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

Defining RCE1 and ICMT as Therapeutic Targets in K-RAS–induced Cancer

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Till Dan och Nila

ABSTRACT

CAAX proteins, such as the RAS and RHO proteins, are recognized by a specific *CAAX* motif at the carboxyl terminus, which undergoes posttranslational modifications. First, a lipid group is attached to the cysteine (the "*C*") of the *CAAX* motif by farnesyltransferase (FTase) or geranylgeranyltransferase-I (GGTase-I); second, the *–AAX* are removed by RAS converting enzyme 1 (RCE1); and third, the cysteine is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT). These modifications are important for the subcellular localization of the protein and for protein-protein interactions.

Several *CAAX* proteins, including RAS and RHO, are involved in the pathogenesis of cancer. Therefore, much effort has focused on exploring the possibility of inhibiting *CAAX* proteins as an anticancer strategy. One potential strategy would be to inhibit the *CAAX* processing enzymes; FTase, GGTase-I, ICMT or RCE1. Previous studies showed that inactivating *Rce1* and *Icmt* in mouse fibroblasts mislocalized RAS away from the plasma membrane and reduced RAS transformation, but nothing was known about the impact of inhibiting these enzymes on cancer development *in vivo*.

The aim of this thesis was to define the impact of inactivating *Rce1* and *Icmt* on the development of K-RAS–induced cancer and thus validate the *CAAX* processing enzymes RCE1 and ICMT as potential therapeutic targets for cancer treatment.

Cre-*lox*P techniques were used to activate an oncogenic K-RAS allele and inactivate *Rce1* or *Icmt* in hematopoietic cells in mice. Activation of the oncogenic K-RAS allele in hematopoietic cells results in a lethal myeloproliferative disease (MPD) with leukocytosis, splenomegaly and autonomous colony growth of hematopoietic cells.

Surprisingly, inactivation of *Rce1* worsened all the phenotypes of the K-RAS–induced MPD and caused the mice to die earlier. On the contrary, inactivation of *Icmt* inhibited the progression of MPD and reduced splenomegaly and autonomous colony growth. Furthermore, inactivating *Icmt* reduced lung tumor development in a K-RAS induced lung cancer model.

The results indicate that inhibiting RCE1 may not be a good strategy for treating RAS-induced hematological malignancies. ICMT, on the other hand, appears to be a promising therapeutic target, and should be further evaluated for the treatment of both hematological malignancies and solid tumors.

LIST OF PUBLICATIONS

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

I ***Rce1*** **deficiency accelerates the development of K-RAS–induced myeloproliferative disease.**

Annika Wahlström, Briony Cutts, Christin Karlsson, Karin Andersson, Meng Liu, Anna-Karin Sjögren, Birgitta Swolin, Stephen G. Young, Martin O. Bergö.

Blood. 2007, **109**:763–768.

II **Inactivating *Icmt* ameliorates K-RAS–induced myeloproliferative disease.**

Annika Wahlström, Briony Cutts, Meng Liu, Annika Lindskog, Christin Karlsson, Anna-Karin Sjögren, Karin Andersson, Stephen G. Young, Martin O. Bergö.

Blood. 2008, **112**:1357−1365.

Om en person du talar med inte tycks lyssna, var tålmodig.
Det kan helt enkelt vara så att han har lite ludd i ena örat.

Nalle Puh - A.A. Milne

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ABBREVIATIONS

AML	acute myeloid leukemia
BFU-E	burst forming unit-erythroid
CD	cluster of differentiation
CFU-E	colony forming unit−erythroid
CFU-G	colony forming unit−granulocyte
CFU-GEMM	colony forming unit−granulocyte-erythrocyte-macrophage-megakaryocyte
CFU-GM	colony forming unit−granulocyte-macrophage
CFU-M	colony forming unit−macrophage
EPO	erythropoietin
ERK	extracellular signal−regulated kinase
FTI	farnesyltransferase inhibitor
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GGTI	geranylgeranyltransferase-I inhibitor
GM-CSF	granulocyte-macrophage−stimulating factor
GTP	guanine triphosphate
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MPD	myeloproliferative disease
PI3K	phosphatidylinositol-3 kinase

INTRODUCTION

Cancer is the leading cause of death in the world and many researchers struggle to solve the mystery behind this complex disease.[1] The development of cancer starts with a single cell that is transformed into a cancer cell, which exhibits uncontrolled proliferation.[2] Transformation can be caused by mutations in genes that encode proteins involved in cell cycle progression, proliferation, differentiation, and motility. Mutations can result in gain of function of oncoproteins, or loss of function of tumor suppressor proteins. Normally, at least three mutation events are required to turn a normal human cell into a cancer cell.[3] Members of the *CAAX* protein family are often involved in the pathogenesis of cancer.[4] Therefore, much effort has focused on exploring the possibility of inhibiting individual *CAAX* proteins, and also their posttranslational modifications, as anticancer strategies.

CAAX proteins

The *CAAX* protein family is a large group of proteins important for many processes in the eukaryotic cell including: growth, proliferation, differentiation and morphology changes (Table 1, page 12).[5] *CAAX* proteins are recognized by the specific amino acid sequence at the carboxyl terminus, the *CAAX* motif, where "*C*" is cysteine, "*A*" an aliphatic amino acid, and "*X*" any amino acid. This *CAAX* motif undergoes a series of posttranslational modifications, which are important for subcellular localization, stability, and protein-protein interactions.[6]

Studies of *CAAX* proteins in yeast

The first findings that led to the discovery of *CAAX* proteins came out of studies on fungal mating factors in the late 1970s. Fungal mating factors are pheromones secreted by yeast to initiate the mating process. There are two mating cell types in the budding yeast *Saccharomyces cerevisiae*: **a**-mating type and α-mating type, which secrete **a**-factor and α-factor pheromones, respectively. The **a**-factor propeptide, but not the α-factor, contains a carboxyl-terminal farnesylated cysteine residue.[7,8] The molecular structure of yeast mating **a**-factor pheromone was identified in 1988 and it was confirmed that the carboxyl-terminal cysteine is farnesylated and that the carboxylgroup of the farnesylated cysteine is methylated.[9]

In the 1980s several groups showed that eukaryotic RAS proteins share a common sequence at the carboxyl terminus[10-12] and that this sequence is modified posttranslationally.[13] It was subsequently established that many other proteins have a cysteine residue four amino acids from the end and might be similarly modified.[14] There are about 280 proteins that fit this profile and approximately 120 of them are predicted to be posttranslationally modified.[15]

Table 1. List of well known CAAX proteins and their main cellular functions.

Protein	Prenylation	Cellular functions
H-RAS	F	Proliferation, cell cycle progression, gene expression, differentiation
N-RAS	F/G	Proliferation, cell cycle progression, gene expression, differentiation
K-RAS	F/G	Proliferation, cell cycle progression, gene expression, differentiation
RAP1	G	Regulation of cell adhesion, proliferation and differentiation
RHEB	F	Regulation of cell cycle progression, cell growth
RAL	G	Proliferation, motility, gene expression, vesicular transport, actin organization
RHOA	G	Actin organization, formation of stress fibers and focal adhesions, microtubule stability, cytokinesis, phagocytosis
RHOB	F/G	Formation of stress fibers, endosomal transport, promoting apoptosis
RND 3	F	Cell migration, loss of stress fibers and focal adhesions, inhibition of cell cycle progression
RHOH	F/G	Negative regulation of proliferation, migration and survival of hematopoietic cells
RAC1	G	Regulation of gene transcription, cytoskeleton reorganization, lamellipodia formation, proliferation, survival
CDC42	G	Filopodia formation, vesicle trafficking, migration, cytokinesis
TC10	F	Filopodia formation, cell signaling, cell growth
RAB	G	Vesicular transport, membrane trafficking
Lamin A	F	Structural component of the nuclear lamina
CENP-E	F	Involved in sister kromatid separation during mitosis
CENP-F	F	Involved in sister kromatid separation during mitosis

F = farnesylation; G = geranylgeranylation

CAAX proteins have been thoroughly studied during the past decades and many research groups have participated in identifying and characterizing the enzymes that carry out the posttranslational modifications. Much effort has focused on evaluating the importance of these modifications for the subcellular localization and function of *CAAX* proteins.

Posttranslational modifications of *CAAX* proteins

The *CAAX* motif triggers three posttranslational modifications, which increase the hydrophobicity of the protein and thereby promote interaction with membranes and other proteins.[6] Each modification step is dependent on the previous step, which means that protein isoprenylation by FTase or GGTase-I is a prerequisite for endoproteolysis by RCE1 and methylation by ICMT (Figure 1).[16]

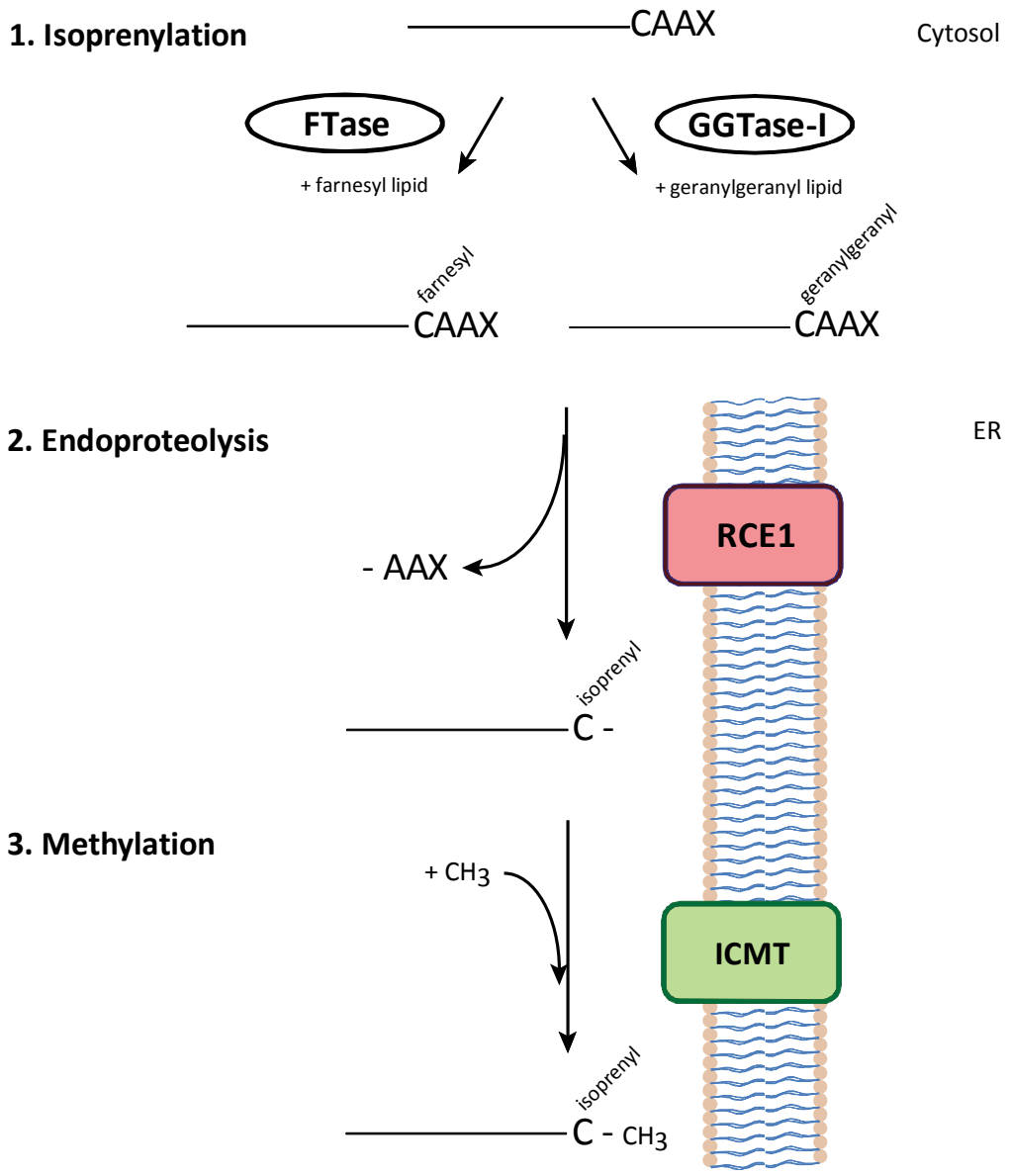


Figure 1. Posttranslational modifications of CAAX proteins*. Proteins with a CAAX motif at the carboxyl terminus undergo three sequential posttranslational modifications. The first modification step is addition of a isoprenylgroup to the cysteine of the CAAX motif by farnesyltransferase (FTase) or geranylgeranyltransferase-I (GGTase-I). The isoprenylated CAAX protein then becomes a substrate for RAS converting enzyme 1 (RCE1), which removes the last three amino acids (the –AAX of the CAAX motif) by endoproteolysis. Finally, the newly exposed isoprenylated cysteine residue is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT).*

Step 1: The cysteine residue is isoprenylated by FTase or GGTase-I

Newly synthesized *CAAX* proteins are first isoprenylated, which means that a lipid group is covalently attached via a thioether linkage to the cysteine residue of the *CAAX* motif.[6] Isoprenylation takes place in the cytosol and is catalyzed by protein farnesyltransferase (FTase) or protein geranylgeranyltransferase type-I (GGTase-I). FTase adds a 15-carbon farnesyl lipid (farnesylation) and GGTase-I adds a 20-carbon geranylgeranyl lipid (geranylgeranylation).[7]

FTase was first identified and purified from the cytosol of rat brain,[17] and GGTase-I was identified and purified from bovine brain cytosol.[18] Both FTase and GGTase-I are heterodimeric proteins consisting of one α-subunit, which they share, and one distinct β-subunit specific for each of the proteins.[19] FTase and GGTase-I are metalloenzymes that require zinc for enzymatic activity; FTase also requires magnesium.[20]

The "*X*" of the *CAAX* motif determines whether the cysteine residue will be farnesylated or geranylgeranylated. Generally, when the "*X*" is leucine, the *CAAX* motif is processed by GGTase-I (*e.g.*, RHOA and CDC42);[7,15,21] when the "*X*" is serine, methionine, glutamine or alanine it is processed by FTase (*e.g.*, RAS, nuclear lamins, RHEB, and centromeric proteins).[5,7,15,22,23] There are also some *CAAX* proteins that can be processed by either FTase or GGTase-I (*e.g.*, K-RAS, RHOB and RHOH).[24] The prediction of prenylation type based on the amino acid at position *X* of the *CAAX* motif is not perfect and it needs to be confirmed by experimental studies.[24]

Step 2: The *–AAX* residues are removed by RCE1

After isoprenylation, the *CAAX* proteins become associated with the endoplasmic reticulum (ER), where they are recognized by the endoprotease RAS converting enzyme 1 (RCE1)[a], which is an integral ER membrane protein with the active site facing the cytosol.[6,25,26] RCE1 removes the last three amino acids (the *–AAX*) from the isoprenylated cysteine residue by endoproteolysis.[27] The isoprenylated *CAAX* proteins are processed by RCE1, regardless of whether they are farnesylated or geranylgeranylated.[16]

The *RCE1* gene was first identified in yeast

In the late 1980s, it was established that farnesylated *CAAX* peptides undergo endoproteolytic processing.[28,29] Ten years later two yeast genes, *RCE1* and *STE24* (*AFC1*), were shown to be involved in the endoproteolytic cleavage of *–AAX* from the farnesylated proteins Ras2p and the precursor of yeast mating pheromone **a**-factor.[27] The identification of *RCE1* and *STE24* was an important breakthrough that made it possible to clone the human

[a] RCE1 is the official name for this enzyme but it has in previous studies been referred to as FACE2 and RACE.

and mouse orthologues for the two genes.[26,30-34] The protein Rce1p is essential for the endoproteolysis of Ras2p and can also cleave the *–AAX* from **a**-factor. Ste24p has two functions: removal of the carboxyl-terminal *–AAX* of **a**-factor and cleavage of an N-terminal extension,[30] but it is not involved in the endoproteolytic processing of the yeast RAS proteins.[27]

Rce1 deficiency is lethal in mice

Kim and co-workers identified the mouse *Rce1* gene and generated mice with a knockout allele (*Rce1*$^{-}$).[26,32] *Rce1* is essential for the embryonic development in mice.[32] *Rce1*$^{-/-}$ embryos started to die after embryonic day (E) 15.5 and the few live-born mice identified were small and did not live past day 10. The precise cause of death is not clear and no abnormalities in morphology or organogenesis was found.[32]

Further investigations showed that RAS proteins in *Rce1*$^{-/-}$ cells exhibited a reduced electrophoretic mobility compared with RAS proteins from *Rce1*$^{+/+}$ and *Rce1*$^{+/-}$ cells, suggesting that the RAS *CAAX* motif is not endoproteolytically processed in the setting of *Rce1* deficiency.[32] If endoproteolysis of RAS proteins is blocked, carboxyl methylation would also be defective in *Rce1*$^{-/-}$ cells, since carboxyl methylation requires removal of the *–AAX*. Kim and co-workers analysed the methylation status of RAS proteins from *Rce1*$^{-/-}$ cells and showed that carboxyl methylation was impaired in the absence of RCE1.[32] Furthermore, prenylated recombinant RAS proteins and two other *CAAX* proteins, farnesylated $G_{\gamma 1}$ and geranylgeranylated RAP1B, could not be proteolytically processed by membranes from *Rce1*$^{-/-}$ fibroblasts.[32] These findings demonstrate that the *Rce1* gene product is essential for the endoproteolytic processing of RAS proteins. Despite the absence of endoproteolysis, the levels of RAS proteins did not seem to be altered, which indicates that there is likely no effect on RAS protein turnover in the setting of *Rce1* deficiency.[32]

Step 3: The newly exposed isoprenylated cysteine is methylated by ICMT

After endoproteolysis, the isoprenylated cysteine residue is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT)[b]; a membrane-bound protein located at the ER with the active site facing the cytosol.[15] Unlike the first two processing steps, methylation by ICMT is potentially reversible under physiologic conditions but the evidence for this is not strong.[35]

The *STE14* gene is responsible for carboxyl methylation in yeast

As mentioned above, the yeast mating hormone **a**-factor was found to possess a methyl group at the carboxyl terminus.[9] Subsequent studies showed that the gene *STE14* was required for the carboxyl methylation of **a**-factor and that *STE14*-deficient yeast mutants lack carboxyl methyltransferase activity.[36,37]

[b] ICMT is the official name for this enzyme. It has in previous studies been referred to as pcCMT, CMT, and PCMT.

Another study demonstrated that Ste14p is also responsible for methylation of Ras1p and Ras2p (and most likely a variety of other yeast proteins),[38] which is in agreement with an earlier study showing that H-RAS is carboxyl methylated in rat embryonic fibroblasts.[39]

The nucleotide sequence of yeast *STE14* was first reported by the laboratory of Jasper Rine,[40] and it was shown that the gene product is not essential for cell growth in yeast.[41] Several orthologs of *STE14* have been cloned: *mam4* in *Schizosaccharomyces pombe* (yeast), *Xmam4* in *Xenopus laevis* (African clawed frog)[42] and *Icmt* in mammalian cells,[43] suggesting that ICMT may be important in all eukaryotic species.

Icmt deficiency is lethal in mice

Icmt deficiency in mice results in embryonical lethality; $Icmt^{-/-}$ embryos died between E10.5 and E12.5.[44] Investigations by another group showed that ICMT may be essential for the earliest stages of liver development suggesting that *Icmt*-deficient embryos died from anemia caused by defects in liver development.[45]

Examination of tissues from male chimeric mice (which were generated by injecting homozygous $Icmt^{-/-}$ embryonic stem cells into wild-type blastocysts) revealed that $Icmt^{-/-}$ cells contribute differently to the development of different tissues. In skeletal muscle the contribution of *Icmt*-deficient cells was high suggesting that ICMT may not be important for the development of skeletal muscle; whereas in brain, liver and testis the contribution was relatively low suggesting that the development of those tissues requires ICMT activity.[44] Northern blot analysis of tissues from wild-type mice showed that expression of *Icmt* in different tissues was inversely correlated to the extent of $Icmt^{-/-}$ cell contribution in the chimeric mice. Thus, for example, expression of *Icmt* was high in brain and liver where the contribution of $Icmt^{-/-}$ cells was low.[44]

Secondary membrane targeting modifications

In addition to the three *CAAX* motif modifications, many *CAAX* proteins possess a second membrane targeting signal that increases the stability of the membrane association.[46] For some proteins (*e.g.* H-RAS, N-RAS and RHOB), this second signal is palmitoylation of cysteine residues upstream of the *CAAX* motif.[24,47] It has been suggested that isoprenylation, but not endoproteolysis and methylation, is required for palmitoylation to occur. This suggestion was verified in experiments showing that palmitoylation of H-RAS and TC10 proceeded in a normal fashion in *Rce1*- and *Icmt*-deficient cells, respectively.[24,48]

Palmitoylation is a reversible reaction in which a 16-carbon palmitate fatty acid is attached to cysteine residues with a thioether linkage.[49] The mechanism behind this reaction has been difficult to elucidate and it was only recently discovered that there are several different protein fatty acyltransferases (PATs) responsible for palmitoylation.[50] DHHC9·GCP16, a

membrane bound protein located in the Golgi apparatus, has been identified as a specific palmitoyltransferase for human H-RAS and N-RAS.[51]

Other *CAAX* proteins, such as K-RAS, are not palmitoylated but have a polybasic region consisting of a stretch of lysine residues, which function as a second signal. The lysine residues are positively charged and bind to the negatively charged phospholipid heads of the inner surface of the plasma membrane.[52] Hancock and co-workers showed that replacing the lysines of the polybasic region of human K-RAS4B with the uncharged amino acids glutamine, resulted in mislocalization of a large proportion of the proteins away from the plasma membrane into the cytosol – despite the fact that they were fully processed at the *CAAX* motif.[52]

The posttranslational modifications of the *CAAX* motif and the second membrane targeting signal are important for the proper localization of *CAAX* proteins, for protein stability, and potentially for protein function.[24,53,54] *CAAX* proteins have diverse functions and are involved in a variety of processes in cells and in the process of turning normal cells into cancer cells, and also in allowing primary tumor cells to metastasize.[55] The *CAAX* proteins have therefore been studied extensively and one important issue has been to investigate their role in the pathogenesis of cancer.

The role of *CAAX* proteins in the pathogenesis of cancer

Cancer is a complex disease and many different proteins, including several *CAAX* proteins, are involved in the pathogenesis. Among the most studied and best characterized *CAAX* proteins implicated in human cancer are the members of the RAS superfamily.[4,56]

The RAS superfamily of small GTPases

The RAS superfamily is divided into five major subgroups, RAS, RHO, RAB, RAN, and ARF, based on sequence and functional similarities.[57] They are small GTPases with a relatively low molecular weight (about 20−35 kDa) and act as molecular switches with high affinity for guanine diphosphate (GDP) and guanine triphosphate (GTP).[58] They cycle between inactive GDP-bound and active GTP-bound states and have low intrinsic GTPase activity that hydrolyses GTP to GDP, and low GDP/GTP exchange activities.[57]

GDP/GTP binding is regulated by two types of proteins: guanine nucleotide exchange factors (GEFs), which promote activation of the proteins by enhancing the release of GDP and the binding of GTP, and GTPase activating proteins (GAPs), which promote inactivation by accelerating the intrinsic GTPase activity.[59] Several GEFs and GAPs can act on the same GTPase protein and allow for very precise regulation of downstream signaling.[57]

The RAS proteins

The first isolated small GTPases were rat sarcoma (RAS) oncoproteins − hence the name RAS superfamily. It was first observed that a murine leukemia virus, isolated from a rat with leukemia, could induce sarcoma in newborn rodents[60] and a few years later the oncogenic retroviruses Harvey and Kirsten murine sarcoma viruses (Ha-MSV, Ki-MSV) were identified by serial passage of murine leukemia viruses through Wister-Furth rats.[61]

The *Ras* oncogenes were identified in the retrovirus strains Ha-MSV and Ki-MSV and it was established that these strains were recombinant viruses with gene sequences derived from the rat genome.[62] Subsequently, it was shown that each of the Ha-MSV and Ki-MSV retrovirus strains contained genes encoding sarcoma proteins and they induced cellular transformation. These genes were named *Ras* (*v-Hras* and *v-Kras*) for rat sarcoma.[63] The *v-ras* oncogenes have human orthologues, *HRAS* and *KRAS*, and a third member of the *RAS* gene family, *NRAS*, has also been identified.[64]

The three human *RAS* genes (*NRAS*, *HRAS* and *KRAS*) encode four different RAS proteins: N-RAS, H-RAS, K-RAS4A and K-RAS4B. K-RAS4A and K-RAS4B are generated by alternative splicing of the fourth exon of the *KRAS* gene. Whereas K-RAS4A is expressed at low levels, K-RAS4B is ubiquitously expressed and accounts for 90-99% of all K-RAS mRNA.[65] In this thesis K-RAS4B will be referred to as K-RAS.

The biological function and subcellular localization of the three RAS isoforms differ.[66] In mice, knockout of *Kras2* (the mouse gene encoding K-RAS) results in embryonic lethality;[67] in contrast, mice lacking *Hras*, *Nras* or both genes, are viable.[68,69] Thus, K-RAS, but not H-RAS and N-RAS, is essential for embryonic development in mice.

Trafficking and localization of RAS proteins

As mentioned earlier, RAS proteins are synthesized and posttranslationally modified in the cytosol. After processing of the *CAAX* motif, N- and H-RAS are transported from the ER to the Golgi, where they are palmitoylated on one or two cysteine residues, respectively. The palmitoylated proteins are transported to the plasma membrane on vesicles through the secretory pathway.[70,71] N- and H-RAS with mutated palmitoylation sites accumulate predominantly in the ER but also in the Golgi.[70,71]

The mechanism behind the trafficking of K-RAS from ER to the plasma membrane is not fully established but it is clearly distinct from the trafficking of N- and H-RAS.[71] K-RAS lacks the cysteine residues upstream of the *CAAX* motif, and is not palmitoylated at the Golgi. However, several mechanisms have been suggested for the trafficking pathway of K-RAS: diffusion of the protein down an electrostatic gradient towards the negatively charged plasma membrane (promoted by the polylysine motif),[72] transport involving an unidentified chaperone, or transport along microtubules.[66] It has been shown that both the polylysine region and carboxyl methylation by ICMT are important for the interaction between K-RAS and microtubules.[73] Thus, one possibility is that K-RAS is transported to the plasma membrane on microtubules.

At the plasma membrane, the lipid anchors of N- and H-RAS promote the association with cholesterol-rich lipid rafts whereas K-RAS, with its polylysine motif, associates with more featureless structures of the membrane.[66,74] The membrane localization of H-RAS is in a dynamic equilibrium that is regulated by GTP loading. GDP-bound H-RAS has a higher affinity for lipid rafts than GTP-bound H-RAS and GTP loading increases the amount of H-RAS in non-raft membrane compartments.[75] The plasma membrane localization of K-RAS appears to be unaffected by cycles of GDP/GTP-binding.[74]

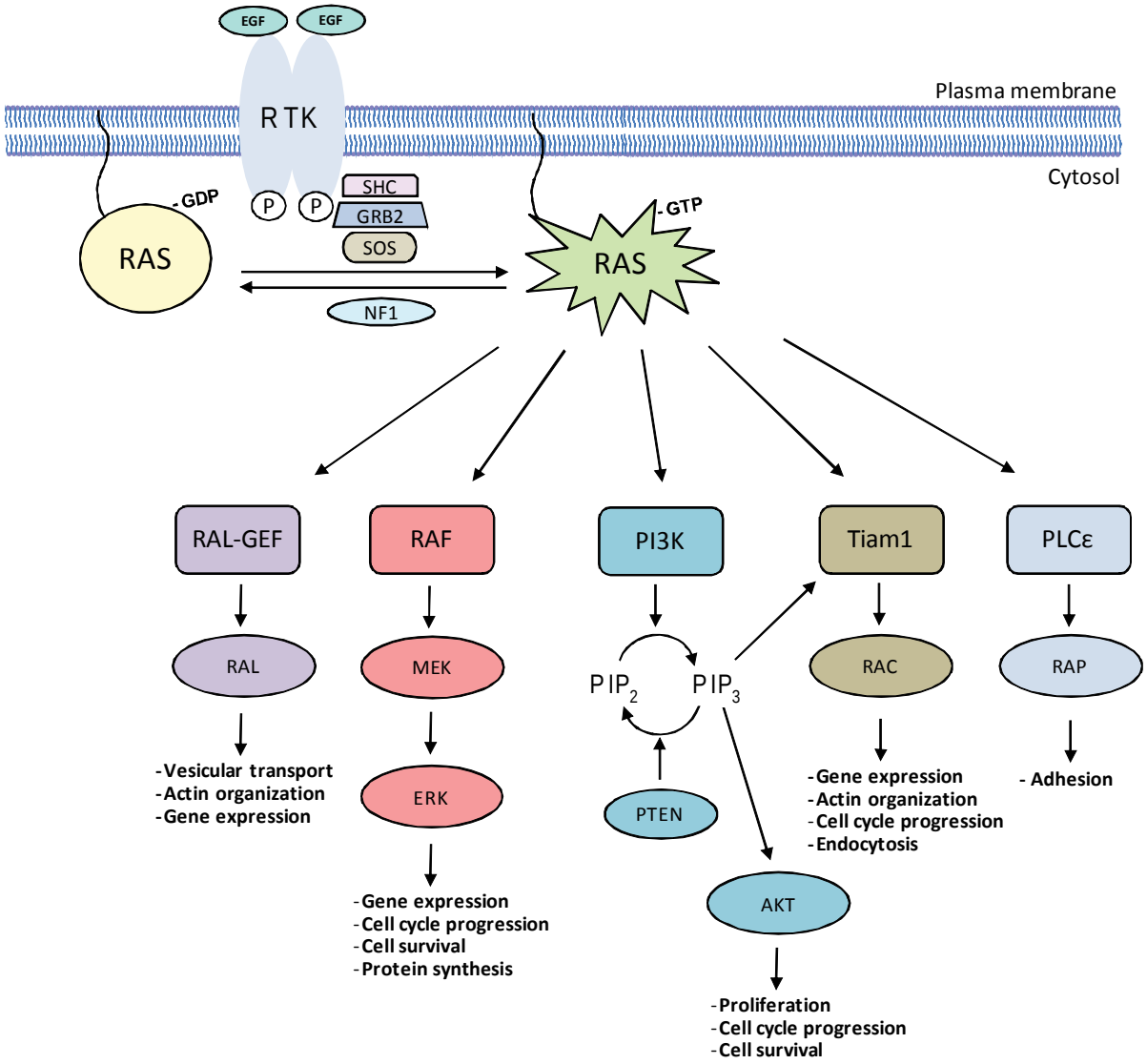


Figure 2. RAS signaling pathways. *RAS is activated at the plasma membrane in response to extracellular stimuli such as epidermal growth factor (EGF) that binds to receptor tyrosine kinase (RTK) resulting in phosphorylation and activation of RTK. Phosphorylated RTK recruits SHC and GRBs, which bind to and activate SOS, a RAS-GEF, which promotes activation of RAS. Activated RAS interacts with several downstream effectors and the most studied RAS signaling pathway is RAF/MEK/ERK.*

RAS signals through several downstream effectors

RAS is activated at the plasma membrane in response to extracellular stimuli, such as epidermal growth factor (EGF) that binds to a receptor tyrosine kinase (RTK), resulting in phosphorylation and activation of the RTK.[76] Phosphorylated RTKs recruit the adaptor proteins Src-homology-2 (SHC) and growth factor receptor bound 2 (GRB2), which bind to and activate son-of-sevenless (SOS).[66] SOS is a RAS-GEF which facilitates GDP release and GTP binding of RAS.

Activated RAS then interacts with several downstream effectors (Figure 2). The most well-studied RAS signaling pathway is the RAF-MEK-ERK mitogen-activated protein kinase (MAPK) cascade, which transfers signals from cell surface receptors and other signaling proteins by

phosphorylation of downstream effectors.[77] The RAS effector RAF exists in three isoforms: A-RAF, B-RAF, and c-RAF-1. RAF phosphorylates MAPK/ERK kinase (MEK) which in turn phosphorylates and activates extracellular signal–regulated kinase (ERK). Phosphorylated ERK regulates many substrates including various transcription factors (*e.g.*, Elk-1 and c-myc) resulting in changes in gene expression. Mutations in B-RAF are common in human cancer and it has been suggested that B-RAF and RAS mutations have similar roles in oncogenesis, due to the fact that they are common in the same types of cancer but they rarely occur together in the same tumor.[78] The most important role of oncogenic RAS might be mediated through RAF activation but there are additional pathways essential for RAS transformation.[55,79]

Another well-characterized RAS pathway is the phosphatidylinositol-3 kinase (PI3K) signaling pathway, which is important for cell growth and survival. PI3K converts phosphatidylinositol (4,5)-biphosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3) by adding a phosphate group, which results in phosphorylation and activation of AKT.[55] AKT can contribute to oncogenesis through many downstream effectors including the tumor suppressor $p21^{CIP1}$. AKT inhibits the function of $p21^{CIP1}$ in the nucleus by phosphorylation of $p21^{CIP1}$, which results in cytoplasmic localization.[80] Phosphatase and tensin homolog (PTEN) is another tumor suppressor that can counteract PI3K by promoting the conversion of PIP_3 to PIP_2, which results in reduced phosphorylation of AKT.[81]

Other downstream RAS effectors are RAL-GEFs, which activate the small GTPases RALA and RALB. It has been suggested that activated RAL-GEFs alone are not sufficient to induce transformation, but they contribute to RAS-induced transformation *in vitro*.[55]

Hyperactive RAS signaling is involved in the pathogenesis of cancer

Mutations in *RAS* genes are involved in many different forms of cancer.[82,83] Mutations most commonly occur in codon 12, 13, and 61 and result in loss of the intrinsic RAS GTPase activity and insensitivity to RAS-GAPs.[82] The mutated RAS proteins are constitutively active and hyperactive RAS signaling can result in cellular transformation.

K-RAS and N-RAS are the isoforms most frequently mutated in human cancer. RAS mutations are found in approximately 30% of all human cancers.[82] K-RAS mutations are common in pancreatic, colon, and lung cancer; N-RAS mutations are common in hematological malignancies; and H-RAS mutations are rare and are found in bladder, skin, and thyroid cancer.[83,84]

Hyperactive RAS signaling can also be caused by mutations in genes encoding proteins that interact with RAS, such as FMS-like tyrosine kinase 3 (FLT3), neurofibromin 1 (NF1), and breakpoint cluster region/Abelson (BCR/ABL).[85] FLT3 and BCR/ABL are tyrosine kinases that activate RAS through GRB2 and SOS. NF1 is a RAS-GAP and deficiency in *Nf1* results in high levels of RAS-GTP, which result in hyperactive RAS signaling.[86]

RAS signaling is complex; new pathways and effector proteins are discovered, and RAS transformation is facilitated by signaling through different pathways in different cell types.[55] Some of the proteins that interact with RAS signaling and cooperate with RAS during oncogenic transformation are other members of the *CAAX* protein family.

Other members of the RAS protein family

RAS-proximate 1 (RAP1) is important for the regulation of cell adhesion, proliferation, and differentiation. The role of RAP1 appears to be dependent on cell type; both defective and constitutively active RAP1 can result in malignancies but in distinct cell types,[87] and it has been suggested that accumulation of RAP1-GTP is associated with myeloid disorders in mice.[88]

RAS homolog enriched in brain (RHEB) proteins are important for cell growth and regulation of cell cycle progression.[89] RHEB acts downstream of PI3K/AKT and activates the mammalian target of rapamycin (mTOR) which activates S6K.[89] It is not clear whether RHEB promotes or antagonizes RAS signaling; one study showed that RHEB might antagonize oncogenic RAS signaling by interaction with RAF,[90] and another study showed that RHEB might promote RAF activation.[91]

Other proteins involved in RAS signaling are the RAS-like (RAL) proteins RALA and RALB. They are activated by RAS through RAL-GEFs and regulate gene expression, actin organization, and membrane trafficking.[55] It has been reported that RALA is required for anchorage-independent growth of human tumor cells and RALB is important for survival of human tumor cells.[55,92] Furthermore, RALA has been shown to increase the metastatic capacity of transformed cells.[93]

The RHO protein family

Another large group of RAS superfamily proteins is the RAS homlogous (RHO) protein family of GTPases. RHO proteins are involved in cell migration, proliferation, survival/apoptosis, polarization, cell adhesion, and they are key regulators of actin cytoskeleton organization.[57,94] Many RHO proteins are involved in pathological processes including cancer cell migration, invasion, and metastasis.[95]

In addition to the GEFs and GAPs, there is a third group of RHO regulator proteins: RHO guanine nucleotide dissociation inhibitors (RHO-GDIs).[95] RHO-GDI binds to RHO and covers the prenyl group at the carboxyl terminus and stabilizes the protein in the cytosol as a RHO-GDI-complex.[96] Thus, the RHO-GDIs inhibit RHO activity.

The mammalian RHO family includes 20 members divided into several subfamilies. The most studied RHO proteins are RHOA, RAC1, and CDC42.[57,94,95,97] RHOA and RAC1 are often upregulated in human cancer and have been implicated in several stages of cancer progression: transformation, migration, and metastasis.[98] The precise role of CDC42 in

cancer is not clear and seems to be tissue specific. On one hand, upregulation of CDC42 has been proposed to contribute to the pathogenesis of some forms of breast cancer;[99] on the other hand, in a liver specific knockout model, loss of CDC42 enhanced the cancer development.[100]

Three other RHO proteins are RHOB, RND3, and RHOH. They have all been suggested to have tumor suppressor functions; RHOB is often downregulated in human tumors and its expression is inversely correlated to tumor aggressiveness;[101-103] RND3 expression inhibits cell cycle progression and RAS-induced transformation[104] and is decreased in prostate cancer;[105,106] and low expression of RHOH is associated with acute myeloid leukemia (AML).[107]

The RAB protein family

The largest subgroup in the RAS superfamily is the RAS-like proteins in brain (RAB) family, which includes about 60 members. RAB proteins regulate vesicular transport and are located in membranes of different subcellular compartments. Most of the RAB proteins end with *CC*, *CX, CXC* or *CCXX* at the carboxyl terminus and only a few of them (RAB8 and RAB13) have a *CAAX* motif.[108] RAB proteins are geranylgeranylated at one or two cysteines at the carboxyl terminus by RAB geranylgeranyltranferase (RGGT or GGTase-II).[109] The *CXC*-RAB proteins are also methylated by ICMT at the carboxyl-terminal cysteine without prior endoproteolysis by RCE1.[44,108] The fact that *CXC*-RABs are substrates for ICMT and not for RCE1, might be important in explaining differences in the phenotypes of *Rce1* versus *Icmt* deficiency.

There are four regulator proteins that control the activity of RAB proteins: GEFs, GAPs, RAB-GDIs, and the RAB escort proteins (REP).[109] REP is required for geranylgeranylation of RAB and there are two proposed mechanisms for this: newly synthesized RAB binds to REP which presents the RAB to GGTase-II, or REP associates with GGTase-II and the complex binds to unprenylated RAB.[108] The prenylation of RAB is essential for the association with the membranes and to be fully active RAB needs to be membrane-associated and GTP-bound.[109]

It was recently suggested that a crucial parameter for cancer initiation and progression is disruption of endocytosis,[110] which might make the RAB proteins interesting as targets for cancer treatment.

The role of other *CAAX* proteins in cancer development

Centromeric protein E (CENP-E) and mitosin (CENP-F) are *CAAX* proteins involved in mitosis and they are both substrates for FTase. CENP-E and CENP-F are attached to the kinetochores and microtubules and play an important role in cancer progression by promoting cell cycle progression and regulating sister chromatid separation during mitosis.[111-113] CENP-F has been shown to be upregulated in breast cancer and high expression of CENP-F was associated with higher risk of cancer death.[114]

Since many *CAAX* proteins contribute to the development of cancer, there has been a lot of focus on finding ways to inhibit these proteins. The majority of those studies have focused on inhibiting RAS. One strategy that has received lots of attention is inhibiting membrane targeting of the RAS proteins by interfering with the processing of the *CAAX* motif.

CAAX processing enzymes– targets for cancer treatment?

Given the central role of RAS proteins and RAS signaling in the pathogenesis of cancer, much effort has focused on targeting this class of proteins as a strategy to treat cancer. However, targeting RAS itself with the goal of blocking RAS activity has proved to be difficult.[115] One potential strategy is to mislocalize RAS away from the plasma membrane and this might be accomplished by interfering with the *CAAX* processing enzymes. Most of these efforts have focused on developing inhibitors of FTase and GGTase-I. Although it is clear that this strategy has clinical value, there have also been some drawbacks, indicating that more research in this field is needed.

FTase inhibitors

Several farnesyltransferase inhibitors (FTIs) have been developed and tested in preclinical trials.[17,23] FTI treatment clearly causes mislocalization of H-RAS and inhibition of RAS-induced transformation in cells.[116-119] However, clinical trials have not shown the same efficacy and have mainly been disappointing.[23,120]

FTI treatment of H-RAS–induced tumors in mice resulted in tumor regression, while N-RAS –transformed cells showed modest tumor regression in response to FTI treatment.[121] The growth of K-RAS transformed tumor cells was inhibited but the tumors did not regress.[122] These differences in sensitivity in response to FTIs between the three RAS isoforms can be explained by a process called alternative prenylation. Alternative prenylation allows N-RAS and K-RAS to be geranylgeranylated by GGTase-I when FTase is inhibited. H-RAS is exclusively farnesylated and cannot be alternatively prenylated.[120,123]

The fact that K-RAS and N-RAS were geranylgeranylated by GGTase-I in the setting of an FTI, was a significant problem, primarily since most human tumors harbor mutations in these RAS isoforms. However, several K-RAS– and N-RAS–transformed tumor cell lines did show a respons to FTI treatment (even though K-RAS and N-RAS were geranylgeranylated in those cells) implying that other farnesylated *CAAX* proteins might be important in RAS transformation.[124-126]

One candidate protein is RHOB, which is present in both farnesylated (RHO-F) and geranylgeranylated (RHO-GG) forms.[102] RHOB-GG is more abundant at normal conditions and account for about 70% of the total RHOB.[127] It has been suggested that RHOB-GG has a growth inhibitory function and that the inhibitory effect of FTI treatment could result from loss of RHOB-F or gain of RHOB-GG or both.[128] In contrast, other studies indicate that RHOB

is not a target for FTIs.[129,130] Another candidate protein that might play a role in the antitumor effect of FTIs is RHEB. RHEB is required for cell growth and cell cycle progression and it has been shown that this function is dependent on RHEB farnesylation.[89] It has also been shown that FTI treatment of cells block the activation of S6K by RHEB.[131] FTIs have also been shown to affect the function of the mitotic proteins CENP-E and CENP-F resulting in G_2-M arrest of the cells.[111]

GGTase-I inhibitors

The disappointing results with FTIs in clinical trials and the fact that other geranylgeranylated *CAAX* proteins are important for oncogenesis prompted the development of GGTase-I inhibitors.[132] GGTIs have shown efficacy *in vitro* but there has been concern regarding potential toxicity. GGTIs induce apoptosis of cultured cells and cause toxicity in mouse models.[133-135] Another study, showed that inactivating the gene encoding the β subunit of GGTase-I (*Pggt1b*) resulted in proliferation arrest but did not seem to affect the viability of the cells; the *Pggt1b*-deficient fibroblasts remained viable for more than three weeks.[136] Inactivation of *Pggt1b* also reduced tumor formation and improved survival in mice with a K-RAS–induced lung cancer. Moreover, no apparent toxicity was seen in tissues or cells without tumors in this study[136] suggesting that GGTIs should be evaluated further.

Previous studies on RCE1 and ICMT

The problems with low efficacy of FTI treatment *in vivo*, and the concerns regarding toxicity of GGTI treatment drew the attention to the other two *CAAX* processing enzymes, RCE1 and ICMT, as potential targets for cancer treatment. Since most or all *CAAX* proteins are processed by RCE1 and ICMT, regardless of prenylation type, the problem with alternative prenylation would no longer be an issue. It has been speculated that it might be too toxic to inhibit RCE1 or ICMT since both these enzymes process far more substrates than either FTase or GGTase-I. It is therefore of great importance to thoroughly evaluate the impact of *Rce1* deficiency and *Icmt* deficiency *in vitro* and *in vivo*.

Rce1 deficiency reduces cell growth and transformation *in vitro*

To investigate the effects of inhibiting RCE1 on cell growth, Bergo and co-workers generated mice with a conditional *Rce1* knockout allele (*Rce1*^{fl}). When *Rce1*$^{fl/fl}$ cells were treated with *Cre*-adenovirus it resulted in knockout of the *Rce1* gene on both alleles, yielding *Rce1*-deficient *Rce1*$^{\Delta/\Delta}$ cells. With this allele it was possible to study and compare the phenotypes of normal *Rce1* expression (in *Rce1*$^{fl/fl}$ cells) and *Rce1* deficiency (in *Rce1*$^{\Delta/\Delta}$ cells generated from *Cre*-adenovirus treated *Rce1*$^{fl/fl}$ cells) in the same cell line.[48]

Rce1 deficiency in fibroblasts resulted in mislocalization of RAS proteins away from the plasma membrane (Figure 3, page 26), and reduced proliferation.[48,137] *Rce1* deficiency also reduced the ability of K-RAS– or H-RAS–transformed cells to form colonies in soft agar,

indicating that *Rce1* deficiency inhibited oncogenic RAS transformation. Treatment with an FTI potentiated the effect of *Rce1* deficiency and completely blocked the colony forming ability of the *Rce1*-deficient RAS-transformed cells.[48]

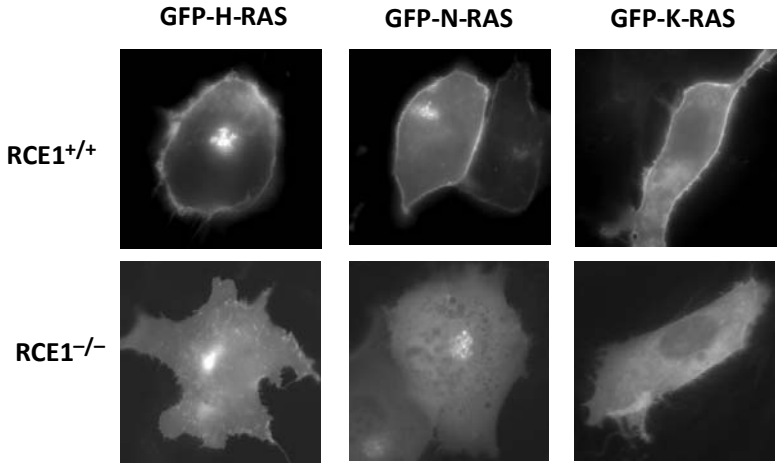


Figure 3. Mislocalization of RAS proteins in* Rce1*-deficient cells. *RAS proteins (tagged with green fluorescent protein (GFP)) are mislocalized away from the plasma membrane in* Rce1*-deficient cells. (Confocal micrographs from Dr Mark Philips)*

These thorough *in vitro* studies on *Rce1* deficiency indicated that *Rce1* might be an interesting anticancer drug target and the next step was to further define the effects of *Rce1* deficiency *in vivo*.

RCE1 is not required for hematopoiesis

Transplantation of *Rce1*$^{-/-}$ fetal liver hematopoietic stem cells into lethally irradiated mice restored hematopoiesis.[32,138] Although livers from *Rce1*$^{-/-}$ embryos contained fewer nucleated cells than *Rce1*$^{+/-}$ and *Rce1*$^{+/+}$ embryos, there were no obvious differences in appearance or function of cells from the different genotypes.[32]

Rce1 deficiency had no effect on the sensitivity of hematopoietic cells to granulocyte-macrophage colony-stimulating factor (GM-CSF); similar numbers of colony forming unit granulocyte macrophage (CFU-GM) was formed from *Rce1*$^{-/-}$, *Rce1*$^{+/-}$ and *Rce1*$^{+/+}$ fetal livers in response to different concentrations of GM-CSF.[32]

Interestingly, wild-type mice transplanted with *Rce1*$^{-/-}$ fetal liver cells developed mild leukocytosis (*i.e.*, incresed white blood cell (WBC) counts) by 3 months after transplantation. The increase in WBCs was due to increased numbers of mature myeloid cells (neutrophils and monocytes).[138] Spleen size and splenic cytoarchitecture of recipient mice were normal and the mice remained healthy until they were killed at 6 months of age. Western blot analysis of bone marrow cells from *Rce1*$^{-/-}$ recipients demonstrated normal ERK signaling in response to GM-CSF.[138]

Thus, the absence of RCE1 did not appear to affect hematopoiesis or be associated with apparent toxicity, which are crucial parameters in the search for inhibitors of potential therapeutic targets. However, the absence of RCE1 resulted in a small but reproducible increase in WBC counts in recipient mice.

RCE1 inhibitors

Several inhibitors of RCE1 have been described and most of them are prenyl peptide–based compounds that work as substrate analogues or substrate mimics.[139] RPI, a tetrapeptide-based competitive inhibitor of RCE1, showed high potency in experiments with membrane suspensions in a microtiter plate assay.[140] Another group of protease inhibitors that have shown promising results are the chloromethyl ketones.[141] Two chloromethyl ketones, BFCCMK and UM96001, were shown to inhibit the anchorage-independent growth of K-RAS–transformed rat and human endometrial cancer cells.[142] A third group of inhibitors, peptidyl (acyloxy) methyl ketones (AOMK), have also been described as potential inhibitors of RCE1, although they were not entirely specific; STE24 and ICMT were also inhibited by the AOMKs.[143] This indicates that further studies on these compounds are needed to obtain specific inhibitors of RCE1.[143]

Carboxyl methylation by ICMT is important for cell proliferation and subcellular localization of RAS

Homozygous *Icmt* knockout (*Icmt*$^{-/-}$) mouse embryonic stem cells lacked the ability to methylate recombinant K-RAS.[34] The knockout of *Icmt* resulted in mislocalization of all three isoforms of RAS, away from the plasma membrane into the cytosol and internal membranes (Figure 4).[24,34,137]

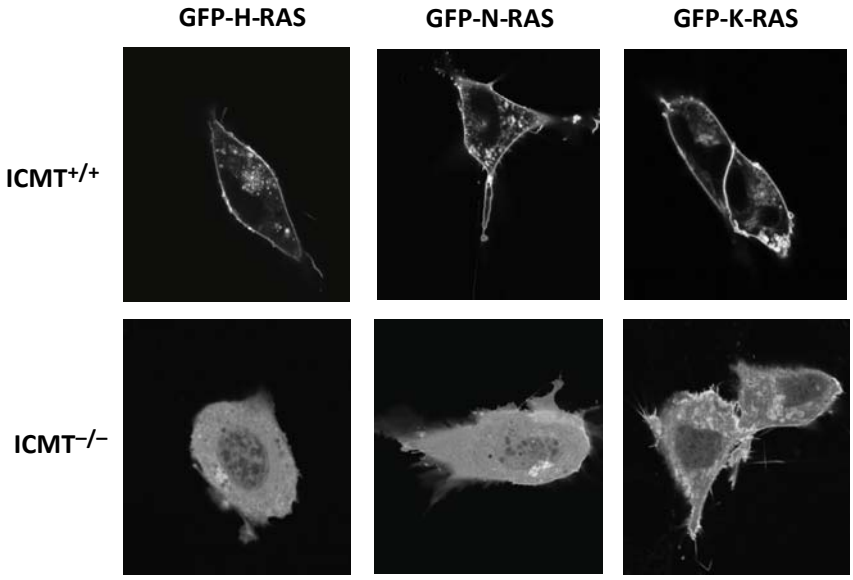


Figure 4. Mislocalization of RAS proteins in* Icmt-*deficient cells. *GFP-tagged RAS proteins are mislocalized away from the plasma membrane in* Icmt-*deficient cells. (Contains confocal micrographs from Dr Mark Philips)*

The impact of *Icmt* deficiency on K-RAS–induced transformation *in vitro*

To further investigate the effects of *Icmt* deficiency on cell growth and oncogenic transformation Bergo and co-workers created a conditional *Icmt* knockout allele ($Icmt^{fl}$) and generated homozygous $Icmt^{fl/fl}$ fibroblast cell lines. $Icmt^{fl/fl}$ fibroblasts expressed *Icmt* and treatment with *Cre*-adenovirus caused excision of exon 1 of the *Icmt* gene yielding $Icmt^{\Delta/\Delta}$ cells. $Icmt^{\Delta/\Delta}$ cells exhibited complete loss of ICMT enzymatic activity and accumulation of ICMT substrate proteins.[53]

The inactivation of *Icmt* reduced cell growth and inhibited transformation induced by retroviral overexpression of oncogenic K-RAS, both in soft agar experiments and in nude mice.[53] Surprisingly, despite mislocalization of the RAS proteins, growth factor–stimulated phosphorylation of ERK1/2 or AKT1 was not affected in the setting of *Icmt* deficiency. This suggested that RAS signaling can proceed from the cytosol or from intracellular membranes, which was supported by studies from the laboratory of Mark Philips.[144] However, the levels of RHOA were significantly reduced as a result of increased protein turnover.[53] The *Icmt*-deficient oncogenic K-RAS–expressing cells (K-RAS-$Icmt^{\Delta/\Delta}$) also exhibited a large RAS/ERK dependent increase in $p21^{CIP1}$, probably caused by the decreased RHOA levels.[53] $p21^{CIP1}$ is an inhibitor of the cell cycle and is upregulated by overexpression of activated RAS.[145] This upregulation of $p21^{CIP1}$ can be antagonized by RHOA.[146] When $p21^{CIP1}$ was deleted in the K-RAS-$Icmt^{\Delta/\Delta}$ cells their capacity to grow in soft agar was no longer inhibited by *Icmt*.[53]

The suggestion that inactivation of *Icmt* inhibited K-RAS transformation through decreased levels of RHOA, indicate that the inhibitory effect of *Icmt* deficiency might not be limited to K-RAS–induced transformation. Indeed, inactivation of *Icmt* also inhibited B-RAF transformation.[53] B-RAF is not a *CAAX* protein and therefore not a substrate for ICMT, and the fact that B-RAF transformation was inhibited in the setting of *Icmt* deficiency suggests that other *CAAX* proteins are important for the transforming ability of B-RAF.

ICMT inhibitors

There are different types of inhibitors suggested to act on ICMT. One type is S-adenosylhomocysteine (AdoHcy or SAH), which is the product of the methylation reaction itself, or compounds that increase intracellular SAH. The methylation reaction converts S-adenosylmethionine (AdoMet or SAM) into SAH, which can bind to ICMT and function as a feed-back competitive inhibitor.[147] SAH is converted to adenosine and homocysteine by SAH hydrolase (Figure 5, page 29).

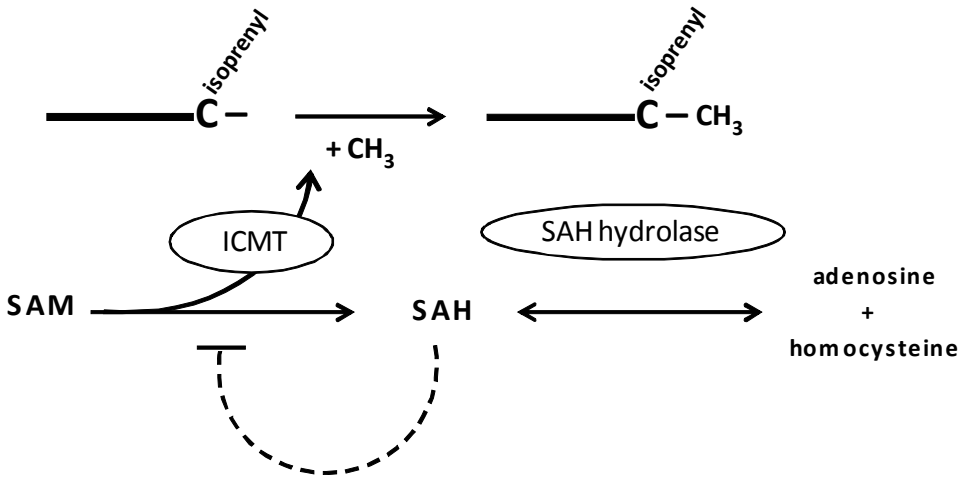


Figure 5. Carboxyl methylation by ICMT. *S-adenosyl methionine (SAM) is the methyldonor in the methylation reaction catalyzed by ICMT. In the methylation reaction SAM releases a methylgroup (CH_3) and is converted into S-adenosyl homocysteine (SAH), and ICMT adds the methylgroup to the isoprenylated CAAX or CXC-RAB protein. SAH can bind to and function as a feed-back competitive inhibitor of ICMT. SAH hydrolase converts SAH into adenosine and homocysteine by a reversible reaction.*

Methotrexate is an antifolate, commonly used in cancer treatment. Methotrexate inhibits the formation of dihydrofolate and tetrahydrofolate from folate resulting in inhibition of DNA synthesis.[148] It has been suggested that one additional mechanism for the antiproliferative effect of methotrexate is inhibition of methylation by ICMT. Methotrexate increases the levels of homocysteine and thereby also the levels of SAH, which results in inhibition of the methylation reaction catalyzed by ICMT.[149] The methylation reaction−with SAM as a methyldonor and SAH as the product−is general for all methyltransferases and not specific for ICMT. It is therefore likely that methotrexate would inhibit many different methylation reactions. Regardless of the mechanism, methotrexate is not a specific inhibitor of ICMT.

A second type of inhibitors is derivates of the substrate prenylcysteine including *N*-acetyl-*S*-farnesyl-L-cysteine (AFC) and *N*-acetyl-*S*-geranylgeranyl-L-cysteine (AGGF). Both AFC and AGGC are substrates for ICMT and thereby act as competitive inhibitors.[15] Several analogs of AFC and AGGC have been developed and evaluated as inhibitors of ICMT but many of them have pleiotropic effects and it is therefore difficult to distinguish their effects on ICMT.[150,151]

Another type of ICMT inhibitor is the indole-based small molecule substrates. The most potent and promising compound is cysmethynil,[152,153] which function as a competitive inhibitor with respect to the isoprenylated cysteine and a noncompetitive inhibitor with respect to SAM. Cysmethynil showed antitumor activity in cancer cells and appeared to be a selective inhibitor of ICMT. Evidence for specificity comes from experiments with *Icmt*-deficient fibroblasts, which were insensitive to the drug.[153,154]

Summary of previous findings on RCE1 and ICMT

The findings that inactivation of *Rce1* or *Icmt* caused mislocalization of all three RAS isoforms, reduced cell growth and inhibited K-RAS–induced transformation *in vitro* support the idea that RCE1 and ICMT might be attractive targets for the treatment of cancer harboring mutations that result in hyperactive RAS signaling. The next step to address this issue would be to evaluate the impact of *Rce1* and *Icmt* deficiency on K-RAS transformation *in vivo*.

AIM AND QUESTIONS

Overall aim

The overall aim of the work for this thesis was to define the impact of inactivating *Rce1* and *Icmt* on the development of K-RAS–induced cancer and thus validate the *CAAX* processing enzymes RCE1 and ICMT as potential therapeutic targets for cancer treatment.

Scientific questions to answer:

- Would inactivation of *Rce1* inhibit K-RAS–induced myeloproliferative disease in mice?

- Would inactivation of *Icmt* inhibit K-RAS–induced myeloproliferative disease and lung cancer in mice?

METHODS

In this thesis both *in vivo* and *in vitro* methods were used. Some of them are described below and more detailed descriptions of all methods are found in the method section of each of the two papers. Animal procedures were approved by the animal research ethics committee in Gothenburg, Sweden.

DNA recombination with Cre-*lox*P techniques

The mouse models we used are based on Cre-*lox*P techniques. The Cre-*lox*P system is commonly used in genetically modified mice and makes it possible to induce recombination of genomic DNA in a tissue- and time-specific manner. *Cre* recombinase is an enzyme from the bacteriophage P1 that recognizes and binds to specific *lox*P (locus of (x) crossing over) sites. The *lox*P site is a 34-base pair (bp) long DNA sequence consisting of two 13-bp inverted repeats flanking an 8-bp spacer region.[155] *Cre* recombinase cleaves the DNA in the spacer regions of two *lox*P sites and the DNA sequence in between, that is "flanked by *lox*P sites" (floxed), is eliminated (Figure 6).

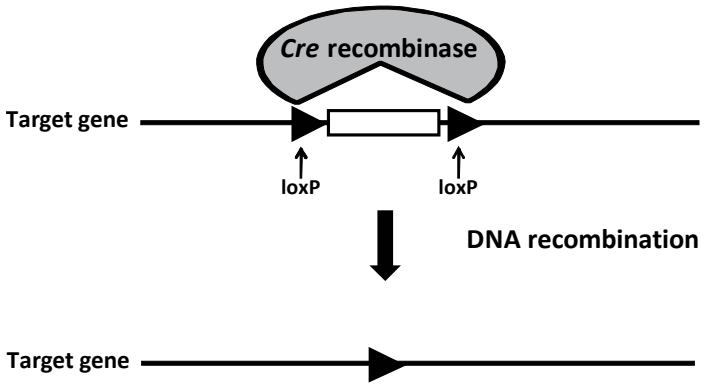


Figure 6. Removal of DNA sequences with Cre-*lox*P techniques. Cre *recombinase recognizes and binds to* lox*P sites flanking a DNA sequence of interest.* Cre*-induced recombination results in removal of the DNA sequence between the* lox*P sites.*

With this technique, genes can be inactivated or activated depending on the construction of the floxed target gene. By expressing *Cre* from a cell type–specific promoter, the recombination event can be directed into a specific tissue or cell type.

A latent *Cre*-inducible oncogenic K-RAS allele (*Kras2*^{LSL})

We used mice heterozygous for a latent oncogenic K-RAS^{G12D} allele (*Kras2*$^{LSL/+}$ mice), generated by the laboratory of Tyler Jacks.[156] The *Kras2*^{LSL} allele is latent but *Cre*-inducible and was engineered to contain two modifications. First, an activating mutation, which results in an amino acid exchange from glycine (G) to aspartic acid (D) in codon 12 (G12D), was inserted into exon 1 of the *Kras2* gene. Second, a STOP cassette flanked by *lox*P sites

(*lox*P-STOP-*lox*P, LSL) was introduced in the promoter region. In the absence of *Cre* recombinase, the STOP cassette prevents transcription of the gene; thus no mutated K-RAS is expressed. Expression of *Cre* recombinase results in removal of the STOP cassette, which turns on expression of the mutated *Kras2*^{G12D} allele (Figure 7).

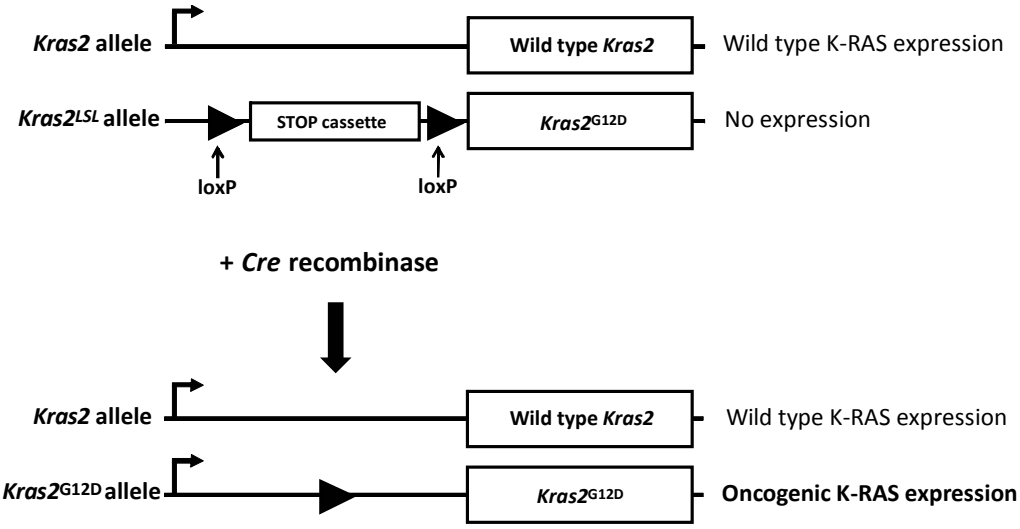


Figure 7. Activation of the latent* Cre-*inducible* Kras2^{LSL} *allele. *The* Kras2^{LSL} *allele has an activating mutation in codon 12 in the* Kras2 *gene, which results in hyperactive K-RAS signaling. A STOP cassette, flanked by* lox*P sites, prevents expression of oncogenic K-RAS. In the presence of* Cre *recombinase, the STOP cassette is removed resulting in expression of mutated oncogenic K-RAS from the endogenous promoter.*

K-RAS^{G12D}, which has no intrinsic GTPase activity and is constitutively active, is a common mutation in human cancer.

The *Kras2* allele has been used to develop a large number of mouse cancer models. Many of these models mirror tumor development in humans and are widely used. One important advantage of this allele is that the expression is driven by the endogenous *Kras2* promoter. Other models, where oncogenic K-RAS is driven by a strong viral promoter usually result in overexpression and might not mirror the natural course of cancer development in humans.

Activating K-RAS^{G12D} in the bone marrow with the Mx1-*Cre* transgene

In the first mouse model (Paper I and II), we used the interferon-inducible Mx1-*Cre* transgene (M). Injection of interferon into the peritoneum (intraperitoneal; i.p.) of the Mx1-*Cre* transgenic mice activates the Mx1 promoter and turns on the expression of *Cre* recombinase, mainly in bone marrow but also in liver, spleen and other tissues.[157] Instead of interferon, polyinosinic-polycytidylic acid (pI-pC), which is double stranded RNA, can be used to activate the Mx1 promoter. Injection of pI-pC mimics a virus infection and activates the immune system and triggers intrinsic interferon release.[157]

The *Kras2*^{LSL}Mx1-*Cre* (*K*^{LSL}M) mouse model was initially published in 2004 by two groups and showed that activation of K-RAS^{G12D} in hematopoietic cells results in a rapidly progressing and fatal myeloproliferative disease (MPD).[158,159] The hallmarks of MPD are: increased WBC counts (leukocytosis), splenomegaly, anemia, and hyperproliferation of one or more lineages of hematopoetic cells that retain the capacity to differentiate.[159] The K-RAS–induced MPD does not progress into an AML, which is a common feature of human MPDs.[160] AML is associated with a rapid expansion of immature myeloid cells such as myeloblasts with impaired ability to differentiate.[159] Other criteria for AML are: > 20% nonlymphoid immature forms/blasts in blood, spleen or bone marrow, rapidly fatal to primary animals, and lethal to sublethally irradiated secondary mice.[161] K-RAS^{G12D} can, however, cooperate with PML-RAR[162] or *Nf1* deficiency (unpublished data) to induce AML. Thus, K-RAS^{G12D} is capable of initiating MPD but can only induce AML in cooperation with other mutations.[159] The *K*^{LSL}M MPD model is well suited as a test model for anti-cancer strategies; it is 100% penetrant, the phenotypes are robust and the disease can be traced by simple blood sampling.

Activating K-RAS^{G12D} in the lung with the LysM-*Cre* allele

In the second mouse model (Paper II), we used an allele with *Cre* recombinase expression driven by the lysosyme M promoter (LysM-*Cre*), which is active in myeloid cells and in type II pneumocytes in the lung.[163] The LysM promoter is not inducible and expression of *Cre* starts during embryogenesis.[164,165]

The *Kras2*^{LSL}LysM-*Cre* (*K*^{LSL}LC) mouse model was published by our group in 2007. The dominant phenotype of the *K*^{LSL}LC mice is an aggressive and lethal lung cancer where all mice die (have to be euthanized) at three weeks of age.[136] Another phenotype of *K*^{LSL}LC mice is a mild myeloproliferation; hematopoietic cells from *K*^{LSL}LC mice exhibit autonomous colony growth *in vitro*.[136] The advantages of using this model for initial validation of potential drug targets are that it is 100% penetrant and rapidly fatal.

Inactivating *Rce1* or *Icmt* with conditional knockout alleles

To inactivate *Rce1* or *Icmt* we used mice with *Cre*-inducible knockout alleles for *Rce1 (Rce1*^{fl}) and *Icmt (Icmt*^{fl}).[48,53] To define the impact of inactivating *Rce1* in a mouse model of K-RAS–induced cancer, we bred *Rce1*$^{fl/fl}$*Kras2*^{LSL} mice with *Rce1*$^{fl/+}$Mx1-*Cre* mice to obtain *Rce1*$^{fl/+}$*K*^{LSL}M and *Rce1*$^{fl/fl}$*K*^{LSL}M mice. When these mice were injected with pI-pC, the expression of K-RAS^{G12D} was switched on in hematopoietic cells in both groups of mice; one *Rce1* allele was inactivated in the case of the *Rce1*$^{fl/+}$*K*^{LSL}M mice, and both *Rce1* alleles were inactivated in the case of the *Rce1*$^{fl/fl}$*K*^{LSL}M mice (Figure 8, page 35). In this way we could define the impact of absent RCE1 activity on the development of K-RAS–induced MPD.

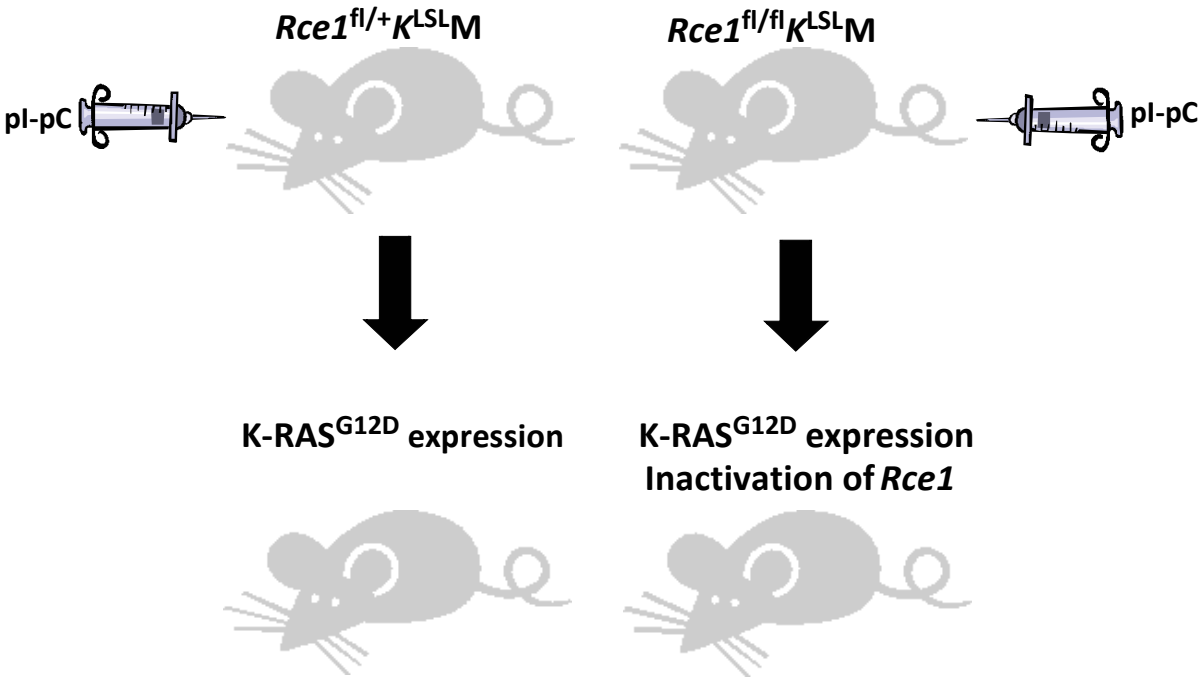


Figure 8. Mouse model for inactivating* Rce1 *in K-RAS induced MPD. *Injection of pI-pC activates the expression of K-RAS^{G12D} in both groups of mice and inactivates one* Rce1 *allele in the* Rce1$^{fl/+}$K^{LSL}*M mice and both* Rce1 *alleles in the* Rce1$^{fl/fl}$K^{LSL}*M mice.*

For Paper II, we used the same breeding strategy to generate *Icmt*$^{fl/+}$*K*^{LSL}M and *Icmt*$^{fl/fl}$*K*^{LSL}M mice, in which pI-pC-injections switched on K-RAS^{G12D} expression in hematopoietic cells and inactivated one and two *Icmt* alleles, respectively. We also bred *Icmt*$^{fl/fl}$*Kras2*^{LSL} mice with *Icmt*$^{fl/+}$LysM-*Cre* mice to generate *Icmt*$^{fl/+}$*K*^{LSL}LC and *Icmt*$^{fl/fl}$*K*^{LSL}LC mice; in these mice, expression of K-RAS^{G12D} and inactivation of the *Icmt*^{fl} allele occured in type II pneumocytes of the lung and myeloid cells. Inactivation of one allele for either *Rce1* or *Icmt*, which results in cells expressing half of the normal levels of RCE1 and ICMT activity, does not produce any apparent phenotypes *in vitro* or *in vivo*.

Identification of specific cell types with fluorescence-activated cell sorting

Fluorescence–activated cell sorting (FACS) can be used to identify specific cell types and to sort heterogenous mixtures of biological cells. Cells can be incubated with antibodies that bind to specific cell surface markers (cluster of differentiation; CD). Specific cell types express specific CD-markers and the expression pattern changes when a hematopoietic cell differentiates. For example expression of CD34, which is a stem cell-specific marker, is lost when the cell differentiates and starts to express more lineage-specific CD-markers. Table 2 (page 36) shows the cell surface markers that were used in our experiments.

Table 2. List of cell surface markers

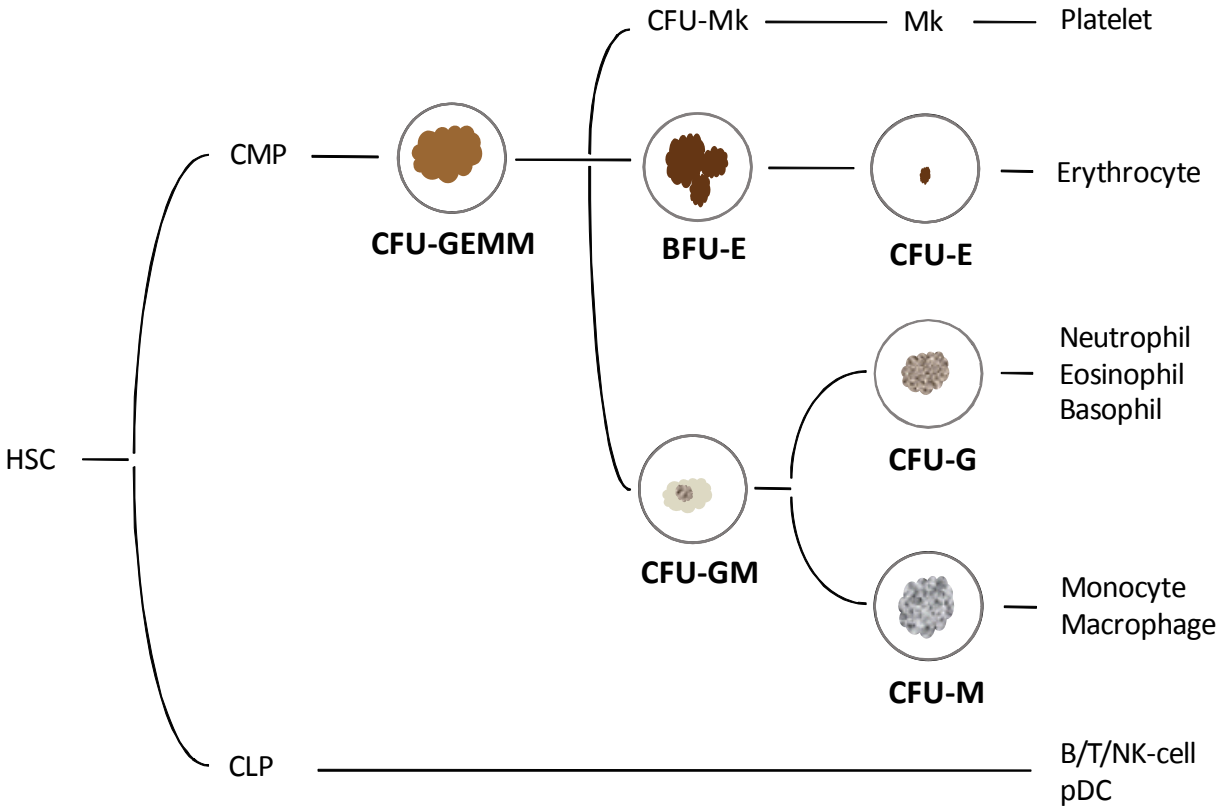
Cell surface markers	Cell types
$CD34^+$	Stem cells
$CD117^+$	Hematopoietic progenitor cells
$CD45^+$	Leukocytes
$CD11b^+$	Myeloid cells
$CD13^+$	Monocytes and macrophages
$CD14^+$	Monocytes
$GR\text{-}1^+$	Granulocytes

Proliferation and differentiation potential of hematopoietic cells can be assayed *in vitro*

Under normal conditions, the production of blood cells (hematopoiesis) takes place in the bone marrow. The hematological system is hierarchically organized and starts with hematopoietic stem cells (HSC), which are primitive and multipotent progenitors with an almost unlimited capacity for self-renewal (Figure 9, page 37).[166] The HSCs give rise to hematopoietic progenitor cells, which are pluripotent but lack the capacity for self-renewal. There are two main types of progenitors: the common myeloid progenitors are committed to the myeloid lineage which produces neutrophils, eosinophils, basophils, monocytes, macrophages, erythrocytes and platelets; and the common lymphoid progenitors are committed to the lymphoid lineage, including B and T lymphocytes and natural killer-cells (NK-cells).[167] Under normal conditions, only differentiated cells are released into the circulation.

Hematological malignancies cause alterations in hematopoiesis, which can result in hyperproliferation of progenitors or a block in the differentiation. Colony assays can be used to evaluate and quantify the ability of hematopoietic cells to proliferate and differentiate, and to distinguish normal from malignant hematopoiesis. A common strategy is to plate single cell suspension of bone marrow cells or splenocytes in methylcellulose medium supplemented with growth factors that trigger differentiation along a specific lineage. A single cell gives rise to a colony forming unit (CFU) consisting of hundreds of mature cells. The colonies are counted under a microscope and classified based on morphology of the cells in the colony. A colony that is composed of two or more cell types (*i.e.* CFU-granulocyte-erythroid-macrophage-megakaryocyte, CFU-GEMM; or CFU-GM) has arisen

from a more primitive progenitor than a colony consisting of cells from only one lineage (*i.e.* CFU-granulocyte, CFU-G; CFU-monocyte, CFU-M; or CFU-erythroid, CFU-E). Normal bone marrow cells do not form colonies in the absence of growth factors but transformed cells can exhibit growth-factor–independent colony growth.



Figur 9. Differentiation of hematopoietic progenitors into mature circulating cells. *Hematopoietic stem cells (HSC) are primitive and multipotent progenitors. The HSCs give rise to two main lineage-specific pluripotent hematopoietic progenitor cells: the common myeloid progenitors (CMP) which are committed to the myeloid lineage and generate platelets, erythrocytes, neutrophils, eosinophils, basophils, monocytes and macrophages; and the common lymphoid progenitors (CLP) which are committed to the lymphoid lineage and generate B-cells, T-cells, NK-cells, and plasmacytoid dentritic cells (pDC). In* in vitro *colony assays, single hematopoietic cells give rise to CFUs which can be counted and typed. CFU-GEMM and CFU-GM are generated from more immature cells than CFU-G and CFU-M, and burst forming unit-erythrocyte (BFU-E) is generated from a more immature cell than CFU-E.*

Experiments with mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) are a very important tool that can provide information about cellular processes that cannot be assessed *in vivo*. Advantages of using MEFs are that there is an almost unlimited supply of cells and these experiments are much less time consuming than *in vivo* studies. One disadvantage is that the cells are isolated away from their natural environment. This means that while the cells adapt to life on plastic plates, important characteristics of the cells may disappear, whereas they may acquire new features

beneficial for their new environment. These limitations should be considered when interpreting results from cell culture experiments.

Potential limitations of the experimental strategies

One limitation with the Mx1-*Cre* mouse model is that *Cre* expression occurs in tissues other than bone marrow. That *Cre* is active in other tissues is evident from the tumors found in gut, skin, thymus, and lung. These other tumors could potentially complicate the interpretation of our experiments. Ideally, oncogenic K-RAS should only be activated in bone marrow cells. One way to get around this problem would be to isolate fetal liver cells from K^{LSL}M mice and transplant them into irradiated recipient mice and then inject those mice with pI-pC. In this way oncogenic K-RAS would only be activated in the transplanted bone marrow of the recipients.

In the case of the K^{LSL}LC model, the initial purpose was to obtain a more specific model for myeloid malignancies, since lysozyme M should only be expressed in myeloid cells. The LysM-*Cre* transgenic mice have been widely used for myeloid cell−specific *Cre* expression. However, we found that the dominant malignancy in K^{LSL}LC mice was lung cancer. In retrospect, this was not surprising; lysozyme M was shown to be expressed in type II pneumocytes decades ago.[163]

It is of great interest to evaluate the impact of *Icmt* in a model with solid tumors, but one disadvantage with this lung cancer model is that it is not perfectly representative of human lung cancer. In human lung cancer only one or a few cells initiate tumor development; in the K^{LSL}LC model, many or all lung cells initiate tumors at the same time. This is a problem in several cancer mouse models and it can be an issue when interpreting the results and predicting the impact on mechanisms and treatment of human cancer.

Another issue with our models is the possibility of partial recombination of the $Rce1^{fl}$ allele (in the $Rce1^{fl/fl}K^{LSL}$M mice), or the $Icmt^{fl}$ allele (in the $Icmt^{fl/fl}K^{LSL}$M or $Icmt^{fl/fl}K^{LSL}$LC mice). For example, partial recombination would produce $Rce1^{fl/\Delta}K^{G12D}$M cells; *i.e.*, cells that express K-RAS^{G12D} and still express RCE1 because of an unrecombined "fl" allele. We developed quantitative PCR (Q-PCR) assays to monitor for partial recombination. The Q-PCR assays quantified recombination of the $Kras2^{LSL}$ and $Rce1^{fl}$ alleles and were performed on genomic DNA isolated from spleen and bone marrow cells.

Regardless of these limitations, the chosen strategies were likely to provide valuable information about the potential of RCE1 and ICMT as therapeutic targets for the treatment of K-RAS−induced malignancies.

SUMMARY OF RESULTS AND DISCUSSION

Paper I

The aim of this study was to determine if inactivation of *Rce1* would inhibit the development of K-RAS–induced MPD. To address this issue we injected pI-pC into *Rce1*$^{fl/+}$*K*^{LSL}M control mice (in which oncogenic K-RAS^{G12D} was activated) and *Rce1*$^{fl/fl}$*K*^{LSL}M mice (in which oncogenic K-RAS^{G12D} was activated and *Rce1* was inactivated) and then assessed MPD phenotypes at various stages.

Inactivation of *Rce1* accelerated the development of K-RAS–induced MPD

As expected from previous studies, the control *Rce1*$^{fl/+}$*K*^{LSL}M mice developed progressive leucocytosis with an increased proportion of myeloid cells. By contrast, the inactivation of *Rce1*, in the *Rce1*$^{fl/fl}$*K*^{LSL}M mice, caused a remarkable increase in WBC counts and reduced survival compared with the control *Rce1*$^{fl/+}$*K*^{LSL}M mice. The number of immature myeloid cells in peripheral blood was also increased in the setting of *Rce1* deficiency.

Histological examinations of tissues from *Rce1*$^{fl/+}$*K*^{LSL}M mice showed mild to moderate infiltration of leukocytes in liver and spleen, partial obliteration of the splenic architecture with extramedullary hematopoiesis, and adenoma formation in the lung. These results were consistent with previous reports using *K*^{LSL}M mice.[158,159] In the *Rce1*$^{fl/fl}$*K*^{LSL}M mice, the leukocyte infiltration into the liver was more severe and resulted in congestion of central veins, areas of necrosis and swelling of hepatocytes, and the cytoarchitecture of the spleen was completely disrupted. These mice also developed large adenomas and diffuse hyperplasia in the lung.

Western blot analysis showed that the levels of phosphorylated ERK were increased in the spleens and livers from the *Rce1*$^{fl/+}$*K*^{LSL}M and *Rce1*$^{fl/fl}$*K*^{LSL}M mice compared with control mice without MPD. The levels of phospho-AKT and p21^{CIP1} were similar in the three groups of mice. We did not detect any differences in activation of downstream RAS effectors between *Rce1*$^{fl/+}$*K*^{LSL}M and *Rce1*$^{fl/fl}$*K*^{LSL}M spleens that could explain the difference in disease severity.

To further investigate the histological findings in spleens, we assessed the ability of splenocytes to form colonies in methylcellulose. Splenocytes from *Rce1*$^{fl/+}$*K*^{LSL}M mice were able to form colonies in the absence of growth factors and this ability was increased in the *Rce1*$^{fl/fl}$*K*^{LSL}M splenocytes. The colonies from both *Rce1*$^{fl/+}$*K*^{LSL}M and *Rce1*$^{fl/fl}$*K*^{LSL}M spleens resembled macrophages and were composed of CD45^{+}/CD11b^{+}/CD13^{+}/CD14^{-} cells.

Previous studies have shown that K-RAS–induced transformation in fibroblasts is reduced in the setting of *Rce1* deficiency. Consequently, we hypothesized that inactivation of *Rce1* would inhibit the K-RAS–induced MPD in our mouse model. That inactivation of *Rce1*

accelerated the development of K-RAS–induced MPD was highly unexpected. It has previously been shown that irradiated mice transplanted with *Rce1*$^{-/-}$ fetal liver cells exhibited a moderate increase in the number of WBCs.[138] This result, together with our findings, suggests that *Rce1* may act as a tumor supressor gene in hematopoietic cells and that *Rce1* deficiency accelerates the proliferation of both normal and K-RAS^{G12D} expressing hematopoietic cells.

One potential explanation for the different results in fibroblasts compared with hematopoietic cells could be that in fibroblasts, oncogenic K-RAS was overexpressed,[48] and in hematopoietic cells, K-RAS expression was driven by the endogenous promoter. To address this issue, we performed proliferation assays with *Rce1*$^{fl/fl}$*K*^{LSL} fibroblasts. These assays showed that inactivation of *Rce1* in fibroblasts expressing K-RAS from the endogenous promoter, reduced K-RAS^{G12D}–induced proliferation, similar to previous studies where oncogenic K-RAS was overexpressed.[48] These results demonstrate that *Rce1* deficiency has a distinct effect in fibroblasts and hematopoietic cells.

The results from Paper I showed that inactivation of *Rce1* accelerates the development and progression of K-RAS–induced MPD. It could be argued that the disease should be classified as an AML, based on the criteria in the "Bethesda proposals for classification of nonlympoid hematopoietic neoplasms in mice" by Kogan and co-workers.[161] When we classified the disease we used the criteria for human AML, which are more restrictive with the requirement of at least 20% blast cells in peripheral blood. We do not yet know, however, if the disease is transplantable into irradiated mice.

Paper II

The aim of this study was to investigate the impact of *Icmt* deficiency on the development and progression of K-RAS−induced MPD and lung cancer.

To address this issue we initially used the same approach as in Paper I: we compared disease phenotypes of pI-pC−injected *Icmt*$^{fl/+}$*K*^{LSL}M and *Icmt*$^{fl/fl}$*K*^{LSL}M mice.

Inactivation of *Icmt* reduced the phenotypes of K-RAS−induced MPD

From 0 to 7 weeks after pI-pC injections, *Icmt*$^{fl/+}$*K*^{LSL}M and *Icmt*$^{fl/fl}$*K*^{LSL}M mice developed signs of MPD with increased WBC counts but the proportion of immature myeloid cells in peripheral blood was only slightly increased. After 7 weeks there was a rapid increase in WBCs in the *Icmt*$^{fl/+}$*K*^{LSL}M compared with the *Icmt*$^{fl/fl}$*K*^{LSL}M mice. At 13−14 weeks the percentage of immature myeloid cells was increased to 25% in the *Icmt*$^{fl/+}$*K*^{LSL}M mice and histological analysis demonstrated myeloid infiltration in the liver and disruption of the splenic architecture. These phenotypes were significantly reduced in the *Icmt*$^{fl/fl}$*K*^{LSL}M mice.

Even though the progression and severity of the MPD phenotypes were significantly reduced, the inactivation of *Icmt* did not improve survival. One possible explanation for the

lack of effect on survival could be tumors in other tissues caused by partial recombination; *i.e.*, activation of the *Kras2*^{G12D} allele and incomplete inactivation of *Icmt*. Both *Icmt*$^{fl/+}$*K*^{LSL}M and *Icmt*$^{fl/fl}$*K*^{LSL}M mice developed lesions in other tissues such as malignant thymic lymphoma, adenocarcinoma of the lung and large papillomas in the gastrointestinal tract. Genotyping of tissues from *Icmt*$^{fl/fl}$*K*^{LSL}M mice showed activation of the *Kras2*^{G12D} allele and almost complete inactivation of *Icmt* in spleen and bone marrow, while all other tissues showed incomplete inactivation of *Icmt*. These data suggest that tumors in other tissues, where *Icmt* failed to be inactivated, contributed to the early death of *Icmt*$^{fl/fl}$*K*^{LSL}M mice.

***Icmt* deficiency reduced growth factor–independent colony growth of hematopoietic cells**

The absence of *Icmt* reduced splenomegaly and growth factor–independent colony growth of splenocytes from mice with K-RAS–induced MPD. FACS analysis of splenocytes from *Icmt*$^{fl/+}$*K*^{LSL}M mice showed increased proportions of CD11b and GR-1 double−positive cells (*i.e.*, immature myeloid cells) compared with both *Icmt*$^{fl/fl}$*K*^{LSL}M and control mice. The ability of *Icmt*$^{fl/fl}$*K*^{LSL}M splenocytes to form colonies in the presence of growth factors was reduced compared with splenocytes from *Icmt*$^{fl/+}$*K*^{LSL}M mice, but it was still higher than with control splenocytes. The splenocyte colonies were mainly CFU-GMs, which are generated by precursors of the granulocytic and monocytic lineages. *Icmt*$^{fl/+}$*K*^{LSL}M splenocytes formed colonies at low concentrations of GM-CSF and were sensitive to increased concentrations of GM-CSF. The absence of *Icmt* inhibited the colony growth at low concentrations and reduced the sensitivity to GM-CSF.

Similar to splenocytes, bone marrow cells from *Icmt*$^{fl/fl}$*K*^{LSL}M mice exhibited reduced colony formation in the absence of growth factors compared with *Icmt*$^{fl/+}$*K*^{LSL}M mice. Interestingly, the colony formation of bone marrow cells in the presence of growth factors was actually increased in the setting of *Icmt* deficiency, both in the presence and absence of oncogenic K-RAS. Furthermore, at low levels of GM-CSF, bone marrow cells from *Icmt*$^{fl/fl}$*K*^{LSL}M mice formed fever colonies than *Icmt*$^{fl/+}$*K*^{LSL}M mice, but at saturated levels, the *Icmt*-deficient bone marrow cells, both with and without K-RAS^{G12D}, formed more colonies than cells from *Icmt*$^{fl/+}$*K*^{LSL}M and control mice.

The reason for the increased colony production of *Icmt*-deficient bone marrow cells is not clear and it can be argued that this could cause unwanted side effects when ICMT inhibitor drugs are used. On the other hand, mice lacking *Icmt* in hematopoietic cells (with wild-type K-RAS) are healthy and exhibit normal WBC counts over several months of observation. One possible explanation for this could be that *Icmt* deficiency only increases colony formation at saturated levels of growth factors, and that proliferation and differentiation of hematopoietic cells *in vivo* are unaffected.

Previous studies have shown that bone marrow cells expressing K-RAS^{G12D} are hypersensitive to EPO and produce BFU-Es in the absence of EPO.[159] EPO-independent formation of BFU-E is a sign of ineffective erythropoiesis and even though inactivation of

Icmt normalized this phenotype, both *Icmt*$^{fl/+}$*K*^{LSL}M and *Icmt*$^{fl/fl}$*K*^{LSL}M mice developed anemia. Bone marrow cells from *Icmt*$^{fl/+}$*K*^{LSL}M mice showed hypersensitivity to EPO and formed BFU-E colonies in the absence and presence of EPO. However, the EPO sensitivity of bone marrow cells from *Icmt*$^{fl/fl}$*K*^{LSL}M mice was normalized and similar to control.

Western blot analysis of splenocytes showed that a substantial proportion of the RAS proteins were in the soluble fraction in the *Icmt*-deficient cells, indicating that they were mislocalized away from the plasma membrane in the setting of *Icmt* deficiency. This finding is in line with a previous study.[34]

Western blot analysis of downstream effectors of RAS showed reduced levels of GM-CSF–stimulated phosphorylation of MEK and ERK in CD11b-positive splenocytes from *Icmt*$^{fl/+}$*K*^{LSL}M mice compared with control. The levels were further reduced in splenocytes from *Icmt*$^{fl/fl}$*K*^{LSL}M mice. The reduction of phopshorylated-ERK in response to endogenous oncogenic K-RAS is consistent with previous studies.[168,169] We do not have an explanation for the further reduction of phosphorylated-ERK in the *Icmt*-deficient splenocytes, but we speculate that it might be related to the reduced spleen weights *in vivo* and reduced colony growth *in vitro*. However, this speculation would be limited to splenocytes since *Icmt* deficiency did not reduce the levels of phosphorylated-ERK in bone marrow cells in this study or in fibroblasts in a previous study.[53]

Inactiviation of *Icmt* reduced K-RAS–induced lung tumor development and myeloproliferation

To further assess the impact of *Icmt* deficiency on the development of K-RAS–induced malignancies, we used a second mouse model and evaluated phenotypes of *Icmt*$^{fl/+}$*K*^{LSL}LC and *Icmt*$^{fl/fl}$*K*^{LSL}LC mice. The *Icmt*$^{fl/+}$*K*^{LSL}LC mice died or became moribund at three weeks of age and showed a massive increase in lung weight caused by extensive lung tumor formation. *Icmt* deficiency improved survival and reduced lung weight. Inactivation of *Icmt* also reduced the autonomous colony growth of K-RAS^{G12D}–expressing splenocytes and bone marrow cells.

The results from Paper II, show that inactivation of *Icmt* reduces the severity and progression of two K-RAS–induced malignancies *in vivo*.

Why did *Rce1* deficiency accelerate and *Icmt* deficiency inhibit K-RAS–induced MPD?

One possible explanation for the opposite results with *Rce1* and *Icmt* could be that in addition to *CAAX* proteins, a subset of the RAB proteins (*i.e.*, the ones terminating with –*CXC*) are processed by ICMT but not by RCE1. It is possible that carboxyl methylation of *CXC*-RAB proteins is important for RAS-induced oncogenesis *in vivo* and that inactivation of

Icmt interferes with this function. This could only explain the inhibitory effect of *Icmt* deficiency and not the accelerating effect caused by inactivation of *Rce1*.

Another potential explanation could be that there are other *CAAX* proteins involved in RAS-induced oncogenesis and that one or several of them behave differently in the setting of *Rce1* deficiency than in the setting of *Icmt* deficiency. This was supported by a recent study showing that the localization and function of RHO family proteins were differently affected in the setting of *Rce1* deficiency compared with *Icmt* deficiency. In this study, some proteins were mislocalized in $Rce1^{-/-}$ cells but not in $Icmt^{-/-}$ cells, and other proteins were mislocalized in $Icmt^{-/-}$ cells but not in $Rce1^{-/-}$ cells, and yet other proteins were equally affected in both cell types.[24] These findings indicate that it is difficult to predict the effects of inactivation of *Rce1* or *Icmt* on specific *CAAX* proteins, and that the effects are likely not general for all *CAAX* proteins.

The simplest potential explanation for the opposite results with *Rce1* and *Icmt* would be that there is a *CAAX* protein with tumor suppressor function that is affected by inactivation of *Rce1* but not affected by inactivation of *Icmt*; such a *CAAX* protein would thus exhibit loss-of-function when it retain the *–AAX*. One candidate *CAAX* protein that could be involved in the acceleration of K-RAS–induced transformation in the setting of *Rce1* deficiency is RHOB, which was mislocalized in $Rce1^{-/-}$ cells but not in $Icmt^{-/-}$ cells.[24] RHOB has been shown to function as a tumor suppressor by antagonizing RAS/PI3K/AKT signaling, and blockade of PI3K/AKT resulted in upregulation of RHOB.[103]

RHOH is another possible tumor suppressor *CAAX* protein that has been shown to antagonize proliferation and survival. Overexpression of RHOH resulted in impaired activation of RAC GTPases, and knockdown of RHOH expression stimulated proliferation, survival and migration of murine hematopoietic progenitor cells.[170] The fact that RHOH is mainly expressed in hematopoietic cells[170] makes it even more interesting, and could help explain why we did not see accelerated K-RAS transformation in the *Rce1*-deficient fibroblasts.

There are many *CAAX* proteins that promote RAS transformation and could be responsible for the inhibitory effect of inactivation of *Icmt*. The fact that *Icmt* deficiency also inhibited B-RAF–induced transformation of fibroblasts, even though B-RAF is not a substrate for ICMT, supports the idea that other *CAAX* proteins are important for RAS-induced oncogenesis.[53] Furthermore, FTIs have shown effect on tumors regardless of whether they possess RAS mutations, which is also in line with this reasoning.[125,171] The findings that some FTIs inhibit tumorigenesis, indicate that the *CAAX* proteins that contribute to RAS-transformation are substrates for FTase. However, this reasoning did not get support from a study using a conditional knockout allele for *Fntb* (the gene encoding the β-subunit of FTase). In this study, genetic disruption of *Fntb* did not affect tumorigenesis.[172] Explanations for these contradicting results could be that the FTIs are not specific and have additional targets, or, as

suggested by Yang and co-workers, that the recombination of the *Fntb* allele failed to eliminate *Fntb* expression and only reduced FTase activity.[173]

If it is true that inhibition of FTase (or inactivation of *Fntb*) does not affect tumorigenesis, the essential *CAAX* proteins involved in RAS transformation should be substrates for GGTase-I. This reasoning draws support from a study showing that inactivation of *Pggt1b* (the gene encoding the β-subunit of GGTase-I) inhibited the growth of K-RAS–transformed fibroblasts.[136] Furthermore, when the K-RAS–transformed fibroblasts were transfected with plasmids encoding farnesylated mutants of RHOA and CDC42, the cells were partially rescued from the growth arrest caused by inactivation of *Pggt1b*.[136]

The impact of GGTase-I deficiency was also evaluated *in vivo* with the lung cancer model that we used in Paper II. The phenotypes and severity of disease were reduced to a greater extent when *Pggt1b* was inactivated compared with when *Icmt* was inactivated, which further suggests that geranylgeranylated proteins are important for K-RAS–induced oncogenesis.[136]

CONCLUSIONS AND FUTURE PERSPECTIVES

The overall aim of this thesis was to evaluate RCE1 and ICMT as potential therapeutic targets for cancer treatment with *in vivo* studies in mice.

- The answer to the scientific question if inactivation of *Rce1* would inhibit K-RAS–induced myeloproliferative disease in mice is – No.

  Inactivation of *Rce1* accelerated the progression of K-RAS–induced myeloproliferative disease in mice. On the contrary, inactivation of *Rce1* inhibits K-RAS transformation in fibroblasts, suggesting that the effects of *Rce1* deficiency are tissue specific. It would therefore be interesting to evaluate the impact of *Rce1* deficiency in other cancer models, with solid tumors, before discarding RCE1 as a possible therapeutic target for cancer treatment. It would also be interesting to determine if *Rce1* deficiency would cooperate with other mutations in hematological malignancies.

- The answer to the scientific question if inactivation of *Icmt* would inhibit K-RAS–induced myeloproliferative disease and lung cancer in mice is – Yes.

  Inactivation of *Icmt* inhibited the progression of K-RAS–induced myeloproliferative disease and lung cancer in mice. These results support the concept that targeting ICMT might be an effective strategy for treatment of RAS-induced malignancies. The next step would be to test ICMT inhibitors (*e.g.*, cysmethynil) *in vivo* and compare the impact of pharmacologic and genetic loss of ICMT activity. It would also be interesting to evaluate the impact of *Icmt* deficiency in cancer models initiated by mutations other than K-RAS, and to define the therapeutic efficacy of simultaneously inhibiting ICMT and FTase or ICMT and GGTase-I.

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