Doctoral Thesis for the Degree of Doctor of Philosophy, Faculty of Medicine

The Importance of Isoprenylation and *Nf1* Deficiency in K-RAS–induced Cancer

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A doctoral thesis at a University in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have already been published or are manuscripts at various stages (in press, submitted, or manuscript).

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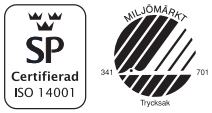


ABSTRACT

The RAS and RHO family proteins contribute to tumorigenesis and metastasis and belong to a family of so called *CAAX* proteins. The membrane targeting and proper function of *CAAX* proteins are dependent on posttranslational isoprenylation by farnesyltransferase (FTase) or geranylgeranyltransferase type I (GGTase-I). Inhibitors of FTase and GGTase-I have been developed to block RAS-induced cancer, but their utility has been difficult to evaluate because of off-target effects, drug resistance, and toxicity. One aim of this thesis was to use genetic strategies in mice to define the physiologic importance of *CAAX* protein isoprenylation and to evaluate FTase and GGTase-I as potential anti-cancer drug targets.

Oncogenic mutations in RAS are common in cancer and result in hyperactive RAS signaling. However, a RAS mutation alone is not sufficient for cancer development in humans. Rather, cancer arises as a consequence of cooperation between several mutational events. The tumor suppressor gene neurofibromatosis type I (*NF1*) is a RAS-inactivating protein. Thus, loss of *NF1* also results in hyperactive RAS signaling and this occurs in some types of cancer. It has been proposed that *NF1* deficiency is functionally equivalent to an oncogenic RAS; but NF1 may operate in other pathways. It is not clear if *NF1* deficiency would be redundant in RAS-induced cancer development or if the two mutations would cooperate. A second aim of this thesis was to define the impact of *Nf1* deficiency on the development of K-RAS–induced cancer in mice.

To approach these aims, Cre/*loxP* gene targeting techniques in mice were used, to simultaneously activate an oncogenic K-RAS allele, to induce lung cancer or myeloid leukemia, and inactivate the genes encoding FTase and GGTase-I, or *Nf1*.

Inactivating the gene encoding the β-subunit of GGTase-I eliminated enzyme activity, blocked proliferation and reduced motility of fibroblasts. Moreover, inactivation of GGTase-I reduced tumor formation and increased survival of mice with K-RAS–induced lung cancer. Finally, several cell types, including lung tumor cells and macrophages remained viable in the absence of GGTase-I.

Inactivating the gene encoding the β-subunit of FTase eliminated farnesylation of HDJ2 and H-RAS, prevented H-RAS targeting to the plasma membrane, and blocked proliferation of fibroblasts. FTase inactivation reduced tumor formation and increased survival of mice with K-RAS–induced cancer to a similar extent as the inactivation of GGTase-I. The simultaneous inactivation of FTase and GGTase-I markedly reduced lung tumors and improved survival.

These data suggest that inhibition of FTase and/or GGTase-I could be useful in the treatment of K-RAS–induced cancer.

In mice, expression of oncogenic K-RAS or inactivation of *Nf1* in hematopoietic cells results in myeloproliferative disorders (MPDs) that do not progress to acute myeloid leukemia (AML). However, the simultaneous inactivation of *Nf1* and activation of oncogenic K-RAS in hematopoietic cells induced AML in mice. The levels of active RAS were not increased in mice with AML, raising the possibility that *Nf1* deficiency may contribute to AML by non-RAS pathways.

This result points to a strong cooperation between *Nf1* deficiency and oncogenic K-RAS and sheds new light on mechanisms of RAS-induced leukemia development.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. GGTase-I deficiency reduces tumor formation and improves survival in mice with K-RAS–induced lung cancer

Sjogren AK, Andersson KM, Liu M, Cutts BA, Karlsson C, Wahlstrom AM, Dalin M, Weinbaum C, Casey PJ, Tarkowski A, Swolin B, Young SG, Bergo MO.

J. Clin. Invest. 2007 May; 117(5):1294–1304.

II. Targeting the protein prenyltransferases efficiently reduces tumor development in mice with K-RAS–induced lung cancer

Liu M*, Sjogren AK*, Karlsson C, Ibrahim M, Andersson KM, Olofsson FJ, Wahlstrom AM, Dalin M, Yu H, Yang S, Young SG, Bergo MO.

Submitted (under revision in *Proc. Natl. Acad. Sci. USA*)

*These authors contributed equally.

III. *Nf1* deficiency cooperates with oncogenic K-RAS to induce acute myeloid leukemia in mice

Cutts BA*, Sjogren AK*, Andersson KM, Wahlstrom AM, Karlsson C, Swolin B, Bergo MO.

Blood 2009 Oct; 114(17): 3629–3632.

*These authors contributed equally.

ABBREVIATIONS

AML	Acute myeloid leukemia
DPI	Dual prenylation inhibitor
ER	Endoplasmic reticulum
ES cells	Embryonic stem cells
FTase	Farnesyltransferase
FTI	FTase inhibitor
FTS	Farnesyl thiosalicylic acid
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGTase-I	Geranylgeranyltransferase type I
GGTI	GGTase-I inhibitor
GTP	Guanosine triphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
ICMT	Isoprenylcysteine carboxyl methyltransferase
MDS	Myelodysplastic syndrome
MPD	Myeloproliferative disease
MPN	Myeloproliferative neoplasm
NF1	Neurofibromatosis type I
Np-RAP1A	Nonprenylated RAP1A
NSCLC	Non-small cell lung cancer
PI3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PP	Pyrophosphate
RCE1	RAS-converting enzyme 1

TABLE OF CONTENTS

De omnibus dubitandum – "Allt bör betvivlas."

René Descartes

INTRODUCTION

Everyone knows someone who has been diagnosed with cancer. In Sweden, 50 000 new cases are reported every year and around 13% of all deaths in the world are cancer related [1, 2]. Although surgery, radiation and cytostatic drugs are still the most common treatment strategies today, increased knowledge about the molecular mechanisms governing tumorigenesis has resulted in rational design of new drugs.

One potential drug target is RAS—the most frequently mutated oncoprotein in human cancer. Mutations in RAS result in hyperactive RAS signaling. Hyperactive RAS signaling can also be caused by mutations in genes that interact with RAS, such as the tumor suppressor gene neurofibromatosis type I (*NF1*). Although knowledge about the biology of RAS proteins is increasing, we do not yet fully understand the physiologic and therapeutic importance of the posttranslational processing of RAS, or the ability of RAS to cooperate with other mutations in cancer development. An overall goal of my thesis is to shed some light on those issues.

CAAX proteins are involved in the pathogenesis of cancer

RAS belongs to the family of *CAAX* proteins. *CAAX* proteins terminate with the amino acid sequence *C-A-A-X*, where "*C*" is a cysteine, "*A*" is often an aliphatic amino acid, and "*X*" can be any amino acid. The family of *CAAX* proteins consists of more than hundred members [3]. RAS is perhaps the most well known *CAAX* protein involved in cancer development, but several other *CAAX* proteins also contribute to tumor development and progression (e.g. RHOA, RAC1, CDC42, RALA, RHOC), by both RAS-dependent and RAS-independent mechanisms [4-9]. Table 1 lists the *CAAX* proteins discussed in this thesis and summarizes their normal functions and potential roles in cancer.

Table 1. *CAAX* proteins

Protein	Function	Cancer association	*CAAX*	F	GG
H-RAS	GTPase/Signal transduction	Oncogene (point mutations) , transformation	CVLS	F	
RHEB	GTPase/Signal transduction	Overexpressed in tumor cells	CSVM	F	
CENP-E	Mitotic protein/kinetochore-microtubule attachments		CKTQ	F	
CENP-F	Mitotic protein/kinetochore-microtubule attachments	Overexpressed in tumors	CKVQ	F	
RND1	GTPase/Organization of actin cytoskeleton		CSIM	F	
RND2	GTPase/Organization of actin cytoskeleton		CNLM	F	
RND3/RHOE	GTPase/Organization of actin cytoskeleton	Cell type-dependent effects	CTVM	F	
HDJ2	Cochaperone/Protein folding		CQTS	F	
Prelamin A	Processed to Lamin A and C/Nuclear lamina		CSIM	F	
PRL1	Tyrosine phosphatase/Cell growth and mitosis	Progression, motility and invasion	CCIQ	F	GG*
PRL2	Tyrosine phosphatase/Cell growth and mitosis	Progression	CCVQ	F	GG*
PRL3	Tyrosine phosphatase/Cell growth and mitosis	Progression, motility and invasion	CCVM	F	GG*
K-RAS	GTPase/Signal transduction	Oncogene (point mutations) , transformation	CVIM	F	GG**
N-RAS	GTPase/Signal transduction	Oncogene (point mutations) , transformation	CVVM	F	GG**
RHOB	GTPase/Endocytic trafficking	Tumor suppressor	CKVL	F	GG
RHOH	GTPase/Signal transduction in hematopoetic cells	Tumor suppressor	CKIF	F	GG
RHOA	GTPase/Actin cytoskeleton, migration, trafficking etc	Overexpressed in tumors, RAS transformation	CLVL		GG
RHOC	GTPase/Stress fibers, focal adhesions, trafficking	Overexpression, invasion and metastasis	CPIL		GG
RAC1	GTPase/Cytoskeleton, transcription, proliferation, migration	Overexpression and mutations, RAS transformation	CLLL		GG
RAC2	GTPase/Cytoskeleton, transcription, proliferation, migration	Overexpressed in tumors	CSLL		GG
RAC3	GTPase/Cytoskeleton, transcription, proliferation, migration	Overexpressed in tumors	CTVF		GG
CDC42	GTPase/Actin cytoskeleton, proliferation	RAS transformation	CVLL		GG
RALA	Vesicle trafficking, cell morphology, motility, transcription	RAS transformation, invasion and metastasis	CCIL		GG
RALB	Vesicle trafficking, cell morphology, motility, transcription	Cell survival, invasion and metastasis	CCLL		GG
RAP1A	Proliferation, differentiation, adhesion, polarity, migration	Cell type-dependent effects on proliferation, metastasis	CLLL		GG

F = farnesylation, GG = geranylgeranylation
* Weak substrate for GGTase-I when FTase is inhibited
** Substrate for GGTase-I when FTase is inhibited

CAAX proteins undergo posttranslational modifications

The *CAAX* motif triggers three posttranslational modifications: isoprenylation, endoproteolysis and methylation (figure 1). These modifications increase membrane affinity, promote protein-protein interactions and can affect the stability of *CAAX* proteins [10-12]. Moreover, many *CAAX* proteins need a second signal to acquire a stable membrane association, such as palmitoylation of upstream cysteine residues or the presence of a polybasic sequence [13, 14].

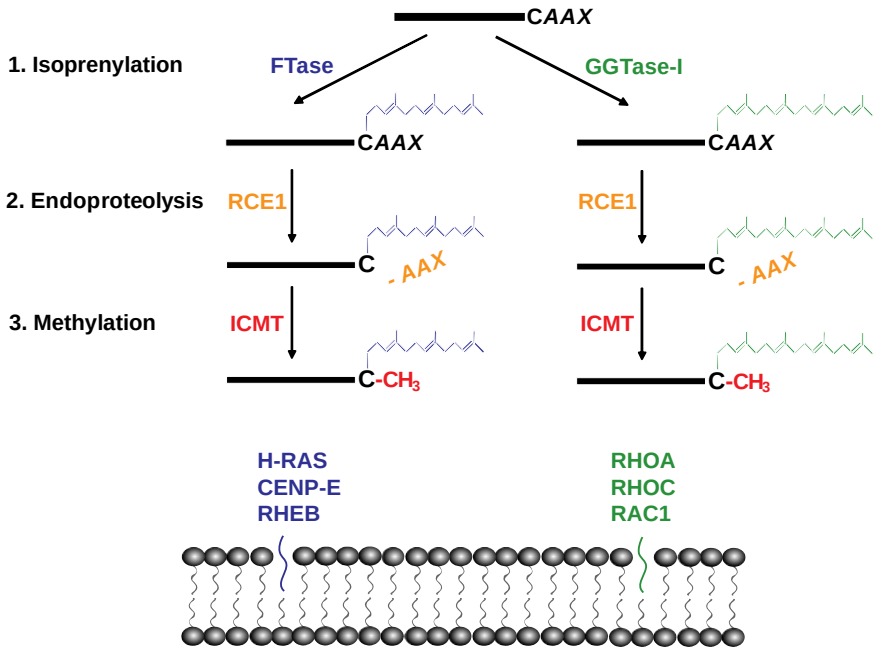


Figure 1. *CAAX* proteins undergo three posttranslational modifications that render them hydrophobic. Immediately after translation, *CAAX* proteins are isoprenylated in the cytosol by farnesyltransferase (FTase) or geranylgeranyltransferase type I (GGTase-I). Isoprenylated *CAAX* proteins are targeted to the endoplasmic reticulum (ER), where RAS-converting enzyme 1 (RCE1) and isoprenylcysteine carboxyl methyltransferase (ICMT) are located. RCE1 is an endoprotease that cleaves off the - *AAX* amino acids. Finally, the newly exposed isoprenylated cysteine (C) is methylated by ICMT. The modified *CAAX* proteins are then transported by various routes to their subcellular locations.

Isoprenylation

Isoprenylation[1] is the covalent attachment of either a farnesyl or a geranylgeranyl isoprenoid lipid to the cysteine residue of the *CAAX* motif. Isoprenylation of *CAAX* proteins is catalyzed by FTase and GGTase-I.

Isoprenoid lipids are intermediates in the cholesterol synthesis pathway

The 15-carbon isoprenoid farnesyl-pyrophosphate (farnesyl-PP) is an intermediate in the cholesterol synthesis pathway (figure 2, page 11) and studies of this pathway led to the discovery of protein isoprenylation in mammalian cells [13]. The rate limiting step in cholesterol synthesis is the formation of mevalonate from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is catalyzed by HMG-CoA reductase. Statins are widely used drugs that lower blood cholesterol levels: they block the formation of cholesterol by inhibiting HMG-CoA reductase.

[1] Farnesylation and geranylgeranylation are collectively called isoprenylation, prenylation or lipidation. In this thesis I use the term isoprenylation.

But statins affect a variety of cellular functions, such as reducing cell proliferation, and many of those effects appear to be unrelated to the lowering of cholesterol. The effect of statins on cell proliferation was restored by supplementing the culture medium with mevalonate, but not cholesterol. By tracing the added mevalonate, it was found that mevalonate was converted into isoprenoids, which were incorporated into proteins [15].

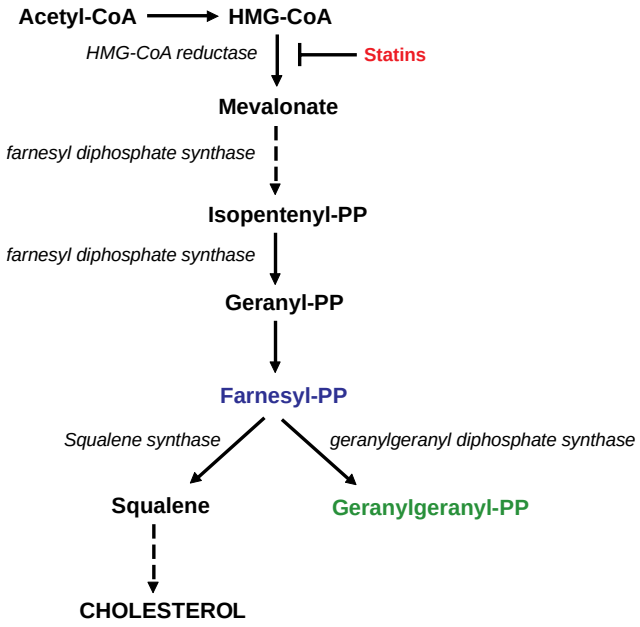


Figure 2. The cholesterol synthesis pathway. First, Acetyl-CoA is converted to HMG-CoA. HMG-CoA is converted to mevalonate by HMG-CoA reductase; this is the rate-limiting step and can be inhibited by statins. Isopentenyl-PP is formed from mevalonate, by phosphorylation and decarboxylation. Next, isopentenyl-PP is converted to geranyl-PP, which condenses with another isopentenyl-PP molecule to yield farnesyl-PP: both these steps are catalyzed by farnesyl diphosphate synthase. Finally, squalene is formed by condensation of two molecules of farnesyl-PP, catalyzed by squalene synthase. Through a series of additional reactions squalene is converted to cholesterol. In addition, geranylgeranyl diphosphate synthase catalyzes the formation of geranylgeranyl-PP from farnesyl-PP.

Enzymology of FTase and GGTase-I

Mammalian FTase and GGTase-I were first identified and isolated from rat brain cytosol in the early 1990: ies [16-19]. FTase and GGTase-I share a common α-subunit, but have unique β-subunits (table 2, page 12). In humans, the common α subunit is encoded by *FNTA* and the β subunits are encoded by *FNTB* and *PGGT1B,* respectively. It is the β subunits that dictate substrate specificity: in general, if the "*X*" residue of the *CAAX* motif is leucine, the protein is geranylgeranylated; otherwise it is farnesylated [3, 20]. However, there are several exceptions to this rule and it is clear that the "*X*" residue only partially explains the substrate specificity.

FTase and GGTase-I are relatively selective towards their respective substrates, but there are examples of cross reactivity. Proteins with a phenylalanine residue (F) at the "*X*" position, for example RHOH, can be substrates for both enzymes. Also, K-RAS and N-RAS end with a methionine (M) and are normally farnesylated by FTase, but when FTase activity is inhibited they can be geranylgeranylated by GGTase-I [21, 22]. Additionally, RHOB is a substrate for both GGTase-I and FTase, despite the C-terminal leucine [3].

Crystal structures of mammalian FTase and GGTase-I, including substrate and product complexes, have increased the understanding of reaction mechanisms and substrate specificity [20, 23-25]. Both FTase and GGTase-I are zinc metalloenzymes and depend on binding a Zn^{2+} ion for catalytic activity [20]. FTase also requires Mg^{2+} for full activity [10].

Table 2. Characteristics of FTase and GGTase-I

	Farnesyltransferase (FTase)		Geranylgeranyltransferase type I (GGTase-I)
Subunits (mammalian)	α (48 kDa) β (46 kDa)	*identical* *25% identity*	α (48 kDa) β (43 kDa)
Genes (mammalian)	*FNTA* (α) *FNTB* (β)		*FNTA* (α) *PGGT1B* (β)
Metal requirements	Zn^{2+} Mg^{2+}	*catalysis, protein binding*	Zn^{2+}
Lipid substrate	Farnesyl-PP 15 carbon		Geranylgeranyl-PP 20 carbon
Protein recognition motif	*CAAX* X=Ala, Gln, Ser, Met, Phe		*CAAX* X=Leu, Phe, sometimes Met
Selected protein substrates	RAS, nuclear lamins, transducin γ subunit, Rhodopsin kinase, centromeric proteins		RHOA, RHOC, RAC, RAP heterotrimeric G protein γ subunits

(Adapted from Lane and Beese, *JLR*, 2006)

Genetic characterization of FTase and GGTase-I

FTase and GGTase-I have been cloned from a number of non-mammalian species. Studies in yeast, fungi, flies and plants yielded diverging results on the impact of disrupting FTase and GGTase-I activity. *RAM2*, a homolog of *FNTA*, is essential in the yeast *Saccharomyces cervisiae* [26] and in *Candida albicans* [27]. In contrast, disruption of *RAM1*, the homolog of *FNTB*, was not lethal but resulted in growth defects [26, 28]. Null mutations in *CDC43*, a homolog of *PGGT1B*, however, were lethal in yeast [29]. Interestingly, *Candida albicans* null *CDC43* mutants were viable, despite the lack of detectable GGTase-I activity, but were morphologically abnormal [30]. Furthermore, null mutations in the β subunit of GGTase-I were lethal in *Drosophila melanogaster* [31] but not in *Arabidopsis thaliana* [32]. Based on these different results, it was impossible to predict the impact of FTase and GGTase-I deficiency in mammalian cells.

A few years ago, Mijimolle et al. developed mice with a conditional knockout allele for *Fntb* [33]. In their study, the inactivation of *Fntb* resulted in embryonic lethality, but the effects in adult tissues were very modest. Some findings were clearly inconsistent with previous studies. Inactivation of *Fntb* appeared to inhibit the farnesylation of HDJ2 and H-RAS, but only partially, and most remarkably, H-RAS remained in the membrane fraction of cells. They also reported that *Fntb*-deficient fibroblasts grew in culture and that the development of K-RAS–induced tumors was unaffected by *Fntb* deficiency.

These findings were surprising for several reasons. First, several studies had established that membrane association of H-RAS is utterly dependent on farnesylation [34, 35]. Second, treating cells with FTase inhibitors (FTIs) typically results in cell cycle arrest and, in mouse models, FTIs are efficacious against many tumors, including those without RAS mutations [36, 37]. Given these unexpected results, we saw an urgent need for reevaluating the role of FTase in mammalian cells and in malignant transformation. The consequences of genetic disruption of *PGGT1B* in mammalian cells had not been studied before the work in this thesis.

Post-isoprenylation (endoproteolysis and methylation)

Membrane association of *CAAX* proteins requires isoprenylation, but the contribution of the post-isoprenylation reactions endoproteolysis and methylation are also significant [10, 38]. The importance of these modifications for subcellular localization and function of *CAAX* proteins have been studied by members of our lab and others, and are described elsewhere [11, 38-43]. Furthermore, our lab defined the impact of *Rce1* and *Icmt* deficiency on K-RAS–induced myeloproliferative disease (MPD) and lung cancer in mice. *Icmt* deficiency reduced all cancer phenotypes, suggesting that ICMT may be a promising drug target for treating RAS-induced cancer [44]. In contrast, *Rce1* deficiency surprisingly accelerated K-RAS–induced MPD development [45].

RAS-induced cancer

The RAS proteins are the most well known *CAAX* proteins associated with cancer. Mutational activation of RAS contributes to tumor formation, progression and metastasis [46]. There are three *RAS* proto-oncogenes[2] in humans: *HRAS*, *NRAS*, and *KRAS*. Activating somatic mutations in these genes, and also mutations in regulators and effectors of the RAS proteins, are prevalent in human cancer, such as pancreas, lung and myeloid malignancies [47].

The interest in RAS started in the 1960s with the discovery of the Harvey and Kirsten rat sarcoma viruses [48, 49]. These retroviruses had hijacked oncogenes from the host (rat) genome, which were responsible for the cancer causing activities of these viruses. These genes were called *Ras* (Rat sarcoma) genes (*Ha-ras* and *Ki-ras*, respectively) [50, 51]. In 1982, it was discovered that the retroviral oncogenes had human homologs (named *HRAS* and *KRAS*) [52-54]. In 1983, a human transforming gene was identified as *NRAS*, the third member of the *RAS* gene family [55, 56].

H-RAS, N-RAS and K-RAS have overlapping but distinct functions

In humans, the three *RAS* genes encode four highly homologous 21 kDa proteins: H-RAS, N-RAS, K-RAS4A and K-RAS4B. K-RAS4A and 4B result from alternative splicing of the same gene; K-RAS4B is the dominant variant and is referred to in this thesis as K-RAS. RAS proteins have the first 85 amino acids in common, which specify binding to GDP and GTP, while they diverge at the C-terminal end (known as the hypervariable region). The hypervariable region contains residues that target RAS proteins to membranes. All RAS proteins are farnesylated at the C-terminal *CAAX* motif. In addition, RAS proteins need a second membrane targeting signal. N-RAS and H-RAS are modified by palmitoylation, while K-RAS has a polybasic stretch of lysine residues [57].

H-RAS, N-RAS and K-RAS have both unique and overlapping functions in different tissues. First, the frequency of mutations in the different *RAS* genes differs and they are associated with different types of cancer. Second, genetic studies in mice have shown that K-RAS is essential for mouse embryonic development [58, 59], whereas mice deficient in H-RAS and N-RAS develop normally and are viable [60, 61].

The relative importance of the RAS isoforms in different tissues may be explained by differences in expression levels. The functional differences between the RAS proteins may also depend on the subcellular localization of the proteins [62]. Once the posttranslational processing is completed in the ER, palmitoylated N-RAS and H-RAS are transported to the plasma membrane by vesicular transport. K-RAS is transported to the plasma membrane via

[2] A proto-oncogene is a normal gene that can become an oncogene by mutations or increased expression.

an uncharacterized route, perhaps via microtubules [63]. An example of the importance of subcellular function is that oncogenic H-RAS has transforming activity when located in ER, but not when located in Golgi [64, 65].

Tight regulation of RAS signaling in normal cells

RAS proteins act as molecular switches that convert extracellular stimuli (growth factors and cytokines) into cellular responses, including proliferation, differentiation and survival (figure 3) [57, 62, 66]. RAS proteins are small GTPases that cycle between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound state. Activation of RAS starts with the binding of a ligand to a cell surface receptor, such as receptor tyrosine kinases and G-protein–coupled receptors. The activated receptor binds to adaptor proteins, which recruit guanine nucleotide exchange factors (GEFs) to the plasma membrane, where RAS is located. The association between RAS and GEFs facilitates nucleotide exchange on RAS, resulting in replacement of GDP with GTP, which is more abundant in the cytosol. GTP-bound RAS has higher affinity for effectors and switches on downstream signaling pathways. The signal is rapidly terminated by the inactivation of RAS through GTP hydrolysis back to GDP, which is stimulated by GTPase activating proteins (GAPs) [57].

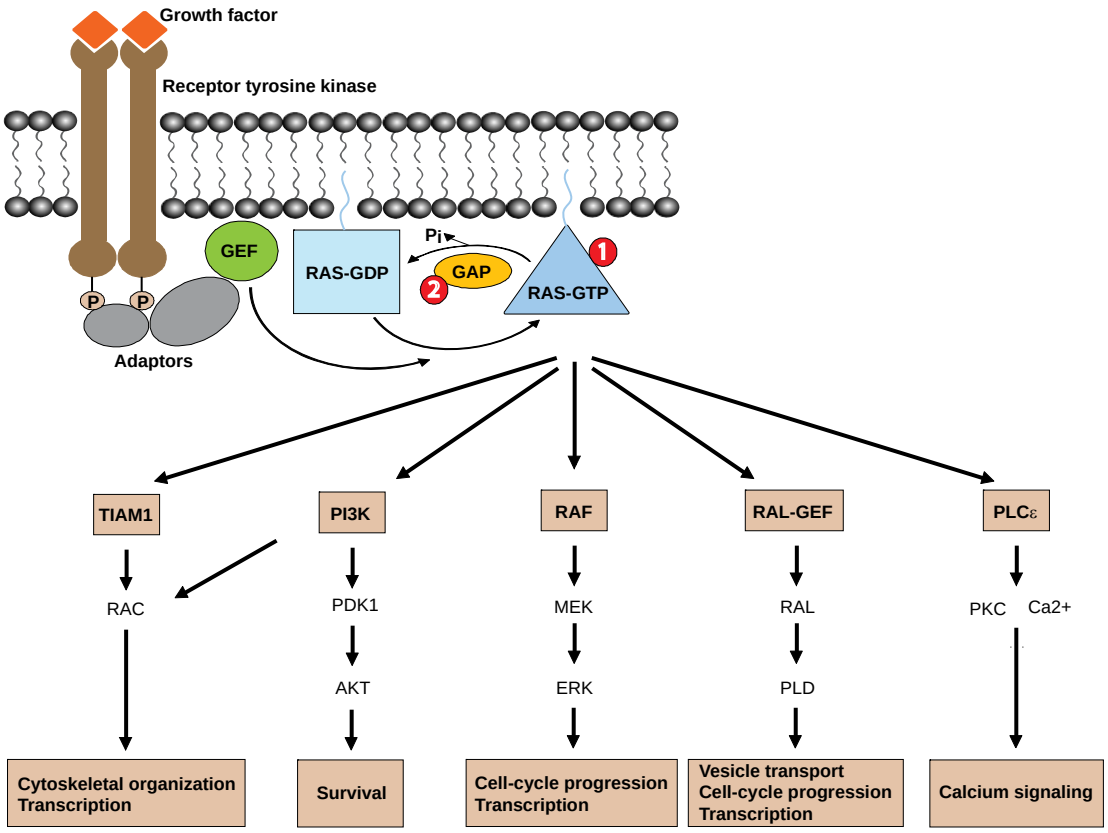


Figure 3. RAS signaling pathways. Ligand-bound, activated receptor tyrosine kinases form complexes with adaptor proteins, like GRB2 and SHC, which recruit GEFs (e.g. SOS) to the plasma membrane. GEFs stimulate the nucleotide exchange on RAS, resulting in increased levels of active GTP-bound RAS. The activation is opposed by the activity of GAPs, which ensure that RAS is rapidly inactivated after stimulation. At least six different RAS-GAPs exist, including p120GAP and NF1. RAS-GTP interacts with several families of effector proteins. The main effector pathways are shown. Numbers 1 and 2 indicate sites for mutations (activating and inactivating, respectively) that result in hyperactive RAS signaling.

RAS-GTP binds to and activates several effectors, which activate a diverse set of signaling cascades. The RAF-MEK-ERK-signaling pathway was the first RAS effector pathway to be characterized and is the one that has been studied the most [62, 67]. Activated ERK stimulates transcription factors, which can activate expression of cell cycle regulatory proteins, such as Cyclin D.

Phosphatidylinositol-3 kinase (PI3K) is another well characterized effector of RAS. Activation of P13K results in generation of the second messenger PIP_3, which activates the kinases PDK1 and AKT. AKT promotes cell survival by phosphorylating, and thereby inactivating, several pro-apoptotic proteins (e.g. BAD). In addition, PI3K activation stimulates RAC, a RHO family GTPase involved in actin cytoskeleton regulation and NF-κB–mediated transcription. RAS can also activate RAC independently of PI3K, by activating the RAC-GEF TIAM1 [68].

RAL-GEFs, such as RALGDS, are yet another RAS effector family. RAL-GEFs stimulate RAL, which activates phospholipase D, an enzyme regulating vesicle trafficking. The RALGDS pathway has been suggested to also contribute to the inhibition of FORKHEAD transcription factors, thereby promoting cell cycle progression and survival. In addition, RAS-GTP can bind to phospholipase C-ε (PLCε) leading to calcium release and activation of protein kinase C (PKC) [69].

Hyperactive RAS signaling in tumor cells

RAS signaling is commonly hyperactivated in tumor cells, which deregulates the downstream signaling pathways and results in aberrant cell growth, survival and differentiation [57].

RAS mutations: Activating point mutations in RAS occur in 20-30% of human cancers [47, 57, 66]. RAS mutations are particularly common in pancreatic (60-90%), colon (35-50%), and lung cancer (20-30%), and in myeloid leukemia (20-30%) [47, 57]. These point mutations cause amino acid substitutions, commonly at codons 12, 13 and 61, which decrease the intrinsic GTPase activity or confer resistance to GAPs. In this way, the mutant RAS protein becomes locked in the active, GTP-bound state and signals continuously (figure 3, page 14). The most frequently mutated *RAS* gene is *KRAS* (85%), followed by *NRAS* (15%) and *HRAS* (<1%) [66]. K-RAS mutations are common in adenocarcinomas of the pancreas, colon and lung [47, 57]. In myeloid leukemia, N-RAS mutations are the most common (but K-RAS mutations are prevalent). H-RAS mutations are found in bladder and thyroid cancer [47, 57].

Inactivating mutations in GAPs: Hyperactive RAS signaling can also be caused by loss of function mutations in negative regulators, such as GAPs. The gene *NFI* encodes a RAS-GAP and functions as a tumor suppressor [70]. Loss of *NFI* results in accumulation of GTP-bound RAS, due to decreased GTP hydrolysis (figure 3, page 14). Patients with an inherited cancer syndrome called NF1 are deficient in one of their two *NF1* alleles, and are predisposed to develop certain tumors (in which both *NF1* alleles are often inactivated). Mutations in *NF1* will be further discussed in subsequent chapters.

Mutations upstream and downstream of RAS: RAS signaling pathways are commonly hyperactivated by alterations in upstream growth factor receptor tyrosine kinases. The epidermal growth factor receptors EGFR/ERBB1 and ERBB2 (also known as HER2/neu) are activated in tumor cells by gene amplification, autocrine or paracrine growth factor production, or activating mutations [71]. C-KIT and FLT3 are other growth factor receptor tyrosine kinases involved in tumorigenesis. Gain of function alterations, such as point mutations, result in ligand-independent activation of these receptors, which can then hyperactivate downstream targets, including RAS. C-KIT is involved in several types of

malignancies, including different forms of leukemia and FLT3 is one of the most frequently mutated genes in acute myeloid leukemia (AML) [72]. Moreover, mutations or amplification of downstream RAS effectors are implicated in human cancer development. One example is BRAF that is frequently mutated in melanomas, which results in activation of ERK. Deletion of the tumor suppressor gene *PTEN*, resulting in activation of the PI3K pathway, is another example [66].

Multiple RAS effector pathways contribute to RAS-induced transformation

Several of the many RAS effectors are expressed in the same cell types. Furthermore, a certain effector is activated differentially by the different RAS isoforms. This, together with the extensive cross-talk between RAS signaling pathways and between RAS and RHO signaling pathways, add to the complexity of regulating cellular behavior. It is a challenge to reveal the contributions of each RAS signaling pathway to RAS-induced cancer.

RAF was the first RAS effector to be identified and by that time it was believed that all consequences of mutations in RAS could be explained by the activation of the RAF-MEK-ERK pathway. Indeed, activation of MEK and ERK is required for RAS-induced transformation of murine cell lines [66]. Also, RAS and B-RAF mutations are found in the same type of cancers, in essentially non-overlapping occurrence [67, 73, 74]. However, some studies have shown that RAF activation alone is not sufficient for tumor development [67].

The discovery of mutations in other RAS effector pathways support the proposal that oncogenic activities of RAS are mediated also by RAF-independent signaling [67]. Mutations in members of the PI3K pathway have been estimated to be found in up to 30% of human cancers [75]. Such mutations are common in breast and colon cancer and include the loss of the tumor suppressor PTEN and point mutations in the catalytic subunit of PI3K [76, 77]. AKT has emerged as a critical mediator for PI3K signaling in tumorigenesis, due to its role in regulating cell survival and cell cycle progression [77].

The RAL-GEF-RAL pathway is probably also important for RAS oncogenesis, even if the details about downstream functions of RAL are still unknown [62]. Studies in rodent fibroblasts suggested a contributory, but yet limited, role for RAL-GEFs in RAS-induced transformation [78]. In human epithelial cells, on the other hand, the activation of the RAL-GEF-RAL pathway alone was enough to induce RAS transformation [79]. In addition, activation of RAL-GEFs alone resulted in metastatic growth of NIH3T3 cells [80].

Finally, activation of TIAM1 may be involved in RAS-induced tumor initiation. Much data point at an extensive collaboration between different GTPase-regulated signaling pathways in controlling cell responses and promoting cell transformation [81]. TIAM1, in its role as a RAC-GEF, is one link between RHO GTPases and oncogenic RAS signaling [67].

Targeting RAS as an anti-cancer strategy

The essential role of RAS in cancer makes it an attractive target for anti-cancer therapy. To target the RAS protein directly, with drugs that interfere with GTP binding or that restore GAP sensitivity, has proven to be very difficult. Attempts are made to directly target gene expression of RAS with antisense techniques, but the usefulness of this strategy is not yet known [82]. Therefore, much effort has been spent on targeting RAS indirectly by inhibiting members upstream or downstream of RAS signaling pathways, or by interfering with RAS membrane association. Targeting RAS membrane association by interfering with the processing of the *CAAX* motif is one aim of this thesis.

Pharmacologic targeting of *CAAX* protein isoprenylation

In 1984, it was found that the C-terminus of RAS was required for membrane association and transformation [35]. Some years later it was realized that RAS proteins are farnesylated [83] and that farnesylation was required for membrane association and transformation of cells [84-86]. This was the starting point for the interest in protein isoprenylation as a target for anti-cancer therapy. Later it became clear that isoprenylation is crucial also for the cellular activities of several other *CAAX* proteins, including geranylgeranylated proteins like RHOA [87]. The post-isoprenylation modifications are also important for proper functions of *CAAX* proteins and all four enzymes: FTase, GGTase-I, RCE1 and ICMT are potential anti-cancer drug targets. This section describes the development and use of inhibitors against FTase and GGTase-I and some of their potential target proteins. I also shortly describe statins, bisphosphonates and farnesyl thiosalicylic acid (FTS), which are drugs that interfere with the membrane association and proper function of *CAAX* protein by alternative mechanisms. Inhibitors of RCE1 and ICMT, the enzymes required for the endoproteolysis and methylation, have also been developed and are described elsewhere (reviewed in [88]).

FTase inhibitors (FTIs)

Shortly after the discovery that RAS is farnesylated, the responsible enzyme, FTase, was characterized and the search for FTIs began [83]. One class of FTIs is the *CAAX* peptidomimetics, such as FTI-276 and FTI-2148, which mimic the *CAAX* motif. Several other FTIs, including lonafarnib (SCH66336) and tipifarnib (R115777), have been identified through library screenings. In early preclinical studies, FTIs blocked tumorigenic growth of many different tumor cell lines [36, 89, 90]. In most cases FTIs induced a G_2/M cell cycle arrest, but some studies reported a G_0/G_1 arrest or no effect on the cell cycle [36, 90, 91]. The ability of FTIs to induce apoptosis seems to be dependent on cell type and on secondary events (e.g. serum depletion) [36, 90]. In vivo, FTIs inhibited tumor growth in xenograft mouse models [92, 93] and in K- and N-RAS transgenic mice [37, 94]. In H-RAS transgenic mice, FTIs even induced tumor regression [95]. Importantly, the antitumor effects of FTIs in mouse models have been achieved with minimal toxicity [96].

FTIs have been evaluated in many clinical trials and have generally been well tolerated; even if some adverse side-effects have been reported, including myelosuppression, diarrhea and vomiting [36, 82]. However, the antitumor effects of FTIs in clinical trials have been disappointing. Good single agent results have been achieved in treatment of hematological malignancies, especially myeloid leukemia, but not for treatment of solid tumors [36]. Still, for the treatment of solid tumors there are some promising data on combining FTIs with chemotherapy.

One likely reason for the lack of potent activity of FTIs is that K-RAS and N-RAS, which are the isoforms most often mutated in human cancers, can be geranylgeranylated in the setting of FTI therapy [21, 22]. This alternative isoprenylation may also explain why FTIs are not as effective in K-RAS and N-RAS transgenic mice, as in H-RAS mouse models. Nevertheless, there is an antitumor effect, despite failure to block RAS isoprenylation, which implicates that the FTI activity is due to inhibition of farnesylation of other proteins. The lack of correlation between antitumor activity and RAS mutations in human cancer cell lines further supports the idea that other *CAAX* proteins are critical targets for FTIs [92, 97].

Potential targets for FTIs:

In theory, the antitumor effect of FTIs can be explained by the loss or change of function of any farnesylated protein(s). Some of the candidate proteins are listed in table 1 (page 9) and discussed further below.

H-RAS

All RAS isoforms are normally farnesylated in vivo. However, as mentioned earlier, K-RAS and N-RAS are isoprenylated by GGTase-I, when FTase activity is inhibited. In contrast, H-RAS is exclusively farnesylated and its membrane association can be completely inhibited with an FTI, resulting in inhibition of H-RAS–dependent oncogenesis [34]. Consequently, tumors harboring H-RAS mutations would be susceptible for FTI treatment, but unfortunately H-RAS mutations are very rare in human cancer. However, FTIs also inhibit wild-type H-RAS, which may have antitumor effects. For example, if RAS signaling is increased due to upstream activating mutations, inhibition of wild-type RAS could stop the signals from reaching the downstream effectors [36, 98]. Targeting a wild-type RAS isoform may also be beneficial in tumors harboring a mutation in another RAS isoform. This idea was suggested by Fotiadou et al., which showed that cell transformation required both wild-type K- and N-RAS, because of distinct downstream signaling branches [99]. In fact, there is growing evidence that the RAS isoforms are functionally different, maybe due to their different subcellular localizations [57].

RHEB

RHEB (Ras homolog enriched in brain) is a farnesylated small GTPase that activates the mTOR signaling pathway and regulates cell growth and the actin cytoskeleton [36]. In *Drosophila,* RHEB is required for cell cycle progression and it is overexpressed in transformed cells and human tumor cell lines [36, 98]. FTI treatment (lonafarnib) in cell culture completely inhibited RHEB isoprenylation and blocked downstream signaling of mTOR [100]. In the same study, lonafarnib enhanced the apoptotic response to the chemotherapeutics tamoxifen and taxane, an effect that was abrogated by expressing a geranylgeranylated form of RHEB (CSVM → CSVL) [100]. In *Schizosaccharomyces pombe*, a geranylgeranylated form of RHEB reversed the cell cycle defect caused by lack of FTase activity. These studies suggest that inhibition of RHEB farnesylation may contribute to the antitumor activities of FTIs, either when the FTI is used alone or when combined with other chemotherapeutics [36, 98].

Centromere proteins (CENP-E and CENP-F)

Inhibiting farnesylation of the mitotic proteins CENP-E and CENP-F may also contribute to the antitumor activities of FTIs. CENP-E and CENP-F are centromere-associated proteins that function in kinetochore-microtubule attachments during mitosis [36, 101]. One study showed that lonafarnib depleted CENP-E and CENP-F from metaphase kinetochores, which disrupted chromosomal maintenance, resulting in mitotic delay [101]. This was consistent with an earlier study demonstrating that farnesylated CENP-F was required for G_2/M progression [102]. Thus, inhibition of CENP-E and/or CENP-F functions may explain the accumulation of tumor cells in the G_2/M phase in response to FTI treatment. Also, CENP-F is upregulated in head, neck and breast tumors, and may be associated with poor prognosis [103, 104].

RHOB

RHOB belongs to the family of RHO GTPases and exists in both farnesylated and geranylgeranylated forms, the latter being more abundant (70%) [105]. RHOB localizes to endosomes and regulates endocytic trafficking [106]. It has been suggested that farnesylated RHOB (RHOB-F) and geranylgeranylated RHOB (RHOB-GG) are functionally different; and that RHOB-GG, which accumulates in FTI-treated cells has an anti-proliferative activity [36, 98]. However, other studies argue against RHOB-F as a crucial FTI target. For example, RHOB-F was shown to be as potent as RHOB-GG in inhibiting cell proliferation in a human tumor cell line [107]. RHOB has even been proposed to act as a tumor suppressor, since it is downregulated in human tumors and since it inhibits tumor growth, cell migration and invasion. Moreover, RHOB-null mice are more susceptible to carcinogen-induced skin tumor formation [5, 108].

PRL/PTP-*CAAX*

The PRL1, 2 and 3 proteins belong to a family of protein tyrosine phosphatases (also called PTP-*CAAX*), which regulate cell proliferation and mitosis. All three PRL proteins are farnesylated, but can undergo some, inefficient, alternative isoprenylation [36]. The PRL proteins, especially PRL3, appear to be involved in cancer progression [109]. Expression of PRL1 and PRL3 has been shown to promote motility and invasion of adenocarcinoma cells. Importantly, treating these cells with FTI-2153 disrupted the subcellular localization of the PRLs and completely inhibited invasion and motility [110]. This suggests that PRL proteins may be important targets for FTI-mediated antitumor effects, especially in metastatic cancers.

RND proteins

The RND (round) proteins RND1, RND2 and RND3 (also called RHOE) are unusual members of the RHO family of small GTPases. They are always bound to GTP and are regulated by expression, localization and phosphorylation instead of GDP/GTP cycling [111, 112]. They are named after the rounded morphology and disrupted actin cytoskeleton observed in cells overexpressing RND1 and RND3. The RND proteins regulate the organization of the actin cytoskeleton. However, it is only RND3 that has been clearly linked to cancer [111], even if its role is not clear and probably cell type-dependent. RND3 is downregulated in some tumors and upregulated in others. RND3 has been shown to inhibit cell cycle progression and RAS-induced transformation, but also to have a pro-survival effect and to promote cell migration and invasion [5, 113]. RND proteins are normally farnesylated and FTI-treatment did not induce alternative isoprenylation [38]. FTI-treatment disrupted the subcellular localization of the RND proteins and reversed the rounded phenotype induced by the ectopic expression of RND1 and 3 [38]. However, the effects of FTI-induced inhibition of membrane association of RND proteins on cancer development remain to be elucidated.

GGTase-I inhibitors (GGTIs)

The isoprenylation of K-RAS and N-RAS by GGTase-I in the setting of FTI therapy was one of the reasons for the development of GGTIs. GGTIs might be used in combination with FTIs to block the isoprenylation of K-RAS and N-RAS. However, it was hypothesized that GGTIs would also be effective on their own, because several geranylgeranylated *CAAX* proteins, such as RHOA, RHOC and RALA, are involved in tumor growth and metastasis [7, 9, 114].

In vitro, GGTIs inhibit proliferation of a variety of human cancer cell lines, by inducing a G_0/G_1 cell cycle arrest [115-118]. The G_0/G_1 arrest may be caused by induction of the cyclin-dependent kinase inhibitor $p21^{CIP1}$ [115, 117]. Most GGTIs induce apoptosis to various

degrees in vitro [116, 118-123]. In vivo, GGTIs inhibit tumor growth in several mouse xenograft models [93, 118, 122, 124, 125]. Treatment with GGTI-2154 and GGTI-2418 not only inhibited tumor growth, but also induced regression of breast tumors in H-RAS and Erb2 transgenic mice, respectively [118, 126]. Some GGTIs induce apoptosis in vivo [122, 126] but some do not [125]. Indeed, one concern about the in vivo use of GGTIs is toxicity. In a study by Lobell et al., two structurally different GGTIs were toxic, at least at higher doses, and caused lethality in mice [123]. On the other hand, other studies have suggested that some GGTIs might not be particularly toxic [118, 124-126]. GGTI-2418 recently became the first GGTI to be evaluated in a Phase I clinical trial [127].

Potential targets for GGTIs:

GGTase-I is responsible for isoprenylating the majority of the RHO family proteins and most isoforms of the γ subunit of heterotrimeric G proteins. So far, there are no reports on alternative isoprenylation by FTase in the setting of GGTase-I deficiency or GGTI treatment. Therefore, all geranylgeranylated *CAAX* proteins are potential targets for GGTIs.

RHOA

Ras homolog gene family member A (RHOA) is a small GTPase that regulates the actin cytoskeleton and the formation of stress fibers. RHOA also affects epithelial polarity, focal adhesion, cell-cell adhesion, cell migration, vesicle trafficking and cytokinesis [128, 129]. These functions are important in tumorigenesis and aberrant RHOA signaling contributes to cancer development. RHOA expression or activity is frequently upregulated in human tumors [5]. In fibroblasts, activation of RHOA is necessary for RAS transformation [129-131]. Active RHOA downregulates the expression of the cyclin-dependent kinase inhibitors $p21^{CIP1}$ and $p27^{KIP1}$, and downregulation of $p21^{CIP1}$ levels is crucial for oncogenic RAS to promote cell cycle entry. Further, RHOA contributes to epithelial disruption during tumor progression and is involved in tumor invasion [129]. However, the precise role of RHOA in tumor invasion is not clear: inactivation of RHOA has been shown to both inhibit and promote migration and invasiveness, depending on cell type [132, 133].

Some studies have attributed the antiproliferative effect of GGTIs to loss of RHOA function. Geranylgeranylation of RHOA has been shown to be required for its ability to form actin stress fibers and focal adhesions, and to promote cell growth and transformation [87]. Also, it has been proposed that nongeranylgeranylated RHOA fails to downregulate $p21^{CIP1}$ and that this contributes to the cell cycle arrest caused by GGTI treatment. However, a study by Solski et al. showed that cells expressing a farnesylated, and thereby GGTI-insensitive, form of RHOA still underwent growth inhibition when treated with a GGTI [134]. This suggests that other GGTase-I substrates are likely to be involved in GGTI-mediated growth inhibition.

RHOC

The small GTPase RHOC is highly homologous to RHOA and RHOB and contributes to the regulation of stress fibers, focal adhesions and endosomal transport [128]. High levels of RHOC is found in some human tumors and appears to promote tumor invasion and metastasis [5]. Clark et al. used a genetic approach to identify RHOC as essential for metastasis [9], which was confirmed by siRNA knockdown of RHOC in vitro [132, 133]. In addition, knockout of RHOC in mice did not affect the development of mammary adenocarinomas, but drastically inhibited metastasis [135]. These results indicate that inhibition of geranylgeranylation of RHOC may contribute to the anti-invasion effects of GGTI treatment in human cancer cell lines [132, 136].

RAC GTPases

The RAC subfamily of RHO GTPases consists of RAC1, RAC2, RAC3 and RHOG, which are all geranylgeranylated [3, 38, 137]. The RAC proteins are involved in pathways that regulate cytoskeletal reorganization, gene expression, cell proliferation and migration. Many RAC functions are cell type-specific and include formation of lamellipodia, focal complexes and membrane ruffles, cell-cell adhesions, cell motility, and activation of NADPH oxidase [106, 128]. Deregulated RAC signaling is implicated in cancer development: RAC1 in particular, but to a lesser extent also RAC2 and RAC3, are upregulated in human tumors. RAC1 is also one of few RHO GTPases that has been shown to be mutated in tumors [5]. In vitro, activation of RAC1 stimulates RAS-transformation and may contribute to cancer cell proliferation by promoting cell cycle progression [138-141]. In vivo, RAC1 was shown to be required for K-RAS–induced lung tumor development [142] and mice lacking the RAC-specific GEF TIAM1 developed fewer skin tumors [143]. The roles of RAC proteins in cancer invasion are likely to be dependent on cell type and expression levels.

RAC1 and RAC3 have been suggested to be important targets for GGTIs. Cox and colleagues showed that GGTI-2166 treatment inhibited RAC1- and RAC3-mediated membrane-ruffling and transformation in NIH3T3 cells. Importantly, expression of GGTI-insensitive farnesylated versions of RAC1 and RAC3 rescued both membrane-ruffling and transformation [137].

CDC42

CDC42 is a RHO family GTPase that controls organization and rearrangement of the actin cytoskeleton, affecting formation of filopodia, cell polarity, migration and chemotaxis [106]. Moreover, CDC42 has been implicated in G_1 cell cycle progression [144]. Expression of constitutively active and dominant negative CDC42 constructs in Rat1 fibroblasts showed that CDC42 expression can stimulate anchorage-independent growth and contribute to RAS-induced transformation [6]. There are conflicting data regarding the role of CDC42 in tumor progression and the role of CDC42 in invasion and metastasis is also unclear [5].

RALA and RALB

The geranylgeranylated RAS-like (RAL) proteins, RALA and RALB, are small RAS GTPases. They participate in several cellular processes, for example vesicle trafficking, regulation of cell morphology and motility, and transcription [8, 145]. RALA and B are activated by RAL-GEFs (e.g. RALGDS), which are direct effectors of activated RAS. RALA and RALB have been found to be hyperactivated in several different tumor samples. Inhibition of RALA with RNAi techniques impaired anchorage-independent growth of cancer cell lines and reduced RAS-induced tumorigenesis in a xenograft model [7, 146]. Moreover, RALA may be important for invasion and metastasis [8, 146]. RALB, on the other hand, may be dispensable for RAS-transformation and tumor formation, but may be implicated in the survival of tumor cell lines and in invasion and metastasis [7, 8, 146]. A role for RAL signaling in survival is supported by a study showing that knocking out RALGDS in mice delayed the onset of tumor formation and decreased metastasis, effects that were associated with increased apoptosis [147].

GGTI studies have suggested that inhibiting the geranylgeranylation of RALA and RALB contributes to the antitumor effects of GGTIs. A study by Sebti and colleagues pointed out RALB as a crucial target for GGTI-induced apoptosis. They showed that a farnesylated RALB mutant made pancreatic cancer cells resistant to GGTI-induced apoptosis. In the same study, GGTI-treatment also inhibited anchorage-independent growth, which was rescued by a farnesylated RALA variant, suggesting RALA as a target for this GGTI-property [148].

RAP1

RAP1 exists in two isoforms (A and B) that belong to the RAS family of small GTPases and are exclusively geranylgeranylated [3]. RAP1 regulates ERK-dependent functions (proliferation and differentiation) and integrin-mediated functions (cell-cell adhesions, cell polarity and migration). The effects of RAP1 signaling on cell proliferation are probably cell context-dependent. In fibroblasts, RAP1 both attenuated and stimulated cell growth [149]. In mouse hematopoietic progenitors, deficiency in SPA1 (a RAP1 GAP) enhanced RAP1 signaling and resulted in enhanced proliferation [150]. RAP signaling has been implicated to also regulate cancer metastasis [151]. In many studies, detection of nonprenylated RAP1A (np-RAP1A) by western blot is used as marker for GGTase-I inhibition [152], but less is known about the functional importance of np-RAP1A.

There are more than 100 *CAAX* proteins and the antitumor effects of FTIs and GGTIs are likely a result of inhibiting several *CAAX* proteins. However, some of the studies discussed above indicate that inhibiting farnesylation or geranylgeranylation of a rather limited set of *CAAX* proteins may explain a great deal of the antitumor effects of FTIs and GGTIs. The conditional knockout mice for FTase and GGTase-I presented in this thesis are well suited for experiments aimed to reveal these target proteins.

Combining FTIs and GGTIs

Because neither an FTI alone nor a GGTI alone inhibits isoprenylation of K- and N-RAS, FTI/GGTI combinations and dual prenylation inhibitors (DPIs) have been evaluated. Indeed, such treatment has been shown to inhibit K-RAS and N-RAS isoprenylation in vitro [93, 123, 153]. In vitro, FTI/GGTI combinations or DPIs have resulted in increased anti-proliferative effects, compared to using either drug alone [93, 122, 153, 154]. In most cases the combined treatment or the use of a DPI resulted in additive or synergistic effects on the rate of apoptosis [116, 122, 123], while one study reported only a moderate increase of apoptosis [153].

The effects of FTI/GGTI combinations differ in vivo. In mice xenografted with human colon cancer cells, cotreatment with the GGTI BAL9611 and the FTI manumycin enhanced the inhibition of tumor growth and enhanced the amount of cell death [122]. No animal toxicity was reported. Similarly, cotreatment with FTI-276 and GGTI-297 was not toxic in nude mice with tumor xenografts; however, the cotreatment did not increase antitumor activity above what was seen with FTI-276 alone [93]. In contrast, in a study by Lobell et al., treatment with distinct FTI/GGTI combinations or DPIs were lethal in mice harboring pancreatic xenograft tumors, at doses required to block K-RAS isoprenylation. Importantly, at these high doses the GGTI compounds (GGTI-1 and GGTI-2) were toxic also on their own [123].

Potential targets of combined FTase and GGTase-I inhibition:

Inhibiting both FTase and GGTase-I would potentially block isoprenylation of all *CAAX* proteins, which make any *CAAX* protein a potential target for FTI/GGTI or DPI treatment. Of course, K-RAS and N-RAS may be critical targets, since they are key oncoproteins that undergo alternative isoprenylation. On the other hand, as discussed earlier, FTIs and GGTIs have antitumor effects regardless of RAS mutation status. Blocking both FTase and GGTase-I would also inhibit isoprenylation of *CAAX* proteins that normally exist in both farnesylated and geranylgeranylated forms, such as RHOB and RHOH [38]. Both RHOB and RHOH, which functions in signal transduction in hematopoietic cells [155], may act as tumor suppressors. RHOH inhibits proliferation and migration and enhances apoptosis [128]. Loss of function of RHOH (by mutation) contributes to malignant progression in lymphomas [5]. Therefore, inhibition of isoprenylation of RHOB and RHOH may not be expected to have an antitumor effect.

Statins, bisphosphonates and FTS

Statins are widely used to lower cholesterol levels and to reduce atherosclerosis and cardiovascular disease. Statins inhibit the formation of mevalonate, the committed step in cholesterol synthesis, resulting in reduced levels of plasma cholesterol. However, statins also have many beneficial effects that are unrelated to cholesterol lowering. These effects involve improving endothelial function, stabilizing atherosclerotic plaques, decreasing oxidative stress and inflammation, and reducing risk of cancer [13, 88, 156]. These effects are thought to be mediated by inhibition of the isoprenoids (farnesyl and geranylgeranyl lipids), which are cholesterol synthetic intermediates (figure 2, page 11).

Lower levels of isoprenoid lipids may result in inhibition of protein isoprenylation. The first consequence of reduced isoprenoid levels is a reduction of cholesterol synthesis, followed by a reduction of geranylgeranyl-PP synthesis. This could result in reduced geranylgeranylation of proteins, thereby inhibiting the membrane targeting and proper function of geranylgeranylated *CAAX* proteins. The importance of geranylgeranylated proteins, such as RHOA and RAC1, are supported by studies showing that the cholesterol-independent phenotypes caused by statin treatment can be restored by the addition of mevalonate or geranylgeranyl-PP, but not farnesyl-PP [88]. The ability of statins to induce apoptosis in tumor cell lines is well-known and more recently the anti-cancer effects of statins have gained attention [13].

Nitrogen-containing bisphosphonates is another class of compounds that can inhibit *CAAX* protein isoprenylation. Bisphosphonates are used for the treatment of diseases characterized by excessive osteoclast-mediated bone resorption, such as osteoporosis and tumor-associated osteolysis [157]. Bone resorption is the process by which osteoclasts break down bone and release minerals. Nitrogen-containing bisphosphonates inhibit farnesyl diphosphate synthase, an enzyme in the cholesterol synthesis pathway (figure 2, page 11). Inhibition of this enzyme results in lower levels of farnesyl-PP and geranylgeranyl-PP, which are essential for the isoprenylation of *CAAX* proteins. Loss of isoprenylated proteins may not only account for the antiresorptive effect of nitrogen-containing bisphosphonates in osteoclasts, but it may also result in antitumor activity. Antitumor effects of nitrogen-containing bisphosphonates have been observed both in vitro and in animal models; but in the latter case it is not clear if the antitumor effect is due to direct inhibition of protein isoprenylation in tumor cells or if it is a result of inhibition of bone resorption per se [158].

FTS is yet another inhibitor that can interfere with *CAAX* protein function [14]. FTS competes with the isoprenoid portion of *CAAX* proteins for membrane binding sites. FTS was shown to inhibit proliferation of both H-RAS transformed Rat-1 cells and K-RAS transformed NIH3T3 cells [159, 160]. The mechanism of action involves disruption of the binding of farnesylated RAS to the plasma membrane. Interestingly, FTS is surprisingly selective towards RAS, and especially active RAS. The FTS Salirasib is currently tested in Phase I/II clinical trials. Initial results indicate that Salirasib is non-toxic and it is now tested in patients with advanced hematological malignancies, pancreatic cancer and lung cancer [14].

Why are genetic studies of FTase and GGTase-I deficiency important?

Publications about the effects of isoprenylation inhibitors reveal many inconsistent results. These differences may be explained by differences in the experimental systems (differences in genetic background, cell type, tissue etc), but also by compound-specific and off-target effects of different compounds. The inhibitors can differ in their selectivity for the target enzyme. For example, GGTI-2166 is 100 fold, whereas GGTI-297 is only 3 fold, more selective for GGTase-I over FTase. This could have effects on tolerability: GGTI-298 is actually toxic at doses required to inhibit RAP1A isoprenylation, while concentrations of GGTI-2166 that block RAP1A isoprenylation are not toxic [124]. As mentioned earlier, one class of GGTIs (GGTI-1, GGTI-2) developed by Merck laboratories were lethal in mice [123]. The authors of this study suggested that the toxicity was related to GGTase-I inhibition, and that the doses of GGTIs used by Sun et al. may have been tolerated because they only partially inhibited GGTase-I [93, 123, 124]. However, even a small dose of GGTI-2, that did not fully inhibit isoprenylation of RAP1A, was lethal [123], suggesting that the toxicity of this compound is an off-target effect. In addition, Sebti and colleagues later showed that GGTI-2154 and GGT-2418 had potent antitumor activities without causing toxicity, at doses that inhibited RAP1A [118, 126]. Further, Tamanoi and colleges showed excellent antitumor activity without toxicity using another type of GGTI compound [125]. They suggested the reason for the lack of toxicity to be the low dose of drug required to inhibit protein geranylgeranylation, compared to other GGTI compounds, such as GGTI-2.

Lack of selectivity could also be a problem for FTIs. Some FTIs from Bristol-Myers Squibb targeted not only FTase but also GGTase-II (the enzyme responsible for geranylgeranylating RAB proteins). In addition, some FTIs have triggered production of reactive oxygen species (ROS), resulting in DNA damage, which is likely an off-target activity of FTIs [161].

The compound-specific and off-target effects of FTIs, GGTIs and DPIs have made it difficult to determine their utility as anti-cancer drugs. This highlights the importance of using a genetic approach to understand the physiologic importance of protein isoprenylation and establish whether or not inhibition of the *CAAX* isoprenylation enzymes could be a useful cancer therapy. One of the aims of this thesis was to use genetic strategies in mice to define the role of *CAAX* protein isoprenylation on the development of RAS-induced cancer.

Lung cancer and myeloid malignancies are associated with hyperactive RAS signaling

The mouse cancer models used in this thesis resemble lung cancer and myeloid malignancies in humans. The mice with K-RAS–induced lung cancer form tumors that are similar to adenocarcinomas in humans [162]. Lung adenocarinoma belongs to the group of non-small cell lung cancer (NSCLC) and is the lung cancer type that has the highest incidence of activating K-RAS mutations (approximately 20-30%) [163-166].

In mice, expression of oncogenic K-RAS or inactivation of *Nf1* in hematopoietic cells results in development of MPD [167-170]. These MPD models resemble human chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML). CMML and JMML are classified as myelodysplastic syndromes/myeloproliferative neoplasms (MDS/MPN), according to WHO (the MPD classification recently changed to MPN to reflect the neoplastic nature) [171]. Simultaneous expression of oncogenic K-RAS and inactivation of *Nf1* in hematopoietic cells in mice resulted in an AML-like disease (described in paper III) [170]. The incidence of K-RAS and N-RAS mutations is approximately 40% in CMML [172] and 20-25% in JMML [173]. Children with the NF1 syndrome have an increased risk of developing JMML and 10-25% of the JMML patients have acquired a somatic mutation to their remaining wild-type *NF1* allele in hematopoietic cells [173]. In AML, activating point mutations in N-RAS and K-RAS have been identified in approximately 20% of cases [174, 175].

In order to relate the mouse work of this thesis to the clinical situation in humans, the next sections provide some general information about lung cancer and some of the myeloid malignancies in humans.

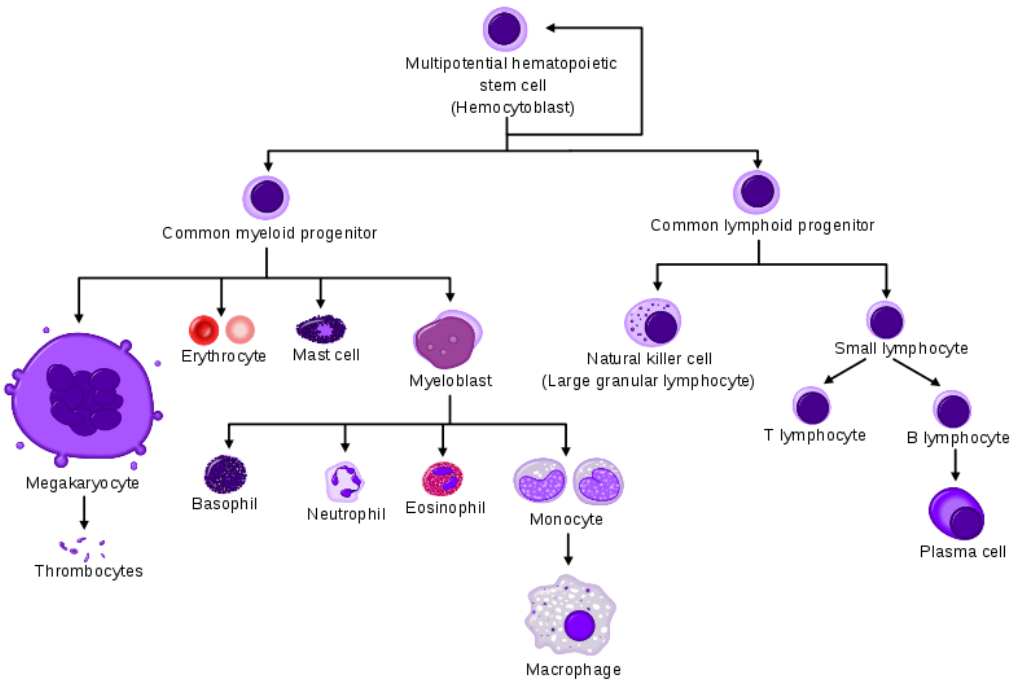
Lung cancer

Lung cancer is the cancer that kills most people in the world; the 5 year survival rate is ~14%. Approximately 90% of lung cancer cases are related to tobacco use. However, the incidence of lung cancer deaths that are not associated with smoking or other environmental factors is increasing. Symptoms of lung cancer include cough, coughing of blood, chest pain and shortness of breath. However, early stage lung cancer is often non-symptomatic, which means that patients are seldom diagnosed before their cancer has reached an advanced stage. Quite commonly, lung cancer is detected incidentally when a chest x-ray is performed for a different reason. Diagnosis is based on bronchoscopy and histological analysis of biopsies. It is possible that computed tomography (CT) can raise survival rates for patients with a high risk for lung cancer by allowing early detection [176].

Lung cancer is divided into two histopathological classes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), accounting for approximately 18% and 80% of lung cancer cases respectively. NSCLC is an aggressive cancer that can be further subclassified into squamous cell carcinoma, large cell carcinoma and adenocarcinoma; adenocarcinoma is the most common form. The size of the primary lesion and extent of metastasis dictate the course of therapy. Early-stage NSCLC is treated with surgery and radiotherapy. When the cancer has metastasized, combination chemotherapy is the treatment of choice. The addition of concurrent radiotherapy may also add survival benefits. However, most patients become resistant to therapy, relapse and die from the disease. Due to the high rate of relapse and toxic side effects under the standard therapies, there is a need for new therapeutic strategies. Advances in the understanding of the molecular events underlying the development of lung cancer have enabled researchers to develop targeted therapies, which are specifically aimed at cancer cells. Targeted drugs include protein kinase inhibitors, antisense oligonucleotides and antibodies [176].

Myeloid malignancies

Hematopoiesis is the formation of blood cells through differentiation of hematopoietic stem cells into mature blood cells (figure 4). Myeloid malignancies are a group of heterogeneous disorders of hematopoietic stem and/or progenitor cells that derive from the myeloid cell lineage. Similarly, neoplasms that derive from the lymphoid linage are called lymphoid malignancies and include lymphomas and lymphocytic leukemias. Myeloid malignancies can be subclassified into MPN, MDS, mixed MDS/MPN, and AML.



http://commons.wikimedia.org/wiki/File:Hematopoiesis_simple.svg (091001)

Figure 4. Hematopoiesis. Hematopoietic stem cells in the bone marrow are multipotential, which means that they can give rise to any of the different types of mature blood cells. Since they can also self-renew, they are not depleted. The common myeloid and lymphoid progenitor cells are committed to their respective pathways and further proliferation and differentiation give rise to mature blood cells, which can enter the blood [177].

MPNs are characterized by overproliferation of one or more myeloid cell types in the bone marrow and the accumulation of these cells in blood. The overproliferating cells retain the capacity to differentiate and are therefore maturing. In contrast, MDSs display abnormal myeloid differentiation and usually also cytopenias (reduced numbers of some type(s) of blood cells). MPNs and MDSs often progress quite slowly, but they are frequently transformed into AML, which is likely due to the acquisition of additional mutations [178]. The hallmark of AML is an increased number of immature myeloid cells/blasts, which results in suppression of normal hematopoiesis [179].

JMML is a rare MDS/MPN that mostly affects children under the age of six. Symptoms include fever, pallor, skin rash, enlarged spleen, liver and lymph nodes. JMML laboratory findings include increased white blood cell counts and monocytosis. Generally the hemoglobin level is low and the number of blast cells is generally less than 5%. The bone marrow is typically hypercellular with myeloid cells of all stages of maturation. An additional criterion is in vitro GM-CSF–hypersensitivity of hematopoietic cells [173, 178, 180]. Deregulated RAS signaling seems to be a central event in JMML, given that 70-85% of the

children with JMML have mutations in *KRAS*, *NRAS*, *NF1* or *PTPN11*. *PTPN11* encodes SHP2, an adapter protein that relays signals from activated growth factor receptors to RAS. Two inherited genetic disorders increase the risk of developing JMML: the NF1 syndrome (germline inactivation of one *NF1* allele) and the Noonan syndrome (associated with *PTPN11* mutations) [173]. The course of JMML is highly variable, but if left untreated 80% of the patients survive less than 3 years. Chemotherapy is ineffective. The only treatment that can cure JMML is bone marrow cell transplantation, which can result in long-time survival in 40-50% of the patients. However the relapse rate is quite high. FTIs have shown some efficacy in a phase II clinical trial [173, 180].

Similar to JMML, CMML exhibits heterogeneous clinical and morphologic features, varying from predominantly myelodysplastic to predominantly myeloproliferative. CMML presents in the age of 65-75 years and common symptoms are weakness and fatigue due to anemia, bruising and bleeding due to thrombocytopenia, and infections due to leukocytopenia. Hallmarks of CMML include persistent monocytosis in the blood, less than 20% blasts in bone marrow and blood, and abnormal differentiation in myeloid lineages [173, 178]. Median survival is 12-24 months [172, 181]. There is no effective therapy for CMML patients: chemotherapy has shown only modest success. Bone marrow transplantation could be curative but is not an option for the majority of patients, since they are old [173].

AML is the most common type of acute leukemia in adults and is diagnosed at a median age of 65 years [179, 182]. AML is most often sporadic, but some inherited disorders (e.g. Down syndrome), MDS and MPN increase the risk of developing AML [179]. In AML, abnormal, immature myeloid cells/blasts accumulate in bone marrow and blood. These leukemia cells can outnumber normal white blood cells, red blood cells and platelets, resulting in infections, fatigue (from anemia) and bleedings. The leukemia cells can also spread to other parts of the body, such as the central nervous system. AML is the diagnosis when at least 20% myeloid blasts are found in bone marrow or blood.

As discussed further in the next section, it has been suggested that two types of mutations are required to cause AML: one that enhances proliferation and/or survival of cells *and* one that impairs differentiation [179]. AML is divided into several subtypes with different treatment options. In general, prolonged remission or cure is rarely achieved, especially not in older people [183]. The most common treatment of AML is chemotherapy. Bone marrow transplantation has a very potent anti-leukemic effect, but treatment-related mortality is around 20%. Targeted therapies are under development: clinical trials with FTIs and FLT3 inhibitors are ongoing [179, 183].

Cooperating mutations in cancer development and progression

Cancer development and progression require two or more cooperating mutations. Cancer can be defined as an uncontrolled accumulation of abnormal cells, which will form a mass (tumor) that can spread to invade distant organs (metastasis). It has been suggested that to become a cancer cell a normal cell needs to acquire six capabilities: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis [184].

According to the widely accepted “multistep model of cancer”, these capabilities are acquired by a stepwise accumulation of genetic changes, where each change (mutation) confers a growth advantage, resulting in the transformation of a normal cell into a malignant cell. This hypothesis is supported by statistical analyses suggesting that four to seven rate-limiting steps are necessary for cancer to arise. Pathological findings of premalignant “intermediate” lesions

also support this hypothesis. In mice, tumorigenesis is likely a multistep process as well, even if the number of required mutations may be less [184].

The cancer platform model is an alternative model that may explain tumor development in some cases [185]. According to this model, cancer will arise if a cell *simultaneously* gains one mutation that promotes proliferation (e.g. RAS) and another mutation that blocks cell death and/or differentiation (e.g. BCL-2) [185, 186]. This creates a "platform" and subsequent interactions between the growing tumor and its environment give rise to additional mutations, resulting in the acquisition of the "hallmarks of cancer" [185].

A similar model has been proposed for hematopoietic malignancies [187]. According to this "two-hit" model, acute leukemia arises as a consequence of cooperation between two mutations: a class I mutation and a class II mutation. Class I mutations confer a proliferative and/or survival advantage and class II mutations impair hematopoietic differentiation. Class I mutations include mutations in receptor tyrosine kinases (e.g. BCR-ABL, FLT3), *RAS* genes (*NRAS*, *KRAS*), *NF1* or *PTPN11*; the presence of a class I mutation in a myeloid progenitor is sufficient to induce myeloproliferative phenotypes. Class II mutations often involve chromosomal translocations targeting transcription factors important for normal hematopoietic development, for example the fusion genes *AML1-ETO* and *PML-RARα* [187, 188]. Cooperation between class I and II mutations have been confirmed in several mouse models of acute leukemia, for example between FLT3 and AML1-ETO in AML [189] and between K-RAS and PML-RARα in acute promyelocytic leukemia (APL) [190]. The two-hit model is supported by the finding that mutations of the same class often are mutually exclusive. AML samples rarely have more than one mutation of each class [188, 189]. Similarly, mutations in *RAS*, *NF1* and *PTPN11*, which all encode components of the RAS signaling pathway, seem to be mutually exclusive in myeloid tumors [191, 192].

Oncogenic RAS and *NF1* deficiency

The non-overlapping pattern of *RAS* and *NF1* mutations supports the idea that *NF1*-deficiency is functionally equivalent with an activating *RAS* mutation. Heterozygous inactivating mutations in *NF1* in the germline cause the NF1 syndrome. NF1 is a familial tumor predisposition syndrome characterized by pigmented skin lesions and benign neurofibromas. Importantly, individuals with NF1 frequently develop certain types of malignant tumors, including neurofibrosacroma and the childhood leukemia JMML. Individuals with NF1 also display noncancerous symptoms, such as learning disabilities and bone deformations. *NF1*-haploinsufficiency seems to be important for the developmental phenotypes, such as learning disabilities, and for abnormal growth and differentiation of melanocytes [57, 70]. In malignant tumors from NF1 patients and *Nf1* haploinsufficient mice, both *NF1* alleles are often inactivated, indicating that *NF1* is a tumor suppressor gene. NF1 is a RAS-GAP and suppresses tumor formation by downregulating RAS signaling. Decreased GAP activity and increased RAS-GTP levels have been found in *NF1*-deficient neurofibrosacroma and myeloid leukemia cells and tumors, as well as JMML samples from children with NF1 [57, 70, 191, 193, 194].

However, other findings suggest that NFI has additional functions, independent of its RAS-GAP activity, at least in some cell types [70, 191, 194]. In neuroblastoma and melanoma cell lines, *NF1*-deficiency has not been associated with increased RAS-GTP levels [195, 196]. Similarly, overexpressing N-RAS in $Nf1^{+/-}$ mice enhanced lymphoma incidence and activated ERK, without affecting the RAS-GTP levels [194]. Further, the GAP-related domain compromises only 10% of the NF1 protein and many mutations found in NF1 patients occur

outside the GAP-related domain [70, 194, 197]. Finally, in Drosophila, NF1 regulates not only RAS, but also adenylyl cyclase - cyclic AMP - protein kinase A (PKA) signaling pathways. In Drosophila, adenylyl cyclase activity is important for learning and memory, neuropeptide response, body size, life span and stress resistance [198-201]. Importantly, human NF1 has also been shown to regulate adenylyl cyclase activity and expression of human NF1 rescued the phenotypes in the NF1 mutant flies [202]. Finally, Corral et al. suggested that NF1 is involved in a RAS-independent pathway that regulates expression of focal adhesion kinase, which may affect cytoskeletal organization [203].

The indications that NF1 is more than a RAS-GAP prompted us to test whether or not *NF1* deficiency was functionally equivalent to oncogenic RAS, by genetically inactivating *Nf1* in mice with a K-RAS–induced MPD.

AIMS OF THE THESIS

The overall research aims for my thesis were to define FTase and GGTase-I as potential drug targets for treating K-RAS–induced cancer, and to define the impact of *Nf1* deficiency in K-RAS–induced cancer.

The specific aims for papers I-III were:

- To test the hypothesis that inactivation of GGTase-I would inhibit K-RAS–induced cancer in mice (I).
- To test the hypothesis that inactivation of FTase, or FTase and GGTase-I, would inhibit K-RAS–induced cancer in mice (II).
- To define the impact of inactivating *Nf1* on the development of K-RAS–induced MPD in mice (III).

EXPERIMENTAL STRATEGIES

Detailed descriptions of methods are found in each of the papers. Here, I describe the genetic strategies and mouse models used in the work for this thesis, to provide a better understanding of their utilities and limitations.

Genetically engineered mice[3] are powerful tools to study gene function, define mechanisms of disease pathogenesis and to test therapeutic strategies for cancer and other diseases. Geneticists have used the mouse as a model organism for more than 100 years, but the interest increased dramatically 30 years ago with the technique for inserting exogenous DNA into the mouse germ line [204-206]. Today, the mouse is the most common experimental animal - thanks to the ease of genetic manipulation; similarities to humans in genetics, organ systems and physiology; their small size; and quick reproducibility [207, 208]. However, it is important to be aware of the limitations of the mouse as a model for human cellular function and pathology. There are many differences in cellular processes between mice and humans; regarding cancer development, we differ in disease susceptibility, tumor types and requirements of genetic changes for cellular transformation [208].

In transgenic mice, exogenous DNA (encoding a gene-of-interest) is incorporated into the mouse genome. Transgenic mice can be produced by viral infection or microinjection of DNA into one of the two pronuclei of a fertilized mouse egg [209, 210]. This technique adds genetic material and has been extensively used to generate mice that overexpress proto-oncogenes or activated oncogenes. Such mice have been valuable for obtaining knowledge about tumor processes and oncogenic cooperation [211-214], but they also have drawbacks. First, pronuclear injection results in random integration of the exogenous DNA into the genome and the number of copies vary, which influences transgene expression levels. Second, there are issues about ectopic expression of oncogenes (e.g. RAS) from exogenous promoters, since this may produce very different results compared to when genes are expressed from their endogenous promoters [168, 210]. These limitations were met by the development of gene-targeted mice.

With gene targeting, an endogenous gene can be modified. DNA is inserted at a specific genomic locus using homologous recombination[4] in embryonic stem (ES) cells [210, 215, 216]. The gene-targeted ES cells are injected into blastocysts and gene-targeted mice are generated. Gene targeting can be used to create “knockout” and “knock-in” mice. In a knockout mouse, the gene-of-interest is functionally eliminated, by inserting a construct with a null mutation. In a knock-in mouse, the sequence of the targeted gene is changed to modify its function (e.g. by insertion of an activating mutation in a proto-oncogene). Transgenic and gene-targeted strategies have one problem in common: the introduced genetic alteration affects all cells of the animal at all times, including embryonic development [217]. For example, homozygous knockout of a vital gene can cause embryonic lethality or severe disruption of development, preventing analysis of its function in adult tissues. In addition, expression of an oncogene or a knockout of a tumor suppressor gene in the whole mouse does not adequately model sporadic tumor development in humans. To address these problems, so called “conditional” strategies have been developed, by which activation or inactivation of a gene can be induced in a tissue-specific and/or time-controlled manner.

[3] Genetic engineering can be defined as a direct manipulation of an organism’s genes.

[4] Homologous recombination is a nucleotide exchange between two similar or identical strands of DNA.

One way to achieve conditional gene targeting is to use bacterial or eukaryotic recombinases, which catalyze recombination between specific nucleotide sequences. The most commonly used recombinase is Cre. Cre has been isolated from bacteriophage P1 and recognizes a 34-bp sequence called *loxP* (locus of crossing "X" over of P1). The mouse models used in this thesis were created using the Cre/*loxP* system. A similar recombinase system is Flp/FRT. Flp, isolated from *Saccharomyces cervisiae*, catalyzes recombination between FRT (FLP recombination target) sites [218]. Another approach to achieve spatial and temporal control is the tetracycline (tet)-dependent systems: "tet-on" and "tet-off". Unlike the Cre and Flp systems, this system allows gene expression to be switched on and off at any time, by administration or withdrawal of doxycycline [219].

Inducible and site-specific gene targeting with Cre/*loxP* techniques

Cre/*loxP*-mediated targeting can be used to create conditional, cell type-specific knockout alleles [220]. Homologous recombination in ES cells is used to insert *loxP* sites (in nonfunctional regions of the gene), so that they flank the endogenous gene-of-interest or an exon essential for gene function. Cre recombinase recognizes the *loxP* sites and induces recombination between them. The recombination results in deletion of the DNA between the *loxP* sites, producing a nonfunctional allele. Tissue-specific knockout is commonly attained by mating the mice containing the "floxed" allele with mice expressing Cre in the desired cell type or tissue. Figure 5 (page 33) shows the strategy that we used to knockout our conditional *Pggt1b* allele in a cell type-specific manner. Cre can also be delivered to a specific tissue (e.g. liver or lung) by infection with a Cre-adenovirus [162, 221]. Furthermore, gene inactivation can be induced at a given time, by using an inducible promoter to control Cre expression, for example the interferon-inducible *Mx1* promoter [222]. In this way, the gene can be inactivated in adult tissues, circumventing potential effects of gene loss on embryonic development.

Moreover, Cre/*loxP* strategies can be used to restrict expression of a gene to a specific tissue. This can be done by inserting a STOP cassette (a transcriptional terminator sequence flanked by *loxP* sites) between the gene to be expressed and its promoter [223]. In Cre-expressing cells the STOP cassette is removed, which switches on expression of the gene. The conditional oncogene LSL-*K*-*ras*G12D (hereafter designated K^{LSL}), used in this thesis, is an example of this strategy [162].

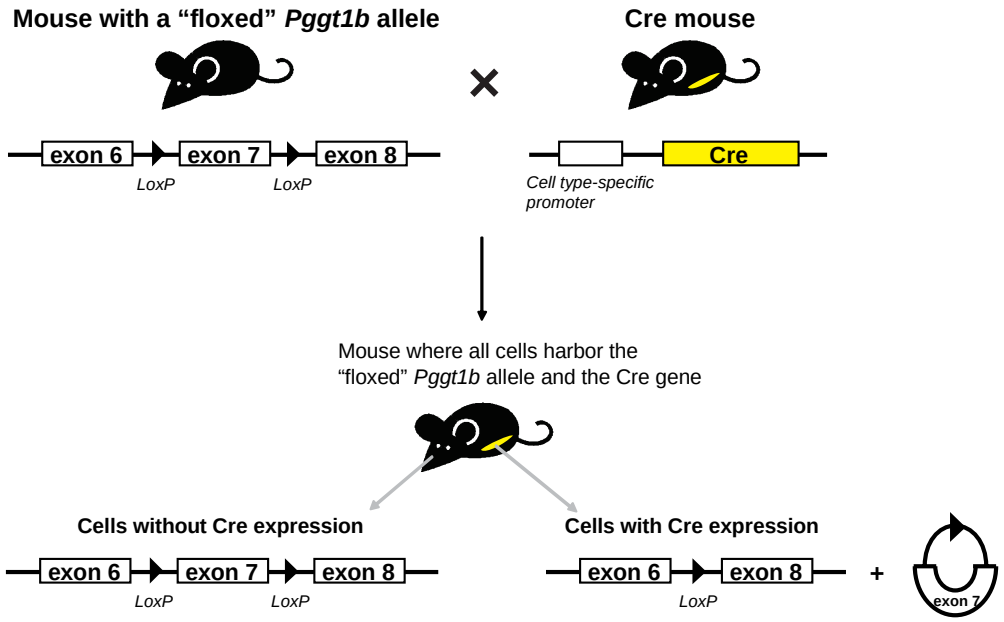


Figure 5. Cell type-specific inactivation of *Pggt1b* using the Cre/*loxP* system. A mouse with a "floxed" *Pggt1b* allele was created by inserting *loxP* sites around exon 7, which is essential for gene function. This *loxP*-containing mouse was bred with a mouse harboring a Cre transgene, controlled by a cell type-specific promoter. In the resulting mouse, Cre is produced only in cells where the Cre promoter is active. In these cells, Cre recombines the *loxP* sites, deleting exon 7 of *Pggt1b*, which inhibits the production of a functional gene product from this allele. In mice that carry the Cre transgene and *two* copies of the floxed *Pggt1b* allele, both copies of *Pggt1b* are inactivated and no functional GGTase-I is produced.

Mouse models for defining the impact of FTase and GGTase-I deficiency on lung cancer and myeloproliferation

In paper I and II we describe the generation and validation of conditional knockout alleles for FTase and GGTase-I, designated *Fntb*^{fl} and *Pggt1b*^{fl}, respectively. We used mice harboring these alleles to define the impact of inactivating *Fntb* and/or *Pggt1b* on tumor development in two different mouse cancer models.

Assessing the impact of inactivating *Fntb* and *Pggt1b* in *K*^{LSL}LC mice

K^{LSL}LC is a model of lung cancer and myeloproliferation and is described in paper I. *K*^{LSL}LC mice carry a copy of the *K*^{LSL} allele [162]. In the *K*^{LSL} construct, a *KRAS* oncogene with an activating mutation (G12D) has been inserted downstream of a floxed STOP cassette. The *K*^{LSL} allele is normally silent, but Cre recombinase excises the STOP cassette, switching on expression of K-RAS^{G12D}. The *K*^{LSL} allele was produced by a knock-in approach, which means that the mutant construct was inserted into the endogenous *KRAS* locus. Thus, K-RAS^{G12D} is expressed from the endogenous promoter. In addition, *K*^{LSL}LC mice harbor a *lysozyme M-Cre* (designated LC) knock-in allele. As a result, *K*^{LSL}LC mice express Cre from the endogenous lysozyme M promoter, which is active in granulocytes, monocytes, macrophages and type II pneumocytes (lung cells) [224-226], resulting in K-RAS^{G12D} expression in these cell types.

The dominant phenotype of the *K*^{LSL}LC mice was a rapidly fatal lung cancer. The disease was 100% penetrant and extremely predictable, with phenotypes that were easy to measure (e.g. survival, lung weight). The lethality and full penetrance made the *K*^{LSL}LC mice well

suited for proof-of-concept studies: would inactivation of FTase and/or GGTase-I have an impact on K-RAS–induced lung cancer in vivo? To answer this question, we bred K^{LSL}LC mice that were homozygous for the "floxed" *Fntb* and/or *Pggt1b* alleles (figure 6 outlines the typical experimental strategy). In the lung and myeloid cells of these mice, Cre simultaneously activated expression of K-RAS^{G12D} an inactivated *Fntb* and/or *Pggt1b*.

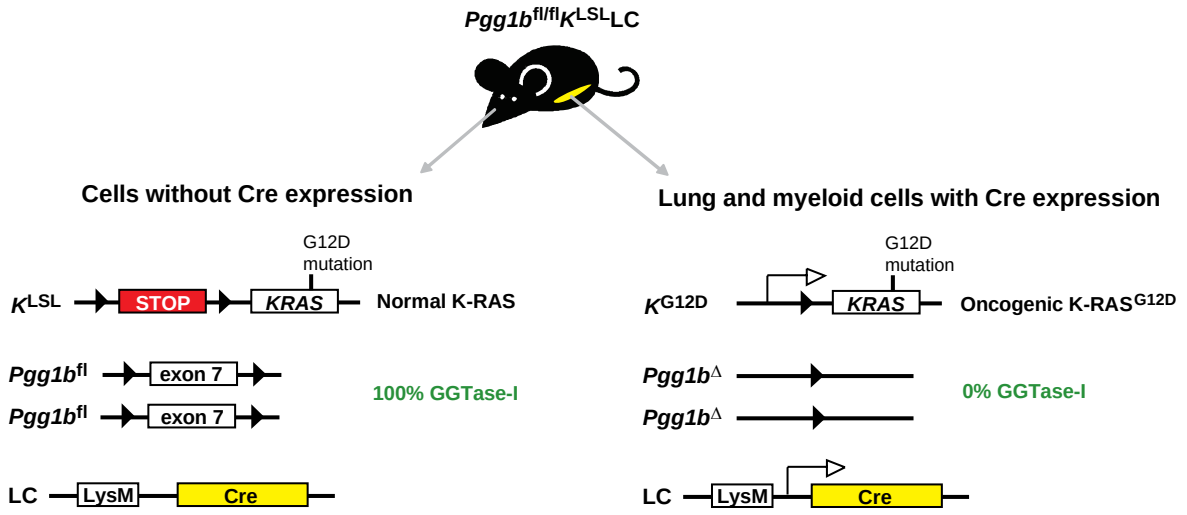


Figure 6. Experimental strategy to define the impact of GGTase-I deficiency on K-RAS–induced cancer. *Pggt1b*$^{fl/fl}K^{LSL}$LC mice were generated, in which K-RAS^{G12D} was expressed and *Pggt1b* inactivated in lung and myeloid cells. As comparison, littermate *Pggt1b*$^{fl/+}K^{LSL}$LC mice, harboring one wild-type allele of *Pggt1b*, were generated; these mice exhibit a fatal lung cancer and mild myeloproliferative phenotypes.

One drawback with the K^{LSL}LC model was that Cre expression was not restricted to one cell type, resulting in the development of not only lung cancer, but also myeloproliferative phenotypes. Furthermore, cancer was initiated already in newborn mice. Finally, Cre expression was very wide-spread; essentially all lung cells were turned into lung cancer cells; in humans only one or a very limited number of cells contribute to the development of the primary tumor. Therefore, it was essential to substantiate our findings with another model of K-RAS–induced cancer, where Cre expression was induced in only a few lung cells.

Inactivation of *Fntb* and *Pggt1b* in a second K-RAS–induced lung cancer model

Lung tumor formation can be induced in adult K^{LSL} mice by inhalation of a Cre-adenovirus through the nose. Cre is expressed in the infected lung cells, the oncogenic K-RAS allele is activated and tumors develop within 4–6 weeks [162]. By controlling the virus dose and the time allowed for tumors to develop, the number and histological grade of tumors can be regulated, so to more closely resemble human lung cancer. In our hands, this model was 100% penetrant, although variations in tumor burden between mice of the same genotype were larger than with the K^{LSL}LC model.

The basis of our genetic strategies is that Cre expression activates K-RAS^{G12D} expression, to initiate tumor formation, and inactivates the genes encoding the isoprenyltransferases, in the same cells at the same time. One general limitation with this experimental strategy is the inability to evaluate the impact of inactivating *Fntb* and/or *Pggt1b* on already established tumors. Nevertheless, this strategy will answer the question of whether tumors would develop in the absence of FTase and/or GGTase-I.

Modeling myeloid malignancies and oncogene cooperation in mice

Myeloid malignancies are often associated with hyperactive RAS signaling. In mice, conditional inactivation of *Nf1* or activation of K-RAS^{G12D} expression in hematopoietic cells results in a fatal MPD with a long [169] or short [167, 168] latency, respectively. In both models, Cre expression is controlled by the interferon-inducible Mx1 promoter, which can be activated by injection of pI-pC (a double stranded RNA that induces an interferon response). In *Nf1*$^{fl/fl}$Mx1-*Cre* mice (designated *NM*), Cre expression inactivates *Nf1* in hematopoietic cells; in *K*^{LSL}Mx1-*Cre* (*KM*) mice, Cre expression activates expression of K-RAS^{G12D} from its endogenous promoter. Both models exhibit leukocytosis, growth factor hypersensitivity of hematopoietic cells, splenomegaly, and infiltration of myeloid cells in the liver—but they do not progress to acute leukemia.

Loss of *NF1* has been viewed as functionally equivalent with an activating K-RAS mutation. However, in mice, the MPD induced by *Nf1* inactivation is much less severe than the K-RAS–induced MPD. We argued that if *Nf1* deficiency and oncogenic RAS mutations were functionally equivalent, inactivation of *Nf1* should have a limited effect on the development of K-RAS–induced MPD. To answer this question, we bred *Nf1*$^{fl/fl}$*K*^{LSL}Mx1-*Cre* (*NKM*) mice and injected them with pI-pC. The *NKM* mice allowed us to define the impact of *Nf1* deficiency on K-RAS–induced MPD.

SUMMARY OF RESULTS

FTase and GGTase-I deficiency reduced tumor development and improved survival in mice with K-RAS–induced lung cancer (Paper I-II)

Paper I and II describe the impact of inactivating FTase and/or GGTase-I on normal and K-RAS^{G12D}–expressing mouse embryonic fibroblasts, and on the development of K-RAS–induced cancer in mice. First, conditional knockout alleles for the β subunits of FTase (*Fntb*^{fl}) and GGTase-I (*Pggt1b*^{fl}) were created and validated. The *Fntb*^{fl} allele was validated by showing that Cre-adenovirus infection converted *Fntb*$^{fl/fl}$ fibroblasts to *Fntb*$^{\Delta/\Delta}$ fibroblasts, which lacked expression of *Fntb*. Furthermore, inactivation of *Fntb* inhibited isoprenylation of the FTase substrates HDJ2 and H-RAS, and blocked membrane targeting of H-RAS. Expression of Cre recombinase in *Pggt1b*$^{fl/fl}$ mouse embryonic fibroblasts converted the *Pggt1b*^{fl} alleles to null alleles (*Pggt1b*$^{\Delta}$) and blocked GGTase-I activity. Thus, the conditional knockout alleles worked as planned.

Inactivation of *Fntb* alone and *Pggt1b* alone in fibroblasts blocked cell proliferation, while simultaneous inactivation induced apoptosis

Inactivation of *Fntb* arrested proliferation of primary and immortalized fibroblasts (figure 7A). FTase-deficient fibroblasts (*Fntb*$^{\Delta/\Delta}$) were large and flat, accumulated in the G_2/M phase of the cell cycle, but remained viable. Western blots showed that p21^{CIP1} was upregulated in primary *Fntb*$^{\Delta/\Delta}$ fibroblasts compared to primary *Fntb*$^{fl/\Delta}$ fibroblasts (having one functional *Fntb* allele). FTase deficiency also induced a G_2/M cell cycle arrest in K-RAS^{G12D}–expressing fibroblasts.

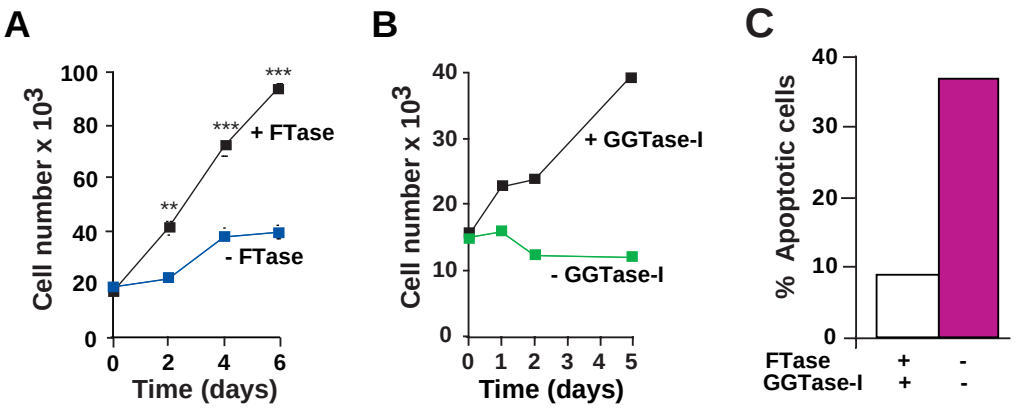


Figure 7. Fibroblasts deficient in either FTase or GGTase-I failed to proliferate, and simultaneous deficiency in both enzymes induced apoptosis. (A) Equal numbers of primary *Fntb*$^{fl/\Delta}$ fibroblasts treated with β-gal (+FTase) and Cre (-FTase) were seeded and subsequently counted at the indicated times. (B) Equal numbers of primary *Pggt1b*$^{fl/fl}$ fibroblasts treated with β-gal (+GGTase-I) and Cre (-GGTase-I) were seeded and subsequently counted at the indicated times. (C) Percent apoptotic cells in cultures of *Fntb*$^{fl/fl}$*Pggt1b*$^{fl/fl}$ fibroblasts 4 days after incubation with β-gal– or Cre-adenovirus. β-gal is a virus that does not induce recombination and was used as a control.

Similarly, inactivation of *Pggt1b* blocked proliferation of primary fibroblasts (figure 7B, page 36), but did not affect cell viability. The *Pggt1b*$^{\Delta/\Delta}$ fibroblasts were arrested in the G_1 phase of the cell cycle and accumulated p21^{CIP1}. Furthermore, GGTase-I–deficient fibroblasts were small and spindled shaped, contained reduced amounts of polymerized actin and migrated poorly. In K-RAS^{G12D}–expressing fibroblasts, inactivation of *Pggt1b* caused cell rounding and proliferation arrest, but not apoptosis. *Pggt1b*$^{\Delta/\Delta}$K-RAS^{G12D} cells also accumulated in the G_1 phase of the cell cycle, but expressed normal levels of p21^{CIP1}. However, *Pggt1b* inactivation prevented the K-RAS^{G12D}–induced increase in Cyclin D1, a protein implicated in cell cycle progression [227]. Finally, coexpression of farnesylated mutants of RHOA and CDC42 in *Pggt1b*$^{\Delta/\Delta}$K-RAS^{G12D} fibroblasts prevented cell rounding and partially restored proliferation. Expression of only farnesylated RHOA or only farnesylated CDC42 did not restore these effects.

Cre-adenovirus treatment of *Fntb*$^{fl/fl}$*Pggt1b*$^{fl/fl}$ fibroblasts induced accumulation of K-RAS in the cytosol, indicating loss of isoprenylation. However, the *Fntb*$^{\Delta/\Delta}$*Pggt1b*$^{\Delta/\Delta}$ fibroblasts underwent apoptosis and died within a few days (figure 7C, page 36).

We used western blots to analyse the impact of inactivating *Fntb* or *Pggt1b* on the activation of RAS effectors. *Fntb* inactivation in normal fibroblasts did not affect levels of phosphorylated MEK or ERK, but delayed serum-induced activation of phosphorylated AKT. Inactivation of *Pggt1b* resulted in a minor decrease of phosphorylated ERK in normal fibroblasts, but did not affect phosphorylated ERK in K-RAS^{G12D}-expressing fibroblasts. Levels of phosphorylated AKT increased slightly as a consequence of *Pggt1b* inactivation in both normal and K-RAS^{G12D}–expressing fibroblasts. Thus, inactivating *Fntb* or *Pggt1b* had only minimal effects on signaling through the classical RAS pathways.

Inactivation of *Fntb* or *Pggt1b* reduced lung tumor growth and prolonged survival, and the simultaneous inactivation of *Fntb* and *Pggt1b* further improved phenotypes

In paper I, we developed a mouse model of K-RAS–induced cancer, by breeding mice harboring the Cre-inducible *K*^{LSL} allele with LC mice. Since LC mice had been used in the past to yield myeloid-specific Cre expression, we hypothesized that the *K*^{LSL}LC mice would develop a myeloid malignancy. Although *K*^{LSL}LC mice displayed some mild myeloproliferative phenotypes, the most dramatic phenotype was a rapidly progressing lung cancer. *K*^{LSL}LC mice developed diffuse hyperplasia, adenomas and adenocarcinomas that compressed and obliterated lung alveoli, resulting in a 10-fold increase in lung weight compared to lung weight of healthy, control mice (figure 8A, page 38). The lung cancer was lethal within three weeks of age (figure 8B, page 38). We showed that oncogenic K-RAS^{G12D} was expressed not only in myeloid cells, but also in type II pneumocytes, which is supported by data in the literature and explains the development of lung cancer.

To determine the impact of GGTase-I deficiency on lung tumor development, K^{LSL}LC mice were bred on a background of homozygosity for the conditional GGTase-I knockout allele (i.e., *Pggt1b*$^{fl/fl}K^{LSL}$LC). In the lung cells of these mice, Cre recombinase simultaneously activated oncogenic K-RAS^{G12D} and inactivated *Pggt1b*. We showed that inactivation of *Pggt1b* reduced formation of lung tumors and dramatically prolonged survival (figure 8A-B). Figure 8 includes data from additional K^{LSL}LC and *Pggt1b*$^{fl/fl}K^{LSL}$LC mice that were studied after the publication of paper I.

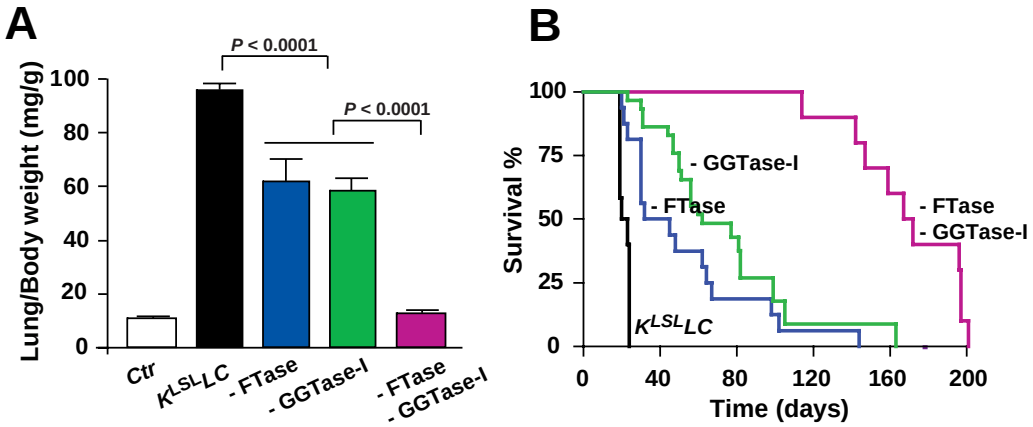


Figure 8. Inactivation of *Pggt1b* and/or *Fntb* reduced lung tumor development and prolonged lifespan of K^{LSL}LC mice. (A) Lung weight in 3-week-old healthy control (Ctr, n=16), K^{LSL}LC (n=27), *Fntb*$^{fl/\Delta}K^{LSL}$LC (-FTase, n=7), *Pggt1b*$^{fl/fl}K^{LSL}$LC (-GGTase-I, n=13) and *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}K^{LSL}$LC (-FTase, -GGTase-I, n=6) mice. (B) Kaplan-Meier curve showing survival of K^{LSL}LC (n=12), *Fntb*$^{fl/\Delta}K^{LSL}$LC (-FTase, n=16), *Pggt1b*$^{fl/fl}K^{LSL}$LC (-GGTase-I, n=22) and *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}K^{LSL}$LC (-FTase, -GGTase-I, n=10) mice.

In paper II, we determined the impact of FTase deficiency on the development of K-RAS–induced lung cancer using the same model as in paper I. Thus, K^{LSL}LC mice were bred on an *Fntb*$^{fl/\Delta}$ background. *Fntb*$^{fl/\Delta}K^{LSL}$LC mice had a reduced lung tumor burden, revealed by histological analyses and lung weight, and lived significantly longer than K^{LSL}LC mice (figure 8A-B). FTase and GGTase-I deficiency reduced lung tumors to a similar extent and there was no difference in survival of the *Fntb*$^{fl/\Delta}K^{LSL}$LC and *Pggt1b*$^{fl/fl}K^{LSL}$L mice (P=0.051, *Pggt1b*$^{fl/fl}K^{LSL}$LC vs. *Fntb*$^{fl/\Delta}K^{LSL}$LC). Notably, *Fntb*$^{fl/\Delta}$LC mice were viable and had normal lung histology.

The next goal was to define the impact of inactivating *Fntb* and *Pggt1b* on the development of K-RAS–induced lung cancer. In the K^{LSL}LC lung cancer model, simultaneous inactivation of *Fntb* and *Pggt1b* dramatically reduced tumor load and improved survival. At three weeks of age, *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}K^{LSL}$LC mice (lacking FTase and GGTase-I in the lung) displayed normal lung histology and lung weights (figure 8A). Western blot analysis showed markedly lower levels of phosphorylated ERK in lung lysates from *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}K^{LSL}$LC mice than in lung lysates from K^{LSL}LC mice. The *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}K^{LSL}$LC mice lived much longer than K^{LSL}LC mice (median 170 days vs. 22 days, P<0.0001), despite wide-spread expression of K-RAS^{G12D}. However, the mice eventually developed lung tumors and had to be euthanized (figure 8B).

We detected lung cells that lacked both FTase and GGTase-I activity in 3-week-old *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC mice. Western blots of lung extracts showed a small proportion of N-RAS (~5%) with reduced electrophoretic mobility, characteristic of nonprenylated N-RAS, indicating the presence of at least some cells lacking both FTase and GGTase-I (figure 9A). In addition, cells that stained for both Prelamin A and np-RAP1A (markers of absent FTase and GGTase-I activity, respectively) were detected in lung sections from 3-week-old *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC mice (figure 9B).

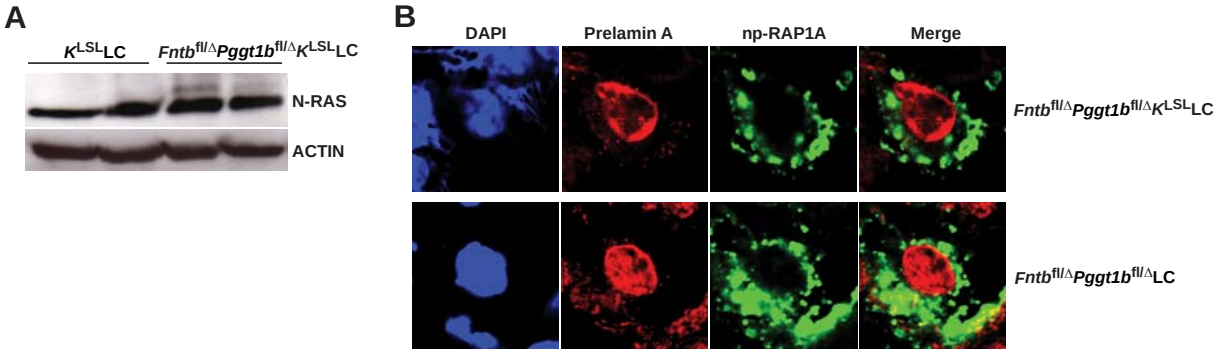


Figure 9. Simultaneous inactivation of *Fntb* and *Pggt1b* inhibited isoprenylation of N-RAS and appeared to be compatible with viability of lung cells. (A) Western blots of lung extracts from 3-week-old *K*^{LSL}LC and *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC mice, incubated with an antibody against N-RAS. Notice the slower migrating form of N-RAS in the *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC extracts. ACTIN was used as loading control. (B) Prelamin A (red, nucleus) and np-RAP1A (green, cytoplasmic) expression in lungs from 3-week-old *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC (upper panel) and *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$LC mice (lower panel), detected by confocal microscopy. Nuclei were stained with DAPI (blue).

We also detected lung cells that were positive for only Prelamin A or np-RAP1A or negative for both. We hypothesized that the lung tumors that eventually developed in *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC mice were derived from such cells: i.e. expressing K-RAS^{G12D}, but with incomplete recombination of *Fntb* and/or *Pggt1b* alleles. Indeed, quantitative PCR analysis of lung tumors isolated from old *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC mice confirmed that the majority of tumor cells had a recombined *K*^{LSL} allele, thus expressing oncogenic K-RAS, but unrecombined *Fntb* and/or *Pggt1b* alleles. This was in contrast to the lung tumors in *Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC, described in paper I, in which both *Pggt1b* alleles were recombined.

Importantly, *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$LC mice were viable and had normal lung weights and histology. Also in these mice, some lung cells stained for both Prelamin A and np-RAP1A, suggesting that normal lung cells were viable in the absence of isoprenylation (figure 9B).

In addition, simultaneous inactivation of *Fntb* and *Pggt1b* inhibited tumorigenesis in a second K-RAS–induced lung tumor model. Cre-adenovirus was intranasally administered to *K*^{LSL} and *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL} mice and 8 weeks later tumor number and size were determined. Cre-treated *K*^{LSL} mice had many large tumors that were visible on the lung surface. In comparison, the Cre-treated *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL} mice showed a 76% reduction in tumor number and a 79% reduction in tumor size.

GGTase-I deficiency was well tolerated in myeloid cells and eliminated K-RAS–induced myeloproliferative phenotypes

Finally, in paper I, we did not only show that fibroblasts and lung tumor cells were viable in the absence of GGTase-I, but also macrophages and CFU-GM colony-forming hematopoietic cells. In addition, inactivation of *Pggt1b* eliminated all myeloproliferative phenotypes of K^{LSL}LC mice, such as elevated levels of neutrophils in the blood, infiltration of myeloid cells in the liver and autonomous colony formation of K-RAS^{G12D}–expressing hematopoietic cells.

In summary, the results in Paper I and II showed that inactivating either FTase or GGTase-I, or both enzymes, reduced tumor development in mice with K-RAS–induced lung cancer.

Nf1 deficiency cooperates with oncogenic K-RAS to induce acute myeloid leukemia in mice (Paper III)

The aim of this paper was to define the impact of *Nf1* deficiency on K-RAS–induced MPD. To approach this issue, we bred *Nf1* conditional knockout mice (*Nf1*$^{fl/fl}$; designated *N*) with K^{LSL} mice (designated *K*) and mice harboring the interferon-inducible Mx1-*Cre* transgene (designated *M*). Injection of pI-pC into the "*NKM*" mice simultaneously induced expression of K-RAS^{G12D} and inactivation of *Nf1* in hematopoietic cells. *NKM* mice were compared with MPD control mice (*KM* and *NM*) and healthy control mice (*Ctr*).

We showed that *NKM* mice developed a myeloid malignancy with a reduced latency and increased severity compared to *KM* and *NM* mice. *Nf1* deficiency in the setting of oncogenic K-RAS^{G12D} expression dramatically increased the total number of white blood cells and the number of immature cells, including myeloblasts (figure 10A and B). Furthermore, the *NKM* mice became anemic and they had a much shorter life span than *KM* mice ($P < 0.0001$) (figure 10C).

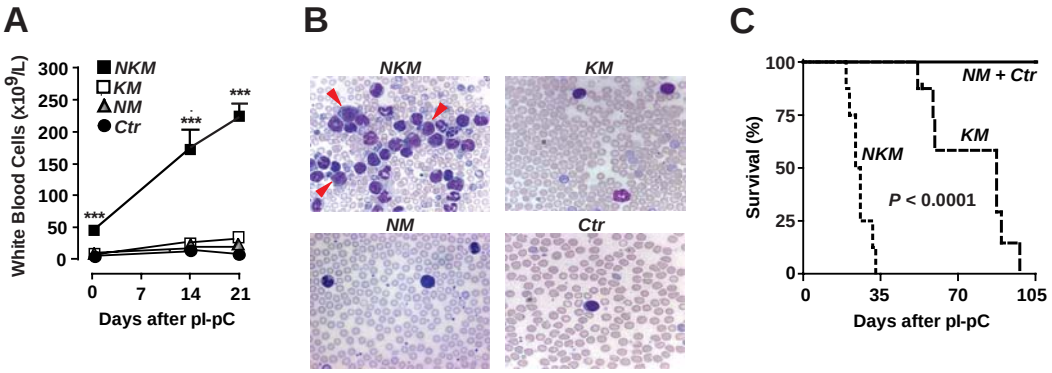


Figure 10. *NKM* mice developed a rapidly fatal myeloid malignancy, with a large number of immature white blood cells in the blood. (A) White blood cell counts of *NKM, KM*, *NM* and *Ctr* mice. *** P < 0.001. (B) Photographs of typical blood smears from *NKM*, *KM*, *NM* and *Ctr* mice three weeks after pI-pC injection. Arrowheads point to myeloblasts. (C) Kaplan-Meier curve showing survival of *NKM*, *KM*, *NM* and *Ctr* mice.

NKM mice displayed a higher degree of myeloid cell infiltration in liver and spleen compared to *KM* and *NM* mice, and the weights of these organs were dramatically increased. In addition, *NKM* splenocytes had an increased proportion of surface markers for immature myeloid cells (Cd11b+/Gr1+, CD34, CD117; determined by FACS analysis) and produced more colonies in methylcellulose both in the absence and presence of growth factors, compared with MPD control mice. Finally, the *NKM* disease was transplantable.

Bone marrow cells from pI-pC injected *NKM* mice were injected into sublethally irradiated recipient mice. All 6 recipients became moribund by 12 weeks after transplantation and three of them had high white blood cell counts and splenomegaly.

Thus, the results showed that the *NKM* mice developed AML. AML in mice is classified based on the following criteria: increased number of nonlymphoid hematopoietic cells in spleen and bone marrow, anemia, spreading of neoplastic cells (leukocytosis and nonlymphoid infiltration in the liver), >20% immature cells in peripheral blood, and rapid fatality in primary animals [170].

Levels of RAS-GTP (active RAS) were not higher in bone marrow cells from *NKM* mice than in bone marrow cells from *KM* mice (figure 11A). Similar results were seen in Cd11b+ hematopoietic cells (figure 11B). These results indicated that mechanisms unrelated to enhanced activation of RAS contributed to the progression of MPD (*KM* mice) into AML (*NKM* mice).

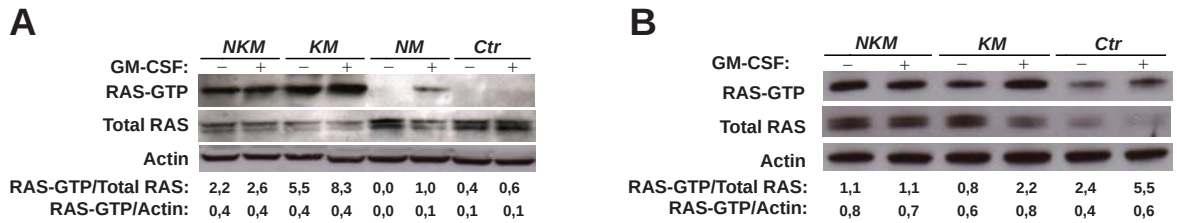


Figure 11. RAS-GTP levels were similar in hematopoietic cells from *NKM* and *KM* mice. (A) Western blots of extracts from serum-starved and GM-CSF stimulated bone marrow cells from *NKM*, *KM*, *NM* and *Ctr* mice. (B) Western blots of extracts from serum-starved and GM-CSF stimulated Cd11b+ cells from *NKM*, *KM* and *Ctr* mice. ACTIN was used as loading control. Band densities were determined and displayed as ratios.

In summary, the results described in paper III showed that *Nf1* deficiency cooperated with oncogenic K-RAS and that the two mutations induced AML in mice.

DISCUSSION

Paper I showed that inactivation of *Pggt1b* blocked proliferation and reduced migration of fibroblasts. Furthermore, inactivating *Pggt1b* reduced tumor development and improved survival of mice with K-RAS–induced lung cancer. In Paper II, we showed that inactivation of *Fntb* prevented membrane targeting of H-RAS and arrested proliferation of fibroblasts. *Fntb* inactivation also improved survival and reduced tumor growth in mice with K-RAS–induced lung cancer. Simultaneous inactivation of *Fntb* and *Pggt1b* potentiated the antitumor effect. In Paper III, we showed that *Nf1* deficiency and oncogenic K-RAS cooperated to induce AML in mice.

The importance of validating conditional knockout alleles

Our findings regarding the consequences of *Fntb* inactivation differ significantly from Mijimolle's previously published findings using another conditional *Fntb* knockout allele [33]. In the latter study, it was suggested that *Fntb* inactivation only partially inhibited isoprenylation of HDJ2 and did not prevent membrane targeting of H-RAS. Also, Mijimolle et al. reported that *Fntb*-deficient fibroblasts could grow in culture. Finally, inactivation of *Fntb* had no significant effect on lung tumor initiation in mice with endogenous expression of K-RAS^{V12}. Although the reason for these discrepancies is not know, a potential explanation is that excision of the floxed exon 3 in Mijimolle's *Fntb* allele resulted in an unexpected splicing event. Characterization of the transcripts from the mutant allele showed that the dominant transcript lacked both exon 3 and 4 sequences; this transcript is predicted to encode a protein with an in-frame deletion, which may retain partial function [228].

Thus, when using Cre/*loxP* techniques to create a conditional knockout allele, it is crucial to validate that the recombination event results in an actual null allele. It is important that this is done by both documenting recombination within the genomic DNA and through analysis of transcripts [228]. We validated our conditional FTase knockout allele by showing that Cre recombination deleted the promoter and exon 1 and blocked *Fntb* expression: no transcripts were detected from the *Fntb* null allele.

Incomplete recombination - a potential limitation of knockout strategies

One potential limitation with conditional gene targeting is the possibility of incomplete recombination. For example, partial recombination in *Fntb*$^{fl/fl}$*K*^{LSL} cells would produce *Fntb*$^{fl/\Delta}$*K*^{G12D} cells, expressing K-RAS^{G12D} but also FTase, due to an unrecombined *Fntb*^{fl} allele. We developed quantitative PCR assays to monitor for partial recombination. The quantitative PCR assays quantified recombination of the *K*^{LSL}, *Fntb*^{fl} and *Pggt1b*^{fl} alleles and were performed on genomic DNA isolated from cells and tissues. In addition, recombination of the *Fntb*^{fl} and *Pggt1b*^{fl} alleles were determined by western blots and immunofluorescence, using antibodies to various *CAAX* protein substrates as markers of FTase and/or GGTase-I deficiency. Moreover, in paper II, we minimized the risk of partial recombination, by using mice harboring one null allele of *Fntb* and/or *Pggt1b* (e.g. *Fntb*$^{fl/\Delta}$*K*^{LSL}LC mice).

In paper I, we used quantitative PCR to show that both *Pggt1b*^{fl} alleles were recombined in 80-90% of myeloid cells and in cells isolated from lung tumors. Recombination of the *Fntb*^{fl} allele was determined by using the characteristic that nonfarnesylated HDJ2 and H-RAS migrate slower in SDS-PAGE gels than their farnesylated counterparts. In this way, it was confirmed that both *Fntb*^{fl} alleles were recombined in fibroblasts; in vivo, approximately 50% of HDJ2 in lung extracts from *Fntb*$^{fl/\Delta}$*K*^{LSL}LC mice migrated slower.

To detect cells with recombined *Fntb*^{fl} and *Pggt1b*^{fl} alleles, we performed western blots with antibodies against K-RAS and N-RAS, which display a retarded electrophoretic mobility when they are nonprenylated. In addition, by using confocal microscopy and immunofluorescence, we detected cells that stained for Prelamin A and/or np-RAP1A. Although a portion of cells stained positive for both Prelamin A and np-RAP1A in lung tissue from 3-week-old *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC mice, other cells stained positive for only Prelamin A or np-RAP1A or were negative for both markers. *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC mice eventually developed tumors and we suspect that these were formed by cells with unrecombined *Fntb*^{fl} and/or *Pggt1b*^{fl} alleles. Indeed, quantitative PCR analysis of lung tumor DNA isolated from *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC mice confirmed incomplete recombination of *Fntb*^{fl} and/or *Pggt1b*^{fl} alleles. Despite this, *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC mice lived much longer than *K*^{LSL}LC mice.

What proteins are responsible for the antitumor effects of inactivating *Fntb* and *Pggt1b*?

The absence of FTase or GGTase-I reduced tumor development induced by oncogenic K-RAS, despite the fact that the isoprenylation of K-RAS was unaffected. This implies that the observed antitumor effects are due to inhibition of isoprenylation of *CAAX* proteins other than K-RAS. Perhaps this could also explain the minor effects of *Fntb* or *Pggt1b* inactivation on the RAS downstream effectors MEK and ERK. In normal fibroblasts, phosphorylated (activated) ERK was not reduced by inactivating *Fntb* and only a minor decrease was seen when inactivating *Pggt1b*.

There are more than 50 geranylgeranylated proteins in human cells [3], which are all potential candidates for mediating the antitumor effects of *Pggt1b* inactivation. Similarly to most GGTI-treated cells, cell cycle arrested *Pggt1b*$^{\Delta/\Delta}$ fibroblasts exhibited increased levels of p21^{CIP1}. Increased p21^{CIP1} levels have been suggested to be a consequence of inhibiting geranylgeranylation of RHOA; therefore we hypothesized that RHOA may be an important target. However, *Pggt1b* inactivation in K-RAS^{G12D}–expressing fibroblasts did not affect p21^{CIP1}. The explanation for this is not known. But GGTIs can also induce a G_1 cell cycle arrest in some tumor cell lines without increasing p21^{CIP1} [115]. Moreover, reduced polymerized actin and impaired migration of *Pggt1b*$^{\Delta/\Delta}$ fibroblasts suggested involvement of proteins regulating actin cytoskeleton, such as RHOA and CDC42.

To test if geranylgeranylation of RHOA and CDC42 was important for cell proliferation, cell cycle arrested *Pggt1b*-deficient fibroblasts expressing K-RAS^{G12D} were transfected with farnesylated mutants of RHOA (fRHOA) and CDC42 (fCDC42). The fact that expression of neither fRHOA nor fCDC42 alone could restore cell proliferation of these cells is consistent with a study showing that farnesylation of RHOA did not prevent GGTI-mediated growth inhibition [134]. However, coexpression of fRHOA and fCDC42 prevented cell rounding and could temporarily restore the proliferation of *Pggt1b*-deficient fibroblasts expressing K-RAS^{G12D}. This indicates that RHOA and CDC42 are the GGTase-I substrates that are crucial for cell proliferation. However, we cannot rule out the possibility that some GGTase-I substrates undergo alternative isoprenylation by FTase. Also, the *Pggt1b*$^{\Delta/\Delta}$K^{G12D} cells transfected with fRHOA and fCDC42 stopped growing after a while, which indicates that other GGTase-I substrates are required for maintaining cell proliferation.

Also, it has been shown earlier that expressing farnesylated versions of RAC and RAL proteins can make cells resistant to different antitumor effects of GGTIs. These studies

suggest that geranylgeranylation of RAC1 and RAC3 is important for transformation [137], and that geranylgeranylation of RALA and RALB is important for cell growth and survival [148].

Similar to GGTase-I, FTase has more than 50 cellular substrates and the antitumor effects of inactivating *Fntb* may be explained by the inhibition of the proper function of any of these *CAAX* proteins [3]. The G_2/M cell cycle arrest of our *Fntb*-deficient fibroblasts may be explained by inhibition of the mitotic proteins CENP-E and CENP-F. This would be supported by FTI studies showing that inhibiting farnesylation of CENP-E and CENP-F disrupts chromosomal maintenance and results in mitotic delay [101]. Also, farnesylation of CENP-F has been shown to be required for cells to progress through G_2/M [102].

FTI studies have also suggested RHEB and PRL proteins to be important targets. In *Schizosaccharomyces pombe*, the cell cycle defect caused by FTase deficiency was reversed by expressing a geranylgeranylated form of RHEB [36, 98]. Expression of geranylgeranylated RHEB could also prevent the enhanced apoptotic response to chemotherapeutics mediated by FTI treatment [100]. Moreover, inhibiting farnesylation of H-RAS, which does not undergo alternative isoprenylation by GGTase-I, may have antitumor effects also in tumors caused by K-RAS mutations. Indeed, wild-type H-RAS and N-RAS have been shown to be required for initiating and maintaining growth of K-RAS–induced tumors [229].

Although the studies above indicate that some *CAAX* proteins may be particularly important, the antitumor effects of inactivating *Fntb* or *Pggt1b* are likely a result of inhibiting several *CAAX* proteins. Our $Fntb^{fl/fl}$ and $Pggt1b^{fl/fl}$ fibroblasts are valuable tools for identifying these substrates. Since Cre recombination results in a cell cycle arrest in these cells, they can be used to test what proteins are crucial for cell proliferation. We plan to transfect $Fntb^{fl/fl}$ fibroblasts with geranylgeranylated mutants of CENP-E and CENP-F and subsequently infect them with Cre-adenovirus. If geranylgeranylated CENP-E or/and CENP-F rescue cell proliferation, these proteins are required for cell cycle progression. Similarly, we plan to define whether farnesylated versions of RAC1 and RAC3 reverse the phenotypes of our *Pggt1b*-deficient fibroblasts. Other suggested FTI and GGTI targets may be tested in the same way.

Similar strategies could be used to produce knock-in mice, replacing genes encoding FTase or GGTase-I substrates by sequences mutated to express geranylgeranylated and farnesylated versions of the same proteins, respectively. We are in the process of generating a knock-in mouse that express farnesylated RHOA. This mouse will be valuable for evaluating the role of RHOA in mediating effects of *Pggt1b* inactivation in vivo.

The enhanced antitumor effect of simultaneously inactivating *Fntb* and *Pggt1b* may be explained by the inhibition of several farnesylated and geranylgeranylated *CAAX* proteins; and/or by inhibition of *CAAX* proteins that can be substrates for both enzymes. K-RAS is of course an obvious target; inhibition of isoprenylation and membrane association of K-RAS may contribute to the enhanced antitumor effect. In our study (Paper II), K-RAS isoprenylation was inhibited in *Fntb*/*Pggt1b*-deficient fibroblasts (lacking both *Fntb* and *Pggt1b*), at least to a certain extent: a substantial portion of K-RAS accumulated in the soluble protein fraction and had a slower electrophoretic mobility. In contrary to the effect of inactivating *Fntb* alone or *Pggt1b* alone, western blots of lung extracts showed that inactivating both *Fntb* and *Pggt1b* dramatically decreased levels of phosphorylated ERK. This implicates that reduced signaling through the RAS-RAF-MEK-ERK pathway may contribute significantly to the observed antitumor effects. As discussed earlier, disrupting activation of

wild-type N-RAS may also result in antitumor effects. Along this line, a small proportion of lung cells in vivo contained nonprenylated N-RAS.

Furthermore, *Fntb*/*Pggt1b*-deficient cells should also fail to isoprenylate *CAAX* proteins that normally exist in both farnesylated and geranylgeranylated forms, such as RHOB and RHOH. RHOB, as well as RHOH, which may be specific for hematopoietic cells, act as tumor suppressors [155]. Therefore, inhibition of RHOB and RHOH may rather promote than inhibit tumorigenesis. However, given the increased antitumor effect of blocking both FTase and GGTase-I, these proteins may play a minor role in cancer development, at least in lung cancer.

Will inhibition of FTase and/or GGTase-I be associated with toxicity?

FTIs have been shown to be remarkably well tolerated in mice and in humans [36, 82]. Similarly, we showed that *Fntb*$^{fl/\Delta}$LC mice, with *Fntb*-deficient myeloid cells and lung cells, were healthy and fertile and did not display any apparent pulmonary abnormalities. In vivo use of GGTIs has raised concerns about severe toxicity [123], even if some studies have suggested that GGTIs may not be particularly toxic [118, 124-126]. *Pggt1b*$^{fl/fl}$LC mice were fertile and appeared healthy, with normal lung histology and normal white blood cell counts. Furthermore, several cell types were viable in the absence of *Pggt1b*, including lung tumor cells and macrophages.

The data about toxicity of combined FTI/GGTI treatment in vivo are conflicting. In some xenograft models, no animal toxicity was reported [93, 122], while other FTI/GGTI combinations and DPIs were lethal in mice [123]. We showed that the simultaneous inactivation of *Fntb* and *Pggt1b* resulted in cell death in fibroblasts, while inactivation of both genes in lung cells appeared to be well tolerated. Despite their lung tumors, *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL}LC and *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$*K*^{LSL} mice looked healthy for several months. Furthermore, no obvious abnormalities were found in lungs from *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$LC mice. However, as judged by immunofluorescence, several lung cells in these mice expressed either FTase or GGTase-I or both, while relatively few cells were deficient in both enzymes. This may indicate that *Fntb*/*Pggt1b*-deficient lung cells are more susceptible to cell death. In the ongoing revision of paper II, we assess if any apoptosis can be detected in lung cells of the *Fntb*$^{fl/\Delta}$*Pggt1b*$^{fl/\Delta}$LC mice.

Since we inactivated *Fntb* and/or *Pggt1b* in a cell type-specific manner, we still know relatively little about toxicity of FTase and/or GGTase-I deficiency in other cell types, organs or whole animals. Furthermore, we have not assessed long term toxicity of inactivating *Fntb* and/or *Pggt1b*. To define the utility of inhibiting FTase and/or GGTase-I as an anti-cancer strategy, assessment of organ and whole body toxicity is crucial. To address this question, *Fntb*$^{fl/fl}$ and *Pggt1b*$^{fl/fl}$ mice could be bred with mice harboring different cell type-, tissue-specific, or ubiquitous Cre transgenes.

Fntb$^{fl/fl}$ and *Pggt1b*$^{fl/fl}$ mice will be useful in many kinds of studies

As discussed earlier, *Fntb*$^{fl/fl}$ and *Pggt1b*$^{fl/fl}$ fibroblasts and mice are valuable tools for identifying FTase and GGTase-I substrates that are important for the antitumor activities of FTIs and GGTIs. The conditional knockout mice may also be bred with cell type-specific Cre mice, to study the functional importance of FTase and GGTase-I in different cell types. Similarly, the tolerability of FTase and/or GGTase-I deficiency in different organs can be defined. *Fntb*$^{fl/fl}$ and *Pggt1b*$^{fl/fl}$ mice can also be used to define the effects of FTase and/or GGTase-I deficiency on the development of other cancers, including cancer not initiated by

oncogenic RAS. Furthermore, *Fntb*$^{fl/fl}$ and *Pggt1b*$^{fl/fl}$ mice can be used to study the importance of protein farnesylation and geranylgeranylation in various other diseases. Finally, the *Fntb*$^{fl/fl}$ and *Pggt1b*$^{fl/fl}$ mice can be used to elucidate which effects of FTIs and GGTIs that are due to enzymatic inhibition and which are due to off-target effects.

The importance of *Nf1* deficiency in K-RAS–induced cancer

In Paper III, we showed that simultaneous inactivation of *Nf1* and expression of oncogenic K-RAS in hematopoietic cells induced AML. This was unexpected, since *Nf1* deficiency has been viewed as functionally equivalent with an oncogenic RAS mutation.

Potentially, the AML phenotype may be caused by hyperactive RAS signaling per se, since *Nf1* deficiency may increase levels of GTP-bound N-RAS and H-RAS. However, we showed by western blots that RAS-GTP levels in hematopoietic cells (total bone marrow and CD11b+ cells) of *NKM* mice were not elevated compared to *KM* mice. This data supports that RAS-independent functions of NF1 contribute to the AML in *NKM* mice.

NFI has been suggested to have other functions than RAS-GAP activity. Actually, many mutations found in NF1 patients occur outside the GAP-related domain [70, 194, 197]. Particularly interesting is the potential involvement of NF1 in the cyclic AMP-PKA pathway described in Drosophila [200, 201]. NF1 has also been shown to regulate the actin cytoskeleton in a RAS-independent way, by acting as a negative regulator of the RAC1/PAK1 pathway [230]. Moreover, Guo et al. suggested that NF1 may function as an effector that mediates signaling important for differentiation [199]. Therefore, it could be speculated that loss of NF1 impairs differentiation, thereby providing "the second hit" by which the RAS-mediated MPD progresses into AML. Another potential explanation for the cooperation between *Nf1* deficiency and RAS signaling could be that NF1 functions as a GAP for GTPases other than RAS, which may be involved in signaling pathways regulating cellular differentiation.

We have planned to determine if the cooperation of *Nf1* deficiency and oncogenic K-RAS is caused by the loss of the RAS-GAP activity or if it is related to a RAS-GAP–independent function of NF1. We will infect *NKM* fetal liver cells with a lentivirus expressing either the RAS-GAP domain of NF1 or a full length NF1 with mutations in the RAS-GAP domain, and then inject the transduced cells into lethally irradiated mice. After repopulation, we will inject pI-pC to induce development of AML. If the RAS-GAP domain can prevent the MPD from developing into an AML, we can conclude that it is the loss of the RAS-GAP activity of NF1 that contributes to the development of AML. Similarly, if the full length NF1 with the mutated RAS-GAP domain restores the MPD, we can conclude that RAS-GAP–independent functions of NF1 are important for progression into AML. We plan to perform similar experiments with dominant negative and constitutively active PKA, to determine if PKA might be involved in the cooperation between *Nf1*deficiency and oncogenic K-RAS.

CONCLUSIONS

In this thesis I used genetic strategies in mice to define the importance of isoprenylation and *Nf1* deficiency in K-RAS–induced cancer. I can now conclude the following:

- Inactivation of FTase and/or GGTase-I reduced the development of K-RAS–induced cancer in mice.

This result validates the idea of targeting FTase and/or GGTase-I as an anti-cancer strategy. However, to conclude that FTase and GGTase-I are suitable drug targets the potential toxicity of inhibiting these enzymes must be further evaluated.

- *Nf1* deficiency cooperated with oncogenic K-RAS to induce AML in mice.

Contrary to our expectations, simultaneous inactivation of *Nf1* and expression of oncogenic K-RAS in hematopoietic cells induced AML in mice. Future experiments are planned to define the mechanisms behind this cooperation.

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