110α, β, and δ) and one regulatory subunit (p85) (Esther Castellano, 2011; Simons and Ikonen, 1997; Simons and Sampaio, 2011). PI3Ks convert phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5) trisphosphate (PIP_3). PIP_3 in turn activates PDK1 that phosphorylates the kinase Akt. Depending on the cellular context, Akt plays a role in an array of signaling pathways including cell growth, survival and migration (Figure 4) (Wong et al., 2010; Yuan and Cantley, 2008). Akt-mediated signaling through the tuberous sclerosis complex (TSC) leads to activation of the mammalian target of rapamycin (mTOR) complex that primarily regulates protein synthesis. These Ras effector pathways have gathered attention as some effector components of these pathways are frequently mutated in cancers and thus can drive cancer formation.

2 Post-translational modifications of Ras GTPases

The wide number of biological functions of the Ras superfamily of small GTPases are dependent on signaling specificity. Together with the activity as molecular switches, signaling specificity of small GTPases is achieved through post-translational modifications. Nearly all members of the Ras, Rab and Rho superfamily small GTPases undergo distinct post-translational modifications in the C-terminus. These modifications modulate the subcellular localization, as well as interactions with negative and positive regulators, downstream effectors and chaperones.

2.1 Ras synthesis, processing and trafficking to the plasma membrane

Ras proteins are synthesized in the cytosol and are subsequently targeted to the cellular membranes by series of post-translational modifications in the HVR (Figure 5).

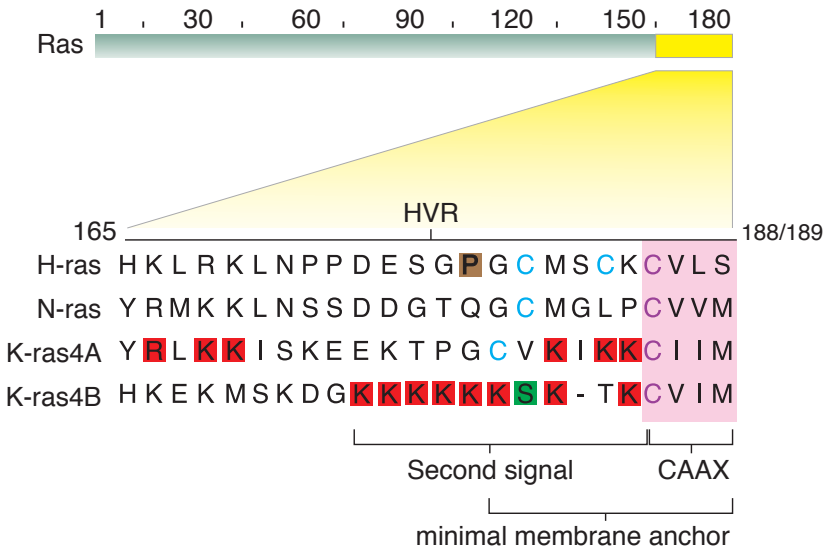

Figure 5. The Ras hypervariable membrane targeting region. The C-terminal hypervariable region (HVR) of the four Ras isoforms is shown. This region of 24-25 amino acids contains all the membrane targeting information required for proper membrane targeting of the specific Ras isoforms. It comprises the C-terminal CAAX box (pink) that dictates the prenylation and post-prenyl processing. Followed by the 'second signals' immediately upstream of the CAAX box, such as palmitoylated cysteine(s) (in blue), polybasic regions (in red) or serine phosphorylation sites (green). For H-, N- and K-ras4A, the second signal is the palmitoylation on the cysteines (blue) or clusters of polybasic regions. In K-ras4B the second signal is provided by a stretch of polybasic lysines (red). S (green) marks the phosphorylated Serine 181 of K-ras4B. P (brown), the Proline 179 involved in the cis-trans isomerization that regulates H-ras depalmitoylation.

For the canonical Ras isoforms, the first step in post translational modifications is the irreversible addition of the 15-carbon farnesyl isoprenoid catalyzed by the enzyme farnesyl transferase (FTase) to the cysteine residue on the CAAX box (C denotes cysteine, A aliphatic amino acid, and X is any amino acid) (Figure 6) (Casey et al., 1989; Hancock et al., 1989). Alternatively, in the presence of FTase inhibitors, K-ras and N-ras become prenylated by the 20-carbon geranylgeranyl chain through the geranylgeranyl transferase I (GGTase I) (Whyte et al., 1997). The isoprenoids offer weak but sufficient membrane affinity for the prenylated proteins and serve as a foundation to promote the next processing steps. Prenylated Ras is targeted to the endoplasmic reticulum (ER) where the 'AAX' amino acids are proteolytically cleaved by the ER-resident metalloprotease RCE1 (Ras-converting enzyme 1). This is followed by the carboxy methylation of the farnesylated cysteine by the isoprenylcysteine carboxymethyltransferase (ICMT) in the ER (Figure 6) (Gutierrez et al., 1989).

These CAAX-processed proteins require at least one 'second signal' to enhance their membrane interactions and trafficking to the plasma membrane (Hancock et al., 1990). There are two types of second signals, in the case of H-ras, N-ras and K-ras4A it is the Cys residues that get palmitoylated and for K-ras4B the second signal is the stretch of polybasic lysines (polybasic region) upstream of the CAAX motif. The covalent addition of acyl chain of a fatty acid to a protein is termed acylation and the addition of a 14-carbon myristoyl chain (myristoylation) or a 16-carbon palmitoyl chain (palmitoylation) to a protein is most common (Resh, 2013). Myristoylation occurs as an irreversible cotranslational modification usually at the N-termini glycine of the proteins (Magee, 1990). Palmitoylation is a reversible post-translational modification that in the case of Ras proteins is catalyzed by protein acyl transferases (PATs) present in the cytoplasmic face of the Golgi. DHHC9 and GCP16 together create a PAT that selectively palmitoylates H- and N-ras (Figure 6) (Swarthout et al., 2005). H-ras is palmitoylated on 2 cysteines (Cys181 and Cys184), K-ras4A and N-ras on cysteine (Cys180 in K-Ras 4A and Cys181 in N-ras) (Laude and Prior, 2008). This palmitoylation together with farnesylation creates a 100-fold higher affinity of Ras to the membranes (Shahinian and Silvius, 1995).

In contrast, K-ras4B is not palmitoylated, instead it has a polybasic region (PBR) that together with the farnesyl group provide electrostatic interactions with the negatively charged phospholipids at the inner leaflet of the plasma membrane (Hancock et al., 1989; 1990). Neither the farnesylation alone, nor the PBR can provide stable membrane association for K-ras4B, but both these interactions together provide enough affinity. K-ras4B does not go to the Golgi instead traffics more directly to the plasma membrane. Recent data show that K-ras4A is unique with a hybrid membrane targeting sequence that has a palmitoylated cysteine flanked by two short polybasic regions (Tsai et al., 2015). It is also shown that either of these two motifs is enough to deliver K-ras4A to the plasma membrane. Furthermore, palmitoylated K-ras4A lacks Golgi enrichment, suggesting that a

plasma membrane-associated PAT is likely involved in the palmitoylation rather than DHHC9/GCP16 (Tsai et al., 2015).

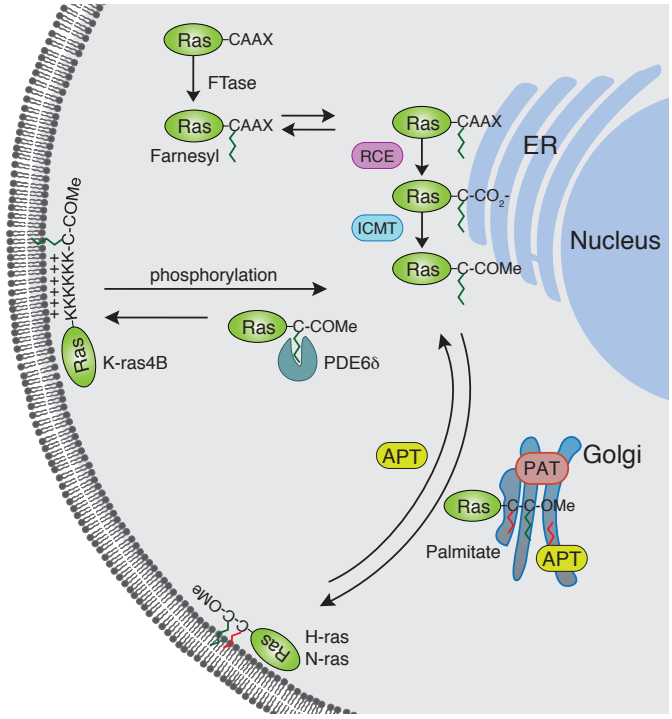

Figure 6. Ras post-translational processing and trafficking. Ras proteins are synthesized on the cytosol and quickly farnesylated (green, lipid anchor) by farnesyl transferase (FTase) at the C-terminal cysteine of the CAAX motif and are transported to the endoplasmic reticulum (ER). In the ER, Ras encounters the Ras-converting enzyme (RCE1), which cleaves the terminal AAX residues, as well as isoprenylcysteine carboxymethyltransferase (ICMT1), which carboxymethylates the C-terminal farnesylated cysteine. K-ras4B, which is not palmitoylated, traffics directly to the plasma membrane and the farnesyl motif together with electrostatic interactions from its polybasic region (PBR) provide stable plasma membrane binding. H-ras and N-ras undergo reversible palmitoylation by golgi-resident protein acyltransferases (PATs) that add palmitoyl groups (red, palmitate) on the cysteines, which enables stable binding to the plasma membrane. Deacylation by the acyl¬protein thioesterases (APT1/2) release Ras from the plasma membrane to be reacylated back to the plasma membrane. The prenyl-binding protein PDEδ controls association of all Ras isoforms with membranes by binding to the farnesyl moiety and solubilizing non-palmitoylated Ras, so that Ras can be restored to the plasma membrane. (Figure modified from Ahearn et al., 2011a)

2.2 Ras spatial organization and regulation

The regulation of Ras membrane-association and trafficking is rather complex and not yet fully understood (Figure 6). The association of Ras with multiple subcellular membranes is dynamic and it is facilitated by the reversible interactions of the polybasic domains and the palmitoyl groups. This is achieved through a dynamic Ras palmitoylation and depalmitoylation cycle consisting of palmitoylation at the Golgi, delivery to the plasma membrane by the secretory pathway, followed by

ubiquitous depalmitoylation and return to the Golgi where they can interact with PATs for palmitoylation (Figure 6). Recent work has demonstrated that acyl protein thioesterases (APT1/2) are responsible for H- and N-ras depalmitoylation. APT1 cleaves the thioester bond and removes the S-palmitoylation leaving the Ras with the farnesyl moiety (Dekker et al., 2010). This depalmitoylation solubilizes the Ras to the cytosol, leaving it to be re-palmitoylated in the Golgi and trafficked back to the plasma membrane. This makes up a unidirectional cycle between the plasma membrane and Golgi. This cycle is critical for ensuring the proper distribution of palmitoylated Ras to the plasma membrane and the Golgi.

This is further enhanced by the prenyl-binding protein PDE6δ (phosphodiesterase of retinal rod subunit δ) that acts as a solubilizing factor that modulates Ras proteins by sustaining their dynamic distribution in cellular membranes. PDE6δ sustains correct intracellular organization of farnesylated Ras proteins, Rheb and other small G proteins (Chandra et al., 2012). Shuttling by PDEδ is regulated by the allosteric interaction with ADP ribosylation factor-like 2/3 (Arl2/3). In fact, the binding of the GTP-bound active Arl2 to PDEδ allosterically expels farnesylated Ras from the binding pocket of PDEδ (Ismail et al., 2011). This expulsion results in enrichment of farnesylated Ras on perinuclear membranes. From these membranes, K-ras with its polybasic region is trapped by negatively charged recycling endosomes (Schmick et al., 2014) and depalmitoylated H/N-ras are re-acylated in the Golgi (Rocks et al., 2010). From both these compartments – recycling endosomes and the Golgi – Ras is trafficked back to the plasma membrane, where it again participates in signal transduction. In addition, depalmitoylation of H-ras was recently shown to be stimulated by the peptidyl-prolyl isomerase activity of 12 kDa FK506-binding protein (FKBP12) (Ahearn et al., 2011). FKBP12 catalyzes the cis-trans isomerization of Gly-Pro at 178-179 of H-ras and accelerates depalmitoylation. Inhibiting or silencing FKBP12 inhibits H-ras depalmitoylation (Ahearn et al., 2011), implicating peptidyl-prolyl isomerization in Ras organization and regulation.

There are also other modifications that can regulate Ras trafficking and signaling. For instance, protein kinase C (PKC)-dependent phosphorylation of Serine 181 in K-ras4B – a process mutually exclusive with calmodulin binding to K-ras4B - interferes with the electrostatic interaction of the HVR with the plasma membrane (Villalonga et al., 2001; 2002). This changes the localization of K-ras4B from the plasma membrane to the cytosol and mitochondria where K-ras4B triggers apoptosis via Bcl-XL (Bivona et al., 2006), thereby converting K-ras4B from a growth-promoting to a growth-suppressing protein (Sung et al., 2013). However, there have also been reports suggesting that phosphorylation of K-ras4B is necessary for its oncogenic properties and tumor growth (Alvarez-Moya et al., 2010; Barceló et al., 2014). Taken together, although the different Ras isoforms share a high degree of sequence identity the mature protein products display distinct patterns of intracellular processing, post-translational modification, and subcellular location, largely depending on the differences in the HVR.

3 Nanoclustering

The plasma membrane was for long thought to act just as a permeable lipid barrier separating the cytosol and the extracellular environment. Now it is clear that the plasma membrane is a complex, heterogeneous and dynamic organelle comprised of a large array of subdomains that are regulated both spatially and temporally. These lipid domains are formed through lipid-lipid, lipid-protein, protein-protein and protein-cytoskeleton interactions.

3.1 Lipid rafts to signaling protein nanoclusters

In 1972 the famous Singer-Nicolson 'fluid-mosaic' model of the plasma membrane integrated transmembrane proteins into a bilayer lipid matrix, where membrane proteins could diffuse freely among homogenous lipids as independent components (Singer and Nicolson, 1972). Already by 1974 experimental evidence suggested that the fluid-mosaic model was probably inaccurate and that lipids were proposed to exist as 'clusters of lipids' that appeared in a 'more ordered-state' surrounded by free lipids (Lee et al., 1974; Wunderlich et al., 1975). In 1982 Karnovsky et al. first formalized the idea of lipid domains (Karnovsky et al., 1982), followed by the classic report from Brown and Rose in the early 1990s, showing the selective partitioning of glycophosphatidylinositol-anchored proteins (GPI-APs) to cholesterol and sphingolipid enriched detergent-resistant fractions (Brown and Rose, 1992). This was followed by studies from Simons et al. in the mid-1990s suggesting the functional significance of lipid raft membrane microdomains would be to act as signaling platforms (Simons and Ikonen, 1997; Simons and Sampaio, 2011). According to these early descriptions, lipid rafts were viewed as stable pre-existing, 100-500 nm wide, rigid, cholesterol-rich liquid-ordered membrane domains into which proteins with appropriate lipid anchors or transmembrane domains partitioned preferentially. Indeed, experiments with artificial bilayers showed existence of such domains, thereby supportive of their existence also in live cell plasma membranes. However, such rafts could not be detected in plasma membranes, leading to the questioning of their very existence in cells. This along with difficulties to visualize rafts in living cells and ambiguities of the approaches used made this model very contentious. More recent and alternative models view rafts as transient, dynamic, nanoscale cholesterol-dependent domains encompassing specific lipids and proteins that influence the formation and stability of the domains.

3.2 Ras nanoscale organization on the plasma membrane

Already in the wake of the raft-model, the application of fluorescence-based methods such as fluorescence resonance energy transfer (FRET) and fluorescence polarization anisotropy (FPA), lead to the identification of transient, nanoscale cholesterol-dependent clusters of GPI-anchored proteins (Goswami et al., 2008; Sharma et al., 2004; Varma and Mayor, 1998). This provided compelling evidence

for the existence of nanoscale assemblies for previously identified raft associated proteins including Ras, Src-kinases and heterotrimeric G-proteins.

The first tentative evidence for raft association of Ras was from membrane fractionation and biochemical experiments (Prior et al., 2001; Roy et al., 1999). This was followed by the direct visualization of the distribution of GFP tagged Ras on intact apical plasma membranes sheets using electron microscopy (EM) combined with spatial mapping (Prior et al., 2003). This EM spatial analysis showed that ~44 % of the Ras proteins on the plasma membrane are distributed in immobile nanodomains called nanoclusters, while the rest exist as freely diffusing monomers (Plowman et al., 2005; Prior et al., 2003). Moreover, these Ras nanoclusters and free monomers are also found to be freely diffusing and not confined to any membrane ultrastructure. Each of these nanoclusters is 12-20 nm in diameter and contains 6-7 Ras proteins. These experiments provided the definitive evidence for existence of Ras nanoclusters (Figure 7). Single particle tracking (SPT) experiments using GFP-Ras in live cells estimated the lifetime of Ras nanoclusters to be ~0.1 to 1 s, suggesting a dynamic turnover of Ras nanoclusters (Murakoshi et al., 2004; Plowman et al., 2005) that are constantly forming and disassembling.

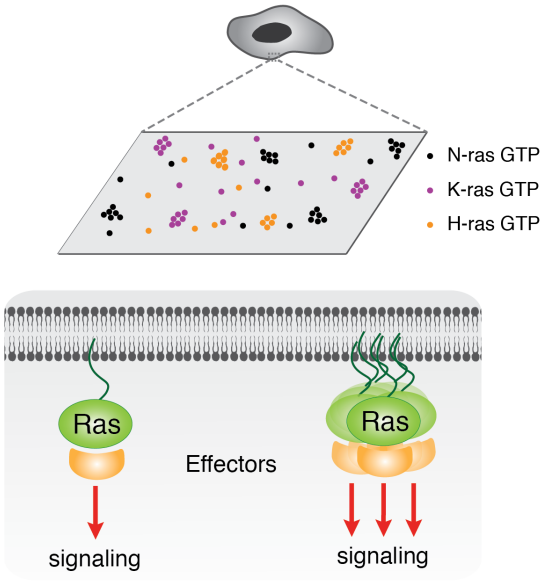

Figure 7. Ras nanoclusters. *Top,* Ras proteins form isoform- and guanine nucleotide-specific spatially segregated and functionally distinct nanoclusters that serve as signaling platforms of downstream effector proteins.

With the combination of quantitative imaging techniques such as EM-mapping, SPT, FLIM-FRET and FRAP it is now clear that the Ras isoforms: H, N and K, all occupy and operate as spatially distinct, non-overlapping nanoclusters (Figure 7). There is further lateral segregation for each Ras isoform based on the activation

state, such that Ras-GTP and Ras-GDP nanoclusters are distinct and non-overlapping for each isoform. This highly dynamic spatiotemporal system of Ras nanoclustering is very similar to the clustering of GPI-APs (Sharma et al., 2004). Similar to Ras, GPI-APs also exist as freely diffusing monomers and transient immobile cholesterol-sensitive nanoclusters (Sharma et al., 2004). Computational modeling and signaling experiments have shown for both GPI-APs and Ras that the fraction of proteins in nanoclusters, i.e. the clustered fraction, is constant over a multi-log range of plasma membrane expression levels. This means that the clustered fraction is insensitive to the expression levels, suggesting that the system is actively held in a non-equilibrium state. For GPIs, this is regulated by the cortical actin cytoskeleton, but in the case of Ras, the spatiotemporal dynamics is a combination of the plasma membrane interactions of the lipid anchors, the HVR and G-domain and the recently characterized lipid components of the plasma membrane (Zhou and Hancock, 2015). Furthermore, the different Ras isoforms with distinct nanocluster composition differentially use these components.

3.3 Ras nanocluster composition

3.3.1 Cholesterol

Cholesterol mediates the lateral segregation of Ras, which is essential for proper effector recruitment and downstream signaling (Prior et al., 2003; Zhou et al., 2010). The first evidence of difference in lipid composition of Ras nanoclusters was from cholesterol depletion experiments with methyl β-cyclodextrin (MβCD) (Prior et al., 2003). These experiments provided evidence for time-dependent loss of GFP-tH clustering in MβCD treated cells, however this treatment had no effects on GDP or GTP bound H-ras. It is now clear that H-ras-GDP forms cholesterol-dependent clusters and H-ras-GTP forms cholesterol-independent clusters (Prior et al., 2003). EM and FLIM-FRET measurements show that MβCD mediated cholesterol depletion disrupts the lateral segregation of H-ras-GTP and H-ras-GDP nanoclusters leading to extensive mixing of GTP and GDP-bound H-ras. These heterotypic clusters significantly compromise H-ras mediated signal transduction (Ariotti et al., 2014; Roy et al., 1999). In contrast to H-ras, GTP bound N-ras is cholesterol dependent, and GDP bound N-ras is cholesterol independent. N-ras-GDP nanoclusters are insensitive to cholesterol depletion while N-ras-GTP clusters are highly sensitive to MβCD treatment (Roy et al., 2005). K-ras-GTP and GDP nanoclusters, however, are both cholesterol-independent structures (Plowman et al., 2005). Taken together, these data clearly show that cholesterol operates distinctly in different Ras nanoclusters.

3.3.2 Acidic Lipids

Apart from cholesterol, the lipid composition of the various Ras nanoclusters was revealed in a recent study (Zhou et al., 2014). In this study, systematic EM mapping

of the lipid content associated with different Ras isoforms was performed using fluorescently tagged lipid-binding probes, such as pleckstrin homology (PH) and C2 domains. Phosphatidic acid (PA), labeled with GFP-Spo20, co-clustered more with K-ras-GTP and H-ras-GTP than H-ras-GDP nanoclusters. Whereas phosphatidylinositol 4,5-biphosphate (PIP2), labeled with GFP-PH-PLCδ, and phosphatidylinositol 3-phosphate (PI_3P), labeled with GFP-PH-FYVE, were found to be enriched in clusters with H-ras-GDP. On the other hand, phosphatidylinositol 4-phosphate (PI4P) preferentially co-clusters with H-ras-GDP and H-ras-GTP nanoclusters but not K-ras nanoclusters. Particularly phosphatidylserine (PS), labeled with GFP-LactC2, co-clustered with all Ras nanodomains tested. Nevertheless, PS is also important for the structural integrity of the nanoclusters, as discussed below. These results clearly indicate that nanoclusters of different Ras isoforms are composed of distinct lipid compositions (Zhou et al., 2014). This is consistent with the observations that different Ras effectors have lipid substrate preferences, like H-ras that efficiently activates PI3Ks, which bind PIP2 via the p110 subunit. K-ras on the other hand extensively co-clusters with PA, and activates Raf that has a PA-binding domain. Thus the lipid composition of each Ras nanocluster also determines the effector recruitment and signaling downstream.

In particular, EM and FLIM-FRET experiments show that PS is required for the structural stability of K-ras-GTP nanoclusters, but not H-ras-GDP or -GTP nanoclusters (Zhou et al., 2014). K-ras interacts with PS through electrostatic interactions with the HVR while H-ras mostly interacts with PS via the palmitate anchors (Hancock, 2003). However, the PS-H-ras interaction is less specific and less sensitive to PS level changes (Zhou et al., 2014). Moreover, PS depletion has been shown to disrupt K-ras nanoclustering and mislocalise K-ras, thus abrogating K-ras signaling (Cho et al., 2012b). Analogous to cholesterol, distribution of PS has significant effects on the lateral segregation of Ras. Proper lateral segregation of K- and H-ras-GTP occurs over a narrow optimal range of plasma membrane PS levels. Like cholesterol, PS levels beyond this optimal range lead to mixing of K-ras and H-ras and these heterotypic clusters compromise Ras signaling (Zhou et al., 2014).

3.3.3 Nanocluster Scaffolds

In addition to cholesterol and lipids, protein scaffolds are also involved in regulating Ras nanoclustering. Galectins are a family of 15 β-galactoside-binding lectins that share a consensus amino acid sequence and members of this family are shown to act as Ras nanocluster scaffolds. Galectins have been implicated in a wide range of biological functions such as homeostasis, apoptosis, and vascular embryogenesis and in pathological conditions such as inflammation, diabetes, atherosclerosis and cancer (Astorgues-Xerri et al., 2015). Several lines of evidence show galectins to have extracellular and intracellular functions (Liu et al., 2002). Galectin-1 (Gal-1), a member of the galectins, was first shown to interact with H-rasG12V and increase Ras activity and support cell transformation (Paz et al., 2001). Now we know that

Gal-1 is an integral component of the H-ras nanoclusters and Gal-1 overexpression enhances H-rasG12V nanoclustering (Belanis et al., 2008). Moreover, H-ras activation increases Gal-1 co-localization with H-ras on the plasma membrane, suggesting that Gal-1 levels can modulate H-ras signaling (Rotblat et al., 2010).

However, Galectin-3 (Gal-3), another member of the β-galactosidase binding lectins, was shown to be a specific binding partner of activated K-ras (Shalom-Feuerstein et al., 2008). K-ras and Gal-3 interaction is GTP dependent and Gal-3 is recruited to the plasma membrane by active K-ras. This K-ras-GTP/Gal-3 association promotes PI3K and C-Raf activation but reduces EGF induced ppErk activation. Moreover, ectopic Gal-3 expression enhances K-ras-GTP nanoclustering, while by suppressing Gal-3 this nanoclustering can be reduced, suggesting that cytosolic Gal-3 levels also are able to modulate K-ras-GTP clustering (Shalom-Feuerstein et al., 2008). This makes Gal-3 an integral component of the K-ras nanoclusters.

Proteins such as nucleolin (NCL) and nucleophosmin (NPM, also known as B23) have been identified to modulate K-ras nanoclustering. NCL and NPM are phosphoproteins that are ubiquitously expressed in the nucleus and cytoplasm. NCL and NPM play key roles in ribosome biogenesis, cell proliferation, cell transformation and they are overexpressed in many cancers. Interestingly, these proteins were found to specifically interact with K-ras, but not H-ras on the plasma membrane (Inder et al., 2009). Unlike Gal-1 and Gal-3, this interaction is independent of the activation state of K-ras. NPM and NCL both increase – through distinct mechanisms – K-ras plasma membrane levels. Indeed, NPM was found to specifically interact with K-ras on the plasma membrane and stabilize both K-ras-GDP and K-ras-GTP nanoclusters (Inder et al., 2009). This increased clustering of K-ras-GTP leads to increased MAPK signaling output. In contrast, NCL is not a recognized nanocluster scaffold, rather it is proposed to act as a chaperone that brings K-ras to the plasma membrane, as NCL overexpression does not affect K-ras nanoclustering. Therefore, NPM and NCL modulate K-ras nanoclustering and signaling through distinct mechanisms.

3.3.3.1 Caveolin and Caveolae

Caveolae affect signaling by altering the organization of the plasma membrane. Caveolae are morphologically identifiable 60-80 nm wide cup-shaped plasma membrane pits found abundantly in many mammalian cell types (Parton and del Pozo, 2013). There are remarkable cell type-specific and quantitative differences in caveolae densities between cells. Caveolae play an important role in endocytosis, vesicle transport, mechanosensing, plasma membrane organization and signaling (Ariotti and Parton, 2013). Caveolae are considered as a subset of lipid rafts, with enrichment of cholesterol, glycosphingolipids and lipid-anchored proteins. The signature components of caveolae are the caveolins and cavins, which are key for caveolae formation. Three mammalian caveolins have been well characterized: Cav1,

Cav2 and Cav3 (Ariotti and Parton, 2013). Cav1 and Cav2 are mostly co-expressed ubiquitously in cells, except for skeletal muscles, which have high Cav3 expression. Cavins are cytoplasmic proteins that are recruited to the caveolae to work together with caveolins in caveola-formation and regulation. Cavin 1 (also known as Polymerase I and transcript release factor, PTRF) was originally identified as a protein involved in dissociation of transcription complexes in vitro (Jansa and Grummt, 1999). Absence of PTRF leads to loss of caveolae in cultured cells, and expression of PTRF induces formation of caveolae in various cultured cells and in zebrafish embryos (Hill et al., 2008).

The role of caveolae in cancer development and progression has been subject of controversy. Initial studies showed that loss of Cav-1 was sufficient to induce anchorage-independent growth and tumor formation (Galbiati et al., 2000). Expression of the oncogenes K-ras, H-ras and Src reduced Cav-1 expression in NIH3T3 cells and re-expression of Cav-1 in transformed murine fibroblasts down-regulated signaling via the Ras-Raf-Erk pathway (Williams and Lisanti, 2005). Consistent with these observations, Cav-1 is down-regulated in several cancers, including breast and ovarian cancer. However, this is not the case with metastatic prostate cancers that have increased Cav-1 expression (Nassar et al., 2013a). Thus, caveolae may have both oncogenic and tumor-suppressive properties but the factors that determine these differential effects are not well understood.

EM immunogold labeling experiments have shown PS to be enriched in the caveolae (Ariotti et al., 2014). Although the mechanism is unclear, caveolae specifically regulate PS distribution and interestingly all members of the cavin family bind PS *in vitro*. As mentioned earlier, PS plays an important role in K-ras nanoclustering and it was recently shown that loss of caveolae perturbs Ras organization in an isoform-specific manner (Ariotti et al., 2014). Loss of Cav-1 or PTRF alters cellular lipid composition and PS distribution in the plasma membrane, thus leading to increased K-ras nanoclustering and MAPK signaling. In contrast, loss of caveolae negatively affects the GTP-dependent lateral segregation of H-rasG12V nanoclusters, possibly due to a role of caveolae in regulating plasma membrane cholesterol. However, in cells expressing both active H- and K-ras, loss of caveolae leads to formation of heterotypic clusters (Zhou et al., 2014). These studies suggest that caveolae provide a scaffold that function to sequester specific lipids and thereby control Ras signaling.

3.4 Ras nanocluster formation

3.4.1 Lipid anchors

Lipid modifications of Ras are required for proper plasma membrane targeting. The minimal membrane anchors of H-ras (tH) and K-ras (tK) constitute the C-terminal CAAX motif in addition to a 'second signal'. The 'second signals' are required for proper plasma membrane localization and they play distinct roles in Ras spatial

regulation and nanoclustering. For instance, EM-mapping data show that dual-palmitoylated H-ras-GTP localizes to cholesterol-independent domains but the mono-palmitoylated mutant H-RasG12V C184S localizes to cholesterol-dependent clusters (Roy et al., 2005). These data together with others suggest that the palmitoylation of Cys184 is essential for proper H-ras-GTP nanocluster formation. In contrast, the polybasic domain together with the farnesyl lipid moiety determines K-ras plasma membrane localization and nanoclustering. Indeed, tK localizes to cholesterol-independent nanoclusters, as the highly polar polybasic region of K-ras prefers the fluid, cholesterol-poor lipid domains and also pull the negatively charged lipids (Zhou and Hancock, 2014). This indicates the presence of a K-ras induced lipid sorting that leads to segregation of the phospholipids.

3.4.2 HVR and G-domain conformation orientation

In addition to Ras membrane anchors that are critical for membrane binding, the orientation of the G-domain with respect to the membrane is now considered a novel codec for Ras membrane organization and isoform-specific signaling (Abankwa and Vogel, 2007; Abankwa et al., 2008). The GTP-dependent lateral segregation of Ras is ascribed to the nucleotide-dependent changes in the G-domain conformational orientation. Molecular dynamics simulations of lipid modified full-length H-ras on a model membrane show GDP/GTP dependent interactions with the bilayer through distinct residues in the catalytic domain. In GTP-H-ras, basic residues R128 and R135 on helix α4 interact with membrane lipids, whereas basic residues R169 and K170 of the HVR stabilize GDP-H-ras. These charged residues in α4 and the proximal HVR that engage in mutually exclusive interactions with the membrane are called the 'switched elements'. GTP binding leads to structural rearrangements in the switch I and II; and also in the switch III region, which is a network of salt bridges involving β2-β3-loop and helix α5. GTP-induced structural changes in switch III (D47 and E49 in the β2–β3 loop, R161 and R164 in helix-α5) leads to the release of R169 and K170 in the HVR from membrane binding, leading to a ~100° rotation of the H-ras G-domain (Abankwa et al., 2008; 2007). At the same time, R128 and R135 in helix-α4 now interact with membrane lipids and stabilize the new orientation. The proper orientation of the G-domain was recently shown to be essential also for scaffold interactions (Guzmán et al., 2014b). The switch III region is found to be partly conserved in several Ras isoforms (Abankwa et al., 2008; 2010). Recent work from our laboratory identified that rare cancer associated mutations in the switch III region increase Ras activity by increasing nanocluster formation. These mutations do not alter the biochemical functions of Ras in solution, rather the increase in nanoclustering leads to increased downstream effector recruitment and tumorigenicity (Solman et al., 2015). These studies suggest that the isoform-specific lateral segregation of the Ras isoforms is attributed to the difference in lipid anchors and conformations, and to the different G-domain conformations of the Ras isoforms.

3.5 Ras dimerization

Thus far we have considered Ras-proteins as a freely diffusing monomer or as transient nanoclusters, but recently the idea that Ras exists partially as a homo-dimer has gained interest. It was proposed over a decade ago that membrane anchored Ras dimerization could mediate C-Raf activation (Inouye et al., 2000). This notion has gathered more attention recently. Molecular simulations show that lipidated N-ras adopts a perpendicular orientation that is stabilized only by dimer formation. The residues involved in this N-ras dimer interface are located to the G-domain. Interestingly, Lin et al., reported H-ras to form dimers through protein-protein interactions (Lin et al., 2014). Using a combination of quantitative biophysical techniques, they mapped the dimerization interface to the switch II region (residues 60-76). Nussinov and colleagues recently showed that the catalytic domain of K-ras-GTP could form stable homo-dimers with two distinct dimer interfaces, one with switch I and effector binding regions and another with the helical interfaces that may promote Raf activation (Muratcioglu et al., 2015). By utilizing super resolution imaging techniques, Chu's Lab showed that endogeneous levels of K-ras-GTP forms dimers and activates signaling (Nan et al., 2015). Indeed, active Ras has been shown to drive formation of C-Raf dimers, trimers and tetramers (Nan et al., 2013). These studies suggest that Ras dimerization can mediate and assist Raf dimerization and signaling. Therefore, it now seems likely that Ras dimers could act as intermediate building blocks for forming higher order nanoclusters.

3.6 Nanocluster and signaling

Active Ras nanoclusters act as signaling sites for recruitment of downstream effectors such as Raf and PI3K to the plasma membrane. The activation of these distinct effector pathways is confined to Ras-GTP nanoclusters. Furthermore, direct relationship between the G-domain orientation and effector binding further supports the link between Ras nanoclustering, activation and effector recruitment (Abankwa et al., 2008; 2010). However, there are marked quantitative differences in the ability of K- and H-ras to activate C-Raf and PI3K (Yan et al., 1998). As mentioned earlier, Ras effectors also have distinct lipid binding domains. For example, C-Raf has PS- and PA-binding domains and binding to these domains is critical for its activation (Ghosh et al., 2003; McPherson et al., 1999). This is further supported by experiments that show C-Raf-RBD with the PS-binding domain (CRD - cysteine-rich domain) has significantly enhanced binding specificity to K-ras-GTP nanoclusters, compared to just the C-Raf-RBD (Abankwa et al., 2010). The recent mapping of lipids in K- and H-ras nanoclusters further supports this notion, as K-rasG12V nanoclusters have high content of the key cofactor PA, as compared to H-rasG12V nanoclusters (Zhou et al., 2014). Therefore, isoform specific signaling stems from Ras nanoclusters acting as signaling platforms, where all the key components such as lipids and cofactors are assembled to promote recruitment and activation of specific effectors.

Spatiotemporal dynamics of Ras nanoclustering are essential for high fidelity signaling (Kholodenko et al., 2010). According to Hancock and colleagues, the non-equilibrium kinetics constantly maintains the fraction of Ras found in nanoclusters (Tian et al., 2010). As a result, this clustered fraction gives a linear relationship between levels of Ras-GTP and the number of Ras-GTP nanoclusters on the plasma membrane (Tian et al., 2007). Each of this Ras-GTP nanocluster responds maximally to low input signals, enabling the nanoclusters to operate at a low threshold. Thus, they function as a digital nanoswitch that could deliver a fixed quantum of activated Erk output into the cytosol. (Harding and Hancock, 2008). This makes the total Erk system output from the plasma membrane to be analog, as the amount of nanoswitches generated is a linear function of the EGF input. This spatiotemporal dynamics of Ras allows for the plasma membrane to be an analog-digital-analog (ADA) converter, which digitizes the analog EGF input signal by forming an appropriate number of Ras nanoclusters, thus regenerating the analog signal into a corresponding level of ppErk output into the cytosol with high-fidelity (Harding and Hancock, 2008; Kholodenko et al., 2010; Tian et al., 2007; Zhou and Hancock, 2015). An important feature of this system is that the output signal response is determined by the clustered fraction. As described earlier, the nanoclusters are regulated by multiple factors, such as lipid anchors, lipid content of the plasma membrane, actin cytoskeleton and scaffolding proteins (Zhou and Hancock, 2015).

Importantly, computation and experimental evidence shows that loss or reduction of nanoclusters result in a reduction in the ppErk response to EGF. This holds true even if the levels of Ras-GTP on the membrane are unchanged, as in the case of oncogenic Ras. Although oncogenic Ras mutant cells have high Ras-GTP levels, reducing the clustered fraction abrogates the Ras signal output (Zhou et al., 2014). This suggests that Ras nanoclusters could be important pharmacological targets, and targeting these nanoclusters would be a novel and viable therapeutic approach to blocking oncogenic Ras signaling in cancers and diseases.

4 Molecular and cellular effects of Ras signaling

4.1 Ras isoforms play critical role in development

Despite the high sequence similarities, the Ras isoforms differ significantly in functions in distinct tissues. It was already reported in the late 1980s through expression analysis of adult mouse tissues that H-ras is highly expressed in skin, brain and muscle, while K-ras is mostly found in lungs, colon and thymus and N-ras in thymus and testis (Leon et al., 1987). Ras isoforms are differentially expressed also during mouse development (Muller et al., 1983). These early studies clearly suggested that the Ras proteins have distinct cell- and tissue-specific functions. Knock-out studies in mice revealed the importance of K-ras4B in embryonal development. The K-ras4B knockouts are embryonic lethal where the embryos die within 2 weeks of gestation with liver defects and anemia (Esteban et al., 2001; Koera et al., 1997). In contrast, mice without H-ras, N-ras, both H- and N-ras, or K-ras4A were viable and do not show any obvious phenotype (Johnson et al., 1997; Umanoff et al., 1995). The importance of Ras signaling during embryo development is further highlighted when microinjection of a dominant negative H-ras17N mutant into the two-cell stage embryos resulted in embryonic lethality, suggesting that Ras signaling is required for progressing through two-cell stage (Yamauchi et al., 1994). Although substituting H-ras for K-ras supports normal embryonic development in mice, the adult mice nevertheless develop with cardiovascular pathology suggesting functional differences (Potenza et al., 2005). The importance of Ras in development is further highlighted in the so-called Rasopathies – a class of developmental disorders caused by germline mutations in the Ras/MAPK pathway (Rauen, 2013). In Costello and Noonan syndromes, germline mutations in the *HRAS* and *KRAS* genes, respectively, give rise to distinct phenotypic features. The phenotypic features include facial abnormalities, heart defects, impaired growth and development, and especially for the patients with Noonan syndrome, a predisposition to specific cancers (Castellano and Santos, 2011). Taken together, this data show that Ras is important for the development and that the distinct Ras isoforms have both specific and overlapping functions during development.

4.2 Ras mutations in cancer

The frequency and distribution of Ras mutations in human cancers are variable (Table 1). According to the recent data in the COSMIC v73 (Catalogue of Somatic Mutations in Cancer) database, the three Ras genes are mutated in almost 27% of all analyzed cancers (http://cancer.sanger.ac.uk/cosmic). These Ras mutations occur in the following preferential frequency: K-ras (19%, in 32,021 of 165,870 samples), N-ras (5.0%, in 4,278 of 85,415 samples) and H-ras (2.6%, in 1426 of 54, 418 samples). It is also worth mentioning that the frequency of these mutations change according to the database used, the dataset used for analysis, and the type of cancers represented in the database.

However, it is clear that more than 99% of these Ras mutations identified in cancer occur in the hot-spot residues G12, G13 and Q61 (Figure 3). The G12 and Q61 mutations lead to impaired GAP-stimulated GTP hydrolysis, resulting in constitutively active Ras. The G12 and G13 mutations are common in K-ras and H-ras, whereas the Q61 mutation occurs predominantly in N-ras. These are the three most common activating Ras mutations that result in aberrant downstream signaling. The functional differences between the different Ras isoforms mentioned before are also reflected in the frequency of their mutations. More than 85% of the Ras cancer mutations occur in K-ras (Cox and Der, 2010; Prior et al., 2012). The highest frequencies of K-ras mutations are found in pancreatic ductal adenocarcinoma (PDAC) around 95%, colorectal cancer (CRC) around 50%, and lung adenocarcinomas around 30%, whereas N-ras is highly mutated in melanoma and myelogenous leukemia. Even though rare overall, H-ras is predominantly mutated in thyroid, bladder and head and neck cancers.

Table 1. Frequency of Ras mutations in selected human cancers

Primary Tissue	KRAS (%)	HRAS (%)	NRAS (%)	Total (%)
Pancreas	60.30	0.00	1.64	61.95
Colon	35.31	0.81	4.25	40.36
Skin	2.29	10.74	16.26	29.30
Biliary tract	26.29	0.00	2.12	28.40
Endometrium	16.86	1.21	4.41	22.48
Small intestine	21.10	0.00	0.00	21.10
Lung	17.51	0.84	0.75	19.09
Cervix	7.71	8.88	1.54	18.13
Haematopoietic and lymphoid tissue	5.75	0.36	11.23	17.34
Ovary	13.89	0.40	1.96	16.25
Urinary tract	4.83	10.06	1.23	16.13
Prostate	8.29	5.53	1.47	15.28
Upper aerodigestive tract	3.01	9.17	2.93	15.11
Salivary Gland	3.18	10.83	1.06	15.07

(Data shown in the table are compiled from the COSMIC v73. Cancers with total Ras mutation frequencies above 15% are listed).

In many of these cancers, Ras mutations are considered to be one of the early events leading to cancer development and progression. One study with active K-ras in mouse models found that activation of K-ras but not N-ras promoted colon cancers (Haigis et al., 2008). Interestingly, they also reported that K-ras activation alone

leads to hyperplasia in the colon and not neoplasia. However, K-ras together with loss of tumor suppressor functions, like APC, lead to enhanced cancer progression. In many cases of CRC, loss of APC is the initiating event followed by a K-ras mutation (Kinzler and Vogelstein, 1996). Furthermore, the well-understood molecular events of PDAC implicate mutational activation of K-ras as a general early event followed by the loss of tumor suppressors CDKN2A and p53 (Eser et al., 2014). This early role of Ras supports its role in cancer initiation and progression. Furthermore, depletion of Ras in cancer cells or suppression of Ras expression in Ras driven mouse models lead to impaired cell growth and tumor recession (Chin et al., 1999; Singh et al., 2009; Ying et al., 2012). These studies suggest that Ras activity is not only needed for tumor initiation and progression, but it is as an essential component of tumor maintenance and they further validate Ras a relevant therapeutic target in human cancers.

One of the puzzling issues for Ras biologists has been the preferential mutation and activation of different Ras isoforms in different cancer tissue types (Table 1). More specifically, the preponderance of K-ras mutations in human cancers compared to H- and N-ras, in spite of the fact that all these isoforms have potent transforming properties in model systems and are expressed widely in adult tissues and tumors. Even though direct experimental evidence is still lacking, some observations account for this. First support for the idea that K-ras has distinct functions came from the role of K-ras in development (as discussed earlier). These studies indicated that it is the genomic locus and the differential expression of K-ras that are critical for development (Potenza et al., 2005). Balmain and colleagues further extended this idea to show that the H-ras knock-in mice with H-ras in the K-ras locus are highly susceptible to urethane-induced lung tumors (To et al., 2008). This argues that the Ras locus and tissue specific expression are essential and not the proteins themselves. Consequently, the tissue specific expression of different Ras isoforms has been thought to account for the association of specific Ras isoform mutations in distinct tissues. An elegant study from Barbacid and colleagues demonstrated the importance of this cellular context of Ras signaling (Guerra et al., 2014). They employed a mouse model with whole body expression of active K-ras12V to understand the tumor induction capacity of endogenous K-ras. Surprisingly, mutated K-ras in these mice failed to induce uncontrolled cell proliferation and had no effect on most tissues. Interestingly, only the lung alveolar cells underwent K-ras mediated transformation and formed lung adenocarcinomas. This study suggested that K-ras driven tumor formation is highly dependent on the cellular context. Following this Jacks and colleagues reported the potential role of K-ras in self-renewal, when they demonstrated that the conditional expression of K-rasG12D could lead to expansion of bronchioalveolar stem cells (BASCs) stem cells in vivo (Kim et al., 2005). This suggested that some Ras mutated cancers could develop from stem cells or progenitor cells.

4.3 Some Ras isoforms can confer stem cell properties

Stem cells are characterized by their cardinal property to self-renew. Self-renewal is the process whereby, upon cell division, a stem cell can give rise to one or two daughter cells that retain the capacity to self-renew, ensuring the maintenance of the stem cell population. In normal adult tissues, these stem cells differentiate to produce the differentiated tissues. In the context of cancer, it was already suggested in the 1980s that cancer cells are differentiated cells generated from tumor 'stem' cells. Today, we understand some of the molecular mechanisms behind stem cell properties, whether normal or in cancerous cells. It is noteworthy that early studies to understand Ras function in embryonic stem cells (ESCs) reported the identification of mouse embryonic-Ras (E-ras) (Takahashi et al., 2003). E-ras was found to share 40% homology to other Ras proteins with amino acid substitutions in the residues corresponding to GTPase activity implying a constitutive active E-ras. In mouse, E-ras was expressed specifically in ESCs and not in differentiated cells. Interestingly, the human orthologue of E-ras (Hrasp), is also reported to be constitutively active (Takahashi et al., 2003). Though the importance of E-ras in human cancers is largely unclear, its expression and epigenetic regulation has been reported in gastric cancer cells (Yashiro et al., 2009).

More recently, one reason for the high frequency of K-ras mutations has been attributed to its role in stem-like or progenitor cells. While trying to understand the preponderance of K-ras mutations in cancers of endoderm-derived tissues such as pancreas, colon and lung, Settleman and colleagues provided the first functional evidence that out of K-, H- and N-ras, only K-ras can endow stem cell-like properties on certain cell types (Quinlan et al., 2008). Using the well-established retinoic acid (RA)-induced stem cell differentiation model of F9 cells, they identified that active K-ras promotes the expansion of the F9 stem cells, expression of stem cell markers and blocking of RA induced differentiation. In contrast, H-ras leads to RA-induced differentiation, while N-ras is inert in this system. Interestingly, the ability of K-ras to confer stem cell properties was not limited to its subcellular localization, as cells expressing the K-rasG12V chimera substituted with the H-ras c-terminal tail do not differentiate and continue to express stem cell markers. Analysis of the effector pathways revealed that K-ras employs distinct PI3K, Raf and also RalGDS signaling pathways for stem cell proliferation and maintenance. These data have been consistent with the reported role of K-rasG12D in the expansion and maintenance of colonic epithelial stem cells in vivo (Haigis et al., 2008). Moreover, K-ras, but not H- and N-ras, is shown to directly bind calmodulin (Villalonga et al., 2001). K-ras and calmodulin interaction is inhibited by the calmodulin kinase II calmodulin-binding domain. This calmodulin binding has been shown to be mutually exclusive with the K-ras phosphorylation at S181 (Bivona et al., 2006; Naujokat and Steinhart, 2012; Villalonga et al., 2002). Recent evidence emerging from Frank McCormick's laboratory suggests that K-ras and not H-ras with these unique properties can impart stem-cell like characteristics to cancer cells (Wang et al., 2014; 2015).

Apart from K-ras, there is also evidence suggesting that N-ras plays a critical role in hematopoietic stem cells (HSCs) (Li et al., 2013). The mutational activation of N-ras results in enhanced proliferation and self-renewal of HSCs in mice. Furthermore, in these HSCs N-ras activates both the Mek-Erk and STAT signaling cascades. Interestingly, N-ras promotes a bimodal response in HSCs, by driving self-renewal in one subset of HSCs and increasing cell division in the other subset of cells. However, it is not known whether this bimodal effect also applies to other Ras isoforms. Nevertheless, there is evidence showing that activation of K-rasG12D in HSCs also initiates acute leukemia in mice, leading to the emergence of distinct self-renewable cell populations that acquire cooperating mutations as the cancer progresses (Sabnis et al., 2009). Thus it is speculated that some cancer cells within Ras mutated tumors would possess inherent self-renewal capacity and survival advantage.

4.4 Cancer stem cells (CSCs)

Cells that make up a tumor exhibit significant heterogeneity in terms of morphology, genetic lesions and functionally. This heterogeneity is also seen in cancers derived from a single cell, as some cancer cells harbor the dynamic ability of co-opting self-renewal mechanisms and can transit from a differentiated to non-differentiated states and vice versa. This may be modulated by specific micro environmental signals and cellular interactions arising in the tumor. Understanding the basis for tumor heterogeneity has interested cancer researchers for decades. Two mutually exclusive explanations or theories were put forward to explain tumor heterogeneity (Figure 8a). According to the clonal evolution theory of cancer, when a mutant cancer cell divides and gives rise to a number of descendants, a few of these descendants may acquire a new mutation or epigenetic change that can give them a selective advantage over other tumor cells. This is called clonal expansion. This clone of cells can now expand and dominate the cancer. By acquiring new mutations these cells can further evolve to make the next clone of cancer cells. According to this theory, all the cancer cells from the dominant clone will have similar tumorigenic potential (Greaves and Maley, 2012).

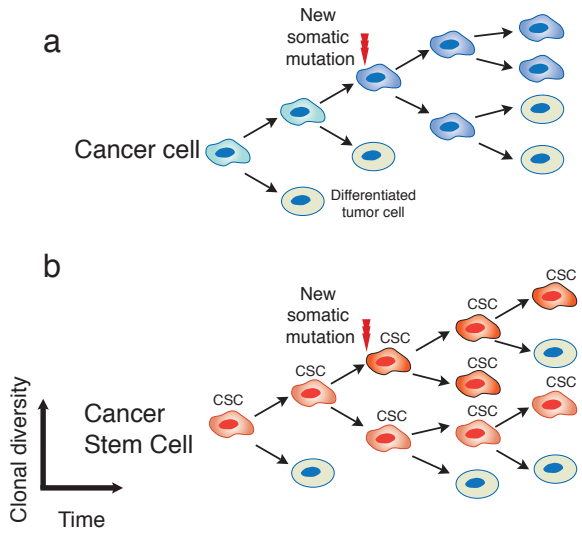

Figure 8. Schematic representation of clonal evolution and cancer stem cell models of tumor growth. a) In the clonal evolution model of tumor growth, all tumor cells are equally tumorigenic and can stochastically self-renew or differentiate, leading to tumor heterogeneity. Acquiring new mutations generates clones diversity that further increases tumor heterogeneity. **b)** In the cancer stem cell (CSC) model, only a small subset of tumor cells have self-renewal capacity and they give rise to progenitors cells that eventually differentiate to form the bulk of the tumor. Acquiring new mutations leads to further clonal diversity.

According to the cancer stem cell model, only a small fraction or population of cancer cells within a tumor have the extensive replicative potential or self-renewal capacity, and can give rise to the tumor (Figure 8b). The cancer stem cell hypothesis was first developed through transplantation experiments with leukemia cells that suggested that only a fraction of cells from the tumor could give rise to new tumors in mice. These small population of cells comprised of self-renewing, tumorigenic cells were termed the cancer stem cells (CSCs) (Dick, 2008). The proof for CSCs in solid tumors was established from the seminal work showing that only 2% of the neoplastic cells taken directly from breast cancers are capable of forming new tumors (Al-Hajj et al., 2003). This work also described that cells with a $CD44^{high}/CD24^{low}$ phenotype are highly enriched for CSCs, and as little as 100 of these putative-CSCs could recapitulate the tumor (Al-Hajj et al., 2003). This was followed by work from others showing identification of CSCs in many tissues (Kreso and Dick, 2014).

Although there has been no consensus over CSCs and their properties, it is now accepted by definition that these cells must have the capacity to self-renew. However, in some cancers it is impossible to distinguish between CSCs and non-CSCs. Recent studies support the possibility that tumor heterogeneity is a complex process and that clonal evolution and CSC models may not be mutually exclusive. A unified model of clonal evolution and cancer stem cells suggests that a CSC may acquire a mutation and give rise to sub clones with self-renewal capabilities. These clones may

over time accumulate distinct mutations and evolve in parallel and give rise to progenitors without self-renewal capabilities (Kreso and Dick, 2014). It is also suggested that some cancer cells can transit from non-CSC and CSC-states resulting in higher cancer cell plasticity, which seems to be governed by transcription factors (Chaffer et al., 2011; 2013; Mani et al., 2008; Morel et al., 2008). The initial evidence for this came from the Weinberg and Puisieux groups showing that induction of epithelial-mesenchymal transition (EMT) may play a role in the conversion of non-CSC cells to CSC-like cells. EMT is a transdifferentiation process during embryogenesis where epithelial cells transdifferentiate to mesenchymal cells. During this conversion, epithelial cells transiently lose their cell-cell junctions, apico-basal polarity, reorganize the cytoskeleton and alter their gene expression and signaling (Polyak and Weinberg, 2009). These EMT processes are activated during cancer and wound healing, and recent evidence links EMT to self-renewal and CSC-state. Using a breast cancer model, Puisieux and colleagues reported that overexpression of oncogenic Ras and activation of MAPK can drive EMT and lead to enrichment of breast epithelial cells with stem-like properties (Morel et al., 2008). In a correlative study, Weinberg and colleagues showed that overexpression of transcription factors Twist or Snail in immortalized breast epithelial cells induced EMT and these cells acquired a $CD44^{high}/CD24^{low}$ phenotype of breast CSCs (Mani et al., 2008). Recent studies from Weinberg and group also show that CSC hierarchy is not valid for all cancers, as they found a subpopulation of breast non-CSCs that can dynamically transition from non-CSC to CSC-states, mediated by the transcription factor Zeb1 (Chaffer et al., 2011).

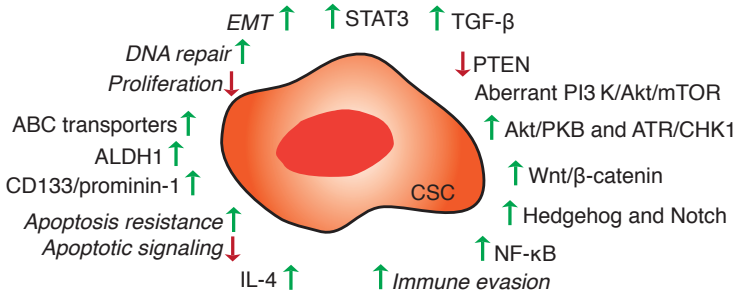

Figure 9. Mechanisms and pathways that contribute to the resistance of CSCs to conventional drugs and radiation therapy. The Wnt/β-catenin, Hedgehog and Notch, and NF-κB pathways are all involved in progenitor cell expansion. The Akt and ATR/CHK1 pathways promote survival e.g. by an amplified checkpoint activation and efficient DNA repair. The ATP-binding cassette (ABC) drug transporter, aldehyde dehydrogenase (ALDH) and CD133/prominin-1 all contribute to drug or radioresistance. CSCs are also resistant against apoptosis and have defective apoptotic signalling, and e.g. autocrine production of interleukin-4 (IL-4) has been shown to protect against apoptosis in CSCs. The PI3K/Akt/mTOR pathway has been shown to be involved in both cell survival, progenitor cell renewal, drug resistance and EMT. Other mechanisms that contribute to resistance include transient dormacy, changes in cell metabolism to a preference for hypoxia, evasion from the immune system and protection by the microenvironment (reviewed in Pattabiraman and Weinberg, 2014).

Notwithstanding these issues, it is now widely accepted that the CSC properties are defined by a collection of genetic, epigenetic and tumor microenvironment factors that confer self-renewal properties to a cell. This is collectively referred to as 'stemness'. There is now strong evidence to support the link between stemness of CSCs and tumor therapy resistance, progression and tumor recurrence (reviewed in (Kreso and Dick, 2014). Several mechanisms and pathways have been shown to contribute to the resistance of CSCs (Figure 9). The clinical relevance of CSCs and stemness in patients is highlighted by studies showing that cancers with stem cell-like gene expression signature are associated with relapse and patient outcome in human breast, gliobastoma, leukemia and ovarian cancers (Cabrera et al., 2015; Eppert et al., 2011; Wicha, 2012). These studies indicate that targeting the CSCs and their stemness features might be the most viable therapeutic strategy against CSCs and targeting CSCs might lead to a more effective clinical response (Figure 10).

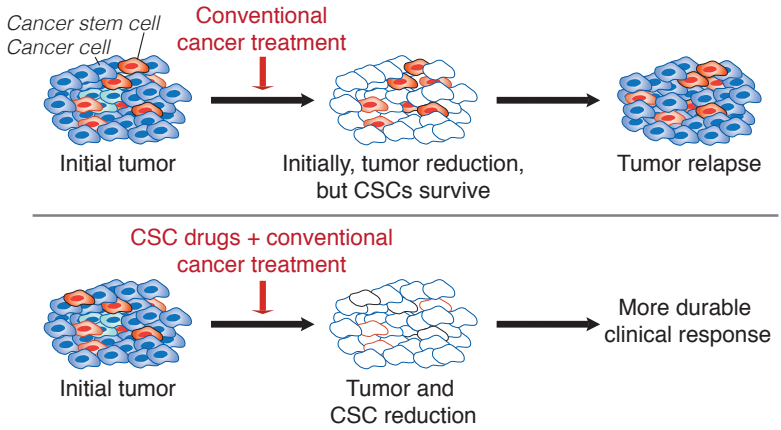

Figure 10. Therapeutic relevance of targeting CSCs. *Top*: Tumor contains a mix of CSCs, progenitor and differentiated cancer cells, and these cells contribute to the heterogeneity. Conventional cancer therapy kills mostly progenitor-like and highly proliferative cells leading to a transient reduction in tumor burden. While the therapy-resistant, relatively dormant CSCs survive and seed a new tumors leading to relapse. *Bottom:* CSC drugs and therapies in combination with conventional therapies would kill or differentiate the CSCs and also kill the proliferative cells. The reduction in CSCs leads to a reduction in tumor recurrence and relapse.

4.4.1 Compounds and drugs targeting CSCs

The understanding of the molecular pathways involved in breast CSCs was first successfully used in a high throughput chemical screen of small molecules when the CSC inhibitor salinomycin was identified by utilizing the differences in the CD44/CD24 ratio between CSCs and non-CSCs (Gupta et al., 2009). Subsequently, similar high-throughput screens have been used to identify CSC inhibitors of AML CSCs (Sachlos et al., 2012), breast CSCs (Carmody et al., 2012), ovarian CSCs (Mezencev et al., 2012) and glioblastoma CSCs (Visnyei et al., 2011). This has lead to the identification and characterization of several drugs that target CSCs (Naujokat and Laufer, 2013; Sachlos et al., 2012). Table 2 shows the various sources of CSC

drugs and their targets in different cancer types and their current status in clinical trials (www.clinicaltrials.gov). In many of these trials the CSC drugs are used in combination with conventional drugs. Many of these clinical CSC drug candidates target pathways that are altered in CSCs (Figure 9). The following section briefly summarizes the activity of some of these compounds, such as the microbial derivatives e.g. salinomycin, plant-derivatives e.g. resveratrol, curcumin, and classical drugs e.g. metformin, tranilast and thioridazine (Naujokat and Laufer, 2013).

Table 2. Compounds and drugs that target CSCs and their molecular targets. The clinical trial identifiers are from www.clinicaltrials.gov (13.12.2015). In case there were several trials only one number is given.

Compound Name	Molecular Targets/Targeted Pathways	Cancer Type	Clinical Trials	Clinical Trial Identifier/ Reference
Antibiotics				
Salinomycin (ionophore antiobiotic)	Wnt pathway, etc	Vulvar carcinoma	No clinical trials, case reports only	Naujokat and Steinhart et al., 2012
IPI-926 (Cyclopamine derivative)	Hedgehog pathway inhibitor	Head and neck, advanced solid tumors	I-II	NCT01383538
Resveratrol	Wnt and hedgehog pathways, p53	Medulloblastoma, glioblastoma, breast, pancreatic, colon	I	NCT00256334
Sulforaphane (naturaal isothiocyanate)	NF-κB, hedgehog, EMT and Wnt/β-catenin pathways	Pancreatic, breast, prostate, chronic myeloid leukemia	II	NCT01228084
Mithramycin	RNA synthesis inhibitor	Breast, lung, esophageal, mesothelioma, gastrointestinal neoplasm, solid tumors, Ewing sarcoma	I, II	NCT01624090
Small molecular inhibitors				
BMS-833923 (XL139)	Hedgehog pathway inhibitor, Gli inhibition, Smo antagonist	Leukemia, lung, stomach and esophaegal neoplasms	I, II	NCT01357655
GDC-0449 (Vismodegib)	Hedgehog pathway, Smo antagonist	Pancreas, medulloblastoma, solid tumors	II	NCT00607724
LDE225 (Sonidegib)	Hedgehog pathway inhibitor, Gli inhibition, Smo antagonist	Ovarian, prostate, hematologic	I, II	NCT02195973
PF-04449913 (Glasdegib)	Hedgehog pathway inhibitor, Gli inhibition, Smo antagonist	Leukemia, myelofibrosis	I, II	NCT02226172

Compound Name	Molecular Targets/Targeted Pathways	Cancer Type	Clinical Trials	Clinical Trial Identifier/ Reference
LEQ506	Hedgehog pathway inhibitor	Solid tumors	I	NCT01106508
RO4929097	γ-secretase inhibitor (Notch pathway)	Pancreatic, breast, lung, glioma, colon, prostate, solid tumors, melanoma, ovarian	I	NCT01122901
MK-0752	γ-secretase inhibitor (Notch pathway)	Breast, pancreatic, refractory CNS cancer	I-II	NCT00645333
Reparixin/ Repertaxin	Chemokine receptor 1/2 inhibitor (IL-8 pathway)	Breast	II	NCT02370238
SL-401	IL-3 receptor	Leukemia	I, II	NCT02113982
PF-04691502	Dual PI3K/mTOR inhibitor	Breast, endometrial	I, II	NCT00927823
VS-5584	PI3K/mTOR kinase inhibitor	Non-hematologic cancers, lymphoma	I	NCT01991938
VS-4718	FAK inhibitor	Metastatic non-hematologic cancers	I	NCT01849744
VS-6063 (Defactinib)	FAK inhibitor	K-ras mutant non-small cell lung cancer, mesothelioma, ovarian cancer, non-hematologic cancers	I, II	NCT01951690
GO-203-2c	MUC1-C	Leukemia, solid tumors	I, II	NCT02204085
BBI608 (Napabucasin)	STAT3 and β-catenin inhibitor	Glioblastoma, colorectal, hepatocellular carcinoma, advanced malignancies	I-III	NCT01325441
BBI503	Multiple stemness kinases inhibitor	Hepatocellular carcinoma, colorectal cancer, advanced solid tumors	I, II	NCT01781455
GW572016 (Lapatinib)	Tyrosine kinase inhibitor, EGFR and ErbB2 pathway inhibition	Breast, colorectal, gastric, head and neck, lung	II, III, FDA approved for use in breast cancer	NCT00486954
AZD3965	Mitochondrial monocarboxylate transporter 1 inhibitor	Prostate, gastric, solid tumors	I	NCT01791595
Antibodies				
CSL362	CD123	Leukemia	I	NCT01632852
IIPOP (Catumaxomab)	EpCam and CD3	Ovarian, gastric	II	NCT01784900
MT110	EpCam and CD3	Lung, gastric, colorectal, breast	I	NCT00635596
OMP-21M18 (Demcizumab)	DLL4 (Notch pathway)	Pancreatic, non-small cell lung cancer	I, II	NCT01189968

Compound Name	Molecular Targets/Targeted Pathways	Cancer Type	Clinical Trials	Clinical Trial Identifier/ Reference
OMP-305B83	DLL4 and the VEGF receptor	Solid tumors	I	NCT02298387
OMP-52 M51	Notch1 receptor	Lymphoid, solid tumor	I	NCT01703572
OMP-59R5 (Tarextumab)	Notch2/3	Pancreatic, small cell lung cancer	I, II	NCT01647828
OMP-18R5 (Vantictumab)	Frizzled 7 receptor (Notch pathway)	Pancreatic, breast, non-small cell lung cancer, solid tumors	I	NCT01957007
OMP-54 F28	Wnt ligands	Liver, ovarian, pancreatic, solid tumors	I	NCT02069145
OMP-131R10	RSPO3 (RSPO-LGR5 pathway, Wnt signaling)	Colorectal cancer	I	NCT02482441
Trastuzumab	HER2/neu receptor	Breast, HER2-positive carcinomas	I, II, FDA approved for breast	NCT02120911
Classical drugs				
Metformin (anti-diabetic drug)	Activates AMPK, suppresses mTOR	Breast cancer, prostate cancer, gynaecological cancers, solid tumors	I-III	NCT01579812
Thioridazine (anti-psychotic drug)	Activates AMPK	Leukemia	I	NCT02096289
Lovastatin (hypolipidemic agent)	HMGCoA reductase inhibition	Leukemia	I, II	NCT00583102
Others				
Fursultiamine (derivative of thiamine)	Inhibits expression of Oct4, Nanog, Sry, ABC	Esophageal squamous cell carcinoma	II	NCT02423811
2-Methoxyestradiol (Panzem, metabolite of estradiol)	HIF inhibitor, microtubule inhibitor	Leukemia, glioblastoma, ovarian, breast, myeloma	I, II	NCT00400348, Hartwell et al., 2013
NVP-BEZ235 (Imidazoquinoline derivative)	Dual PI3K/mTOR inhibitor	Breast, renal, prostate, solid tumors	I, II	NCT00620594
Curcumin, analogs GO-Y030, difluorinated-curcumin (CDF)	Amplifying E-cadherin/β-catenin negative feedback	Pancreatic, rectal, breast	I, II	NCT00745134

4.4.1.1 Microbial-derived compounds

Salinomycin is a polyether antibiotic isolated from *Streptomyces albus*, which has been used for more than 30 years to treat coccidiosis in poultry (Callaway et al., 2003). It is a very selective potassium ionophore that acts in cytoplasmic and

mitochondrial membranes, promoting efflux of K^+ ions and interfering with their transmembrane potential (Naujokat et al., 2010). Weinberg and colleagues demonstrated that salinomycin was 100-fold more potent than standard chemotherapeutics against breast CSCs (Gupta et al., 2009). Pre-treatment of these cells with salinomycin resulted in a more than 100-fold reduction in tumor-seeding capacity. Moreover, gene expression analysis showed that salinomycin down-regulated genes involved in expansion of mammary stem cells, formation of mammospheres and genes inversely correlating with overall survival of breast cancer patients. In addition, treatment of mice bearing breast CSC-derived tumors with salinomycin reduced the tumor mass and metastasis as well as the number of tumor CSCs and induced their differentiation (Gupta et al., 2009). A follow-up study reported that salinomycin induced apoptosis in multiple cancer cell types but not in normal cells. Furthermore, salinomycin activates a distinct apoptotic pathway independent of p53, cell cycle arrest and caspase activation (Fuchs et al., 2009). A subsequent study demonstrated that salinomycin treatment overcame ABC-transporter mediated drug resistance and induced apoptosis of AML stem cells (Fuchs et al., 2010). A series of studies have shown salinomycin to target CSCs in different cancers including gastric, lung, colorectal, and prostate cancer (reviewed in (Naujokat and Steinhart, 2012)).

Despite the increasing number of such reports, the exact molecular target of salinomycin has not been identified. A number of recent studies have, however, reported mechanisms that have increased the understanding of the mode of action of salinomycin. One of these mechanisms is the induction of apoptosis and cell death. However, there are reports suggesting salinomycin to induce both apoptotic and nonapototic cell death depending on the cell types (Fuchs et al., 2009; 2010; Ketola et al., 2012; Kim et al., 2011). The increase in apoptosis in certain cancer cells could be attributed to the ionophoric potassium efflux activity of salinomycin (Naujokat and Steinhart, 2012). Furthermore, salinomycin has been shown to target various molecular complexes and pathways that are critical for the development and maintenance of CSCs or that confer resistance and survival of CSCs, such as the ABC transporter, the Wnt/ β-catenin pathway and acquisition of EMT. There is evidence showing that salinomycin is a potent inhibitor of the multidrug resistance ABC-transporter P-glycoprotein in cancer cells (Riccioni et al., 2010). Salinomycin has also been shown to inhibit Wnt signaling by inhibiting coreceptor LRP6 and down-regulating the Wnt target genes in chronic lymphocytic leukemia cells (Lu et al., 2011). In addition, oxidative phosphorylation has been linked to mesenchymal stem cell transformation, and salinomycin has been shown to inhibit mitochondrial oxidative phosphorylation (Mitani et al., 1976). The original finding by Gupta et al, also reported that salinomycin induces differentiation of CSCs and up-regulates genes involved in breast epithelial cell differentiation (Gupta et al., 2009). Taken together, salinomycin may target CSCs and cancer cells via multiple mechanisms.

4.4.1.2 Plant-derived compounds

Resveratrol is a natural phytoalexin found in red wine grapes and peanuts. It is shown to induce p53, cell cycle arrest and apoptosis and to inhibit Wnt and Hedgehog signaling pathways (Naujokat and Steinhart, 2012). A number of studies have reported the activity of resveratrol against CSCs of medulloblastoma, colon, breast and pancreas. Interestingly, resveratrol inhibits pancreatic CSCs *in vitro* through apoptosis, inhibition of EMT, loss of multi-drug resistance and lower self-renewal capacity (Shankar et al., 2011). These multiple effects lead to inhibition of pancreatic tumor formation and growth in K-rasG12D mice that form spontaneous PDAC (Shankar et al., 2011). Resveratrol is now being tested by several studies for clinical use (Singh et al., 2015).

Curcumin, found in the Indian spice plant turmeric, has been widely studied for its anti-inflammatory, anti-cancer, anti-viral and immunomodulatory activities (Naujokat and Steinhart, 2012). Initial studies with glioblastoma CSCs reported that curcumin treatment lead to differentiation, apoptosis and reduced clonogenicity of glioblastoma CSCs. Subsequent studies have shown that curcumin and curcumin analogues inhibit breast, colon and pancreatic CSCs, and curcumin is currently investigated by various clinical studies (Gupta et al., 2013).

4.4.1.3 Classical drugs

In addition to the microbial and plant-derived compounds, a few classical drugs that have been used for decades to treat metabolic, immune and psychiatric diseases have been found to be active against CSCs.

Metformin is a commonly used drug for treating type II diabetes. The mechanisms of action of metformin in reducing insulin and glucose are well known. In cancer cells metformin has been shown to inhibit ATP production, activate AMP-Kinase, suppress PI3K and mTOR activity and also reverse EMT (Martin-Castillo et al., 2010). Metformin was also shown to have anti-proliferative properties and inhibit growth of several cancer cell lines (Martin-Castillo et al., 2010). A number of recent studies have shown that metformin selectively targets breast, pancreatic and thyroid CSCs. Particularly, metformin significantly reduces the $CD44^{high}/CD24^{low}$ breast CSC populations found in various breast cancer types (Hirsch et al., 2009). Moreover, metformin reduces expression of EMT markers such as Twist, Slug and Zeb1, together with the stem cell marker Oct4 in breast CSCs (Bao et al., 2012; Jung et al., 2011; Vazquez-Martin et al., 2010). In pancreatic CSCs, metformin was recently shown to inhibit expression of transcription factors, NANOG, Oct4 and Notch1, which are associated with CSC self-renewal and maintenance (Bao et al., 2012). Consistent with these results, metformin treatment in a K-rasG12D driven pancreatic mouse model significantly reduced tumor weight and suppressed the expression of the CSC markers CD44, CD133 and ALDH1 (Mohammed et al., 2013).

Thioridazine, identified from a HTS for targeting neoplasic human pluripotent stem cells (hPSCs) is a well-known anti-psychotic drug (Sachlos et al., 2012). Earlier studies have shown that thioridazine targtes PI3K and mTOR signaling and causes cell cycle arrest and apoptosis (Kang et al., 2012). Recently thioridazine was shown to induce differentiation of hPSCs and AML SCs, and it was shown to be specifically active against cells expressing dopamine D2 receptors (Sachlos et al., 2012). These studies suggest that dopamine D2 receptors could be a novel anti-CSC target (Walsby et al., 2010). It is worth noting that in the original study, thioridazine was found to be active at very high concentrations that can not be translated to *in vivo* testing, indicating that thioridazine may work independently of D2 signaling for its anti-CSC effect. Nevertheless, it is currently investigated in a phase I study in combination with cytarabine for relapsed or refractory AML (Clinical trial ID - NCT02096289).

Tranilast is an anti-allergy drug approved for use in Japan. The anti-tumor potential of tranilast has gathered considerable interest. Tranilast has been shown to inhibit breast CSC tumorspheres, reduce expression of stem cell markers Oct4 and CD133, and prevent *in vivo* lung metastasis of breast CSCs (Prud'homme et al., 2010).

Taken together, these studies highlight the critical role of CSCs and their role in cancer. The activation of the EMT and its relationship to CSC has further increased our understanding of CSCs. Since CSCs are regarded as the major culprits in therapy resistance and relapse following conventional therapy, the CSC drugs and inhibitors highlight the possibility of selectively targeting CSCs (Figure 10).

5 Therapeutic strategies for targeting Ras

As earlier described, the Ras proteins are important oncogenes that contribute to the formation and maintenance of cancer. Thus finding drugs that specifically target Ras has been the focus of intense efforts in the past decades. This section summarizes the recent attempts to develop Ras inhibitors in academia and industry (Figure 11, Table 3).

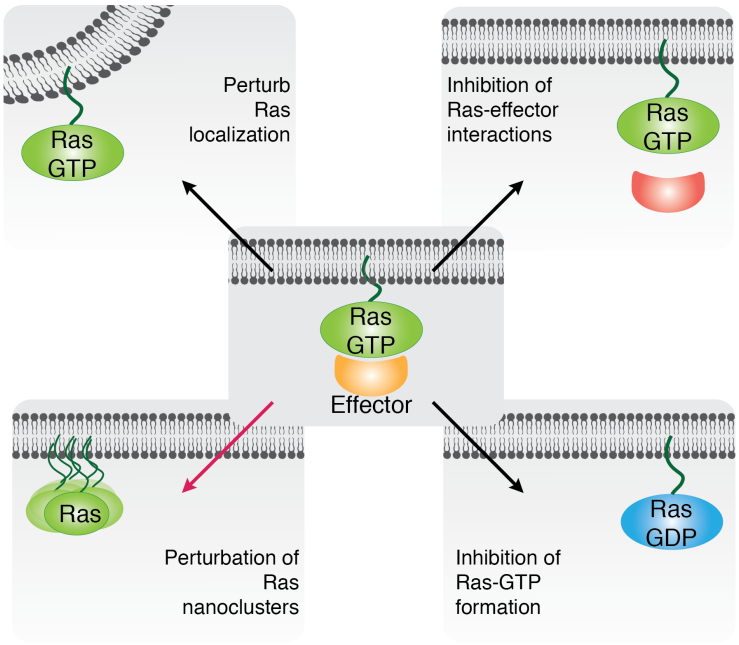

Figure 11. Strategies for targeting Ras. Some of the strategies to target Ras involve the impairment of Ras localization, inhibition of Ras-effector interactions and inhibition of Ras-GTP formation. Another potential therapeutic strategy is the perturbation of Ras nanoclusters. It should be noted that in addition to these strategies, major efforts so far have been directed towards targeting Ras downstream signaling pathway members.

5.1 Inhibiting the post-translational processing of Ras

Ras-dependent signaling requires the correct membrane localization of Ras predominantly in the plasma membrane. This critical need for Ras to associate with cellular membranes has been appreciated for decades (Jackson et al., 1990; Willumsen et al., 1984). As described earlier, nascent Ras proteins undergo a well-described series of post-translational modifications, which include cysteine *S*-farnesylation, proteolysis and carboxy-methylation at the C terminus. In addition, H-ras and N-ras are subjected to cysteine *S*-palmitoylation (Ahearn et al., 2012). Therefore, impairment of Ras localization has been explored as a mean to inhibit oncogenic Ras signaling, and each of these modifications represents viable targets for therapeutic intervention. It was one of the most promising directions towards an anti-Ras drug and two decades of extensive research involved targeting Ras

lipidation. These efforts were, however, followed by their dramatic and disappointing results in the clinic.

Farnesylation is the first step of the CAAX processing and farnesyl transferase (FTase) has been a target of numerous and intensive rational drug design and screening efforts to identify FTase inhibitors (FTIs) (Figure 12). This resulted in the development of many FTIs that fall into two categories: non-peptidomimetics such as tipifarnib (Beaupre et al., 2004; Zujewski et al., 2000), lonafarnib (Wang and Johnson, 2003), and BMS-214662 (Rose et al., 2001), and CAAX peptidomimetics including FTI-276 (Sun et al., 1998), FTI-277 (Lerner et al., 1995), L-744832 (Song et al., 2000), B956 (Nagasu et al., 1995) and FTI-2153 (Crespo et al., 2001). A number of publications have described their pre-clincal efficiency showing inhibition of H-ras farnesylation, impaired downstream signaling and tumor growth in animal models (Adjei, 2001; Berndt et al., 2011; Haluska et al., 2002). Particularly, lonafarnib, tipifarnib and BMS-214662 reached advanced clinical trials and were tested in over seventy clinical trials, but failed to show clinical efficacy against solid tumor such as pancreatic and colon cancer or with AML (Berndt et al., 2011). This was shown to be due to a process of alternative geranylgeranylation of K-ras and N-ras by geranlygeranyl transferase I (GGTase I), in the presence of FTIs. One potential solution was treatment with GGTase inhibitors or dual prenylation inhibitors to block both FTase and GGTase. The dual prenylation inhibitor (L-778123) even reached phase III clinical trails and was tested on a variety of hematologic and solid tumors (Holstein and Hohl, 2012). Disappointingly, neither the agent alone nor in combination, displayed clinical efficacy as the intended target K-ras was not affected. However, a number of recent studies have reported promising results with FTIs as single agents and in combination with normal chemotherapeutics in a subset of elderly patients with hematological malignancies (Witzig et al., 2011). An alternative strategy to disrupt Ras prenylation is through inhibition of isoprenoid pyrophosphate substrates, which are intermediates in the cholesterol biosynthetic pathway. Statins, which are used to treat hyperlipidemia also limit global protein prenylation in cells by depleting isoprenoids downstream of mevalonate. However, use of statins for anti cancer therapy is limited by the very high doses required to block prenylation (Holstein and Hohl, 2012).

Loss of postprenylation processing enzymes RCE1 and ICMT has been shown to cause mislocalization of Ras proteins and to reduce Ras-induced transformation in cells, making these enzymes attractive drug targets (Bergo et al., 2002; Gysin et al., 2011). A high-throughput screen with yeast Rcep1, lead to the identification of RCE1 inhibitors with low micromolar range activity (Manandhar et al., 2007). Moreover, studies have also found ICMT inhibitors such as Cysmethyil (Figure 12) (Winter-Vann et al., 2005). This compound was shown to mislocalize Ras, impair MAPK signaling and inhibit cell growth. However, the myriad of other substrates of these two enzymes (RCE1 and ICMT1), the lack of sufficiently selective and potent small molecule inhibitors, perplexing context dependent roles for RCE1 and ICMT (Bergo

et al., 2001; 2002), and toxicity associated with loss of RCE1 (Bergo et al., 2004) have hampered the pharmacological development of inhibitors.

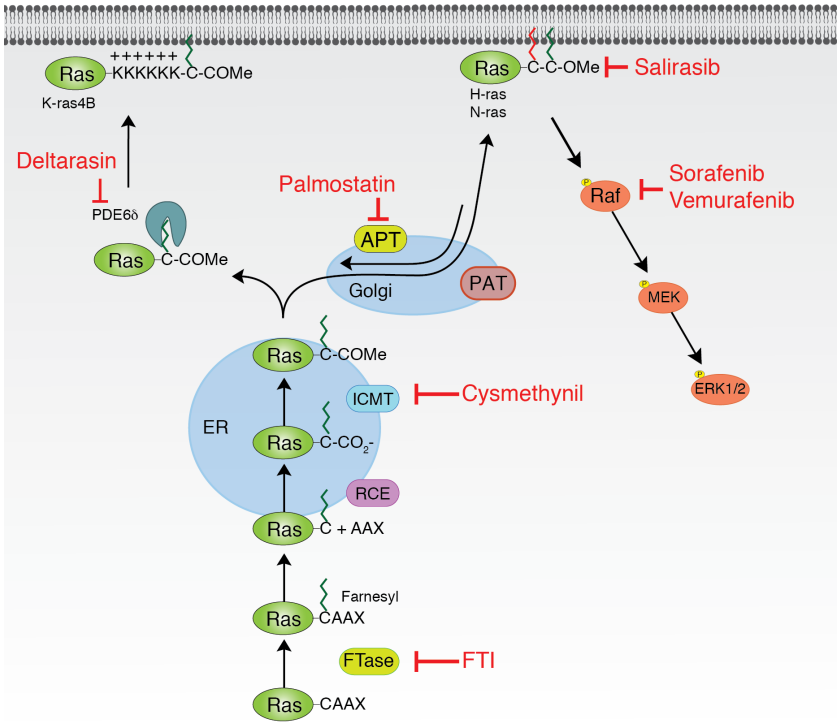

Figure 12. Schematic representation of selected inhibitors targeting Ras post translational processing and signaling.

Non-specific palmitoylation inhibitors such as the palmitate analog 2-bromopalmitate (2BP) have been widely used as a tool compound and its inhibitory effects on palmitoylation of H-ras and other proteins have been described (Draper and Smith, 2009; Webb et al., 2000). The identification of DHHC9/ GPC16 as the protein acyl transferase (PAT) that modifies N-ras and H-ras (Swarthout et al., 2005) suggests the possibility of targeting palmitoylation of Ras more selectively. Recent evidence implicating the palmitoylated K-ras splice variant, K-ras4a in colorectal cancer suggests palmitoylation could indeed be an attractive target for Ras-mutated cancers (Tsai et al., 2015).

Recently, depalmitoylation of Ras from cellular membranes was shown to be required for dynamic cycling and ultimate enrichment of palmitoylated H- and N-ras on the plasma membrane (Rocks et al., 2010; 2006). Blocking the depalmitoylation machinery breaks the cycle and leads to Ras mislocalization. Therefore, inhibiting depalmitoylating acyl protein thioesterase 1 (APT1) and APT2 with covalent b-lactone inhibitors palmostatins B and M leads to redistribution of

palmitoylated H- and N-ras to the endomembranes (Figure 12). Palmostatin has been shown to inhibit proliferation of myeloid progenitor cells expressing oncogenic N-ras in treated mice and partially reverse oncogenic phenotype of H-ras transformed MDCK cells (Dekker et al., 2010). In addition to palmostatins, non-covalent APT1/2 inhibitors have also been identified (Adibekian et al., 2012; Zimmermann et al., 2012). Moreover, H-ras depalmitoylation is stimulated by FKBP12 that binds rapamycin and other rapalogs (Ahearn et al., 2011).

Using an analogous approach of interfering with the N-/H-ras palmitoylation cycles, K-ras4B shuttling by prenyl-binding protein PDEδ was targeted, and inhibitors of the K-ras–PDEδ interaction have recently been identified (Chandra et al., 2012; Zimmermann et al., 2013). Deltarasin, a *bis*-benzimidazole compound with high affinity for PDEδ, effectively impairs the K-Ras4B–PDEδ interaction and induces relocalization of Ras family proteins (Figure 12). In addition, deltarasin reduces proliferation and tumor growth in xenografts of K-ras transformed pancreatic cancer cells (Zimmermann et al., 2013). However, further structural optimization of deltarasin would be required, considering its nonspecific inhibitory activity toward other small GTPases such as Rheb (Chandra et al., 2012). These studies highlight that modulation of Ras processing and trafficking can open new opportunities for modulating Ras signaling.

Table 3. Classification of inhibitors targeting Ras and downstream effectors.

Compound	Target/mechanism of action	Ras specificity	Reference
Compounds that inhibit Ras-effector interactions			
Sulindac (NSAID)	Direct binding in vicinity of H-ras switch I	H-ras	Waldmann et al., 2004; Muller et al., 2004
Methylcyclopropene (MCP)	Ras-Raf interaction	K-ras	Kato-Stankiewicz et al., 2002; González-Pérez et al., 2010
Cu^{2+} or Zn^{2+} cyclens	Ras-effector interaction. Shifts H-ras-GTP conformational equilibrium towards state 1	Ras	Rosnizeck et al., 2010
Kobe-family inhibitors	Stabilizes Ras in open state 1 conformation.	H-ras (T35S)-GTP	Shima et al., 2013
Compounds that inhibit exchange of GDP for GTP			
SCH54292 and water-soluble SCH54292	Suggested to bind Ras between the switch II and helix 4 regions.	H-ras	Taveras et al., 1997; Palmioli et al., 2009
Peptide HBS3	Interferes with Ras-SOS interaction	Ras	Patgiri et al., 2011
4,6-dichloro-2-methyl-3-aminoethyl-indole (DCAI)	Blocks K-ras/SOS interaction by binding to the hydrophobic pocket located between the switch II region and the core β-sheet.	K-ras	Maurer et al., 2012
Indole derivative	Blocks K-ras/SOS interaction by binding to the hydrophobic pocket	K-ras	Sun et al., 2012

	located between the switch II region and the core β-sheet.		
SML-10-70-1	Mimics GDP when bound to K-ras	K-rasG12C	Lim et al., 2013
Acrylamide-12 (AA12)	Binds irreversibly to K-rasG12C and disrupt switches I and II	K-rasG12C	Ostrem et al., 2013
Bisphenol A	Inhibits SOS-mediated nucleotide exchange by direct binding.	K-ras, H-ras	Schopel et al., 2013
Cyclorasin 9A and 12A (cyclic peptides)	Bind K-ras in the switch I and II regions.	K-ras	Wu et al., 2013; Upadhyaya et al., 2015
Compounds that alter Ras plasma membrane localization			
Tipifarnib, lonafarnib, and BMS-214662 (non-peptidomimetics)	FTase inhibitors. Inhibits farnesylation of Ras. Changes localization of Ras.	H-ras	Beaupre et al., 2004
FTI-276, FTI-277, L-744832, B956 and FTI-2153 (CAAX peptidomimetics)	FTase inhibitors. Inhibits farnesylation of Ras. Changes localization of Ras.	H-ras	Sun et al., 1998; Lerner et al., 1995
Statins	Inhibits Ras prenylation through inhibition of isoprenoid pyrophosphate substrate. Changes localization of Ras.	-	Hindler et al., 2006
RPI, prenylcysteine chloromethylketone derivatives, S-adenosylhomocysteine, prenylcysteine derivates (AFC, AGGC)	Targets RCE1 and ICMT. Changes localization of Ras.	Ras	Hollander et al., 2000; Manandhar et al., 2007
2-Bromopalmitate	Inhibits palmitoylation, but is not specific. Changes localization of Ras.	H-ras, N-ras	Draper et al., 2009; Webb et al., 2000
Palmostatins B and M	B-lactone inhibitors. Blocks depalmitoylation. Changes localization of Ras.	H-ras, N-ras	Dekker et al., 2010
Deltarasin	Inhibits the K-ras–PDE6d interaction. Changes localization of Ras.	K-ras	Zimmermann et al., 2013
Salirasib (farnesylcysteine mimetic)	Inhibits the prenyl-binding interactions between Ras and farnesyl-binding proteins. Disrupts Ras membrane anchorage.	H-ras	Marom et al., 1995; Kloog et al., 2013
Compounds that target Ras downstream signaling			
LY3009120	Pan-Raf inhibitor	-	Peng et al., 2015
PLX8394	B-Raf inhibitor	-	Zhang et al., 2015
PLX7904	B-Raf inhibitor	-	Zhang et al., 2015
CCT196969	Pan-Raf and Src-family kinase inhibitor	-	Girotti et al., 2015

Pictilisib (GDC-0941)	Pan-PI3K inhibitor	-	Sarker et al., 2015
Buparlisib	Pan-PI3K inhibitor	-	Maira et al., 2012
Alpelisib (BYL719)	p110α selective-PI3K inhibitor	-	Fritsch et al., 2014; Furet et al., 2013
Taselisib (GDC-0032)	p110α selective-PI3K inhibitor	-	Ndubaku et al., 2013
MLN1117	p110α selective-PI3K inhibitor	-	Jenssen et al., 2011
AZD8186	p110β selective-PI3K inhibitor	-	Hancox et al., 2015
Idelalisib (GS-1101)	p110δ selective-PI3K inhibitor	-	Fruman et al., 2014
NVP-BEZ235	Dual PI3K/mTOR inhibitor	-	Maira et al., 2008

Finally, a number of farnesyl-derived compounds were designed to inhibit the prenyl-binding interactions between Ras and farnesyl-binding proteins. Salirasib, also known as farnesylthiosalicylic acid (FTS), a farnesylcysteine mimetic, was proposed to target galectins and to compete for interaction with Ras proteins (Elad et al., 1999; Elad-Sfadia et al., 2002; Kloog et al., 2013; Paz et al., 2001). Salirasib has been shown to disrupt Ras membrane anchorage, leading to an inhibition of tumor cell growth (Marom et al., 1995). Although, Salirasib was tested in small clinical trials, no objective responses were seen either as monotherapy against advanced K-ras-mutant lung adenocarcinoma or in combination with gemcitabine in pancreatic cancer (Laheru et al., 2012; Riely et al., 2011).

5.2 Drugging Ras structurally

The disappointing results with developing FTIs as anti-Ras drugs, lead to the notion that Ras is 'undruggable'. Designing drugs to bind Ras directly seemed futile due to the lack of apparent binding domains or pockets for small molecule binding in the Ras tertiary structure (Marcus and Mattos, 2015). However, the recent developments with the Ras crystal structures and high-throughput screening have given hope that small molecules can bind in Ras pockets. The following section provides an overview of some of accomplishments with inhibitors that directly target Ras and its effector interactions.

Vos et al., in 1988 and Pai et al., in 1989 reported the first tertiary structure of H-ras-GDP and H-ras-GppNHp respectively (de Vos et al., 1988; Pai et al., 1989). H-ras consists of a hydrophobic core of 6-stranded β-sheets and five α-helices with ten connecting loops. Comparison of the GDP and GTP bound H-ras revealed a guanine nucleotide exchange driven conformation change in the predominantly flexible regions, Switch I (residues 32-28) and Switch II (residues 60-75). This was followed by NMR spectroscopy data that revealed the existence of at least two distinct

conformational states that are in dynamic equilibrium. H-ras-GppNHP adopts two conformations in solution, state 1 and state 2 (Geyer et al., 1996; Spoerner et al., 2005) regardless of the presence or absence of the oncogenic mutations. State 1 corresponds to an inactive conformation with greatly impaired ability to bind effectors and it is recognized by GEFs. Effector proteins recognize state 2 and it represents the active conformation, due to an effector-binding induced equilibrium shift towards state 2. Crystal structures of H-ras GTP alone or bound to effector correspond to this state. Crystal structures of H-ras mutants in state 1 (Geyer et al., 1996) revealed that state 1 corresponds to an open conformation with the loss of hydrogen bonding across the switches I and II and guanine nucleotide found in state 2. This results in the surface pocket suitable for accepting small-molecule compounds. Different approaches have been used to directly target Ras: inhibiting the exchange of GDP for GTP, increasing GTP hydrolysis rates or inhibiting effector interactions.

5.3 Inhibiting the exchange of GDP for GTP

Some previous studies have reported potential small molecule association with Ras. However, there was little knowledge on the binding site of the inhibitors and no defined binding pocket was reported. Tavares *et al*., first highlighted the concept of targeting Ras by small molecules when they characterized the Ras-binding compounds SCH54292 (Taveras et al., 1997) and its water-soluble derivative (Palmioli et al., 2009). *In silico* mapping suggested that these molecules bind to Ras between the switch II and helix 4 regions and do not displace the nucleotide. In the cellular context, the exact mechanism of action is not known, hindering identification of similar inhibitors. Inhibitors to block the nucleotide exchange reaction were developed using modified peptides based on the Ras-binding domains of GEFs (Patgiri et al., 2011; Sacco et al., 2012). Peptide HBS3, a cell-permeable synthetic α-helix mimic of SOS developed using the hydrogen bond surrogate (HBS) approach was shown to interfere with Ras-SOS interaction and down regulate Ras signaling in response to epidermal growth factor (EGF)-stimulation in HeLa cells (Patgiri et al., 2011).

Ras guanine nucleotide-binding site is a known allosteric site for potential modulation. In 2012, two groups independently reported the use of an NMR-based fragment screening approach to identify inhibitors that block the K-ras/SOS interaction (Maurer et al., 2012; Sun et al., 2012). These inhibitors: 4,6-dichloro-2-methyl-3-aminoethyl-indole (DCAI) (identified by the Genentech group) and indole derivative compound (identified by Fesik's group) bound to the same hydrophobic pocket, located between the α2 helix (switch II region) and the core β-sheet of K-ras and thereby interrupted K-ras/SOS association. These fragments make very different contacts inside the pocket (Maurer et al., 2012; Sun et al., 2012). However, there was no evidence for their inhibitory effect on Ras mutated cancer cells.

Lim et al., and Ostrem et al., found an alternative strategy of inhibiting GDP-exchange (Lim et al., 2013; Ostrem et al., 2013). Lim et al., used a synthetic chemistry approach to design and screen GDP derived analogues. They identified analogue SML-10-70-1 that mimics GDP when bound to K-ras and can covalently modify cysteine 12 of K-rasG12C. This modification is K-rasG12C specific and not found in wild type K-ras. SML-10-70-1 blocked the proliferation of K-rasG12C mutated cancer cells (Lim et al., 2013). Ostrem and colleagues used a disulphide-fragment-based screening approach called tethering, which relies on the formation of a disulfide bond between the ligand and a cysteine residue in the protein of interest (Erlanson et al., 2000; Ostrem et al., 2013). By taking advantage of the nucleophilicity of the cysteine thiols, they used K-rasG12C as a target for identifying small molecules. Notably, the mutant cysteine 12 in K-ras sits in the proximity to both the nucleotide-binding region and the switch regions that are involved in Ras effector interactions. The fragment-based screening led to the identification of inhibitors that irreversibly binds to K-rasG12C and disrupt switches I and II. Crystallographic studies of the complex revealed a novel drug-binding pocket. Structure based optimization resulted in the most potent compound acrylamide 12 (AA12) that is specifically active against mutated K-rasG12C, but not wild type K-ras *in vitro,* and it induced apoptosis of lung cancer cells carrying K-rasG12C (Ostrem et al., 2013). However, the effect of this compound in either H- or N-rasG12C is not known, though the mutations are rare in these Ras isoforms.

By using an NMR-based screening approach against a related GTPase Rheb, it was found that Bisphenol A directly binds to Rheb and also K-ras (Schöpel et al., 2013). Bisphenol A inhibits the SOS-mediated nucleotide exchange activity of both K- and H-ras. Again the binding site of Bisphenol A in K-ras was identical to the one described earlier for the structurally different compounds (Schöpel et al., 2013). More recently, a combinatorial peptide library screening against K-ras found cyclic peptides (cyclorasin 9A and 12A) that strongly bind K-ras (Upadhyaya et al., 2015; Wu et al., 2013). The binding site of the cyclorasin was mapped to the switch I and II regions, similar to the ones reported before by Genentech and the Fesik group (Maurer et al., 2012; Sun et al., 2012).

5.4 Inhibiting Ras-effector interactions

Blocking the interactions of Ras-GTP with downstream effectors is another strategy for inhibiting constitutively active Ras. This Ras-effector targeting has produced few low-affinity inhibitors. It was first reported using a phenotypic screening approach that sulindac, a NSAID (nonsteroidal anti-inflammatory drug) strongly inhibited H-ras-induced malignant transformation of MDCK-F3 cells and the Ras-dependent Raf activation (Muller et al., 2004; Waldmann et al., 2004). NMR analysis of H-ras with the inhibitor revealed a direct binding of sulindac in the vicinity of H-ras switch I (Muller et al., 2004), however these observations were not studied further. Methylcyclopropene (MCP) compounds were identified as inhibitors of Ras-C-Raf

interaction using a yeast two-hydrid system. MCP compounds inhibited anchorage-independent growth of cancer cells harboring a K-ras mutation. However, the direct target of the inhibitor has been ambiguous. Rosnizeck et al., showed inhibition of Ras-effector protein interactions using Cu^{2+} and Zn^{2+} cyclens as a way to deregulate over active GTP-bound Ras (Rosnizeck et al., 2010). The authors hypothesized that the presence of these metal cyclen derivatives - capable of shifting the conformational equilibrium of H-ras-GTP toward state 1 - caused a disruption of the nucleotide-binding site in the presence of GTP leading to weak effector interactions. However, these cyclen derivatives have low binding affinity for Ras-GTP, weak inhibitory effect on Ras-Raf and lack evidence for cellular efficacy hindering their further optimization (Rosnizeck et al., 2010).

The identification of state 1 of H-ras-GTP lead to the identification of the Kobe-family inhibitors that block Ras activation by stabilizing Ras in the open state 1 conformation (Shima et al., 2013). The Kobe0065-family of inhibitors was identified using an *in silico* screening approach coupled with computer docking simulations. Kobe0065 and its analogue Kobe2602 compounds inhibited the Ras-Raf-Mek-Erk pathways and nucleotide exchange activity of SOS. NMR structure revealed that the compound inserted into the surface pocket of H-ras (T35S)-GTP. Furthermore, the compounds inhibited Ras-mutated cancer cell lines and reduced tumor growth in a xenograft model (Shima et al., 2013).

5.5 Targeting Ras downstream effectors

Given the difficulties associated with targeting mutant Ras proteins directly, search began for drugs targeting the downstream effector pathways, particularly the Raf-MAPK and PI3K pathways. The Raf-Mek-Erk pathway, was the first Ras effector-signaling pathway to be identified and is required for Ras-mediated transformation and tumorigenesis (Kyriakis et al., 1992). Most of the attempts to target Raf-Mek-Erk signaling pathway were largely focused on finding Raf and Mek inhibitors. The identification of *BRAF* mutations in cancer (~8% of all cancers and 50% of all melanomas) makes it an ideal anti-cancer target (Dong et al., 2003). This lead to the identification of several Raf inhibitors and some were clinically approved like vemurafenib and dabrafenib. For instance, vemurafenib binds and inhibits all three Raf kinases in biochemical assays and it effectively inhibits Erk signaling and proliferation of the B-Raf V600E melanoma cell lines and mouse xenograft models. These inhibitors also showed unprecedented clinical activity in patients with B-Raf V600E or V600K mutant melanomas (Chapman et al., 2011; Hauschild et al., 2012).

However, a notable property of these B-Raf inhibitors is that they lead to paradoxical activation of Raf kinase especially in Ras mutant cancers, leading to increased Erk signaling precluding their use for Ras mutated cancers (Chapman, 2013; Poulikakos et al., 2011). This is because, in the presence of oncogenic Ras, B-Raf inhibitors can drive the formation of B-Raf/C-Raf hetero- and homodimers, which contain one

inhibitor-bound partner and one partner that is inhibitor-free. The inhibitor-bound partner drives activation of the inhibitor-free partner through scaffolding or conformational functions, thus activating C-Raf and, consequently, stimulating Mek and Erk activation (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). This 'Raf inhibitor paradox' has gathered a lot of research interest in the past few years, and it has lead to the recent identification of next-generation Raf inhibitors dubbed 'paradox breakers'. Zhang and colleagues reported that the paradox-breaking B-Raf inhibitors PLX7904 and PLX8394 can inhibit downstream Erk signaling in cultured cells with B-Raf mutations (Zhang et al., 2015). Importantly, these inhibitors suppress Erk phosphorylation in *BRAF*-mutant melanoma cells but do not stimulate Erk signaling in *RAS*-mutant cells. Moreover, PLX7904 did not enhance B-Raf/C-Raf heterodimerization in Ras-mutant cells and in vemurafenib-resistant cells. PLX8394 is currently in clinical trials (www.clinicaltrials.gov, NCT02428712). These inhibitors are predicted to have increased safety and clinical efficacy compared to first-generation and pan-Raf inhibitors. Recently, Peng and colleagues characterized a new pan-Raf inhibitor, LY3009120, which inhibits all Raf family members with similar potencies. LY3009120 inhibits monomeric B-Raf V600E as well as WT and mutant Raf dimers with minimal paradoxical activation. In addition, LY3009120 was shown to have anti-tumor activity in cancer models carrying oncogenic K-ras, N-Ras, or B-Raf mutations (Peng et al., 2015).

Mek and Erk inhibitors do not have this paradoxical activation. Nevertheless, their early clinical efficacy was limited by toxicity and failing to select patients with tumors containing mutations in the Erk pathway (Samatar and Poulikakos, 2014). The first MEK inhibitor to be approved by FDA for clinical use was trametinib, a potent Mek1/2 inhibitor that preferentially binds to unphosphorylated Mek1/2 and prevents Raf-dependent Mek phosphorylation. It was approved for treatment of metastatic melanoma with the B-Raf (V600E/K) mutation and its efficacy was proved in a phase III clinical trial of patients with B-Raf mutant melanoma who had not received prior B-Raf therapy (Samatar and Poulikakos, 2014). However, trametinib treatment was unsuccessful for patients who relapsed after therapy with either vemurafenib or dabrafenib. Though the reasons for this acquired resistance are not clear, one possibility is the reduced dependence of the resistance tumors on Erk signaling for growth. Cobimetinib, another allosteric inhibitor of Mek1/2 with sustained inhibition of Erk/MAPK signaling is currently in clinical trials (Choo et al., 2012). A phase I trial (CoBRIM) trial of cobimetinib combined with B-Raf inhibitor (vemurafenib) in patients with B-Raf V600E/K mutations confirmed an improved response to combination therapy. This was further confirmed by a phase III trial of vemurafenib plus cobimetinib in advanced B-Raf mutant melanoma (Larkin et al., 2014). Cobimetinib was very recently approved by the FDA for treatment of patients with unresectable or metastatic melanoma with a B-Raf V600E/K mutation, in combination with vemurafenib (www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm472193.htm).

Cobimetinib is now under regulatory review for the same indication in several countries (Garnock-Jones, 2015). Similar to Raf inhibitors, Mek inhibitors are also limited by drug resistance that typically involve the loss of Erk-driven negative feedback loops. Nevertheless, Mek inhibitors such as GDC-0623, which stabilizes the Raf-Mek complex, has shown greater efficacy in K-ras mutated cancers than conventional Mek inhibitors.

Until recently, development of Erk inhibitors was lagging behind Raf and Mek inhibitors, largely due to the assumption that there is no additional benefit of targeting Erk, since inhibition of Raf and Mek would be sufficient to inhibit Erk. However, most resistance mechanisms result in reactivation of Erk. Active Erk inhibitors, such as VTX-11e and SCH772984, have been described before (Aronov et al., 2009; Morris et al., 2013). SCH772984 has been found to be active against both N-ras and B-Raf mutated cancer cell lines. In addition, SCH772984 was reported to act synergistically with vemurafenib against B-Raf mutant cell lines (Wong et al., 2014). Moreover, Erk1/2 inhibitors, including CC-90003, GDC-0994, MK-8353 and BVD-523 (ulixertinib), are currently in phase I/II clinical studies. GDC-0994 was reported to have significant activity in multiple *in vivo* cancer models, including K-ras-mutant and B-Raf-mutant human xenograft tumors in mice (Robarge et al., 2014). Similarly, BVD-523 inhibits tumor growth in B-Raf-mutant melanoma and colorectal xenografts as well as in K-ras-mutant colorectal and pancreatic models. BVD-523 was found to inhibit the growth of cells resistant to dabrafenib, trametinib, or their combination (Germann et al., 2015). The preclinical and clinical studies of these inhibitors will help in better understanding whether they may be used as novel agents in MAPK directed therapeutic strategies.

The identification and characterization of such clinically available Raf, Mek and Erk inhibitors with different biochemical and pharmacologic properties are predicted to potently inhibit signaling and avoid toxicities. As mentioned above, which inhibitor as single agent or in combination is 'better', will depend on tumor type and tumor genetic context.

The PI3K pathway is another well-studied signaling cascade that is often up-regulated in tumor cells, indicating its importance in cancer. Ras mutations drive PI3K activity, and mutations in Ras and PI3K often coexist e.g. in colorectal and endometrial cancers (Esther Castellano, 2011). Currently there are several classes of potent and selective small molecule PI3K inhibitors under development. They are classified into pan-PI3K and isoform-selective inhibitors, depending on their specificities to the four isoforms of the p110 catalytic domains α, β, γ, and δ (Table 3). Pictilisib (GDC-0941) was the first oral, potent, pan-PI3K inhibitor shown to be active in glioblastoma and ovarian cancer xenograft models (Raynaud et al., 2009). A recent first-in-human phase I study confirmed the activity of pictilisib in inhibiting PI3K in patients with solid tumors (Sarker et al., 2015). Alpelisib (BYL719) is a p110α-selective PI3K inhibitor that has shown activity in a variety of cancer cell

lines, especially in those harboring PIK3CA mutations. *In vitro* studies have predicted alpelisib to be most potent in head and neck, hematological, and breast cancers and alpelisib is currently investigated in phase I-II clinical trials (Massacesi et al., 2015). Synergistic activity of combined binimetinib (Mek1/2 inhibitor) and alpelisib was recently reported in human neuroblastoma cell lines. This combined treatment suppresses the activation of MAPK, PI3K/Akt and mTOR (Hart et al., 2015). However, inhibitors targeting the PI3K pathway are generally limited by the feedback loops that activate upstream signaling and their poor therapeutic index. *In vitro* data suggest oncogenic Ras to be a strong predictor of resistance to PI3K inhibitors (Gysin et al., 2011; Ihle et al., 2009). Therefore, targeting PI3K alone is likely insufficient for Ras mutated cancers. In light of the crosstalk between the Raf-Mek-Erk and PI3K pathways, it has been proposed that dual inhibition of both pathways may be necessary to treat Ras-driven tumors (Sabbah et al., 2011). In support of this assumption, combined inhibition of Mek (by Selumetinib) and PI3K/mTOR (by NVP-BEZ235) has been shown to be effective at suppressing tumor growth of mice with K-ras driven lung cancers (Engelman et al., 2008). Moreover, there are several ongoing clinical trials testing the dual inhibition of the Raf-Mek-Erk and PI3K pathways (Britten, 2013; Saini et al., 2013).

5.6 Targeting Ras nanoclusters

Perturbing the spatiotemporal dynamics of Ras nanoclusters disrupts Ras signaling. This makes nanoclustering a viable therapeutic target for pharmacological targeting and to inhibit aberrant Ras signaling in human cancers. Work from our laboratory provided the first proof-of-concept study to identify potential Ras nanocluster inhibitors (Köhnke et al., 2012). By using a FRET-based screening approach, Köhnke et al., identified ionophoric antibiotic macrotetrolides to specifically disrupt H-ras nanoclustering and EGF-induced Ras signaling (Köhnke et al., 2012). However, the mechanism of action of these macrotetrolides or similar ionophoric compounds on Ras nanoclustering and signaling is unknown. Recent work from John Hancock and colleagues has further validated Ras nanoclusters as anti-cancer drug targets. Using a high-content imaging based screening method, they identified two known compounds, fendiline and staurosporine (STS) to mislocalize K-ras (Cho et al., 2012b; van der Hoeven et al., 2013). Fendiline is an FDA approved drug, originally found as a L-type calcium channel blocker. Fendiline specifically decreases K-ras but not H-ras nanoclustering, leading to impaired downstream signaling and growth of K-ras mutated cancer cells. Though the exact molecular mechanism for this is not completely characterized, it is unrelated to the activity of fendiline as a calcium channel blocker (van der Hoeven et al., 2013). STS and its analogues are well-known PKC-inhibitors that were found to deplete PS from the inner leaflet of the plasma membrane to the endomembranes, thus disrupting K-ras nanoclustering and signaling (Cho et al., 2012b). These effects were unrelated to STS-mediated inhibition of PKC.

In addition to drugs that change plasma membrane lipid composition, drugs that change lipid and membrane properties have also been found to affect Ras nanoclustering (Zhou et al., 2010). Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen and indomethacin have been reported to specifically stabilize cholesterol-dependent H-ras-GDP and N-ras-GTP nanoclusters, but they have no effect on cholesterol-independent nanoclusters (Zhou et al., 2012). However, this stabilization of cholesterol-domains leads to formation of heterotypic clustering between H-ras-GDP and H-ras-GTP resulting in lower MAPK signaling. It is therefore suggested that the chemo-preventive action of NSAIDs could be partly related due to the effect on Ras nanoclustering. It is now understood that Raf-inhibitors, in a Ras-dependent manner, can lead to paradoxical activation of the Raf-Mek-Erk pathway by inhibitor-induced homodimerization of B-Raf or heterodimerization of B- and C-Raf (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2011). It was recently shown that Raf inhibitors like sorafenib and SB590885 specifically enhance K- and N-rasG12V but not H-rasG12V nanoclustering (Cho et al., 2012a). Mechanistically, Raf inhibitor-treated cells form stable Raf dimers with two RBDs that drive Ras dimerization that leads to higher K- and N-rasG12V nanoclustering (Cho et al., 2012a).

Ras membrane lipid composition and lipid interactions with Ras are critical for nanoclustering and proper lateral segregation, suggesting that nanoclustering and lateral segragation must be a general characteristic of all lipid-anchored proteins. However, this possibility remains to be investigated. Given the importance of Ras nanoclusters in signal transduction and cancer, targeting Ras nanoclustering and lateral segregation is an attactive therapeutic target for drug development.

AIMS OF THE STUDY

Due to their eminent role in cancer, Ras proteins have been considered a major drug target for more than 30 years. Numerous attempts have been made to find a true anti-Ras drug, yet not a single drug has emerged from these efforts. These past failures in the search for drugs to target Ras have driven some researchers to jump ship, deeming Ras 'undruggable'. Nevertheless, there is currently a growing optimism that this could change in the near future as we gain new insights into Ras function. Now it is clear that Ras forms nanoscale signaling complexes on the plasma membrane, termed nanocluster. Ras nanoclustering is essential for oncogenic Ras signaling. The focus of my PhD thesis was to explore nanoclusters as novel anti-cancer drug targets and to identify chemical modulators of nanoclustering. For the identification of nanocluster inhibitors a cell-based high-throughput amenable Ras FRET-biosensor screening platform was established. Diverse chemical libraries were acquired and screened for potentially novel chemical modulators of Ras. Potential hit molecules were validated on full-length oncogenic proteins using a combination of imaging methods such as FLIM-FRET, confocal and super resolution microscopy, and biochemical methods. The selected hit compounds were then functionally validated on different Ras isoforms using cellular transformation and tumor growth assays.

The specific aims of the thesis were:

- To utilize FRET-biosensors as a small molecule screening platform to identify nanoclustering and lipid modification inhibitors.
- To perform a small molecule chemical screen to identify H-ras nanocluster modulators.
- To perform differential screens with H- and K-ras biosensors to find novel inhibitors of K-ras nanoclustering.
- To design and validate FRET-biosensors that report on membrane targeting of N-myristoylated proteins.

EXPERIMENTAL PROCEDURES

More detailed information on the experimental procedures is available in the original publications. For more information on the use of the flow cytometry-based Fluorescence Resonance Energy Transfer (FRET)-biosensors assays see (Najumudeen et al., 2015).

Cell lines

Name (company)	Publication
BHK21, baby hamster kidney cells (Sigma-Aldrich)	I, II, III
HEK293-EBNA, HEK293 cells expressing the EBNA-1 gene	I, II, III
MEF, wild-type mouse embryonic fibroblasts	I
Du-315-6 (H-ras$^{-/-}$, N-ras$^{-/-}$) mouse embryonic fibroblasts	I
PC12, rat adrenal pheochromocytoma cells	I
MDA-MB-231, Human breast adenocarcinoma cells	I, II
MDCK, Madin-Darby canine kidney cells, stably expressing either mGFP-K- or H- rasG12V (gift from J. Hancock)	II
NCI-H1395, OVSAHO, NCI-H1975, Calu-6, A2780, SK-MEL-2, SNU-398, COLO320, MeWo, NCl-H196, A375, U-2OS, HGC27, MDA-MB-231, HS675T (GenScript)	II

Antibodies

Name	Application	Publication
Erk1/2 (Cell Signaling Technology, Cat. No. 9102)	WB	I, II
phospho- ppErk1/2 (T202/Y204; Cell Signaling Technology, Cat. No. 9101)	WB	I, II
Akt (Cell Signaling Technology, Cat. No. 9272)	WB	I
phospho-pAkt (T308; R&D Systems, Cat. No. 658320)	WB	I
β-actin (Sigma Aldrich, Cat. No. A1978)	WB	I, II
Pan-Ras	WB	I
Cav-1 (BD biosciences, Cat. No. 610060)	STED	II
GFP (BioVision, Cat. No. 3999-100)	WB	II
APC-conjugated anti-CD44 (clone G44-26, BD Biosciences)	Flow cytometry	II
PE-conjugated anti-CD24 antibody (clone ML5, BD Biosciences)	Flow cytometry	II

Methods

Name	Publication
Chemical library screening with H-ras-NANOPS, K-ras NANOPS and Yes-NANOMS	I, II, III
Immuno-electron microscopy	I
Confocal microscopy	II
Fluorescence resonance energy transfer (FRET) confocal microscopy	III
Flow cytometric FRET on BD LSRII (BD Biosciences)	I, II, III
Flow cytometric analysis of stem cell markers LSRII (BD Biosciences)	II
Fluorescence lifetime imaging microscopy (FLIM)-FRET	I, II
Fluorescence recovery after photobleaching (FRAP)	I
STED microscopy	II

FACS and FRAP data analysis with IGOR Pro 6 (WaveMetrics)	I, II
Image analysis using ImageJ	I, II
Western blotting (WB)	I, II
Quantification of immunoblot, ImageLab™ software (BioRad)	I, II
PC12 cell differentiation assay	I
Mammosphere assay	I
In ovo tumor growth assay	I
Cell proliferation assay	II
Drug sensitivity profiling (performed through GenScript)	II
siRNA knockdown	I, III
Quantitative reverse transcription PCR	III
In silico analysis of co-expressed genes (conducted by Aittokallio group)	II
In silico analysis of cell lines (conducted by Aittokallio group)	II
In silico analysis of clinical samples (conducted by Aittokallio group)	
Dose-response analysis (nonlinear regression analysis) with GraphPad PRISM software	I, II
Statistical analysis (t-test, ANOVA) with GraphPad PRISM software	I, II, III

Chemical libraries

Name and description	Publication
Enzo® Screen-Well® Natural Product library (Enzo Life Science Inc.), 502 naturally derived compounds	I
In house collection of 157 natural compounds (Brunhofer et al., 2012; Narwal et al., 2012)	I
Ionophore collection (Cherry-picked)	II
Microbial Screening Technologies (MST) Library	II

Reagents

Reagents (manufacturer)	Application	Publication
jetPRIME (Polyplus transfection)	Cell transfection	I, II, III
FuGene6 (Roche)	Cell transfection	III
Lipofectamine 3000 (Thermo-Fischer Scientific)	Cell transfection	I
Lipofectamine RNAiMax (Invitrogen)	Cell transfection	III
DMEM, Dulbecco's modified Eagle's Medium (Invitrogen, Sigma-Aldrich)	Cell culture	I, II, III
RPMI-1640, Roswell Park Memorial Institute medium (Invitrogen)	Cell culture	I
FBS, fetal bovine serum (Invitrogen)	Cell culture	I, II, III
Horse serum (Invitrogen)	Cell culture	I
L-glutamine (Sigma-Aldrich)	Cell culture	I, II, III
Penicillin (Sigma-Aldrich)	Cell culture	I, II, III
Streptomycin (Sigma-Aldrich)	Cell culture	I, II, III
DMSO (Sigma-Aldrich)	Compound solvent	I, II, III
Rat tail collagen I (Gibco)	Cell culture	I
B27 supplement (Gibco)	Cell culture, sphere	I
Epidermal growth factor (EGF; Sigma)	Cell culture, sphere	I
Fibroblast growth factor (FGF; Sigma)	Cell culture, sphere	I
PFA, paraformaldehyde (Sigma-Aldrich)	Microscropy	I, II, III
Mowiol 4–88 (Sigma)	Microscopy	I, II, III

4.5 nm gold nano-particles coupled to anti-GFP	Electron microscopy	I
Fluorescein	FLIM-FRET standard	I, II
FITC beads (Bangs Laboratories)	FRET standard	I, II, III
Ringers buffer	FRAP	I
Clarity™ Western ECL blotting (BioRad)	WB	I
Matrigel (BD Biosciences)	*In ovo* tumor growth	I
AlamarBlue (Invitrogen)	Cell proliferation and viability	II

Inhibitors (used in the thesis)

Name	Concentrations	Publication
Cycloheximide (Fischer Scientific)	0.18 µM, 10 µM	I
Anisomycin (Fischer Scientific)	2 µM, 10 µM	I
Harringtonine (Fischer Scientific)	2 µM, 10 µM	I
Doxorubicin (Sigma-Aldrich)	100 ng/ml, 2 µM	I, II
Salinomycin (BioAustralis)	1.3 µM, 2 µM	I, II
Nigericin (BioAustralis)	1.3 µM	II
Lasalocid sodium (BioAustralis)	1.7 µM	II
Staurosporine (STS) (BioAustralis)	10 nM	II
Avermectin (BioAustralis)	0.2, 2 µM	II
Ivermectin (BioAustralis)	0.2, 2 µM	II
Congoblatin A (BioAustralis)	0.2, 2 µM	II
Ophiobolin A (BioAustralis)	0.2, 2 µM	II
Leptomycin B (BioAustralis)	0.2, 2 µM	II
Kazusamycin B (BioAustralis)	0.2, 2 µM	II
Streptonigrin (BioAustralis)	0.2, 2 µM	II
DDD85646	4 µM	III
Myristoleic Acid (Sigma-Aldrich)	0.3 µM	III
Tris (dibenzylideneacetone) dipalladium (TDP; SantaCruz)	1.0 µM	III
FTI-277 (Calbiochem)	0.3 µM	III
2-bromopalmitate (Sigma Aldrich)	100 µM	III
2-fluoropalmitate (United Bioresearch)	100 µM	III

Plasmid constructs

Name	Publication
mCFP and mCitrine-CTH or H-ras-NANOPS (Kohnke et al., 2012), C-terminal hypervariable region of human H-ras	I, II
pmGFP-H-rasG12V (Abankwa et al., 2008), mutated human H-ras	I, II
pmGFP-K-ras4BG12V (Abankwa et al., 2010), mutated human K-ras	I, II
mRFP-C-Raf-RBD (Abankwa et al., 2008), Ras-binding domain of C-Raf	I, II
pcDNA3-Gal-1 (Paz et al., 2001), Galectin-1	I
pcDNA3-asGal-1 (Paz et al., 2001), Galectin-1 knockdown	I
pmCherry-H-rasG12V	I, II
pmCherry-K-rasG12V	I, II
pmGFP-wt-H-ras, wild-type H-ras	I
mCFP and mCitrine-CTK or K-ras-NANOPS, C-terminal hypervariable region of human K-ras	II

mGFP-LactC2, mRFP-LactC2 (gift from J. Hancock)	II
PTRF-Flag (Hill et al., 2008), Polymerase I And Transcript Release Factor	II
Cav1-HA (Hill et al., 2008), Caveolin-1 or Cav-1	II
pN_Src16_mCit-N1 and pN_Src16_mCFP-N1 or Src-NANOMS, N-terminus from human c-Src	III
pN_mutSrc16_mCit-N1 and pN_mutSrc16_mCFP-N1 or mutant Src-NANOMS, glycine 2 mutated to alanine in N-terminus of c-Src	III
pN_Yes17_mCit-N1 and pN_Yes17_mCFP-N1 or Yes-NANOMS, N-terminus from Yes1	III
pN_mut Yes17_mCit-N1 and pN_mutYes17_mCFP-N1, mutated Glycine 2 to Alanine and Cysteine 3 to Serine in N-terminus of Yes1	III
pN_Gi2.mCit-N1 and pN_Gi2.mCFP-N1 or Gi2-NANOMS (Abankwa & Vogel, 2007)	III
pmCherry-H-rasG12V and pmCherry-K-rasG12V (Constructed by replacing pmGFP from the pmGFP-H-rasG12V and pmGFP-K-rasG12V plasmids with pmCherry from the pmCherry-C1 vector (Clontech Laboratories Inc., CA, USA).	I, II
Yes- and Src-NANOMS (Constructed by adding specific N-terminal membrane targeting regions to the N-termini of the pmCit-N1 or pmCFP-N1 vectors)	III

siRNA

Name (manufacturer)	**Publication**
H-ras (Life technologies)	I
NMT1	III
NMT2	III

RESULTS AND DISCUSSION

1 Cellular FRET-biosensors as a screening platform to identify nanoclustering and lipid modification inhibitors (I-III)

Ras controls signaling pathways important for cell growth, differentiation, and survival. Ras has been shown to initiate cancers and the continued expression of Ras is necessary for tumor maintenance, as shown in model organisms. This has paved the way for targeting Ras, and there is significant amount of evidence to suggest that therapeutic targeting of Ras is a viable strategy. However, so far the attempts to find a safe drug to inhibit Ras activity have failed. Nanoclustering of proteins has emerged as a novel anti-cancer target as drugs targeting nanoclusters can hamper their activation and signaling. As a part of my thesis project I set out to identify new inhibitors of Ras-nanoclustering by using a FRET-based screening approach. The following chapter describes the results from screening different chemical libraries (I-III). The functional validation and characterization of the hits from these screens is described in chapters 2 and 3 of Results and Discussion.

1.1 Ras-derived FRET biosensors report on nanoclustering and membrane anchorage (I-II)

We have previously reported the application of cytometric FRET measurements for detecting nanoclustering and lipid modification of membrane bound proteins (Abankwa and Vogel, 2007; Najumudeen et al., 2015). In this method, lipid anchors of membrane proteins are genetically fused to the fluorescent proteins mCFP (donor) and mCit (acceptor), and expressed in mammalian cells. We exploit the fact that these lipid-modified proteins in cellular membranes organize into nanoclusters and display high FRET, which is measured using a BD LSRII flow cytometer equipped with a 96-well high-throughput sampler. The FRET measured on a two-dimensional membrane or on biological membranes depends on the ratio of donor-acceptor fluorophores and the concentration of these fluorophores. In our cytometer-based method, FRET is measured in a high throughput fashion by adapting the sensitized acceptor-emission method (Gordon et al., 1998). In this method, at a constant donor-acceptor ratio of 1:1, the FRET efficiency increases with the increase in the acceptor expression levels, towards a maximal FRET value termed FRET E_{max} (Abankwa and Vogel, 2007). This E_{max} is characteristic for different membrane bound proteins and it can be used to report on functional membrane targeting and nanoclustering.

H- and K-ras FRET-biosensors report on the functional membrane anchorage and nanoclustering of H- and K-ras, respectively. These biosensors are designed with the C-terminal hypervariable region (HVR) of H- and K-ras, genetically fused to the fluorescent proteins mCFP and mCit, and named as H- and K-ras-NANOPS

(Nanoclustering and Prenylation Sensor), respectively (Abankwa et al., 2008; Köhnke et al., 2012). The HVR has been used as a surrogate marker to report on the nanoclustering of the full-length proteins (Abankwa et al., 2008; Köhnke et al., 2012; Rotblat et al., 2010). The Ras-NANOPS undergoes the same post-translational modifications (i.e., farnesylation and/or palmitoylation) in the C-terminus like the full-length protein (Figure 5). Thus, they can also report on the steady-state activity of prenyltransferases and acyltransferases in the cell. The close packing of the prenylated Ras into nanoclusters on the membrane is measured as FRET E_{max}. A reduction in this FRET value corresponds to either loss of membrane anchorage due to inhibition of the protein prenylation machinery, or due to inhibition of nanoclustering of the Ras-NANOPS. This makes our Ras-NANOPS FRET-assay a highly sensitive and robust system to search for chemical modulators of Ras lipid modification, membrane localization and nanoclustering (Figure 13).

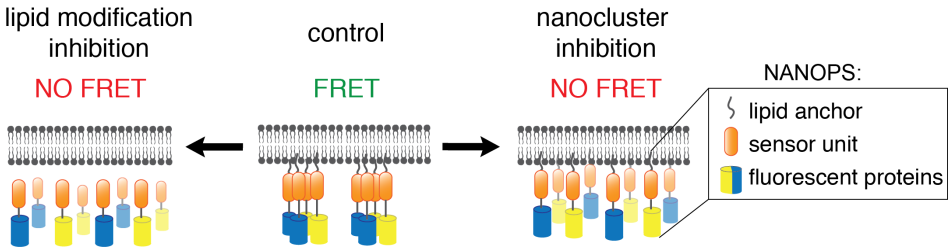

Figure 13. Schematic representation of the nanoclustering-based FRET biosensors. *Centre,* Tight packing of membrane anchors with fluorescently tagged donor (mCFP)- and acceptor (mCit)-fluorophores (blue and yellow cylinders, respectively) in nanocluster leads to FRET. *Left,* FRET can decrease due to loss of membrane anchorage due to changes in lipid modification and subcellular distribution. *Right,* FRET can decrease due to inhibition of nanoclustering of lipid-modified proteins.

1.2 Small molecule chemical screen with H-ras-NANOPS identifies H-ras nanocluster modulators (I)

In order to identify novel chemical modulators of H-ras nanoclustering, we performed a chemical screen of 659 small-molecules from two chemical libraries (Enzo Natural Product library (Enzo Life Science Inc.) and a chemically diverse in-house natural compound collection). The compounds were used at an average concentration of 10 µM on BHK cells expressing the H-ras NANOPS (I: Figure 1A). A good dynamic range for the screens was observed with qualitative parameters such as a Z' value of 0.6 and coefficient of variation (% CV) of 5%. By setting the hit selection criteria or hit limit at >15% change in FRET from the average we identified 38 primary hits (I: Figure 1B). These hits were filtered out of auto-fluorescent molecules and then cross-validated in a human cell line (HEK293). Results obtained from both HEK and BHK cells showed a good correlation (I: Supplementary Figure 1a). We confirmed twelve hits after cross-validation (I: Figure1C), with six compounds that decreased (negative deviators) the FRET E_{max}. Surprisingly, we also

identified six hits that increased (positive deviators) the FRET E_{max}. The negative deviators mevastatin, dihydrotanshinone (DHTS), dihydroxyacetophenone, tubericidin, b-lapachone and A23187 reduced the E_{max} of H-ras-NANOPS. The loss of FRET with the Ras-NANOPS could for most part be attributed to either loss of membrane anchorage due to inhibition of the protein prenylation machinery, or to inhibition of nanoclustering of H-ras-NANOPS on their cognate membranes (Köhnke et al., 2012). Not surprisingly, two of these negative deviators – mevastatin and DHTS - have already been identified to inhibit farnesyl transferases. These two inhibitors block farnesylation and thereby inhibit the membrane anchorage of H-ras-NANOPS, leading to the loss of FRET (Figure 13).

Mevastatin - found in the early 1970s from microbial metabolites- was 'a blockbuster drug' approved for clinical use for treating hypercholesterolemia (Clendening and Penn, 2012). Mevastatin is a well-established hydroxymethylglutaryl coenzyme A reductase (HMGCR) inhibitor (Brown et al., 1978; Endo et al., 1976). HMGCR is the rate-limiting enzyme of the mevalonate pathway that is crucial for generation of cholesterol and other lipid precursors. It has also been observed that statin-use reduced the risk of cancers in many patients (Nielsen et al., 2012). Now, it is well established that some anticancer properties of statins are related to the inhibition of the mevalonate pathway. With respect to Ras signaling, the key isoprenoid intermediates necessary for Ras lipid modification – farnesyl pyrophosphate and geranylgeranyl pyrophosphate - are products of the mevalonate pathway. As Ras and other small GTPases require isoprenylation for proper membrane anchorage, loss of HMGCR activity impairs Ras function. Although a number of clinical trials have been conducted to test the efficacy of statins in anticancer therapy, it is now clear that the statin anticancer efficacy is dependent on many factors including the cancer tissue type and type of statin used. For example, lipophilic statins such as fluvastatin, are potent against breast cancers, whereas the hydrophilic pravastatin is active against hepatic cancers (Clendening and Penn, 2012). However, statins in combination with approved anticancer agents have shown variable results in Ras-mutated cells. A recent study showed that Atorvastatin-treatment can overcome gefitinib (EGFR inhibitor) resistance of K-ras mutated non-small cell lung carcinoma cells both in vivo and in vitro (Chen et al., 2013). A drug combinations study from National Cancer Institute suggested that statins in drug combinations with B-Raf therapeutics were sensitive towards K-ras mutant melanomas (Held et al., 2013). Statin therapy has also been suggested to have therapeutic benefit for thyroid cancers (Bifulco, 2008). On the other hand, statin therapy for patients with colorectal cancers treated with Cetuximab (EGFR inhibitor) showed no improvement in patient survival (Krens et al., 2014). It is noteworthy that lovastatin was found to specifically inhibit leukemia stem cells (LSCs) while sparing normal hematopoietic stem and progenitor cells (HSPCs) via inhibition of HMGCR (Hartwell et al., 2013). The authors concluded that the anti-LSC effect of lovastatin could be independent of Ras function.

Dihydrotanshinone (DHTS) – a tanshinone isolated from *Salvia miltiorrhiza* –is another known FTase inhibitor (Lee et al., 1998). DHTS has been reported to have FTase inhibitory activity with an *in vitro* assay using purified FTase from Rat brain (Lee et al., 1998). Nevertheless, this activity of DHTS had not yet been reported in cells. Our identification of DHTS as a negative regulator of H-ras-NANOPS corroborates the original finding from Lee et al., and suggests that DHTS could have potent FTI activity also in cells. Even though the mechanism of action of DHTS on FTase is unknown, it is interesting to speculate that DHTS may have a similar mechanism of action as nonpeptidomimetic FTIs, such as tipifarnib and lonafarnib. Since DHTS-like plant-derived FTIs have been found to inhibit the binding of farnesyl diphosphate to the FTase (Khan et al., 2010; Shaikenov et al., 2001). In addition, a number of other molecular targets have been reported for the action of DHTS, such as activation of the apoptosis cascade, inhibition of hypoxia-inducible factor-1 (HIF-1) and inhibition of topoisomerase I (Chen et al., 2014). DHTS has been shown to have anti-cancer properties with dose and time-dependent inhibition of cancer cell growth, invasion and migration (Dong et al., 2011). These data suggest that the anti-cancer potential of DHTS could be a combination of these activities, which warrants further characterization. However, the identification of these known Ras FTase inhibitors validates the sensitivity of the assay and the possibility to identify novel Ras lipid modification and membrane trafficking inhibitors. Furthermore, it highlights the potential of the Ras-NANOPS screening assay to identify not only pharmacologically relevant inhibitors, but also compounds that can help us better understand Ras functioning.

A surprising finding from this screen was the identification of 6 compounds that increased the FRET E_{max} of H-ras-NANOPS (I: Figure 1C). Intriguingly, 5 out of these 6 compounds were structurally divergent protein synthesis inhibitors (PSI). The effect of protein synthesis inhibition on Ras is not known. The functional characterization and validations of these positive deviators is discussed in chapter 2 of Results and Discussion.

1.3 Differential screen with H- and K-ras-NANOPS identifies novel CSC inhibitors (II)

Cancer stem cells (CSC) possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor. These CSCs are believed to contribute to anti-cancer therapy resistance and relapse after therapy (Kreso and Dick, 2014). However, given the importance of Ras signaling in cancers, the role of Ras in CSC is not well understood. Our laboratory had previously utilized the Ras-NANOPS assay described above to identify macrotetrolides as potential Ras nanocluster inhibitors (Köhnke et al., 2012). Macrotetrolides are ionophoric antibiotics that can insert into cellular membranes and alter the membrane organization. Here, we postulated that such inhibitors or compounds that affect

membrane organization could have potential nanocluster inhibitor properties. We therefore made a small collection of 13 known ionophoric compounds (II: Supplementary Table 1). We performed a differential screen with these compounds using two Ras-derived biosensors H-ras-NANOPS (Köhnke et al., 2012) and K-ras-NANOPS (K-ras4B derived) (Abankwa et al., 2008) expressed in mammalian cells (II: Figure 1B). From this chemical screen, we identified three hit compounds that specifically altered the nanoclustering associated FRET E_{max} of K-ras but not H-ras-NANOPS (II: Figure 1C). These hit compounds; salinomycin, nigericin and lasalocid were used for further validation. Salinomycin and nigericin are potent K^+ ionophores that are commonly used in poultry for their antibiotic and anticoccidal (against intestinal parasites) activities. Nonetheless we found that other compounds in the library with similar ionophoric activity do not affect K-ras-NANOPS in the same manner, suggesting a mechanism of action that does not involve the ionophoric activity. Interestingly, salinomycin and nigericin have already been reported by Weinberg and colleagues to target breast CSCs (Gupta et al., 2009). This has lead to repurposing these inhibitors as potential anti-cancer agents; however, their mechanism of action is not well understood. Our efforts to understand the K-ras specific activity of these inhibitors are described and further discussed in detail in chapter 3 of Results and Discussion.

In addition to the above screen, we performed a small molecule screen of ~400 diverse microbial metabolites and related semi-synthetics from the MST library, to identify novel K-ras specific nanocluster inhibitors. We screened these metabolites using both H-ras-NANOPS and K-ras-NANOPS expressed in BHK cells (II: Supplementary Figure 5a). With a hit selection of >10% change in FRET from the average, we identified 39 primary hits (II: Supplementary Table 5). In accordance with the previously published Ras targeting compounds (Köhnke et al., 2012), we identified nactins/macrotetrolides as H-ras-NANOPS specific hits. We also found the recently described K-ras localization inhibitors antimycin and neoantimycin (Salim et al., 2014). In addition, we also confirmed that the tool compound staurosporine (STS), and a number of STS analogues, oxostaurospoine, stauprimide and UCN-01 that affect phosphatidylserine (PS) and PS/Ras clustering (Cho et al., 2012b). The identification of these already reported inhibitors, together with established HMG-CoA inhibitors, such as lovastatin and simvastatin further corroborates and highlights the potential of our Ras-NANOPS screening platform. Among the primary hits, we also found avermectin and its derivative ivermectin to specifically affect K-ras-NANOPS. Interestingly, Weinberg and colleagues had previously identified avermectin as Abamectin (a mixture of avermectin B1a and B1b) to target breast CSCs (Gupta et al., 2009). From these screening efforts we identified conglobatin A and ophiobolin A as two potential CSC inhibitor candidates. The functional validation, characterization and anti-CSC property of these compounds are discussed in detail in chapter 3 of Results and Discussion.

1.4 Myr-NANOMS report on membrane targeting of N-myristoylated proteins (III)

The essential role of Ras nanoclustering in cell signaling and cancers is now well demonstrated (Solman et al., 2015; Tian et al., 2007; Zhou and Hancock, 2015). It is postulated that nanoclustering might be a more common, or even the dominant feature of plasma membrane protein organization (Garcia-Parajo et al., 2014). Similar to Ras proteins, Rho and heterotrimeric G proteins have been shown to laterally segregate into distinct membrane nanodomains (Abankwa and Vogel, 2007; Crouthamel et al., 2010). Lipid modifications of these proteins are critical for their anchoring to the membrane and nanoclustering. In addition to farnesylation and palmitoylation, many membrane proteins are myristoylated by N-myristoyl transferases (NMT) (Selvakumar et al., 2007). N-myristoylation is the irreversible co-translational addition of a myristoyl group to the N–terminal glycine by NMT, which is preceded by proteolysis of the N-terminal methionine by methionine aminopeptidases (Met-AP1 and 2) (Wright et al., 2010). Some of the well-characterized myristoylated proteins include the oncogenic Src family kinases, Gα proteins and apoptosis regulator BID (Selvakumar et al., 2007; Wright et al., 2010).

Despite the importance of myristoylated proteins in cancer, there is a lack of suitable assays to identify specific myristoylation inhibitors. Using the principle of the Ras-NANOPS FRET assay, we developed FRET-biosensors to detect nanoclustering and membrane anchorage of myristoylated proteins. To design biosensors that report on the functional membrane anchorage of myristoylated proteins, we utilized the N-terminal membrane targeting sequences of three myristoylated proteins: heterotrimeric G protein subunit $G\alpha_{i2}$, Yes- and Src-kinases. Similar to the Ras-biosensors, these sequences were fluorescently tagged and were named $G\alpha_{i2}$, Yes- or Src- NANOMS (NANOclustering and Myristoylation Sensors) (III: Figure 1). When expressed in cells, these NANOMS become lipid modified and display high FRET. A comparison of the FRET values of the three NANOMS in BHK cells revealed that Yes-NANOMS have a high FRET E_{max} of >0.4, followed by Src- (~0.3) and by Gi2-NANOMS (~0.2), indicating comparatively strong nanoclustering of Yes-NANOMS (III: Figure 2a). Treatment of Yes-NANOMS expressing cells with the N-myristoyl transferase (NMT) inhibitor DDD85646 (Frearson et al., 2010) resulted in the cytoplasmic redistribution of the biosensor and in a significant loss of FRET E_{max} (III: Figure 2a). This value was similar to the E_{max} values of a mutant non-myristoylatable Yes-biosensor, where myristoylated glycine is replaced by alanine (III: Figure 2a). Consistent with the Yes-NANOMS, the other biosensors Src- and Gi2 also showed a significant decrease in FRET E_{max} values after treatment with DDD85646 (III: Figure 2a). To confirm the specificity of the biosensor response to NMT inhibitors, we tested FTI and statins that did not induce a response (III: Supplementary Figure 3a). Furthermore, weaker NMT inhibitors such as myristoleic acid (MA) and Tris (dibenzylideneacetone) dipalladium (TDP) or palmitoylation inhibitors had little or no effect on the biosensors in BHK or HEK cells (III: Supplementary Figure 2b, c, 3b-d). We further validated that the Yes- and

Gi2-NANOMS report on NMT by siRNA mediated knockdown of the human NMT1 and NMT2, which lead to a significant loss of E_{max} for both biosensors (III: Figure 3). These data confirm that NANOMS respond potently to chemical and genetic loss of NMT or inhibition of myristoylation, and it is a suitable system to monitor myristoylation in cells.

Next we explored whether the Yes-NANOMS is a suitable system for chemical screening to identify novel membrane targeting inhibitors of myristoylated proteins. To this end, we collected compounds that are chemically similar to the previously reported Met-AP inhibitors fumagillin and bengamide and derivatives (III: Figure 4a) and tested their efficacy with Yes-NANOMS expressed in BHK cells. These inhibitors have been successfully used to block membrane anchorage and activity of myristoylated proteins. The chemical screen with Yes-NANOMS had a good dynamic range with a Z'-score of 0.60 and DDD85646 as a positive control. From this screen we identified fumagillin, homoserine lactones and reveromycin B to reduce the FRET E_{max} values. Consistent with its effect against Met-AP2, only fumagillin significantly decreased the E_{max} (III: Figure 4b).

1.5 Conclusions and future perspectives: FRET-biosensors are valuable tools for screening molecules that alter membrane targeting and nanoclustering (I-III)

In conclusion, the data on the FRET-biosensors designed and employed above indicate that they are a suitable platform for screening chemical libraries (I: Figure 1; II: Figure 1). The FRET-biosensor assay integrates the essential features of most high-content imaging based screening approaches that is subcellular localization (Cho et al., 2012b) and it is customizable to be done at high-speeds needed for large screens. Compared to imaging methods, this method allows monitoring of the full expression range of the biosensors on cells and combines the subcellular distribution of membrane anchors into a single, quantitative FRET-parameter E_{max}. Our screening results with Ras-NANOPS (I-II) clearly demonstrate the capacity of the NANOPS-assay to identify novel Ras nanoclustering and signaling inhibitors. Furthermore, the identification of already known FTase inhibitors, HMG-CoA inhibitors (mevastatin and simvastatin), together with Ras localization inhibitors such as STS and analogues, further corroborates the accuracy and reproducibility, and highlights the potential of the relatively simple Ras-NANOPS screening platform. Our results with the Myr-NANOMS (III) show that the FRET-biosensor assay principle could be applied for other lipid-modified membrane proteins. For instance, we have previously developed Rab-NANOPS to provide first evidence that Rab GTPases also form nanoclusters (Köhnke et al., 2012; Najumudeen et al., 2015). These Rab-NANOPS have also been utilized to identify novel RabGGTase inhibitors that inhibit Rab membrane localization (Coxon et al., 2014). The assay can be adapted for other cell lines with high expression levels for the biosensors to measure E_{max} (II: Figure 1). As future perspectives, the Ras-NANOPS screening platform has

the potential for application in genetic screening or RNAi-library applications. Our screening results here show the advantage of using both H- and K-ras-NANOPS for performing differential RNAi screens in detecting isoform-specific upstream modulators of H- and K-ras. Both these biosensors respond to known and novel nanocluster modulators in a similar manner as the full-length Ras proteins. In the context of genetic screens, our preliminary results with H-ras-NANOPS show that RNAi mediated knockdown of Galectin-1 can specifically disrupt H-ras-NANOPS (unpublished results), which is consistent with the role of Gal-1 as a H-ras nanocluster scaffold. Such observations, and the critical role of nanocluster modulators in MAPK signaling, suggest that the NANOPS-assay has a strong potential for future application in RNAi-screening.

2 Protein synthesis inhibitors increase H-ras nanoclustering and tumor growth (I)

2.1 Protein synthesis inhibitors enhance H-ras but not K-ras nanoclusters

From the chemical screen with H-ras-NANOPS, along with the negative deviators discussed above, we identified six compounds – cycloheximide, anisomycin, harringtonine, emetine dihydrochloride, diacetoxyscirpenol and actinomycin D - that positively up regulated H-ras nanoclustering (I: Figure 1 C). Intriguingly, five out of these six compounds were structurally divergent protein synthesis inhibitors (PSI). A table of known activities and targets of these PSIs is shown in (I: Supplementary Table 3). Many of these PSIs are routinely used as tool compounds in cell biological research to block protein translation initiation, elongation and RNA synthesis (I: Supplementary Table 4). Moreover, these inhibitors affect cell signaling and have been reported to induce neuronal differentiation (Greenberg et al., 1986; Grollman, 1967; Louis et al., 1994). Nevertheless, a Ras isoform specific effect of protein synthesis inhibition is not known; therefore, we decided to further understand the specific effect of PSIs on Ras nanoclustering using cycloheximide (CHX) as a representative PSI.

We examined the effect of CHX on the nanoscale organization of Ras using electron microscopy (EM). This method allows for high-resolution quantification of Ras on the membrane and enables the direct visualization of the distribution of mGFP-tagged Ras on intact apical plasma membrane sheets using EM combined with spatial mapping (Prior et al., 2003). BHK cells transiently expressing mGFP-tagged constitutively active H-rasG12V, wild-type H-ras and K-rasG12V were treated with CHX for 24 h and their membrane sheets were labeled using anti-GFP 5 nm gold nanoparticles and imaged using EM. This EM spatial analysis showed that CHX significantly increases the nanoclustering of both constitutively active and wild-type full length H-ras (I: Figure 2A). However, CHX does not affect the nanoscale spatial

distribution of K-rasG12V (I: Figure 3A). These EM data indicate that the effect of CHX is H-ras isoform specific.

We next validated the EM results using FLIM-FRET measurements between mGFP- and mCherry tagged H-rasG12V and K-rasG12V. The FRET emerging due to the dense packing of mGFP- and mCherry-tagged Ras into nanoclusters is used as readout for nanoclustering. Cells expressing these fluorescent FRET-pairs were treated with CHX and the changes in fluorescence lifetimes were expressed as apparent FRET efficiency (%). These FRET measurements show that CHX treatment at both high (10 μM) and low concentrations (0.18 μM) significantly increased H-ras nanoclustering (I: Figure 2B). Moreover, this effect was comparable to Gal-1 overexpression, which was used as a positive control for H-ras nanoclustering (I: Figure 2B). Similarly to CHX, two other PSI (anisomycin and harringtonine) also significantly increased H-ras nanoclustering (I: Supplementary Figure 3A). However, nanoclustering of K-rasG12V was not affected by CHX treatments (I: Figure 3B). We found that Gal-1 overexpression could have a negative effect on K-ras nanoclustering (I: Figure 3B) indicating a Gal-1 independent effect of CHX on K-ras.

We next investigated CHX effects on H-ras nanoclustering using FRAP (Fluorescence recovery after photobleaching). FRAP is used to the measure lateral diffusion and mobility of fluorescently tagged Ras proteins (Guzmán et al., 2014a). It is known that at steady state, 40% of Ras proteins on the plasma membrane exist as immobile clusters (Zhou and Hancock, 2015). Gal-1 overexpression has previously been shown to specifically stabilize cholesterol-independent H-ras nanoclusters (Prior et al., 2003) and to increase the immobile fraction of H-rasG12V (Rotblat et al., 2010). An increase in the immobile fraction of Ras quantified using FRAP therefore corresponds to an increase in Ras being immobilized in nanoclusters. Consistent with EM and FLIM-FRET data, CHX significantly increased the immobile fraction of H-rasG12V (I: Figure 2C). This increase is at a similar and comparable level to that of Gal-1 overexpression (I: Figure 2C). Taken together, EM immunogold spatial mapping, FLIM-FRET and FRAP data firmly establish that CHX specifically increases H-ras nanoclusters and has no effect on K-ras nanoclusters.

It is surprising to find small molecules that increase Ras nanoclustering from screens for nanocluster inhibitors. However, this is an important observation from a signaling standpoint, as recent studies have shown that Raf-inhibitors could lead to 'paradoxical activation' of Raf. This Raf activation leads to increased proliferation and in some cases induce tumorigenesis *in vivo* of Ras-mutated cells (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). In fact, these Raf inhibitors have been shown to increase K- and N-ras nanoclustering and signaling, but not H-ras nanoclustering (Cho et al., 2012a). Therefore, characterization of inhibitors that specifically activate H-ras warrants further investigation. Notably, H-ras is mutated

in cancers of the skin, bladder and salivary glands (Table 1) and it also been implicated in differentiation of adipocytes and neuronal cells (Bar-Sagi and Feramisco, 1985; Qui and Green, 1992). Moreover, active H-ras has been shown to specifically mediate F9 stem cell differentiation leading to cessation of proliferation (Quinlan et al., 2008; Yamaguchi-Iwai et al., 1990). However, the role of wild-type H-ras in stem cells is not known. Gal-1 also positively and specifically regulates H-ras nanoclustering (Belanis et al., 2008; Paz et al., 2001; Rotblat et al., 2010). Our results here show that Gal-1 overexpression has a negative effect on K-rasG12V nanoclustering. This could be attributed to the phosphatidylserine (PS) mediated negative impact that H-ras on K-ras nanoclustering, as H-ras can sequester plasma membrane PS and consequently affect K-ras nanoclustering (Zhou et al., 2014). This negative effect would be therefore further increased by Gal-1 overexpression.

It is well known that cholesterol plays an important role in the proper lateral segregation of H-ras nanoclusters (Prior et al., 2003). H-ras-GDP forms cholesterol dependent nanoclusters, whereas H-ras-GTP is cholesterol independent. Drugs that affect this segregation such as indomethacin and aspirin (non-steroidal anti inflammatory drugs) have been shown to increase H-ras-WT but decrease H-rasG12V nanoclustering (Zhou et al., 2010; 2012). However, our results with EM clearly show that CHX treatment specifically increases nanoclustering of H-ras-WT, H-rasG12V and tH (a surrogate marker for H-ras-GDP nanoclusters), but not K-rasG12V. This suggests that the CHX effects on H-ras nanoclustering are very likely cholesterol independent and H-ras specific. However, changes in other lipids that affect nanoclustering cannot be ruled out. On the other hand, it could be reasoned that the increase in nanoclustering of H-ras found with CHX could be due to loss of H-ras depalmitoylation. It has been shown earlier that CHX inhibits FKBP12 stimulated H-ras depalmitoylation (Ahearn et al., 2011). This loss of FKBP12 activity corresponds to an increase in H-ras membrane residence and signaling. Considering the role of palmitoylation on H-ras nanoclustering (Roy et al., 2005), it is plausible that the increase in H-ras nanoclustering seen with CHX could be due to increased palmitoylated H-ras on the PM. However, all other PSIs found in our screen are structurally diverse and have different mechanism of action for protein synthesis inhibition (I: Supplementary Table 4). Moreover, we show that blocking protein translation (I: Supplementary Figure 2) is the common denominator of these compounds that increase H-ras nanoclustering (I: Supplementary Figure 3a). These results suggest that protein translation inhibition through a yet unknown mechanism feedbacks specifically towards H-ras nanoclustering.

2.2 Increased H-ras nanoclustering drives differentiation and tumor growth (II)

Ras activation and nanoclustering is transmitted as downstream signaling through the activation of downstream effectors. Active GTP-bound Ras nanoclusters on the plasma membrane act as signaling platforms to facilitate effector recruitment and

activation. This Ras-effector interaction reports directly on the Ras nanocluster function and rate of effector recruitment from cytoplasm to the membrane bound Ras (Tian et al., 2007). To investigate whether the induction of H-ras nanoclustering by CHX correlates with the recruitment of downstream effectors, we used our well-established effector-recruitment FLIM-FRET assay to analyze the interaction of mGFP-tagged Ras and mRFP-tagged RBD of c-Raf in BHK cells treated with CHX (Abankwa et al., 2010; Guzmán et al., 2014b). Using this assay we could show that CHX treatment significantly increases effector recruitment of H-rasG12V but not of K-rasG12V (I: Figure 4A and B). This is in line with our EM and nanoclustering-FLIM data indicating that only H-ras nanocluster mediated signaling is affected by CHX. Since effector recruitment initiates the MAPK signaling cascade, we performed western blot analysis of the MAPK cascade and found that CHX and other PSIs such as anisomycin and harringtonine significantly increased ppErk and pAkt signaling in BHK cells (I: Figure 4B, Supplementary Figure 3). Nevertheless, it could be contended that the increase in MAPK signaling is unassociated to increased H-ras activity. To corroborate the H-ras specificity, we compared ppErk and pAkt levels on MEFs and their Rasless counterparts that lack H-ras$^{-/-}$ and N-ras$^{-/-}$ (Drosten et al., 2010). Indeed, CHX failed to activate ppErk and pAkt signaling in Rasless MEFs, whereas Rasless MEFs transiently expressing H-ras-WT show a significant increase in ppErk and pAkt signaling. Collectively these signaling data indicate that H-ras has a distinct role in CHX- and PSI-mediated increase in MAPK signaling.

However, increase in MAPK (ppErk) signaling is mostly, but not always, associated with increased cellular proliferation and tumorigenicity. Oncogenic Ras is known to induce morphological differentiation of pheochromocytoma (PC12) cells by inducing neurite formation (Bar-Sagi and Feramisco, 1985). Ras activity can induce PC12 cell differentiation independent of growth factor stimulation and this makes the PC12 differentiation assay one of the widely used model system for MAPK activity (Bar-Sagi and Feramisco, 1985). We used the PC12 differentiation assay to investigate whether CHX induced H-ras activation increases differentiation. Interestingly, previous studies have reported protein synthesis inhibitors to induce differentiation in primary culture of neurons from the central nervous system (Louis et al., 1994) but the mechanism of action has been unclear. Expression of H- and K-rasG12V induces differentiation of PC12 cells, as seen by neurite formation, and the addition of CHX significantly increased the percentage of differentiated cells expressing H-rasG12V (I: Figure 4C) but not K-rasG12V (I: Figure 4C). This is concordant with our results that CHX augments H-ras nanoclustering and signaling, leading to differentiation.

We then examined whether CHX induced H-ras nanoclustering and signaling would also increase tumorigenicity or tumor growth in a H-ras dependent manner. To determine the CHX induced tumorigenicity, we tested CHX on tumorsphere formation. The tumorsphere or mammosphere assay is a standard clonogenic assay for the detection of self-renewal and tumorigenic capacity of cancer stem cells (CSC)

that are enriched in the non-adherent spheres (Clarke et al., 2006; Dontu et al., 2003). MDA-MB-231 breast cancer cells are naturally enriched for CSCs and form mammospheres in non-adherent conditions. Compared to treatment with chemotherapeutic like doxorubicin or CSC inhibitor salinomycin that decreased tumorsphere formation, CHX treatment significantly increased the number of spheres suggesting that CHX increases the tumorigenicity of these cells (I: Figure 5B). Since CHX increases the number of tumorspheres, we questioned whether this is H-ras dependent. Surprisingly, siRNA mediated loss of H-ras together with CHX treatment significantly reduces the number of spheres (I: Figure 5C). These data suggest that H-ras is essential for CHX mediated action and that depletion of H-ras fully reverses the response compared to control treated cells. This corroborates the idea that H-ras exclusively effects CHX mediated Ras signaling activation. To further validate the increase in mammospheres, we utilized an *in ovo* tumor growth assay as a surrogate xenograft model. In this assay, MDA-MB-231 cells are grown on the chorion allantois membrane (CAM) of a chicken embryo and treated with CHX. Concurrent with the mammosphere data, CHX treatment significantly increased the tumor mass as compared to the control (I: Figure 5D).

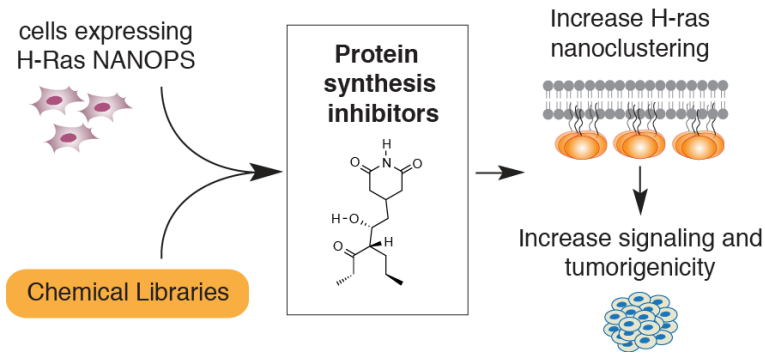

Figure 14. Schematic abstract showing protein synthesis inhibitors increase H-ras nanoclustering and induce tumor growth (Najumudeen et al., 2015).

Protein synthesis, is the most energy-consuming process in the cell and it is frequently dysregulated in cancers. Results from this study show PSIs such as CHX could specifically increase H-ras nanoclustering, signaling and drive tumor growth (Figure 14). These results are reminiscent of the recently identified paradoxical Raf activation by Raf inhibitors (Cho et al., 2012a; Poulikakos et al., 2010). As with Raf inhibitors, protein synthesis inhibitors might have these effects only under certain cellular or genetic context, as seen with the mammospheres derived MDA-MB-231 cells. These cells are K-ras mutated and enriched in CSC-like populations with increased drug resistance and self-renewal capacity (Kreso and Dick, 2014). Little is known about the role of the different Ras isoforms and their associated signaling in CSC. Others and we (II) have provided evidence for the divergent role of Ras isoforms in CSC. Interestingly, our results here show CHX treatment increases H-ras signaling, mammospheres and tumor growth. However, depletion of H-ras and

CHX treatment leads to synthetic lethality in the K-ras mutant MDA-MB-231 mammospheres. Others have shown that loss of wild-type H-ras and N-ras can sensitize K-ras mutant cells to DNA damaging agents (Grabocka et al., 2014). Our data indicate the significance of PSI and similar inhibitors as tool compounds to investigate synthetic lethality.

Protein metabolism is controlled and maintained by the rates of protein synthesis and degradation in cells. Previous reports have indicated the half-live of endogenous Ras proteins to be ~9-12 hours under CHX (Kim et al., 2009). However, the observed changes in H-ras nanoclustering by PSIs are unlikely to be related to changes in Ras stability or de novo Ras synthesis, since our results with Rasless MEFs overexpressing wild-type H-ras show no significant changes in pan Ras levels after 24 hour CHX treatment (I: Figure 5a). This suggests that CHX and PSI induced increase in H-ras nanoclustering is unrelated to changes in the steady-state Ras levels. Activation of the Ras/Erk or Ras/PI3K/Akt pathway activates mechanisitic target of rapamycin (mTOR). mTOR is activated by different cues such as amino acids, insulin and growth factors. mTOR controls various cellular processes and protein synthesis is one of the well-characterized effect of mTORC1. mTORC1 controls lipid and protein synthesis, which is required for cell growth, proliferation and survival (Laplante and Sabatini, 2009). For instance, mTORC1 to a large extent controls lipid biogenesis to generate membranes for proliferating cells, by activating SREBP1/2 (sterol regulatory element-binding protein 1) transcription factors that control genes involved in lipid and cholesterol synthesis. Deregulation of mTORC1 leads to reduced SREBP levels that disrupts expression of genes required for lipogenesis (Laplante and Sabatini, 2012b). Membrane lipids such as phosphatidylserine and cholesterol play an essential role in proper lateral segregation of Ras nanoclusters and changing the compositon on the plasma membrane has been shown to affect Ras nanoclustering (Cho et al., 2012b; Prior et al., 2003; Zhou et al., 2014). It is plausible that changes in protein synthesis rates and mTOR activity could alter the plasma membrane lipid profile and thereby affect Ras nanoclustering.

Activation of the Ras/Erk or Ras/PI3K/Akt pathway activates mTORC1 that phosphorylates eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and the p70 ribosomal S6 kinase 1 (S6K1). Phosphorylation of 4E-BP1 leads to activation of eIF4E, which leads to cap-dependent translation. Activation of S6K1 leads to translation initiation and elongation (Laplante and Sabatini, 2012a). Phosphorylated eIF4E is associated with oncogenic signaling (Hsieh and Ruggero, 2010). Previous studies and our unpublished results show that blocking protein synthesis results in hyperphosphorylation of S6K1 (Krieg et al., 1988). It is known that oncogenic stress-induced cellular senescence (OIS) stops overactive MAPK signaling. OIS is a permanent cell growth arrest caused by an activated oncogene in primary untransformed cells and it acts as an *in vivo* tumor suppression mechanism (Adams, 2009). However, it is likely that non-oncogene induced stresses like high

intracellular amino acid levels, deregulation of mTOR could elevate S6K1 or eIF4E levels and bypass OIS. It has already been shown that ectopic expression of eIF4E in primary HMECs could bypass senescence (Avdulov et al., 2004), this together with a cooperating second-hit induces neoplastic-like properties in immortalized HMECs (Larsson et al., 2007). In fact, our unpublished data suggest that signaling stresses such as increased intracellular amino acid levels (as seen with PSI treatment) or inhibition of components downstream of the mTORC1 complex can specifically increase H-ras nanoclustering and signaling. How this specific feedback with H-ras is achieved is not yet understood. Interestingly, recent reports have indicated an association between the translation machinery and MAPK signaling in cancer drug resistance. Several mechanisms of resistance towards therapies targeting the MAPK or PI3K pathways are known to converge on mRNA translation (Lito et al., 2013; Silvera et al., 2010). One such mechanism involves the formation of the eIF4F complex (Boussemart et al., 2014). Boussemart et al. show that an increase in eIF4F complex-formation significantly associated with the resistance to anti-B-Raf, anti-Mek, and combination therapy. However, inhibition or loss of eIF4F complex synergises with B-Raf inhibition to act on cancer cells. A comprehensive understanding of the coupling between the Ras pathway and the protein synthesis machinery could create novel drug targeting opportunities.

3 Cancer stem cell inhibitors target K-ras signaling in a stemness context (II)

3.1 CSC inhibitors affect K-ras nanoscale organization and signaling (II)

The differential screen with H- and K-ras-NANOPS identified three hit compounds that specifically altered the nanoclustering associated FRET E_{max} of K-ras-NANOPS (II: Figure 1C). The hit compounds: salinomycin, nigericin and lasalocid were selected for further analysis. To understand the K-ras specific mechanism of these inhibitors, we performed further validation with the constitutively active full length K- or H-rasG12V. We used the consitutively active full-length Ras isoforms for further validation as nanoclustering of active Ras is most relevant in the context of signaling output (Tian et al., 2007). The isoform-specific nanoclustering of H- and K-ras was quantified using FRET from FLIM-FRET measurements between mGFP- and m-Cherry tagged K- or H-rasG12V. BHK cells transiently overexpressing the FRET-pairs treated with the compounds showed that all three hit compounds significantly reduced nanoclustering FRET of K-rasG12V, but nigericin also affected H-rasG12V nanoclustering (II: Figure 2A). This indicated that some of these compounds reduced the nanoclustering-FRET in a K-ras specific manner. This loss of FRET observed from the nanoclustering-FRET assays could be attributed to either loss of membrane anchorage or inhibition of K-ras nanoclustering on the membrane

(II: Figure 1A). To check whether this reduced nanoclustering-FRET is due to changes in subcellular Ras localization, we did confocal imaging with MDCK cells stably expressing and BHK cells transiently expressing H- or K-rasG12V treated with the hit compounds. This revealed that all compounds induced a loss of plasma membrane localization of both H- and K-ras (II: Figure 2D, Supplementary Figure 1A). From these observations one can extrapolate that these hit compounds induce changes to Ras localization and nanoscale membrane organization.

To understand the implications of the reduced nanoclustering and plasma membrane localization by the hit compounds we followed the downstream signaling events. Ras nanoclusters act as signaling platforms to facilitate effector recruitment and activation. Using our well-established Ras-effector recruitment FRET assay between fluorescently tagged Ras and RBD of C-Raf in cells, we quantified that all three hit compounds significantly reduced the RBD recruitment to K-rasG12V (II: Figure 3A) measured as loss of FRET; and they had no effect on the FRET between RBD and H-rasG12V (II: Figure 3B). This data is in line with our nanoclustering-FLIM data indicating that all the hit compounds affect K-ras nanoclustering and RBD recruitment. Since effector activation initiates the MAPK cascade, we measured the downstream signaling output of BHK cells transiently overexpressing K- and H-rasG12V and treated with the hit compounds. In accordance with the K-ras specific activity of the compounds, we observed a significant reduction in downstream MAPK signaling in K-rasG12V (II: Figure 3C) and not in H-rasG12V (II: Figure 3D) transfected cells. This decrease in K-ras mediated downstream MAPK signaling is reflected as significant reduction in proliferation of BHK cells by salinomycin and nigericin (II: Figure 3E). Using Ras-transformed 3T3 cells we could show that K-ras transformed cells were more sensitive to salinomycin treatment than H-ras transformed cells (unpublished data). These results are consistent with previously reported activity of salinomycin and nigericin in arresting cell growth and proliferation. However, our results indicate a previously unknown specificity of these inhibitors towards K-ras activity.

Taken together our data show that the CSC inhibitor salinomycin most specifically disrupts K-ras nanoclustering and effectively reduces MAPK signaling and proliferation. This is in agreement with the fact that K-ras is involved in stem-cell self-renewal (Quinlan et al., 2008). With respect to CSCs, salinomycin has been shown to be active against many different CSC types (Naujokat and Laufer, 2013), however the exact molecular target has not been determined. There is accumulating evidence showing the importance of K-ras in stem cells; early studies on colonic epithelial cells of mice with activated K-rasG12D expressed are locked in a poorly differentiated state and express stem-cell markers. This results in widespread hyperplasia (no benign adenomas), and it cooperates with loss of *Apc* to induce adenocarcinomas (Haigis et al., 2008). However, expression of N-rasG12D in these mice does not induce hyperplasia or cooperates with loss of *Apc* (Haigis et al., 2008). More recent reports show that K-rasG12D expression in Lgr5+ stem cells leads to

clonal expansion and competition with the normal crypt cells (Snippert et al., 2013); yet, these mice do not develop neoplasias. Thus suggesting different roles for K- and N-ras in colon cancers. Recent work from Kevin Haigis and colleagues shows that similar to N-rasG12D in hematopoietic stem cells (Li et al., 2013), K-rasG12D has a bimodal activity on quiescent intestinal stem cells (Gierut et al., 2015). Bimodal activity refers to the ability of mutant Ras to increase the proliferation kinetics of one subset of stem cells, and increase the self-renewal potential of another subset (Gierut et al., 2015). In this context, it would be interesting to know if this 'bimodal' response of Ras activation is specific to the Ras family of genes or also found in other oncogenic mutations.

3.2 Salinomycin targets K-ras and PS nanoscale organization (II)

It is well known that lipid composition of the plasma membrane is critical for Ras membrane anchorage and clustering (Zhou et al., 2014). Specifically, the negatively charged phosphatidylserine (PS), which is a common constituent of all Ras nanoclusters was shown to be an essential structural component of K-ras nanoclusters (Zhou et al., 2014). Recently, staurosporine (STS) was shown to strongly redistribute PS from the plasma membranes affecting K-ras nanoclustering and localization (Cho et al., 2012b). Furthermore, it was reported that Fendiline - a FDA approved calcium channel blocker - reduces K-ras4B nanoclustering by mislocalizing K-ras from the plasma membrane. Though, the exact molecular mechanism is not currently known, it is shown to be independent of its calcium channel blocking activity (van der Hoeven et al., 2013). The activity of these inhibitors is reminiscent to that of our hit compounds. These studies also indicate that changing the lipid composition of the plasma membrane affects Ras nanoclustering and localization and this leads to changes in MAPK signaling and can have biological implications (Zhou and Hancock, 2015). PS interacts with the polybasic region of K-ras and it is required for the structural stability of K-ras nanoclusters (Plowman et al., 2008; Zhou et al., 2014). Using the fluorescently tagged C2 domain of lactadherin (LactC2) as probe for subcellular PS, we examined the PS nanoscale organization and coclustering with Ras using FLIM-FRET. All the hit compounds and STS significantly reduced PS nanoclustering-FRET (II: Supplementary Figure 1B). However, our unpublished data show that salinomycin and nigericin do not induce a clearly predominant intracellular pool of PS, as seen after STS treatment (Cho et al., 2012b), suggesting that the Ras mislocalization by these inhibitors is not a direct consequence of loss of PS from the plasma membrane. Interestingly, only salinomycin specifically reduced PS-K-rasG12V coclustering FRET, while the other compounds affected PS coclustering to both H- and K-rasG12V. These data collectively suggest that the hit compounds perturb Ras-nanoscale organization by decreasing PS-clustering and salinomycin specifically affects K-ras/PS coclustering. This specific effect on K-ras is significant; as it has been shown that H-rasG12V can negatively affect assembly and segregation of K-ras nanoclusters remotely via PS (Zhou et al., 2014).

Moreover, PS is known to be enriched particularly in sites abundant in caveolae (Fairn et al., 2011). In the context of Ras nanoclustering and plasma membrane lipid composition, Ariotti et al., recently proposed that caveolae can remotely control Ras nanoclustering and signaling (Ariotti et al., 2014). They show that loss of caveolae results in an increase in PS-clustering and as a consequence leads to increase in K-ras nanoclustering and signaling, while H-ras remains unaffected (Figure 15) (Ariotti et al., 2014).

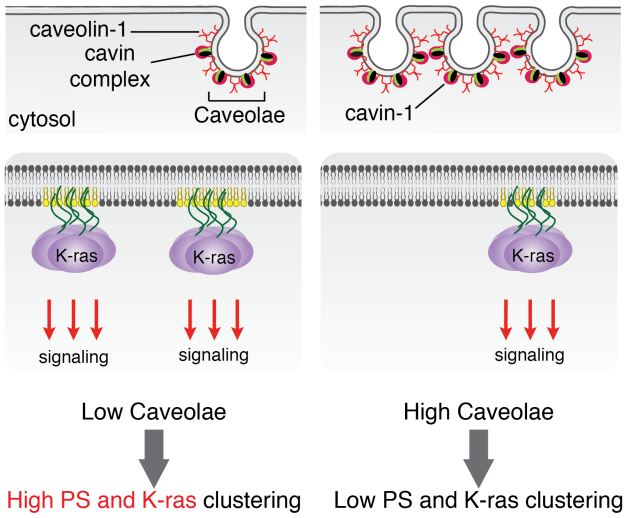

Figure 15. Schematic representation of the model of K-ras activity modulation by presence or absence of caveolae, based on work from Ariotti et al., 2014. *Left,* Absence or loss of caveolae increases the plasma membrane PS (in yellow) clustering. Consequently, this increase in PS clustering increases K-ras clustering and signaling. *Right,* Increase in caveolae levels leads to PS enrichment in caveolae that lowers PS and K-ras clustering.

It is also shown that PS binds to all four cavin members *in vitro* (Bastiani and Parton, 2010; Fairn et al., 2011; Hill et al., 2008). While trying to understand the cell type dependent activities of salinomycin and other hits using Ras-NANOPS, we identified that the hit compounds lost activity in HEK cells. We therefore focused on the possible role of caveolae in this context, as the quantitative comparison of caveolae with STED super resolution microscopy suggested that HEK cells have a significantly lower caveolae density than BHK cells (II: Figure 4A, B). Transient overexpression of the caveolae structural proteins Cav1 and PTRF together in HEK cells significantly increased the caveolae density, close to the same level as BHK cells (II: Figure 4A, B). Consistent with Ariotti et al., HEK cells reexpressing the caveolar proteins showed significantly reduced K-rasG12V nanoclustering-FRET and RBD recruitment (II: Figure 4C, E). This would be attributed to the enrichment of PS in the caveolae that negatively affects K-ras nanoclustering (Figure 15). Furthermore, salinomycin significantly reduced the K-rasG12V nanoclustering and RBD recruitment in HEK cells reexpressing the caveolar proteins; whereas H-rasG12V

nanoclustering and RBD recruitment were unaffected by caveolar reexpression or inhibitor treatments (II: Figure 4D, F). Consistent with the above results, Cav1 and PTRF expression also desensitized HEK but not BHK cells to salinomycin (Figure 4G, H). Taken together our results show that caveolae reexpression in HEK cells can restore salinomycin activity towards K-ras and it can differentiate between caveolae expression levels.

The proper lateral segregation of the Ras isoforms is maintained within a very narrow PS concentration range (Zhou et al., 2014). Therefore, increasing caveolae density sequesters the mobile PS from the plasma membrane, resulting in lower PS levels in the membrane and therefore an overall reduction in K-ras nanoclustering. This is reflected as reduced salinomycin sensitivity, as there is less PS and K-ras for salinomycin activity. This is similar to the effect of activated H-ras that negatively regulates PS and thereby K-ras nanoclustering (II: Figure 3E). Thus, one could speculate that the PS-mediated K-ras inhibitory activity of H-ras could also affect cell sensitivity and cell fate. The most important implication from the above results is that caveolae can modulate the sensitivity of cells in certain contexts to the CSC drug salinomycin. Interestingly, low caveolae and Cav-1 levels have been implicated in stem cells and differentiation (Baker and Tuan, 2013). Studies with Cav-1 null mice show that loss of Cav-1 leads to an increased adult mammary stem cell population and high expression of stem cell markers Sca-1, keratin-5 and keratin-6 (Sotgia et al., 2005). The same group also provided evidence showing that Cav-1 loss leads to hyper-proliferation and expansion of intestinal crypt stem cells, with a simultaneous upregulation of β-catenin (Li et al., 2005). In addition, Cav-1 deletion has recently been shown to impair hematopoietic stem cell self-renewal and differentiation (Bai et al., 2014). Interestinly, a recent study from Lawson et al., utilizing a single-cell gene expression analysis identified Cav-2 expression to be specifically repressed in low-burden metastatic cells (Lawson et al., 2015). Consistent with these studies, loss of Cav-1 has been associated with poorer prognosis (Witkiewicz et al., 2009). A similar role has been associated with PTRF in prostate cancer (Nassar et al., 2013b). These studies support a more critical role of caveolae (via PS) in cancer and stem cells. In accordance with these studies, our results show that caveolae play a significant role in the sensitivity of cancer cells to the CSC drug salinomycin.

3.3 K-ras nanoclustering associated gene signature predicts salinomycin sensitivity and patient survival (II)

In addition to the K-ras specificity of salinomycin and the differences in caveolae levels between BHK and HEK cells, we found that the lack of caveolae – that leads to free-PS and high dependence on K-ras signaling – makes the HEK cells almost 8-fold more sensitive to salinomycin (IC_{50} – 0.63 μM) than BHK cells (IC_{50} – 4.9 μM). Therefore, it seems very likely that there is a relationship between salinomycin

potency and components of K-ras nanoclustering. Along with caveolae, there are a number of other known proteins that impact K-ras nanoscale organization and signaling (Review of literature 3.3, II: Supplementary Table 2). We therefore analyzed the available gene expression patterns of H-, K- and N-ras isoforms, six known nanoclustering modulators and EGFR (II: Supplementary Table 2) in embryonal stem cells (ESCs) from the stem cell database ESTOOLS (Kong et al., 2013). Interestingly, this analysis revealed that the expression of these genes in ESCs is basically inversed to the expression in fibroblasts. Moreover, we could find three distinct sets of ESC expression signatures that are characteristic of naïve and primed ESC, and those in transit between these two states (II: Supplementary Figure 3a).

We expanded the initial ten gene set by searching ESTOOLS for co-regulated genes (II: Methods for details). From this analysis, we identified vimentin (VIM), caveolin-2 (CAV-2) and integrin α5 (ITGA5) to be among the co-regulated genes. Vimentin is an intermediate filament protein that is a well-known EMT marker (Thompson et al, 1992). During EMT epithelial cells undergo complex reprogramming to lose epithelial markers and gain expression of mesenchymal markers like N-cadherin and vimentin among others. Vimentin expression is specifically associated with mesenchymal stem cells and in the invasive metastatic properties of cancer cells (Ye et al., 2015). Cav-2 is a caveolin isoform that is expressed together with Cav-1 in a wide range of tissues (Bastiani and Parton, 2010). Importantly, Cav-2 positively regulates Cav-1 mediated caveolae formation, suggesting that Cav-2 plays a regulatory role in caveolae dynamics (Kirkham et al., 2008; Mora et al., 1999; Sowa et al., 2003). Integrin α5 is implicated in cancer and its expression is correlated with cancer progression in many tissue types (Seguin et al., 2015). Integrin α5 forms heterodimers with β1 subunit to mediate adhesion to the extracellular matrix protein fibronectin. It is upregulated in various cancer types and it is crucial for angiogenesis. These studies suggested that vimentin, cav-2 and ITGA5 to be the most plausible genes to be associated with K-ras and stemness regulation. Interestingly, unsupervised clustering of these genes was consistent with high K-ras expression in ESC and low in differentiated cells (II: Figure 5a). This motivated us to check whether salinomycin targets K-ras in cancer cells with a gene expression similar to ESCs. If this were true, it is predicted that cells that respond to salinomycin would be resistant to standard chemotherapeutics (Gupta et al., 2009). To derive cancer cell lines that have an ESC-like or fibroblast-like signature, we correlated the expression of these 13 genes predominantly associated with K-ras nanoclustering (hereafter K-ras-nanoclustering signature) in cancer cell lines, ESCs and fibroblasts. From this we identified a panel of cancer cell lines that have a K-ras nanoclustering signature either more like ESCs or fibroblasts (II: Figure 5b).

We then tested this panel of cancer cell lines for sensitivity to the CSC inhibitor salinomycin compared to the conventional chemotherapeutic Staurosporine (STS). Consistent with our prediction, cancer cell lines that have an ESC-like signature showed higher sensitivity to salinomycin (II: Figure 5c), whereas the fibroblast-like

cells were more sensitive to STS (II: Figure 5d). Correlating the drug response profiles with the gene expression further confirmed that responsiveness to salinomycin positively correlated with K-ras expression and negatively with caveolae genes (II: Figure 5e). Standard chemotherapeutic STS mostly showed opposite association. Taken together these data suggest that our K-ras-nanoclustering-signature could be used to predict cancer cell lines responsiveness to CSC inhibitor salinomycin.

Though the idea of CSC is still contentious, the evidences from others and our data here indicate that a K-ras centric mechanism in the context of CSCs is very plausible. Several reports have shown that EMT induction can generate breast cancer cells with CSC-like characteristics (Ansieau, 2013; Gupta et al., 2009; Mani et al., 2008). EMT induction has also been linked to drug-resistance (Singh and Settleman, 2010). Recently, mutant K-ras has been implicated to promote mesenchymal features in basal-breast cancers through EMT. Moreover, K-ras is shown to be preferentially activated in basal-breast cancer cells compared to luminal-breast cancer cells (Kim et al., 2015). Therefore, it is very likely that our K-ras centric mechanism would also be relevant in the context of CSC and CSC characteristics.

Besides, other reports suggest that some K-ras mutated cells can be independent on K-ras signaling, while some K-ras wild-type cells are K-ras dependent and there is a differential K-ras dependency in K-ras mutated cancer cells. These reports also suggest that gene expression signature of the Ras pathway is a better predictor of Ras dependency and signaling compared to K-ras mutation status (Loboda et al., 2010; Singh et al., 2009). These studies further support the notion that a measure of all Ras-associated signaling is a better measure of Ras dependent signaling compared to just Ras mutation statuses. In this context, our K-ras nanoclustering gene signature integrates information transmitted upstream of K-ras nanoclustering and activation, and correlates with drug sensitivity, and it is able to predict response to inhibitors targeting K-ras in CSCs.

Interestingly, almost 8% (605 / 7536) of all the patient tumor samples in the The Cancer Genome Atlas (TCGA) database have an ESC-like K-ras-nanoclustering signature that, according to our data, would be sensitive to CSC inhibitors. This signature was associated with poor overall survival of patients with this fraction of tumors (II: Figure 5f). Interestingly, this poor patient survival was more significant using ESC-like cancer cell line expression data. This confirms our assumption that tumors with the K-ras-nanoclustering-signature contain a high number of CSCs or CSC-like cells. This might be of particular importance to stratification of these patients that have low overall survival and would be high responders to salinomycin and other CSC inhibitors. Moreover, analysis of the actual tumor types that had the ESC-like signature revealed an enrichment of this signature in cancers of the female reproductive tissue such as breast invasive carcinoma, ovarian serous cystadenocarcinoma and uterine corpus endometrial carcinoma (II: Supplementary

Table 4). This was followed by a relatively high enrichment of ESC-like signature in acute myeloid leukemia. This is significant as our K-ras-nanoclustering signature is conserved across a number of tumor types, including tumors that generally have low Ras mutation frequencies, such as breast cancer. The identification of breast cancer enrichment is in line with the original observation by Weinberg and colleagues who identified salinomycin by using transformed mammary cells (Gupta et al., 2009). The enrichment of AML cancer types is consistent with the reported activity of salinomycin against AML SCs (Fuchs et al., 2009). Our data shows that salinomycin targets CSC with low caveolae, and this could be the reason behind the general toxicity observed with salinomycin. However, the understanding of such CSC susceptibilities would be of great importance for future cancer therapy.

3.4 Ophiobolin A is a novel candidate CSC inhibitor (II)

Salinomycin has gathered lot of research interest due to its strong activity against CSCs (Naujokat et al., 2010). However, the pleiotropic effects of salinomycin have hindered its application in the clinic. To demonstrate the suitability of our K-ras-nanoclustering focused screening platform to identify novel CSC inhibitors. Among the primary hits, we identified previously reported CSC inhibitor avermectin and its derivative ivermectin (Gupta et al., 2009) and stauprimide a highly selective inducer of ESC differentiation (Zhu et al., 2009) to have activity towards K-ras FRET-NANOPS. The identification of these compounds further support the link between K-ras and stemness.

From the primary hits, we identified five substance classes that were largely represented (leptomycins, avermectins, ophiobolins, conglobatin and streptonigrin) with some selectivity towards K-ras-NANOPS (II: Supplementary Table 5). One representative compound from each class was taken for further validation, giving a total of seven new compounds that were analyzed as potential CSC inhibitors (II: Supplementary Table 6). We next validated these inhibitors using FLIM-FRET measurements between mGFP- and mCherry tagged K- or H-rasG12V. These FLIM-FRET analyses show that all compounds significantly reduced K-rasG12V nanoclustering-FRET (II: Figure 6a), however only leptomycin and streptonigrin also affected H-ras nanoclustering-FRET (II: Figure 6b). We then analyzed whether these compounds affected the Ras effector-recruitment through our effector-recruitment FRET-assay. Using this analysis we found that all the compounds significantly reduced K-rasG12V-RBD recruitment FRET, but not H-rasG12V-RBD (II: Supplementary Figure 5b). The reduction in K-ras nanoclustering quantified in these FRET assays could be also due to perturbation of Ras membrane anchorage. To check whether the hit compounds induce changes in subcellular Ras localization, we did confocal imaging with MDCK cells stably expressing H- or K-rasG12V and treated with the hit compounds. These imaging experiments revealed that these hit componds do not affect H- and K-ras subcellular distribution (II: Supplementary

Figure 5c).

We then analyzed the anti-CSC potential of these compounds in breast CSC using the well-studied $CD44^+/CD24^-$ enriched CSC population found in breast cancer cell lines. We used flow cytometry to analyze the $CD44^+/CD24^-$ CSC-population of MDA-MB-231 breast cancer cells treated with the inhibitors. Using this analysis, we established that conglobatin A and ophiobolin A significantly reduced the $CD44^+/CD24^-$ population, similarly to salinomycin (II: Figure 6C, D). Testing of the other ophiobolin analogues ophiobolin B and C on $CD44^+/CD24^-$ populations showed that ophiobolin A and B are highly active and dose dependently reduced the $CD44^+/CD24^-$ cells, whereas ophiobolin C is inactive. Structural comparison of these ophiobolin analogues suggested that the oxygen 13 is required for the activity of ophiobolin A and B (II: Supplementary Figure 6B).

To conclude, from this second screen we have identified two compounds that specifically affect K-ras-nanoclustering and signaling, which facilitated the identification of two new potential CSC inhibitors. The MST library used in the second screen consists of a diverse collection of compounds with antibiotic, antifungal, nematocidal and anti-tumor properties. It is interesting to note that some of the hits from this second screen such as leptomycin and staurosporine analogues have already been reported to be highly toxic against mammalian cells. However, our results also show that metabolites with little or no toxicity such as avermectins and conglobatin are active against K-ras nanoclustering. This suggests that toxicity *per se* is not sufficient for a compound to be found as active in the screen.

Our results also show that of all the validated hits, Ophiobolin A is found to be the most effective breast CSC inhibitor. These results are supported by the specific effect of ophiobolin A on K-ras nanoclustering and effector recruitment. Corroborating the effect on $CD44^+/CD24^-$ breast CSCs, our unpublished data show that ophiobolin A treatment significantly affects the self-renewal and tumorgenicity of breast CSCs (MDA-MB-231) measured using mammospheres assay. Our data also show that Ophiobolin A dose-dependently reduces the mammosphere formation in other breast cancer cells with high CSC populations (data not shown). Concurrent with this effect on CSCs, ophiobolin A is less potent against breast cancer cells (like MCF7) that have a low CSC population (data not shown).

Although at this moment the exact mechanisms underlying the elimination of CSCs by ophiobolin A are poorly understood, our results above show that one potential mechanism could be the perturbation of K-ras nanoclustering and signaling. It is also evident that the effects of ophiobolins on K-ras are not due to changes in the subcellular localization or distribution. This suggests that ophiobolin could be targeting a K-ras specific interaction. Interestingly, previous studies have shown that ophiobolin A is a strong calmodulin (CaM) antagonist that covalently binds calmodulin on lysines on position 75 or 77 and 148 thus inhibiting CaM activity (Au

et al., 2000; Kong Au and Chow Leung, 1998). The Lys-75 was shown to be the primary inhibitory site that is responsible for all the inhibitory activity of ophiobolin A binding. The Lys-75 CaM mutant is partially resistant to ophiobolin A, whereas mutation on all three Lys renders the CaM completely resistant to ophiobolin A (Kong Au and Chow Leung, 1998). Other reports have shown ophiobolin A to induce apoptosis in mouse leukemia cells and inhibit growth of human cancer cells (Au et al., 2000; Bencsik et al., 2014). Recently, ophiobolin A was shown to induce cell death in glioblastoma cells (Bury et al., 2013). However, the precise mechanism of action on CaM inhibition and the action on mammalian cancer cells has remained unclear. In the context of Ras signaling, CaM is already shown to specifically bind to active K-ras4B and not H- or N-ras (Villalonga et al., 2001). Moreover, it has been shown that the HVR of K-ras provides the major CaM binding site, but both the HVR and catalytic domain are involved in CaM binding affinity (Abraham et al., 2009). The binding of CaM to K-ras4B is shown to be mutually exclusive with the S181 phosphorylation of K-ras4B and this modulates oncogenic and non-oncogenic K-ras activity and functions (Alvarez-Moya et al., 2010; 2011). Therefore, it is likely that ophiobolin A exhibits its anti-CSC properties through inactivation of CaM, leading to a deregulated K-ras nanoclustering and signaling. In fact, we have preliminary experimental evidence showing that loss of CaM by siRNA can lead to loss of tumorspheres or CSCs. The exact molecular mechanism behind these observations and the relationship to K-ras still needs to be worked out. Nevertheless, these observations are consistent to the very recent report from the McCormick group that proposed blocking the K-ras/CaM interaction as a novel approach to specifically interfere with K-ras stemness signaling in cancer cells (Wang et al., 2015). The results above could lead to a better understanding of the mode of action of ophiobolin in CSC.

3.5 Future perspectives

Ras proteins play a critical role in normal cell cycle, growth and differentiation. They cluster into nanoscale complexes on the plasma membrane called nanoclusters. Nanoclustering of oncogenic Ras proteins has emerged as a novel therapeutic target. The results obtained from this thesis suggest that nanoclusters indeed could be a potential avenue to find novel drugs that modulate activity of oncogenic proteins. There is now a growing understanding that not all Ras isoforms are created equal and that the different isoforms have distinct roles and function in normal and cancer cells. Consequently, strategies targeting Ras need to be specific for the different isoforms. The work presented in this thesis suggests that a nanocluster-directed differential screening approach could identify compounds that are able to specifically modulate the distinct Ras isoforms. In the context of K-ras, our identification of previously known CSC drugs and potentially novel CSC inhibitors from a small library of microbial metabolites suggests that natural products and metabolites could be rich sources of similar compounds. Although the clinical relevance of these compounds is currently unclear, they can be chemically modified

to generate analogues with specific activity towards CSCs that are well tolerated. Moreover, several compounds and natural products are being repurposed and clinically tested for activity against CSCs. However, the goal of future cancer therapy should be to eradicate all cancer cells in a patient, including CSCs. Similarly to cancer cells, CSCs, may also show various cell specific mutations. Since the CSCs represent a heterogeneous population, finding a "silver bullet" that kills all CSCs might prove difficult. CSCs that are not targeted by a particular CSC-directed therapy might escape, and subsequently therapy-resistant CSC clones might arise. Therefore, finding new molecular pathways and gene signatures to successfully target CSCs is important. The finding of gene signatures similar to our K-ras nanoclustering associated gene signature would be of clinical relevance in the selection of patients that would respond maximally to a specific CSC-directed therapy.

As cancer therapy becomes more individualized, it also underlines the need for better mechanistic understanding of molecular drivers and molecular mechanisms involved in cancer. For instance, the reason for high frequency of K-ras mutations compared to N- and H-ras mutations in various cancer types is still not resolved. Several studies argue for different reasons behind this. One of the reasons for this preponderance of K-ras mutations is attributed to the unique property of K-ras in bestowing cells with stem cell properties. The work presented in this thesis and work from others suggests that K-ras, indeed, plays an important role in CSCs. The advances in genetically engineered mouse models now offer ways to tissue-specifically delete genes. These models could offer new insights into the role of K-ras in adult stem cells in tissues such as stomach and colon. What is the K-ras dependency of these stem cells *in vivo* and what would be the fate of these cells if they were to lose the K-ras allele? These are some of the questions that need to be answered.

CONCLUDING REMARKS

Since their discovery three decades ago, Ras proteins have been widely studied and implicated in human cancers, but therapeutic targeting of Ras remained elusive. When I began my PhD thesis project, efforts to therapeutically target Ras in cancers were making a coming back. This was largely due to the developments made in the past decade on computer modeling, crystallography and novel ways of screening for drug compounds. In addition, recent years have enabled a better understanding of the complexity in Ras subcellular regulation and function. The combination of this and afore mentioned tools have now renewed the hope of finding a safe drug to curb Ras activity. It has become clear in recent years that not all Ras isoforms are created equal. Particularly, studies pointing to the important role of K-ras in conferring stem cell properties had started to accumulate, however, how this is regulated is not fully understood. It was now widely accepted that the Ras proteins form nanoscale-signaling complexes called nanoclusters on the plasma membrane that dictate downstream signaling. The work from others and us has confirmed the importance of Ras nanoclustering in cancers, and nanoclusters have emerged as potential anti-cancer drug targets. In this thesis, a FRET-based small molecule screening approach is used for finding novel chemical modulators of Ras nanoclustering. This screening method was previously shown to identify potential nanocluster inhibitors. In the first study, we utilized this method to screen for H-ras specific nanocluster modulator compounds and found that PSIs such as CHX and others, specifically induced H-ras nanoclustering, effector recruitment and downstream signaling. Interestingly, CHX also increased the CSC-enriched mammospheres and tumor growth of breast cancer cells. Depletion of H-ras and CHX treatment significantly reduced mammospheres, suggesting an exquisite synthetic lethality. These results provides insights into the possibility of identifying Ras-isoform specific modulators using nanocluster-directed screening, as we also show that PSI do not increase K-ras nanoclustering or signaling. In the second study, we used this nanocluster-directed differential screening approach to show that the CSC inhibitor salinomycin specifically inhibits K-ras nanocluster organization and this activity is sensitive to the presence of caveolae. We use this insight to derive a K-ras nanoclustering associated and stem cell derived gene signature that predicts the sensitivity of cancer cells to salinomycin. We show that 8% of samples in TCGA database with the above gene signature have a significantly higher mortality. Using this mechanistic insight, we identify ophiobolin A and conglobatin A to specifically inhibit K-ras nanoclustering and act as potential breast CSC inhibitors. The third study investigates the application of the above FRET-based assay to design biosensors that can report on the membrane anchorage of N-myristoylated proteins. We show that these biosensors can potentially report on inhibitors of membrane anchorage and nanoclustering of myristoylated proteins.

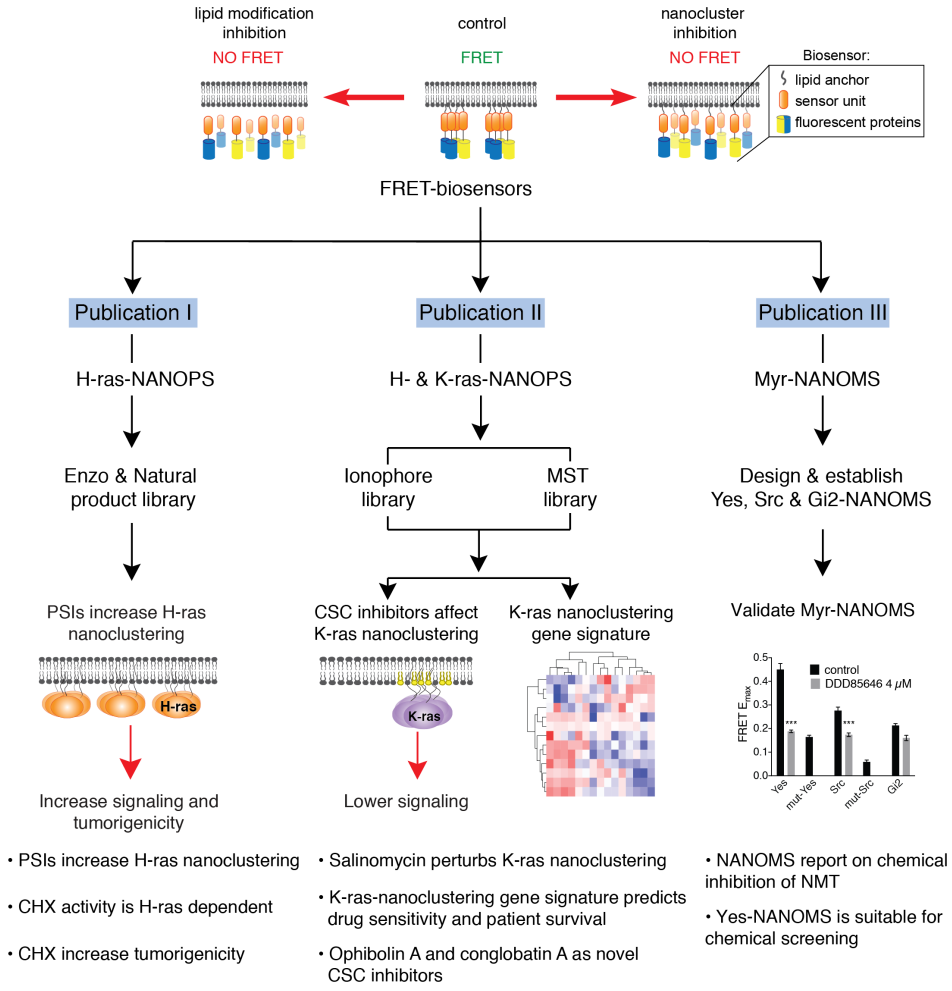

Figure 16. Schematic summary of all results presented in this thesis.

The results presented in this thesis and its original publications (Figure 16), provide valuable insights into targeting nanoclusters of different oncogenic proteins and the perturbation of nanoclustering as a tractable new therapeutic target. It gives new understanding to the role of PSI and CSC inhibitors on different Ras isoforms. The future years hold great promises and challenges for understanding and targeting Ras in the context of Ras isoforms, membrane architecture, cell type and tissues.

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Arafath Kaja Najumudeen

Targeting oncogenic signaling proteins

- new insights using a FRET-based chemical biology approach

This thesis describes a new approach to disrupting signaling of oncogenic proteins, specficially of Ras proteins. Ras is one of the major oncogenes, and it is mutated in 20% of all human cancers. Despite numerous efforts, targeting Ras directly in cancer has proven unsuccessful. We have taken a new approach to targeting Ras, namely targeting its localization and organization into nanoclusters in the cell membrane. By utilizing Förster resonance energy transfer (FRET)-based methods, we have screened chemical libraries to identify compounds that affect Ras nanoclustering. One of the main findings is that known cancer stem cell (CSC) inhibitors affect K-ras nanoclustering. We identify a K-ras-associated gene signature predicts response of cancer cells to CSC inhibitors. In addition, we were also able to identify novel compounds that target CSCs. The results give new insights into the role of Ras nanoclusters as promising new molecular targets in cancer and in stem cells.

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