Liprin-α1 in cancer cell adhesion machinery and tumor progression

Henna Pehkonen

Research Programs Unit and Medicum, Faculty of Medicine, Doctoral Programme in Biomedicine
University of Helsinki
Finland

Academic Dissertation

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**Supervisor**

Docent Outi Monni, Ph.D.
Research Programs Unit and Medicum, Faculty of Medicine
University of Helsinki
Finland

**Pre-examiners appointed by the Faculty**

Professor John Eriksson, Ph.D.  
Associate Professor Vesa Hütönen, Ph.D.
Turku Center for Biotechnology  
Faculty of Medicine and Health Technology
University of Turku  
University of Tampere
Finland  
Finland

**Opponent appointed by the Faculty**

Professor Ivan De Curtis, Ph.D.
San Raffaele Scientific Institute and
University Vita-Salute, Milan
Italy


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To my family
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ABSTRACT

The main purpose of my thesis was to study the function of liprin-α1 protein in adhesion and tumor cell progression in head and neck squamous cell carcinoma and breast cancer. Liprin-α1 is encoded by PPFIA1 which is located at the 11q13 region. 11q13 region is frequently amplified in several cancers and it often leads to poor prognosis of the patients. Liprin-α1 belongs to the family of LAR protein tyrosine phosphatase-interacting proteins (liprins). Liprin-α1 is related to cell spreading, motility and invasion, and is involved in the regulation of focal adhesion signaling and disassembly. Previous results have shown controversy in the role of liprin-α1 in tumor migration and progression in epithelial cancer cells. Therefore, the aim of my PhD project was to clarify the cellular functions of liprin-α1 in cancer progression to improve our understanding of the role of liprin-α1 in the biology of head and neck and breast cancer. We first studied cellular localization of liprin-α1 in primary and metastatic cancer cell lines. Interestingly, liprin-α1 localized to different adhesion and cytoskeletal structures depending on the invasiveness of the cells. In cell lines originating from primary tumors, liprin-α1 localized to adhesion rings of invadosomes, whereas in metastatic cell lines, liprin-α1 was mostly located near the leading edge or at the lamellipodia together with adhesion proteins. Invadosomes were able to degrade the extracellular matrix, which is important in cancer cell progression. Liprin-α1 influenced the invasive growth properties of the cancer cells depending on their migratory capabilities. In invasive breast cancer cells, liprin-α1 had a significant impact on mesenchymal cancer cell invasion and the formation of outgrowths in three-dimensional collagen. In non-invasive cells without 11q13 amplification and where liprin-α1 was located within the invadosome structures, liprin-α1 did not have an effect on cell invasive growth. On the other hand, in the 11q13 amplified cells liprin-α1 knockdown inhibited cohesive growth behavior in cells with limited invasive capacity. We showed that liprin-α1 contributes to cancer cell invasion by regulating intermediate filament vimentin, thereby contributing to cytoskeletal organization as well as to cellular structures related to adhesion in epithelial cancers. These data indicate that the effect of liprin-α1 in cancer progression and invasion is context- and cell type-
dependent. The aim of the second part of the thesis was to identify cellular pathways modulated by liprin-α1 and the structures where liprin-α1 is located. RNA sequencing and gene set enrichment analysis after liprin-α1 silencing revealed several pathways important in cancer cell signaling including the regulation of membrane microdomains, the positive regulation of cell death and the regulation of anchoring junctions. Most importantly, liprin-α1 knockdown led to upregulation of CD82 transmembrane protein, which is a well-known metastasis suppressor in several cancer types, and a correlation of high liprin-α1 and low CD82 expression on a tissue microarray of oral squamous cell carcinomas supported these results. The interplay between liprin-α1 and CD82 was verified by knocking down CD82 in shPPFA1 HNSCC cells, which resulted in enhanced invasive properties, partly restoring the invasive growth capacity of the cells. To study the potential role of liprin-α1 as a drug response modulator due to its importance in regulating cancer cell invasive growth, a drug screen of 527 anticancer compounds was carried out in cells with liprin-α1 silencing. Liprin-α1 sensitized cells to MEK inhibitors and drugs regulating cell cycle or metastatic growth of the cells. Taken together, my thesis show that liprin-α1 is an important player in epithelial cancer cell invasion and its effect in cancer progression and cell invasive growth is context and cell type-dependent. In addition, thesis provides insight into the liprin-α1 mediated cell signaling pathways related to adhesion and cytoskeletal elements. Furthermore, as liprin-α1 has an important role in cancer cell adhesion and invasion, it may be an indicator for several drugs that inhibit targets contributing to the metastatic growth of cancer cells.
YHTEENVETO

ABBREVIATIONS

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP2 – activating protein 2
ATP – adenosine triphosphate
BFB – breakage-fusion-bridge
CAMKII – Ca²⁺/calmodulin-dependent protein kinase II
CASK – calcium/calmodulin dependent serine protein kinase
CD82 – CD82 Antigen, Metastasis Suppressor Kangai-1
Cdc42 – Cell Division Cycle 42
DBM – destruction box motif
DNA – deoxyribonucleic acid
DRCR – double rolling-circle replication
DSS – drug sensitivity score
ECM – extracellular matrix
EEA1 – early endosome antigen 1
EGF – epidermal growth factor
EGFR – epidermal growth factor receptor
ERC1 – ELKS-Rab6-interacting protein-CASK
FA – focal adhesion
FAK – focal adhesion kinase
FoSTeS – fork stalling and template switching
GIT1 – G Protein-Coupled Receptor Kinase Interacting ArfGAP 1
GRIP – glutamate receptor interacting protein
GSEA – gene set enrichment analysis
GTPase – a large family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP)
HER2 - human epidermal growth factor receptor 2
HNSCC – head and neck squamous cell carcinoma
HPV – human papilloma virus
IE - intractable epilepsy
IF – intermediate filaments
ING4 – inhibitor of growth 4
JunB – transcription factor Jun-B
LAR – leukocyte antigen protein tyrosine phosphatase
LH – liprin homology
LL5 – pleckstrin homology like domain family B member 1 and 2
MMP – matrix metalloproteinase
MTT – (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
OPSCC - oropharyngeal squamous cell carcinoma
p53 – tumor protein p53
PDZ – PDZ binding sequence
PPFIA1 - PTPRF Interacting Protein Alpha 1: Protein Tyrosine Phosphatase, Receptor Type, F Polypeptide (PTPRF), Interacting Protein (Liprin), Alpha 1
PTPase – protein tyrosine phosphatase
Rac1 – Rac Family Small GTPase 1
Rho – family of GTPases
RIM – Regulating Synaptic Membrane Exocytosis 1
Rock – Rho-associated protein kinase
RNA – ribonucleic acid
RT – radiotherapy
SAM – sterile alpha motif
SCCHN – squamous cell carcinoma of the head and neck
scRNAseq - single cell RNA sequencing
Src – proto-oncogene tyrosine-protein kinase
syd-2 – synapse-defective-2
TAM - tumour associated macrophage
TGF-β – transforming growth factor beta
TLE - intractable temporal lobe epilepsy
TNFα – tumor necrosis factor alpha
UICC - The Union for International Cancer Control's
UPS – ubiquitin-proteasome system
VEGF – vascular endothelial growth factor
2D – two dimensional
3D – three dimensional
5'FU – fluorourasil
1. LIST OF ORIGINAL PUBLICATIONS

This thesis work consists of the following original articles.


2. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) and breast cancer can be life-threatening conditions if not diagnosed at the early stages of the disease. It is crucial and important to study molecular background of these malignancies to gain insight into how these tumors arise and develop. Genetic aberrations, including mutations and structural alterations, are common in cancer and they accumulate during the development of tumor into severe disease. Gene amplifications, which often lead to overexpression of growth-promoting genes, commonly occur in the development of cancer. One of the most commonly amplified region is 11q13, which often leads to poor prognosis of cancer patients. The 11q13 region contains genes with known functions to cell cycle and invasion, but the role of many genes in cancer progression has not been studied in detail. One of these genes is PPFIA1, which is located at the core of the 11q13 region. PPFIA1 encodes the liprin-α1 protein, and has been described as an adhesion and invasion related protein with cellular functions in neuronal and non-neuronal cells.

Adhesion and invasion are important factors in tumor cell progression. Tumor cells use adhesive and invasive structures to attach to the surrounding environment and to pass through the extracellular matrix to invade into the surrounding tissue or environment. Adhesive and invasive structures consist of large amount of proteins, and several factors are involved in the stability and composition of these dynamic structures called focal adhesions and invadosomes. Crucial factors for the formation of adhesive or invasive structures include the tumor cell environment and composition, matrix availability and mechanosensing of the environment. These aspects have an impact on how cell-cell contacts and cell-extracellular matrix contacts are developed.

In this study, a number of molecular biology methods and in vitro cell models were applied to investigate the role of liprin-α1 in cancer progression and processes related to cell adhesion and invasion. Whole transcriptome analysis was utilized to identify liprin-α1 mediated regulators of tumor progression and cell invasive growth. By using multiple approaches in molecular biology and applied tumor genomics we provide novel information about molecular mechanisms by which liprin-α1 contributes to tumor progression and invasion.
3. REVIEW OF THE LITERATURE

3.1 Head and neck squamous cell carcinoma (HNSCC)

3.1.1 Epidemiology and clinical features

Head and neck squamous cell carcinoma (HNSCC) comprises the sixth most common malignancy worldwide\(^27\) (Cancer Facts and Figures 2012, American Cancer Society), and consists of a heterogeneous group of cancers arising from the upper aerodigestive tract\(^1\). In Finland, the number of new cases per year is around 800\(^28\). The aerodigestive tract has crucial roles in respiration, speech and swallowing\(^29\). HNSCC originates from the nasal cavity, paranasal sinuses, oral cavity, tongue, salivary glands, pharynx and larynx, and the pharynx includes the nasopharynx, oropharynx and hypopharynx\(^27,30\). The different regions of head and neck cancer are illustrated in Figure 1. The five-year survival rate of HNSCC depends on the anatomical site of the cancer\(^27,31,32\) which has implications on the prognosis, survival and the choice of treatment\(^27\). Treatments commonly deteriorate the life quality of the patients, and thus it is crucial to identify the anatomical site of origin\(^27\). Staging of oral cancer is done according to the classification system of The Union for International Cancer Control's (UICC) (Table 1), where T defines how deep into the tissue the tumor has grown, N is whether the tumor has spread to the lymph nodes and M describes whether the tumor formed a metastasis\(^33,34\). Advanced metastatic spreading of the disease to lymph nodes complicates better survival\(^35\). HNSCC can be divided into HPV negative and positive cancers\(^36,37\). These two groups differ by the survival expectations for patients\(^38-40\), as well as in molecular profile\(^41-44\). The HPV negative group\(^36\) has a less favorable survival status, whereas the prognosis for HPV positive cases is better\(^38\). Precancerous lesions, such as leukoplakias or erythroplakia\(^45\), may occur before invasive cancer. In general, HNSCC is thought to arise from several precancerous genetically altered cells in a process called “field cancerization”, with a high risk of developing into premalignant and malignant lesions\(^46\).
**Table 1.** Definition of TNM classification. T=tumor, N=node, M=metastasis; the stages in HNSCC, table adapted and modified from Sobin *et al.*, 1997, Brierley *et al.*, 2017 33,34.

<table>
<thead>
<tr>
<th>Stage</th>
<th>TNM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>T1N0M0</td>
</tr>
<tr>
<td>Stage II</td>
<td>T2N0-1M0, T1N1M0</td>
</tr>
<tr>
<td>Stage III</td>
<td>T3N0-2M0, T1-2N2M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>T4N0-2M0, any T N3M0, any T any N M1</td>
</tr>
</tbody>
</table>

**Figure 1.** Anatomical sites of head and neck area where cancers can arise. These include parasanal sinuses, nasal cavity, oral cavity, tongue, salivary glands, larynx and pharynx. Pharynx can be divided into nasopharynx, oropharynx, and hypopharynx27. Simplified illustration adapted and modified from the images at http://www.nhsctcancerservices.hscni.net/head-and-neck/ (Cancer Research UK) and https://training.seer.cancer.gov/head-neck/anatomy/overview.html (National Cancer Institute).

### 3.1.2 Molecular genetic and epigenetic changes in HNSCC

Several genetic aberrations are related to the development of HNSCC, and the earliest model for HNSCC development was introduced in the 1990’s47. The initial stages of cancer development include loss of heterozygosity at 3p, 9p and 17p, whereas gains in 11q, 4q and 8p occur in the more advanced stages of
tumor progression\textsuperscript{47}. TP\textsubscript{53} mutations together with other genetic abnormalities promote progression of HNSCC \textsuperscript{48-51}. It is thought that cancer initiation can begin with mutations occurring in a single stem cell in cancer and in HNSCC \textsuperscript{52-54}. Stem cells typically stay for a longer time in the tissue, where they self-renew and give rise to progenitors and differentiated cells. It has been suggested that the longer a cell persists in a tissue, the higher the likelihood that this cell will accumulate the necessary mutations required to become tumorigenic\textsuperscript{54}. A commonly amplified gene in HNSCC is epidermal growth factor receptor (\textit{EGFR}), and it is commonly considered as a driver gene\textsuperscript{36,51}. Gene amplifications are discussed in more detail later in the text (Chapter 6). In addition, epigenetic changes influence cancer progression, and different methylation subtypes have been characterized for HNSCC. For example, the genes that are silenced by hypermethylation in OPSCC are related to signaling pathways including apoptosis, DNA repair, cell cycle and WNT signaling\textsuperscript{55}. In HNSCC, several molecular genetic events lead to either inactivation of tumour suppressor genes or activation of proto-oncogenes (Figure 2)\textsuperscript{51}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{genomic_instability.png}
\caption{Adapted and modified from references (Califano, \textit{et al.}, 1996, Argris \textit{et al.}, 2008, Perez-Ordonez \textit{et al.},2006) \textsuperscript{47,51,56}. In the progression of head and neck cancer, different genetic alterations occur. 11q13 amplification can be seen in the later stages of the carcinoma progression.}
\end{figure}
3.1.3 Risk factors and treatment in HNSCC

The most common risk factors for HNSCC include tobacco smoking and alcohol consumption for cancers originating from the oral cavity, oropharynx, hypopharynx and larynx. HPV infections are a common risk factor for certain subtypes of HNSCC. Head and neck cancers originating from the tonsil have the highest association with HPV, while cancers arising from the rest of the oropharynx have a milder association, and the oral cavity and larynx have the weakest association with HPV. High risk HPV types HPV16, which is the most common type, and HPV18 to a lesser extent are causal factors for HPV positive squamous cell carcinoma of the head and neck (SCCHN). In situ hybridization and p16 staining for immunohistochemistry are used for detection of HPV subtypes related to oncogenic transformation. HPV16 and HPV18 cause cancer through the viral oncoproteins E6 and E7 by inactivation of tumour suppressor proteins (p53, PRb). Gene expression profiles and chromosomal aberrations have shown differences between HPV negative and HPV positive cancers, and patients with HPV positive tumors typically have a better prognosis than those with HPV negative tumors. For the prevention of HNSCC in the future, it is important to reduce alcohol consumption and tobacco smoking, and HPV vaccinations are also thought to decrease the risk.

The treatment options for HNSCC include surgery, chemotherapy and radiotherapy (RT). Typically, the first and standard treatment choices for early or localized carcinoma are surgical operation or radiation therapy for the oral cavity, pharyngeal and laryngeal cancers. Commonly used chemotherapy agents for primary therapy for head and neck cancers with usually simultaneous radiation are cisplatin, carboplatin, and 5-FU. Immunotherapy is emerging as a promising and possible alternative treatment for HNSCC. Immune system alterations include among others molecular changes in immune checkpoint molecules with an increase in the proportion of immunosuppressive regulatory T cells, abnormal regulation of T cells, alterations in cytokines, myeloid dendritic cells (MDC) and decreasing the number of natural killer cells. Therapies that restore the activity of the immune system are under research for future treatments which include immune checkpoint inhibitors.
3.1.4 Intratumoural heterogeneity

A factor which poses challenges to the treatment of HNSCC is intratumoural heterogeneity, as this may lead to differential responses to therapeutic agents. Tumors have different genetically altered landscapes, and cancer stem cells may also be present in the cancer tissue. More data using single-cell transcriptomics will provide information on HNSCC heterogeneity, and mathematical models of intratumoural heterogeneity and its effect on clinical outcomes have been generated and explored. Intratumoural heterogeneity as well as the tumor microenvironment have effects on cell-cell and molecular interactions, and heterogeneity may be result of genetic change, or changes in environment. There is evidence that high intratumoural genetic heterogeneity is associated with poor clinical outcome in HNSCC patients.

4. Breast cancer

4.1 Breast cancer subtypes

Breast cancer is the most common cancer in women, but the overall mortality is decreasing due to improved mammographic screening and treatment. Breast cancer can be divided into different subtypes, luminal A (good prognosis) and luminal B (poor prognosis), HER2-positive, basal-like, and claudin-low subtype. In addition, additional subtypes based on molecular drivers have been characterized. HER2 cancers have an amplification of HER2 and are commonly highly proliferative, as basal-like cancers usually have poorer prognosis than luminal types. Gene expression profiling is used for the classification of breast cancer and it underlines the complexity of the disease. A previous study showed that several additional driver alterations in HER2-positive tumors add complexity to HER2-amplified cancer, where HER2 amplification is heterogeneously expressed. To improve patient treatment outcomes, subtyping classification based on gene expression has been used. Different breast cancer subtypes are considered to arise from various cells of origin. The breast epithelium forms a ductal network, and is composed of two different cell types, secretory luminal
and basal/myoepithelial cells\textsuperscript{111}. Stem cell properties are found in the subpopulation of basal epithelial cells that can give rise to the mammary epithelial network\textsuperscript{112,113}.

5. Tumour microenvironment

In general, tumor microenvironment includes multiple cell types such as cancer associated fibroblasts, immune and inflammatory cells, the blood and lymphatic vascular networks, adipose cells and neuroendocrine cells\textsuperscript{114}. In addition to different cell types, the extracellular matrix of tumour microenvironment is complicated containing cytokines, growth factors and hormones from tumour and stromal cells\textsuperscript{114}. The tumour microenvironment has a crucial role in the development of cancer as well as in the response to therapies\textsuperscript{115}. Gene expression profiles related to the microenvironment have shown its importance from a prognostic point of view\textsuperscript{116-118}. Cells related to immunity, immune cells\textsuperscript{119}, and cells called cancer–associated fibroblasts\textsuperscript{120} are the main classes of cells that can be found in the tumour microenvironment. Cells that have a positive effect on tumour development as well as metastasis are called tumour associated macrophages (TAMs), although specific immune cells can also have a negative effect on tumour progression\textsuperscript{121}. Gene expression signatures and the activation of specific cell signaling pathways are influenced by the environment and cell culture conditions. If cells are cultured in the three dimensional cell culture platform, contacts with other cells and the microenvironment are more essential and available as compared to the two-dimensional cell culture\textsuperscript{122,123}. In a previous study, 3D cell culture conditions slowed down the cell proliferation rate and viability of the cells and the expression of genes related to cellular metabolism differed between cells cultured in 2D and 3D environment\textsuperscript{124}. Because these methodological factors affect cell signaling, taking into account this variation is important to get the reliable overview of the gene expression patterns in a cell\textsuperscript{122,123}. In addition, intratumoural heterogeneity and individual characteristics of tumours in vivo poses additional challenge for studying cell signaling events in cancer cells\textsuperscript{99,114}.
6 Gene amplification in cancer

Gene amplification refers to an increase in copy number, which often leads to the overexpression of the genes that are amplified. Gene amplification is a common event in cancer and constitutes a defined region in a chromosome. The majority of the amplicons include several genes rather than just a single gene. The structure of an amplicon is often complex including several core regions. Although amplicons typically consist of several genes, only some of the genes provide a selective growth advantage to the cells. Amplicon drivers are gene or genes located in the amplicon that provide a growth advantage to cancer cells, and driver genes are typically both overexpressed and amplified. Methods, such as integrative genomic and transcriptomic profiling, have been able to identify such potential drivers.

6.1 Mechanisms of gene amplification

There are four hypotheses that have been proposed to explain the formation of amplicons: extrareplication and recombination, the breakage-fusion-bridge (BFB) cycle, double rolling-circle replication (DRCR), and replication fork stalling and template switching (FoSTeS). The most common mechanism, the breakage-fusion-bridge mechanism, initiates with the loss of a telomere and the fusion of sister chromatids. The formation of a dicentric chromosome leads to the formation of a bridge during anaphase, which then re-breaks, and as the cycle proceeds, the daughter cells may receive uneven chromatid. Thus, genes that were originally located in different sister chromatids result in the same chromatid.

6.2 11q13 amplification

It has been suggested that 11q13 amplification in HNSCC occurs following DNA breakage and sister chromatid fusion due to a break at the chromosomal fragile site FRA11F. In addition to 11q13 amplification in HNSCC, the loss of the 11q distal part of the chromosome may lead to more severe disease. Loss of distal 11q in HNSCC is suggested to lead to chromosomal instability, tumor cell progression, and resistance to cancer therapy. In addition to HNSCC, 11q13 amplification is a
common event in breast cancer\textsuperscript{11,126,129,146-152}, ovary\textsuperscript{153,154}, lung\textsuperscript{155}, skin (melanoma)\textsuperscript{156-163}, esophagus\textsuperscript{164-166}, pancreas\textsuperscript{167,168} and bladder cancer\textsuperscript{169,170}. 11q13 amplification includes a core of frequently amplified genes, such as \textit{PPFIA1}, \textit{CCND1} and \textit{CTTN}\textsuperscript{10,13,165,171,172}. The core region of 11q13 is shown in Figure 3. \textit{CCND1} encodes the cyclin D1 protein that promotes G1/S transition of the cell cycle\textsuperscript{171} and \textit{CTTN}\textsuperscript{165} which encodes the cortactin that contributes to tumor progression and invasion. One of the genes at the 11q13 amplification that have not been studied in detail include \textit{PPFIA1}, that encodes liprin-\textalpha protein. A previous study described 11q13 amplified genes including \textit{PPFIA1} as important genes for cancer progression\textsuperscript{173}. Another study showed that in metastatic OSCC, there was a high correlation between DNA copy number alteration at 11q13.2-11q13.3 and gene expression, and \textit{PPFIA1} was among the most significantly altered genes\textsuperscript{10}. Recently, a study evaluated the potential drivers associated with poor prognosis in advanced oropharyngeal squamous cell carcinomas (OPSCC)\textsuperscript{12}. Integrative analysis of gene copy number and expression identified 10 commonly altered genes in the 11q13 region to be cancer-related\textsuperscript{12}. \textit{PPFIA1} was among one of these genes, and study showed that in addition to HPV status, \textit{PPFIA1} overexpression was an independent prognostic marker\textsuperscript{12}.

| Genes | ND7/F18 | SH1A | EMAP3 | GAL | MTB1 | OPTIA | MPP5 | TPCK2 | W1052/CCND1 | ORAG5 | PG18 | PSYD | PFF1 | TME1 | MANO1 | PADO | PPFIA1 | CTHN | BAN2 | C2HC7 | NADYM | MDM1 |
|-------|---------|------|-------|-----|------|-------|------|-------|---------|-------|-------|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| UT-SCC-1 | | | | | | | | | | | | | | | | | | | | | | | | |
| UT-SCC-19 | | | | | | | | | | | | | | | | | | | | | | | | |
| UT-SCC-47 | | | | | | | | | | | | | | | | | | | | | | | | |
| UT-SCC-47 | | | | | | | | | | | | | | | | | | | | | | | | |
| UT-SCC-19B | | | | | | | | | | | | | | | | | | | | | | | | |
| UT-SCC-27 | | | | | | | | | | | | | | | | | | | | | | | | |
| SCC-4 | | | | | | | | | | | | | | | | | | | | | | | | |
| SCC-25 | | | | | | | | | | | | | | | | | | | | | | | |

**Figure 3.** The 11q13 amplification core. Genes that were amplified in each cell line are marked in red. \textit{PPFIA1} is located at the core region of 11q13 amplification\textsuperscript{13,174}. DNA copy number Log2 ratios were extracted from the Agilent oligo array-based Comparative Genomic Hybridization (CGH) data using Agilent CytoGenomics Software (v4.0.3.12) and ADM-2 algorithm for genes mapping to 11q13 region. (Data provided by Oncogenomics Lab, by O. Monni)\textsuperscript{13,174}
7. Liprin protein family

Liprin-α1 belongs to the liprin protein family, which consists of the liprin-α and liprin-β proteins that have been classified based on sequence similarities and binding characteristics\textsuperscript{14,175}. In invertebrates (\textit{C. elegans, Drosophila}), liprin-α gene, syd-2 (synapse-defective-2),\textsuperscript{176} and Dliprin\textsuperscript{177} have homology to liprin family. In vertebrates, four different liprin-α isoforms are found, liprin-α1 (LIP.1), -α2, -α3 and -α4\textsuperscript{175}. The human liprin-α1 and worm syd-2 show 40% conservation of amino acid identity\textsuperscript{178}. The structure of liprin-α consists of three SAM (sterile alpha motif) domains, which are the C terminal domain, forming the LH domain, and the N terminal region, which is predicted to form coiled-coil structures\textsuperscript{14,17,175,178,179} (Figure 4). All the liprin-α proteins are expressed in all regions of the brain. In addition, liprin-α1 is expressed in various tissues, and liprin-α2 and liprin-α3 are predominantly expressed in brain tissue and liprin-α4 has the highest expression in muscle tissue\textsuperscript{178}. Liprins were first identified as LAR leukocyte antigen protein tyrosine phosphatase-interacting proteins\textsuperscript{17,175}, and phosphorylation of liprin regulates binding to LAR\textsuperscript{180}. Initially, LAR and liprin-α1 (LIP.1) were shown to co-localize at the discrete ends of focal adhesions (FAs)\textsuperscript{17}. The liprin-α1/LAR complex was proposed to be involved in the interactions between actin and integrin as well as cadherin and catenin promoting connection of cytoskeleton to extracellular matrix through protein tyrosine phosphatase activity\textsuperscript{175}. Liprin-α1 consists of 32 exons in both human and mouse and it has five alternative splice variants with different tissue distribution and temporal expression\textsuperscript{178}. Liprin-α1 with exon 10 was detected solely in the adult brain, while transcripts lacking exon 18 were abundant at a specific developmental stage, and transcript with exons 24 and 25 were strongly expressed in the adult brain\textsuperscript{178}. Interestingly, it has been shown that liprin has the ability to autophosphorylate, and through this can self-regulate interactions through an intrinsic enzymatic activity\textsuperscript{180}. 
The liprin-α1 protein consists of N terminal coiled-coil domains, SAM domains which form the C terminal liprin homology LH domain, DBM motif and C-terminal PZD binding sequence. Liprin-β1 shares partial homology with liprin-α1. Simplified image is adapted and modified from references (de Curtis I., 2011, Serra-Pages et al., 1998) 14,175.

**Figure 4.** The liprin-α1 protein consists of N terminal coiled-coil domains, SAM domains which form the C terminal liprin homology LH domain, DBM motif and C-terminal PZD binding sequence. Liprin-β1 shares partial homology with liprin-α1. Simplified image is adapted and modified from references (de Curtis I., 2011, Serra-Pages et al., 1998) 14,175.

7.1 Physiological role of liprins

Liprin-α proteins carry out important functions related to synapse assembly and function181-183. Liprin-α proteins are expressed and enriched at the active zones of neurons181. Liprin-α proteins have crucial functions in the nervous system, which include the development and maintenance of dendritic spines and excitatory synapses, the assembly, organization and function of presynaptic active zones and in vesicle trafficking178. Liprin-alphas have shown to be important in synapse maturation182 and in active zones183. Liprin-alphas have also been associated with the trafficking of synaptic vesicles184. In addition, liprin-α has been shown to be localized at the dendrites, hippocampal neurons, and postsynaptic sites185-187, with a postsynaptic activity related to glutamate receptor interacting protein (GRIP), and targeting of AMPA receptors187. ERC1 (ELKS-Rab6-interacting protein-CASK) is needed
at the active zone, and together with liprin-α and RIM, has regulatory role in neurotransmitter signaling188. It has been shown that liprin-α1 is recruited to synaptic membranes by ERC1188, GIT1 has been shown to be an interaction partner for liprin-α185,188, and GIT proteins are related to migration, adhesion and trafficking189. GIT proteins are involved in focal adhesions, and GIT2 has been shown to have negative role in lamellipodial protrusions and Rac1 and Cdc42-related focal adhesion signaling190. At postsynaptic sites, the protein complex formed by GIT1 and liprin-α is crucial for AMPA receptor targeting185. Also, liprin-α interacts with CASK (calcium/calmodulin dependent serine protein kinase), through the SAM1 domain191. CAMKII phosphorylation has been shown to lead to liprin-α degradation, and, additionally, its expression is regulated by ubiquitin-proteasome system (UPS) in hippocampal neurons192. A study showed that liprin-α1 mutants, which were not degraded by CAMKII, led to inhibition of dendritic targeting of LAR receptors and finally to reduced synapse amount192. Introduction of liprin-α homolog mutants in C. elegans (syd-2) led to an unusual distribution of presynaptic proteins, prolonged active zones and deteriorated synaptic transmission176. Recently, PPFFIA1 has been identified as a potential risk gene for Alzheimer’s disease193. Also, observations show that liprin-α1 is upregulated in the temporal neocortex of patients with intractable epilepsy (IE), while the same study showed that liprin-α1 was upregulated in the hippocampus and cortex of the intractable temporal lobe epilepsy (TLE) rat model compared to the control group194. Liprin-α proteins appear to have several important physiological functions in the regulation of neuronal cells. Kinesin-3/KIF1A is important in the vesicular transport of synaptic vesicles, and it interacts with liprin-α186. Liprin-α and kinesin 1 interact in Drosophila and in C. elegans, and SYD2/liprin-α has been shown to interact with UNC-104/kinesin, and disturbances in these interactions lead to impairments in vesicle transport through axons184,195. In neuronal cells and perhaps also in non-neuronal cells, it has been suggested that liprin-α and interacting protein complexes might have a role in cargo transport14.
7.2 Liprins in cell motility and adhesion

Liprins have been shown to function in cell motility, regulating cell spreading, β1 integrin signaling and recycling. Liprin-α1 contributes to the assembly/disassembly of focal adhesions, and stabilizes inactive integrins at the cell membrane for further activation, which leads to novel adhesion sites. Our study has shown that liprin-α1 localizes at invadosome structures along with other focal adhesion proteins in HNSCC cells. Liprin-α1 regulates extracellular matrix degradation, cell invasion and cell invasive growth in motile breast cancer cells. In colon cancer cells, liprin-α1 promotes cell migration and spreading, and in these cells liprin-α1 interacts with ING4. In vivo, liprin-α1 is associated with the metastatic potential of cancer cells. Liprin-α1 belongs to the adhesome proteome, which consists of proteins involved in integrin adhesion complexes. Liprin-α1 is involved in α5β1 recycling and in the vascular phenotype of zebrafish via fibronectin. In endothelial cells and zebrafish embryos, depletion of PPFIA1 led to impaired vascular morphogenesis, as zebrafish embryos had cardiovascular defects such as enlarged heart chambers and impaired blood flow. Liprin-α1 is a member of a polarized and dynamic structure, which is important in cell migration, together with the ERC1 and LL5 proteins, and in focal adhesion turnover. A recent study showed that liprin-α1 participates in the invadosome development, and although it does not prevent invadosome formation, it is important in the regulation of the motility and maturation of invadosome structures. It has been suggested that liprin-α proteins might behave differently in different types of cancer cells depending on the protein-protein interactions and protein composition of the cells. In addition, the tumor cell environment may play a crucial role in defining the role of liprins in cancer cell signaling.
8. Adhesive and invasive structures in cells

8.1 Focal adhesions and focal contacts

Previously, liprin-α has been identified as a LAR interacting protein with a focal adhesion association175. Focal adhesions are complex multiprotein complexes (Figure 5), where integrin transmembrane proteins connect the actin cytoskeleton to the extracellular matrix, and these sites contain proteins like vinculin, talin, GTPases, kinases and phosphatases24,204-210. Different definitions for focal adhesions exist, and they can be classified as focal complexes, focal adhesions, fibrillar adhesions and three-dimensional (3D) matrix adhesions205,211. Regulation of focal adhesions or complexes can occur through Rac and Cdc42211,212, and Rho activity controls focal adhesions211,213. Defined as complex signaling structures, focal adhesions consist of several protein-protein interactions, but how the cell regulates different interactions at different states of cell migration or the cell cycle is not fully known in detail. Additionally, matrix availability has effects on the cellular composition of adhesion structures211. Proteins that have important functions in migration, in adhesion, focal complexes and focal adhesions include among others FAK211,214-216, Src211,217,218, paxillin210,211, talin209,211, vinculin209,219, and transmembrane protein integrins220,221. Focal adhesion size is an important factor for determining their impact on cell migration21. Focal adhesions formed in cells are not stable, but transient complexes, crucial for cell spreading and motility as well as in cell survival and proliferation and associations of adhesions with the extracellular matrix are dynamic with constant assembly and disassembly222-226. Focal adhesions are mechanosensing complexes, and interactions of several proteins such as vinculin and talin are important227,228. The vinculin association to talin is suggested to be regulated by tension forces229-231. Sensing of environmental cues at the cell surface has a major effect on the participation of specific integrins into the adhesion complexes, and this greatly influences the activation of different integrins (such as α5β3, or α4β1), and the process of focal adhesion assembly and disassembly23,211,232-235.
8.2 Cytoskeletal elements, intermediate filaments and adhesion

Actin and microtubules have long been known to interact with focal adhesions. Intermediate filaments, which are members of the dynamic cytoskeletal protein family, have been shown to be present at sites of adhesions of focal adhesions. Vimentin intermediate filaments are important in the epithelial to mesenchymal transition and in migratory properties of the cells. Intermediate filaments have been shown to be related to maintaining tension forces of the actin cytoskeleton, sensing stretching of the cells, and they deliver information about mechanical forces inside the cell through integrins. Evidence of molecular links between focal adhesions and intermediate filaments exist, and post-translational modifications are crucial when considering intermediate filaments signaling. It is suggested
that signaling between focal adhesions and intermediate filaments is bidirectional\textsuperscript{245,247,248}. A study has showed that anchorage of intermediate filaments at focal adhesion sites is needed for focal adhesion dynamics and if this is impaired it has impact on focal adhesion assembly and cell motility\textsuperscript{249}. In addition phosphorylation of vimentin regulates modifications of lamellipodia formation\textsuperscript{240}, and a study has showed that phosphorylation of vimentin at S71 inhibits migration of cells as a result of sphingolipid treatment related to Rock activity and focal adhesion\textsuperscript{250}. In endothelial cells, the number of focal contacts with vimentin association is known to increase during stress\textsuperscript{251}. Interesting observation is that p53 protein associates with the vimentin cytoskeleton in senescent fibroblasts with decreased cell signaling of the focal adhesion proteins and smaller focal adhesions\textsuperscript{252}. Importantly, intermediate filaments have been linked in vesicle trafficking, and possibly modulation of the surface expression of integrins\textsuperscript{241,253-257}. Apart from malignant cells, overproduction of vimentin leads to the senescence of fibroblasts\textsuperscript{258}. In addition, senescent cells have a dense and long vimentin network, with long and thin actin fibers\textsuperscript{258}. In addition, in epithelial cells, increased motility can be linked to the lack of a specific keratin intermediate filament network\textsuperscript{259,260}. Keratins are involved in adhesion and interactions between focal adhesions and keratins are speculated to be transient, and include the binding of plectin, a focal adhesion protein\textsuperscript{257,261-263}.

### 8.3 Invadosomes

We have shown that liprin-α1 is part of an invadosome complex occurring spontaneously in HNSCC cells from primary tumors\textsuperscript{18}. In addition liprin-α1 associates with invadosomes in cells where invadosomes can be induced by c-Src overexpression\textsuperscript{203}. The term invadosome includes structures called invadopodia in cancer cells, and podosomes in non-transformed cells, and are actin rich adhesion structures\textsuperscript{264}. Podosomes can be found in dendritic cells, macrophages, endothelial cells, vascular smooth muscle cells and osteoclasts\textsuperscript{265-271}. Invadosome structures are mechano-adhesive, and important in invasion processes\textsuperscript{20,22,26,272,273}. Invadosomes can be present as aggregates, individual dots, rosettes or linear structures\textsuperscript{25} (Figure 6).
Invadosomes are also called microdomains, and are commonly formed at the ventral surface of the cell\textsuperscript{25,274,275}. Invadosomes have a capacity to interact with and degrade the extracellular matrix using matrix metalloproteinases (MMP’s)\textsuperscript{276}. Invadosome structures have been observed \textit{in vivo}\textsuperscript{277-279}.

\textbf{Figure 6.} Simplified image of invadosome organization in x-y and z directions in cancer cells. A. Invadosomes with adhesion rings. Green illustrates core of invadosome, which consists of F-actin and cortactin. Red illustrates adhesion ring, which consists of adhesion proteins. B. Image illustrates the degradating capabilities of invadosomes into extracellular matrix (ECM shown in violet/dark green). Image is adapted and modified from (Di Martino et al., 2016, and Hoshino et al., 2013)\textsuperscript{25,280}

Invadosomes are able to function as mechanosensors\textsuperscript{20}, and they can sense and respond to the stiffness of the extracellular matrix\textsuperscript{20,281-285}. The properties of invadosomes vary (size, half-life), as well as their number inside individual cells, and formation of invadosomes is dependent on the surrounding environment and the cell type\textsuperscript{19,276,286-288}. In certain cells (including macrophages, dendritic cells and osteoclasts) invadosomes form when adhesion is occurring\textsuperscript{280,290}. Endothelial cells can form invadosomes after the introduction of a stimulant, such as expression of active Cdc4\textsuperscript{271}, activation of c-Src, introduction of phorbol esters or sodium fluoride\textsuperscript{291-294}, or for example cytokines (transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF) or tumour necrosis factor α (TNF-α))\textsuperscript{295,296}. In cancer cells, invadosomes can be induced for example by epidermal growth factor (EGF) or TGF- β, and they can be either inducible or constitutively
expressed in cancer cells^{297,298}. Substrate structure has an effect on the number of invadosomes^{281,299}, their maturation, the distances where they are formed in relation to each other and how they are formed into rosette structures^{282}. Degradative capabilities are dependent on the rigidity of the environment^{300}. When cells are grown in a soft environment, this results in lower contractility^{301,302}, a decreased density of invadosomes and ECM degradation, when these conditions are compared to conditions in a stiffer matrix environment^{281,299}. An environment that mimics the *in vivo* environment in cancer cells has been shown to be sufficient to enable invadosome formation with a high density of fibrillar collagen I^{303}. Other environmental cues can, in addition, induce the formation of invadosomes such as topological cues, static pressure and injury^{304}. Invadosomes can sense modifications of actin dynamics and changes in myosin tension leading to modified organization and degradation of the extracellular matrix^{281,283}. Invadosomes have the ability to sense the intrinsic mechanosensitivity, which has been shown with single invadosomes, which produce protrusion forces that are enhanced with the rigidity of the ECM^{305}. This is dependent on the actin filament structure of the invadosomes that interact with formins and other cross-linkers^{306}. The majority of studies exploring invadosomes have been done *in vitro*, but there is evidence that invadosomes also exist in the *in vivo* environment^{25}. Imaging techniques for studying invadosomes limit the identification of invadosome structures, but methods such as intravital multiphoton microscopy can be used to observe cells *in vivo* with the surrounding matrix^{307-309}. Invadosome-like and invadopodia-like structures have been studied in several animal models^{278,310-318}. Invadosome-like structures are often related to the degradation of the basement membrane^{25}. There is evidence that after cytokine exposure, human umbilical vein endothelial cells formed rosettes and had proteolytic functions^{294,295,319}, and *ex vivo* studies for angiogenesis have revealed that rosettes co-localize with cortactin in endothelial cells^{320}. In addition, invadosome rosettes have been shown to be induced after VEGF-A treatment in the mouse endothelium, and invadosome structures were shown to be crucial for the branching of blood vessels as well as for pathological angiogenesis, where $\alpha_6\beta_1$ integrins were needed for the formation and
stabilization of the structures\textsuperscript{317}. It has been shown that silencing Tks5, (an invadosome marker), or inhibition of matrix metalloproteinases (MMPs), leads to prevention of invadosome formation, and Tks5 positive protrusions degraded extracellular matrix proteins. This study clarifies the potential relevance of invadosome structures in the \textit{in vivo} environment \textsuperscript{316}.

8.4 Other proteins related to cell adhesion and invasion

8.4.1 KAI1/CD82, adhesion and integrins

Tetraspanin membrane protein CD82 has been linked to integrin signaling\textsuperscript{321-326}, and CD82 has been shown to be involved in integrin trafficking by several studies, and to associate with integrins such as $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha L\beta_2$\textsuperscript{326-331}. A study has shown that CD82 expression contributes to the $\alpha_6$ integrin internalization, and through this specific integrin, to the adhesion properties of cells\textsuperscript{326}. Additionally, another study showed that KAI1/CD82 is an important modulator of $\beta_1$ integrin maturation, and its overexpression reduced the migratory capabilities of the cells\textsuperscript{323}. CD82 also has an important function in hematopoietic stem cells\textsuperscript{333}. Understanding how CD82 expression is regulated and activated is crucial in order to develop its potential use in therapies\textsuperscript{334}. Despite limited number of data, the available information suggests that loss of CD82 expression is caused by mutation, loss of heterozygosity, mutation of the promoter or hypermethylation of the promoter\textsuperscript{335-338}. In addition, a dominant negative protein might occur due to a splice variant\textsuperscript{334}. CD82 can be regulated transcriptionally, and a study showed that p53, JunB and AP2 regulated KAI1/CD82\textsuperscript{339,340}. Post-transcriptional regulation occurs as well, since CD82 has been shown to be regulated by ubiquitination, palmitoylation and glycosylation, and these modifications are needed for the proper function of CD82\textsuperscript{326,341}. It has been speculated that without these modifications, the effect of CD82 to suppress metastasis will be diminished or missing\textsuperscript{334}. KAI1/CD82 has been considered as a metastatic suppressor for solid tumors\textsuperscript{342}, and a metastatic suppressor role has been described in laryngeal\textsuperscript{343}, breast\textsuperscript{344}, prostate\textsuperscript{345-347}, colon\textsuperscript{348,349}, pancreatic\textsuperscript{350}, gastric\textsuperscript{351}, hepatocarcinoma\textsuperscript{352} and thyroid\textsuperscript{353} cancer. CD82 has been shown to
regulate oral cancer cell migration and invasion, and in a xenograft model, overexpression was shown to reduced the volume of the tumors\textsuperscript{354}. Downregulation of CD82 has been shown to be related to reduced survival of human oral cancer patients\textsuperscript{355,356}. Furthermore, overexpression of CD82 has been shown to reduce the \textit{in vitro} migration and \textit{in vivo} metastasis of breast cancer\textsuperscript{344}. Finally, downregulation of CD82 has been related to prostate cancer progression\textsuperscript{336,344}. 
9. AIMS OF THE STUDY

Liprin-α1 is encoded by PPFA1, which is located at the commonly amplified region at 11q13 in head and neck squamous cell carcinoma and breast cancer. Previously, the role of liprin-α1 in cancer cell progression has not been established in detail. Our aim was to study the localization and role of liprin-α1 in processes related to cancer cell signaling and progression. The specific aims of my study were:

1) To determine the cellular localization of liprin-α1 in cancer cells and its role in cancer cell adhesion and invasion processes

2) To study the cellular structures and signaling pathways mediated by liprin-α1 expression

3) To study the expression of liprin-α1 and CD82 and their correlation in clinical HNSCC samples

4) To explore the potential of liprin-α1 as a drug response indicator in metastatic cells
10. MATERIALS AND METHODS

10.1 Cell lines (Part I, II, III)

The cell lines used in this study were the head and neck cancer cell lines UT-SCC-19A, UT-SCC-19B, UT-SCC-24A, UT-SCC-24B, UT-SCC-42A, UT-SCC-42B and UT-SCC-95, which were derived from HNSCC patients (the cell lines have been provided by Prof. Reidar Grènman, Department of Otorhinolaryngology, Head and Neck Surgery, University of Turku), SCC-25 (ATCC), and the breast cancer cell lines MDA-MB-231 and Hs578T (ATCC).

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Table 2. Cell lines used in studies I-III. Abbreviations: UT-SCC = University of Turku Squamous Cell Carcinoma, A = primary tumor, B = metastasis. *ATCC = American Type Culture Collection, SCC-25, MDA-MB-231 and Hs578T cell lines were ordered from ATCC.

10.1.1 Cell culture (I, II, III)

The UT-SCC and MDA-MB-231 cell lines were cultivated in DMEM medium (Lonza), supplemented with penicillin/streptomycin (100 U/ml) (Lonza), 2 mM L-glutamine (Lonza), 0.1 mM non-essential amino acids (NEAA, Lonza) and 10% fetal bovine serum (FBS, Gibco). The SCC-25 cell line was cultivated in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium, with 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, penicillin/streptomycin (100 U/ml), 0.5 mM sodium pyruvate from Lonza and 10% FBS from Gibco. The Hs578T cell line was cultivated with RPMI-1640 medium supplemented with penicillin/streptomycin (100 U/ml) (Lonza), 2 mM L-glutamine (Lonza), 0.1 mM non-essential amino acids (NEAA, Lonza), and 10% FBS (Gibco).
10.2 Antibodies (Part I, II, III)

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Table 3. The most important antibodies used in studies I-III.
10.3 Constructs (Part I, II, III)

The shRNA constructs for *PPFIA1*, *VIM* and *CTTN* were obtained from the TRC1 or TRC2 libraries (Sigma-Aldrich). The constructs are shown in Table 4. The hairpin sequence is cloned into pLKO.1 vector. The pLKO.1 vector contains a puromycin resistant gene for mammalian selection. As a control, an shScramble/non-target construct was used (Sigma-Aldrich, Functional Genomics Unit). The pLenti6/V5 DEST vector and an ORF for *PPFIA1* was used for overexpression of *PPFIA1* (clone ID 4794300, Open Biosystems). The pENTR221 plasmid with a selected ORF was recombined with the destination vector and pLenti6/V5 DEST (Invitrogen). The lentiviral expression vector pLenti6/V5 DEST (Invitrogen) carrying the *PPFIA1* ORF was generated by Gateway cloning in collaboration with Genome Biology Unit at the University of Helsinki (www.biocenter.helsinki.fi/bi/gbu) according to the manufacturer’s protocol (Invitrogen). The vector includes a blastidicin resistance gene for mammalian selection.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Construct</th>
<th>TRC</th>
<th>Clone ID</th>
</tr>
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<td>1</td>
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</tr>
</tbody>
</table>

Table 4. The shRNA constructs that were used from The RNAi Consortium library.

10.4 Western blot (Part I, II, III)

Briefly, the cells were in general grown to subconfluency overnight, and lysed with lysis buffer (RIPA, Sigma-Aldrich). Protease and phosphatase inhibitors (Roche) were added into RIPA buffer. Laemmli buffer and β-mercaptoethanol were added. Reduced samples were boiled and nonreduced samples were prepared without β-mercaptoethanol and boiling. Ready-made SDS page gels were used (BioRad) (10μg), and proteins were transferred to PVDF membranes with transferblot turbo equipment (BioRad). For blocking, 5% milk or 5% BSA in TBST buffer were used. Membranes were washed with TBST and incubated with primary antibody (1:1000) overnight at +4°C, and washes were followed with TBST. The incubation time for the secondary antibody was 1h at RT, and the membranes were washed with TBST. Detection of membranes was done with detection reagents and X-ray films with a film machine developer (Millipore) or using Chemidoc (BioRad).
10.5 Immunoprecipitation (Part I)
Briefly, the cells were counted, plated and incubated +37°C o/n. Cells were washed with cold PBS and collected. Protease inhibitors were added to lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM EDTA and 1%TX-100). Pre-clearing was performed with immobilized protein A beads (GE Healthcare). Pre-cleared cell lysates were incubated with rotation with liprin-α1 antibody for endogenous protein and beads for 2 h at +4°C. The tubes were centrifuged, supernatant removed and beads washed in lysis buffer. The loading buffer (5% β-mercaptoethanol, 2 × Laemmli buffer, BioRad) was added to the final supernatant. The samples were boiled, centrifuged and collected.

10.6 Migration assay (Part I)
Briefly, 24-well plates with cell culture inserts were used to perform Transwell cell migration (BD Biosciences) (8 μm membrane pore size in the membrane, BD Biosciences). Matrigel without growth factors was used to coat the upper surface of the membrane (200 μg/ml) and incubated (1 h, +37°C). PBS was used to wash the cells, and the cells were then resuspended in the medium (10% FBS) and incubated (+37°C, 2 h). Washes with PBS followed and the cells were resuspended in serum-free medium (with 0.1% albumin solution (BSA, Sigma-Aldrich)). Medium with 10% FBS was added to the bottom wells of the chambers, and cell suspension was added to the upper part of the inserts. Migrated cells were fixed with methanol, stained (Hoechst) and quantified with Cellomics ArrayScan 4.5 (Thermo Scientific). Cotton swabs were used to clean the upper surface of the insert. Images of 30 fields were captured from each membrane and the number of cells migrating through the insert was counted. The experiment was repeated three times, and the average of duplicates for experimental conditions was used. Three different experiments were used to determine the standard deviations.

10.7 Gelatin degradation assay (Part I)
Briefly, coverslips were coated with poly-L-lysine (50 μg/ml, Sigma-Aldrich), washed with PBS, and fixed with 0.5% glutaraldehyde (Sigma-Aldrich) and washed with PBS. The coverslips were coated with 1 mg/ml Oregon green gelatin mixed with 2 mg/ml denatured collagen I, washed with PBS, incubated with 5 mg/ml sodium borohydride (Sigma-Aldrich) and washed with PBS. Coverslips were incubated in complete medium overnight, after which the cells were counted and seeded to coverslips. The cells were incubated in complete medium, and fixed with 4% PFA in PBS.
10.8 Focal adhesions and quantification of degradation of extracellular matrix (Part I)

ImageJ software was used for the quantification of the focal adhesions and the degradation of extracellular matrix. ImageJ was used to quantify the focal adhesions after thresholding the background and vinculin as a marker. The average shape or size of the focal adhesions was quantified from 3 x 30 cells from different experiments. The images were inverted to black and white and the focal adhesions were defined by the intensity threshold. The total area was defined from 0 to 1 for circularity and the area of the focal adhesions was defined from five to infinity. Circularity was set from 0 to 0,5 to quantify focal adhesions with an elongated shape. The background was defined by the intensity threshold for the quantification of extracellular matrix degradation, and was set for circularity from 0 to 1 and for degradation area one to infinity. The analysis was done for >50 cells/experiment to quantify the extracellular matrix. Three experiments were performed to calculate the standard deviations. For statistical analysis, unpaired student’s t-test was used and the result was significant if $P < 0.05$. A Zeiss Meta 780 laser scanning microscope with Zeiss 40x/1.4 N.A. plan-apochromat oil objective at room temperature was used to collect confocal images.

10.9 RNA interference by lentiviral particles (Part I, II, III)

Briefly, the cells were counted and incubated in 12 well-plates overnight. Polybrene (1 μg/μl), fresh medium were added, lentiviral particles were added and the cells were centrifuged. The cells were incubated for 4-8 hours (+37°C), and the medium was changed. The cells were incubated for 72h, and the selection marker puromycin (1 μg/ml) or blastidicin S Hcl (2 μg/ml) (Invitrogen) was added, and after selection, the cells were used for different purposes. All the viruses used in this study were generated at the Biomedicum Functional Genomics Unit (University of Helsinki).

10.10 Microscopy (Part I, II, III)

Zeiss Meta 780/880 laser scanning microscopes (Zeiss 40x or 63x/1.4 N.A. plan-apochromat oil objective) were used for confocal imaging. The Zeiss Zen 2010 Lite program was used for acquisition. Adobe Photoshop CS6, ImageJ, and Illustrator CS6 software were used for adjusting, quantification and/or background correction. The images were acquired using a Zeiss Axio Imager Upright epifluorescence microscope with ApoTome for optical sectioning with structured illumination. Image acquisition was done with a Hamamatsu Orca R2 camera and Zen software.
10.11 Immunofluorescence (Part I, II)

The cells were grown on coverslips until they reached desired confluency. PBS was used for washing, and fixation was done with 4% PFA/PBS, and PBS was used for washing. 0.12% glycine/PBS incubation preceded washing with PBS. The blocking solution was 3% BSA in PBS. The cells were incubated with primary antibody for 1h (1:100) in 1% BSA in PBS at RT, and coverslips were washed with PBS three times. The cells were then incubated with secondary antibody for 1h in 1% BSA in PBS at RT (1:400). The cells were washed three times with PBS and two times with milliQ water. Mounting of the microscopy slides were carried out with mowiol, DABCO and DAPI. To carry out immunofluorescence from three-dimensional cell culture, the cells were first counted and embedded into three-dimensional collagen as collagen drops in 12 or 24 well plates. Collagen I from rat tail (Sigma-Aldrich or Gibco) was used. The protocol has been described previously18,357. Briefly, the cells were counted and mixed with collagen I suspension according to the manufacturer's instructions, and the collagen I cell suspension was incubated at +37°C. After that, medium was added, and the medium was changed every day of the experiments. The cells were washed with PBS and fixed with 4% PFA in PSB. The drops were washed with PBS, and blocking was done with either 3% BSA in PBS or 15% FBS, 0.25% Tx-100 and PBS for 1h-4h. The drops were washed with 0.25% Tx100 in PBS for 4h-overnight, and the washing buffer was changed 4-9 times. Primary antibody was added and the sample was incubated overnight, after which washes were done with 0.25% Tx-100 in PBS. Secondary antibody was added and incubated in 0.25%Tx-100 in PBS, after which washes were done with 0.25% Tx-100 in PBS, followed by washes with PBS and/or milliQ water. The collagen drops were embedded in mowiol + DAPCO + DAPI.

10.12 Microarray analysis (Part I)

Microarray analysis was performed at the Biomedical Functional Genomics Unit, University of Helsinki, from the metastatic UT-SCC-24B HNSCC cell line, cultured in 2D and knocked-down with shPPFIA1. Scramble (shScr) was used as a control. Three replicates from shPPFIA1 and shScr each using the same construct were performed. RNA was extracted using the miRNA easy Minikit from Qiagen according to the manufacturer's instructions. The microarray data was normalized and the expression values were transformed to the log2-scale using Chipster. The microarray data in part I has been described in detail, deposited and in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE75756 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE75756).
10.13 Collagen contraction assay (Part II)
The cells were counted suspended with collagen I and pipetted into low adhesion plates, after which the contraction of collagen I was monitored for 1-6 days. The collagen I gels were imaged and quantified with ImageJ software.

10.14 Spheroid formation assay (Part I and II)
Briefly, the cells were counted and plated on low adhesion plates with U-shaped bottoms, without the extracellular matrix. The cells were incubated, after which spheroid formation was measured. The spheroids were imaged with a microscope, and analysis was done with ImageJ.

10.15 MTT-assay and soft agar assay (Part I)
Briefly, the cells were calculated (5000 cells/well) and plated on a 96-well plate, and incubated with several different lengths of time. MTT reagent (5 mg/ml) was added and incubated for 2h after which cells were lysed with lysis buffer (10% SDS, 10 mM HCl, H2O), and measured at an absorbance of 540 nm. Soft agar for colony growth formation has been described earlier18.

10.16 3D-collagen culture (Part II)
Briefly, the cells were calculated and embedded into 3D collagen according to the manufacturer's instructions (Gibco). 500 μl collagen I and the cell suspension were pipetted onto a 24-well plate and incubated for five days with changes of the medium, after which the cells/collagen were extracted by Trizol reagent. RNA was extracted from the cells and subjected to RNA sequencing.

10.17 RNA isolation and removal of ribosomal RNA
Briefly, trizol (Life Technologies) was added to the cell/collagen I mixture and mixed with Precellys ceramic beads in a homogenizer (Precellys). Chloroform (Fisher Scientific, Hampton, NH, USA) was added to the samples, followed by mixing. The samples were centrifuged, the upper phase was kept, isopropanol (Fisher Scientific) was added and the solution was mixed. The samples were incubated centrifuged and 75% ethanol (Etax) was added. The samples were mixed and centrifuged. The pellets were dissolved in Rnase-free water (Lanza AccuGENE Molecular Biology Grade Water). Isolated RNA was purified using the Qiagen RNeasy Mini kit according to the instructions of the manufacturer (Qiagen, Hilden, Germany). Ribosomal RNA was removed using the RiboZero Complete Gold Human kit following the instructions of the manufacturer (Illumina, San Diego, CA, USA).Second strand cDNA synthesis (NEBNext, Illumina) and purification of double-stranded cDNA by 1.8X Agencourt AMPure XP beads were carried out according to instructions provided by the manufacturer (Illumina).
10.18 Library preparation and RNA sequencing
NEBNext Directional RNA library kit was used with instructions provided by the manufacturer for library preparation (Illumina). The PCR amplified library was purified using Agencourt AMPure XP beads with the instruction provided by the manufacturer (Illumina). The quality of the sequencing library was assessed using Bioanalyzer (Agilent High Sensitivity Chip). Library denaturation and dilution were made according to the NextSeq 500 system guide (Illumina). The Illumina NextSeq 500 High 75 bp reagent cartridge for RNA sequencing was used.

10.19 RNA sequencing data analysis and GSEA (Part II)
Briefly, the RNA-seq data was pre-processed using QualiMap, FastQC, STAR aligner, and trimmomatic software. Differential expression analysis was performed with the DESeq2 package in R v3.2.3 (logarithmic transformation), and for the statistical significance of the gene expression changes, the Wald test and Benjamini-Hochberg (corrected values) were used. The RNA sequencing data have been deposited in The National Center for Biotechnology Information’s (NCBI’s) Gene Expression Omnibus (GEO, Series accession number GSE108392). The Venny 2.1 analysis program was used to visualize the number of common genes that were differentially expressed between the groups (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Briefly, gene set enrichment analysis was used to study whether sets of genes showed statistically significant differences between the two groups investigated\textsuperscript{358,359}.

10.20 RNA interference by siRNA (Part II)
Briefly, CD82 was silenced by siRNA according to the instructions given by the manufacturer (Sigma Aldrich), knockdown of the protein was confirmed by western blot, and the cells were processed for knockdown experiments. Control siRNA was run with the knockdown experiments. Briefly, the cells were plated, and the amount of siRNA, Opti-Mem and medium were optimized according to the instructions of the manufacturer. siRNA and the transfection reagent RNAimax were first mixed with the medium, after which they were combined and added to the cells. The cells were incubated for 24h and transferred to collagen I, and incubated for seven days, after which they were fixed and colony growth was quantified with ImageJ software. The images were captured with a confocal microscope.

10.21 Drug screen for cell viability and cytotoxicity (Part III)
A panel of anti-cancer drugs was studied with the MDA-MB-231 shScr and shPPFIA1 cell lines. A set of 527 FDA approved and oncology compounds were tested with two different assays: cell viability (CellTitre Glo) and cell
cytotoxicity (CellTox Green). The screen was carried out at the Institute of Molecular Medicine (FIMM, University of Helsinki).

10.22 Immunohistochemistry for paraffin sections and TMA samples (PART III)

Tumor and healthy cheek mucosa samples from patients diagnosed with oral or oropharyngeal cancer were obtained from Helsinki University Hospital and Tampere University Hospital, Finland. The Ethics Committee of Helsinki University Hospital, Finland approved the study, and the Ministry of Social Affairs and Health, Finland gave permission to use the tumor tissue for this work. Before embedding the pieces into paraffin, they were fixed with 4% paraformaldehyde followed by sectioning by microtome. Deparaffinization and antigen retrieval of the sections were performed by heating the samples in sodium citrate buffer (10 mmol/L, pH 6.0, autoclave). Blocking solution used for the sections was (5% horse serum, 0.3% Triton X-100 in PBS) and primary antibodies were diluted in 1% BSA, 0.3% Triton X-100 in PBS at +4°C overnight. Double immunofluorescence staining of samples with selected antibodies was performed to better visualize the localization. Selected proteins using antibodies (TMA) were detected from the sections with BrightVision Poly-HRP-Anti Rb and -Anti Ms kits (ILimmunoLogic) and DAB (ImmPACT DAB). Washes were performed (0.3-1% Triton X-100 in PBS), and the sections were incubated with fluorescently labeled secondary antibodies (Molecular Probes/Invitrogen) and DAPI in MQ-water.

10.23 Immunofluorescence for frozen tissue sections (PART III)

Tumor and normal cheek mucosa samples were provided, 4% paraformaldehyde was used for fixing, followed by incubation of 30% sucrose in PBS overnight, and mounting was done with cryomatrix (Thermo Scientific). Cryostat was used for sectioning of the samples. The blocking solution for the sections was 5% horse serum with 0.3% Triton X-100 in PBS, and incubation with selected primary antibodies was done in 1% BSA and 0.3% Triton X-100 in PBS at +4°C. The incubation period was overnight. The samples were washed with PBS, and the sections were incubated with secondary antibodies (fluorescent label, Molecular Probes/Invitrogen) and DAPI in MQ-water.

All the protocols used in this work have been described and/or cited in the original publications in detail\(^{18,36}\), or in the third manuscript (Pehkonen H., \textit{et al}, unpublished data). The ethics approval for use of patient material were given in Dnro 16/13/03/02/2014 (Helsinki University Hospital) and ETL R16098 and ETL R16098 (Tampere University Hospital).
11. RESULTS

11.1 Part I

11.1.1 Liprin-α1 regulates cancer cell invasive growth into 3D collagen I
The first aim of this study was to evaluate the role of liprin-α1 in cell invasive growth in cancer cells from different origins. HNSCC cell lines with and without 11q13 amplification (SCC-25 and UT-SCC-95) originating from the primary tumor as well as metastatic breast cancer cell lines (Hs578T and MDA-MB-231) were grown in three-dimensional collagen I and invasive growth was followed. Invasive and motile breast cancer cell lines showed reduced mesenchymal invasive properties and outgrowths in three-dimensional collagen I after liprin-α1 knockdown. HNSCC cell lines from primary tumors only poorly invaded into the collagen I matrix, which was in line with their significantly weaker motility through matrigel coated inserts as compared to the invasive breast cancer cells. In addition, the HNSCC cell line with no 11q13 amplification and liprin-α1 knockdown did not show reduced invasion capabilities as compared to cells from breast cancer. The SCC-25 HNSCC cell line with 11q13 amplification showed a decreased cell-cell adhesive growth pattern after liprin-α1 knockdown, and the cells turned to a phenotype with less prominent cell-cell contacts and differential invasive properties when compared to breast cancer cells in 3D collagen. Our data suggests that liprin-α1 is required for expansive growth behavior in the 11q13-positive SCC-25 cell line from primary tumors, whereas in highly metastatic breast cancer cells, liprin-α1 promotes cancer cell invasion.

11.1.2 Liprin-α1 localizes to different adhesive structures in cancer cells
Localization of liprin-α1 was explored in primary HNSCC cells as well as in metastatic breast cancer cells. In the UT-SCC-95 cell line originating from a primary tumor of the tongue, liprin-α1 showed prominent localization to adhesion rings of invadosome structures. In addition liprin-α1 was located close to the cell edge or near focal adhesions together with other adhesion
proteins vinculin, talin, paxillin and β1 integrin. Liprin-α1 was mostly located at the outer part or around the adhesion ring, where it co-localized with β1 integrin. Cortactin, a well-known invadosome and invadopodia marker, and actin localized at the core of the adhesion ring in these structures. Invadosomes were mostly detected with adhesion rings, but in addition, adhesion rings with different sizes and prominent or less prominent adhesion ring complexes were observed, which were confirmed with other invadosome markers in UT-SCC cell lines from primary tumors. In cell lines from primary HNSCC, which spontaneously formed invadosomes, immunoprecipitation with the adhesion protein talin showed co-immunoprecipitation with liprin-α1, which was supported by localization of these two proteins to the same structures by immunofluorescence. The localization pattern was different in motile and invasive breast cancer cells, as liprin-α1 localized after the leading edge, and close to focal adhesions, although not necessarily directly co-localizing with adhesion structures (Figure 7). In addition, motile breast cancer cells had no detectable adhesion rings studied by the adhesion proteins vinculin and cortactin. Similar to metastatic breast cancer cells, liprin-α1 localized close to the focal adhesion or after the leading edge in metastatic HNSCC cells, showing that the localization was dependent on the invasiveness of the cells. These observations highlight the context-dependency of liprin-α1 in cancer cell invasion and the importance of delineating these structures in different cell types to determine the function of liprin-α1 in different environments.
11.1.3 Liprin-α is involved in controlling focal adhesions and degradation of the extracellular matrix

The morphology of focal adhesions was examined after liprin-α1 knockdown in HNSCC and breast cancer cells. Liprin-α1 knockdown altered the shape and/or size of focal adhesions. Vinculin was used as a marker in Hs578T and metastatic UT-SCC-42B cell lines to quantify the size and the shape of the focal adhesions after liprin-α1 knockdown. The focal adhesions were smaller and/or different in shape in motile cell lines. Invadosomes, in which liprin-α1 was localized into an adhesion ring, were able to degrade the extracellular matrix. However, in these poorly invading cells (UT-SCC-95) into collagen I, liprin-α1 knockdown did not significantly have impact on the ECM degrading capability.
of the cells or it was not able to prevent the formation of these structures despite being strongly associated with the invadosomes. HNSCC cell lines from different patients with primary tumor and corresponding metastatic/primary persistent tumors were tested for invasive properties into three dimensional collagen I. Compared to cell lines from primary tumor, metastatic tumors and primary persistent samples were more invasive. Adhesion rings with liprin-α1 positivity were spontaneously only observed in cell lines obtained from primary tumor, which is likely due to the different invasive characteristics of these cell lines.

11.1.4 Liprin-α1 as a modulator of vimentin

To gain more thorough insight into liprin-α1 function, we carried out microarray analysis after liprin-α1 knockdown or overexpression. Vimentin showed upregulation after liprin-α1 knockdown in a cell line with low endogenous expression of liprin-α1, whereas the expression of vimentin was downregulated in liprin-α1 overexpressing cells. Western blot confirmed the upregulation of vimentin in several HNSCC cell lines after liprin-α1 knockdown. As previously explained, liprin-α1 localized to invadosome structures and to the outer side of the adhesion ring. Vimentin expression was detected around adhesion ring near liprin-α1 in the UT-SCC-95 cell line. On the other hand, in UT-SCC-24B cells with liprin-α1 silencing vimentin localized either near the nucleus or it was accumulated at the cytoplasm. In addition, liprin-α1 knockdown led to vimentin bridge structures suggesting defects in cell division. In HNSCC, vimentin was not endogenously expressed in high levels, but was rather expressed at the edges of the cell colonies. Breast cancer cells endogenously express high levels of vimentin. Liprin-α1 knockdown led to accumulation of vimentin close to the nucleus. When breast cancer cells were embedded into three-dimensional collagen I, they showed invasive growth pattern with mesenchymal outgrowths. Liprin-α1 knockdown significantly suppressed these outgrowths in three-dimensional collagen I. This may be explained by the vimentin network localization around the nucleus rather than elongating into the collagen I matrix, into a proper network in liprin-α1 knockdown cells. In addition, it has been shown
previously with MDA-MB-231 cell line, that after liprin-α1 knockdown cells degrade less extracellular matrix\textsuperscript{15}. On the other hand, UT-SCC-95 cell line showed adhesion ring structures and limited invasive capabilities in three-dimensional collagen I which might explain that it was poorly motile through matrigel-coated inserts. The expression of other proteins related to the epithelial-to-mesenchymal transition were also studied after liprin-α1 knockdown in HNSCC and breast cancer cells, but no differences in the expression of these proteins could be observed. Several cell lines showed less metabolic activity after liprin-α1 knockdown. On the other hand, no effect on cell invasive growth or differences in metabolic activity were observed in a UT-SCC-95 cell line from a primary tumor. Invasive Hs578T cell line showed reduced growth in three-dimensional soft agar after liprin-α1 knockdown.

11.2 Part II

11.2.1 Liprin-α1 promotes invasive cell growth of metastatic HNSCC

The purpose of this part of the study was to clarify the role of liprin-α1 in the growth of motile and invasive HNSCC cell lines. The metastatic UT-SCC-42B cell line showed reduced colony size after liprin-α1 knockdown in three-dimensional collagen I, supporting the previous results with metastatic breast cancer cell lines. Similarly, UT-SCC-19B showed less invasive morphology of the colonies after liprin-α1 knockdown, although the reduction in colony size was not statistically significant. However, morphological changes of the colonies after liprin-α1 silencing were clear. A less significant effect of liprin-α1 silencing could be explained by high-level amplification of 11q13 in the UT-SCC-19B cell line and the higher liprin-α1 expression level as compared to UT-SCC-42B without 11q13 amplification. In these UT-SCC-42B cells, liprin-α1 partly co-localized with vinculin in the adhesion or invadosome-like structures at the edges of the colonies. Liprin-α1 silencing also led to clearly visible actin cytoskeleton re-arrangement in UT-SCC cells cultured in 3D collagen I, further strengthening its role in modulating adhesion related cytoskeletal elements in cancer cells.
11.2.2 Liprin-α1 has several crucial functions related to cell-cell or cell-substrate junctions as well as on the composition of the cell membrane

To explore mechanisms by which liprin-α1 regulates cell invasive growth, RNA sequencing was carried out in cells with liprin-α1 silencing. In HNSCC cells, GSEA showed enrichment of gene sets related to cell-cell junctions, vacuolar transport, signaling related to phosphatase complexes and protein localization to the membrane. In breast cancer cells with liprin-α1 silencing, enriched gene sets included regulation of cell death, signaling of membrane microdomains, signaling related to the morphogenesis of the epithelium and proteolysis. Among others ARDCC3, TP63 and SORL1, were differentially expressed as well as SMURF1 which is involved in integrin signaling. Interestingly, VIM was upregulated in HNSCC cells grown in 3D environment after liprin-α1 knockdown supporting our results from the 2D cell culture\(^6\).

11.2.3 CD82 is upregulated due to liprin-α1 knockdown and is located in cell membrane or vesicle-like structures

The CD82 transmembrane protein was upregulated after liprin-α1 knockdown in MDA-MB-231, UT-SCC-42A, and UT-SCC-42B cells, shown by RNA sequencing, western blotting and immunofluorescence. The localization of CD82 in HNSCC and breast cancer cells was explored in both the 2D and 3D cell culture platforms after shPPFIA knockdown. In 2D cell culture, CD82 was located partly at the cell membrane and in vesicle-like structures. To identify whether CD82 was located in early endosomes or vesicles, the cells were stained with caveolin-1 recognizing endosomal vesicles as well as with early endosome antigen 1 (EEA1). In the cells with liprin-α1 knockdown, CD82 did not co-localize with EEA1. On the other hand, caveolin-1 partly co-localized with CD82 in shPPFIA1 cells. In 3D collagen I, CD82 localized into the cell membrane, which was confirmed with phalloidin and ZO-1 staining in the UT-SCC-42A cell line. As in 3D cell culture, liprin-α1 localized to adhesion-like or invadosome-like structures and/or near the cell edge in HNSCC cells. As CD82 was upregulated in cells with liprin-α1 knockdown, we explored whether CD82 silencing in liprin-α1 knockdown cells would at least partially restore the invasive capabilities of the UT-SCC-42B metastatic HNSCC cell line in 3D.
collagen I. Partial knockdown of CD82 in the UT-SCC-42B cell line with liprin-α1 knockdown made cells more invasive as quantified by phalloidin staining. These results support the interplay of liprin-α1 and CD82 in the invasive growth of metastatic cancer cells.

11.2.4 PPFIA1 amplification associates with poor survival of HNSCC and breast cancer patients

Because gene expression analysis revealed gene expression changes in cellular pathways related to cancer cell signaling and CD82 was upregulated in HNSCC and breast cancer cells after liprin-α1 knockdown, TCGA data was explored to study the frequency of PPFIA1 amplification and its impact on survival in clinical HNSCC and breast cancer samples. Patients with PPFIA1 amplification had reduced survival in months on average as compared to non-amplified cases based on the TCGA data set analysis. This result underlines the importance of studying gene amplification and the mechanism behind PPFIA1/liprin-α1 function in patient data.

11.3 Part III

11.3.1 Expression of liprin-α1 is associated with low CD82 levels in HNSCC patient samples

Based on our previous findings on the overexpression of CD82 after liprin-α1 silencing, we explored the correlation and frequency of liprin-α1 and CD82 expression in clinical patient samples. The hypothesis was that the inverse correlation of these two proteins could be observed in clinical patient material as well. Cryosections and tissue microarrays were used to study the expression of liprin-α1 and CD82. In cryosections from four different oral tongue squamous cell carcinoma patients, liprin-α1 was expressed either in a weak or moderate level or it was ubiquitously expressed, whereas CD82 was expressed heterogeneously or at a low level. On the other hand, individual cells with strong CD82 expression showed less liprin-α1 expression. Tissue microarray from 76 oral tongue squamous cell carcinomas showed variable liprin-α1 expression as well. In 51% of patients, liprin-α1 expression was weak (scored 0-1), whereas in 49% samples the expression was strong (scored 2-3). On the
other hand, expression of CD82 was weak or negative in most of the samples (79%; scored 0-1) and strong in 21% of the samples (scored 2-3). Correlation between high liprin-α1 and low CD82 expression was statistically significant when measured by the chi-square test ($P < 0.001$). All the tissue microarray samples were collected from patients with oral tongue squamous cell carcinoma.

11.3.2 Liprin-α1 sensitizes cancer cells to several MEK inhibitors and drugs related to invasive cell growth

Because liprin-α1 has been observed as an important regulator of cell invasive growth in HNSCC and breast cancer cell lines$^{15,18,360}$, we aimed to study the possibility of liprin-α1 as a potential drug response indicator. The effect of liprin-α1 expression in drug response was tested with a panel of 527 drugs. The MDA-MB-231 breast cancer cell line was selected for the experiment, because liprin-α1 had an effect to cell invasive growth in this cell line. Several MEK inhibitors, such as pimasertib, trametinib and cobimetinib effectively inhibited cell proliferation of liprin-α1 expressing cells, when DSS was set over 10 for control (shScr) and under 5 for shPPFIA1 knockdown cells. Similarly, several drugs related to cell invasive growth or cell cycle were more effective in cells with liprin-α1 expression. These drugs included, among others, PLK1 inhibitor (TAK-960), met signaling related c-met-axl-ron-tyro-3 inhibitor (BMS-777607), CDK inhibitor (PHA-793887) and aurora kinase inhibitor (PF-03814735). Aurora kinase inhibitor (PF-03814735) treatment led to significant morphological changes in shScr and shPPFIA1 MDA-MB-231 cells. As mentioned above, PF-03814735 reduced cell viability more effectively in liprin-α1 expressing control cells (shScr) than in shPPFIA1 cells. Due to the role of liprin-α1 in invasion, drugs related to cell invasive growth, cell cycle and metastasis may be potent in the treatment of patients with 11q13 amplification and/or overexpression of liprin-α1.
12. DISCUSSION

12.1 Liprin-α1 in cancer cell invasive growth

In head and neck cancer cells, depletion of liprin-α1 enhances migratory properties\textsuperscript{172}, whereas in breast cancer cells its depletion leads to decreased migration and extracellular matrix (ECM) degradation\textsuperscript{15}. In colon carcinoma cells, liprin-α1 has a positive effect on cell motility, which has been linked to interaction with the tumor suppressor protein \textit{ING4}\textsuperscript{199}. We showed that mesenchymal and highly invasive breast cancer cell lines formed less invasive colonies and mesenchymal outgrowths after liprin-α1 knockdown in three-dimensional collagen I. Similar to metastatic breast cancer cells, liprin-α1 silencing reduced the invasive growth of the metastatic HNSCC cell line or invasive morphology of the cell colonies. On the other hand, in less invasive primary HNSCC cell lines liprin-α1 knockdown led to differential outcomes in invasion compared to metastatic cell lines: 1) the HNSCC cell line with 11q13 amplification turned into a more invasive phenotype in 3D collagen with less cell-cell contacts after liprin-α1 knockdown; 2) the HNSCC cell line with no 11q13 amplification showed no effect on cell invasion after liprin-α1 knockdown. Migratory properties of different cancer cell lines were explored using matrigel coated inserts and as expected the breast cancer cell line MDA-MB-231 was significantly more motile through matrigel coated inserts as compared to primary HNSCC cell lines. This underlines the context dependency of liprin-α1 function\textsuperscript{15,18,172,360}. Furthermore, 11q13 amplification may explain the observed differences in liprin-α1 dependent migratory phenotype in primary HNSCC cell lines. Immunofluorescence showed liprin-α1 to co-localize partly with vinculin in the 3D environment in the structures observed as potential adhesion- or invadosome-like structures near the edges of the colonies. It has been shown previously that the assembly and protein composition of invadosomes depend on the environment which explains the observed linear invadosomes when cultured in a collagen environment\textsuperscript{25}. Due to the mechanosensing, dynamic and transient nature of invadosomes, the shapes of these structures may vary depending on the state of the cells at a specific moment in time\textsuperscript{22}. Our results strongly support the role of liprin-α1 in
invasive machinery of the cells as knockdown reduced invasive capabilities of a metastatic HNSCC cell line\textsuperscript{366}, and this has previously also shown in breast cancer\textsuperscript{45}.

12.2 Liprin-α1 as a part of the invadosome complex

Invadosomes are considered important complexes regarding cell invasion and the degradation of the extracellular matrix with matrix metalloprotease activity\textsuperscript{19,276}. Similar to invasive capabilities, liprin-α1 localization depended on the invasive properties of the cells. In HNSCC cells originating from primary tumors, liprin-α1 localized to invadosome structures and more prominently into the outer part of the adhesion ring. Invadosome structures were verified by immunofluorescence staining of β1 integrin in addition to other adhesion proteins including vinculin and paxillin. Cortactin, a marker for the invadosome core and a protein encoded by the \textit{CTTN} gene at the 11q13 amplicon, was located as expected with actin in UT-SCC HNSCC cell lines from the primary tumor\textsuperscript{274,297,165}. Invadosome complexes degraded the extracellular matrix when coated onto fluorescent gelatin. This data shows that liprin-α1 is an important player in the invadosome and adhesive complexes of cancer cells. Although the knockdown of liprin-α1 did not alter the capability of HNSCC cell lines from primary tumors to degrade the extracellular matrix or did not prevent the occurrence of invadosomes, it probably has important functions regarding invadosome dynamics. As previously discussed, it has been shown that in motile breast cancer cells, liprin-α1 knockdown reduces the extracellular matrix degradation capability, and, in addition, it recently has been linked to the maturation process of invadosomes\textsuperscript{15,203}. This evidence suggests that the role of liprin-α1 may be in the regulation of the dynamics and the stabilization or duration as well as maturation of these transient complexes, which are constantly assembled and disassembled in a cell\textsuperscript{22,264}. In addition, invadosomes have been regarded as mechanosensing complexes, which may require overexpression of Cdc42\textsuperscript{271} or src \textsuperscript{203,294}. These aspects, such as context dependency and invadosomes as being dynamic structures, pose challenges in the studies of these structures. On the other hand, as liprin-α1 localized close to focal adhesions or in invadosomes in primary cell lines or after the leading edge in more motile cell lines, likely explains differences in
invasion properties of the cells. Data from motile cells indicate that liprin-α1 functions in regulating the shape and/or size of focal adhesions, which may have an impact on cell motility and cell spreading in these cells. Intriguingly, liprin-α1 has been shown to regulate the spreading of the cells and to be involved in integrin signaling\textsuperscript{196,197}. This implies that liprin-α1 is an important player in invasion through its association with invadosome and adhesive structures during cancer cell progression.

12.3 Liprin-α1 in the modulation of cytoskeletal elements related to adhesion

Gene expression profiling using microarrays and RNA sequencing revealed upregulation of vimentin expression in metastatic shPPFIA1 HNSCC cells with low endogenous vimentin expression in control (shScr) cells. In addition, liprin-α1 silencing led to a distinct disorganized vimentin network in breast cancer cells including decreased mesenchymal invasion and displacement of the vimentin network close to the nucleus. Vimentin is an important mediator in the epithelial-to-mesenchymal transition, and belongs to the intermediate filament family of cytoskeletal proteins\textsuperscript{240}, and it can be modulated by for example by specific phosphorylation\textsuperscript{250}. We showed that vimentin surrounded the liprin-α1 positive adhesion rings in the HNSCC cell line from a primary tumor. In addition, as discussed earlier, liprin-α1 knockdown influenced the size of the focal adhesions, when quantified from motile HNSCC or breast cancer cells, and where liprin-α1 was localized after the leading edge or near focal adhesions. The modulation of vimentin intermediate filaments possibly occurs through adhesive structures, and it is possible that liprin-α1 participates in the maturation and dynamics of focal adhesions in these cell lines, where cytoskeletal elements are crucially involved. This data implies that liprin-α1 is an important modulator of cell invasive capabilities through focal adhesions and invadosome structures, which are related to cytoskeletal elements, including intermediate filaments.
12.4 Contribution of liprin-α1 to cancer cell signaling and in modulation of tumor suppressor CD82

To get a better overview of cancer cell signaling and to identify pathways and biological processes that are differentially expressed in cells with liprin-α1 silencing, HNSCC and breast cancer cells were cultured in a 3D environment followed by RNA sequencing and GSEA analysis. Liprin-α1 knockdown led to enriched gene sets associated with several crucial pathways related to cell death, protein trafficking and cell-cell contacts, as well as cell-membrane or substrate contacts and membrane microdomain modulation. These data further supported the previous results showing liprin-α1 as an important player in cancer cell progression as well as in cell-cell communication. Importantly, the CD82 transmembrane protein was upregulated in shPPFIA1 cells. CD82 has been shown previously to act as a tumor suppressor for several solid tumors342, and in OSCC, its expression has been shown to reduce tumor volume354. These results were systematically observed in different cell lines and using different constructs to silence liprin-α1. In shPPFIA1 cells, CD82 was detected in endosomal-like vesicles as well as partly at the cell membrane in a 2D environment. However, specific marker for these vesicles could not be found, although partial co-localization was observed with caveolin-1. These vesicle-like structures may be very specific, or possibly endosomes with no EEA1 positivity. The availability of the extracellular matrix could at least partly explain the membrane localization of CD82 in 3D collagen I in shPPFIA1 cells. Silencing of CD82 in shPPFIA1 cells led to enhanced invasive potential in the metastatic HNSCC cell line, partly restoring the invasive growth capacity of the cells. As a conclusion, liprin-α1 is an important player in modulating CD82 expression and cell signaling events leading to cancer cell progression. Importantly, PPFIA1 amplification was associated with reduced survival in clinical HNSCC cases when TCGA data was studied. This data underlines the importance of studying the molecular signaling and pathways related to liprin-α1 function.
12.5 Association of liprin-α1 and CD82 expression in SCC of the oral cavity

Because liprin-α has a role in regulating the invasive growth capabilities of cells and it has shown to associate with the tumor suppressor CD82, it was important to study the expression of these proteins in clinical tumor samples. In the samples from the oral cavity, liprin-α1 and CD82 showed an inverse staining pattern of strong liprin-α1 and low or negative CD82 staining. Tumor samples with high liprin-α1 expression may be positive for 11q13 amplification and are possibly invasive or aggressive in nature. Several other genetic alterations are likely to appear as well as the genetic landscapes of the tumours vary individually, in addition to intratumoral heterogeneity. This data indicates the presence of high liprin-α1 expression in a subset of patients, where CD82 is low or negative. Correlation between the expression levels of liprin-α1 and CD82 was measured to be statistically significant at \( P<0.001 \). The correlation between survival and the expression of these proteins with clinical data would give more information, whether this would potentially have an effect on patient prognosis and survival. What would be interesting to study, would be the presence of invadosomes in patient tissue, and better resolution imaging for the fine structure would be needed in future studies. To study invadosomes \textit{ex vivo} or \textit{in vivo} might be challenging, due to the transient and dynamic character of the invadosomes, and these structures may form only at the site where the tumour cell invades into the surrounding environment or tissue. Invadosomes might appear only at the invasive front of the cells and be induced by the mechanosensing properties which are sensed from the environment\textsuperscript{20,273}. In addition, invadosomes may vary in structure, and be in the form of linear invadosomes, invadosomes with adhesion rings or as rosettes\textsuperscript{25}.

12.6 Effect of liprin-α1 in drug response to anticancer drugs

Because liprin-α1 has been shown to be expressed in patient samples and to have an effect on the invasive properties of cancer cell lines\textsuperscript{15,18,172,360}, it was important to study if liprin-α1 has an effect on the response to different anticancer drugs. Drug sensitivity and resistance testing was performed in a
metastatic breast cancer cell line with control cells and liprin-α1 knockdown cells and drug responses were measured by both cell viability and cell cytotoxicity assays. Cells with liprin-α1 expression were more sensitive to certain MEK inhibitors when compared to shPPFIA1 cells with both cytotoxicity and cell viability assays by DSS scores. In addition, shScr cells were more sensitive with several drugs related to cell cycle control and metastatic growth compared to shPPFIA1 cells. In addition, liprin-α1 knockdown sensitized cells to IAP inhibitors when compared to control cells, which are related to metastasis and migration, which may explain the better response in liprin-α1 knockdown cells. These data verify that liprin-α1 may be a possible indicator of drug response in metastatic breast cancer cells. Regarding the fact that liprin-α1 regulates cell invasion, this may explain why drugs inhibiting metastatic growth are more efficient in cells with liprin-α1 expression. BMS-777607, which is a c-met inhibitor and has activity towards c-met-axl-ron-tyro3, may potentially function through CD82, which was upregulated after liprin-α1 knockdown. Met has been shown to be related to CD82 function through c-src and c-met, and possibly BMS-777067 shares a similar mechanism in reducing the cell invasive properties related to cell invasive growth. The Aurora-kinase PF-03814735 inhibitor, CDK inhibitor (PHA-793887) and PLK-1 inhibitor, TAK-960 target cell cycle progression, and possibly have an effect on cell invasive growth. This data from the DSRT reveals vulnerabilities which could be of benefit for patients with high liprin-α1 expression. However, these results need to be further validated and potentially combinatorially tested using different compounds.
13. CONCLUSIONS AND FUTURE PROSPECTS

PPFIA1, which encodes the liprin-α1 protein, is located at the common 11q13 region showed to be amplified in cancer\(^{10,12,13,129}\). Liprin-α belongs to the LAR interacting protein family\(^{175}\), and has been shown to have functions in neuronal cells, as well as functions in non-neuronal cells\(^{14}\). Liprin-α has been shown to interact with LAR at focal adhesions\(^{17}\), and to have functions in the adhesive properties of cells\(^{16,196,197}\) as well as on the invasive capabilities of cancer cells\(^{15,200}\). The focus of this thesis was to clarify the function of PPFIA1 in different types of cancer cells. The first part of this thesis revealed that liprin-α1 is directly located in the invadosome structures in cell lines derived from primary tumors of HNSCC patients, and that these structures are able to degrade the extracellular matrix, which is important for cancer cell invasion. In motile cells, liprin-α1 localized after the leading edge and close to focal adhesions although it did not necessarily directly co-localized with adhesion proteins. The first part of this thesis revealed the connection of liprin-α1 with adhesion structures through vimentin intermediate filaments. Importantly, liprin-α1 was able to regulate invasive growth into the three-dimensional matrix, which was dependent on the cell line. The second part of this thesis corroborated the results from the first part. More specifically, in addition to breast cancer, liprin-α1 was found to be an important regulator of cell invasive growth into three dimensional collagen in cells from metastatic HNSCC as well. Gene set enrichment analysis revealed liprin-α1 to be an important part of the cell signaling events related to cancer cell signaling. Importantly, liprin-α1 was shown to modulate the CD82 transmembrane protein. CD82 has been shown to be a tumor suppressor in several solid tumors, and for activation may require different transcriptional or post-transcriptional mechanisms including palmitoylation or glycosylation\(^{326,341,342}\). Knockdown of liprin-α1 resulted in changes in the overall landscape of gene expression of several important gene sets, as this implies modifications in several signaling pathways, which may depend on the cell line and cell type. TCGA data clarified that PPFIA1 amplification leads to reduced survival in patients, and, in addition, in a recent study based on a TCGA data, PPFIA1 amplification has
been related to poorer survival in OSCC patients, which strengthens the results obtained from our study. Interestingly, strong liprin-α1 expression was detected in a subset of samples from HNSCC patients, which may implicate the presence of 11q13 amplification, and possibly indicates a more aggressive tumor. In the future, it would be important to study the role of liprin-α1 in more detail in drug response to validate the results from our drug screen or to study combinatorial drug experiments. In addition, invadosome structures should be studied in patient data with high-resolution microscopy, although this might be challenging due to the transient and dynamic nature of these structures. An interesting future experiment would be studying with vesicle markers the vesicle-like structures where CD82 was localized, and it may be related to endoplasmic reticulum regulation, which was one of the gene sets from GSEA results. Because liprin-α1 has been shown to be important in neuronal and non-neuronal cells, it would be important to study what kind of effect liprin-α1 has during development. Clinical data from the tissue microarray of HNSCC should be studied to determine if liprin-α1 and CD82 are important for the evaluation of individual patients for prognosis or survival. Other cancer types should be tested with patient data, most importantly breast cancer and different subtypes such as HER2 and luminal B.
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15. REFERENCES

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