Investigation of the mechanisms terminating growth factor induced Ras activation

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Akt	protein kinase B
BTK	bruton tyrosine kinase
Cpm	counts per minute
DAG	diacylglycerin
DH	dbl homology
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EM	electron microscopic
ER	endoplasmatic reticulum
Erk	extracellular-signal-regulated kinase
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GppNHp	guanosine-5'-[(β,γ)-imido]triphosphate
Grb2	growth factor receptor-bound protein 2
GST	*glutathione S-transferase*
GTP	guanosine triphosphate
hvr	hyper variable region
IP	Immunoprecipitation
IP3	inosit-1,4,5-trisphosphat
MAPK	mitogen-activated protein kinase
MEK	mitogen/extracellular signal-regulated kinase
mRNA	messenger RNA
MS	mass spectrometry
NF1	neurofibromin 1
NGF	nerve growth factor
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PKC	protein kinase C
PLC	phospholipase Cε
PLD1	phospholipase D1
PM	plasma membrane
PR	proline-rich
RA	Ras association
Ras	rat sarcoma
RasGRF	Ras guanine nucleotide releasing factor
RasGRP	Ras guanine nucleotide releasing protein
RBD	Ras binding domain
REM	Ras exchange motif
Rsk	ribosomal S6 kinase
RT	room temperature
RTK	receptor tyrosine kinase
SH2/3	src homology 2/3
siRNA	small interfering RNA
SLO	streptolysin O
Sos	son of sevenless
STAT	Signal Transducer and Activator of Transcription

4 Abstract

Transient Ras activation induced by growth factors is crucial for normal cell proliferation. Ras activity is regulated by opposing actions of two classes of Ras regulatory enzymes. Guanine nucleotide exchange factors (GEFs) promote GTP-bound Ras state by enhancing exchange of GDP with GTP. GTPase activating proteins (GAPs) enhance the otherwise slow intrinsic Ras GTPase activity rate, promoting conversion into the inactive GDP-bound state of Ras. The rise in RasGTP levels following exposure to growth factors is well understood. It proceeds via stimulation of Sos, a GEF which drives the accumulation of active, GTP-loaded Ras. The mechanism of the ensuing deactivation of Ras is not yet fully understood, but is inferred to involve feedback inhibition of Sos. This study focuses on understanding of how Ras signalling is terminated in the context of a mitogenic environment to limit and control Ras signalling in time.

An experimental strategy involving cell permeabilisation enabled the loading of cells with radioactive $[\alpha\text{-}^{32}P]GTP$ in order to monitor both the rate of $[\alpha\text{-}^{32}P]GTP$ uptake as well as the $[\alpha\text{-}^{32}P]GTP$ / $[\alpha\text{-}^{32}P]GDP$ ratio on Ras at the pre-steady-state. Combining both pieces of information allowed important conclusions on GEF activity and the contribution of GAPs. The downstream activation of canonical Ras-MAPK signalling pathway was analysed by Western Blotting.

It was observed that growth factors induce a characteristically transient Ras activation in HeLa cells. Strikingly, GEF activity is continuously high at all time points tested, even if RasGTP levels have dropped back to the basal state. Experiments with non-hydrolysable GTP analogues and mathematical modelling confirmed the presence of high GAP activity at late stages of growth factor stimulation. Finally we identified a crucial activation of the GAP protein NF1 to counteract GEF activity and to deactivate Ras. Furthermore, Rsk1/2 inhibition led to a sustained Ras activation whereas the GEF activity was not affected. Collectively, the findings emerged from this thesis identified a new molecular mechanism terminating growth factor induced Ras activation characterised by the involvement of Rsk and NF1 in a feedback loop. For the first time a biochemical stimulation of the GAP activity associated with growth factor signalling pathways was demonstrated. Secondly, the acquired data revealed a central role of the tumour suppressor NF1 as an executing target protein of a MAPK induced feedback pathway. Prospectively it is of medical importance to figure out the biochemical link between Rsk and NF1 activation in growth factor induced Ras deactivation to improve therapeutic strategies for patients with RASopathy syndromes.

5 Zusammenfassung

Transiente Ras-Aktivierung induziert durch Wachstumsfaktoren ist entscheidend für normale Zellproliferation. Ras-Aktivität wird durch entgegengesetzte Eigenschaften zweier Ras regulatorischer Enzyme kontrolliert. Die Guanin-Nukleotid-Austauschfaktoren (GEFs) fördern dabei den GTP-gebundenen Zustand von Ras durch den beschleunigten Austausch von GDP mit GTP am Ras-Molekül. Die GTPase-aktivierende Proteine (GAPs) erhöhen die geringe intrinsische GTPase Aktivitätsrate, welches den inaktiven GDP-gebundenen Zustand fördert. Bei der Ras-Aktivierung, welche durch ein rasch ansteigendes RasGTP Level nach mitogener Stimulation gekennzeichnet ist, handelt es sich um ein im Detail verstandenen Mechanismus. Die Stimulation von Sos, einem GEF, treibt dabei die Ansammlung von aktivem GTP-beladenem Ras an. Der Mechanismus der anschließenden Ras-Deaktivierung ist hingegen noch nicht vollständig aufgeklärt. Es wird jedoch vermutet, dass eine Rückkopplungshemmung auf Sos involviert ist. Im Rahmen meiner Arbeit galt es zu verstehen, wie das Ras-Signal nach mitogener Stimulation herunterreguliert wird, um die Aktivierung der Ras-Signalwege zeitlich zu begrenzen und zu kontrollieren.

Wir verwendeten eine Permeabilisierungstrategie, die es uns ermöglichte, Zellen mit radioaktivem [α-^{32}P] GTP zu laden, um erstens die Rate der [α-^{32}P] GTP-Aufnahme, und zweitens den [α-^{32}P] GTP/[α-^{32}P] GDP Quotienten von Ras im *pre-steady-state*, zu verfolgen. Die kombinierte Betrachtung der beiden Informationen erlaubt Rückschlüsse auf die GEF-Aktivität und den Beitrag der GAPs. Die Aktivität des kanonischen Ras-MAPK-Signalweges wurde mittels Western Blot analysiert.

Wir beobachteten, dass Wachstumsfaktoren eine charakteristische transiente Ras-Aktivierung in HeLa Zellen induzieren. Erstaunlicherweise war die GEF-Aktivität zu jeder Zeit konstant hoch, auch wenn die RasGTP Level bereits wieder auf Grundniveau zurückgegangen waren. Experimente mit nicht-hydrolysierbaren GTP-Analoga und mathematische Modellierung bestätigten und rationalisierten die Anwesenheit von hoher GAP-Aktivität in den späten Phasen der hormonellen Stimulation. Wir zeigten eine Aktivierung des GAP-Proteins NF1, um der GEF-Aktivität entgegenzuwirken und Ras zu deaktivieren. Darüber hinaus führte eine Rsk1/2 Hemmung zu einer anhaltenden Ras-Aktivierung, wobei die GEF-Aktivität jedoch nicht beeinflusst wurde. Zusammenfassend zeigen unsere Ergebnisse einen neuartigen molekularen Mechanismus zur Regulation des mitogen aktivierten, transienten Ras-Signals gekennzeichnet durch die Beteiligung von Rsk und NF1 innerhalb einer Rückkopplungsschleife.

Wir konnten somit erstmalig einerseits eine Stimulation der GAP Aktivität im Zusammenhang mit Wachstumsfaktorsignalwegen demonstrieren und dokumentieren. Andererseits enthüllen diese Daten eine zentrale Rolle des Tumorsuppressors NF1 als ausführendes Zielprotein von MAPK-induziertem Rückkopplungssignalweg. Prospektiv ist es von medizinischer Bedeutung die biochemische Verbindung zwischen Rsk und NF1-Aktivierung in Wachstumsfaktor-induzierter Ras Deaktivierung herauszufinden, um die Therapiestrategien für Patienten mit RASopathy Syndromen zu verbessern.

6 Introduction

6.1 Signal Transduction

Cells communicate with each other through extracellular signalling molecules or cell-cell contacts. In this manner cells coordinate physiological multi-cellular processes such as proliferation, differentiation, migration, survival, and apoptosis. Cytokines, growth factors, and hormones are so called chemical messengers and transmit messages between the cells. Not all molecules can pass through the lipid bilayer of a cell, and so signal transduction systems are used in order to relay an external signal to the cell interior.

Typically, cellular signalling cascades involve phosphorylation events through various kinases that lead to a large amplification of the signal and finally activate an effector or regulatory protein and consequently regulate cellular functions. These enzymatic cascades are tightly controlled in order to prevent disastrous consequences for proper cell functioning. The stimulating signal that initiates a cellular response routinely also initiates a mechanism for shutting down that response. For example, the activation of a kinase often triggers the activation of a corresponding phosphatase.

Its important to understand signalling networks since mutations in genes, which encode for proteins involved in signal transduction, are enriched in malignant cells and tumours. Generally, an extracellular signal activates many different signal transduction pathways that lead to a number of cellular responses, in this work we focus on the growth factor induced Ras signalling pathways.

6.2 Ras a small GTPase

Ras is a key regulator in various signal transduction pathways and ensures survival of the cell through its involvement in cellular proliferation, differentiation, apoptosis and cytoskeletal reorganisation (Hall, 1990a, Crespo and Leon, 2000).

Beside heterotrimeric G proteins, small GTPases are a type of G-Proteins. The Ras superfamily of small GTPases comprises over 150 human members and exhibit 5 subfamilies: Ras, Rho, Rab, Arf and Ran (Takai et al., 2001, Colicelli, 2004b, Wennerberg et al., 2005). All GTPases are molecular switches that alternate between an active GTP bound state and an inactive GDP bound state.

Arf family proteins play a role in vesicular transport (Nie et al., 2003) and members of the Rho family regulate organelle development, cytoskeletal dynamics and cellular movement (Burridge and Wennerberg, 2004). Rab GTPases regulate the intracellular vesicle transport (Stenmark and Olkkonen, 2001) whereas Ran proteins control nuclear transport systems (Dasso, 2006).

The best-studied members of the Ras GTPases are Harvey-Ras (H-Ras), Kirsten-Ras (K-Ras), and neuroblastoma-Ras (N-Ras). They have a molecular weight of 20 to 25 kDa and control various cellular functions such as cell proliferation, cell differentiation, and cell death (Shimizu et al., 1983, Colicelli, 2004a). The three Ras isoforms were firstly discovered as transforming oncogenes of murine sarcoma viruses (DeFeo et al., 1981) and later identified in eukaryotes from yeast to humans. All isoforms are highly related, sharing approximately 85% protein identity (Barbacid, 1987), whereas the major variability exist in the carboxyl terminus. Ras proteins are expressed ubiquitously in mammalian cells and display biologically significant differences despite their high degree of homology: K-Ras is essential for normal mouse embryogenesis (Johnson et al., 1997, Koera et al., 1997, Esteban et al., 2001), whereas H- and N-Ras double knockout mice were shown to be viable (Umanoff et al., 1995). Studies in so-called Rasless MEFs (carrying *null H-Ras* and *N-Ras* alleles along with a floxed *K-Ras*) illustrate that the Ras isoforms are essential for proliferation and migration, but not for survival. Further they showed that the activation of the Raf/MEK/Erk pathway but not the PI3K pathway was sufficient to sustain normal proliferation and migration in Rasless MEFs (Drosten et al., 2010). In this thesis, we will not differentiate between the Ras isoforms, so the term Ras will be used for all three Ras isoforms collectively.

6.2.1 *Ras a bimolecular switch*

Ras proteins are essential for cells to leave a quiescent state (G_0) as well to pass through the G_1/S transition of the cell cycle (Taylor and Shalloway, 1996, Peeper et al., 1997, Crespo and Leon, 2000). Thus, Ras proteins show two peaks of activation during the cell cycle. The first Ras activation during G1 phase progression is transient (Marshall, 1995a, Coleman et al., 2004) whereas the second Ras activation in cycling cells at mid-G1 is prolonged (Dobrowolski et al., 1994, Taylor and Shalloway, 1996). The latter is uncoupled from Erk activation and is dependent on RNA and protein synthesis (Taylor and Shalloway, 1996).

In conclusion, quiescent cells require at least two peaks of Ras activation in order to run through one round of cell division. Its still a debate if Ras targets other pathways in mid-G1 phase to push the progression into S phase, since functional links for Ras and the retinoblastoma tumour-suppressor protein (Rb) for G1 exiting has been suggested. In the present thesis we will only pay attention on the first short-lived Ras activation peak.

The sequential steps for a Ras cycling between the active GTP-bound and inactive GDP-bound state are: 1) GTP binding, 2) GTP hydrolysis, 3) phosphate release, and 4) GDP release. Thereby the rate-limiting step for Ras protein activation is the exchange of bound GDP with GTP. In the late 80s several groups measured the rate constants for the nucleotide dissociation step and in most cases this is a very slow step (around 4×10^{-4} s^{-1}) (Neal et al., 1988). In contrast the Ras activation in the cell takes place within seconds to minutes after growth factor induced RTK stimulation. That means the intrinsic reaction rates of Ras are too slow for efficient Ras activation after stimulation and favouring an inactive steady state conformation of Ras even in the presence of a high cellular GTP/GDP ratio (Buday and Downward, 2008). The intrinsic GTPase activity (the rate limiting step for Ras inactivation) of Ras is also low (4.2×10^{-4} s^{-1}), which would tend to prolonged signal transduction (Neal et al., 1988). Obviously, Ras kinetics is modified by two classes of auxiliary proteins namely GEFs and GAPs (Figure 1).

RasGTP accumulation is triggered by GEF binding. The GEF catalysed release of GDP will be immediately followed by the rapid binding of GTP, just because GTP is abundant in the cell (10fold higher) relative to GDP (Vetter and Wittinghofer, 2001). EGFR signalling requires the recruitment of Sos (a prominent RasGEF) to the plasma membrane in order to induce Ras activation which is manly assisted by adaptor proteins like Shc and Grb-2 (Buday and Downward, 2008). It is important to note that Ras activation is not exclusively the outcome of GEF activity (Hennig et al., 2015). There are further levels of Ras activation such as: 1) mono-ubiquitination, which possibly interfere with GAP function (Jura et al., 2006, Hobbs et al., 2013), 2) the movement of Ras molecules between raft and non-raft microdomains, which probably affect access of Ras for GEFs and GAPs (Prior et al., 2001) or 3) signalling molecules like nitric oxide or cyclopentenone prostaglandins, which directly modify specific cysteine residues of the C-terminal Ras region (Oliva et al., 2003, Heo et al., 2005).

Ras inactivation is controlled by GAPs, which stimulate the low intrinsic GTPase activity thereby increasing the rate of GTP hydrolysis to GDP. In contrast to GEF mediated Ras activation, the molecular mechanisms of GAP activity and regulation upon mitogenic signalling are still a controversial topic.
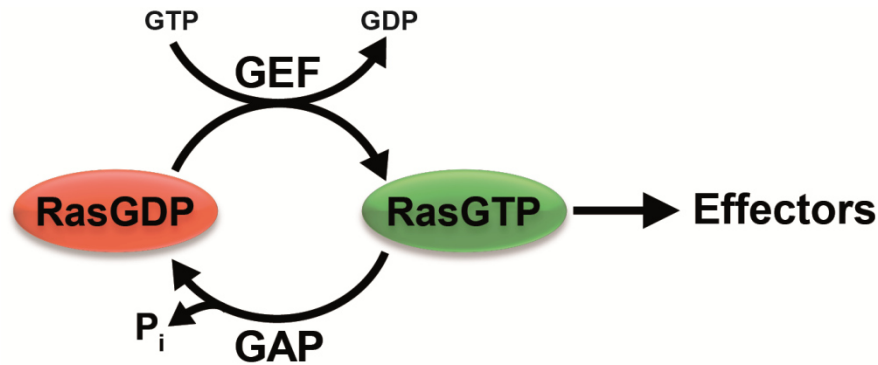
Figure 1: The Ras cycle by GEF and GAP proteins.
Ras cycle between an active (GTP-bound) and inactive (GDP-bound) state controlled by GEFs and GAPs. GEFs stimulate the exchange of GDP for GTP, whereas GAPs bind to Ras and stimulate its intrinsic GTPase activity.

6.2.2 Structure of Ras

The potential of the molecular switch is based on two fundamental properties of Ras: 1) Ras binds guanine nucleotides with high affinity and 2) Ras hydrolyses bound GTP to GDP and inorganic phosphate (Figure 1).

The crystal structure of the Ras protein as well as its interactions with nucleotides was identified in the late 80s by Sung-Hou Kim (de Vos et al., 1988) and Alfred Wittinghofer (Pai et al., 1989, Wittinghofer and Pai, 1991). Ras contains a G-domain made of five conserved polypeptides loops (G1-G5) and a C-terminal CaaX box mediating interaction with a lipid bilayer (Figure 2). The G-Domain carries out basic functions of nucleotide binding and hydrolysis. In the GTP bound form the γ-phosphate of the GTP and two invariant amino acids of the switch I (G2 motif) and switch II (G3 motif) form two hydrogen bonds. The P-loop (G1) is a glycine-rich loop and binds the α/β-phosphate of the GTP as well as coordinates the divalent magnesium ion, which helps to coordinate nucleotide binding. G4 and G5 motif are responsible for the specific guanine binding with G4 carrying a guanine binding motif. As soon as a water molecule has been optimally positioned for nucleophilic attack on the γ-phosphate of GTP the switch regions relax which causes a conformational change and the hydrolysis of GTP (McCormick et al., 1985, Vetter and Wittinghofer, 2001).

Since the GDP release is comparable slow this process is triggered via interaction with a Ras nucleotide exchange factor, mainly Sos. The Ras/Sos interaction takes place in the switch I and II region leading to a sterically displace of the magnesium ion which results in GTP binding. Then the GTP-bound conformation allows binding of downstream effectors (Vetter and Wittinghofer, 2001).
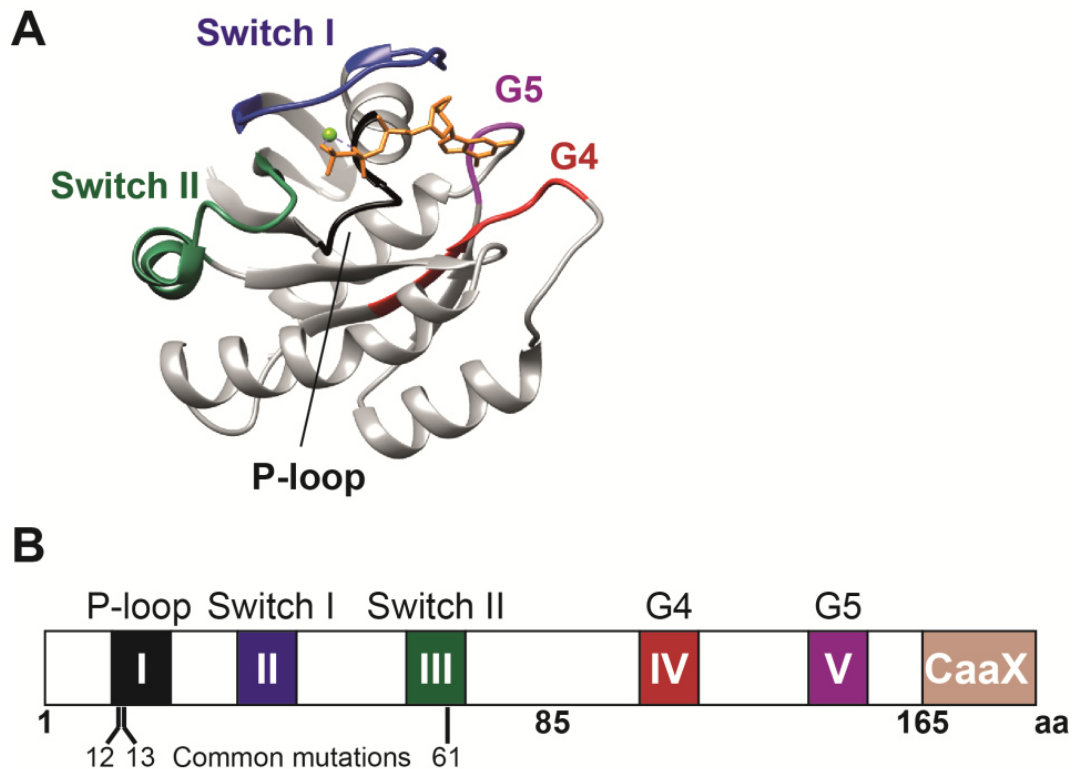
Figure 2: Structure of Ras
A) Crystallographic structure of H-Ras loaded with non-hydrolysable GTP is shown. G-domains are coloured. **B)** Schematic diagram of the Ras protein domains. The catalytic units G1-G5 are involved in GDP/GTP binding and the CaaX motif at the C-terminus is essential for membrane binding and trafficking. The most common oncogenic mutations occurred at codon 12, 13, and 61, the region that is identical among the 3 isoforms. Figure 2A adapted from (Hennig et al., 2015).

6.2.3 Post-Translational Modification of Ras

The Ras proteins are membrane-associated proteins due to post-translational modifications, which add a hydrophobic anchor, a process that is required for Ras activation and signalling. The C-terminal CaaX motif undergoes sequentially post-translational modification by three enzymes: farnesyltransferase (FTase), Ras-converting enzyme 1 (RCE1) and isoprenylcysteine carboxylmethyltransferase (ICMT). First Ras gets prenylated, involving the transfer of either a farnesyl moiety to CaaX cystein in the cytosol that directs Ras to the endoplasmatic reticulum (ER) (Figure 3). Next the tripeptide aaX is cleaved from the C-terminus by the specific endoprotease RCE1 bringing the farnesylcysteine into the C-terminal position. The now C-terminal prenylcysteine of Ras is methylated by ICMT a methyltransferase (Choy et al., 1999, Wright and Philips, 2006) (Figure 3). N-Ras and H-Ras undergo an isoform specific palmitoylation step within the hyper variable region (hvr), a region of 20 amino acids upstream of the CaaX sequence, which enables a Golgi apparatus mediated vesicle transport to the plasma membrane (Hancock et al., 1990) (Figure 3). In particular, we have previously shown that the palmitoylation of N-Ras is necessary for activation by agonists in growth factor signalling (Song et al., 2013). Conclusively, the recirculation to the Golgi and the ER, which is initiated by depalmitoylation, may contribute to the shutdown of Ras signalling. In contrast, K-Ras have two splice variants K-Ras4A and K-Ras4B. The latter one, which is not palmitoylated, displays a positively charged basic sequence with six contiguous lysines and traffic directly to the plasma membrane via a poorly understood pathway (Apolloni et al., 2000) (Figure 3). Mutations in the CaaX motif or loss of a modifying enzyme result in defective Ras processing and aberrant localisation of Ras within cells (Kim et al., 1999).

Shown by our own group endogenous growth factor induced RasGTP is predominantly localised at the plasma membrane (Augsten et al., 2006). Here, Ras isoforms are not distributed randomly. Ras proteins are localised in plasma membrane domains, e.g. lipid rafts and caveolea. This distribution is Ras isoform specific (Abankwa et al., 2010). Especially, H-Ras is associated in microdomains with cholesterolrich regions (lipid rafts) whereas K-Ras operates from actin-dependent, cholesterol-independent nanoclusters (Prior et al., 2003) and N-Ras is a prominent endomembranous component.

Interestingly, segregation of GTP-bound H-Ras from rafts is essential for efficient Raf activation (Prior et al., 2001, Rotblat et al., 2004, Guzman et al., 2014).
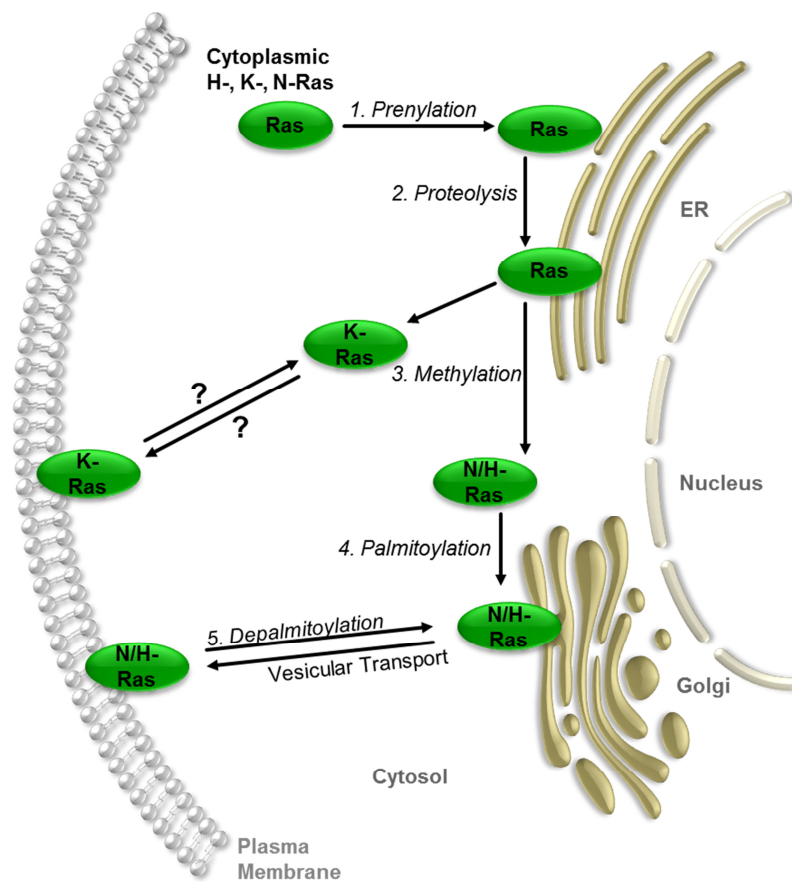
Figure 3: Overview of Ras post-translational modification and membrane trafficking within the cell compartments.
CaaX processing is initiated in the cytosol by prenylation followed by proteolysis and a methylation step at the ER. K-Ras then traffics to the plasma membrane through a poorly characterised pathway. H-Ras and N-Ras, after palmitoylation pass the Golgi and reach the PM with the aid of vesicles. Depalmitoylated H-Ras and N-Ras cycle through the ER for repalmitoylation.

6.3 Regulators of the small GTPase Ras

The status of Ras activity is under the control of two major regulators GEFs and GAPs. This chapter of the thesis describes the structure, regulatory mechanisms of GEFs and GAPS as well as their involvement in human cancer or other malignancies.

6.3.1 Guanin nucleotide exchange factors (GEFs) of Ras

RasGTP formation results from the growth factor sparked stimulation of nucleotide exchange on Ras. This process is triggered by RasGEFs, which promote the dissociation of otherwise tightly bound nucleotides from Ras proteins.

Mammals express 3 classes of RasGEFs (Figure 4). The expression of the 2 GEF families RasGRF and RasGRP is largely restricted to neurons and leukocytes (Kawasaki et al., 1998, Dower et al., 2000, Pierret et al., 2000, Fernandez-Medarde and Santos, 2011b), whereas Ras activation in other cell types is mostly driven by the ubiquitously expressed GEF Sos. There are 2 known members of the mammalian Sos family (Sos1 and Sos2). Upon growth hormone receptor stimulation, Sos that is normally cytoplasmic, gets recruited to the plasma membrane thereby reaching the vicinity of Ras. To achieve this, the PH domain of Sos serves a membrane-targeting function (Chen et al., 1997) and the C-terminal proline-rich (PR) region serves as the recognition site for SH3 domains on Grb2 to mediates their interaction (Rozakis-Adcock et al., 1993).

In general GEFs are multidomain proteins (Bos et al., 2007) (Figure 4). The REM (Ras exchange motif) and cdc25 domain form the catalytic module and are common to almost all Ras-family GEF structures. The Dbl homology (DH) /pleckstrin homology (PH) domains in tandem, binds Ras and opens it for GDP/GTP exchange (Soisson et al., 1998). The PH and C1 domains are responsible for lipid binding, whereas IQ and EF domains facilitate calcium binding. Due to those putative calcium-binding properties, second messengers like DAG and Ca^{2+} and their role in Ras activity have been discussed extensively (Farnsworth et al., 1995, Ebinu et al., 1998, Cullen and Lockyer, 2002, Walker et al., 2003). Indeed the calcium-binding on the EF module plays a crucial role in stimulating RasGRP1 activity (Iwig et al., 2013). Furthermore the DAG binding C1 domain is mandatory to recruit the RasGRPs to the plasma membrane in order to activate Ras (Ebinu et al., 1998, Johnson et al., 2007).

Sos is able to induce GDP/GTP exchange on all 3 Ras isoforms. Upon growth factor stimulation, Sos is recruited by Grb2 to the plasma membrane and activate Ras (Aronheim et al., 1994). Once Sos is activated, the interaction of the REM domain with the switch 2 region of Ras mediates the anchoring to RasGDP, whereas the interaction of the cdc25 domain with the switch 1 region of Ras leads to GDP dissociation (Rojas et al., 2011).

There are diverse regulatory mechanisms of Sos activity (Cherfils and Zeghouf, 2013). Under resting conditions in the cytosol, Grb2 provokes negative regulation and so reduces the stimulus-independent Ras activation (Zarich et al., 2006). In addition the DH-PH unit is a autoinhibitory domain and block the allosteric binding site for Ras (Sondermann et al., 2004).

It is also described that the binding of phospholipase D2 generated phosphatidic acid to the PH-domain of Sos is necessary for the localisation of Sos to the membrane and activation of Ras (Zhao et al., 2007, Litosch, 2015).
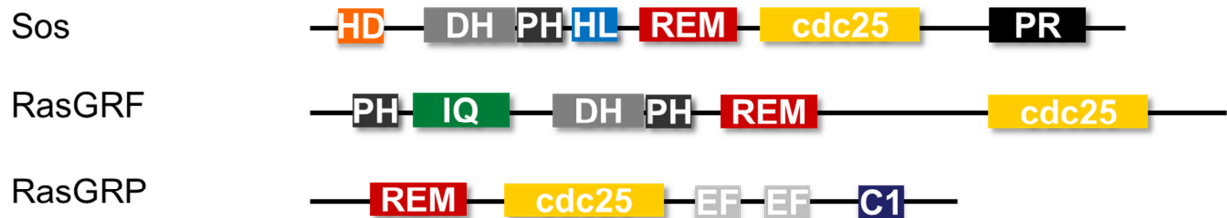
Figure 4: Domain structure of the RasGEF classes.
The REM-cdc25 tandem module harbours the catalytic domain that exhibits nucleotide exchange activity towards Ras. Domain nomenclature according to SMART database (http://smart.embl-heidelberg.de/): C1, DAG-binding C1 domain; cdc25, catalytic GEF domain; DH, Dbl homology; EF, Ca^{2+}-binding EF hand; IQ, calmodulin-binding motif; HD, histone domain; HL, helical linker; PH, Pleckstrin homology; PR, proline-rich region; REM, Ras exchanger motif. Figure based on (Hennig et al., 2015).

GEFs in malignancies

Mutational activation of the RasGEF Sos has been identified in human genetic syndromes such as the Noonan syndrome, which is characterised by short stature, facial dysmorphia, congenital heart defects and skeletal anomalies (Tartaglia et al., 2007, Pierre et al., 2011). Noonan syndrome is an autosomal dominant disorder that frequently shows mutations in *PTPN11* (Tyrosine-protein phosphatase non-receptor type 11) and in several members of the Ras/Erk pathway such as *K-RAS* and *RAF* (Roberts et al., 2007, Shannon and Bollag, 2007). PTPN11 mutations are mostly missense mutations and lead to increased Ras/Erk pathway activity (Ostman et al., 2006). Additional a mutation in the *SOS1* gene causes hereditary gingival fibromatosis type 1 (Hart et al., 2002) (Figure 7). Further ablation of Sos1 in mice is embryonic lethal (Qian et al., 2000, Baltanas et al., 2013) and cannot be compensated by Sos2 (Qian et al., 2000). Many germline mutations are located in the amino-terminal region (HD, DH, PH, HL) of Sos and perturb intramolecular interactions, which are necessary for Sos autoinhibition (Sondermann et al., 2004). Aberrant RasGRP1 and 4 activities are reported in leukemogenesis and the overactive RasGRP3 is involved in the formation and maintenance of prostate cancer. Overexpression of RasGRP3 is also observed in human breast tumour tissue samples as well as in multiple human breast cancer cell lines (Ksionda et al., 2013).

6.3.2 GTPase activating proteins (GAPs) of Ras

The human genome predicts 170 GAPs for small GTPases, of which only 6 classes are bona fide RasGAP proteins (Bernards, 2003, Bos et al., 2007) (Figure 5).

All GAPs are composed of numerous domains including a finger-like domain (Wittinghofer et al., 1997). The highly conserved finger-like domain is usually an arginine finger which interacts with the phosphate-binding loop (P-loop) of Ras. This interaction increases the GTP hydrolysis more than 1000-fold by stabilising the transition state (Scheffzek et al., 1997). GAPs reduce in this way the number of RasGTP molecules available for interaction with the downstream effectors, leading to an attenuation or termination of downstream signalling. At least GAPs are able to control the rise and fall of RasGTP levels, but its still unknown if GAP activity is constitutive or subject to cellular fine-tuning and regulation.

The sub-cellular localisation of Ras signalling complexes, protein-protein interactions, protein degradation and second messenger as well post-translational modifications makes the GTPase activity control highly complex and is intensely debated by (Bos et al., 2007) and (Grewal et al., 2011). The control and regulation mechanisms consider that RasGAPs are critical elements in growth factor signalling and bridge the link between activated receptor tyrosine kinases and the small GTPase Ras. p120GAP was the first characterised GAP protein with an N-terminal SH3 domain flanked by two SH2 domains (Figure 5) that directly recognise phosphorylated tyrosines on activated growth factor receptors and cytoplasmic proteins (Pamonsinlapatham et al., 2009). Further it is postulated that the PH domain mediates the interaction with Ras (Drugan et al., 2000). For the C2 domain it has been proposed that it mediates protein-protein interactions with Ca^{2+}-dependent membrane-binding proteins. p120GAP is also known as a multi-interacting protein in downstream signalling due to its associations with Akt, Aurora or p190RhoGAP (a GAP protein for the Rho family of small GTPases) (Pamonsinlapatham et al., 2009).

NF1 is a large tumour suppressor protein harbouring a central RasGAP region (Martin et al., 1990). Intriguingly, NF1 lacks other functional protein domains that could potentially serve as a link to cell surface receptor signalling, except of the phospholipid-binding Sec14-PH module juxtaposed to the RasGAP domain (Welti et al., 2007) (Figure 5). Studies reported that specific lipids like arachidonic acid and phosphatidic acid have strong inhibitory effects on NF1 catalytic activity but do not influence the Ras/NF1 interaction (Tsai et al., 1989, Bollag and McCormick, 1991,

Han et al., 1991). NF1 is known to interact with all four mammalian syndecans (Hsueh et al., 2001), transmembrane proteins, which enables recruiting NF1 to specialised domains of the plasma membrane to gain proximity to activated Ras or other functional proteins. However the significance of this observation remains unclear. Further it was shown that NF1 is phosphorylated at the C-terminal region by protein kinase A. Such a phosphorylation encourages the association with 14-3-3 proteins and correlates with a decreased GAP activity (Izawa et al., 1996, Feng et al., 2004). Interestingly, alternative splicing of the NF1 pre-mRNA influences the NF1 mediated GTP hydrolysis intensity. Herein exon 23a is an alternative exon inside the GAP-related domain and exon 23a exclusion displays a 10 times higher GAP activity (Zhu et al., 2008, Barron and Lou, 2012, Hinman et al., 2014). Furthermore proteasomal degradation seems to be important in the regulation of NF1 abundance (Cichowski et al., 2003). But nothing is really known about NF1 function and regulatory mechanisms in Ras activation/deactivation. Although biochemical studies have failed to integrate NF1 in specific signalling pathways or its precise role in controlling Ras signalling pathways, NF1 remains a noteworthy candidate. In addition to its role of resolving Ras signalling, studies in *Drosophila melanogaster* have shown that NF1 affects adenylyl cyclase activity and consequently cAMP levels (Guo et al., 2000, Walker et al., 2013), although the underlying molecular mechanism remains elusive.

In mammalian cells the GAP1 family comprises four proteins: GAP1m, $GAP1^{IP4BP}$, CAPRI (Ca^{2+}-promoted Ras inactivator) and RASAL (Ras-GTPase activating-like protein) (Yarwood et al., 2006). They all share conserved structural modules, including tandem C2 (calcium-dependent phospholipid-binding) domains, the catalytic RasGAP domain, and a PH domain next to a Bruton tyrosine kinase (BTK) motif (Figure 5). Thus they are controlled by Ca^{2+} oscillations and inositol phosphate lipid levels. For example plasma membrane localisation and concomitant GAP activity of RASAL and CAPRI correlate with the Ca^{2+} concentration in the cell (Lockyer et al., 2001, Walker et al., 2004). In contrast, $GAP1^{IP4BP}$ is constitutively membrane bound though requires binding of a Phospholipase C to its PH domain for GAP activity (Cullen and Lockyer, 2002).

SynGAP is only expressed in neurons and has been implicated in the inactivation of Ras activity at excitatory synapses (Kim et al., 1998). SynGAP catalytic activity is regulated through phosphorylation via the cyclin-dependent kinase 5 and the Ca^{2+}/calmodulin-dependent protein kinase II (Oh et al., 2004, Walkup et al., 2015). Remarkably GAPVD1 (GTPase activating protein and VPS9 domains 1) can act via the RasGAP domain as a GAP and via the VPS9 domain as a GEF for Rab31 (Lodhi et al., 2007).

In addition GAPs are considered to have dual functions in the cell depending on the domain variety. On the one hand the down regulation of the Ras/MAPK pathway after growth factor stimulation (signal terminator) and on the other the transduction of downstream signals (signal transmitter) to regulate cellular processes involved in apoptosis, proliferation and cell migration (McCormick, 1989, Hall, 1990b, Pamonsinlapatham et al., 2009).
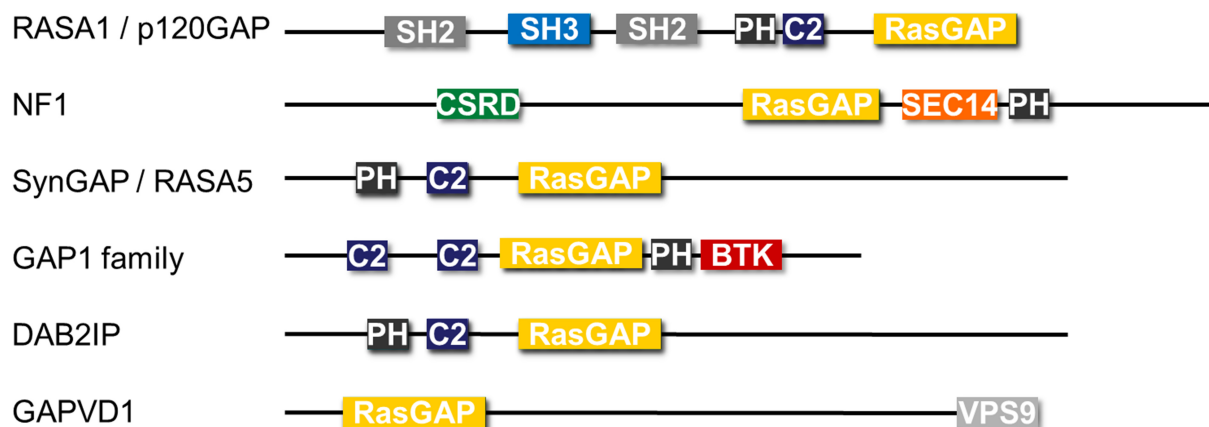
Figure 5: Domain architecture of RasGAPs.
The six RasGAP families with the functional and catalytically domains are shown. Domain nomenclature according to SMART database (http://smart.embl-heidelberg.de/): BTK, Bruton's tyrosine kinase Cys-rich motif; C2, protein kinase C conserved region 2; CSRD, cysteine and serine rich domain; PH, Pleckstrin homology; RasGAP, catalytic domain of GTPase activating protein for Ras; SEC14, lipid-binding domain; SH2, Src homology 2 domain; SH3, Src homology 3 domain; VPS9, domain present in VPS9 protein. Figure based on (Hennig et al., 2015).

GAPs in malignancies

Based on the Ras-inactivating nature of GAP proteins, mutations and aberrant regulation leading to loss of GAP function have putative oncogenic potential. Fibroblasts derived from *P120GAP* deficient mice showed an increased and prolonged activation of Ras MAPK pathway, but they showed no abnormal or excessive cell proliferation, arguing that p120GAP is not required for mitogenic

signalling. This might be due to compensating effects by alternative GAPs, or due to reorganisation of signalling networks (Henkemeyer et al., 1995, van der Geer et al., 1997). Furthermore, the $P120GAP^{-/-}$ null mice have vascular defects and display neuronal apoptosis (Henkemeyer et al., 1995). Therefore loss of function of *P120GAP* seems to be involved in angiogenesis and the development of capillary and arteriovenous malformations (Tidyman and Rauen, 2009) (Figure 7).

Contemporary reports have considered DAB2IP as a tumour suppressor in prostate cancer with growth inhibitory and pro-apoptotic activities via Ras-dependent and -independent pathways, which supports its role in maintaining cell homeostasis (Figure 7) (Chen et al., 2002, Chen et al., 2003, Xie et al., 2009). DAB2IP expression levels were also found to be lower in samples of patients with hepatocellular carcinoma and hepatocellular carcinoma cell lines (Calvisi et al., 2011, Zhang et al., 2012). In addition, various studies have shown that the *DAB2IP* gene is inactivated in several cancers due to aberrant promoter hypermethylation, e.g. in gastrointestinal tumour (Dote et al., 2005), lung (Yano et al., 2005), prostate (Chen et al., 2003) and breast cancers (Dote et al., 2004).

For NF1 several independent groups have reported that primary cells (fibroblasts, Schwann cells and neurons) derived from *NF1* mutant embryos revealed widespread developmental abnormalities (Jacks et al., 1994). The hallmarks of neurofibromatosis type 1 include development of benign nerve and skin tumours and an increased risk of developing other malignancies (Thiel et al., 1995, Upadhyaya et al., 2008, Gulhane and Kotwal, 2015, Nishida et al., 2015). Consistent with the RasGAP function, loss of NF1 results in elevated levels of RasGTP and activation of its corresponding downstream effectors leading to aberrant proliferation and differentiation in multiple cell types (Basu et al., 1992, DeClue et al., 1992, Bollag et al., 1996, Guha et al., 1996, Wu et al., 2006).

Studies targeting *NF1* heterozygously indicated that these mice do not have neurofibromatosis (Brannan et al., 1994, Vogel et al., 1999) whereas chimeric animals harbouring, heterozygous and *NF1* null cells developed neurofibromatosis implicating NF1 wild type allele loss is rate-limiting in tumourigenesis (Cichowski et al., 1999, McClatchey and Cichowski, 2001). Likewise, several other GAP species are implicated in human diseases, but are beyond the scope of this thesis.

For further information Cichowski et al. have recently reviewed the role of RasGAPs in cancer (Maertens and Cichowski, 2014).

6.4 Ras signalling

6.4.1 Activation of Ras by receptor tyrosine kinases

Cell surface receptors are generally divided into 3 classes: 1) the ion channel-linked receptor 2) the enzyme-linked receptor and 3) G protein-coupled receptor.

The intracellular domain of the enzyme-linked receptor has a catalytic function which catalysis receptor autophosphorylation and phosphorylation of substrates. The largest group of enzyme-linked receptors are tyrosine kinase receptors. Several types of receptor tyrosine kinases (RTKs) are known (Choura and Rebai, 2011).

The EGFR (ErbB-1) was the first discovered member of the RTK class also named ErbB family (Lemmon and Schlessinger, 2010). Many ligands are able to activate EGFR such as the epidermal growth factor (EGF). EGF binding causes receptor dimerisation and leads to an activation of the EGFR (Heldin, 1995, Schlessinger, 2002). EGFR activate a number of different intracellular signalling pathways for instance the Ras-MAPK (Jorissen et al., 2003), PI3K (phosphoinositide 3-kinase), Phospholipase C (PLC) (Chattopadhyay et al., 1999), and the Jak/STAT pathways (Quesnelle et al., 2007). In this work we focus on Ras signalling.

EGFR exhibits an intrinsic protein-tyrosine kinase activity that transfers the γ-phosphate of bound ATP to the tyrosine (Y) residues of exogenous substrates and the C-terminal receptor domain. Phosphotyrosine binding domain (SH2)-containing proteins like Src, Shc and Grb2 (growth factor receptor-bound protein 2) bind the receptor and serve as an intracellular docking site for SH2 effector proteins (Normanno et al., 2006). In this way adaptor proteins are localised to tyrosine-phosphorylated sites and mediate further intracellular reactions.

The strength and duration of intracellular signalling from the EGFR are controlled by receptor internalisation and degradation which can be triggered through receptor association with intracellular signalling molecules; e.g. c-Cbl, a tyrosine phosphorylation substrate accelerates desensitisation by increasing receptor poly-ubiquitination (Levkowitz et al., 1999, Marmor and Yarden, 2004, Ravid et al., 2004). Furthermore protein tyrosine phosphatases are able to inhibit EGFR signalling (Ostman and Bohmer, 2001). This tight regulation ensures cell homeostasis and is frequently compromised in cancer cells (Ostman et al., 2006).

6.4.2 Ras downstream effector signalling

Growth factor stimulated EGFR signalling induces a robust accumulation of GTP-loaded Ras which represents the active form of that small GTP-binding protein. The activation of Ras is mediated by the binding of Grb2 either directly at the activated EGFR (Batzer et al., 1994) or indirectly, by binding to EGFR-associated, tyrosine phosphorylated Shc (Sasaoka et al., 1994). The N-terminal SH3 domains of the adapter protein Grb2 bind Sos1/2 (Sos) (Lowenstein et al., 1992, Li et al., 1993, Rozakis-Adcock et al., 1993). Sos functions as a guanine nucleotide exchange factor (GEF) and initiate the conversion of inactive GDP-bound Ras to the active GTP-bound form (Overbeck et al., 1995) (Figure 6). Ras inactivation is mediated by GAPs (GTPase-activating proteins) which enhance the intrinsic GTPase activity of Ras proteins and thereby returning Ras to the inactive GDP-bound state (Bos et al., 2007) (Figure 6).

Ras is a central signal transduction molecule due to its ability to activate downstream signalling pathways like Raf/MEK/Erk, PI3K/Akt/mTOR, Rac, Ral and the Protein kinase C (PKC) pathway (Malumbres and Barbacid, 2003) (Figure 6). In addition Ras is able to stimulate p38 and JNK, two other mitogen-activated protein kinases beside Erk (Cano and Mahadevan, 1995). Most effectors contain a Ras binding domain (RBD) or a Ras association (RA) domain. Despite the high-affinity Ras-effector binding, their interaction is rather temporary. The GTP hydrolysis and subsequent inactivation of Ras signalling provokes the release of effector molecules and termination of further downstream signalling events (Marshall, 1996).

The Raf/MAPK pathway is evolutionarily conserved and one of the most intensively studied signal transduction processes downstream of Ras (Perrimon, 1994, Sternberg and Han, 1998). The essential elements in this pathway belong to a family of protein-serine/threonine kinases called the MAP kinases. The first mammalian effector of Ras is the serine/threonine-specific protein kinase Raf (Dickson et al., 1992, Warne et al., 1993). Immediately after Ras activation Raf binds RasGTP via its RBD. Further phosphorylation events lead to entire Raf kinase activation (Rajalingam et al., 2005). Raf phosphorylates MAPK1/2 (MEK1/2), which subsequently activates extracellular-signal-regulated kinase 1/2 (Erk1/2). MAPKs like Erk phosphorylate substrates with the consensus sequence Px(S/T)P. The activated Erk has many substrates in the cytosol and the nucleus, including signal transducers and transcriptional regulators respectively (Yang et al., 1998, Roskoski, 2012).

Well-known Erk substrates are Rsk, cPLA2, MNK1/2, c-Fos and Elk-1. The diversity in cellular responses due to Erk signalling suggests that many Erk substrates have not been identified yet (Kosako et al., 2009).

The 90 kDa ribosomal S6 kinases (Rsk) are a family of widely expressed Ser/Thr kinases characterised by two non-identical, catalytic domains connected by a regulatory linker region and a carboxy-terminal docking site for Erk (Anjum and Blenis, 2008). These domains gain full activity upon sequential phosphorylation events (Thr573, Ser380, Ser363, Thr359 and Ser221) following MAPK docking (Dalby et al., 1998). In mammals, four Rsk genes have been identified (Rsk1, Rsk2, Rsk3 and Rsk4). With the exception of Rsk4, expression of the Rsk mRNAs has been shown to be ubiquitous in every human tissue (Zeniou et al., 2002). RxRxxS/T or RRxS/T are the target sequences in known Rsk substrates (Leighton et al., 1995). The Rsk consensus phosphorylation motif is shared by other basophilic protein kinases, such as Akt, suggesting a functional overlap. Activated Rsk enzymes are mainly described to act further downstream to regulate cell survival (Bonni et al., 1999, Shimamura et al., 2000) and cell growth by simultaneously regulating substrates that are involved in gene transcription and mRNA translation (Carriere et al., 2008, Romeo et al., 2012).

The phosphoinositide 3-kinases (PI3K) catalyses the phosphorylation of membrane associated lipids thereby converting phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 propagates through binding of phosphoinositide-dependent kinase 1 (PDK1) the activation of the protein kinase B (PKB/Akt) which phosphorylates and activates other proteins. Moreover PI3K signalling activates the small GTPase Rac which is involved in cytoskeleton reorganisation (Castellano and Downward, 2011).

Further Ras effector proteins are not part of this thesis work and will not be further described.
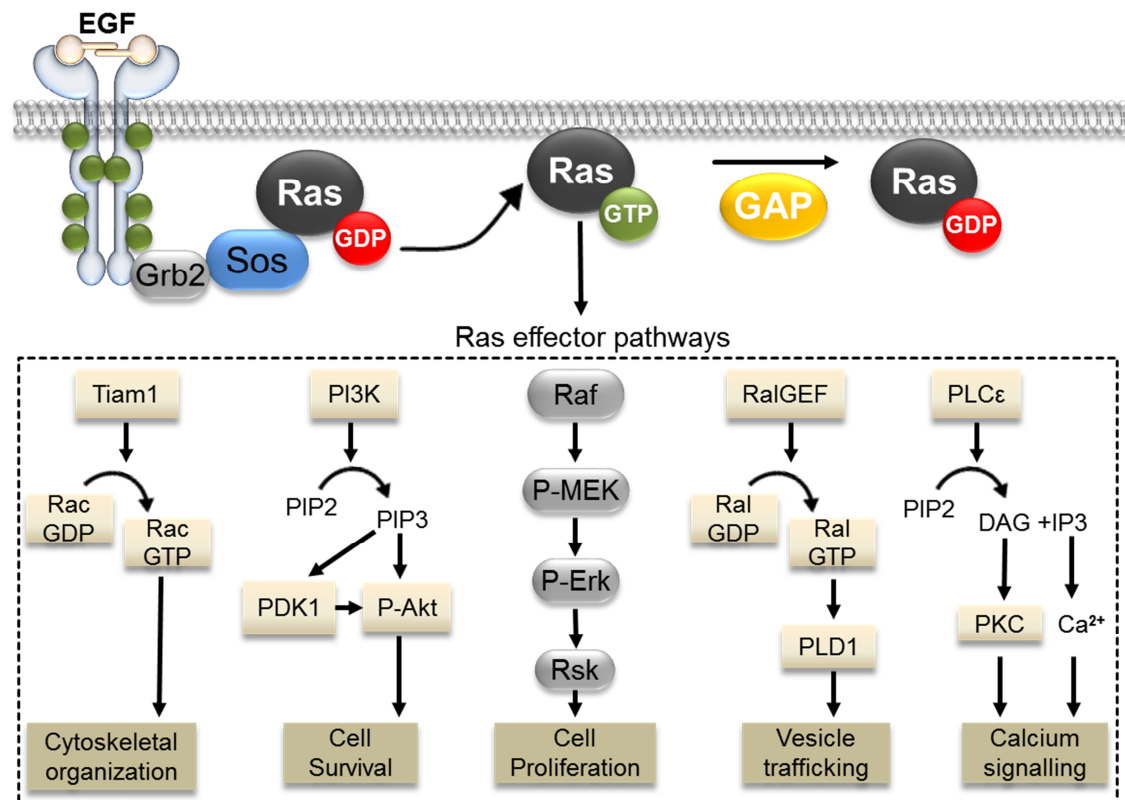
Figure 6: Ras activates multiple downstream effector pathways
Ras is activated by epidermal growth factor receptor signalling at the cellular membrane by the ubiquitous expressed guanine nucleotide exchange factor Sos. Thereby GAPs stimulate the hydrolysis of GTP on Ras, returning them to their inactive state. The major Ras effector pathways are shown. The best-studied pathway activated by Ras is the Raf/MEK/Erk signalling cascade which leads to cell proliferation. PI3K take part in cell survival and proliferation. In addition active Ras stimulates RacGTP and RalGTP formation through TIAM1 a RacGEF and RalGEF. Figure based on (Schubbert et al., 2007).

6.5 Ras signalling in tumourigenesis

Proto-Oncogenes like *RAS* are genes, which drive oncogenesis upon gain-of-function mutations. This leads to aberrant signalling and the dysregulation of the cellular functions such as cell proliferation and differentiation lead to cancer formation and tumour growth. Around 30% of all human cancers contain activating Ras mutations (Prior et al., 2012). Ras gain-of-function mutations occur frequently as point mutations in codon 12, 13 (P-loop) or 61 (Switch II) leading to amino-acid substitutions (Bos, 1989) (Figure 2). Interestingly these three major mutations are located in the nucleotide binding area. The mutations reduce the intrinsic GTPase activity of Ras and trigger a resistance towards the catalytic GAP activity (Trahey and McCormick, 1987, Zhang et al., 1990, Scheffzek et al., 1997). The subsequent attenuated activation of Ras and its effector pathways lead to cell transformation in culture and formation of human tumours.

Specific Ras genes are mutated in different malignancies (Bos, 1989) (Figure 7). K-Ras is the most common mutational activated oncoprotein and prevalent in pancreatic (95% frequency) (Hirai et al., 1985, Almoguera et al., 1988) as well in combination with N-Ras mutations in myeloid malignancies (Schubbert et al., 2007). Whereas *N-RAS* and *H-RAS* mutations have been detected in melanoma (Balmain and Pragnell, 1983) and bladder cancer (Fernandez-Medarde and Santos, 2011a), besides mutations in the *H-RAS* gene cause the Costello syndrome (Aoki et al., 2005) (Figure 7).

In addition to mutations of *RAS* there are several other classes of mutations that affect components of the Ras signalling for example the 1) growth-factor-receptor activation, 2) GAP deletion, or 3) overexpression of positive and negative regulators like Sos and Spred1 and 4) mutation or amplification of Ras effectors. All of these dysregulations were shown to cause several developmental disorders and/or cancer (Fernandez-Medarde and Santos, 2011a) (Figure 7). The genetic syndromes including Noonan-, Costello-, cardio-facio-cutaneous-, Legius syndrome and Neurofibromatosis type 1 are called RASopathies and show overlapping phenotypic features and symptoms that include facial abnormalities, heart defects, impaired growth and development (Simsek-Kiper et al., 2013).

Many efforts have been made to develop cancer therapeutic agents targeting Ras or components of the Ras signalling pathway to treat various diseases, including cancer. For instance Farnesyltransferase inhibitors to block Ras membrane localisation, antisense oligonucleotides against Ras and Raf, kinase inhibitors targeting Ras effector pathways as well as small molecule tyrosine kinase inhibitors and monoclonal antibodies raised against the EGFR upstream of Ras (Downward, 2003, Saxena et al., 2008). Taken together EGFR and the Ras/MAPK signalling cascade are important drivers of oncogenesis and in turn attractive targets for anticancer therapies. However, no clinically relevant direct Ras modulator has been yielded in the past and Ras proteins have been finally considered as "undruggable" (Cox et al., 2014). New directions in targeting of Ras pathways are excellently discussed elsewhere (Singh et al., 2015).
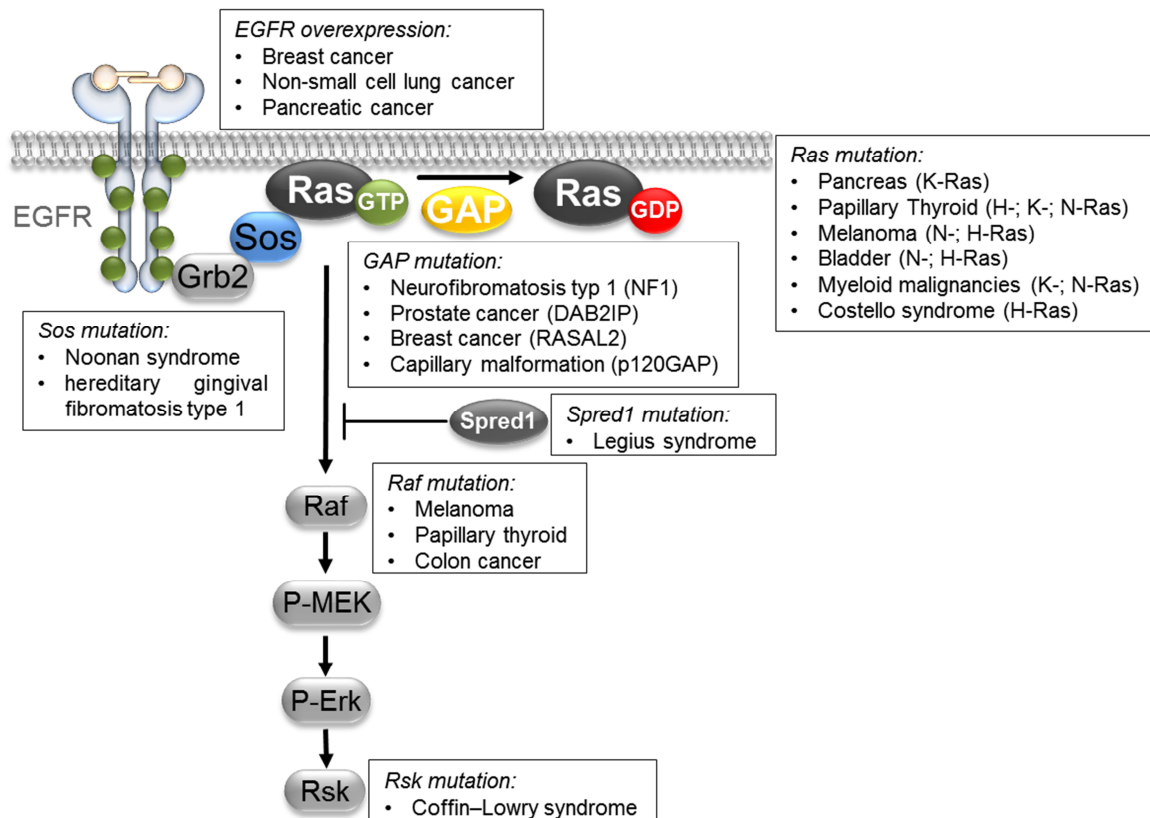
Figure 7: Overview of the main developmental disorders and tumours associated with aberrant Ras signalling.
Gene mutations in the Ras/MAPK cascade and overexpressed EGFR causes diverse human cancers and developmental syndromes, the so called RASopathies. Here we display only a selection of all malignancies which are caused by aberrant Ras/MAPK signalling. Figure based on (Bentires-Alj et al., 2006).

6.6 Ras feedback mechanism and negative regulator in Ras signalling

Early studies in the 90s proposed that the duration of the Ras/Erk signalling plays a critical role in cell fate decision (Traverse et al., 1992, Marshall, 1995a, von Kriegsheim et al., 2009). Nowadays it is still a matter of debate how the activation of the same canonical signalling cascades dictates distinct biological outcomes. Chris Marshall and co-workers delivered the first evidences in PC12 cells. They suggest that the duration and magnitude of Erk activation is critical for cell signalling decisions. NGF (nerve growth factor) and PDGF stimulated cells underwent cell differentiation in correlation with a prolonged Erk signal (Heasley and Johnson, 1992, Traverse et al., 1992). In contrast, EGF induced short-term Erk activity (Traverse et al., 1994), which led to cell proliferation (Nguyen et al., 1993, Marshall, 1995b). Later it was shown that EGF stimulation initiates a negative feedback loop, whereas NGF stimulation induces further signal amplification due to a positive feedback on the MAPK cascade (Brightman and Fell, 2000, Santos et al., 2007). One mechanism of

how cells sense the Erk signal duration is the accumulation of immediate early gene products in response to Erk activity (Murphy et al., 2002, Amit et al., 2007).

A similar dynamic manner shows the Ras activity duration. Here the transient Ras activation induces proliferation or differentiation whereas a long term or sustained Ras activation induced by overexpression or constitutive active Ras in primary cells can induce senescence or growth arrest (Ridley et al., 1988, Serrano et al., 1997, Narita and Lowe, 2005). However, oncogene-induced senescence combined with other genetic lesions of a tumour suppressor protein like p53 or p16 promotes tumour progression (Serrano et al., 1997, Ferbeyre et al., 2002). This seems to be a major hurdle for cancer cells to achieve full transformation. At least for normal cell proliferation a transient Ras activation is necessary. Nevertheless how cells ensure a timely controlled, transient activation of Ras during mitogenic signalling is not yet clear. To terminate the Ras signal at the right time seems to be important for the cellular response. Whereas the Ras activation is triggered by enhanced exchange of guanine nucleotides on Ras, the mechanism responsible for the desensitisation of Ras (Ras deactivation) has remained obscure. The regulation of the active Ras is likely to occur at different levels, via inhibition or interruption of the upstream signals or negative regulation via a feedback loop starting downstream of Ras, here the signal induces or activates its own negative regulators.

Previous studies demonstrated that insulin and several other agents (platelet-derived growth factor, serum, and phorbol ester) elicit a growth factor dependent phosphorylation of Sos and ensue disassociation of the Grb2-Sos complex (Waters et al., 1995a, Waters et al., 1995b, Dong et al., 1996). For the reason that Sos phosphorylation was correlated with the desensitisation phase of Ras inactivation they concluded that Grb2-Sos complex dissociation interrupt the ability of Sos to catalyse nucleotide exchange on Ras and thereby terminate the RasGTP formation. Finally, it has been postulated that Sos phosphorylation and following destabilisation of Sos-Grb2 complex occur by a MEK-dependent pathway (Figure 8A).

Recently, a second phospho-dependent Ras-MAPK feedback loop targeting Sos has been suggested. Here Rsk phosphorylates Sos and in turn generates a 14-3-3-docking site (Figure 8B). 14-3-3 binding has be suggested to reduce Sos catalytic activity or prevent its interaction with Ras or diminish the capacity for Sos to bind to the plasma membrane and thereby attenuate Ras-MAPK signalling (Saha et

al., 2012). These data demonstrated a further molecular feedback mechanism from MAPK pathway to the exchange factor Sos.

Furthermore the Erk pathway is able to induce the transcriptional upregulation of sprouty proteins (Ozaki et al., 2001, Murphy et al., 2002, O'Donnell et al., 2012). Sprouty proteins are known to bind Grb2 and are thought to impede the activation of Sos proteins thereby decreasing Ras activation (Casci et al., 1999, Reich et al., 1999, Hanafusa et al., 2002) (Figure 8C). Additionally, Cbl proteins are able to downregulate RTKs through Grb2 mediated Cbl-RTK binding and subsequent multi-ubiquitylation (Thien and Langdon, 2001, Huang and Sorkin, 2005, Lee et al., 2014). In this context endocytosis of RTKs has been considered to regulate MAPKs as well (Dikic, 2003, Wiley, 2003, Marmor and Yarden, 2004).

Further negative regulators of MAPK signalling are the Spred proteins. Spred1 inhibits the activation of MAP kinase by suppressing phosphorylation and activation of Raf (Wakioka et al., 2001) (Figure 8D).

The Ras activation process depends on its membrane localisation and subsequent spatial vicinity to membrane receptors (Zhou and Hancock, 2015). At least the mechanisms that drive Ras in microdomains are unknown but could be another possible regulatory tool in the context of Ras activity regulation (Rotblat et al., 2010) (Figure 8E).

RasGAPs like NF1 are known as negative regulators of Ras due to their intrinsic catalytical GTPase activating domain (Figure 8F). Despite indisputable advances in our understanding of the functional characteristics of GAP enzymes and based on the huge awareness of the tumour suppressive roles of DAB2IP and NF1, there is little knowledge on their molecular mechanisms and cellular regulation. During the last years RasGAPs have been in the shadow of GEF biology as the important driver and regulator of Ras activity. An important goal is to determine the extent of GAP activity contributing to the deactivation of Ras signalling upon growth factor stimulation. It has not been shown how GAP activity shapes the amplitudes and duration of Ras signalling. Furthermore, the signalling events downstream of activated growth factor receptors which induce and fine-tune RasGAP engagement as well as GAP activity have not been resolved yet. Taken together, we still lack solid evidences, which convincingly document the modulation of growth factor signalling by GAPs.

In this context open and interesting questions are: Are there Ras GAPs acting downstream of an activated growth factor receptors? And which signalling events drive the interaction of GAPs and Ras? In this study it was our ambitions to answer these questions.
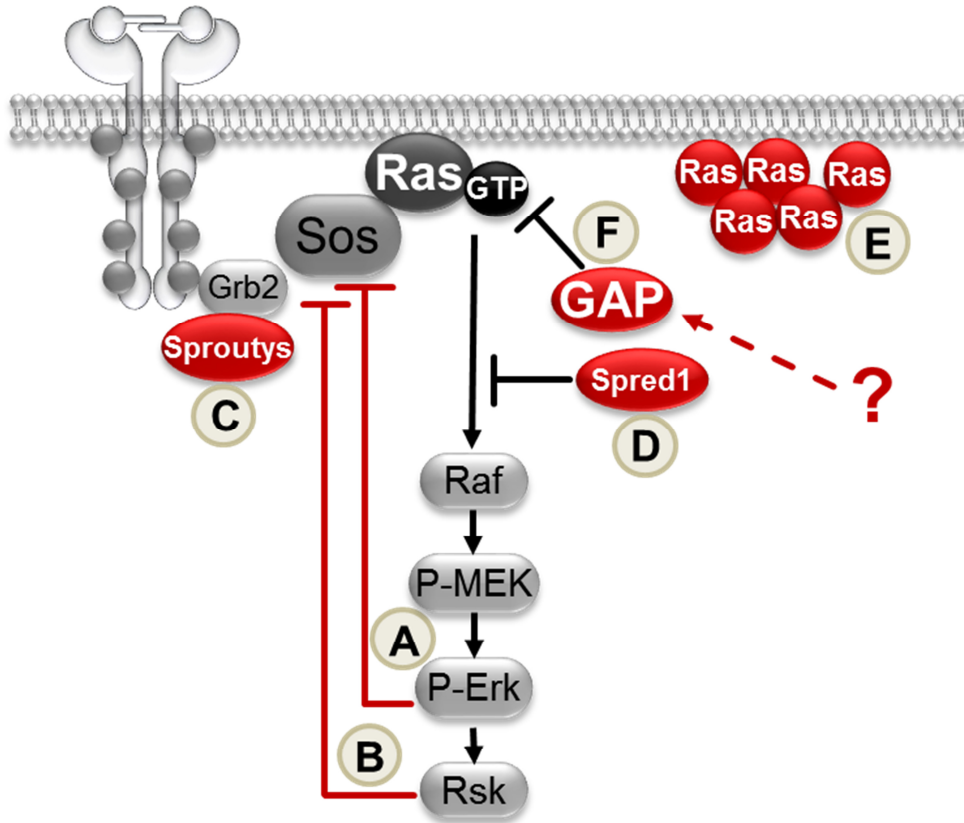
Figure 8: Possible Ras feedback mechanisms.
The Ras pathway is highly regulated and exhibits a negative feedback signalling network. This figure includes many, but not all, negative regulators and proposed feedback loops in growth factor signalling (shown in red). **A+B)** Erk and Rsk are suggested kinases to initiate the negative feedback loop downstream of Ras via Sos phosphorylation. **C)** Sprouty proteins are immediate early gene products of Erk and proposed to decrease the Sos activity through Grb2 binding. **D)** Spred1 diminishes the MAPK signalling by directly disruption of the Ras-Raf binding. **E)** The reorganisation of Ras molecules on the plasma membrane is another thinkable Ras downregulating mechanism. **F)** GAP proteins function as a negative regulator by direct interaction with Ras molecules for GTP hydrolysis. Whereas the GTP hydrolysis on Ras is described in detail, the mechanisms how GAPs become activated after growth factor stimulation are not known.

7 Aim of the work

The duration of Ras activity determine cell fate decisions. Growth factor-induced Ras activation must be transient to drive cells into the cell cycle and to promote a proper proliferative response. Mutations in Ras lead to constitutive Ras activation and promote senescence. In combination with another oncogene or inactivation of a tumour suppressor, oncogenic Ras triggers cell transformation. Hence, many studies focused on investigating the role of mutated, oncogenic Ras in cell signalling and tumour development. In contrast, the mechanodynamics of transient Ras activation in growth factor signalling in normal cell function is largely understudied. The current understanding is that Ras activation in quiescent cells arises through the activation of Sos, which consequently results in the stimulation of nucleotide exchange on Ras. The principle behind the decline of RasGTP levels, further referred as Ras deactivation, is poorly understood. Current models argue for a negative feedback on Sos, based on the observation that Sos becomes phosphorylated following the activation of Ras/MAPK pathway. However, this assumption was never tested experimentally. In theory, the restoration of basal RasGTP level could be mediated by diverse mechanisms: 1. Suppression of GEF activity, herein GAPs play a rather passive role. 2. Activation of GAP(s). 3. A combination of GEF inhibition and GAP activation could synergise to switch off Ras. The current thesis work aims to address following questions:

a) Which mechanisms terminate growth factor induced Ras activation?

b) How do feedback loops contribute to Ras deactivation? Do these feedbacks impact Sos activity, as originally proposed, or do they engage GAPs?

c) If Ras deactivation is driven by a GAP system, what is the identity of the relevant GAP?

d) Are GAPs constitutively active enzymes or targets of cellular signalling? What are the mechanisms leading to GAP activation?

8 Materials and Methods

8.1 Material

8.1.1 Reagents

Table 1: List of reagents

Reagent	Company
Acetic acid	Carl Roth GmbH & Co. KG
Acrylamide/Bis-acrylamide 30% (37.5:1)	Carl Roth GmbH & Co. KG
[alpha-^{32}P]GTP	Hartmann Analytic
Amersham Hyperfilm MP	GE Healthcare
Ammonium formate	Carl Roth GmbH & Co. KG
Ammonium persulphate (APS)	SERVA Electrophoresis GmbH
Bovine serum albumin (BSA)	PAA
Bromophenol blue	Carl Roth GmbH & Co. KG
Calcium chloride (CaCl$_2$)	Sigma-Aldrich
Coomassie Brillant Blue R250	Sigma-Aldrich
Deoxycholic acid sodium salt	Carl Roth GmbH & Co. KG
Diethylpyrocarbonat (DEPC)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Dithiothreitol (DTT)	AppliChem GmbH
DMEM	Sigma-Aldrich
Epidermal growth factor	Life Technologies
Ethylene glycol tetraacetic acid (EGTA)	AppliChem GmbH
Ethylene diamine tetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG
Fetal calf serum (FCS)	Biowest
GammaBind–Sepharose	Amersham Biosciences
GeneJuice® Transfection Reagent	Novagen
Glutathione	Sigma-Aldrich
Glutathione–Sepharose	JenaBioscience GmbH
Glycine PUFFERAN®	Carl Roth GmbH & Co. KG
GppNHp	JenaBioscience GmbH
GST–Raf-1-RBD	Lab-intern
Guanosine diphosphate (GDP)	Sigma-Aldrich
Guanosine triphosphate (GTP)	Sigma-Aldrich
HEPES	AppliChem GmbH
Hydrochloric acid	Carl Roth GmbH & Co. KG
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Calbiochem
Leupeptin-hemisulphate	AppliChem GmbH
Lysozym	Sigma-Aldrich
Magnesium chloride (MgCl$_2$)	AppliChem GmbH
2-Mercaptoethanol	Carl Roth GmbH & Co. KG
Microycystin-LR	Enzo Life Sciences GmbH
N,N,N´,N´-Tetramethylendiamine (TEMED)	Carl Roth GmbH & Co. KG
Nonylphenylpolyethylenglycol (NP)-40	Merck KGaA
PageRuler™ Plus Prestained Protein Ladder	Thermo Fisher Scientific Inc.
PageRuler™ Prestained Protein Ladder	Thermo Fisher Scientific Inc.
Pefabloc SC® (AEBSF hydrochloride)	AppliChem GmbH
Pepstatin A	AppliChem GmbH
PMSF (Phenylmethylsulfonylfluoride)	Sigma-Aldrich
Polyethylenimine (PEI) branched	Sigma-Aldrich
Polyvinylidendifluoride (PVDF) membrane	GE Healthcare
Potassium acetate	Carl Roth GmbH & Co. KG

Potassium chloride (KCl)	Carl Roth GmbH & Co. KG
Potassium L-glutamate	Sigma-Aldrich
Saint Red	Synvolux Therapeutics
Sepharose CL-4B	Sigma-Aldrich
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG
Sodium Deoxycholate	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	AppliChem GmbH
Sodium orthovanadate	Sigma-Aldrich
Sodium-β-glycerophosphate	SERVA Electrophoresis GmbH
Streptolysin O (SLO)	Aalto Bio Reagents Ltd.
TLC PEI Cellulose F	Merck
Tris ultrapure	AppliChem GmbH
Triton X-100	Carl Roth GmbH & Co. KG
Trypsin-EDTA	Life Technologies
Tween®20	Serva
Western Lightning ECL, Enhanced Chemiluminescent Substrate	PerkinElmer
Whatman filter paper	Bio-Rad Laboratories Inc.

8.1.2 Buffers and solutions

Table 2: Buffers for Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

10x SDS running buffer	2x SDS sample buffer	5x SDS sample buffer
250 mM Tris 2 M Glycine 35 mM SDS	20% Glycerol 4% SDS 10% 2-Mercaptoethanol 0.02% Bromophenol blue 124 mM Tris pH 6.8	33% Glycerol 5% SDS 25% 2-Mercaptoethanol 0.02% Bromophenol blue 85 mM Tris pH 6.8
Coomassie staining solution	**Stacking gel buffer**	**Separating gel buffer**
0.5% Coomassie Brilliant Blue R250 in absolute ethanol mix 1:1 with 20% acetic acid	0.5 M Tris pH 6.8	2 M Tris pH 8.8

Table 3: Buffers for Western blotting and immunodetection

Transfer buffer pH 10	Stripping buffer pH 6.7	10x TBS-Tween pH 7.6
48 mM Tris 39 mM Glycine 0.037% SDS 15% Methanol	100 mM 2-Mercaptoethanol 62.5 mM Tris 2% SDS	100 mM Tris 1 M NaCl 1% Tween 20
Blocking solution	**ECL**	
1x TBS-Tween 1% BSA	50% Oxidizing Reagent Plus 50% Enhanced Luminol Reagent Plus	

Table 4: Buffer for Ras activity assay

Lysis buffer	
50 mM Tris pH 7.5 140 mM NaCl 5 mM MgCl$_2$ 1 mM EGTA 1% NP-40	*Protease inhibitors* 42 mM Pefabloc 2 µM Leupeptin 100 µM PMSF 1.5 µM Pepstatin A
Add freshly 100 µM GDP 2 mM DTT 25 µg/ml purified GST-Raf-1-RBD	*Phosphatase inhibitors* 100 µM Sodium vanadate 1 µM β-Glycerophosphate 3.4 nM Microcystin

Table 5: Buffers for Nucleotide exchange assay

Lysis buffer	Permeabilisation solution	Washing solution
50 mM Hepes pH 7.5 100 mM NaCl 10 mM MgCl$_2$ 1% NP-40	50 mM Hepes pH 7.5 23 mM NaCl 3 mM MgCl$_2$ 100 nM CaCl$_2$ 1 mM EGTA	50 mM Hepes pH 7.5 500 mM NaCl 5 mM MgCl$_2$ 0.1% TX-100 0.005% SDS
Add freshly 100 µM GDP 100 µM GTP 2.5 µg/ml Y13-259 *Protease inhibitors* *Phosphatase inhibitors*	*Add freshly* 107 mM K-glutamate 1 mM ATP 2 mM DTT *9 MBq* [α-^{32}P]GTP 15 IU/ml SLO	
Elution solution	**TLC mobile Phase**	
5 mM DTT 5 mM EDTA 0.2% SDS 0.5 mM GDP 0.5 mM GTP	1.24 M Ammonium formiate 3.7% HCl	

Table 6: Buffer for Nanocluster assay

KOAc buffer	Staining solution	Fixative solution
25 mM Hepes pH 7.4 115 mM K-acetate 2.5 mM MgCl$_2$	0.6% Uranyl acetate 1.8% Methyl cellulose	1x KOAc buffer 0.1% glutaraldehyde 4% paraformaldehyde
Blocking solution		
1x KOAc buffer 0.2% Fish skin gelatine 0.2% BSA		

8.1.3 Antibodies

Table 7: List of antibodies for western blot analyses

Primary Antibodies				
Name	**Source**	**Company**	**Dilution**	**Cat. No.**
Akt	rabbit	Cell Signaling Technology®	1:2000	9272
DAB2IP	rabbit	Abcam	1:1000	ab87811
Ras-GAP (clone 13/RAS-GAP)	mouse	BD Transduction Laboratories™	1:1000	610040
EGF receptor	rabbit	Cell Signaling Technology®	1:2000	4267
FLAG® M2	mouse	Sigma Aldrich®	1:1000	F3165
GFP Gold-conjugated	sheep	Prepared and kindly provided by Alison Beckett	7-10 µg/ml	
GSK-3β (27C10)	rabbit	Cell Signaling Technology®	1:1000	9315
K-Ras F234	mouse	Santa Cruz Biotechnology®	1:1000	sc-30
Neurofibromin (D)	rabbit	Santa Cruz Biotechnology®	1:1000	sc-67
N-Ras F155	mouse	Santa Cruz Biotechnology®	1:500	sc-31
p44/42 MAPK (ERK1/2)) (137F5)	rabbit	Cell Signaling Technology®	1:1000	4695
P-Akt Substrate (RXRXXS/T)(110B7)	rabbit	Cell Signaling Technology®	1:1000	9614
pan-Ras C-4	mouse	Santa Cruz Biotechnology®	1:500	sc-166691
p-EGF Receptor (Y1068)	rabbit	Cell Signaling Technology®	1:1000	2236
p-ERK1/2 (Y204)	mouse	Santa Cruz Biotechnology®	1:1000	sc-101761
Phospho-(Ser/Thr) Akt Substrate	rabbit	Cell Signaling Technology®	1:1000	9611
Phospho-GSK-3β (Ser9) (D85E12) XP®	rabbit	Cell Signaling Technology®	1:2000	5558
Phospho-p90RSK (Ser380)	rabbit	Cell Signaling Technology®	1:1000	9341
pS473-Akt	rabbit	Cell Signaling Technology®	1:2000	4060
RSK1/RSK2/RSK3 (32D7)	rabbit	Cell Signaling Technology®	1:1000	9355
SOS1 (clone 25/SOS1)	mouse	BD Transduction Laboratories™	1:1000	610095
Spred 2	rabbit	Sigma Aldrich®	1:1000	S7320
Y13-259 used for IP anti-Ras	rat	Lab-intern	2.5 µg/ml	

Secondary Antibodies			
Name	**Source**	**Provider**	**Dilution**
HRP-labelled anti-mouse	goat polyclonal	KPL®	1:10000
HRP-labelled anti-rabbit	goat polyclonal	KPL®	1:10000

8.1.4 Inhibitors

Table 8: List of inhibitors

Inhibitor	Name	Provider	Final concentration	Cat. No.
AKT inhibitor	Wortmannin	Sigma Aldrich®	100 nM	W1628
EGFR inhibitor	AG1478	Merck Millipore	250 nM	658552
ERK inhibitor	FR108204	Sigma Aldrich®	50 µM	SML0320
MEK inhibitor	U0126	Enzo Life Sciences	10 µM	BML-EI282
RSK inhibitor	BI-D1870	Enzo Life Sciences	10 µM	BML-EI407
p38 inhibitor	SB202190	Sigma Aldrich®	10 µM	S7067

8.1.5 siRNA

All siRNAs containing a mixture of four SMARTselection designed siRNAs targeting one gene, purchased from Dharmacon and dissolved in DEPC-water with a concentration of 250 ng/µl. siRNA concentration and integrity were analysed spectrophotometrically with a NanoPhotometer (Thermo Fisher Scientific Inc.).

Table 9: List of siRNAs

Target gene	Cat. No.
Human DAB2IP	L-008249-01-0005
Human NF1	L-003916-00-0005
Human RASA1 (5921)	L-005276-00-0005
Human RPS6KA1 (Rsk1)	L-003025-00-0005
Human RPS6KA3 (Rsk2)	L-003026-00-0005

8.1.6 Real-time qPCR primer

Table 10: List of primers

cDNA	Orientation	Sequence 5´ to 3´
Rps6ka1 (RSK1)	forward	ccggctctcaaaagaggtg
	reverse	agctcggccaggtaaaactt
Rps6ka3 (Rsk2)	forward	gcatgataagctgtttaaatgtcc
	reverse	caaaatgtcatatactttgctagttgc
Rps6ka2 (RSK3)	forward	ttaagcgccatcccttctt
	reverse	ggtttgaacggtggcttg
Rps6ka6 (Rsk4)	forward	ggtggtcatatggtgttcttatgt
	reverse	ggtctcatttctgtctttaccttga

The primer sequences were adapted from Romeo et al. 2013 (Romeo et al., 2013).

8.1.7 Plasmids

Table 11: List of plasmids

Name	Vector	Source
DAB2IP	pcDNA3.1	Kindly provided by Prof. Jer-Tsong Hsieh (University of Texas Southwestern, USA)
FLAG-Spred2	pCMV2-FLAG(N)	Kindly provided by Akihiko Yoshimura (Keio University School of Medicine, Japan)
GFP-CAPRI	pEGFP-C2	Kindly provided by Prof. Peter Cullen (University of Bristol, UK)
GFP-GAP1[IB4BP]	pEGFP-C1	
GFP-GAP1m		
GFP-RASAL		
GFP-NF1	pCDH-EF1a-EGFP-C2-IRESPuro	Kindly provided by Yan Cui (FLI, Jena, Germany)
HA-p120GAP	pcDNA3	Kindly provided by Christian Widmann (University Lausanne, Switzerland)

8.1.8 Kits

Table 12: List of Kits

Name	Company	Cat. No.
RNA isolation kit	Macherey-Nagel	740955
First Strand cDNA Synthesis Kit	Thermo Scientific	K1611
QuantiTect SYBR Green PCR Master Mix (2X)	Qiagen	204141

8.2 Methods

8.2.1 Cell line and stimulations

Cervical cancer (HeLa) cells, Mouse embryonic fibroblasts (MEFs), Human Embryonic Kidney 293 T (HEK293T) cells (kindly provided by Yan Cui, FLI, Jena, Germany) and H-Ras$^{-/-}$, N-Ras$^{-/-}$, K-Ras$^{lox/lox}$ MEFs (kindly provided by Mariano Barbacid, Madrid, Spain) were cultured at 37°C and 5% CO_2 atmosphere in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FCS.

EGF was added to a final concentration of 10 ng/ml. Inhibitors such as U0126, FR108204, BI-D1870, Wortmannin, SB202190 and AG1478 were pre-incubated for 30 min before stimulation.

- U0126 is a highly selective non-ATP competitive inhibitor of both MEK1 and MEK2 (Favata et al., 1998).

- FR180204 is an ATP competitive inhibitor that prevents the catalytic activity of ERK1/2 but not its threonine and tyrosine residue phosphorylation (Ohori et al., 2005).

- BI-D1870 is a specific ATP competitive inhibitor of RSK isoforms (Sapkota et al., 2007).

- Wortmannin is an allosteric, non-ATP competitive inhibitor of the PI3K pathway (Powis et al., 1994, Wymann et al., 1996).

- AG1478 inhibits EGFR tyrosine kinase activity and receptor autophosphorylation by competitively binding to the ATP pocket of EGFR (Han et al., 1996).

- SB202190 is a specific inhibitor of p38alpha and p38beta and binds within the ATP pocket of the active kinase (Davies et al., 2000).

8.2.2 Transfection of cell lines

<u>Transient transfection</u>

Transient cell transfection with plasmid DNA was performed using branched Polyethylenimin (PEI). Briefly, cells were seeded in 6 well plates. HeLa cells can be efficiently transfected at 70-80% confluency. PEI reagent (10 µg/µl stock) was diluted 1:20 in pre-warmed DMEM (25 µl to 500 µl), mixed and further diluted in DMEM 1:25 (60 µl to 1500 µl). Further 1 µg DNA per well was dissolved in 250 µl plain DMEM and 250 µl of the PEI-DMEM solution were added, mixed thoroughly and allowed to complex by incubating 30 min at RT. The cell culture medium of the 6-well plate was replaced by 1 ml of plain DMEM. Then 500 µl of the PEI-DNA mix were added drop-wise per well. The cells were incubated and the medium was exchanged after 3 hours to 2 ml DMEM supplemented with 10% FCS to overcome cellular toxicity by PEI-particles. After 24 hours cells were serum-starved overnight and prepared for western blotting.

<u>Acute knockdown via siRNA transfection</u>

For siRNA-mediated acute knockdowns ON-TARGETplus siRNA- SMARTpool™ from Dharmacon were used. Cells were seeded at a density of 30-40% confluency and transfected with 0.5 µg siRNA per well except of Rsk 2 where 1 µg was used.

siRNA transfections were conducted with Saint-Red transfection reagent from Synvolux Therapeutics according to the manufacturer's protocol. Cells were incubated for 48 h and starved overnight in the absence of serum.

Stable knockdown via lentiviral transduction

Neurofibromin was stably knocked down in HEK293T cells by Yan Cui (Leibniz Institute on Aging – Fritz Lipmann Institute (FLI), Jena) using the targeting sequence GCTGGCAGTTTCAAACGTAA.

8.2.3 RNA isolation, cDNA synthesis and quantitative real-time PCR analysis

Total RNA isolation and purification was performed using RNA isolation kit according to the manufacturer's protocol. The RNA concentration and integrity was determined by spectrophotometric measurements at 260 nm and 280 nm.

cDNA synthesis was performed using cDNA Synthesis Kit with 50 ng/µl of total RNA per sample and Oligo-dT-based priming.

For quantitative real-time PCR (qRT-PCR) analysis, the QuantiTect SYBR Green PCR Master Mix (2x) was used. The master mixes contained 0.125 µM of each GAPDH or RpS6Ka1, RpS6Ka3, RpS6Ka2, RpS6Ka6 primer. The analysis was performed in triplicates using 1 µl of the cDNA preparation in a total reaction volume of 12 µl (Table 13). The PCR was performed in an Eppendorf MasterCycler RealPlex4 under the following conditions: 10 min of initial denaturation at 95°C followed by 45 cycles of [15s at 95°C, 30 s at 55°C and 40s at 72°C]. Melting curve analysis was subsequently performed. Relative transcript levels were determined by calculating 2ΔCt values, using GAPDH expression levels for normalisation.

Table 13: qRT-PCR Reaction Mix

Standard reaction	Volume
Template DNA	1 µl
2x SYBR Green PCR Master Mix	6 µl
Sense primer (10 µM)	0.37 µl
Antisense primer (10 µM)	0.37 µl
RNase-free water	4.26 µl
total	**12 µl**

8.2.4 SDS-PAGE

Electrophoretic separation of proteins was achieved by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a vertical slab electrophoresis system (Hofer scientific, California, #SE400) and 5 to 12.5% polyacrylamide gels. For analysis of proteins with a molecular weight in a range of 100-250 kDa the separating gel contained 5% acrylamide. Small proteins like Ras (21 kDa) as well proteins with a molecular weight up to 90 kDa were resolved in a 12.5% separating gel. SDS gels were run with SDS running buffer at a constant current of 25-30 mA per gel. Following SDS-PAGE, Coomassie staining was used for visualisation of the GST-Raf-1-RBD affinity probe. Then Western blotting and immunodetection were used for specific detection of proteins of interest.

8.2.5 Western Blotting and immunodetection of immobilised proteins

Western blotting by semidry transfer was applied for protein detection after separation via SDS-PAGE. Proteins were blotted with transfer buffer onto PVDF membranes. Generally, a 0.45 µm pore-sized PVDF membrane was used except for Ras-detection which was achieved by blotting onto a 0.20 µm pore sized membrane. Proteins were transferred under distinct conditions due to their different molecular weights which are listed in Table 14.

After protein transfer, the membrane was incubated in blocking solution for 30 min at room temperature (RT). The blocked membranes were incubated in primary antibody solution at 4°C overnight. Next day, membranes were washed three times with 1x TBS-Tween for 10 min and placed in the secondary antibody solution for 45 min at room temperature. Following three further washing steps with 1x TBS-Tween for 10 min, membranes were covered with the HRP substrate (Western Lightning ECL) and the chemiluminescent signal was detected with the Fujifilm LAS-2000 documentation system. After protein detection, stripping buffer was added for 30 min at 50°C and the membrane was washed again before adding 30 min blocking solution and another primary antibody. All incubation and washing steps were performed under shaking and all antibodies were diluted in blocking solution.

Table 14: Western Blot transfer conditions

Protein	Condition	Molecular weight
p38, Erk1/2, Gsk3ß, Akt	1 mA/cm^2, 50 min	38–56 kDa
N-; K-Ras	0.8 mA/cm^2, 45 min	18-21 kDa
NF1	1.5 mA/cm^2, 75 min	250 kDa
Rsk	1 mA/cm^2, 60 min	90 kDa
p120GAP, DAB2IP, EGFR, Sos1	1.5 mA/cm^2, 60 min	120-170 kDa

8.2.6 Expression and purification of the Raf-1-Ras binding domain

The pGEX plasmid containing GST-Raf-1-Ras binding domain (RBD) was expressed in *Escherichia coli* BL21DE3pLysS. Expression was induced at an OD600 of 0.4 to 0.6 with 0.1 mM IPTG for 2 h at 37°C. The cell pellet was then resuspended in Tris buffer (pH 7.5) containing 150 mM NaCl, 10 mM 2-Mercaptoethanol and freshly added protease inhibitors. The cell suspension was lysed by 3 freeze-thaw cycles and by adding of lysozyme, 1 mM EDTA and 1% Triton X-100. The lysate was clarified by centrifugation (30 min, 30 000 rpm and 4°C). GST-Raf-1-RBD proteins were purified directly from bacterial lysates via fast protein liquid chromatography (FPLC) by using a GSTrap™ FF (GE Healthcare) and by adding glutathione to the elution buffer. The purification method is based on the high affinity of GST for glutathione. Eluted proteins were further concentrated with Amicon® Ultra Centrifugal Filters (Merck Millipore) and free glutathione were removed via size exclusion by using pre-equilibrated gel filtration column with PBS. The protein-containing fractions were visualised by Coomassie staining following SDS-PAGE (Figure 9). The fractions containing the most RBD protein were collected afterwards. Finally, the protein concentration was determined by Bradford protein assay.
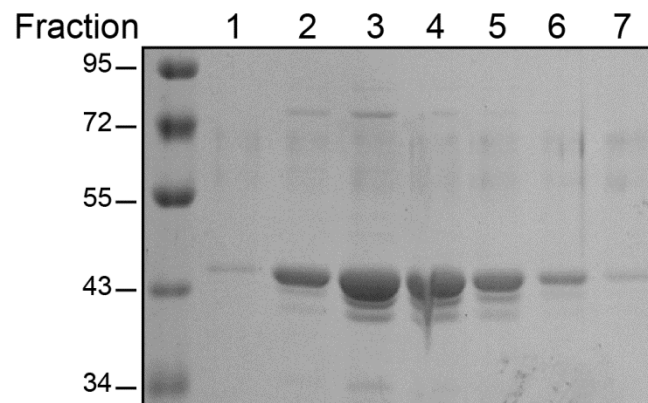
Figure 9: Coomassie stained SDS-PAGE of fractions from RBD purification.
Gel lanes were loaded with fractions collected after gel filtration. Predicted molecular weight of the RBD is 43 kDa.

8.2.7 Ras activity assay

Activation of Ras was analysed by a RasGTP pulldown assay. For this purpose, active GTP-bound Ras was purified from cell extracts and analysed by Western Blotting. This approach was advantageous for measuring RasGTP levels at steady state conditions.

Cells seeded in 6 well plates were deprived of serum overnight and treated as appropriate and lysed in 0.5 ml of ice-cold lysis solution supplemented with protease and phosphatase inhibitors, 100 µM GDP, 2 mM DTT and 25 µg/ml of soluble recombinant GST–Raf-1-RBD protein. The fusion protein was purified as explained above. GDP and GST–Raf-1-RBD were included in the lysis buffer to quench post-lytic GTP-loading and GAP-dependent Ras-bound GTP hydrolysis. Cell material was scraped off and lysates were cleared by centrifugation (20 min, 14 000 rpm, 4°C). GST-Raf-1-RBD/RasGTP complexes were collected on glutathione-sepharose (30 min at 4°C on a rotating wheel), washed once with 750 µl lysis buffer lacking GDP and GST–Raf-1-RBD and processed for Western Blotting. However, this Ras activity assay is unsuitable to directly probe the activity of the regulatory proteins (GEFs and GAPs) in the cell (Figure 10A).

A Ras activity assay

GEF

GTP

measures RasGTP levels at **steady state**

GAP

B Nucleotide uptake in permeabilised cells

GEF

GTP

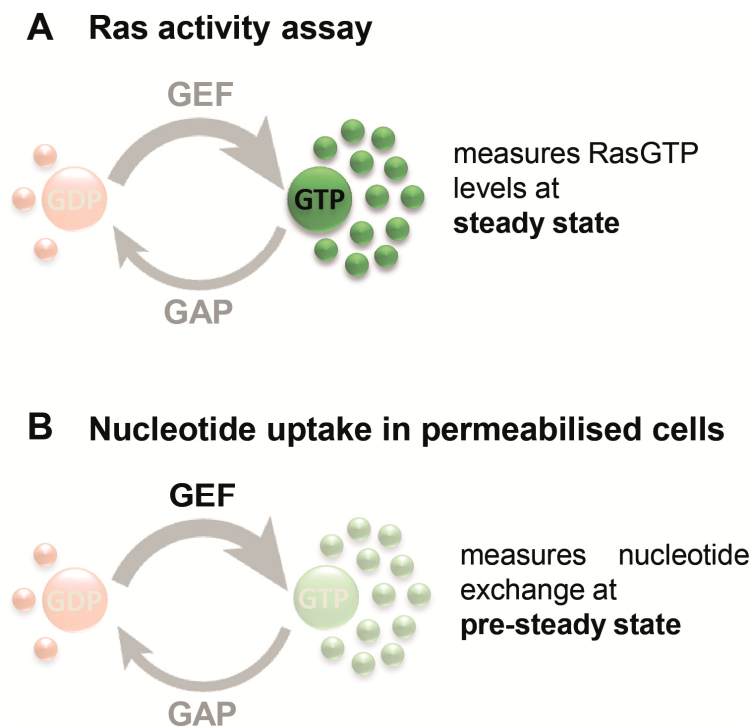measures nucleotide exchange at **pre-steady state**

GAP

Figure 10: Comparison of the applied assays.
A) RasGTP affinity purification was used to assess the steady state level of RasGTP in the cell lysate.
B) The permeabilisation-based approach was performed to measure at pre-steady state the nucleotide exchange on Ras catalysed by GEFs. This figure is adapted from (Hennig et al., 2015).

8.2.8 Nucleotide exchange assay and Ras immunoprecipitation

The Nucleotide exchange assay aimed to monitor, first, the rate of [α-^{32}P]GTP uptake by Ras at pre-steady-state as readout for the activity of GEFs, and second, the [α-^{32}P]GTP/[α-^{32}P]GDP ratio on Ras, again at pre-steady-state (Figure 10B).

Before cell permeabilisation serum-starved cells were seeded in 6-well plates and stimulated as appropriate. The plasma membrane permeabilisation were started by replacing the medium with 0.6 ml/well permeabilisation solution supplemented with 9 MBq [α-^{32}P]GTP and 15 unit/ml freshly thawed streptolysin O (SLO). The SLO, which is a bacterial toxin forms pores in the cell membranes and at same time perfuse the intact cells with [α-^{32}P]GTP. For treated cells, this permeabilisation solution was supplemented with the relevant drug. At this point kinetics were started and due to the nucleotide exchange Ras binds rapidly [α-^{32}P]GTP, which was immunoprecipitated and analysed. The higher the exchange rate, the more labelled Ras accumulated. The reactions were quenched by lysis with 1 ml of ice-cold lysis buffer supplemented with Y13-259 Ras-antibody per well for immunoprecipitation. Ras monoclonal antibody Y13-259 bound GTP and GDP-bound Ras proteins and prevented changes in the Ras loading state. Lysates were cleared by centrifugation (20 min, 14 000 rpm, 4°C) and supernatant was added to a final concentration of 500 mM NaCl, 0.5% sodium deoxycholate and 0.05% SDS. Immunocomplexes were collected on GammaBind–Sepharose by 45 min incubation at 4°C under rotation. After six rounds of washing with 1 ml of ice-cold washing solution, immunoprecipitates were subjected to Cerenkov counting. Afterwards, Ras nucleotides were eluted from the samples and analysed by Thin Layer Chromatography (TLC).

8.2.9 Thin layer chromatography

Eluted Ras associated nucleotides were separated by thin layer chromatography (TLC) as reported by Rubio et al. (Rubio et al., 2003, Rubio et al., 2006). 30 µl of elution solution were added to the Ras-bound sepharose to elute the RasGDP/GTP from the samples. After 20 min incubation at 68°C in the thermoshaker, the samples were spun down and 16 µl of the supernatant were loaded stepwise (2 µl each step) on a PEI cellulose coated plate. The plate was placed for nucleotide separation in the mobile phase. For visualisation of the labelled nucleotides the TLC was exposed for 4 weeks on film for autoradiography.

After sufficient exposure amounts of GTP and GDP bound to Ras-encoded protein were quantitated by densitometry using the Multi Gauge software.

8.2.10 Mathematical modelling

The ordinary differential equation (ODE) models were built in cooperation with Graham Ladds (University Cambridge) and Manuel A. Esparza-Franco (University of Warwick). In the model Ras cycles between GDP and GTP bound states with Michaelis-Menten kinetics, the balance of which depends on the GEF/GAP ratio. To simulate EGF stimulation, receptor recruits GEF to form receptor-GEF complex (R-GEF) increasing the rate of Ras-GTP formation. Downstream signalling from RasGTP enforces the feedback after a small time delay to account for the MAPK cascade. In the GEF-only model, feedback catalyses the separation of R-GEF complexes into free receptors and inactive GEF. In the GAP-only model, feedback catalyses the activation of GAP molecules. The parameters of each model will be available soon from Hennig, A. et al. in the journal Cell Communication and Signaling.

8.2.11 Nanocluster assay

The Nanocluster assay was performed in cooperation with Ian Prior and Alison Beckett (University Liverpool, UK) to determine the Ras molecule distribution at the inner leaflet of the plasma membrane by a combination of an electron microscopic (EM) approach coupled to sophisticated image analysis (Prior et al., 2003, Beckett and Prior, 2015). PM sheets were ripped off from adherent HeLa cells directly onto EM grids. For this purpose cells were grown on glass coverslips and transfected with GFP-H-Ras in 6-well plates with GeneJuice® Transfection Reagent following the manual instructions and incubated overnight in serum-free medium. Electron microscope grids were covered with pioloform and coated with poly-L-lysine. The coverslip with GFP-H-Ras transfected cells (70–80% confluent) was washed with 0.1 M phosphate buffer (pH 7.35) then overlaid on the grids and pressure was applied using a silicon bung for 1 to 2 seconds. When the grid was removed large areas of the plasma membrane sticked to the grid. The grids were then washed by transferring them to a drop KOAc buffer on Parafilm M®. The plasma membrane sheet was fixed for 10 min with fixative solution followed by quenching free aldehyde groups by washing with 20 mM Glycine in for PBS three times 15 min each.

After blocking the non-specific antibody-binding sites with blocking solution for 10 min the plasma membrane sheets were immunolabeled with GFP antibody conjugated to gold particles for 30 min. The labelled grids were washed with blocking solution five changes for 5 min each and five times in water for 2 min each. All of the previous steps were carried out at RT. After washing, the ripped-off plasma membranes were incubated on ice for 10 min in a staining solution and picked up in 5-mm cooper wire loops and left to dry in the loops before analysing the H-Ras distribution by a transmission electron microscope (JEOL 1010). To analyse the complete gold patterns statistically, methods for point pattern analysis were used. Ripley's K-function analysis (Ripley, 1977, 1979; Philimonenko et al., 2000) evaluates an exhaustive map of all interparticle distances over the study area and compares the observed distribution with that expected from complete spatial randomness.

9 Results

9.1 EGF induces transient Ras activation

It has been demonstrated that growth factors induce short-lived Ras activation in cells emerging from quiescence (Muroya et al., 1992, Qui and Green, 1992, Langlois et al., 1995b). Previous reports showed that this transient Ras activation is important for the induction of mitogenic programs (Marshall, 1995b, Crespo and Leon, 2000). However, the mechanisms terminating growth factor induced Ras activation is still unclear.

In order to study Ras activity, an *in vitro* Ras activity assay was performed taking advantage of the Ras binding domain (RBD) to bind RasGTP. As a model system immortalised mouse embryonic fibroblasts (MEF cells) and a cervical cancer cell line (HeLa cells) were used due to easy manipulation and widely acceptance in this field of research. Robby Markwart (former Diploma student) showed that EGF stimulated HeLa cells display a well-defined transient Ras activation kinetic (Figure 11A) characterised by a strong increase in RasGTP levels within few minutes followed by a decline to basal levels after 5 min of EGF stimulation (Figure 11C). To examine whether this effect is mechanistically conserved, the Ras activity assay was repeated in MEF cells, and observed a similar transient pattern of RasGTP formation (Figure 11B).

The MAPK pathway is one of the downstream signalling cascades initiated by active Ras. The activity of this pathway can be measured by the phosphorylation of the downstream effector kinases for example Erk. The phospho-Erk level was probed for the indicated time points. In both cell lines, a sustained activation of Erk was observed exceeding the temporal profile of Ras activation (Figure 11).

Previous studies have demonstrated that prolonged EGF stimulation results in EGFR internalisation or degradation and subsequent interruption of the upstream signalling of the Ras/MAPK pathway. To assess the EGFR activity different antibodies were used to detect various EGFR phosphorylation sites. In HeLa cells a continuous activation of the EGFR tyrosine autocrossphosphorylation sites Y1045 and Y1068 (Figure 11) was detected. Thus, Ras activity decreases even in the presence of EGF receptor signalling.
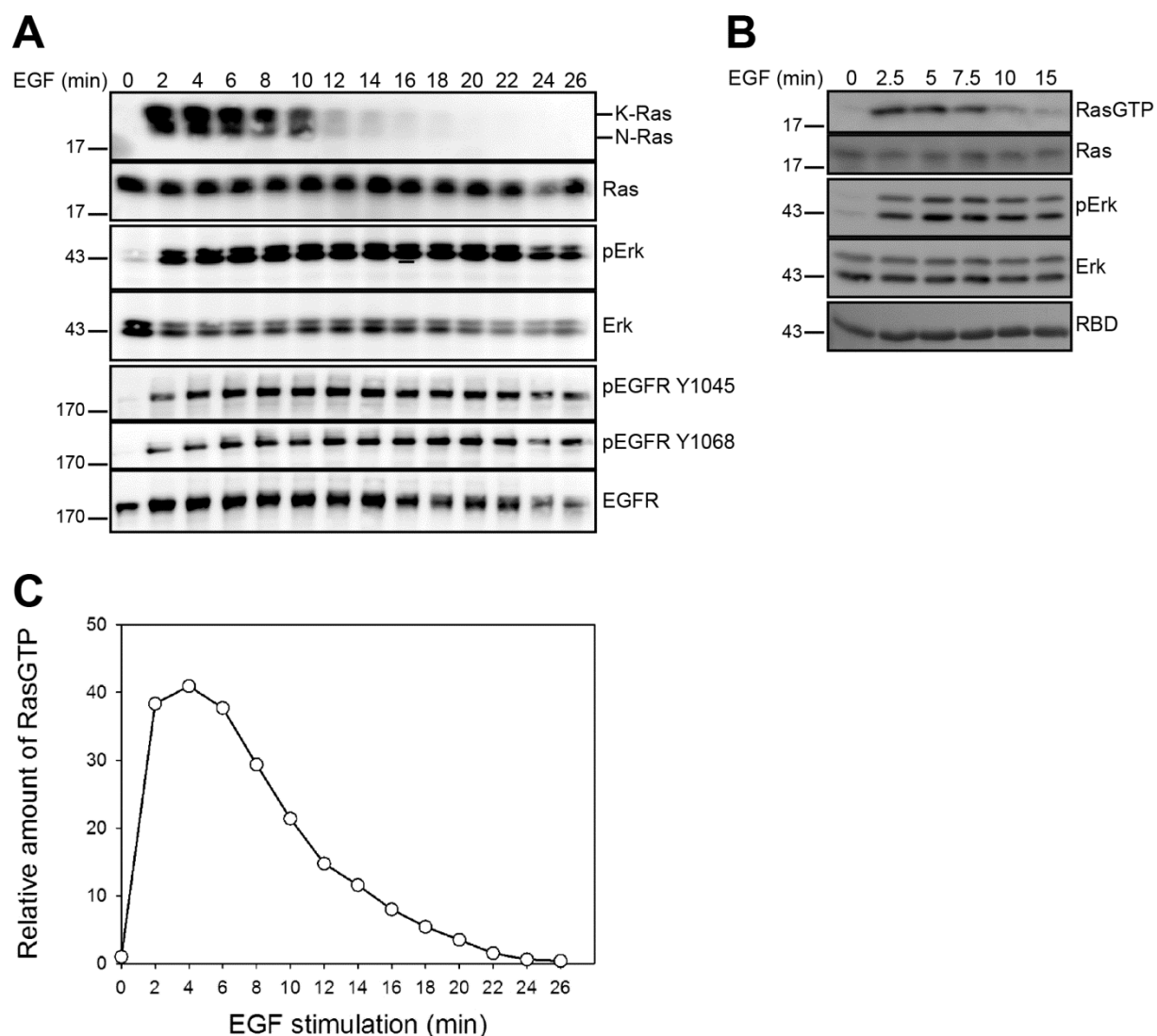
Figure 11: EGF induces transient Ras activation.
A) Transient Ras activation in HeLa cells. Serum-starved HeLa cells were challenged with 10 ng/ml EGF and Ras activation was determined via RasGTP activity assay. The two bands correspond to K-Ras and N-Ras (collectively referred to as Ras from here on). EGFR and Erk phosphorylation were determined using phosphosite-selective antibodies. RBD: Coomassie stain of Ras binding domain used for collecting RasGTP. **B)** MEF cells challenged with EGF were processed for Ras and Erk activity assays as in (A). **C)** A quantification of the RasGTP kinetics is shown. Figure A and C adapted from Robby Markwart.

9.2   Mathematical model describing the feedback stimulation of GAP activity

The central dogma for Ras deactivation has been the downregulation of Sos, which is downstream of the growth factor receptor activation and drives the activation of Ras. In contrast, the role of RasGAPs downstream of active Ras is less characterised. To understand the contribution of GEFs as well of GAPs in Ras activation/deactivation a cooperation with Manuel A. Esparza-Franco (University of Warwick, UK) and Graham Ladds (University of Cambridge, UK) was started. They designed a mathematical model describing sequential growth factor-induced Sos activation, RasGTP formation and a RasGTP-driven feedback loop for Sos-inhibition (Figure 12A). This model is called GEF-only model for the reason that just one feedback loop to decrease GEF activity is included. On the basis of this model, they simulated Ras activation kinetic in the background of absent, low or high basal GAP activity (Figure 12B). The simulation demonstrated that a high basal GAP activity is needed to diminish the RasGTP level, as well if the system parallel invoke a feedback inhibition of Sos. In contrast, no or low GAP activity form a prolonged RasGTP level.

To define the basal GAP activity in HeLa cells the basal GAP activity was manipulated via combined or single siRNA mediated knockdown of three major GAPs (p120GAP, DAB2IP and NF1) or GFP-NF1 / HA-RASA1 GAP overexpression in quiescent HeLa cells. In the Ras activity assay the basal RasGTP level did not change in GAP diminished cells, whereas the overexpression of NF1 triggered a slight decrease of the basal Ras level (Figure 12C). This experiment provided first-hand evidence for a low basal GAP activity in HeLa cells.
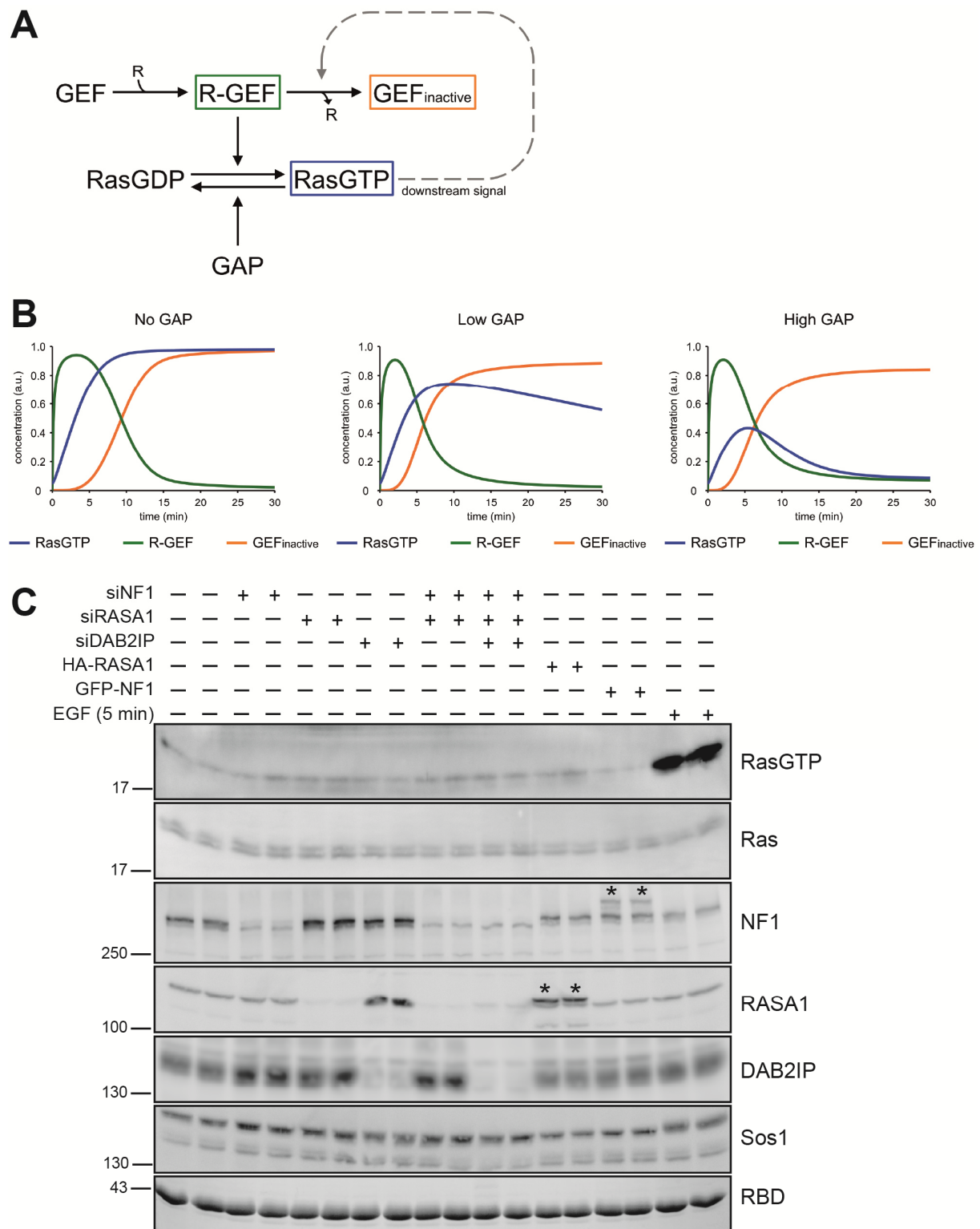
Figure 12: Mathematical model and basal GAP activity
A) Minimal Ras model describing Ras deactivation as induced by RasGTP-dependent feedback inhibition of Sos (GEF-only model). **B)** Simulations of Ras activation/deactivation using the model from (A) in a background of absent, low or high basal GAP activity. **C)** Basal RasGTP amount following manipulation of RasGAP levels via combined or single knockdown or GAP overexpression. 5 min EGF stimulation is shown as positive control after stimulation. The illustrations shown in A and B were provided by Manuel A. Esparza-Franco.

9.3 EGF mediates sequential engagement of Sos

9.3.1 Sos is phosphorylated downstream of Ras

Previous studies indicated the presence of negative feedback loops sparked downstream of Ras where the Ras effector kinases MEK, Erk and Rsk phosphorylate Sos after growth factor stimulation and thereby interfere with the ability of Sos to activate Ras (Waters et al., 1995b, Holt et al., 1996, Douville and Downward, 1997, Kim et al., 2006). As shown by Katharina Wolff (Master student), the phosphorylation of Sos (indicated by a electrophoretic mobility shift) follows EGF stimulation in HeLa cells. This phenomenon was abolished by treatment with a MEK or Erk specific inhibitor, whereas the pan-Rsk inhibitor treated cells showed Sos1 protein levels similar as untreated cells (Figure 13).
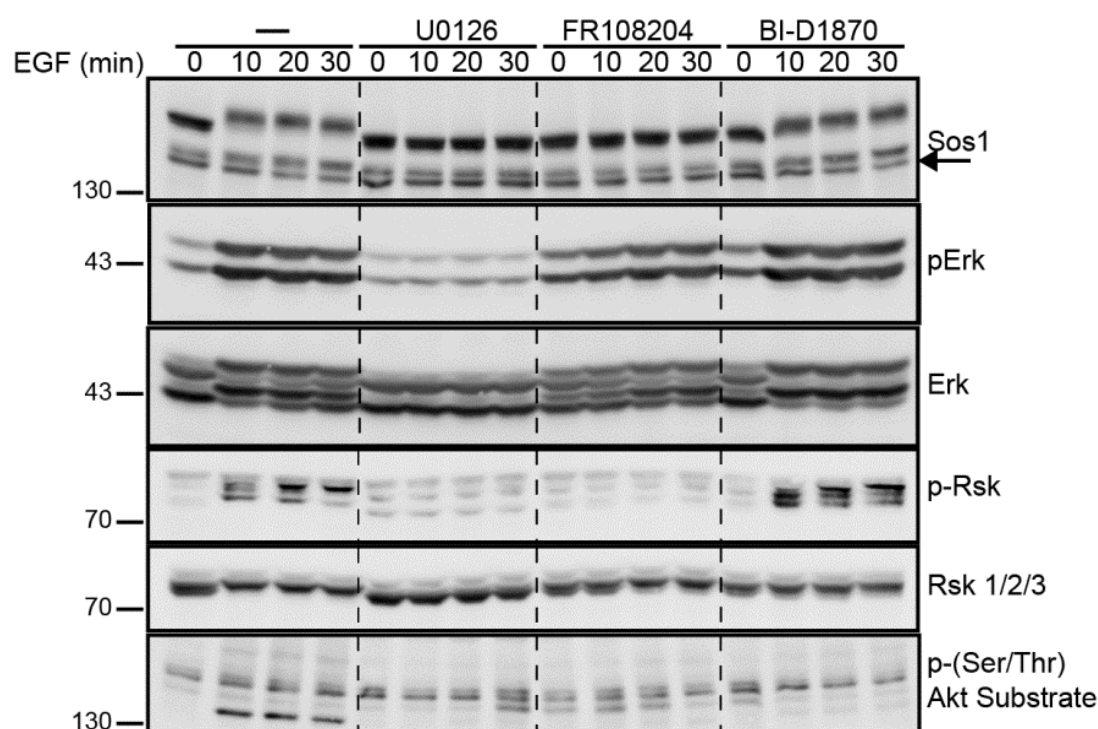
Figure 13: EGF induces a mobility shift in Sos.
HeLa cells were treated with inhibitors for MEK (U0126), Erk (FR108204) or Rsk (BI-D1870) prior to stimulation with 10 ng/ml EGF. Extracts were processed via western blotting using the indicated antibodies. The detection of pErk protein level served as MEK inhibitor control, whereas the pRsk signal was used to monitor the Erk-inhibition. p(Ser/Thr) Akt substrate protein levels validated the potency of the Rsk inhibitor. The arrow marks an unspecific doublet band. Figure was modified from Katharina Wolff.

9.3.2 Sos activity does not decay with decline in RasGTP levels

As a next step it was important to prove the assumption that Sos phosphorylation concomitantly downregulates Sos activity. The conventional Ras activity assay (Figure 10A) is limited by the assessment of the RasGTP level at steady state, the

nucleotide exchange assay was used for this purpose. This approach enabled to assay the nucleotide exchange at the pre- steady state. This is particularly relevant in view of the fact that EGF induced RasGTP accumulation could be caused either by an increase of nucleotide exchange on Ras or by the decrease of the GTP-hydrolysis activity of Ras. Therefore, the readout of the Ras activity assay is not suitable to distinguish between altered GEF and GAP action. The nucleotide exchange assay was technically achieved by streptolysin O (SLO) permeabilised cells and the measurement of direct binding of [α-32P]GTP to Ras over time. Afterwards the nucleotides bound to Ras were separated by thin layer chromatography (TLC), to monitor the RasGTP level under different conditions. The combined results of the applied permeabilisation assay and the TLC aimed to shed light on the actual status of GAP activity in cells. Up to know an assay to explore direct GAP activity is not feasible. Pretests validated that the radioactivity is specifically associated to Ras in [α-32P]GTP loaded HeLa cells. In the nucleotide exchange assay only the combination of the Ras-IP antibody together with SLO is able to raise the measured radioactivity. This effect was reversed by excess of cold GTP. Further, the specificity of the permeabilisation assay was shown before in other cell types (Rubio 2000).

In order to investigate the effect of SLO permeabilisation on Ras activation, HeLa cells were permeabilised with SLO in the presence of EGF, followed by a Ras activity assay. This experiment revealed that Ras as well as its downstream targets such as Erk flow out of the cell over time, which affected the intensity of RasGTP levels but not the transient kinetic (Figure 14A).

In the following experiments the nucleotide uptake at three different time points was examined to determine the role of GEFs in the feedback deactivation of Ras. At first, the nucleotide exchange of the unstimulated cells was measured, corresponding to the basal level of Ras nucleotide exchange activity. Next, 5 min post EGF stimulation, when RasGTP levels peaked (Figure 11C) it was measured. The EGF stimulation at this time point increased markedly the rate of nucleotide uptake by Ras and an equal accumulation of RasGTP in the same samples could be shown in the TLC (Figure 14A). These data confirmed the commonly accepted view of GEFs being involved in EGF-induced Ras activation. In order to address the GEF activation at the time where the RasGTP level decreases the third measurement was set at 20 min after constant EGF exposure. Surprisingly, the data revealed a similar high nucleotide exchange rate on Ras at this late time point compared to 5 min post EGF stimulation

(Figure 14B and C). In contrast, the GTP level in the TLC dropped back (Figure 14B and C) as detected by the conventional Ras activity assay (Figure 11A). Furthermore, these findings were also verified in MEF cells (Figure 14D) underlining the importancy of this observation suggesting a general mechanism. Thus, the modulation of Sos activity is not the major driver of restoring basal RasGTP levels within this early window of growth factor stimulation. In line, we suggest that GAPs play an essential role in counteracting the high GEF activity and shut down of Ras activity.
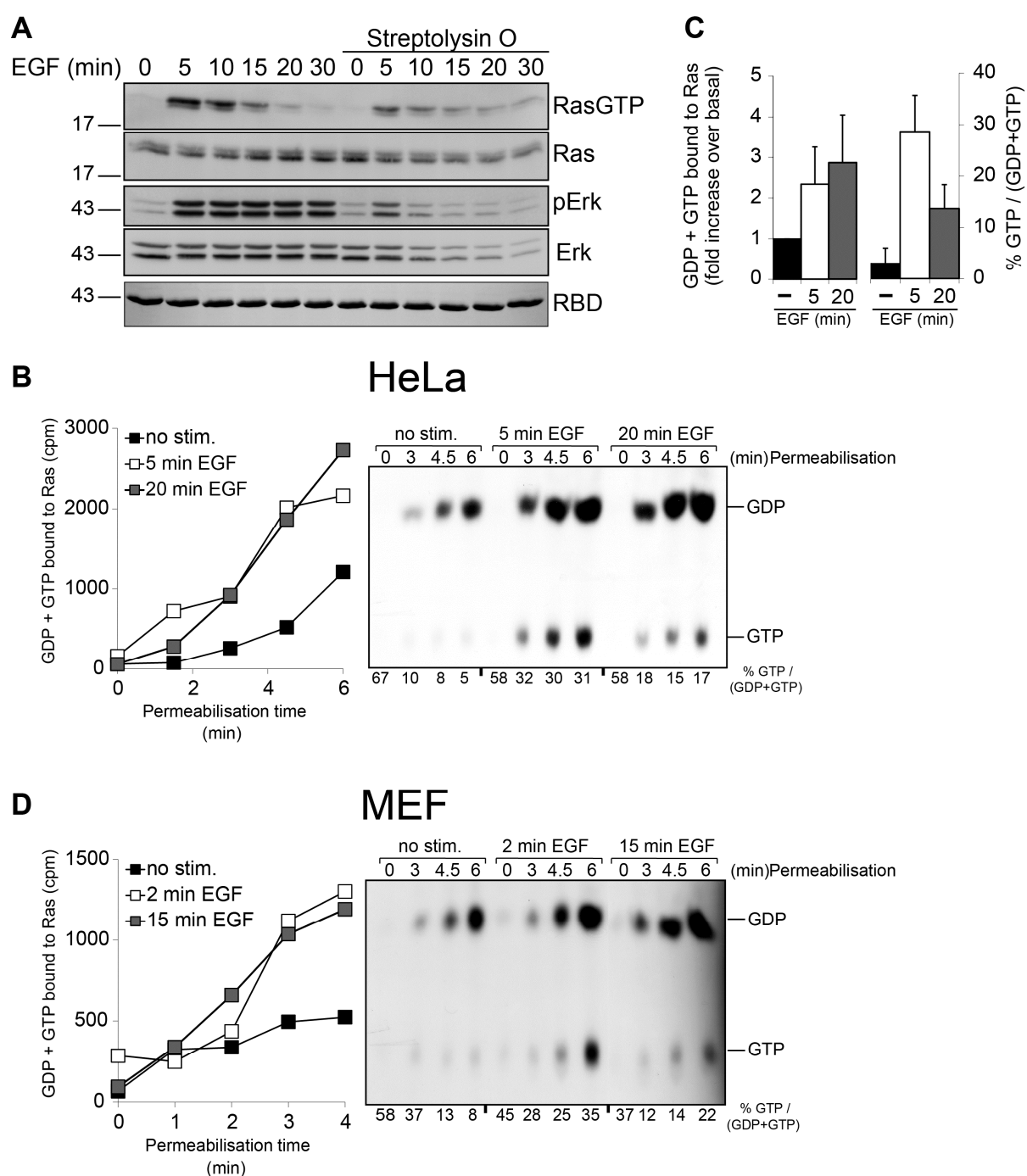

Figure 14: Please see legend on following page.

Figure 14: EGF induces transient RasGTP accumulation and prolonged up-regulation of nucleotide exchange.
A) HeLa cells were permeabilised or not with SLO followed by EGF stimulation and subjected to a Ras activity assay. SLO was added simultaneously with EGF. **B)** Nucleotide exchange assay in permeabilised HeLa cells at 0, 5 and 20 min after EGF administration. Nucleotides associated to Ras-IPs were additionally eluted from Ras and separated via TLC (on the right). %GTP/(GDP +GTP) values were determined by densitometry and marked under the panel. Of note, initial values start off high and level off only at later time points. This pattern is due to the difference in time required for single Ras proteins versus the entire Ras population to achieve steady-state nucleotide turnover. **C)** Quantification of nucleotides bound to Ras-IPs 6 min after permeabilisation (as recorded in A). On the left, the amount of GDP + GTP bound to Ras was plotted as the fold increase of radioactivity bound to Ras in EGF-stimulated versus unstimulated cells. On the right, the amount of GTP expressed as percentage of GDP + GTP was plotted. Values are means ± S.E.M. for three independent experiments. **D)** Same experiment as in (B) performed in MEF cells.

The previous data suggest that Ras deactivation is driven by increased GAP activity in a background of ceaseless high nucleotide exchange. To confirm this prediction a combination of the permeabilisation approach with the Ras activity assay was performed. For this purpose, the cells were loaded with GTP or with the non-hydrolysable GTP analogue GppNHp along with SLO permeabilisation and EGF stimulation. HeLa cells were lysed and the fraction of active Ras was assayed by the Ras activity assay. In GTP loaded cells the Ras activation is transient and returns to the basal state after 20 min EGF stimulation. In comparison, active Ras was accumulated after 5 and 20 min of EGF stimulation in GppNHp loaded cells (Figure 15). This indicates first, an early high GEF activity that drives fast GppNHp loading of Ras, and second, proofed the presence of high GAP activity due to the fact that Ras-GppNHp is insensitive to GAP action. Taken together, the findings strongly supported the initial hypothesis that Ras deactivation is triggered by a timely gradual upregulation of GAP(s) activity in a background of continuous high nucleotide exchange.
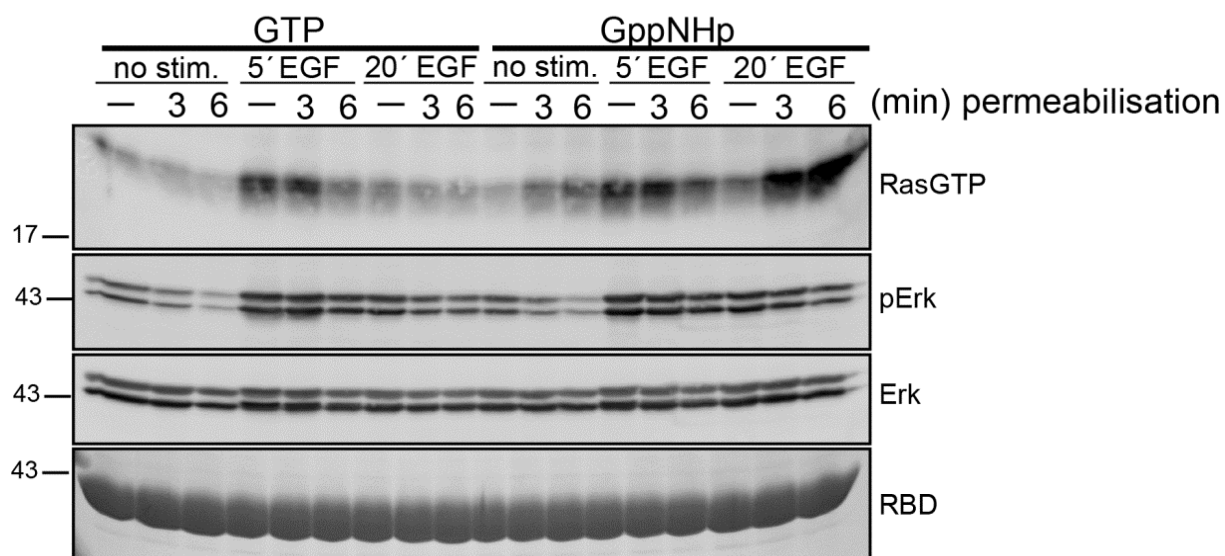
Figure 15: GppNHp but not GTP promotes strong Ras activation at late time points of EGF stimulation.
HeLa cells were permeabilised for the indicated time frames in the presence of GTP or GppNHp before (no stim.), 5 min or 20 min after EGF stimulation. Reactions were stopped by cell lysis and cell extracts were subjected to biochemical analysis of Ras and Erk activation.

9.4   A feedback mechanism limits the duration of Ras activation

As mentioned earlier, previous studies proposed negative feedback loops induced downstream of Ras by the MAPK pathway to terminate Ras activity (Langlois et al., 1995a, b, Waters et al., 1995a, Waters et al., 1995b, Dong et al., 1996, Douville and Downward, 1997). To re-evaluate the negative feedback loop in the Ras/Erk pathway specific downstream kinase inhibitors or siRNA mediated knockdown approaches were used.

9.4.1   Inhibition of MEK and Erk prolongs RasGTP level

By using U0126, a highly specific MEK inhibitor, Erk phosphorylation was abolished and sustained Ras activation in HeLa cells was observed (Figure 16A). This finding suggests that an activation of MEK is involved in Ras deactivation. Similarly, this regulatory role of MEK was detected in MEF cells (Figure 16A). To look further downstream of MEK the cells were treated with FR108204, a potent Erk inhibitor. To monitor inhibition, an antibody specific for phospho-MAPK substrates was used. According to the results obtained with the MEK inhibitor the RasGTP level is prolonged through Erk inhibition (Figure 16B). Taken together, the Ras deactivation seems to be triggered via the MEK/Erk pathway, however the molecular mechanisms remained unknown.

In addition, an experiment in MEF cells which only express K-Ras was performed. These cells showed a similar time course of prolonged Ras activity in response of MEK inhibition (Figure 16C).
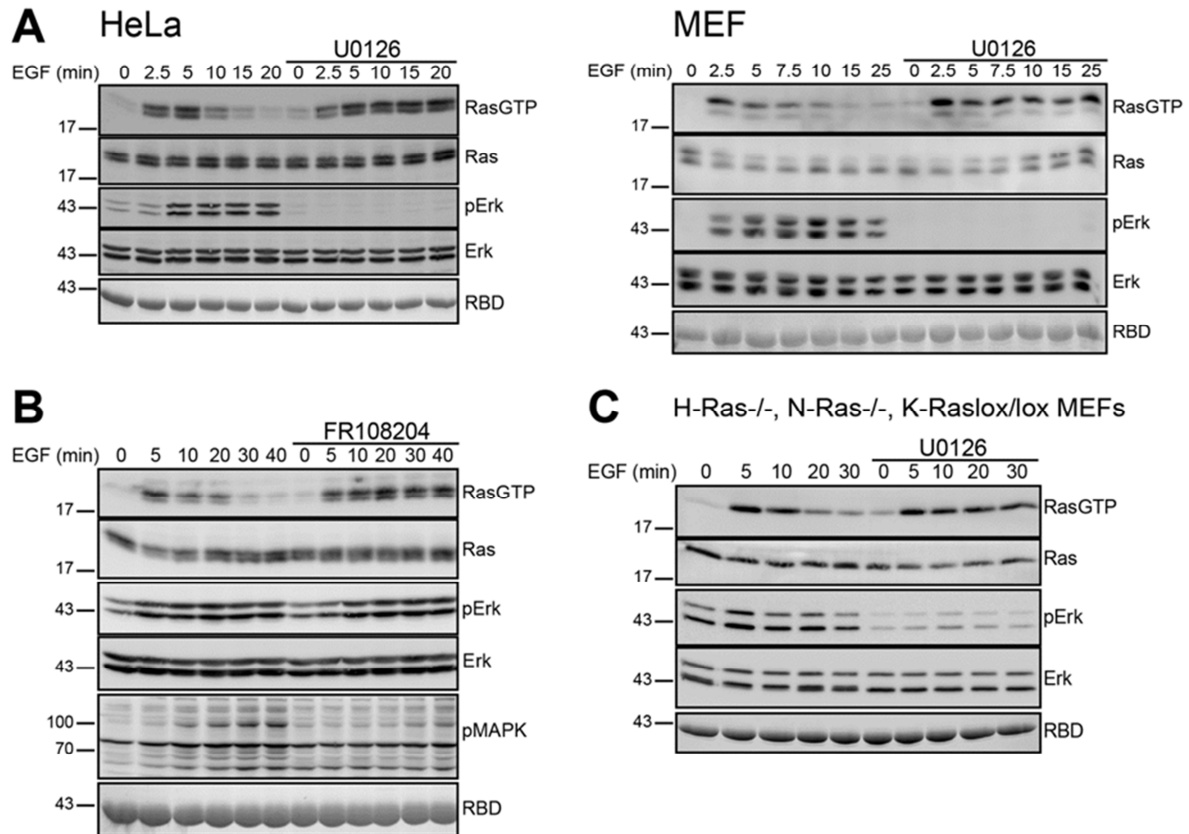
Figure 16: Inhibition of the MEK/Erk pathway prolongs Ras activation.
A) Resting HeLa or MEF cells were left untreated or treated with the MEK inhibitor U0126, followed by EGF stimulation and subjected to a Ras activity assay. **B)** HeLa cells treated with the Erk inhibitor FR108204 or untreated were challenged with EGF and analysed for Ras and Erk activity pMAPK: Ab against the phosphorylated Erk consensus motif. **C)** Same experiment as in (A) performed in H-Ras-/-, N-Ras-/-, K-Raslox/lox MEFs expressing only K-Ras. The results are representative for at least three independent experiments.

9.4.2 Inhibition of Rsk also leads to prolonged RasGTP accumulation

Accumulating evidence suggests the existence of an Rsk-initiated negative-feedback loop that reduces Ras activation (Douville and Downward, 1997). To verifiy the possibility of Rsk activity being involved in modulating Ras activity a pan-Rsk inhibitor was used. Comparable to the inhibition of MEK or Erk, the block of Rsk activity using the BI-D1870 inhibitor led to increased RasGTP level (Figure 17A). qRT-PCR analysis revealed that Rsk 1 and 2 are the predominantly expressed isoforms in HeLa cells (Figure 17B). The siRNA-mediated depletion of Rsk1 and 2 mimicked the inhibitor effects in terms of prolonged of RasGTP accumulation (Figure 17C).

Overall, pharmacological inhibition as well as protein ablation of the MAPK-activated kinase Rsk caused a prolonged Ras activity, suggesting that the Ras deactivation is triggered by an Rsk-dependent feedback loop. However, these findings do not explain whether this process involves changes in the activity of GEFs, GAPs or both.
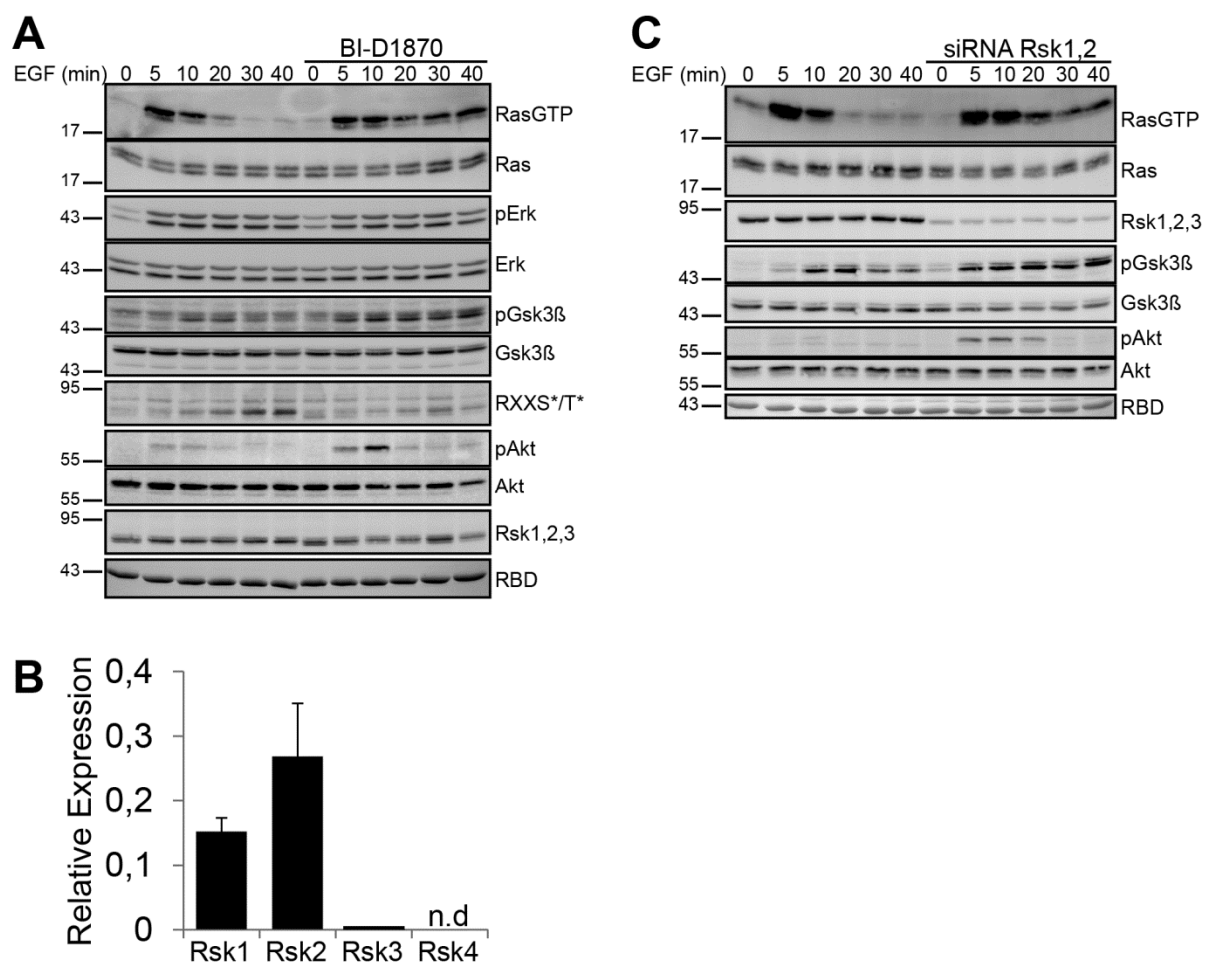
Figure 17: Rsk inhibition leads to RasGTP accumulation at late EGF stimulation time point.
A) Starved HeLa cells were pre-treated with the pan-Rsk inhibitor BI-D1870 and challenge with EGF for the indicated periods of time. The activation status of Erk and Rsk was monitored using phospho-site specific antibodies against Erk, Rsk and the phoshorylated Rsk/Akt-consensus motif (RXXS*/T*). Note that phosphorylation of the common Rsk and Akt substrate GSK3ß did not decline, possibly due to a feedback activation of Akt in response to Rsk inhibition. The results are representative for at least three independent experiments. **B)** Real-time PCR analysis of Rsk isoform expression in HeLa cells. **C)** Rsk1 and Rsk2 were simultaneously silenced via siRNA in HeLa cells followed by stimulation with EGF and biochemical analysis of Ras activation.

9.4.3 PI3K/Akt and p38 inhibition do not influence the transient Ras kinetics

In addition to the MAPK pathway, Ras activity initiates other signalling cascades in a coordinated intracellular signalling network. With the intention to address whether the Ras deactivation is connected to one of these pathways the PI3K/Akt pathway was inhibited with Wortmannin and the p38 pathways with SB202190. The interruption of both effector branches did not affect RasGTP levels especially on late time EGF stimulation (Figure 18). Overall, after testing several pharmacological inhibitors within the Raf/MEK/Erk pathway the data disclose that the Ras deactivation originates from the MAPK-pathway, but the mechanisms behind the feedback loop are not known yet.
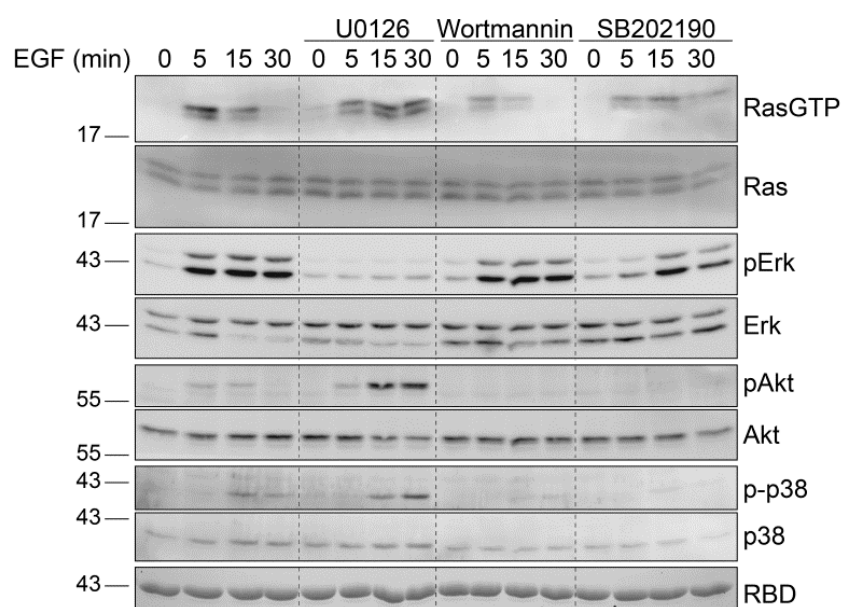
Figure 18: EGF induces PI3K and p38 independent transient Ras activation.
HeLa cells pre-treated with the MEK inhibitor U0126, PI3K inhibitor Wortmannin or the p38 inhibitor SB202190 were challenged with EGF and subjected to a biochemical Ras activation assay.

9.5 Sos activity is not affected by the feedback mechanism

To clarify whether the MAPK pathway regulates Sos activity, GAP activity or both the nucleotide exchange assay was performed in the background of MEK (Figure 19A) and Rsk inhibition (Figure 19B). The chromatographic separation of Ras-bound GDP/GTP showed that the inhibitor treatment restored RasGTP accumulation at 20 or 40 min post-EGF stimulation (Figure 19A/B). These findings were consistent with the western blot analysis of the RasGTP pull-down (Figure 16A and Figure 17A) and confirmed the existence of the feedback loop. Importantly, the nucleotide uptake rate was sustained at a high level following EGF stimulation and was not affected by the MEK/Rsk inhibition (Figure 19). Since Sos is the only relevant GEF expressed in

HeLa cells, these findings suggest that despite its phosphorylation Sos activity in terms of Ras activation is not affected by the MAPK pathway.
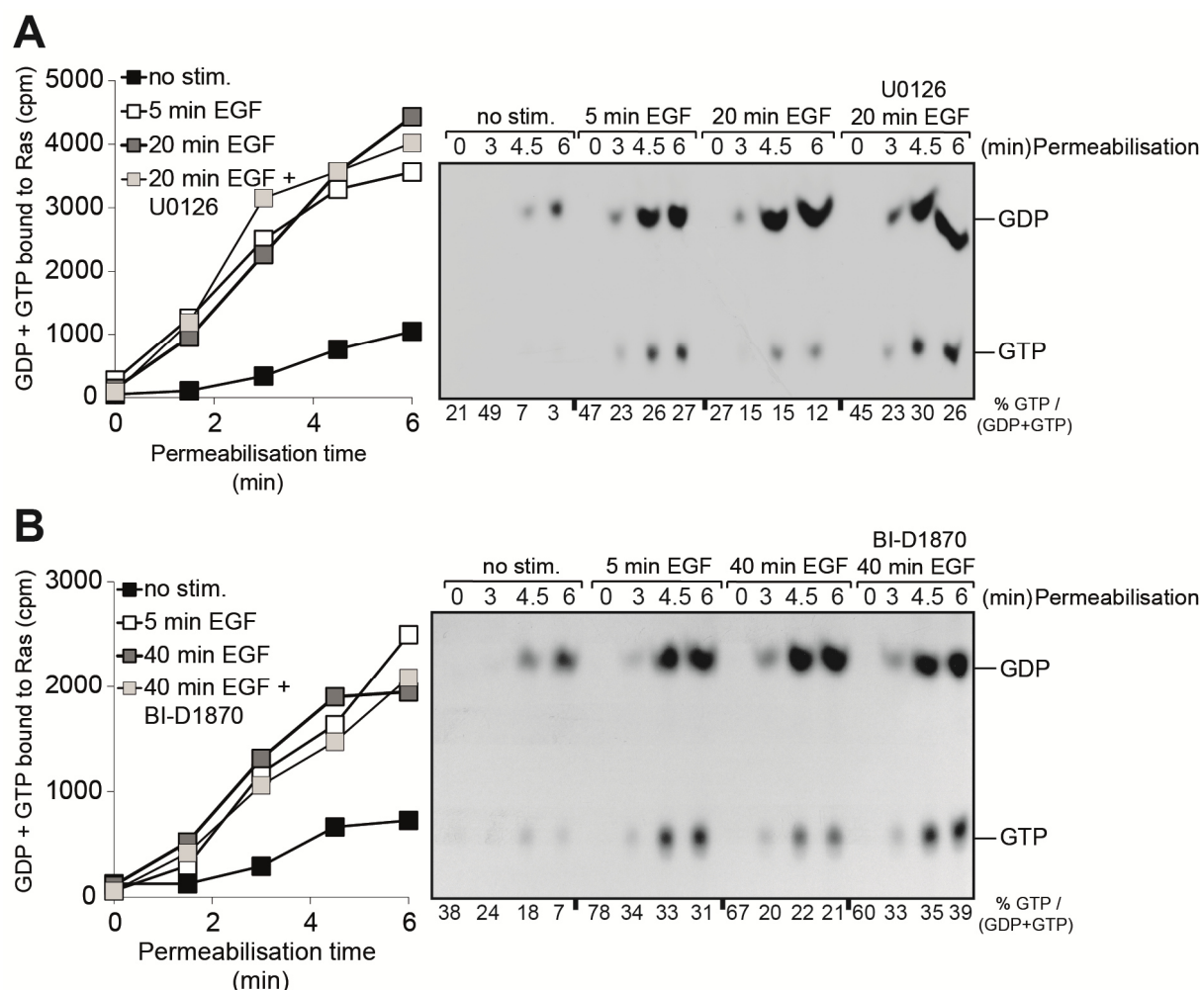
Figure 19: Feedback deactivation of Ras is mediated via GAP up-regulation.
A) Quiescent HeLa cells were pre-treated with U0126 as indicated, challenged with EGF and subjected to analysis of Ras nucleotide exchange. **B)** Same experiment as in (A) performed in cells treated with the pan-Rsk inhibitor BI-D1870. %GTP/(GDP +GTP) values were determined by densitometry and marked under the panel.

9.6 NF1 is the responsible GAP within the feedback loop

6 major classes of GAPs have been described in literature so far. To figure out which RasGAP is responsible for keeping the Ras activation transient their respective expression patterns were assessed by immunoblot detection (Figure 20A). Tagged-overexpression plasmids and siRNA mediated knockdown for NF1 served as controls for antibody specificity. The western blot analysis showed expression of NF1, DAB2IP and RASA1 also known as p120GAP. The GAP1 family members CAPRI, GAP1m and RASAL1 were poorly expressed endogenously, while the

plasmid-encoded overexpression was clearly detected. For GAP1[IB4BP] the immunodetection failed due to a non-functional antibody.

Additional public gene expression datasets, which are placed at GEO (GSE6783), were checked for GAP expression levels. In this study serum-starved HeLa cells were stimulated with EGF for the indicated time intervals up to 480 minutes. By transcriptome analysis p120GAP (RASA1) and NF1 were detected in HeLa cells (Figure 20B) but likewise no appreciable levels of the GAP1 family members (GAP1m, GAP1[IP4BP], CAPRI, RASAL) (Amit et al., 2007).
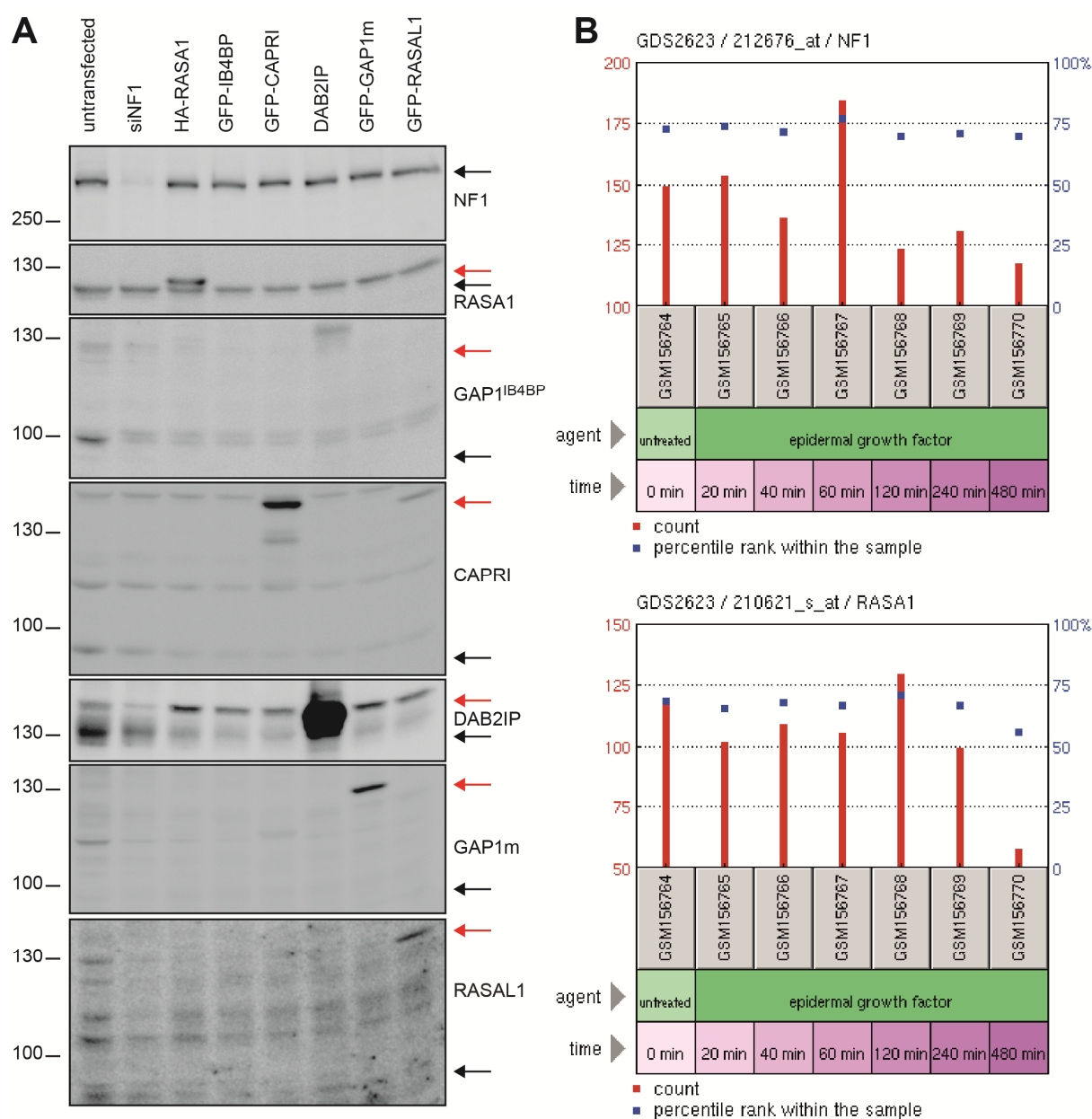
Figure 20: Please see legend on following page.

Figure 20: Immunoblot detection and GEO search for GAP protein expression in HeLa cells
A) Western blot analysis showing GAP expression level in HeLa cells. Red arrows mark overexpressed proteins, black arrows mark predicted mobility level of endogenous protein.
B) The cervical carcinoma HeLa cells gene expression datasets from the GEO dataset ID GSE6783 with the platform ID GPL96 were used to identify expression of GAP species. In this study serum-starved HeLa cells were stimulated with EGF for the indicated time intervals up to 480 minutes (Amit et al., 2007). Depicted are the expression for the both detected GAPs NF1 and RASA1.

9.6.1 siRNA mediated NF1 knockdown prolongs RasGTP accumulation

All three GAPs, NF1, p120GAP and DAB2IP, were abundant in HeLa cells during EGF stimulation and no changes were detected in their expression level (Figure 21A, lower panels). In order to address the involvement of each of the three GAP proteins in Ras deactivation, individual knockdowns were performed by using respective siRNA. The knockdown of the p120GAP (RASA1) showed no remarkable changes on RasGTP levels compared to untransfected cells (Figure 21A). Further, it was verified that the siRNA treatment had no influence on the integrity of the MAPK mediated feedback mechanism by combining siRNA knockdown with MEK inhibitor treatment. Likewise, the DAB2IP knockdown did not affect the decrease in RasGTP level at late time points after EGF stimulation (Figure 21B). In contrast, NF1 depletion led to sustained RasGTP level similar to the effects of MEK inhibition (Figure 21C). Moreover, NF1 knockdown showed no changes on investigated GAP levels. This result was reproduced in HEK293T cells stably transfected with shRNA for NF1 (Figure 21D).
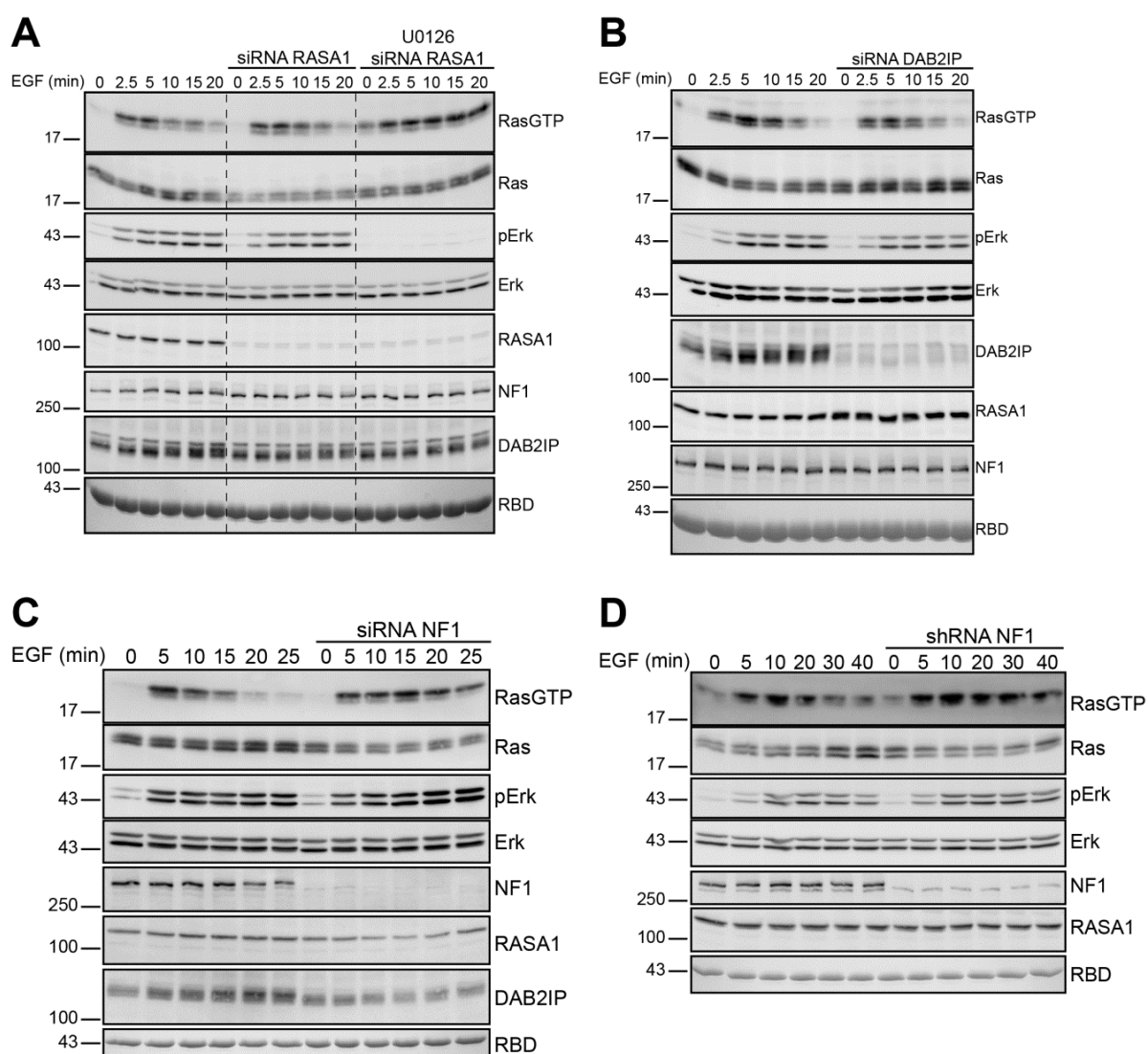
Figure 21: Feedback mediated stimulation of NF1 mediates Ras deactivation.
A) EGF induced Ras activation in HeLa cells subjected to previous siRNA-mediated silencing of RASA1. siRNA transfected cells were additionally treated with the MEK inhibitor U0126. **B)** DAB2IP-silenced HeLa cells were treated with EGF for indicated time points. **C)** Same experiment as in (B) performed in NF1-silenced HeLa cells. **D)** Time course of EGF-driven Ras activation in HEK293T cells and a derivative line with stable shRNA-mediated knockdown of NF1. The results are representative for at least three independent experiments.

9.6.2 NF1 acts in a background of high nucleotide exchange activity

Previous experiments displayed similar prolongation of RasGTP formation after MEK/Rsk inhibition and NF1 knockdown. To understand the precise role of NF1 at the time where the Ras level declines, the permeabilisation assay to measure the GTP uptake by Ras was performed in NF1 depleted HeLa cells. On the one hand, the TLC figured out that the RasGTP level is sustained after 20 min EGF stimulation upon NF1 knockdown, similar to the western blot of the Ras activity assay. On the other hand, the nucleotide exchange is continuously proceeding, indicating that the GEF activity is not affected by NF1 depletion.

Hence, NF1 is the major regulatory protein that decreases RasGTP levels at late time points of growth factor signalling.
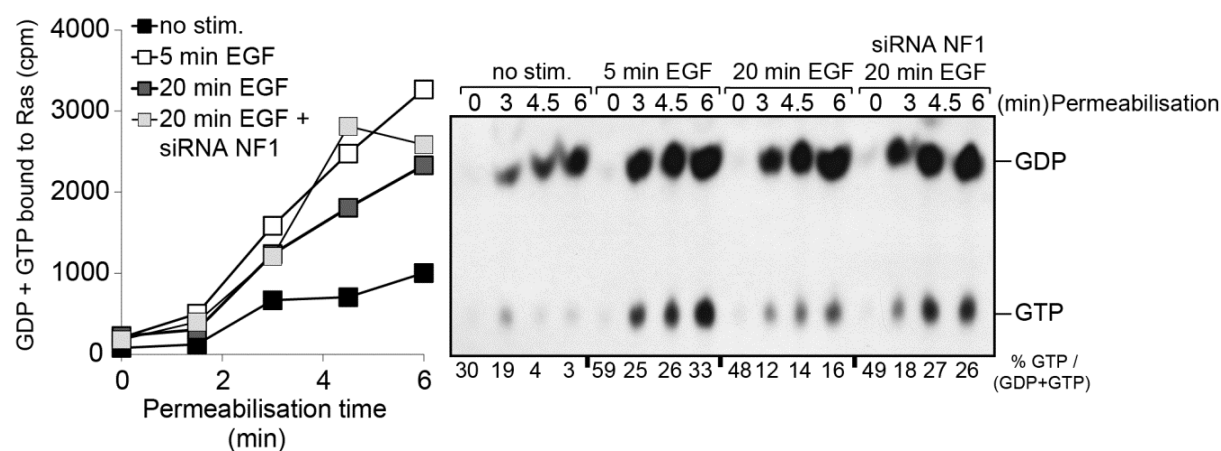
Figure 22: NF1 activation mediated Ras deactivation
HeLa cells subjected to siRNA mediated silencing of NF1 were challenged with EGF. Cells were permeabilised at 0, 5 and 20 min after EGF stimulation and processed for the analysis of nucleotide exchange on Ras. Nucleotides associated to Ras-IPs were additionally eluted from Ras and separated via thin layer chromatography (TLC, on the right). %GTP/(GDP +GTP) values were determined by densitometry and marked under the panel.

9.7 NF1 harbours conserved Erk and Rsk phosphorylation sites

The former results unveil an engagement of Rsk kinase signalling likewise NF1 in the downregulation of RasGTP level after growth hormone receptor signalling. At present, little is known about the regulation of NF1 and its precise role in controlling Ras signalling pathways. In order to elucidate a biochemical link between Rsk signalling and NF1 the NF1 protein sequence were checked for specific Erk and Rsk phosphorylation sites. The multiple sequence alignment of human NF1 with the NF1 amino acid sequence from a variety of other species revealed only one MAPK motif (PxSP) in humans at the C-terminal end from amino acid 2758 to 2761 (Figure 23). This phosphorylation motif is highly conserved amongst mammals (*Homo sapiens*, *Mus musculus* and *Rattus norvegicus*) and *Drosophila melanogaster*. In contrast, NF1 contains three Rsk phosphorylation site consensus motifs (RxRxxS/T), which are conserved within mammals but not in fly.

To investigate the possibility of direct phosphorylation of NF1 by Erk two different antibodies directed against the phosphorylated Erk substrate motif or phospho-Threonine-Proline were used, to detect a signal in NF1 IPs 20 min post-EGF stimulation. Since NF1 is a huge protein with innumerable phosphorylation sites, additional phosphorylations after EGF stimulation are difficult to predict in

western blot analysis and no changes were detected within the Erk phosphorylation sites (data not shown). To this end, mass spectrometry (MS) analysis was performed by Katharina Wolff in cooperation with Dr. K-H. Gührs (Leibniz Institute on Aging, FLI, Jena). For MS analysis, GFP-NF1 was overexpressed in HeLa cells. Notably, many reported studies (Martin et al., 1990, Klose et al., 1998) used only partial domains of NF1 for their analysis based on complications to clone full-length NF1 (f.e. GAP domain). Strikingly, Yan Cui from the Leibniz Institute on Aging in Jena was able to clone the full length NF1 with few modifications in the cDNA and kindly provided us a GFP tagged NF1 plasmid for transient overexpression. GFP-NF1 was purified by immunoprecipitation and separated by SDS-PAGE. Silver stained protein bands containing GFP-NF1 or candidate interactors were excised and subjected to proteolytic trypsin in-gel digestion. Because of weak NF1 overexpression and low capacity to immunoprecipitate tagged or untagged NF1 the MS analysis and the attempt to directly compare the phosphorylation sites at different EGF stimulation time points was not possible (extensive described in the Master thesis of Katharina Wolff). Similarly, efforts to mutate the Erk phosphorylation site Thr2738Ala were not feasible. Thus, the question if Thr2738Ala-mutated NF1 may abrogate Ras deactivation in HeLa cells depleted for their endogenous NF1 remained open. Moreover, defining a direct Rsk phosphorylation on NF1 with a phosphorylated Rsk/Akt-consensus motif (RXXS*/T*) antibody in HeLa lysates was not achieved.

```
Drosophila    AKSALFLKYFTLFMNLLNDCIDSSEAEKEMNNTPLLPPRPRMAAGKLTALRNATILAMSN    1195
Homo          AKSQLFLKYFTLFMNLLNDCSEVEDESAQT------GGRKRGMSRRLASLRHCTVLAMSN    1151
Pan           AKSQLFLKYFTLFMNLLNDCSEVEDESAQT------GGRKRGMSRRLASLRHCTVLAMSN    1151
Mus           AKSQLFLKYFTLFMNLLNDCSEVEDENAQT------GGRKRGMSRRLASLRHCTVLAMSN    1153
Rattus        AKSQLFLKYFTLFMNLLNDCSEVEDENAQT------GGRKRGMSRRLASLRHCTVLAMSN    1153
              *** ************** : .: . :          * *  : :*::**..*:****

Drosophila    FGISKVKSAAVTAFRSSCRHPTDKWLGNERVTQPLPADRERLSLPSLEVITDALLEIMEA    2255
Homo          FGISKVKSAAVIAFRSSYRDR---------SFSPGSYERETFALTSLETVTEALLEIMEA    2215
Pan           FGISKVKSAAVIAFRSSYRDR---------SFSPGSYERETFALTSLETVTEALLEIMEA    2215
Mus           FGISKVKSAAVIAFRSSYRDR---------SFSPGSYERETFALTSLETVTEALLEIMEA    2217
Rattus        FGISKVKSAAVIAFRSSYRDR---------SFSPGSYERETFALTSLETVTEALLEIMEA    2196
              ********** ***** *.          .*    :** ::* ***.:*:*******

Drosophila    GDESAP------VPPSPRPYNLSSSLSSLTLGSPTDKAFSSESLDFYDNCPGSVSSLRRA    2785
Homo          GIDEETSEESLLTPTSPYPPALQSQLSITA---NLN-------------LSNSMTSLATS    2788
Pan           GIDEETSEESLLTPTSPYPPALQSQLSITA---NLN-------------LSNSMTSLATS    2788
Mus           GIDEETSEESLLTPTSPYPPALQSQLSITA---NLN-------------LSNSMTSLATS    2790
Rattus        GIDEETSEESLLTPTSPYPPALQSQLSITA---NLN-------------LSNSMTSLATS    2769
              * :.        .* ** *  *.*.**  :     :         *::**  :

Drosophila    SHSKSRAKHRINDSPSH----------------------------------    2802
Homo          QHSPGIDKENVELSPTTGHCNSGRTRHGSASQVQKQRSAGSFKRNSIKKIV    2839
Pan           QHSPGIDKENVELSPTTGHCNSGRTRHGSASQVQKQRSAGSFKRNSIKKIV    2839
Mus           QHSPGLDKENVELSPTAGHCNSGRTRHGSASQVQKQRSAGSFKRNSIKKIV    2841
Rattus        QHSPGIDKENVELSPTTGHCNSGRTRHGSASQVQKQRSAGSFKRNSIKKIV    2820
              .** .  *..:: **:

*  -- all residues or nucleotides in that column are identical
:  -- conserved substitutions have been observed
.  -- semi-conserved substitutions have been observed
   -- no match.
```

Figure 23: Multiple sequence alignment of NF1 proteins from different species
The alignment depicted shows sequence regions containing possible Erk and Rsk specific phosphorylation sites. Erk sites are highlighted in blue and Rsk sites in yellow. Alignment was generated by the online software tool Clustal Omega at http://www.ebi.ac.uk/Tools/msa/clustalo/.

9.8 Spred proteins and Ubiquitin are possible NF1 interaction partners

Despite few NF1 interaction partners have been identified during the last decade the cellular role of NF1 remains unclear. Stowe et al. (Stowe et al., 2012) showed that Spred1 is an NF1 interacting protein and that NF1 is necessary for Spred1's inhibitory function as a negative regulator of the Ras/MAPK pathway. They showed that Spred1 binding induces the plasma membrane localisation of NF1. Recently suppressor effects of Spred2 on the Ras–MAPK pathway were confirmed as well (Nobuhisa et al., 2004). In order to decipher if Spred proteins are involved in a NF1 containing Ras deactivation feedback loop, Katharina Wolff could verify the interaction of overexpressed Flag-tagged Spred1 and 2 with NF1 by co-immunopreciptiation (IP). Of note, precipitating for NF1 revealed only interaction with Spred2 but not with Spred1 (data not shown). To establish the role of Spred proteins on RasGTP levels a usual Ras activity assay was performed in HeLa cells overexpressing Flagged-Spred proteins. Cells with high levels of Spred2 showed

diminished RasGTP level while keeping a transient kinetic following EGF stimulation (Figure 24). Similar results could be achieved by overexpression of Spred1 (data not shown). By performing these experiments in the presence of pharmacological MEK inhibition, it was shown that the feedback loop itself was not abolished by the overexpression of Spred proteins or the transfection procedure. Furthermore, a decreased Sos activity dependent on Spred2 overexpression was obtained to reduce RasGTP level (Figure 24B). In conclusion, Spred2 decreases the GEF activity rather than inducing GAP activity. Based on these findings we stopped focusing to identify the role for Spred proteins as mediators of Rsk-dependent NF1 activation.
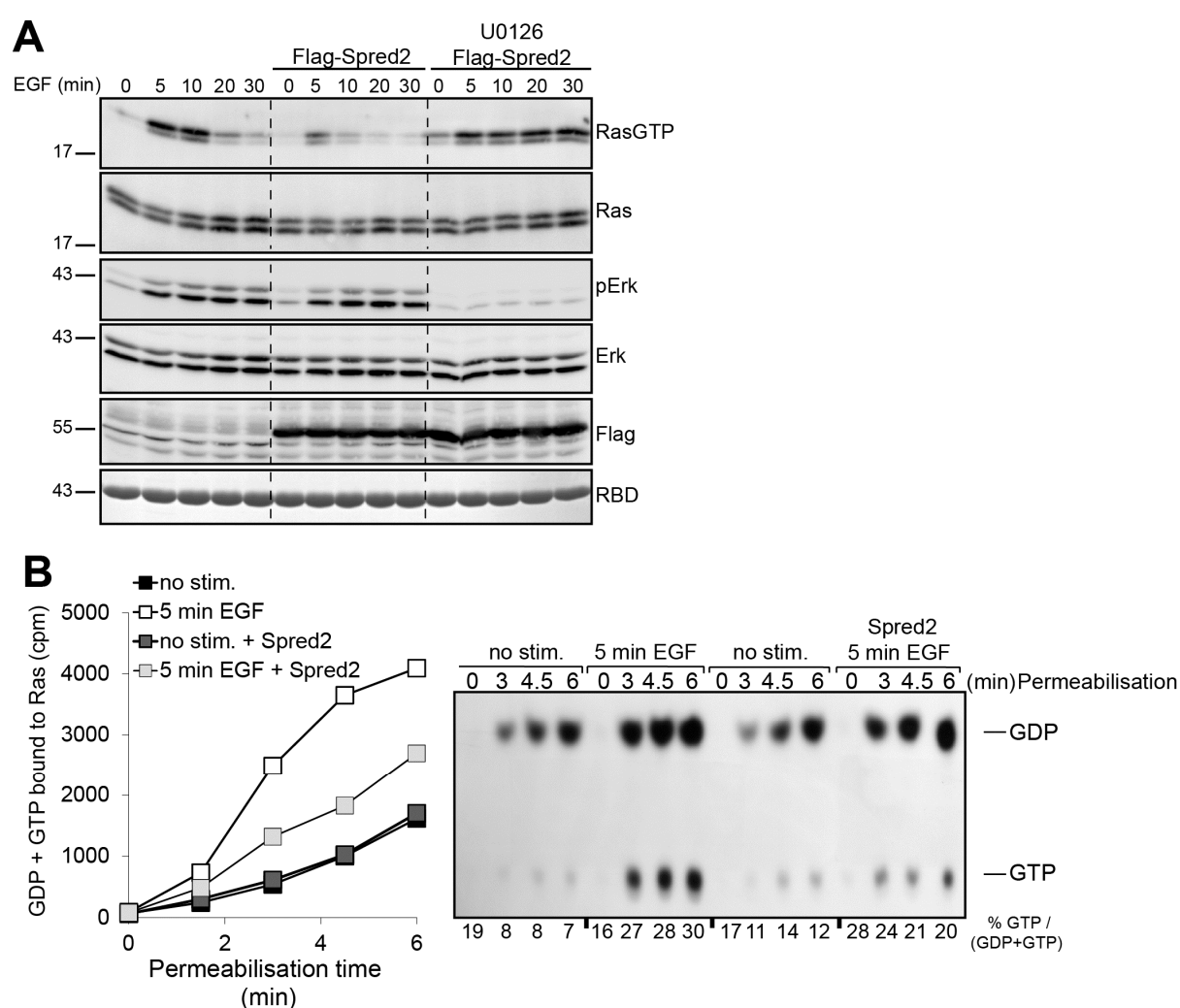
Figure 24: Spred2 overexpression reduces RasGTP levels and nucleotide exchange activity.
A) HeLa cells transfected with Flag-Spred2 were stimulated with EGF in the presence or absence of the MEK inhibitor U0126 and processed for RasGTP analysis and Flag-Spred2 expression.
B) Nucleotide exchange assay in HeLa cells transfected with Flag-Spred2. Cells were permeabilised prior to or 5 min after EGF stimulation and processed for the analysis of nucleotide exchange on Ras. Nucleotides associated to Ras-IPs were additionally eluted from Ras and separated via thin layer chromatography (TLC, on the right). %GTP/(GDP +GTP) values were determined by densitometry and marked under the panel.

A previous report showed that NF1 is destabilised by growth factors via the proteasome and that ubiquitin can interact with NF1. It is likely that these features play a role in the proposed Ras feedback loop. To investigate the role of ubiquitin Katharina Wolff overexpressed HA-tagged ubiquitin and performed the same experimental set-up as described before. However, we could not detect any ubiquitination on NF1 in HeLa cells during EGF stimulation (data not shown). Taken together it is unlikely that ubiquitin affects NF1 activity.

9.9 MEK inhibition increases Ras nanoclustering

Previous studies have established that the distribution of Ras molecules to distinct micro- and nanodomains at the inner leaflet of the plasma membrane (PM) has an impact on the downstream signalling capacity of Ras (Prior et al., 2001, Prior et al., 2003). These observations raise the possibility that Ras itself influences the feedback signal. One opportunity could be that MEK/Erk feeds back on the nanocluster distribution of Ras making it more accessible to NF1 action. In this context a cooperation with Prof. Ian Prior from the University of Liverpool was initiated. His group has great experience in imaging Ras signalling domains by electron microscopy to characterise the dynamic of Ras. The preliminary data performed by Alison Beckett the technical assistant in the Prior lab demonstrated that MEK1/2 inhibition via UO126 or combined ablation of MEK1 and MEK2 via siRNA increased the H-Ras nanoclustering in HeLa cells independently of EGF stimulation (Figure 25). Based on a work stay in Liverpool further nanocluster experiments at 5 min and 20 min post-EGF were conducted to confirm the impact of MEK and NF1 knockdown on Ras nanoclustering at the PM of HeLa cells but the data show inconsistent results and are not shown here. Finally MEK inhibition affects the distribution of Ras to microdomains and nanoclusters but we don't know yet how this distribution is affected in response to growth factors.
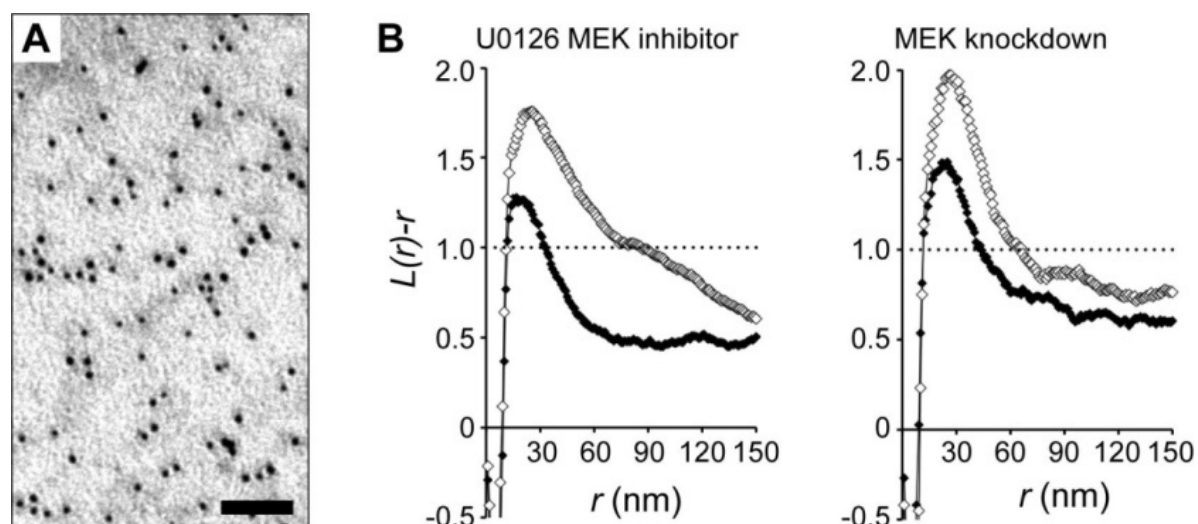
Figure 25: Loss of MEK activity alters H-Ras nanoclustering.
A) Immuno-gold labelling of GFP-H-Ras on isolated plasma membranes reveals Ras nanocluster distributions. **B)** Ras nanocluster distribiution analysed by spatial statistics. L(r)-r values above 1.0 indicate significant clustering. H-Ras nanoclustering (♦) is increased upon loss of MEK activity (◊) by either MEK1/MEK2 inhibition or knockdown. Bar = 50 nm. Figure created by Alison Beckett.

9.10 Modelling of a GAP-mediated feedback

The observations suggest that an Rsk dependent feedback regulates Ras deactivation via activating NF1 GAP activity. To get some testable predictions that could give more insight Manuel A. Esparza-Franco modeled the pathways and potential effects on GEF and GAP blockage. The minimal mathematical models described the feedback stimulation of GAP activity in the presence of continuously high GEF activity. All values they used are based upon published data with the exception of the NF1 parameters where no data was available. The assumptions he has made and the outputs from these models are outlined in the next sections.

9.10.1 Late time GAP blockade via Erk inhibition

On the assumption that Rsk promotes NF1 activity, they concluded that the adding of an Erk inhibitor (FR180204) blocks the GAP activity in the cells. Therefore the first model mimics the GAP blockage at late stimulation and predicts a fast increase of RasGTP levels (Figure 26A). To validate this prediction Erk was inhibited acutely at 35 min post-EGF treatment for which re-raising RasGTP levels were detected (Figure 26B). Thus, inhibition of Erk signalling after GAP mediated Ras inactivation, increases RasGTP levels most likely due to continuous GEF activity of Sos in the presence of abolished GAP activation.

Figure 26: Late time Erk inhibition blocks the GAP activity and increases the RasGTP level.
A) Model simulating late time Erk inhibition. In the model the concentration of RasGTP, the receptor-GEF complex (R-GEF) and GAP activity are plotted against the time of EGF stimulation. Receptor-GEF complex mimic the activity of Sos. **B)** Ras activation was initiated in HeLa cells by 10 ng/ ml EGF addition and Erk activity was quenched 35 min later with 50 µM FR180204. Samples were processed in a RasGTP activation assay. Figure A created by Manuel A. Esparza-Franco.

9.10.2 Late time GEF blockade via EGFR inhibiton

In the second model they simulated a late time GEF activity blockade by interruption the pathway upstream of Ras to approve a GAP-mediated feedback. For this purpose they used an EGFR inhibitor (AG1478), which is supposed to obstruct the EGFR triggered activity of Sos. Theoretically, late time RasGTP levels are triggered by GAP activity in the background of high GEF activity and thus a block of the high GEF activity at this time point predicts a further drop in RasGTP levels (Figure 27A). To proof this hypothesis in an experimental set up HeLa cells received an EGFR inhibitor 35 min post-EGF stimulation. The predicted drop in the anyway low RasGTP level was observed by the following Ras activity assay (Figure 27B). Erk activity declined in parallel as a consequence of EGFR inhibition. To validate that EGFR inhibition reduces the remaining nucleotide exchange activity a permeabilisation assay was accomplished under these conditions. Importantly, exchange curve of EGFR inhibitor treated HeLa cells was similar to the basal condition despite the continuous EGF stimulation (Figure 27C). This highlights the simultaneous action of Sos GEF and GAP proteins on Ras following growth factor receptor stimulation, with Sos being the major driving force and GAP proteins representing the physiological brake. Whereas Sos activity in the sense of Ras activation seems to be primarily regulated upstream of Ras, GAP activity are highly correlative with MAPK, e.g. Rsk signalling.
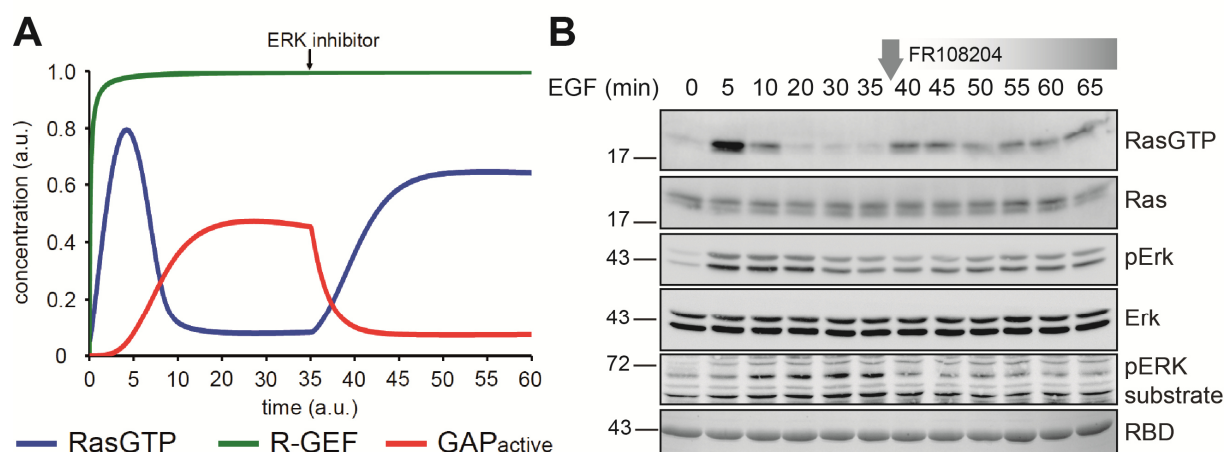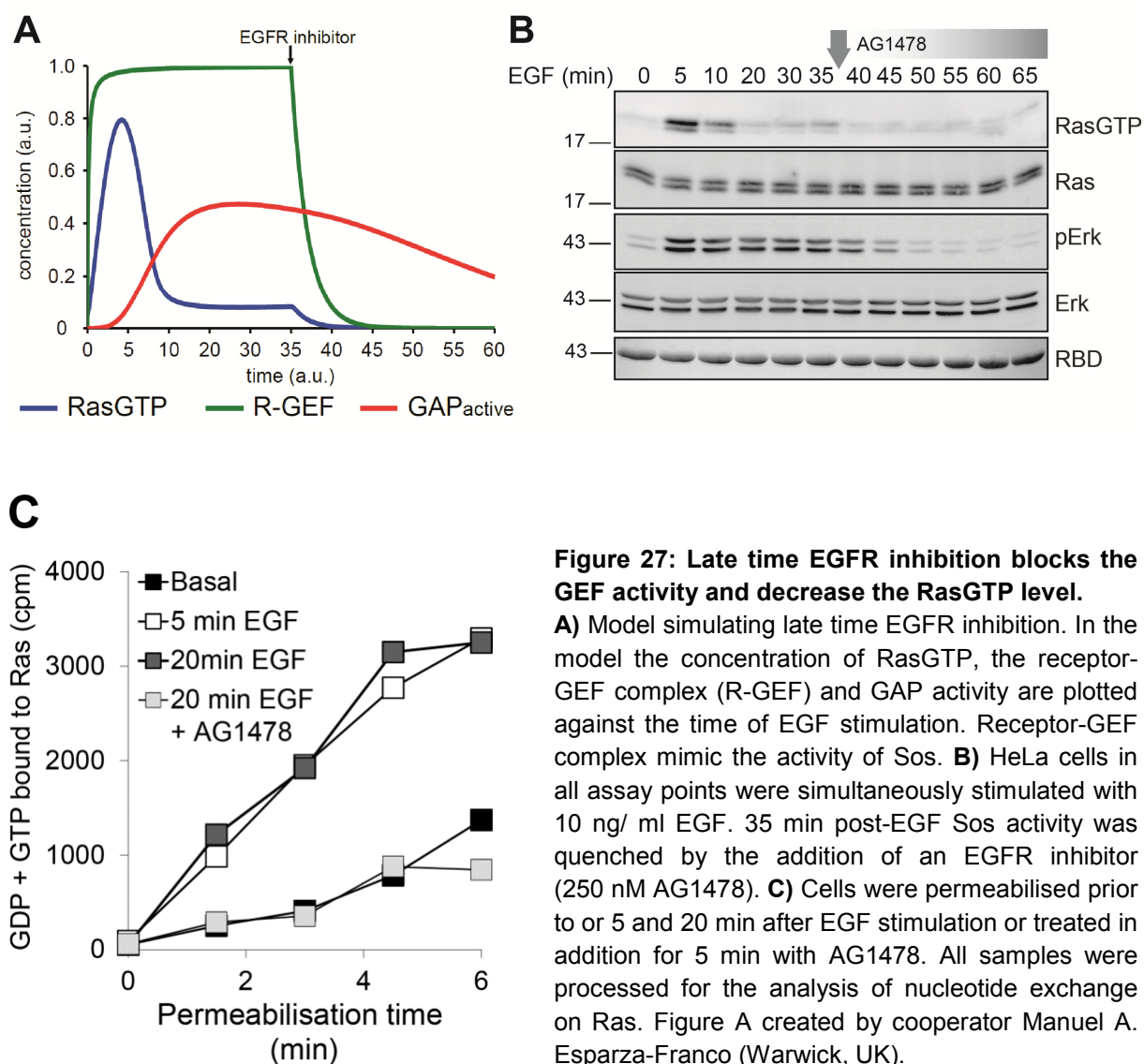
Figure 27: Late time EGFR inhibition blocks the GEF activity and decrease the RasGTP level.
A) Model simulating late time EGFR inhibition. In the model the concentration of RasGTP, the receptor-GEF complex (R-GEF) and GAP activity are plotted against the time of EGF stimulation. Receptor-GEF complex mimic the activity of Sos. **B)** HeLa cells in all assay points were simultaneously stimulated with 10 ng/ ml EGF. 35 min post-EGF Sos activity was quenched by the addition of an EGFR inhibitor (250 nM AG1478). **C)** Cells were permeabilised prior to or 5 and 20 min after EGF stimulation or treated in addition for 5 min with AG1478. All samples were processed for the analysis of nucleotide exchange on Ras. Figure A created by cooperator Manuel A. Esparza-Franco (Warwick, UK).

10 Discussion

Growth factor induced Ras-MAPK pathway activation leads to cell proliferation and differentiation. Aberrant Ras signalling can be found in many malignancies as nearly 30% of all human cancers are due to a point mutation in *RAS* (Bos, 1989). Because of its important role in carcinogenesis Ras itself as well as the upstream and downstream signalling molecules are prominent targets for anticancer therapy. The last three decades of intensive research yielded profound structural information of Ras and its regulators (GEFs and GAPs), including detailed information about catalytically Ras/GAP/GEF interactions (Scheffzek et al., 1997, Wittinghofer et al., 1997, Ahmadian et al., 2003). Despite constant efforts, the treatment of Ras driven cancers has remained largely ineffective (Cox et al., 2014). The complexity and the huge amount of feed-forward and feedback mechanisms often lead to drug resistance and hence combinatorial drug treatment is the method of choice (Vera et al., 2010). Nevertheless, the targeting of Ras downstream signalling effectors, such as Raf or MEK inhibition has been the most favourable approach, even if their mechanism of actions is far from being completely understood.

Growth factors induce a transient Ras activation to drive cells into the cell cycle and evoke cell proliferation. The activation of Ras is triggered through increased nucleotide exchange activity on Ras. The termination of the Ras signal can be caused by three theoretical ways (Figure 28): First, via GEF inactivation while GAPs play only a minor role to keep a steady-state of Ras-bound GTP hydrolysis. Second, via GAP activation in order to counteract the action of GEFs or third, a combination of both. Nevertheless it is important to switch off Ras rapidly after growth factor stimulation for the sake of transient (i.e. mitogenic) signal transmission. One major aim of this thesis was to reveal the mechanisms behind Ras deactivation and the underlying signalling network.
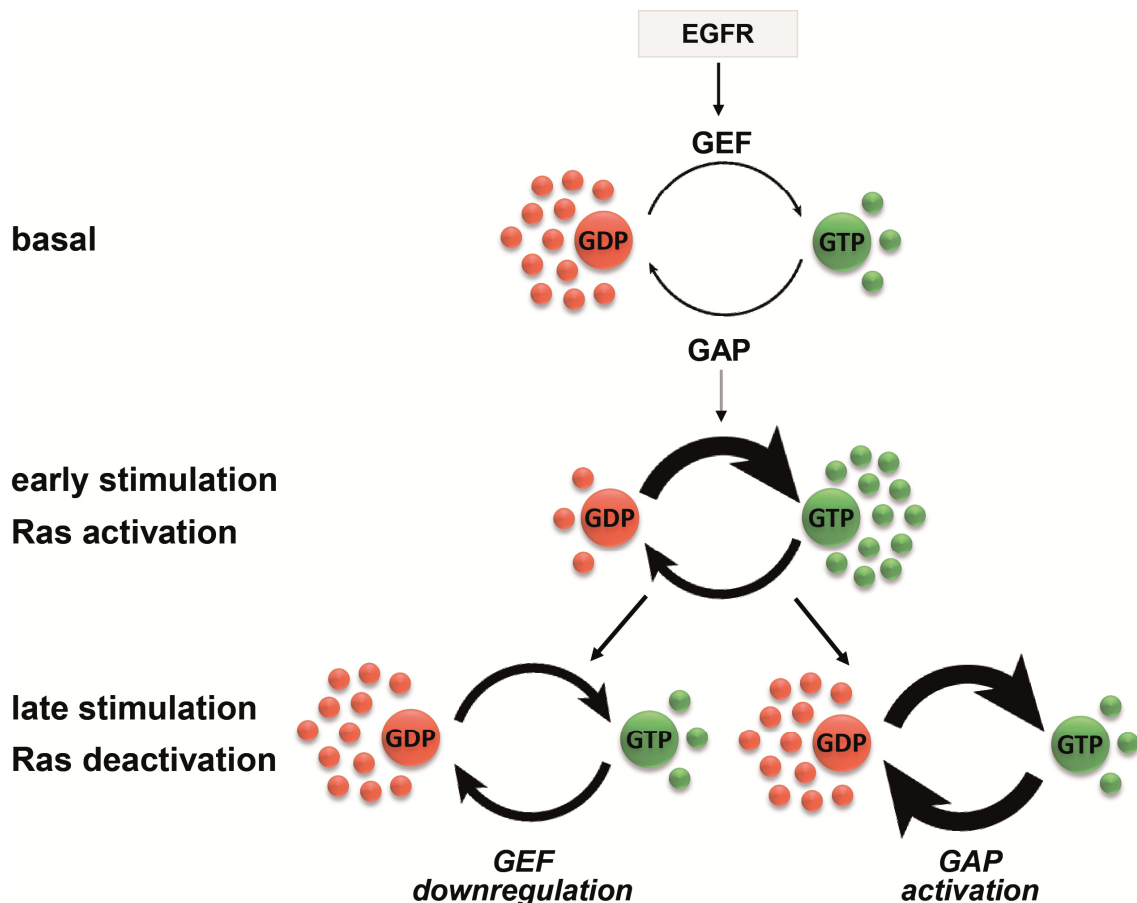
Figure 28: Ras activation/deactivation can occur via changes in GEF and/or GAP activity.
Growth factor induced Ras activation is of transient nature. Further, Ras activation is known to be mediated by GEF activation. However, various models exist accounting for Ras deactivation in response to growth factors: 1. downregulation of GEFs, 2. activation of GAPs or 3. A combination of both (the activity of GAPs and GEFS is symbolised by the thickness of the arrows).

10.1 Ras activation is of transient nature

The kinetics of Ras activity were approached upon growth factor stimulation in two different cell types by a conventional Ras activity assay, measuring the RasGTP levels at steady state. In line with previous studies (Muroya et al., 1992, Traverse et al., 1992, Langlois et al., 1995b), growth factor stimulation induced a rapid increase in RasGTP levels. Interestingly, Ras activity was observed to decline back to basal levels already after several minutes depending on the stimulus and the cell typ. The termination of the Ras signal was achieved despite the continuous stimulation of receptor tyrosine kinase activity. In accordance with Waters et al. (Waters et al., 1996) we detected a persistent EGFR phosphorylation arguing against the possibility of fast EGFR degradation or dephosphorylation triggering the transient Ras activation. In addition to the continuous upstream signalling, the activity of the downstream signalling visualised by phosphorylated Erk, is also sustained indicating

ongoing kinase activity downstream of Ras apart from the strong decline in RasGTP levels.

Many publications report considerations about growth factor and mitogen mediated Ras activation catalysed by activation of GEF (mainly Sos) by measuring the nucleotide uptake in permeabilised cells (Table 15) (Buday and Downward, 1993b, a, Graziani et al., 1993, Medema et al., 1993). The data presented within this thesis confirmed the observation that EGF triggers Sos accelerating GTP-loading of Ras, since an enhanced nucleotide uptake was measured in HeLa and MEF cells after stimulation. Notably, the permeabilisation assay was advantageous enabling the measurement of GEF activity at pre-steady state.

At the bottom line Ras activation is generated by GEF activation as supposed before. However, we cannot exclude that GAP inactivation may also play a role in EGF induced Ras activation. Generally. direct assessment of GAP activity is experimentally unfeasible. To reveal the influence of GAPs on basal Ras activity we performed a Ras pulldown experiment in resting cells with a single or combined GAP knockdown and identified a marginal increase in RasGTP levels. This experiment gave us elementary evidence that Ras activation is not based on GAP inhibition and that RasGAPs possibly act first of all downstream of growth factor stimulation to downregulate Ras.

It has been hypothesised that the negative feedback on Sos initiated downstream of Ras, shapes the dynamics of RasGTP accumulation (Langlois et al., 1995a, Rozakis-Adcock et al., 1995, Waters et al., 1995a, Corbalan-Garcia et al., 1996b, Douville and Downward, 1997). However, a comprehensive discussion including the particular role of GAPs was not addressed within this reports. In cooperation with Graham Ladds a mathematical model was created to simulate the involvement of GAPs. The obtained model supposed that the fast decay of RasGTP after stimulation can occur in the background of high GAP activity only. In respect of lower GAP activity the RasGTP level would stay high or drop just slowly because of the low intrinsic hydrolysis rate. Likewise several computational models described earlier predicted the need of high GAP activity (Wolf et al., 2007). Moreover simply switching off Sos was considered as insufficient to efficiently deactivate Ras without invoking high GAP activity (Markevich et al., 2004). As the available data for RasGAPs is limited and the direct GAP activity measurements are technically unfeasible, a great interest exists to profoundly understand the underlying biochemical mechanisms.

Stimulus	Cells	Reference
EGF	Gab1-/- MEF	Yamasaki et al. (2003)
	Rat-1 cells	Medema et al. (1993)
		Buday and Downward (1993b)
		Buday and Downward (1993a)
	COS-7	Rubio et al. (2003)
	PC12	Basu et al. (1994)
	NIH-3T3 derivate	Medema et al. (1993)
	Rat-1 derivate	Medema et al. (1993)
Phorbol ester	Cardiac myocytes	Montessuit and Thorburn (1999)
	COS-7	Rubio et al. (2006)
	Pancreatic acini	Duan et al. (1995)
LPA	COS-7	Rubio et al. (2003)
NGF	PC12	Basu et al. (1994)
Cholecystokinin (CCK)	Pancreatic acini	Duan et al. (1995)
Insulin	NIH-3T3 derivate	Medema et al. (1993)
	Rat-1 derivate	Medema et al. (1993)
HGF	A549 cells	Graziani et al. (1993)
PDGF	Swiss-3T3	Nanberg and Westermark (1993)
	Human foreskin fibroblasts	Nanberg and Westermark (1993)
	PAE cells	Arvidsson et al. (1994)

Table 15: Studies documenting a stimulation of nucleotide exchange on Ras in permeabilised cells in response to growth factors and mitogens.
Table is adapted from a review article (Hennig et al., 2015).

10.2 Sos phosphorylation does not reduce nucleotide exchange activity on Ras

In case of Sos mediated Ras activation it is published that Sos is recruited to the plasma membrane by the RTK adapter protein Grb2 to catalyse the GDP/GTP exchange on Ras (Li et al., 1993, Rozakis-Adcock et al., 1993). In theory, attenuation of the Ras signal in the cell could be easily achieved by the plasma membrane detachment of Sos, thus abolishing proximity of Sos to Ras. In agreement with this hypothesis, studies reported a MEK or Erk-dependent Sos phosphorylation early after growth hormone receptor stimulation, which leads to the dissociation of Sos from Grb2 (Waters et al., 1995a, Waters et al., 1995b, Dong et al., 1996, Zhao et al., 1998). Further it has been speculated that Grb2-Sos dissociation acts inhibitory on the Ras nucleotide exchange and in this way modulates the duration of Ras activation (Langlois et al., 1995a, Rozakis-Adcock et al., 1995, Waters et al., 1995b, Corbalan-Garcia et al., 1996b, Porfiri and McCormick, 1996, Kamioka et al., 2010). In addition to Erk and MEK, p90 Rsk was supposed to be a further mitogen activated kinase targeting Sos (Douville and Downward, 1997). Furthermore, Roux et al. (Saha et al., 2012) hypothesised that Rsk dependent Sos phosphorylation creates

14-3-3 binding sites and therefore negatively regulates MAPK signalling. However, the GEF activity of Sos was never tested in these studies. In our work we confirmed the phosphorylation of Sos after growth factor stimulation. Moreover, Sos phosphorylation was blocked by pharmacological inhibition of MEK and Erk by the use of U0126 (Duncia et al., 1998) and FR108204 (Ohori et al., 2005), respectively. Though, if the pan-Rsk inhibitor BI-D1870 (Sapkota et al., 2007) was applied phosphorylation of Sos remained uneffected. In conclusion these observations revealed that the EGF mediated Sos phosphorylation is MEK/Erk-dependent, nonetheless Rsk-independent.

The Sos-negative feedback models described in literature are conclusive but have never been proved experimentally, because measuring Sos driven nucleotide exchange in live cells is experimentally challenging. Similar to previous studies (Buday and Downward, 1993b, Medema et al., 1993, Rubio et al., 2003), the nucleotide exchange at pre-steady state was measured by using the permeabilisation-based approach. Surprisingly, the nucleotide exchange rate on Ras was continuously high as well at the time point where RasGTP amount dropped down. This result disproved the previous observations of the MAPK-mediated negative feedback inhibition of Sos activity, at least in the case of the particular cellular models analysed. Taken together, the results gave evidence for a growth factor dependent Sos phosphorylation, but these phosphorylation had no influence on the Sos nucleotide exchange activity, indicating that regulation of Sos activity is dispensable for the Ras feedback inhibition. Furthermore, they point to GAP activation as the mechanism of signal termination. These results are in line with previous publications demonstrating that neither the dissociation of Sos from Grb2 nor the phosphorylation of Sos correlate with Ras activity state (de Vries-Smits et al., 1995, Corbalan-Garcia et al., 1996a). Growth factor dependent feedback phosphorylation of Sos potentially acts on different signalling pathways, that may be related to the control of Rac and the actin cytoskeleton (Sini et al., 2004).

Conclusively, the rapid decline of RasGTP level does not rely on the inhibition of Sos activity. This statement is supported by the observation that permeabilised HeLa cells loaded with non-hydrolysable GTP displayed sustained RasGTP levels in contrast to cells loaded with GTP which is sensitive for GAP action. In addition, this experimental set-up proved the presence of RasGAP activity during Ras deactivation.

10.3 Ras deactivation is driven by Rsk-dependent feedback activation

In comparison to previous reports, our results demonstrated that regulation of the nucleotide exchange activity of Sos is not the major driver of the inactivation of Ras. Nevertheless, several reports claimed feedback regulation of the MAPK cascade (Brightman and Fell, 2000, Cirit et al., 2010, Kamioka et al., 2010). In accordance, our own findings showed that Ras activity is sustained after MEK/Erk/Rsk inhibition, which indeed presumes a feedback loop operating via MEK/Erk/Rsk in order to downregulate Ras activity.

In regard of the fact that the existing Ras isoforms differ biologically and functionally (Castellano and Santos, 2011), we could show that the feedback proceeded in MEFs expressing K-Ras but not N-Ras and H-Ras. So it is suggested that the feedback is maintained at least in the presence of K-Ras.

Moreover, the permeabilisation assay did not reveal any changes in the nucleotide exchange activity during the MEK or Rsk inhibitor treatment, indicating that the MAPK mediated phosphorylations on Sos do not influence its GEF activity. Notably Rsk inhibition interrupts the feedback deactivation of Ras without affecting the phosphorylation state of Sos, providing further evidence that Sos phosphorylation does not correlate with the fast decline in RasGTP level.

In accordance with other expression studies (Carriere et al., 2008) we detected Rsk1 and Rsk2 as the main isoforms in HeLa cells. To strengthen the assumption that Rsk1/2 are mediators of GAP feedback activation, a combined knockdown of Rsk1 and Rsk2 was performed and showed an equally prolonged Ras activation. In agreement with a Rsk mediated Erk inhibition in *Drosophila* (Kim et al., 2006), our results corroborate the hypothesis that the growth factor stimulated feedback loop is propagated via Rsk1 and Rsk2.

p38 kinase is most strongly activated by proinflammatory cytokines (Lee et al., 1994) and less efficiently by Ras (Lin et al., 1995). Nevertheless the p38 pathway was shown to provide negative feedback for Ras proliferative signalling as well (Chen 2000). Hence, it is likely that even this moderate activation could have an important impact on Ras signalling. Solely the inhibition of MEK/Erk or Rsk and not PI3K or p38, resulted in prolonged Ras activation, further demonstrating that the PI3K pathway is not involved in the EGF-stimulated feedback on Ras. The concomitant detection of enhanced phospho-Akt level after MEK and Rsk inhibition was possibly caused by a normal, compensatory cell response after Rsk inhibition, since Rsk and

Akt share substrate specificity. This reflects the complexity of MAPK signalling including several pathway crosstalks, which amplifies the mitogenic stimuli (Kiyatkin et al., 2006).

The fact that the kinases Rsk1 and Rsk2 stimulate the GAP activity in a kind of a positive feedback loop to terminate Ras activation is a totally new paradigm, especially due to the reason that active Rsk enzymes are mainly described to act further downstream.

Ultimately, Ras itself engages a GAP system for the purpose of restraining the duration of its own activation in response to growth factors. That implies that RasGAPs play a precise role in Ras deactivation.

10.4 NF1 in the agonist-dependent control of Ras deactivation

The experimental and modelling data show that RasGAP activity is induced in the context of a feedback loop initiated by activated Ras and modulated by Rsk1 and Rsk2. Results obtained from a proteomic screen (Amit et al., 2007) and western blot analysis validated the abundant expression of p120GAP, NF1 and DAB2IP in EGF stimulated HeLa cells. For this reason the efforts were focused on p120GAP, NF1 and DAB2IP. The negligibility of the GAP1 family members was corroborated by immunoblotting results showing very low protein levels in the analysed cell model. In addition this was supported by the fact that EGF is a weak PLC activator evoking poor Ca^{2+} release, which consequently leads to less GAP1 activity. SynGAP proteins were equally unlikely candidates because they are a known as neuronal restricted GAP species (Kim et al., 1998).

To identify the responsible RasGAP we monitored the EGF permitted Ras kinetic in GAP diminished HeLa cells. The knockdown of NF1 alone sustained RasGTP level after growth factor stimulation, which was comparable to the level after Rsk inhibition. This experiment provided the first evidence for NF1 functioning as the RasGAP responsible for attenuating Ras activity in mitogenic signalling.

The chromatograms from the permeabilisation assay in NF1 knockdown cells showed prolonged RasGTP in the presence of an unaltered nucleotide exchange, comparable to the rate obtained using the Rsk inhibitor. Hence, NF1 is a likely candidate to deactivate Ras via an Rsk-dependent feedback mechanism. It has been known for a long time that NF1 is ubiquitously expressed and its tumour suppressor activity is supposed to base on the ability to downregulate RasGTP level via the RasGAP domain (Bollag et al., 1996, Guha et al., 1996, Klose et al., 1998).

Now in this study it is firstly demonstrated that NF1 possesses crucial functions in growth factor signalling. Future work may address the identification of precise biochemical links. Three plausible mechanisms may be suggested to regulate NF1 activity in growth factor controlled Ras deactivation and will be discussed in the next sections.

10.4.1 Direct NF1 phosphorylation as possible regulation mechanism

NF1 is a large protein consisting of 2,818 amino acids with several phosphorylation sites. Firstly, a potential mechanism may involve Rsk kinase mediated phosphorylation of NF1 to increase its GAP activity in order to drive RasGTP hydrolysis (Figure 29). The EGF dependent NF1 phosphorylation level was tested by using different approaches. First, a modest mobility shift was detected after EGF stimulation on standard western blots. To improve the detection various methods were applied. 1) A special, modified phosphate-binding acrylamide using manganese(II) ion-aided complex formation which acts as a selective phosphate tag and enhanced the mobility shift in western blots. 2) An agarose based approach (PhostagTM Agarose) where di-nuclear zinc(II) complex is attached as a selective phosphate-binding tag molecule. Technically this strategy is based on an immobilised metal affinity chromatography. Both approaches were insufficient to yield conclusive findings (data not shown), either due the large protein size of NF1 or vast extent of phosphorylation sites that possibly mask Rsk-dependent phosphorylation events. Furthermore, difficulties in molecular manipulation of the NF1 coding sequence were a hurdle for approaches such as site-directed mutagenesis to substantiate the specific phosphorylation site. Analysis of mass spectra was not successful to determine any phosphorylation event within the three Rsk-consensus motifs (RxRxxS/T) because of dissatisfactory quality of the spectra. Finally, more rigorous characterisation of NF1 including approaches like immunoprecipitation or stably transfected mutants may aid in revealing interaction partners and growth-factor induced modifications to this large RasGAP.

10.4.2 NF1 regulation via unknown intermediate proteins

A second possibility is that one or more unknown protein(s) get engaged by Rsk to regulate NF1 activity (Figure 29). Many substrates have been defined for Rsk (Frodin and Gammeltoft, 1999) which adds further complexity to this effector pathway.

Efforts to screen for Rsk-phosphorylated proteins acting on NF1 after growth factor stimulation were inconclusive as the huge number of candidates precluded further work in this direction. In contrast, literature proposes various proteins as NF1-interacting proteins (Ratner and Miller, 2015).

a) Spred proteins as negative regulator of the MAPK pathway and NF1-binding proteins

Evidence that Spred proteins are possible candidates derives from the fact that the Legius syndrome, a rasopathy and developmental disorder caused by germline mutations in the *Spred1* gene, has an overlapping phenotype (facial abnormalities, learning disabilities, pigmental changes) with neurofibromatosis type 1 (Brems et al., 2007, Pasmant et al., 2009, Spurlock et al., 2009). In this respect a common regulatory mechanism seems likely. Further evidence along this line, is given by another publication which showed that NF1 is a Spred1-interacting protein and suggested that this interaction is essential for the inhibitory function of both Spred1 and NF1 on Ras (Courtois-Cox et al., 2006, Stowe et al., 2012).

Spred proteins are known to directly negate the Ras/MAPK pathway after various mitogenic stimulations (Bundschu et al., 2007, Ullrich et al., 2011). The hypothesis of Rsk mediated phosphorylation of Spred leading to increased NF1 GAP activity seems conclusive. Reports about tyrosine phosphorylation of Spred exist (Mason et al., 2004, Lock et al., 2006), whereas nothing is known about Ser/Thr phosphorylation. Finally, it is conceivable that the phosphorylation of Spred by protein kinases is a possible mechanism to regulate Spred activity (King et al., 2005). However, Spred proteins exhibit neither an Erk phosphorylation site nor an Rsk phosphorylation motif weakening a potential connection to Rsk.

Furthermore, it has been reported that Spred proteins are able to act upstream and downstream of Ras. Studies proposed that the mode of action is downstream of Ras where Spred inactivates the MAPK pathway by suppressing the phosphorylation and activation of Raf. In this context, Wakioka and co-workers described an enduring Spred-Ras interaction. However, they published that the interaction has no influence on Ras activation or the Raf plasma membrane localisation (Wakioka et al., 2001). In contrast, King et al. (King et al., 2005) evidenced that Spred regulates Ras upstream by affecting the RasGTP levels, although the detailed mechanism have not been clarified.

An additional level of Ras-independent regulatory mechanisms is the localisation of Spred1 in cholesterol-rich membrane rafts/caveoli fractions and that Spred1 inhibits Erk activation in conjunction with Caveolin-1, a main component of the caveolae plasma membranes (Nonami et al., 2005).

As observed by Ras activity assay, Spred2 overexpression facilitates a considerable reduction in RasGTP levels. Spred2 overexpression thereby affects the Sos activity rather than GAP activity demonstrated by diminished nucleotide exchange activity in the permeabilisation assay. Thus, Spred2 contributes to the regulation of Ras activity but not in the context of NF1 mediated Ras downregulation.

At least Spred proteins are signalling inhibitors with proposed tumour suppressive functions, but we found no correlation between Spred and NF1 GAP interaction and Ras deactivation after growth factor stimulation. We still cannot answer the question if the negative regulator Spred is involved in a fundamental mechanism of negative feedback or a kind of fine-tuning of the signal. The data regarding Spred proteins leave room to perform further experiment to unveil the emerging role regarding Sos inhibition.

b) Is the EGF induced NF1 activity regulated by ubiquitin-dependent proteolysis?

Ubiquitin ligases have been described as NF1- interacting proteins. It is known that the binding of ubiquitin molecules to the target protein induces rapid degradation by the 26S proteasome and that this degradation process is involved in diverse cellular processes (Kornitzer and Ciechanover, 2000). To observe if the protein level of disposable NF1 is responsible for transient Ras activation the NF1 protein level was detected by western blot analysis. NF1 immunoblots showed high NF1 abundance at every time point tested during EGF stimulation. This observation is inconsistent with a publication claiming that Ras dynamic is regulated as a consequence of Erk mediated NF1 accumulation (Cichowski et al., 2003, Tan et al., 2011, Hollstein and Cichowski, 2013). Also in *Yeast* it was shown that an ubiquitination pathway can negatively regulate NF1 (Phan et al., 2010). Further, it was postulated that ubiquitination functions as mediator of signal transduction in addition to its role in cellular proteolytic processes (Pickart and Eddins, 2004). Ubiquitin overexpression studies performedin HeLa cells did not confirm NF1 ubiquitination even following growth factor stimulation. These findings do not confirm the theory of regulating Ras activity via ubiquitination and proteolysis as well as fast growth factor induced

re-elevation of NF1 at least in the case of the cell types studied here. Moreover, considering the large size of NF1, it is difficult to conceive the possibility of rapid NF1 synthesis and degradation as a regulatory mechanism of Ras deactivation.

10.4.3 EGF dependent NF1 and Ras distribution

Previous studies argued that also membrane targeting of GAPs can influence the catalytic activity of RasGAPs (Huang et al., 1993, Sot et al., 2013). As mentioned before, Spred1 is able to attract NF1 to the plasma membrane and thus in turn may downregulate Ras activity (Stowe et al., 2012). On the other side, it is predictable that the Sec14-PH module of NF1, which is relevant for phospholipid-binding (D'Angelo et al., 2006, Welti et al., 2007), is liable for NF1 membrane localisation and this way may reach high proximity to Ras. It is known that lipids like arachidonic acid and phosphatidic acid are negative regulators of the NF1 GAP activity, but no lipid was identified to stimulate the NF1 GAP activity and thereby switch off Ras (Tsai et al., 1989, Tsai et al., 1990, Golubic et al., 1991, Han et al., 1991).

Another mechanism to localise NF1 at the membrane has been described in neurons. Here the interaction of NF1 with syndecan, a transmembrane protein, has been supposed to facilitate NF1 translocation (Hsueh et al., 2001). For neuronal cells it has been further reported that an EGF-dependent PKC phosphorylation on NF1 increases its association with the actin cytoskeleton and the NF1 GTPase activity (Mangoura et al., 2006). In addition, NF1 seems to link the Ras pathway with PKA-associated signals, due to the binding of 14-3-3 protein to a PKA-dependent phosphorylation site which reduces NF1 GAP activity (Feng et al., 2004). Altogether, it is conceivable that in response to EGF a Rsk-mediated phosphorylation or lipid modification of NF1 can influence the NF1 localisation and accessibility to Ras. Vice versa, a translocation of Ras within the plasma membrane may also lead to the formation of a Ras/NF1 complex. In this context, studies have demonstrated that 40% of Ras proteins are distributed to distinct micro- and nanoscale domains at the plasma membrane, and that the exact pattern of distribution has major impact on the downstream signalling capacity of Ras (Prior et al., 2003, Rotblat et al., 2004, Plowman and Hancock, 2005, Roy et al., 2005, Zhou and Hancock, 2015). Initial nanocluster assays without EGF show that MEK inactivation led to a marked increase in H-Ras nanoclustering. This observation indicated a role of MEK in Ras nanoorganisation. Ras nanocluster distribution after growth factor stimulation and its importance for MEK or Erk activity has never been addressed before.

It was only reported that B-Raf inhibition enhances clustering of K-Ras and N-Ras but not H-Ras with consequent enhancement of MEK-Erk activation. At least the demonstration that effectors other than Raf regulate Ras clustering is novel. However, a MEK-dependent nanocluster rearrangement to increase the Ras accessibility for NF1 to decline RasGTP level is straight forward. Unfortunately, the attempts to address changes in Ras nanoclustering after EGF stimulation did not reveal a possible regulation mechanism. In accordance, SLO permeabilised cells feature similar transient RasGTP level, even though cholesterol containing microdomains are destroyed and consequently Ras distribution is likely to be affected.
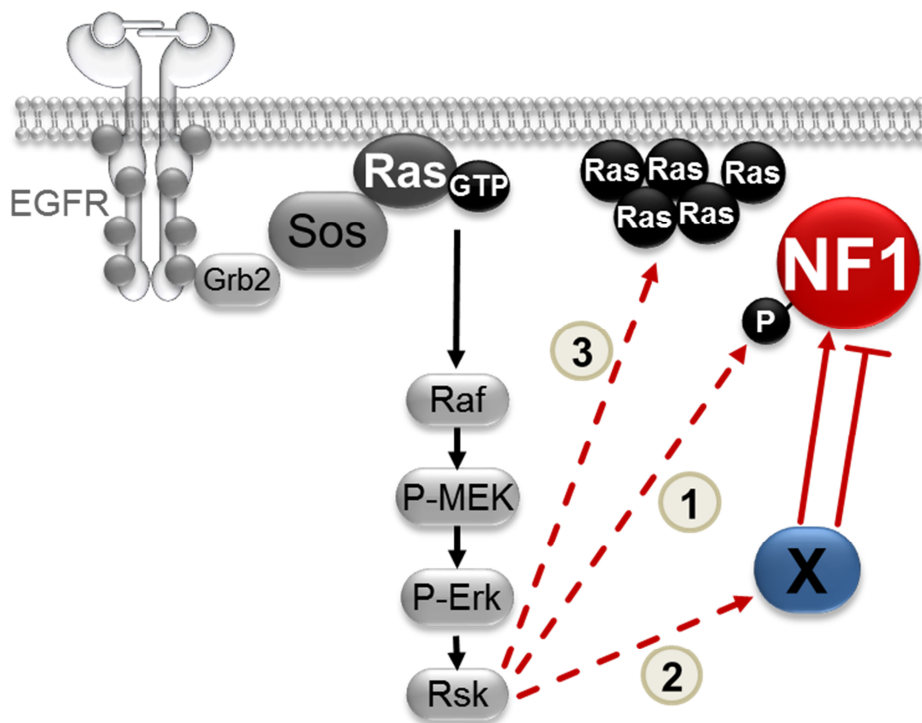
Figure 29: Possible mechanisms to initialise the decline of RasGTP levels.
1) Rsk directly activates NF1 via phosphorylation. **2)** One or more unknown interacting proteins get engaged by Rsk and regulate NF1 GAP activity; here also NF1 interacting proteins like Spred or ubiquitin-dependent NF1 proteolysis play a role. **3)** A NF1 independent mechanism describes a MAPK dependent Ras rearrangement after growth factor stimulation.

10.5 Combination of biological data and mathematic modelling

Ras activation and subsequent desensitisation is controlled at multiple steps and several connected pathways that are simultaneously active. It was postulated that a cross-talk between the kinase Rsk and NF1 somehow leads to Ras deactivation after growth factor stimulation. It was attempted to link and confirm the obtained results, so computational modelling was realised in cooperation with Graham Ladds (University Cambridge) and Manuel A. Esparza-Franco (University of Warwick).

One generated model was the so called GEF-only model which postulates that transient Ras activation mediated by RasGTP-dependent feedback inhibition of Sos requires high basal GAP activity. Previous simulations (Ozaki et al., 2005, Wolf et al., 2007) and results emerged from the permeabilisation assay verified that assumption, since the intrinsic nucleotide exchange and GTP-hydrolysis rate of Ras compensate each other (Hennig et al., 2015). This implies that RasGTP levels would drop very slowly if Sos became inactivated in a background of null basal GAP activity. The finding that RasGTP levels were not increased in GAP diminished cells reveal that HeLa exhibit a low basal GAP activity. This observation further indicates that there might be a RasGTP driven pathway that turns on GAP activity after growth factor stimulation. The importance of GAP activity was further highlighted by the fact that GEF activity does not decay during Ras deactivation. Moreover, we could show that Ras deactivation was indeed MEK/Erk- and even Rsk-dependent, but not modulated by Sos as proposed before (Brightman and Fell, 2000, Cirit et al., 2010, Kamioka et al., 2010).

To validate our assumption that a high GAP activity evokes the decline of RasGTP level, Manuel A. Esparza-Franco created two mathematical models based on our experimental data. We combined computational modelling with biochemical experiments to elucidate the dynamics of these feedback mechanisms. The modelling predicted fast changes of RasGTP levels at late time GEF/GAP blockade. In the 1st experiment the GAP activity was blocked by adding an Erk inhibitor late post-EGF stimulation and we observed an immediate rise in RasGTP levels due to the maintained high Sos activity. In the 2nd experiment an EGFR inhibitor was applied to the same late stimulation time point to block Sos activity. We observed a drop in RasGTP levels most likely due to continuous high GAP in the absence of GEF activity. At least we could confirm all predictions of the models in our cells and sum up all present findings in a revised minimal mathematical model that represent

transient Ras activation in the background of RasGTP-dependent GAP activation (Figure 30).
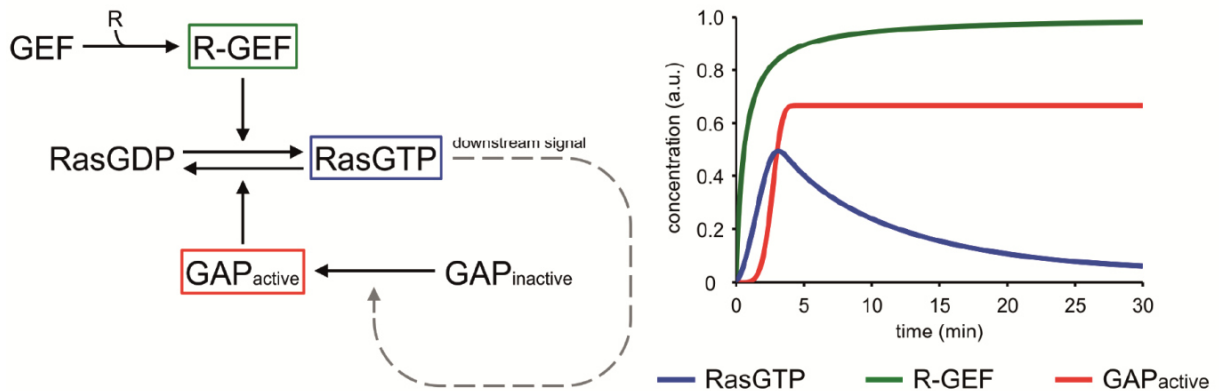
Figure 30: Mathematical model describing the feedback stimulation of GAPs.
Minimal mathematical model describing Ras activation/deactivation mediated by a positive feedback stimulation of RasGAP. Figure created by Manuel A. Esparza-Franco.

Taken together, the findings indicate that Ras deactivation is not initiated by merely Sos inhibition. In addition to the involvement of Rsk in a feedback loop mediating Ras signal termination, we identified NF1 as the GAP species counteracting continuously high Sos activity (Figure 31). Furthermore, our data reveal for the first time a biochemical evidence for a role of the tumour suppressor NF1 controlling the growth factor-induced Ras activity.
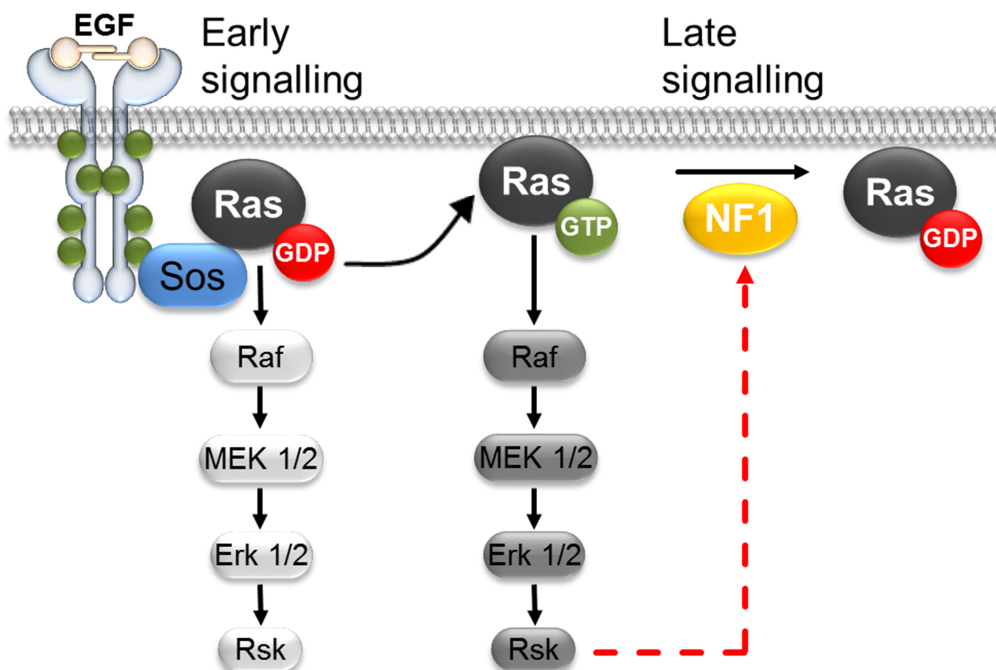
Figure 31: Schematic cartoon of the mechanism of Ras activation/deactivation.
After EGF stimulation Sos promotes activation of Ras at the cellular membrane by facilitating exchange of GDP for GTP. Active Ras binds Raf and initiates the MAPK signalling. The termination of active Ras at late time stimulation point is caused by Rsk dependent feedback stimulation of NF1 in the background of prolonged Sos activation.

10.6 Clinical Relevance of Rsk dependent NF1 activation

The tight control of Ras activation/deactivation is important in regulating cell growth. Gain of function mutations within the Ras pathway or the dysregulation of its connected negative regulators lead to various developmental disorders collectively termed RASopathies. These syndromes often reveal overlapping phenotypic features caused by moderate over-activation of MAPK signalling. Patients with such syndromes are predisposed to cancer (Simsek-Kiper et al., 2013). NF1 was identified more than 20 years ago as a large transcript disrupted in neurofibromatosis type I patients (Wallace et al., 1990). However the critical affected pathways involved in the pathogenesis of neurofibromatosis are still unknown. Various interacting factors like Spred proteins, ubiquitin, lipids and tubulin have been described but none of them seems to be causative for the disease progression (Bollag et al., 1993, Hsueh et al., 2001, De Schepper et al., 2006, Stowe et al., 2012, Hollstein and Cichowski, 2013). In accordance with our experiments, Larribere et al. (Larribere et al., 2015) recently published that *NF1* loss induces senescence during melanocyte differentiation as well as in patient derived cafe-au-lait macules.

Interestingly, Stowe and colleagues postulated that the Legius syndrome, caused by *SPRED1* mutation, and neurofibromatosis-1 might have some molecular mechanisms in common (Stowe et al., 2012). In our study we could not determine any functional link between Spred1 and NF1 in growth factor mediated signalling. But our data convincingly suggests that Rsk is able to induce NF1 activity in order to downregulate the RasGTP level in response to mitogen signalling. The data provide a rational explanation of the overlapping clinical manifestation of neurofibromatosis and the Coffin-Lowry syndrome, which is caused by mutations in *RSK2* (Trivier et al., 1996). The Coffin–Lowry syndrome is likewise associated with mental retardation as well as growth, cardiac and skeleton abnormalities (Delaunoy et al., 2006), partially reflecting pathophysiology of RASopathies. Interestingly, initial diagnoses of Noonan syndrome had to be revised to Coffin-Lowry syndrome or neurofibromatosis-1 (Chen et al., 2014) Different cell types may simply invoke various components of the MAPK pathway reflecting distinct features of each disorder. Finally, it is likely that the *RSK2* gene is a new addendum to the RASopathy genes, for which we offer new mechanistic insight.

The clinical use of Erk and MEK inhibitors in anti-cancer therapy is often depending on combinatorial treatment with other relevant drugs (Poulikakos and Solit, 2011, Hatzivassiliou et al., 2012, Sun et al., 2014). This is due to reactivation of the Raf-Erk pathway leading to resistance to Erk or MEK inhibitor treatment alone (Sanchez-Laorden et al., 2014). Based on our data set, it can be assumed that MEK inhibition based anti-cancer treatment leads to reduced NF1 activity followed by prolonged and enhanced Ras activation. This in turn favours the development of drug resistance and aggravation of the disease for example elevated tumour formation. Our data provide comprehensive explanations how MEK inhibition as a single agent treatment represents a viable therapy for Neurofibromatosis patients, which was shown before by other groups (Chang et al., 2013, Jessen et al., 2013).

10.7 Concluding remarks

The cellular response of the growth factor induced Ras-MAPK signalling depends on the duration of the Ras activity. Meaning, Ras proteins act as a critical switch and determine the cell's fate. Numerous studies showed that a transient Ras activation mediates cell proliferation, whereas prolonged activation of the Ras/Erk pathway drives the cells in senescence (Serrano et al., 1997, Lee et al., 1999) or shifts the balance towards differentiation (Qui and Green, 1992, Traverse et al., 1992, Halfar et al., 2001). In accordance, *NF1* loss promotes senescence in cells to hold back tumorigenesis by preventing progression of benign tumours to malignancy (McGillicuddy et al., 2009, Larribere et al., 2015). The complexity of this cellular behaviour requires a tight regulation of Ras activity. In our study we provide the experimental basis to convincingly propose a central role of the Ras negative regulator GAP namely NF1 instead of GEFs in playing a central role in Ras deactivation. We observed that the Ras signalling pathway features a more complex regulatory mechanism in terms of temporal Ras activity modulation which is coordinated through partially unknown feedback loops, specific cross talks of certain mediators and scaffold proteins. In this respect it is important to note that kinetics of the Ras activity depend on the cell type and specific kind of stimulus. At least in our EGF stimulated cells we showed that Ras initiate its own deactivation mechanism via feedback activation of NF1 possibly through Rsk. Taken together, these data provide the first evidence that NF1 activity is regulated by growth factor signalling, although the exact biochemical process linking Rsk and NF1 activation remains an enigma.

The large size of the tumour suppressor NF1 and the technically limitations in handling NF1 plasmids have all contributed to the hurdles that still make the research of NF1 function and its precise role in controlling Ras signalling pathways experimentally challenging. An important goal of the next few years will be to better understand the relevance of possible NF1 interaction partners such as Spred proteins or to identify new NF1 interaction partners. For this purpose, studies with full-length NF1 are necessary because most biochemical studies have been performed with fragments comprising the catalytic domains of NF1. Future work should also resolve the influence of potential Rsk- or Erk-dependent NF1 phosphorylation. Additional efforts are needed to entirely understand the role of NF1 that may differ depending on the cell type specific features. Further it is of high relevance to improve therapeutic options of patients suffering from RASopathies that is based on the complete knowledge about the complex Ras signalling network.

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13 Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: PD Dr. I.Rubio, Dr. G. Ladds

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, 17.12.2015

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14 Curriculum vitae

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