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NOTCH IN CANCER AND CANCER METABOLISM:
SIX DEGREES OF INTRACELLULAR TURBULENCE

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Stockholm 2016

Cover image shows the contact surface between the plasma membranes of two neighboring cells where Notch receptors and ligands interact.

Published by Karolinska Institutet.
Printed by E-Print AB

ISBN 978-91-7676-349-0

Notch in Cancer and Cancer Cell Metabolism: Six Degrees Of Intracellular Turbulence

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Science is not about what's true or what might be true; science is about what people with originally diverse viewpoints can be forced to believe by the weight of public evidence.
– Lee Smolin

Dedicated to Family, Friends, and Loved Ones

Abstract

Notch signaling is an evolutionarily conserved cell-to-cell contact-dependent signaling mechanism in multicellular organisms directing cellular fates both in early development and adult tissues. In metazoans the Notch pathway consists of multiple paralogs of receptors and ligands constituting a complex juxtacrine communications network orchestrating organismal homeostasis. Binding of receptors on signal-receiving cells to the ligands on signal-sending cells leads to proteolytic cleavage and release of the intracellular domain of Notch (NICD). NICD subsequently translocates to the cell nucleus to activate Notch downstream gene expression machinery by binding to the Notch-dependent transcriptional regulator CSL. Notch is highly context-dependent, and the nature of Notch-mediated outcomes is governed by multiple factors such as crosstalk with other signaling pathways, post-translational modifications, and CSL-binding type preference. Notch is ultimately a cell fate decider with a temporal specificity, where context and time can determine whether Notch inhibits or promotes a cellular outcome. The importance of the Notch pathway is further emphasized by the dramatic effects of dysregulated Notch signaling, which often leads to life-threatening diseases and cancer, such as CADASIL and T-ALL.

In this thesis I have glimpsed behind the veil into the unknowns of Notch signaling and investigated several novel aspects and peculiarities relating to Notch deregulation in cancer, and to Notch regulation via post-translational modifications.

“When Notch and Pim Unite”, Notch1 ICD undergoes post-translational phosphorylation by Pim kinases occurring at the nuclear localization signal within the PPD-domain, thus modulating the nuclear transport and transactivation of N1ICD. This impacts tumor growth and metabolism in breast cancer, and migration in prostate cancer.

In **“A Metabolic Turn of Events”** we discover that Notch signaling is able to reprogram the metabolism in breast cancer where high Notch levels induce the PI3K/Akt pathway leading to a shift towards aerobic glycolysis, while low Notch leads to a forced switch to glycolysis following mitochondrial oxidative phosphorylation defects. The Notch deficiency subsequently sensitizes the cancer cells for low glucose conditions.

Next we unleash **“Systematic KOs”,** when we knockout CSL in MDA-MB-231 breast cancer cells which leads to increased tumor growth and an activated hypoxic response. Furthermore, comparison of the Notch wild-type and CSL knock-out transcriptomic signatures reveals an upregulation of over 1700 genes not part of the Notch gene signature, suggesting that CSL transcriptionally controls a number of genes not part of the canonical Notch signature.

Lastly, we are **“Falling Into Hypoxity”** as canonical Notch1 is shown to induce HIF2α and trigger a HIF1α-to-HIF2α switch in medulloblastoma. However, Notch1 remains tumor suppressive in CAM-xenographs and the genetic removal of HIF2α increases tumor growth.

Taken together, this thesis contributes new puzzle pieces to building a complete picture of the Notch signaling pathway, its role in cancer, and provides new vistas for future anti-Notch therapies.

List of publications

I. When Notch and Pim Unite:

Phosphorylation of Notch1 by Pim kinases promotes oncogenic signaling in breast and prostate cancer cells
Niina M. Santio*, Sebastian K.-J. Landor*, Laura Vahtera, Jani Ylä-Pelto, Elina Paloniemi, Susumu Y. Imanishi, Garry Corthals, Markku Varjosalo, Ganesh babu Manoharan, Asko Uri, Urban Lendahl, Cecilia Sahlgren# and Päivi J. Koskinen#
Oncotarget 2016

II. A Metabolic Turn of Events:

Hypo- and hyperactivated Notch signaling induce a glycolytic switch through distinct mechanisms
Sebastian K.-J. Landor, Anders P. Mutvei*, Veronika Mamaeva*, Shaobo Jin, Morten Busk, Ronald Borra, Tove Grönroos, Pauliina Kronqvist, Urban Lendahl and Cecilia Sahlgren
Proc Natl Acad Sci USA. 2011. 108:18814-9.

III. Systematic KOs:

Loss of CSL unlocks a hypoxic response and enhanced tumor growth potential in breast cancer cells
Eike-Benjamin Braune*, Yat Long Tsoi*, Yee Peng Phoon*, Sebastian Landor, Helena Silva Cascales, Daniel Ramsköld, Qiaolin Deng, Arne Lindqvist, Xiaojun Lian, Cecilia Sahlgren, Shao-Bo Jin# and Urban Lendahl#
Stem Cell Reports. 2016.

IV. Falling into Hypoxity:

Notch signaling upregulates HIF2α expression in tumor cells
Anders P. Mutvei, Sebastian K.-J. Landor, Cecilia Sahlgren, Shaobo Jin, and Urban Lendahl
Manuscript

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Article not included in thesis

Inhibiting Notch activity in breast cancer stem cells by glucose functionalized nanoparticles carrying γ-secretase inhibitors

Mamaeva V., Niemi R., Beck M., Özliseli E., Desai D., Landor S., Grönroos T., Kronqvist P., Pettersen I.K., McCormack E., Rosenholm J.M., Lindén M., Sahlgren C.

Mol Ther. 2016 Feb 26.

List of abbreviations

ADAM	A Disintegrin And Metalloproteinase
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
CSL	CBF1/Suppressor or hairless/LAG-1
DAPT	5-fluorophenylacetyl-L-alanyl-2-phenylglycine-1,1-di-methylethyl ester
DHPCC-9	1,10-dihydropyrrolo[2,3-*a*]carbazole-3-carbaldehyde
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
FIH	Factor inhibiting HIF1α
GSI	Gamma-secretase inhibitor
MMTV	Mouse mammary tumor virus
NECD	Notch extracellular domain
NICD	Notch intracellular domain
NLS	Nuclear localization signal
PI3K	Phosphatidylinositol 3-kinase
Pim	Proviral Integration site for Moloney murine leukemia virus
PKCζ	Atypical protein kinase C ζ
PTEN	Phosphatase and tensin homolog
RAM	RBP-J associated molecule
T-ALL	T-cell acute lymphoblastic leukemia
TAN1	translocation-associated Notch homolog

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Review of the literature

Background

All forms of life are complex biochemical systems that propagate themselves in time and space, within zones where heat and liquid water can exist. One of these so called habitable zones exists in our solar system on our planet Earth. All life, including our own human biochemistry is the product of millions of years of evolution, naturally selected to sustain the changes in planetary environments. However, the starting point for all life is a small building block called a cell. Cells can exist alone or together giving rise to unicellular and multicellular organisms respectively. Bacteria are the most simple unicellular organisms consisting of just one prokaryotic cell per organism, while humans are multicellular organisms of $10^{\wedge 14}$ eukaryotic cells per individual. In multicellular beings cells come in different shapes and sizes which organize to give rise to tissues and organs.

Science is the search for true knowledge, by empirical collection of observations and data from our surroundings. It is the fundamental human aspiration to understand and grasp the four or more dimensions we are set to exist in. One of the big questions science is trying to decipher is the biomechanisms of life, but we humans as a species have only just begun to unravel the complex interactions that occur in every cell. As human technology advances, so does our knowledge of life.

Introduction

The history of cancer

Cancer is an ancient menace of multicellular lifeforms. The oldest evidence of cancer comes from tumor masses found in fossilized dinosaur bones dating back ~70 million years, as well as from the first human cancer victims whose bodies were mummified ~1500 BC. (1). Already Hippocrates –"The Father of Medicine" (460 BC – 377 BC) described in his Hippocratic corpus with the words "karkinos" and "onkos" (in Greek: crab and mass) the existence and treatment of lumps and lesions with both benign and malignant outcomes. During the rise of the Roman Empire notably Aulus Cornelius Celsus (25 BC – 50 AD) stressed early diagnosis and distinguished inoperable "carcinomas" from resectable "cacoethes". Arguably the most accomplished medical practicioner of antiquity was Galen of Pergamon (129 AD – 216 AD) who advanced various scientific disciplines in the footsteps of Hippocrates. Despite promoting humorism and the black bile theory of cancer, both Hippocrates and Galen were among the first to establish a rational approach to medicine over the prevailing ancient intertwinement of medicine and God. After the fall of the Roman civilization, medical practitioners of the Byzantine Empire followed the footsteps of Galen and organ specific description of different cancers started to take place.

During the renaissance the Italian anatomist Gabriele Fallopius (1523 – 1562) described for the first time the palpable clinical differences between benign and malignant cancers, still applicable today. This was also the first time for the humoristic black bile theory to be challenged and the era when modern science started to raise its head with the development of the modern scientific method. Shortly thereafter the discovery of microorganisms by Robert Hooke and Antoni Van Leeuwenhoek in the middle of the 17th century laid a foundation for microbiology. In the 18th century the first correlations between cancer and environmental factors started to emerge, when physician John Hill and surgeon Pervical Pott linked cancer to usage of tobacco snuff and to the occupation of chimney sweep. In 1859 the release of The Origin of Species by Charles Darwin gave humans a glimse of their origins (2). Soon thereafter humanity received its follow-up lesson in genetics by Gregor Mendel in the 1860s giving rise to the now well established Mendelian genetics (3).

The puzzling nature of cancer remained a mystery from the ancient times all the way to the 19th century. During the beginning of the 20th century discovery of antibiotics in 1928 - 1941 by Fleming, Chain, and Florey opened up new venues for cancer treatment. Notably actinomycin D was used widely to treat pediatric tumors in the 1950-1960s (4). Similarly, in the field of nutritional research, folate, or vitamin B9 was synthesized for the first time in 1937, and folate antagonists eg. Methotrexate showed unquestionable efficacy in treating children with leukemia (5, 6). At the same time, cancer theory was being reformed by progress in bacteriology, parasitology, and virology, and microorganisms were falsely labeled as the culprits of all that was cancer. This ultimately resulted in a Nobel Prize for Johannes Fibiger in 1926 for his discovery of the *Spiroptera carcinoma*, a worm which he interpreted as being the cause of stomach cancer, a hypothesis later proven to be false. However, today several other parasites are *de facto* known to cause cancer. Similarly the paradigm of viruses as causative agents in oncogenesis started to expand with the discovery of the Rous sarcoma virus (RSV) (7), and the mouse mammary tumor virus (MMTV) (8). With RSV came also the first discoveries of viral genome encoded tumor forming oncogenes (v-src), homologues of which were later discovered in the avian genome (9).

In the 1930s knowledge of health risks associated with cancer were accumulating, and new tools allowed researchers to systematically explore the nature of cancer. As health risks became known the US congress enacted the National Cancer Act of 1937, leading to the forming of the National Cancer Institute (NCI) in 1939. At the end of World War II the first cancer drug was derived from mustard gas widely used in chemical warfare (5). With the discovery of DNA, which paved the way into the molecular era of biology, cancer was widely thought of as one unique disease with a specific pathophysiological mechanism easily treated once identified. In the early seventies, cancer had become the second leading cause of death in the US. This led US president Nixon to declare “war” on cancer in 1971 by signing of the National Cancer Act with the aim of eradicating cancer as a major cause of death (10). After the start of the national cancer crusade many expected quick results, however, to this day, the battle wages on. As it turns out the war would not be won in one strike, but in many small skirmishes.

Our knowledge of the universe and us as a species is constantly evolving. With the completion of The Human Genome Project we now know the sequence of our DNA and can approximate the human genome to contain ca. 21000 genes. The ENCODE project currently challenges the paradigm of Junk DNA, by suggesting that also this DNA is important. Furthermore, we are also currently going beyond genetics in what is termed epigenetics to study inherited traits independent of the genetic code; a renaissance of Lamarckism. As basic research methods improve so do clinical applications. With the huge advancements in state of the art STED nanoscopy by Stefan Hell et al. we are now able to visualize life processes at a molecular scale with breathtakingly high resolutions separating objects only a few nanometers apart. With the development of improved screening methods for individual mutations and the onset of personalized medicine we are that much closer to developing targeted therapies, which like homing missiles seek and destroy cancer specifically. Scientific breakthroughs pave the way for the modern view on cancer. It is now known that cancer isn't just one disease, easily triumphed by the one right drug, but an umbrella term for a myriad of life-threatening diseases of dysregulated cell growth. A scourge of the multicellular.

In the 21st century cancer is viewed as a deregulation of the intertwined cell signaling pathways present in each cell in an organism. Modern cancer therapy is based on investigating the countless cell signaling components that are involved in mediating cancer formation, ultimately allowing us to target specific pathways important in diverse cancer forms. A myriad of cell signaling components capable of inducing cancer exist. One of these pathways is Notch.

Historical background of The Notch Signaling Pathway

The first mention of Notch originates from a study on a mutant *Drosophila melanogaster* strain from the 1910s, which was observed by John Smith Dexter working in the laboratory of Thomas Hunt Morgan to exhibit notched or beaded wings in a partial loss of function phenotype (11-14). Later, in the beginning of the 1940s, Donald F. Poulson kicked off the Notch field in *D. melanogaster* by observing and documenting for the first time the embryonic lethality in homozygous null Notch mutants (15, 16). Notch was cloned in the mid 1980s independently by two research groups, i.e. by Artavanis-Tsakonas' and Michael Young's group thus reinvigorating the Notch field in metazoans (17-20). At the same time light was shed on the protein itself as Notch was identified to be a trans-membrane receptor (21). The identity of two ligands, namely Delta and Serrate were soon to follow (22-24).
Following the initial discovery of the Notch gene, dramatic effects on the pathways deregulation started to surface. At the eve of the 1990s, a chromosomal translocation giving rise to a truncated form of the mammalian Notch receptor was identified in <1% of patients with T-cell acute lymphoblastic leukemia (T-ALL) (25). The subsequently named translocation-associated Notch homolog (TAN1) was able to, when ectopically expressed in bone marrow, to develop T-cell neoplasms (26). Yet, the effect of Notch on the development of T-ALL seemed at the time small and insignificant. However, years later the field experienced a paradigm shift when Andrew P.

Weng together with Jon C. Aster discovered that over 50% of T-ALL cancers harbor activating mutations in the Notch1 receptor (27).

At the end of the 1980s evidence of a role for Notch in breast cancer was also accumulating. The identification of the integration site for the mouse mammary tumor virus (MMTV) led to the discovery of another truncated Notch paralog, named INT3 (integration site 3), later to be known as Notch4 (28). Similarly, overexpressing Notch4 in mammary tissue of transgenic mice led to the development of mammary tumors in 100% of the cases (29).

Today we know that Notch is involved in a significant number of processes both in development and adult tissue homeostasis (30-32). The development of the Notch pathway is thought to stretch hundreds of millions of years into the past, being associated with the rise of the metazoan multicellular organisms (33, 34). At the eve of transition from unicellularity to multicellularity, the fundamental units of life, namely the cells needed to develop means for communication, coordination, and organization between each other. These functions were mediated by signal-transduction pathways, which allowed cells to orchestrate differentiation programs for development of specialized cells and organs. In metazoans, less than 20 different pathways developed to mediate developmental processes but out of these only 7 form the "crème de la crème", controlling most of the cell communication that occurs during development (33, 35). The seven major cell-cell signaling pathways are: Wnt; Transforming Growth Factor β (TGF-β); Hedgehog; Receptor Tyrosine Kinase (RTK); Jak/STAT; nuclear hormone receptor; and Notch (30).
Notch is indeed one of the "heavy-hitters" in metazoan developmental, as well as in postnatal signaling, regulating multiple processes which include: proliferation, apoptosis, cell polarity and more, giving rise to tissue-broad regulation such as lateral inhibition and induction, stem cell maintenance, patterning, and binary cell fate decisions (30, 36). Inhibition and induction of differentiation, as well as lineage specification at different branch points in development display the context-dependent signature function of Notch. In one context, precursors of equipotency can be steered towards differential cell fates upon receiving unequal levels of Notch signal, while in another context Notch can simply induce terminal differentiation (37). Furthermore, Notch has recently been described as an inducer of transdifferentiation during adult tissue homeostasis, where in the adult lung Notch levels can lead to direct conversion of cellular fates from mucus secreting club and goblet cells, to mucus-transporting ciliated cells, and *vice versa* (38), thus further expanding the reach of the Notch pathway. All these functions continue to have relevance in self-renewing tissues of the adult vertebrate organisms but also in tumorigenesis.

Developmental processes involving Notch signaling

Lateral Inhibition

The most classical developmental process controlled by Notch is lateral inhibition during neuronal development. Starting with a cell population with a random expression pattern of Notch ligands and receptors, the cells will slowly undergo a change towards a 'checkers' or 'salt-and-pepper' -pattern, where cells that end up only expressing Notch will remain undifferentiated and later commit to a epithelial fate, while cells with Jagged-ligand expression will differentiate to form neurons. This allows for neurons to develop intertwined in supportive glial cells. Thus, Notch signaling allows a full spectrum of cells to develop, separating early-born cell types from late-born cell types.

The classical view of lateral inhibition suggests that in neurons expression of the Notch ligand Dll1 is induced by the proneural genes Mash1 and Ngn2. The ligands then bind to Notch expressed on neighboring cells and via subsequent release of NICD activate the Notch downstream gene reponse in these cells. The NICD-RBPjκ complex in turn induces the expression of Hes1 and Hes5, which suppress the proneural genes and subsequently the Notch ligand genes. The modern view of lateral-inhibition on the other hand suggests the Ngn2-Dll1-Hes1 axis oscillates dynamically in neural progenitors, in a manner optimizing neural progenitor cell proliferation and neuronal differentiation (39). Moreover, the developing nervous system is partitioned into many compartments by boundaries, where compartment cells may oscillate Hes1 while boundary cells express Hes1 in a sustained manner giving rise to neuron-free zones (40). Sustained or oscillatory Hes1 expression patterns may thus also contribute to differential characteristics in undifferentiated neural progenitor cells (39). Furthermore, the dualistic nature of Notch also aids the plasticity in the adult brain, where Notch helps maintain stem cells and transit-amplifying (TA) cells, whereas inhibition of Notch leads to an increase in TA cells and neurons (41, 42).

Binary cell fate decisions

Another well-defined developmental mechanism involving Notch is that of asymmetric cell division which can give rise to sibling cells of distinct fates and characteristics. Asymmetric daughter fates can be determined by asymmetrically distributed cell fate determinants, which segregate to only one of the two daughter cells (43). Fate determinants, such as Numb and Sanpodo, which interact with Notch, can through their asymmetric distribution also affect the distribution of Notch (see section on Numb and Sanpodo). While Numb antagonizes Notch levels and vice versa, Sanpodo potentiates the effects of either low Notch or high Notch in the two different settings (44).

Lateral induction

Lateral induction represents the third mode of Notch action in development, where Notch and ligand expression on adjacent cells results in positive feedback which elevates expression of both Notch and ligand on both cells (45). Thus, instead of inhibiting each other, the cells cooperate to meet their fates together (46). For example the formation of terminally differentiated secondary lens fibers from the monolayer of epithelial cells on the lens surface in the vertebrate ocular lens, relies on FGF-mediated switching from lateral inhibition to lateral induction (47). Another example involves the formation of arteries, specifically the smooth muscle cell (SMC)–layers surrounding the endothelial vessel lumen. Expression of Jagged on endothelial cells induces Notch and subsequently Jagged on the first SMC-layer, an effect which propagates via lateral induction to the following SMC layer (48). Similar lateral induction has also been described in the development of the inner ear(49, 50).

Molecular basis of The Notch Pathway

The Notch

In the bilaterian metazoans of the animalian kingdom the Notch pathway of juxtacrine signaling varies in complexity depending on the species and whether they are invertebrates or vertebrates. Classic canonical Notch signaling is mediated via DSL (Delta, Serrate, Lag-2) ligand binding to the Notch receptors, which leads to cleavage of the receptor and release of the Notch intracellular domain (NICD). NICD subsequently translocates to the cell nucleus binding to the transcriptional regulator CSL (CBF-1 in humans, suppressor of hairless in *Drosophila melanogaster*, LAG-1 in *Caenorhabditis elegans*) thus activating Notch target gene expression. Much of the core mechanistic knowledge of Notch today comes from genetic studies in *C. elegans* and *D. melanogaster*. In *C. elegans* the Notch-related receptors LIN-12 and GLP-1 are activated by binding to the ligands LAG-2, APX-1, ARG-1, and DSL-1 while downstream Notch signaling is mediated via the transcriptional regulator LAG-1 (51). Likewise in *D. melanogaster*, a single Notch receptor is activated by two different ligands, namely Serrate and Delta, and where NICD ultimately binds suppressor of hairless (52, 53). Despite differences in nomenclature, the core units exhibit conserved functionality among species. The desired goal of course is to understand the functionality of Notch in *Homo sapiens*, i.e. humans.

In humans, the Notch pathway involves 4 receptors (Notch1-4), with the encoding genes on chromosomes 9, 1, 19, and 6 respectively (54-56), as well as ligands (Jagged1&2, Dll1,3,4) on chromosomes 20, 14, 6, 19, and 15 respectively (57-61). Both receptors and ligands exhibit redundant overlapping functions as well as distinct properties. A classic canonical Notch signaling cascade starts with two neighboring cells making physical contact with each other. This allows receptors on the plasma membrane of the signal-receiving cell to bind DSL ligands expressed on the signal-sending cell. Of course in reality, all cells are both signal-sending and

signal-receiving to different degrees, yielding bidirectional signaling complexity. A mechanical "tug of war" follows which subsequently leads to the endocytosis of the ligand and cleavage of the receptor. The Notch receptor has 3 cleavage sites, S1 is an early cleavage event catalyzed by Furin convertases occurring in the trans-Golgi apparatus allowing for heterodimerization and correct assembly of the receptor (62). After assembly the signal-sensitive receptor is transported to the plasma membrane in wait for activation. S2 cleavage is the first activating event which is characterized by the formation of a truncated Val1721 receptor. S2 cleavage is catalyzed by ADAM (A Disintegrin And Metalloproteinase) family metalloproteases, notably by ADAM-17 and ADAM-10. The third cleavage event at S3/S4 is mediated by gamma-secretase and leads to the release of the 1744Val N-terminal intracellular domain of Notch (NICD). Also other cleavages occur, but the 1744 cleavage generates the most active and stable form of NICD (63). Following cleavage at S3, NICD subsequently translocates to the nucleus where it binds CSL to activate gene expression.

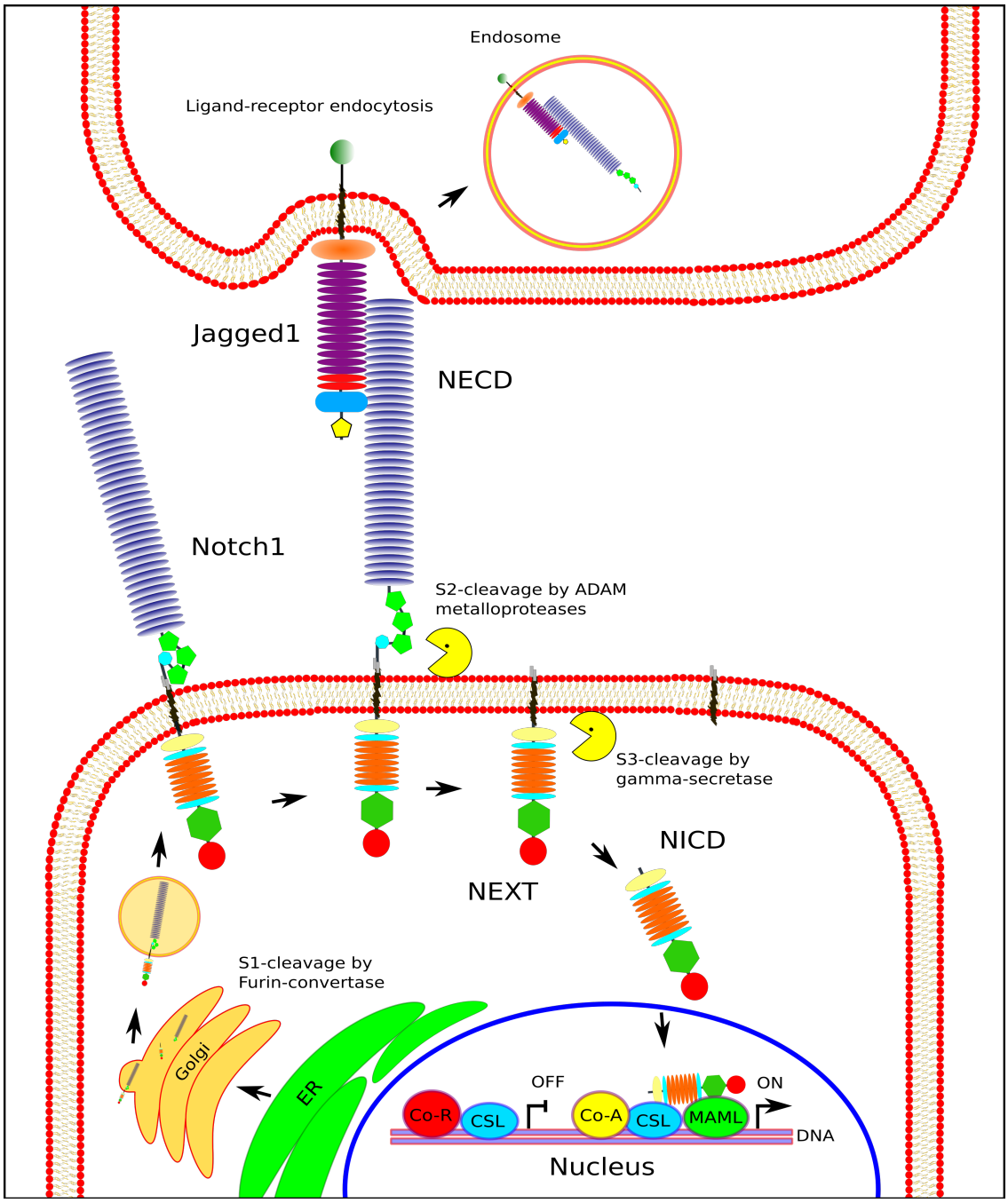

Figure 1.The canonical Notch signaling pathway. The Notch receptor is translated in the ER and undergoes cleavage at S1 by Furin convertase and subsequent heterodimerization in the Golgi apparatus. From the Golgi, the mature Notch receptor is transported to the plasma membrane. At the membrane, the active Notch1 receptor can bind a Notch ligand (Jagged1) presented by a neighboring cell. This leads to endocytosis of the ligand-receptor complex, which reveals S2 for cleavage by ADAM metalloproteases. This event subsequently reveals S3 for cleavage by the gamma-secretase complex, and releases NICD. NICD then translocates to the nucleus and binds CSL-MAML in a complex, displacing the corepressors (Co-R) and instead recruits coactivators (Co-A) for initiation of Notch downstream gene expression.

The domains of the Notch receptor

Notch receptors are type I transmembrane glycoproteins out of which the prototypical *Drosophila* Notch weighs around 300kDa. The receptors are synthesized as precursor proteins and proteolytically cleaved in the trans-Golgi complex by a furin-like convertase at S1 (62, 64), followed by a non-covalent heterodimerization of the extracellular 180kDa portion to the 120kDa transmembrane and intracellular component of Notch (62). After processing in the Golgi and the endoplasmid reticulum (ER), which includes both glycosylation and fucosylation of several EGF repeats (65) (66, 67), the mature receptors are transported to the plasma membrane. Extracellular binding between Notch ligands and receptors is mediated via the calcium-dependent EGF (epidermal growth factor-like) repeats (68, 69). Mammalian Notch1 and Notch2 proteins contain 36 tandemly arranged EGF-repeats, while Notch3 and Notch4 have 34 and 29, respectively (70). Out of these, EGF-repeats 11-12 are most important for productive interactions with ligands (71, 72). The ligand-binding extracellular portion of Notch is attached to the NRR which maintains metalloprotease resistance in the absence of ligand binding. The importance of this regulation is demonstrated by the fact that most point mutations and insertions that lead to constitutive activation of Notch1 in T-ALL are found within the NRR. (27) The NRR consists of three cysteine-rich LIN-12-Notch repeats (LNR) and the heterodimerization domain (HD) containing both the S1 (furin) and S2 (metalloprotease) cleavage sites both preceding the transmembrane portion (73). The LNR, together with the HD domain, cover the S2 metalloprotease site in the autoinhibited state. Following ligand association and endocytosis a pulling force is exerted on the receptor which according to the mechanotransduction model of Notch activation leads to stripping of the LNR off of the S2 site, revealing it for proteolytic processing by Kuzbanian/ADAM10 and ADAM17/TACE (tumor necrosis factor α converting enzyme) (74) (75). An alternative hypothesis for the mechanotransductive regulation of S2 cleavage exists and is called the allosteric model where allosteric regulation of Notch yields a reconfiguration of the molecule subsequently allowing ADAMs to cleave at S2. The S2 cleavage creates the membrane-tethered Notch extracellular truncation (NEXT), which is a substrate for γ-secretase that progressively cleaves NEXT within the transmembrane domain from the intramembrane layer towards the middle of the transmembrane domain, from site S3 to S4. Cleavage at S3 is sufficient to release NICD, while the subsequent cleavage at S4 releases the transmembrane Nβ peptide. Different NICD species can be formed at S3 cleavage, however, the most active and stable one is the NICD cleaved at valine 1744 (63, 74). The γ-secretase enzyme complex consists of four membrane proteins, namely the catalytic component Presenilin and three cofactors: Nicastrin, Pen2 and Aph1, in a 1:1:1:1 stoichiometry (76). Mammalian cells exhibit at least two presenilin (PS1/2) and two Aph isoforms, potentially allowing for at least four different γ-secretase complexes to form, and especially the PS1/2 switch can contribute differentially to Notch signaling (74, 77, 78). Furthermore, the enzyme complex can be reduced to the functional trimeric core of PS1/Pen2/Aph1 and still remain 50% active, yet nicastrin is required for optimal stability and activity (79).

The active Notch fragment NICD translocates to the nucleus with the help of nuclear localization sequences. Previous research has indicated that NICD contains two nuclear localization sequence domains (NLS1-2), located N-terminally and C-terminally of the ANK repeats, respectively (80), although up to four distinct potential nuclear localization sequences have been observed (81). The N-terminal NLS1 contains 2 basic monopartite sequences for nuclear localization at 1779-1783, and 1820-1825, while the C-terminal NLS2 has two closely spaced sequences of basic amino acids at 2156-2160 and 2177-2182 previously thought to function together as a bipartite NLS (82) after mutational removal of the two clusters of basic amino acids and the linker region. However, the two basic sequences have later via mutational studies been shown to not mediate nuclear localization (81). Classical nuclear localization sequences (cNLS) are recognized by Importin-α which via its 2 binding pockets; the minor and major groove can bind a monopartite NLS singly or the two clusters of a bipartite sequence, normally separated by a 9-12 amino acid linker (83). Recent findings however show the existence of longer linker sequences with functionality in either direct binding to the Importin-α backbone or in regulating binding affinity to Importin-α (83). Post-translational modification of the linker sequence or sequences close to the bipartite NLS may also directly affect the conformation needed for the bipartite NLS binding to the minor and major groove (83-85). Nuclear transport of Notch has been shown to be mediated by Importin-α (81).

Binding of NICD to CSL occurs mechanistically via a bipartite functional entity termed RAMANK and is constituted by the stable high-affinity interaction of RAM (86, 87) and weak interaction of the seven ankyrin repeats (ANK) domain to CSL (82). The N-terminal RAM domain of NICD binds via its short (≤25 residues) ΦWΦP motif to the BTD pocket of CSL, and substitutions in this motif significantly reduces binding affinity (86-89). While the RAM domain mediates docking to CSL the ANK repeat domain is alone capable of mediating transactivation of CSL via weak interactions (82). Formation of the ternary complex of CSL-NICD-MAML is however ANK-dependent and occurs independently of RAM (88, 90). Neither NICD nor CSL alone is able to bind MAML, however when complexed together the two proteins cooperate in binding MAML with high affinity, suggesting that the function of the NICD-CSL complex and ANK repeat domain is to allow MAML association (73). MAML is the essential cofactor required for initiation of the Notch downstream response, and the CSL-NICD-MAML complex subsequently recruits the p300 histone acetyltransferase for activation of transcriptional responses (91).

Structurally CSL contains a 420 amino acid core encompassing the N-terminal domain (NTD), the β-trefoil domain (BTD) and the C-terminal domain (CTD) (86), and binds DNA as a monomer with the NTD and BTD at the core consensus site (C/A/T)(G/A)TG(G/A/T)GAA (92). Also other weaker consensus sites exist (93). Interestingly, increased complexity is added to the system by the fact that several Notch responsive genes, including Hes and Hey related genes have dual CSL binding sites, and sequence paired sites (SPS) which can be arranged either in a head-to-head or head-to-tail configuration (93-95). Different CSL configurations favor different NICDs, for example N1ICD prefers paired sites while N3ICD performs best on single sites (94, 96).

CSL is considered to function in the absence of NICD as a repressor of its target genes with the help of a myriad of corepressors such as CIR, FLH1C/KyoT2, SPEN aka. SHARP/MINT, histone demethylase Lid/KDM5A, and NCoR/SMRT (97-99) that can form several different corepressor (CoR) complexes with different binding modalities. For example, KyoT2 binds with high affinity to CSL via the BTD with a similar ΦWΦP motif as found in the RAM domain of NICD (100), while binding studies with SHARP/MINT, which is emerging as the most critical Notch corepressor in vivo, suggests different mechanisms of interaction (99, 101, 102). CoR complexes are further able to recruit histone deacetylases (HDACs) and histone methylases to modulate the chromatin environment. At the event of NICD, MAML and other coactivators such as histone acetyltransferase (HAT) complexes p300/CBP and p300/CBP-associated factor (PCAF) are recruited to displace corepressors, and activate target gene expression. This dual mode of action allows for tight control of Notch downstream genes. The view of CSL as a static repressor in the absence of NICD is however being challenged by data indicating that CSL may be dynamically recruited to DNA-binding sites in response to Notch activation (103). Alternatively preloaded complexes may be exchanged.

Following NLS2 is the evolutionary divergent transactivation domain (TAD) which in murine Notch1 is located at amino acids 2194-2398 and is capable of Notch paralog-dependent autonomous transactivation of CSL (104, 105). The nuclear protein EBNA2 encoded by the Ebstein-Barr virus also possesses a similar but distinct TAD domain capable of CSL transactivation (104). A complete TAD domain can only be found in Notch1 and Notch2, and out of the two Notch1 exhibits stronger activity. (104, 106). Notch3 possesses a shorther and much weaker TAD requiring a zinc-finger binding site near the CSL site for functionality (94), which partially explains the weaker transactivation seen by N3ICD compared to N1ICD and N2ICD (106, 107). N4ICD completely lacks a TAD (108). The TAD domain also contains a C-terminal PEST motif rich in proline (P), glutamic acid (E) serine (S), and threonine (T), which via post-translational modifications controls half-life and degradation of NICD (30).

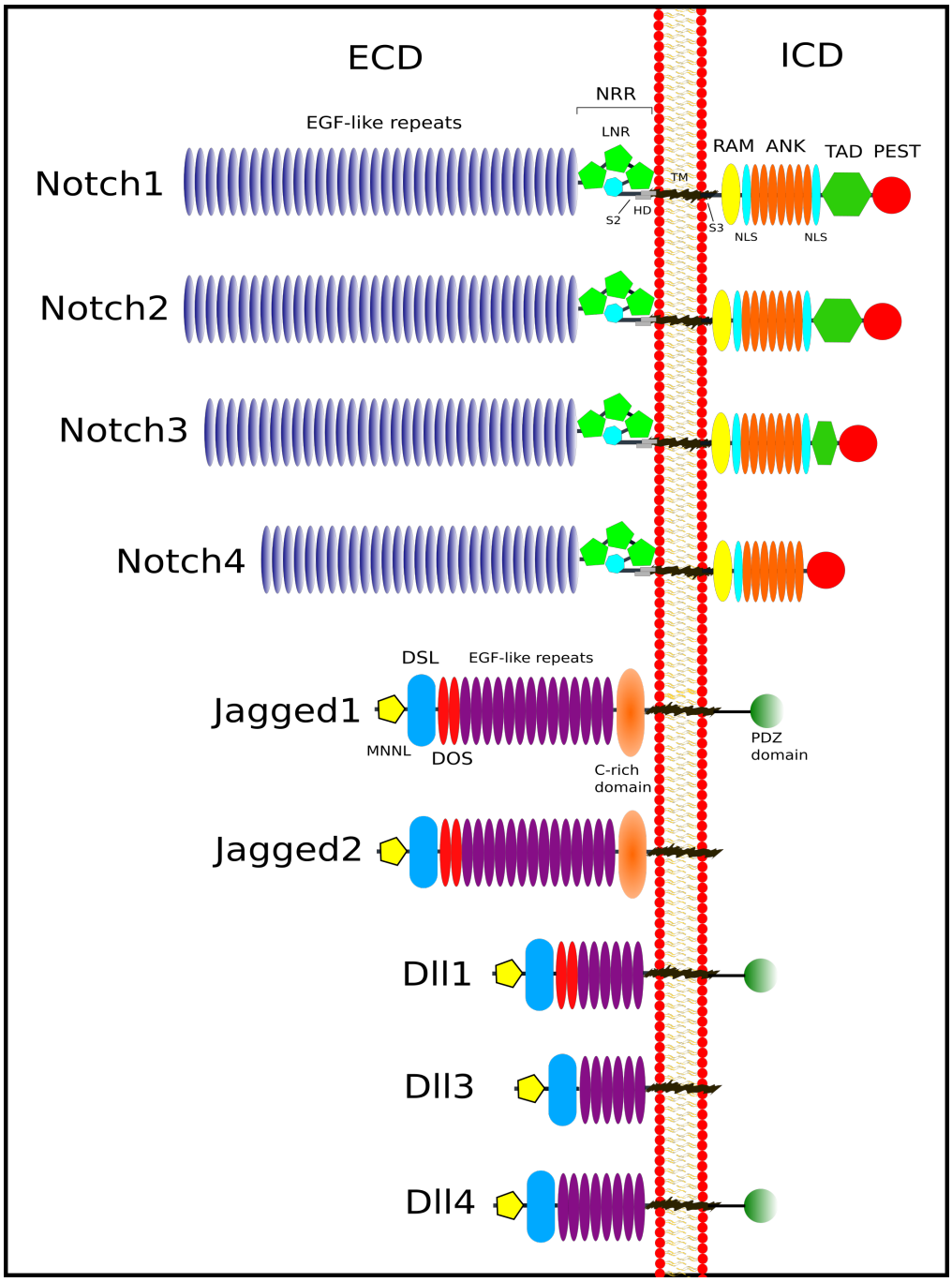

Figure 2. The domains of the mammalian Notch receptors and ligands. Notch receptors and ligands are divided into extracellular and intracellular domains (ECD and ICD respectively). The Notch ECD contains 29-36 tandemly arranged epidermal growth factor like repeats (EGF), and a negative regulatory region (NRR) containing the LIN-12-Notch repeats (LNR), and the heterodimerization domain (HD), which encompasses the S2 cleavage site. The S3 cleavage site is located immediately after the transmembrane domain (TM). The Notch ICD consists of the RBP-J associated molecule (RAM) domain, two nuclear localizations sequences (NLS), 7 ankyrin repeats (ANK), a transactivation domain (TAD), and a proline, glutamic acid, serine, and threonine-rich domain (PEST). Note the exceptions in Notch3 and Notch4, where Notch3 exhibits a smaller TAD domain, while Notch4 is lacking the TAD and the second NLS. The

Notch ligands contain on their ECD the MNNL (Module at the N-terminus of Notch ligands) module, DSL (Delta/Serrate/LAG-2) motif, and DOS (Delta and OSM-11-like proteins) domain, which participate in ligand binding. The Jagged ligands also contain a cysteine-rich area close to the transmembrane domain. At the ICD, Jagged1, Dll1/4 also contain a PDZ (post synaptic density protein [PSD95], *Drosophila* disc large tumor suppressor [Dlg1], and zonula occludens-1 protein [zo-1]-binding motif, which participates in intracellular protein-protein binding.

Notch ligands

The Notch ligands are also type I transmembrane proteins with a similar architecture as the receptors containing an extracellular domain (ECD), a singular transmembrane domain, and an intracellular domain (109). The ECD of the ligands contains an N-terminal MNNL (Module at the N-terminus of Notch ligands) module, a DSL (Delta/Serrate/LAG-2) motif, and may also contain a specialized tandem EGF-repeat called the DOS (Delta and OSM-11-like proteins) domain, as well as several other tandem EGF-repeats (109). The MNNL, DSL and DOS domains are all involved in receptor binding (73, 74). Recently, the MNNL has also been described to contain a C2-domain which through calcium loading can bind different phospholipid moieties (110). The canonical DSL ligands Jagged1, Jagged2 and Dll1, Dll3, and Dll4 in mammals correspond to the Serrate and Delta ligands in *Drosophila* respectively. The mammalian Notch ligands have so far been documented to have overlapping functional redundancy with the exception of Dll3. Dll3 is the most divergent of the ligands with inability to efficiently localize to the plasma membrane and to signal in trans, and has subsequently been hypothesized to act as an inhibitor of ligand-induced Notch signaling (30, 111-113). What separates the Jagged from the Dll ligands is the cysteine-rich motif close to the plasma membrane found only in Jagged ligands. The ICD of Jagged1, Dll1/4 contains a PDZ (post synaptic density protein [PSD95], *Drosophila* disc large tumor suppressor [Dlg1], and zonula occludens-1 protein [zo-1]-binding motif, which has been suggested to have a role in allowing binding of PDZ-binding domain containing proteins (114), and subsequent bidirectional signaling (115).

Downstream effectors

A large number of Notch downstream target genes have been identified, and the best characterized ones are the transcriptional repressors of the basic helix-loop-helix (bHLH) type of transcriptional repressors. These include the Hes (Hes1-7) and Hey families (Hey1, Hey2, HeyL, HesL/HeIT, Dec1/BHLHB2, Dec2/BHLHB3) (116-118). Hes1 acts as a tumor suppressor in epithelial cells by inhibiting proliferation, while being in turn downregulated by 17β-estradiol in ER-positive breast cancer thus increasing proliferation (119). Other well-characterized Notch downstream targets are c-myc, nuclear-factor-kappa (NF-κB), vascular endothelial growth factor (VEGF), p21, p27, Akt, Slug, and Snail (120-123). Notch1 also controls the expression of Notch3 (124), and is involved in regulating the cell cycle by induction cyclin D1 and CDK2 thereby promoting S1 entry (125). With the development of more and more powerful RNA-

sequencing techniques (126), detailed information about the various Notch-transcriptomes is emerging. The immediate Notch response is considerably larger and more diverse than previously thought with the appearance of distinct cell-type and tissue specific sets of only partially overlapping transcriptomes (30). Furthermore, Notch-dependent long non-coding RNA expression profiles have also been unraveled (127).

Post-translational modifications of Notch

Post-translational regulation of Notch is an emerging field of research with the potential to further elucidate the pathway's context-dependent pleiotropism. Several of the domains of NICD are targeted by different enzymes to modulate the outcome of NICD.

Phosphorylation

Protein phosphorylation is one of the most important regulatory mechanisms in eukaryotic cells, and it is estimated that one third of all eukaryotic proteins undergo reversible phosphorylation (128). Indeed, Notch is included in the phospho-protein family and contains multiple paralog-specific phosphorylation sites with different regulatory functions (120).
Glycogen synthase kinase-3β (GSK-3β/Shaggy) is able to phosphorylate N2ICD at several residues C-terminally of the ANK domain thus negatively regulating its transcriptional activity (129). However, GSK-3β in turn enhances the stability of N1ICD and is required for Hes-1 expression (130). Granulocyte colony-stimulating factor (G-CSF) can phosphorylate N2ICD at multiple sites, including S2078, thus inactivating the molecule (131). Cyclin-dependent kinase 8 (CDK8) interacts directly with MAML and phosphorylates N1ICD at multiple residues in the PEST domain thus strongly enhancing the PEST-dependent degradation of N1ICD by Fbw7/Sel10 ubiquitin ligase (132). Nemo like kinase (NLK) is able to phosphorylate N1ICD C-terminally of the ANK domain between amino acids 2126-2282 decreasing transcriptional activity by interfering with formation of Notch ternary complex (133). On the other hand N3ICD activity is increased by NLK phosphorylation (133). PKC zeta phosphorylates membrane bound NEXT and full length Notch receptors, and depending on activation state, either enhances NICD formation or triggers Notch receptor recycling (134). More recently, Notch4 has been observed to be targeted for Akt phosphorylation, and subsequent 14-3-3 association thus restricting nuclear translocation of N4ICD (135).

Ubiquitination

Various components of the Notch signaling pathway, including both receptors and ligands, are modified by E3 ubiquiting ligases (136). The prototypical E3 Notch modulator F-box and WD-40 (Fbxw7/Sel10/cdc4) ubiquitinates NICD at CDK8 phosphorylated sites within the PEST domain thus regulating its half-life via ubiquitin-proteasome-mediated degradation (132, 137-

139). Even higher levels of control over degradation exist, for example serum- and glucocorticoid-inducible kinase 1 (SGK1) phosphorylation of Fbxw7 at serine 227 functions as a switch to allow Fbxw7 to ubiquitinate N1ICD (140). The significance of regulating NICD half-life is underlined by the fact that both activating gain of function mutations of NOTCH1, as well as loss-of-function mutations of FBXW7 can be found in T-ALL (141, 142). Over 50% of T-ALL cases harbor activating NOTCH1 mutations within the HD domain and/or the PEST motif (27), while a high percentage of FBXW7 mutations further amplifies NICD lifetime in the cells and mediates γ-secretase resistance (142, 143). Activating NOTCH1 mutations together with the loss of NUMB, a negative regulator of Notch, have also been observed in non-small-cell lung cancer (144). Another E3 ubiquitin ligase affecting Notch is Deltex which by direct association to NICD via the ANK domain (145), as well as via the β-arrestin protein Kurtz, which leads to ubiquitination of NICD, positively regulates Notch(146). Deltex is thought to direct endosomal trafficking of Notch leading to both positive and negative outcomes in regards to NICD formation (147, 148). Also other non-E3 ubiquitin ligases exist which can associate with NICD and affect Notch signaling (30).

Hydroxylation

The HIF asparaginyl hydroxylase, factor inhibiting HIF1α (FIH) is able to hydroxylate HIF1α, and also N1-3ICD, but not N4ICD (149, 150). The identified hydroxylation sites on N1ICD are found at N1945 and N2012, located within the ANK repeats domain, and FIH hydroxylation seems to affect Notch signaling diversity (30, 149, 150).

Acetylation

Acetylation of Notch has also been implicated in NICD stability and subsequently in Notch downstream gene expression. Recently 14 acetylation sites targeted by PCAF and p300 were identified on N1ICD prolonging N1ICD half-life, while the deacetylase SIRT1 was shown to oppose this stabilization (151).
On the other hand, N3ICD undergoes N-terminal acetylations and deacetylations at K1692 and K1731, within the RAM domain, by p300 and HDAC1 respectively (152). Acetylation primes N3ICD for subsequent ubiquitination and proteasomal degradation, thus also affecting the transcriptional activity of the protein (152).

Other modifications and regulators of Notch receptors and ligands

Glycosylation

Post processing after S1 cleavage in the trans-Golgi network leads to addition of both O-linked fucose and O-linked glucose to the EGF-repeats of the Notch extracellular domain (NECD) by

Protein O-fucosyltransferase (POFUT1/Ofut1) and Protein O-glucosyltransferase (POGLUT1/Rumi), respectively (65, 67). Two new forms of NECD glycosylation called O-GlcNac'ylation and O-Xylosylation by Rumi have also recently been discovered in *Drosophila* (153, 154). O-fucosylation is believed to support correct functioning of all Notch paralogs, while the O-fucose modified EGF-repeats can further be elongated by the Fringe family of glycosyltransferases by addition of N-acetylglucoseamine to O-fucose (66, 67) to modulate Notch signaling activity (155). In *Drosophila*, Fringe increases Notch sensitivity to Delta while decreasing sensitivity to Serrate by addition of GlcNac to O-fucose (155, 156). Three Fringe homologs exist in mammals, namely Lunatic Fringe, Manic Fringe, and Radical Fringe (157, 158). Distinct functions for all three Fringes have been observed in a wide variety of contexts in mammalian cells, however, the overall significance of Fringe modulation is still largely unknown (159).

Numb & Sanpodo

Numb is a membrane associated protein whose expression inversely correlates with that of Notch, and Numb thus functions as a negative regulator of Notch output. For example during sensory organ development, in the sensory organ precursor cells (SOP) in *Drosophila*, Numb localizes along the anterior-posterior axis of the fly yielding an asymmetric cell division of pI cells, thus generating characteristically distinct pIIa (Notch ON) and pIIb (Notch OFF) cells (160-162). Numb antagonizes Notch by binding Notch in complex with Sanpodo, as well as the endosomal protein α-adaptin, which is required in cells supporting high levels of clathrin-mediated endocytosis (163-166). Sanpodo is a four-pass transmembrane protein discovered in *Drosophila*, which potentiates the effects of Numb during asymmetric cell division. In pIIa cells Sanpodo binds Presenilin, a part of the γ-secretase complex, while in pIIb cells Sanpodo mediates internalization of the Notch receptor (167). Numb induces the endocytosis of Sanpodo (168), and is specifically found localized in endosomes controlling endosomal trafficking and recycling of Notch/Sanpodo complexes (169). Numb is thus believed to inhibit Notch/Sanpodo complex recycling to the membrane, instead stalling the internalized endosomes in the cytosol (169).

Mammalian homologues of Numb have been observed to recruit ubiquitination machinery directly to the plasma membrane thereby promoting ubiquitination of the Notch receptor and subsequent degradation of NICD (170). Numb has also been observed to disrupt the formation of the murine double minute 2 (MDM2) and p53 complex, thus protecting p53 from degradation, subsequently leading to inhibited Notch activity (171, 172). Numb governs the endocytic trafficking of the Notch1 receptor, either yielding recycling back to the cell membrane or degradation in lysosomes (173). Overall, several isoforms of mammalian Numb with arguably redundant functions have been identified, and are believed to govern not only asymmetric cell division in the CNS, but the proper development outside the CNS as well (162).

Non-canonical Notch

Non-canonical Notch signaling is an umbrella term to describe CSL-independent Notch activation and downstream signaling through other pathways than the classical Notch target genes (174). The first reports of non-canonical Notch signaling came already during the 1990s when Notch was found to inhibit muscle cell differentiation independently of CSL (175, 176). Questions that still baffle the non-canonical Notch field is how non-canonicity is mediated by transcription factors other than CSL (177, 178), and how it is mediated by interactions that occur in the cytoplasm (174). According to a recent review by Ayaz & Osborne 2014, non-canonical Notch can be divided into three logical categories, namely: 1) γ-secretase mediated activation of Notch occurring independently of ligand interaction, 2) NICD activity independent of CSL, and 3) membrane bound Notch signaling in the absence of γ-secretase cleavage, sometimes also without ligand activation (179). One example of physiological non-canonical Notch signaling comes from *Drosophila* and mammalian neural stem cells where PTEN-induced kinase 1 (PINK1) has been shown to recruit full-length Notch to the mitochondria (180).

A prime example of when canonical and non-canonical Notch signaling unite is during the crosstalk with Wnt signaling, an event with both synergistic and antagonistic effects and the potential to orchestrate the outcome of many developmental fates (181, 182). Notch and Wnt have been shown to converge synergistically when β-catenin interacts with canonical Notch bound to CSL in induction of arterial fate in vascular progenitors (183). Similar synergy is also reported in tumorigenesis and proliferation of intestinal cells (184), as well as in maintenance of hematopoietic stem cells (185). By contrast, ligand/CSL-independent Notch i.e. non-canonical Notch signaling, is often reported to antagonize Wnt/β-catenin (174). For example, in *Drosophila*, Notch downregulates armadillo/β-catenin independently of transcriptional activity (186). Also Numb has been hypothesized to have a role in the crosstalk with both Notch and Wnt (174).

Notch receptor and ligand trafficking

Endocytosis and recycling of Notch receptors and ligands in both signal-sending and signal-receiving cells has been observed to be critical for directing and regulating Notch activity. Trafficking and recycling of Notch receptors is observed as a constitutively occurring event in cells, where numerous regulators of endocytic trafficking have in the past 15 years been identified as being essential for activation of Notch signaling (187, 188). Numb is a known regulator of Notch1 trafficking which when active will redirect receptors from recycling to lysosomal degradation (173). Regulation of Notch receptor trafficking is also important during cleavage of NICD at S3 as this is thought to occur both at the plasma membrane and in endosomes as they transition to become lysosomes.

Ligand endocytosis and recycling is a poorly understood process, however, ligand internalization is believed to be induced by monoubiquitination of the ligands by the E3 ubiquitin ligases

Neuralized and Mindbomb (74). Current models suggest that the subsequent recycling of the ligand produces a more active cell surface ligand, however, exact modifications occurring during this event are still under debate. Suggested modifications include: post-translational modification, clustering of ligands, and localization into specific plasma membrane microdomains (189, 190).

Notch signaling in disease

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a progressive central nervous system degenerative disorder linked to Notch3 mutations (191), causing mainly vascular defects. Onset of the disease is highly variable, and clinical presentations vary from patient to patient but include: Migraines with aura, transient ischaemic attacks, dementia, apathy, and mood changes (192). Furthermore, leukoencephalopathy showing white matter pathology is observed on MRI, and CADASIL leads to a bedridden terminal stage within a mean of 25 years (192). First identified in 1977 by two different groups (193, 194) and later mapped to chromosome 19 (195), CADASIL is now known to be caused by over 70 different mutations in the extracellular domain of Notch3 (196, 197), leading to odd numbers of cysteine residues causing impaired formation of cysteine disulfide-bridges in the EGF-repeats domain (191). However, the exact molecular mechanisms behind CADASIL remain still largely unknown.

Alagille syndrome is a multisystem developmental disorder caused by Notch signaling pathway abnormalities. The disease involves characteristic facial features and abnormalities in several organs including liver, heart, eye, and skeleton with additional minor involvement of the renal and vascular system (198). Over 94% of cases are caused by mutations in Jagged1 causing Jagged1 haploinsufficiency(199, 200), however, a small subset with Notch2 mutations also develop Alagille syndrome (201).

Spondylocostal dysostosis (SD) is a rare autosomal dominant or autosomal recessive axial skeletal growth disorder caused by vertebral malsegmentation due to disruption of the segmentation clock (198, 202). Innactivating mutations in the DLL3 gene have been shown to cause autosomal recessive form of SD (60), where the normal function of Dll3 is to inhibit canonical Notch signaling by cis-inhibition in the cis-Golgi (113). Furthermore, Lunatic Fringe has been observed to cause similar clinical manifestations as loss of DLL3 (202-204).

Notch in cancer

Deregulated Notch signaling is associated with a number of different forms of cancer, conferring both solid tumors and cancers of hematopoietic origin (205). Depending on the tissue type, Notch and its different paralogs can have tumor promoting or tumor suppressing activities. Notch

deregulation is evident in many cancer forms, including lung and cervical carcinomas, neuroblastoma, medulloblastoma, breast cancer, prostate cancer, pancreatic and colorectal cancer, melanoma and different leukemias (206).
Notch mutations are present in many cancers both in primary tumors, as well as in established cancer cell lines, albeit in higher frequency in vitro (207). The most classical case is T-ALL, where over 50% of the cases harbor activating NOTCH1 mutations within the HD domain and/or the PEST motif (27).

Notch in breast cancer

Breast cancer is the second most commonly occurring cancer and the fourth leading cause of cancer deaths in the world, according to the GLOBOCAN study done in 2012 by The World Health Organization (208). Despite being a heterogenous disease, breast cancer is molecularly classified into 5 major subtypes, based on estrogen receptor, progesterone receptor, and HER2-receptor status. These five subtypes are: Luminal A, Luminal B, HER2+, Basal-like, and Claudin-low (209, 210). The claudin-low subtype, which is characterized by decreased expression of the tight-junction protein Claudin (211), exibits more heterogenous, and mixed features compared to the other four classical subtypes, and is thus hard to classify into any previously existing subtype (210, 212).

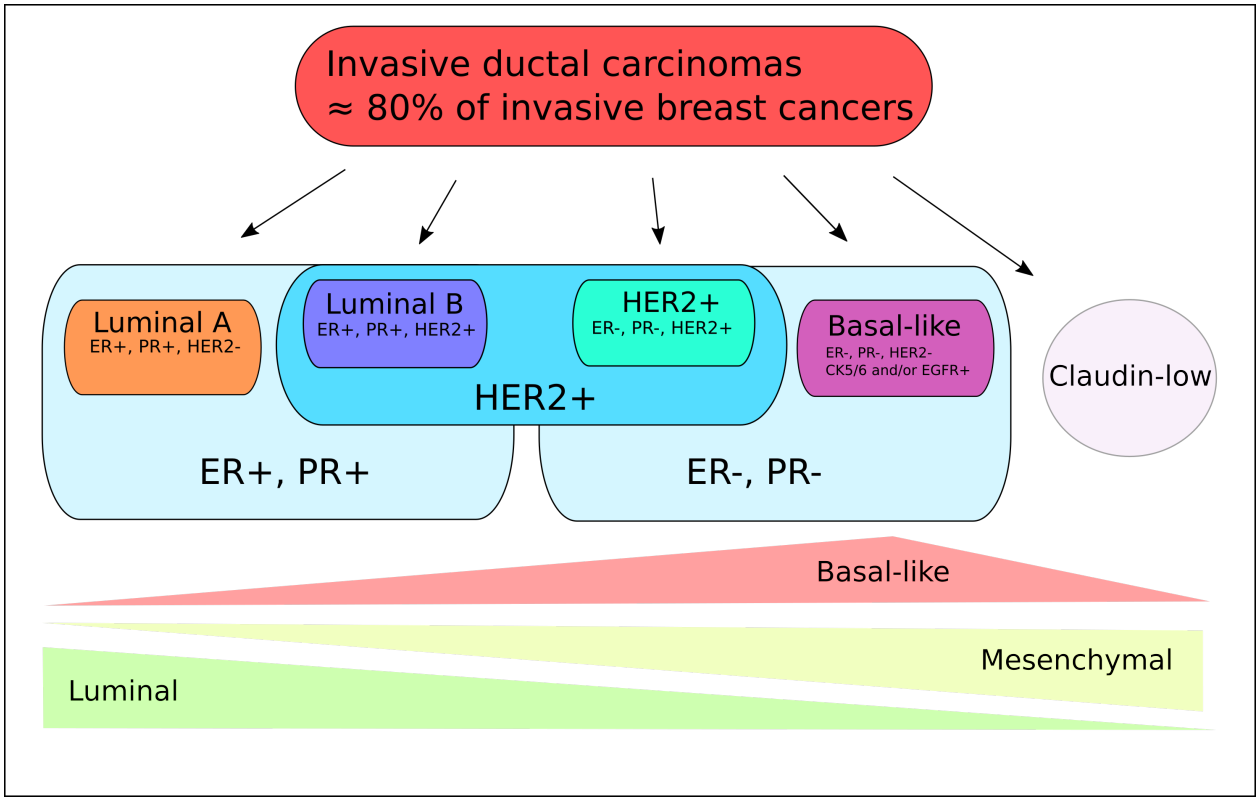

Figure 3. The molecular subtypes of breast cancer. The five subtypes are Luminal A, Luminal B, HER2+, Basal-like, and Claudin-low. Adapted from Prat and Perou 2009, and Sandhu et al. 2010.

The first data describing the oncogenic potential of Notch in solid tumors came from animal studies aimed at characterizing the "int3" insertion site of the mouse mammary tumor virus (MMTV) (28). This site was later identified as the Notch4 locus (213), and MMTV insertion was shown to drive expression of an extracellularly truncated Notch4 transcript (214). Despite not being essential for embryogenesis, and having a limited role in normal mammary development (215-217), overexpressed Notch4 promotes breast tumorigenesis by signaling upstream of c-kit and PDGFR (218). The MMTV also places genomic insertions in the Notch1 locus, although with lower frequency, which lead to the expression of similar extracellularly truncated transcripts as with Notch4 (219, 220).

Notch1 has to date been intensively studied within the breast cancer field and overactivation not only correlates with highly aggressive forms of the disease, but has also been found to crosstalk with a large number of oncogenic signaling pathways (121, 221). In Ras-positive tumors Notch1 has been identified as a downstream effector of Ras (222), while oncoproteins such as c-myc and Notch4 have been established as direct target genes of Notch1 (219, 222-224). Also, in ~50% of breast cancer cases Notch deregulation is linked with the loss or silencing of the Notch antagonist Numb (221, 225). Overall, high levels of NOTCH1 and JAGGED1 lead to poor survival in breast cancer patients (226). The most aggressive basal (triple-negative) type of breast cancer also often possess a specific Notch1 genetic signature (226, 227), which can include gene arrangements and fusions generating a gain of function in Notch1 (228). Notch1 has also been shown to drive migration and invasion by inducing epithelial-to-mesenchymal transition (EMT) via Slug and subsequent repression of E-cadherin(123, 229), as well as by regulation of extracellular matrix metalloproteinases (230).

The effects of estradiol on Notch activity are conflicting. Estradiol has been reported to increase Notch1 protein levels while reducing transcriptional activity and nuclear localization (231) Another study shows increased Notch1 activity in response to estradiol (232). On the other hand reduction of estrogen receptor (ER) or ErbB2 activity, notably by tamoxifen or trastuzumab respectively, yields elevated Notch1 signaling activity (231).

Notch2 has in several studies been identified with tumor suppressing potential. Better patient survival is linked with high Notch2 expression in breast cancer (233), while ectopic activation of Notch2 has been linked to increases apoptosis in MDA-MB-231 breast cancer (234). Notch3 upregulation in mammary glands has been shown to lead to the formation of mammary tumors in vivo (235), and Notch3 is a important driver of proliferation in ErbB2-negative breast cancer cell lines eg. MCF-7 (236). However, Notch3 has recently been observed to have tumor suppressing functions when introduced into Notch3-null breast cancer cell lines (237). In this context Notch3 was able to induce senescence via the cell cycle inhibitor p21 (237).

Overall, Notch1 and Notch4 as well as Jagged1 are reported to be oncogenic, while Notch2 has a tumor suppressive function in breast cancer (238). Notch3 appears to have context-dependent roles as oncogenic and as tumor suppressive (237). Upregulated Notch is also connected to the appearance of cancer stem cells in breast cancer (239).

Notch in prostate cancer

Both Notch1 and Jagged1 have been linked to prostate cancer growth, migration, and invasion via the downstream effectors Akt, mTOR, and NF-κB (240-243), with prostate cancer frequently metastasizing to the bone and lymph nodes (244). Many in vitro cultured prostate cancer cell lines exist with different Notch signatures, however, the exact expression signatures are still unclear. Well known prostate cancer cell lines like PC-3 and LNCaP have previously both been confirmed to express high Notch1, Notch2, and Jagged1, Jagged2 levels (244). However, the expression status of Notch3 remains unclear with some studies observing the loss of Notch3 (244), while others report Notch3-positivity in PC-3 cells (245). Notch3 has even been found to be activated by hypoxia in LNCaP cells (246).

Notch and cancer metastasis

Notch is one of many well documented inducers of epithelial-to-mesenchymal transition (EMT), a process indispensable in such embryonic processes as gastrulation, as well as in adult tissue repair requiring cell motility (247). Similarly in the cancer setting, a plethora of signaling pathways, including Notch, can drive EMT subsequently leading to tumor progression and metastasis. Notch is observed to induce metastasis via Slug-mediated repression of E-cadherin (123, 229) and via Snail-1 (122). Furthermore, Notch has been found to regulate extracellular matrix metalloproteases (230). Feedforward amplification has also been documented where activation of the EMT-inducer ZEB1, leads to increased Notch signaling (248).

Non-canonical Notch in cancer

Many signaling crosstalks have already been identified which may mediate non-canonical Notch in cancer (179). In breast cancer, non-canonical Notch1 has been shown to regulate Il-6 via IKKα/β and p53 (249), while in ovarian cancer Notch1 helps drive migration and invasiveness via upregulation of lysyl oxidase (122). Also non-canonical Notch4 signaling has been implicated in formation of mammary tumors (250). On the other hand, non-canonical Notch3 has been suggested to drive cancer via the NF-κB pathway in leukemia (251).

Cancer metabolism

Metabolic reprogramming is today considered a key event in the development of cancer. The term aerobic glycolysis was coined in the 1920s by Otto Warburg when he observed that cancer cells, despite access to an ample oxygen supply, preferentially metabolize glucose through the fermentation-like process involving purely glycolysis. This process, now known as The Warburg Effect, in order to be complete also involves conversion of the pyruvate to lactate and the export of lactate from the cell. The classic hallmarks of cancer have been previously defined (252-254)

and today metabolic reprogramming is considered one of the necessities of cancer development and maintenance. The reasons for a cancer cell to utilize aerobic glycolysis are multifaceted. Firstly, glycolysis on its own is a simple reaction with seemingly straightforward kinetics compared to a combined oxidative phosphorylation. Thus aerobic glycolysis in an environment with ample supply of glucose can be even faster than complete oxidation through the electron transport chain. Secondly, the cancer phenotype is a proliferating phenotype, which means that anabolic biosynthetic pathways are upregulated. This also affects the function of the citric acid cycle, which through cataplerosis loses many of its intermediates for biosynthesis of membrane lipids and nucleic acids. Other advantages include avoidance of the mitochondrial intrinsic apoptotic pathway and lowered ROS levels, invasion of nearby tissue with the help of the acidic byproduct lactate, and priming of certain tumors for survival in hypoxic niches within the heterogenic tumor.
The molecular mechanisms controlling the metabolic switch, which is linked to tumor growth and progression, are still poorly understood. Defects in OXPHOS have been implicated in the Warburg phenotype but recent evidence suggests that oncogenic activation and loss of tumor suppressors are also critical modulators of tumor metabolism. Activation of the PI3K//Akt pathway, Ras, Src, Myc and loss of p53 induce glycolytic phenotypes similar to that observed by Warburg (255). According to Warburg's original theory, permanent mitochondrial damage was the cause of upregulated glycolysis, however, more recently it has been shown that tumor mitochondria do respire, although with lower efficacy. The reserve capacity for OXPHOS observed in cancers suggests that glycolysis is preferred to support cell growth and not to compensate for faulty mitochondria.

Notch interacting partners in cancer

PI3K/Akt

Akt (Also known as protein kinase B, or PKB) is a cytoplasmic serine/threonine kinase family, consisting of three closely related isoforms, namely Akt1-3. The Akt paralogs exhibit functional redundancy but also have both tissue specific and organelle specific localization and function (256). The Akt pathway functions as a nexus connecting extracellular signals to generate diverse intracellular outcomes and is the second most frequently mutated signaling pathway in cancer (257). In breast cancer alone, Akt mutations are observed in over 70% of cases (257).
The PI3K/Akt pathway influences many cellular processes involved in carcinogenesis including proliferation, angiogenesis, and EMT (258). Akt is activated by a class of intracellular lipid kinases called the phosphatidylinositol 3-kinase (PI3K). PI3K is in turn classically activated by growth factor receptor tyrosine kinases (RTKs) such as insulin receptor (IR), insulin like growth factor receptor (IGF-R), epidermal growth factor receptor (EGFR), and platelet derived growth factor receptor (PDGFR), as well as G-coupled receptors such as Ras. PI3K phosphorylates phosphatidylinositol 4,5-diphosphate (PIP_2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP_3) which can then bind the pleckstrin homology (PH) domains of both 3-phosphoinositide-

dependent kinase 1 (PDK1) and Akt. This leads to PDK1 autophosphorylating itself and transphosphorylating Akt, making it active. The generation of PIP_3 is counter-balanced by phosphatase and tensin homologue deleted on chromosome ten (PTEN), a phosphatase that reverses the action of PI3K. Also other kinases have been reported to activate Akt (257).
Notch regulates PI3K/Akt signaling. Activated Notch1 has been observed to inhibit apoptosis by stimulation of anti-apoptotic proteins via a Notch1-$p56^{lck}$-PI3K-Akt pathway in T cells (259). Similar regulation of Akt by Notch1, as well as a reciprocal relationship between the two proteins has been seen in T-ALL (260, 261). Downregulation of Notch1 and Jagged1 in prostate cancer has been detected to inhibit cancer growth and survival, as well as to induce apoptosis via inactivation of Akt, mTOR, NF-κB, and FoxM1 (242, 262). Notch has also been shown to prevent apoptosis in breast epithelial cells by induction of Akt (263).
PTEN expression is negatively regulated by Hes1 and mutational loss of PTEN has been observed to induce resistance to Notch-inhibition in T-cell leukemia, giving rise to what Palomero et al. describe as a "shift in oncogene addiction" from Notch to PI3K/Akt subsequently leading to PI3K inhibitor sensitization GSI resistance (264, 265).

HIF

The most valuable commodity in the body is the energy packed molecule ATP, which functions as the universal fuel and currency for travel, transport, construction, and any other type of activity within the mammalian organism.
In normal oxygen conditions, or normoxia, our bodies utilize aerobic metabolism to generate energy in the form of ATP. Aerobic metabolism involves the mitochondria, oxidative phosphorylation, and the electron transport chain, where oxygen functions as an electron acceptor in a reaction yielding water and 32 molecules of ATP per glucose molecule. This is where our fundamental need for a heart pumping oxygenated blood comes from. However, during certain physiological conditions, such as exercise or high altitudes, or during pathological conditions like stroke or myocardial infarction oxygen levels may become reduced. A reduced oxygen environment is defined as hypoxic when O_2 levels drop below 2%. In human tissues oxygen levels range from 2-9% while normoxic atmospheric oxygen levels are around 21% (266). During low oxygen supply a new set of rules apply to metabolism, called anaerobic glycolysis. During hypoxia, pyruvate is directly reduced to lactate in a seemingly inefficient reaction yielding 2 molecules of ATP per glucose molecule. The fifteen-fold decrease in ATP synthesis efficiency is an inevitable adaptation to low oxygen envionments, the presence of which is constantly monitored by the organism. In the 1950s, radiation oncologists were the first ones to describe tumor hypoxia, in conjunction with failed radiation therapy treatments of solid tumors. However, the machinery for oxygen sensing was not identified until the 1990s (267). Hypoxia inducible factors (HIFs) are the central regulators of oxygen-dependent gene expression, which is an essential part of normal cell and tissue functioning, as well as cancer biology. HIFs are heterodimeric proteins consisting of 3 constantly transcribed and translated, oxygen-labile alpha subunits (HIF1-3α), and 3 stably expressed beta subunits (HIF1-3β) (268) (269). Under normoxic conditions the oxygen-labile subunits are hydroxylated at specific prolyl

residues resulting in subsequent ubiquitination and proteasomal degradation of the α subunit by the 26S proteasome (270). The ubiquitination event is mediated by the von Hippel-Lindau (VHL) tumor suppressor protein, which via direct association to the α subunit recruits a E3 ubiquitin ligase protein complex (271). Beyond being regulated at the level of protein stability, HIF1-2α are also regulated by FIH1 by hydroxylation of the C-terminal transactivation domain of the HIFs leading to repression of HIF transactivation activity (272, 273).

HIF1 is ubiquitously expressed in all tissues, HIF2 in select cell types and tissues, including endothelial cells, glial cells, kidneys, heart, lungs, and small intestine (269, 274), and HIF3 in the Purkinje cells of the cerebellum and corneal epithelium in the eye, as well as in tissues of the thymus, lung, heart, kidney, and liver (274, 275). The role of HIF3α remains largely unknown, although it has been observed to function as a negative regulator of HIF-mediated gene expression (275). Over a hundred downstream target genes of HIFs have been identified, however, the targets vary with HIF1 exclusively targeting glycolytic enzymes, as well as erythropoietin, while HIF2 activates c-myc, TGFα, lysyl oxidase, Oct4 and Cyclin D1 (276) (274, 277). HIF1 and HIF2 also exhibit redundancy in the control of several downstream genes, eg. VEGF (Vascular endothelial growth factor), and may also have contrasting function (276, 277). Furthermore, HIF1α has been shown to functionally interact with NICD during hypoxia thereby increasing Notch target gene expression (278).

P53

The p53 tumor suppressor is the most frequently mutated pathway in cancer and a complex cross talk between Notch and p53 has been observed. In breast and prostate cancer p53 has been shown to correlate with Notch1 expression (279). Notch1 is a known target gene of p53 (280), and furthermore p53 is known to associate with the Notch1 transcriptional complex (NTC) in a MAML-dependent manner thus inhibiting Notch1-dependent transcription (172). Furthermore, p53 has been reported to associate with CSL within the NTC (281). Numb is able to via p53/MDM2 regulate Notch-levels (172) but there are also reports of direct MDM2 ubiquitination of Notch1 and subsequent augmentation of Notch1 activity (282).
Likewise p63 and p73, which are part of the p53-gene family, have been reported to induce Jagged1 and Jagged2 thus mediating crosstalk with the Notch family during development (283).

Pim

The Pim-kinase family of small molecule Serine/Threonine kinases was first identified in the 1980s when the kinase coding DNA sequence was found to be the proviral integration site of the Moloney murine leukemia virus. The Pim family has 3 isoforms: Pim1, Pim2, and Pim3, which are ubiquitously expressed, constitutively active synergizing oncogenes regulated at the transcriptional, translational, and proteasomal level with short half-lives (<5 min) (284). The Pim genes also contain multiple transcription initiation sites giving rise to alternative splice forms.

The Pim kinases contain over 35 potential recognition sites for other kinases suggesting that Pim is under significant upstream regulation from as of yet unconfirmed phosphorylation events (284).

Pim1 is expressed as both the 33kDa and 44kDa isoforms with distinct cellular localization, where the 44kDa contains an N-terminal proline motif which binds to the SH3 domain of ETK on the cell membrane (285). Pim1 is significantly protected from degradation by Hsp90, while association of Pim1 with Hsp70 leads to degradation (286) (287). Pim2 is expressed as 3 splice isoforms while Pim3 only has a single protein-yielding transcript. The Pim proteins exhibit 61-71% sequence homology amongst each other but show different tissue distribution. Pim1 is expressed at the highest level in hematopoietic cells and in a number of solid tumors, Pim2 in brain and lymphoid tissue, and Pim3 in kidney, breast, and brain (288-291).

Pim genes are generally induced by transcription factors, such as Jak/STAT and NF-κB involved in growth factor signaling pathways, such as intereferon-α and interleukins (292, 293). Also hypoxia (294-296) and Krüppel like factor 5 (KLF-5) (297) have been observed to induce Pim. However, deficiency in Pim kinases leads to very mild phenotypes, including a reduction in body size and an impaired hematopoietic growth factor response (291). Despite the lack of a profound phenotype, Pim kinases are important and show paralog redundancy during development and adult life.

All three Pim kinases have been identified as potent oncogenes and drivers of tumorigenesis in both tissue culture and animal models (298). Similarly its been documented that all Pim paralogs associate and can cooperate with C-myc and N-myc to induce leukemias and lymphomas (298-300). Pim kinases also function as inhibitors of apoptosis via phosphorylation and blocking of the pro-apoptotic BAD protein (301-304).

Outline and aims of the thesis

The collective aim of this thesis has been to study Notch and its interacting partners in cancer, as well as the role of Notch in regulation of cancer metabolism. **"When Notch and Pim Unite"** deciphers the role of PIM kinases in regulation of Notch signaling output of breast and prostate cancer cells and the subsequent impact on tumorigenesis. **"A Metabolic Turn of Events"** delves into the intricacies of Notch-mediated regulation of cancer cell metabolism. **"Systematic KOs"** unravels the consequences of CSL-knockout in cancer, while **"Falling Into Hypoxity"** deciphers the Notch-HIF2α crosstalk axis.

Key aims:

- **Analyze Pim kinase modulation of Notch activity in breast and prostate cancer**
- **Analyze the influence of Notch on the metabolic state of breast cancer cells**
- **Analyze the effect of CSL knock-out on tumor growth in breast cancer**
- **Study the Notch and HIF2α interaction in medulloblastoma and breast cancer**

Results and discussion

I. **When Notch and Pim Unite:** Phosphorylation of Notch1 by Pim kinases promotes oncogenic signaling in breast and prostate cancer cells

Post-translational modification (PTM) of Notch is an emerging field in Notch-research attempting to explain the pleiotrophism of Notch output. A review of the existing evidence of Notch PTMs can be found in Andersson et al. 2011 (30).

In paper I, we started by screening cancer cells for both Notch1 and Pim1 expression. Both the breast cancer cell line MCF-7 and the prostate cancer cell line PC-3 exhibited robust levels of Notch1 and Pim1 protein. Subsequently, we wanted to know if the endogenous Notch1 and Pim1 colocalize in these cell lines. Thus, we performed colocalization microscopy, and proximity-ligation assays in MCF-7 cells to find out that these proteins did indeed interact. Fluorescence-lifetime imaging (FLIM) with ectopically expressed Notch1 and Pim1 also showed physical interaction in PC-3 cells.

Next, we proceeded with measuring Notch activation using the 12xCSL luciferase reporter when inhibiting or transiently overexpressing Pim kinases. The specific Pim inhibitor 1,10-dihydropyrrolo[2,3-a]carbazole-3-carbaldehyde (DHPCC-9) has previously been shown to inhibit all 3 Pim isoforms *in vitro* by binding specifically to the ATP binding site (305, 306). In a MCF-7 breast cancer background we found that inhibiting Pim kinases with DHPCC-9 lowered endogenous Notch activity in the 12xCSL luciferase assay, while overexpression of Pim1 yielded augmented Notch activation. Similarly, inhibiting Pim kinases in a Notch1 ectopically overexpressed background lowered Notch activation. To confirm that the effects were Pim kinase specific, Notch activity was measured using the 12xCSL luciferase reporter assay in combination with siRNA silencing of Pim1,2,3.

To answer the ultimate question whether Pim kinases can phosphorylate Notch intracellular domains we performed in vitro kinase assays with 32-P-linked ATP. Bioinformatic sequence alignment of N1ICD with the consensus sequence for Pim kinases had revealed 2 potential phosphorylation sites for Pim on N1ICD. All 3 Pim isoforms were found to phosphorylate murine N1ICD and N3ICD but not N2ICD, and subsequent, mass spectrometric analysis performed on in vitro phosphorylated N1ICD identified Serine 2152 as the phosphorylation site. For N3ICD, sequence analysis and mutagenesis of 2 potential phosphorylation sites revealed serine 1673 to be phosphorylated. In human Notch proteins the sequence of interest was highly conserved and the phosphorylation sites were mapped at S2162 for Notch1 and S1672 for Notch3. Bioinformatic study of human N1ICD sequence and domain structure revealed that for N1ICD the phosphorylation site at S2162 was located in the PPD domain at the second NLS domain within the linker between the 2 clusters of a possible bipartite NLS (82). Mutation of the entire sequence including the two basic amino acid clusters and the linker has previously

revealed an increased cytoplasmic localization of N1ICD (82). However, a later study showed that mutational loss of the two basic amino acid clusters did not affect nuclear localization (81), suggesting that nuclear transport may be mediated by the linker sequence. Indeed, modulation of classical bipartite NLS linkers or sequences around such basic amino acid clusters have previously been shown to affect either the direct association of the linker to the backbone of Importin-α, or the conformation of the NLS allowing for binding to the minor and major groove of Importin-α (83, 84). Furthermore, N1ICD nuclear localization has been shown to be mediated by Importin-α 3, 4, and 7 (81). Likewise, phosphorylation of NICD has recently been shown to impact nuclear localization (135).

However, in *Drosophila* the sequence around the second NLS has been redefined as the potentially phosphorylated domain (PPD) where the N-terminal basic sequence has been shown to also mediate direct binding to Su(H) (307). In mammals the PPD is not observed to bind to BTD of CSL, but may instead bind other regions of CSL (87). Interestingly, the phosphorylated residue at S2162 in human Notch1 is in *Drosophila* Notch substituted for a Lysine (K) with a larger side chain comparable to that of a phosphorylated Serine. Thus it could be hypothesized that the constitutive binding of NICD to Su(H) in *Drosophila* is in other species a phosphorylation-dependent dynamic process (307).

In Notch, both of the NLS domains are located in so called low complexity regions (LCRs) (74), and being nearly impossible to crystallize, these regions are often regarded as simple linkers between orderly domains. LCRs have recently however been proposed to function as signaling hubs contributing to regulatory functions of proteins (308, 309). For example, phosphorylation events may take advantage of target proteins disordered region interfaces (309-311).

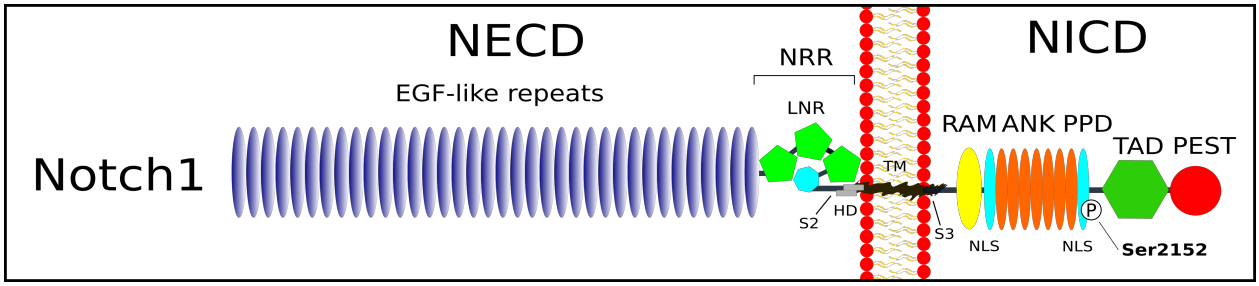

Figure 4. Notch1 is phosphorylated by Pim kinases at a distinct site on its intracellular domain (NICD). The phosphorylation site on N1ICD at serine 2152 is localized in the second nuclear localization sequence (NLS), also defined as the potentially phosphorylated domain (PPD).

To dissect the specific effect of either phosphorylation event we designed and constructed phosphodead and phosphor-mimicking mutant plasmids by replacing the phosphorylated serine with alanine or glutamate (SA and SE mutants) in N1ICD. We found N1ICD-SA to both significantly lower nuclear localization as well as abolish Notch activation in the 12xCSL luciferase assay.

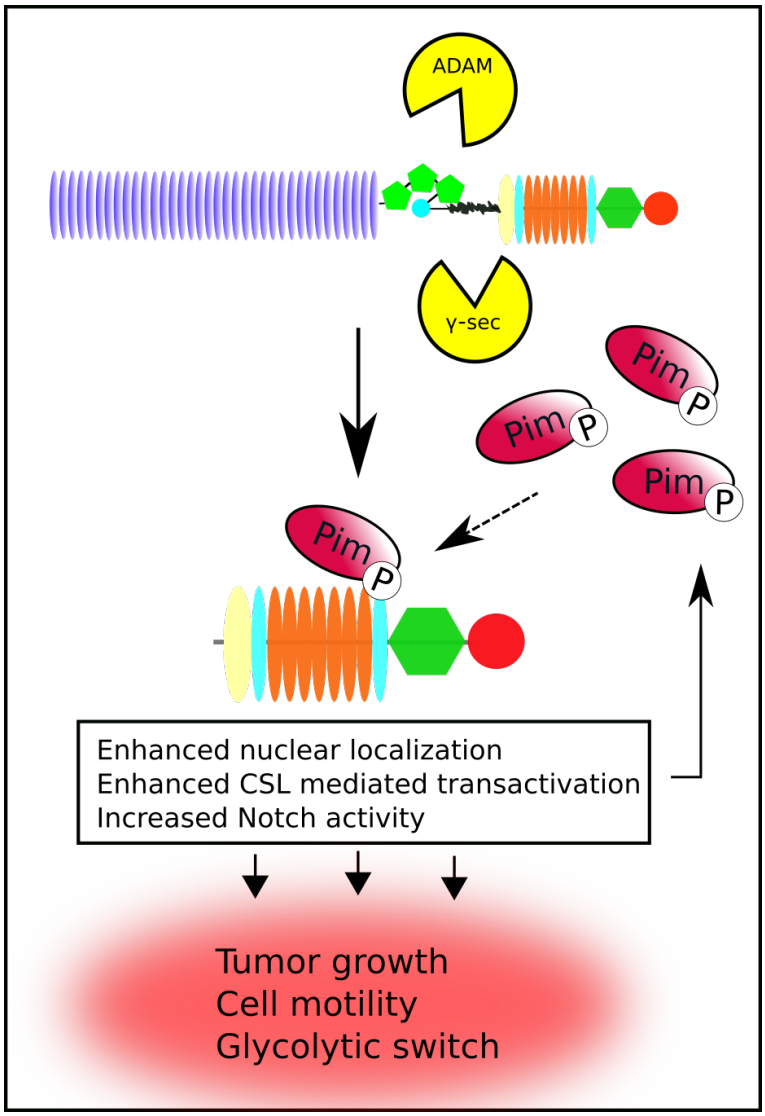

Figure 5. Pim-mediated phosphorylation of N1ICD potentiates Notch1 activity. Phosphorylation of N1ICD by Pim-family kinases enhances N1ICD nuclear localization and CSL association leading to increased Notch1 activity. Notch1 also upregulates Pim protein levels via a feed-forward loop. Collectively Pim and Notch1 synergize in mediating tumor growth, cell migration, and cancer metabolism.

Notch has previously been identified as an EMT promoter via the induction of Snail-1 and Slug (122, 123, 229) as well as via regulation of matrix metalloproteases (230). We studied N1ICD phosphorylation in conjunction with PC-3 cell migration in a scratch wound assay. Jagged1-mediated induction in wound healing showed dependence on N1ICD phosphorylation. Jagged1 was able to increase wound healing in the control setting, but not in cells treated with DHPCC-9. Similar results were obtained with N1ICD-SA.

As in paper II we studied glucose uptake, lactate production and mitochondrial membrane potential in MCF-7 cells transiently transfected with N1ICD and Pim. Pim slightly elevated glucose analog 2-NBDG uptake while DHPCC-9 treatment yielded a 2-fold increase in 2-NBDG

uptake compared to control levels. Likewise, the relative lactate production was increased when DHPCC-9 was used. The N1ICD-SA mutant also significantly increased glucose uptake and lactate production, showing that the results were mediated via phosphorylation of N1ICD.

To ascertain the *in vivo* effects of the Notch-Pim crosstalk in an MCF-7 and PC-3 background we transplanted tumors of both cell lines onto the chicken CAM. MCF-7 cells were transplanted with transient transfections of N1ICD-WT, N1ICD-SA, N1ICD-SE and allowed to form tumors in the presence and absence of Estradiol (E2) and DHPCC-9. MCF-7 is an estrogen-dependent breast cancer cell line (312, 313), and furthermore Pim1 has been shown to be a ERα target gene (314). In the CAM model with MCF-7 breast cancer and in the presence of E2, N1ICD-WT and N1ICD- SE exhibited increased tumor formation in comparison to N1ICD-SA, and DHPCC-9 attenuated tumor growth in N1ICD-WT tumors. In all cases N1ICD-SA lowered tumorigenesis.

In addition, PC-3 CAM tumor xenographs were treated with increasing dose of DHPCC-9 in combination with DAPT. A significant combinatorial effect was observed with DAPT and DHPCC-9 treatments suggesting a potential new vista for combinatorial therapy for certain cancer forms in the future. Taken together this paper reveals a novel post-translational modification of Notch1 mediated by Pim kinases which potentiates Notch1-mediated protumorigenic effects.

II. **A Metabolic Turn of Events:** Hypo- and hyperactivated Notch signaling induce a glycolytic switch through distinct mechanisms

The hallmarks of cancer proposed by Hanahan and Weinberg (252, 253) represent a set of perks acquired during carcinogenesis. The transition from a healthy cell to a neoplastic state involves that the cell acquires a succession of these capabilities giving diverse advantages in cell production and survival. As discussed above, Otto Warburg had in the 1920s discovered that despite an ample oxygen supply cancer cells would still prefer utilizing what he termed “aerobic glycolysis” to meet the energy demands of the cancer cells. Today metabolic reprogramming is considered an emerging hallmark of cancer (253, 254)

In paper II we wanted to decipher the impact of the Notch signaling pathway on breast cancer metabolism. It is known that different cancers utilize varied levels of glycolysis (255, 315). When comparing the estrogen-dependent MCF-7 and the triple-negative MDA-MB-231 claudin-low breast cancer, their glycolytic phenotypes correlate with their Notch1 expression levels. MCF-7 cells exhibit a low glycolytic phenotype with low Notch1 levels whereas MDA-MB-231 is highly glycolytic with high Notch1 levels.

To answer the question whether Notch could influence the glycolytic phenotype in cancer we designed 3 stable GFP-tagged MCF-7 cell lines expressing different levels of Notch, in an attempt to either boost or lower the glycolytic phenotype in combination with elevated or inhibited levels of Notch. 12xCSL luciferase reporter assay confirmed that MCF-7 N1ICD-GFP had high Notch1 levels, MCF-7 GFP normal levels, and MCF-7 dnCSL expressed a dominant negative form of CSL yielding a blocked Notch phenotype. These are subsequently referred to as

^{high}Notch, ^{normal}Notch, and ^{low}Notch cells.

The 3 stable cell lines were orthotopically xenografted into the fat pad of athymic nude mice and allowed to form tumors for a specific length of time after which the tumors were weighed and analysed by immunohistochemistry (IHC), aswell as with ^{18}F-FDG PET. After 8 weeks ^{high}Notch cells had formed significantly bigger tumors than the ^{normal}Notch controls, however ^{low}Notch cells had regressed. The growth of ^{high}Notch tumors was also visible in the H&E and Ki67 stainings showing invasive highly proliferative tumor morphology compared to control. ^{18}F-FDG PET experiments showed after 5 weeks an increased ^{18}F-FDG uptake not only in the hyperactive Notch tumors, but also in the hypoactive Notch tumors. A similar effect was seen in vitro with the stable Notch cells when glucose uptake was normalized to ATP production. By activating naive MCF-7 cells with immobilized FC-Jagged ligands a matching increase in glucose consumption was evident. Similarly by inhibiting Notch in the highly glycolytic MDA-MB-231 cells the glycolytic phenotype as well as lactate production was reduced. To corroborate our findings we compared expression of the glucose transporter GLUT1, a marker of glycolytic cancers, with N1ICD expression in patient samples of basal versus non-basal breast cancers. A positive correlation was seen between N1ICD and GLUT1 expression.

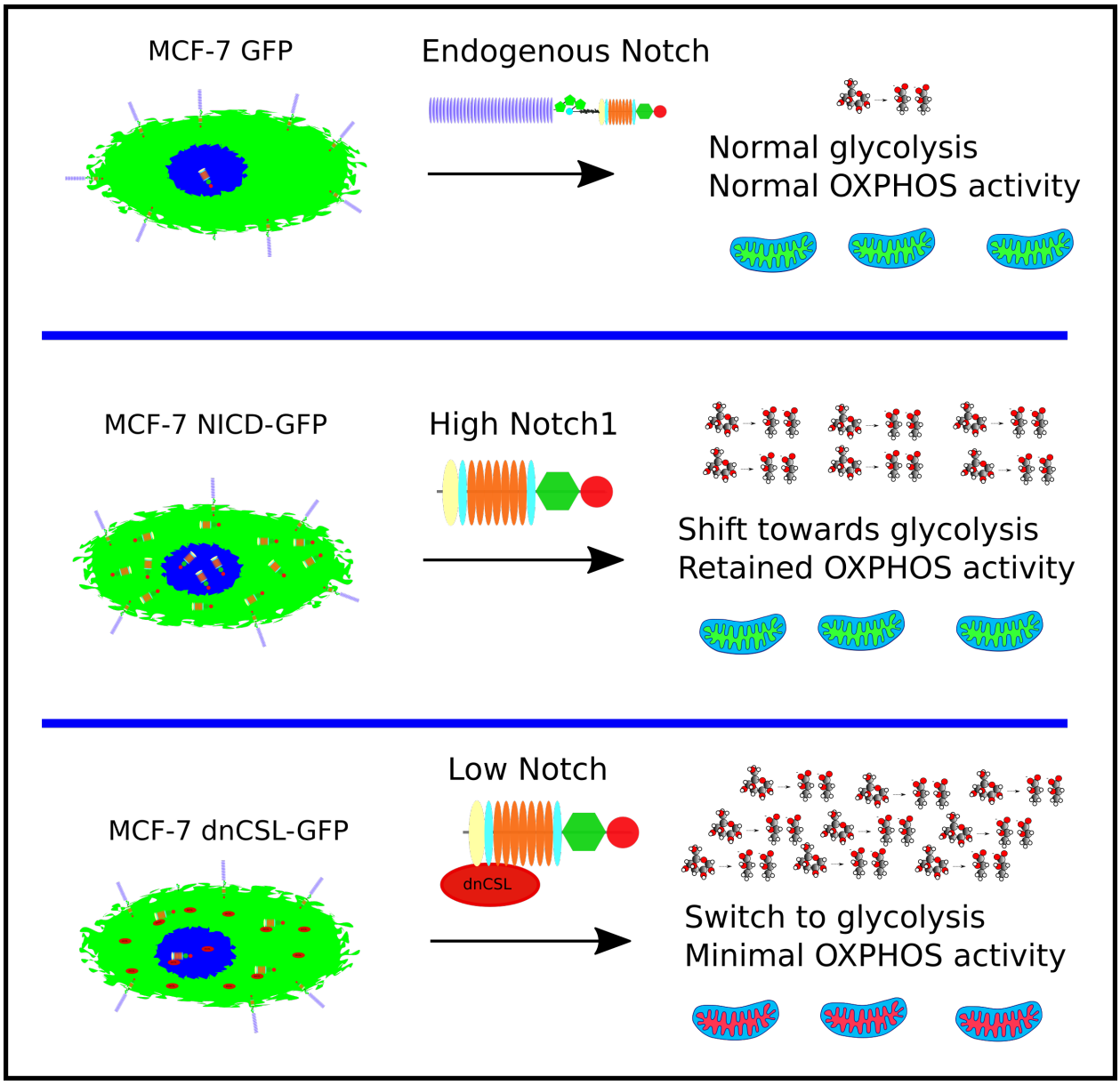

Figure 6. Notch modulation gives rise to phenotypical changes in MCF-7 breast cancer metabolism. Endogenous levels of Notch activity exhibit a normal MCF-7 cell-type specific cellular metabolism. Overexpression of Notch1 shifts cell metabolism towards glycolysis while still retaining active oxidative phosphorylation (OXPHOS). Inhibiting Notch with dominant negative CSL (dnCSL) leads to a complete switch to glycolysis and minimal OXPHOS activity.

We next studied the downstream mechanisms of how Notch signaling could mediate changes in glycolytic phenotype. In ^{high}Notch cells the levels of phosphorylated Akt S473 were increased in similar fashion as previously reported (259-263) and the induced glycolytic phenotype could be rescued with the addition of the PI3K inhibitor LY294002. The ^{high}Notch cells also exhibited increased mRNA expression of HK2, GLUT1, ALDOA, and PDK2.

The ^{low}Notch cells only showed an increase in PDK2 mRNA. The ^{low}Notch cells, however,

exhibited a glycolytic phenotype different from that seen in ^{high}Notch cells involving increased mitochondrial membrane potential and sensitivity to complex I, and V (ATPase) inhibition with Rotenone and Oligomycin respectively, suggesting an electron transport chain defect. Similarly in naïve MCF-7 cells DAPT treatment increased mitochondrial membrane potential as well as sensitized the cells for Oligomycin. Functional assays of complex I and IV showed decreased activity in ^{low}Notch cells, and lowered complex IV activity correlated with lowered complex IV, and COXII, a subunit of complex IV protein levels. Not surprisingly, oxygen consumption and ATP production was lower in ^{low}Notch cells, despite the ATPase still maintaining a functional ATP hydrolysis, excluding any functional error in complex V. When comparing lactate accumulation with oxygen consumption both ^{high}Notch and ^{low}Notch cells showed a higher dependency on glycolysis. The ^{low}Notch cells also showed a higher sensitivity for glucose deprivation further supporting the notion of glucose addiction. Finally p53 protein levels were found to correlate with Notch levels, and ^{low}Notch cells were significantly destitute of p53 protein, as also previously reported (279). Introducing wild-type p53 into ^{low}Notch cells rescued the glycolytic dependence.

Drosophila Notch has earlier been observed to be required for proper functioning of the electron transport chain, specifically the activity of NADH oxidase and NADH dehydrogenase. (316). Furthermore, Notch has been shown to control the expression of flavoproteins linked to the activity of the respiratory chain (317). Similarly, a patient with CADASIL (Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), caused by mutations in Notch3, exhibited mitochondrial impairment (318). Oddly, full-length Notch has also been reported localized to the outer mitochondrial membrane in conjunction with PINK1 (180), further strengthening the case for Notch in regulation of mitochondrial metabolism.

More recently, metabolic reprogramming has been linked to therapy resistance in another Notch-driven tumor type namely T-ALL. T-ALLs exhibiting activating NOTCH1 mutations have been observed to rely on glutaminolysis for anaplerosis, and combined Notch and glutaminolysis inhibition shown to attenuate growth of primary T-ALL xenographs (124). However, GSI resistance is often observed in T-ALL and one of the metabolic escapes is the loss of PTEN and constitutive activation of PI3K/Akt (124, 319).

III. **Systematic KOs:** Loss of CSL unlocks a hypoxic response and enhanced tumor growth potential in breast cancer cells

Canonical signaling from the entire family of Notch receptors is transmitted through the DNA-binding protein CSL. However, despite functioning as an effector of NICD-mediated gene output, in the absence of NICD, CSL effectively quenches all Notch target gene expression. Phenotypical changes at the event of CSL removal have thus been hard to predict. However, recent research has demonstrated formation of keratinocyte tumors and mesenchymal field cancerization (320) in different layers of the skin at the event of CSL removal (281, 321). Loss of CSL has also been observed to drive the transition from normal fibroblasts to cancer-associated fibroblasts (CAF) resulting in cellular senescence. Simultaneous loss of p53 however, has been

shown to engage unimpeded expansion of the CSL-deficient CAF cells (281). In breast cancer, loss of CSL, either via shRNA or genetic ablation (322), has been reported to increase tumorigenicity (323). Furthemore, 33% of invasive breast carcinomas inherently exhibit CSL depletion (323).

In paper III, we studied the effects of genetical removal of CSL in the breast cancer setting using CRISPR/Cas9 technology. A Cas9-induced double-strand break within the CSL locus and subsequent Non-Homologous End Joining (NHEJ), allowed a 1bp insertion to disrupt the reading frame of the CSL transcript in MDA-MB-231 cells. These cells that endogenously express high levels of Notch, were thus engineered to become a CSL-deficient MDA-MB-231 $CSL^{-/-}$ genotype. Two clones were selected for further studies. The MDA-MB-231 $CSL^{-/-}$ cells expressed no detectable CSL protein and showed no activation of downstream Notch 12xCSL luciferase reporter constructs. After transplantation into mouse mammary fat pad, as well as onto CAM of fertilized chicken eggs, tumor growth was analyzed. In both cases the MDA-MB-231 $CSL^{-/-}$ cells formed larger tumors compared to the control MDA-MB-231 WT cells. In the mice xenographs MDA-MB-231 $CSL^{-/-}$ clones exhibited a 2.8 fold increase in tumor mass compared to WT already after 5 weeks. Histological analysis of mouse tumors revealed that MDA-MB-231 $CSL^{-/-}$ were more proliferative by Ki67 staining. Similarly proliferation was increased in MDA-MB-231 $CSL^{-/-}$ cells in vitro as analyzed by EdU staining. Apoptosis was inhibited in MDA-MB-231 $CSL^{-/-}$ cells as assayed by measurement of cleaved Caspase-3. In a transwell migration assay clone #1 showed enhanced migration whereas clone#2 did not. However, both clones showed more aggressive invasive properties in a Matrigel-coated transwell invasion assay. In conclusion, removal of both alleles of the CSL gene yielded enhanced tumor growth in vivo and increased invasiveness in vitro.

The MDA-MB-231 $CSL^{-/-}$ cells also exhibited a multinucleated giant-cell phenotype suggestive of a mitosis defect. This phenotype was characterized by cells having a large volume with either a giant nucleus or a polyploid nucleus, with the large cells often surrounded by smaller cells. Single cell analysis revealed that the cells indeed presented with aberrant mitosis, where parent cells divided into multiple daughter cells or exited mitosis without cytokinesis, suggesting that loss of CSL may affect mitotic progression.

To study the molecular mechanisms downstream of CSL derepression, we analyzed the hypoxic response, and specifically the HIF1α levels. Hypoxia is a known driver of aggressive metastatic cancer (324), with HIF1α having well documented interactions with the Notch pathway (122, 150, 273, 278). Loss of CSL yielded increased levels of HIF1α in normoxia, which resulted from stabilization and increased half-life of the HIF1α protein. HIF1α mRNA levels remained unchanged between MDA-MB-231 WT and MDA-MB-231 $CSL^{-/-}$ cells. Increased HIF1α in MDA-MB-231 $CSL^{-/-}$ cells was also accompanied by hypoxic downstream gene upregulation, although with a different signature between the two clones. Clone #1 upregulated VEGF-A gene expression while clone#2 elevated the expression of STC2 and KLF8.

Ultimately, we wanted to assess the transcriptional signature of CSL-deficiency. By culturing MDA-MB-231 WT cells in vitro on immobilized Jagged1 ligands, and using the γ-secretase inhibitor DAPT to quench Notch cleavage, we were able to define the Notch transcriptional signature that was both ligand and γ-secretase-dependent. Subsequent RNA-sequencing analysis revealed this to contain 139 genes, including well-established Notch downstream targets, such as HES1, HES4, and NRARP. On the other hand, transcriptomic analysis of MDA-MB-231 $CSL^{-/-}$ cells revealed 1768 genes that were upregulated, and comparison between the 1768 genes and the 139 genes in the Notch signature revealed only 47 genes common in both categories. To analyze in vivo transcriptome we utilized the S3-technology (325) to bioinformatically sort out the human mRNAs from the tumor-stroma mixture and observed similar distinct transcriptomes in vivo as in the in vitro situation between the MDA-MB-231 WT and MDA-MB-231 $CSL^{-/-}$ tumors. Overall, the data suggest that CSL transcriptionally controls a number of genes not part of the canonical Notch signature.

IV. **Falling into Hypoxity:** Notch signaling upregulates HIF2α expression in tumor cells

The hypoxia machinery is known to interact with the Notch pathway with HIF1α stabilizing NICD in the canonical pathway (278), and FIH1 acting as a negative regulator of both HIF1α and NICD (150, 273). Evidence also exists that NICD can non-canonically stabilize and augment the effects of HIF1α (122).

In paper IV we set out to study if the canonical Notch pathway is capable of regulating another HIF paralog, namely HIF2α. Indeed, already in normoxia we observed increased HIF2α mRNA expression when Notch was activated in eight different cancer cell lines (derived from brain, blood, lung, breast, and renal cancer). Similarly an upregulation of HIF2α mRNA was evident in both primary glioblastoma and breast cancer cells, and in non-tumorigenic primary mesenchymal cells. As in normoxia, HIF2α mRNA upregulation was also evident in hypoxic conditions, and transcriptome analysis comparing Jagged1-mediated active Notch signaling with HIF2α expression correlated positively. NRARP (Notch-regulated ankyrin repeat-containing protein) was used as a control for active Notch-mediated transcription.

To decipher whether nuclear NICD was required for increasing HIF2α expression we utilized a Notch1 ICD-Estrogen receptor fusion construct (NERT2) which is normally localized to the cytoplasm, but translocates to the nucleus with the addition of Tamoxifen (249). When transiently expressed in Tamoxifen-treated DAOY medulloblastoma cells, NERT2 induced the expression of HIF2α and NRARP, while simultaneous expression of a dominant negative MAML (dnMAML) abolished the effect, suggesting that the upregulation of HIF2α is mediated via canonical Notch signaling. To test whether HIF2α is a direct transcriptional target of Notch we tested activation with a 2kb HIF2α promoter-luciferase reporter. However, no activation of reporter was observed with cells cultured with Jagged1 ligand. To corroborate this, we blocked protein translation in combination with Notch activation, and measured mRNA of both HIF2α and NRARP. While NRARP was potently induced, HIF2α was not suggesting that HIF2α is not a

direct Notch target mediated by Notch ICD/CSL.

The protein level of HIF2α was observed elevated already in normoxia both in primary breast cancer and medulloblastoma cell lines D324 and DAOY following N1ICD expression. A more moderate expression of Notch1 using immobilized Jagged1 did not significantly increase HIF2α in primary breast cancer or MDA-MB-231, suggesting dose dependency. However, both during hypoxia and $CoCl_2$ treatment as a chemical mimic of hypoxia, Notch was able to induce HIF2α in primary breast cancer, MDA-MB-231, as well as in D324 and DAOY respectively. Interestingly, Notch induction also led to a prototypical HIF1α-to- HIF2α switch (326) in D324, primary breast cancer, and MDA-MB-231 cells.

To compare the transcriptomes of Notch and HIF2α, we started by checking whether VEGRα and AREG (Amphiregulin), two genes known to be upregulated by HIF2α, were in fact induced by Notch. Indeed, both were upregulated by Notch and the induction was halted by DAPT. Following this, we extended the transcriptional screening to a genome-wide level in DAOY cells stably expressing NERT2 with Tamoxifen treatment under both normoxic and hypoxic conditions. 59 of 547 genes upregulated by Notch in hypoxia were inhibited by HIF2α siRNA knockdown, suggesting that circa 10% of the Notch transcriptome requires active HIF2α.

Notch1 and Notch2 have previously been shown to have different roles in medulloblastoma tumors (327), however a controversy has risen from a report indicating that Notch is dispensable in Sonic Hedgehog-driven medulloblastomas (328). In our paper, DAOY cells were transiently transfected with Notch1 and Notch2 ICD and enhanced tumor growth was observed in the CAM tumor model only in Notch2 cells keeping with the data from Fan et al. 2004 (327). Tumor suppressive functions for HIF2α have previously been reported in both human breast cancer and rat gliomas (329, 330). When comparing NERT-DAOY and NERT-DAOY HIF2α-deficient cell tumors on CAM, activation of Notch1 did not induce tumor growth, however the HIF2α-deficient tumors promoted rapid growth, suggestive of a tumor suppressive role for HIF2α also in this context.

Conclusions

Collectively this thesis presents six novel degrees of Notch pleiotropism in different forms of cancer (Figure 7). We have expanded the pool of Notch post-translational modifications to include Pim-mediated phosphorylation of Notch1. The synergy between Pim and Notch enables optimized Notch activation during tumorigenesis in both breast and prostate cancer. We show that both high and low Notch can induce glycolysis and that Notch is required for functional oxidative phosphorylation. We expand on the metabolism saga by showing that knocking out CSL yields a hypoxic response via HIF1α in breast cancer thus increasing tumorigenicity. In medulloblastoma, however, Notch1 induces HIF2α, which exhibits tumor suppressive properties. Finally the knock-out of CSL in breast cancer also influences a vast population of genes independently of Notch. These six findings expand the Notch-verse and bring us closer to complete understanding of The Notch.

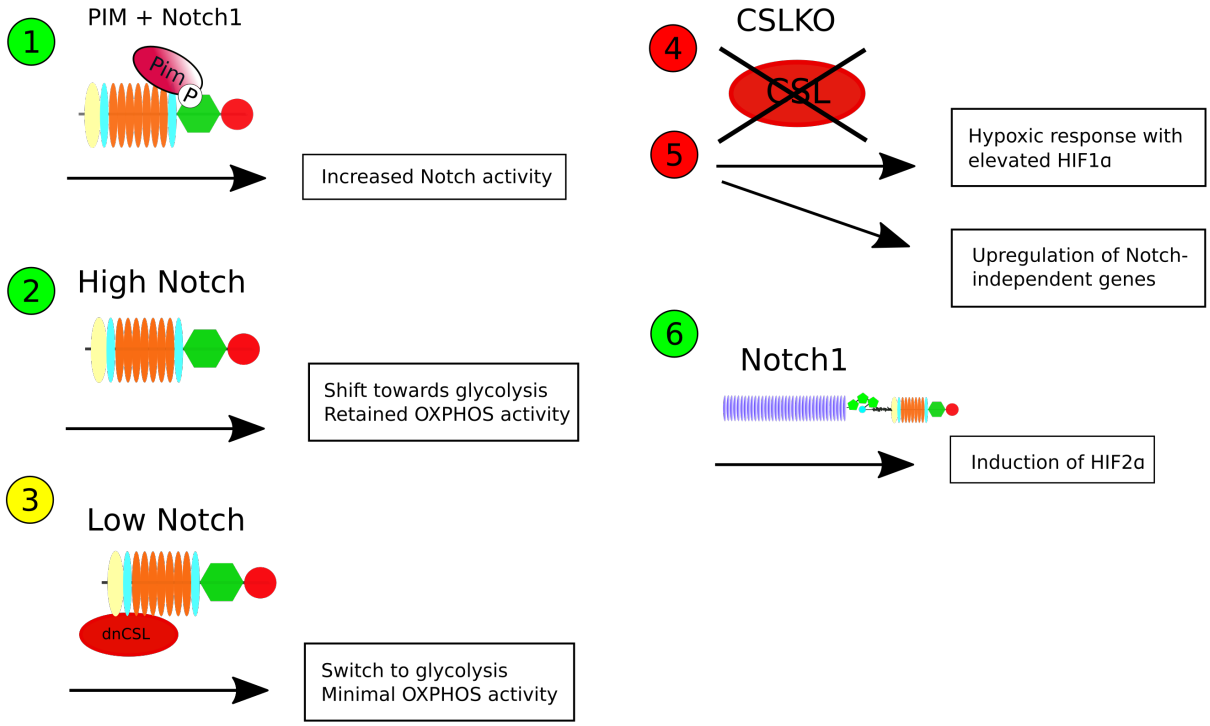

Figure 7. Six Degrees of Intracellular Turbulence.

Future perspectives

As a follow-up to the Notch-Pim interaction in paper I, we will continue to study the interaction between Pim and Notch by utilizing CRISPR methodology to create both knock-outs and knock-ins of the phosphorylation site of Notch1 in both MCF-7 and PC-3 cell lines. Knock-ins will replace the phosphorylated serine with alanine in both N1ICD to gain endogenous insight into the Pim – Notch crosstalk axis. We will also widen the paradigm to include triple-negative MDA-MB-231 breast cancer.

Furthermore also utilizing CRISPR, we will expand our investigations to involve Notch3 and decipher both the physiological and oncogenic role of Notch3 phosphorylation. For Notch3 the phosphorylation site has already been pinpointed to the RAM domain inside the binding groove between RAM and CSL, where the added phosphate interferes with binding of WxP to RAM (87, 88). N3ICD-SA and N3ICD-SE mutants have been devised, and our data indicate that Pim-phosphorylation inhibits the activity of N3ICD as measured by the 12xCSL luciferase assay, and subsequently lowers the levels of p21 in MCF7 cells. Supporting this notion is the observation that Notch3 has previously been identified as a tumor suppressor in breast cancer in connection to p21 expression (237).

Modern day cancer treatment is evolving towards combinatorial therapy where monotherapy has failed due to therapy resistance or high drug dose toxicity. With several drugs targeting both Notch and Pim currently in clinical trials (ClinicalTrials.gov), our discovery of direct Notch-Pim pathway crosstalk opens up a new possible combinatorial regimen for cancers expressing high

levels of both Notch1 and Pim.

Similarly our data indicate that blocking the Notch paralogs sensitizes breast cancer cells to glucose deprivation. 2-deoxy-D-glucose (2-DG) is a glucose analog currently in clinical trials which when taken up by cancer cells, competes with glucose and halts glycolysis. Thus a plausible combination therapy could consist of a γ-secretase inhibitor and 2-DG to treat certain cancers.

The future also involves deciphering the significance of the Notch-independent genes affected by CSL removal. The mode of action for CSL is undergoing a paradigm shift, where newly surfaced data argue for Notch-mediated dynamic recruitment of CSL to target sites (103). In this model CSL remains only loosely, or not at all associated to DNA in the repressor-state. However, Castel et al. also show static binding of CSL to Notch-independent sites. Whether the Notch-independent genes regulated by CSL-removal are separate individual genes, or if the change in gene expression is the result of a broader modification of the chromatin landscape remains to be determined.

We have touched upon the intricacies of Notch- HIF crosstalk, yet many questions remain unanswered. The controversial data implicating HIF2α in both tumor suppressive and tumor promoting roles hint of dose-dependency. Furthermore, whether the other Notch paralogs can regulate HIF2α, and how HIF3α fits into the cross-talk, remain to be seen (331).

Article not included in thesis

Inhibiting Notch activity in breast cancer stem cells by glucose functionalized nanoparticles carrying γ-secretase inhibitors

The cancer stem cell (CSC) paradigm suggests that a small subset of so called cancer stem cells in tumors exhibit similar self-renewal potential as normal stem cells (332). This hijacking of the stem cell characteristics by cancer cells aids not only in tumor initiation, but also in metastasis, recurrence, and therapy resistance (333). Interestingly, signaling pathways involved in controlling self-renewal of both stem and progenitor cells, can when dysregulated contribute to oncogenesis (332). Thus, in concordance with the cancer stem cell model of cancer, specific targeting of this subpopulation should prove more effective than classical cancer therapies. One of the most promising new targeted CSC-therapeutics is nanoparticle drug delivery.

In this paper we started off by utilizing the previously characterized ^{high}Notch, ^{normal}Notch, and ^{low}Notch cells to explore the influence of Notch on breast CSC population. As previously reported, ^{high}Notch cells outgrew the ^{normal}Notch, and ^{low}Notch counterparts, however, ^{high}Notch cells also expressed increased levels of CD44, a known marker for CSCs. To confirm the presence of CSCs we plated ^{high}Notch, ^{normal}Notch, and ^{low}Notch cells on low adherence plates in serum free conditons with supplemental growth factors. The ^{high}Notch cells formed significantly higher number and bigger spheroids than ^{normal}Notch cells, whereas ^{low}Notch cells were unable to form spheroids. The presence of CSCs in ^{high}Notch cells was further supported by xenotransplantation of 1000 cells of each cell line into nude mice, as only CSCs are capable of initiating cancer growth when only a small number of cells is transplanted. The ^{high}Notch cells formed tumors in 3/5 mice, whereas ^{normal}Notch, and ^{low}Notch cells did not form any tumors even after 3 months.

MCF-7 cells express the ER-receptor and are thus dependent on estrogen for optimal growth in vitro and in vivo. The ^{normal}Notch cells exhibited higher sensitivity for estrogen depletion compared to ^{high}Notch cells. Immunolabeling revealed decreased ER-receptor expression in ^{high}Notch compared to ^{normal}Notch cells. To further elucidate if the estrogen independence was linked to the CSC population, we grew tumors derived from dissociated spheroids of ^{high}Notch cells in gonadectomized mice and compared it to the control group where were tumors grown in normal mice. Expression of α6 integrin in the spheroids confirmed the CSC phenotype. Initial growth of tumors in gonadectomized mice was slower, reaching a comparable size to the control tumors only after 7 weeks. Yet, 4/6 mice transplanted with ^{high}Notch cells developed tumors also in gonadectomized mice. Immunohistochemistry revealed high expression of CD44, the proliferation marker Ki67, as well as cytokeratin 5/6, indicators of basal like cancer phenotype.

Based on our previous data that ^{high}Notch cells exhibit increased glucose uptake we also wanted to know the metabolic phenotype of the ^{high}Notch CSCs. Indeed, after enriching for CSCs from

the ^{high}Notch tumors we saw increased glucose uptake with ^{18}F-FDG PET. Similarly, spheroids generated from endogenous MCF-7 and MDA-MB-231 cells grown in non-adherent conditions also displayed increased glucose uptake compared to their adherent counterparts. Since MDA-MB-231 cells and normally more glycolytic than MCF-7 cells, the difference between each cell line's CSCs and the heterogeneous cancer cell population was more pronounced in MCF-7. In addition, when measuring extracellular acidification rate (ECAR, measured in milli-pH/min) in ^{low}Notch and MDA-MB-231 cells using the Seahorse analyzer we observed a higher glycolytic reserve capacity in MDA-MB-231 compared to ^{low}Notch cells. Cells with lower glycolytic reserve capacity are more dependent on glycolysis.

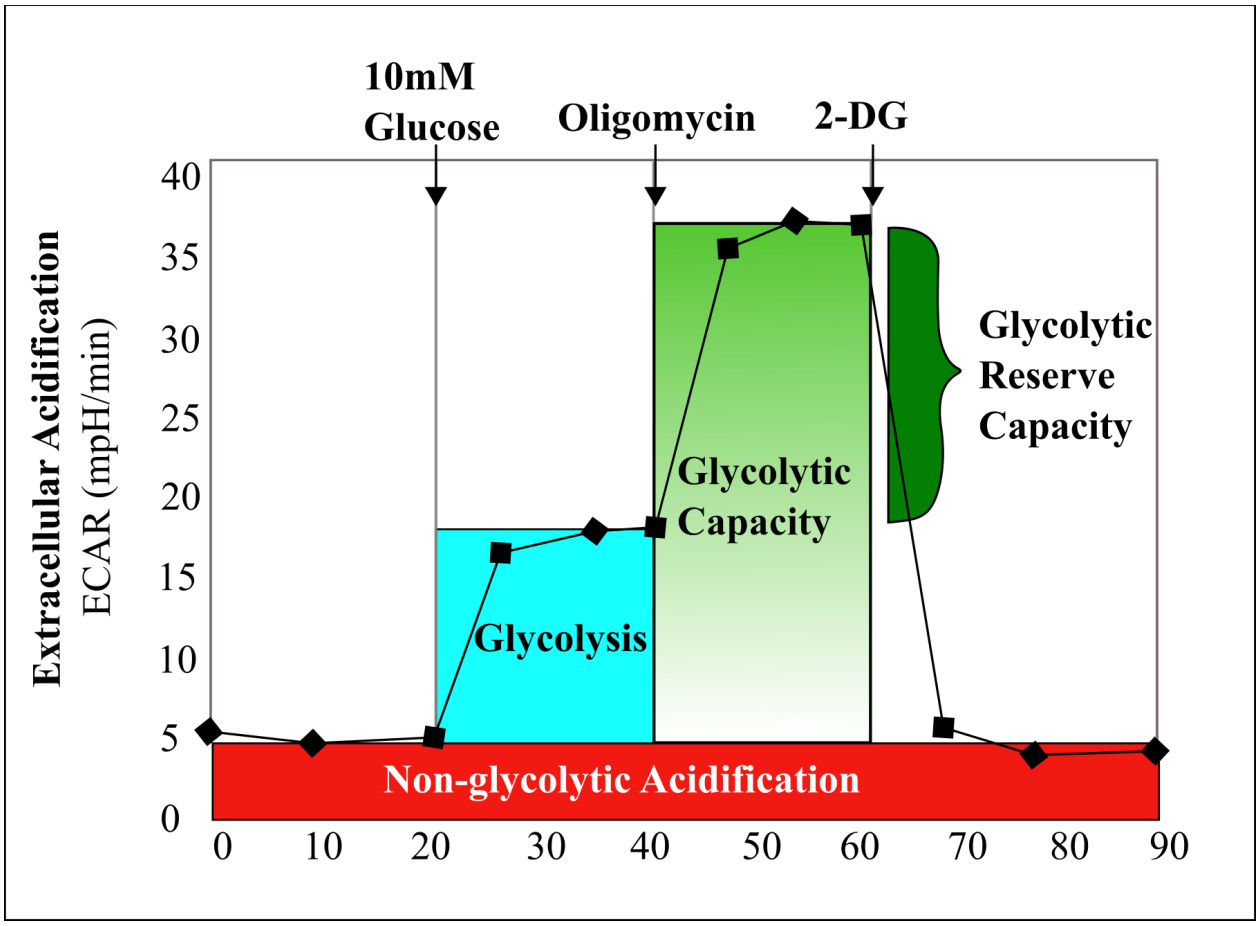

Figure 8. Extracellular acidification (ECAR) profile showing results of a hypothetical glycolytic measurement using the Seahorse XF analyzer. Addition of 10mM glucose induces glycolysis in cells increasing ECAR above the level of non-glycolytic acidification. Oligomycin inhibits oxidative phosphorylation forcing cells to utilize their glycolytic reserve capacity. Addition of 2-DG inhibits glycolysis and stops cellular glycolysis. *Picture adapted from Seahorse Bioscience glycolysis stress test profile.*

Since Notch signaling is required for self-renewal of CSCs and as CSCs express a glycolytic phenotype characterized by increased glucose uptake, we sought to target CSCs using glucose functionalized nanoparticles loaded with gamma-secretase inhibitors (GSI). Mesoporous silica nanoparticles (MSN) have been demonstrated to function well as drug deliverers in vitro (334),

and we have previously shown MSNs to be good deliverers of GSI with good therapeutic efficacy (335). In our study different conjugation methods were devised where glucose was attached to MSN either directly or via the polyethyleminine (PEI) linker. Particles were also functionalized with fluorescein isothiocyanate or with the fluorescent probe Atto647. Subsequently, the best uptake of particles in MDA-MB-231 cells after 4h of incubation was obtained with directly conjugated MSN-Gluc, and MSN-PEI-Gluc particles conjugated under organic conditions. Subsequent experiments were thus conducted with these particles.

We next sought to analyze uptake in the CSC population. CSCs were identified using the aldehyde dehydrogenase-1 (ALDH1) activity. MSN-PEI-Gluc particles exhibited higher uptake with glucose functionalization compared to control particles lacking glcose both in cancer cells and CSCs. However, MSN-PEI particles were also readily taken up by cancer cells. Particle uptake was further examined by confocal microscopy where glucose functionalized particles exhibited a intracellular vesicular pattern while non-glucose functionalized particles aggregated at the cell border. The particle uptake was also more pronounced in MDA-MB-231 cells than healthy MCF-10 mammary epithelial cells. However, increased uptake in CSCs with MSN-Gluc was only observed in MCF-7 but not MDA-MB-231 cells.

In order to verify the tumor targeting functionality of the particles in vivo, we orthotopically transplanted $3x10^6$ MDA-MB-231 cells into the mammary glands of female NOD SCID mice, however MSN outperformed MSN-Gluc particles in uptake. The therapeutic efficacy MSN-PEI-Gluc particles was also tested on CAM xenographs where MSN-PEI-Gluc particles were loaded with DAPT. Control treatment with free DAPT, as well as DAPT loaded particles reduced number of cancer cells per mg tissue. Similarly the the number of CSCs was also reduced in both DAPT and MSN-PEI-Gluc-DAPT-particle treated tumors. However, therapeutic efficacy was also obtained with MSN-PEI-DAPT particles.

Experimental procedures

12xCSL luciferase reporter

The 12xCSL luciferase reporter, first described by Honjo et al. (336) has been utilized before (122) and in several of the projects presented in this thesis (337). The reporter is based on a TP-1 promoter containing six copies of a 50-mer oligonucleotide each containing two CSL binding sites (338), and allows real time analysis of Notch activation (338).

CRISPR/cas9

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), and CRISPR associated (Cas) system was developed in 2013 to rival the TALEN and Zinc finger gene editing systems. CRISPR was originally identified in bacteria as a defense system against foreign DNA, either plasmid or viral. Today, three different types of bacterial CRISPR systems have been described, out of which type II is the one widely used in genome engineering and often simply referred to as CRISPR.
The CRISPR/Cas9 system consists of 2 components, namely a guide RNA (gRNA), and an endonuclease, the Cas9. The gRNA is designed to contain a targeting sequence complementary to the genomic DNA, a scaffold sequence for Cas9, and the Protospacer Adjacent Motif (PAM) sequence immediately following the target sequence. The PAM signals the site for Cas9 cleavage, generating a double-stranded break (DSB). Following the Cas9 DSB, repair can occur via Non Homologous End Joining (NHEJ) or Homology Directed Repair (HDR) pathway. The NHEJ can result in insertions or deletions in the DSB which often lead to frameshifts or premature stop codons. On the other hand, the HDR pathway requires the presence of a repair template to fix the DSB. This method can be used to introduce specific nucleotide changes.

CAM-model

The chorioallantoic membrane (CAM) model developed in 1911 by Rous & Murphy (339) is a multipurpose in vivo tool to study various physiological phenomenon in the developing chick embryo. Thanks to the highly vascularized CAM membrane, the model can be used to study tumor xenograph growth, response to therapy, angiogenesis, hemodynamics, immune cell trafficking and more.

Figure 9. Chorioallantoic membrane (CAM) tumor model. MCF-7 breast cancer xenografts on CAM incubated in a De Rycke Savimat MG 200 egg incubator

Proximity ligation assay – PLA

The proximity ligation assay (PLA) is a method allowing for detection of endogenous protein interactions at single-molecule resolution. Originally developed by Fredriksson et al. (340) and later adapted for i*n situ* use by Söderberg et al. (341), this technique utilizes oligonucleotide labeled antibodies to detect protein-protein interactions only 30-40nm apart. A PLA protocol consists of fixing of the samples and incubating with two primary oligonucleotide labeled antibodies. Being bound in close proximity, the two oligonucleotides can hybridize. This leads to binding of two additional connector oligonucleotides, which are ligated together to form a spherical DNA molecule. Subsequent amplification by rolling circle amplification (RCA) leads to the formation of a long single stranded DNA molecule, which collapses into a bundle. Finally, the bundle is detectable by hybridization of fluorescently labeled complementary oligonucleotides (342).

The Best of Times: Acknowledgements

Sooo… you skipped straight to the acknowledgements. Yes you did, I saw you do it, don't even try to deny it! Well, I guess I can't stop you from reading this part first, but hey, check out the rest of the book as well, its good stuff!!!

The doctoral thesis presented herein was conducted mainly at the Centre for Biotechnology, and Åbo Akademi at Biocity in Turku. I'd like to express my gratitude to both institutions and the personnel affiliated with both. Without you this work would not have been possible. I am also extremely grateful for Karolinska Institutet for enrolling me as a PhD-candidate, and granting me affiliation and financing to do my thesis. Speaking of financing, I would like to thank all my financial sources: **Urban Lendahl** and The NotchIT FP7 ESR-fellowship, Knut and Alice Wallenberg foundation, The Swedish Cultural Foundation of Finland, K. Albin Johansson Foundation, Magnus Ehrnrooth Foundation, and the Sigrid Juselius Foundation.

First and foremost I would like to thank both of my "bosses", i.e. **Urban Lendahl**, and **Cecilia Sahlgren**, both of whom I saw surprisingly little of during my PhD. Perhaps a fitting fate for someone with my status as a vagabond PhD-student, endlessly having to explain my affiliation.

Urban, you were quite the urban legend for me, only seeing you maybe once or twice a year. Still you managed to have an immense influence on the process of me growing into a scientist. During our meetings, I could have listened to you in awe for hours, just baffled by your knowledge, at the same time trying desperately to remember every last detail that was being discussed. After leaving your office I always felt inspired to tackle even the toughest challenges. Now that I think back, I should have visited you more to avoid those numerous phases of existential despair. I am also extremely grateful to you for enrolling me in the NotchIT-consortium, which allowed me to participate in several different international courses and conferences across Europe, all in all, a breathtakingly amazing experience that quite frankly changed me as a person.

My deepest gratitude goes to **Cecilia**, for believing in the silent man who came to your group at the end of 2009 and after a few preliminary flow cytometry runs, eventually taking him under your wing and giving him a crazy metabolism project. I'm also very grateful to you for throwing me to the lions quite early in the game, when it came to presenting data to a big audience. I wasn't too thrilled about it then, but needless to say, it developed character. The time I have spent in your research group has matured me not only as a scientist, but also as a human being.

I would also like to thank all my collaborators and coauthors. Special thanks to **Päivi Koskinen** and **Niina Santio** for the collaborative effort deciphering the Notch and Pim kinase interaction.

"All work and no play makes Sebastian a dull boy?" The completion of this thesis would not have been possible without all the unique friendships within the group, all the hilarious moments and memorable social events and game nights during the years. I consider myself one of the

lucky ones to have been blessed with such awesome and fun co-workers. Thank you **Marika**, **Daniel**, **Christian**, **Rasmus**, **Valeriy**, **Ezgi**, **Veronika**, and **Adolfo**. I'd also like to thank the alumni members **Martina** and **Natalie**.

Special thanks to **Marika** for having the patience to take care of 3 guys in the lab, and for all the laughs and funny coffee break discussions. **Daniel "The Wolf of Tykistökatu-Street" Antfolk** for teaching me so much about investing, and for fun times spent playing poker, snooker, and disc golf. **Christian** for sharing the room both in Mykonos and New York, and for repeatedly letting everyone know the dimensions of Copag poker sized playing cards during our poker nights. **Rasmus "Get to da choppa!" Niemi** for your uncanny sense of humor, cooking tips, and gym enthusiasm second to none.

I've had the pleasure to get to know many astonishing people in the biocity community. These include (but are not limited to): **Josef**, **Iris** & **Erik**, **Julia**, **Anders**, **Ilari**, **Jenny**, **Anna A**, **Käthe**, and **Alexandra**. Furthermore, I've had the privilege to get to know the Abankwa lab members very well so thank you **Arafat**, **Maja** + **Tony**, **Olga**, **Camillo** + **Johanna**.

Thank you all members of the Lendahl lab who have made me feel welcome when I've been visiting KI., especially **Anders "Lord of The Worms" Mutvei**, **Shao-Bo**, **Heather**, and **Tanya.**

There is still one person missing from the above list, and that is of course **Elina**, my co-pilot during the last years of working on my PhD, and also in life. Since the get-go I've been amazed by your kindness and good-heartedness. Not only that, your work is highly professional, but always with a tongue-in-cheek spice to it. You've taught me numerous "tricks of the trade" that have made my life so much easier in the lab. Your humoristic side also translates to your personal life, which makes you a very funny companion for a man to have. You also never fear to throw down the gauntlet and really challenge me in almost every activity and hobby. Love you for that! Thank you for bearing with this maniac, and taking good care of him both on his good and bad days.

Finally, I wish to convey my gratitude to my parents **Marjatta** and **Kaj Landor**, who have supported me throughout my life to the point where I have felt completely spoiled. I am truly lucky to have been born into such a loving family. I love you both to bits!

Kiitos teille molemmille tuestanne vuosien aikana. Parempia vanhempia ei voisi toivoa. Nyt ollaan sitten käyty koulua pyöreät 24 vuotta, eiköhän olisi aika etsiä uusi harrastus? ;) Tack för stödet under åren, jag kunde inte ha önskat mig bättre föräldrar.

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