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Analysis of signaling pathway activity in single cells using the *in situ* Proximity Ligation Assay

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Abstract

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A cell that senses signals from its environment uses proteins for signal transduction via post translational modifications (PTMs) and protein- protein interactions (PPIs) from cell membrane into the nucleus where genes controlling cell proliferation, differentiation and apoptosis can be turned on or off, i.e. changing the phenotype or fate of the cell. Aberrations within such proteins are prone to cause diseases, such as cancer. Therefore, it is important so study aberrant signaling to be able to understand and treat diseases.

In this thesis, signaling aberrations of PTMs and PPIs were analyzed with the use of the *in situ* proximity ligation assay (*in situ* PLA), and the thesis also contain method development of rolling circle amplification (RCA), which is the method used for signal amplification of *in situ* PLA reaction products.

Paper **I** considers the integrity of RCA products. Here, the aim was to generate a smaller and more compact RCA product, for more accurate either visual or automated analysis. This was achieved with the use of an additional so called compaction oligonucleotide that during RCA was able to bind and pull segments of RCA products closer together. The compaction oligonucleotide served to increase the signal to noise ratio and decrease the number of false positive signals.

The crosstalk between the Hippo and TGFβ signaling pathways were studied in paper **II**. Activity of the Hippo signaling pathway is regulated by cell density sensing and tissue control. We found differences in amounts and localization of interactions between the effector proteins of the two pathways depending on cell density and TGFβ stimulation.

In paper **III** the NF-κB signaling pathway constitutively activated in chronic lymphocytic leukemia (CLL) was studied. A 4 base-pair frameshift deletion within the NFKBIE gene, which encodes the negative regulator IκBε, was found among 13 of a total 315 cases by the use of targeted deep sequencing. We found reduced levels of IκBε protein, decreased p65 inhibition, and increased phosphorylation, along with increased nuclear localization of p65 in NFKBIE deleted cases compared to healthy cases.

Crosstalk between the Hippo and Wnt signaling pathway are studied within paper **IV**. Here, we found differences in cellular localization of TAZ/β-catenin interactions depending on colon cancer tumor stage and by further investigate Hippo/WNT crosstalk in cell line model systems we found an increase of complex formations involved in the crosstalk in sparse growing HEK293 cells compared to dense growing cells. Also, active WNT3a signaling was affected by cell density. Since cell density showed to have a big effect on Hippo/WNT crosstalk we continued to investigated the effect of E-cadherin, which has a function in cell junctions and maintenance of epithelial integrity on Hippo/WNT crosstalk. Interestingly, we found that E-cadherin is likely to regulate Hippo/WNT crosstalk.

Keywords: cell signaling, Wnt, Hippo, TGFB

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To My Friends and Family

List of Papers

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

I Clausson, C.M.*, **Arngården, L**.*, Ishaq, O., Krzywkowski, T., Koos, B., Brismar, H., Wählby, C., Nilsson, M., Söderberg, O. (2015) Compaction of rolling circle amplification products increases signal strength and integrity. *Scientific Reports.* 5, 12317; doi: 10.1038/srep12317

II Grannas, K.*, **Arngården, L.*,** Lönn, P., Mazurkiewicz, M., Blokzijl, A., Zieba, A., Söderberg, O. (2015) Crosstalk between Hippo and TGFβ - Subcellular localization of YAP/TAZ complexes. *Journal of molecular biology.* 427(21):3407-3415

III Mansouri, L., Sutton, L.A., Ljungström, V., Bondza, S., **Arngården, L.**, Bhoi, S., Larsson, J., Cortese, D., Kalushkova, A., Plevova, K., Young, E., Gunnarsson, R., Falk-Sörqvist, E., Lönn, P., Muggen, A.F., Yan, X.J., Sander, B., Enblad, G., Smedby, K.E., Juliusson, G., Belessi, C., Rung, J., Chiorazzi, N., Strefford, J.C., Langerak, A.W., Pospisilova, S., Davi, F., Hellström, M., Jernberg-Wiklund, H., Ghia, P., Söderberg, O., Stamatopoulos, K., Nilsson, M., Rosenquist, R. (2015) Functional loss of I B leads to NF-κB deregulation in aggressive chronic lymphocytic leukemia. *The Journal of Experimental Medicine*. 1;212(6):833-843

IV **Arngården, L.**, Löf, L., Grannas, K., Raykova, D., Zieba, A., Grabek, A., Oelrich, J., Figueiredo, J., Dahlin, J., Kamali, M., Seruca, R., Söderberg, O. Crosstalk between Wnt and Hippo signaling pathways changes upon colon cancer stage and is affected by cell density and loss of or mutated E-cadherin protein. *Manuscript.*

* Shared first author

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Related Work by the author

Original Articles

Koos B, Cane G, Grannas K, Löf L, **Arngården L**, Heldin J, Clausson CM, Klaesson A, Hirvonen MK, de Oliveira FM, Talibov VO, Pham NT, Auer M, Danielson UH, Haybaeck J, Kamali-Moghaddam M, Söderberg O. (2015) Proximity-dependent initiation of hybridization chain reaction. *Nature Communications*. 6:7294

Fristedt Duvefelt C, Lub S, Agarwal P, **Arngården L**, Hammarberg A, Maes K, Van Valckenborgh E, Vanderkerken K, Jernberg Wiklund H. (2015) Increased resistance to proteasome inhibitors in multiple myeloma mediated by cIAP2--implications for a combinatorial treatment. *Oncotarget* 6(24):20621-35

Book chapters

Koos B, **Andersson** L, Clausson CM, Grannas K, Klaesson A, Cane G, Söderberg O. (2014) Analysis of protein interactions in situ by proximity ligation assays. *Curr Top Microbiol Immunol*. 377:111-26

Contents

Abbreviations

AML	Acute myeloid leukemia
BcR	B cell receptor
CSC	Cancer stem cell
iRCA	Immuno rolling circle amplification
CTC	Circulating tumor cells
Co-IP	Complex-immunoprecipitation
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FRET	Förster resonance energy transfer
HRP	Horseradish peroxidase
HCR	Hybridization chain reaction
IMS	Imaging mass spectrometry
IF	Immunofluorescence
IHC	Immunohistochemistry
iRCA	Immune rolling circle replication
MS	Mass spectrometry
MET	Mesenchymal-epithelial transition
iRCA	Immuno rolling circle amplification
RCA	Immuno rolling circle amplification
PCP	Planar cell polarity
proxHCR	Proximity hybridization chain reaction
in situ PLA	*In situ* proximity ligation assay
PTM	Post translational modification
PPI	Protein-protein interactions
RCA	Rolling circle amplification
RCR	Rolling circle replication
SNR	Signal to noise ratio

Introduction

It is fascinating to reflect upon how life has developed with the four elementary components of our genome, Adenine, Thymine, Guanine and Cytosine holding the genetic information through evolution. Triplets of these four bases within our genome encode amino acids that are the building blocks of proteins. We know that the development of life is strictly programmed from the fertilized egg, through development and differentiation into all the cell types that composes the human body. However, errors can arise within our genes or proteins throughout this developmental process. In the germline the genetic errors are the fundament for evolution, but they can also cause diseases such as cancer.

Proteomics is a highly dynamic and complex world. It is created with the genome as a foundation, but it is dynamic due to environmental factors that can change protein activity through post translational modifications (PTMs) of proteins and changes in protein-protein interactions (PPIs). Changes in protein activity can in turn regulate what genes that are expressed and to what extent each gene should be expressed, processes that shape the phenotype of the cell.

I will herein guide you through the dynamic and complex world of proteomics, from genome to proteins and how aberrations of proteins may cause disease.

My research revolves around detection of protein functions, with the aim to generate a better understanding of underlying mechanisms of cancer at a proteomic level. Ultimately, the molecular mechanisms causing cancer are essential clues for how to finally treat diseases.

The biological studies I have conducted have involved analyses of PTMs and PPIs in signaling pathways such as the Hippo, TGF-β, Wnt and NFκB signaling pathways, which are pathways commonly deregulated in various types of cancers. However, to be able to study proteomics we need reliable molecular tools, suited for generating the molecular information we search for. My thesis work has also involved improvements of such a method, that is, enhancement of proximity ligation assays (PLA).

Proteomics

The term proteomics refer to the characterization of proteins. This characterization is a difficult task since the proteome is very complex and dynamic. Modulations and alterations in our genome are due to mutations and epigenetics, but there is also enormous complexity at the level of proteins. The Human Genome Organization estimate that our genome consists of around 21 000 genes and many of those genes generate multiple proteins. [1]. Several biological processes may intercede in the transfer of information from gene to a mature protein. One such process is alternative splicing, where introns are removed and exons joined together in different constellations to form a variety of mRNA molecules resulting in proteins with different composition although the proteins are encoded by the same gene. Moreover, distinct transcripts may be joined together during splicing so called trans-splicing[2] or adjacent transcription units can be transcribed together, called tandem chimerism[3]. The amount of mRNA and protein does not necessarily correlate; mRNA can quickly be degraded or get its translation regulated. To respond to external stimuli, the pool of proteins constantly needs to change in order to adapt to new conditions. In order to remove proteins a process called ubiquitination may be used to conjugate proteins with ubiquitin, targeting them for proteasomes and finally protein degradation. Proteins are often altered by PTMs to regulate their functions, such as phosphorylation, methylation, acetylation, glycosylation, oxidation and nitrosylation, catalyzed by enzymes. As an example, in phosphorylation a protein kinase adds a phosphate group to the protein either on the amino acids serine, threonine, tyrosine or histidine. Dephosphorylation is the removal of a phosphate group from the protein. Both phosphorylation and dephosphorylation can turn the activity of a protein either on or off. The phosphate group is negatively charged, which contributes to a negative charge of the protein causing conformational changes in the protein structure. This can result in exposure of active sites to which other proteins can bind. PTMs also influence the transport of proteins to different locations in a cell. The above may serve to illustrate some of the complexity of protein characterization. However, knowledge of human proteomics together with genome and mRNA data is necessary to better understand biological processes that help us understand the cause of diseases.

Cell signaling

I would define cell signaling as the ability of cells to process information from the surrounding environment into a response, such as cell proliferation, differentiation or apoptosis. Cell signaling can be initiated by environmental factors or by signaling molecules emanating from a cell itself or from sur-

rounding cells. A cell can produce intracrine signals that stay within the cell or send out autocrine signals that affect receptors on its own surface, paracrine signals that bind to cell surface receptors of nearby cells or endocrine signals in the form of hormones that can be transported with the blood stream to distantly located cells[4]. Cells also communicate by direct cell to cell contact - so called juxtacrine signaling[5]. A protein on its own cannot achieve much; it typically needs to physically interact with other proteins or molecules to start a biological process that can generate a cellular response. Upon binding of an extracellular signal to a cell surface receptor, the receptor changes conformation, a process that recruit other proteins located intracellularly to initiate the signaling transduction cascade through PTMs and PPIs. The signaling cascade finally arrives in the nucleus where transcription factors bind their target genes to initiate transcription of genes responsible for processes such as proliferation, differentiation or apoptosis. Since cell signaling controls these crucial functions, aberrant activities in any of these pathways are prone to cause diseases such as cancer, and the aberrant activity is thereby a potential target for anti-cancer therapy[6].

In the following sections I will describe a few of these signaling pathways. I have selected these particular pathways because they are the topic of three of four papers included in my thesis.

Hippo signaling

The Hippo signaling pathway controls organ growth by regulating cell proliferation and apoptosis. The pathway itself is controlled by cell density. Cell density causes mechanotransduction - that is translation of mechanical forces and deformations into biochemical signals that affect cells to make essential decisions such as cell proliferation, differentiation and apoptosis[7]. When cells grow in a dense cell population YAP/TAZ, effector proteins of the Hippo pathway, are located in the cytoplasm, where YAP/TAZ are subjected to proteasomal degradation. YAP/TAZ proteins are translocated to the nucleus in cells growing in sparse cell culture conditions, where these effector proteins can bind transcription factors for initiation of transcription[8]. This pathway is not mediated by a dedicated surface receptor; instead many different upstream pathways regulate Hippo signaling. When activated, MST1/2 kinase binds Sav1, which gets phosphorylated. Phosphorylated Sav1 in turn, activates LATS1/2, which can also be phosphorylated by Mob1. When activated, LATS1/2 phosphorylates the transcriptional coactivators YAP/TAZ[9]. This phosphorylation provides a priming signal for the kinase CK1 to add an additional phosphorylation onto YAP/TAZ protein. Upon phosphorylation by CK1, YAP/TAZ are recognized by the E3 ubiqui-

tin ligase β-TrCP which catalyzes YAP/TAZ degradation[10,11]. Also, it has been reported that Taz degradation depends on phosphorylated β-catenin that bridges TAZ to the ubiquitin ligase β-TrCP[12]. This process occur in a large protein complex called the destruction complex, which is part of Wnt signaling (**Figure 1a**). The whole picture of YAP/TAZ phosphorylation for further degradation in not fully understood. There might be additional molecular mechanisms contributing to YAP/TAZ degradation. In the off-state there is no phosphorylation of Sav1, which prevents signaling. This results in accumulation of YAP/TAZ in the cytoplasm that can be transported to the nucleus for transcriptional activation (**Figure 1b**)[9].

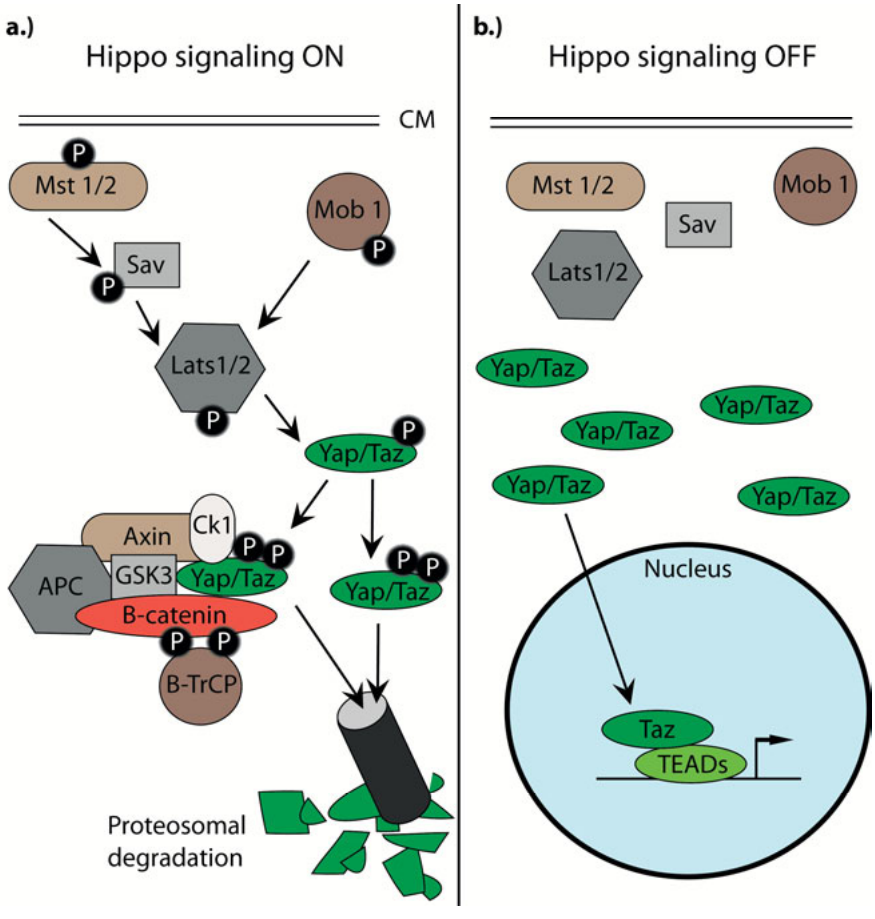

Figure 1. Illustration of Hippo signaling pathway.
a.) Active signaling is initiated by phosphorylated Mst1/2 that mediate phosphorylation of Sav, which in turn phosphorylates Lats1/2. Lats1/2 can also become phosphorylated via activated Mob1. When phosphorylated, Lats1/2 activate the effector proteins YAP/TAZ. Phosphorylated YAP/TAZ becomes degraded by proteosomal degradation via an additional phosphorylation by CK1 that makes YAP/TAZ recognizable by β-TrCP. This process is thought to occur both with and without YAP/TAZ association to the destruction complex, part of WNT signaling. b.) When Hippo signaling is inactive there is no phosphorylation of Lats1/2 and thereby no signal transduction. YAP/TAZ accumulates in the cytoplasm and is translocated to the nucleus where YAP/TAZ can initiate transcription.

Wnt signaling

There are three known pathways activated by the WNT receptor, namely the WNT/$^{Ca2+}$ pathway, the canonical WNT/β-catenin cascade, and the non-canonical WNT pathway (the non-canonical planar cell polarity (PCP) pathway)[13]. Among these pathways, the canonical pathway has been the subject of most research and is best known.

When canonical WNT/β-catenin signaling is in an OFF state (**Figure 2a**), cytoplasmic β-catenin levels are kept low by continuous proteasome-mediated degradation, controlled by the β-catenin destruction complex: GSK-3, APC, Ck1 and Axin. Axin serves as a scaffold for the other proteins. Ck1 and GSK3 are responsible for β-catenin phosphorylation, which makes β-catenin recognizable by β-TrCP and thereby targeted for ubiquitination and degradation by the proteasome[14,15]. A recent study has shown that it is essential for YAP/TAZ, the effector proteins of Hippo signaling, to be present within the destruction complex for recruitment of β-TrCP[16].

WNT signaling is initiated when the WNT ligand binds the extracellular N-terminal domain of the transmembrane receptor Frizzled (**Figure 2b**). The Frizzled receptor forms a complex with the transmembrane protein LRP, which results in the recruitment of the scaffolding protein Dvl. Dvl activity is critical for WNT signaling and its activation has been demonstrated to depend upon CK1δ/ε activity[17]. When activated, Dvl phosphorylates LPR6, a process that recruits Axin to the receptor complex[18], which inhibit further β-catenin degradation. B-catenin accumulates in the cytoplasm and is translocated into the nucleus where it binds to the transcription factors TCF/LEF that activate transcription of WNT target genes[13,19].

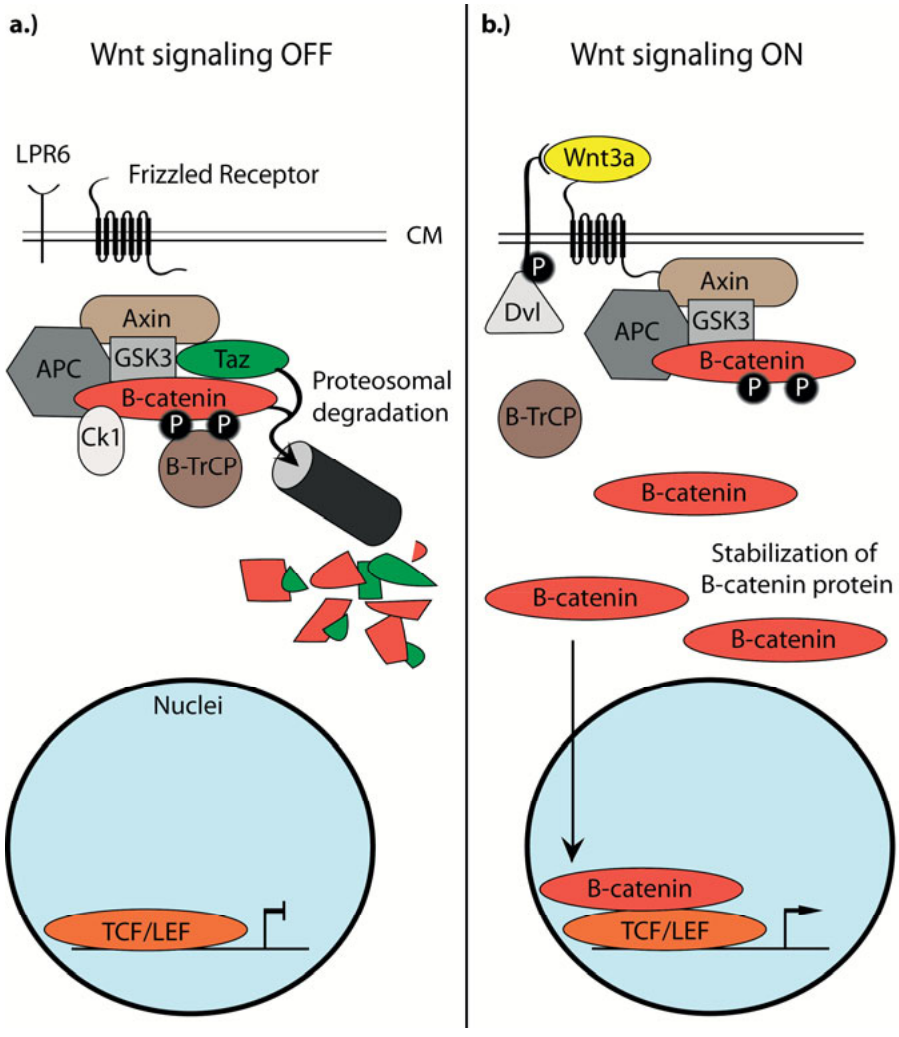

Figure 2. Illustration of Wnt signaling pathway.
a.) When Wnt signaling is inactive, β-catenin protein levels are kept low by proteosomal degradation via the destruction complex, composed of GSK-3, APC, Ck1 and Axin. Ck1 and GSK3 are responsible for β-catenin phosphorylation, which makes β-catenin recognizable by β-TrCP and thereby targeted for ubiquitination and degradation by the proteasome. b.) Active Wnt signaling is initiated when Wnt3a ligand bind the Frizzled receptor, that form a complex with the transmembrane protein LPR6. This receptor complex formation recruits Dvl, which phosphorylates LPR6, which further recruits Axin to the receptor complex, resulting in inactivation of the destruction complex. B-catenin accumulates in the cytoplasm and translocate to the nucleus where it can bind transcription factors for initiation of transcription.

TGFβ signaling

TGFβ signaling is initiated when the TGFβ cytokine bind the TGFβ type II and type I serine/theorine kinase receptors at the cell surface. This result in a formation of a TGFβ-activated heterotetrameric ligand-receptor complex[20]. Upon activation of the type I kinase receptor, the receptor phosphorylates receptor-regulated R-Smads, Smad 2/3, which in turn induce heterodimeric complex formation of Smad2/3-Smad4. Smad2/3-Smad4 complexes are then transported to the nucleus where they are able to initiate transcription of target genes (**Figure 3a**). During TGFβ-induced signaling, Smad-independent signaling pathways are also activated, including MAP kinase, Rho-like GTPase and PI3K/AKT pathways, allowing extensive variation of TGFβ family responses[21,22]. In an OFF state, no receptor complexes are formed that can phosphorylate Smad2/3 and no complex formation between Smad2/3-Smad4 occur. Smad proteins are located in the cytoplasm (**Figure 3b**)[23].

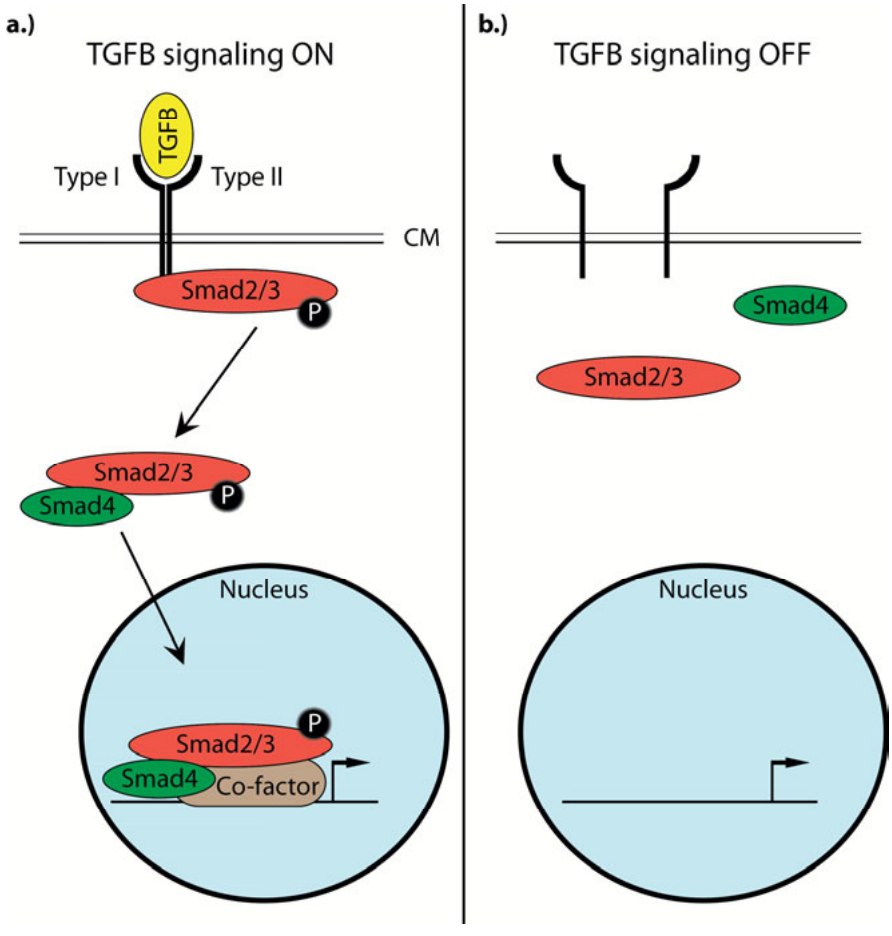

Figure 3. Illustration of TGFβ signaling pathway. a.) In active signaling the TGFβ cytokine bind the TGFβ type II and type I serine/theorine kinase receptors that form a ligand-receptor complex. Upon this receptor complex formation, Smad2/3 become phosphorylated and form complexes with Smad4 for further translocation to the nucleus where initiation of transcription of target genes occur. B.) In inactive TGFβ signaling there is no receptor complex formation. Smad proteins are located in the cytoplasm.

NF-κB signaling

The NF-κB signaling pathway regulates cellular processes such as cell cycle progression, differentiation and apoptosis[24]. There are two NF-κB activation pathways, both initiated by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and pathogen-associated molecular patterns (PAMPs). The classical pathway is best known (**Figure 4a**). Upon stimulation of the classical signaling pathway, cell surface receptors such as toll like receptors (TLRs), the antigen receptors TCR/BCR, or lymphocyte coreceptors such as CD40, CD30, or receptor activator of NF-κB (RANK) activate the IKK complex. This complex involves IKKα, IKKβ and IKKγ, where IKKγ is essential for IKKβ to catalyze phosphorylation of the NF-κB inhibitor κBs (IκBs). The phosphorylation results in polyubiquitination of IκBs for further degradation. Degradation of IκBs releases NF-κB1(p105)/RelA (also known as p65) heterodimers. The complex is further activated by PTMs that prime part of the p105 protein for proteasomal degradation resulting in release of the p50 protein. A heterodimer composed of NF-κB1(p50)/RelA is now able to translocate to the nucleus for further initiation of transcription[24]. The other pathway is the alternative pathway (**Figure 4b**), which is activated via cell surface receptors that belong to the TNF cytokine family, including CD40, the lymphotoxin β receptor, and the BAFF receptor. Here, it is only the IKKα homodimers that phosphorylates the C-terminus of NF-κB2(p100), a process that partly degrade p100 resulting in formation of the p52 protein[25]. NF-κB2(p52) in a complex with RelB is then translocated into the nucleus[26]. This is a simplified version of the NF-κB signaling pathways. In reality there are both homo- and hetero dimers formed between the five transcription factors NF-κB1/2, NF-κB2, RelA, RelB and c-Rel[27]. There are studies indicating that the two pathways have different functions, where the classical pathway is mostly involved in innate immunity[28,29] and the alternative pathway might have a function in adaptive immunity[30,31].

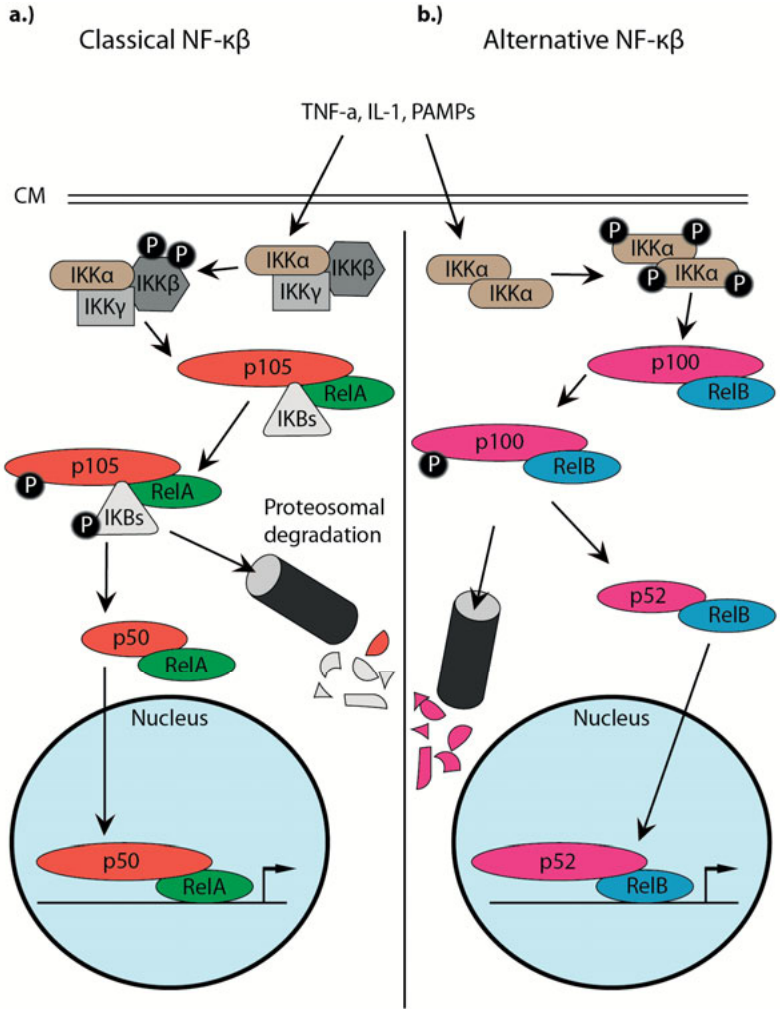

Figure 4. Illustration of NF-κB signalingsignaling pathway. NF-κB signaling is initiated by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and pathogen-associated molecular patterns (PAMPs). a.) In the classical NF-κB signaling pathway the activated IKK complex, composed by IKKα, IKKβ and IKKγ mediate phosphorylation of the NF-κB inhibitor κBs (IκBs). The phosphorylation results in polyubiquitination of IκBs for further degradation. Degradation of IκBs releases NF-κB1(p105)/RelA (also known as p65) heterodimers. The complex is further activated by PTMs that prime part of the p105 protein for proteasomal degradation resulting in release of the p50 protein. A heterodimer composed of NF-κB1(p50)/RelA is now able to translocate to the nucleus for further initiation of transcription. b.) In the alternative NF-κB signaling pathway activated IKKα homodimers phosphorylate NF-κB2(p100), a process that partly degrade p100 resulting in formation of the p52 protein. NF-κB2(p52) in a complex with RelB is translocated into the nucleus for further initiation of transcription.

Tumor heterogeneity

A tumor is developed from one single cell subjected to an oncogenic mutation, resulting in higher proliferation rate and genetic instability, something that increases the risk of new mutations to occur, thus opening up for the generation of tumor cells with a broad genetic and epigenetic diversity[32].

There are two main concepts for how tumors develop. The clonal evolution model is based on the notion that cancer cells over time acquire mutations, and by natural selection of the fittest and most aggressive cells tumors may develop[33]. The second concept is the Cancer Stem Cell (CSC) hypothesis, where CSCs are thought to be the only cells contributing to tumor progression. This is in contrast to the clonal evolution theory where all tumor cells are thought to have the potential to drive cancer progression. However, a cell with a mutation must proliferate to cause disease. The proliferation capacity decreases through cell differentiation, i.e. a stem cell have a higher proliferation rate and thereby also a higher risk of gaining mutations that cause cancer progression, rather than a mature cell with low or no proliferation capacity. The CSC concept was first established in acute myeloid leukemia (AML) over 20 years ago[34]. Since then CSCs have been found in a variety of solid tumors[35]. Depending on the model distinct therapeutic strategies may be better suited to cure cancer. Cancer derived through the CSC hypothesis might be efficiently cured if a specific population of cells are targeted, namely the CSC cells, while a therapeutic strategy to cure a cancer developed by clonal evolution would need to target multiple cell populations[36,37].

In the end, the malignant tumor consists of tumor sub-populations with different molecular profiles, morphology, and expression of specific markers. For instance, in patients with gastric cancer, measurement of levels of HER2 is essential for efficient selection of patients that benefit from therapies with trastuzumab, a therapeutic antibody that targets HER2. However, the amount of HER2 protein expression in these tumors varies between subpopulations, which may potentially contributed to inaccurate assessment of HER2 status[38]. Simultaneous treatment with several drugs targeting different tumor sub-populations might be necessary in some tumors to achieve a successful treatment.

In personalized medicine a fundamental idea is to base the choice of treatment on the unique molecular profile of each patient, but the analysis may be prone to errors if the analysis is based on a single biopsy sample from a tumor. Analyses of mutations in biopsy samples obtained from primary renal carcinomas and associated metastatic sites reveal that 63-69% of all somatic mutations were undetectable across every tumor region[39]. The fact that intratumoral heterogeneity is so diverse creates a huge challenge for introducing personalized medicine into health care.

Tumor microenvironment

The tumor microenvironment refers to the area of the tumor, but also its surrounding, including blood vessels and stromal cells, signaling molecules, immune cells and extracellular matrix (ECM). These factors, together or alone may support processes such as tumor growth and invasion, by protecting the tumor cells from host immunity and provide niches for metastases to thrive.

All tissues and organs have their own unique microenvironment and each cell in the human body has differentiated to assume a particular molecular profile, fulfilling the requirements for survival in its tissue or organ microenvironment. To obtain malignant growth the tumor cells need to interact with both genetically alternated "tumor" cells and "healthy" cells, as well as the dynamic microenvironment in which they live[40]. One type of "healthy" cells that the tumor cells have to avoid is the host´s immune system that aims to eliminate tumor cells. However, in many cancers the tumor cells succeed to escape host immunity. Tumor cells might survive due to selection of clones capable of escaping identification by the host immunity or by suppressing the immune response[41].

Tumor cells often grow uncontrollably due to mutations causing loss of apoptotic signals and because of short circuits of signaling pathways, rendering the cell independent on upstream signaling. These fast growing tumor cells are in need of oxygen and nutrition, which results in less oxygen and nutrition for surrounding tumor and non-tumor cells. The high demand for oxygen and nutrients makes angiogenesis a requirement for tumor growth. Despite the ability of tumor cells to induce angiogenesis the high growth rate of a tumor creates big tumor masses that grow further away from blood vessels, resulting in hypoxia within the tumors. Hypoxia results in a further increase of angiogenic growth factors[42] and it is associated with genetic instability of tumor cells such as downregulation of DNA repair mechanisms[43] causing tumor progression and an increase in tumor heterogeneity[39].

Some tumor cells differentiate into motile mesenchymal cells, a process called epithelial-mesenchymal transition (EMT). Upon EMT the cells lose their junctions and apical/basal polarity and generate a migratory and invasive phenotype. EMT is an essential process in for example mesodermal formation, neural tube formation, tissue repair and stem cell behavior. The phenomenon of EMT is also found in circulating tumor cells (CTCs) that cause metastasis[44,45]. A tumor cell that has undergone EMT has to change back to its original phenotype by mesenchymal-to- epithelial transition (MET) to be able to give rise to a new tumor. However, when the MET is completed the cell might have ended up in a new microenvironment where

the cell does not obtain the traits needed to survive. The tumor cell would probably die, but some might be able to adapt to the new environment and thereby survive, or alternatively, the cell would change the environment to fit its needs. CTCs can also return to its original tumor, a phenomenon called self-seeding which has been confirmed in various experimental models[46]. Tumor metastasis can also be initiated by exosomes[47,48]. Exosomes are cell-derived vesicles consisting of a lipid bilayer membrane surrounding a small cytosol that contain various molecular constituents from its cell of origin, including proteins and nucleic acids[49]. Exosomes are present in most biological fluids and can also be used for paracrine signaling[50] and they may have pro-tumorigenic effects on many different cell types within the tumor microenvironment[51]. In conclusion, the microenvironment plays an important role in tumor development, survival and metastasis. It would be impossible to understand the biology of cancer without taking the microenvironment into account.

Methods for detection of single proteins and protein-protein interactions

There are several available methods for protein detection, each of them having their advantages and constraints. The most suitable choice of method for a biological application depends on many parameters such as sample material, available reagents and instruments, whether the target of interest is of high or low abundance, what the requirements are for resolution, and depending on the specific biological question to be addressed.

Immunofluorescence (IF) and immunohistochemistry (IHC) are methods based on antibody recognition used for protein detection *in situ*. The antibodies can either be labeled with fluorophores or enzymes, e.g. horseradish peroxidase (HRP), and they are visualized by microscopy or in the case of IF also through flow cytometry. The limit of detection is affected by the resolution of the microscope used. To be able to detect single antibodies bound to a sample using a standard epi fluorescence microscope it is necessary to amplify the signal and it is of particular importance to be able to increase the signal to noise ratio (SNR) if biological samples have high auto fluorescence. An increased SNR is achieved with methods such as immune rolling circle amplification (iRCA) [52] for single protein detection and using the *in situ* proximity ligation assay (*in situ* PLA) [53] for detection of either single proteins with improved specificity, or of PPIs and PPMs *in situ*. Both methods are based on rolling circle amplification (RCA) for signal amplification. RCA requires a circular DNA template to which a primer DNA sequence is hybridized for the creation of a free 3´end to which the phi29 DNA polymer-

ase binds and initiates replication. The RCA product will be a concatemeric molecule of several copies of the reverse complementary sequence of the original DNA circle. Several hundred such repeats can be present in a single molecule. By utilizing a fluorophore-labeled oligonucleotide that is complementary to the RCA product each RCA product can be labeled with several hundred fluorophores. Detection of iRCA and *in situ* PLA signals can be performed either by microscopy or flow cytometry. *In situ* PLA can provide increased specificity of detection of PTMs or single proteins, compared to e.g. immunofluorescence or immunohistochemistry, because *in situ* PLA is based on two antibody binding events in order to generate a signal rather than just one. There are other alternatives to *in situ* PLA that do not require enzymes for signal generation, such as the Proximity hybridization chain reaction (proxHCR)[54] for detection of single proteins or PPIs and PPMs *in situ.* This method involves signal amplification via a hybridization chain reaction (HCR)[55] instead of RCA. *In situ* PLA signals are easier to quantify than products of proxHCR due to the distinct, countable dots generated from a target detection events, while proxHCR generate signals that are quantified by total immunofluorescence. HCR presents advantages over RCA however, in that HCR does not require enzymes for signal generation, making it less expensive and simplifying storage of reagents.

Other methods also used for detection of PPIs are Förster resonance energy transfer (FRET) and CO-IP. FRET can be used for measuring PPIs with the help of a pair of antibodies labeled with a donor and an acceptor fluorophore, respectively. When antibodies carrying donor fluorophores bind in proximity to antibodies carrying acceptor fluorophores, energy is transferred from excited donor fluorophores to acceptor fluorophores and light is emitted from the acceptor at longer wavelengths than those of the donor's emission spectra. As with IF and IHC, FRET has a low signal to noise background but the advantage of FRET is that it can be used *in vivo* [56]. Co-immunoprecipitation (Co-IP), or pull down as it is sometimes called, is also used for detection of protein- protein interactions, but only in cell lysates. Agarose beads coupled with antibodies are incubated together with the cell lysate. The antibody binds its target epitope. The beads are washed and remaining proteins are the target protein of the antibody and any possible interaction partners of that protein. The immunoprecipitate may be denatured to dissociate interacting proteins, for further fractionation by SDS PAGE, followed by transfer onto a membrane. The membrane is incubated with HRP or fluorophore labeled antibodies detecting interacting proteins[57]. In addition, it is possible to find new, unknown protein interactions by analyzing pulled down proteins by mass spectrometry (MS)[58]. In MS the whole protein sample is fragmented into peptides by e.g. trypsin digestion and subsequently ionized by an electron beam, generating charged fragments that

can be separated according to their mass and charge ratio by acceleration in an electric or magnetic field[59]. MS is able to detect and identify in principle all abundant proteins but the sensitivity is limited for low abundant proteins. It is possible to combine MS with imaging to provide spatial information of the analytes, so called imaging mass spectrometry (IMS)[60]. This combined method divides a tissue section into spots down to 100 μm in diameter for MS measurement followed by an image reconstruction of the tissue section where MS data is linked to the spatial localization spot within the tissue section analyzed. The advantages of this method are that it is label free and it allows multiplex analysis of hundreds to thousands of molecules. To further increase the spatial resolution, it is possible with the help of mathematical predictions to combine IMS measurement with a resolution of 100 μm with optical microscopy maps obtaining a resolution of 10 μm[61]. However, single cell resolution or protein localization within a cell is not possible. To detect proteins at the single cell level it is possible to use a single cell mass cytometer. This method stains cells with antibodies labeled with lanthanides, where each antibody is labeled with metals of distinct masses. The cells are then nebulized into single cell droplets and each cell is vaporized by argon plasma to generate ionization of the cells atomic constituents for detection via MS. So far 34 parameters have been measured with this method in single cells[62]. It is also possible to use antibodies labeled with lanthanides to detect protein localization in single cells[63].

Antibodies as affinity binders

Many of our techniques that detect proteins rely on affinity binders. This imposes certain demands on the affinity binders. First of all we want the binders to be specific, recognizing the correct target and only to a minimal extent any other proteins. Secondly they should have a high affinity in order to generate a strong and stable binding to the target antigen. The most commonly used binders today are antibodies. Antibodies are produced in different ways. Polyclonal antibodies are produced by antibody enrichment from blood of animals immunized with the target antigen [64]. Monoclonal antibodies are produced by fusion of a myeloma cell line with B-lymphocytes from animals immunized with the antigen. This generates an immortal cell line that produces the desired antibody[65]. Polyclonal antibodies rely on one single animal for production and no more lots can be produced after the animal's death. This is in contrast to monoclonal antibodies that are a replenishable resource. In addition, the antibody profile changes over time, which means that blood samples obtained at different time points from the same animal can differ. Also, polyclonal antibodies are the products of several B cell clones, where clones may differ in affinity and target different epitopes on the target antigen. Monoclonal antibodies, by contrast, derive from a single

clone. Binders can also be produced entirely through *in vitro* technologies - so called recombinant affinity reagents. These are produced by phage din situ PLAy where a degenerated library of Ig gene segments or other scaffolds such as DARPins[66] are expressed as a vast repertoire of proteins to ensure that individual molecular clones with affinity against any possible target can be found. By doing multiple rounds of selection, the phages with highest affinity can be selected from the pool [67].

As already mentioned it is common with an insufficient lot-to-lot consistency of polyclonal antibodies. To fully trust results generated from antibody based assays they need to be properly validated to determine if the antibody cross-reactivity is acceptable. Validation can be performed using biological samples with different expression of the target antigen of the antibody, such as protein knock-outs or by stimulation of a certain receptor on a cell line resulting in up or down regulation of the target antigen. Another type of antibody validation is to use technical approaches, such as MS on products immunoprecipitated from a cell lysate by an antibody to determine which protein the antibody binds to. If there is an existing well validated antibody for the same target antigen as a newly produced antibody, the new antibody can be validated by comparison with the old antibody.

I believe that with higher demands of antibody validation, where the requirements of the antibody are high specificity and affinity for its target antigen, along with requirements for consistency between different lots, recombinant antibodies will be more commonly used in the future.

The *in situ* proximity ligation assay (PLA)

The *in situ* proximity ligation assay (PLA) is a technique used to detect single proteins with high specificity as well as PPIs and PTMs of proteins. The method requires two antibodies that are conjugated to DNA oligonucleotides, so-called PLA probes. When two PLA probes bind their target molecule in proximity (**Figure 4a**) the conjugated DNA strands can template hybridization and ligation of two added oligonucleotides, creating a circular DNA template (**Figure 4b**). This circular DNA template next serves as a template for an RCA reaction (**Figure 4c**). One of the PLA probes has a free 3´end from which phi29 DNA polymerase can start amplification of the circular DNA template. The other probe is blocked with a 2´O-methyl group to prevent priming. Phi29 DNA polymerase can amplify the circular DNA template, creating a long DNA strand with up to 1000 copies of the complement of the DNA circle, folded up in a ball with a size of approximately 1 µm. To detect the amplified DNA template, fluorophore labeled detection

oligonucleotides are hybridized to a complementary sequence in the RCA product (**Figure 1d**). These detection oligonucleotides are then locally visualized in cells by flow cytometry or microscopy [53].

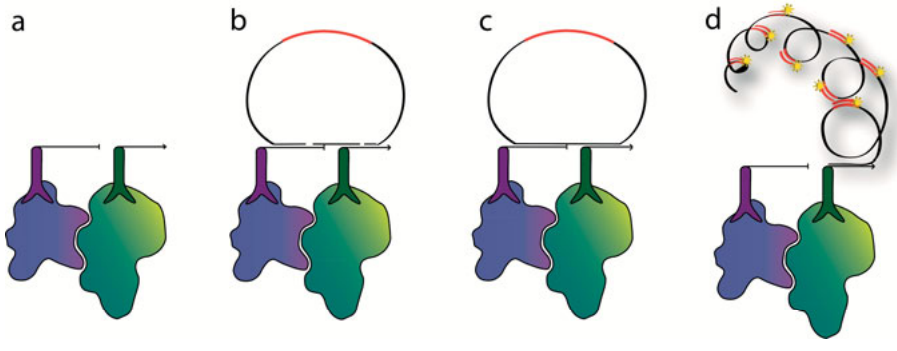

Figure 5. Illustration of the *in situ* proximity ligation assay (in situ PLA). a.) PLA probes bind to their target epitope. b.) One long and one short circularization oligonucleotide hybridize to the PLA arms of the PLA probes. The red part of the long circularization oligonucleotide represents a sequence complementary to the fluorophore labelled oligonucleotide that are added later in the procedure. c.) The two circularization oligonucleotides are ligated together by T4 ligase to form a DNA circle. d.) The enzyme phi29 amplify the DNA circle and at the same time, fluorophore labelled oligonucleotides bind to their complementary site (red part) on each DNA circle.

Present investigations

Paper I: Compaction of rolling circle amplification products increases signal integrity and signal-to-noise ratio.

Introduction

RCA is used for signal amplification of PLA detection events. The dynamic range of PLA has been a limiting factor. Therefore, a new oligonucleotide design was previously developed, which uses several unique circularization oligonucleotides at different ratios, with different sequences to which the detection oligonucleotides hybridize [68]. Another limiting factor of PLA is that even if each single detection event is amplified by RCA, still some tissues, for example those from brain that suffer from high autofluorescence, making it difficult to distinguish signals from the background autofluorescence. In paper I we present a solution to improve the dynamic range and SNR by the use of compaction oligonucleotides.

Aim

The aim of paper I was to generate more compact RCA products while retaining all fluorescence. The RCA products will hence be smaller and brighter, providing a higher SNR.

Procedures, findings and discussion

The compaction oligonucleotides are designed so that both ends hybridize to the same complementary hybridization site of the RCA product, bringing these regions closer together to form smaller and more intensely fluorescent RCA products. We also found that regular RCA products may disintegrate something that was prevented with addition of the compaction oligonucleotide during RCA. This finding prevents detection of false positive signals arising from cleavage or elongated distribution of individual RCA products.

Paper II: Crosstalk between Hippo and TGFβ: Subcellular Localization of YAP/TAZ/Smad Complexes.

Introduction

The Hippo signaling pathway is regulated by cell density and it controls organ growth by regulating cell proliferation and apoptosis. TGFβ is a multi-functional cytokine that activates downstream intracellular signaling of Smad proteins by interacting with the TGFβ type II and type I serine/ threonine kinase receptors. Aberrations within Hippo and TGFβ signaling pathways are linked to tumorigenesis. These pathways are well studied on their own, but there are many missing pieces of the molecular mechanisms behind the crosstalk between Hippo and TGFβ signaling. The effector proteins of the Hippo signaling pathway YAP/TAZ have been shown to interact with Smads in the TGFβ signaling pathway. Interactions between YAP/TAZ and Smads have been shown to influence the nuclear and cytoplasmic shuttling of Smads by sequestering Smads in the cytoplasm of confluent cells[69]. Smad signaling has also been shown to be reduced upon TGFβ stimulation in dense growing cells[69].

Aim

The aim of paper II was to investigate formation and cellular localization of YAP-Smad2/3 and TAZ-Smad3/3 protein complexes in relation to cell density and TGFβ stimulation.

Procedures, findings and discussion

In situ PLA was used to study YAP-Smad2/3 and TAZ-Smad3/3 interactions in HaCaT and HT29 cells with regard to cell density and TGFβ treatment. We found that YAP/TAZ-Smad2/3 complexes were abundant in sparsely growing HaCaT cells and that they were predominantly located in the nucleus. In dense cultures of cells the complexes were fewer and mainly located to the cytoplasm. No YAP/TAZ-Smad2/3 complexes were found in HT29 cells, which do not express the Smad 4 protein. Smad4 forms complexes with Smad2/3 proteins upon TGFβ treatment. These complexes are further translocated to the nucleus, where they orchestrate transcription together with transcriptional activators or repressors. To examine if the deficiency of Smad4 could account for the absence of YAP/TAZ-Smad2/3 complexes in HT29 cells, we treated HaCaT cells with siRNA targeting Smad4. However, after siRNA treatment of HaCaT cells, complexes were still formed, which excluded Smad4 deficiency as a possible mechanism for the absence of YAP/TAZ-Smad2/3 in HT29 cells.

Paper III: Functional loss of IκBε leads to NF-κB deregulation in aggressive chronic lymphocytic leukemia.

Introduction

The NF-κB signaling pathway regulates processes such as cell cycle progression, differentiation and apoptosis. This pathway is constitutively active in chronic lymphocytic leukemia (CLL) and the molecular mechanisms behind are largely unknown. In 2014, Damm *et al.*[70] found a recurrent 4-bp truncating mutation in the NFKBIE gene, encoding IκBε, which is a negative regulator of NF-κB signaling. However, the consequence and function of this 4-bp truncating mutation is unexplored.

Aim

The aim of paper III was to investigate the NF-κB signaling pathway in CLL for protein aberrations that could underlie the constitutively active NF-κB signaling in CLL.

Procedures, findings and discussion

A targeted gene panel for deep sequencing of 18 members of the NF-κB pathway was used to screen 315 CLL cases. The NFKBIE gene was the gene most frequently mutated. A 4-bp deletion was found in 13 of these 21 cases carrying mutations in the NFKBIE gene. Compared with healthy patients, the NFKBIE-deleted cases showed reduced IκBε protein levels by western blot analysis and a decrease of IκBε/p65 interactions by Co-IP, along with increased phosphorylation and nuclear translocation of p65, which is potentially underlying a more active state. In addition, by studying interactions between p65 and all IκBs (IκBα, IκBβ, IκBε) using *in situ* PLA in six *NFKBIE* WT unstimulated CLL cases, IκBε exhibited the greatest number of interactions with p65 per cell analyzed, supporting its important role in CLL.

B cell receptor (BcR) signaling has a central role in CLL pathobiology. Aggressive disease with poor prognosis is seen in patients with distinctive stereotyped BcRs[71]. The reduced levels of IκBε protein was primarily found in CLL patients with aggressive disease, which could be a possible mechanism behind the aggressive subsets of CLL.

Paper IV: Crosstalk between Wnt and Hippo signaling pathways changes upon colon cancer stage and is affected by cell density and loss of or mutated E-cadherin protein.

Introduction

This paper investigates the crosstalk between Hippo and Wnt signaling pathways. As described in paper II, the Hippo signaling pathway is regulated by cell density, and it controls organ growth by regulating cell proliferation and apoptosis. The effector proteins of Hippo signaling, YAP/TAZ has been proven to interact with the destruction complex, part of the Wnt signaling pathway. YAP/TAZ has been shown to be essential for β-catenin proteasomal degradation and vice versa[12,16].

The E-cadherin protein is located in cell junctions and can form complexes with β-catenin. B-catenin protein levels are regulated by the destruction complex via proteasomal degradation. Patients with gastric cancer often have mutated E-cadherin protein, which has been shown to change the tendency for E-cadherin and β-catenin to interact[72]. However, there are no reports as to how or if loss of or mutated E-cadherin has any effect on the crosstalk between Hippo and Wnt signaling.

Aim

The aim of paper IV was to investigate the crosstalk between Hippo and Wnt signaling pathways, in regard to cell density, Wnt treatment and the absence of E-cadherin protein, along with different E-cadherin mutations associated with gastric cancer.

Procedures, findings and discussion

In situ PLA was used to study localization and formations of protein interactions that are involved in the Hippo/WNT signaling crosstalk. We found differences in cellular localization of TAZ/β-catenin interactions depending on colon cancer tumor stage and continued to investigate the crosstalk in several model systems mimicking different cancer circumstances. We found large increases in complex formations of protein interactions involved in the WNT/Hippo crosstalk of sparse growing HEK293 cells compared to dense growing cells. Cell density also affected the cellular response to WNT3a treatment. To further investigate the role of cell densities on Hippo/WNT crosstalk we aimed to investigate the role of E-cadherin protein, which is an

important protein for maintenance of epithelial integrity via cell contact inhibition on the Hippo/WNT crosstalk. CHO cells which naturally do not express E-cadherin were transfected with WT E-cadherin and E-cadherin carrying missense mutations, associated to hereditary diffuse gastric cancer (HDGC) cases. These mutations cause a more invasive pathogenesis in comparison to cases expressing the wild-type (WT) E-cadherin. We found that protein complex formations part of WNT/Hippo crosstalk were affected by loss of or mutated E-cadherin and suggest that E-cadherin dependent cell-cell adhesion is likely to regulate Hippo/WNT crosstalk.

Conclusions

My research revolves around detection of protein functions, with the aim to generate a better understanding of underlying mechanisms of cancer at a proteomic level. However, to be able to study proteomics we need reliable molecular tools, suited for generating the molecular information we search for. My thesis work has also involved improvements of such a method, that is, enhancement of proximity ligation assays (PLA).

Paper I deals with development of *in situ* PLA. *In situ* PLA is used to study proteins, PTMs, and PPIs. The use of compaction oligonucleotides for *in situ* PLA, as presented in paper I, create more compact and smaller RCA products with increased signal to noise ratio and minimizing risks of false positive signals during analysis.

I have used *in situ* PLA in papers II, III and IV to study aberrations of cell signaling.

In paper II the crosstalk between Hippo and TGFβ signaling was studies in regard to cell density and treatment with TGFβ. We found that YAP/TAZ-Smad2/3 complexes were abundant in sparsely growing HaCaT cells and were predominantly located in the nucleus, while in dense cultures the complexes were fewer and mainly located to the cytoplasm. No YAP/TAZ-Smad2/3 complexes were found in HT29 cells.

In paper III a recurrent 4-bp deletion in the NFKBIE gene was found by deep sequencing in 13 out of 315 CLL cases. Cases with the 4-bp deletion showed reduced IκBε protein levels and decreased p65 inhibition, along with increased phosphorylation and nuclear translocation of p65 compared to healthy patients.

In paper IV the crosstalk between Hippo and Wnt signaling was studied. We found differences in cellular localization of TAZ/β-catenin interactions depending on colon cancer tumor stage and by further investigate Hippo/WNT crosstalk in cell line model systems we found an increase of complex formations in sparse growing HEK293 cells compared to dense growing cells. Also, active WNT3a signaling was affected by cell density. Since cell density showed to have a big effect on Hippo/WNT crosstalk we continued to

investigated the effect of E-cadherin, which has a function in cell junctions and maintenance of epithelial integrity on Hippo/WNT crosstalk. We found that E-cadherin is likely to regulate Hippo/WNT crosstalk.

Future perspectives

In situ PLA is used to study single proteins as well as PTMs and PPIs in signaling pathways. However, there is a need to simultaneously monitor several events in different signaling pathways in a cell or tissue. A recent modification of *in situ* PLA concerns parallel analysis of multiple protein complexes. It is now possible to introduce unique tags to each *in situ* PLA reaction product and thereby monitor multiple events in parallel[73]. Since, *in situ* PLA uses fluorophores to detect and distinguish the events, we are limited in multiplexing of the assay. This is because fluorophore emission spectra often overlap. A solution to this problem is achieved by read out via mass cytometry where lanthanides are used instead of fluorophores[74]. Alternatively, the readout may be based on repeated cycles of hybridization of detection oligonucleotides and recording of signals[75] or by the usage of RCA products subjected to *in situ* sequencing by ligation[76].

When antibodies are converted to PLA probes it is not possible to control where on the antibody the DNA strands are attached or how many strands that are conjugated to each antibody. Conjugations may also occur at the antigen binding site of the antibody, impairing antigen binding thus decreasing the efficiency of the assay. By using recombinant binders it would be possible to direct the conjugation to a specific site, so as the availability of such binders increases, it may prove possible to use them as a source for PLA probes. To increase the sensitivity of *in situ* PLA, that can be caused by for example non-circularizing ligation events, we currently investigate alternative oligonucleotide designs that will reduce the formation of linear ligation products that cannot be amplified by RCA. Hopefully this will further improve the efficiency of the *in situ* PLA method in the near future.

As mentioned earlier, tumors are typically characterized by extensive cellular heterogeneity. To properly diagnose and treat cancer patients efficiently it would be an advantage to use single cell analysis. Today most publications use *in situ* PLA to quantify the RCA products per cell as an average of signals per cell. This forgoes the advantage of PLA that information is acquired on the level of single cells. But to present single cell data with sometimes many different parameters we need to develop new ways to present the data

to account for where in a tissue a cell is located[77]. The way to get there is via bioinformatics, e.g. presenting single cell data by a computational approach such as spanning-tree progression analysis of density-normalized events (SPADE)[62,78] to identify different cell populations based on their molecular profiles. This would facilitate analysis of how cells communicate and interacts with the microenvironment.

Today, *in situ* PLA is mainly used in research. There are *in situ* PLA assays under development with the goal to reach the clinic. An advantage of *in situ* PLA is that the readout can be performed either by microscopy of flow cytometry, methods that today are used for diagnostics in areas such as transplantation, oncology, hematology, genetics and prenatal diagnosis. To analyze cell cultures and primary cells in a fast and cost effective way more automated way to run PLA assays will be needed and the method may be applied in lab on a chip formats with automated microscopy scanning.

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