Role of intracellular FGF1 interaction partners, and HSP90 as a therapeutic target in FGFR1 driven malignancy

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AB</td>
<td>Acidic box</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens junction</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>BAIAP2L1</td>
<td>BAI1-associated protein 2-like 1</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BCR</td>
<td>Breakpoint cluster region</td>
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<tr>
<td>BCR-ABL</td>
<td>Break-cluster-region-Abelson</td>
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<tr>
<td>CBL</td>
<td>Ubiquitin ligase</td>
</tr>
<tr>
<td>CDC37</td>
<td>Cell division cycle 37</td>
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<tr>
<td>Cdc42</td>
<td>Cell division control protein 42</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein kinase 2</td>
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<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
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<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DLT</td>
<td>Dose-limiting toxicitie</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERBB2/HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<tr>
<td>FOP1/FOP2</td>
<td>FGFR1 oncogene partner 1/2</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<tr>
<td>FHF</td>
<td>FGF homologous factors</td>
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<tr>
<td>FIBP</td>
<td>FGF1 intracellular binding protein</td>
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<tr>
<td>FRS2</td>
<td>Fibroblast growth factor receptor substrate 2</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumor</td>
</tr>
<tr>
<td>GAB1</td>
<td>GRB2-associated binding protein 1</td>
</tr>
<tr>
<td>GAR</td>
<td>Glycine/arginine rich</td>
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<tr>
<td>GAS</td>
<td>Gamma activated site</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>GRB2</td>
<td>Growth factor receptor-bound 2</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphate hydrolase</td>
</tr>
<tr>
<td>Hdm2</td>
<td>Human double minute 2</td>
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<tr>
<td>HMW</td>
<td>High molecular weight</td>
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<tr>
<td>HSF1</td>
<td>Heat shock factor 1</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>Kpn</td>
<td>Karyopherin</td>
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<tr>
<td>LRRC59</td>
<td>Leucine rich repeat containing 59</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MKP3</td>
<td>MAPK phosphatase 3</td>
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<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
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<tr>
<td>MTOC</td>
<td>Microtubule organizing center</td>
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<tr>
<td>NES</td>
<td>Nuclear export sequence</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
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<tr>
<td>NSCLC</td>
<td>Non-small-cell lung carcinoma</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase/Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PtdIns(4,5)P2</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P3</td>
<td>Phosphoinositide-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>RAF</td>
<td>Rapidly accelerated fibrosarcoma</td>
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<tr>
<td>RAS</td>
<td>Rat sarcoma</td>
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<tr>
<td>RBP</td>
<td>RNA binding protein</td>
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<tr>
<td>Rho</td>
<td>Ras homology gene family</td>
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<tr>
<td>RRM</td>
<td>RNA recognition motifs</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<tr>
<td>RSK2</td>
<td>Ribosomal protein S6 kinase 2</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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</tbody>
</table>
SCLL  Stem cell leukemia/lymphoma
Sef   Similar expression to fgf genes
SH2   Src homology 2
siRNA small interferering RNA
SOS   Son of sevenless
SPR   Surface plasmon resonance
STAT Signal transducer and activator of transcription
TACC Transforming acidic coiled-coil
TGF   Transforming growth factor
TJ    Tight junction
TKI   Tyrosine kinase inhibitor
ZNF198 Zinc finger gene ZNF198
17-AAG 17-alkylamino-17demetohxygeldanamycin
List of papers

Paper I

Nucleolin regulates phosphorylation and nuclear export of fibroblast growth factor 1 (FGF1)

Torunn Sletten, Michal Kostas, Joanna Bober, Vigdis Sorensen, Mandana Yadollahi, Sjur Olsnes, Justyna Tomala, Jacek Otlewski, Małgorzata Zakrzewska, Antoni Wiedlocha

PloS ONE, in press.

Paper II

Different anti-leukemic activity of the HSP90 inhibitor NVP-AUY922 during in vitro and in vivo treatment of human leukemic KG-1a cells

Torunn Sletten, Skjalg Bruheim, Antoni Wiedlocha

Paper III

The ER-protein LRRC59 is involved in regulating epithelial cell polarization, directed migration, and lumen expansion in Caco-2 cysts

Torunn Sletten, Antoni Wiedlocha, Vigdis Sorensen
Introduction

Fibroblast growth factors (FGFs) are ligands involved in a great variety of essential cellular processes. FGFs were first discovered in fibroblasts, thereby their name. However, several FGFs were later found to be expressed in other cell types such as epithelial and neuronal cells (Burgess and Maciag, 1989). FGFs are highly conserved from invertebrates to vertebrates. They bind fibroblast growth factor receptors (FGFRs) on the cell surface, and upon downstream signaling events, regulate proliferation, differentiation, migration, apoptosis and growth arrest. FGF signaling regulates several developmental processes such as mesoderm patterning in early embryo and development of multiple organ systems, including limb, midbrain and lung, as well as development of the nervous system (Beenken and Mohammadi, 2009; Guillemot and Zimmer, 2011; Mason, 2007; Thisse and Thisse, 2005; Turner and Grose, 2010). In adult life, FGFs are crucial regulators in inflammation, wound healing, tissue repair and angiogenesis (Powers et al., 2000).

FGF induced signaling is specific and highly regulated, and disrupted FGF signaling causes a wide spectrum of severe pathological conditions, such as skeletal disorders, dwarfism, mood disorders, metabolic disorders, Kallmann syndrome, as well as cancer (Eswarakumar et al., 2005; Hardelin and Dode, 2008; Itoh and Ornitz, 2011; Su et al., 2008; Turner et al., 2012; Turner and Grose, 2010). As FGF signaling orchestrate a plethora of functions, it is highly important to understand the mechanisms of FGF signaling in order to understand severe conditions such as cancer.

The fibroblast growth factor family

The FGF family consists of 22 members in humans, comprising FGF1-FGF23, where FGF15 is not identified in humans. The FGF family encodes structurally related polypeptides with high affinity to heparin, and they are classified according to their differences in sequence homology, mechanisms of action, and phylogeny (Itoh and Ornitz, 2004). The first FGFs detected were FGF1 and FGF2. They were isolated from a bovine brain and the pituitary gland almost four decades ago, and were employed as mitogens for cultured fibroblast (Gospodarowicz, 1975; Gospodarowicz et al., 1978). The different FGFs consists of ~150-300 amino acids, where a
A conserved core comprising 120 amino acids shows a ~30-60 % amino acid identity (Itoh and Ornitz, 2004).

Based on phylogenetic analysis, the FGF family has been divided into seven subfamilies. The subfamilies FGF1/2, FGF4/5/6, FGF3/7/10/22, FGF9/16/20 and FGF8/17/18 function in a paracrine fashion, whereas the subfamily FGF19/21/23 functions in an endocrine manner, while the subfamily FGF11/12/13/14 functions in an intracrine fashion (Figure 1) (Itoh and Ornitz, 2011). *FGF15* and *FGF19* are orthologous genes, where *FGF15* is expressed in mice and not humans, while *FGF19* is expressed in humans but not in mice (Ornitz and Itoh, 2001).

![Figure 1. The FGF family](image)

The phylogenetic tree shows the evolutionary relationship between the 22 FGF genes. The 22 FGF genes are divided into seven subfamilies, each consisting of 2-4 FGF genes. The branch length illustrates the proportional distance between each gene (from Itoh and Ornitz et al. 2011).

FGF11-14 are called FGF homologous factors (FHFs). They lack a signal sequence for secretion through the endoplasmatic reticulum (ER)-Golgi secretory pathway, and
are not secreted from the cells they were produced in. They express intracrine activity and act independently of FGFRs (Kelleher et al., 2013; Schoorlemmer and Goldfarb, 2001). Due to the FGFR independent mechanisms of action, their affiliation to the FGF family has been disputed (Goldfarb, 2005). FGF11-14 has been shown to control excitability throughout the central nervous system by binding to and modulating intracellular domains of voltage gated sodium channels (Goldfarb et al., 2007), and calcium channels (Hennessey et al., 2013). Furthermore, FGF11-14 interact with the neuronal MAPK scaffold protein, islet-brain-2 (Schoorlemmer and Goldfarb, 2002).

Many FGFs mediate paracrine signaling, which means that they are secreted from the cell they were produced in to act on FGFRs on neighboring cells. For instance, ligands produced in epithelial cells are secreted to act on FGFRs on mesenchymal cells and vice versa. The subfamily FGF4/5/6, FGF3/7/10/22, and FGF8/17/18 contains a cleavable N-terminal signal sequence that direct secretion through the classical ER-Golgi secretory pathway (Itoh and Ornitz, 2011; Powers et al., 2000). The subfamily FGF9/16/20 lacks a signal sequence on the N-terminal, but is nonetheless secreted through the classical ER-Golgi pathway (Itoh and Ornitz, 2011; Powers et al., 2000). The FGF1/2 subfamily does not hold a signal sequence and are synthesized on cytosolic ribosomes (Burgess and Maciag, 1989). Nevertheless, FGF1 and the 18 kDa isoform of FGF2, produced upon initiation of translation at the 5’ AUG start codon (Mignatti et al., 1992), can be released from the cell in unconventional manners independent of the ER-Golgi pathway. FGF1 is released upon stress such as heat shock, hypoxia or serum deprivation (Jackson et al., 1992; Mouta et al., 2001; Shin et al., 1996). FGF1 secretion depends on complex formation with the calcium binding protein S100A13 and binding to the cytoplasmic C2A domain on the vesicle protein synaptotagmin (Landriscina et al., 2001; Mohan et al., 2010; Tarantini et al., 1998). In contrast to the stress induced release of FGF1, FGF2 is constitutively released. The 18 kDa isoform interacts with heat shock protein 27 (HSP27), and this interaction is probably crucial for HSP27 facilitated secretion of the 18 kDa isoform of FGF2 (Piotrowicz et al., 1997). Secretion of the 18 kDa isoform of FGF2 is also dependent on the multidrug resistant-associated proteins, as probeinuid and ovabain inhibition impaired secretion, and on the α-subunit of Na⁺-K⁺-ATPase (Florkiewicz et al., 1998; Gupta et al., 1998). Also, translokin together with the KIF3A kinesin has been shown to play an important role in secretion of the 18 kDa isoform (Meunier et
al., 2009). Additionally, unfolding of FGF2, is not crucial for its secretion (Backhaus et al., 2004).

The paracrine FGFs have strong affinity to heparan sulfate proteoglycans (HSPGs) and heparin, a highly sulfated glycosaminoglycan. When secreted, the paracrine FGFs bind to HSPGs present on the cell surface and in the extracellular matrix (ECM). HSPGs in the ECM function as a reservoir for secreted FGFs. Heparinases, proteases or specific FGF binding proteins can release FGFs from the HSPG, and released FGFs can bind to FGFRs on the cell surface of neighboring cells. HSPGs also interact with the FGFR and FGF and stabilize the ligand-receptor complex.

The subfamily 15/19/21/23, on the other hand, does not interact with HSPGs, which enables them to diffuse and function in an endocrine fashion. These FGFs have low affinity to the FGFRs, and the interaction with FGFRs requires the transmembrane co-receptor klotho (Goetz et al., 2007; Zhang et al., 2006).

In addition to acting in a paracrine manner, FGF1, FGF2, and FGF3 also function in an intracrine fashion (Wiedlocha and Sorensen, 2004). FGF1 possesses nuclear localization motifs, one monopartite nuclear localization sequence (NLS) on the N-terminal part, and one bipartite NLSs on the C-terminal part, important for its translocation into the nucleus (Imamura et al., 1990; Wesche et al., 2005). Exogenous FGF1 and endogenous FGF1 translocates into the nucleus (Cao et al., 1993; Sano et al., 1990; Wiedlocha et al., 1995). The low molecular weight variant of FGF2 (18 kDa) contains a bipartite arginine rich NLS on its C-terminal part (Sheng et al., 2004), and can translocate into the nucleus before secretion (Choi et al., 2000; Claus et al., 2003). It can also translocate into the nucleus after internalization (Bouche et al., 1987). High molecular weight (HMW) FGF2 isoforms (22, 22.5, 24, and 34 kDa) are produced upon translation initiation upstream of the 5’ AUG start codon, namely on in-frame CUG codons, and this upstream area harbors an additional NLS. The endogenous HMW isoforms of FGF2 are not secreted from the cells they were produced in, but localize to the nucleus in an NLS mediated fashion upon methylation of arginine residues on the NLS localized in the upstream area (Pintucci et al., 1996). The 34 kDa FGF2 isoform possesses yet an additional NLS (arginine rich type), and nuclear import is suggested to occur by binding to importin β (Arnaud et al., 1999).
FGF3 also possess an NLS on the C-terminal part, and can also localize in the nucleus (Antoine et al., 1997; Kiefer et al., 1994).

**The fibroblast growth factor receptor family**

The FGFR family comprises four closely related genes which encode four tyrosine kinase FGFRs (FGFR1-4). All FGFRs are single-pass transmembrane proteins composed of a transmembrane domain, a split tyrosine kinase domain localized on the cytosolic side, and an extracellular part (Figure 2). The extracellular part includes a signal peptide, and up to three immunoglobulin (Ig)-like domains (D1-D3). Domain D2 and D3 binds to FGF ligands, and a conserved positively charged region in the D2 domain serves as a binding site for HSPGs/heparin. Interaction with HSPGs/heparin is crucial for the generation of a stable FGF/FGFR signaling complex (Schlessinger, 2000). An acidic stretch containing seven to eight acidic residues and a serine rich region between D1 and D2 is called the acidic box (AB). Receptor auto-inhibition is achieved through interactions between the acidic box and the positively charged heparan sulfate binding region in D2 (Kalinina et al., 2012; Schlessinger, 2003).

Due to alternative splicing of the FGFR mRNAs, a variety of FGFR isoforms exist, including soluble secreted FGFR isoforms, FGFRs with a truncated C-terminal domain, and FGFRs containing two or three Ig-like domains. Further diversity is made by alternative splicing in the D3 domain of FGFR1-3, which leads to receptor variants with different ligand-binding specificities (FGFR1b/c, FGFR2b/c, and FGFR3b/c). These receptor variants are produced with different exons encoding for the C-terminal half of D3, where exon 8 encodes the FGFRb isoforms, while exon 9 encodes the FGFRc variants (Eswarakumar et al., 2005). The FGFRb is expressed mostly in epithelial cells, while FGFRc is expressed mostly in mesenchymal cells. Paracrine signaling is generated by epithelial cells producing ligands which activate the mesenchymal FGFRc isoforms, and mesenchymal cells producing ligands which activate the epithelial FGFRb isoforms. For instance, FGFR2b expressed by epithelial cells, binds FGF7 and FGF10 produced in mesenchymal cells, but not FGF2, which is produced in epithelial cells. FGFR2c expressed by mesenchymal cells, binds FGF2 and FGF18 produced in epithelial cells, but not FGF7 and FGF10, which is produced in mesenchymal cells (Eswarakumar et al., 2005). This creates a regulatory
mechanism which permits communication between the epithelial and mesenchymal layers during development (De et al., 2000; Eswarakumar et al., 2002; Mohammadi et al., 2005; Orr-Urtreger et al., 1993). Each of the FGFRs interacts with an exclusive subset of FGFs. Exceptionally, FGF1 binds all receptor isoforms (Powers et al., 2000).

Figure 2 A schematic presentation of the FGFR

The N-terminal part of the FGFR consists of a signal peptide and three Ig-like domains (D1-D3) facing the ECM. FGFs bind to D2 and D3, while the receptor associates with HSPG/heparin via the D2 domain. The acidic box (AB) is localized between the D1 and D2 domain. Alternative splicing of the D3 domain of FGFR1, FGFR2, and FGFR3 generates the FGFRb and FGFRc isoforms. The juxtamembrane region is localized between the transmembrane domain (TMD) and the kinase domain. The juxtamembrane region, the kinase domain, and the C-terminal tail are localized within the cytosol (adapted from Goetz and Mohammadi et al., 2013).

The cytosolic part consists of a variety of regulatory residues. It holds a split-tyrosine kinase domain, and transphosphorylated tyrosines serve as docking sites for intracellular signaling proteins. The phosphorylated tyrosine kinase domains also phosphorylates the downstream signaling molecule phospholipase Cγ (PLCγ) and the scaffolding protein fibroblast growth factor receptor substrate 2 (FRS2), both critical
contributors of FGF downstream signaling. The C-terminal tail is rich in serines, and ERK phosphorylation of Ser777 was found to be crucial for inhibition of FGFR1 signaling (Zakrzewska et al., 2013). Moreover, the juxtamembrane region of FGFRs are highly conserved and is responsible for FRS2 binding (Ong et al., 2000). Furthermore, the intracellular part contains conserved lysine residues, which can be ubiquitinated upon FGF stimulation, leading to sorting of FGFRs to lysosomes for degradation (Haugsten et al., 2005; Haugsten et al., 2008). Specific amino acids in the C-terminal part of FGFR1 and FGFR4 were found to be important for nuclear translocation of exogenous FGF1 (Sorensen et al., 2006).

Activation of fibroblast growth factors and cell signaling

Downstream signaling events are activated upon binding of FGF ligands to FGFRs. Paracrine FGF ligands binds with high affinity to FGFRs (K_D=10^{-11} M), and with lower affinity to HSPG (K_D=10^{-8} M) (Burgess and Maciag, 1989). FGF and FGFR, including HSPG, form a FGF:HSPG:FGFR dimeric 2:2:2 ternary complex (Schlessinger et al., 2000). This complex formation stabilizes the ligand-receptor interaction, and enables receptor dimerization and a conformational shift in the FGFR structure, which induce transphosphorylation of the tyrosine kinase domain (Eswarakumar et al., 2005). Seven tyrosine phosphorylation sites were discovered in FGFR1 (Tyr463, Tyr583, Tyr585, Tyr653, Tyr654, Tyr730 and Tyr766) (Schlessinger, 2000), which are phosphorylated sequentially and in a strictly ordered three stage fashion (Furdui et al., 2006; Lew et al., 2009). First, Tyr653 localized in the activation loop of the catalytic core of the kinase is phosphorylated. This initiates a 50- to 100-fold increase in the activity, switching from a low state activity to an active state. This is followed by the second stage, where four tyrosines are phosphorylated sequentially, starting with Tyr583 located at the kinase insert region, followed by Tyr463 situated in the juxtamembrane region, then Tyr766 in the C-terminal tail, and finally the Tyr585 in the kinase insert region (Furdui et al., 2006). The third stage transphosphorylation occurs on Tyr654, the second tyrosine in the activation loop, and induces a 10-fold increase of the tyrosine kinase activity (Lew et al., 2009). The seventh tyrosine that is phosphorylated, Tyr730 is located in the second half of the conserved kinase (Mohammadi et al., 1996).
Activated FGFR kinases, in turn phosphorylate and activate the three main signaling pathways RAS/MAPK, PI3K/AKT and PLCγ/Protein kinase C (PKC), which initiate several cellular responses (Figure 3) (Eswarakumar et al., 2005; Powers et al., 2000; Turner and Grose, 2010).

FRS2 is a key mediator of signaling via FGFRs, and plays a significant role in FGF induced RAS/MAPK stimulation and PI3K/AKT stimulation (Eswarakumar et al., 2005; Goetz and Mohammadi, 2013; Turner and Grose, 2010). FRS2 possesses a phosphotyrosine binding (PTB) domain and myristylation sites at the N-terminal part, and several tyrosine phosphorylation sites on the C-terminal part (Kouhara et al., 1997). FRS2 is constitutively associated with the juxtamembrane region of the receptor through its PTB domain, and localizes to the plasma membrane via its myristylation sites (Gotoh, 2008). Upon activation and phosphorylation of tyrosines on the FGFR, FRS2 is phosphorylated on its tyrosine sites and activated. Phosphorylated FRS2 recruits and binds the adaptor protein growth factor receptor-bound 2 (GRB2). GRB2 forms a complex with either the guanine nucleotide exchange factor, son of sevenless (SOS) or the GRB2-associated binding protein 1 (GAB1). Upon complex formation with SOS, SOS stimulates GDP-GTP exchange and activation of RAS GTPase and the downstream RAF and MAP kinases (Figure 3a). The RAS-MAPK cascade mainly induces cell proliferation, but can also initiate differentiation, migration, or other cellular processes (Goetz and Mohammadi, 2013). Complex formation with GAB1, on the other hand, leads to activation of the PI3K/AKT-dependent anti-apoptotic pathway (Figure 3b) (Eswarakumar et al., 2005; Goetz and Mohammadi, 2013; Turner and Grose, 2010).

The downstream signaling molecule PLCγ is recruited to the transphosphorylated Tyr766 in the C-terminal FGFR1 tail through its Src homology 2 (SH2)-domain (Mohammadi et al., 1991). Upon interaction with the receptor, the phosphorylated tyrosine kinase activates PLCγ. This leads to hydrolysation of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) and production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Figure 3c). DAG activates PKC, a kinase family involved in several intracellular processes. IP3 binds to receptors on ligand gated calcium channels on the ER, triggering the opening of these ion channels and releasing calcium from the ER lumen. Calcium release activates the calcium dependent proteins
such as the phosphatase calcineurin, the calmodulin dependent protein kinase, as well as some members of the PKC family (Eswarakumar et al., 2005). The activation of the PLC\(\gamma\)/PKC pathway leads to expression of proteins involved in cell motility, as well as reinforcement of RAF signaling (Hartwig et al., 1992; Turner and Grose, 2010).

**Figure 3 FGFR signaling pathways**

a) FGF binding to FGFRs and activation of FRS2 activates GRB2, which forms a complex with SOS. This activates the RAS/RAF/MAPK pathway, initiating the activity of the transcription factor FOS, which induce transcription of genes important for cell proliferation. b) Complex formation between GRB2 and GAB1 initiates the PI3K/AKT signaling pathway, promoting cell survival. AKT activity inhibits pro-apoptotic effectors such as Caspase 9, BCL-2 antagonist of cell death (BAD) and BCL-2-associated X protein (BAX), as well as the activity of the transcription factor forkhead box class O (FOXO). c) FGF binding and activation of the FGFRs also activates PLC\(\gamma\) resulting in hydrolysis of PtdIns(4,5)P\(_2\) into IP\(_3\) and DAG. DAG activates PKC, which stimulates myristolated Ala-rich C kinase substrate (MARCKS), an important regulator of cell migration. IP\(_3\) stimulates Ca\(^{2+}\) release, and activation of calcium dependent proteins such as calcineurin, which then stimulates nuclear translocation of nuclear factor of activated T-cells (NFAT). NFAT is a transcription factor that initiates transcription of genes required for cell migration (from Goetz and Mohammadi et al., 2013).

Other FGFR signaling pathways include activation of the ribosomal protein S6 kinase 2 (RSK2) pathway downstream of MAPK (Kang et al., 2009; Xian et al., 2009), the p38 MAPK pathway, a kinase activated downstream of RAS, and the Jun N-terminal kinase (JNK) pathways (Turner and Grose, 2010). Additionally, FGFR signaling also
activates SRC (Salotti et al., 2013). The activities of these pathways are usually cell context dependent, and often occurs in cancer (Turner and Grose, 2010).

In some cell types, and especially in cancer, active receptor tyrosine kinases (RTKs), including FGFRs activate signal transducer and activator of transcription (STAT) signaling (Harada et al., 2007; Hart et al., 2000; Hart et al., 2001; Turner and Grose, 2010; Wu et al., 2014). EGFR-STAT3 signaling induce the formation of malignant peripheral nerve sheath tumors (Wu et al., 2014). Possibly, STAT is recruited to FGFRs upon phosphorylation of tyrosine residues. STAT, harboring a SH2 domain, binds to the phosphorylated receptor tyrosine kinase, resulting in phosphorylation on a single residue on STAT. This phosphorylation activates STAT, which then dissociates from the receptor and dimerize following translocation into the nucleus (Turner and Grose, 2010). In the nucleus, STAT binds to regulatory regions of target genes, specifically to a gamma activated site (GAS) family of enhancers thereby initiating or enhancing transcriptional responses (Kisseleva et al., 2002; Murray, 2007). It is not fully understood how STAT signaling is activated by RTKs, and it seems to vary within different contexts. For instance in glioblastoma, the epidermal growth factor receptor (EGFR) is translocated into the nucleus where it directly activates STAT (Fan et al., 2013).

FGFR signaling is attenuated by endocytosis followed by receptor degradation in lysosomes (Haugsten et al., 2008), and by negative feedback mechanisms (Cabrita and Christofori, 2008; Furthauer et al., 2002; Tsang et al., 2002; Turner and Grose, 2010). FRS2 is important for inhibition of FGFR signaling via endocytosis. It recruits ubiquitin ligase CBL through the adaptor molecule GRB2, which leads to degradation of both FRS2 and FGFRs (Wong et al., 2002). MAPK signaling phosphorylates FRS2 on multiple threonine residues and this attenuates ligand induced receptor signaling through a negative feedback mechanism, which reduces FRS2 tyrosine phosphorylation and thereby the recruitment of GRB2 to the signaling complex (Lax et al., 2002). Moreover, upon FGFR activation, sprouty proteins are phosphorylated and interacts with GRB2, which then inhibits complex formation between GRB2 and FRS2 (Hanafusa et al., 2002). Also, inducton of MAPK phosphatases such as MAPK phosphatase 3 (MKP3) attenuates downstream signaling (Zhao and Zhang, 2001). Additionally, similar expression to \textit{fgf} genes (Sef) family members attenuates FGFR
phosphorylation following ERK activity (Kovalenko et al., 2003). Another negative feedback mechanism for FGFR signaling involves ERK-mediated phosphorylation of FGFR1 on Ser777, which results in lower receptor kinase activity, and reduced signaling. Additionally, activation of MAPKs via other RTKs can result in phosphorylation of FGFR1 on Ser777, revealing a crosstalk regulation of FGFR activity by other signaling pathways (Zakrzewska et al., 2013).

Translocation of exogenous FGF1 into the nucleus

FGF1 influence cellular behavior by several mechanisms. For instance, exogenous FGF1 act by binding to and activation of FGFR, activating intracellular signaling cascades, as well as via an intracrine pathway upon translocation into the cytosol and nucleus (Imamura et al., 1990; Imamura et al., 1994; Wiedlocha and Sorensen, 2004; Wiedlocha et al., 2005). The role of translocated exogenous FGF1 in the nucleus remains to be elucidated, but the mechanism for its translocation has been thoroughly investigated and will be described in the following section.

More than 20 years ago, exogenous FGF1 was found to translocate into the cytosol and nucleus due to a putative NLS located on a basic region enclosed on residues 21-27 of FGF1 (Imamura et al., 1990; Imamura et al., 1992). When the NLS sequence was deleted, the deletion mutant was not able to translocate into the nucleus. Neither was it able to stimulate a full mitogenic response, although it was able to bind and activate receptors and the transcription factor *c-fos*. Several other publications supported the finding that FGF1 could localize in the nuclear compartment and function by a dual mechanism (Cao et al., 1993; Friedman et al., 1994; Imamura et al., 1994; Sano et al., 1990). In addition to the monopartite NLS identified on the N terminal part of the FGF1 molecule (Imamura et al., 1990), FGF1 translocation into the nucleus is also regulated by a bipartite NLS localized on the C-terminus (Wesche et al., 2005). Wiedlocha et al., published two reports indicating that a dual signaling mechanism exists for FGF1, both nuclear translocation of extracellular FGF1 and signaling via FGFRs. FGF1 fused to the N-terminal end of a diphtheria toxin A fragment (FGF1-DT-A) was added to cells without FGFRs. FGF1-DT-A translocated into the cells via the toxin entry pathway, and an increase in DNA synthesis but no effect on proliferation was observed in the cells (Wiedlocha et al., 1994). The report
showed that a mitogenic response of FGF1 was activated after translocation into the nucleus. Further, it was found that both FGF1 stimulation of FGFRs on the cell surface and nucleocytoplasmic translocation was required for full mitogenic response (Wiedlocha et al., 1996).

Over the years the translocation mechanism of exogenous FGF1 has been shown to be a highly regulated process. FGF1 translocation was found to be dependent on the FGFRs, however binding to HSPG was not crucial for this process (Klingenberg et al., 1998; Klingenberg et al., 2000a; Wiedlocha et al., 1995; Wiedlocha et al., 1996). The C-terminal end of FGFR4 containing 57 amino acids was found to be required for translocation. This indicates that components within the C-terminal end is essential for FGF1 translocation (Klingenberg et al., 2000a). Later, FGF1 translocation was found to be dependent on binding to either FGFR1 or FGFR4, and not FGFR2 and FGFR3 (Sorensen et al., 2006). Mutational analysis showed that variation in the cytoplasmic tail influenced the ability of the different FGFRs to mediate translocation of FGF1 into the cytosol and nucleus. A few specific amino acid residues on FGFR1 and FGFR4 was important for translocation of FGF1 (Sorensen et al., 2006). Interestingly, upon deletion of the tyrosine kinase domain or inhibition of the kinase activity, FGF1 was still translocated into the nucleus (Klingenberg et al., 2000a; Sorensen et al., 2006; Zakrzewska et al., 2011). Altogether these studies highlight the importance of the C-terminal receptor tail.

It was shown that treatment with Bafilomycin A1, blocking the vacuolar proton pumps, completely inhibited translocation of FGF1 (Malecki et al., 2002). Vacuolar proton pumps are only localized in endosomes, including the lysosomes, and the Golgi apparatus, and this study shows that translocation of FGF1 to the cytosol probably take place in an intracellular membrane bound endosomal compartment. Treatment with Brefeldin A, which disrupts the Golgi apparatus, had no effect on FGF1 translocation into the cell, indicating that FGF1 does not employ the retrograde transport route through the Golgi and ER (Malecki et al., 2002). Acidification of the cytosol was performed to inhibit the endocytic uptake from coated pits, however, this did not influence translocation of FGF1 into the cell interior, suggesting an alternative endocytic pathway required for FGF1 translocation (Citores et al., 1999). Disruption of microtubules and actin filaments by nocodazole, did not influence the translocation
of FGF1 into the cytosol (Malecki et al., 2002). Lysosomes are vesicles that belong to the degradative pathway, and trafficking of early endosomes towards lysosomes occurs on these cytoskeletal structures. Thereby, these findings indicate that FGF1 translocation across the membrane occurs in early endosomes and not lysosomes as they depend on these cytoskeletal structures. Although proteins crossing cellular membranes usually require extensive unfolding of the protein, this was not required for translocation of FGF1 over membranes (Wesche et al., 2000).

Several intracellular molecules were found to be important in regulation of cellular uptake of FGF1. PI3 kinase was found to play a role in endocytosis (Klingenberg et al., 2000b), while p38 MAPK was found to play a role in translocation (Sorensen et al., 2008). The molecular chaperone heat shock protein 90 (HSP90) was also found to be important for translocation of both FGF1 and FGF2 from endosomes to the cytosol, as a specific inhibitor of HSP90 blocked translocation of FGF1 and FGF2 from the endosomes (Wesche et al., 2006). Also, the ER-protein leucine rich repeat containing 59 (LRRC59) was found to be strictly required for nuclear import of exogenous FGF1 (Zhen et al., 2012). Translocation of exogenous FGF1 into the nucleus was also dependent on RAN GTPase and the importins α1 and β1, also called karyopherin α1 (Kpnα1) and karyopherin β1 (Kpnβ1), recognized as crucial regulators for nucleocytoplasmic shuttling (Figure 4) (Zhen et al., 2012). FGF2 (18 kDa) did not require LRRC59 for its translocation, but unlike FGF1, depended on translokin (Bossard et al., 2003).

In the nucleus, FGF1 is phosphorylated by PKCδ on Ser130 (Wiedlocha et al., 2005). This event modifies the conformation of FGF1, which leads to exposure of a nuclear export sequence (NES). This NES binds exportin-1/CMR1 and phosphorylated FGF1 is rapidly exported from the nucleus in an NES-mediated manner (Nilsen et al., 2007). After export to the cytosol, FGF1 is dephosphorylated and degraded (Wiedlocha et al., 2005). In paper I we identify nucleolin as a binding partner of FGF1, and show that nucleolin is involved in nucleocytoplasmic trafficking of FGF1 by regulating phosphorylation and nuclear export of FGF1 (Figure 4).

Malecki et al., found a correlation between translocation of exogenous FGF1 and the cell cycle G1-phase (Malecki et al., 2004). That translocation was cell cycle
dependent was also suggested by others (Imamura et al., 1994). However, another report showed later that FGF1 translocation is cell cycle independent (Zakrzewska et al., 2011). Zakrzewska et al., observed FGF1 in the nucleus every 24 h, and claimed that translocation of FGF1 into the nucleus is a periodic event, independent of the cell cycle, and speculated that nuclear localization of FGF1 is a process involved in circadian rhythms.

The biological role of exogenous FGF1 translocated into the nucleus is not fully understood. However, intracellular FGF1 has been shown to inhibit the p53 pathway by interfering with components important for the p53-dependent apoptosis pathway, in addition to negatively influence p53 stability (Bouleau et al., 2005). Furthermore, impaired nuclear translocation of FGF1 in neuronal cells inhibited neurotrophic activity as well as protected cells from p53-dependent apoptosis (Rodriguez-Enfedaque et al., 2009). It is not clear if the actions described above are performed by exogenous FGF1 translocated into the nucleus, or due to nuclear translocation of endogenous non-secreted FGF1. However, endogenous FGF1 has been shown to act, via an intracrine pathway, as a survival factor and a factor involved in differentiation (Raguenez et al., 1999; Renaud et al., 1994; Renaud et al., 1996). As described above, exogenous FGF1 and exogenous FGF2 (18 kDa) employs different trafficking mechanisms for translocation, which indicate different functions inside the cell.
Figure 4 Model of nucleocytoplasmic trafficking of exogenous FGF1.

Upon FGF1 interaction with the FGFR1/4 situated in the plasma membrane (PM) the FGF1-FGFR1/4 complex is internalized into endosomes. HSP90 and p38 MAPK activity is required for translocation of FGF1 from the endosomes to the cytosol. Then, FGF1 binds LRRC59 situated in the ER-membrane. LRRC59 together with RAN GTPase and the importins Kpnα1 and Kpnβ1 assist in the NLS mediated nuclear translocation of FGF1. In the nucleus, FGF1 interacts with nucleolin (as shown in this thesis), and PKCδ phosphorylates FGF1 resulting in exposure of an NES that interacts with exportin-1, which then mediates nuclear export. Phosphorylated FGF1 in the cytosol is de-phosphorylated and degraded.

Interaction partners of intracellular FGF1

In an attempt to understand the role of intracellular FGF1, it has been of great interest to detect its intracellular interaction partners in order to reveal possible cellular processes where FGF1 could play a role. FGF1 has been shown to interact with several intracellular proteins, for instance FGF1 intracellular binding protein (FIBP) (Kolpakova et al., 1998). Interestingly, FIBP was later found to be critical for
development of FGF-dependent left-right asymmetry in zebrafish (Hong and Dawid, 2009). FGF1 has also been reported to interact with GRP75/mortalin, a member of the HSP70 family involved in regulation of glucose response, antigen processing and cell mortality (Mizukoshi et al., 1999). FGF1 also interacts with p53, and as mentioned above, nuclear translocation of FGF1 was found to be necessary for neurotrophic activity as well as protection from p53-mediated apoptosis (Rodriguez-Enfedaque et al., 2009). Moreover, casein kinase 2 (CK2) and p34/LRRC59 (described below) were found to interact with FGF1, and the interaction between CK2 and FGF1 was associated with its mitogenicity (Skjerpen et al., 2002a; Skjerpen et al., 2002b). LRRC59 was found to be strictly required for nuclear import of exogenous FGF1 together with the classical nuclear import factors importin α1 (Kpnα1) and β1 (Kpnβ1), in a RAN GTP-dependent fashion (Zhen et al., 2012). In this thesis we show that FGF1 interacts with the nuclear multifunctional protein nucleolin, and that nucleolin is required for phosphorylation of FGF1 by PKCδ and nuclear export (Paper I).

**Nucleolin**

Nucleolin was first discovered in Chinese hamster ovary (CHO) cells and Novikoff hepatoma cells (Bugler et al., 1982). It is the most abundant non-ribosomal protein in the nucleolus, and is a multifunctional RNA binding protein (RBP) involved in various biological processes such as chromatin remodeling, ribosome biogenesis, gene silencing, DNA metabolism, senescence, and cell cycle regulation. Nucleolin is also located in the cytoplasm and on the cell surface (Abdelmohsen and Gorospe, 2012; Ginisty et al., 1999; Mongelard and Bouvet, 2007; Srivastava and Pollard, 1999; Tajrishi et al., 2011).

Nucleolin consists of three structurally multifunctional domains, reflecting the multiple functions and its wide subcellular localization (Abdelmohsen and Gorospe, 2012). The N-terminal domain holds several acidic stretches and several phosphorylation sites. The C-terminal domain includes a glycine/arginine-rich domain (GAR-domain), which mediates association with target mRNA, ribosomal proteins and other proteins (Abdelmohsen et al., 2011; Bouvet et al., 1998; Ghisolfi et al., 1992). The central domain harbors four RNA binding domains designated RNA
recognition motifs (RRM) interacting with mRNAs and pre-rRNA (Abdelmohsen et al., 2011; Serin et al., 1996).

The involvement of nucleolin in ribosome biogenesis has been elucidated extensively over the years, and spans from transcription of ribosomal (r)RNA, rRNA maturation, and ribosome assembly, to nucleocytoplasmic trafficking of ribosomal proteins and subunits. The N-terminal domain of nucleolin is involved in rRNA transcription and associates with the pre-rRNA processing complex (Roger et al., 2003). Nucleolin acts as an RNA-chaperone and assists the folding of pre-rRNA by interacting with the stem-loop structure on the pre-rRNA via its RNA binding domains (Allain et al., 2000). It also exerts a role in the catalysis of the first processing step of pre-rRNA and plays a role in the cleavage of the precursor transcript of rRNA, which takes place at the 5´ external transcribed spacer (5´-ETS) (Ginisty et al., 1998; Ginisty et al., 2000). Nucleolin has strong affinity for ribosomal proteins and is able to assemble ribosomal subunits in the nucleus (Bouvet et al., 1998), and also contributes to the nucleocytoplasmic transport of newly assembled ribosomal subunits (Abdelmohsen and Gorospe, 2012; Tajrishi et al., 2011; Xue and Melese, 1994). Nucleolin play several roles in DNA metabolism, including DNA replication, telomere maintenance, DNA repair and recombination (Khurts et al., 2004; Pollice et al., 2000; Seinsoth et al., 2003; Yang et al., 2002; Yang et al., 2009). Additionally, nucleolin assists in nucleocytoplasmic trafficking of proteins such as endostatin, lactoferin, the US11 protein of herpes simplex virus 1, as well as the type I transforming growth factor β receptor (TGFβ receptor) (Chandra et al., 2012; Greco et al., 2012; Legrand et al., 2004; Song et al., 2012).

Nucleolin, being a regulator of cell proliferation and cell growth, is highly expressed in proliferating cells such as cancer cells, and many oncogenes are under positive regulation by nucleolin. For instance, overexpression of nucleolin in the cytosol of chronic lymphocytic leukemia cells is shown to be crucial for the oncogenic stability of the mRNA encoding BCL2 (Otake et al., 2007). Further, it was shown that nucleolin stabilizes BCL2 in breast cancer cells (Soundararajan et al., 2008). In addition, nucleolin negatively influences the translation of TP53 mRNA, which results in an anti-apoptotic response (Takagi et al., 2005). Moreover, several reports show that nucleolin is localized on the cell surface of cancer cells such as leukemia cells and gastric cancer cells, where it serves as a receptor for tumorigenic factors
(Watanabe et al., 2010b). When expressed on the cell surface, nucleolin is used as a marker for diagnosis of gastric cancer and is considered to be a potent target for anti-cancer therapy (Watanabe et al., 2010a).

**Fibroblast growth factor receptors in cancer**

The complex intercellular FGFR signaling network governs several basic cell functions essential for biological processes, including embryonic development, organogenesis, angiogenesis, wound healing, tissue repair, and tissue homeostasis in adults (Beenken and Mohammadi, 2009; Itoh and Ornitz, 2011; Powers et al., 2000; Turner and Grose, 2010). Thus, deregulated FGFR signaling is a crucial factor in pathological conditions, including tumor growth and maintenance of malignancy (Beenken and Mohammadi, 2009; Haugsten et al., 2010; Kelleher et al., 2013; Turner and Grose, 2010). Several FGF/FGFR signaling mechanisms contribute to the development of cancer, and below some of these mechanisms will be presented.

**Excessive FGFR signaling in cancer**

Elevated FGFR signaling can be caused by cellular or genetic alterations such as chromosomal rearrangements, point mutations in the genes encoding FGFRs, dysregulated FGFR-gene promoters or gene amplification, or imbalance in deregulation mechanisms of FGFR signaling. Overexpression of FGFRs are found in different human cancers such as cancer of the brain, head and neck, lung, breast, stomach, prostate, sarcomas, and multiple myeloma (Chin et al., 2006; Toyokawa et al., 2009; Behrens et al., 2008; Kwabi-Addo et al., 2004; Chang et al., 2005; Allerstorfer et al., 2008; Freier et al., 2007; Baird et al., 2005).

A gene amplification, appearing in 10 % of human breast cancers, are located in the chromosomal region which includes FGFR1, 8p11-12 (Chin et al., 2006; Fearon et al., 2013; Gelsi-Boyer et al., 2005; Letessier et al., 2006). However, this region is rich in genes, and FGFR1 is not always overexpressed even if it is amplified. Nevertheless, growth of breast cancer-derived cell lines have been shown to be dependent on FGFR1, thus implying FGFR1 as a potential therapeutic target (Reis-Filho et al., 2006). Overexpression of FGFR2 has also been implicated in breast cancer cells, giving rise to constitutive signaling and inhibition of apoptosis (Easton et al., 2007;
Hunter et al., 2007; Meyer et al., 2008; Turner et al., 2010). Overexpression of FGFR3, due to chromosomal translocation in the t(4;14), is found in almost 25% of multiple myeloma, and is an independent prognostic factor for poor outcome of multiple myeloma (Chang et al., 2005; Chesi et al., 1997; Keats et al., 2006). Chesi et al., found that transcription of the FGFR3 gene after chromosomal translocation is controlled by a strong IgH enhancer, which results in overexpression of FGFR3 (Chesi et al., 1998). Overexpression of FGFRs, as well as FGFs are also involved in both maintenance and progression of prostate cancer (Acevedo et al., 2007; Freeman et al., 2003; Hamaguchi et al., 1995; Haugsten et al., 2010; Kwabi-Addo et al., 2004). For instance, FGFR1 and FGFR4 are found to be selectively overexpressed in prostate tumors at different stages (Sahadevan et al., 2007). Moreover, overexpression of FGF2 together with FGFR1 and FGFR2, both responding to FGF2, was identified in prostate cancer, resulting in paracrine stimulation and prostate cancer progression (Giri et al., 1999). Overexpression of FGFs in tumor cells and the surrounding tissue is identified in melanoma, liver, colon, and lung carcinomas. In addition, an increased release of FGFs from the ECM influence FGFR signaling and carcinogenesis (Haugsten et al., 2010; Turner and Grose, 2010).

Increased FGFR signaling due to isoform switches, includes a shift in splicing of the third Ig-like domain that generates isoforms with altered FGF-binding capacity, or a shift to a more oncogenic isoform, as the various isoforms show different oncogenic potentials (Ahmad et al., 2012; Haugsten et al., 2010; Knights and Cook, 2010; Wesche et al., 2011). For instance, a disruption of the regulatory system where the FGFR isoforms b and c, important for paracrine communication between epithelial and mesenchymal cells are switched, results in autocrine signaling and epithelial to mesenchyma transition (EMT) in rat models of prostate and bladder cancer (Oltean et al., 2006; Savagner et al., 1994; Yan et al., 1993).

Other mechanisms giving rise to elevated FGFR signaling includes impaired termination of FGFR signaling, constitutively active fusion proteins (described later) and various activating mutations found in the FGFRs. These include mutations in the kinase domain resulting in constant activation, and mutations promoting dimerization and subsequent activation of the receptor kinase, as well as mutations leading to impaired degradation of the receptor. All four FGFRs are found to hold mutations in
different types of cancer such as bladder cancer, endometrial cancer, and lung cancer (Ahmad et al., 2012; Haugsten et al., 2010; Turner and Grose, 2010; Wesche et al., 2011).

Although the majority of elevated FGFR signaling leads to oncogenic behavior, FGFR2 is assumed to hold tumor suppressor activities in certain cancers (Gartside et al., 2009).

**FGFR fusion proteins derived from chromosomal translocations**

FGFR fusion proteins can be a product of chromosomal rearrangements. Some fusion proteins are potent oncogenes able to drive proliferation in cancer cells. A fusion protein which holds functional features encoded by two original genes may result in a protein with oncogenic properties. It is often found that the tyrosine kinase domain of the FGFR is juxtaposed to a fusion partner, which holds structural properties that lead to constant dimerization of the kinase domain of the receptor. This creates a fusion protein with a constitutive active tyrosine kinase (Jackson et al., 2010). FGFR fusion proteins identified in cancer to date are soluble and localize to the cytosol, and possibly nucleoplasm, where they may escape normal attenuation mechanisms such as lysosomal degradation.

Several FGFR1 fusion partners with oncogenic properties have been identified, including the zinc finger gene ZNF198 (ZNF198), FGFR1 oncogene partner 1/2 (FOP1/FOP2), and breakpoint cluster region (BCR) (Jackson et al., 2010). Most of the FGFR1 fusion proteins identified to date are found in patients with the myeloproliferative disorder stem cell leukemia/lymphoma syndrome (SCLL/8p11 myeloproliferative syndrome), classified as hematopoietic and lymphoid neoplasms with *FGFR1* abnormalities (Jackson et al., 2010; Macdonald et al., 1995; Macdonald et al., 2002). For instance was the oncoprotein FOP2-FGFR1, studied in this thesis, identified in 8p11 myeloproliferative syndrome (Grand et al., 2004). SCLL is a rare condition, which occurs slightly more often in men than in women, and might develop into forms of leukemic/lymphomic disorders such as acute myeloid leukemia, a disease which is resistant to conventional chemotherapy (Abruzzo et al., 1992; Goradia et al., 2008).
FGFR fusion proteins have also been identified in patients and tumor samples, including cholangiocarcinoma, breast cancer, prostate cancer, lung squamous cell cancer, bladder cancer, thyroid cancer, oral cancer, glioblastoma, and head and neck squamous cell cancer (Wu et al., 2013). A chromosomal rearrangement resulting in FGFR3-BAI1-associated protein 2-like 1 (BAIAP2L1) is shown to be an oncogenic FGFR3 fusion protein in bladder cancer (Williams et al., 2013). Also, fusion of the $FGFR1$ gene and the transforming acidic coiled-coil ($TACC$) domain $TACC1$ or the $FGFR3$ gene and the $TACC2$ gene, result in an oncogenic activity of the FGFR-TACC fusion protein in 3 % of human glioblastoma (Singh et al., 2012).

It appears that fusion proteins play a significant role in several types of cancer, and this argues that chromosomal rearrangements resulting in FGFR fusion proteins can give rise to oncogenic drivers that are potential drug targets in cancer therapy.

**Therapeutic approaches**

In present clinical trials, several targeting agents such as tyrosine kinase inhibitors (TKIs) are being tested on various cancers. Some of these drugs target oncogenes identified in cancer, such as breast cancer, prostate cancer, non-small-cell lung carcinoma (NSCLC), and colon cancer (Fearon et al., 2013; Knights and Cook, 2010). For instance, several TKIs have been developed to target oncogenes such as ERBB2 in HER2-positive breast cancer or NSCLC (Garassino and Torri, 2014; Keating, 2014; Tolaney, 2014), BCR-ABL or BCR-ABL/SRC in chronic myeloid leukemia (Baccarani et al., 2014), or members of the janus kinase-STAT (JAK-STAT) pathway, often dysregulated in hematological malignancies (Furqan et al., 2013). Although the target agents are specific, challenges regarding the use of TKIs involve the development of resistance.

**HSP90 inhibitors in cancer treatment**

Clinical inhibition of HSP90 is emerging as a promising therapeutic approach. HSP90 is a molecular chaperone regulated by the transcription factor heat shock factor 1 (HSF1) that also regulates other HSP members crucial for the heat shock response (Dai et al., 2007; Santagata et al., 2011; Whitesell and Lindquist, 2005). HSP90 is
essential for the stability and function of many proteins. It is critical for maturation and folding of nascent polypeptides and protein domain folding and stabilization, correct assembly of multimeric protein complexes, as well as protein trafficking (Trepel et al., 2010). Numerous proteins involved in cell signaling and stress responses, as well as oncogenes, are dependent on HSP90 activity for their correct folding. This includes kinases, transcription factors, protein regulators of the cell cycle, signal-transduction proteins, antiapoptotic proteins, and telomerases (Garcia-Carbonero et al., 2013; Trepel et al., 2010; Whitesell and Lindquist, 2005). Therefore, HSP90 is an essential regulator in processes critical for cell survival. The heat shock response is the most evolutionary conserved protective mechanism found in nature (Ritossa, 1996). HSP90 is a highly conserved chaperone among multicellular organisms, and facilitates folding of about 50% of kinases in yeast (Mandal et al., 2007).

HSP90 is an ATPase that exerts its function together with heat shock protein 70 (HSP70) and other co-chaperones, including CDC37, forming a complex called the HSP90 machinery (Trepel et al., 2010). Post translational modifications, such as phosphorylation, nitrosylation and acetylation regulate HSP90 activity (Scroggins and Neckers, 2007; Scroggins et al., 2007; Trepel et al., 2010). HSP90 is localized in the cytoplasm, however, it has also been found to localize on the cell surface, as well as being secreted to the extracellular environment (Eustace et al., 2004; Sidera and Patsavoudi, 2008).

Tumors exhibit proteotoxic stress, one of many hallmarks of cancer (Luo et al., 2009). Proteotoxic stress is a result of altered balance between growth and survival signals, often due to aneuploidy and unfolded protein aggregates in tumors (Ganem et al., 2007; Torres et al., 2008; Williams et al., 2008). The heat shock response is often constitutively active in cancer and contributes to the malignant transformation (Dai et al., 2007). The increased expression of HSPs are a common feature in cancer and are detected in both solid tumors and hematological malignancies (Chant et al., 1995; Ciocca et al., 1993; Conroy et al., 1998; Kaur and Ralhan, 1995; Kimura et al., 1993; Ralhan and Kaur, 1995; Santarosa et al., 1997; Yufu et al., 1992). The mutated and therefore less stable kinases, regulatory proteins and signaling molecules found in cancer cells are considered to be more dependent on HSP90 for their stability,
compared to proteins in normal cells. The mutated oncoproteins take advantage of the HSP90 machinery and escape degradation, a property found to be important for the survival of oncogenic activity in cancer cells (Calderwood et al., 2006; Gray, Jr. et al., 2008; Whitesell and Lindquist, 2005; Workman, 2004). Therefore cancer cells could detriment from HSP90 inhibition. Unlike specific TKIs, targeting one specific kinase and thus one signaling pathway, HSP90 inhibition can potentially suppress multiple oncogenic signaling pathways simultaneously, thereby reducing the possibility for molecular feedback loops and mutations leading to drug resistance.

Twenty years ago, HSP90 was found to be a molecular target for geldanamycin (Whitesell et al., 1994). Geldanamycin was first isolated from *Streptomyces higroscopicus var geldanus* in 1970, and is a naturally occurring benzoquinone anamycin antibiotic (Garcia-Carbonero et al., 2013). Geldanamycin showed antitumor activity in cellular studies on several cell lines (Garcia-Carbonero et al., 2013). However, geldanamycin has poor solubility and stability, being easily modified in the liver, and therapeutical doses resulted in liver toxicity in animal studies (Powers and Workman, 2007; Supko et al., 1995). Several geldanamycin analogues were developed in order to overcome this problem, and 17-alkylamino-17demethoxygeldanamycin (17-AAG; tanespimycin) was the first HSP90 inhibitor with improved pharmacological properties and toxicity profiles to enter clinical trials (Banerji et al., 2005; Garcia-Carbonero et al., 2013; Kelland et al., 1999).

At present, about 15 HSP90 inhibitors are evaluated in clinical trials, including geldanamycin derivatives, resorcinol derivatives, purine analogues and other compounds (Garcia-Carbonero et al., 2013; Jhaveri et al., 2012; Neckers and Workman, 2012). The drugs are tested on cancers such as, HER2-positive breast cancer, myeloma, acute myeloid leukemia (AML), prostate cancer melanoma, ovarian cancer, rectal cancer, NSCLC, chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL), and gastrointestinal stromal tumor (GIST) (Garcia-Carbonero et al., 2013). Although structurally unrelated, most of the drugs binds the ATP binding site on HSP90 with higher affinity than ATP, thereby blocking ATP binding and hydrolysis. HSP90 inhibition leads to degradation of client proteins via the ubiquitin-proteasome pathway (Whitesell and Lindquist, 2005).
In this thesis we tested the antitumor activity of NVP-AUY922 in FOP2-FGFR1 driven malignancy. NVP-AUY922, is a resorcinol derivative, identified upon high-throughput screening (Jhaveri et al., 2012; Neckers and Workman, 2012). The drug has shown antitumor activity via cytostasis, apoptosis, invasion and angiogenesis on human endothelial cells, also illustrated by reduced microvessel density in tumor xenografts (Eccles et al., 2008). NVP-AUY922 is at present considered the most potent HSP90 inhibitor (Brough et al., 2008). A phase I clinical trial showed that NVP-AUY922 was well tolerated when administrated to patients, where maximum tolerated dose (MTD) was 70 mg/m², and dose-limiting toxicities (DLT), included diarrhea, asthenia/fatigue, anorexia, atrial flutter, and visual symptoms (Sessa et al., 2013). NVP-AUY922 is currently being tested in clinical trials, for instance, a phase II study investigates the effect of the agent on patients with HER2-positive and estrogen-receptor-positive breast cancer (Garcia-Carbonero et al., 2013; Zagouri et al., 2013). At present, NVP-AUY922 in combination with trastuzumab is tested on patients harboring HER2-positive tumors with trastuzumab refractory metastasis [www.clinicaltrials.gov; NCT01271920], (Garcia-Carbonero et al., 2013; Zagouri et al., 2013). The antitumor effects of NVP-AUY922 is also tested on other cancer types such as NSCLC, lymphoma and colorectal cancer, as well as in combination with other drug agents (Garcia-Carbonero et al., 2013; Garon et al., 2013).

**LRRC59**

LRRC59 was brought to our attention when it was identified as an interaction partner of FGF1, suggested to be involved in mediating its mitogenic activity (Skjerpen et al., 2002b). Later, our laboratory revealed that LRRC59 played an essential role in nuclear import of exogenous FGF1 (Zhen et al., 2012).

The *LRRC59* gene encodes a protein of 307 amino acids, which is a tail-anchored (type II) membrane protein positioned in the ER membrane. LRRC59 consists of a small ER-luminal domain, and a larger cytosolic part (Ohsumi et al., 1993). The cytosolic N-terminal part harbors the leucine-rich-repeat (LRR) domain, and a putative coiled-coil (CC) domain (Figure 5). Many proteins possess a structural LRR-domain, which is assumed to take on a β strand-turn-α helix based horseshoe shape, possibly involved in the formation of protein-protein interactions (Kobe and Kajava,
LRRC59 was first described as a protein abundant in microsomal membrane and assumed to be an ER ribosome receptor, and designated ribosome binding protein p34 (Ichimura et al., 1992; Ohsumi et al., 1993; Tazawa et al., 1991). The role of p34/LRRC59 as a ribosome receptor was later refused (Kalies et al., 1994).

Figure 5 Schematic representation of LRRC59.
The schematic representation of LRRC59 illustrates the LRR domain constituted by amino acids 38-131, the coiled-coil (CC) domain constituted by amino acids 148-216, the transmembrane domain (TM) constituted by amino acids 245-269, and the ER-lumenal tail (adapted from Zhen et al. 2012).

The biological role for LRRC59 is still largely undefined. However, based on information available in gene expression databases and published large scale studies, it is clear that LRRC59 is a conserved and widely expressed protein (www.proteinatlas.org; www.genecards.org), (Luo et al., 2008; Mukherji et al., 2006; Nagaraj et al., 2011; Terp et al., 2012). It is a widely expressed protein in human organs and tissues (www.proteinatlas.org). LRRC59 is highly expressed in epithelial and neuronal cells, while mesenchymal cells (stroma/muscle) and hematopoietic cells express moderate to low levels of LRRC59 (www.proteinatlas.org). This distinct expression pattern suggests specialized roles for LRRC59 in epithelial and neuronal cells. Large scale analysis has connected LRRC59 to cell viability, migration and cancer (Luo et al., 2008; Mukherji et al., 2006; Nagaraj et al., 2011; Terp et al., 2012). Genetic screening of several cell lines, identified LRRC59 as an essential protein for cancer cell viability (Luo et al., 2008). Also, the expression level of 44 proteins, including LRRC59 was found to correlate with metastasis efficiency of breast cancer cells (Terp et al., 2012). LRRC59 has also been identified as one out of 268 genes that are essential for the progression of the cell cycle (Mukherji et al., 2006). These findings suggests that the expression of LRRC59 is essential for proliferating cells, and that LRRC59 could be involved in maintaining the malignant phenotype of cancer cells.
In Paper III we show that LRRC59 is required for polarization and directed cell migration of epithelial cells, as well as lumen expansion in 3D cysts of the Caco-2 cancer cell line.

**Epithelium**

The epithelium is a tissue that lines all organs, cavities and surfaces in the body. An epithelium consists of polarized epithelial cells characterized by an asymmetric division of the plasma membrane, termed the apical-basal polarity. This asymmetric composition of the cells lining the organs of the body creates a barrier that is crucial when facilitating absorption, secretion and transcellular transport. The epithelial cell polarity and morphogenesis of the epithelium are highly regulated (Bryant and Mostov, 2008). Disrupted epithelial cell polarity causes diseases such as polycystic kidney disease, cystic fibrosis as well as metastatic cancer (McConkey et al., 2009; Willenborg and Prekeris, 2011; Wilson, 1997; Wilson, 2011). Carcinomas are defined as cancer that develops from epithelial cells, and about 80-90 % of cancers are carcinomas. It is important to understand the biology of epithelial cells, in order to understand the development and behavior of carcinomas.

The apical and basolateral domain of the plasma membrane in epithelial cells have distinct lipid and protein compositions. The apical surface faces the external environment, for instance the lumen in the intestine, while the basolateral surface associates to the adjacent cells and the underlying connective tissue. The lateral membrane domain of these cells connects to adjacent cells (Bryant and Mostov, 2008). The distinct composition of lipids and proteins in the apical and basolateral membrane is maintained by polarized trafficking (Apodaca et al., 2012; Cao et al., 2012), and tight junctions (TJs), which serve as barriers between the apical and basolateral membrane (Shin et al., 2006). The TJs are also responsible for linking the neighboring cells together, creating a tight epithelial cell layer functioning as a barrier between the lumen and the connective tissue. Adherens junctions (AJs) consist of cadherins and nectins, and are located on the lateral domain, basal from the tight junctions on the epithelial cell. AJs are connected to the actin cytoskeleton, and facilitate mechanical stability of the cell, maintain the integrity of the apical and basolateral membrane as well as connecting neighboring cells (Ivanov and Naydenov,
Localized on the basal membrane are integrins which are ECM receptors, interacting with the underlying basement membrane.

How cell polarity is established is not fully understood. As reviewed by others, TJ formation is important to initiate the cell polarity process, and three polarity complexes are involved in regulating polarity in mammalian cells, the CRB (Crumbs) complex, the PAR (Partitioning defective) complex and the SCRIB (Scribble) complex (Willenborg and Prekeris, 2011). These polarity complexes are localized at distinct areas in the plasma membrane (Chen and Zhang, 2013; St and Ahringer, 2010; Willenborg and Prekeris, 2011). Also phosphoinositides show distinct localization, where PtdIns(4,5)P$_2$ positions to the apical membrane (Martin-Belmonte et al., 2007), while PtdIns(3,4,5)P$_3$ localizes basolateraly where it exerts a regulatory role in basolateral membrane formation (Gassama-Diagne et al., 2006). Positive feedback mechanisms involving Rho GTPases such as Cdc42 and Rac are also involved in organizing cell polarity (Etienne-Manneville and Hall, 2002; Etienne-Manneville, 2004; Srinivasan et al., 2003).
Aim of the studies

Paper I: Identify interaction partners of FGF1 and the role of the interactions

FGF1 can act by binding to FGFRs on the cell surface and thereby activate intracellular signaling cascades. Additionally, non-secreted as well as exogenous FGF1 translocated into the cytosol and nucleus are acting via an intracrine pathway. In this study we aimed at identifying intracellular proteins interacting with FGF1, as well as elucidating the role of the interaction.

Paper II: Study the potential antitumor effects of the HSP90 inhibitor NVP-AUY922 in the human leukemic KG-1a cell line in cell culture and in vivo

The fusion protein FOP2-FGFR1 is a product of chromosomal translocations of the FGFR1OP2 gene and the FGFR1 gene, and is the driver oncoprotein in the KG-1a acute myeloid leukemic cell line. Since the discovery that this oncoprotein is a HSP90 client addicted to the activity of the molecular chaperone HSP90, it has been of interest to study the effect of HSP90 inhibition on this cell line. NVP-AUY922 is a novel HSP90 inhibitor with an improved toxicity profile in vivo. The purpose of this study was to study the antitumor activity of NVP-AUY922 on FOP2-FGFR1 driven malignancy in a mice model injected with KG-1a. We also analyzed the effect of the HSP90 inhibitor on the activity of downstream signaling molecules and proliferation of the KG-1a cell line in a cell culture.
Paper III: **Study the ER-protein LRRC59 in epithelial cells**

LRRC59 is an ER-bound protein, and its biological functions are not fully elucidated. LRRC59 was drawn to our attention when identified as an interaction partner of FGF1, and later found to be required for nuclear import of exogenous FGF1. LRRC59 is highly expressed in epithelial and neuronal cells, and 80-90 % of carcinomas are developed from epithelial cells. The aim of this study was to characterize the role of LRRC59, with emphasis on its involvement in regulating epithelial cells.
Summary of papers

Paper I: Nucleolin regulates phosphorylation and nuclear export of fibroblast growth factor 1 (FGF1)

By pull down experiments and mass spectrometry, we identified the nuclear protein nucleolin as an interaction partner of FGF1. The interaction was verified by several pull down experiments \textit{in vitro} and \textit{in vivo}. We also identified nucleolin as an interaction partner of FGF2, by pull down experiments. We analyzed the strength of the interactions by surface plasmon resonance (SPR)-analysis. Mutational analysis and SPR-analysis identified that the heparin binding site on FGF1 is the interaction site for nucleolin. With the knowledge that nucleolin regulates nuclear shuttling of proteins and ribosomal subunits, we investigated if nucleolin had a role in nuclear trafficking of FGF1 and FGF2. Nucleolin showed no effect on nucleocytoplasmic trafficking of FGF2, nor did it influence nuclear import of FGF1. However, our studies did reveal a role for nucleolin in nuclear export of FGF1. Exogenous FGF1 translocated into the nucleus is phosphorylated by PKC\(\delta\) on the Ser130 residue. This phosphorylation event results in exposure of a NES on FGF1 allowing interaction with Exportin-1 and nuclear export. Upon nucleolin depletion, however, FGF1 was not phosphorylated by PKC\(\delta\), and FGF1 remained in the nucleus. The findings show a role for nucleolin in phosphorylation and nuclear export of FGF1.

Paper II: Different anti-leukemic activity of the HSP90 inhibitor NVP-AUY922 during \textit{in vitro} and \textit{in vivo} treatment of human leukemic KG-1a cells

Our previous work identified the fusion protein FOP2-FGFR1 as an oncoprotein dependent on the action of HSP90 for its stability and activity. FOP2-FGFR1 is a fusion protein composed of the tyrosine kinase domain of FGFR1 and a coil coiled domain of the FOP2 protein. The coil-coiled domain of FOP2 results in constant dimerization of the kinase domain, resulting in a constantly active kinase. When the HSP90 inhibitor NVP-AUY922, a derivative of resorcinol, was shown to harbor a low toxicity profile, we pursued the effect of HSP90 inhibition using NVP-AUY922 on FOP2-FGFR1 stability in cell culture as well as in an \textit{in vivo} model. We found that treatment of KG-1a cells with NVP-AUY922 strongly reduced the stability of FOP2-FGFR1, as well as RAF1, another client of HSP90, and abrogated downstream
signaling cascades involving STAT1 and PLCγ. In line with the effects on the activity of signaling molecules, we also observed an inhibition of proliferation of KG-1a cells. The results in cell culture indicate that NVP-AUY922 inhibits the stability as well as the kinase activity of the FOP2-FGFR1 fusion protein. NVP-AUY922 is an inhibitor with an acceptable toxicity profile, and we studied the effect of this drug on survival in a systemic AML mouse model. KG-1a cells were injected into mice to develop a systemic AML model, and the xenografts were treated with NVP-AUY922. With the dose scheduling tested we observed no antitumor activity of NVP-AUY922 in vivo. NVP-AUY922 has been shown to have low stability in plasma, compared to in solid tumors. Further, KG-1a cells treated with NVP-AUY922 had an increased expression of HSP70, which can result in a reduced apoptotic response and a compensatory effect of the HSP90 inhibition. We suggest that the treatment schedule should be optimized, and that the effect of NVP-AUY922 on FOP2-FGFR1 driven malignancy should be further investigated.

Paper III: The ER-protein LRRC59 is involved in regulating epithelial cell polarization, directed cell migration, and lumen expansion in Caco-2 cysts

In this paper, we investigated the role of the ER-bound protein LRRC59 in epithelial cells. Microarray analysis showed that LRRC59 expression was high in both normal and colorectal cancer samples, but significantly higher in the colorectal cancer samples. Upon depletion of LRRC59 by siRNA, we discovered a morphological change illustrated by an increased cell spread area compared to control cells. We continued our studies investigating the role of LRRC59 on cell polarity. Polarized cells position their Golgi between the leading edge and the nucleus, however, after LRRC59 depletion RPE cells failed in orienting their Golgi and microtubule organizing center (MTOC) correctly in a wound healing assay. In order for a cell to migrate in an oriented and organized fashion, it needs to be polarized. We analyzed the migration of LRRC59 depleted cells by live cell imaging, and our results showed that LRRC59 depletion impaired the directed cell migration. Also, LRRC59 depleted cells showed impaired lamellipodia formation in RPE cells and impaired lumen formation in Caco-2 cysts in a 3D matrigel. Our results indicate that LRRC59 depletion impairs cell polarity and suggest a role for LRRC59 in the apical membrane function. We suggest that the ER-localization of LRRC59 is important in regulating
expression or secretion of proteins important for regulating cell polarity, an important aspect of epithelial cell function.
Experimental considerations

Cell lines

Cell lines are an established model system well suited for basic research, and are often a first step experiment before proceeding with animal experiments. Therefore, studies on cell cultures may bridge basic and translational research. However, a cell line is a simplified model system, and does not entirely reflect the complexity of cells in tissues in an organism. Additionally, cell lines may harbour genotypic and phenotypic transformations. In the projects in this thesis we have used cell lines that we thought were suitable for each individual study.

In paper I we used the osteosarcoma U2OS cell line stably transfected with FGFR1 (U2OSR1), as translocation of exogenous FGF1 requires FGFR1 or FGFR4. U2OS cells are cancer cells that are easy to maintain in the laboratory and possible to stably transfect. It is advantageous to use several cell lines, to show that the effects studied are not cell line specific or due to abnormalities. In paper I we also used the human foreskin (BJ) fibroblast cells, the immortalized mouse fibroblast cell line NIH3T3, and the human embryonic kidney 293 (HEK 293) cell line, adapted to grow in cell culture. These are non-cancer cell lines, which better represent the physiological state in vivo.

In paper II we used the human KG-1a leukemic cell line, as this cell line holds the driver oncoprotein, FOP2-FGFR1 (Gu et al., 2006), which we wanted to study. As LRRC59, studied in paper III, is highly expressed in epithelia tissue we used the immortalized hTERT RPE (retina, eye; pigment epithelium) cell line. We also used the HeLa cell line. HeLa cells have an epithelial origin, are cancer cells used for multiple purposes, and are the most frequently used cell line. However, HeLa cells are not an optimal cell line as it has accumulated mutations, and is preferably used together with other cell lines. In paper III we also used the Caco-2 cell line, which originate from a human colon adenocarcinoma. Caco-2 cells harbour low metastatic properties (Flatmark et al., 2004), and like normal epithelial cells, they are less motile. Upon culturing the Caco-2 cells differentiate and polarize, expressing cell-cell contacts via tight junction, and acquires the typical epithelial morphology consisting of an apical and basolateral plasma membrane. These characteristic features are
typical for normal epithelial cells such as the enterocytes lining the small intestine. Therefore, Caco-2 cells are a good model for normal epithelial colon cells. In paper III these cells were grown in a 3D environment to allow polarization into a 3D cyst, reflecting epithelial cells facing the lumen of the intestine on their apical side and the connective tissue on their basolateral side. To avoid genotypic and phenotypic transformations and occurrence of senescence, we used a few number of passages of all the cell lines described. In addition, all the cell lines used in this thesis were mycoplasma tested, as mycoplasma contamination can influence cell growth and cell characteristics.

**siRNA transfection**

Transfection of cells by small interfering RNA (siRNA) is a technique used to knock down a gene of interest, and has become a common tool to study protein function. siRNAs are double stranded RNAs, and upon transfection into cells, the complementary endogenous mRNA is targeted and degraded by a specific RNA interference pathway. Upon siRNA transfection with siRNA targeting a specific mRNA it is common to transfect cells with scrambled control siRNA, which consists of random sequences that do not specifically target any genes. As siRNAs has been shown to activate immune responses (Sledz et al., 2003) the inclusion of the scrambled siRNA verifies that the observed effects of the targeting siRNA is due to knock down of a specific mRNA, and not due to the siRNA level in the cell *per se*.

The biggest concern related to the use of siRNA is off-target effects. This can occur if the siRNA matches not only the mRNA of interest, but also interferes with other mRNAs in the cell. Several approaches can be performed to avoid this problem. The siRNA sequence should have minimal homology to mRNAs other than the target, and this can be checked by genome analysis. A pool of siRNAs where the siRNAs target different sequences on the same mRNA increases the likeliness of knock down. However, siRNA pools may increase the chance of unspecific targeting. To increase the reliability of the studies, more than one siRNA targeting different sequences within the mRNA of interest is ideal to ensure that the observed effects are due to knock down of the gene of interest, and not due to off-target effects. In the studies were siRNA was applied, we used two different siRNAs targeting dissimilar sequences within the mRNA of interest.
Although it is experimentally challenging, resque experiments where the phenotype obtained by gene knock down is rescued by transfection of cells with a siRNA resistant cDNA of the knocked down gene, will verify that a gene-specific phenotype was studied. The reliability of the findings obtained by siRNA-mediated knock down will be strengthened if the findings are supported by other experimental approaches. For instance, in paper I we use both siRNA (nucleolin depletion) and mutations (of the nucleolin binding site of FGF1) to verify our results. The siRNAs used in paper III, were validated in a previous study (Zhen et al., 2012). In paper III however, two different siRNAs were only used in some experiments and it will be of importance to check all the effects observed with both siRNAs.

**Affinity pull-down experiments and surface plasmon resonance**

Pull-down experiments are used to extract a protein from a solvent or lysate. In affinity pull-down a “bait” protein is linked to a tag, and the bait and the tag are immobilized on a ligand (support beads). The bait-tag forms a complex with the ligand and this is incubated with a protein source such as a cell lysate. Proteins in the cell lysate interacting with the bait can then be pulled down. In this thesis, we performed affinity pull-down experiments with FGF1/FGF2 using different tags linked to FGF1/FGF2. Using this approach we keep the surface of FGF1/FGF2 free to interact with other proteins (target). The disadvantage with pull-down experiments is that the tag might bind to the targets on its own, and thereby give false positive hits. Therefore, it is important to do control experiments. A tag can be linked to a protein known to not interact with the interacting targets, or the tag can be used alone. Another approach is to use different tags. In paper I we used three different tags and beads to verify our results.

SPR can be used to study direct protein-protein interactions without the usage of tags, as well as to study the strength of the interactions. By SPR protein-protein interactions are analysed by immobilizing a protein of interest on a chip. The chip is, by a micro-flow system, exposed to a solution consisting of other molecules which are allowed to interact. In paper I all the approaches mentioned above were applied, all verifying the direct interaction between FGF1/FGF2 and nucleolin.
Experimental considerations

**FGF1 in vivo-phosphorylation assay**
The phosphorylation assay was developed to investigate if exogenous FGF1 can cross cellular membranes and translocate into the cytosol and nucleus (Olsnes et al., 2003). The assay takes advantage of the protein activity of PKCδ, which only exists in the cytosol and nucleus and not in endosomes or other cytoplasmic compartments. Since FGF1 is phosphorylated by PKCδ on the Ser130 residue (Wiedlocha et al., 2005), *in vivo*-phosphorylation of FGF1 shows that FGF1 was present in the cytosol/nucleus. The assay is performed by radiolabelling the cellular ATP pool with [33P]phosphate, before stimulation with recombinant, unlabelled FGF1 in the presence of heparin. Cells are then lysed and [33P]FGF1 extracted by binding to heparin-sepharose. Unlike other proteins, FGF1 is highly resistant to trypsin due to its increased stability upon interaction with heparin. To avoid background from other proteins, the heparin-sepharose beads are treated with trypsin, removing almost all proteins other than FGF1. Phosphorylated FGF1 is then analyzed by SDS-PAGE and fluorography. Typical pitfalls of this method are too heavy washing, which results in uneven loading, due to loss of beads, or too rough trypsination, which leads to loss of FGF1. Too little washing or too little trypsination, will give rise to high background (Wiedlocha et al., 1992; Wiedlocha et al., 1995).

**Cell fractionation**
Upon fractionation of cells into a nuclear, cytosolic, and membrane (including endosomes) fractions it is important to avoid that proteins belonging to the cytosolic or membrane fraction contaminate the nuclear fraction and *vice versa*. In paper I we used cell fractionation as a method to determine translocation of FGF1/FGF2. We used digitonin to separate the cytosol from the remaining of the cell. Digitonin is a detergent that binds to cholesterol on the plasma membrane. Digitonin treatment of cells creates holes in the plasma membrane allowing the cytosol to leak out. It is important to remove the unbound digitonin before harvesting the cytosol, to avoid that it binds to the nuclear membrane and disrupt the nucleus, contaminating the cytosolic fraction with nuclear components. The detergent Triton-X 100 was used to separate the nucleus from the membrane fraction. The nucleus is resistant to Triton-X 100 treatment, due to a dense lamina, and the nuclear fraction needs to be sonicated in order to break up the lamina and release nuclear FGF1/FGF2. In paper I we also used Bafilomycin A1 (BafA1) as a negative control for cytosolic and nuclear translocation.
of FGF1/FGF2. BafA1 inhibits translocation, but not endocytosis, by selectively inhibiting the V-type H⁺-ATPase localized in the endosomal membrane (Malecki et al., 2002). In the presence of BafA1, FGF1/FGF2 should not be present in the cytosolic or nuclear fractions. Thus, BafA1 exerts a good negative control for translocation.

**Use of chemical inhibitors**

Chemical inhibitors are used in paper I and II. Chemical inhibitors are fast and easy tools to study inhibitory effects compared to siRNA treatment, which is performed over longer time periods. Both inhibitors and siRNA transfections are specific, however, long time treatment can result in compensatory effects. If possible, it is preferable to use both inhibitors and siRNA transfection to confirm the results. Upon use of inhibitors it is critical to use concentrations recommended to avoid unspecific effects, or side effects as a consequence of treatment. In all papers, we have used concentrations recommended by the company providing the compound.

**Confocal microscopy**

The confocal microscope gives the opportunity to observe and study the different structures, compartments and areas of a cell. For instance, in paper III, we observed the apical membrane and the basolateral membrane of a Caco-2 cyst, after immunostaining with antibodies against proteins that localize to these different membrane domains. When analyzing proteins with a confocal microscope, it is important to bear in mind that bleed-through effects might occur and give a false result. Bleed-through occurs upon spectral overlap when combining fluorophores and it is crucial to use a combination that gives minimal spectral overlap. For instance, fluorophores that are far from each other in the spectrum, and fluorophores with similar intensity will strongly reduce bleed-through effects. Another aspect that is important when doing analysis on a confocal microscope is to avoid saturation of pixels which could result in false co-localization. Moreover, when comparing two different samples such as siRNA treated and scrambled control treated cells, the images should be scanned with identical settings. In order to obtain objective results, an appropriate number of cells should be analyzed for statistical significance. All these aspects were considered in experiments where confocal microscopy was applied.
The cell migration assay
A cell migration assay was performed by using a BioStation IM (Nikon), which images cells over a long time period. The cells are then analyzed by the ImageJ software and the Chemotaxis tool. This assay allows analysis of several migrating cells at one time, and the software gives the opportunity to analyze speed and persistence of the migrating cells. We stimulated migration by mechanically removing the neighbouring cells in a confluent monolayer with a pipette tip. In order to make the samples comparable, we aimed at getting the wound size as identical as possible. To achieve statistical significance, about 50 cells or more were analyzed in each experiment for each condition, and the experiment was repeated three times.

Due to their ability to acquire a transient polarized morphology, cells are able to migrate in vitro. However, cell migration on glass coverslips does not reflect the migrating environment in an organism. The in vivo milieu consists of ECM, different cells, secreted factors and signalling molecules. There is an ongoing debate regarding how well a 2D-system can be compared to a 3D environment (Meyer et al., 2012). Therefore, it was important to verify the in vitro (2D) cell migration data with other experiments. In paper III, simultaneously with the observed defect in directed migration we observed a defect in cell polarity upon LRRC59 depletion. Consistent with this we observed an impaired polarity also in 3D Caco-2 cysts, upon LRRC59 depletion.

Animal models for acute myeloid leukemia (AML)
Animal models are used to achieve an understanding of gene functions and treatments in a physiological context. Medicine developed for humans are first tested and analyzed on animals in order to understand their effects, safety and toxicity. For instance, mice models are extensively used when studying cancer, as they provide a physiological model, have high degree of homology to humans and short generation time. In paper II we injected the human leukemic KG-1a cells into immunodeficient NOD Scid gamma mice to develop a systemic AML xenograft. This xenograft will mimic the leukemic condition in humans, and reflect the tumor activities of the human KG-1a cell line, harvested from a leukemic patient. The fusion protein FOP2-FGFR1 is the oncogenic driver in KG-1a cells, and our model does not only reflect the phenotypic features of AML, but also the genotypic features. FOP2-FGFR1 is an oncogene dependent on the activity of HSP90, and we tested the antitumor activities
of the HSP90 inhibitors NVP-AUY922 and 17-AAG on animal survival. KG-1a cells represent an uncommon subtype of AML which develops after a chronic phase of myeloid hyperplasia called 8p11 myeloproliferative syndrome/stem cell leukemia/lymphoma syndrome (EMS/SCLL). It can usually not be eradicated by conventional chemotherapy and is associated with poor outcome. Therefore, it is of interest to test the effect of novel drug candidates for therapy. The immunodeficient NOD Scid gamma mouse is a well suited strain for modeling of human leukemia because it allows more consistent development of systemic growth of leukemic cells, than for example the nude mouse, which is less severely immune compromised. In the case of NVP-AUY922, the discrepancy between effects in cell culture and activity in the KG-1a in vivo model suggests that the dosing schedules used in paper II are suboptimal. In leukemia it seems ideal to administer this drug as a constant infusion, which currently is not feasible in an animal model. This remains a challenge for further preclinical studies.

The activity of NVP-AUY922, as well as 17-AAG was tested on cell cultures, before entering in vivo studies. In mice treatment experiments, a minimal number of animals were used to achieve appropriate statistical strength. The Institute for Cancer Research (Oslo University Hospital), where the experiments were conducted, provides a new animal facility approved for work with immunodeficient animals.
Discussion

A novel role for nucleolin in phosphorylation and nuclear export of FGF1

After the discovery that exogenous FGF1 could translocate into the cytosol and nucleus, we have investigated the translocation mechanisms of FGF1, and aimed at identifying interaction partners of FGF1 that could give us insight into which regulatory roles FGF1 possesses intracellularly.

In this thesis (paper I) we show, by different approaches, that nucleolin interacts with FGF1, and that this interaction is important for phosphorylation of FGF1 on Ser130, as well as for nuclear export of FGF1. We found that nucleolin interacts with the HSPG binding site on FGF1, which shows that this site has a dual function. When FGF1 is located extracellularly this site constitutes a binding site for HSPGs, and intracellularly it serves as a binding site for nucleolin. Endostatin is another protein that functions both extracellularly and intracellularly by translocating into the nucleus in an NLS-mediated and nucleolin dependent fashion (Song et al., 2012). Consistant with our findings, nucleolin also binds to the heparin binding site of endostatin (Fu et al., 2009). The C-terminal part of nucleolin includes a positively charged GAR-domain, which mediates association with the negatively charged target mRNA (Abdelmohsen et al., 2011; Bouvet et al., 1998; Ghisolfi et al., 1992). Since the heparin binding site in FGF1 is positively charged, FGF1 most likely interacts with negatively charged regions outside the GAR domain, in an electrostatic fashion. The FHFs have high sequence and structural homologies with secreted FGFs. FHFs are non-secreted and play their roles intracellularly. Interestingly, they have high affinity to heparin in vitro (Beenken and Mohammadi, 2009). Since neither heparin, nor HSPGs are localized intracellularly, and as the heparin binding site is conserved between FGFs and FHFs, it is tempting to speculate that the heparin binding site serve as an intracellular binding site for nucleolin or other proteins on FHFs.

In our work we show that nucleolin also interacts with FGF2, but in contrast to what was observed for FGF1, nucleolin was not required for the nucleocyttoplasmic trafficking of FGF2. FGF1 and the 18 kDa isoform of FGF2 undergo nuclear translocation in similar fashions, both requiring HSP90 and vacuolar proton pumps.
for their translocation from endosomes (Malecki et al., 2002; Wesche et al., 2006). However, the two growth factors also depend on different proteins upon nuclear translocation. Nuclear import of FGF1 requires LRRC59, while FGF2 is not dependent on LRRC59 for its nuclear import (Zhen et al., 2012). On the other hand, FGF2 is dependent on translokin, while translokin was not required for nuclear translocation of FGF1 (Bossard et al., 2003). Also, FGF1 and FGF2 use dissimilar mechanisms for secretion. FGF2 is continuously secreted, requiring proteins such as HSP27, the α-subunit of the Na⁺-K⁺-ATPase, as well as translokin and the translokin partner, the kinesin KIF3A (Florkiewicz et al., 1998; Meunier et al., 2009; Piotrowicz et al., 1997). In contrast, FGF1 is secreted upon stress such as heat shock, hypoxia and serum starvation (Jackson et al., 1992; Mouta et al., 2001; Shin et al., 1996). Secretion of FGF1 requires complex formation with synaptotagmin and the calcium binding protein S100A13, which localizes close to the plasma membrane (Landriscina et al., 2001; Mohan et al., 2010; Tarantini et al., 1998). This underlines the possibility that FGF1 and FGF2 have distinct intracellular roles, and nucleolin possibly interacts with FGF1 and FGF2 for different reasons. This would be consistent with the fact that nucleolin play roles in a variety of cellular processes (Abdelmohsen and Gorospe, 2012; Tajrishi et al., 2011).

The biological role for translocated exogenous FGF1 in the nucleus is not clear. Intracellular FGF1 has been shown to inhibit p53 induced apoptosis as well as to exert neurotrophic activity in a nuclear localization dependent fashion (Bouleau et al., 2005; Rodriguez-Enfedaque et al., 2009). Nucleolin regulates apoptosis, both on the translational level and the protein level. For instance, nucleolin binds as a homodimer to the 5’-3’-UTR interaction site on the p53 mRNA, which suppresses the translation of p53 mRNA. Upon stress such as DNA damage, the ribosomal protein RPL26 interacts with nucleolin on the same site in the p53 mRNA, resulting in heterodimerization of nucleolin and RPL26 and enhanced translation of p53 (Chen et al., 2012; Takagi et al., 2005). Furthermore, upon cellular stress, nucleolin binds to the ubiquitin protein ligase Hdm2, and thereby impairs downregulation of p53, resulting in increase stability and levels of p53 (Saxena et al., 2006). We hypothesize, that FGF1 together with nucleolin regulates the p53-mediated apoptotic response.
Upon phosphorylation of FGF1 on Ser130 by PKCδ, a conformational change leads to exposure of an NES, which binds exportin-1/CMR1 (Nilsen et al., 2007). Interaction between exportin-1 and FGF1 leads to export from the nucleus through nuclear pores (Nilsen et al., 2007). The phosphorylation of FGF1 resulting in nuclear export most likely finalize the nuclear activity of FGF1. This assumption is also strengthened by the fact that phosphorylated FGF1 exported to the cytosol is degraded. Therefore, we can speculate that nucleolin is involved in terminating the activity of FGF1 in the nucleus. Nucleolin has previously been reported to be involved in nucleocytoplasmic trafficking of several proteins, including endostatin, US11 protein of herpes simplex virus 1, lactoferrin, and type I TGF-β receptor (Chandra et al., 2012; Greco et al., 2012; Legrand et al., 2004; Song et al., 2012). Nucleolin facilitates internalization of both endostatin and lactoferrin (Legrand et al., 2004; Song et al., 2012). After internalization, nucleolin further mediates nuclear import of endostatin together with importin α1 and importin β1 in an NLS mediated fashion (Song et al., 2012). Similarly, nucleolin together with importin β1, Smad2/3, and RAN GTPase activity mediates nuclear import of the TGF-β receptor in HER2 transformed breast cancer cells, where the TGF-β receptor potentially regulates RNA processing (Chandra et al., 2012). Nucleolin interacts with US11, a viral protein known to localize to the cytoplasm and nucleolus. Upon nucleolin depletion, the levels of US11 in the nucleolus increased, suggesting a role for nucleolin in nuclear export of US11, and for the outcome of US11 mediated infection (Greco et al., 2012).

As mentioned in the introduction, and reviewed by others, nucleolin is a key player in essential cellular processes, including transcription of rRNA, rRNA maturation and ribosome assembly, as well as chromatin remodeling and several aspects of DNA metabolism (Abdelmohsen and Gorospe, 2012; Ginisty et al., 1998; Ginisty et al., 1999; Tajrishi et al., 2011). The rRNA synthesis occurs in the nucleolus. We cannot exclude that FGF1 localize in the nucleolus. We have observed FGF1 in the nucleolus (unpublished data), but this has to be investigated more carefully as it could be artifacts from the cell fixation processes. The 18 kDa isoform of FGF2 localize in the nucleolus (Sheng et al., 2004) and has been shown to interact with the upstream binding factor (UBF), which is a transcription factor involved in rRNA transcription. Upon nuclear translocation of the 18 kDa isoform of FGF2, FGF2 together with UBF exerts roles in rRNA synthesis by binding to the rRNA gene promoter (Sheng et al.,
2005). It is not unlikely that translocated FGF1 play roles in rRNA synthesis, maturation, or assembly, or in regulating DNA metabolism or other cellular processes located within the nucleus or nucleolus.

**HSP90 as a therapeutic target in FOP2-FGFR1 driven malignancy**

FOP2-FGFR1 is the driver oncogene in the human KG-1a leukemic cell line. In this thesis (paper II) we show that the stability of the fusion protein FOP2-FGFR1 is impaired in KG-1a cells upon treatment with the HSP90 inhibitor NVP-AUY922. Also, the downstream signaling by STAT1 and PLCγ, as well as cell proliferation is abrogated upon NVP-AUY922 treatment of KG-1a cells. These results are consistent with our previous findings showing a reduced stability of FOP2-FGFR1 upon treatment with the HSP90 inhibitors geldanamycin and radicicol (Jin et al., 2011). The data show that FOP2-FGFR1 is dependent on the activity of HSP90 for its stability and activity.

The complexity of human cancer reflected by a vast genetic and epigenetic diversity has resulted in a great interest in targeting cellular pathways that are universally utilized by cancer cells but not by their normal counterparts (Dai et al., 2012). Therefore, targeting the heat shock response represents a potentially promising strategy. The heat shock response, also called the stress response, is one of the most evolutionary conserved cytoprotective mechanisms found in living organisms (Eckl and Richter, 2013; Hahn, 2009; Pearl et al., 2008; Ritossa, 1996). The stress induced heat shock response is initiated by the transcription factor heat shock factor 1 (HSF1), which regulates the heat shock response via the expression of HSPs (Westerheide and Morimoto, 2005). HSPs such as HSP90 serve as regulators for proteostasis, including protein synthesis, folding, trafficking, aggregation, disaggregation, and degradation (Trepel et al., 2010). Disrupted proteostasis leads to proteotoxic stress, which is considered a hallmark of cancer (Luo et al., 2009). Factors such as dysregulation of the protein translation machinery, imbalanced protein synthesis due to aneuploidy or gene-amplification, accumulation of mutated oncoproteins and enhanced protein damages upon oxidative stress all contributes to the stress phenotype of cancer. Mutated proteins and oncogenes are often less stable than normal proteins, and are
more dependent on the chaperone activities provided by the HSPs. HSP90 protects the mutated and overexpressed proteins essential for cancer cell growth and survival from misfolding and degradation (Trepel et al., 2010). As reviewed by others, this makes the heat shock response a critical contribution to the cancer cell survival and maintenance of malignancy (Dai et al., 2012; Trepel et al., 2010; Whitesell and Lindquist, 2005).

Several HSP90 inhibitors are tested in clinical trials on various cancers (Garcia-Carbonero et al., 2013). It has been suggested that cancer driven by driver oncogenes that are dependent on HSP90 activity such as HER2, EGFR, anaplastic lymphoma kinase (ALK), and BRAF, will show the greatest antitumorigenic responses when treated with HSP90 inhibitors (Neckers and Workman, 2012; Trepel et al., 2010). The second generation HSP90 inhibitor, NVP-AUY922 holds an improved toxicity profile compared to other HSP90 inhibitors, and is the most potent HSP90 inhibitor yet described (Eccles et al., 2008). NVP-AUY922 has previously been shown to have antitumor activity in human gastric cancer cells and a gastric cancer xenograft (Lee et al., 2011; Wainberg et al., 2013). NVP-AUY922 treatment also inhibited proliferation of breast cancer cells in vitro, and showed antitumor activity on a estrogen-receptor and HER2-positive breast cancer model (Jensen et al., 2008; Wainberg et al., 2013). Additionally, NVP-AUY922 has been shown to inhibit growth of NSCLC cells in vitro, as well as to reduce cell growth in a NSCLC KRAS mutated xenograft (Garon et al., 2013). NVP-AUY922 also affects the growth stability in a NSCLC xenograft model harboring EGFR mutations giving resistance to TKI (Garon et al., 2013). NVP-AUY922 is being tested on several cancers, such as NSCLC in a phase two trial, and on HER2-positive and estrogen-receptor-positive breast cancer in a phase two extension study (www.clinicaltrials.gov), (Garcia-Carbonero et al., 2013).

In our cell culture studies, NVP-AUY922 showed antitumor effects by inhibiting cell proliferation and reducing the stability of the driver oncoprotein FOP2-FGFR1 in KG-1a cells. Despite these promising effects, and although FOP2-FGFR1 is addicted to the activity of HSP90 (Jin et al., 2011), we did not observe antitumor activities on a KG-1a xenograft model treated with NVP-AUY922. The reason for this might be due to suboptimal drug scheduling and/or HSP70 induced resistance. NVP-AUY922 has been shown to have low stability in plasma, compared to solid tumors, liver, heart,
lung, and muscle as the levels of NVP-AUY922 in plasma was below the detection level after 48 hours (Jensen et al., 2008). The low stability of NVP-AUY922 in plasma is a possible reason for the lack of antitumor effects in our study. A drawback with animal models is that the drug cannot be administrated continuously by infusion. It is not given that an increase in concentration of the administrated drug will show antitumor effects on our AML systemic model, as the drug will be administrated only once a day or every other day. In addition to the low stability of NVP-AUY922 in plasma, non-optimal schedule time of treatment might also be a reason for the lack of antitumor activity of NVP-AUY922 in vivo.

Another possible explanation for the lack of antitumor activities of NVP-AUY922 in vivo is due to HSP70 induced resistance. Upon HSP90 inhibition, expression of other HSPs such as HSP70 and HSP27 are induced (Erlichman, 2009). Consistent with this, KG-1a cells treated with NVP-AUY922 showed an increase in HSP70 expression, which also is consistent with other studies (Garon et al., 2013; Jensen et al., 2008; Lee et al., 2011; Wainberg et al., 2013). Increased expression of HSP27 has been shown to induce resistance to the HSP90 inhibitor 17-AAG (McCollum et al., 2006). Increased expression of other HSPs such as HSP70 seen in our studies, might explain our lacking antitumor activity of both NVP-AUY922 and 17-AAG treated KG-1a xenografts. HSP70 have anti-apoptotic activity, and an increase in HSP70 levels results in apoptotic resistance and tumorigenesis (Rerole et al., 2011; Wood et al., 2011). A combined inhibition of HSP70 isoforms have shown to inhibit HSP90, and induce a tumor specific apoptotic response (Powers et al., 2008). It is of great interest to study the antitumor activity of NVP-AUY922 when combined with other HSP inhibitors. Inhibition of HSP70 has shown to induce myeloma cell death and HSP90 inhibition combined with HSP70 inhibition is emerging as a therapeutic approach to overcome the compensatory expression of HSP70 that reduce the antitumor effects of HSP90 inhibition (Evans et al., 2010; Goloudina et al., 2012; Zhang et al., 2013). Also, treatment of trastuzumab resistant gastric cancer xenografts models with trastuzumab in combination with NVP-AUY922 resulted in better antitumor activity, compared to xenograft models treated with either drug alone (Wainberg et al., 2013). It will be of interest to study the antitumor activities of NVP-AUY922 in combination with other drugs on the KG-1a xenograft model.
A biological role for the ER-bound protein LRRC59 in epithelial cell polarity

The biological functions of the ER-bound LRRC59-protein are still unknown. However, LRRC59 is quite abundant in all tissues, and especially in epithelial and neuronal tissue. Thus, LRRC59 might perform key functions in epithelial and neuronal cells. In this thesis (paper III) we show that LRRC59 depletion impairs the organization of epithelial cell polarity. For instance, LRRC59 depletion resulted in an increased cell spread area, impairment of both Golgi orientation towards a migratory front and lamellipodia formation. Also, cells that lacked LRRC59 showed a reduced ability to undergo directional migration. All of these findings support a defect in cell polarity upon LRRC59 depletion. The results were additionally strengthen by the fact that LRRC59 depleted cells also failed in forming an apical lumen in a spherical cell mass (cyst) developed from Caco-2 cells in a 3D matrigel.

We have previously shown that LRRC59 is involved in nucleocytoplasmic shuttling of FGF1, where LRRC59 is strictly required for nuclear import of exogenous FGF1. LRRC59 facilitated nuclear import of FGF1 together with the importins α1 (Kpnα1) and β1 (Kpnβ1) (Zhen et al., 2012). LRRC59 localizes to the ER-membrane via its C-terminal part, while the coiled-coil domain and the LRR domain faces the cytosol. LRRC59 also localize to the nuclear envelope (NE), and it has been suggested that LRRC59 can localize to the inner nuclear membrane (INM) in a Kpnβ1-dependent manner (Zhen et al., 2012).

Transmembrane proteins holding an LRR domain play central roles in development and organization of neural connectivity, including axon guidance and target selection, and formation of synapses in neurons (Ko, 2012). The LRR domains holds great variation and serve as binding sites for a diverse group of interaction partners, including pathogens (de et al., 2011; Ewing et al., 2007; Ko, 2012; Offord and Werling, 2013). The characteristic LRR structure enables efficient protein-protein interactions, and makes the LRR containing proteins well suited for regulation of intracellular communication and cell adhesion (de et al., 2011).

Cell polarity is important for functionality in both epithelial and neuronal tissue/cells.
The defects observed in cell polarity upon LRRC59 depletion in epithelial cells might be explained by various scenarios, and three different possibilities are listed below. First, the effects might be due to a lack of transport through the secretory pathway, as LRRC59 is an ER-protein and might be involved in secretion of proteins via the ER to their appropriate locations where they regulate polarity. The second possible explanation is that LRRC59 might be a regulator of translation. LRRC59 was originally identified as a ribosome binding protein (Ichimura et al., 1992; Ohsumi et al., 1993; Tazawa et al., 1991). Like ribosomes, LRRC59 localizes to the rough ER, where it might regulate translation of transmembrane proteins or proteins that later are to be secreted and exert regulatory roles in cell polarity, as translation occur on the rough ER. Finally, LRRC59 might influence establishment of cell polarity via its nuclear transport mechanisms. LRRC59 was found to be strictly required for import of FGF1 into the nucleus (Zhen et al., 2012). It is possible that LRRC59 is involved in nuclear trafficking of other proteins than FGF1, such as components important for transcription or translation of proteins required for cell polarization.

In cancer, many proteins are overexpressed resulting in an imbalance in cell communication and function. We found an increased expression of LRRC59 mRNA in colorectal cancer samples (paper III). Consistent with this, LRRC59 has been shown to be overexpressed in some cancers (www.oncomine.org). Further, large scale analyses identified LRRC59 as an essential protein for cancer cell viability, as well as metastatic efficiency (Luo et al., 2008; Terp et al., 2012).

In conclusion, defining the roles of proteins with unknown functions is vital to understand cellular processes as well as to develop well functioning targeted therapy for diseases such as cancer. Considering the expression pattern of LRRC59, and our recent findings suggesting LRRC59 as a regulator of epithelial cell polarity, this may contribute to new knowledge particularly relevant for understanding the biology of epithelial cells and the development and behavior of carcinomas.
Conclusions and perspectives

FGF signaling is specific and extensively regulated. Although FGF signaling has been thoroughly studied over the years, many of the signaling mechanisms and actions of the different FGFs are not fully elucidated. In paper I we show that FGF1 interacts with nucleolin, and that nucleolin is important for phosphorylation of FGF1 by PKCδ, thus preventing nuclear export. Nucleolin is a protein that exerts multiple functions and localizes to several cellular compartments. The role of the interaction between FGF1 and nucleolin needs to be studied further in order to reveal the role of translocated exogenous FGF1 in the nucleus. We hypothesize that FGF1 together with nucleolin localize into the nucleus and regulate p53 induced apoptosis. Given that some of the FGF1 mutants designed in our studies cannot bind nucleolin, and are unable to be phosphorylated by PKCδ, these FGF1 mutants may serve as tools to study the role of FGF1 in apoptosis or in rRNA synthesis studies.

HSP90 is emerging as an excellent target for cancer therapy. However, the challenge concerning resistance is a major factor that needs to be overcome. Our results in paper II illustrate that the HSP90 inhibitor NVP-AUY922 impairs the stability of the driver oncoprotein FOP2-FGFR1, and attenuates proliferation and the activity of downstream signaling molecules such as STAT1 and PLCγ in human KG-1a leukemic cells. However, NVP-AUY922 showed no antitumor activity in a KG-1a xenograft model. We suggest that the lacking antitumor activities of NVP-AUY922 in our experiments are due to suboptimal dose scheduling and/or HSP70 induced resistance. Several reports show that a combination of two drugs gives greater antitumor activities than either drug alone. Further, it will be interesting to test the effects of NVP-AUY922 in vivo, employing different time schedules for drug administration and a combination of NVP-AUY922 and the cytotoxic drug cytarabine used in treatment of AML.

At present, we know the sequence of all human protein-encoding genes, and a vast amount of information is available on their expression patterns in tissues and during biological processes. Still, many of the proteins known to exist in cells have unknown or poorly understood functions. LRRC59 is one such protein. Here we show that LRRC59 depletion results in impaired epithelial cell polarization and directed cell
migration, as well as impaired lumen formation in a 3D Caco-2 cyst, and suggest that the ER-localization of LRRC59 is crucial for its role in regulating epithelial cell polarity. We were not able to find the regulatory mechanism for how LRRC59 depletion impaired cell polarization. It will be of interest to perform affinity pull-down experiments using LRRC59 as bait in order to detect protein interaction partners of LRRC59 that might reveal the exact regulatory role of LRRC59 in establishment of epithelial cell polarity.
References


References


References


References


References


Wu, J, Patmore, D M, Jousma, E, Eaves, D W, Breving, K, Patel, A V, Schwartz, E B, Fuchs, J R, 
formation of malignant peripheral nerve sheath tumors. Oncogene 33, 173-180.

Wu, Y M, Su, F, Kalyana-Sundaram, S, Khazanov, N, Ateeq, B, Cao, X, Lonigro, R J, Vats, P, Wang, 
Discov. 3, 636-647.

Xian, W, Pappas, L, Pandya, D, Selfors, L M, Derksen, P W, de, B M, Gray, N S, Jonkers, J, Rosen, J M, 
and Brugge, J S (2009). Fibroblast growth factor receptor 1-transformed mammary epithelial cells 
are dependent on RSK activity for growth and survival. Cancer Res. 69, 2244-2251.

Xue, Z and Melese, T (1994). Nucleolar proteins that bind NLSs: a role in nuclear import or ribosome 

Yang, C, Maiguel, D A, and Carrier, F (2002). Identification of nucleolin and nucleophosmin as 


Zagouri, F, Sergentanis, T N, Chrysikos, D, Papadimitriou, C A, Dimopoulos, M A, and Psaltopoulou, 

FGF1 into cytosol and nucleus is a periodic event independent of receptor kinase activity. Exp. Cell 
Res. 317, 1005-1015.

specificity of the fibroblast growth factor family. The complete mammalian FGF family. J. Biol. Chem. 
281, 15694-15700.

Zhao, Y and Zhang, Z Y (2001). The mechanism of dephosphorylation of extracellular signal-regulated 

Zhen, Y, Sorensen, V, Skjerpen, C S, Haugsten, E M, Jin, Y, Walchli, S, Olsnes, S, and Wiedlocha, A 
(2012). Nuclear import of exogenous FGF1 requires the ER-protein LRRC59 and the importins 
Nucleolin regulates phosphorylation and nuclear export of fibroblast growth factor 1 (FGF1)

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Abstract

Extracellular fibroblast growth factor 1 (FGF1) acts through cell surface tyrosine kinase receptors, but FGF1 can also act directly in the cell nucleus, as a result of nuclear import of endogenously produced, non-secreted FGF1 or by transport of extracellular FGF1 via endosomes and cytosol into the nucleus. In the nucleus, FGF1 can be phosphorylated by protein kinase C δ (PKCδ), and this event induces nuclear export of FGF1. To identify intracellular targets of FGF1 we performed affinity pull-down assays and identified nucleolin, a nuclear multifunctional protein, as an interaction partner of FGF1. We confirmed a direct nucleolin-FGF1 interaction by surface plasmon resonance and identified residues of FGF1 involved in the binding to be located within the heparin binding site. To assess the biological role of the nucleolin-FGF1 interaction, we studied the intracellular trafficking of FGF1. In nucleolin depleted cells, exogenous FGF1 was endocytosed and translocated to the cytosol and nucleus, but FGF1 was not phosphorylated by PKCδ or exported from the nucleus. Using FGF1 mutants with reduced binding to nucleolin and a FGF1-phosphomimetic mutant, we showed that the nucleolin-FGF1 interaction is critical for the intranuclear phosphorylation of FGF1 by PKCδ and thereby the regulation of nuclear export of FGF1.

Introduction

Fibroblast growth factor 1 (FGF1) belongs to the heparin binding fibroblast growth factor family, which consists of 22 members involved in a variety of cellular responses during embryonic development and in adult organisms. FGF1 regulates proliferation, differentiation, cell survival, and apoptosis [1]. FGF1-activity is usually mediated in a paracrine fashion by binding to and activation of high affinity, tyrosine kinase FGF receptors (FGFR1-4) on the cell surface. The activation of FGFRs leads to activation of downstream signaling cascades such as the PLCγ/PKC, PI3K/Akt, and Ras/MAP kinase pathways [2].

In addition to activation of FGFRs and their downstream signaling pathways, extracellular FGF1 can cross cellular membrane and translocate to the
cytosol and nucleus [3,4]. Also endogenously produced, non-secreted FGF1 can be found in the cell nucleus [5,6]. Nuclear FGF1 has been implicated in DNA synthesis and proliferation [7], and it has been shown to play a role in cell differentiation, survival and in modulating p53-induced apoptosis [5,6,8]. In addition to FGF1, exogenous FGF2, epidermal growth factors (EGFs), cytokines, as well as receptors such as EGF receptors, FGFR1, and FGFR2, can be transported to the nucleus where they regulate cellular activities such as proliferation, survival and tumor progression [3,4,9–12].

The translocation of extracellular FGF1 into the cell is a regulated process and requires binding to cell surface FGFR1 or FGFR4 [13–15]. Also, the activity of several intracellular proteins such as PI3K [16] and p38 MAPK [17] is necessary for this process. Furthermore, it was shown that translocation of endocytosed FGF1 to the cytosol depends on a vesicular transmembrane electric potential indicating that FGF1 is translocated to the cytosol from an endosomal compartment [18]. The nuclear import of FGF1 is regulated by two nuclear localization sequences (NLS), one monopartite [19] and one bipartite [20]. Inside the nucleus, FGF1 is phosphorylated by PKC\(\dot{\theta}\) on serine 130 [21]. Exportin-1 binds phosphorylated FGF1, and FGF1 is then rapidly exported in a nuclear export sequence (NES)-mediated fashion to the cytosol where it is subsequently degraded [21,22].

More studies on the mechanism of action of intracellular/nuclear FGF1 are necessary to elucidate the role of intracellular FGF1, and we have aimed at identifying intracellular binding partners of FGF1. Previously, we have shown that FGF1 interacts with several intracellular proteins including casein kinase 2 (CK2) [23], and FGF1 intracellular binding protein (FIBP) [24], a protein found to be crucial for FGF-dependent left-right asymmetry patterning in zebrafish [25]. Furthermore, FGF1 interacts with LRRC59/ribosome binding protein p34 [26], which is required for translocation of FGF1 from the cytosol to the nucleus [27]. FGF1 has also been found to interact with GRP75/mortalin [28] and p53 [6]. We show here that FGF1, as well as FGF2, interacts with nucleolin, a multifunctional nucleolar protein involved in cellular processes such as growth, cell cycle regulation, transcription, apoptosis, ribosome biogenesis, and nucleocytoplasmic trafficking of ribosome particles [29] as well as other proteins [30–33]. It has previously been published that nuclear FGF2 interacts with and stimulates CK2, which leads to phosphorylation of nucleolin [34]. Here, we explore the role of the FGF1-nucleolin interaction in intracellular trafficking of FGF1 and demonstrate that nucleolin regulates phosphorylation of FGF1 by PKC\(\dot{\theta}\) in the nucleus and thereby regulates nuclear export of FGF1.

Materials and Methods

Antibodies and reagents

\(^{[35]}\)S\)methionine, \(^{[33]}\)P\)phosphate, and \(^{\gamma-33}\)P\)ATP were obtained from Amersham Pharmacia Biotech. Recombinant FGF1 and \(in vivo\) transcribed \(^{[35]}\)S\)methionine labelled FGF1 (\(^{35}\)S-FGF1) were produced as described previously [16]. Leptomycin B (LMB) and thapsigargin were from Sigma-Aldrich. Rottlerin and bafilomycin A1 (BafA1) were from Calbiochem. The following primary antibodies were used with the catalogue numbers indicated in parentheses: mouse anti-nucleolin (anti-C23) (sc-8031), goat anti-FGF1 (sc-1884), goat anti-FGF2 (sc-74412), and rabbit anti-PKC\(\dot{\theta}\) (sc-937) from Santa Cruz Biotechnology, mouse anti-lamin A (ab8980) and mouse anti-GAPDH-HRP (ab9482) from Abcam, rabbit anti p44/42 MAPK (#9102) and rabbit anti-phospho-PKC\(\dot{\theta}\) (Thr 505) (#9374) from Cell Signalling Technology, mouse anti-Hsp90 (610419) from BD Transduction Laboratories, and mouse anti-\(\gamma\)-tubulin (T6557) from Sigma. Secondary antibodies conjugated to HRP were from Jackson Immune-Research Laboratories. Recombinant active PKC\(\dot{\theta}\) was from
SignalChem and full length FGF1 was from Abcam. Heparin-Sepharose CL-6B affinity resin and Glutathione Sepharose 4 Fast Flow affinity resin was from GE Healthcare, and Ni-NTA Superflow was from Qiagen. Protease inhibitor cocktail tablets (EDTA-free, Complete) were from Roche Diagnostics and phosphatase inhibitor cocktails were from Sigma-Aldrich. Dynabeads M-280 Streptavidin was purchased from Invitrogen and anti-c-Myc antibody (Agarose) was from Abcam. 4-20% Precise Protein Gels were from Thermo Scientific and mini-PROTEAN TGX precast gels from Bio-Rad. Immobilon-P membranes were from Millipore and Trans-Blot Turbo 0.2 μm PVDF from Bio-Rad.

Cell lines and bacterial strains

Human normal foreskin fibroblast cell line BJ, human embryonic kidney 293 (HEK 293) cell line and mouse fibroblast cell line NIH3T3 were from ATCC. Fibroblast cell lines were grown in Quantum 333 medium (PAA laboratories GmbH). The HEK 293 cell line and the previously described U2OSR1 cell line [35] were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10 % FBS (PAA Laboratories GmbH). Both the Quantum 333 medium and DMEM medium were supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, from Gibco) and the cells were grown in a 5 % CO₂ atmosphere at 37°C. The cells were seeded into tissue culture plates the day preceding the start of the experiments. E. coli strains BL21(DE3)pLyS Rosetta and BL21(DE3)pLyS were from Merck, and BL21(DE3)-RIL was from Stratagene.

Plasmids

The nucleolin coding sequence was obtained from GenScript. Sequences encoding FGF1 (Ala-residues 21-154) [36], FGF2 (residues 1-154) and a fragment of nucleolin (residues 284-707, nucleolin-C) [37] were cloned into a pDEST15 expression vector (with an N-terminal GST). The sequence encoding FGF1 was also cloned into a pET-SBP expression vector (with an N-terminal SBP), a pDEST17 expression vector (with an N-terminal hexahistidine peptide) and a pcDNA3 vector (with an N-terminal myc sequence).

siRNAs and transfection

The nucleolin targeting siRNA was obtained from Qiagen (SI02654925). LRRC59 targeting siRNA was described previously [27]. Scrambled control siRNA was obtained from Thermo Scientific Dharmacon (CD-001810-01-20). For siRNA transfection studies, U2OSR1 cells (1x10^5 cells/ml) were seeded out and after 24 h the cells were transfected with 50 nM of the nucleolin targeting siRNA and control targeting siRNA, and 75 nM of the LRRC59 targeting siRNA using Lipofactamine RNAiMax Transfection Reagent (Invitrogen) according to the procedure given by the company. Seven hours after transfection, 10 % FBS was added to the cells, and the cells were cultured for 72 h before further experiments.

Transient expression of myc-FGF1 was performed by transfecting HEK 293 cells with plasmid DNA using Escort IV Transfection Reagent (Sigma) in Minimum Essential Medium (MEM, Gibco) according to the manufacturer’s protocol. After 7 h the medium was changed for DMEM supplemented with 10 % FBS and the cells were grown for 24 h.

Design of mutations

Potential interaction sites on the surface of the FGF1 molecule were determined using the bioinformatic web servers meta-PPISP [38], ConSurf [39] and SWAKK [40]. 18 surface mutations disturbing putative binding sites were selected and introduced using the Quick Change Site-Directed Mutagenesis protocol (Stratagene).
Protein expression and purification

The C-terminal fragment of nucleolin (residues 284-707, nucleolin-C) was expressed as a fusion protein with an N-terminal GST in the E. coli Bl21(DE3)pLysS Rosetta strain. The protein was purified from the bacterial lysate using a Glutathione Sepharose 4 Fast Flow column, followed by rTEV protease cleavage and tandem GSH-Sepharose HiTrap and Heparin-Sepharose HiTrap chromatography using the Äkta Prime system (GE Healthcare). Purity and molecular mass were verified by SDS-PAGE and matrix-assisted laser desorption/ionization time-of-flight MS on an Applied Biosystems 4800 (Life Technologies).

FGF1 and FGF2 wild type proteins or mutants were produced with an N-terminal GST tag in E. coli Bl21(DE3)pLysS or Bl21(DE3)-RIL strains and then purified on a Glutathione Sepharose 4 Fast Flow column. To obtain tag-free proteins, fusion proteins were cleaved by rTEV protease and subjected to tandem GSH-Sepharose HiTrap and Heparin-Sepharose HiTrap chromatography using the Äkta Prime system (GE Healthcare). We obtained 17 soluble proteins among 18 FGF1 mutant constructs. His-FGF1 as well as SBP-FGF1 were produced in E. coli Bl21(DE3)pLysS or Bl21(DE3)-RIL strains and then purified on a Heparin-Sepharose CL-6B column. Protein homogeneity and identity were checked by SDS-PAGE and mass spectrometry (Applied Biosystems). To verify the native conformation of purified FGFs, circular dichroism (Jasco J-715 spectropolarimeter) and fluorescence (Jasco FP-750 or FP-8500 spectrofluorimeter) measurements were applied as described previously [36, 41]. FGF1 and FGF2 were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific Pierce) in a 1:1 molar ratio for 5 min.

Pull-down assays

BJ, NIH3T3, or transfected HEK 293 cells were lysed in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 supplemented with a protease inhibitor cocktail) and sonicated for 3×10 s. Cellular debris was pelleted by centrifugation. Cleared HEK 293 lysate was incubated with 20 μl of anti-c-Myc antibody (Agarose) for 1 h at room temperature. Cleared BJ or NIH3T3 cell lysates were incubated with 73 pmol of recombinant GST-FGF1, GST-FGF2 or GST protein alone (negative control) for 1 h followed by incubation with 30 μl of Glutathione Sepharose 4 Fast Flow for 1 h at room temperature. Cell lysates were also incubated with recombinant SBP-FGF1, biotinylated FGF2 or His-FGF1 followed by incubation with 50 μl of Streptavidin-coated Dynabeads or 30 μl of Ni-NTA Superflow for 1 h. In all cases, the resins were washed four times in PBS with 1 % Triton X-100 before the protein complexes were eluted by 10 min boiling in SDS sample buffer. Protein complexes were subjected to SDS-PAGE and western blotting.

A similar procedure was applied to test for direct binding of recombinant nucleolin to FGF1 and FGF2. 73 pmol of FGFs were incubated with an equal molar amount of nucleolin.

Mass spectrometry analysis

To identify proteins interacting with FGF1 we used recombinant SBP-FGF1 and cell lysate from NIH3T3 cells. 10 μg of SBP-FGF1 was incubated with 100 μl of Streptavidin-coated Dynabeads at 4°C. After 2 h the beads were washed three times with PBS with 1 % Triton X-100 and incubated with lysed cells for 2 h at 4°C. Then, the beads were washed four times in PBS with 1 % Triton X-100 before the protein complexes were eluted by 10 min boiling in SDS sample buffer. The proteins were analyzed by SDS–PAGE followed by Coomassie blue staining. In the control experiment cell lysate was incubated with Streptavidin-coated Dynabeads alone. Protein bands different from the control were cut from the gel, trypsinized and analyzed by MS. For selected samples MS/MS experiments were performed. Mass spectra were
acquired on an Ultraflex II MALDI-TOF/TOF instrument from Bruker Daltonics (Bremen, Germany) controlled by FlexControl software (version 2.4, Bruker Daltonics) at the Core Facility for Proteomics and Mass Spectrometry at Oslo University Hospital-Rikshospitalet, Institute of Immunology, Oslo, Norway.

Surface plasmon resonance

The interaction between nucleolin and FGF1 or FGF2 was investigated by surface plasmon resonance (SPR) analysis using a Biacore 3000 system (GE Healthcare). Recombinant nucleolin-C (10 μg/ml in 10 mM sodium acetate buffer, pH 4.0) was covalently immobilized on a carboxymethylated dextran sensor chip (CM4, GE Healthcare) using an amine coupling kit (GE Healthcare) on the level of ~ 540 response units (RU). A reference flow cell was prepared by subjecting a surface to the amine coupling procedure in the absence of nucleolin. HEPES-buffered saline-P (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005 % P20, pH 7.4) was used as immobilization running buffer. 60 μl aliquots of FGF1 or FGF2 in running buffer (PBS with 0.1 mg/ml BSA, 0.005 % P20, pH 7.3) were injected at 25°C at a flow rate of 30 μl/min and specific protein-protein interactions were measured. Dissociation of growth factors from nucleolin was monitored over a 4 min period. Binding of selected FGF1 variants to nucleolin was examined upon their injection at the concentration of 654 nM onto a CM5 sensor chip (GE Healthcare) with immobilized nucleolin (on the level of ~ 5970 RU). Regeneration of the sensor chip surface was performed with 1.0 M NaCl. The data were analyzed using the BIAevaluation 3.1 software (GE Healthcare). Final sensorgrams were generated by subtracting the response in the reference flow cell from the responses in the sample flow cell. Interaction Maps and calculations of kinetic parameters for the interaction of the C-terminal fragment of nucleolin with FGF1 and FGF2 were made by Ridgeview Diagnostics AB, Uppsala, Sweden.

Cell fractionation

Cells incubated with FGFs were washed in a high salt/low-pH-buffer (HSLP, 2 M NaCl, 20 mM sodium acetate, pH 4.0) to remove surface bound FGFs and HEPES medium before fractionation. For fractionation of cells into cytoplasmic and nuclear fractions cells were lysed in lysis buffer (0.15 M KCl, 40 mM Tris, pH 7.2, 1 % Triton X-100, 2 mM EDTA) supplemented with protease and phosphatase inhibitor cocktails. The Triton X-100 soluble fraction was collected as the cytoplasmic fraction and the insoluble fraction obtained by centrifugation of lysates was collected as the nuclear fraction. The nuclear fraction was washed in lysis buffer and sonicated. For fractionation of cells into membrane, cytosolic and nuclear fractions we used a digitonin fractionation method, described previously [21]. Cells incubated with FGFs for 6 or 10 h (in addition to inhibitors LMB (5 ng/ml), thapsigargin (1 μg/ml), rottlerin (10 μM) or BafA1 (10 nM) were washed with HSLP-buffer to remove surface bound FGFs. The cells were permeabilized with 20 μg/ml digitonin in PBS and incubated at 25°C for 5 min and on ice for additional 30 min to allow the cytosol to diffuse into the buffer. The buffer was collected and denoted the cytosolic fraction. The remainder of the cells was lysed in lysis buffer, and the Triton X-100 soluble fraction was designated the membrane fraction. The insoluble material was sonicated and designated the nuclear fraction. FGFs were extracted from the subcellular fractions by adsorption to Heparin-Sepharose beads, washed and then eluted and analyzed by SDS-PAGE and fluorography and/or immunoblotting. In addition, samples of the fractions were loaded directly onto SDS-PAGE for analysis by immunoblotting.
In vivo phosphorylation of FGF1

The method was previously described in [16,27]. Briefly, U2OSR1 cells were starved for 24 h and the cellular ATP pool was radiolabelled by incubation with 25 μCi/ml \([^{33}P]\)phosphate in phosphate free medium overnight. Then the cells were stimulated with 100 ng/ml recombinant FGF1 and 10 U/ml heparin for 6 h. All inhibitors/drugs were added at the same time as FGF1 and were present during the entire incubation. After 6 h the cells were washed with a HSLP-buffer and HEPES medium containing heparin to remove surface bound FGF1. The cells were then lysed and sonicated (for analysis of total cell lysate) or fractionated into cytoplasmic (containing both cytosol and membranes) and nuclear fractions. The cell lysate/subcellular fractions were incubated with Heparin-Sepharose beads to bind FGF1 and the beads were washed extensively. FGF1 is highly resistant to trypsin when bound to heparin, and the beads (with bound FGF1) were treated with 2 μg/ml TPCK- treated trypsin (Sigma-Aldrich) to remove most proteins other than FGF1. FGF1 was eluted from the beads in an SDS-buffer and subjected to SDS-PAGE and western blotting. The blots were exposed to fluorography to detect \([^{33}P]\)-phosphorylated FGF1 (\(\text{33P-FGF1}\)). \(\text{33P-FGF1}\) represents the fraction of internalized FGF1 that was translocated to the cytosolic/nucleus since the specific phosphorylation of FGF1 by PKC8 can only occur in the cytosolic and nuclear compartment due to the intracellular localization of PKC8. The total amount of internalized FGF1 (which includes material in endosomes) was detected on the same blot by anti-FGF1 and immunoblotting. For analysis of proteins other than FGF1, an aliquot of each lysate/fraction was withdrawn before binding to Heparin-Sepharose and analyzed separately by SDS-PAGE and immunoblotting.

In vitro phosphorylation of FGF1 by PKC8

Recombinant wild type or mutant FGF1 (200 ng) was incubated with recombinant active PKC8 (50 ng) for 30 min at 30°C in a buffer containing 40 μCi [γ-\(^{33}\)P]ATP, 25 mM MOPS pH 7.2, 12.5 mM β-glycerol-phosphate, 25 mM MgCl₂, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT, and 50 μM unlabelled ATP. The FGFs were thereafter adsorbed to Heparin-Sepharose beads, washed, and separated by SDS-PAGE and analyzed by fluorography and anti-FGF1 by immunoblotting.

Results

FGF1 and FGF2 interacts with nucleolin

To identify intracellular interaction partners for FGF1, affinity pull-down assays using cell lysates and recombinant FGF1 tagged with streptavidin binding peptide (SBP-FGF1) as a bait were performed. FGF1-interacting proteins were separated by SDS-PAGE (Figure S1), then extracted, trypsinized and analyzed by mass spectrometry (MS). The most frequently identified protein was nucleolin. The identity of nucleolin was confirmed by MS/MS experiments. Other proteins found using this approach were lamin A, nucleophosmin, lamin A/C, annexin A2, Ap-2 complex subunit alpha-2, carbamoyl-phosphate synthase, and major vault protein.

The interaction between FGF1 and nucleolin was verified by in vitro binding experiments followed by immunoblotting using a nucleolin-specific antibody. To ensure the independence of the results from experimental settings we applied three constructs of FGF1 fused to different tags (SBP-FGF1, hexahistidine-tagged FGF1 (His-FGF1) and glutathione S-transferase-tagged FGF1 (GST-FGF1)), three different types of resins (Streptavidin-coated Dynabeads, Glutathione Sepharose or Ni-NTA-Agarose) for growth factor immobilization, and lysates from two different cell lines (BJ and NIH3T3) as a source of cellular
proteins. We also used biotinylated FGF2 (Biot-FGF2) and glutathione S-transferase-tagged FGF2 (GST-FGF2) to investigate whether nucleolin binding is specific for FGF1. As shown in Figure 1A, nucleolin was present in the fraction pulled down from the cell lysates by each of the FGF1 or FGF2 constructs. As a negative control we used either beads alone or beads incubated with GST. These experiments show that FGF1 or FGF2 can form a complex with nucleolin. To provide more physiological conditions, we also tested whether FGF1 could interact with endogenous nucleolin in mammalian cells. Similarly to previously shown pull-down experiments with recombinant FGFs, we were able to pull-down nucleolin from HEK 293 cells by precipitating transiently overexpressed myc-FGF1 using myc-agarose (Figure 1B). Nucleolin was not detected in untransfected cells upon incubation with myc-agarose. This result confirms that nucleolin is an interaction partner of FGF1 in mammalian cells.

To test if the interaction between FGF1 or FGF2 and nucleolin is direct, we performed in vitro binding assays using recombinant Biot-FGF2 or SBP-FGF1 and a recombinant C-terminal fragment (residues 284-707) of nucleolin (nucleolin-C). In agreement with a study of Yang et al., we were not able to express the full-length nucleolin since its N-terminal domain is very acidic and hinders the solubility of the recombinant protein [35]. We found that FGF1 and FGF2, but not beads alone, bound to nucleolin-C, indicating that there is a direct binding between these proteins (Figure 1C). Next, to quantify the strength of the interactions, we applied the SPR technique using the Biacore system. Concentration-dependent responses for FGF1 and FGF2 (concentration range 10-320 nM) upon binding to immobilized nucleolin-C confirmed an in vitro interaction of these proteins (Figure 1D). Despite the monomeric state of FGFs, association and dissociation curves did not fit well to the simple 1:1 Langmuir binding model and thus exhibited a complexity in the interactions. In order to assess the binding parameters the method called Interaction Map was applied [42], which allows for the resolution of the contributing interactions without knowledge about kinetic constants or degree of heterogeneity (K. Andersson, and M. Malmqvist, Method for the analysis of solid biological objects, patent application WO 2010033069, 2008). Interaction Map calculations confirmed the complexity of the interaction between growth factors and nucleolin-C (Figure 1E). In the case of FGF1-nucleolin binding, analysis of the sensorgrams suggested the existence of two parallel binding processes, one of apparent affinity \( K_D = 4.0 \times 10^{-8} \text{ M} \) (\( k_a = 1.8 \times 10^{4} \text{ M}^{-1}\text{s}^{-1} k_d = 7.0 \times 10^{-4} \text{ s}^{-1} \)) and one of approximate affinity \( 4.8 \times 10^{-7} \text{ M} \) (\( k_a = 1.7 \times 10^{6} \text{ M}^{-1}\text{s}^{-1} k_d = 8.3 \times 10^{-1} \text{ s}^{-1} \)). The weaker interaction seems to be approximately three times as abundant as the stronger interaction (Table S1). The FGF2-nucleolin sensorgrams also indicated the contribution of two components in the overall binding, one of approximate affinity \( K_D = 2.2 \times 10^{-8} \text{ M} \) (\( k_a = 1.7 \times 10^{4} \text{ M}^{-1}\text{s}^{-1} k_d = 3.8 \times 10^{-4} \text{ s}^{-1} \)) and one of approximate affinity \( 1.3 \times 10^{-7} \text{ M} \) (\( k_a = 3.5 \times 10^{6} \text{ M}^{-1}\text{s}^{-1} k_d = 4.4 \times 10^{-1} \text{ s}^{-1} \)). The two interactions contributed equally to the detected signal (Table S1).

In experiments with recombinant FGF1 we used its truncated form (Ala-FGF121-154), which is commonly used in FGF1 research and well characterized [43]. There was no difference in the proliferative response of cells to full length FGF1 and its truncated form (Figure S2A). We also compared the ability of both FGF1 forms to bind to nucleolin and found no difference (Figure S2B).

**Identification of residues of FGF1 involved in nucleolin binding**

An in vitro binding assay with recombinant nucleolin-C and biotinylated FGF1 (Biot-FGF1) was performed in the presence of heparin, a negatively charged polyanion that binds to positively charged amino acid residues on the surface of FGF1. As shown in Figure 2A and Figure S3, the presence of heparin remarkably...
diminished the nucleolin-C interaction with FGF1 suggesting that the residues involved in nucleolin binding are localized within the heparin-binding region of the FGF1 molecule.

To identify more precisely residues of FGF1 responsible for the interaction with nucleolin, we constructed and tested 17 mutants with disturbance in putative binding sites on the surface of the FGF1 molecule (Figure S4A). SPR experiments using FGF1 mutants at concentration of 654 nM showed a significant decrease in the binding response for three variants: K126A/K127A, R136E and K142E (Figure S4B, Figure 2 B). For the K126A/K127A and the K142E variants we observed significantly weaker binding to nucleolin than for the wild-type, whereas for the R136E mutant we could not detect any response even at 1 μM concentration. All of these amino acid substitutions are located within the positively charged heparin-binding site of FGF1, suggesting an electrostatic nature of the FGF1 binding to nucleolin. As a control we used an L147A variant with a substitution outside the heparin binding region. Figure S5 shows elution profiles of selected mutants from a Heparin-Sepharose column with a linear NaCl gradient.

**Nucleolin is not involved in nuclear import of exogenous FGF1**

Endocytosed FGF1 can translocate to the cytosol by crossing endosomal membranes and thereafter be imported into the nucleus [18]. Nucleolin has been shown to play a role in nucleocytoplasmic trafficking of proteins and ribosomal subunits [29–33,44], and we therefore investigated if nucleolin is involved in any of the intracellular transport steps of externally added FGF1.

To study the nuclear import of FGF1, we used U2OS cells stably transfected with FGFR1 (U2OSR1) that were depleted for nucleolin by transfection with nucleolin-specific siRNA. Since nucleolin is an essential protein, the siRNA parameters were chosen so as to give partial nucleolin depletion to ensure cell viability. The cells were serum starved for 24 h before stimulation with FGF1 for 6 h to allow endocytosis and entry into the nucleus [15]. Then, the cells were washed to remove surface bound FGF1, lysed and fractionated into a cytoplasmic fraction, which included membranes and endosomes, and a nuclear fraction. The fractions were analyzed for FGF1 by immunoblotting. In scrambled siRNA treated cells as well as in nucleolin depleted cells, FGF1 was detected in both the nuclear and the cytoplasmic fractions (Figure 3). In accordance with previous reports, nuclear entry of FGF1 was efficiently inhibited by BafA1, which inhibits vacuolar proton pumps and thereby represses translocation of FGF1 from endosomes to the cytosol [18], and by depletion of the LRRC59 protein thereby inhibiting transport of FGF1 from the cytosol to the nucleus [27]. BafA1 treatment and LRRC59 depletion were used here and in later experiments as negative controls for FGF1 translocation into the cytosol/nucleus. Our data indicates that nucleolin is not involved in the endocytic uptake or nuclear translocation of exogenous FGF1.

**Nucleolin is required for intracellular phosphorylation of FGF1**

Translocated FGF1 can be phosphorylated by PKCδ on serine 130. This phosphorylation event has been shown to occur in the nucleus and to regulate the availability of a NES and thereby nuclear export of FGF1 [22]. To study if nucleolin is involved in nuclear phosphorylation of FGF1, U2OSR1 cells depleted for nucleolin were serum starved, treated with [33P]phosphate to label the cellular ATP pool, and then incubated with unlabelled recombinant FGF1 for 6 h. Total cell lysates were analyzed for intracellularly [33P]-phosphorylated FGF1 (33P-FGF1). As shown in Figure 4A, 33P-FGF1 was detected in control cells (scrambled or no siRNA treated), however, for nucleolin depleted cells, as well as for LRRC59 depleted or BafA1 treated cells, no phosphorylated FGF1 was observed. This result was confirmed by siRNA against nucleolin
obtained from two different companies with non-overlapping sequences (Figure S6). This result suggests that nucleolin is involved in regulating intracellular FGF1 phosphorylation. We also confirmed our results using full length FGF1. Nucleolin depletion inhibited intracellular phosphorylation of the long as well as the short form of FGF1 (Figure S7), and this experiment also demonstrated that the long form of FGF1 was converted into a shorter form before translocation into the nucleus.

We analyzed if nucleolin depletion affected the activity of PKCδ, the kinase responsible for FGF1 phosphorylation. Cells were stimulated with FGF1 and analyzed for activated (Thr505 phosphorylated) PKCδ by immunoblotting. As shown in Figure 4B, activation of nuclear PKCδ appeared to be similar in nucleolin depleted and scrambled siRNA treated cells. Thus, nucleolin does not seem to influence the presence or activation of PKCδ in the nucleus.

To further test the role of nucleolin in the phosphorylation of FGF1, we studied phosphorylation of three FGF1 mutants with highly reduced ability to interact with nucleolin (FGF1 K126A/K127A, R136E and K142E, as described above) and one control mutant with no disturbance in the FGF1-nucleolin interaction (FGF1 L147A). All of these FGF1 mutants could be phosphorylated by PKCδ in an in vitro assay (Figure 4C). However, within U2OSR1 cells, no phosphorylation of the mutants, except the control variant, could be detected, although their nuclear import was comparable to that of wild type FGF1 (Figure 4D). This result suggests that an interaction between nucleolin and FGF1 is necessary for intracellular phosphorylation of FGF1 by PKCδ.

We have previously observed that when cells are treated with thapsigargin, a drug that can be used to inhibit nuclear import of proteins, translocated FGF1 is phosphorylated in the cytosol, due to inhibited nuclear import of FGF1 as well as PKCδ [21,27]. We therefore analyzed if FGF1 could be phosphorylated in the cytosol in nucleolin depleted cells in the presence of thapsigargin. As shown in Figure 4E, nucleolin depletion abolished phosphorylation of FGF1, while with the concomitant treatment with thapsigargin phosphorylated FGF1 could be detected in the cytoplasmic fraction. This finding corroborates our previous result that the activity of PKCδ is unaffected by nucleolin depletion while it also indicates that the regulatory role of nucleolin in the process of PKCδ mediated phosphorylation of FGF1 is restricted to a nuclear localization.

To test if nucleolin functions as an enhancer for the PKCδ mediated phosphorylation of FGF1, we studied the in vitro phosphorylation of FGF1 by PKCδ in the presence or absence of recombinant nucleolin, but we were unable to detect a stimulatory effect of nucleolin in phosphorylation of FGF1 (Figure S8). The role of nucleolin in PKCδ mediated phosphorylation of FGF1 thus appears to be specific for an intracellular and nuclear location.

**Nucleolin regulates nuclear export of FGF1**

Since nucleolin depletion abolished in vivo phosphorylation of FGF1, and phosphorylation regulates nuclear export of FGF1 [21], we investigated the nuclear export of FGF1 in nucleolin depleted cells. Previous studies have demonstrated that the amount of FGF1 in the nucleus reaches a peak about 6 h after the addition of FGF1 to the cell medium, and FGF1 is thereafter exported from the nucleus and degraded in the cytosol [21]. Scrambled siRNA treated and nucleolin depleted cells were incubated with FGF1 for 2-10 h and then the nuclear fractions were analyzed for FGF1. Figure 5A shows that a peak amount of nuclear FGF1 was observed at 6 h in scrambled treated cells, while the amount of nuclear FGF1 was reduced after 8 h and undetectable after 10 h, which is in accordance with previously published data [21]. In nucleolin depleted cells, the amount of nuclear FGF1 did not decline notably between 6 h and 10 h after FGF1 addition,
suggesting that nucleolin depletion inhibits nuclear export and thereby prolongs the localization of translocated FGF1 in the nucleus (Figure 5A).

To study in more detail the intracellular localization of FGF1 in nucleolin depleted cells we investigated the amount of FGF1 in membrane, cytosolic and nuclear fractions. Cells were treated with in vitro \[^{35}\text{S}\]methionine-labelled FGF1 (\[^{35}\text{S}-\text{FGF1}\]) for 6 or 10 h (Figure 5B). In scrambled treated cells and nucleolin depleted cells, FGF1 was found in the nucleus, as well as in the cytosol, after 6 h. In scrambled treated cells, no FGF1 was detected in the nucleus after 10 h, but some was still found in the cytosol, presumably due to the efficient export from the nucleus. In nucleolin depleted cells, on the other hand, FGF1 was located in the nuclear fraction and not in the cytosolic fraction after 10 h, indicating that nucleolin depletion inhibited FGF1 nuclear export. A similar effect was observed when the nuclear export was blocked by LMB, a specific inhibitor of exportin-1, or when the phosphorylation of FGF1 was inhibited by rottlerin, an inhibitor of PKC\(\delta\). When nuclear import was blocked by thapsigargin, translocated FGF1 was detected only in the cytosolic fraction (6 h and 10 h). These data indicate that nucleolin is a crucial factor for regulation of nuclear export of FGF1.

Similarly to FGF1, exogenous FGF2 can translocate into the nucleus \([11]\), however, FGF2 does not contain a PKC\(\delta\) phosphorylation site and the mechanisms for its nuclear export is unknown. Since nucleolin also interacts with FGF2, we investigated if nucleolin plays a role in nuclear export of FGF2 as observed for FGF1. As can be seen in Figure 5C, the amount of nuclear FGF2 after incubation for 2-10 h is the same in nucleolin depleted cells as in scrambled treated cells. This indicates that nucleolin does not influence nuclear shuttling of FGF2 within the time frame studied.

**Nucleolin regulates nuclear export of FGF1 via phosphorylation**

To determine if nucleolin played a role in nuclear export of FGF1 by regulating its phosphorylation only, or if nucleolin was involved directly in nuclear trafficking, we studied the nuclear export of FGF1 S130E, a mutant which mimics FGF1 phosphorylated on serine 130 \([45]\). Cells depleted for nucleolin were stimulated with FGF1 S130E or wild type FGF1 for 6 h or 10 h and then fractionated into membrane, cytosolic, and nuclear fractions (Figure 6). Wild type FGF1 was found in the nuclear fraction and not in the cytosolic fraction after 6 h as well as 10 h in nucleolin depleted cells. However, the FGF1 S130E mutant was present in the cytosolic fraction and not in the nuclear fraction after 10 h, both in scrambled and nucleolin depleted cells, indicating that it was efficiently exported from the nucleus. This result indicates that nucleolin is not directly involved in the nuclear export of FGF1, but rather regulates FGF1 nuclear export via regulating its phosphorylation by PKC\(\delta\).

**Discussion**

In this study we show that FGF1 interacts with the nuclear protein nucleolin, and that nucleolin regulates nucleocytoplasmic trafficking of FGF1. We found that nucleolin is required for phosphorylation of FGF1 on serine 130 by PKC\(\delta\) in the nucleus. This phosphorylation is a key regulatory event for nuclear export of FGF1 and nucleolin is thus a crucial regulator of FGF1 nuclear export.

The ability of exogenous FGF1 to translocate into the nucleus was discovered more than two decades ago \([19,46]\), while the mechanism for translocation and the intracellular action of FGF1 are continuously being investigated \([12]\). To elucidate the role of nuclear FGF1 we aimed to identify intracellular targets for FGF1 by mass spectrometry-based proteomic studies. We identified nucleolin as an interaction partner of
FGF1 and confirmed the direct in vitro binding between FGF1, and also FGF2, and nucleolin by SPR technique. The SPR kinetic studies displayed a complex interaction suggesting two parallel binding processes between FGF1/FGF2 and nucleolin. The $K_D$ was estimated to in the range from $4.0 \times 10^{-8}$ M to $4.8 \times 10^{-7}$ M for FGF1 and in the range from $2.2 \times 10^{-8}$ M to $1.3 \times 10^{-7}$ M for FGF2. FGF2 has previously been suggested to interact with nucleolin [34,47], but the direct FGF2-nucleolin and FGF1-nucleolin interactions are for the first time presented here.

Bioinformatic analysis enabled us to obtain 17 FGF1 variants with mutated putative interaction sites. SPR analysis revealed that most of them did not exhibit any significant change in nucleolin binding. However, four amino acid residues were found to be involved in nucleolin binding, K126, K127, R136 and K142. These are located at the heparin-binding site, indicating a dual function of the positively charged patch on the FGF1 molecule. This suggests that the heparin-binding sites in FGF1 responsible for heparin interaction outside the cell are engaged again after translocation of FGF1 into the nucleus, then constituting a binding site for the intracellular protein nucleolin. As FGF1 is a relatively small protein, our finding points to a great economy of the FGF1 structure. Interestingly, nucleolin has previously been shown to bind to the heparin-binding site on endostatin which leads to internalization of endostatin and stimulation of antiangiogenic activities [48].

Nucleolin is a multifunctional protein implicated in a variety of cellular processes including ribosome biogenesis, proliferation, and differentiation [29,44,49]. Also, nucleolin has been found to regulate nucleocytoplasmic shuttling of several proteins including endostatin [33], lactoferrin [32], transforming growth factor β receptor [30], and the US11 protein of Herpes Simplex Virus 1 [31], as well as ribosomal proteins and subunits [29]. We therefore studied the uptake and intracellular trafficking of exogenous FGF1 in nucleolin depleted cells. We found that the intranuclear phosphorylation of FGF1 was abolished and that the nuclear export of FGF1 was severely impaired. Nucleolin is an essential protein in the cell and inhibition or depletion of nucleolin has previously been shown to lead to cell cycle arrest, defects in centrosome duplication and nucleolar disruption [50]. Therefore, as suggested before, we studied the role of nucleolin in cells only partially depleted for nucleolin [51]. These partially depleted cells appeared viable, and although the effect on FGF1 phosphorylation was severe, the nucleolin depletion had no apparent effect on the endocytic uptake of FGF1 or the translocation of FGF1 from endosomes into the cytosol and nucleus. Nucleolin depletion had also no detectable effect on the activity or the nuclear import of PKCδ, suggesting that nucleolin rather has a direct role in facilitating PKCδ-mediated phosphorylation of FGF1. One possibility is that FGF1 requires binding to nucleolin in order to be a substrate for PKCδ. To verify if this was the case, we tested FGF1 mutants that had a reduced or abolished binding to nucleolin (in vitro) and found that they were not phosphorylated intracellularly, although their nuclear import was comparable to that of wild type FGF1, and they could be phosphorylated by PKCδ in vitro. Furthermore, we found that wild type FGF1 could be phosphorylated in nucleolin depleted cells when the cells were treated with thapsigargin, a drug that inhibits nuclear import of FGF1 as well as PKCδ and thereby allows for PKCδ-FGF1 interaction in the cytosol. We therefore hypothesize that nucleolin stabilizes the interaction between FGF1 and PKCδ, and that this action is specific for a nuclear location. Possibly, nucleolin acts as a scaffolding protein and may induce a conformational change in the FGF1 molecule, allowing S130 of FGF1 to be exposed and available for the action of PKCδ.

Phosphorylation of FGF1 alters its conformation leading to exposure of a functional leucine-rich type NES at the C-terminus, and thereby regulates nuclear export of FGF1 [22]. We found that in nucleolin depleted cells, FGF1 remained in the
nucleus several hours longer than in control cells, indicating a requirement of nucleolin for nuclear export of FGF1. By stimulating nucleolin depleted cells with an FGF1 mutant which mimics FGF1 phosphorylated on serine 130 (FGF1 S130E), we could distinguish if nucleolin was only involved in phosphorylation of FGF1 or if it was involved directly in the nuclear export process. We found that nucleolin depletion did not significantly inhibit nuclear export of FGF1 S130E, indicating that nucleolin controls FGF1 nuclear export mainly via regulating its phosphorylation by PKCδ. Although nucleolin was found to interact with FGF2 as well as FGF1, we did not observe any change in the nucleocytoplasmic trafficking of exogenous FGF2 upon nucleolin depletion. Unlike FGF1, translocated FGF2 binds to the protein translokin, which is important for nuclear import of FGF2 [52,53]. In the nucleus FGF2 can bind and stimulate CK2, which induces phosphorylation of nucleolin [34]. Nuclear FGF2 can also interact with the transcription factor Upstream Binding Factor (UBF), and directly regulate rRNA transcription [47]. Possibly, the lack of nucleolin mediated trafficking of FGF2 is due to the lack of a PKCδ phosphorylation site on FGF2.

Nucleolin consists of three functional domains, an N-terminal domain with several acidic stretches, a C-terminal domain rich in glycine/arginine, and a central domain containing two to four RNA recognition motifs [49]. Further, nucleolin goes through several post translational modifications which targets nucleolin to different cellular compartments where it carries out different functions [29]. We showed here that FGF1 interacts with a fragment of nucleolin consisting of residues 284-707, which constitutes the C-terminal domain and the four RNA binding motifs [37]. Nucleolin has been shown to interact with several other proteins including ErbB4 receptor [54], Hdm2 [55], ribosomal protein L26 [56], and p53 [57], reflecting its many functions in the cell. The interaction between FGF1 and nucleolin suggests that FGF1 could regulate the functions of nucleolin or vice versa. Interestingly, both nucleolin and nuclear FGF1 have been implicated in regulation of p53. Nucleolin is involved in translational regulation of p53 mRNA [58], and stabilization of the p53-protein [55,59], while nuclear FGF1 protects cells from p53-regulated apoptosis [5,6]. Therefore it is possible that the interaction of FGF1 with nucleolin is involved in the anti-apoptotic function of translocated FGF1. FGF1 mutants defective in nucleolin binding are not phosphorylated and exported from the nucleus, and therefore may act longer by a potential nuclear pathway to protect the cells from apoptosis. More studies are necessary to clarify the biological role of FGF1 in the nucleus and how the roles of FGF1 and nucleolin intersect. Nevertheless, nuclear export of FGF1 is probably important in the regulation of its intracellular activity and the data presented here clearly implicate nucleolin as a regulator of the nuclear export of FGF1.

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Reference List


Figure legends

Figure 1. Nucleolin binds to FGF1 and FGF2. A) Proteins from cleared cell lysates (BJ or NIH3T3 cells) were pulled down by SBP-FGF1, Biot-FGF2, His-FGF1, GST-FGF1, GST-FGF2, GST protein, or no protein (-), and respective resins (Streptavidin-coated Dynabeads, Ni-NTA Superflow or Glutathione Sepharose™ 4 Fast Flow). Protein complexes were subjected to SDS-PAGE and immunoblotting (IB) with an anti-nucleolin antibody. B) HEK 293 cells transiently transfected with myc-FGF1 were lysed and then subjected to immunoprecipitation followed by SDS-PAGE and immunoblotting (IB) with an anti-nucleolin antibody. Non-transfected cells were used as controls. C) Recombinant nucleolin-C was incubated with recombinant SBP-FGF1 or Biot-FGF2 and Streptavidin-coated Dynabeads, then washed and analyzed by IB with an anti-nucleolin antibody. D) SPR analysis was performed using nucleolin-C immobilized on a CM4 sensor chip at the level of ~540 RU and recombinant FGF1 or FGF2 were injected as analyte on the chip at increasing concentrations (10 nM to 320 nM) in duplicates (solid lines). Running buffer was injected in duplicates as a control (dashed lines). E) Interaction Maps from kinetic SPR data obtained for FGF1 and FGF2 binding to nucleolin-C. The contribution of a particular ka or kd in the overall binding event is shown by colors, from black (no contribution) to red (strong contribution).

Figure 2. The positively charged heparin-binding site of FGF1 is responsible for binding to nucleolin. A) Biot-FGF1 was incubated with recombinant nucleolin-C and Streptavidin-coated Dynabeads in the presence or absence of heparin, then washed and analyzed by SDS-PAGE and immunoblotting with an anti-nucleolin antibody. B) SPR analysis of the binding of FGF1 mutants (K126A/K127A, R136E, K142E and L147A) to nucleolin-C immobilized on a CM5 sensor chip at the level of ~5790 RU. FGF1 wild-type or its variants were injected as analytes at a concentration of 654 nM.

Figure 3. Nucleolin is not involved in nuclear import of exogenous FGF1. U2OSR1 cells were transfected with siRNA against nucleolin (ncl) or LRRC59 (lrrc59) or scrambled (scr) RNA or left non-transfected (-). The cells were serum starved for 24 h and incubated with 100 ng/ml FGF1 and 10 U/ml heparin, and in one case also 10 nM BafA1, for 6 h. The cells were washed, lysed, and fractionated into a cytoplasmic fraction (CP) and a nuclear fraction (N). FGF1 was extracted from both fractions by adsorption to Heparin-Sepharose and analyzed by SDS-PAGE and immunoblotting (IB) with anti-FGF1. The cellular fractions were also analyzed for nucleolin, HSP90, and lamin A by IB as indicated.

Figure 4. Nucleolin is required for intracellular phosphorylation of FGF1. A) U2OSR1 cells were transfected with siRNA as indicated, serum starved for 24 h and labelled by [33P]phosphate, and thereafter stimulated with 100 ng/ml unlabelled, recombinant FGF1 in the presence of 10 U/ml heparin, and 10 nM BafA1 where indicated, for 6 h. FGF1 was extracted from total cell lysates by adsorption to Heparin-Sepharose and analyzed by SDS PAGE and fluorography to detect in vivo phosphorylation, and immunoblotting (IB) to detect FGF1. The lysates were also analyzed for nucleolin and HSP90 by IB. B) U2OSR1 cells transfected with siRNA as indicated were serum starved for 24 h, stimulated with FGF1 and heparin for the indicated time and then lysed and fractionated into a cytoplasmic (CP) and a nuclear (N) fraction before analysis by SDS-PAGE and IB for phospho-PKCδ and total PKCδ. The fractions were also analyzed for nucleolin, lamin A, and ERK1/2 by IB. C) Recombinant FGF1 or FGF1 mutants as indicated, were incubated with recombinant, active PKCδ and [γ-32P]ATP and thereafter analyzed for in vitro phosphorylation by fluorography, and IB to show loading. D) U2OSR1 cells were serum starved and labelled with [33P]phosphate and stimulated with FGF1 or FGF1 mutants as indicated for 6 h. The cells were
fractionated into cytoplasmic and nuclear fractions. FGFs were extracted from the fractions by binding to Heparin-Sepharose and analyzed by fluorography to detect \textit{in vivo} phosphorylation and immunoblotting (IB) to detect FGFs. Fractions were also analyzed for marker proteins by IB as indicated. E) U2OSR1 cells were transfected with siRNA as indicated, serum starved for 24 h and labelled with $[^{33}\text{P}]$phosphate and stimulated with FGF1 in the absence or presence of 1 μg/ml thapsigargin. The cells were fractionated and analyzed as described in (D).

**Figure 5. Nucleolin is required for nuclear export of FGF1, but does not influence nuclear localization of FGF2.** A) U2OSR1 cells were transfected with siRNA as indicated, serum starved for 24 h, and incubated with 100 ng/ml FGF1 and 10 U/ml heparin for 2, 4, 6, 8 and 10 h, and also 10 nM BafA1 where indicated. The cells were lysed and fractionated into a cytoplasmic fraction (CP) and a nuclear fraction (N), and FGF1 was extracted from the fractions by adsorption to Heparin-Sepharose and analyzed by SDS-PAGE and immunoblotting (IB) with anti-FGF1. The cellular fractions were also analyzed for nucleolin, GAPDH, and lamin A by IB as indicated. B) U2OSR1 cells were transfected with siRNA as indicated, and serum starved for 24 h before stimulation with $^{35}\text{S}$-FGF1 for 6 h (left panel) or 10 h (right panel) in the absence or presence of 10 nM BafA1, 5 ng/ml LMB, 10 μM rottlerin or 1 μg/ml thapsigargin, as indicated. The cells were fractionated into membrane (M), cytosolic (C) and nuclear (N) fractions. $^{35}\text{S}$-FGF1 was extracted from all fractions by adsorption to Heparin-Sepharose and analyzed by SDS-PAGE and fluorography. The fractions were also analyzed for nucleolin, ERK1/2 and lamin A by IB. C) The experiment was performed as in (A) except the cells were stimulated with 100 ng/ml FGF2 instead of FGF1.

**Figure 6. Nucleolin regulates nuclear export of FGF1 via phosphorylation.** U2OSR1 cells were transfected with siRNA as indicated, and serum starved for 24 h before stimulation for 6 h (left panel) or 10 h (right panel) with 100 ng/ml FGF1 (wt) or 100 ng/ml FGF1 S130E and 10 U/ml heparin. Cells were also treated with 10 nM BafA1 or 5 ng/ml LMB, where indicated. The cells were fractionated into membrane (M), cytosolic (C) and nuclear (N) fractions, and FGFs were extracted from the fractions by adsorption to Heparin-Sepharose and analyzed by SDS-PAGE and immunoblotting (IB). The fractions were also analyzed for nucleolin, γ-tubulin and lamin A by IB, as indicated.

Supporting Information Legends

Table S1. Kinetic parameters of FGF1 and FGF2 binding to recombinant nucleolin-C.

Figure S1. Coomassie stained SDS-PAGE gel of FGF1-interacting proteins.

Figure S2. Comparison of truncated form of FGF1 and full length FGF1.

Figure S3. SPR shows that heparin prevents FGF1 binding to nucleolin.

Figure S4. Mutational analysis of FGF1 binding to nucleolin.

Figure S5. Elution profiles of FGF1.

Figure S6. Non-overlapping siRNA sequences against nucleolin inhibits phosphorylation of FGF1 by PKCδ.
Figure S7. Nucleolin is required for intracellular phosphorylation of full length and truncated forms of FGF1.

Figure S8. Nucleolin does not influence phosphorylation of FGF1 by PKCδ in vitro.
Figure 1

A

BJ cells

IB: nucleolin

NIH 3T3 cells

IB: nucleolin

B

IB: nucleolin

C

IB: nucleolin

D

FGF1-nucleolin

EGF2-nucleolin

E

FGF1-nucleolin Interaction Map

FGF2-nucleolin Interaction Map
Figure 2

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B

- FGF1 WT
- FGF1 L147A
- FGF1 K142E
- FGF1 K126A/K127A
- FGF1 R136E

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**IB: FGF1**

**CP**

**IB: nucleolin**

**CP**

**IB: HSP90**

**N**

**IB: FGF1**

**N**

**IB: lamin A**

**N**
### Figure 5

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**Western Blots**

- **IB: FGF1**
- **IB: nucleolin**
- **γ-tubulin**
- **IB: FGF1**
- **IB: lamin A**

Legend:
- M
- C
- N
Figure S1. Coomassie stained SDS-PAGE gel of FGF1-interacting proteins isolated from affinity pull-down assay using NIH3T3 cell lysate and recombinant FGF1 tagged with streptavidin binding peptide (SBP-FGF1) as a bait. As a negative control NIH3T3 cell lysate was incubated with Streptavidin-coated Dynabeads. Nucleolin was identified by MS analysis in the gel fragment marked by rectangle box.
Figure S2. Comparison of truncated form of FGF1 (short, 21-154) and full length FGF1 (long, 1-154). (A) FGF1-induced cell proliferation of NIH 3T3 cells. AlamarBlue reagent was added to serum-starved cells that had been stimulated for 48 h with indicated concentrations of FGF1 (truncated (amino acids Ala-21-154) and full length (amino acids 1-154) in the presence of heparin (10 U/ml). The fluorescence corresponding to the number of cells was measured using EnVision multimode plate reader (PerkinElmer). The graph represents the mean ± SEM of four independent experiments. (B) FGF1 binding to nucleolin. SPR analysis was performed using nucleolin-C immobilized on a CM4 sensor chip at the level of ~540 RU and recombinant FGF1 was injected as analyte on the chip at 200 nM concentration. Running buffer was injected as a control.
Figure S3. SPR shows that heparin prevents FGF1 binding to nucleolin. Nucleolin-C was immobilized on a CM4 chip at the level of ~ 540 RU. FGF1 was injected as an analyte at 50 nM concentration, in the absence or presence of heparin at a concentration of 76.4 U/ml. The applied heparin concentration was calculated to keep the heparin:FGF1 ratio of 10 U heparin per 100 ng FGF1, as used in the experiments performed on cells.
Figure S4. Mutational analysis of FGF1 binding to nucleolin. A) FGF1 residues potentially involved in binding with protein partners identified by bioinformatic analysis using the following web servers: meta-PPISP [36], ConSurf [37] and SWAKK [38]. Residues of FGF1 involved in nucleolin binding are marked with red. B) SPR measurements of interaction between 17 FGF1 mutants and the C-terminal fragment of nucleolin. Nucleolin-C was immobilized on a CM sensor chip at the level of ~5790 RU. Wild-type FGF1, as well as its mutational variants, were injected on the chip as analytes at concentrations of 654 nM.
Figure S5. Elution profiles of FGF1 and its mutants from a Heparin-Sepharose column with a linear NaCl gradient.
Figure S6. Non-overlapping siRNA sequences against nucleolin inhibits phosphorylation of FGF1 by PKCδ. U2OSR1 cells were transfected with siRNA against nucleolin obtained from Santa Cruz Biotechnology (sc-29230) (2ncl) or Qiagen (SI02654925) (1ncl), as indicated (non-overlapping sequences), serum starved for 24 h and labelled by $[^{33}\text{P}]$phosphate, and thereafter stimulated with 100 ng/ml unlabelled, recombinant FGF1 in the presence of 10 U/ml heparin, and 10 nM BafA1 or 10 U/ml heparin alone, where indicated, for 6 h. The cells were fractionated into cytoplasmic (CP) and nuclear (N) fractions. FGF1 were extracted from the fractions by binding to Heparin-Sepharose and analyzed for phosphorylated FGF1 ($^{33}\text{P}$-FGF1) by SDS-PAGE and fluorography, and immunoblotting (IB) to detect total FGF1. Fractions were also analyzed for marker proteins by IB as indicated.
Figure S7. Nucleolin is required for intracellular phosphorylation of full length and truncated forms of FGF1. U2OSR1 cells were transfected with siRNA as indicated, serum starved for 24 h and labelled by \[^{33}\text{P}\]\text{phosphate}, and thereafter stimulated with 100 ng/ml unlabelled, recombinant FGF1, either full-length (long, l-FGF1) or the truncated version (short, s-FGF1), in the presence of 10 U/ml heparin for 6 h. 10 nM BafA1 was added where indicated. The cells were lysed and fractionated into cytoplasmic (CP) and nuclear (N) fractions. Samples of the fractions were analysed by SDS-PAGE and immunoblotting (IB) as indicated. A sample of l-FGF1 and s-FGF1 was loaded directly on each gel (10 ng each on the CP gel and 5 ng each on the N gel, in lanes to the right) to indicate their difference in molecular weight/migration in SDS-PAGE. For the remainder of CP and N fractions, FGF1 was extracted by adsorption to Heparin-Sepharose and analyzed by SDS-PAGE and fluorography to detect the \textit{in vivo} phosphorylation of FGF1 (\[^{33}\text{P}\]-FGF1). As can be discerned by their mobility in SDS-PAGE, only the short form of FGF1 is detectible in the nucleus and as a phosphorylated protein, indicating that the full-length FGF1 was truncated (by a BafA1 sensitive process) before translocation into the nucleus.
Figure S8. Nucleolin does not influence phosphorylation of FGF1 by PKCδ in vitro. Mixtures of varying amounts of recombinant nucleolin-C, FGF1, bovine serum albumin (BSA), and active PKCδ, as indicated, were incubated with [γ-33P]ATP for 30 min at 30°C. The protein mixtures were then analyzed by SDS-PAGE and fluorography to detect phosphorylated proteins that were identified by their molecular weight.