The expression and molecular functions of LRIG proteins in cancer and psoriasis

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The simple things are the most extraordinary things, and only the wise can see them
- Paulo Coelho

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Abstract

The leucine-rich repeats and immunoglobulin-like domains (LRIG) family consists of three integral membrane proteins that are important in human cancer. LRIG1 is a negative regulator of growth factor signaling. Its expression is associated with longer survival in several cancer types, and the gene has been shown to function as a tumor suppressor. The roles of LRIG2 and LRIG3 are less well known. The aim of this thesis was to improve our understanding of the expression and function of the LRIG protein family in psoriasis and cancer.

To investigate their expression in psoriasis, the mRNA levels and subcellular localization of the LRIG proteins were analyzed and compared between normal and psoriatic human skin. There were no differences in the LRIG mRNA levels between psoriatic and normal skin samples. However, the subcellular localization of all three LRIG proteins differed between psoriatic and normal skin.

To study the physiological and molecular functions of Lrig2, we generated *Lrig2E12*$^{-/-}$ mice. These mice were viable and born at a Mendelian rate, but *Lrig2E12*$^{-/-}$ mice had an increased rate of spontaneous mortality and a transient reduction in growth rate compared to *Lrig2* wild-type (wt) mice. In an orthotopic platelet-derived growth factor (PDGF)B-driven brain tumor mouse model, we studied the effect of *Lrig2* on gliomagenesis. All *Lrig2* wt mice developed tumors; 82% developed grade II/III tumors, and 18% developed grade IV tumors. Only 77% of the *Lrig2E12*$^{-/-}$ mice developed tumors, and they were all grade II/III tumors. Thus, Lrig2 increased the incidence and malignancy rates of PDGFB-driven gliomas. We then analyzed the effect of Lrig2 on Pdgf receptor (Pdgfr) signaling. Lrig2 had no effect on Pdgfr steady-state levels, the starvation-induced up-regulation of Pdgfrs, the phosphorylation of Pdgfrs, primary cilium formation or the PDGFBB-induced phosphorylation of Akt or Erk1/2. However, the kinetics of induction of the immediate-early genes *Fos* and *Egr2* were altered, resulting in a more rapid induction in *Lrig2E12*$^{-/-}$ cells.

We then analyzed the clinical and biological importance of LRIG1 in lung cancer. In a human lung cancer tissue micro-array (TMA), LRIG1 expression was found to be an independent positive prognostic factor for adenocarcinoma. To study the importance of Lrig1 regarding lung cancer development *in vivo*, we used an inducible EGFR^{L858R}-driven mouse lung

cancer model. The mice developed diffuse lung adenocarcinoma, and the tumor burden was greater in *Lrig1*$^{-/-}$ mice than in *Lrig1*$^{+/+}$ mice ($p = 0.025$) at 60 days. The human lung cancer cell line H1975, with either normal or Tet-induced expression of *LRIG1*, was injected into the flanks of Balb/cA nude mice. Tumors formed by LRIG1-overexpressing cells were smaller than those formed by parental cells, further indicating that LRIG1 is important during lung tumor formation or growth. *In vitro*, LRIG1 suppressed the proliferation of H1975 cells and down-regulated the phosphorylation of MET and RET.

To investigate the molecular functions of LRIG proteins further, we performed a yeast two-hybrid (YTH) screen using a peptide from the cytosolic tail of LRIG3 as bait. This screen identified LMO7 and LIMCH1 as prominent interaction partners for LRIG3. Proximity ligation assays showed that LMO7 interacted with all of the LRIG proteins at endogenous expression levels. *LMO7* and *LIMCH1* were expressed in all human tissues analyzed. Their expression was dramatically decreased in lung cancer compared to normal lung tissue. The expression of LMO7 was analyzed in a human lung cancer TMA. LMO7 was expressed in respiratory epithelial cells in normal lungs. However, LMO7 was only expressed in a quarter of the lung tumors. LMO7 expression was found to be an independent negative prognostic factor for lung cancer.

In summary, we found that the LRIG proteins were redistributed in psoriatic skin. In a mouse glioma model, *Lrig2* promoted oligodendroglioma genesis. LRIG1 was an independent positive prognostic factor in human lung cancer. *Lrig1* ablation increased the tumor size in an EGFR^{L858R}-driven lung cancer mouse model. LRIG1 expression decreased the tumor growth of human lung cancer cells in a xenograft mouse model. LMO7 interacted with all three LRIG proteins and was an independent negative prognostic factor in human lung cancer. These data demonstrate the importance of LRIG proteins in human disease.

List of papers

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

Paper I:

Karlsson T, Mark EB, Henriksson R, Hedman H. Redistribution of LRIG proteins in psoriasis. J Invest Dermatol. 2008 May;128(5):1192-5.

Paper II:

Rondahl V, Holmlund C, **Karlsson T**, Faraz M, Henriksson R, Hedman H. *Lrig2*-Deficient Mice are Protected Against *PDGFB*-Induced Glioma. Manuscript.

Paper III:

Kvarnbrink S**, Karlsson T**, Forssell J, Edlund K, Holmlund C, Faraz M, Botling J, Feng M, Lindquist D, Micke P, Henriksson R, Johansson M and Hedman H. LRIG1 is a prognostic biomarker and tumor suppressor in non-small cell lung cancer. Manuscript.

Paper IV:

Karlsson T, Kvarnbrink S, Botling J, Micke P, Johansson M, Henriksson R, Hedman H. LMO7 interacts with LRIG proteins, and is a negative prognostic marker in lung cancer. Manuscript.

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Abbreviations

ALK	Anaplastic lymphoma kinase
AR	Androgen receptor
BMP	Bone morphogenetic protein
CH	Calponin homology
CML	Chronic myeloid leukemia
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EML4	Echinoderm mictotubule-associated protein-like 4
ER	Estrogen receptor
FGFR	Fibroblast growth factor
GIST	Gastrointestinal stromal tumor
GWAS	Genome wide association study
HF	Hair follicle
IFE	Inter follicular epidermis
ISC	Intestinal stem cell
KO	Knock out
LMO7	LIM domain only 7
LRIG	Leucine-rich repeats and immunoglobulin-like domains
LRR	Leucine-rich repeats
MRI	Magnetic resonance imaging
NSCLC	Non-small cell lung cancer
OPC	Oligodendrocyte progenitor cell
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PLCγ	Phospholipase C-γ
RCAS	Replication competent ALV splice acceptor
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SG	Sebaceous gland
SRF	Serum response factor
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TMA	Tissue micro-array
UFS	Urofacial syndrome
VEGF	Vascular endothelial growth factor
WHO	World health organization
WT	Wild type

Sammanfattning på svenska

LRIG är en proteinfamilj som består av tre proteiner: LRIG1, LRIG2 och LRIG3. Dessa proteiner finns i de flesta av kroppens vävnader och de har alla visat sig vara involverade i cancer. Av dessa tre proteiner har LRIG1 studerats mest, och man har sett att LRIG1 kan hämma cancertillväxt genom att stänga av och nedreglera vissa proteiner som kan orsaka cancer.

I denna avhandling har målet varit att öka förståelsen av hur LRIG-proteiner fungerar och i vilka sjukdomar de är viktiga. Betydelsen av LRIG-proteinerna har studerats i psoriasis och cancer, sjukdomar där tillväxtfaktorsignalering är viktig.

I hud från friska personer och psoriasispatienter kunde vi se att det inte var någon skillnad i nivå av LRIG1, LRIG2 eller LRIG3. Däremot såg vi att alla tre proteiner ändrade plats inuti hudceller från psoriasispatienter jämfört med celler från frisk hud. Detta tyder på att LRIG-proteiner kan vara involverade i psoriasis.

Vi skapade möss där vi tagit bort genen för Lrig2, så kallade Lrig2 knock-out (ko) möss. Dessa möss föddes levande, såg normala ut, och det föddes lika många honor som hanar. Däremot dog fler Lrig2 ko möss än vad som är normalt, och de hade en övergående viktreduktion jämfört med normala möss. Tidigare studier har visat att LRIG2 är en negativ prognostisk markör för oligodendrogliom, en typ av elakartad hjärntumör. Vid studier av gliom i möss kunde vi se att möss som saknade Lrig2 bildade färre och mindre elakartade hjärntumörer. Detta tyder på att Lrig2 främjar både bildandet och aggressiviteten av oligodendrogliom. I ett försök att ta reda på varför så stimulerades celler med tillväxtfaktorn PDGF-BB. Vi kunde då se att Lrig2 ko celler hade en snabbare induktion av genuttrycket av de ”omedelbara-tidiga” generna *Fos* och *Egr2*, men det fanns ingen effekt på mängden Pdgf-receptorer, aktiviteten av dessa eller de nedströms aktiverade proteinerna Akt eller Erk1/2.

Vi studerade uttrycket av LRIG-proteiner i humana lungcancertumörer och fann då att LRIG1 var en oberoende positiv prognostisk faktor vid lungcancer, d.v.s. patienter som uttryckte höga nivåer av LRIG1 levde längre tid efter sin lungcancerdiagnos än de som inte hade något uttryck av LRIG1. För att studera betydelsen av Lrig1 i lungcancerutveckling så använde vi en musmodell där lungcancer kan induceras i möss som antingen var normala för Lrig1 eller möss där vi hade tagit bort genen för Lrig1. Vi såg att möss

som hade Lrig1 utvecklade mindre tumörer 60 dagar efter tumörinduktion än de som saknade Lrig1, men 90 dagar efter tumörinduktion så var tumörstorleken lika stor i normala möss som i Lrig1 ko möss. Detta indikerar att Lrig1 hämmar tumörtillväxt i lungcancer, åtminstone i ett tidigt skede av sjukdomen. Humana lungcancerceller med normal eller ökad nivå av LRIG1 implanterades under huden på möss. Tumörer som bildades av celler med ökad LRIG1-nivå var mindre än tumörer som bildades av celler med normal nivå av LRIG1. I cellförsök såg vi även att LRIG1 kunde hämma lungcancercellers tillväxt och att LRIG1 kunde nedreglera aktivering av tillväxtfaktorreceptorerna MET och RET. Dessa försök visar att LRIG1 har en betydelse vid lungcancer.

För att söka efter proteiner som samverkar med LRIG-proteinerna gjordes en ”jäst-två-hybrid” analys. I denna fann vi att LRIG3 interagerade med två proteiner: LMO7 och LIMCH1. LMO7 kolokaliserade med alla tre LRIG proteinerna. LMO7 och LIMCH1 uttrycktes i alla vävnader vi undersökte men uttrycket var dramatiskt sänkt i lungcancertumörer jämfört med normal lunga. I en analys av LMO7 i humana lungtumörer såg vi att LMO7 var en negativ prognostisk faktor i lungcancer, d.v.s. att patienter som uttryckte LMO7 levde kortare tid än patienter som saknade uttryck av LMO7.

Sammanfattningsvis har vi sett att LRIG-proteinerna ändrar subcellulär lokalisation i hud från psoriasispatienter jämfört med hud från friska individer. Lrig2 ko möss föddes levande men hade en ökad dödlighet och i en musmodell ökade Lrig2 incidensen och maligniteten av gliom. LRIG1 var en oberoende positiv faktor i lungcancer och i musmodeller hämmade Lrig1 tumörtillväxt i lunga. LMO7 band till alla tre LRIG-proteinerna och var en negativ prognostisk faktor vid lungcancer. LRIG-proteinerna tycks alltså ha en viktig roll vid tillväxtfaktorberoende sjukdomar som psoriasis och cancer.

Introduction

Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are single-pass transmembrane receptors that are involved in cellular signaling (*1*). RTKs control multiple cellular functions, both during development and in adulthood, such as proliferation, migration, metabolism, differentiation and survival. There are 58 human RTK proteins, which have been categorized into 20 different subfamilies based on their protein structures (*1*). All RTKs, with the exception of the insulin receptor family members, exist as inactive monomers in the cell membrane. Ligand binding to the extracellular portion of the RTK induces conformational changes which stabilize the dimerization of the receptors. The tyrosine kinase domain, which is located in the intracellular portion of the receptor, is then autotransphosphorylated, which generates docking sites for cytoplasmic signaling molecules. This initiates signaling cascades by activating downstream signaling molecules, resulting in the transcription of genes that are regulated by RTKs (Figure 1) (*1*). In normal cells, RTK signaling is controlled by several molecular mechanisms. However, aberrant RTK signaling is frequently found in cancer, where RTKs and their regulatory proteins often act as oncoproteins (*1*).

The epidermal growth factor receptor family

The epidermal growth factor (EGF) receptor (EGFR) family is one of the subfamilies that the RTK family is comprised of. EGFR family members are important regulators of cell proliferation, migration, adhesion, differentiation, angiogenesis and apoptosis. The EGFR family consists of four members: EGFR (ERBB1 or HER2), ERBB2 (HER2 or NEU), ERBB3 (HER3) and ERBB4 (HER4). The receptors consist of an extra-cellular ligand binding domain, a transmembrane domain and a cytoplasmic tail that contains the tyrosine kinase domain. Eleven ligands, including EGF and amphiregulin, have been described for the EGFR family members. These ligands bind to EGFR, ERBB3 and/or ERBB4. However, no ligand has been described to bind to ERBB2. Upon ligand binding, the receptors go through a conformational change from a closed inactive state to an open conformation. This allows homo- or heterodimerization of the receptors to occur and causes autotransphosphorylation of the tyrosine kinase domain, resulting in

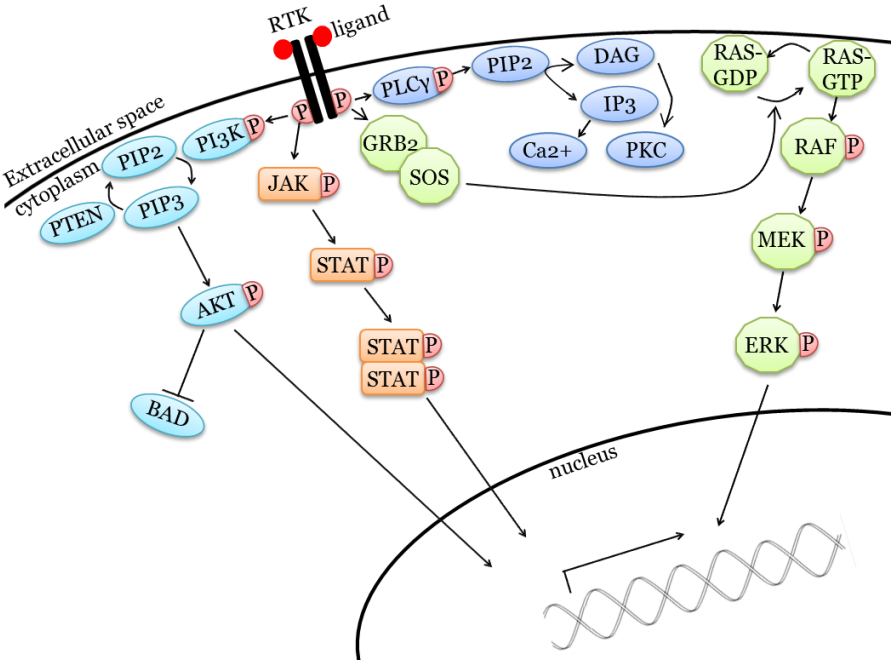

Figure 1: Simplified overview of common RTK signaling pathways. Upon ligand binding, RTKs hetero- or homo- dimerize and autotransphosphorylate the tyrosine residues that are located in the kinase domain. Docking proteins, such as GRB2, PI3K, PLCγ and JAK, bind to the phosphorylated receptors and induce signaling cascades that regulate multiple cellular processes, including cell survival, proliferation, metabolism, differentiation and migration. Activated PI3K phosphorylates PIP2 to generate PIP3, which recruits PDK1 and AKT, leading to the phosphorylation and activation of AKT. Activated AKT phosphorylates various substrates, resulting in inactivation of apoptotic signaling pathways via BAD inhibition, as well as the regulation of genes that are involved in cell proliferation (*2*). Phosphorylated JAK activates STAT, which dimerizes and translocates to the nucleus upon phosphorylation, where it regulates gene transcription (*3*). SOS interacts with GRB2 when bound to the phosphorylated receptor and activates RAS. RAS activates RAF, which phosphorylates MEK, leading to ERK phosphorylation. Activated ERK is transported into the nucleus, where it regulates gene transcription (*4*). PLCγ binds to the phosphorylated receptor, resulting in PLCγ phosphorylation, which cleaves PIP2 into DAG and IP3. DAG activates PKC, which also activates MAPK signaling. IP3 increases intracellular Ca^{2+} concentrations (*5, 6*).

multiple phosphorylated tyrosine residues (*7*). ERBB3 has impaired intrinsic kinase activity and becomes phosphorylated when dimerized to another EGFR family member, preferably ERBB2 (*8, 9*). Multiple effector proteins interact with the phosphotyrosine residues on the EGFR family members through the SH2- or PTB domains, resulting in the initiation of signaling cascades. The main signaling pathways that are activated by the EGFR family are the MAPK pathway, the PI3K pathway, the JAK-STAT pathway and the PLCγ pathway (Figure 1) (*7*). Activation of the MAPK pathway results in the transcription of genes that promote cell proliferation, differentiation, cell migration and adhesion (*4*). Activation of the PI3K pathway results in inhibition of apoptosis, and hence, survival, but it also stimulates cell proliferation and angiogenesis (*2*). The JAK-STAT pathway is involved in regulation of cell differentiation, proliferation, angiogenesis and apoptosis (*3*). The PLCγ pathway mainly regulates cell migration (*5*). EGFR family member signaling is highly regulated, and following the initiation of signaling, both early and late attenuators that aid in the down-regulation of signaling are induced (*7*). LRIG1 is a late attenuator of EGFR family signaling, as will be discussed later.

Role of the EGFR family in cancer

Aberrant EGFR family member signaling is a common feature in many solid cancers, including non-small cell lung cancers (NSCLC) (*10*), breast cancers (*11*) and gliomas (*12, 13*). Aberrant EGFR signaling may result from several mechanisms, including EGFR gene amplification, EGFR ligand overexpression, or activating mutations of *EGFR*, resulting in constitutive EGFR signaling that is independent of ligand binding (*7*). Several oncogenic mutations in the *EGFR* gene have been described. The EGFRvIII mutation is present in approximately 25% of glioblastomas. EGFRvIII has an 801 bp in-frame deletion of exons 2-7, which results in the deletion of a large part of the extracellular domain of the receptor. The truncated EGFR is constitutively active and has a prolonged half-life (*7, 14*). In NSCLC, different *EGFR* deletions and point mutations in the tyrosine kinase domain are common events. The $EGFR^{L858R}$ mutation is frequently found in NSCLC. In this mutated EGFR, the amino acid leucine (L) is replaced by arginine (R) at amino acid position 858. This mutation is located within the tyrosine kinase domain and results in constitutive EGFR activation. Small in-frame deletions in exon 19 of the *EGFR,* which result in ligand-independent constitutive EGFR activation, are also common features of NSCLC. Both the $EGFR^{L858R}$ mutation and exon 19 deletions are most commonly found in never-smokers and are associated with sensitivity to EGFR tyrosine kinase inhibitors (TKIs) (*15-17*). The $EGFR^{T790M}$ mutation, which is a point mutation at amino acid

790, is associated with resistance to EGFR-TKIs in NSCLC. The T790M amino acid is located within the catalytic pocket of EGFR. This mutation results in higher ATP affinity and lower EGFR-TKI affinity, resulting in resistance to EGFR-TKIs (*18*).

Treatment of cancers that are driven by aberrant EGFR family signaling

Several inactivating agents that interfere with EGFR signaling have been developed to potentially treat EGFR-driven cancers. Small molecule TKIs, monoclonal antibodies, peptide vaccines and antisense oligonucleotides have been developed. Today, EGFR TKIs and anti-EGFR monoclonal antibodies are widely used to treat diseases such as NSCLC (*19, 20*), breast cancers (*21, 22*), colorectal cancers (*23*) and squamous cell carcinomas (SCC) of the head and neck (*24*). The EGFR TKIs erlotinib (Tarceva) and gefitinib (Iressa) compete with ATP for binding to the catalytic site of EGFR tyrosine kinase, thereby inhibiting the catalytic activity of EGFR. These inhibitors are approved for clinical use in NSCLC and are widely used in the palliative setting to treat metastatic disease (*19, 20, 25*). Cetuximab (Erbitux) and panitumumab (Vectibix) are monoclonal antibodies that bind to EGFR and induce internalization of the receptor. They are mainly used clinically for the treatment of metastatic colorectal cancer (*23-25*). Cetuximab is also approved for the treatment of metastatic SCC of the head and neck (*24, 25*). Many gliomas display aberrant EGFR signaling. Despite this, clinical trials using erlotinib and gefitinib have not demonstrated improved survival in glioma (*26*). Trastuzumab (Herceptin) is a monoclonal antibody that binds to the extracellular domain of ERBB2. Trastuzumab inhibits dimerization of ERBB2, thereby arresting breast cancer cells in the G1 phase of the cell cycle. In addition, trastuzumab inhibits angiogenesis by inhibiting the expression of proangiogenic factors, such as VEGF, and inducing antiangiogenic factors, such as thrombospondin. Trastuzumab is used for the treatment of ERBB2 positive breast cancer in the adjuvant and palliative settings (*22, 25, 27, 28*). Pertuzumab (Perjeta) is another monoclonal antibody that binds to the extracellular portion of ERBB2 and inhibits receptor dimerization. Combination therapy of ERBB2 positive breast cancer using trastuzumab and pertuzumab has shown promising results (*29*). Several other agents that are directed against EGFR signaling are currently undergoing clinical trials.

The platelet-derived growth factor receptor family

The platelet-derived growth factor (PDGF) receptor (PDGFR) family is another subfamily of the RTK family. The PDGFRs play important roles during embryonic development, where they control the formation of vessels and organs. In adults, PDGFR signaling is involved in the wound healing process and the control of interstitial fluid pressure (*30*). The PDGFR family consists of PDGFRα, PDGFRβ, KIT, CSF1R and FLT3 (*1*). Four ligands have been described for PDGFRα and PDGFRβ: PDGFA, PDGFB, PDGFC and PDGFD. These ligands form homo- and heterodimers with one another: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. The different ligand dimers interact with PDGFRα and/or PDGFRβ, and upon ligand binding, the receptors form either homo- or heterodimers: PDGFRαα, PDGFRαβ or PDGFRββ (Figure 2). Dimerization results in autotransphosphorylation of the tyrosine kinase domain and the activation of signaling cascades as a result of the phosphorylation of cytoplasmic substrates. The main signaling pathways that are activated in response to PDGFR signaling include the MAPK pathway, the PI3K pathway, the PLCγ pathway and the JAK/STAT pathway (*31*).

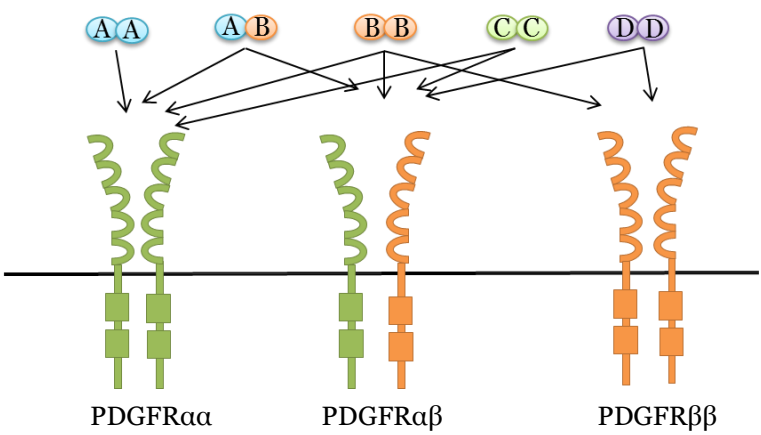

Figure 2: Ligand binding to PDGF receptors. The ligand PDGF-AA interacts with PDGFRα to form a PDGFRαα homodimer. PDGF-AB interacts with PDGFRα or PDGFRβ to form PDGFRαα or PDGFRαβ dimers. PDGF-BB interacts with PDGFRα or PDGFRβ to form PDGFRαα, PDGFRαβ or PDGFRββ dimers. PDGF-CC interacts with PDGFRα and PDGFRβ to form PDGFRαα or PDGFRαβ dimers. PDGF-DD interacts with PDGFRα or PDGFRβ to form PDGFRαβ or PDGFRββ dimers (*31*).

Roles of the PDGFRs in diseases

Aberrant PDGFR signaling is common to several types of cancers, including gastrointestinal stromal tumors (GIST), leukemias and gliomas (*13, 32-34*). Aberrant PDGFR signaling has also been observed in several other diseases, including inflammation, pulmonary fibrosis, and atherosclerosis (*35, 36*). In mice, PDGFB expressing retroviruses can induce gliomas (*37, 38*), as will be further discussed later on in this thesis. Aberrant PDGFR signaling is also a main feature of the proneural group of human gliomas, and nearly 30% of gliomas show increased PDGFR signaling (*13*). Aberrant PDGF expression contributes to the growth of tumors via autocrine signaling. However, aberrant PDGF expression also affects non-transformed stromal cells, such as pericytes, smooth muscle cells and fibroblasts, via paracrine signaling, which results in angiogenesis and alterations in interstitial fluid pressure (*36, 39, 40*).

Treatment of cancers that are driven by aberrant PDGFR signaling

Several small molecule inhibitors that inhibit multiple RTKs, including the PDGFRs, have been developed, *e.g.,* imatinib mesylate (Gleevec), dasatinib (Sprycel), sunitinib (Sutent) and sorafenib (Nexavar) (*25, 41-44*). However, to date, no inhibitor has been developed that only inhibit PDGFRs. Imatinib mesylate is an inhibitor of multiple tyrosine kinases, including PDGFRα, PDGFRβ, BCR-ABL and c-KIT. It is widely used for the treatment of chronic myeloid leukemia (CML), which is characterized by the activation of the BCR-ABL fusion oncoprotein (*41*). Imatinib has also been shown to have beneficial effects in patients with advanced GIST (*45*). PDGFR signaling is often de-regulated in gliomas. However, clinical trials using imatinib mesylate for glioblastoma treatment have been disappointing, with low response rates. Nevertheless, several responses in patients with glioblastomas and anaplastic gliomas have been reported, indicating a possible role for PDGFR inhibitors in the treatment of a subset of glioma patients (*46*). Dasatinib is an inhibitor of several tyrosine kinases, including BCR-ABL, SRC, c-KIT, PDGFRβ and EPHA2. This inhibitor is used for treatment of CML, including imatinib-resistant CML (*42*). Clinical trials are ongoing to study the efficacy of this inhibitor for the treatment of breast cancers and gliomas (*47, 48*).

LRIG proteins

In this thesis, we studied the leucine-rich repeats and immunoglobulin-like domains (LRIG) proteins and analyzed their expression and functions in psoriasis, glioma and lung cancer.

Structure and evolution of the LRIG proteins

The human LRIG protein family consists of three paralogs: LRIG1, LRIG2 and LRIG3. These proteins are integral, single-pass, transmembrane proteins, consisting of 15 leucine-rich repeats that are flanked by cysteine-rich N- and C-terminal flanking domains, three immunoglobulin-like domains, a transmembrane domain and a cytosolic tail (Figure 3). The ectodomains, transmembrane domains and membrane-proximal portions of the cytosolic tails of LRIG proteins are relatively well conserved. However, the membrane-distal portions of the cytosolic tails of these three proteins are less well conserved (*49*). The leucine-rich repeats domain of LRIG1 is shaped like an arch (*50*).

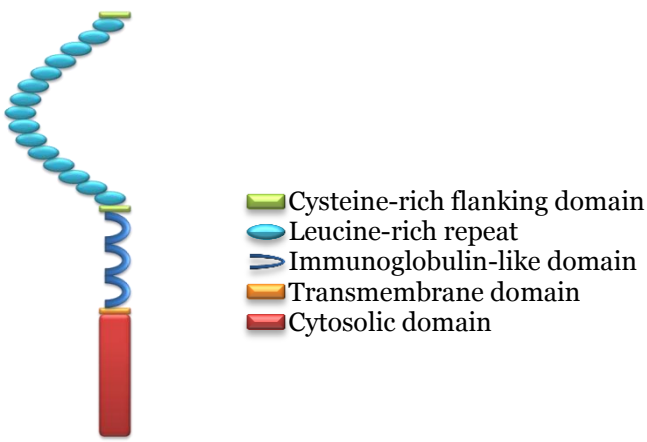

Figure 3: Domain organization of LRIG proteins. The LRIG proteins are highly similar. The extracellular, or luminal, domains consist of 15 leucine-rich repeats that are flanked by cysteine-rich flanking domains, and three immunoglobulin-like domains. The LRIG proteins also contain a transmembrane domain and a cytosolic tail.

The LRIG proteins have been conserved throughout evolution. *Mus musculus* expresses orthologs to the three human LRIG proteins. The mouse *Lrig1*, *Lrig2* and *Lrig3* are highly similar to the human LRIG proteins, sharing 80%, 87% and 86% amino acid sequence identity between the respective mouse and human orthologs. Structurally, the mouse *Lrig* proteins contain the same number and order of protein domains as the human proteins. The pufferfish, *Fugu,* also contains three orthologous LRIG proteins that share high amino acid sequence identity with the human proteins: 69%, 65% and 75%, respectively. However, the nematode *Caenorhabditis elegans* (*C. elegans*) and the tunicate *Ciona intestinalis* each only contain one Lrig ortholog (*49, 51*). The *C. elegans* Lrig ortholog, SMA-10, consists of the same number and order of protein domains as the human LRIG proteins. The extracellular, or luminal, domains show sequence similarities to human LRIG. However, the cytosolic tail of SMA-10 is short and is not conserved between *C. elegan*s and human (*51*).

Expression of LRIGs in tissues and cells

All of the LRIG proteins are ubiquitously expressed in human tissues. The expression of LRIG1, LRIG2 and LRIG3 has been analyzed in a number of tissues, and the LRIGs are expressed at the mRNA and protein levels in all of the tissues that have been analyzed thus far. *LRIG1* mRNA is highly expressed in the lungs, brain and stomach, whereas low levels are expressed in the liver, placenta and spleen (*52*). *LRIG2* mRNA is highly expressed in the heart, pancreas and thymus, whereas low levels are expressed in the placenta, liver and lungs (*53*). *LRIG3* mRNA is highly expressed in the skin, stomach and thyroid, whereas low levels are found in adrenal glands, blood and skeletal muscles (*49*). At the subcellular level, all three LRIG proteins have been detected at the cell membrane, in the perinuclear area and in cytoplasmic vesicle-like structures (*53-55*).

Molecular functions of LRIG1 and its role in cancer

Of the three LRIG proteins, LRIG1 is the most studied. LRIG1 is a multifunctional protein that is involved in different molecular processes, including the negative regulation of RTKs, the regulation of epidermal stem cell quiescence and tumor suppression. In several cancer types, high LRIG1 expression is a positive prognostic factor. In addition, LRIG1 has been reported to function as a tumor suppressor in the mouse intestine.

Lrig1 is a regulator of growth factor signaling

LRIG1 is a negative regulator of several RTKs and has been shown to down-regulate EGFR family members, the MET receptor and RET receptor signaling (*56-58*). LRIG1 is a multifunctional protein, which down-regulates receptor signaling using different methods. For the negative regulation of EGFR, LRIG1 functions as a late attenuator in a c-Cbl-dependent manner. Binding of EGF to EGFR results in downstream signaling and the transcription of target genes, including the *LRIG1* gene. The ectodomain of LRIG1 physically interacts with EGFR, and the juxtamembrane region of LRIG1 recruits the E3 ubiquitin ligase c-Cbl, resulting in ubiquitylation of both LRIG1 and EGFR. Both proteins are subsequently degraded, thus constituting a negative feedback loop (*56, 59*). The mutated and constitutively active variant EGFRvIII is also down-regulated by LRIG1. LRIG1 interacts physically with EGFRvIII and promotes lysosomal degradation in a c-Cbl independent manner (*60*). ADAM17 is a metalloprotease that can proteolytically cleave LRIG1 (*61*), generating a shed and soluble form of LRIG1 (sLRIG1), which corresponds to the complete ectodomain of LRIG1. sLRIG1 can negatively regulate EGFR signaling both *in cis* and *in trans (61)*. The leucine-rich repeat domain can inhibit EGF binding to EGFR on its own and thereby negatively regulate EGFR signaling (*50*).

LRIG1 negatively regulates the MET receptor in a c-Cbl independent manner (*57*). LRIG1 physically interacts with the MET receptor, both under basal and receptor-activated conditions, and this interaction induces lysosomal degradation of phosphorylated and non-phosphorylated MET receptors, independent of c-Cbl (*57*).

In the case of RET receptor regulation, LRIG1 is involved in a negative feedback loop. LRIG1 is transcriptionally up-regulated in response to cellular stimulation with the RET ligand GDFN (*58*). LRIG1 physically interacts with RET and hinders the translocation of the receptor into lipid rafts, where it exerts its signaling functions. The LRIG1-RET interaction also restricts the binding of GDNF to RET, which thus inhibits the activation of RET. The down-regulation of RET signaling is thus not associated with receptor degradation (*58*).

SMA-10 was identified in a screen for genes that regulate body size in *C. elegans*. SMA-10 is the only LRIG protein that is present in the nematode, and it promotes bone morphogenetic protein (BMP) signaling, both in the nematode and in mammalian cells. SMA-10 physically interacts with the BMP receptors SMA-6 and DAF-4, but not with the BMP ligand (*51*).

LRIG1 expression is regulated by sex steroid hormones

In a prostate cancer cell line, androgen stimulation induced both LRIG1 mRNA and protein expression (*62*), indicating that LRIG1 expression is regulated by androgen. LRIG1 is also regulated by estrogen, and *LRIG1* is a direct transcriptional target of ERα (*63*). In meningioma, LRIG1 expression is correlated with ERα status (*64*).

LRIG1 regulates contact inhibition

A recent study showed that LRIG1 regulates contact inhibition in lung cancer cell lines. These cell lines express low endogenous levels of LRIG1. Ectopic overexpression of LRIG1 did not affect proliferation in non-confluent cells. However, in confluent cells, LRIG1 overexpression resulted in significant reduction in cell proliferation. Upon cell-cell contact, the EGFR co-localized with E-cadherin and LRIG1 at cell-cell contacts, and LRIG1 formed a ternary complex with E-cadherin and EGFR. LRIG1 was required for growth inhibition upon cell-cell contact (*65*).

LRIG1 regulates epidermal stem cell quiescence

The epidermis, the stratified epithelial cell layer that protects organisms from the environment, is constantly remodeled. Deletion of *Lrig1* in mice results in psoriasis-like lesions on the snouts and tails (*66*), indicating that Lrig1 has important epidermal functions. Stem cells in the basal layer of the epidermis are responsible for cell renewal in the epidermis. The proliferation of the stem cells in the interfollicular epidermis (IFE) is highly regulated and is dependent upon input from several signaling pathways, such as Notch, Egfr and Wnt (*67-69*). LRIG1 is expressed by stem cells in the human IFE and is a stem cell marker for human IFE (*70*). Expression of LRIG1 in these cells renders the cells unresponsive to EGF stimulation, which results in stem cell quiescence. Therefore, LRIG1 regulates epidermal stem cell quiescence.

The pilosebaceous unit of the mouse epidermis consists of the hair follicle (HF) and the adjacent sebaceous gland (SG). Lrig1 is expressed in a multipotent stem cell compartment that is located in the junctional zone between the SG, bulge and infundibulum of the HF. These Lrig1-positive cells can give rise to IFE, SG and HF, but lineage tracing experiments indicate that these stem cells are only bipotent and give rise to cells in the SG and IFE (*71*).

LRIG1 in cancer

LRIG1 expression and its prognostic value in cancer

LRIG1 is located on chromosome 3p14, a region that is deleted in various cancers (*72*). Many studies have investigated the role of LRIG1 in tumor cell lines and tumor tissues. *LRIG1* mRNA is down-regulated in lung, colon and prostate cancer cell lines compared to the normal corresponding tissues (*73*). Increased copy numbers of the *LRIG1* gene were observed in 34% of breast tumors (*74, 75*). Loss of *LRIG1* gene heterozygosity has been observed in several cancer cell lines and lung cancer lesions (*65*). LRIG1 RNA and protein expression has been extensively studied, and LRIG1 expression was found to be decreased in clear cell renal cell carcinoma (*76, 77*), cutaneous SCC (*78*), breast cancers (*79*), ERBB2 positive breast cancer (*79*), ERα positive breast cancer (*63*), and astrocytic tumors (*80*) compared to the normal corresponding tissues. In human cutaneous SCC tumors, LRIG1 expression was correlated with tumor differentiation. LRIG1 was expressed in well and moderately differentiated tumors, but little or no LRIG1 was expressed in poorly differentiated tumors (*78*). In cutaneous SCC, LRIG1 expression was also found to be a prognostic marker: high LRIG1 expression was correlated with longer patient survival (*81*). High LRIG1 expression has also been found to be a positive prognostic factor for other cancer types, including early stage epithelial uterine cervical SCC (*82*), ER positive breast cancers (*63*) and cervical adenocarcinomas (*83*). LRIG1 protein expression was, paradoxically, found to be either a positive or negative prognostic factor for prostate cancer. High expression of LRIG1 was determined to be a risk factor in tumor samples that were collected from untreated patients and a positive prognostic factor for prostatectomized patients. The regulation of LRIG1 by androgen may explain the differences in survival that were observed in patients with high expression levels of LRIG1 in the two different sample sets (*62*).

LRIG1 is a stem cell marker and tumor suppressor in the mouse intestines

The villus and crypts of the intestine are lined with intestinal epithelium, which is constantly remodeled. Stem cells that reside at the bottom of the crypts are responsible for the highly regulated remodeling of the epithelium. There are at least two different intestinal stem cell (ISC) compartments. One of these ISC compartments expresses Lgr5, contains highly proliferative cells and is located between Paneth cells at the bottom of the crypts (*84*). The other ISC compartment resides at the +4 position in the crypts and is constituted of slowly cycling cells that express Bmi1, mTert and Hopx. The

rapidly proliferating cells are responsible for the daily generation of the intestinal epithelium, whereas the quiescent cells are activated upon tissue injury. Different studies have shown that Lrig1 is expressed by either the rapidly proliferating Lgr5 expressing stem cells (*85, 86*) or slowly cycling stem cells (*84*). Thus, which ISC compartment the Lrig1 expressing cells belong to remains controversial.

Genetic deletion of *Lrig1* in two different *in vivo* mouse studies has implicated *Lrig1* as a key regulator of Erbb signaling in ISCs. Deletion of *Lrig1* in mice that have a FVB/N background resulted in grossly enlarged intestines that were caused by enlarged crypts (*85*). The crypts were enlarged due to increased numbers of stem cells, progenitor cells and Paneth cells, and the mice had to be euthanized ten days after birth due to the severity of the phenotype. Erbb1-3 and Met were overexpressed and activated in the intestinal epithelium, resulting in increased MAPK and cMyc signaling (*85*). Deletion of *Lrig1* in mice with mixed genetic backgrounds resulted in viable and fertile mice. However, these mice developed low grade duodenal adenocarcinomas at 5-6 months of age, which had progressed into more advanced tumors by 13-14 months of age (*84*). These tumors contained high levels of Erbb1-3 and phosphorylated Erk1/2, indicating that Lrig1 behaves as a tumor suppressor in the mouse intestine by negatively regulating Erbb signaling. Thus, deletion of Lrig1 disrupts the negative feedback loop of Erbb signaling, resulting in tumor formation in the intestines (*84*).

Role of LRIG1 in lung

Mice express *Lrig1* in the trachea and the first bronchial divisions. Ablation of *Lrig1* in mice resulted in epithelial thickening in both the trachea and the first bronchial divisions(*65*). Thickening of the epithelium was caused by increased cell proliferation, and these cells displayed increased Egfr phosphorylation. However, there was no change in the proportion of basal, Clara and ciliated cells. These data indicate that Lrig1 regulates the proliferation of epithelial cells and tissue homeostasis in the upper airways, but not cell differentiation (*65*). In humans, LRIG1 is also expressed in the bronchial epithelium in normal lungs. However, in human lung carcinoma *in situ* samples, both LRIG1 mRNA and LRIG1 protein were down-regulated compared to normal samples from the same patients (*65*). Because LRIG1 was down-regulated during these early stages of lung cancer formation, loss of LRIG1 appears to be an early event during lung cancer genesis. Together, these data indicate that LRIG1 may act as a tumor suppressor in the lung epithelium (*65*). Smoking is the main risk factor for the development of lung

cancer. Interestingly, LRIG1 is down-regulated in the epithelium of current smokers compared to former smokers (*87*).

Can LRIG1 be used for cancer treatment?

In an attempt to investigate whether LRIG1 can be utilized as a treatment modality for cancer, athymic mice were subcutaneously injected with the human T24 invasive bladder cancer cell line (*88*). After tumor establishment, the mice were treated with LRIG1 using adenoviral-mediated administration via injection into the tumors. The mice that were treated with LRIG1 displayed significantly smaller tumors compared to the control mice (*88*). In another study, sLRIG1 was delivered locally in an orthotopic glioblastoma xenograft mouse model in which patient-derived glioblastoma tumors were implanted into mouse brains with encapsulated cells that either did, or did not, express and secrete sLRIG1 (*89*). The sLRIG1-expressing encapsulated cells inhibited glioma growth, and mice with sLRIG1-expressing encapsulated cells had a median survival time that was 32% longer than mice that were treated with encapsulated control cells (*89*). These data indicate that LRIG1 plays an important role during cancer formation, and that LRIG1 may be used as a treatment modality.

Functions of Lrig2 and its role in cancer

LRIG2 is the least studied protein in the LRIG family. However, some studies have indicated that LRIG2 may function as a negative prognostic marker in certain types of cancer.

Biological role of LRIG2

Knowledge about the molecular functions and biological role of LRIG2 is lacking. However, the results of one study indicated that LRIG2 plays roles in cell proliferation, migration and invasion in a glioma cell line (*90*). Recently, whole exome sequencing of individuals with human urofacial syndrome (UFS) identified mutations in LRIG2 in 21% of these individuals (*91*). How these mutations contribute to UFS remains unclear.

LRIG2 in cancer and adenomas

Several studies have indicated that LRIG2 plays a role in cancer. *LRIG2* is located at chromosome 1p13. This region is frequently deleted in human cancers, such as oligodendrogliomas (*72*). In contrast to LRIG1, which is a positive prognostic marker that is associated with longer patient survival, LRIG2 expression appears to be a negative prognostic marker. High LRIG2 expression in invasive early-stage squamous cervical cancer is correlated with poor patient survival (*92*). Pituitary adenomas, which are normally benign tumors, are invasive in a small percentage of patients. LRIG2 expression is elevated in invasive, but not in non-invasive, pituitary adenomas (*93*). In oligodendrogliomas, cytoplasmic expression of LRIG2 is a negative prognostic marker (*94*). Patients with no cytoplasmic LRIG2 expression had a median survival time of 120 months, compared to a median survival time of 74 months for patients with cytoplasmic LRIG2 expression. However, in astrocytic tumors, perinuclear LRIG2 expression was correlated with longer patient survival (*95*). In meningiomas, LRIG2 expression is correlated with histological subtypes and ER status (*64*).

Functions of Lrig3 and its role in cancer

The molecular functions of LRIG3 are not fully understood. However, data indicate that LRIG3 is involved in the regulation of RTKs and Wnt signaling. LRIG3 has also been implicated to be a positive prognostic factor for certain cancer types.

Molecular function of LRIG3

LRIG3 is required for neural crest (NC) formation in developing *Xenopus* embryos. Knockdown of LRIG3 resulted in inhibition of multiple NC marker genes, severe anterior defects and delayed, or prevented, neural fold closure. Wnt signaling is necessary during NC formation, and LRIG3 enhances Wnt signaling in animal caps, resulting in the upregulation of several fibroblast growth factor (*FGF)* genes. FGF signaling is also required during NC formation. LRIG3 physically interacts with *Xenopus* FGFR1 via the ectodomain, resulting in the downregulation of FGFR1 and the subsequent downregulation of the FGFR1 signaling cascade (*96*).

Ablation of *Lrig3* in mice results in craniofacial defects such as a shortened snout and loss of the lateral semicircular canal in the inner ear (*97*). During lateral canal formation in the otic vesicle, Lrig3 is expressed in the lateral

pouch and restricts netrin1 (Ntn1) expression to the center of the pouch where Lrig3 is not expressed. In the center of the pouch, Ntn1 down-regulates the basal lamina, allowing fusion to occur and the semicircular canal to form. Repressive interactions between Lrig3 and Ntn1 coordinate the timing and location of the fusion (*97*). *Lrig3* ablation results in enhanced Ntn1 expression throughout the lateral pouch, resulting in fusion of the whole pouch and no formation of the lateral canal (*97*). Lrig3 was found to physically interact with the TKRs Erbb, Erbb2 and Erbb4 (*98*).

Several studies have analyzed the effects of up- and down-regulation of LRIG3 in cell lines. Down-regulation of LRIG3 in a glioma cell line increased proliferation, adhesion and invasion, and decreased apoptosis (*99*). However, both up- and down-regulation of LRIG3 in a bladder cancer cell line decreased proliferation and increased apoptosis (*100, 101*).

LRIG3 appears to be involved in the regulation of both heart function and blood cholesterol levels. In a genome wide association study (GWAS), a loci that was close to the *LRIG3* gene was found to be associated with risk of heart failure (*102*), and another GWAS study identified a SNP that was close to the *LRIG3* gene that was associated with high-density lipoprotein cholesterol levels in the blood (*103*). Recent experiments that were conducted by our group have demonstrated that *Lrig3*-deficient mice develop cardiac hypertrophy (Hellström M, personal communication).

LRIG3 in cancer

Knowledge regarding the function of LRIG3 in cancer is incomplete. However, LRIG3 has been implicated to play a role in cancer in several studies. *LRIG3* is located at chromosome 12q13.2, a region that is often altered in various cancers (*72*). When aptamer technology was used in an attempt to identify biomarkers for NSCLC that can separate high risk individuals (heavy smokers) who do not have cancer from NSCLC patients, LRIG3 was one of twelve biomarkers that could correctly classify blood samples as controls or cases with 91% sensitivity and 84% specificity (*104*). In a pituitary adenoma cell line, LRIG3 was down-regulated compared to normal tissue (*105*). Perinuclear expression of LRIG3 in astrocytic tumors has been correlated with longer patient survival and a low proliferation index (*95*), while a high fraction of LRIG3 positive cells in cervical adenocarcinomas has been correlated with longer patient survival (*83*). These findings indicate that LRIG3 may be of importance in cancer.

LMO7

In the work that was conducted in support of this thesis, we discovered that LRIG proteins interact with LMO7.

LMO7 is a multifunctional protein that has several different functions. LMO7 shuttles between the cell membrane, cytoplasm and nucleus and functions both as a stabilizer of adherence junctions and a transcription factor (*106, 107*). It is expressed in the majority of human tissues and is highly expressed in the lung. Several isoforms of LMO7 have been described to result from alternative splicing. The protein consists of a CH domain, a PDZ domain and a LIM domain, and some of the isoforms also contain an F-box domain (*106, 108-111*).

Figure 4: LMO7 domain organization. LMO7, isoform 1, consists of 1349 amino acids and contains an N-terminal CH domain, a PDZ domain and a C-terminal LIM domain.

Molecular functions of LMO7

LMO7 localizes to epithelial adherence junctions where it interacts with afadin and α-actinin. The interaction of LMO7 with afadin couples LMO7 to the nectin-system, and the interaction of LMO7 with α-actinin couples LMO7 to the E-cadherin-catenin system. LMO7 thereby functions as a connector between these two systems and stabilizes adherence junctions (*106*). LMO7 shuttles between the nucleus and the cytoplasm. When LMO7 is located in the nucleus, it can physically interact with the nuclear membrane protein emerin at the inner nuclear envelope, or function as a transcription factor and regulate the transcription of many genes, including *EMD*, *PAX3*, *PAX7* and *MYF5* (*112, 113*). Emerin is both a positive and negative regulator of LMO7. It is required for the nuclear localization of LMO7, allowing LMO7 to function as a transcriptional regulator of genes. However, when LMO7 interacts with emerin, the activity of LMO7 is inhibited (*107*). LMO7 is also involved in the transcriptional up-regulation of genes that are activated by the transcription factor serum response factor (SRF). When inactive, the

myocardin-related transcription factor (MRTF) interacts with actin in the cytoplasm. LMO7 inhibits the actin-binding ability of MRTFs, resulting in export of the MRTF from the cytoplasm into the nucleus, where it binds to the SRF and subsequently activates the transcription of genes (*113*). LMO7 also plays an important role during heart development in fish. Knockdown of LMO7 in zebrafish results in heart defects in the embryos, including bradycardia, arrhythmia and delocalization of the heart (*114*).

LMO7 and cancer

LMO7 is located at chromosome 13q21-q22, a region that is associated with hereditary breast cancer (*115*). LMO7 expression was up-regulated in several tumors, including breast and colon tumors (*111, 116*), and it has been associated with lymph node metastases in human breast cancer (*117*). Ablation of *Lmo7* in mice results in viable mice that appear normal. However, 20% of *Lmo7*-ablated mice develop spontaneous lung cancer at high age, especially adenocarcinomas (*118*). In a study of LMO7 expression in human lung adenocarcinomas, LMO7 expression was associated with T-stage: LMO7 expression was higher in lower stages, while LMO7 expression was lower in higher stages (*119*).

Diseases that were examined in this work

In this thesis, we studied LRIG1, LRIG2 and LRIG3 in three different diseases in which aberrant RTK signaling is important for disease initiation or progression: psoriasis, oligodendroglioma and lung cancer. Mice ablated for *Lrig1* develop psoriasis-like lesions; therefore, we were interested in studying the role of LRIG proteins in this disorder. Oligodendroglioma is a rare type of brain tumor, and the cytoplasmic expression of LRIG2 in oligodendrogliomas is associated with poor prognoses. Therefore, we were interested in studying the role of LRIG2 in this disease. Lung cancer is responsible for the most cancer related deaths worldwide, and because EGFR signaling is of importance in this disease, we were interested in studying the role of LRIG proteins in lung cancer.

Psoriasis

Psoriasis is a chronic and systemic disease. It affects the skin and other organs and has a major impact on quality of life. The prevalence of psoriasis worldwide is approximately 2-3%, with higher prevalence in temperate

countries and lower incidence in subtropical and tropical countries (*120*). The onset of psoriasis can occur at any age. However, 75% of patients are diagnosed before the age of 40 (*121*). There are different subtypes of psoriasis. The most common type is psoriasis vulgaris, which affects approximately 90% of psoriatic patients. This type of psoriasis affects the scalp, hands, elbows, knees, back and thighs and is associated with red, scaly plaques that are well-delineated from the surrounding skin (*122*). Psoriasis does not only affect the skin. It is also associated with systemic disorders, including cardiovascular disease, Chron's disease, psoriatic arthritis, obesity, diabetes, depression, and a 3 – 4 year reduction in life expectancy (*123-129*). The etiology of psoriasis is under debate. However, the disease is characterized by hyperproliferation of epidermal keratinocytes and inflammation.

Normal and psoriatic epidermis

The epidermis, the stratified epithelium that covers our bodies and protects us from the environment, is constantly remodeled and consists of several different cell layers: the basal layer, spinous layer, granular layer and the cornified layer (Figure 5) (*69*). In the basal cell layer, epidermal stem cells are responsible for the constant renewal of keratinocytes, which constitute the majority of the cells in the epidermis. Epidermal stem cells divide asymmetrically and produce transit amplifying (TA) cells. These cells go through a few rounds of cell divisions before migrating up through the cell layers, with increasing differentiation (*69*). During the migration and differentiation processes, the cells exit the cell cycle and begin to express epidermal differentiation markers, such as keratins and involcurin. The cells then lose their nuclei and organelles and become dead corneocytes, which are shed from the surface of the body (*69*). In psoriatic skin, the epidermis becomes thickened due to hyperproliferation of the keratinocytes. The migration of keratinocytes from the basal layer to the cornified layer is shortened from 40 days in normal skin to only 6-8 days in psoriatic skin. The differentiation of the keratinocytes is altered with aberrant keratin expression and reduced or missing granular layer. Parakeratosis may also occur (*130, 131*). In the normal epidermis, few immunological cells are present. In psoriatic skin, active inflammation occurs and is accompanied by a massive increase in the number of inflammatory cells, most notably T cells and dendritic cells. The inflammatory cells produce cytokines, which increase inflammation, resulting in a vicious cycle. In healthy individuals, skin inflammation subsides. However, in psoriatic patients, inflammation does not subside (*132*).

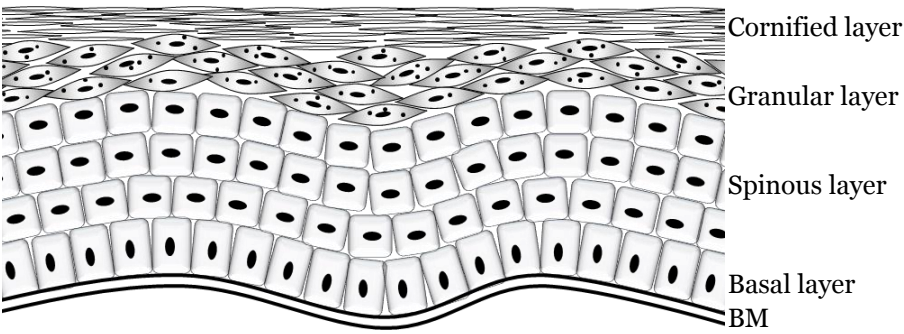

Figure 5: Organization of the epidermis. The basal layer of epithelial cells is located on top of the basement membrane (BM). Keratinocytes migrate from the basal layer into the spinous layer, where they start to express differentiation markers. The cells then migrate further, into the granular layer, where they further differentiate and begin to lose their organelles and nuclei. The cells then differentiate into dead corneocytes in the cornified layer.

Role of EGFR in normal and psoriatic epidermis

As previously discussed, EGFR signaling is involved in multiple cellular responses, including cell proliferation, migration, adhesion, differentiation and apoptosis. In the skin, EGFR has multiple functions, including keratinocyte proliferation and survival, inhibition of differentiation, wound healing and the regulation of immune homeostasis in the skin. The importance of EGFR signaling in the skin was discovered as early as 1933, when mice, called waved-1, had "coats which looked exactly as though the animals had been to the hairdresser and had had a permanent wave treatment" (*133*). Many years later these mice were discovered to carry a point mutation in the EGFR ligand TGF-α. Over the years, evidence accumulated indicating that EGFR signaling is important in the skin. The EGFR is expressed by cells in the epidermis and the majority of the receptors are located in the basal layer (*134*). EGFR ligands are also expressed in the epidermis, where they act as growth factors for keratinocytes and control proliferation via both auto- and paracrine regulation (*135, 136*). In addition, the EGFR regulates the production of various cytokines in the skin and is involved in skin inflammation (*137*). Genetically engineered mice that have deletions in the *EGFR* gene, or EGFR signaling ligands, display skin alterations (*133, 138*). Psoriasis-like phenotypes have been observed in genetically modified mice that overexpress the EGFR ligand amphiregulin and in mice in which the negative regulator of EGFR signaling, *LRIG1*, has

been ablated *(66, 139)*. Anti-EGFR therapies have been used to treat several malignancies. Common side effects of these drugs include rashes, dry skin, itching and hair and periungual alterations, which further demonstrate the role of EGFR in skin homeostasis (*140*). In psoriatic skin, there is an increased number of EGFRs and altered receptor distribution with EGFRs located throughout the epidermal cell layers (*141*). The amount and distribution of EGFR ligands are also altered in psoriatic skin *e.g.* in healthy skin, the EGFR ligand HB-EGF is mainly located in the basal layer, but is located throughout the epidermis in psoriatic skin (*142*). The resulting increase in EGFR signaling promotes the increased hyperproliferation of the keratinocytes, resulting in epidermal thickening. Increased EGFR signaling also induces the expression of several cytokines that are involved in skin inflammation (*137*).

Gliomas

Tumors in the central nervous system (CNS) are rare and constitute approximately 2% of all human tumors. Gliomas are the most common primary cancers in the CNS and account for 31% of all brain tumors. The most common type of glioma is glioblastoma, which constitutes 54% of all gliomas. Glioblastomas are heterogeneous, invasive and highly aggressive tumors. Despite new treatment modalities, glioblastoma carries a very poor prognosis, and patients have a median survival after diagnosis of 15 months. Fewer than 3% of glioblastoma patients are alive 5 years after diagnosis (*143-148*).

Histological classification of gliomas

Gliomas are histologically classified according to the WHO into subgroups depending on malignancy and which glial cell type the tumor cells resemble. Astrocytomas constitute approximately 80% of all gliomas and resemble astrocytes. Oligodendroglial tumors constitute approximately 10% and resemble oligodendrocytes. Ependymomas constitute approximately 10% and resemble ependymal cells (Table 1). Mixed forms, such as oligoastrocytomas that consist of a mixture of malignant cells that resemble both astrocytes and oligodendrocytes, constitute approximately 3% of all gliomas (*148*). The tumors are graded according to the WHO classification on a four step scale, where grade I is benign and grades II-IV are malignant, diffusely infiltrating tumors, which differ in malignancy grade. Grade I tumors are slowly growing tumors that are often cured by surgery (*147*). Grade II tumors are well-differentiated tumors with low mitotic activity.

However, these low grade tumors have a tendency to transform into high grade tumors. Grade III tumors are aggressive and have high mitotic activity and infiltrative cells. Grade IV tumors are classified as glioblastomas. These aggressive tumors are highly invasive and heterogeneous and are composed of poorly differentiated astrocytic cells with nuclear atypia and frequent mitotic activity. These tumors are highly vascularized, but often, large necrotic areas are found within the center of the tumor (*147*). The majority of glioblastomas, approximately 90%, are primary glioblastomas, which develop rapidly, without a previous history of low grade glioma. The remaining 10% are secondary glioblastomas, which develop gradually from a low grade glioma. Primary and secondary glioblastomas are histologically indistinguishable but contain different genetic aberrations (*143*).

Glioma	Grade I	Grade II	Grade III	Grade IV
Astrocytic	x	x	x	x
Oligodendroglial		x	x	
Oligoastrocytic		x	x	
Ependymal	x	x	x	

Table 1: Glioma subtypes. A simplified overview of the different subtypes of glioma and the malignancy grades that are represented in each subtype is presented. Astrocytic tumors range in malignancy grade from I-IV, oligodendroglial and oligoastrocytic tumors range from grades II-III, and ependymal tumors range from grades I-III (*147*).

Molecular profiling of gliomas

Recently, an alternative to the WHO classification of gliomas has been proposed, which is based on molecular profiling. In this classification, glioblastomas are classified into four groups: the proneural, classical, mesenchymal and neural groups (*13*). The proneural molecular group is dominated by point mutations in the *IDH1* gene and aberrant PDGFR signaling. Mutations or loss of *TP53* and *PTEN* are frequent events, as are the high expression of oligodendroglial markers, such as *PDGFRA* and *OLIG2*, and the high expression of pro-neural markers, such as *SOX* and *DCX*. The classical molecular group is characterized by chromosome 7 amplification that is paired with chromosome 10 loss, together with amplification and activating mutations of *EGFR*. In glioblastomas, *TP53* mutations are the most common mutations. However, *TP53* mutations are not detected in the classical group. Deletion of the *CDKN2A* gene and

deletion or inactivating mutations of *PTEN* are frequent events. The classical group expresses the neural precursor and stem cell marker *NES*, and the Notch and Sonic hedgehog pathways are activated. The mesenchymal molecular group harbors the most aggressive tumors, which contain *NF1* deletions or mutations, frequent *TP53* mutations and deletions or inactivating mutations of *PTEN*. These tumors express mesenchymal markers, such as *MET*, and astrocytic markers, such as *CD44* and *MERTK*. The neural group displays mutations in *EGFR*, *TP53* and *PTEN* and expresses neural markers, such as *NEFL* and *GABRA1* (*13*).

Molecular Group	Major Genetic Alterations	Tissue Lineage Gene Signature
Proneural	*IDH1, PDGFR*	Oligodendrocytes
Classic	*EGFR*	Astrocytes
Mesenchymal	*NF1, TP53*	Astrocytes
Neural	*EGFR*	Neurons

Table 2: Molecular profiling of gliomas. The four molecular groups, the major molecular alterations within each group and the tissue linage gene signatures that are expressed in each group are presented.

Oligodendroglial tumors

Oligodendroglial tumors are classified into WHO grade II (oligodendrogliomas) and grade III (anaplastic oligodendrogliomas). Oligodendrogliomas are rare, primary malignant tumors, which constitute 2.5% of all primary brain tumors and 77% of all oligodendroglial tumors (*149*). This tumor type mainly arises in adults, with a peak incidence between 40-45 years. Oligodendrogliomas are well-differentiated tumors, which are diffusely infiltrating. They have moderate cellularity, low mitotic activity and monomorphic cells that have uniform, round nuclei. These tumors most often arise in the cortex and white matter. Anaplastic oligodendrogliomas constitute approximately 1% of primary brain tumors and 23% of all oligodendrogliomas. Diagnoses peak between 45 and 50 years of age. Compared to oligodendrogliomas, anaplastic oligodendrogliomas are more aggressive tumors that have high mitotic activity, microvascular proliferation with or without pseudopalisading, diffusely infiltrating cells, rounded hyperchromatic nuclei and perinuclear halos. Necrosis may also be present

(*147*). Unlike anaplastic astrocytomas, anaplastic oligodendrogliomas are often chemosensitive and are characterized by a longer median survival (*150*). However, the tumors are heterogeneous, and median survival varies between 3.5 and 15 years for oligodendrogliomas and between 2 and 5 years for anaplastic oligodendrogliomas (*151, 152*). The five year survival for oligodendrogliomas and anaplastic oligodendrogliomas is 66% and 38%, respectively (*149*).

Molecular profiling of oligodendroglial tumors

Deletion of the 1p/19q chromosome arms

One of the most common genetic alterations in oligodendroglial tumors is the complete loss of the 1p and 19q chromosome arms. Approximately 70 - 80% of oligodendroglial tumors display 1p/19q loss, and this is considered to be the genetic hallmark of oligodendroglial tumors. These deletions occur regardless of grade and are considered to be an early event during oligodendroglioma genesis (*153, 154*). Complete loss of the 1p and 19q chromosome arms is associated with improved chemosensitivity to alkylating agents and better prognoses. In the clinic, these deletions are now used as prognostic and predictive markers for response to treatment of oligodendroglial tumors (*155*).

Point mutations in IDH1/IDH2

The majority (up to 80%) of oligodendroglial tumors contain mutually exclusive point mutations in the gene encoding isocitrate dehydrogenase (IDH) 1 or IDH2. The *IDH* genes encode three enzymes, IDH1, IDH2 and IDH3, which catalyze the oxidative carboxylation of isocitrate into α-ketoglutarate, resulting in the production of nicotinamide adenine dinucleotide phosphate (NADPH). Mutations in *IDH* result in decreased levels of α-ketoglutarate and increased levels of 2-hydroxyglutarate. The most common mutation is a guanine to adenine transition in codon 132, resulting in an arginine to histidine substitution, IDH1R132H. *IDH1* mutations are highly associated with deletions of the 1p and 19q chromosome arms, *MGMT* hypermethylation and improved prognoses (*156-158*).

MGMT hypermethylation

The *MGMT* gene encodes a DNA mismatch repair enzyme that removes alkyl groups from the O^6 position of guanine in DNA following alkylation. In oligodendroglial tumors, the *MGMT* promoter is hypermethylated in 55% – 80% of cases and is associated with tumors that have 1p and 19q deletions (*159*). Methylation results in silencing of *MGMT* gene transcription, and hence, decreased MGMT expression. In glioblastomas, *MGMT* promoter hypermethylation predicts response to temozolomide treatment (*145*).

Genetic alterations in RTKs and downstream signaling

Several RTKs are implicated in oligodendroglioma genesis. De-regulated EGFR signaling is an early event in oligodendroglioma genesis. Approximately one-quarter of oligodendroglial cases display non 1p/19q deleted oligodendroglial tumors with EGFR amplification, which results in EGFR overexpression and is associated with poor prognoses, higher rates of necrosis and older age of the patients (*160*). De-regulated PDGFR signaling is common in oligodendroglial tumors. PDGFRα, as well as ligands for this receptor, are frequently overexpressed in both oligodendrogliomas and anaplastic oligodendrogliomas (*161*). Expression of the PDGFRβ on the surface of endothelial cells suggests that PDGF ligands that are expressed by glioma cells can stimulate both tumor cells and non-transformed cells via autocrine and paracrine stimulation (*31*). *PIK3CA*, a catalytic subunit of PI3K, is mutated in approximately 14% of oligodendroglial tumors (*162*). *PTEN*, a negative regulator of PI3K signaling, is frequently deleted or inactivated by mutations. CIC is a DNA binding transcriptional repressor that is downstream of the MAPK signaling pathway. Inactivating mutations in the gene encoding *CIC* have been found in approximately half of all oligodendroglial tumors (mainly in tumors with 1p/19q loss). The majority of the mutations are located in exon 5, which codes for the DNA binding portion of CIC (*163, 164*).

Genetic alterations that regulate cell cycle control

The tumor suppressor gene *TP53*, which regulates cell cycle control, DNA repair and apoptosis, is the most commonly mutated gene in gliomas. However, in oligodendroglial tumors, mutations in the *TP53* gene are rarely observed (*154*). Other mutations in genes that regulate cell cycle control are nevertheless observed. Retinoblastoma (*RB*), a negative G1-S phase cell cycle regulator, is frequently mutated in oligodendroglial tumors (*165*). *CDKN2A*

and *CDKN2B*, which encode $p16^{INK4a}$ and $p16^{INK4b}$, are negative regulators of the G1-S transition. These genes are inactivated by hypermethylation or homozygous deletion in up to 40% of oligodendroglial tumors (*166*).

Other genetic alterations

FUB1 is a transcriptional regulator of c-Myc expression. Inactivating mutations in *FUB1* have been detected in one-quarter of oligodendroglial tumors, almost exclusively in 1p/19q deleted tumors (*163, 167*). Deletion of chromosome 10q predicts poor progression-free and overall survival, irrespective of 1p/19q status in both oligodendrogliomas and anaplastic oligodendrogliomas (*168*). Deletion of chromosome 9 and amplification of chromosome 7 are also frequent genetic alterations that are found in oligodendroglial tumors (*165*).

Enrichment of the proneural molecular subgroup

The four gene expression subtypes that are found in gliomas (proneural, classical, mesenchymal and neural) are all found in astrocytic tumors. However, the proneural subtype was enriched in oligodendroglial tumors, with three-quarters of the tumors displaying proneural gene expression profiles. The mesenchymal gene signature was not detected in oligodendroglial tumors, but classic or neural gene signatures were detected in one-quarter of oligodendroglial tumors. Patients with oligodendroglial tumors harboring the proneural gene signature had increased survival rates compared to the non-proneural gene expression subtypes (*169*).

Lung cancer

Lung cancer accounts for most cancer related deaths worldwide. Almost one in five cancer related deaths are due to lung cancer, and in 2008, 1.38 million humans died from this disease. The majority of the cases occur in industrialized countries, and worldwide, lung cancer incidence is higher in men than it is in women (*146*). Smoking is heavily associated with lung cancer incidence, and countries with high frequencies of smoking among the population also experience high incidences of lung cancer. The relative risk for lung cancer in smokers is tenfold or more compared to non-smokers. Non-smokers who are subjected to passive smoking have a 20% increased risk of lung cancer (*146*). In Sweden, smoking habits are similar for men and women, and lung cancer incidence is equal. At the time of diagnosis, most

patients have locally advanced or metastatic disease, which is correlated with poor prognoses, overall median survival of 7-11 months and a 5 year survival rate that is less than 10% (*170*).

Histological subtypes of lung cancer

The 2004 WHO classification is currently used for the classification of lung cancer. Lung cancer is classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 85% of all lung cancer cases and is further divided into adenocarcinomas, squamous cell carcinomas (SCC) and large cell carcinomas (Figure 6). These subgroups can be further divided into more than 50 different recognized histological classifications of lung cancer (*146, 170*). Adenocarcinomas of the lung consist of epithelial tumors that arise from the small bronchi, bronchioles or alveoli. These tumors are characterized by mucus production and/or distinct growth patterns (*171, 172*). Adenocarcinomas are often peripherally located in the lung, but may also be found centrally. The incidence of adenocarcinoma is increasing, and adenocarcinoma has now replaced SCC as the most common type of lung cancer (*146, 172*). Approximately 40% of lung cancer patients harbor tumors that display histology that is characteristic of adenocarcinomas. Both smokers and non-smokers may develop this disease. However, adenocarcinomas are the most common lung cancer subtype among non-smokers (*173*). SCC consists of epithelial tumors that arise from the major bronchi and are often located centrally within the lung. These tumors are characterized by keratinization and/or intracellular bridges (*170*). The incidence of SCC is decreasing, and approximately one-quarter of all lung cancer cases display histology that is consistent with SCC. Mainly smokers develop SCC (*173*). Large cell carcinoma is an exclusion diagnosis that is made when the histology does not fit the criteria of either adenocarcinoma or SCC (*170*). This is a heterogeneous group of tumors, but they consist of poorly differentiated cancer cells, and their prognoses are poor. Approximately 10% of all lung cancer cases display large cell histology, and mainly smokers develop this disease (*173*). SCLC is an epithelial tumor type that is characterized by small tumor cells that have neuroendocrine features and high proliferative activities. These tumors are often centrally located, and 2/3 of these patients have advanced disease at diagnosis, which is associated with poor prognoses (*170*).

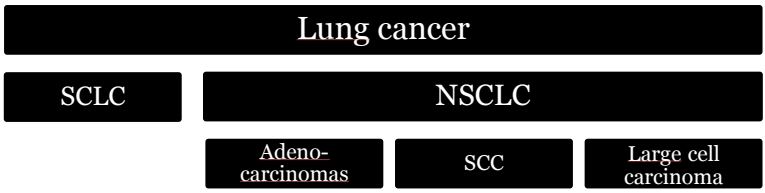

Figure 6: Histological subtypes of human lung cancers. Lung cancer is divided into SCLC and NSCLC. NSCLC is further divided into adenocarcinomas, SCC and large cell carcinomas. These subtypes can be further divided into more than 50 different histologic subgroups.

Molecular profiling and targeted therapies for lung adenocarcinomas

Approximately 30 years ago, *KRAS* was discovered to be mutated in lung cancer. Nearly 10 years ago, *EGFR* was found to be mutated in another, mutually exclusive, set of lung cancers (*15, 174*). Massive sequencing over the past few years has identified many mutations that drive lung tumor formation. Several of these mutations are mutually exclusive. The mutations differ based on histological subtypes, ethnicity, gender, and smoking status.

KRAS

KRAS is mutated in approximately 15-30% of lung adenocarcinomas. These mutations are more frequently found in smokers than in non-smokers, in white (20-30%) than East Asian patients (5%), and in patients with adenocarcinomas versus those with SCC (*175*). The most common mutation is the replacement of glycine at exon 2, codon 12, with another amino acid, such as valine (G12V) or aspartic acid (G12D). These mutations are activating, rendering the GTPase in an active state with the potential to activate the downstream RAF-MEK-ERK signaling pathway, resulting in the positive regulation of cell proliferation (*175*). Attempts have been made to treat these patients with therapeutic strategies that target the KRAS signaling pathway, including KRAS farnesylation and MEK (*176, 177*). These trials have all failed. However, in a phase II clinical trial, sorafenib (Nexavar) was used as a treatment for NSCLC. Sorafenib is a multi-kinase inhibitor which inhibits kinases, including c-Raf, b-Raf, VEGFRs and PDGFRs. Treatment using sorafenib was associated with disease stabilization (*178*). However, to date, there are no clinically approved treatment options that target *KRAS* mutations in these patients.

EGFR

The *EGFR* is frequently mutated in lung adenocarcinoma patients, and these mutations are mutually exclusive to *KRAS* mutations (*17, 179*). Approximately 10% of lung adenocarcinoma patients harbor mutations in the *EGFR* gene, but the frequency of *EGFR* mutations is more common in East Asian patients compared to other ethnic groups, in females than in males, in non-smokers than in smokers, and in tumors that display adenocarcinoma histology compared to other lung cancer histologies (*180*). The most common mutations are small, in-frame deletions of exon 19 and two missense mutations in exon 21: L858R and L861Q. These mutations affect the kinase domain of the receptor and render the EGFR active, independent of ligand binding (*15*). Erlotinib and gefitinib are small molecular inhibitors of EGFR. They bind with higher affinity than phosphate to the kinase domains of the EGFR, which inactivates the receptor (*19, 20*). Treatment of lung adenocarcinoma patients harboring activating EGFR mutations using these inhibitors was the first personalized treatment that was developed for lung cancer. Compared to standard treatment, anti-EGFR treatment has increased progression free survival (PFS) (*181-183*). However, after a median of 10-14 months on these treatments, resistance occurs (*184*). In more than half of resistant tumors, a secondary mutation, T790M, has occurred in *EGFR*. This mutation reduces the effectiveness of EGFR-TKI treatment by decreasing the affinity of EGFR-TKI for the binding pocket in EGFR (*18*). Other resistance mechanisms include MET amplifications (in up to 22% of cases) and the transformation from adenocarcinoma histology to SCLC histology (in up to 14% of the cases). The mechanisms underlying resistance in the remaining patients remain unknown (*184-186*). Today, there is no standard treatment for advanced NSCLC patients with EGFR mutations and induced TKI resistance. However, afatinib (Tomtovok) is a small molecular inhibitor of ERBB family receptors that has activity against both wt and mutated EGFR receptors, including exon 19 deletions and the T790M mutation. Clinical trials using afatinib have shown promising results, with increased PFS and response to treatment in patients harboring $EGFR^{T790M}$ mutations (*187*). As the prevalence of EGFR-TKI resistance increases, the need for a better understanding of the mechanisms underlying resistance and how to treat these patients increases.

EML4-ALK

In 2007, a gene fusion between *EML4* and anaplastic lymphoma kinase (*ALK*), *EML4-ALK*, was described in NSCLC (*188*). ALK is an RTK that is

expressed in neural cells, and its downstream signaling is mediated through the MAPK, PI3K and JAK-STAT pathways (*189*). The fusion protein is composed of the N-terminal part of EML4 and the intracellular part of ALK, including the kinase domain. The fusion protein results in constitutive activation of the ALK kinase domain, downstream signaling pathways and cellular proliferation and survival (*188*). The *EML4-ALK* translocation is found in approximately 3-4.5% of lung adenocarcinoma patients. It is mutually exclusive to mutations in the *EGFR* or *KRAS* genes and more common in younger patients, non-smokers/light smokers and patients with adenocarcinoma histology (*190*). Crizotinib (Xalkori) is a small molecular inhibitor of multiple TKs, such as MET and ALK. Treatment of EML4-ALK-positive advanced NSCLC patients with crizotinib has shown promising results and is now approved for locally advanced or metastatic NSCLC (*25, 191, 192*). However, resistance to crizotinib inevitably occurs months or years after treatment initiation. Resistance is mediated by different mechanisms including secondary resistance mutations in the kinase domain of the *EML4-ALK* fusion gene, amplification of the *EML4-ALK* fusion gene, activation of other oncogenes such as *EGFR* or *KIT* and additional unknown causes (*193*).

Genes regulating the cell cycle

Mutations in the *TP53* gene are the most common alterations of tumor suppressor genes in lung adenocarcinoma. Approximately 65-70% of lung adenocarcinoma patients harbor mutations in this gene (*172*). The cell-cycle checkpoint kinases *ATM* and *RB1* are mutated in 7% and 4% of patients with lung adenocarcinoma, respectively (*179*).

ROS1 rearrangements

ROS1 is an RTK in the insulin receptor family. *ROS1* rearrangements containing the kinase domain of ROS1 and different translocation partners have been detected in approximately 2% of patients with NSCLC. These rearrangements are more commonly found in non-smokers.

Other mutations found in lung adenocarcinoma

The following mutations have also been found in lung adenocarcinomas: the oncogenes *MET* (14%), *ERBB2* (2-4%), *ERBB3* (2%), *ERBB4* (4-5%), *PDGFRA* (4%), *KDR* (2-5%), *EPHA3* (5%) and *MAP2K1* (5%) and the tumor suppressor genes *STK11* (20-30%), *LRP1B* (9%) and *NF1* (7%) (*172, 179*).

Molecular profiling of SCC of the lung

In contrast to adenocarcinoma, the driving mutations in lung SCC are poorly understood, and to date, no targeted therapies exist for this disease. Nevertheless, recent studies have permitted insight into the molecular alterations of lung SCC. These studies have identified targetable mutations in up to 96% of lung SCC cases, giving hope for future targeted therapies (*194, 195*).

Cell cycle control

The regulation of the cell cycle control is altered in a majority of lung SCC patients. Mutation of the *TP53* gene is found in 81% of lung SCC patients (*195*). *CDKN2A* is inactivated in 72% and *RB1* is mutated in an additional 7% of lung SCC tumors (*195*).

RTKs and downstream signaling

Mutations of different RTKs and downstream signaling molecules are present in a majority of the lung SCC samples analyzed. The fibroblast growth factor 1 (*FGFR1*) is mutated or amplified in approximately 7-20% of lung SCC samples. Treatment with an FGFR1 inhibitor in a mouse xenograft model results in tumor regression, and several FGFR1 inhibitors are now in early phase clinical testing (*195, 196*). *FGFR2* and *FGFR3* are mutated in 3% and 2% of SCC patients, respectively (*195*). Approximately 4% of lung SCC patients have mutations in the discoidin domain receptor 2 (*DDR2*). Dasatinib has shown promising results as an inhibitor of DDR2 and is now being evaluated in a phase II study (*195, 197*). *EGFR*, *ERBB2* and *ERBB3* are amplified in 9%, 4% and 2%, respectively, of SCC tumors. However, mutations of these genes are rare (*195, 198*). *PDGFRA* is amplified or mutated in another 9% of SCC patients (*195*), and the downstream signaling molecule *BRAF* is mutated in 0-4% of patients (*195, 198*). Mutations have also been identified in several genes involved in the PI3K pathway. *PIK3CA* is mutated in 8-16% of lung SCC patients (*195, 198*). The tumor suppressor gene *PTEN* is mutated or deleted in approximately 15-28% of lung SCC patients, and it has been shown to be methylated in 35% of these tumors (*194, 195*).

Response to oxidative stress

Genes regulating the response to oxidative stress are altered in a third of SCC tumors, with mutations and copy number alterations of *KEAP1* in 12%, *NFE2L2* in 19% and mutations or deletions in *CUL3* in 7% of patients (*195*).

Genes regulating squamous differentiation

Genes involved in squamous cell differentiation are altered in almost half of lung SCC patients. *SOX2* and *TP63* are amplified in 21% and 16% of cases, respectively. *NOTCH1*, *NOTCH2* and *ASCL4* are mutated in 8%, 5% and 3% of cases, respectively, and *FOXP1* is deleted in 4% of lung SCC patients (*195*).

Aims

The aim of this thesis was to improve our understanding of the roles that LRIG proteins play in psoriasis and cancer. Specifically, we sought to accomplish the following:

- Investigate whether LRIG mRNA or protein expression is de-regulated in psoriasis.

- Investigate the effects of *Lrig2* deletion in mice, and analyze whether Lrig2 plays a role in gliomagenesis in mice.

- Investigate the clinical importance and biological role of LRIG1 in lung cancer.

- Identify cytosolic interacting protein partners for LRIG3, and analyze their role in lung cancer.

Materials and methods

Human skin and lung samples (I)

Punch biopsies from 25 psoriatic patients and 15 age- and sex-matched controls were taken and treated as described previously (*199*). A human lung cancer tissue micro-array (TMA) containing surgical material from 363 patients, with two cores from each patient, was studied. All major subgroups of lung cancer were represented, but the TMA mainly consisted of low stage adenocarcinoma and SCC samples. A database containing information on patient age, performance status, smoking habits, gender, histological subtype and survival was included.

RNA samples (I, II, IV)

The extraction of total RNA from normal and psoriatic skin (*200*) and lung (*201*) has previously been described. Total RNA from the following normal human tissues were analyzed: ovary, adrenal gland, bladder, small intestine, testicle, thymus, stomach, colon, spleen, kidney, placenta, heart, liver, brain, thyroid and lung. Total RNA from cell lines was prepared using the RNAqueous kit and rendered DNA-free using the TURBO DNA-free kit.

Quantitative real-time RT-PCR, primers and probes (I, II, IV)

Quantitative real-time RT-PCR was performed as previously described with the following primers and probes: *LRIG1*, *LRIG2*, *LRIG3* and *Rn18S* (*49, 52*); *LMO7*, Hs01009229_m1; *LIMCH1,* Hs00971627_m1; *Fos*, Mm00487425_m1; *Egr1*, Mm00656724_m1 and *Egr2,* Mm00456650_m1. We used the ThermoScript™ RT-PCR System or qScript 1-Step qRT-PCR Kit. Standard curves were generated from the following sources: *LMO7* and *LIMCH1*, *in vitro* transcribed RNA; *LRIG1*, *LRIG2* and *LRIG3*, total RNA from RS; *Rn18s,* total RNA from HEL2; *Fos*, *Egr1* and *Egr2*, total RNA from MEFs. Expression levels were normalized to *Rn18s*. All samples were run in triplicate.

Immunohistochemistry (I, II, III, IV)

For immunohistochemistry (IHC), human skin biopsies were fixed in formalin, embedded in paraffin, sectioned and stained using a Ventana staining machine as previously described (*199*), except that the antigen retrieval was performed in a 10 mM citrate buffer, pH 7.3. Sections were stained for LRIG1, LRIG2 and LRIG3 using polyclonal rabbit anti-LRIG antibodies at the following concentrations: LRIG1 3 µg/ml, LRIG2 5.8 µg/ml and LRIG3 11 µg/ml (*53-55*). Mice brains were sectioned and stained with hematoxylin and eosin using standard methods. The human lung TMA was stained with the following antibodies and concentrations: rabbit anti-LRIG1: 0.5 µg/ml; rabbit anti-LRIG2: 1 µg/ml; rabbit anti-LRIG3: 2.2 µg/ml and rabbit anti-LMO7-1250: 6 µg/ml.

Mouse strains and models

Mice were kept under controlled conditions with a 12-hour day/night cycle. Water and pellets were provided *ad libitum*. *Ntv-a* mice were obtained from Eric Holland and Lene Uhrbom. Mice harboring the human *EGFR*^{L858R} gene (L858R EGFR [L57]) were obtained from Katerina Politi and Harold Varmus (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). The CCSP-rtTA mouse strain carrying the *rtTA* transgene (CCSP-rtTA(cc10)) was provided by Jeffrey A. Whitsett (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA).

Generation of Lrig1$^{-/-}$ and Lrig2E12$^{-/-}$ mice

Construction of the targeting vectors and generation of the mice with floxed and deleted *Lrig1* or *Lrig2E12* was performed at Ozgene (Bentley DC, WA, Australia). Briefly, the 5´, loxP and 3´ homology arms were generated by PCR amplification from genomic DNA of 129Sv/J mice. Targeted vectors were electroporated into 129Sv/J mouse embryonic stem (ES) cells, and the transformed ES cells were injected into C57BL/6 blastocysts. To generate knockout (ko) mice, *Lrig1* and *Lrig2E12* floxed mice were crossed with Oz-Cre transgenic mice. The *Cre* gene was removed by back-crossing the mice against C57BL/6.

Study of glioma using the Ntv-a RCAS/PDGFB mouse model

To study the genesis of gliomas, we used the Ntv-a RCAS/PDGFB mouse model. Replication-competent ALV splice acceptor (RCAS) is an avian retrovirus that can be used as a carrier of oncogenes such as PDGFB. Transfection of RCAS/PDGFB into a chicken fibroblast cell line, DF-1, generates infected cells that produce RCAS/PDGFB retroviruses. These viruses can only infect cells that express the avian retroviral receptor Tv-a. This receptor is not expressed by mammalian cells. Therefore, normal mouse cells cannot be infected. However, mouse cells that are genetically modified to express the Tv-a receptor can be infected by RCAS. Several different mouse strains have been developed that are engineered to express Tv-a under the control of various tissue specific promoters. These strains include the Ntv-a, Ctv-a and Gtv-a mouse strains. The Ntv-a mouse strain expresses the *Tv-a* gene under the control of the *Nestin* promoter, resulting in Tv-a receptor expression by neuroepithelial stem cells and early neural progenitor cells. Injection of RCAS/PDGFB-producing DF-1 cells into the brain of newborn mice results in infection followed by the production of the oncoprotein PDGFB by the neuroepithelial cells and early neural progenitor cells. This process results in the formation of grade II and III oligodendroglial-like tumors and glioblastomas (*37, 202*).

Newborn Ntv-a mice were intracranially injected in the right frontal region, using a 10-μl Hamilton syringe, with 2 μl of RCAS-PDGFB-HA-producing DF-1 chicken fibroblasts, as previously described (*202*). Twelve weeks later, the mice were sacrificed, and the tumors analyzed.

Study of lung cancer using the L858R57/rtTA mouse model

To study the role of Lrig1 in EGFR-driven lung cancer, we used the L858R57/rtTA mouse model. Transgenic mice carrying the human $EGFR^{L858R}$ mutation, under the control of a TetO promoter, were cross bred with CCSP-rtTA mice. The CCSP-rtTA mice express the *rtTA* gene under the control of the *Scgb1a1* promoter, which is only active in bronchial and type II epithelial cells in the lungs. When fed a doxycycline diet, L858R57/rtTA mice express rtTA in their lung endothelial cells, which activates the expression of $EGFR^{L858R}$ in these cells. This process generates diffusely infiltrating adenocarcinoma lung tumors (*203*). To study the effect of Lrig1 on lung tumor formation, L858R57/rtTA mice were cross bred with $Lrig1^{+/-}$ mice. Lung tumor formation was studied using magnetic resonance imaging (MRI) in L858R57/rtTA mice that were either $Lrig1^{-/-}$ or $Lrig1^{+/+}$.

Study of lung cancer using a mouse xenograft model

Balb/cA nude mice were subcutaneously implanted with $5x10^6$ parental or LRIG1-inducible H1975 cells. Parental cells were implanted on the right flank and LRIG1-inducible cells on the left flank. Mice were fed standard or doxycycline-containing feed pellets. Tumor volumes were analyzed by MRI.

Western blotting (II, IV)

Cells were grown in six-well plates and lysed in a 1% Triton x-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl lysis buffer supplemented with a complete protease inhibitor. For the analysis of the phosphorylated proteins, the cells were lysed in lysis buffer supplemented with 10 mM NaF, 1 mM EGTA, 1 mM NaVP4, 20 mM beta-glycerophosphate, and an EDTA-free protease inhibitor cocktail. Proteins were separated with SDS-PAGE and analyzed using Western blot. The primary antibodies used were the following: mouse anti-Actin, rabbit-anti-p44/42, rabbit anti-Akt, rabbit anti-phospho-Akt, mouse anti-phospho-p44/42, rabbit anti-phospho-EGFR, rabbit anti-phospho-Met, mouse anti-actin, mouse anti-FLAG M2, rabbit anti-PDGFRα, rabbit anti-PDGFRβ, rabbit anti-EGFR, mouse anti-Met, rabbit anti-LMO7-55, rabbit anti-LMO7-1250, rabbit anti-LIMCH1-6, rabbit anti-LIMCH1-945, rabbit anti-mLrig2-147 and LRIG1-151. Appropriate secondary HRP-conjugated antibodies were used. Images were quantified using the Chemidoc XRS system and Quantity One software.

Plasmids (II, III, IV)

The vectors *p3XFLAG-LRIG1*, *p3XFLAG-LRIG2* and *p3XFLAG-LRIG1* encoding the FLAG-tagged LRIG proteins were generated by cloning PCR-amplified *LRIG1, LRIG2* and *LRIG3* cDNAs, respectively, into the *p3X-FLAG-CMV* vector. The *pMH-LMO7* and *pMH-LIMCH1* expression vectors were generated by cloning PCR-amplified *LMO7* and *LIMCH1*, respectively, into the pMH vector, resulting in fusion proteins tagging LMO7 and LIMCH1 C-terminally with HA. The *pLVX-LRIG1-IRES-ZsGreen* and *pLVX-LRIG2-IRES-ZsGreen* plasmids were generated by cloning PCR-amplified *LRIG1* and *LRIG2*, respectively, into the *pLVX-IRES-ZsGreen1* vector, resulting in a fusion protein encoding either LRIG1 or LRIG2 tagged with 3xFLAG, together with ZsGreen. The *pLVX-LRIG1-TRE3G* vector was generated by cloning PCR-amplified *LRIG1* into *pLVX-TRE3G*, resulting in doxycycline-inducible expression of LRIG1. The expression vector encoding myc-tagged LRIG1 [11] was kindly provided by Colleen Sweeney (UC Davis, Sacramento,

CA, USA). The expression vector *pcDNA3-PDGFRα* was a kind gift of Carl-Henrik Heldin (Ludwig Institute, Uppsala, Sweden).

Cell lines (II, III, IV)

Cells were cultured at 37°C in a humidified incubator with 5% CO_2. Cells were cultured in the following cell culture media: COS-7, DF1, HEK293 and Lenti-X 293T were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin; H460, H1975, and H1819 (kindly provided by John D Minna (UT Southwestern Medical Center, Dallas, TX, USA)) were cultured in RPMI medium 1640 supplemented with 10% FBS and 50 μg/ml gentamicin. Primary MEF cells were isolated from E12.5 or E13.5 Lrig2E12$^{+/-}$ crosses and cultured in DMEM supplemented with 10% FBS and 50 μg/ml gentamicin, MEM non-essential amino acids and 50 μM 2-mercaptoethanol; Primary *Ntv-a* cells were established as previously described (*202*) and cultured in DMEM as follows: Nutrient Mixture F-12 supplemented with N2 supplement, 100 units/ml penicillin, and 100 μg/ml streptomycin with a daily addition of 10 ng/ml bFGF.

Confocal laser scanning microscopy (II, IV)

Cells were grown on cover slips, fixed and permeabilized using 4% paraformaldehyde, and blocked in PBS containing 5% FBS and 0.05% saponine. The primary antibodies used were the following: mouse-anti-FLAG M2, mouse-anti acetylated-tubulin, rat-anti-PDGFRα, rat-anti-PDGFRβ, rabbit anti-LMO7-1250 and high affinity rat anti-HA. The appropriate secondary AlexaFluor-conjugated antibodies were used. Nuclei were visualized using Pro Long Gold. Actin filaments were stained using AlexaFluor 647 Phalloidin. The 3D images were acquired using confocal laser scanning microscopy.

Proximity Ligation Assay (II, IV)

Cells were grown and treated on glass cover slips and then fixed and permeabilized using ice-cold methanol at -20°C for ten minutes. The cells were washed three times using ice-cold PBS and blocked using PBS containing 5% FCS and 0.1% Tween for one hour. The proximity ligation assay (PLA) was then performed using the DuolinkII kit. For the PDGFR phosphorylation experiments, cells were starved for 24 hours and then

stimulated with 10 or 50 ng/ml of PDGF-BB for ten minutes prior to fixation. The primary antibodies used were rabbit anti-PDGFRα, rabbit anti-PDGFRβ, mouse anti-phospho-tyrosine, rabbit anti-LRIG1 30-45, rabbit anti-LRIG1 151-165, rabbit anti-LRIG2 LIG2-151, rabbit anti-LRIG2 LIG2C, rabbit anti-LRIG3 32-47, rabbit anti-LRIG3 207-221, mouse anti-LMO7, mouse IgG2a kappa and rabbit polyclonal IgG.

Gene transduction

To generate stably transduced cell lines, the Lenti-X Lentiviral Expression System was used. Lenti-X 293T cells were cotransfected with the Lenti-X HT Packaging Mix and the relevant expression vectors using the Xfect transfection reagent according to the manufacturer's instructions. Forty-eight hours post transfection, the lentiviruses were harvested from the supernatant and used for the transduction of the human lung cancer cell line H1975. Stably transduced cells were selected using puromycin 1 μg/ml and G418 1 mg/ml

siRNA

Nine thousand cells were transfected in a twelve-well chamber with 15 nM of LMO7 siRNA or 15 nM of the silencer negative control siRNA vector using the siPORT NeoFX transfection reagent. Forty-eight hours post transfection, the cells were fixed or lysed.

Statistics (I, II, III, IV)

All statistical analyses were performed using the SPSS statistical package. The Wilcoxon signed-rank test was used to analyze the mRNA levels in clinically normal vs. psoriatic skin (I). The differences in *Lrig* expression in the brain were analyzed using the Kruskal-Wallis test (II). The differences in organ weights were analyzed using Student's t-test for independent samples (II). Survival was estimated using Kaplan-Meier plots, and the differences between curves were analyzed using the log rank test (II, III, IV). Differences in body weight, *Fos* and *Egr2* expression, and levels of total and phosphorylated proteins were analyzed using Student's paired t-test (II). The differences in brain tumor incidence and grade were analyzed using the chi-square test for independence (II). Multivariate survival analysis for the TMA data was performed using Cox regression (III, IV). To compare the means of *in vitro* and *in vivo* data, the Mann-Whitney test was used (III). The

significance level was set to $p<0.05$ for all analyses except for the analysis of relative organ weights, for which the significance level was set to $p<0.01$ because of the large number of tests (51 pairwise tests).

Ethics

All experiments were approved by the relevant local ethics committees as follows: Human RNA (I), R 597-98 Göteborg; Human epidermis (I), 04-035 M Regional Ethics Committee of Umeå; Glioma formation *in vivo* (II), A12-07, A5-2010, A 25-07 and A52-10 centrala försöksdjursnämnden; Lung tumor formation *in vivo* (III), A 42-10 jordbruksverket; Lung TMA (IV), 2006/325 and 2012/532 Uppsala, Xenograft model (III), A167-12 Umeå. All mice were housed and maintained, and all experiments were performed, in accordance with the European Communities Council Directive (86/609/EEC).

Results and discussion

LRIG in normal and psoriatic skin (I)

The ablation of *Lrig1* in mice results in the development of psoriasis-like lesions on the snouts and tails (*66*). To analyze whether LRIG mRNA or protein expression was de-regulated in psoriasis, we analyzed both the levels of mRNA and the intensity of LRIG immuno-reactivity, and the subcellular localization of the LRIG proteins in healthy and psoriatic skin.

Levels of LRIG in normal and psoriatic skin

The mRNA levels of *LRIG1, LRIG2* and *LRIG3* were analyzed in healthy and psoriatic skin from five psoriatic patients, and the intensity of LRIG immuno-reactivity was investigated by staining epidermal sections from 25 psoriatic and 15 age- and sex-matched controls for LRIG1, -2 or -3. There was no difference in either the mRNA levels or in the intensity of the LRIG immuno-reactivity between psoriatic and normal skin. This result contrasts with a study in which LRIG1 was decreased in psoriatic skin compared to normal skin (*66*). The antibody used in that study recognized the ectodomain of LRIG1, whereas ours recognized the cytosolic tail. This distinction may indicate that the ectodomain of LRIG1 is cleaved in the psoriatic epidermis.

Subcellular localization of LRIG proteins in normal and psoriatic skin

The immuno-reactivity of the LRIG proteins was readily detected in both normal and psoriatic skin. However, the distribution of all LRIG proteins was altered in psoriatic versus normal skin. In psoriatic skin, the LRIG expression was enhanced, with more LRIG-positive cells in the spinous layer, and the cell surface localization of both LRIG1 and LRIG2 was absent. The LRIG proteins are transmembrane proteins, and LRIG1 has been shown to interact with several RTKs, including the EGFR, and to down-regulate their signaling (*56*). In psoriatic skin, EGFR and the EGFR ligands are overexpressed, and there is increased signaling of the EGFR pathway, which results in keratinocyte hyperproliferation (*130*). The lack of LRIG1 at the cell surface in psoriatic skin may indicate that LRIG1 no longer has the potential to physically interact with RTKs and to down-regulate their signaling, at

least not in the cell membrane. Psoriatic skin is characterized by inflammation and hyperproliferation of keratinocytes. The mechanism of the altered localization of the LRIG proteins is not known. It is possible that inflammatory cytokines released by immuno-inflammatory cells in the psoriatic skin result in altered subcellular localization of the LRIG proteins in keratinocytes. No experiments to analyze this hypothesis have been performed. Both LRIG1 and LRIG3 were frequently located in the nuclei, in normal and psoriatic skin. The function of nuclear LRIG proteins is not known. Nevertheless, nuclear localization has been detected in other cells and tissues, including COS-7 cells, prostate, skin and the cerebral cortex (*54*). We detected nuclear LRIG1 expression in scattered cells within the basal layer of both normal and psoriatic skin. Studies have shown that LRIG1 is expressed by epidermal stem cells located in the basal layer and that LRIG1 regulates epidermal stem cell quiescence (*70*). The LRIG1-positive cells we detected may represent epidermal stem cells. However, further experiments are needed to determine whether these LRIG1-positive cells are stem cells. In this study, we found LRIG1-positive cells both in the basal and spinous layers. Previous studies used different antibodies, which may explain the difference in protein localization.

Generation of *Lrig2E12* ablated mice (II)

To study the functions of Lrig2 *in vivo*, we generated *Lrig2E12* ko mice in a C57BL/6 background. The *Lrig2* gene was ablated by deleting exon 12 via homologous and Cre-mediated recombination. The deletion of exon 12 resulted in a frame shift of the reading frame and generated multiple immediate stop codons. Southern blot, cDNA sequencing, quantitative real-time RT-PCR and Western blotting confirmed the deletion of exon 12 and the loss of the full-length Lrig2 protein. There remains, however, a possibility that a truncated protein consisting of leucine-rich repeats was expressed. Due to a lack of reagents, *e.g.*, an antibody against the mouse ectodomain of Lrig2, we have not been able to address this possibility. Despite the possibility of a truncated protein, we observed a clear phenotype attributable to the deletion of *Lrig2E12*.

Phenotype of *Lrig2E12* ablated mice (II)

Lrig2E12$^{-/-}$ mice were viable, born at Mendelian frequencies and had no obvious macroscopic defects or behavioral abnormalities. However, these mice had an increased mortality rate and a transient reduction in growth rate.

Anatomy

Lrig2E12$^{-/-}$ mice were transiently smaller than wt mice but appeared anatomically normal. Eleven organs, including the brain, lungs and liver, from healthy female and male *Lrig2E12* wt, heterozygous and ko mice were collected and examined. There were no macroscopic differences in the organs between the genotypes. The organs were weighed, but there were no relative differences in organ size between the genotypes. Nevertheless, the possibility remains that *Lrig2E12*$^{-/-}$ mice may have subtle changes in their organs that were not detected in the routine necropsy performed. Further studies at the cellular and molecular levels are needed to determine whether the organs and tissues in the *Lrig2E12*$^{-/-}$ mice are normal.

Increased spontaneous mortality

To study the survival of *Lrig2E12*$^{-/-}$ mice, 153 male and 159 female mice were followed for 200 days. Fifty days after birth, 12% of the *Lrig2E12*$^{-/-}$ male mice had died. They were either found dead in their cages or were euthanized due to the sudden onset of severe disease detected as emaciation, hackled fur and/or crouched body position. Between 100 and 130 days of age, another 12% of the male mice died. Fifty days after birth, 20% of the *Lrig2E12*$^{-/-}$ female mice had died. All wt and heterozygous mice, except one female wt mouse, were alive at the end of the experiment. Hence, there was an increased mortality rate in both male and female *Lrig2E12*$^{-/-}$ mice compared to the wt mice, and the time of death differed between the genders. Five mice that had to be euthanized due to sudden illness were sent to the National Veterinary Institute (SVA) for necropsy. These mice were all emaciated. One mouse suffered from purulent cystitis and pyelonephritis, and another male had a moderate accumulation of degenerative neutrophils in the lumen of the accessory genital glands. Except for emaciation, the remaining mice showed no anatomical explanation for the sudden onset of severe disease. Due to the severity of the phenotype with sudden severe disease, it is surprising that the *Lrig2E12*$^{-/-}$ mice were grossly anatomically normal. Therefore, the reason for the increased mortality in the *Lrig2E12*$^{-/-}$ mice remains unresolved. However, in humans, mutations in LRIG2 cause urofacial syndrome. This syndrome is a rare autosomal disease that results in facial grimaces when smiling and urinary tract problems including incontinence, urinary tract infections and kidney failure (*91*). If left untreated, this disease may be lethal. Interestingly, one of the *Lrig2E12*$^{-/-}$ mice that underwent necropsy suffered from purulent cystitis and pyelonephritis, a condition detected in humans with urofacial syndrome. Nevertheless, this syndrome would only explain the death of one of the five

mice. This situation calls for thorough examinations of organs such as kidney, heart and brain of the *Lrig2E12*$^{-/-}$ mice to determine the reason for the increased mortality.

Transiently reduced growth rate

At E13.5 and birth, there was no weight difference between the *Lrig2* genotypes. However, 5 days after birth, the *Lrig2E12*$^{-/-}$ mice were significantly smaller than the wt mice. The mice were weighed weekly, and male and female *Lrig2E12*$^{-/-}$ mice were significantly smaller than their littermates until 15 or 12 weeks of age, respectively. There are many studies documenting weight reduction in genetically modified mice, and it is often an idiopathic phenomenon. The reason for the transiently reduced growth rate in *Lrig2E12*$^{-/-}$ mice is not known. Many factors are involved in the regulation of body weight. It is possible that the *Lrig2E12*$^{-/-}$ mice suffered from a transient de-regulation of hormones that regulate appetite or nutritional uptake from the intestines. Another possibility is that the mice were born with malfunctioning intestines. It would be of interest to analyze hormone levels in the blood of *Lrig2E12*$^{-/-}$ mice to determine whether, for example, pituitary hormones are responsible for the transiently reduced growth rate. A thorough examination of the mouse intestines would also reveal possible microscopic defects of the intestines not discovered in the macroscopic analysis.

Lrig2 promoted glioma formation *in vivo* (II)

LRIG2 has been shown to be an independent negative prognostic factor for oligodendroglioma patients (*94*). To study the role of *Lrig2* in gliomagenesis, we utilized an experimental glioma mouse model. Newborn Ntv-a mice of the different *Lrig2E12* genotypes were injected with RCAS/PDGFB-producing DF-1 cells. In these mice, the RCAS retrovirus can integrate stably into the genome of neuroepithelial stem cells and early neural progenitor cells, which will then overexpress the *PDGFB* oncogene. All *Lrig2E12* wt mice developed tumors; 18% of the tumors were grade IV, and the remaining were oligodendroglioma-like type II/III tumors. All *Lrig2E12*$^{+/-}$ mice also developed tumors; 8% of the tumors were grade IV, and the remaining were oligodendroglioma-like grade II/III tumors. Among the *Lrig2E12*$^{-/-}$ mice, only 77% developed tumors, and they were all oligodendroglioma-like grade II/III tumors. These data strongly indicate that *Lrig2* promoted both the incidence and malignancy of glioma *in vivo*. These data are also in line with

the previous finding that cytoplasmic LRIG2 expression is a negative prognostic factor for oligodendroglioma patients (*94*).

Effect of *Lrig2* on PDGFR signaling (II)

To investigate whether Lrig2 has any effect on Pdgfr signaling, we studied Pdgfr signaling in mouse embryonic fibroblasts (MEF) of the different *Lrig2E12* genotypes. We analyzed the levels of Pdgfrα and Pdgfrβ after serum starvation, measured primary cilium formation, and assessed the phosphorylation status of the Pdgfrs and the downstream effector molecules Akt and Erk1/2 after stimulation with PDGF-BB. We also analyzed the effects of PDGFR levels after LRIG2 overexpression. We found no difference between the *Lrig2E12* genotypes in any of these analyses. However, when we investigated the induction of the immediate-early genes *Fos* and *Egr2* in response to PDGF-BB stimulation, we discovered that the kinetics differed between wt and *Lrig2E12*$^{-/-}$ MEFs. The induction of both *Fos* and *Egr2* was faster in the *Lrig2E12*$^{-/-}$ compared to the wt MEFs. In the *Lrig2E12*$^{-/-}$ MEFs, there was an increased induction of *Fos* at 40 minutes of PDGF-BB stimulation relative to the wt MEFs ($p = 0.004$). For the *Egr2* induction, we observed a significant increase in the induction of the *Lrig2E12*$^{-/-}$ MEFs at both 20 and 40 minutes of stimulation ($p = 0.027$ and $p = 0.007$, respectively). For *Fos* and *Egr2* we also observed a trend towards a decrease in the induction of *Lrig2E12*$^{-/-}$ MEFs compared to wt at 60 and 120 minutes, respectively. These data indicate that Lrig2 affects the induction of immediate-early genes in MEFs in response to PDGF-BB stimulation. The induction of the immediate-early gene *Egr1* after stimulation with PDGF-BB was also analyzed. *Lrig2E12*$^{-/-}$ MEFs had a trend for increased induction after 40 minutes of stimulation ($p=0.078$) and a non-significant decreased induction after 60 minutes relative to the wt (unpublished observations). These data further demonstrate the involvement of Lrig2 in the induction of immediate-early genes. However, we do not yet understand the mechanism underlying this effect. The apparent lack of effect by Lrig2 on the levels or activation of Pdgfrs and their downstream signaling molecules Akt and Erk1/2 may be explained as follows. Lrig2 may have an effect on molecules between Akt or Erk1/2 and on the induction of immediate-early genes. It could also be that Lrig2 induces subtle differences in Pdgfr signaling that may be difficult to study at the level of individual phosphorylation steps, but these subtle differences may result in altered kinetics of induction. Pdgfr signaling is complex, and Lrig2 may affect other signaling pathways influenced by Pdgfr, such as the JAK/STAT pathway, which was not analyzed in this study. To fully understand the effects of Lrig2 on Pdgfr signaling, further investigations are needed.

Importance of LRIG proteins in human lung cancer (III)

LRIG1 is a tumor suppressor, at least in the mouse intestine, and high LRIG1 expression correlates with better patient survival in several cancers, such as cervical carcinoma and breast cancer (*63, 95*). However, the biological role of LRIG1 in lung cancer is not clear, and the role of LRIG2 and LRIG3 in lung cancer had not been previously studied. We stained a human lung tumor TMA consisting of samples from 363 patients with antibodies specific for LRIG1, LRIG2 and LRIG3 and evaluated the staining intensity, the proportion of stained cancer cells, and the subcellular localization of the immuno-reactivity. LRIG1 was expressed in 47% of the tumors, and all LRIG1-positive cells expressed LRIG1 in the nucleus. Kaplan Meier analysis showed that patients with high LRIG1 expression had a significant survival benefit compared to LRIG1-negative patients ($p = 0.012$). The median survival was 991 days longer in patients with high LRIG1 expression compared to those with no expression. Stratification of histological subtypes indicated that LRIG1 expression was most important in patients with lung adenocarcinoma. Multivariate Cox regression comparing patients with high LRIG1 expression to those with no expression and adjusted for clinical prognostic factors showed a decreased risk of death in the LRIG1 high expressing group ($p = 0.004$), which revealed LRIG1 to be an independent prognostic factor in human lung cancer. LRIG2 and LRIG3 were expressed in 85% and 33% of the tumors, respectively. The expression pattern was mainly cytoplasmic for both proteins, and the Kaplan Meier analysis showed no survival effect for either protein. These data imply that LRIG1, but not LRIG2 or LRIG3, is important in lung cancer. Patients expressing high levels of LRIG1 had a 2.7 year survival benefit compared to non-expressing patients, which demonstrates a clear effect of LRIG1 on human lung patient survival. The importance of LRIG1 as a prognostic marker for human lung cancer will be discussed later.

Phenotype of *Lrig1* ablated mice (III)

To generate *Lrig1*$^{-/-}$ mice, we crossed and intercrossed C57BL/6 *Lrig1*$^{+/-}$ mice with mice from a mixed background. *Lrig1*$^{-/-}$ mice were born viable, at a Mendelian rate and without obvious macroscopic defects. This phenotype was in sharp contrast to other *Lrig1* ko mice that have previously been described. Lrig1 ko mice in a mixed background developed psoriasis-like lesions on their snouts and tails (*66*) and hyperplasia of the epithelial cells in the airways (*65*). In an FVB/N background, *Lrig1* ko mice developed grossly enlarged intestines and had to be euthanized 10 days after birth due to the severity of the phenotype (*85*). In a background mix of C57BL/6 and Sv129,

Lrig1 ko mice developed duodenal adenocarcinoma (*84*). The different *Lrig1* ko phenotypes in the different genetic backgrounds show the importance and effect of the genetic background.

Effect of *Lrig1* on EGFR^{L858R} induced lung tumor formation *in vivo* (III)

To study the importance of Lrig1 in lung tumor formation *in vivo*, we used a mouse model with inducible induction of lung tumor formation. The mouse model carried the human *EGFR*^{L858R}, which is one of the most common genetic alterations of the *EGFR* in NSCLC, coupled to a pneumocyte-specific promoter with a Tet-On transcriptional activation system. Upon treatment with doxycycline, these mice develop diffusely infiltrating EGFR^{L858R}-driven lung tumors. To study the effect of Lrig1 in these tumors, the mice were cross-bred with C57BL/6 *Lrig1*$^{+/-}$ mice, and the tumor formation in *Lrig1*$^{+/+}$ and *Lrig1*$^{-/-}$ mice was analyzed by MRI 60 and 90 days after doxycycline treatment initiation. Tumor formation was quantified using a semi quantitative approach, which showed that *Lrig1* wt mice harbored smaller tumors compared to *Lrig1*$^{-/-}$ mice at 60 days ($p = 0.025$), but not at 90 days. These data indicate that *Lrig1* suppressed early lung tumor growth or tumor initiation. These data are in line with a previous study where *Lrig1*$^{-/-}$ mice demonstrated an increased proliferation of bronchial epithelial cells (*65*).

Effects of ectopic *LRIG1* expression on human EGFR$^{L858R+T790M}$ lung cancer cells *in vivo* and *in vitro*

To further study the possible tumor suppressive effects of LRIG1 in lung cancer, we studied tumor formation in an *in vivo* xenograft model. H1975 is a lung cancer cell line derived from human lung adenocarcinoma that harbors the EGFR$^{L858R+T790M}$ double mutations. The T790M mutation confers EGFR-TKI resistance and is frequently found in patients who have developed treatment resistance. The H1975 cell line was transduced with an empty vector or a doxycycline-inducible LRIG1 expression vector. Sixteen Balb/cA nude mice were injected subcutaneously with $5x10^6$ H1975 parental cells on the right flank and inducible LRIG1 overexpressing H1975 cells on the left flank. Eight mice were put on a doxycycline diet to induce LRIG1 expression in LRIG1 doxycycline-inducible cells. Eighteen days after injection, once large tumors had formed, the tumors and lungs of the mice were scanned using MRI. Analysis of the MRI data showed that there was a significant difference in the tumor volumes of the LRIG1-inducible tumors between the mice that had or had not been treated with doxycycline ($p =$

0.045) where the LRIG1-induced tumors were smaller. Comparing the tumor volume ratio in doxycycline-treated mice between parental and LRIG1 induced tumors showed a significant difference (p=0.025), where tumors formed by induced LRIG1 overexpressing cells were smaller than tumors formed by parental cells. To study the mechanisms underlying the tumor suppressive effects of ectopically expressed LRIG1, we analyzed cell proliferation *in vitro*. H1975 cells were stably transduced with LRIG1, LRIG2 or the empty *pLVX-IRES-ZsGreen* vector as a control. Stably transduced cells thus expressed a green fluorescent protein, and the frequency of stably transduced cells within a cell population could be followed over time by flow cytometry. The frequency of stably transduced cells in mixed cell populations containing both non-transduced and transduced cells was followed for 20 days. The frequency of empty vector control or LRIG2-transduced cells did not change during the 20 days. However, the frequency of LRIG1-transduced cells declined dramatically over time, suggesting that LRIG1 suppressed the proliferation of H1975 cells. To further study the molecular reasons for the suppressive effect of LRIG1 on H1975 tumor growth, we analyzed phosphorylation of RTKs in the H1975 cell line, in both non-induced and LRIG1-induced cells. In non-induced cells we detected high phosphorylation levels of EGFR, ERBB2 and MET and low phosphorylation levels of RET. The induction of LRIG1 expression resulted in reduced phosphorylation levels of MET and RET. These results suggest that LRIG1 down-regulates MET and RET signaling, but not EGFR- or ERBB2 signaling, in H1975 cells. LRIG1 has previously been shown to down-regulate both MET and RET signaling, as well as ERBB signaling (*56-58*). EGFR-TKI resistance in NSCLC is mediated by several mechanisms where the $EGFR^{T790M}$ mutation is a frequent event. However, the amplification of MET is detected in 22% of EGFR-TKI-resistant NSCLC tumors (*185*). It is compelling to speculate that the growth inhibition of LRIG1 in H1975 cells was caused by down-regulation of MET and RET signaling. It would be of interest to investigate whether MET and/or RET inhibitors would generate the same growth inhibitory effect as does LRIG1. Taken together, these results show that LRIG1 can inhibit human EGFR-mutated lung cancer cells that are resistant to clinically used EGFR-TKIs. These results further support the tumor suppressor effect of LRIG1 in human lung cancer. If further studies validate that LRIG1 is a tumor suppressor in lung cancer, it would be of interest to analyze if LRIG1 can be of therapeutic value in human lung cancer. One could speculate that systemic administration of LRIG1, or a part of LRIG1, to lung cancer patients could have effect on patient survival.

LRIG protein interacting partners (IV)

Identification and cloning of LMO7 and LIMCH1

Our understanding of the function of LRIG3 is limited. To increase our knowledge of the protein partners interacting with LRIG3, we performed a YTH screen using part of the cytosolic tail of LRIG3 as bait. Out of the 25 isolated clones, 13 encoded the C-terminal part of LIMCH1, and 11 clones encoded the C-terminal part of LMO7. These proteins are homologous to each other and contain both a CH and a LIM domain. LMO7 also contains an F-box and a PDZ domain. Nucleotide sequencing identified 11 and 8 independent clones as LIMCH1 or LMO7, respectively. The minimal bait dependent cDNA clone obtained for LIMCH1 encoded the amino acids 931-1083 (NP_055803) and for LMO7 the amino acids 1153-1349 (NP_005349). All clones contained the complete LIM domain of the respective protein, indicating that the LIM domains of LMO7 and LIMCH1 could be mediating the interactions with LRIG3. *LMO7* and *LIMCH1* cDNAs were PCR-cloned from human brain and lung cDNA libraries. The cloned *LMO7* cDNA corresponded to the transcript variant 1 (NM_005358). The cloned *LIMCH1* cDNA corresponds to the transcript variant 3 (NM_001106189), which generates an isoform with deletions of amino acid 761 and amino acids 980-1005.

Expression of cloned LMO7 and LIMCH1 in cells

The isolated cDNAs were cloned into the mammalian expression vector pMH, which C-terminally tags hemagglutinin (HA) of cloned genes, generating the pMH-LMO7 and pMH-LIMCH1 constructs. The transfection of these plasmids into COS-7 cells, and the subsequent analysis by Western blotting revealed multiple protein bands, including protein bands with predicted sizes for full length proteins. This finding indicates that both LMO7 and LIMCH1 are posttranslationally modified. There are numerous possible posttranslational modifications of proteins, and the Western blot bands of different size could, in principle, be due to processes such as acetylation, ubiquitylation, phosphorylation or proteolytic cleavage. It is also possible that alternative translational start sites were used. Possibly, the different sized Western blot bands represented protein variants with different functions. For example, it is likely that the loss of the CH domain would result in altered function of the protein. In the present study, no attempts were made to analyze the cause of the different sized bands or the function of the different protein variants.

Generation of antibodies against LMO7 and LIMCH1 (IV)

Polyclonal antibodies against LMO7 and LIMCH1 were developed by immunizing rabbits with the synthetic peptides CSKKDIILRTEQNSG (LMO7 -55), CTQSPTPRSHSPSAS (LMO7-1250), LGLEALQPLQPEPC (LIMCH1-6) or CQTSNPTHSSEDVKP (LIMCH1-945). The sera were affinity purified, and the specificity of the antibodies was analyzed using Western blotting on lysates from COS-7 cells transiently transfected with LMO7 or LIMCH1. LMO7-55, LMO7-1250 and LIMCH1-945 appeared to be specific for LMO7 and LIMCH1. In IHC, the LMO7-1250 antibody generated specific staining. Regrettably, the LIMCH1 antibodies did not generate specific IHC staining and could therefore not be used in the IHC analysis. The Human Protein Atlas, a project in which antibodies against all human proteins are being generated, has developed an antibody against LMO7. In normal lungs, this antibody generates strong immuno-reactivity in respiratory epithelial cells and pneumocytes (*204*). In another study, an LMO7 antibody generated robust immuno-reactivity in respiratory epithelial cells (*119*). Our LMO7-1250 antibody also generated strong immuno-reactivity in respiratory cells, but not in pneumocytes. The reason for this discrepancy is not known, but could possibly be due to differential antibody recognition and the expression of the various isoforms of LMO7 discussed above.

Subcellular localization and endogenous interactions between LRIG1-3 and LMO7 (IV)

To study the subcellular localization of the LRIG proteins and LMO7, COS-7 cells were transiently transfected with LRIG and LMO7 expression plasmids. Protein localization was studied using antibodies and confocal laser scanning microscopy. Studies of the subcellular localization of LMO7 by others show that LMO7 co-localizes with F-actin (*113*). In our subcellular localization experiments using the LMO7-1250 antibody, we also detected co-localization between LMO7 and F-actin. These results further indicate that we have generated a specific antibody for LMO7. In addition, LMO7 localized to the cell border, the perinuclear region, the cytoplasm and, to a lower extent, the nucleus. LMO7 co-localized with all three LRIG proteins at the cell border and in the cytoplasmic and perinuclear regions. The proteins did not co-localize everywhere in the cells but rather only in specific areas. Through these experiments we showed that sub-pools of LMO7 and the LRIG proteins were in proximity to each other in the cell membrane, cytoplasm and also in the nucleus. It is thus possible that LMO7 and the LRIG proteins interact in these areas. However, these co-localization experiments were based on the transient overexpression of LMO7 and the LRIG proteins, which may have

altered the localization and interactions of the proteins. Therefor we used PLA to study endogenous interactions between LRIG3 and LMO7. This assay demonstrated that LMO7 and LRIG3 interacted at endogenous expression levels in several cell lines. Furthermore, LRIG1 and LRIG2 also interacted with LMO7 at endogenous levels. The specificity of the assay was confirmed by down-regulation of LMO7 using siRNA. The juxtamembrane part of the cytosolic tail of LRIG3 was used as bait in the original YTH screen. The amino acid sequence is similar between the LRIG proteins in this area, and therefore it was not surprising that all three LRIG proteins interacted with LMO7. LMO7 is a suggested transcription factor for multiple genes and has been shown to be a connector between the cadherin and nectin systems via physical interactions to afadin and α-actinin, thereby stabilizing adherence junctions. The biological function of the LMO7-LRIG interactions remains to be explored. Nevertheless, our results show that the LRIG proteins, which have been described as membrane proteins that interact with RTKs, also interact with a transcription factor within the nucleus. The interaction with LMO7 may explain the nuclear localization of the LRIG proteins. However, the function of the potentially nuclear LRIG protein is not known.

Expression levels of LMO7 and LIMCH1 in normal human tissues and lung cancer tissues (IV)

To analyze *LMO7* and *LIMCH1* mRNA expression levels in human tissues, we used quantitative real-time RT-PCR. A panel of different human tissues was analyzed and expression levels normalized to *Rn18s*. High expression levels of *LMO7* were detected in the small intestine and lung, whereas low expression levels were found in the adrenal gland, spleen, liver and thymus. High expression levels of *LIMCH1* were found in the lung and placenta, whereas low levels were found in the colon and liver. These results were consistent with previous studies, which demonstrated high expression of *LMO7* in normal human lungs (*106, 108*). The deletion of *Lmo7* in mice results in spontaneous lung cancer formation (*118*). We found high expression of both *LMO7* and *LIMCH1* in normal human lungs. To investigate the expression levels in human lung cancer tissues, we performed quantitative real-time RT-PCR on ten different lung tumor tissue samples. Four adenocarcinomas, one carcinoma in situ, four SCC and one undifferentiated carcinoma all expressed dramatically lower levels of both *LMO7* and *LIMCH1* mRNA than those in normal lung tissue. To corroborate these findings, we analyzed the expression of *LMO7* and *LIMCH1* in normal lungs, lung adenocarcinomas and lung SCC samples in the cancer genome atlas (TCGA) database. Again, in the TCGA database there was a dramatic difference in the gene expression between the normal and lung cancer

samples for both *LMO7* and *LIMCH1*. High expression levels were detected in normal samples, but the majority of the lung cancer samples expressed lower levels. The down-regulation of *LMO7* and *LIMCH1* in lung tumors would be consistent with LMO7 and LIMCH1 being tumor suppressors that are down-regulated in human lung cancers.

Clinical importance of LMO7 in human lung cancer (IV)

To study the clinical role of LMO7 and LIMCH1 in human lung cancer, we aimed to analyze both proteins in a human lung tumor TMA. However, the LIMCH1 antibody we generated did not function satisfactorily in IHC. Thus, we only performed the study for LMO7. We used the same TMA as that used to study LRIG proteins in lung cancer (III). The 363 lung cancer patient samples, together with 17 normal lung samples, were stained for LMO7 using the LMO7-1250 antibody. In normal lungs, LMO7 was expressed at moderate intensity in the cytoplasm of respiratory epithelial cells. Seventy-five percent of the lung cancer samples showed no expression of LMO7, 22% showed weak expression, 2% showed moderate expression and 1% showed strong expression. The subcellular localization of LMO7 in the tumor cells was mainly cytoplasmic. To investigate whether LMO7 expression had any effect on patient survival, we performed Kaplan Meier and log rank analyses. When studying all samples, there was a trend towards shorter survival in the LMO7-expressing group ($p = 0.088$). The stratification of histological subtypes showed no survival effect in adenocarcinoma patients ($p = 0.495$), whereas there was a trend of reduced survival among LMO7-expressing SCC patients ($p = 0.071$). Multivariate Cox regression analysis adjusted for LRIG1 and clinical prognostic factors showed LMO7-expressing patients to have a statistically significantly higher risk of death ($p = 0.013$) compared to non-LMO7-expressing patients. The stratification of histological subtypes showed the survival of adenocarcinoma patients to be independent of LMO7 expression ($p = 0.265$). However, SCC patients with LMO7-expressing tumors had a 1.84-fold increased risk of death ($p = 0.016$). These data indicate that LMO7 is a negative prognostic factor in human lung cancer. The expression of LMO7 in 57 adenocarcinoma lung tumor samples has been analyzed previously. Multivariate Cox regression analysis showed no effect on survival in these patients (*119*). These data are consistent with our results, showing that LMO7 has a survival effect in the SCC patient population only.

LRIG1 and LMO7 in combination as prognostic factors in human lung cancer

To analyze whether LRIG1 and LMO7 expression in combination could be used as prognostic factors in lung cancer patients, we analyzed the survival of patients with different combinations of $LRIG1^{high}$ or $LRIG1^{negative}$ and $LMO7^{positive}$ or $LMO7^{negative}$ tumors. Lung cancer patients with tumors expressing high levels of LRIG1 and no LMO7 had a mean survival of 123 months, compared to 58 months for patients with tumors expressing LMO7 but no LRIG1. This dichotomy corresponded to a survival benefit of more than 5 years for the $LRIG1^{high}$ and $LMO7^{negative}$ patients. A stratification analysis of adenocarcinoma and SCC indicated that the combination of LRIG1 and LMO7 as prognostic factors was important in both cancer types. Both patients with adenocarcinoma and SCC $LRIG1^{high}$ and $LMO7^{negative}$ patients had a survival benefit of more than 5 years relative to the $LRIG1^{negative}$ and $LMO7^{positive}$ patients ($p = 0.014$, $p = 0.013$, respectively). These data indicate that the combined expression of LRIG1 and LMO7 is a powerful prognostic factor in in this lung cancer cohort. In the future, it would be of interest to validate these findings in another cohort of lung cancer patients. If LRIG1 and LMO7 are validated as prognostic markers for lung cancer, they may prove to be useful in the clinical context. Patients harboring $LRIG1^{negative}$ and $LMO7^{positive}$ tumors have a much shorter survival compared to patients with $LRIG1^{high}$ and $LMO7^{negative}$ tumors. The $LRIG1^{negative}$ and $LMO7^{positive}$ group of patients may be speculated to benefit from additional adjuvant treatment.

Conclusions

In this thesis we analyzed the expression and role of LRIG proteins in psoriasis, glioma and lung cancer. We found the following:

The LRIG proteins were differentially distributed in psoriatic compared to normal skin, whereas the mRNA and protein expression levels were not altered.

Lrig2 ablated mice were grossly normal but suffered from a transiently reduced growth rate and an increased rate of spontaneous mortality. However, these mice were protected against PDGFB-induced glioma, which might be explained by an observed altered regulation of immediate-early gene induction.

LRIG1 was an independent positive prognostic marker in human NSCLC.

Our *Lrig1* ablated mice appeared grossly normal. Compared to the wt mice, the *Lrig1* ablated mice developed larger lung tumors in an $EGFR^{L858R}$-inducible lung tumor mouse model. Additionally, LRIG1 expression suppressed subcutaneous tumor growth of EGFR mutated human lung cancer cells.

LMO7 interacted with all three LRIG proteins.

LMO7 was highly expressed in normal lungs but was dramatically decreased in lung cancers. LMO7 was an independent negative prognostic factor in NSCLC.

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