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DYSREGULATED NOTCH SIGNALING IN BREAST CANCER AND LIVER DISEASE

Indira V. Chivukula

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Dysregulated Notch Signaling in Breast Cancer and Liver Disease
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Cover image shows a pink ribbon for breast cancer awareness and a Blue Denim Genes Ribbon™ for rare and genetic disease awareness, over the human *JAG1* mRNA sequence (NCBI Ref Seq: NM_000214.2).
Ribbon design inspired by Wendy at clker.com; Family icon by OCHA from flaticon.com

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By

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For Dad and Mom,
my first mentors.

Take chances,
make mistakes,
get messy!

– Ms. Frizzle
The Magic School Bus

ABSTRACT

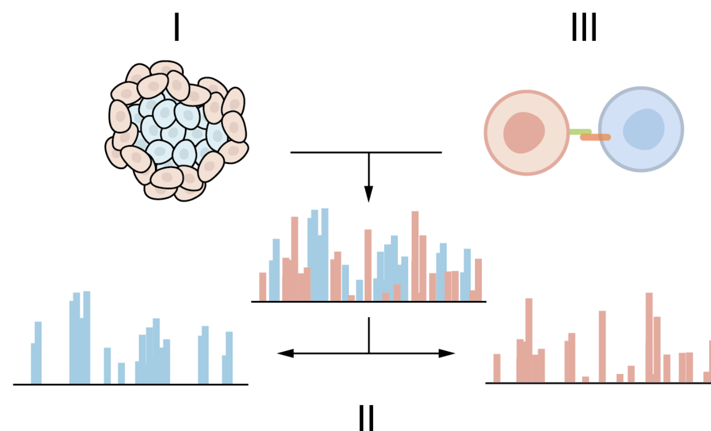
The evolutionarily conserved Notch signaling pathway regulates crucial aspects of development and tissue homeostasis. This thesis contributes research towards understanding a role of non-canonical Notch signaling in the tumor-stroma interaction of breast cancer, provides a bioinformatics-based technology to study these interactions, and proposes a novel mouse model of the liver disease in Alagille syndrome.

In **Paper I**, we report a novel target for non-canonical Notch signaling in breast cancer, the cytokine IL-6. In human breast cancer cell lines, we observe increased IL-6 mRNA and protein levels when Notch signaling is amplified, in turn activating the JAK/STAT pathway in a p53-dependent, but CSL-independent fashion, via IKKα and IKKβ of the NF-κB pathway. These data add a new facet to the existing body of knowledge on hyperactivated Notch signaling in promoting inflammation in breast tumors.

In **Paper II**, we present and validate a new bioinformatics-based approach of species-specific sequencing (S^3). Using an intermixed human tumor and mouse stroma cell population from xenografted cells, we demonstrate a way to decode transcriptomes, separated by their species-specific differences, with 99% accuracy. This technique circumvents current problems in mechanically separating mixed tissue, and paves the way to efficiently analyze *in vivo* cell-cell interactions.

In **Paper III**, we characterize a mouse strain, with a missense mutation in the Jagged1 gene, as a potential model for the rare genetic disorder Alagille syndrome. We show that this model recapitulates pathologies in the liver, heart, lens and kidney observed in Alagille patients, and identify dysregulated biliary morphogenesis caused by this mutation. We also use the S^3 technology, developed in Paper II, to investigate signaling specifically in receptor-expressing cells by wild type and mutated Jagged1.

In summary, the work presented in this thesis sheds new light on the role of Notch signaling in breast cancer and liver disease, and provides a novel technology to facilitate the detailed study of cell-cell interactions.



LIST OF SCIENTIFIC PAPERS

I. Jin S*, Mutvei AP*, **Chivukula IV**, Andersson ER, Ramsköld D, Sandberg R, Lee KL, Kronqvist P, Mamaeva V, Östling P, Mpindi J-P, Kallioniemi O, Screpanti I, Poellinger L, Sahlgren C and Lendahl U.
Non-canonical Notch signaling activates IL-6/JAK/STAT signaling in breast tumor cells and is controlled by p53 and IKKα/IKKβ.
Oncogene (2013) 32:4892–4902.

II. **Chivukula IV***, Ramsköld D*, Storvall H, Anderberg C, Jin S, Mamaeva V, Sahlgren C, Pietras K, Sandberg R and Lendahl U.
Decoding breast cancer tissue–stroma interactions using species-specific sequencing.
Breast Cancer Research (2015) 17:109.

III. Andersson ER*, **Chivukula IV***, Hankeova S*, Ramsköld D, Musilova J, Huch M, Fischler B, Ellis E, Strom S, Nemeth A, Clevers H, Sandberg R, Bryja V, Lendahl U.
A Jagged1 missense mutation links Notch receptor-selective ligand interaction and hypomorphic Notch signaling to an Alagille syndrome-like phenotype in mice.
Manuscript.

* Equal contributors

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LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
AGS	Alagille syndrome
ANK	Ankyrin repeats
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
CADASIL	Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CAF	Cancer-associated fibroblast
CLL	Chronic lymphocytic leukemia
CSL	CBF1/Suppressor of Hairless/Lag-1
DAPT	5-difluorophenylacetyl-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester
DLL	Delta-like
DNA	Deoxyribonucleic Acid
DOS	Delta and OSM-11-like proteins
DSL	Delta/Serrate/Lag-2
ECD	Extracellular domain
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
GSI	Gamma-secretase inhibitor
HD	Heterodimerization domain
HES	Hairy/enhancer of Split
HEY	Hairy/enhancer of Split related with YRPW motif
HNF	Hepatocyte nuclear factor
ICD	Intracellular domain
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor
IKK	IκB kinase
IL	Interleukin
JAG	Jagged
JAK	Janus kinase
LNR	Lin12-Notch repeats
LPS	Lipopolysaccharide
mRNA	Messenger RNA

ncRNA	Non-coding RNA
NECD	Notch extracellular domain
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
NLS	Nuclear localization sequence
NRR	Negative regulatory region
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEST	Proline/glutamic acid/serine/threonine-rich motifs
PR	Progesterone receptor
qPCR	Quantitative real-time PCR
RAM	RBP-Jκ association module
RBP-Jκ	Recombination signal binding protein for immunoglobulin kappa J region
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
siRNA	Small interfering RNA
STAT	Signal transducers and activators of transcription signaling
T-ALL	T cell acute lymphoblastic leukemia
TGF-β	Transforming growth factor-beta
TMD	Transmembrane domain
TNBC	Triple-negative breast cancer
TNF-α	Tumor necrosis factor-alpha
tRNA	Transfer RNA
Wnt	Wingless-related integration site
ZO	Zona Occludens

INTRODUCTION

As with all living things, the human body is on a constant quest for homeostasis, a state where many variables are balanced so conditions remain stable. The Swedish term *lagom*, meaning 'just the right amount', aptly encapsulates this concept, where a homeostatic body represents just the right balance of conditions to achieve the state of health. Diseases can occur when this balance is not achieved, when there is too little or too much. Cancer is an example of 'too much', where cells undergo uncontrolled growth, but there are also disorders of 'too little' where loss or misplacement of cell types or structures is seen.

The struggle for achieving and maintaining optimal homeostasis begins before birth, during development, when the body plan is being laid out. Dysregulation of cues necessary for normal body plan formation can lead to an overproduction or underproduction of specific factors leading to a plethora of diseases of varying severity, affecting diverse organs. The struggle continues post-birth when achieving and maintaining the 'just right' homeostatic balance of cells in each organ. Dysregulation of cues responsible for this balance can lead to an equal plethora of diseases where cells are lost or overproduced.

Information essential to homeostasis, and thus health, is exchanged within and between cells via signaling pathways. Notch signaling is a critical line of this cellular communication and has been implicated in both cancer and developmental disorders. This thesis contributes to understanding roles of Notch signaling in maintaining homeostasis, specifically how its dysregulation (a) mediates the interaction of tumor cell and stroma in breast cancer, and (b) leads to bile duct breakdown in the liver disease of Alagille syndrome, as well as (c) providing a useful technology to study these roles.

Each topic covered in this introduction is a field of biology in and of itself; therefore for the sake of clarity and brevity, I will highlight aspects of each topic as it pertains to the papers included in this thesis.

CELL COMMUNICATION

No man is an island, entire of itself; every man is a piece of the continent, a part of the main.

– Meditation XVII (excerpt) by John Donne c.1624

A core concept of nature is that all aspects of a system are connected. Specific to biology, cells live in a community, acting on and reacting to their surroundings, i.e. no cell is an island.

Cells have evolved efficient diversity generating mechanisms, which we call signaling pathways, to relay this information. Cell communication starts with a signal-sending component, called a ligand (often a molecule, protein, lipid or ion) that interacts with a signal-receiving component called a receptor (molecule that responds to specific ligands), which initiates a signal transduction (biochemical chain of events in response to an initial signal, creating a targeted response).

These signals can be classified as being within, among or between cells, as follows:

- Intracrine[A] signals produced by the cell stay within the cell itself.
- Autocrine signals produced by the cell are secreted (exit the cell membrane), and affect the cell itself, or a neighbor of the same cell type, via receptors on the cell surface. An example of this is shown in **Paper I**.
- Juxtacrine signals produced by the cell interact with adjacent, touching cells. This can be via ligand-receptor interactions or gap junctions, and are capable of affecting either cell, or both. The canonical Notch signaling pathway, introduced in the next section, uses this mode of signaling with membrane-anchored ligands and receptors. **Papers I-III** address different aspects of this pathway.
- Paracrine signals produced by the cell affect other cells in close proximity. Examples include neurotransmitters, the major signaling molecules for the nervous system, and cytokines for the immune system. The latter is shown in **Paper I**.
- Endocrine signals produced by the cell affect distant cells, transported by the cardiovascular or lymphatic system. Hormones are the major signaling molecules for the endocrine system.

In this thesis, several of these modes of signaling are addressed, with Notch signaling as the common denominator. In **Paper I**, we study the signaling in breast cancer, and identify the cytokine interleukin[B]-6 (IL-6) as a target of non-canonical Notch signaling. In **Paper II**, we present a novel RNA-Seq-based bioinformatics tool that distinguishes the transcriptomes of signal-receiving cells from signal-sending cells, in a mixed species scenario. In **Paper III**, we

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[A] *intra*, within (Latin); *krinein*, to separate (Greek); *autós*, self (Greek); *iuxta*, alongside (Latin); *pará*, next to (Greek); *éndon*, inside (Greek)
[B] Interleukin: coined in 1979 by Dr. Vern Paetkau; derives from *inter*, between (Latin) and *leukin*, produced by and acts on leukocytes (Green, 1988)

elucidate the effects of a specific mutation in the Jagged1 ligand of the Notch pathway, similar to what is seen in the human condition, Alagille syndrome.

'THE TEMPLE OF NOTCH'[C]

The Notch pathway is a member of the 'Signaling Seven' of evolutionarily conserved signaling mechanisms crucial for early embryonic development, but with such importance comes great responsibility, as dysregulation of these pathways are also critical drivers of disease. The other six members are Wnt, TGF-β, Hedgehog, JAK/STAT, nuclear hormone receptors and receptor tyrosine kinase pathways (Barolo and Posakony, 2002; Penton et al., 2012).

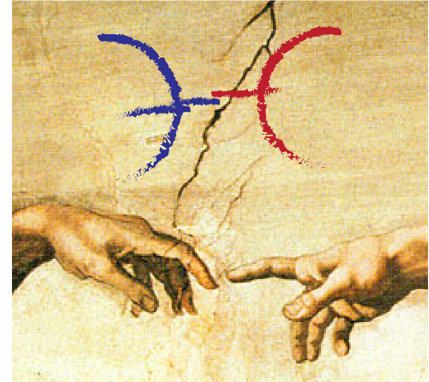

Figure 1: Adapted from *The Creation of Adam* (Michelangelo, 1508-12)

Discovery

Notch signaling is a cell-cell contact dependent pathway (Figure 1[D]) and is a major regulator of cell fate during development. In *Drosophila*[E], there is one receptor (Notch) and two ligands (Serrate and Delta), and in *C. elegans*[F] there are two receptors (Lin-12 and Glp-1) and one ligand (Lag-2). In mammals, there are four Notch receptors (Notch 1-4) and five canonical ligands: Jagged (Jag) 1, 2 and Delta-like (Dll) 1, 3, 4 (Kopan and Ilagan, 2009).

First described in 1914 by John S. Dexter as a heritable abnormality of 'perfect notched wings' in *Drosophila melanogaster* (Dexter, 1914), the alleles of the Notch gene were identified three years later by Thomas Hunt Morgan (Morgan, 1917). Almost 70 years later, the laboratories of Spyros Artavanis-Tsakonas (Artavanis-Tsakonas et al., 1983) and Michael W. Young (Kidd et al., 1983) independently deciphered the first Notch receptor gene sequence by cloning the *Drosophila* Notch locus. *Drosophila* Delta (Vassin and Camposortega, 1987) was the first ligand to be sequenced, followed by *Drosophila* Serrate (Fleming et al., 1990) and *C. elegans* Lag-2 (Henderson et al., 1994). In 1991, Leif W. Ellisen and colleagues first demonstrated the existence of Notch signaling in humans, connected to human health, when they discovered that the unknown gene displaying chromosomal translocations in T cell acute lymphoblasic leukemia (T-ALL) was the human homolog of the *Drosophila* Notch receptor (Ellisen et al., 1991). Since this time, the roles and functions of Notch signaling have become an ever-increasing field of study, and nearly 25 years later, new roles of Notch signaling are still being discovered. Detailed knowledge of

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[C] The name of a 'world' by Disco, dedicated to Minecraft's creator Markus Persson
[D] Figure 1: also conceptualized by Prof. Isabella Screpanti at the first international Notch meeting, EMBO Workshop on Notch Signalling in Development and Cancer (2005)
[E] *Drosophila*: fruit fly, model organism
[F] *Caenorhabditis elegans*: roundworm, model organism

the canonical and non-canonical roles of this pathway could unlock novel insights into human development and disease.

Canonical Notch signaling in mammals

The Notch pathway is unique, in that it does not use an amplifying signal, but rather relies on sequential steps to convey extracellular input to intracellular gene expression. Notch signaling is often viewed as a gatekeeper against differentiation, maintaining cells in a more stem-cell-like state, although in some cellular contexts, such as skin, it can act in a differentiation-promoting manner (Artavanis-Tsakonas et al., 1999).

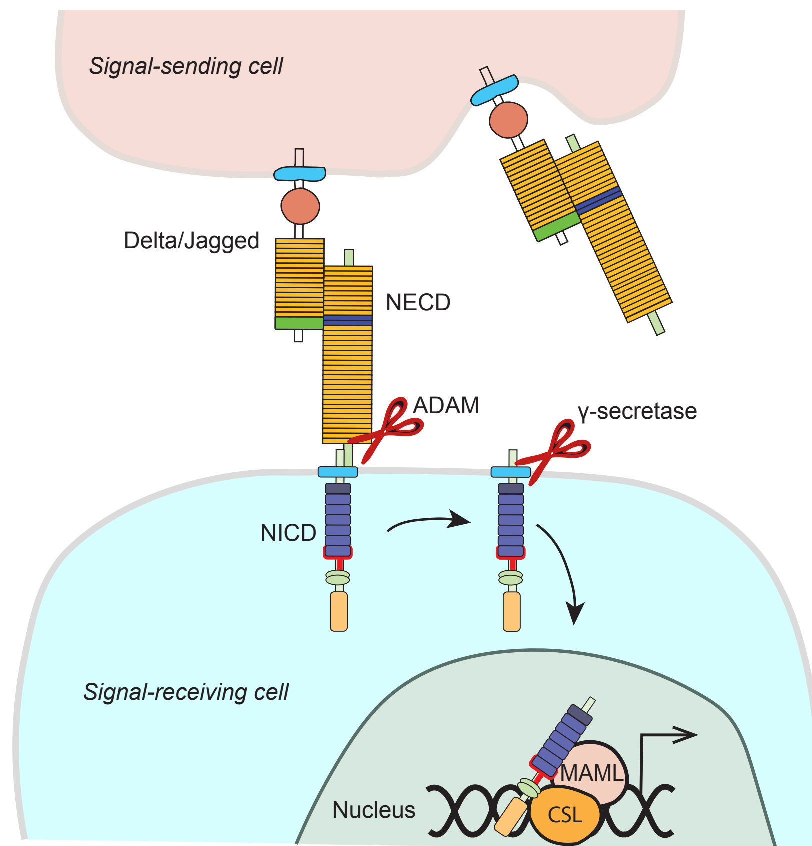

Figure 2: The canonical Notch signaling pathway

In the canonical Notch pathway (Figure 2), the signal-sending cell expresses transmembrane DSL (Delta/Serrate/Lag-2) ligands that bind to the transmembrane Notch receptor on a neighboring, signal-receiving cell. DSL ligands can activate signaling through the Notch receptors in *trans* (cell-cell interaction) or inhibit signaling in *cis* (interaction within in the same cell) (D'Souza et al., 2008). Ligand binding leads to the cleavage of Notch by ADAM (a disintegrin and metalloprotease) proteases at site 2 (S2). The Notch extracellular domain (NECD), along with the activating ligand, is shed and endocytosed into the signal-sending cell. The Notch intracellular domain (NICD) is released in the signal-receiving cell upon cleavage by the γ-secretase complex at site 3 (S3) near the inner plasma membrane, and at site 4 (S4) within the transmembrane domain. The NICD translocates to the nucleus, where it interacts with the DNA-binding protein CSL (CBF1/Suppressor of Hairless/Lag-1), also referred to as RBP-Jκ in mammals (Furukawa et al., 1992), and acts as a transcription co-

factor along with other co-activators, such as Mastermind-like (MAML1) (Kopan and Ilagan, 2009).

We have only recently begun to understand the diversity in the Notch downstream responses. Transcriptional targets of Notch signaling tend to be context-specific, with indicative target genes differing dependent on the cell type studied, but traditional transcriptional targets include the Hes (Iso et al., 2001) and Hey (Maier and Gessler, 2000) gene families, which encode basic helix-loop-helix transcription factors (recommended reviews: Andersson et al., 2011; Kopan and Ilagan, 2009). We have developed a tool, presented in **Paper II**, which can be used to learn more about the downstream consequences of Notch signaling by performing RNA-Seq on a human and mouse mixed-species co-culture of ligand-expressing and receptor-expressing cells.

Evolutionary conservation of Notch receptors and ligands

The Notch signaling pathway is highly evolutionarily conserved, with true receptors and ligands emerging in Metazoa (Gazave et al., 2009). *Drosophila*, *C.elegans* and mammalian Notch receptors and ligands share most of the same functional domains (further explained in the *Ligand-receptor interaction* section). The basic difference between *Drosophila* Notch (dNotch) and the four mammalian Notch paralogs (mNotch1-4) is the number of epidermal growth factor-like (EGF)-repeats (29-36) that each receptor contains, with dNotch and mNotch1-2 highly similar (Kopan and Ilagan, 2009). The differences between the species are indeed important for function, but the mammalian Notch components share very high sequence similarity, for example human and mouse receptors and ligands of the Notch pathway share 83-97% similarity in protein sequences (Table 1-2). Human and mouse fragments are used to experimentally activate the Notch pathway (expanded in *The Notch Toolbox* section), and are able to be interchanged because they are considered to be functionally identical.

		NCBI accession number (Number of amino acids, aa)		**Identity**[G]	
	Protein	*H.sapiens*	*M.musculus*	Protein	DNA
Receptors	**Notch1**	NP_060087.3 (2555 aa)	NP_032740.3 (2531 aa)	91.6%	86.0%
	Notch2	NP_077719.2 (2471 aa)	NP_035058.2 (2473 aa)	92.6%	87.1%
	Notch3	NP_000426.2 (2321 aa)	NP_032742.1 (2318 aa)	91.0%	84.3%
	Notch4	NP_004548.3 (2003 aa)	NP_035059.2 (1964 aa)	82.4%	83.2%

Table 1: Notch receptors. Data compiled from Pairwise Alignment Scores of NCBI HomoloGene (ncbi.nlm.nih.gov/homologene)

[G] Identity: proportion of pairs of identical residues between two aligned sequences over the length of the coverage area.

Table 2: Notch ligands.

		NCBI accession number (Number of amino acids, aa)		Identity	
	Protein	*H.sapiens*	*M.musculus*	Protein	DNA
Ligands	**Jagged1**	NP_000205.1 (1218 aa)	NP_038850.1 (1218 aa)	96.6%	90.2%
	Jagged2	NP_002217.3 (1238 aa)	NP_034718.2 (1247 aa)	90.5%	87.4%
	Delta-like1	NP_005609.3 (723 aa)	NP_031891.2 (722 aa)	88.8%	85.3%
	Delta-like3	NP_058637.1 (618 aa)	NP_031892.2 (585 aa)	83.6%	81.7%
	Delta-like4	NP_061947.1 (685 aa)	NP_062327.2 (686 aa)	87.0%	86.1%

Ligand-receptor interaction

The Jagged and Delta ligands are characterized by a DSL domain, EGF-repeats and transmembrane domain (TMD). The signal activating interaction between Notch ligands and the Notch receptors occurs by non-covalent association of EGF-repeats 11-12 of the receptor with the DSL domain of the ligand (Rebay et al., 1991; Shimizu et al., 1999), but it is important to note that, although the DSL domain is required for binding, it is not sufficient for promoting signal initiation (Cordle et al., 2008). An evolutionarily conserved valine in EGF-repeat-8 is essential for ligand selectivity between Serrate/Jagged and Delta family ligands (Yamamoto et al., 2012), and Dll4 binds with greater affinity than Dll1 to Notch1 EGF-repeats 6–15 (Andrawes et al., 2013). Figure 3 illustrates the domains of the Notch receptors and ligands. It would be valuable to know the downstream effects of each specific ligand-receptor combination, but information is limited in this area, foremost due to the plethora of cell-specific responses and a dearth of ligand- and receptor-specific detection tools (Andersson and Lendahl, 2014).

It is hypothesized that a mechanical pulling force is needed for the Notch extracellular domain (NECD) to be released from the cell membrane after ligand interaction (Meloty-Kapella et al., 2012; Nichols et al., 2007), therefore the use of soluble ligands is controversial in the field. The signaling activity from these soluble ligands has been attributed to the fact that the ligand is actually tethered to extracellular matrix (ECM) components, such as microfibril-associated glycoprotein 2 (MAGP2), providing the stabilization needed for the required pulling forces (D'Souza et al., 2008; Nehring et al., 2005). The changes in protein structure due to mechanical pulling force can be thought of as a coil being pulled, revealing spaces and structural components that can be accessed by molecules and affect subsequent steps in the signal transduction.

Bidirectional signaling, where receptor-binding causes activation of signaling in the ligand-expressing cell as well (e.g. the Eph-ephrin pathway) (Pasquale, 2008) is less definitive in Notch signaling. This may be due to the relatively small intracellular domain of the ligands, or because current tools require some knowledge of specific transcription factors or downstream events. Without this knowledge, it is a bit like finding a needle in a haystack to verify whether this occurs. The tool developed in **Paper II** could provide a way to investigate the possibility of Notch backward-signaling in distinct cell contexts, by specifically generating the transcriptome of the ligand-expressing cell.

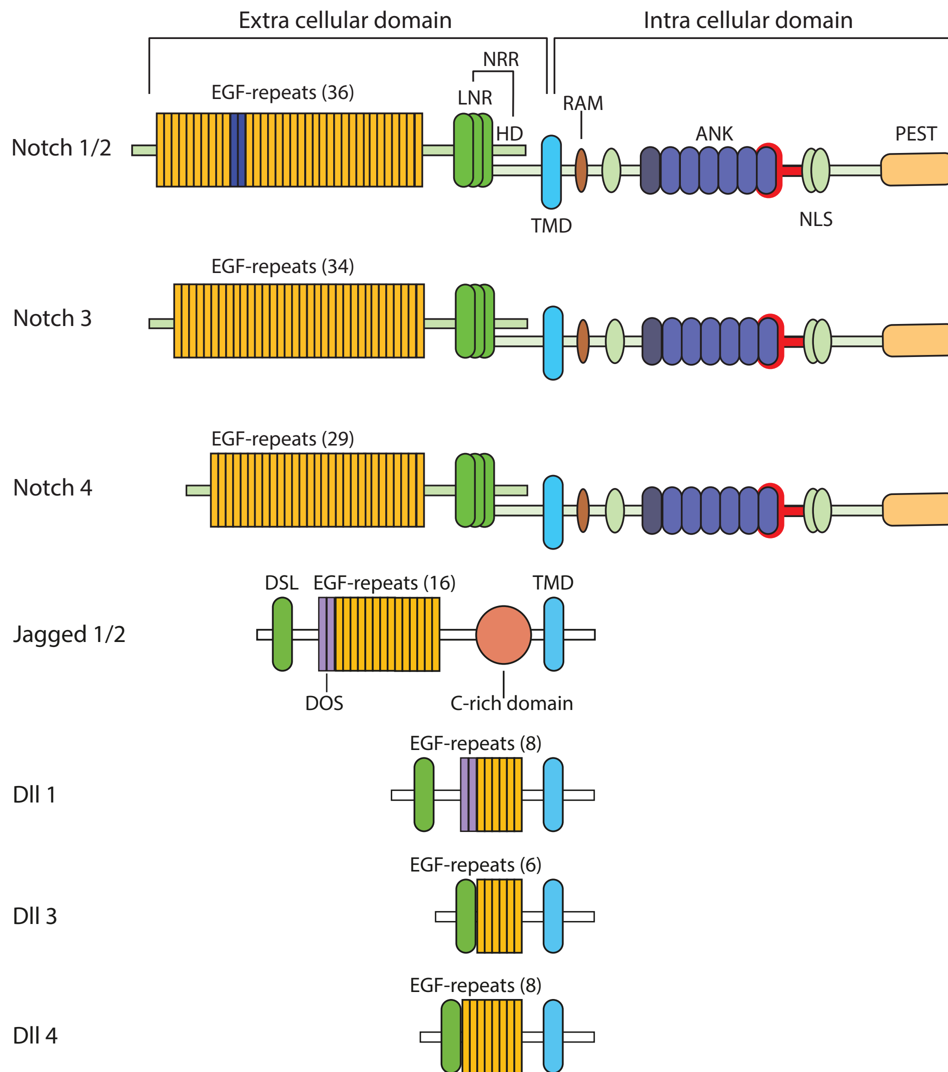

Figure 3: Receptors and ligands of the canonical Notch pathway.
EGF, epidermal growth factor-like; LNR, Lin12-Notch repeats; NRR, negative regulatory region; HD, heterodimerization domain; TMD, transmembrane domain; RAM, RBP-Jκ association module; ANK, ankyrin repeats; NLS, nuclear localization sequence; PEST, proline/glutamic acid/serine/threonine-rich motifs; DSL, Delta/Serrate/LAG-2; DOS, Delta and OSM-11-like proteins; C, cysteine
(adapted from Kopan and Ilagan, 2009)

Non-canonical Notch signaling in mammals

Non-canonical Notch is broadly classified as any DSL-independent, non-DSL ligand interaction, CSL-independent or non-nuclear-NICD signaling, as well as direct interaction with components of other signaling pathways (Andersen et al., 2012; D'Souza et al., 2010; Heitzler, 2010).

The number of reports of non-canonical Notch signaling in mammals is growing, ranging from direct interaction of NICD with Smad3 in Notch-TGFβ crosstalk (Blokzijl et al., 2003) or NICD with components of the hypoxia pathway (Poellinger and Lendahl, 2008), to non-nuclear NICD involved in abrogating Bax-induced apoptosis (Perumalsamy et al., 2010), and $CD4^+$ T cell activation and proliferation regulated by Notch1 independent of CSL (Dongre et al., 2014).

Non-canonical Notch signaling has been linked to breast tumor progression, for example, hyperactive Notch4 in CSL-deficient mammary tissue induces mammary tumors (Raafat et al., 2008). We contribute another example in **Paper I**, where we show that CSL-independent and non-nuclear-NICD-initiated Notch signaling activates IL-6 expression and JAK/STAT signaling in p53-mutated breast cancer cells.

Crosstalk with other pathways

There are thought to be just under 20 signal transduction pathways responsible for generating every cell type, pattern and tissue in metazoans (Barolo and Posakony, 2002; Gazave et al., 2009; Gerhart, 1999; Pires-daSilva and Sommer, 2003). Each contains a unique set of components, all of which are encoded in the genomes of every animal. These pathways increase the diversity of their output by interaction, or crosstalk, with each other.

The Notch pathway is simple, with relatively few components, yet it participates in many diverse processes. One way versatility is achieved is through extensive interaction with other pathways (Poellinger and Lendahl, 2008), therefore studying this crosstalk contributes to our knowledge of Notch's role in development as well as understanding disease progression and designing combination therapies. NICD interacts with intracellular components of many pathways, including nuclear factor (NF)-κB (Shin et al., 2006; Wang et al., 2001), BMP/TGFβ (Blokzijl et al., 2003; Dahlqvist et al., 2003; Itoh et al., 2004), Wnt (Axelrod et al., 1996; Espinosa et al., 2003; Hayward et al., 2005) and Hedgehog pathways (Dave et al., 2011; Schreck et al., 2010; Xie et al., 2013), as well as the cellular hypoxic response (Coleman et al., 2007; Gustafsson et al., 2005; Sahlgren et al., 2008; Zheng et al., 2008).

Tumor protein p53

p53 is a tumor suppressor whose activation is triggered by acute DNA damage or hyperproliferative signals, and responds by activating pathways of cell cycle arrest, DNA repair, apoptosis and senescence (Bieging et al., 2014).

Since Notch plays an important role in cancer, and p53, considered the 'guardian of the genome' (Lane, 1992), is mutated in over 50% of cancers (Vogelstein et al., 2000), it is not difficult to see that these two major biological players influence one another. The transactivating form of another tumor protein, p73α, is known to physically interact with NICD thereby inhibiting CSL-mediated transcription (Hooper et al., 2006). Recently, it was shown that compromised CSL and p53 function promotes cancer-associated fibroblast activation (Procopio et al., 2015). In **Paper I**, we show a link between Notch, NF-kB, p53 and IL-6 (expanded in the *Cytokine signaling in tumor and stroma* section).

The Notch Toolbox

The present-day scientist has a comprehensive set of tools for studying the Notch signaling pathway, including gene expression plasmids, pathway activity reporters, inhibitors, recombinant proteins, modified cell lines and mouse models, all of which are utilized in the work of this thesis. Future tools could include Notch-related genome engineering by the CRISPR/Cas9 system (Jinek et al., 2012), as already shown for *Notch* in *Drosophila* via conditional mutagenesis (Xue et al., 2014).

Plasmids

Expression constructs of Notch pathway components can be transfected into cells to activate or inhibit signaling. In addition to the full-length form of the Notch receptor (NotchFL), many genetically modified versions have been developed, for example the NotchΔE (lacking the ECD) is constitutively cleaved by γ-secretase in the absence of ligand and thus sensitive to γ-secretase inhibitors (GSIs), and NICDΔRAM (lacking the RAM domain) cannot interact with CSL. Dominant negative mutants of the Notch transcriptional activation complex have also been developed, for example the dominant negative CSL (R218H, arginine-to-histidine replacement at position 218) that can bind to NICD but not to DNA, and dominant negative MAML that cannot recruit other transcription complex components thereby effectively blocking Notch signaling.

Notch transcriptional reporter constructs are frequently used to monitor Notch activity in cells and tissues. Commonly used promoters to drive Notch-dependent reporters, such as luciferase or fluorescent proteins, are based either on endogenous targets (e.g. Hes, Hey) or multimeric CSL-binding sites (e.g. 12xCSL-Luciferase (Kato et al., 1997)) (Ilagan and Kopan, 2014).

Inhibitors

Manipulating the Notch pathway at any point from receptor maturation to receptor-ligand interaction, NICD release or cofactor recruitment would affect its signaling output. Current therapeutic interventions focus on inhibiting receptor-ligand interactions and interfering with the proteolytic processing of the receptors (Groth and Fortini, 2012; Shao et al., 2012). Notch inhibitors have been developed as potential therapeutic interventions, but are also valuable tools when studying the signaling pathway. The γ-secretase complex is responsible for releasing the NICD, and GSIs, such as DAPT, are used to block this cleavage, abrogating

canonical Notch signaling in the cell (Berezovska et al., 2000). GSIs are pan-Notch-receptor inhibitors, which can sometimes be disadvantageous if studying specific receptor roles in cells or tissue expressing several Notch receptors (De Kloe and De Strooper, 2014). In contrast to GSIs, blocking antibodies able to inhibit Notch signaling can target individual Notch receptors (Gordon and Aster, 2014) or ligands (Koga and Aikawa, 2014).

The first drug to undergo a phase I clinical trial was a GSI in 2006 (Deangelo et al., 2006), but was discontinued due to gastrointestinal toxicity in patients. In addition to other GSIs, meant to block the release of the NICD by the γ-secretase complex, current drug development approaches target specific Notch receptors and ligands with antibodies designed to block interaction or lock the structure from proceeding with the signal cascade (Wu et al., 2010). There is yet to be a successful 'Notch drug' on the market, let alone a candidate continuing to later-phase clinical trials (Andersson and Lendahl, 2014).

Recombinant proteins

The immobilized ligand assay is used to present DSL ligands to Notch-expressing cells for *in vitro*[H] analysis of the activated pathway. Engineered ECDs of ligands fused to the Fc domain of immunoglobulin G (IgG) antibodies are tethered to Protein-G-coated tissue culture plates (Varnum-Finney et al., 2000) (Figure 4). In this setting, the orientation of the immobilized ligands, more so than ligand density, is an important factor for active signaling (Andersson and Lendahl, 2014; Goncalves et al., 2009; Toda et al., 2011). Also, the Fc domain of the fusion proteins induces dimerization, which may explain why soluble ligands have been reported to activate Notch signaling (Czajkowsky et al., 2012).

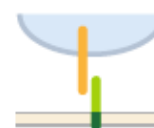

Figure 4: Immobilized ligand presentation

Modified cell lines

Expression constructs for all receptors and ligands, as well as many constituent components, of the Notch pathway have been used to transfect cell lines. Co-culturing receptor-expressing cells with ligand-expressing cells (Figure 5) can be used to activate, measure and study ligand-induced Notch signaling, **Paper II** explores the distinct transcriptomes between Notch activation by immobilized ligand and by co-culture.

Figure 5: Co-culture

Mouse models

There are a large number of different transgenic mouse lines generated to cause constitutive or conditional loss or gain of function of many components of the Notch pathway, as well Notch signaling reporter lines to visualize Notch-responsive cells in mice (Gridley and

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[H] *in vitro*: in glass, (Latin) refers to studying molecules or cells outside their biological context or organism from which they originate

Groves, 2014). For example, the R26-STOP-NICD mouse (Murtaugh et al., 2003) can be used to study the role of elevated Notch signaling in a cell- or tissue-directed manner when crossed with a promoter-specific Cre mouse (e.g. WAP-Cre to activate whey-acidic-protein-expressing cells in the mouse mammary gland). Mice can also model Notch-driven diseases. There are 4 Jagged1 point mutations that cause inner-ear defects in mice: Headturner (G289D, glycine-to-aspartic-acid replacement at position 289) (Kiernan et al., 2001), Slalom (P269S, proline-to-serine replacement at position 269) (Tsai et al., 2001), Ozzy (W167R, tryptophan-to-arginine replacement at position 167) (Vrijens et al., 2006) and Nodder (H268Q, histidine-to-glutamine replacement at position 268) (Hansson et al., 2010). The Nodder missense mutation sits in the Delta and OSM-11-like protein (DOS) domain (Figure 3), at EGF repeat-2 of Jagged1. **Paper III** explores the hypomorphic[I] signaling of this Jagged1 mutation and its effects on the development of many of the same organs symptomatic in Alagille syndrome.

[I] Hypomorph: a type of mutation causing a partial loss of gene function through reduced expression or functional performance (Muller, 1932).

NOTCH SIGNALING IN DISEASE

Since the first indication of dysregulated Notch signaling driving disease was shown when Notch mutations were associated with T-ALL, the list has grown to include cancer, immune disorders, developmental syndromes, stroke and cognitive symptoms. Activating *NOTCH1* mutations have been implicated in chronic lymphocytic leukemia (CLL) (Fabbri et al., 2011; Puente et al., 2011). Mutations in *DLL3* (Bulman et al., 2000) and associated Notch pathway components (Sparrow et al., 2006; 2008; Whittock et al., 2004) cause skeletal defects in spondylocostal dysostosis. Cognitive dysfunctions, such as CADASIL syndrome, can be caused by *NOTCH3* mutations (Joutel et al., 1996). Mutations in *JAG1* (Li et al., 1997; Oda et al., 1997), in the majority of cases, or in *NOTCH2* (McDaniell et al., 2006), can cause Alagille syndrome, the subject of **Paper III**.

Alternative splice variants occur when exons are combined in different ways to produce more than one gene product from the same locus. Alternative splicing of Notch pathway components is seen in normal mammalian development, but it also plays a role in disease progression. Alternative splicing of *NOTCH2* and CSL (*RBPJ* in mammals) is frequently observed in genome-wide studies of AML (acute myeloid leukemia) (Adamia et al., 2014; Lobry et al., 2014). Table 3 lists the total number of variants, as well as the number of protein-coding transcripts for each receptor and ligand of the canonical Notch pathway. With many genes listed having fewer than half of their transcripts as protein-coding, it would be interesting to study if the non-protein coding variants also have a regulatory role.

	Gene (human)	Total # of transcripts (splice variants)	# of protein-coding transcripts
Receptors	*NOTCH1*	3	1
	NOTCH2	8	4
	NOTCH3	6	3
	NOTCH4	5	1
Ligands	*JAG1*	9	2
	JAG2	5	2
	DLL1	2	2
	DLL3	5	3
	DLL4	3	1
Selected Components	*RBPJ*	31	22
	NUMB	32	19
	MAML1	4	1
	MAML2	2	2
	MAML3	3	3

Table 3: Data compiled using Ensembl (Flicek et al., 2014)

Poor patient prognosis in breast cancer has been linked to many dysregulated components of the Notch pathway: loss or inactivation of the Notch negative regulator *NUMB* (Pece et al., 2004; Stylianou et al., 2006), elevated *JAG1* expression (Reedijk et al., 2005), enhanced Notch signaling after tamoxifen treatment (Rizzo et al., 2008), activating gene rearrangements in Notch receptors (Robinson et al., 2011), *NOTCH1* and *NOTCH4* overexpression in triple-negative breast cancer (TNBC) (Speiser et al., 2012). Notch in breast cancer is studied in **Paper I and II**.

BREAST CANCER

Cancer is a complex disease, with tumors having heterogeneous gene expression, histology and clinical outcome. A recent study found a strong correlation between the number of cell divisions of normal self-renewing cells in a particular tissue with the lifetime risk of cancer in that tissue, with only one third of the variation in a tissue's cancer risk attributable to environmental factors or inherited predispositions, leaving the majority due to 'bad luck', i.e. random mutations (Tomasetti and Vogelstein, 2015).

Douglas Hanahan and Robert Weinberg proposed that the complexities of this disease can be described in terms of a set of six underlying principles, or 'acquired capabilities' during tumor development, dubbed the 'hallmarks of cancer' (Hanahan and Weinberg, 2000): self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis (shown with Notch in breast epithelial cells (Meurette et al., 2009)), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. In addition, they identified cancer-specific metabolism and immune evasion as emerging hallmarks, and genomic instability and tumor-promoting inflammation as enabling characteristics (Hanahan and Weinberg, 2011). In order to successfully investigate these capabilities in all cancer types, with the goal of finding novel therapeutic targets to allay and eventually ablate tumorigenesis, robust and reproducible models must be in place.

The normal human mammary gland has a system of ducts and lobules. The lobules are organized into approximately 20 lobes, drained by collecting ducts that converge at the nipple. Each lobule is made of acini that form the terminal duct lobular units (TDLUs), which are the functioning secretory units of the mammary gland. The acini and ducts consist of a surrounding basement membrane, an outer layer of myoepithelial cells, an inner layer of polarized luminal epithelial cells, and a central lumen. Outside the basement membrane is the stroma, which consists of connective tissue, blood vessels, fat and nerves (Nilsson et al., 2001). Breast cancer is a disease in which malignant neoplasia forms in the ducts and lobules of the breast.

As with most glandular organs, the mammary gland is in a stroma of mesenchymal cells, such as fibroblasts, adipocytes, immune cells and ECM. The ECM provides structural support, and directs the organization of the cytoskeleton through transmembrane receptor signaling to keep tissue integrity intact (Bissell et al., 2005). For example, myoepithelial cells from ductal carcinoma in situ (DCIS) show gene expression variation and epigenetic changes

compared to myoepitheial cells from normal breast tissue (Allinen et al., 2004; Hu et al., 2005). Furthermore, normal and malignant mammary cells grown in two-dimensional monolayer cultures on tissue culture plastic without any ECM lose their tissue-specific function and morphological organization (Weigelt and Bissell, 2008).

The basement membrane in breast tissue, composed mainly of laminin and type IV collagen, is a specialized form of the ECM that functions as structural support, and plays a role in regulating proliferation, organization and differentiation (Leblond and Inoue, 1989).

Regardless of the histological type of the tumor, breast malignancies arise mainly in the TDLU. Carcinomas show a loss of epithelial polarity and tissue organization. Changes in cellular composition associated with a normal mammary gland progressing to an invasive carcinoma include the loss of myoepithelial cells, an increased number of in myofibroblasts and immune cells in the stroma, and enhanced vascularization. (Weigelt and Bissell, 2008).

Clinical overview

According to the latest (2012) World Health Organization GLOBOCAN project report, estimating cancer incidence, mortality and prevalence worldwide, breast cancer is the second most common cancer in the world, comprising 25% (1.67 million new cases) of all cancers diagnosed and over 0.5 million deaths in 2012. The mortality rate in less developed regions is 1.6 times higher than in more developed regions (Ferlay et al., 2015).

Breast cancer is not a single disease, but rather a diverse collection of histopathologies, genetic and genomic variations, and clinical outcomes. Several genetic perturbations are important for breast cancer, including loss of or mutation in p53, mutations in BRCA genes (which codes for the breast cancer type susceptibility protein) and the signaling status of human epidermal growth factor receptor 2 (HER2) (Vargo-Gogola and Rosen, 2007), as well as the expression levels of cell-cell junction proteins such as Claudin and E-cadherin (Perou, 2011).

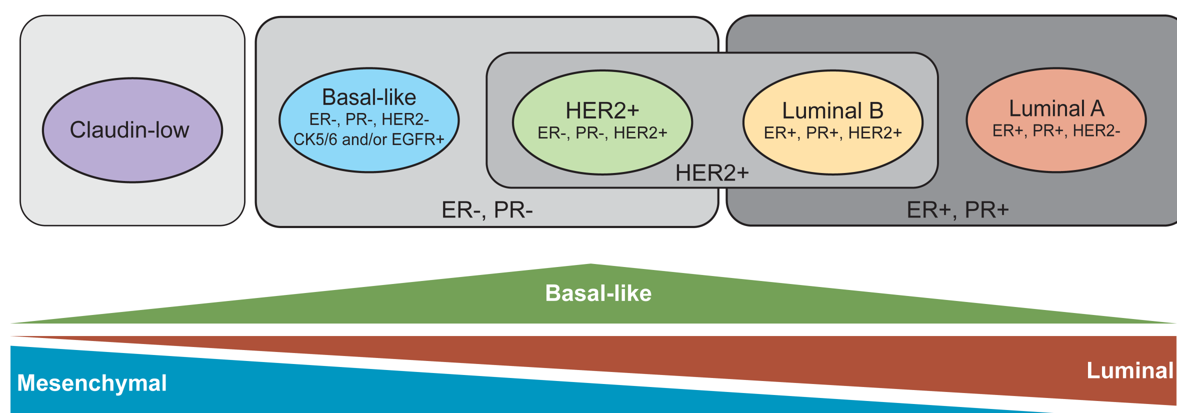

Figure 6: Molecular subtypes of breast cancer.
ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; CK: cytokeratin; EGFR: epidermal growth factor receptor.
(adapted from Prat and Perou, 2009; Sandhu et al., 2010)

One way to classify breast carcinomas into specific subtypes is according to their gene expression profile: Luminal A, Luminal B, HER2-enriched, Claudin-low, Basal-like and Normal Breast-like (Prat and Perou, 2011). The molecular subtypes of breast cancer are illustrated in Figure 6. Each subtype's prognosis and treatment response is different (Sørlie et al., 2001). The luminal A and B subtypes are sensitive to hormone therapy like tamoxifen (a small molecule antagonist of the estrogen receptor), while luminal B and $HER2^+$ subtypes can be treated with the trastuzumab (Herceptin®), a monoclonal antibody that interferes with the HER2/neu receptor. Basal and claudin-low subtypes, more aggressive and difficult to treat, are associated with poor prognosis (Badve et al., 2011; Herschkowitz et al., 2007).

There are four types of standard treatment, used often in combination: surgery, radiation therapy, hormone therapy, and chemotherapy. Hormone therapies include estrogen receptor antagonists and aromatase inhibitors. There are several promising chemotherapeutic strategies designed as adjuvant therapies, including monoclonal antibodies and tyrosine kinase inhibitors (National Cancer Institute, 2015).

In humans, breast cancer metastasizes mostly to the lung, bone and liver. Less common sites are the brain, kidney, spleen and heart (Weigelt et al., 2005). Between 60% and 70% of women who succumb to breast cancer have had it eventually spread to their lungs, and in 21% of cases, the lung is the only site of metastasis (Horak and Steeg, 2005; Minn et al., 2005).

Notch plays an important role in cell fate decisions, promoting cell-fate commitment along the luminal lineage of the developing mammary gland (Bouras et al., 2008; Politi et al., 2004; Raafat et al., 2011). It is becoming increasingly evident that dysregulated Notch signaling is linked to breast cancer, where Notch is rarely mutated but frequently hyper-activated (Mutvei et al., 2015). For example, upregulation of Notch signaling is found in several forms of breast cancer (Pece et al., 2004; Stylianou et al., 2006), and tamoxifen treatment in breast cancer therapy may in fact elevate Notch signaling as an unwanted side effect (Rizzo et al., 2008). Notch negative regulation by Numb is relevant for normal mammary tissue homeostasis, and when skewed, contributes to oncogenesis, as shown by a "high Notch/low Numb" signature in breast cancers (Pece et al., 2004; Stylianou et al., 2006). In the mouse, directed overexpression of activated Notch in the mammary gland results in breast tumors (Raafat et al., 2004). There are emerging hallmarks of cancer now being explored in the context of breast cancer, including cellular energetics (Hanahan and Weinberg, 2011), where dysregulated Notch activity leads to a glycolytic switch in breast tumor cells, and elevated Notch signaling correlates with more invasive growth in breast tumor xenograft experiments (Landor et al., 2011). The role of Notch signaling in tumor-promoting inflammation is an emerging hallmark yet to be understood.

Tumor-stroma interactions

When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil.

– Stephen Paget (Paget, 1889)

Paget's seed and soil hypothesis speaks to the importance of studying the 'congenial soil', or stroma, of a tumor. Cancer-associated fibroblasts, endothelial cells, pericytes, lymphocytes, myeloid cells and ECM populate a tumor's microenvironment (Pietras and Östman, 2010). Not only the primary tumor, but also the site of its metastasis, requires a permissive site for colonization (Horak and Steeg, 2005). Understanding communication between tumors and their stroma, for example by cell-cell interactions and cytokine signaling, can lead to developing treatment strategies to make this stroma less habitable.

Cytokine signaling in tumor and stroma

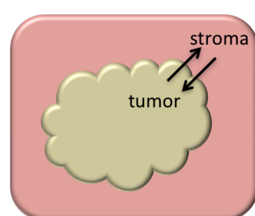

Figure 7: tumor-stroma crosstalk

One of the first identified cytokines, IL-6, has a pro-inflammatory function in stimulating immune responses during infection and inflammation. It acts as an anti-inflammatory myokine (cytokine produced from muscle) in response to muscle contraction (Febbraio and Pedersen, 2005). IL-6 is mainly produced by innate immune cells and B cells, but is also secreted by many non-leukocyte cell types such as fibroblasts and malignant cells (Hirano, 1998), and its production is regulated by NF-κB signaling (Libermann and Baltimore, 1990). Elevated levels of IL-6 are associated with chronic inflammation, including rheumatoid arthritis and Crohn's disease (Rincon, 2012), as well as liver and colon cancers (Mauer et al., 2015). In breast cancer patients, elevated serum IL-6 levels correlate with poor outcome (Bachelot et al., 2003; Sansone et al., 2007; Sethi et al., 2011; Wang et al., 2005). Since hyperactivated Notch and elevated IL-6 levels are seen in breast cancer, we explore if Notch signaling can lead to IL-6 expression in **Paper I**.

Breast cancer models

Models commonly used to investigate breast cancer include cell lines, xenografts and genetically engineered mice (Polyak, 2007; Vargo-Gogola and Rosen, 2007), the first two of which are discussed below as they pertain to the thesis.

Cell Lines

Using cell lines for cancer research has a number of advantages, including the fact that they are easy to handle, continuously divide so they can be grown in vast quantities, and can be revived from cryogenic freezing to restart experiments years later. Also, by working with commonly used cell lines, new findings can be compared to those reported in the field.

That being said, these same points have disadvantages as well. Cell lines grown in continual culture are prone to phenotypic and genotypic drift, because of the selective advantage of more rapidly growing clones within the cell population (Burdall et al., 2003). Also, cell lines are sensitive to culture conditions, especially the inclusion or exclusion of growth factors, which can alter their cell phenotype, differentiation, signaling pathway activation and gene expression (Birgersdotter et al., 2005; Streuli et al., 1991; Yamada and Cukierman, 2007). Databases, such as the Broad Institute's Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012), are helpful resources for detailed genetic characterization of human cancer cell lines.

The pros and cons above also hold true for breast cancer cell lines. An additional limitation is that patient biopsies of breast tumors are heterogeneous, yet relatively homogeneous cell lines are routinely used in breast cancer research. Overall, when chosen appropriately and cultured correctly, cell lines can continue to be used as powerful experimental tools (Geraghty et al., 2014; Holliday and Speirs, 2011).

The MCF-10A (from the Michigan Cancer Foundation) (Soule et al., 1990) immortalized normal breast epithelial cell line, and the breast cancer cell lines MCF-7 (Soule et al., 1973) and MDA-MB-231 (from the MD Anderson Cancer Center) (Cailleau et al., 1974) are among the most common tools used in breast cancer research. Additional cell lines used in **Paper II** include: MDA-MB-175 VII (Cailleau et al., 1974), Sk-Br-3 (Engel and Young, 1978), T-47D (Engel and Young, 1978; Keydar et al., 1979), HCC38 (Gazdar et al., 1998), and Hs578T (Hackett et al., 1977). Table 4 summarizes several characteristics of the breast cancer cell lines used in this thesis.

Cell Line	Type of cancer	Subtype	Original tissue	ER status	PR status	HER2 enriched	p53 status
MCF-7	IDC	L	M (PE)	+	+	–	WT
T-47D	IDC	L	M (PE)	+	+	–	MUT
MDA-MB-175 VII	IDC	L	M (PE)	+	–	–	WT
Sk-Br-3	IDC	L / HER2	M (PE)	–	–	+	MUT
MDA-MB-231	IDC	B	M (PE)	–	–	–	MUT
Hs578T	CS	B	P	–	–	–	MUT
HCC38	DC	B	P	–	–	–	MUT
MCF-10A	nc	B	Breast	–	–	–	WT

Table 4: Characterization of breast cancer cell lines used in this thesis. IDC: invasive ductal carcinoma; CS: carcinosarcoma; nc: not cancer (immortalized normal breast epithelium); L: luminal; B: basal; M (PE): metastasis (pleural effusion[J]); P: primary tumor; ER: Estrogen receptor (+, present; -, absent); PR: Progesterone receptor (+, present; -, absent); HER2: oncogene encoded by *ERBB2* gene (+, amplified; -, absent or low); WT: wildtype; MUT: mutant. (compiled from Finn et al., 2009; Lacroix and Leclercq, 2004; Lacroix et al., 2006; Subik et al., 2010)

[J] Pleural effusion: excess fluid accumulation in the space around the lung

Xenografts

Although 'mice are not little humans' (Jonkers and Derksen, 2007), the laboratory mouse is the best compromise as a mammalian model organism for studying human disease, because of physiological and genetic similarities, and relative ease of genetic manipulation. Mice for studying human cancer fall into either transplantation or transgenic models. Transplantation can be done by allografting[K] (mouse cells into mouse) or xenografting[L] (human cells into mouse) (Jonkers and Derksen, 2007), the latter of which is used in **Paper II**.

Both the transplantation and transgenic models are useful, but neither is ideal to study the entire cancer pipeline from gene to drug discovery. Each has its advantages and limitations, which should be considered when interpreting their data.

Allograft and transgenic, or genetically engineered, models share the benefit of not exhibiting graft versus host immune reactions (Outzen and Custer, 1975; Ozzello et al., 1974), which is a problem when using xenografts, but come with the drawback that mice tend to develop mesenchymal tumors, whereas human age-related cancers mostly are of epithelial origin (DePinho, 2000). Xenografts are often used to test cancer drugs to study their effect on human, rather than mouse, tumors (Richmond and Su, 2008).

Orthotopic[M] (grafting tissue in its natural position) xenografts of human tumors are popular in cancer research, but the technique is restricted to immune-compromised (e.g. athymic nude mice lacking certain T cell responses) or immunodeficient (e.g. SCID mice lacking T- and B-cell responses) animals to avoid the graft versus host reactions, mentioned above. Consequently, the role of an intact immune system in tumor formation and progression cannot be studied in these mice. Xenografts of human tumors in humanized mice have been developed (Kuperwasser et al., 2004; 2005) to provide a more realistic tumor microenvironment, but tend to be expensive and technically complicated.

[K] *állos*, other (Greek)
[L] *xenos*, foreign (Greek)
[M] *ortho*, correct (Greek)

ALAGILLE SYNDROM

First described in the 1970s by Daniel Alagille (Alagille et al., 1975), Alagille syndrome (AGS) is an autosomal dominant genetic disorder, diagnosed primarily by clinical findings followed by sequence analysis to detect the precise genetic aberration. The disease is traditionally defined clinically by cholestasis (due to insufficient bile ducts in the liver) and congenital heart disease, in addition to abnormalities in the skeleton, eye, kidney and a characteristic facial phenotype. Other, less frequently seen, features target the ear, intestine and pancreas (Crosnier et al., 2000; Dhorne-Pollet et al., 1994; Hingorani et al., 1999; Kamath et al., 2004; Li et al., 1997; Martin et al., 1996; Oda et al., 1997). Figure 8 illustrates the variety of organs affected in AGS.

Clinical overview

AGS is caused mainly by missense and nonsense mutations (almost 90% of cases) or deletions (approximately 5-7% of cases) in *JAG1* (AGS type 1), but also by mutations (approximately 1% of cases) in *NOTCH2* (AGS type 2) (McDaniell et al., 2006; Online Mendelian Inheritance in Man, 1986; Spinner et al., 2000b; Turnpenny and Ellard, 2012).

Categorized as a rare disease, AGS afflicts approximately 1 in 30-70,000 newborns, based on liver disease diagnoses in infants. This may be an underestimated prevalence, as some AGS patients do not develop liver disease during infancy (Kamath et al., 2010; National Library of Medicine US, 2015). Approximately 30-50% of AGS patients have an inherited mutation, while about 60-70% have a *de novo*[N] mutation (Spinner et al., 2000a). Palliative care treatment for AGS patients is focused on surgical and medical treatments for liver and heart disease.

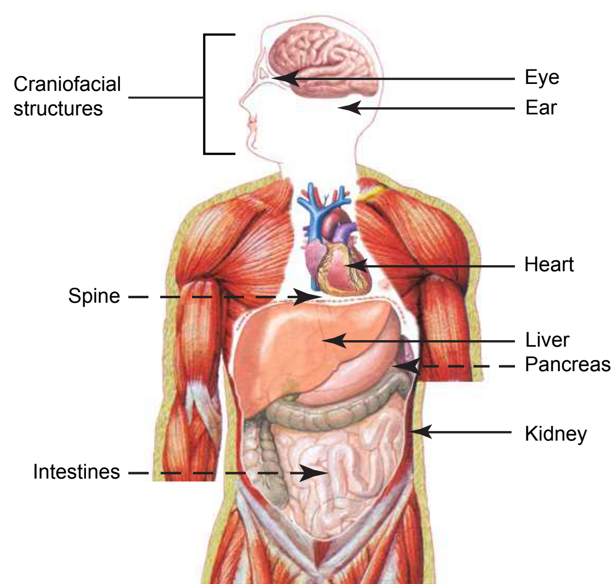

Figure 8: Organs affected in Alagille syndrome. Solid lines highlight organs also affected in the Nodder mouse (Paper III). (body image courtesy of organs_harvard-wm.org)

[N] *de novo*: anew, beginning again (Latin). A *de novo* mutation is a gene alteration present for the first time in one family member as a result of a germ line mutation (Pagon et al., 1993).

AGS models

There are currently five transgenic mouse models identified as exhibiting Alagille syndrome-associated phenotypes:

- inner ear phenotype by a missense mutation in *Jag1* on a C3HeB/FeJ background: Headturner (*$Jag1^{Htu/+}$*) (Kiernan et al., 2001), Slalom (*$Jag1^{Slm/+}$*) (Tsai et al., 2001) and Ozzy (*$Jag1^{Ozzy/+}$*) (Vrijens et al., 2006).
- liver, heart, eye and kidney phenotypes in a *Jag1/Notch2* double heterozygous mouse (*$Jag1^{dDSL/+}$*, *$Notch2^{del1/+}$*) on a mixed C57BL/6J-129S1/SvImJ background (Lozier et al., 2008; McCright et al., 2002).
- liver phenotype by vascular smooth muscle-specific deletion of *Jag1* (*SM22-Cre:$Jag1^{flox/flox}$*) on a C57BL/6J background (Hofmann et al., 2010).
- heart phenotype by endothelial-specific deletion of *Jag1* (*VE-Cadherin-Cre:$Jag1^{flox/flox}$*) on a C57BL/6J background (Hofmann et al., 2012).
- craniofacial phenotype by cranial neural crest-specific deletion of *Jag1* (*Wnt1-Cre:$Jag1^{flox/flox}$*) on a C57BL/6 background (Humphreys et al., 2012).

A recent study this year introduced a mouse model heterozygous for *Jag1* (*$Jag1^{dDSL/+}$*) on a C57BL/6J background, with congenital cholangiopathy due to impaired intrahepatic bile duct development (IHBD), which could be rescued by glycosyltransferase *Poglut1* (*Rumi*) deletion (Thakurdas et al., 2015).

The Nodder mouse, named after the nodding behavior and balance defects in the heterozygous state (*$Jag1^{Ndr/+}$*) on a pure C3HeB background, is a Jagged1 mutant identified from a large-scale ethylnitrose urea (ENU) mutagenesis screen. Its mutation is located at the second EGF-like repeat of the Jagged1 ECD, a location which carries missense mutations in AGS (Krantz et al., 1998; Li et al., 1997; Oda et al., 1997; Spinner et al., 2000a), and encodes a histidine-to-glutamine replacement at position 268 (H268Q). The $Jag1^{Ndr}$ ligand has specifically lost its capacity to bind to Notch1 in *trans* and activate signaling (Hansson et al., 2010). In **Paper III**, we propose the homozygous Nodder mouse (*$Jag1^{Ndr/Ndr}$*) on a mixed C3HeB/FeJ-C57BL/6J background as a novel model for AGS type 1 that recapitulates the liver, heart, eye and kidney phenotypes of the disease, with Jagged1 as the sole driver mutation.

Notch in bile duct development

In the human liver, hepatocytes (liver bulk), the biliary tree and the hepatic portal vein system are arranged in hexagonal functional units called lobules. Before bile ducts develop, only hepatoblasts (hepatocyte progenitor cells) surround the portal mesenchyme and associated endothelial cells. Bile duct development starts with ductal plate formation, a single layer of cholangiocyte (epithelial cells of the bile duct) precursors, which are derived from the hepatoblasts in direct contact with the portal mesenchyme. The ductal plate then duplicates, and small pockets (lumen precursors) develop between the two layers, which then form bile ducts with mature cholangiocytes (Figure 9).

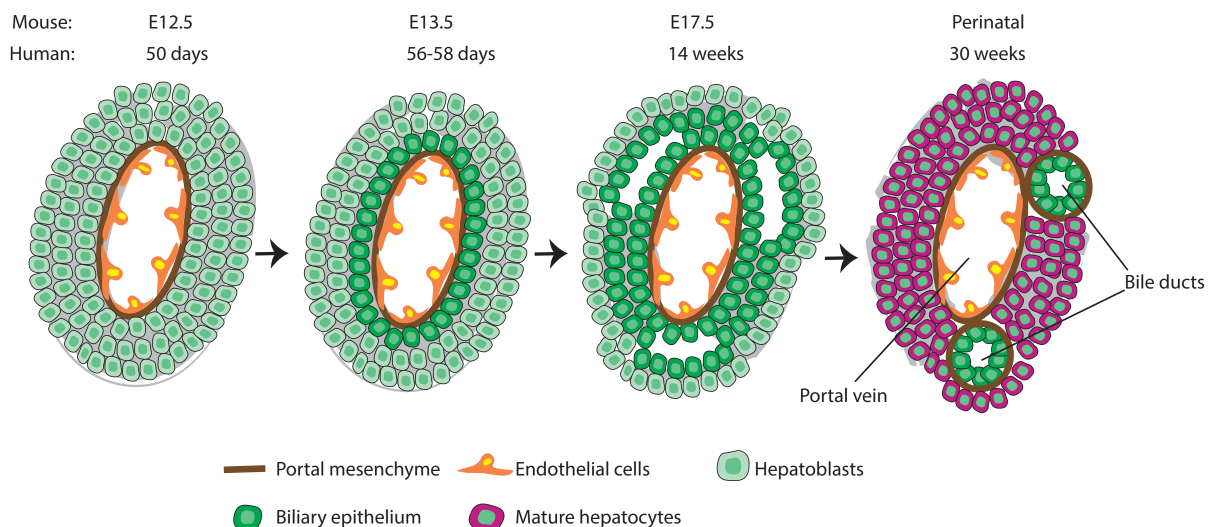

Figure 9: Intrahepatic bile duct formation.
(adapted from Gordillo et al., 2015; Zorn, 2008)

Notch signaling coordinates hepatoblast to cholangiocyte differentiation and tube formation during bile duct development (Lemaigre, 2010). In mice, inhibiting Notch signaling results in reduced biliary differentiation, whereas activating Notch promotes biliary differentiation from hepatoblasts (Zong et al., 2009). When Hes1 is inactivated, a ductal plate develops but does not form tubes (Kodama et al., 2004), and when CSL is inactivated after ductal plate formation, tubulogenesis is impaired and leads to bile duct paucity (Zong et al., 2009). Notch2 has a dominant role in bile duct formation, promoting biliary differentiation and tubulogenesis (Sparks et al., 2010; Tchorz et al., 2009), and intact Notch signaling is necessary for post-natal maintenance of bile duct structures (Sparks et al., 2011).

‘Rise of the organoids’ *(Willyard, 2015)*

All cells in the body are in a three-dimensional environment, but two-dimensional culturing of cells on plastic plates in a growth factor-enriched medium has been the staple of basic and

biomedical research. There is a strong argument for the importance of assaying cells *ex vivo*[O] in a physiologically relevant environment, to recapitulate cell-cell interactions and maintain morphology (Weigelt and Bissell, 2008). A popular substrate-of-choice is Matrigel®, a laminin-rich extracellular matrix (lrECM) extracted from basement-membrane-producing Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells (Kleinman and Martin, 2005).

From cells to organs, the 21st century brought with it an inspired concept of growing rudimentary mini-organs in the dish, where researchers could study everything from development to cancer therapeutics. Laboriously tinkering with needed growth factors and culture conditions, scientists have grown organoids for components of many tissues, including the cerebral cortex, intestine, optic cup, pituitary gland, kidney, liver, pancreas, neural tube, stomach, prostate, breast, heart and lung (Willyard, 2015).

In **Paper III**, we generate bile-duct-enriched liver organoids (Huch et al., 2013; 2015) as a model to study bile duct morphogenesis defects in the Nodder mice. In the biliary organoid culture set-up (Figure 10), isolated bile duct fragments are embedded in Matrigel® and medium supplemented with a precise growth factor cocktail. Less than 24 hours after isolation, the edges of the cylindrical ducts fuse and the organoids swell, ballooning out. Organoids are passaged by manual disruption, broken apart into tiny sheets of cells, which are resuspended in Matrigel®. Again, these sheets fuse into a spheroid structure and begin to expand.

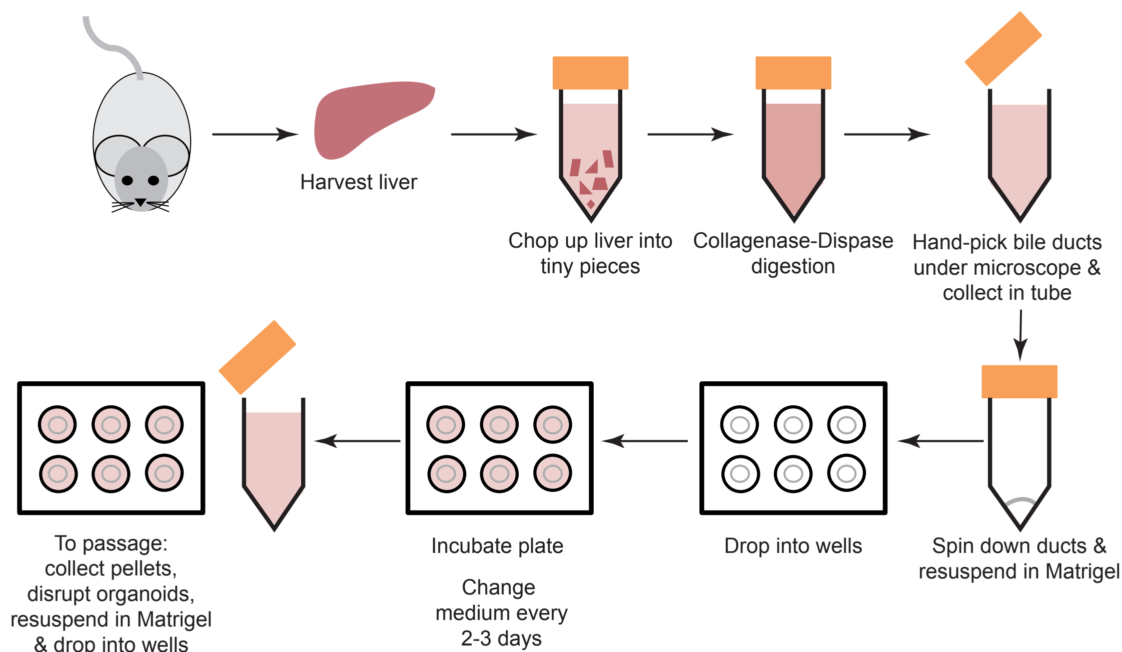

Figure 10: Biliary organoid culture

[O] *ex vivo*: from living, (Latin) refers to tissue from an organism cultured in an external environment

RNA-SEQUENCING

The central dogma of molecular biology (Crick, 1970) explains the flow of genetic information in an organism (Figure 11). It states that DNA encodes RNA (transcription), which encodes protein (translation). The traditional techniques to detect these components have been Southern blot (Southern, 1975) for DNA, Northern blot (Alwine et al., 1977) for RNA and Western blot (Towbin et al., 1979) for protein. Although an immense amount of information has been gathered using these techniques, it limits the user to probing their sample one factor at a time. With these tools, science tended towards a reductionist approach to understand the complexity of biology, simplifying models to individual genes or proteins.

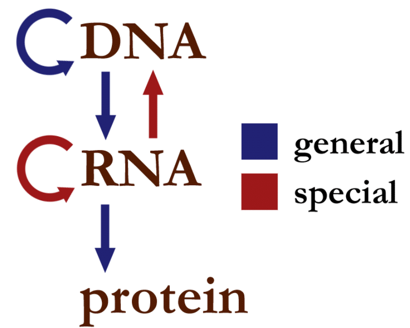

Figure 11: Crick's central dogma (blue) and special cases (red). Image courtesy of Daniel Ramsköld.

The nearly 13-year endeavor that was the Human Genome Project (HGP) predominantly used an automated version of Sanger sequencing (Sanger et al., 1977), a chain-termination method incorporating radioactively labeled dideoxynucleotides of each base pair[P], which used fluorescent dyes to tag each nucleotide (Adams, 2008). The HGP revealed that the over 3 billion base pairs of the human genome contained only approximately 20,500 protein-encoding genes (Clamp et al., 2007; NIH National Human Genome Research Institute, 2005).

The current iteration is called next-generation sequencing (NGS), which refers to non-Sanger-based high-throughput DNA sequencing technologies, where millions of DNA strands can be sequenced in parallel using the following general methodology: template preparation, sequencing and imaging, and data analysis (Grada and Weinbrecht, 2013).

Sequencing the human genome brought with it not only the use of genomics to understand health and disease, but also a drive to generate technologies that increase the speed and lower the cost to do so. With the advent of more complex computing and data management, the ‘omics’ revolution, which started with genomics, spawned a growing list of cell components treated to this high-throughput systematic approach, including transcriptomics, proteomics, lipidomics, metabolomics, epigenomics and pharmacogenomics. Instead of looking for a factor of interest, one could evaluate all factors in a sample at once, like taking a high-resolution snapshot instead of searching for a pixel.

[P] The four nucleotides found in DNA: adenine (A), thymine (T), cytosine (C) and guanine (G), form base pairs A-T and C-G.

Introduction to RNA-Seq

Cells in the body contain the exact same DNA sequence and therefore the same genes (genome), but it is the expression[Q] of these genes that determines the differences between cells of each tissue of the body. Transcriptomics is the study of all RNA molecules (mRNA, rRNA, tRNA and ncRNA)[R] produced by the genome of a specific cell or cell population under defined circumstances. The mRNA population represents the 'active' genes in a cell, whose transcripts are most likely on their way to instructing the synthesis of a specific protein, the major functional unit of a cell's activity.

Currently the most common method for gene expression analysis is quantitative real-time PCR (qPCR) (Gibson et al., 1996; Weis et al., 1992), where complementary DNA (cDNA) reverse transcribed from RNA is used as a template and is amplified in a polymerase chain reaction (PCR). Synthetic primers are designed towards the specific DNA fragment to be amplified by the PCR process. The qPCR reaction is quantified by detecting the intensity of a DNA probe generating a fluorescent signal. Although relatively cost-effective, qPCR only allows for one gene at a time to be analyzed in a group of samples, and the quality of results is highly dependent on the cDNA primer design.

Gene expression microarrays allow for high-throughput analysis (Schena et al., 1995). Here, a library of cDNA probes is printed onto a surface and fluorescently labeled target sequences, which bind to the probe sequence, generate a signal. Limitations include the contents of the probe library and accounting for cross-hybridization of mRNA over multiple probes in the array.

In 2008, RNA-Sequencing (RNA-Seq) was introduced as a way to directly sequence all mRNA in cells, and allowed for gene expression to be quantified (Cloonan et al., 2008; Mortazavi et al., 2008). The basic RNA-Seq workflow starts with extracting RNA from a sample (tissue, cell pellet, single cells). Next is the cDNA library preparation, where poly(A)+ RNA (only mature mRNA ready for translation) is enriched from total RNA, is fragmented and is converted into a library of cDNA fragments with attached adaptors and barcodes[S] specific to each sample. The fragments are PCR-amplified, sequenced, aligned to a reference genome, and analyzed for many factors, including whole-transcriptome gene expression, isoform detect, and strand information (Wang et al., 2009). The abundance of target transcripts are indicated by read counts of that transcript, normalized for the RNA

[Q] Expression: when, and how much of, the DNA 'message' is copied and made into a specific protein (molecules that perform the majority of cellular functions)
[R] m: messenger, r: ribosomal, t: transfer, nc: non-coding
[S] Barcodes: unique DNA sequences (also called indexes or tags) ligated to the cDNA fragments of a sequencing library, used for downstream sorting and identification by bioinformatic software

length and the sequencing depth of the sample, with the unit RPKM (reads per kilobase per million)[T].

RNA-Seq provides quantitative approximations of the abundance of target transcripts in the form of counts. However, these counts must be normalized to remove technical biases inherent in the preparation steps for RNA-Seq, in particular the length of the RNA species and the sequencing depth of a sample. For example, expectedly, deeper sequencing results in higher counts, biasing comparisons between different runs with different depths. Similarly, longer transcripts are more likely to have sequences mapped to their region resulting in higher counts, biasing comparisons between transcripts of different lengths.

Experimental considerations

As is the case with effectively using any technique or technology, it is vital to know its limitations to appropriately apply its data to the broader context. There are many aspects to consider when planning RNA-Seq experiments, including the study design, sample preparation, sequencing and data analysis protocols (Kratz and Carninci, 2014).

At the start of any experiment, irrespective of technique or technology, the experimental and control groups have to be assessed to ensure that as many variables as possible are accounted for. Examples of experimental considerations for RNA-Seq include ensuring cell-type homogeneity, consistent time points and sufficient sequencing depth.

It is less of an issue when sequencing cell lines, but as variations of RNA-Seq are used for translational medicine where tissue biopsies are used, it is important to compare homogeneous samples since gene expression profiles differ greatly between cell types. There is currently a move away from bulk towards single-cell RNA-Seq in order to obtain a cell-type-specific transcriptome instead of a tissue-specific one. Potential medical applications of this new avenue are discussed in the *'Base pairs to bedside'* section.

mRNA expression is inherently transient, as it changes depending on a cell's response to both internal and external cues, therefore it is important to remember that RNA-Seq data only offer information of one point in time of a cell's transcriptome. When comparing sample groups to control groups within and among experiments, variables should be considered, such as drug treatment vehicles or a patient's age at the time of biopsy, especially important when studying pediatric diseases such as Alagille syndrome.

RNA-Seq is a powerful tool to detect single nucleotide polymorphisms (SNPs) (Piskol et al., 2013) or splice-site mutations, but its data will only provide information for expressed genes. For example, mutations in non-expressed genes cannot be detected and genetic markers can only be used if they are expressed (Schneeberger, 2014). Therefore, one must consider if and how RNA-Seq can address the experimental question.

[T] RPKM = [# of mapped reads] / ([length of transcript]/1000) / ([total reads]/10^6)

Although often veiled in the 'black box' of Bioconductor packages (Huber et al., 2015) or Java applets, the analysis tools and statistical methods applied to RNA-Seq data are essential considerations for objective data interpretation. With a growing number of large repositories such as the Encyclopedia of the regulatory elements (ENCODE) (Rosenbloom et al., 2013) and The Cancer Genome Atlas (TCGA) (Robbins et al., 2013), uniformly analyzed data allows users to compile results from many sources, but this is difficult to maintain, due to the multitude of ever-evolving algorithms and pipelines. The least that should be done is providing detailed descriptions of the study samples and a clear pipeline of analysis methodology used for any published RNA-Seq data, apart from depositing data at the Sequence Read Archive (for raw sequence reads) (Leinonen et al., 2011) and the NCBI Gene Expression Omnibus (for processed gene expression values) (Barrett et al., 2013; Edgar et al., 2002).

Species-specific sequencing

Xenografting, elaborated in the section above of the same name, is a unique instance when the mixed-species nature of the sample can be used to glean biologically relevant information. A few previously published studies illustrated this: one in which cross-species hybridization of microarrays studied the human tumor transcriptome of brain metastasis in the mouse (Park et al., 2011), and another which used species-specific qPCR analysis to study the mouse angiogenic response across a panel of human tumor xenografts with known differences in vascular and stromal architecture (Farren et al., 2012). Aside from each technique being quite labor-intensive, neither approach allowed for a large-scale analysis of the transcriptomes from the two species. Recent reports have described related technologies to decode mixed-genome transcriptomes (Bradford et al., 2013; Conway et al., 2012; Raskatov et al., 2012; Rossello et al., 2013). **Paper III** presents our parallel effort in contributing a species-specific sequencing technology, which we call S^3 ('S-cube'), using RNA-Seq to quantify all mRNA transcripts in human tumors and surrounding mouse stroma, in an unbiased manner.

Xenograft models inherently provide many of the structural and molecular cues necessary for a tumor to exhibit characteristic growth as in the human body, but this mélange of cell types contributes to the difficulty in interpreting the data to assign transcriptional changes to particular cell types of the same species (as mentioned in *Experimental considerations* above). To supplement RNA-Seq data from xenografts, our S^3 approach can be used to study the interaction of individual components of the stromal compartment on tumor cells, by co-culturing these two cell types in a dish or embedded in a synthetic ECM. Apart from tumor biology, basic molecular pathway analysis can benefit from a cleaner read-out of signal-sending and signal-receiving cells, an example of which we also present in **Paper III**.

'Base pairs to bedside'

The translation of basic science sequencing endeavors to biomedical applications, or 'base pairs to bedside' (Green et al., 2011), is the concept of effectively using NGS technologies to

get unprecedented insight into the expanse of biology that covers the human experience. A therapeutic 'holy grail' is personalized medicine, and genomic medicine is an enticing chalice.

Applications of NGS are now routinely used for many studies, ranging from basic to biomedical research. Developmental and evolutionary biologists use whole-genome sequencing (WGS) for comparative biology studies; clinical researchers use WGS to identify regulatory elements and RNA-Seq for gene expression studies to understand pathological processes; epidemiologists identify novel virulence factors by studying the sequences of bacterial and viral species; and microbiologists study microbiomes of environments and organisms at an unprecedented level. Whole-exome[U] sequencing has been developed as a rapid tool for gene and mutation discovery that clinicians use to directly diagnose a patient displaying symptoms with an unidentified genetic cause. When specific genes or genomic regions are suspected in a disease, targeted sequencing is a more rapid and less analysis-intensive approach to 'target hotspots for disease-causing mutations' (Grada and Weinbrecht, 2013).

Crowned the method of the year for 2013 (Nature Methods Editorial, 2014), Single-cell sequencing is not only complementary to existing NGS methods, but is also a discovery tool able to capture the diversity of individual cell states within a seemingly homogenous tissue, and identify yet-unknown cell types of the developing and diseased body. Scientists have studied entire organisms, the organs themselves and their comprising tissue, but can now look forward to peering into each cell itself, relating it to its neighborhood or community, and not just the entire cell population. For example, imagine a clinician able to identify the 'pathology-important cells' (Eberwine et al., 2014) after single-cell sequencing a patient's tissue biopsy and prescribing a drug cocktail that has been designed to target specific disease-driving factors.

With high-throughput advances and falling prices in NGS technologies, it is absolutely exciting to utilize these techniques to gain higher resolution of your organism or system of interest, but first and foremost it is essential to consider the limitations of each technology in order to use it as a tool, along with other tools, to understand your system in context.

…one of these key principles of science is that no study is definitive of any piece of evidence of any finding on its own. We are in a process of uncertainty reduction when we are doing science, and each study contributes additional evidence to reduce that uncertainty.

– Prof. Brian Nosek
Center for Open Science
(Science Podcast, 2015)

[U] Exome: the protein-coding regions, comprising around 1% of the genome

PRESENT INVESTIGATION

AIMS

The overall aim of the research presented in this thesis is to investigate dysregulated Notch signaling in two diseases where this cell-contact-dependent communication system is implicated: breast cancer and the liver disease of Alagille syndrome (AGS). We focused on:

- A mechanism where hyperactivated Notch signaling crosstalks with other signaling pathways in breast cancer.
- A method to separate transcriptional information using species-specific differences of the cell types involved: tumor and stromal tissue in breast cancer xenografts, and signal-receiving cells with signal-sending cells affected by an AGS-like mutation.
- A characterization of a Jagged1 mutant mouse as a potential model for the liver disease of AGS.

Experimental tools used in this thesis:

Paper I: immunohistochemistry of patient breast tumors, qPCR, p53-luciferase, IL-6 promoter luciferase, κB-luciferase, tamoxifen-induced expression plasmid, Western blot, transfection, gene silencing by siRNA targeting, immobilized ligand assay, co-culture assay, adenoviral infection, ELISA, microarray, analyzing published data sets

Paper II: transfection, 12xCSL-luciferase, immobilized ligand assay, co-culture assay, mammary fat pad xenografting, cDNA library preparation, RNA-Seq, bioinformatics

Paper III: immunohistochemistry of mouse liver, serum chemistry analysis, transfection, qPCR, 12xCSL-luciferase, biliary organoid culture, immobilized ligand assay, co-culture assay, cDNA library preparation, RNA-Seq, bioinformatics

PAPER I: NOTCH & IL-6 IN BREAST CANCER

Notch signaling is often hyper-activated in breast cancer, but how this influences the course of the disease is still relatively unknown. We discover a role of non-canonical Notch signaling in activating the JAK/STAT cascade via IL-6, and identify a role for p53 in the Notch and cytokine response.

IL-6 acts as a pro-inflammatory cytokine, secreted by B cells, T cells and macrophages to stimulate an inflammatory response to tissue damage. IL-6 signaling is dysregulated in a variety of diseases, for example breast cancer, where high serum levels of IL-6 in patients correlate with a poor clinical prognosis (Sethi et al., 2011; Wang et al., 2005).

We analyzed a panel of breast tissue from patients for expression of markers of basal ($CD44^{high}$, $CK5/6^{high}$, ER^-, PR^-) or non-basal breast cancers ($CD44^{low}$, $CK5/6^{low}$, ER^+, PR^+), and found that the basal-type breast cancers expressed higher levels of nuclear Notch1 ICD (N1ICD), an indication of active Notch signaling, compared to the non-basal-type breast cancers. Studying published patient transcriptome data (Pawitan et al., 2005), we also found elevated *IL-6* and *JAG1* mRNA expression in basal, compared to luminal, breast cancers. In a panel of breast cancer cell lines *in vitro*, active Notch signaling lead to increased *IL-6* mRNA levels in basal-like (including MDA-MB-231), significantly more than in luminal (including MCF7), breast cancer cell lines.

We went on to study the link between Notch signaling and IL-6 in breast cancer, in the basal-type breast cancer cells MDA-MB-231, and observed that Notch signaling upregulates IL-6 mRNA and protein expression in two assays: (1) infection with adenoviral vector expressing NICD, and (2) immobilized ligand stimulation, and this upregulation could be abrogated by DAPT. Notch seems to regulate *IL-6* expression transcriptionally, as nascent (pre-splicing) and mature (spliced) *IL-6* mRNA was upregulated to the same extent. This transcriptional increase in turn led to increased protein levels of IL-6 in the cell culture medium, as determined by ELISA (enzyme-linked immunosorbent assay). IL-6 produced in response to upregulated Notch signaling in MDA-MB-231 cells activated JAK/STAT3, and the downstream target Bcl-xL. Conditioned medium from MDA-MB-231 cells over-expressing N1ICD was sufficient to induce pSTAT3 in naïve MCF7 cells, and this induction was blocked by IL-6 blocking antibody. This data together points to *IL-6* as a novel Notch target gene in basal breast cancer cells, and the Notch-induced increase in *IL-6* expression results in autocrine and paracrine activation of JAK/STAT signaling.

To identify the attributes of basal-like breast cancers causing their susceptibility to Notch-mediated IL-6 upregulation, we studied two components, ER and p53 status, differing in MCF7 (ER^+, wild type p53) and MDA-MB-231 (ER^-, mutated p53) cells. In MDA-MB-231 cells, re-introduction of ERα did not alter *IL-6* induction, although it did activate an estrogen-responsive-element-luciferase reporter construct. In contrast, *IL-6* expression dramatically reduced with the introduction of wild-type p53, while *HES1* expression did not change. Mouse embryonic fibroblasts genetically deficient for p53 showed an *Il-6* upregulation when

overexpressing N1ICD, which was abrogated upon introduction of wild type p53. MCF7 cells transfected with a mutated form of p53 now showed *IL-6* upregulation when the N1ICD was overexpressed. Therefore, these data convinced us that the Notch-mediated *IL-6* upregulation we observed was dependent on the cellular p53 status.

The conventional image of the Notch pathway is activating Notch signaling leads to cleavage of the Notch receptor, where the intracellular portion of the receptor then translocates to the nucleus and upregulates downstream genes. In this work, we found evidence that Notch signaling regulates IL-6 in an atypical way, that cytoplasmic N1ICD also could upregulate *IL-6* transcriptionally. Also, NICDΔRAM activated *IL-6* but not *HES1* in MDA-MB-231 cells, and dnCSL did not interfere with N1ICD-mediated *IL-6* upregulation. Therefore, we deduce that non-canonical CSL-independent Notch signaling is implicated in regulating IL-6.

We found that the Notch pathway communicating with the NF-κB cascade led to transcriptional activation of *IL-6* in an unconventional manner, requiring IKKα and IKKβ function but not canonical NF-κB signaling. Pharmacologically inhibiting IKKβ, or siRNA knockdown of IKKα or IKKβ, reduced Notch-mediated *IL-6* upregulation. TNF-α and LPS, but not the N1ICD, activated a κB-luciferase reporter construct, used to monitor downstream NF-κB signaling.

To test whether Notch-mediated IL-6 upregulation could affect tumor-associated macrophages, we exposed RAW264.7 cells, a mouse macrophage-like cell line, to conditioned medium from Notch-activated MDA-MB-231 cells and saw elevated levels of phosphorylated STAT3. Treating RAW264.7 cells with recombinant IL-6 increased *Notch1* and *Jag1* expression, and transfecting N1ICD or NICDΔRAM increased *Il-6* mRNA expression. As *Il-6* and Notch signaling are reciprocally upregulated in RAW264.7 cells, these data suggest the existence of a feed-forward loop for maintaining Notch and *IL-6* expression in macrophages.

In sum, we show that Notch activates IL-6 secretion and JAK/STAT signaling, p53-dependently but CSL-independently via IKKα and IKKβ. Our data link Notch and the tumor inflammation microenvironment in breast cancer, where tumor-stroma cytokine interactions and tumor-associated macrophages are known to instigate cancer progression.

PAPER II: S^3 RNA-SEQ TO STUDY TUMOR-STROMA INTERACTIONS

It is becoming increasingly evident that not only the genetic aberrations in the tumor are important for tumor development and patient prognosis, but also the interplay between the tumor and the surrounding tissue, the stroma. It is likely that there is a complex interaction between the tumor and stroma at many different levels, and it would be important to better understand the molecular consequences of this at a genome-wide transcriptome level. To address this in a breast cancer model, we use the xenografting strategy of mammary fat pad injection to take advantage of the species difference (i.e. human cells introduced into the mouse), and carry out RNA-Seq of both the primary (human) tumor and the surrounding (mouse) stroma. We call this new strategy species-specific sequencing (S^3). With S^3, both the tumor and stroma transcriptomes can be obtained with minimal manipulation of the tissue before analysis, and the gene regulation events in both tumor and stroma can be analyzed for the vast majority of genes based on their species-specific differences.

The S^3 protocol entails (1) obtaining material containing mixed-species cells or tissue stored in conditions to stabilize RNA, (2) disrupting and homogenizing the tissue, extracting total RNA, making a cDNA library, (3) sequencing, demultiplexing each sample, converting data to FASTQ format, aligning sequences to genomes of both species with STAR software, discarding overlapping reads after species separation, and calculating gene expression levels from normalized data.

In order to establish S^3 as a technology, we first needed to evaluate the ability to separate mouse and human cDNA transcripts after RNA-Seq of a mixed sample *in silico*[V]. The strategy is to align all sequence reads to both genomes and discard the overlapping reads, which are conserved and therefore cannot be distinguished between the two species. After testing three human and three mouse samples from previously published RNA-Seq data, we found that approximately 0.15 - 0.30% of the reads from one species were mapped to the other species' genome, rendering them un-available in a mixed scenario. This left over 99% of the reads able to be separated by species.

We were curious if our S^3 pipeline could be applied to a three-species comparison, which could be helpful in studying, for example, xenografted rat cancer-associated fibroblasts (CAFs) with human cancer cells into a mouse to understand tumor-CAF-stroma interactions. In theory, the same strategy of aligning reads to all genomes and discarding the overlapping reads can be applied, but when doing so we found many rat reads misassigned as mouse, although mouse reads were not misassigned as rat. This misassignment of rat reads could be due to the lower quality of the rat genome assembly or annotation, compared to the well-established human and mouse genomes. As genomes from more organisms are better

[V] *in silico*: in silicon, meaning via computer simulation

sequenced with improved technologies, S^3 can be used to decipher transcriptome changes in complex experimental settings.

It is an open question in the Notch field as to how a ligand ought to be presented when designing experiments in study biologically relevant pathway induction, also discussed in the ligand-receptor interaction section in the thesis introduction. To shed light on this, we put the S^3 technology to use in cell culture experiments to decode transcriptional effects of the mode of ligand presentation in Notch signaling. There are essentially two ways to activate ligand-induced Notch signaling experimentally: placing receptor-expressing cells on immobilized ligand fragments, or co-culturing receptor-expressing cells with ligand-expressing cells. We use human MDA-MB-231 cells, which physiologically express high levels of *NOTCH1*, as the receptor-expressing cells, and use mouse 3T3-L1 cells transiently transfected with *DLL4* as the ligand-expressing cells or immobilized Fc-DLL4 ligand. Activation of Notch signaling after each mode of ligand stimulation and abrogation by the γ-secretase inhibitor DAPT was verified using a 12xCSL-luciferase reporter assay. Studying the transcriptomes of the receptor-expressing cells after co-culture or on immobilized ligand, we find marked differences. The 'Notch signatures', genes upregulated after ligand presentation but reduced after DAPT treatment, were compared. Both signatures shared only one common gene, exemplifying how different the transcriptional output can be depending on how a ligand is presented. The co-culture and immobilized ligand assays also differed in the intensity of activation of selected canonical Notch target genes and to their sensitivity to inhibitor, with cells on immobilized ligand showing greater target gene activation and responsiveness to a 6-hour DAPT treatment. The co-culture assay may seem less responsive in a traditional Notch sense, but this could be due to the fact that this assay set-up is more 'noisy', with responding signaling factors from the ligand-expressing cell, but may be more physiologically representative. This distinction would need to be investigated further, with additional time points and other Notch ligands.

The technique that inspired the S^3 approach, engrafting human tumor cells into mouse mammary tissue has been valuable to understand breast tumor growth and to study differences in breast cancer subtypes *in vivo*. We wanted to test the ability of S^3 to capture transcriptome changes in both the tumor and stroma of xenografted luminal (MCF7) and basal-type (MDA-MB-231) breast tumor cells. There were several differing parameters between the two tumor models, including the mouse background, mouse immune status, status of mammary fat-pad clearing and proportion of tumor to stroma excised. In spite of this, our technique is able to capture the distinct transcriptomes of the MCF7 and MDA-MB-231 cells grown *in vivo* compared to cells grown in a dish, and most exciting, it is able to separate the stromal transcriptomes of each xenografted subtype.

In conclusion, we show that our bioinformatics-based transcriptome analysis technique S^3 faithfully assigns 99% of the genes to be of mouse or human origin *in silico*, in cultured cells

and *in vivo*[W]. Many other questions in breast cancer can be addressed using S^3, including studying cells with different levels of Notch expression, xenografting mice with different genetic backgrounds (e.g. p53-deficiency) to explore the consequences for stroma and tumor expression, comparing transcriptomal changes between a primary tumor and its metastatic tumor, xenografting primary breast cancer cells representing well-defined subsets of breast cancer (e.g. ER^+/ER^-, PR^+/PR^-, $BRCA^+/BRCA^-$), and xenografting human cell lines that form tumors at similar rates but are likely to have different effects on the surrounding stroma (e.g. stromal metaplasia, invasion/metastatis, angiogenesis). Essentially, S^3 can be done on any system with samples containing cells from multiple species, as long as each species' genome information is of equally high quality.

[W] *in vivo*: in living (Latin), refers to studying molecules or cells in an organism

PAPER III: NODDER, A MOUSE MODEL OF ALAGILLE SYNDROME

The physiological effects of a gene mutation during development manifest itself in tissues where the gene has a functional role, not compensated for or rescued by complementary factors. Studying models lacking normal gene function help us identify the essential factors necessary for normal organ formation and maintenance, and insights can lead to clinical interventions. Modeling developmental disorders, like Alagille syndrome (AGS), is all the more challenging due to the multiple organ systems affected in these diseases.

Previous attempts to model AGS have proven difficult since *Jag1* knockout mice do not survive past embryonic day (E)11.5 due to vascular defects (Xue et al., 1999). Mouse models for AGS-associated liver disease (described also in the *AGS models* section) have included a *Jag1/Notch2* double heterozygous mouse (Lozier et al., 2008; McCright et al., 2002), conditional ablation of *Jag1* in the portal vein mesenchyme (Hofmann et al., 2010), and a *Jag1* heterozygous mouse (Thakurdas et al., 2015). These models help our understanding of AGS-associated liver defects, but also have several shortcomings: the first model requires both mutated *Jag1* and *Notch2* in displaying AGS-like phenotypes, while *NOTCH2* mutation are observed in only 1% of AGS patients; the second model does not recapitulate AGS features in other organs; and the third model does not exhibit jaundice from bile duct paucity, and other affected organs were not reported.

We characterize a mouse model (*Jag1*$^{Ndr/Ndr}$) with a missense mutation in the gene encoding Jagged1 (H268Q, named Nodder), which presents many AGS-like phenotypes including liver, heart, lens and kidney pathologies in the mouse. This mutation results in intrahepatic bile duct paucity in these mice, and we identify biliary morphogenesis and maintenance, rather than biliary differentiation, to be disrupted. RNA-Seq of Alagille patient liver biopsies shows decreased expression of polarity markers compared to non-Alagille liver biopsies. We also begin to address the molecular mechanism by which this mutation acts, showing a transcriptional hypomorphic Notch signaling response and receptor-selective interaction with Notch2 and Notch3, but not Notch1.

The Nodder mice were originally developed in a C3H background, where the homozygous mutation is embryonically lethal, but the *Jag1*$^{Ndr/Ndr}$ mice survive into adulthood when outbred to a C3H/C57BL6 mixed genetic background, though far fewer than the expected 25% Mendelian ratio survive and those that do are of a considerably smaller size.

The surviving *Jag1*$^{Ndr/Ndr}$ mice present with jaundice from postnatal day (p)1, the first indication of a liver phenotype. These mice display both atrial and ventricular septal defects, also observed in AGS patients, at E15.5 and p0, and progressively irregular eye morphology from E13.5. The *Jag1*$^{Ndr/Ndr}$ mice also exhibit renal symptoms of jaundiced and atrophied kidneys at p10, and altered craniofacial proportions.

Immunohistochemistry with staining for WGA (wheat germ agglutinin; marker of portal mesenchyme) shows a near-complete loss of bile ducts in the *Jag1*$^{Ndr/Ndr}$ mice at p10, and expression analysis from whole-liver extracts reveals a significant downregulation of the

Notch target gene *Hes1* and the bile duct determinant *Hnf1b* (Hepatocyte nuclear factor 1-β). Expression of *Sox9* (Sry-related HMG box 9; marker of biliary cells in the developing liver) and developmental markers along the hepatoblast to hepatocyte lineage were not affected as assessed by qPCR, RNA-Seq and immunohistochemistry. In *Jag1*$^{Ndr/Ndr}$ mice that survive to adulthood, the jaundice seen at neonatal stages is not present, and pan-cytokeratin reveals bile ducts, although with abnormal morphology. ASMA (α-smooth muscle actin; marker of vascular smooth muscle cells) staining shows an absence of hepatic arteries. Blood serum analysis for liver function reveals no significant difference between adult *Jag1*$^{+/+}$, *Jag1*$^{+/Ndr}$ and *Jag1*$^{Ndr/Ndr}$ mice in the enzymes and metabolites tested.

To further investigate if the Jag1^{Ndr} mutation caused morphogenesis or differentiation defects, we cultured biliary organoids (protocol summarized in *Rise of the organoids* section) from *Jag1*$^{Ndr/Ndr}$ and *Jag1*$^{+/+}$ mice. Organoids from isolated bile duct fragments of *Jag1*$^{Ndr/Ndr}$ mice did not thrive as well as those from *Jag1*$^{+/+}$ mice, and some abruptly collapsed after 5-6 days in culture, indicating compromised structural stability, although *Notch2*, *Hes1*, *Hnf4a*, *Sox9* and *Hnf1b* expression were not significantly altered in organoids of *Jag1*$^{Ndr/Ndr}$ mice. A similar structural-collapse phenotype was seen in human Alagille organoids (Huch et al., 2015).

RNA-Seq of AGS patient liver biopsies does not show a decrease in biliary markers (*KRT19*, *HNF1B*, *SOX9*), but several polarity markers of the apical face of biliary cells are lost. Although not transcriptionally downregulated in the patient biopsies, ZO-1 (Tight Junction Protein Zona Occludens-1) does not show normal localization to intercellular tight junctions in the biliary structures of adult *Jag1*$^{Ndr/Ndr}$ mice.

To explore the effects of the Nodder mutation on Notch signaling at the molecular level, we employed a co-culture strategy of human HEK293 Flp-In[X] cells stably expressing wild type Jagged1 ligand (Jag1^{WT}) or Jag1^{Ndr} cultured with mouse myoblast C2C12 control cells or stably expressing the full-length Notch1 receptor (C2C12-FLN1), and carried out the S^3 pipeline (developed in **Paper II**) to obtain the transcriptomes from the receptor-expressing cells co-cultured with Jag1^{WT} or Jag1^{Ndr}. A principal component analysis (PCA), of C2C12 control and C2C12-FLN1 seeing HEK293 control, Jag1^{WT} or Jag1^{Ndr}, shows the transcriptome landscape of C2C12 and C2C12-FLN1 resulting from interaction with Jag1^{Ndr} mid-way between the transcriptomes resulting from interaction with Jag1^{WT} or control cells, although more pronounced in the C2C12-FLN1 cells. Also, several established Notch target genes show intermediate expression levels from interaction with Jag1^{Ndr}, compared to the levels observed after interaction with Jag1^{WT} or control cells. These data allude to Jag1^{Ndr} acting as a Notch-activation hypomorph, with reduced but not complete loss in gene expression. Since this mutation is not signaling-dead, it indicates that Notch signaling is

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[X] Flp-In™ system (ThermoFisher Scientific): allows integration and expression of your gene-of-interest in mammalian cells at a specific genomic location; used for generating constitutive expression cell lines.

proceeding, and indeed, Jag1^{Ndr} exhibits Notch receptor-specific binding to Notch2 and Notch3, but does not bind to Notch1.

Overall, we validate the *Jag1$^{Ndr/Ndr}$* mouse as a model of AGS-like phenotypes in the liver, heart, lens and kidney, which are likely caused by hypomorphic Jagged1 signaling, and implicate biliary cell polarity as a target of Notch signaling, dysregulated in our model and in Alagille patients.

FUTURE PERSPECTIVES

Combination therapy is a cancer treatment strategy to overcome hurdles, such as resistance associated with single-agent therapies and toxicity from high drug dosage. Because of off-target effects and intestinal drug toxicity, γ-secretase-based Notch pathway inhibitors seem to be less helpful as a mono-therapy as first hoped (Brennan and Clarke, 2013), but there are several GSIs, including MK0752 and RO4929097 (ClinicalTrials.gov identifiers NCT00106145 and NCT01151449, respectively), in early clinical trail phases to test safety and efficacy (Andersson and Lendahl, 2014). Along these lines, the Janus kinase inhibitor ruxolitinib combined with the HER2/neu blocking antibody trastuzumab is being tested in a Phase I/II clinical trail to treat metastatic $HER2^+$ breast cancers (ClinicalTrials.gov identifier: NCT02066532). In **Paper I**, an IKKβ inhibitor (TPCA-1) abrogated Notch-mediated *IL-6* expression, pointing to another potential drug target for breast cancer, but no IKK-β inhibitors have reached clinical development, primarily due to concerns over hepatic toxicity seen in IKK-β knockout mice (Deng et al., 2015).

With the non-canonical Notch and IL-6 link established in $HER2^-$ MDA-MB-231 cells in **Paper I,** we can test new therapeutic targets on breast tumor cells, tapping into existing drug treatments for inflammatory diseases, such as tocilizumab (Actemra®), a humanized monoclonal antibody against the IL-6 receptor (IL-6R), used mainly for the treatment of rheumatoid arthritis (Hennigan and Kavanaugh, 2008). For instance, it would be interesting to test the therapeutic effects of a DAPT and tocilizumab combination treatment on MDA-MB-231 xenografted tumors, and analyze drug-induced transcriptional changes using the S^3 pipeline.

The S^3 technology could also be used to further investigate tumor-CAF-stroma interactions, in a three-species approach similar to that tested in **Paper II**, but instead of xenografting human primary breast cancer cells with CAFs from one mouse background into a mouse of another background, such as CAST/EiJ and C57BL/6J, a combination successfully used in (Deng et al., 2014).

Emergent technologies, such as single-cell RNA-Seq and targeted genome editing by CRISPR-Cas9 (Jinek et al., 2012) could provide the high-throughput, high-resolution platforms necessary to be able to capture the pleiotropic Notch pathway's context-dependent signaling diversity. Single-cell sequencing of biopsies from **Paper III**'s *Jag1*$^{Ndr/Ndr}$ mouse livers and AGS patients' livers would allow us to obtain transcriptomes of each represented cell type to detect nuances lost in the traditional bulk sequencing method. We can also venture to rescue the structural collapse we see in *Jag1*$^{Ndr/Ndr}$ biliary organoids, or by correcting the Nodder mutation in the organoids using CRISPR-Cas9 targeted genome editing. This rescue could also be attempted in organoids from AGS patients (Huch et al., 2015), as was successfully done in organoid cultures from colon stem cells of cystic fibrosis patients (Schwank et al., 2013). Another interesting avenue is to perform immune characterization of the *Jag1*$^{Ndr/Ndr}$ mouse, as immune dysregulation is a new feature of the AGS phenotype (Tilib Shamoun et al., 2015).

POPULAR SCIENCE SUMMARY

Rube Goldberg was an American inventor and cartoonist known for depicting creations such as the 'Self-Operating Napkin' (Figure 12), a deliberately over-engineered contraption to accomplish a simple task, like wiping ones mouth, usually including a chain reaction. This led to coining of the term 'Rube Goldberg machine'[Y].

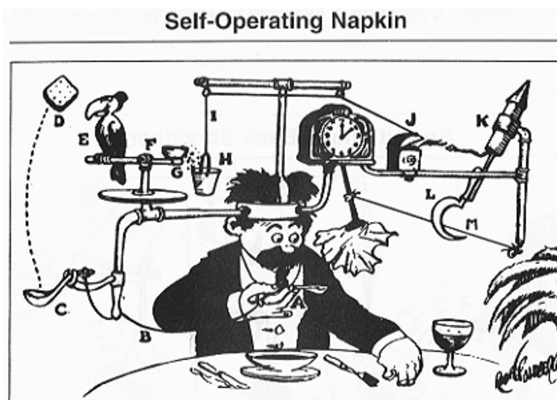

Figure 12: Rube Goldberg's self-operating napkin (1915)

A signaling pathway can seem like a Rube Goldberg machine at first glance, but this complex sequence of events is inevitable, given that a cell has a fixed set of machinery that must be reused to generate and perceive signals from an ever-evolving environment.

For me, health and disease are reflected with 'the Goldilocks principle', when too much or too little of anything leads to disease and 'just right' brings the balance of health. Since the day we were conceived, our bodies have tried, and continue today, to maintain a healthy balance, 'a homeostatic environment', that each cell should be in the right place and doing the right thing. DNA is the instruction manual that keeps our machine running. Depending on what the cell needs, RNA takes sentences of instructions and strings them together to make proteins, which have specific functions and carry out most activities of the cell. If words are misspelled in the manual, the resulting protein can be incorrectly made, and depending on which instructions are affected, this can cause the cell to copy itself uncontrollably, which is what is known as cancer.

My PhD studies have concentrated on two diseases brought about by damaged DNA, breast cancer and the lesser known genetic disease Alagille syndrome, which in its most severe forms leads to heart and/or liver failure, requiring transplantation. What ties these two diseases together is the protein Notch. Notch is a protein displayed on the surface of cells of the body that help the cells to decide what is going on in their environment. Like reaching out to shake hands, Notch reaches out trying to find partners to interact with. When Notch binds (shakes hands) with a neighboring cell the Notch signaling pathway becomes active in the Notch-displaying cell, typically either causes the cell to decide to divide to produce two cells or could tell the cell to turn into a different type of cell. In some forms of breast cancer there is too much Notch signaling, there is too much hand shaking, too many cells are deciding that they need to divide to produce more cells. In Alagille syndrome there is not enough Notch

[Y] Rube Goldberg machine: a deliberately over-engineered device, usually including a chain reaction, designed to perform a simple task in a complicated fashion; named after American cartoonist and inventor Rube Goldberg (1883–1970).

signaling, not enough hand-shaking, and certain types of cell that require a certain amount of Notch signaling are not produced in the developing baby making those organs weak.

In the first paper in my thesis (**Paper I**), I find that Notch signaling drives cancer cells not only to produce more cancerous cells but makes the cells produce another protein called IL-6, which causes inflammation. The ability of Notch signaling to cause inflammation in this way has never been shown and is surely a driver in the creation of the toxic environment in which cancer cells thrive. In my second paper (**Paper II**), I investigate how the communication between cancerous cells and 'healthy' cells (cells without damaged DNA), that surround cancerous cells, may respond to proteins produced by the cancer (like IL-6) to create an environment that actually promotes growth of the DNA damaged cancerous cells. It would be helpful to understand what the tumor (DNA damaged cancerous cells) and stroma (surrounding cells without DNA damage) are 'saying' to each other, in hopes to find a way to cut or change their communication. In order to do this we developed a method, based on the differences between the DNA of humans and mice, for 'reading' the 'internal thoughts' of human tumor cells injected into the mouse mammary gland. We showed that this new tool can be used to study human tumors grown in mice, and perhaps discover new signals or proteins that are druggable (for which anti-cancer drugs can be developed).

In **Paper III** of this thesis, I show that a single 'misspelling' in a mouse gene called Jagged1 can cause a disease in the mouse similar to the human condition Alagille syndrome. It is fascinating and heartbreaking at once to know that just a single 'typo' in a book with 3 billion characters could lead to such a severe condition requiring a liver and/or heart transplant, however being able to model this human disease in mice gives us great opportunities for understanding the disease and developing therapies for it. The Jagged1 gene actually makes one of the proteins that Notch 'shakes hands' with. The Notch protein on one cell binds to proteins called Jagged or Delta on neighboring cells to activate Notch signaling. We show that this single misspelling in the Jagged1 gene decreases the amount of Notch signaling in the developing baby mouse, which causes the liver to develop abnormally, just like in the human disease. So even though there is actually nothing wrong with the Notch protein, the Jagged1 protein in the developing liver is unable to reach out and grab on to the Notch protein in a manner sufficient for creating a normal liver.

As scientists, we will endeavor to continue in simplifying the biological world away from 'Rube Goldberg Machines', removing the daunting and confusing unknowns that obscure the understanding of, and thus treatment potential for, human diseases. With each scientist, each PhD, and each study, a piece of the puzzle is put in place towards the progress to cures.

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[Z] Reference: page 2

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