Identification and Investigations of Leucine-rich Repeats and Immunoglobulin-like Domains Protein 2 (LRIG2)

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Till Moa, Jacob & Elin
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>List of Papers</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>Abstract</strong></td>
<td>2</td>
</tr>
<tr>
<td><strong>Sammanfattning på svenska</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>Abbreviations</strong></td>
<td>6</td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>7</td>
</tr>
<tr>
<td>Cancer</td>
<td>7</td>
</tr>
<tr>
<td><strong>Receptor tyrosine kinases</strong></td>
<td>7</td>
</tr>
<tr>
<td><strong>Platelet-derived growth factor signaling</strong></td>
<td>8</td>
</tr>
<tr>
<td>PDGFs and their receptors</td>
<td>8</td>
</tr>
<tr>
<td>PDGFR signaling</td>
<td>9</td>
</tr>
<tr>
<td>Control of PDGFR signaling</td>
<td>10</td>
</tr>
<tr>
<td>PDGFs and PDGFRs in animal development</td>
<td>10</td>
</tr>
<tr>
<td>PDGFR signaling in cancer</td>
<td>12</td>
</tr>
<tr>
<td><strong>Leucine-rich and immunoglobulin-like domains protein (LRIG)</strong></td>
<td>13</td>
</tr>
<tr>
<td>LRIG1, structure, expression and role in skin homeostasis</td>
<td>13</td>
</tr>
<tr>
<td>LRIG1 as a negative regulator of growth factor signaling</td>
<td>15</td>
</tr>
<tr>
<td>LRIG1 and cancer</td>
<td>16</td>
</tr>
<tr>
<td>LRIG3</td>
<td>18</td>
</tr>
<tr>
<td><strong>Oligodendroglioma</strong></td>
<td>19</td>
</tr>
<tr>
<td>Histology</td>
<td>19</td>
</tr>
<tr>
<td>Molecular etiology of oligodendroglioma</td>
<td>20</td>
</tr>
<tr>
<td>Treatment</td>
<td>23</td>
</tr>
<tr>
<td><strong>Mouse models of oligodendroglioma</strong></td>
<td>24</td>
</tr>
<tr>
<td><strong>AIMS</strong></td>
<td>27</td>
</tr>
<tr>
<td><strong>Results and Discussion</strong></td>
<td>28</td>
</tr>
<tr>
<td>Cloning and characterization of human LRIG2</td>
<td>28</td>
</tr>
<tr>
<td>Characterization of the LRIG2 protein</td>
<td>29</td>
</tr>
<tr>
<td>Expression of LRIG2 during development and in adult tissues</td>
<td>29</td>
</tr>
<tr>
<td>Phenotype of LRIG2-gene-ablated mice</td>
<td>31</td>
</tr>
<tr>
<td>LRIG2 in oligodendroglioma</td>
<td>34</td>
</tr>
<tr>
<td>Is the molecular function of LRIG2 to stabilize PDGFRα?</td>
<td>36</td>
</tr>
<tr>
<td><strong>Concluding Remarks</strong></td>
<td>38</td>
</tr>
<tr>
<td><strong>Acknowledgements</strong></td>
<td>39</td>
</tr>
<tr>
<td><strong>References</strong></td>
<td>41</td>
</tr>
</tbody>
</table>
LIST OF PAPERS

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

**Paper I:**

**Paper II**

**Paper III:**

**Paper IV:**

Additional publication not included in this thesis:


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ABSTRACT

Receptor tyrosine kinases (RTKs) constitute a family of proteins controlling cell growth and proliferation and whose activities are tightly controlled in normal cells. LRIG1 is a negative regulator of RTK signaling and is a proposed tumor suppressor. The aim of this thesis was to identify and study possible paralogs of LRIG1. By using the basic local alignment search tool and cDNA cloning, a human mRNA sequence with similarity to LRIG1 was identified and named LRIG2. By fluorescence in situ hybridization analysis, LRIG2 was found to reside on chromosome 1p13. The LRIG2 amino acid sequence was 47% identical to LRIG1, and the predicted protein domain organization was the same as that of LRIG1. Antibodies against LRIG2 were developed and the apparent molecular weight of the protein was determined to be 132 kDa by SDS-polyacrylamide gel electrophoresis and Western blot analysis. The sub-cellular localization was studied by cell surface biotinylation experiments and confocal fluorescence laser microscopy, which revealed that LRIG2 resided at the cell surface and in the cytoplasm.

The expression patterns of LRIG2 mRNA, during development and in adult tissues, were evaluated using whole-mount in situ hybridization and quantitative real-time RT-PCR, respectively. In E10.5, E11.5 and E12.5 mouse embryos, the Lrig2 expression domains were both overlapping and unique as compared to the expression domains of Lrig1 and the third family member, Lrig3. In adult human tissues, the most prominent LRIG2 mRNA expression was found in skin, uterus and ovary. To study the developmental and physiological role of LRIG2, Lrig2 knock-out mice were generated. The knock-out mice were born at Mendelian frequencies without any apparent morphological abnormalities. However, Lrig2 knock-out mice showed reduced body weight between 5 days and 12-15 weeks of age, increased mortality, and impaired reproductive capacity.

To study the role of LRIG2 as a prognostic factor in oligodendroglioma, LRIG2 expression was analyzed in 65 human oligodendrogliomas by immunohistochemistry. Cytoplasmic LRIG2 expression was an independent prognostic factor associated with poor oligodendroglioma patient survival. The possible functional role of LRIG2 in oligodendroglioma biology was further investigated using the RCAS/tv-a mouse model. Tumors resembling human oligodendroglioma were induced by intracranial injection of PDGFB carrying RCAS retroviruses into newborn Ntv-a mice. Lrig2 wild-type animals
developed tumors at a higher frequency and of higher malignancy than the Lrig2 knock-out mice. This result supports the notion that LRIG2 promotes PDGF-induced oligodendroglioma genesis. A possible molecular mechanism was revealed as LRIG2 overexpression increased PDGFRα levels in transfected cells. In summary, we identified a new gene named LRIG2, showed that it is expressed in a variety of tissues during development and in adulthood, knocked it out and found that it was required for proper animal growth, health, and reproduction. We also found that Lrig2 expression promoted PDGF-induced oligodendroglioma genesis and was associated with poor oligodendroglioma patient survival, possibly via a PDGFRα stabilizing function.
SAMMANFATTNING

Cancer är sjukdomar som karaktäriseras av okontrollerad celltillväxt. Normalt har celler flera olika kontrollmekanismer för att reglera celltillväxt och celldelning och därmed förhindra uppkomst av cancer. För att en normal cell ska tillväxa och dela sig måste den först ta emot utifrån kommande signaler vilket bland annat sker genom att tillväxtfaktorer binder till och därmed aktiverar specifika receptorer på cellytan. Förändringar i de gener som kodar för dessa receptorer leder till en rubbad tillväxtsignalering, vilket är av stor betydelse vid uppkomsten av ett flertal tumörsjukdomar. LRIG1 är ett relativt nyligen upptäckt som hämmer aktiviteten hos en del af cellens tillväxtfaktorreceptorer. Mycket tyder på att LRIG1 är av betydelse vid uppkomst av vissa tumörer.

Målsättningen med denna avhandling var att först undersöka om det hos människa fanns fler LRIG-proteiner än LRIG1. Här beskriver vi identiferingen och kloningen av LRIG2, samt studier av dess uttrycksmönster och funktioner. LRIG2-genen lokaliserades till ett område på kromosom 1, 1p13, som ofta är förändrad i olika tumörformer. Vi fann att LRIG2-genen kodar för ett protein som uppvisar stora strukturella likheter med LRIG1. LRIG2-genen uttrycktes i flera vävnader under musens embryonalutveckling. Hos vuxna människor uttrycktes LRIG2-genen i flera typer av normal vävnad men dock i varierande mängd. För att undersöka den fysiologiska betydelsen av LRIG2 utvecklade vi LRIG2-knockout möss, d.v.s. möss hos vilka LRIG2-genen var utslagen. LRIG2-knockout-mössen verkade till utseendet normala men de var något mindre än möss med intakt LRIG2-gen. LRIG2-knockout-mössen visade också en viss överdödlighet och en försämrad fertilitet. Dessa resultat tyder på att LRIG2 har en viktig funktion under djurens utveckling. Vi visade också att LRIG2 var en prognostisk markör i en viss typ av hjärntumörer, så kallade oligodendrogliom. Patienter med ett högt uttryck av LRIG2 i oligodendrogliom hade en sämre överlevnad än de med ett lågt uttryck. För att ytterligare undersöka vilken betydelse LRIG2 har för uppkomst av hjärntumörer använde vi oss av en experimentell djurmodell i vilken tillväxtfaktorn PDGF orsakar oligodendrogliom-liknande hjärntumörer. Andelen möss som fick oligodendrogliom var lägre i gruppen med LRIG2-genen utslagen än i gruppen med en intakt LRIG2-gen. Dessa resultat gå i linje med de kliniska data där patienter med mycket LRIG2 protein i tumören hade en sämre överlevnad. PDGF tillväxtfaktorer och deras receptorer är av stor betydelse för uppkomsten av oligodendrogliom även hos män-
niska. En möjlig funktion av LRIG2 skulle kunna vara att stabilisera receptor-torn för PDGF och på så sätt öka dess signalering vilket kan leda till en ökad risk att utveckla oligodendrogliom.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ETS</td>
<td>E6 transformation specific</td>
</tr>
<tr>
<td>IDH</td>
<td>Isocitrate dehydrogenase</td>
</tr>
<tr>
<td>IFP</td>
<td>Interstitial fluid pressure</td>
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<td>LRIG</td>
<td>Leucine-rich repeats and immunoglobulin-like domains</td>
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<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
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<td>MGMT</td>
<td>O6-methylguanine DNA methyltransferase</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>RAS</td>
<td>Rat sarcoma viral oncogene homolog</td>
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<td>RB</td>
<td>Retinoblastoma</td>
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<tr>
<td>RCAS</td>
<td>Replication competent ALV splice acceptor</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SH2</td>
<td>Src homology-2 domain</td>
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<td>TP53</td>
<td>Tumor suppressor protein 53</td>
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<tr>
<td>TEL</td>
<td>E-26 transforming specific (ETS)-related gene (also known as ETV6, ets variant 6)</td>
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<tr>
<td>Tv-a</td>
<td>Receptor for subgroup A avian sarcoma and leukemia virus</td>
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<td>WHO</td>
<td>World health organization</td>
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</table>
INTRODUCTION

Cancer

Cancer is a class of diseases caused by genetic aberrations in different genes resulting in uncontrolled cell growth. Normal cells are regulated by many control mechanisms that modulate their proliferation, differentiation, life and death. A cell needs to overcome these regulatory systems to become cancerous. Common to all cancers is that they develop through additional and stepwise genetic alterations. There are at least six essential alterations in the cell’s physiology that are required for malignant transformation. These alterations are described by Hanahan and Weinberg as “the hallmarks of cancer”, which are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evading apoptosis, limitless replicative potential, sustained apoptosis, tissue invasion, and metastasis\(^1\). The genomic alterations that are typically involved in tumorigenesis affect proto-oncogenes and tumor-suppressor genes. Mutations leading to gain of function of proto-oncogenes or loss of function of tumor suppressor genes drive tumorigenesis. Various forms of cancer differ in their biology, resulting in large differences in outcome for the patients. Considerable progress has been made in our knowledge of cancer, which has resulted in more effective and efficient detection, earlier diagnosis, and better treatment. Despite these improvements, however, cancer is still the second most common cause of death in Sweden\(^2\).

Receptor tyrosine kinases

In cancer cells, certain classes of genes are more frequently mutated than others. Among the most commonly altered genes in human cancers are those encoding protein kinases, to which the receptor tyrosine kinases (RTKs) belong. RTKs are cell surface receptors for a variety of growth factors, cytokines, and hormones. The human RTK family consists of 58 members, divided into 20 subfamilies that regulate normal cellular processes such as migration, metabolism, survival, proliferation, and differentiation\(^3\). The activities of the RTKs are tightly controlled in normal cells, but when overexpressed or mutated, the RTKs and their regulatory proteins can become oncoproteins. For example, the epidermal growth factor receptor (EGFR, \textit{ERBB1}) is commonly mutated in non small-cell lung cancer\(^4\) and ampli-
fied, overexpressed and/or mutated in about half of all glioblastomas\textsuperscript{7-8}. Platelet-derived growth factor (PDGF) receptors (PDGFR) are also overexpressed in many gliomas\textsuperscript{9-10}. In breast cancer, \textit{ERBB2} is amplified in about one third of all cases\textsuperscript{11}. In fact, most of the 58 human RTKs have been implicated as oncoproteins in various forms of cancer\textsuperscript{3}.

**Platelet-derived growth factor signaling**

\textit{PDGFs and their receptors}

PDGF was one of the first mammalian cell growth factors to be identified. It was discovered as the serum component responsible for the proliferation of arterial smooth muscle cells, as well as a serum growth factor for fibroblasts and glia cells\textsuperscript{12-14}. PDGF was later found to be involved in tumorigenesis, as the transforming gene in the simian sarcoma virus (SSV), v-sis, was found to be a viral homolog of mammalian PDGFB\textsuperscript{15-16}. PDGFs act primarily as paracrine growth factors and they are major mitogens for a number of cell types. Furthermore, they are implicated in various physiological and pathological processes, including wound healing, development, and tumorigenesis, (for reviews see\textsuperscript{17-19}). The PDGF family comprises four different isoforms, A, B, C, and D, that exerts their biological effects by activating two structurally related RTKs, PDGFR\textalpha{} and PDGFR\textbeta{}. All PDGFs form homodimers and PDGF-A and PDGF-B can also form heterodimers with each other. PDGF-A and PDGF-B are secreted as active ligands, whereas PDGF-C and PDGF-D require cleavage of N-terminal domains to become active. Upon PDGF binding, the PDGFRs homo or hetero-dimerize. The cellular response to PDGF stimulation is determined by the type and amount of stimulating PDGF, as well as the types and amounts of PDGFRs the cell expresses. \textit{In vitro} studies have demonstrated multiple PDGF/PDGFR interactions (Figure 1)\textsuperscript{18}.

PDGFs are synthesized by a large number of cell types, including fibroblasts, vascular endothelial cells, vascular smooth muscle cells, and certain glial cells. The PDGFRs are also expressed by many different cell types. For example, fibroblasts and vascular smooth muscle cells express both PDGFR\textalpha{} and PDGFR\textbeta{}, platelets and oligodendrocyte progenitor cells express only PDGFR\textalpha{}, and macrophages and mammary epithelial cells express only PDGFR\textbeta{}, (for review see\textsuperscript{17}).
**INTRODUCTION**

**Figure 1.** PDGF-AA binds only PDGFRα, thus creating a PDGFRαα homodimer, whereas PDGF-BB binds both PDGFRα and PDGFRβ, thus having the ability to induce all three receptor dimer combinations, PDGFRαα, PDGFRββ and PDGFRαβ. PDGF-CC binding creates PDGFRαα homodimers and PDGFRαβ heterodimers, and PDGF-DD binding creates PDGFRββ homodimers and PDGFRαβ heterodimers.

**PDGFR signaling**

PDGFRs are single transmembrane glycoproteins composed of an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic domain containing the kinase domain. Because PDGFs are bivalent, binding of PDGFs to their receptors induces receptor dimerization, which is followed by trans-phosphorylation on specific tyrosine residues in the intracellular domains. These phosphorylated tyrosine residues provide binding sites for various signal transduction molecules. The docking of signal transduction molecules to the PDGFRs involves protein-protein interactions through specific domains, e.g., Src homology-2 (SH2) or phosphotyrosine binding (PTB) domains. Following binding to the receptors, these molecules may be further activated by phosphorylation events leading to the initiation of further downstream signaling. The three different PDGFR dimers transduce overlapping but not identical down-stream signals, thus resulting in partly different cellular responses. Important signaling molecules that are activated
by the PDGFRs include RAS, phosphatidylinositol 3-kinase (PI3K), and phospholipase C gamma (PLC-γ)\textsuperscript{18}. PDGF-induced activation of the PI3K pathway promotes actin reorganization, directed cell movement, stimulation of cell growth, and inhibition of apoptosis. Activation of the RAS-MAPK pathway is important for the mitogenic effect of PDGFs (as well as differentiation and migration) and activation of PLC-γ promotes cell growth and motility\textsuperscript{18}. As discussed below, PDGF signaling is important both during embryonic development and in tumorigenesis.

**Control of PDGFR signaling**

The activities of receptor tyrosine kinases are controlled by negative feedback mechanisms. It was previously shown that ligand binding to PDGFRs induces receptor degradation\textsuperscript{21}. The degradation of PDGFRs is regulated by Cbl, an E3 ubiquitin ligase. Ligand-induced ubiquitination results in the subsequent internalization of the receptor-ligand complex into endosomal compartments, followed by lysosomal degradation\textsuperscript{22-23}. Protein tyrosine phosphatases (PTPs) are enzymes that remove phosphate groups from phosphorylated tyrosine residues, and thus regulate the phosphorylation state of various signaling molecules. The SH2-containing protein tyrosine phosphate (SH-PTP2) interacts with a specific phospho-tyrosine residue on activated PDGFRβ (Tyr 1009) through its SH2 domain, resulting in dephosphorylation and inactivation of the receptor\textsuperscript{24}. Recently, a relationship between primary cilia and signaling by PDGFRα was shown. Most cells possess a single non-motile cilium called the primary cilium. This cilium functions as a sensory organelle that receives mechanical and chemical signals both from other cells and the environment\textsuperscript{25}. Schnneider et al. showed that PDGFRα, but not PDGFRβ, is localized to the primary cilium in growth-arrested fibroblast and that mutants that fail to form cilia cannot activate PDGFRα\textsuperscript{26}. Regulation of the primary cilium may, thus, also represent a mechanism for the regulation of PDGFRα signaling.

**PDGFs and PDGFRs in animal development**

Both PDGFRα and PDGFRβ are essential during normal animal development (for reviews, see\textsuperscript{18,27-28}). PDGFs drive mesenchymal proliferation in the early embryo as well as migration and differentiation of PDGFR expressing cells during later embryonic stages. PDGF-A and PDGFRα seem to play a more general role than PDGF-B and PDGFRβ.
Complete loss of PDGFRα or PDGFRβ is both embryonic lethal. The phenotypic abnormalities observed in PDGFRα knock-outs include early embryonal neural tube defects and abnormal patterning of the somites, which results in skeletal abnormalities during later stages of embryonic development. Targeted mutations affecting different PDGFRα signaling pathways have revealed that PI3K is an important PDGFRα signaling pathway during development. The developmental roles of PDGF-A/PDGFRα are significant. Generally, during organogenesis, PDGF-A is expressed in the epithelium, and PDGFRα is expressed in the adjacent mesenchyme. PDGFRα signaling is essential for the development and normal function of a wide variety of cell types and tissues including, lung, intestine, testis, kidney, proper patterning of the skeleton, and myelination of the central nervous system (CNS). PDGF-A is the major mitogen for oligodendrocyte progenitor cells. Indeed, PDGFA-deficient mice show a severe reduction in the number of oligodendrocyte progenitor cells, resulting in a subsequent myelination deficiency in all parts of the brain.

PDGFRα signaling is implicated in both male and female fertility. In males, PDGFRα signaling is required for prenatal and postnatal testis development. PDGFRα-deficient male gonads show a severe reduction in the number of fetal Leydig cells, whereas PDGF-A knockouts show a loss of adult Leydig cells, reduced testicular size, and reduced testosterone levels. A recent study by Schmahl et al. showed the importance of PDGFRα signaling in the development of theca cells, i.e., the estrogen- and progesterone-producing cells in females. They found that mutations in PDGF target genes resulted in sterile females due to loss of estrogen production.

PDGFB- and PDGFRB-deficient mice die before birth or at birth due to abnormal kidney development and cardiovascular complications. PDGFB and PDGFRβ play an important role during the development of the vascular support cells (i.e., vascular smooth muscle cells and pericytes), which support and stabilize new vessels. PDGFB is expressed by cells in the vascular endothelium, driving the proliferation of PDGFRβ-expressing vascular smooth muscle cells and pericytes and their subsequent migration along newly formed endothelial sprouts. The development of kidney glomeruli is also dependent on PDGF-B/PDGFRβ signaling.
INTRODUCTION

**PDGFR signaling in cancer**

PDGFR signaling is implicated in various types of cancers, both in solid tumors and in hematological malignancies. PDGFs drive tumorigenesis either by autocrine or paracrine growth stimulation of tumor cells and/or tumor stroma cells, whereas PDGFRs may drive tumorigenesis by overexpression or by gain-of-function mutations. The PDGFs and PDGFRs are expressed in the tumor cells, the surrounding tumor stromal fibroblasts, or in tumor blood vessels.

Activating mutations of *PDGFRA*, the gene encoding PDGFRα, resulting in hyperactive and oncogenic receptors have been reported. In-frame deletion of exons 8 and 9 of *PDGFRA* (PDGFRαΔ8,9), identified in a human glioblastoma, results in a mutant receptor that is active in the absence of ligand and is capable of transforming Rat1 cells. Gain of function mutations in *PDGFRA* are found in gastrointestinal stromal tumors (GISTs), which are mesenchymal tumors of the stomach and small intestine. Most GISTs have mutant KIT receptors and mutation in *PDGFRA* are found exclusively in the subset of tumors that lack *KIT* mutations.

Fusion proteins, due to translocations involving PDGFR genes that result in chimeric proteins with constitutively active tyrosine kinases, are associated with rare hematological malignancies. In chronic myelomonocytic leukemia, a chromosomal translocation results in the fusion of the *PDGFRB* gene with a member of the ETS family of transcription factors, *TEL*, generating the *TEL-PDGFRβ* (also called ETV6- PDGFRβ ) fusion protein. Additionally, chronic eosinophilic leukemia is associated with a constitutively active PDGFRα fusion protein, *FIP1L1-PDGFRα*, as a result of a specific chromosomal deletion between *PDGFRA* and *FIP1L1*. Finally, a translocation between the *PDGFB* and collagen 1A1 (*COL1A1*) genes is associated with the rare skin tumor dermatofibrosarcoma.

Most or all solid tumors are dependent on the tumor stroma for their survival and growth. The tumor stroma is to various degrees composed of extracellular matrix, fibroblasts, endothelial cells, and immune cells. Many solid tumors display paracrine PDGF signaling between PDGF-producing tumor cells and PDGFR-expressing stromal cells. In glioma, paracrine signaling by PDGF-B/PDGFRβ promotes tumor growth and angiogenesis by stimulating vascular endothelial growth factor expression and the recruitment of pericytes to newly formed blood vessels. Furthermore, PDGF signaling is im-
important for the recruitment of cancer associated fibroblasts. A recent study, using a mouse model of melanoma, showed that PDGF-CC/PDGFRα paracrine signaling was important for the recruitment of cancer-associated fibroblasts\textsuperscript{49}. Also, PDGF regulates the interstitial fluid pressure (IFP). In solid tumors, the IFP is increased, reducing the uptake of drugs into the tumor. PDGFRβ signaling increases the IFP in the tumors, and by using a rat colon-cancer model, it was shown that inhibition of PDGFRβ reduces the IFP. This resulted in an increased capillary-to-interstitium transport of low molecular mass compounds into the tumor\textsuperscript{50}.

Paracrine PDGF signaling is also implicated in other tumor types such as breast cancer, where elevated PDGF plasma levels and PDGFRβ expression in tumor stromal fibroblasts are associated with a poor survival\textsuperscript{51-52}. The presence of autocrine PDGF signaling in glioma is supported by several reports that have demonstrated co-expression of PDGF and PDGFR in both glioma cell lines\textsuperscript{53-55} and in glioma tumor specimens\textsuperscript{9,10,55-56}. Similarly, the presence of autocrine PDGF growth stimulation is found in fibroblast-derived skin tumors\textsuperscript{57}.

**Leucine-rich and immunoglobulin-like domains protein (LRIG)**

*LRIG1, structure, expression and role in skin homeostasis*

Leucine-rich and immunoglobulin-like domains protein 1 (LRIG1) is a recently discovered protein that functions as a negative regulator of RTK signaling. The first LRIG1 transcript to be described was mouse Lrig1 (previously named LIG-1). Lrig1/LIG1 was identified in a screen for genes involved in neuronal differentiation\textsuperscript{58}. Due to its structural similarities with the Drosophila protein Kekkon1, an inhibitor of EGFR in insects, our laboratory set out and successfully identified human LRIG1\textsuperscript{59-60}. The LRIG1 gene is located on chromosome 3p14\textsuperscript{59}, a region frequently deleted in various human cancers\textsuperscript{61}. LRIG1 encodes an integral membrane protein with an extracellular or luminal region, consisting of 15 leucine-rich repeats (LRR) and three immunoglobulin-like domains, followed by a transmembrane, and a cytoplasmic domain (Figure 2).
The levels of *LRIG1* mRNA and protein in different mammalian tissues vary considerably\(^{59-60,62}\). The most prominent expression of *LRIG1* in adult humans is in the brain, liver, stomach, and small intestine, whereas bladder, blood, placenta and spleen express low levels. *LRIG1* is also highly expressed in skin. The mammalian epidermis consists of the interfollicular epidermis, hair follicles, and sebaceous glands. Lrig1 is expressed in scattered epidermal basal cells and in hair follicle cells\(^{63-66}\). Two recent studies have shown that Lrig is a novel marker for a multipotent stem cell population in the interfollicular epidermis, where it keeps epidermal stem cells in a quiescent and non-dividing state, thereby regulating skin homeostasis\(^{65-66}\). *Lrig1*-deficient mice, which are born without any apparent morphological defects, develop skin defects on their tails, snouts, and ears after 3 weeks to 4 months of age. The affected tail skin shows psoriasis-like epidermal changes, including epidermal hyperplasia\(^{63}\).

LRIG1 localizes to different subcellular compartments including the plasma membrane, cytoplasm, perinuclear, and nuclear regions. Ectopically expressed LRIG1 co-localizes with early endosomes, the trans-Golgi network,
and the plasma membrane. The sub-cellular localization of LRIG1 is also of clinical importance in astrocytic tumors (see below). In psoriasis, the sub-cellular distribution of LRIG protein is altered compared to normal epidermis.

**LRIG1 as a negative regulator of growth factor signaling**

LRIG1 is a negative regulator of growth factor signaling. The first RTKs shown to be regulated by LRIG1 were the EGFR family members. LRIG1 belongs to the group of negative regulators that are transcriptionally induced following receptor activation, i.e., so called late attenuators. Attenuation of EGFR signaling by LRIG1 is initiated by a physical interaction between the extracellular domains of LRIG1 and EGFR (Figure 3). This is followed by the recruitment of the E3 ubiquitin ligase, c-Cbl, to the LRIG1/EGFR complex, and subsequent polyubiquitination (i.e., the attachment of a chain of ubiquitin) of both EGFR and LRIG1. The LRIG1/EGFR complex is thereby targeted for internalization into lysosomal compartments and degradation. LRIG1 also interacts with and destabilizes EGFRvIII, a mutated and constitutively active form of EGFR found in many glioblastomas. However, LRIG1-mediated destabilization of EGFRvIII seems to be independent of c-Cbl. It has also been shown that a recombinant soluble fragment corresponding to the LRR-domain of LRIG1 can interact with EGFR and compete with EGF for binding, thereby suppressing EGF signaling and the proliferation of EGFR-expressing cells.

LRIG1 is also implicated as a negative regulator of the MET and RET RTKs. Inhibition of MET by LRIG1 is associated with enhanced receptor degradation, in a c-Cbl independent manner. LRIG1 destabilizes the MET receptor in both the presence and absence of its ligand, hepatocyte growth factor (HGF). The mechanism of MET suppression is not yet fully understood, LRIG1 likely acts by facilitating the interaction of MET with the protein degradation machinery. Conversely, inhibition of RET by LRIG1 is not associated with receptor degradation. Instead LRIG1 attenuates RET tyrosine kinase activity by reducing ligand binding to RET, resulting in reduced RET kinase activity and MAPK activation. In summary, LRIG1 negatively regulates growth factor signaling from several RTKs, both by enhancing receptor degradation rates and by inhibiting ligand binding.
**INTRODUCTION**

*Figure 3.* Schematic model of LRIG1 mediated downregulation of EGFR. The extracellular domains of LRIG1 and EGFR physically interact. This is followed by the recruitment of the c-Cbl E3 ubiquitin ligase and the subsequent polyubiquitination of both EGFR and LRIG1, which targets the LRIG1-EGFR complex for internalization into lysosomal compartments and degradation.

**LRIG1 and cancer**

LRIG1 has long been proposed to be a possible human tumor suppressor. This hypothesis was based on its structural similarity to Kekkon-1, an EGFR inhibitor in insects, and the localization of LRIG1 at chromosome band 3p14, a chromosomal region frequently deleted in human cancers. Indeed, LRIG1 was found to be downregulated in renal cell carcinoma, as well as in various other types of human cancer (reviewed by Hedman and Henriksson). In a study of cutaneous squamous cell carcinoma, LRIG1 expression was significantly higher in early-stage than in late-stage tumors and was thus inversely correlated with metastasized disease. In squamous cell carcinoma of the cervix, LRIG1 expression is significantly lower in early stage than in late stage tumors and its expression correlates with a significantly better prognosis. Regarding LRIG1 and survival, a recent meta-analysis suggest that low LRIG1 expression is associated with poor survival.
INTRODUCTION

of patients across multiple tumor types, including breast and lung cancer, glioblastoma and malignant melanoma. LRIG1 is also implicated in prostate cancer. Thomasson et al. showed that ectopically expressed LRIG1 reduces proliferation of prostate cancer cells in vitro. However, they also reported higher LRIG1 expression in prostate tumor specimens compared to non-neoplastic tissue. Interestingly, androgen stimulation induces LRIG1 expression, and thus the authors speculated that androgen could be a putative regulator of LRIG1 expression.

LRIG1 may also be involved in breast cancer. Apparent downregulation of LRIG1 was reported in the majority of ErbB2 overexpressing breast cancers analyzed. On the contrary, an increased LRIG1 gene copy number is found in 34% of breast cancer tumors. This implies that breast tumors with increased LRIG1 gene dosage simultaneously have decreased expression of the LRIG1 protein. The authors of the latter studies pointed out that this apparent paradox might be explained by several possible mechanisms. For example, tandem gene duplications could result in the generation of an oncogenic fusion gene, LRIG1 could be amplified with loss of function, or LRIG1 could be located in the proximity to another breast cancer-related gene.

In colorectal cancer, a great heterogeneity of LRIG1 expression is observed. A study of 30 colorectal cancers reported both overexpression and underexpression of LRIG1 in tumor tissue. Recently, it was suggested that LRIG1 is regulated by two microRNAs, MIR-15a and MIR-16-1, in chronic lymphocytic leukemia (CLL). Hanlon et al. reported significantly lower levels of LRIG1 in CLL patients with deletions of the MIR-15a/MIR-16-1 locus at chromosome band 13q14. LRIG1 expression is also altered in nasopharyngeal carcinoma and homozygous deletions of the LRIG1 locus are reported in nasopharyngeal carcinoma. A meta-analysis of publicly available gene expression datasets revealed that LRIG1 is also downregulated in lung carcinomas. In addition, gene expression profiling of normal lung tissues from smokers and ex-smokers and from squamous cell lung carcinomas shows that LRIG1 is downregulated in the normal bronchial epithelium of current smokers as compared with ex-smokers, and even further down-regulated in lung tumors. In summary, several studies on clinical materials suggest that LRIG1 expression is down-regulated in many tumors, that LRIG1 may function as a tumor suppressor in these tissues, and that LRIG1 expression has a positive effect on patient survival.
**LRIG3**

Two human paralogs of *LRIG1*, *LRIG2* and *LRIG3*, were cloned and characterized by our laboratory in 2004\textsuperscript{62,88}. LRIG2 is the subject of the current thesis and is discussed in the Results section, below. Similar to *LRIG1*, *LRIG3* is widely expressed in human and mouse tissues. The highest *LRIG3* expression is found in the stomach, thyroid, and skin. LRIG3 is less well-studied than LRIG1. However, studies have shown that Lrig3 plays an important role during the morphogenetic development of the mammalian inner ear. Lrig3 was identified as a candidate gene in a large gene trap based screen that was designed to identify genes involved in hearing and balance\textsuperscript{89-92}. Lrig3 mutant mice exhibit craniofacial defects with a shortened snout and impaired balance due to a truncation of the lateral semicircular canal of the inner ear\textsuperscript{90,92}. The sensory organs for hearing and balance in the inner ear are formed from a sphere of epithelium called the otic vesicle. The semicircular canals, which are required for balance and spatial orientation, are sculptured after a complex remodeling of the otic vesicle. During semicircular canal formation, part of the otic epithelium first fuses and then disappears creating a hollow duct. A key regulator of this process is Netrin1. Netrin1 is expressed in restricted domains of the otic vesicle where epithelial fusion occurs and is required for the local disruption of the basement membrane\textsuperscript{93}. Lrig3 regulates Netrin1 expression and thereby regulates both the timing and the location for of epithelial fusion. In Lrig3-deficient mice, the control of Netrin1 expression is deficient, and thus expanded fusion occurs, resulting in defective canal formation\textsuperscript{90}. Lrig3 interacts with ErbB receptors; however, this interaction is unlikely to play a role in Lrig3-dependent inner ear morphogenesis\textsuperscript{92}.

In *Xenopus* development, Lrig3 is implicated in the formation of the neural crest. The neural crest is a group of multipotent cells that are pinched off during the formation of the neural tube, the precursor of the spinal cord. The neural crest cells migrate to various parts of the embryo and give rise to multiple cell types. The formation of neural crest requires a precisely balanced combination of various signals, including Fgf and Wnt signaling. Zhao et al. showed that Lrig3 modulates both Fgf and Wnt signaling and is thereby required for neural crest formation during early embryogenesis in *Xenopus*\textsuperscript{94}. The sub-cellular localization of LRIG3 also appears to be of clinical importance. In a study of 404 astrocytic tumors, perinuclear LRIG3 (and LRIG2) expression was associated with a low grade according to WHO (World
Health Organization) better survival of the patients\textsuperscript{95}. Recently, a genome-wide analysis of single nucleotide polymorphisms associated with cardiovascular disease, showed that certain LRIG3 alleles are associated with high plasma levels of high-density lipoprotein cholesterol\textsuperscript{96}.

**Oligodendroglioma**

Oligodendrogliomas are glial tumors that account for approximately 2.5% of all primary brain tumors and 5 to 6% of all gliomas\textsuperscript{97}. Gliomas are categorized into three major subtypes: oligodendrogliomas, astrocytomas, and ependymomas. Each subtype has its own characteristic histological features, and it is classified according to which cell type the tumor cells resemble most. Thus, oligodendrogliomas are composed of cells resembling oligodendrocytes, astrocytomas of cells resembling astrocytes, and ependymomas of cells resembling ependymal cells, whereas oligoastrocytomas are composed a mixture of cells resembling both oligodendrocytes and astrocytes\textsuperscript{97}. Glial tumors are also classified according to their histologic grade according to the WHO grading system. Glioma malignancy grades range from WHO grade I to IV, grade IV being the most malignant\textsuperscript{97}. Oligodendrogliomas are either grade II (diffuse oligodendroglioma) or grade III (anaplastic oligodendroglioma), whereas all grade IV gliomas are classified as glioblastomas. Oligodendrogliomas are predominantly tumors of adulthood with a peak in incidence between 40 and 60 years of age. Data from two population-based studies indicate a 5-year survival rate for patients with diffuse oligodendroglioma of 66 and 78%, and 30 and 38% for patients with anaplastic oligodendroglioma\textsuperscript{98}.

**Histology**

According to the WHO definition, diffuse oligodendrogliomas are “a well-differentiated, diffusely infiltrating tumor of adults that is typically located in the cerebral hemisphere and composed predominantly of cells resembling oligodendroglia”\textsuperscript{97}. Diffuse oligodendrogliomas are composed of tumor cells with round nuclei, which are slightly larger than those of normal oligodendrocytes, they show an increase in chromatin density, and a low to moderate cellularity. A typical morphological feature of oligodendroglioma is the “fried egg” appearance on paraffin sections, showing tumor cells with clear cytoplasm and a well-defined plasma membrane. Other histological features are microcalcifications and a dense network of branching capillaries\textsuperscript{97}. Ac-
According to the WHO definition, anaplastic oligodendrogliomas are “an oligodendroglioma with focal or diffuse histological features of malignancy and a less favorable prognosis”. In addition to the characteristic features of low-grade oligodendroglioma, anaplastic oligodendrogliomas show a significant mitotic activity and prominent vascular proliferation, and necrosis may also be present.

**Molecular etiology of oligodendroglioma**

**Loss of 1p and 19q**

The histological grading of oligodendrogliomas on its own has not been as reliable as the grading of astrocytomas in determining prognosis. An important step forward was the discovery of the combined loss of the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) as a characteristic genetic lesion of oligodendroglioma. The deletions of 1p and 19q usually involve loss of the entire chromosome arms, most likely due to unbalanced translocations, i.e., the exchange between parts of non-homologous chromosomes that results in loss of genetic material. A combined loss of 1p and 19q is observed in 64 to 89% of oligodendrogial tumors (Figure 4). The presence of 1p/19q loss is associated with a longer survival and a better response to chemotherapy, independent of tumor grade. There is also a clear association between 1p/19q loss and a pure oligodendroglioma type.

The loss of 1p/19q in the majority of oligodendrogliomas may thus indicate the presence of putative tumor suppressor genes at 1p and 19q. One of the suggested tumor suppressor candidate genes on 1p is CDKN2C, which encodes a member of the INK4 family of cyclin-dependent kinase inhibitors (see below). A small subset of anaplastic oligodendrogliomas harbor mutations or homozygous deletions of CDKN2C. Additional reported candidate genes located on 1p include CAMTA1, DFFB, TP73, and SHREW1. These genes demonstrate decreased expression, sometimes due to promoter hypermethylation or homozygous deletions. However, no mutations have been reported in these genes. Various tumor suppressor candidate genes mapped to 19q have also been suggested, such as EMP3, p190RhoGAP, ZNF342 and PEG3. However, it remains to be established which of the putative tumor suppressor genes at 1p and 19q are responsible for the development of oligodendroglioma.
INTRODUCTION

RTK/PTEN/PI3K pathway

Altered RTK signaling is common in oligodendroglioma. EGFR is reported to be overexpressed in ~50% of diffuse and anaplastic oligodendroglomas. However, EGFR amplification is a rare event in oligodendroglioma. PDGF and PDGFR overexpression frequently occurs in both diffuse and anaplastic oligodendrogliomas. Di Rocco et al. demonstrated increased expression of PDGF-A in 16/17 oligodendrogliomas and increased PDGF-B, PDGFRα and PDGFRβ expression in 17/17 of the cases. In another study, by Shosan et al., PDGFRα overexpression was demonstrated in 7/7 of the oligodendroglioma cases. Amplification of PDGFRA has been reported in 15 to 19% of anaplastic oligodendroglioma. As discussed above, signaling by RTKs results in the activation of the PI3K pathway. Direct activation of this pathway by activating mutations of the gene encoding the catalytic subunit of PI3K, PIK3CA, is found in 14% of anaplastic oligodendrogliomas. Similarly, the gene encoding PTEN, a phosphatase that counteracts the activity of PI3K is deleted in 12 to 24% of anaplastic oligodendrogliomas and is also associated with poor prognosis.

p16INK4a/CDK4/RB1 pathway

Alterations in the retinoblastoma (RB) and the tumor suppressor protein 53 (TP53) pathways are common in anaplastic oligodendroglioma. Overall, ~70% of anaplastic oligodendroglioma show alterations in one or both of the RB or TP53 pathways. The RB1 protein controls the cell cycle. The CDKN2A locus encodes two different gene products, p16INK4a and p14ARF, due to alternative reading frames, which play essential roles in cell-cycle control. p16INK4a induces G1 cell cycle arrest by binding to CDK4 and thereby inhibiting the CDK4/cyclinD1 complex, which in turn inhibits RB1. The CDKN2B locus encodes p15INK4b, which is structurally homologous to p16INK4a. Thus, inactivation of the RB1 pathway may result from loss of p16INK4a/p15INK4b expression, CDK4 amplification or loss of RB1. Homozygous deletions or promoter hypermethylation of CDKN2A are found in up to 47% of anaplastic oligodendrogliomas and of CDKN2B in up to 40%. Promoter hypermethylation of RB1 is reported in 10% of anaplastic oligodendrogliomas, and CDK4 amplification is reported in 20%.
TP53/MDM2/p14Arf pathway

TP53, which is encoded by the TP53 gene, is an important tumor suppressor that regulates the cell-cycle and prevents the cell from replicating damaged DNA. TP53 promotes DNA repair by arresting the cell cycle, or in cases of severe DNA damage, induce apoptosis. MDM2 is a key regulator of TP53. In normal cells, MDM2 binds to TP53, inhibits the function of TP53 as a transactivator and induces its degradation. p14Arf, one of the two proteins encoded by the CDKN2 locus, stabilizes both TP53 and MDM2. Thus, loss of TP53, loss of p14Arf, or MDM2 amplification will each contribute to inactivation of the TP53 pathway. Promoter hypermethylation of p14Arf is reported in 21% of low-grade oligodendrogliomas. In anaplastic oligodendroglioma, homozygous deletions or promoter hypermethylation of p14Arf is reported in 40% of the tumors. However, in contrast to many other tumors, TP53 mutations and MDM2 amplifications are rare in both diffuse and anaplastic oligodendroglioma.

MGMT

The DNA repair enzyme, O6-methylguanine DNA methyltransferase (MGMT), plays a significant role in the resistance of tumor cells to alkylating agents. Gene silencing due to promoter hypermethylation of the MGMT gene is found in various cancer types, including gliomas. In oligodendrogliomas, a combined loss of 1p/19q is significantly associated with MGMT promoter hypermethylation, suggesting that transcriptional silencing of MGMT can contribute to the chemosensitivity of oligodendroglioma with 1p/19q co-deletions.

IDH1

Recent studies have found mutations in the gene encoding isocitrate dehydrogenase 1 (IDH1) in the majority of oligodendrogliomas. Mutations in IDH1, or the closely related IDH2, are present in approximately 80% of diffuse and anaplastic oligodendrogliomas. All mutations identified to date are heterozygous missense mutations at a single analogous amino acid residue, R132 in IDH1 and R172 in IDH2. IDH1 catalyzes the conversion of isocitrate to α-ketoglutarate. The mutated form of IDH1 has lost this enzymatic activity and instead gained an ability to catalyze the conversion of α-ketoglutarate to the proposed oncometabolite 2-hydroxyglutarate. The
The presence of IDH mutation correlates with 1p/19q loss, which is observed only in tumors having mutations in \textit{IDH1} or \textit{IDH2} \cite{131,133,134,136}.

\textbf{Treatment}

Initial studies showed that, in general, anaplastic oligodendrogliomas were sensitive to chemotherapeutic treatment with a combination of procarbazine, lomustine, and vincristine (PCV) \cite{105,138}. Despite this finding, chemotherapy has not proven to have a major impact on overall survival in oligodendroglioma. Today, the standard-of-care therapy for patients with anaplastic oligodendroglioma is maximal surgical resection, with the aim to reduce symptoms, and postoperative radiotherapy to delay tumor relapse. Chemotherapy is mainly used as a second-line treatment for progressive disease. There are no studies showing an overall survival benefit of concomitant or adjuvant chemotherapy as an initial treatment for these patients \cite{139,140}. For diffuse oli-
INTRODUCTION

godendroglioma the treatment is mainly surgery. Radiotherapy is used mainly at relapse, as a postoperative treatment, or as monotherapy. Chemotherapy for diffuse oligodendroglioma is not routinely used but may be of benefit in progressive disease after radiotherapy. Although diffuse oligodendroglioma and anaplastic oligodendroglioma are considered to have slightly better prognoses than glial tumors of astrocytic tumors, new treatments for oligodendroglioma are needed.

Mouse models of oligodendroglioma

Animal models provide experimental systems to study tumor biology in physiological settings that may resemble the situation in human disease. Previously, xenograft models were the most commonly used animal models. Typically, human xenograft tumor cells are injected or transplanted into immunocompromised mice, either subcutaneously (i.e., under the skin) or orthotopically (i.e., into the type of organ from which the tumor originates). However, most xenograft models suffer from important limitations, for example, a lack of representative tumor cell lines and the need to use mice with compromised immune systems. For oligodendroglioma, suitable cell lines have been almost completely lacking. More recently, mouse models with germ-line genetic modifications or mice with genetic modifications in somatic cells that mimic the genetic aberrations seen in human tumors have been developed. One way to induce the expression of an oncogene in somatic cells is by using retroviruses. In the RCAS/tv-a mouse model, the avian RCAS (replicon-competent ALV splice acceptor) retrovirus is used as an oncogene carrier. The oncogene of interest is cloned into the RCAS retroviral vector, which is transfected into DF-1 chicken fibroblasts, thus generating retrovirus-producing cells (Figure 5A). These cells are used to infect mice that express Tv-a, the gene encoding the RCAS receptor, under the control of cell-specific promoters (Figure 5B). The advantage of this system is that mammalian cells do not express the Tv-a receptor and thus are not infected unless they ectopically express a Tv-a transgene (Figure 5C). The combinations of different genetic aberrations can be explored by crossing Tv-a transgenic mice with mice carrying targeted deletions or other genetic aberrations.
The effects of various oncogenes, including PDGFB, have been studied using Ntv-a, Gtv-a, and Ctv-a mice together with RCAS viruses encoding the PDGF-B chain. In the Ntv-a mice, RCAS-PDGFB induces tumors resembling human low-grade oligodendroglialomas and a few high grade oligodendroglialomas. In the Gtv-a mice, RCAS-PDGFB induces tumors resembling both human low-grade oligodendroglialomas and oligoastrocytomas, but at a lower incidence than in the Ntv-a mice. In the Ctv-a mice, RCAS-PDGFB induces tumors resembling human low-grade oligodendroglialomas.

The tumor incidence and grade of malignancy can be influenced by additional genetic manipulations. For example, by elevating the expression levels...
of PDGFB in Ntv-a mice, the tumor incidence and the malignancy of the tumors increase\textsuperscript{146}. By pharmacological inhibition of the kinase activity of PDGFR, the malignancy grade was reduced from high to low grade, showing that the levels of PDGFR signaling are important for the grade of these oligodendrogliomas\textsuperscript{146}. In another retroviral model, intracranial injection of a PDGFB-expressing murine retrovirus resulted predominantly in the generation of glioblastoma-like tumors\textsuperscript{147}. Transgenic models with GFAP promoter-driven expression of PDGFB yielded, in one case, spinal oligoastrocytomas\textsuperscript{148}. In another case, no tumors were induced unless the mouse homolog to the human TP53 gene, Trp53, was deleted, which then resulted in the development of glioblastoma-like tumors\textsuperscript{149}. Furthermore, loss of Ink4a and loss of Ink4a together with loss of Arf increase the tumor incidence and grade of malignancy in PDGFB-driven glioma in Ntv-a, Gtv-a and Ctv-a mice\textsuperscript{143-145}. Taken together, in genetic mouse models, PDGF-B has the potential to induce oligodendrogliomas and glioblastomas by transforming glial progenitor cells, astrocytes, and oligodendrocytes.
AIMS

The work of the present thesis was initiated in an effort to identify paralogs of human LRIG1. The LRIG1 protein is a negative regulator of growth factor signaling and implicated in many human cancers. The aims of the present thesis were therefore to (i) identify new members of the LRIG family of proteins, (ii) characterize the LRIG2 gene, transcript, and protein, (iii) investigate the expression pattern of LRIG2 during development and in adult tissues, (iv) analyze the role of Lrig2 in mammalian development and physiology, (v) investigate the expression pattern of LRIG2 proteins in oligodendroglioma and its possible correlation to clinical parameters, and (vi) elucidate the possible molecular function of the LRIG2 protein.
RESULTS AND DISCUSSION

Cloning and characterization of the human LRIG2 gene

Human LRIG2 was identified in a search for paralogs of the previously identified LRIG1 (Paper I). By using the Basic Local Alignment Search Tool (BLAST), we identified an mRNA sequence in GeneBank with homology to LRIG1 (KIAA0806, GeneBank accession number AB018349). Based on the sequence of KIAA0806, we isolated an LRIG2 cDNA by cloning from a human brain cDNA library. To determine the chromosomal localization of LRIG2, we used fluorescence in situ hybridization (FISH). This showed that LRIG2 resided on chromosome 1p13. The chromosomal localization was later confirmed by analyzing the sequenced human genome by BLAST. By analyzing the human genome sequence with BLAST, it was also revealed that the LRIG2 gene consisted of 19 exons spanning approximately 50 kb of genomic DNA. Chromosome band 1p13 is a chromosomal region that is frequently deleted in human cancers. For example, as discussed above, the combined loss of the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) is the most common cytogenetic chromosomal alteration found in oligodendrogliomas.

The open reading frame of the isolated LRIG2 cDNA encoded 1065 amino acids. The predicted amino acid sequence of LRIG2 revealed the same domain organization as that described for LRIG1, with a signal peptide, an extracellular or luminal region containing 15 leucine-rich repeats (LRR) and three immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic tail[59]. In a parallel study, we also identified a third member of the LRIG family, named LRIG3[62]. Thus, the human LRIG gene family comprises three paralogs, LRIG1, LRIG2, and LRIG3. The amino acid sequence of LRIG2 was 47% identical to human LRIG1 and mouse Lrig1 (Paper I) and 54% to human LRIG3[62]. Comparison of the three different LRIG polypeptides revealed a strong conservation in the extracellular, transmembrane, and the membrane proximal portion of the cytoplasmic tail. However, in the distal part of the cytoplasmic tail, no significant similarities were observed (Paper I)[62].

The LRIG gene family is well conserved during evolution. Orthologs of human LRIG1, 2, and 3 exist in mouse, rat, and fish. A single LRIG homolog is found in nematodes, insects, and tunicates[62,67]. The conservation of a protein sequence during evolution implies that the sequence is functionally im-
RESULTS AND DISCUSSION

Important. For example, LRR motifs are identified in a large number of proteins that are mainly involved in protein-protein interactions. Thus, we propose that regions of the LRIG proteins are functionally conserved and have retained their functions (and/or interactions) since the time of the last common ancestor of humans, nematodes and insects.

Characterization of the human LRIG2 protein

To study the LRIG2 protein, antibodies were generated against intracellular peptides of LRIG2 (Paper I and III). The apparent molecular weight of LRIG2 protein was 132 kDa, as determined by SDS-polyacrylamide gel electrophoresis and Western blotting (Paper I). The LRIG2 antibody showed no cross reactivity with LRIG1. The subcellular distribution of LRIG2 was investigated by performing cell surface biotinylation experiments and confocal fluorescence laser microscopy (Paper I). This revealed that the LRIG2 protein was localized to the cell surface and to the cytoplasm. However, the subcellular localization of LRIG2 appeared to be cell-type specific. In COS-7 and Vero cells, LRIG2 localized both to the cell surface and to the cytoplasm, whereas in HEK-293 derived Phoenix cells, LRIG2 primarily localized to the cytoplasm. The presence of asparagine-linked oligosaccharides was investigated using the enzyme N-glycosidase F. Treatment of LRIG2 protein with N-glycosidase F resulted in a reduction of its apparent molecular weight, showing that LRIG2 was a glycoprotein carrying N-linked oligosaccharides.

Expression of LRIG2 during development and in adult tissues

We studied the temporal and spatial gene expression patterns of Lrig1, Lrig2, and Lrig3 during mouse embryonic development by using whole-mount in situ hybridization (Paper II). All three Lrig mRNAs were extensively expressed in all investigated embryonic stages, i.e., E10.5, E11.5, and E12.5. Some embryonic regions displayed extensive overlap in expression of two or three Lrig mRNAs. Unique expression of a single Lrig mRNA was observed for all three Lrigs in different tissues at certain developmental stages. Lrig1 and Lrig3 showed overlapping expression more frequently than Lrig2, whereas Lrig2 showed unique expression domains most frequently. For example, distinct Lrig2 expression was found in several regions in the developing heart and in the neural tube, which could imply important roles of Lrig2 in these regions.
We also analyzed LRIG2 transcript levels in human adult tissues by Northern blotting and quantitative real-time RT-PCR (Paper I). The transcript size of LRIG2 was 4.8 kb in the brain, placenta, skeletal muscle, kidney, pancreas, liver, lung, spleen, and thymus. In the heart, however, the LRIG2 transcript size was slightly smaller, corresponding to a transcript size of 4.6 kb. This could imply tissue-specific alternative splicing of LRIG2 in the heart, which, in light of the fact that Lrig2 was uniquely expressed in the developing heart (Paper II), could be of particular interest. Thus, the possible presence of different Lrig2 isoforms could be of interest to study, but this issue was not further addressed in the current study.

LRIG2 mRNA expression was analyzed in 25 human tissues using quantitative real-time RT-PCR (Paper I). LRIG2 expression was found at various levels in all tissues analyzed. The absolute levels of LRIG2 were relatively high and were in the same levels as the house keeping gene GAPDH in many organs. The highest expression was found in skin, uterus, and ovary. High levels were also found in placenta, kidney, brain, small intestine, adrenal gland, and stomach. Thus, LRIG2, like LRIG1 and LRIG3, was widely expressed in human tissues. Many human tissues showed similar absolute levels of the three LRIG mRNAs. However, LRIG2 expression levels were significantly higher than the levels of LRIG1 and LRIG3 in the adrenal gland, ovary, placenta, and uterus. In the mammary gland, pancreas, and thyroid, LRIG2 levels were much lower than the levels of LRIG1 and LRIG3. The difference in expression of the LRIG mRNAs shows that the LRIG genes are differentially regulated, which could imply differences in their functions as well.

Protein expression was analyzed in tissue lysates from stomach, prostate, lung, and fetal brain. All of the tissues analyzed expressed LRIG2 protein with a particular prominent expression in fetal brain (Paper I). LRIG2 protein expression was also analyzed by immunohistochemistry in human normal brain and peripheral nerve (Unpublished). This revealed an association between LRIG2 expression and axons and nerve fibers in several regions of the CNS (Figure 6). To determine whether LRIG2 was present in the axons or in their myelin sheaths (i.e., whether LRIG2 was expressed by neurons or by oligodendrocytes) we performed co-immunofluorescence analyses of LRIG2 and the myelin protein proteolipid 1 (PLP1) in human optic nerve (Figure 7). This revealed that LRIG2 and PLP1 co-localized in the optic nerve, which showed that LRIG2 was present in the myelin sheaths and thus was expressed by oligodendrocytes.
RESULTS AND DISCUSSION

Phenotype of LRIG2-gene-ablated mice

To study the developmental and physiological role of LRIG2, we generated Lrig2 knock-out mice and investigated their survival, health, body weight, anatomy, and reproductive capacity (Paper II). Lrig2 knock-outs were generated by gene targeting through homologous recombination. The Lrig2 gene was considered to be too long (approximately 50 kb) to be conventionally ablated. Therefore, the targeting vector was constructed to remove exon 12 of the Lrig2 gene, resulting in a shift of the reading frame followed by an

Figure 6. Immunohistochemical analysis of LRIG2 in normal brain and peripheral nerve. Tissue sections were stained for LRIG (brown) followed by nuclear counterstaining (blue). (A) LRIG2 labeling of the cerebrum reveals high expression associated with cerebral white matter. (B) LRIG2 labeling of the medulla oblongata reveals expression associated with axons. (C) LRIG2 labeling of peripheral nerves reveals expression in the myelin sheaths (black arrow). Scale bars: 30 µm

Figure 7. Immunofluorescence co-localization analysis of PLP1 and LRIG2 proteins in human optic nerve. Human optic nerve tissue section was fluorescently labeled with antibodies against PLP1 and LRIG2 and analyzed by confocal immunofluorescence laser microscopy. (A) PLP1 fluorescence (green) reveals optic nerve myelin. (B) LRIG2 fluorescence (red). (C) Merged picture of A and B reveals co-localization of PLP1 and LRIG2. Scale bars: 16 µm.
immediate stop. Despite the fact that \textit{Lrig2} displayed extensive and unique expression in several areas in the developing embryo (\textbf{Paper II}), \textit{Lrig2} knock-out mice were born viable and at Mendelian frequencies with an apparently normal external morphology. However, they displayed a transient but significant reduction in body weight, impaired fertility, and increased mortality (\textbf{Paper II}), as discussed below.

\textit{Reduction in body weight}

There was no difference in body weight between the genotypes in embryos or newborn mice. However, \textit{Lrig2} knock-outs displayed a significantly reduced body weight compared to heterozygous and wild-type mice at 5 days of age and until 12 weeks of age for females and 15 weeks of age for males. To address the role of the genetic background for this phenotype, mice of mixed C57BL/6/FVB/5 background were analyzed; a weight difference at 5 days was also evident C57BL/6/FVB/5 mice. Impaired growth is a common and often idiopathic phenotype in mutant mice, which is reported in numerous studies\textsuperscript{151}. Impaired growth could in principle be due to various causes, with lack of nourishment being one possible explanation. \textit{In situ} hybridization during development revealed unique \textit{Lrig2} expression in the mandible which might suggest a defect in the function of the jaws in the knock-out mice. Therefore, the food pellets were softened in water and made easily accessible for all the animals; nevertheless, the growth differences persisted. No differences in the morphology of the jaws were observed, making it unlikely that this was the cause of the growth impairment. Thus, the \textit{Lrig2} knock-out was associated with an idiopathic and transient reduction in body weight.

\textit{Increased mortality}

\textit{Lrig2} knock-out mice showed an increased mortality compared to their wild-type and heterozygous littermates. By 50 days of age, 20\% of the female and 12\% of the male knock-out mice had died or been euthanized due to disease. Most deaths occurred during the first week after weaning. In most cases, these animals were found dead in their cages, without any previous signs of illness. However, some mice suddenly developed general symptoms of severe illness. They were in poor condition and showed symptoms, including lethargy, hacked fur, and crouched body position, and were therefore euthanized. No other signs were found that could explain the causes of death. To further investigate the causes of death, autopsies were performed on five of the sick and euthanized mice but no obvious cause of death was revealed.
Another possible cause of increased mortality and reduced body weight could be that Lrig2 knock-out mice suffered from disturbed metabolism. Glucocorticoids are steroid hormones that are synthesized and released by the adrenal gland. These hormones regulate a variety of physiological functions, including metabolism, immune functions, and growth. Thus, mice with a specific loss of glucocorticoid receptor in hepatocytes display reduced body weight that is more pronounced in males\(^{152}\). Interestingly, LRIG2 mRNA levels in the human adrenal gland are relatively high. Whether this is also the case in mice has not been addressed. Therefore, it is of interest to investigate the glucocorticoid system in the knock-out mice, as a possible cause of their increased mortality and impaired growth.

In cases of sudden death, one could also suspect heart failure. Lrig2 mRNA was found in the embryonic heart regions. However, necropsy examination of the mice did not reveal any characteristic signs of heart failure. Because arrhythmias due to defects or injuries in the conduction system not always are detected by standard necropsy examinations, it is still possible that Lrig2 plays a role in heart physiology. Therefore, more detailed analyses of the function of the cardiovascular system in the Lrig2 knock-out mice may be warranted.

To examine whether Lrig2 knock-out mice displayed any anatomical aberrations, organs were collected from apparently healthy knock-out, heterozygous and wild-type mice. There was no macroscopically obvious anatomical difference between mice of the different Lrig2-genotypes. However, in females, the weight of the brain was significantly higher in knock-outs compared to Lrig2 wild-type mice. This result could imply a role of Lrig2 in brain development or physiology. However, histological analysis of the brains from Lrig2 wild-types and Lrig2 knock-outs revealed no differences.

**Impaired fertility**

Mating experiments were performed in order to evaluate the reproductive capacity of Lrig2 knock-out mice. The results showed that both male and female Lrig2 knock-out mice had impaired reproductive capacities, but the males exhibited a more severe reproduction phenotype than the females. Only 2 of 11 Lrig2 knock-out males were successful in yielding offspring with wild-type females. Eight of 10 Lrig2 knock-out females were successful in yielding offspring with wild-type males. When wild-type males were mated with wild-type females, all of the tested pairs yielded offspring. The
RESULTS AND DISCUSSION

majority of females mated with Lrig2 knock-out males showed plug formation, which indicates an appropriate mounting behavior and release of seminal vesicle secretion. Lrig2 knock-out males did not show any macroscopically or histologically detectable defects of testes, epididymis, or seminal vesicles. This might therefore indicate a defect that affects the function of sperm, such as motility or the ability of sperm to bind to and fuse with the oocyte. Interestingly, there are high Lrig2 transcript levels in both the testes and ovaries of the adult mouse. Notably, although both Lrig1 and Lrig3 are highly expressed in the reproductive organs, there is no reproduction-associated phenotype reported in Lrig1 or Lrig3 knock-out mice, which might indicate a more important role of Lrig2 in reproduction. A large number of genes are involved in reproduction and there are numerous reports of genetically modified mouse strains with reproductive defects (for review see). Moreover, balanced levels of hormones, such as testosterone and luteinizing hormone, are critical for normal fertilization. Therefore, it would be interesting to investigate any possible association between Lrig2 and hormones with known functions in reproduction.

Due to the extensive expression of Lrig mRNAs in the developing embryo, one could have expected more severe phenotypes of the Lrig mutants. Thus, the relatively mild phenotypes of the Lrig knock-outs might suggest functional redundancy (i.e., the remaining Lrig proteins might compensate for the knocked-out ones). However, our Lrig2 knock-out mice showed a relatively severe phenotype as compared with the Lrig1 and Lrig3 knock-outs. By studying double and triple knock-out mice one could perhaps clarify whether there is functional redundancy among the Lrig proteins. In summary, the Lrig2 knock-out phenotype demonstrated that the Lrig2 protein is important during development. However, more studies are needed to clarify the specific functional role of Lrig2 in development.

LRIG2 in oligodendroglioma

We decided to analyze the role of LRIG2 in oligodendroglioma for several reasons: (i) LRIG2 was located at chromosome 1p13, a region frequently deleted in oligodendroglioma (Paper I); (ii) LRIG2 was prominently expressed by oligodendrocytes (Unpublished, see above); and (iii) the LRIG2 paralog LRIG1 is a known regulator of growth factor signaling and a proposed tumor suppressor. In order to evaluate possible associations between LRIG2 and clinical parameters in oligodendroglioma patients, LRIG2 pro-
tein expression was analyzed in 63 oligodendroglial tumors collected in a tissue microarray by immunohistochemistry. When we compared the LRIG2 staining pattern with survival data, cytoplasmic LRIG2 expression was found to correlate with decreased patient survival. Patients with no cytoplasmic LRIG2 immunoreactivity had a median survival time of 120 months, whereas patients with cytoplasmic LRIG2 immunoreactivity had a median survival time of only 74 months. In a Cox multivariate analysis, cytoplasmic LRIG2 expression was found to be an independent prognostic factor associated with poor oligodendroglioma patient survival. However, because LRIG2 is located on chromosome 1p and a combined loss of 1p and 19q is associated with a favorable outcome for the patients, we wanted to determine if the observed association between low cytoplasmic LRIG2 expression and long survival was secondary to the loss of 1p. Regrettably, it was not possible to determine the 1p/19q status of the tumors included in the tissue microarray study. Instead, an additional set of oligodendrogliomas (n=26) was analyzed for LRIG2 expression and the allelic status of 1p. In this small series of tumors, no correlation between LRIG2 expression and 1p status was found, supporting the concept that LRIG2 is an independent prognostic marker in oligodendroglioma.

As discussed above, LRIG1 is suggested to be a tumor suppressor in certain tumor types. Intriguingly, in the present study, cytoplasmic expression of LRIG2 was associated with a poor survival of oligodendroglioma patients, which was an unexpected observation. However, similar results have been obtained in a series of uterine cervical carcinomas, where cytoplasmic LRIG2 expression was a significant predictor of poor prognosis in early-stage cervical cancer (Hedman et al. submitted). Conversely, in the same tumor series, expression of LRIG1 was associated with good prognosis, suggesting opposing functions of LRIG1 and LRIG2 in cervical cancer, at least.

The possible functional role of LRIG2 in oligodendroglioma genesis was investigated by using a mouse model of oligodendroglioma (Paper IV). Tumors that resembled human oligodendrogliomas were induced by intracranial transduction of mice that expressed the Tv-a RCAS receptor under the control of the nestin promoter (Ntv-a mice) with PDGFB encoding RCAS viruses. The tumor initiating capacity of PDGFB has previously been shown. Ntv-a mice infected with PDGFB carrying RCAS viruses developed gliomas at high frequency, predominantly of oligodendroglioma histology. Interestingly, Lrig2 wild-type mice developed tumors at a higher frequency
and of higher malignancy than \textit{Lrig2} knock-out mice. Tumors arising in the \textit{Lrig2} wild-type mice were both of grade II/III (82\%) and of grade IV (18\%). In contrast, the \textit{Lrig2} knock-out animals developed tumors only of grade II/III (77\%) or no visible tumors at all (23\%). We established mouse embryonic fibroblast (MEF) cell lines from wild-type and \textit{Lrig2} knock-out mice and used these cells to address the role of \textit{Lrig2} in PDGF-induced cell proliferation (\textbf{Paper IV}). Notably, the \textit{Lrig2} knock-out MEF cells showed a reduced sensitivity to PDGF-induced proliferation, which implies a role of LRIG2 in PDGFR signaling. To investigate if LRIG2 influences the levels of PDGFR, we co-transfected LRIG2 and PDGFR\textalpha{} or PDGFR\textbeta{} into HEK-293 cells and examined the protein levels by Western blotting. Interestingly, LRIG2 appeared to stabilize PDGFR\textalpha{}. Taken together, these results indicate that LRIG2 could have a different function than LRIG1 and positively regulate PDGFR\textalpha{}.

\textbf{Is the molecular function of LRIG2 to stabilize PDGFR\textalpha{}}?

Clearly, in our experiments, LRIG2 was important for PDGF-induced oligodendroglioma genesis and cell proliferation. Presently, we can only speculate on the mechanism behind the apparent positive regulation of PDGFR\textalpha{} by LRIG2, but our results suggest that the regulation of PDGFR levels is important. One possible mechanism could be that LRIG2 functions similar to a decoy receptor; that LRIG2 interacts with PDGFR\textalpha{} at the plasma membrane and thereby prevents PDGFR\textalpha{} from interacting with its negative regulator LRIG1. Another possible mechanism could be that LRIG2 interacts with LRIG1 and thereby restraints LRIG1 from exerting its function as a negative regulator of growth factor receptors. This model, however, seems inconsistent with our observation that LRIG2 stabilized PDGFR\textalpha{} only, and not the other LRIG1 target receptors, EGFR, ErbB2, and PDGFR\textbeta{}. Both of the above mentioned models would decrease the degradation rate of PDGFR\textalpha{} and thus result in increased levels of the receptor. The PDGFR\textalpha{} levels would, thus, be influenced by the ratio of LRIG1 and LRIG2 in the cell. A prediction of both of these models, where LRIG2 functions by modulating the activity of LRIG1, is that LRIG2 would not influence receptor levels in LRIG1-deficient cells. This is a testable hypothesis which can experimentally be address by comparing the effects of LRIG2 on PDGFR\textalpha{} in \textit{Lrig1} knock-out and wild-type MEF cells, respectively. In addition, as discussed above, PDGFR\textalpha{} localizes to the primary cilium in growth arrested cells and mutant cells that fail to form cilia do not activate PDGFR\textalpha{}\textsuperscript{26}. Thus, it could be speculated that LRIG2 promotes PDGFR\textalpha{} signaling by mediat-
ing cilia formation and/or that LRIG2 is important for transport of the receptor to the plasma membrane and localization to cilia.

Our Lrig2 knock-out phenotype is also consistent, at least partly, with effects on PDGF signaling. For example, Lrig2 knock-out mice displayed an impaired reproductive capacity and as discussed above, several studies have shown the importance of PDGFRα signaling in both male and female fertility.\textsuperscript{33-34,39} PDGF-A is the major mitogen for oligodendrocyte progenitor cells and determines the number of progenitor cells both in the embryo and in the adult mice.\textsuperscript{32,38,154} However, despite that LRIG2 affects PDGFRα levels and is expressed in oligodendrocytes, there was no myelin phenotype observed in the Lrig2 knock-out mice. Interestingly, it has been claimed that heterozygous PDGFA mutants become normally myelinated in the CNS despite that only half the number of oligodendrocyte progenitor cells are formed.\textsuperscript{18,28} Thus, although Lrig2 knock-out mice does not show a myelin phenotype it would be interesting to analyze if Lrig2 may have an effect on the number of progenitor cells in CNS. In summary, our results are consistent with a role for LRIG2 in the regulation of PDGF signaling, however, other functions of LRIG2 cannot be excluded at present.
CONCLUDING REMARKS

This thesis describes the cloning and characterization of a novel member of the LRIG protein family, named LRIG2. LRIG2 expression was demonstrated in a variety of tissues during development and in adults. Lrig2 knock-out mice were born at Mendelian frequencies with an apparent normal external morphology. However, they showed transiently reduced body weight, an increased mortality, and an impaired reproductive capacity, which demonstrates that Lrig2, is important during development. Furthermore, in human oligodendroglioma, LRIG2 expression was associated with poor patient survival and in a mouse tumor model, LRIG2 promoted PDGFB induced oligodendroglioma genesis. Recent studies have established LRIG1 as a negative regulator of growth factor signaling. However, the results presented in this thesis imply opposite functions of LRIG1 and LRIG2. Finally, this thesis suggests a possible functional role of LRIG2 as a PDGFRα stabilizing protein. Further studies are needed to elucidate the role of LRIG2 in oligodendroglioma genesis and the underlying molecular mechanism behind its apparently positive regulation of PDGFRα.
ACKNOWLEDGEMENTS

During my research studies I have received plenty of support and encouragement from many persons in my surrounding and without your help this thesis would not have been accomplished. Therefore I would like to thank everybody who in some way has supported me. In particular, I would like to thank:

First and most importantly, **Håkan Hedman**, my supervisor who made this thesis possible. Thanks for all your encouragement and never-failing support that you have given me over the years. Your scientific knowledge, enthusiasm, motivation and guidance, make you a great supervisor! Thanks for always being supportive and optimistic even when my experiments failed, so many times, you have the patience of an angel!

**Roger Henriksson**, my co-supervisor. Although, we may not have had such a close supervisor/student relationship I have always felt your support. Thanks for always being optimistic and inspiring and for believing in me.

**Tommy Bergenheim**, for introducing me to Håkan a long time ago…

Thanks to all my co-authors for your contribution to this work. Special thanks to: **Hannu Haapasalo**, for taking such good care of me during my visit. **Olivera Casar Borota**, for your kindness and helpfulness. **Veronica Janson**, for interesting discussions and for being very supportive. I really appreciate all the help you have given me the last couple of weeks.

**Pia, Carina** and **Monica** for helping me out with all sorts of administrative matters, what would I have done without you?

To everyone at the oncology lab, for helping me out with all kind of matters in the lab, for that I’m very grateful. Special thanks to **Lotta**, for friendship and providing me with moral support during the animal experiments, you are great! **Annika H**, for helping out whenever needed and for teaching me RT-PCR. **Yvonne**, for keeping track of all the animals and for keeping me informed about my rights as an employee ☺. **Kerstin**, for IHC staining and your fantastic “lussebulle”. **Mikael K**, for providing help when ever needed; your words of wisdom still light up my day. **Ulrika**, for support and encouragement no matter what, sharing both happiness and sorrows. Ior is now a dear friend, can I keep him? **Marcus**, for many pep-talks and for proof reading part of this thesis. **Terese**, for interesting discussions, you are a great lab mate! **Annika N**, my present personal computer support. Many thanks for your help with awkward figures and lay-outs; you have saved me many gray hairs ☺. **Calle**, my previous computer support, your knowledge about computers have made my life much easier! **Jonas, Guo, and Yi We**, my old LRIG companions. **Wang**, thanks for all the help in the lab, particularly for feeding my cells during
ACKNOWLEDGMENTS

weekends. Anna, for nice long chats, I miss your company. Farrah, for putting in a lot of hard work to help me out when I ran out of time. Soma, for being a great room-mate and for providing me with chocolate. Emma, your optimism and support the last couple of weeks have been great. Ashwin, welcome to the lab!

Agneta, for friendship and for always making me feel that things are going to be ok!

Mikael J, you have been very encouraging, I really appreciate your support! Thanks for reading and commenting on this text.

To the people in the “old” coffee-room (nobody mentioned nobody forgotten) for pleasant company.

Kjell, Parviz. Britta, David, and Jasmine, thanks!

Theresé A, it has been great to share this last couple of weeks with someone in the same boat. I´ll meet you at the rehab center later…

All the people at the department for Medical Biosciences for all your kindness, especially Åsa Lundsten, for helping me out with all kind of practical stuff when moving to the new lab. I don´t know what I would have done without you?

Thanks to the staff in the animal facilities for always helping me out when needed.

To the guy at UMDAC, who saved my Endnote library one week before printing, you are my hero☺.

To all my loved ones, you know who you are, and that you are very important to me!

Mina underbara barn, Moa, Jacob och Elin, tack för att ni har varit så fantaskiskt tålmodiga med mig de sista månaderna. Ni är det som är det viktiga här i livet! Joachim, utan ditt stöd och outtrötliga support hade jag aldrig klarat detta! För att inte tala om den otroliga markservice jag fått de sista månaderna, du har varit fantastisk! Trots många sena kvällar på jobbet så blev det till slut ”bara” en avhandling och ingen ny Nationalencyklopedi☺.
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