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Judith Sophia Johanna Maria Hounjet

Promoter:	Prof. dr. M.A.G.G. Vooijs
Co-promoter:	Dr. ing. A.J. Groot
Assessment committee:	Prof. dr. L. de Windt (UM, Chair)
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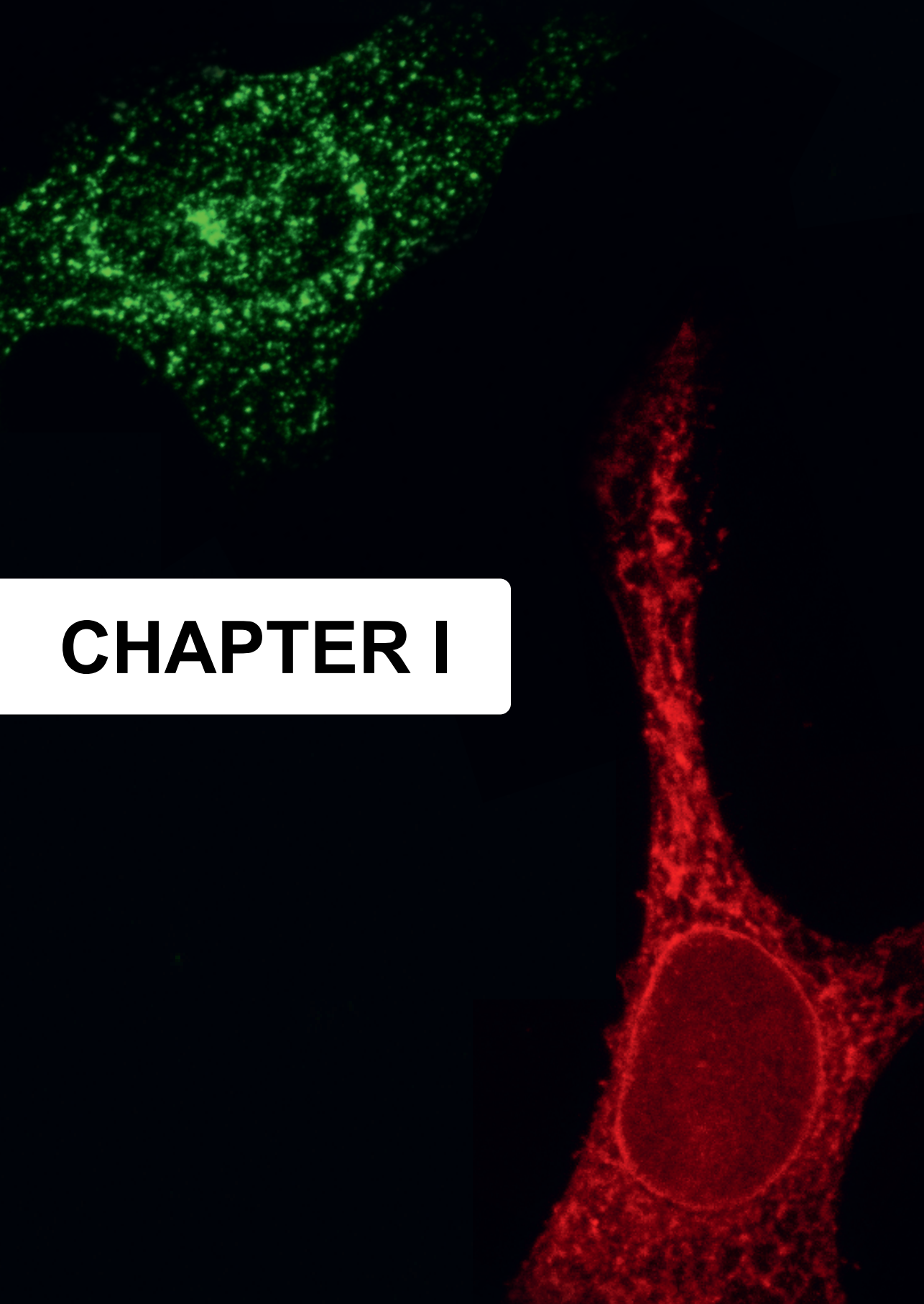
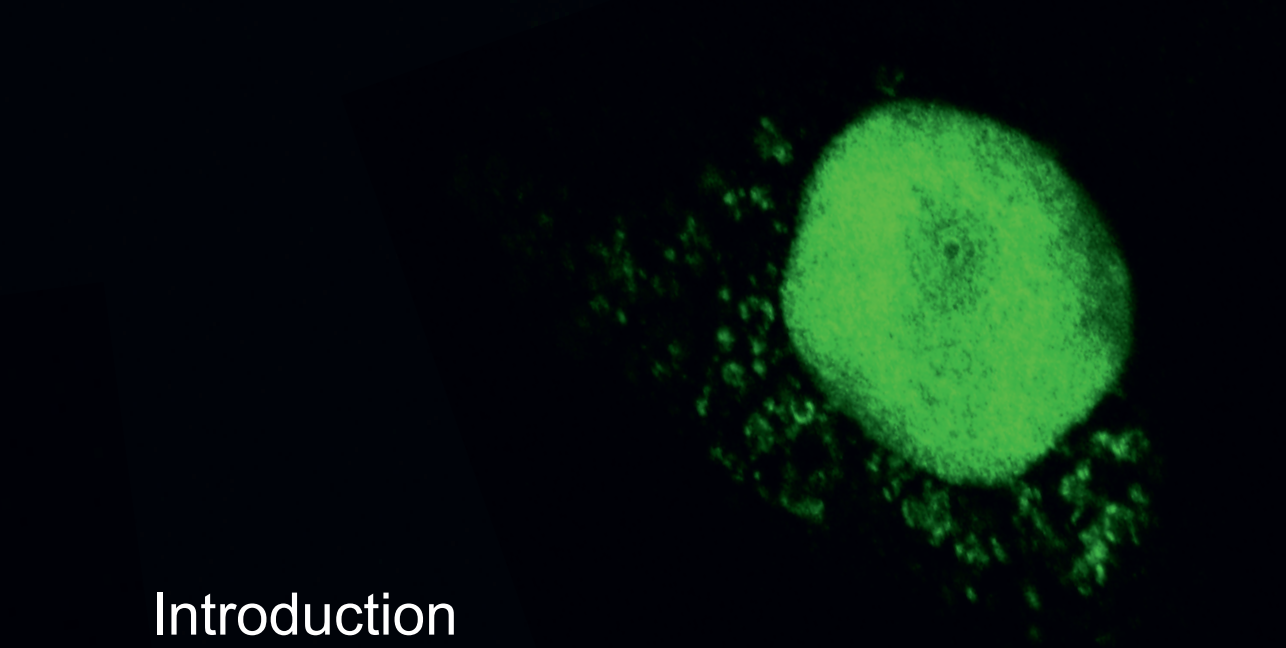


CHAPTER I



Introduction

Judith Hounjet

Department of Radiation Oncology (Maastro), GROW School for Oncology, Maastricht University Medical Centre+, Maastricht, The Netherlands

Maastro clinic, Maastricht, The Netherlands

Cancer

Cancer is the second leading cause of death worldwide. Each year approximately 10 million people die of cancer (World Health Organization). Since cancer has such a great impact on human mortality, worldwide billions were spent on cancer research over the last 50 years since the National Cancer Act of 1971 was signed. The National Cancer Act declared the “war on cancer” to find a cure for cancer by increasing cancer research in understanding the molecular biology and developing more effective cancer treatments. Although mortality rates are (slightly) declining in most higher-income countries, these trends are lacking in lower-income countries (World Cancer Report, WHO). How can it be that one disease kills so many people each year? Why is this disease not cured after spending millions each year?

Most importantly, cancer is often depicted as one disease. However, cancer is a name for a collection of over a hundred distinct, but related, diseases in which cells of a specific tissue of the human body show uncontrolled growth. As cancer can affect almost each tissue of the human body and within specific tissues different cancers can arise, cancer is not one disease and can simply not be cured with a single drug. Besides, tumor cells deregulate processes in the human body that are used during development and maintenance of adult tissues. As these processes are also required by normal tissues for proper homeostasis, extensive research is required to be able to develop “smart” therapeutics targeting cancer cells, without damaging normal tissues.

Hallmarks of cancer

Cancer research has generated comprehensive knowledge on the molecular mechanisms of cancer over the last decades. The foundation in the understanding of cancer has been set by the discovery of cancer specific mutations leading to gain-of-function of oncogenes, stimulating proliferation and growth, and the loss-of-function of tumor suppressors, which inhibit uncontrolled proliferation and stimulate cell death. Although each cancer acts differently general cancer hallmarks, which are essential and common to all cancers for the initiation and progression of tumor cells, have been introduced 20 years ago. These cancer hallmarks include: self-sufficiency

in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastases (Hanahan and Weinberg, 2000). Genetic reprogramming of cancer cells results in increased proliferation by enhanced production of growth signals, cell cycle checkpoint inhibition by suppression of p53, and reduced induction of programmed cell death, due to increased anti-apoptotic signals. In addition, angiogenesis, the formation of new blood vessels, is activated by an angiogenic switch during tumor progression to supply oxygen and nutrients for further tumor growth and survival. During tumor progression tumor cells can invade and metastasize to distant sites, due to modulation of the surrounding tissues including degradation of cell-cell adhesion molecules and modulation of the extracellular matrix.

Over the last decades, additional hallmarks of cancer were discovered, including reprogramming of energy metabolism and genomic instability to sustain proliferation of tumor cells. Additional hallmarks of cancer cells are evasion of the immune system by modulation of T-cells, B-cells, macrophages, and natural killer cells leading to reduced anti-tumor immune responses. Lastly, tumor cells promote inflammation resulting in increased production of growth, survival, and pro-angiogenic factors and invasion stimulating processes, supporting tumorigenesis and tumor progression (Hanahan and Weinberg, 2011).

Tumor microenvironment

Despite the fact that these general hallmarks of cancer make the molecular understanding of tumors complex an additional layer of complexity is applied by the tumor microenvironment. The tumor microenvironment is composed of proliferating tumor cells, surrounding blood vessels, extracellular matrix and non-malignant cells, including: stromal cells, fibroblasts, immune cells, pericytes and adipocytes, which all contribute to the hallmarks of cancer. Stromal cells and cancer-associated fibroblasts (CAFs) produce mitogenic signals, including growth factors and chemokines, which stimulate the proliferation and survival of tumor cells (Hanahan and Coussens, 2012), leading to poor patient prognosis (Räsänen and Vaheri, 2010).

In addition, CAFs remodel the extracellular matrix (ECM) by producing proteolytic enzymes, including matrix metalloproteinases (MMPs), and regulation of ECM synthesis, promoting tumor growth and invasion and induce angiogenesis by the secretion of pro-angiogenic factors and ECM degradation (Hanahan and Coussens, 2012; Kalluri and Zeisberg, 2006).

The activation of angiogenesis results in the formation of abnormal, disorganized, and leaky blood vessels compared to the normal vasculature, which results in poor oxygen and nutrient supply. As a result, tumor cells become hypoxic due to the lack of oxygen, leading to increased cell survival, decreased apoptosis, and resistance to therapy (Trédan et al., 2007). Moreover, endothelial cells produce growth factors stimulating tumor growth and progression (Butler et al., 2010). Infiltrating immune cells in the tumor environment produce cytokines, chemokines and growth factors, including vascular endothelial growth factor (VEGF), which stimulate proliferation and promote angiogenesis (Balkwill and Mantovani, 2012; Hanahan and Coussens, 2012). Infiltrating immune cells also produce proteolytic enzymes degrading the ECM resulting in stimulation of tumor growth and invasion and bind to adhesion molecules to prevent tumor cell death by loss of tissue integrity (Chen et al., 2011). Tumor-associated macrophages can have both tumor promoting or suppressing functions and can produce cytokines suppressing immune responses against tumor cells (Qian and Pollard, 2010).

The Notch signaling pathway

Notch signaling is a highly conserved signaling pathway involved in proliferation and differentiation during embryonic development and in adult tissues (Siebel and Lendahl, 2017). During embryonic development Notch signaling plays an essential role in the development of somite-derived organs, including muscle cell differentiation (Buas et al., 2010; Hubaud and Pourquié, 2014; Kopan et al., 1994). Moreover, Notch signaling controls vasculogenesis and vascular branching during angiogenesis (Fischer et al., 2004; Krebs et al., 2000). Besides controlling vascular outgrowth, Notch signaling also regulates cardiac development, like coronary arterial differentiation (Farber et al., 2019), including the regulation of endocardial stem cells

and proliferation of the myocardium (Grego-Bessa et al., 2007; Kokubo et al., 2005). In addition, Notch signaling regulates hematopoietic stem cell differentiation (Hadland et al., 2004; Han et al., 2002; Radtke et al., 1999) and maintains stem cells of the embryonic central nervous system during development (Gao et al., 2009; Imayoshi et al., 2010). Notch signaling also regulates epidermal proliferation and differentiation in the skin (Massi and Panelos, 2012), kidney development, hair cell differentiation in the cochlea (Brooker et al., 2006; Hartman et al., 2010; Kiernan et al., 2006) and balances the differentiation in the pancreas, in which loss of Notch signaling promotes endocrine differentiation (Apelqvist et al., 1999; Lammert et al., 2000).

During adult life Notch signaling also plays an essential role in tissue homeostasis. First of all, Notch signaling maintains stem cells in the intestinal crypts and promotes enterocyte differentiation by blocking secretory cell differentiation (Milano et al., 2004; van Es et al., 2005). Notch signaling does not only regulate cell-fates decisions in the intestine, but also controls differentiation of B- and T-cells (Han et al., 2002; Pui et al., 1999), regulates cell fate decisions in skeletal muscle (Buas et al., 2010; Nofziger et al., 1999), lung (Mori et al., 2015; Rock et al., 2011), skin (Blanpain et al., 2006; Rishikaysh et al., 2014) and liver (Ortica et al., 2014), and maintains the endothelium of the vasculature (Bhattacharyya et al., 2014; Joutel et al., 2004; Sweeney et al., 2004).

The Notch signaling pathway mediates cell-to-cell communication and is activated by Notch ligand binding of a signal-sending cell to a Notch receptor on a signal-receiving cell. Subsequent proteolytic cleavages lead to the translocation of the cleaved receptor to the nucleus, where it activates its downstream targets. Mammals express four different Notch receptors, Notch1-4 (**Figure 1**). Notch receptors are single-pass transmembrane proteins composed of a series of specific protein domains. The extracellular region of the Notch receptor consists of a series of up to 36 EGF-like repeats and a negative regulatory region (NRR), which includes three Lin12/Notch repeats (LNRs) and a heterodimerization domain (HD). The intracellular region of the Notch receptor consists of a protein-binding RPBJ-associated molecule (RAM) domain, seven ankyrin repeats (ANK), a transcriptional

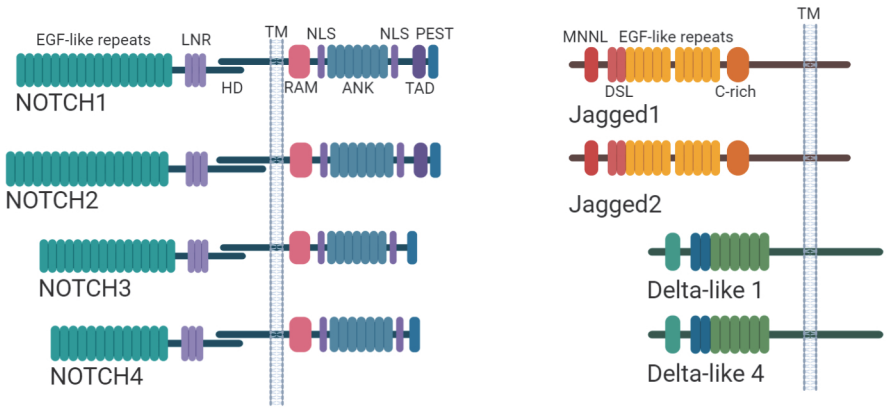


Figure 1. Mammalian Notch receptors and ligands. *Mammals express four Notch receptors, including Notch1-4 and four Notch ligands, including Jagged1, Jagged2, Delta-like 1, and Delta-like 4. LNR: Lin12/Notch repeats, HD: heterodimerization domain, TM: transmembrane, RAM: protein-binding RPBJ-associated molecule, ANK: ankyrin repeats, TAD: transcriptional activation domain, PEST: C-terminal region rich in proline, glutamate, serine and threonine (adapted from Biorender).*

activation domain (TAD), and a C-terminal region rich in proline, glutamate, serine and threonine (PEST domain). Notch receptors require ligands to activate Notch signaling. In mammals four ligands are expressed, which are also single transmembrane proteins. Delta-like 1 (Dll1) and Delta-like 4 (Dll4) are members of the Delta family of ligands and Jagged1 (Jag1) and Jagged2 (Jag2) are members of the Serrate family of ligands.

The Notch signaling pathway is initiated with the transcription of the Notch receptor in the nucleus (**Figure 2**). Next, the Notch receptor is transported from the ER to the Golgi apparatus, where it undergoes its first cleavage at the S1-site by furin-like protease. After this first proteolytic cleavage the heterodimeric NOTCH receptor (TMIC) is transported towards the plasma membrane, where it awaits ligand binding (Kopan and Ilagan, 2009). In the absence of ligand binding, the negative regulatory region (NRR) masks the S2-cleavage site, which results in auto-inhibition of the Notch receptor (Gordon et al., 2009). Upon ligand binding, the NRR unfolds and unmasks the S2-cleavage site of the Notch receptor (Mumm et al., 2000). The exposure of the S2-cleavage site recruits Adam10, which subsequently cleaves the

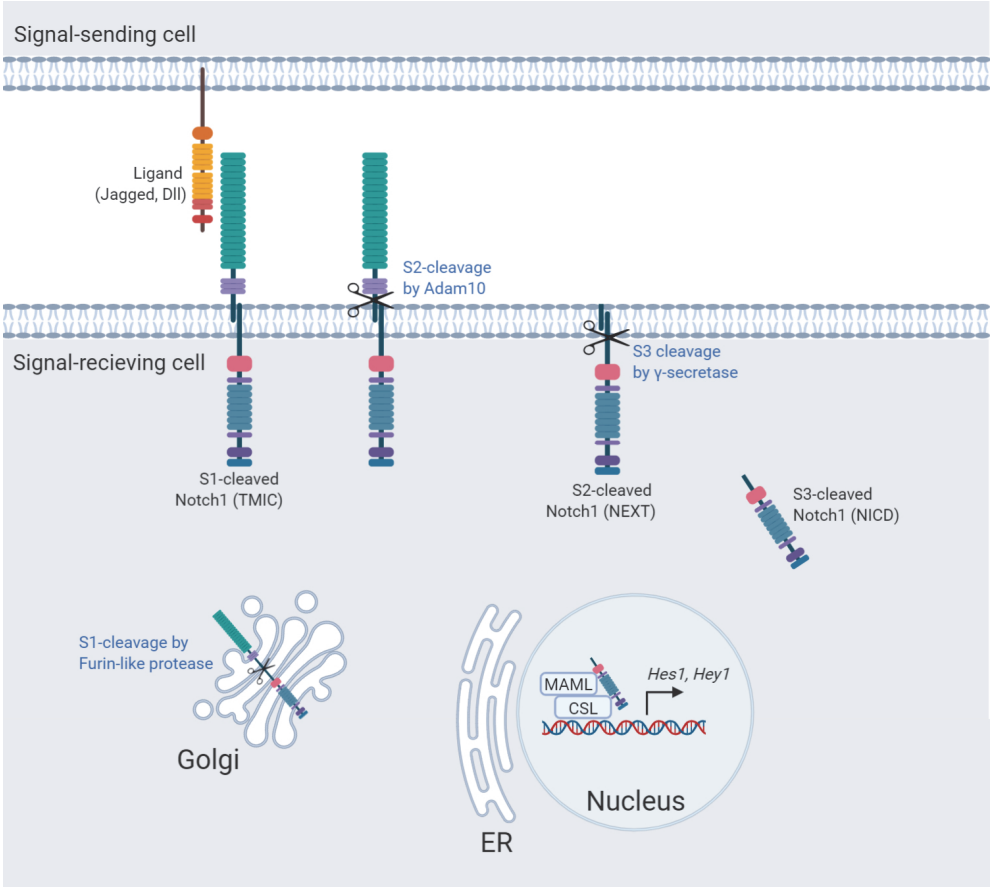


Figure 2. The Notch signaling pathway. *Endogenous Notch signaling requires ligand binding, expressed by a signal-sending cell and three sequential cleavages to release the active form of Notch, which activates Notch downstream targets. TMIC: transmembrane intracellular fragment, NEXT: Notch extracellular truncation, NICD: Notch intracellular domain.*

Notch receptor at Val1711, resulting in a membrane-tethered truncated Notch receptor (NEXT)(van Tetering et al., 2009). Finally, the Notch receptor is cleaved at Val1744 (S3-cleavage site) by the γ-secretase complex, which consists of four subunits including: presenilin 1 or 2, APH-1a or b, PEN-2 and nicastrin, and releases the Notch intracellular domain (NICD)(Kimberly et al., 2003). The NICD is transported towards the nucleus where it forms the Notch Transcription Complex

(NTC) together with DNA-binding factor RBPJ (also known as CSL) and co-activators of the mastermind-like (MAML) family and binds to canonical DNA binding sites in the promoters and enhancers of Notch target genes (Kopan and Ilagan, 2009; Wu et al., 2000).

Endocytic trafficking

Endocytosis is the internalization of molecules by invagination of the plasma membrane and formation of intracellular vesicles, which is essential for the regulation of various pathways in the cell. Endocytosis is used to sort, process, recycle, store, and degrade internalized cargo to fine-tune the activation or silencing of signaling pathways. Endocytosis is initiated with the formation of an early endocytic vesicle from clathrin-coated pits, however, additional clathrin-independent internalization mechanisms exist (Mayor and Pagano, 2007). The majority of internalized cargo is recycled back to the plasma membrane, either direct via the small GTPase family member Rab4 or indirect via recycling endosomes regulated by Rab11 (Huotari and Helenius, 2011; Steinman et al., 1983)(**Figure 3**). A small proportion of endocytic vesicles fuse with each other and mature into late endosomes during which Rab5 is the main regulator. During endosome maturation Rab5 is exchanged for Rab7, which is the major regulator of intracellular trafficking in late endosomes and lysosomes. During maturation, early endosomes can form intraluminal vesicles, which require the recruitment of the components of the endosomal sorting complexes required for transport (ESCRT), leading to the formation of multivesicular bodies (MVBs) with double membranes (Huotari and Helenius, 2011). Sorting of internalized proteins is regulated by ubiquitination. ESCRT complexes recognize ubiquitinated cargo proteins and sort them into intraluminal vesicles in the MVBs. The formation of MVBs is important for the proper sorting of cargo for degradation in lysosomes and endosome maturation. Due to the formation of MVBs internalized cargo is retained from the cytosol and easily accessible for hydrolases once delivered to the lysosomes. During endocytic trafficking, the microenvironment of endosomes and lysosomes changes, due to ion in- and efflux (Scott and Gruenberg, 2011). Upon

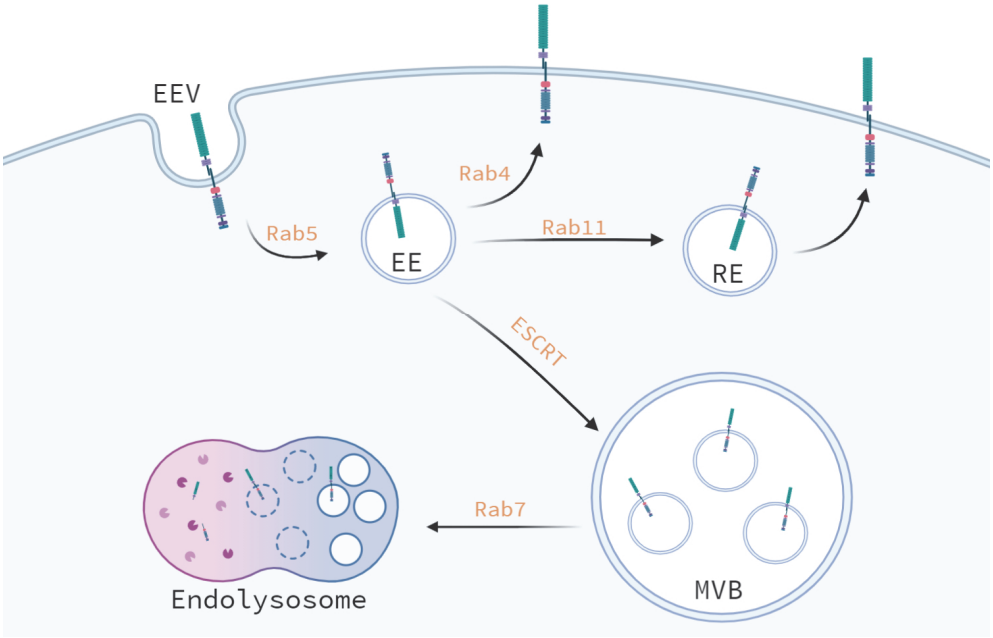


Figure 3. Endocytosis and intracellular trafficking. *Membrane-tethered proteins are continuously internalized. While the majority of membrane-tethered proteins are directly transported back to the plasma membrane, the minority of membrane-tethered proteins are further transported towards the endocytic compartments and either recycled to the plasma membrane via recycling endosomes or degraded in the lysosomes. EEV: early endosomal vesicles, EE: early endosomes, RE: recycling endosomes, MVB: multivesicular bodies.*

endosome maturation, endosomes become gradually more acidic due to increased H^+-ion influx by the V-ATPase (Marshansky and Futai, 2008; Yan et al., 2009). These changes in pH affect protein-protein interactions, enzymatic activity and protein degradation during endocytic trafficking.

Intracellular trafficking of the Notch signaling

Although the Notch signaling pathway is already quite complex, due to the requirement of ligand-binding from an adjacent cell and subsequent proteolytic cleavages, an additional layer of complexity is added by the intracellular trafficking of Notch ligands and receptors. Intracellular trafficking has been shown to be essential for Notch signal activation in both signal-sending and signal-receiving cells

as defects in endocytic trafficking lead to enhanced or reduced Notch activation dependent on the site of endocytic inhibition.

Currently, there are two models on the role of ligand endocytosis in the activation of Notch signaling: the “pulling force” and the “dissociation” model. The “pulling force” model states that endocytosis following the interaction between Notch ligand and receptor generates a mechanical force leading to conformational changes in the Notch receptor, revealing the S2-cleavage site for Adam cleavage and further Notch activation (Gordon et al., 2015). On the other hand, the “dissociation” model suggests that ligand endocytosis upon receptor binding induces mechanical forces, which physically dissociate the Notch receptor after which S2-cleavage and Notch receptor activation can proceed (Nichols et al., 2007).

Endocytic trafficking of Notch receptors can result in both activation or suppression of Notch signaling. Notch receptors, like other transmembrane bound proteins, are internalized for activation or degradation (Huotari and Helenius, 2011). Notch receptors are continuously internalized by clathrin- and dynamin dependent endocytosis (Chastagner et al., 2008; Vaccari et al., 2008). While the majority of receptors is transported back to the plasma membrane to await ligand binding, the minority of internalized Notch receptors travel further in the endocytic pathway, where they can either be activated in maturing endosomes or degraded in lysosomes. We review the endocytic trafficking of the Notch receptor in both *Drosophila* and mammals in more detail in **chapter II**.

Chloroquine: disrupter of intracellular trafficking

To study the role of intracellular trafficking in Notch activation and signaling we used chloroquine to disrupt intracellular trafficking and studied its effects on oncogenic Notch signaling in **chapter III**. The anti-malarial drug chloroquine was first produced in 1934 and was selected for its anti-malaria activity. Chloroquine acts on the early stages of malaria infection which enhances clearance of parasites, preventing further parasite production and development of clinical illness (Gregson and Plowe, 2005). In 1951 chloroquine became also a standard therapy for lupus erythematosus, due to its anti-inflammatory effects (Wolf et al., 2000), which resulted in further testing of

chloroquine treatment in non-malarial diseases, showing effectiveness in a variety of dermatologic, immunologic, and rheumatologic disorders.

Chloroquine, which is a weak base, has been shown to affect several processes in the cell by elevating the pH in intracellular vesicles, including lysosomes (Ohkuma and Poole, 1978; Wolf et al., 2000). Various physiologic functions that depend on an acidic pH are affected by chloroquine, including immunomodulation by inhibition of antigen-presentation, cytokine production, surface-receptor recycling, and apoptosis of immune cells. Chloroquine has also been shown to reduce inflammation, increase nitric oxide production, block DNA, RNA and protein synthesis, and induce rapid degradation of ribosomes (Wolf et al., 2000).

An additional catabolic mechanism which is inhibited by chloroquine is autophagy, due to blockage of the fusion of auto-phagosomes with lysosomes. Autophagy allows the cell to recycle intracellular organelles and macromolecules to sustain growth and survival during stress (Rubinsztein et al., 2012; White, 2012). However, autophagy is also a survival mechanism for cancer cells. The anti-cancer efficacy of chloroquine has been shown in a broad range of malignancies, especially in combination with chemotherapy or radiotherapy in preclinical studies (Ding et al., 2011; Liang et al., 2014; Ratikan et al., 2013; Rouschop et al., 2010; Verbaanderd et al., 2017). As a result, clinical trials are ongoing to evaluate the safety and efficacy of hydroxychloroquine, a less toxic derivative of chloroquine (Browning, 2014), in a broad range of cancers, including brain, breast, bone, prostate, lung and colorectal cancer (Shi et al., 2017; Verbaanderd et al., 2017). However, chloroquine exhibits also autophagy independent effects on tumor progression, including tumor vessel normalization by the induction of Notch signaling in endothelial cells reducing tumor invasion and metastasis (Maes et al., 2014). Although chloroquine affects various physiological processes by elevating the cellular pH, short-term administration rarely causes severe side effects. However, long term exposure shows more severe adverse effects in some cases, including: cardiomyopathy (Tönnesmann et al., 2013), retinal toxicity (Costedoat-Chalumeau et al., 2015), and induction of leukemia (Nagaratnam et al., 1978). Since chloroquine targets intracellular trafficking in vesicles and both Notch signaling and pH in vesicles is important, other processes

that regulate vesicular pH may impact Notch signaling as well. One such process may be iron metabolism and transport, key to all living organisms.

Iron homeostasis

Iron is an essential nutrient for all organisms, facilitating proliferation and growth. Iron regulates oxygen transport by hemoglobin, oxygenation of muscles, oxidative phosphorylation, and DNA synthesis. Moreover, iron is an essential cofactor for many enzymes. Upon intestinal absorption, Fe^{3+} (non-heme) iron is reduced to Fe^{2+} by cytochrome B, which is transported by the proton-coupled Divalent metal transporter 1 (Dmt1) across the apical membrane (Canonne-Hergaux et al., 1999; Fleming et al., 1997)(**Figure 4**). After intracellular trafficking, Fe^{2+} is exported by ferroportin across the basolateral membrane into the circulation accompanied by the re-oxidation of Fe^{2+} to Fe^{3+} to enable binding to transferrin (Abboud and Haile, 2000; Gkouvatsos et al., 2012). Ferroportin expression is regulated by hepcidin, a hormone, which binds to ferroportin and induces its internalization and degradation (Nemeth et al., 2004). Cellular iron uptake is mediated by the interaction of transferrin-bound iron to the transferrin receptor 1 (TfR1) and the subsequent internalization of this complex. The gradual acidification of the endosome triggers a conformational change in transferrin, resulting in the release of Fe^{3+} (Klausner et al., 1983). Following a second reduction to Fe^{2+}, iron is transported by Dmt1 across the endosomal membrane to the cytosol and the Tf-TfR1 complex is recycled back to the plasma membrane, where Tf is released into the circulation. Iron in the cytosol is believed to enter the labile iron pool (LIP) where it provides iron for metabolic processes and proliferation. Cellular iron which is not immediately required for metabolic processes or enzymatic reactions, is sequestered in the cytosol within ferritin for storage (Gkouvatsos et al., 2012). Iron uptake, transport and storage are tightly regulated, which is essential as excess of iron is toxic due to its ability to catalyze the formation of free radicals via the Fenton reaction leading to cellular damage. Iron homeostasis is regulated by the iron response element (IRE)/iron regulatory protein (IRP) system. Most proteins involved in iron homeostasis are encoded by mRNAs containing one or more IREs in their untranslated regions

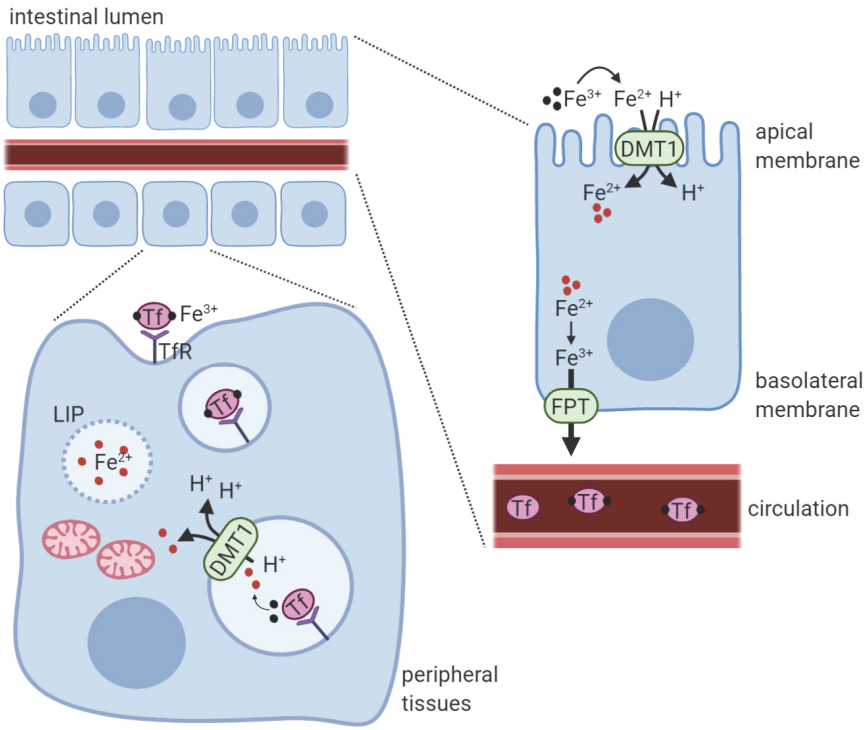


Figure 4. Iron homeostasis. *Iron is absorbed in the intestine via transport across the apical membrane by Divalent metal transporter 1 (DMT1). Ferroportin (FPT) exports iron from the basolateral membrane of the enterocyte into the circulation. In the blood iron is bound to transferrin (Tf), which transports iron to adjacent cells via transferrin receptor (TfR)-mediated endocytosis. Iron is released into the cytosol by DMT1. LIP: labile iron pool.*

(UTRs). These IREs are binding sites for iron regulatory proteins (IRP1 and IRP2), which sense iron levels in the labile iron pool and are activated in iron starved cells (Eisenstein, 2000; Rouault, 2006).

Iron and cancer

Although iron is essential for proper functioning of various processes in the cell, including proliferation and growth, iron can also have detrimental effects on cellular homeostasis due to its ability of forming free radicals, which may result in oxidative stress, inflammation, and DNA damage. Therefore, it is not surprising that iron

homeostasis is often deregulated in malignant cancers (Kwok and Richardson, 2002; Steegmann-Olmedillas, 2011). Iron deficiency and iron deficiency-related anemia are reported in 40-70% of all cancer patients (Ludwig et al., 2015), which result from the excessive need of iron by tumor cells. Mouse models for mammary carcinoma show loss of iron storage in the liver and spleen due to accumulation of iron in tumor and stromal cells, especially in invasive, angiogenic, and necrotic regions (Freitas et al., 2007). Mechanistically, tumor cells do not only require iron to sustain their proliferative mode (Kwok and Richardson, 2002), but also use iron to remodel the extracellular matrix and increase their motility to promote invasion and metastasis (Fischer-Fodor et al., 2015). In contrast, an iron-deficient diet prior to breast cancer cell implanting in mice resulted in increased tumor volumes and metastasis due to increased Notch target gene expression leading to the activation of epithelial-to-mesenchymal transition, which was reversed by iron therapy (Jian et al., 2013). However, only the expression of Notch2, Notch3, and Notch4 receptors was assessed and elevated. Changes in Notch1 receptor expression upon iron-deficient diet were not reported.

To sustain their increased iron demand, tumor cells adjust their intracellular iron metabolism by regulating the expression of iron regulatory genes to increase iron uptake and storage and decrease iron export. Increased expression of TfR1 has been reported in several cancers (Basuli et al., 2017; Högemann-Savellano et al., 2003). Elevated expression levels of the TfR1 lead to increased uptake of transferrin bound iron (Habeshaw et al., 1983; Prutki et al., 2006; Shindelman et al., 1981). As a result, TfR1 expression is correlated with poor patient prognosis (Greene et al., 2017; Habashy et al., 2010). Increased iron levels have also been reported in colorectal cancers with concomitant overexpression of Dmt1 and TfR1 (Brookes et al., 2006). In addition, tumor cells also block iron efflux by ferroportin by up-regulation of hepcidin, which increases the degradation of ferroportin. Hepcidin expression has been reported to be elevated in a broad range of human malignancies, including prostate cancer (Tesfay et al., 2015), lung cancer (Chen et al., 2014), breast cancer (Ciniselli et al., 2015), multiple myeloma (Maes et al., 2010), and renal cell carcinoma (Kamai et al., 2009). The increased levels of hepcidin result in increased degradation

of ferroportin, which is reported in breast (Pinnix et al., 2010), prostate (Tesfay et al., 2015), and ovarian cancer (Basuli et al., 2017), leading to decreased iron export in tumor cells compared to normal tissues, which correlates with poor patient outcome (Pinnix et al., 2010).

Since iron metabolism is deregulated in many cancers, several iron-targeted cancer therapies have been proposed. Iron chelators have been extensively studied as novel cancer therapeutics. In clinical trials iron chelators showed mild to moderate adverse effects, including gastrointestinal symptoms and fatigue (Cohen et al., 2000; Gattermann et al., 2010; Knox et al., 2007; Minden et al., 2014; Neufeld et al., 2012; Nutting et al., 2009; Yamasaki et al., 2011). Iron chelators showed promising anti-leukemic effects in advanced leukemia with 70% of patients showing a 50% reduction in white blood cell counts by triapine treatment (Giles et al., 2003). Additional iron chelators also showed improved patient outcome (List et al., 2012; Minden et al., 2014). However, solid tumors including metastatic renal cell carcinoma and head and neck tumors showed low anti-tumor efficacy in patients (Knox et al., 2007; Nutting et al., 2009). Although partial responses were reported in hepatocellular carcinoma (Yamasaki et al., 2011) and prostate cancer (Dreicer et al., 1997). Moreover, iron chelators have been combined with chemotherapy to increase therapeutic responses. In neuroblastoma and acute myeloid leukemia combining iron chelators with chemotherapy showed promising results (Donfrancesco et al., 1993; Zeidner et al., 2014), although this combination was ineffective in advanced non-small cell lung cancer (Traynor et al., 2010). Lastly, inhibition of hepcidin expression by neutralizing antibodies or inhibition of inflammatory pathways and ferroportin stabilizers to reactivate iron efflux in tumor cells are also being tested as a therapeutic approach (Gardenghi et al., 2010; Jayatilaka et al., 2017). Altogether, iron-targeting in cancer may be a promising therapeutic approach, however, anti-tumor efficacy may depend on the level of iron-addition of the tumor and proper patient selection might be required to increase the therapeutic efficacy.

Notch signaling affecting the hallmarks of cancer

Notch signaling is a cell-cell communication pathway, which can promote or suppress cell proliferation, cell death, and induce specific cell fates or differentiation processes during development and self-renewing of adult tissues. As Notch signaling controls cell proliferation and differentiation, it is not surprising that deregulation of Notch signaling, which results in aberrant cell growth, is found in a broad range of malignancies. Notch signaling can affect all hallmarks of cancer depending on the tissue type, acting as a tumor oncogene of suppressor (Aster et al., 2017). First of all, Notch signaling sustains proliferation of tumor cells by activating c-Myc, which stimulates cell cycle progression and proliferation and inhibits cell death (Palomero et al., 2006). Moreover, Notch signaling activates other pro-growth signaling pathways, including PI3K-Akt signaling by repression of Pten (Palomero et al., 2007). Akt signaling also activates MDM2, which results in TP53 degradation and prevents the activation of the DNA damage response and a cell cycle arrest upon DNA damage further sustaining tumor growth and enhancing genomic instability (Gottlieb et al., 2002). In addition to sustaining tumor growth, Akt signaling also promotes tumor cell survival by increasing the expression of NF-κβ and inhibition of cell death (Manning and Cantley, 2007). Moreover, stromal cells expressing Notch ligands in the microenvironment may support proliferation of tumor cells in the metastatic niche by activation of Notch signaling.

In addition to sustaining proliferation, survival, and inhibition of tumor cell death, Notch signaling has also been reported to promote epithelial-to-mesenchymal transition, by inducing the expression of TGF-β and Snail (Sahlgren et al., 2008; Timmerman et al., 2004), promoting tumor invasion and metastasis. Moreover, Notch signaling has an essential role in angiogenesis and vessel maintenance during development (Gridley, 2007) and suppression of Notch signaling by targeting Dll4 has been shown to result in excessive, non-functional tumor angiogenesis, reducing tumor growth (Kuhnert et al., 2011). Furthermore, accumulating evidence suggests that Notch signaling maintains cancer cells in the stem cell niche in a broad range of malignancies, including glioblastoma, ovarian cancer, and breast cancer. Notch inhibition in these malignancies results in decreased expression of stem cell markers,

reduces tumor growth, and induces treatment sensitivity (D'Angelo et al., 2015; Fan et al., 2010; McAuliffe et al., 2012; Yahyanejad et al., 2016; Zhu et al., 2011).

Notch signaling in human malignancies

Aberrant Notch signaling has been found in a broad range of human malignancies. First of all, T-cell acute lymphoblastic leukemia (T-ALL) is well-known for harboring mutations in NOTCH1 leading to ligand-independent NOTCH1 signaling (Weng et al., 2004). Approximately 60% of all human T-ALL have activating mutations in the heterodimerization (HD) and PEST domain of *NOTCH1.* Due to the mutations in the HD domain, the Notch receptor undergoes conformational changes, which unmask the S2-cleavage site making it accessible for cleavage in the absence of ligand binding. HD domain mutations are often combined with PEST domain mutations in the *NOTCH* receptor, which prevent the rapid degradation of the active NICD, prolonging the constitutive ligand-independent Notch activity. Despite *NOTCH1* mutations in T-ALL, inactivating mutations or deletions in the F-box and WD repeat domain-containing (*FBW7*) gene have also been reported in a selected group of T-ALL patients. The *FBW7* gene encodes for a ubiquitin ligase, which regulates the degradation of NICD (Malecki et al., 2006; O'Neil et al., 2007; Thompson et al., 2007). Therefore, the loss of FBW7 results in the expression of a constitutively active NICD independently of γ-secretase activity in T-ALL. Aberrant Notch signaling has also been reported in other types of leukemia, where Notch can act as an oncogene or tumor suppressor depending on the type of malignancy (Aster et al., 2017).

Secondly, deregulation of *NOTCH1* and *NOTCH2* due to genomic rearrangements has also been reported in breast cancer, from which the majority was derived from triple-negative breast cancers (Robinson et al., 2011). These rearrangements in *NOTCH1* result in a truncated membrane-tethered NOTCH1 receptor, which remains dependent on γ-secretase activity, however, acts independently of ligand binding and S2-cleavage. In *NOTCH2* the rearrangements result in a truncated cytoplasmic NICD, which acts independently of γ–secretase cleavage. Besides, approximately 10% of all non-small cell lung cancers (NSCLC)

also harbor gain-of-function mutations in *NOTCH1*, which also localize to the HD and PEST domain (Westhoff et al., 2009).

Lastly, Notch signaling is deregulated in a broad range of human malignancies, although mutations or rearrangements in *NOTCH* are only found in T-ALL and a selective group of breast and lung cancer patients. In the majority of cancers, NOTCH deregulation is not due to genetic alterations in *NOTCH* itself, but results from mutations or deregulations in the negative regulators of NOTCH signaling. For example, in approximately 50% of all human breast cancers and in 30% of all human NSCLC NUMB signaling is lost (Pece et al., 2004; Westhoff et al., 2009). Loss of NUMB, a negative regulator of Notch signaling and tumor suppressor, results in NOTCH signaling activation.

Therapeutic targeting Notch signaling in cancer

As a consequence of the high frequency of aberrant NOTCH signaling in human malignancies, γ-secretase inhibitors (GSI) have been used as small molecule inhibitors to target the NOTCH1 signaling pathway. Unfortunately, GSI showed limited anti-leukemic activity as a result of dose-limiting toxicities in normal tissues as shown in animal models (Milano et al., 2004; Wong et al., 2004). One of the main dose-limiting toxicities induced by GSI is gastrointestinal toxicity, which results from severe goblet cell metaplasia in the intestine leading to diarrhea, nausea, mucositis, and dehydration. This excessive secretory differentiation results from the simultaneous inhibition of Notch1 and Notch2, which function redundantly in regulating the proliferation and differentiation in the intestinal crypt (Milano et al., 2004; Riccio et al., 2008; van Es et al., 2005; Wong et al., 2004). In addition to intestinal toxicity, adverse effects of the inhibition of Notch signaling have also been reported in the skin, causing moderate dermatitis, cell hyperplasia, and inclusion cysts in preclinical studies, due to Notch-mediated keratinocyte differentiation by Notch signaling (Rangarajan et al., 2001). Although Notch inhibition showed adverse side effects on the immune system in preclinical trials (Wong et al., 2004), no severe toxicities are reported in patients. Lastly, inhibition of Notch signaling can also lead to vascular abnormalities and induce vascular tumors (Yan et al., 2010). Indeed, in

patients, NOTCH inhibition also showed vascular toxicities accounting for cardiac arrest, tachycardia, ventricular dysfunction, and congestive heart failure (Chiorean et al., 2015; Smith et al., 2014).

A lack of predictive biomarkers for patient selection, a shortage of robust pharmacological markers to measure on target activity (Krop et al., 2012), and a lack of specificity of current inhibitors targeting multiple Notch family members lead to low anti-tumor activity and toxicity in normal tissues. Therefore, these current challenges need to be solved to be able to use oncogenic NOTCH signaling as a therapeutic target and improve patient outcome. Importantly, chronic uninterrupted GSI treatment results in high toxicity, while intermittent scheduling remarkably reduces dose-limiting toxicities and shows strong modulation of the NOTCH signaling pathway resulting in anti-tumor activity in phase I clinical trials (Krop et al., 2012; Tolcher et al., 2012).

Additional Notch targeting strategies have been developed, including the use of monoclonal antibodies against Notch ligands and receptors. Monoclonal antibodies against Dll4, which regulates tumor initiation and angiogenesis (Fischer et al., 2011; Noguera-Troise et al., 2006; Ridgway et al., 2006), were well tolerated and showed anti-tumor activity in phase I clinical trials (Chiorean et al., 2015; Smith et al., 2014). Besides, the development of a Notch1 receptor specific monoclonal antibody showed promising preclinical results, including inhibition of tumor growth, deregulation of angiogenesis, and reduced goblet cell metaplasia compared to pan-Notch inhibition (Wu et al., 2010). Additional monoclonal antibodies that specifically target activated Notch receptors have also shown promising preclinical results, however, phase II clinical studies are awaited (Tiyanont et al., 2013). Tarextumab, a monoclonal antibody targeting both Notch2 and Notch3, showed inhibition of NOTCH signaling in a phase I trial and was well tolerated using intermittent scheduling (Smith et al., 2019), although combined with chemotherapy failed to improve patient outcome in pancreatic adenocarcinoma (Hu et al., 2019).

Altogether, over the last two decades targeting of NOTCH signaling as a therapeutic approach in human malignancies has evolved from a bulk inhibition with

severe side effects and dose-limiting toxicity to more specific and better tolerable strategies.

Outline of this thesis

Although extensive progress has been made over the last two decades in the therapeutic targeting of NOTCH signaling, it still remains essential to obtain a better understanding in the molecular mechanisms of NOTCH signaling and its key regulators. Therefore, the aim of this thesis was to extend our knowledge on NOTCH signaling, especially in the differences between NOTCH signaling in cancer and in normal tissues. These differences could be exploited allowing to specifically target oncogenic NOTCH signaling without affecting physiologic NOTCH signaling in adult tissues, leading to enhanced tumor control, reduced toxicity, and improved patient outcome. In this thesis we used two different approaches to address this aim. First, we used chloroquine, an anti-malarial drug which is cheap and easy to produce, as a repurposed drug in the treatment of NOTCH-driven T-ALL in combination with GSI. Secondly, we performed an shRNA silencing screen to discover novel regulators of Notch signaling and potential targets to broaden our therapeutic landscape for Notch inhibition.

In **chapter II** we review the current knowledge on intracellular Notch receptor trafficking and activation in both *Drosophila* and mammals. We focus on ligand-independent Notch signaling in human malignancies, elaborate on the current (proposed) mechanisms of ligand-independent Notch signaling, and discuss the essential role of intracellular trafficking. Finally, we discuss the essence of specific targeting of ligand-independent Notch signaling in cancer and its current challenges.

In **chapter III** we show that the anti-malarial drug chloroquine (CQ), an inhibitor of endosomal and lysosomal function, sensitizes oncogenic NOTCH1 driven human T-ALL to γ-secretase inhibition (GSI). Chloroquine decreases human T-ALL cell viability and proliferation, which is even further enhanced when combined with GSI. Mechanistically, chloroquine impairs the redox balance, inducing DNA damage and activating the DNA damage response leading to a block in cell cycle progression. Besides, we show that chloroquine interferes with ligand-independent NOTCH

signaling by deregulating NOTCH receptor trafficking and processing. We show that chloroquine treatment results in enhanced protein levels of full length and cleaved NOTCH receptors and leads to the accumulation of the NOTCH receptors in intracellular vesicles, especially when combined with GSI. These effects of chloroquine were not observed in GSI-resistant T-ALL cells.

In **chapter IV** we performed a shRNA screen in Notch Adam protease-deficient mouse embryonic fibroblasts to identify novel rate-limiting steps in the Notch1 signaling cascade. We identified Divalent metal transporter 1 (Dmt1) as a novel regulator of Notch-mediated cell fate decisions in muscle, neural, and colorectal cells. We report that the four isoforms of Dmt1 differentially regulate Notch signaling processing and activation. Inhibition of Dmt1b-ire isoforms results in a loss-of-function of Notch signaling, while knockdown of Dmt1b+ire isoforms results in an activation of Notch signaling. Moreover, we show that a complete loss of Dmt1 isoforms results in attenuated ligand-induced Notch signaling, which may result from disturbed intracellular trafficking.

To conclude, we summarize our findings of the previous chapters in **chapter V** and outline the remaining challenges to be addressed in future research.

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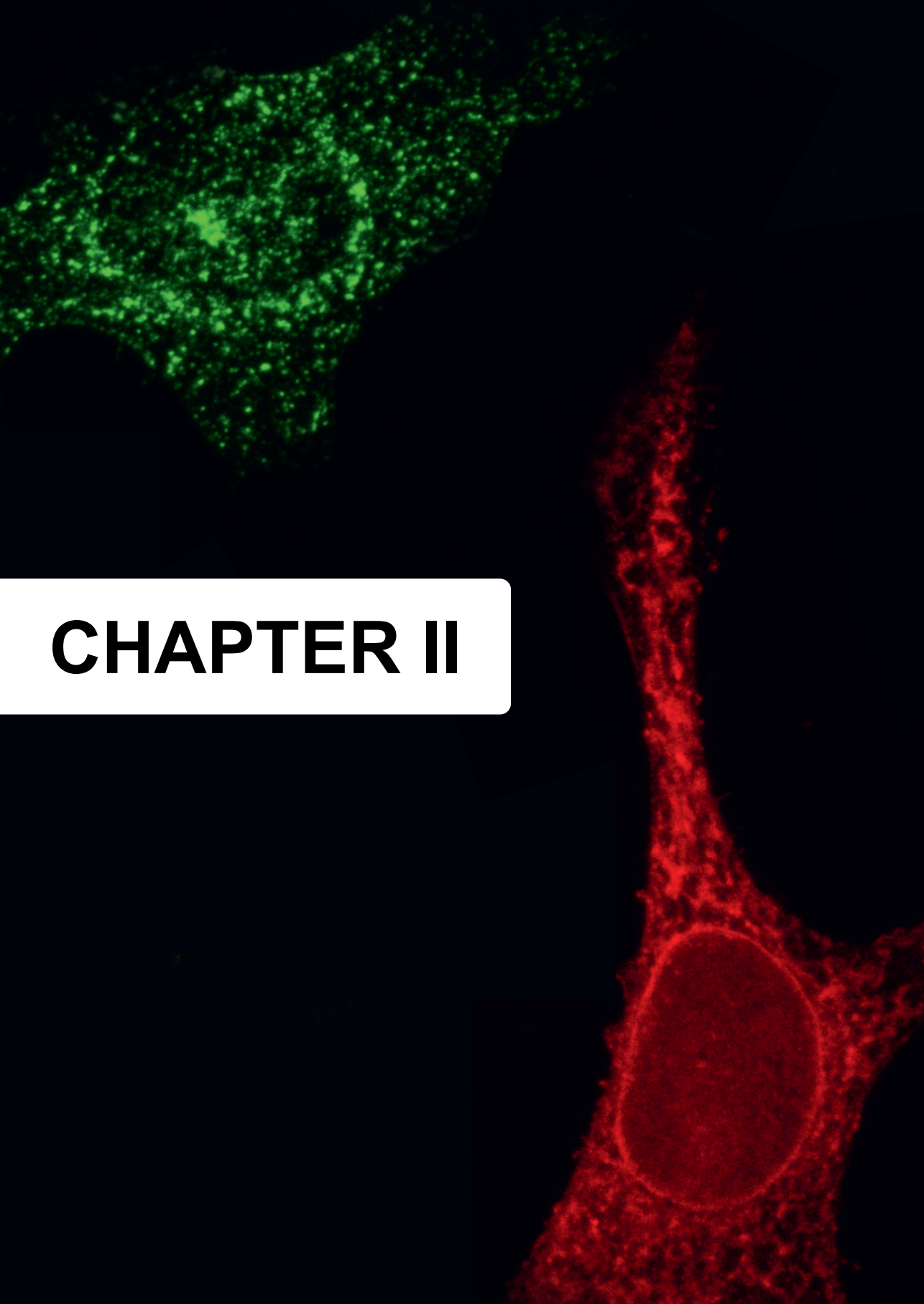
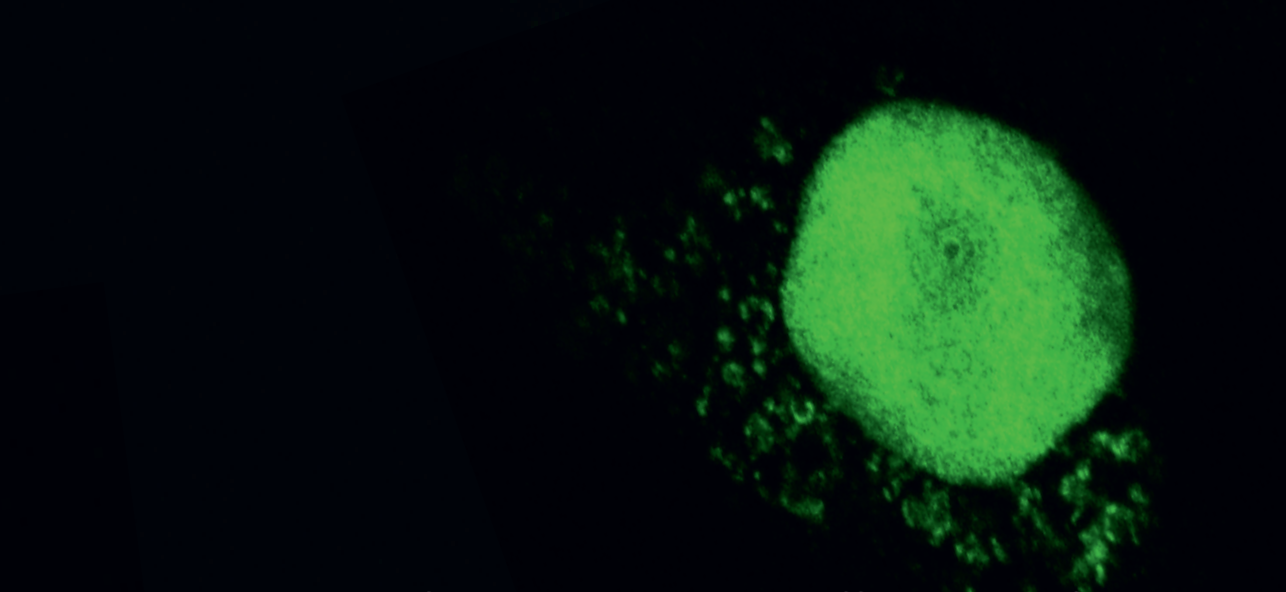


CHAPTER II



The role of intracellular trafficking of Notch receptors in Notch signaling activation (review)

Judith Hounjet and Marc Vooijs[#]

Department of Radiation Oncology (Maastro), GROW School for Oncology, Maastricht University Medical Centre+, Maastricht, The Netherlands

Maastro clinic, Maastricht, The Netherlands

[#]Corresponding author: marc.vooijs@maastrichtuniversity.nl, Tel: +31 (0)433882912

In preparation

Abstract

Notch signaling is a highly conserved signaling pathway involved in proliferation and differentiation during embryonic development and adult homeostasis. Canonical Notch signaling involving ligand-activation, Adam metalloprotease and γ-secretase cleavage, and the role of endosomal trafficking is well described in *Drosophila*. However, the mechanisms of ligand-independent Notch signaling and the role of endosomal trafficking in mammals are still elusive. In this review, we discuss ligand-independent Notch signaling in mammals in both physiological and pathological conditions with cancer prone gain-of-function alleles. In addition, we focus on the proposed mechanisms involved in ligand-independent Notch signaling, including the role of intracellular trafficking, and the current challenges of therapeutic targeting of Notch signaling.

The endocytosis-independent NOTCH signaling pathway

NOTCH receptor signaling is governed by three successive proteolytic cleavages (S1-S3) (**Figure 1**). In the Golgi apparatus, the Notch receptor is cleaved at the S1-site by a furin-like protease creating a non-covalently associated NOTCH heterodimer composed of the NOTCH extracellular domain, the transmembrane, and intracellular domain (TMIC), which is transported towards the plasma membrane, where it awaits ligand binding (Kopan and Ilagan, 2009). In the absence of ligand, the extracellular S2-cleavage site is masked by the negative regulatory region (NRR) composed of the heterodimerization (HD) domain and Lin12-NOTCH repeats (LNR), leading to the auto-inhibition of the Notch receptor (Gordon et al., 2009a). Upon ligand binding, the NRR unfolds and reveals the S2-site, which is subsequently cleaved by the metalloprotease Adam10 resulting in a membrane-tethered truncated Notch receptor (NEXT)(Mumm et al., 2000; van Tetering et al., 2009). To complete the Notch signal transduction route, the Notch receptor is cleaved at Val1744 (S3) in the Notch transmembrane domain by the intra-membrane γ-secretase complex, releasing the Notch-intracellular domain (NICD). The NICD is transported towards the nucleus where it associates with a transcriptional complex containing the RBP-jκ/CSL DNA binding protein to activate its downstream targets (Kopan and Ilagan,

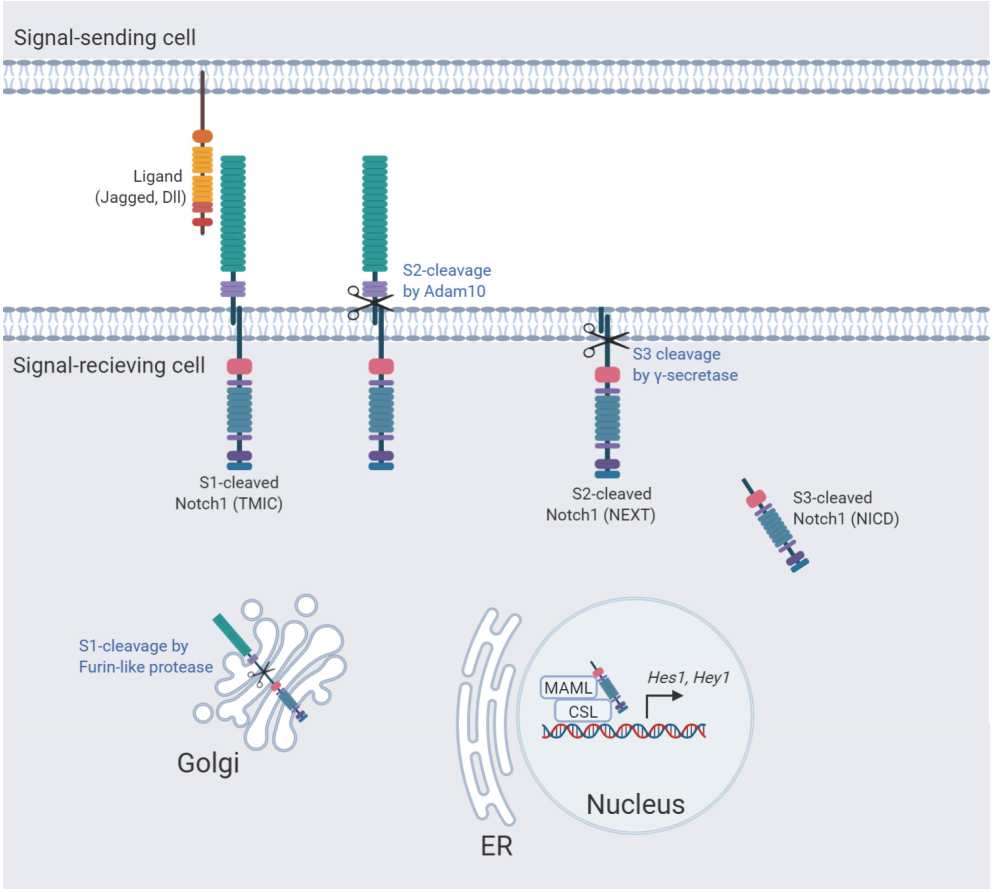


Figure 1. Endocytosis-independent Notch signaling pathway. *Notch signaling requires ligand binding, expressed by a signal-sending cell and three sequential cleavages to release the active form of Notch, which activates Notch downstream target genes. TMIC: transmembrane intracellular fragment, NEXT: Notch extracellular truncation, NICD: Notch intracellular domain.*

2009). For many years this was thought to be the predominant route of Notch activation, however, evidence of intracellular trafficking regulating Notch receptor activation at several steps of the signal transduction route is increasing.

Intracellular trafficking of transmembrane proteins

One of the first demonstrations of receptor-mediated endocytosis was the degradation of the low density lipoprotein (LDL) (Goldstein and Brown, 1974). LDL

was shown to be degraded by a mechanism of clustering at the cell surface, binding to surface protein clathrin, and subsequently pinching an internalization of vesicles targeted for degradation. Clathrin-mediated endocytosis starts with the adaptor protein 2 (AP2) complex, which recruits clathrin to the plasma membrane initiating the formation of a clathrin-coated pit (Cocucci et al., 2012; Kirchhausen et al., 2014). The AP2 complex and additional clathrin-associated sorting proteins act as adaptor proteins and regulate cargo recognition and sorting (McMahon and Boucrot, 2011; Traub and Bonifacino, 2013). Next, the clathrin-coated pit grows and matures into a clathrin-coated vesicle, also called early endocytic vesicle (EEV) (**Figure 2**). Subsequently, the EEV is pinched of by the GTPase dynamin, which releases the clathrin-coated vesicle into the cytosol (Schmid and Frolov, 2011; Traub and Bonifacino, 2013). Next, the released clathrin-coated vesicle is uncoated by the ATPase heat shock cognate 70 (HSC70) and auxilin (Rothman and Schmid, 1986; Ungewickell et al., 1995) and the EEV can fuse with other EEVs or early endosomes (EEs).

However, clathrin-independent mechanisms of endocytosis have also been reported. One clathrin-independent pathway is caveolae-mediated endocytosis, which is involved in transcytosis across endothelia, mechano-sensing, and lipid regulation (Parton and Simons, 2007). Caveolae are invaginations in the plasma-membrane, which are present on the majority of eukaryotic cells and are also called lipid rafts (Pelkmans and Helenius, 2002). These membrane domains are enriched in cholesterol and sphingolipids (Harder and Simons, 1997; Murata et al., 1995). Caveolae-mediated endocytosis is initiated by ligand-binding to cargo receptors (Parton and Simons, 2007), however, the exact process of endocytosis remains largely unclear. Budding of caveolae-coated vesicles is regulated by kinases and phosphatases, although the exact regulators remain to be determined (Kiss, 2012). Like clathrin-mediated endocytosis, caveolae-coated vesicles are also pinched off by dynamin (Henley et al., 1998). Additional clathrin-independent mechanisms of endocytosis have been reported, involving: endophilin, RhoA, Rac1, Cdc42, Arf6, and flotillins (Elkin et al., 2016). To date, it remains unclear whether these endocytic

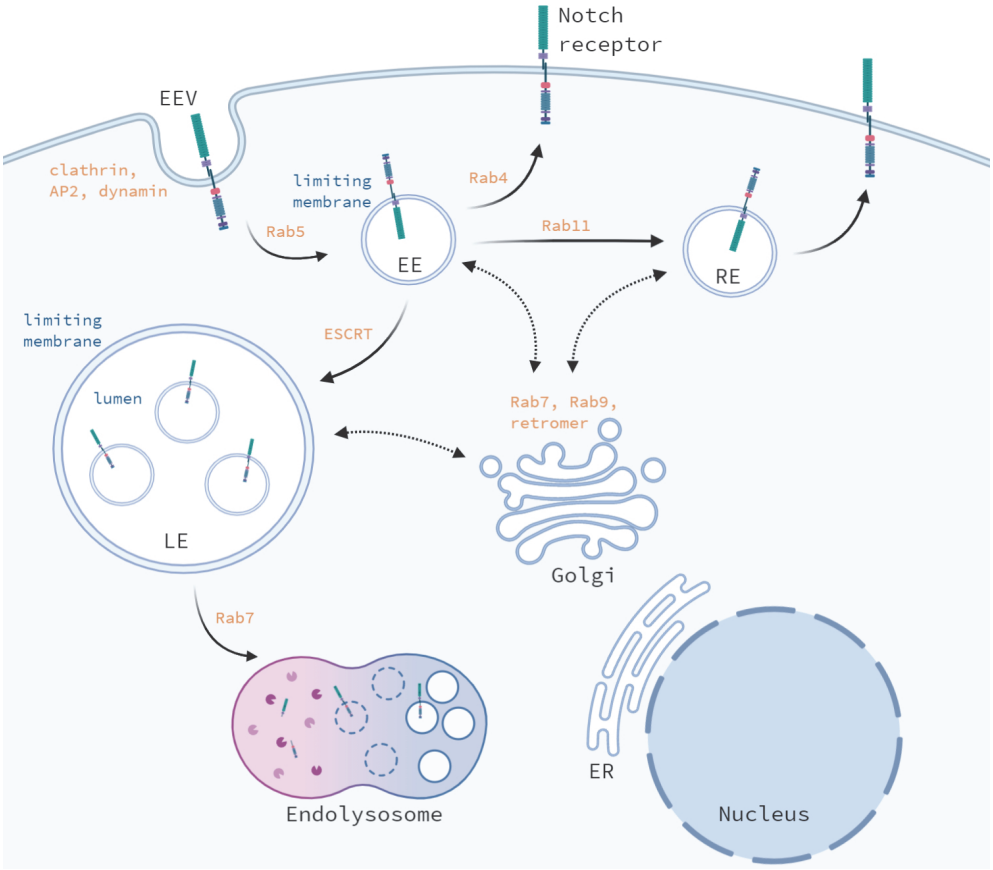


Figure 2. Endocytosis-dependent Notch signaling pathway. *The Notch receptor is continuously internalized. While the majority of internalized Notch receptors are directly transported back to the plasma membrane, the minority of internalized Notch receptors are further transported towards the endocytic compartments and either recycled to the plasma membrane via recycling endosomes or degraded in the lysosomes. EEV: early endocytic vesicle, EE: early endosomes, RE: recycling endosomes, LE: late endosomes.*

pathways are mechanistically distinct and future research has to prove to which extent they contribute to the endocytic capacity of cells.

Subsequent intracellular trafficking of vesicles is regulated by Rab proteins, which are a large family of small GTPases. Early endosomes are weakly acidic (pH 6.8-5.9) (Maxfield and Yamashiro, 1987) and function as the main sorting station, in which cargo is retained, sorted, or accumulated by the regulation of Rab5, before

fusing with late endosomes (Maxfield and McGraw, 2004). During endosome maturation cargo sorting and the formation of intraluminal vesicles is regulated by ESCRT complexes. Moreover, intracellular vesicles expressing endosomal and lysosomal proteins, are continuously exchanged between endosomes and the trans-Golgi system, which is regulated by Rab7, Rab9, and the retromer complex during endosome maturation (Bonifacino and Rojas, 2006; Pfeffer, 2009). Furthermore, Rab5 recruits Rab7 leading to the formation of Rab5/Rab7 expressing endosomes from which Rab5 is subsequently removed during the “Rab switch”. This Rab switch enables the proper association and dissociation of adaptor proteins regulating the endocytic pathway, in which Rab7 is the major regulator in late endosomes and lysosomes. Mature late endosomes (LEs) exist of a limiting membrane expressing LAMP1 and intraluminal vesicles and acid hydrolases in the lumen. Late endosomes travel from a cytosolic region to a perinuclear region, where they fuse with other late endosomes, lysosomes, or endo-lysosomes for cargo degradation (Huotari and Helenius, 2011; Luzio et al., 2007).

However, early endosomes can also directly recycle back to the plasma membrane via Rab4, or can first fuse with a recycling endosome and subsequently fuse with the plasma membrane, a process requiring Rab11 (Grant and Donaldson, 2009). Recycling of unbound Notch ligands and endocytosis of Notch ligands bound to the extracellular domain of the Notch receptor play a critical role in Notch receptor processing and activation (Meloty-Kapella et al., 2012; Parks et al., 2000). Ligand-endocytosis may play a role in both endocytosis-dependent and independent Notch receptor signaling, if ligand and receptor are transported on vesicles in close proximity. In this review we focus on the role of endosomal trafficking of the Notch receptor instead of its ligands.

Endosomal trafficking of the Notch receptor; a comparison between *Drosophila* and mammals

In *Drosophila,* several endocytic regulators tightly control the intracellular trafficking of the Notch receptor and interruption of the different steps of intracellular trafficking have different effects on both ligand-dependent and independent Notch signaling

activation. Although Notch signaling is a highly conserved pathway, there are significant differences between Notch signaling in *Drosophila* and mammals. First, the *Drosophila* genome encodes only one Notch receptor, while mammals express four distinct Notch receptors. In addition, unlike mammalian Notch receptors, the majority of Notch receptors at the cell surface are full length receptors in *Drosophila,* which do not require furin-cleavage for their activation (Kidd and Lieber, 2002). In mammals, loss of furin-cleavage of Notch1 receptors results in decreased surface expression and ligand-induced Notch activity, while loss of furin-cleavage of Notch2 receptors does not affect surface expression or activity of Notch2 receptors (Gordon et al., 2009b). Despite these differences in Notch signaling between *Drosophila* and mammals, is the intracellular trafficking and regulation of the Notch receptor conserved between these species? While intracellular trafficking of the Notch receptor is well studied in *Drosophila,* the endocytic regulation of Notch signaling in mammals is less clear. In mammals, endocytic regulation is more complex because most regulators known in *Drosophila* have multiple orthologs in mammals. Moreover, many of these regulators are essential for maintaining homeostasis and therefore loss-of-function often leads to lethality and cannot be studied. An overview of the key regulators involved in endosomal and lysosomal trafficking and the effect on Notch signaling upon their loss-of-function is summarized (**Table 1**).

Internalization of the Notch receptor

The Notch receptor is continuously internalized by clathrin- and dynamin-dependent endocytosis (Chastagner et al., 2008; Jékely and Rørth, 2003; Vaccari et al., 2008; Windler and Bilder, 2010). Although Notch ligands and receptors are predominantly transported by clathrin-mediated endocytosis in mammals, the Notch receptor can also be internalized by clathrin-independent endocytosis (Okamura and Saga, 2008; Vetrivel et al., 2005). In mammals, the majority of γ-secretase complexes are sequestered in lipid rafts, preventing the processing of Notch1 receptors (Vetrivel et al., 2005). However, Notch1 receptors can also be found on lipid rafts expressing caveolin-1 (Okamura and Saga, 2008). Loss of Pofut1, which mediates the O-

Table 1. *Key regulators of intracellular trafficking and their effects on Notch signaling upon loss-of-function in both Drosophila and mammals.*

	Protein	***Function***	***Loss of function Drosophila***	***Loss of function Mammals***
Internalization	Dynamin	Endosomal vesicle formation	↓ Notch signaling, ↑ Notch receptor expression at the plasma membrane	**Dynamin 1** = ↓ NOTCHΔE internalization, no effect on Val1744 levels in the nucleus (HeLa cells), γ-secretase-mediated NEXT cleavage **Dynamin 2** = ↓ γ-secretase cleavage of Notch1-ΔE-TM
	Clathrin	Endosomal vesicle formation	↓ Ligand-dependent Notch signaling	**Clathrin heavy chain** = ↓ NOTCHΔE internalization - no effect on Val1744 levels in the nucleus
	Crumbs	Prevents Notch receptor internalization and activation	↑ Internalization of Notch ↑ Ectopic ligand-independent Notch signaling	
Recycling	Rme-8	Endosomal sorting and recycling	↑ Notch in enlarged Rab4+ endosomes ↓ Notch signaling	
	Numb	Stimulates Notch receptor trafficking towards MVBs (degradation) instead of recycling endosomes	↓ Notch in the late endosomes, ↑ Notch at the plasma membrane ↓ Ubiquitination of NICD, ↑ Notch signaling	~50% of all human breast cancers, ↑ Notch signaling ~30% of all human NSCLC, ↑ Notch signaling
	Lgl	Inhibits Notch signaling by controlling the asymmetric localization of Numb	↑ Notch signaling, ↑ NICD	↑ Notch signaling, ↑ NICD
Early endosomes	Rab5, Avl	Entry into early endosomes	↓ Notch signaling, ↑ Notch receptor expression at the plasma membrane	

	Protein	*Function*	*Loss of function Drosophila*	*Loss of function Mammals*
Early endosomes	Deltex (dx)	E3-ligase, mono-ubiquitination of Notch-ICD Endosomal stabilization and activation of Notch	↓ Notch signaling, ↓ Notch internalization	↑ Notch signaling $DTX1^{-/-}2^{-/-}$ mutant mice = no defects in Notch dependent T-cell development
	Suppressor of Deltex (Su (dx))	E3-ligase, poly-ubiquitination and lysosomal degradation of Notch	↑ Notch signaling	Itch mutant mice = severe autoimmune disease Itch deficiency humans = severe autoimmune disease and morphologic and developmental abnormalities
Late endosomes	ESCRT	Maturation of early endosomes into MVBs	↑ Notch in endosomes ↑ Ligand-independent Notch signaling	**CHMP5** (ESCRT-III) - No activation of Notch signaling **Vps25** (ESCRT-II) - No activation of Notch signaling **Tsg101** (ESCRT-I) - ↑ ligand-independent Notch signaling (*in vitro*), early embryonic lethal (*in vivo*) **BBS1/3/4** - ↑ ligand-independent Notch signaling, accumulation of Notch in late endosomes
	Lgd	Maturation of early endosomes into MVBs	↑ Notch in early endosomes, ↑ Ligand-independent Notch signaling	↑ Endosomal size in intestinal Cc2d1a mutant mice ~ Notch signaling Loss of both orthologs is not validated yet
	Hrs	Component ESCRT-0, recognizes ubiquitinated proteins and facilitates transport from early to late endosome	↑ Notch in early endosomes, ↓ Notch signaling	
Nuclear translocation	Importin α Importin β1	Nuclear import of NICD	↓ Nuclear localization of Notch ↑ Notch in the cytoplasm	↓ Nuclear localization of Notch

fucosylation of the Notch receptor, results in an accumulation of Notch receptors in the ER, but also in intracellular vesicles yet to be identified. Moreover, loss of Pofut1 showed increased caveolin-mediated endocytosis of Notch receptors. Furthermore, inhibition of caveolin-1 results in increased γ-secretase activity, Notch processing, and target gene expression, due to increased co-localization of γ-secretase on clathrin-coated vesicles. In contrast, ectopic expression of caveolin-1 increased the co-localization of γ-secretase (Nicastrin and Presenilin 1) with calveolin-1-coated vesicles (Kapoor et al., 2010). Interestingly, the catalytic domains of the γ-secretase complex; Psen1 and Psen2, also showed a distinct intracellular localization (Sannerud et al., 2016). Although the majority of PSEN1-containing complexes were expressed at the cell surface, PSEN2-expressing complexes were located to intracellular vesicles, which transport route may also be differentially regulated by using either calveolin-1 or clathrin-expressing vesicles for transport. Although caveolin-1 is also expressed in *Drosophila* during development and in adult tissues, including the brain, muscles, and intestine (Zhang et al., 2016), calveolin-1 mediated endocytosis has not been associated with the internalization of Notch receptors. Together these data imply that Notch activation is tightly regulated by the spatial separation of Notch receptors and the γ-secretase complex by caveolin-mediated endocytosis in mammals.

In general, loss of function of regulators of the early endosomal formation and trafficking, including *Dynamin*, *Clathrin*, *Rab5* or *Avl*, lead to an accumulation of the Notch receptor at the plasma membrane and a loss of Notch activation in *Drosophila* (Chapman et al., 2016; Seugnet et al., 1997; Vaccari et al., 2008). Mammals express three orthologs of Dynamin; Dynamin 1, 2 and 3. Loss of Dynamin1 results in decreased Notch1ΔE (truncated membrane-tethered Notch1 without S2-cleavage site) internalization without affecting NICD-Val1744 cleavage and translocation to the nucleus in Hela cells (Sorensen and Conner, 2010). However, Notch target gene activation was not assessed and Dynamin1 is predominantly expressed in the brain and therefore studying its role in cervical cancer cells may be less relevant. In contrast, loss of Dynamin2, which is ubiquitously expressed, prevents S3-cleavage of Notch1ΔE by γ-secretase thereby blocking

Notch1 signaling (Gupta-Rossi et al., 2004). Inhibition of clathrin heavy chain also resulted in decreased Notch1ΔE internalization without affecting Val1744 levels or its nuclear localization (Sorensen and Conner, 2010), however, expression of downstream targets of Notch was not assessed. These data suggest that the endocytosis-dependent Notch pathway is disturbed upon loss of clathrin in mammals, however, activation of endocytosis-independent Notch signaling may mask these affects. Dynamin triple knockout fibroblasts show severe defects in clathrin-mediated endocytosis, however, no analysis on Notch signaling has been reported yet (Park et al., 2013). In mammals, four different Clathrin orthologs are known, including: clathrin light chain a and b and clathrin heavy chain 1 and 2. Therefore, loss of the different orthologs could have different effects on Notch signaling and may compensate partially for loss of these orthologs in a context dependent manner.

An additional key regulator of Notch receptor internalization in both *Drosophila* and mammals is Crumbs. Crumbs is a transmembrane protein, which regulates epithelial polarity and reduces Notch activity by preventing the internalization of Notch receptors, retaining them at the plasma membrane (Nemetschke and Knust, 2016), and reduces γ-secretase cleavage of Notch receptors in *Drosophila* (Herranz et al., 2006). Upon loss of Crumbs in *Drosophila,* internalization of Notch receptors is increased, leading to ligand-independent activation of Notch signaling. In mammals, three orthologs of Crumbs exist, which all decrease Notch signaling activation upon ectopic expression (Ohata et al., 2011). Mechanistically, all three Crumbs orthologs encode EGF-like repeats, which are similar to Notch ligands. These EGF-like repeats of Crumbs bind to the extracellular domain of the Notch receptor at the plasma membrane, preventing its internalization and subsequent activation. Interestingly, this inhibition of Notch signaling was observed when Crumbs was expressed on the same cell as the Notch receptor (cis), while Crumbs expression on the adjacent cell (trans) did not inhibit Notch activity.

Recycling of the Notch receptor

The majority of internalized Notch receptors are directly transported back to the

plasma membrane mediated by Rab4 (**Figure 2**). A fraction of the internalized Notch receptors in early endosomal vesicles fuse with early endosomes via Rab5 or are indirectly transported back to the plasma membrane via Rab11-positive recycling endosomes. The receptor-mediated protein 8 (Rme-8), or DNAJC13 (human ortholog), regulates intracellular trafficking by cargo sorting, transport from the endosomes towards the Golgi network, and receptor recycling in both *Drosophila* and mammals (Fujibayashi et al., 2007; Girard et al., 2005; Shi et al., 2009). In *Drosophila,* depletion of Rme-8 leads to an accumulation of both full length and S3-cleaved Notch receptors in enlarged Rab4+ endosomes. This disturbance in Notch receptor trafficking results in a decreased number of Notch receptors at the plasma membrane and reduced Notch activity (Gomez-Lamarca et al., 2015). However, the effect on Notch receptor trafficking upon loss of DNAJC13 in mammalian cells remains unstudied.

Numb is an endosomal adaptor protein, which regulates the balance between internalized Notch receptors in recycling endosomes and late endosomes. Upon loss of Numb signaling, Notch receptor entry into late endosomes decreases, which leads to an accumulation of Notch receptors at the plasma membrane, due to increased recycling, and decreased degradation, leading to increased Notch signaling activation (Johnson et al., 2016; Luo et al., 2019). Numb regulates the distribution of α-adaptin, which controls Notch receptor-mediated endocytosis and lysosomal degradation (Berdnik et al., 2002). In *Drosophila,* the lethal giant larvae (Lgl) gene encodes a myosin II-binding protein, which acts a tumour suppressor by regulating proliferation and tissue integrity. Lgl acts upstream of Numb and regulates the localization and activation of Numb in daughter cells during cell division, regulating cell fate by local inhibition of Notch signaling in *Drosophila* (Langevin et al., 2005). In mammals, Numb also negatively regulates Notch signaling activation by promoting the ubiquitination of Notch1 receptors at the plasma membrane and their subsequent degradation (McGill and McGlade, 2003). Loss of Numb has been reported in human malignancies, including breast and lung cancer, leading to increased Notch signaling (Pece et al., 2004; Westhoff et al., 2009). In mammals, asymmetric localization of Numb is lost upon loss of Lgl expression, which results in

increased Notch processing and target gene activation (Klezovitch et al., 2004). Moreover, recycling of Notch2 receptors at the cell surface is regulated by the Commd9 and retromer complexes, in which Commd9 prevents lysosomal degradation of Notch2 receptors (Li et al., 2015). Upon loss of Commd9, Notch2 receptors accumulate in Rab5+ endosomes, cell surface expression is decreased, and expression in recycling Rab11+ endosomes is reduced. Moreover, Notch target gene activation is inhibited by disproportional sorting of Notch2 receptors to the lysosomes resulting in increased lysosomal degradation.

Early endosomal trafficking

Once incorporated into early endosomes the Notch receptor is localized on the limiting membrane, in which the Notch intracellular domain (NICD) protrudes towards the cytoplasm (Dobrowolski and De Robertis, 2012; Hori et al., 2011; Schneider et al., 2013; Wilkin et al., 2008)(**Figure 2**). During the maturation of early endosomes into late endosomes, membrane-bound proteins can selectively be packaged into intraluminal vesicles by ESCRT proteins leading to the formation of multivesicular bodies (MVBs). This selection process is regulated by ubiquitination of Notch by *Deltex (Dx)*, *Suppressor of Deltex (Su(dx))* and *Kurtz* (*Krz*) in *Drosophila* (Hori et al., 2011; Shimizu et al., 2014; Wilkin et al., 2004). Mono-ubiquitination of the NICD by *Dx* blocks transport towards MVBs, resulting in the stabilization of Notch receptors in the maturing endosomes, which leads to the activation of Notch signaling. Therefore, in *Drosophila* Dx is a positive regulator of endocytosis-dependent Notch signaling. However, Dx may also inhibit Notch signaling although the exact mechanism remains elusive. Dx-mediated Notch signaling is tissue specific and whether its effects results in inhibition or activation of Notch signaling may depend on its interacting partners (Fuwa et al., 2006). First of all, Dx activates Notch signaling independent of ligand binding and transports the Notch receptor from the cell surface towards the late endosomes, which requires Rab5 expression. Loss of Dx results in a decreased number of Notch receptors locating to the endosomes (Hori et al., 2004). However, Deltex, which binds to the ankyrin repeats present in the intracellular domain of the Notch receptor, can also form a protein complex consisting of Notch, Deltex, and

Kurtz (β-arrestin), which targets the Notch receptor for degradation (Mukherjee et al., 2005).

In mammals, *Dx* has five orthologs, from which Dtx1, Dtx2 and Dtx4 bind the NICD of Notch1 receptor (Chastagner et al., 2017; Kishi et al., 2001). While Dx is a positive regulator of Notch signaling in *Drosophila*, Dtx1 and Dtx2 are negative regulators of ligand-dependent Notch signaling in mammals. All three Deltex homologues (Dtx1, Dtx2, and Dtx4) are expressed by thymocytes and are able to block Notch signaling in mammals (Lehar and Bevan, 2006). Although the exact mechanism of Notch signaling inhibition has not been proven. Whether Deltex may also activate mammalian Notch signaling remains unclear. Notably, mammalian Dtx1 was shown to induce the transcription of Notch target genes by direct binding to the transcriptional co-activator p300 in the nucleus (Yamamoto et al., 2001). Loss of function studies in mammals showed that loss of Dtx1 does not affect T-cell development, which may result from a redundancy between the three Deltex homologues. However, loss of both Dtx1 and Dtx2 in mice showed also no effect on Notch dependent T-cell development, which suggests that Dtx1 and Dtx2 are negative regulators of Notch signaling in T-cells, but are not essential for Notch signaling during T-cell development (Lehar and Bevan, 2006). Loss of both Dtx1 and Dtx2 resulted in an increase in Dtx4 expression, which may have masked the effects on T-cell development, however, additional loss of Dtx4 showed also no significant changes in T-cell development. Dtx4 was shown to be a potent enhancer of ligand-dependent Notch1 signaling by ubiquitination of the NICD of Notch1 upstream of Adam10 (Chastagner et al., 2017) and Triple-DTX mutant mice showed the most potent activation of Notch. Other protein interacting partners may induce differences in Notch activation in *Drosophila* and mammals.

However, poly-ubiquitination of the NICD of the Notch receptor by *Su (dx)* or *Krz* is recognized by ESCRT proteins, resulting in the transport of the Notch receptor into multivesicular bodies, in which the NICD of the Notch receptor no longer protrudes into the cytosol (**Figure 2**). MVBs can either fuse with lysosomes mediated by Rab7, leading to the degradation of the vesicular-bound proteins or fuse with the plasma membrane leading to the secretion of extracellular vesicles called exosomes

(Doherty and McMahon, 2009). While loss of *Dx* results in loss of Notch internalization and signaling (Yamada et al., 2011), inhibition of *Su (dx),* an E3 ligase regulating *Dx* degradation, leads to increased Notch signaling in *Drosophila* (Brennan and Gardner, 2002; Kanwar and Fortini, 2004). Similar effects on Notch signaling were observed upon loss of DNedd4, a second member of the *Drosophila* Nedd4 family (Wilkin et al., 2004). In *Drosophila,* Nedd4 binds and ubiquitinates the intracellular domain of the Notch receptor, which results in Notch receptor trafficking into early endosomes and targets Notch and Deltex for degradation (Sakata et al., 2004). Loss Nedd4 reduces Notch receptor internalization and activates Notch signaling independently of ligand. The mammalian ortholog of *Drosophila Su (dx)*, AIP4/Itch, also poly-ubiquitinates the NICD of Notch1 in the absence of ligand by indirect binding, leading to its lysosomal degradation (Chastagner et al., 2008). This indirect binding was not enhanced by Dtx or Numb overexpression, although Numb and Itch interact with each other to promote the ubiquitination of Notch1 receptors localized at the plasma membrane (McGill and McGlade, 2003). Therefore, other factors or post-translational modifications might facilitate the interaction between Notch and Itch. AIP4/Itch also partially co-localizes with Dx in endocytic vesicles and poly-ubiquitination of Itch by Dx targets it for lysosomal degradation (Chastagner et al., 2006). However, loss of Itch showed no ectopic activation of the Notch receptor (Chastagner et al., 2008), although increased Notch signaling was reported in *Itch*$^{-/-}$ mice (Matesic et al., 2006), which might be due to the loss of the negative regulation of Notch signaling.

Late endosomal and lysosomal trafficking

In contrast, inhibition of the maturation of early endosomes into MVBs, for example by loss of *ESCRT* or *lethal giant discs (lgd)* expression, results in an accumulation of the Notch receptor in the endosomes, leading to ligand-independent activation of Notch in *Drosophila* (Childress et al., 2006; Vaccari et al., 2008). *Lgd* is a tumor suppressor gene in *Drosophila* and a member of an uncharacterized protein family. In *Drosophila, lgd* mutant cells have enlarged mature endosomes, show decreased degradation of transmembrane proteins, and activation of endocytosis-dependent

Notch signaling (Schneider et al., 2013). This ectopic activation of Notch signaling requires both γ-secretase and V-ATPase activity and localizes to the lysosomes. Schneider et al suggest that the endocytosis-dependent activation of Notch also requires the fusion of mature endosomes with lysosomes since Rab7 depletion reduces the ectopic Notch activation in Lgd mutant cells. In contrast, Rab7 depletion upon loss of ESCRT protein expression has no effect on endocytosis-dependent Notch signaling, indicating that *ESCRT* and *Lgd* mutants activate endocytosis-dependent Notch signaling by different mechanisms.

A similar regulation of endocytosis-dependent Notch signaling by *ESCRT or Lgd* has not been observed in mammals. *Drosophila* Lgd has two orthologs in mammals: Cc2d1a and Cc2d1b. Loss of Cc2d1a, but not Cc2d1b, in the mouse intestine leads to weak endosomal defects, resulting in an increased size of endosomes (Drusenheimer et al., 2015). However, no effect on Notch signaling was observed on secretory differentiation (i.e. goblet cell number) or target gene expression. Nevertheless, it is important to note that only intestinal Notch signaling was investigated, which may differ in other organs and loss of both orthologs of Lgd was not analyzed. Several loss-of-function studies on ESCRT proteins were performed. While loss of CHMP5 (required for ESCRT-III activity) and Vps25 (component of ESCRT-II) did not activate Notch signaling, loss of Tsg101 (component of ESCRT-I) results in ligand-independent activation of Notch *in vitro* (Leitch et al., 2014). However, ligand-independent activation of Notch upon loss of Tsg-101 *in vivo* could not be studied due to early embryonic lethality, which might be a Notch phenotype. Moreover, loss of basal body proteins (BBS1, BBS3, and BBS4), which all interact with Tsg101, leads to increased endocytosis-dependent Notch signaling due to decreased Notch receptor expression at the cell surface and increased localization in the late endosomes (Leitch et al., 2014). This disruption of intracellular trafficking of the Notch receptor results in increased Notch signaling and decreased Notch receptor recycling and degradation in the lysosomes.

Ligand-independent activation of Notch signaling upon disruption of endosomal and lysosomal trafficking is localized to the lysosomes, were Notch receptors are not targeted to the lysosomal lumen for degradation, but are localized

to the limiting membrane of the lysosomes (Wilkin et al., 2008). Since γ-secretase complexes are also expressed on the lysosomes, the Notch receptor is cleaved in the lysosomes, which releases the NICD into the cytoplasm, which results in Notch target gene activation. A similar phenomena has been observed in mammals and a highly conserved dileucine sorting motif in the Notch receptor was identified. This sorting motif targets the Notch receptor from the endosomes to the limiting membrane of the lysosomes and prevents Notch receptor degradation in the lysosomal lumen (Zheng et al., 2013).

However, interference with a later step of endocytic trafficking, including the acidification of endosomes and lysosomes by the loss of *V-ATPase* or *Big brain* expression, results in an accumulation of Notch in enlarged late endosomes and lysosomes and a decrease in both endogenous and ectopic Notch signaling (Fortini and Bilder, 2009; Kobia et al., 2014; Vaccari et al., 2010; Yan et al., 2009). Although Lgl negatively regulates Notch activation by regulating the localization and activity of Numb, Lgl also inhibits endocytosis-dependent activation of Notch signaling by reducing endosomal vesicle acidification. Mechanistically, Lgl interacts with Vap33, which binds and inhibits V-ATPase activity, reducing endocytosis-dependent Notch activity (Portela et al., 2018). In line with these findings, loss of *Lgl* expression showed increased Notch activity, due to decreased endosomal pH and increased γ-secretase activity (Justice et al., 2003; Parsons et al., 2014; Portela et al., 2015). In mammals, similar effects on Notch signaling activation were observed upon intravesicular pH disturbances. In immortalized human non-tumorigenic breast epithelial cells (MCF10-A) inhibition of V-ATPase activity by Bafilomycin A1 decreased Notch signaling (Kobia et al., 2014), but did not affect cell growth. In contrast, growth of human breast cancer (HCC2218 and HCC1599) and T-ALL cells, expressing a ligand-independent Notch1 (membrane-tethered, active Notch), is inhibited by Bafilomycin A1 or GSI (Kobia et al., 2014). However, breast cancer cells harboring a truncated Notch2, which does not include the S3-cleavage site and is constitutively active (HCC1187), do not depend on endocytosis-dependent Notch signaling and are unaffected by V-ATPase inhibition.

Secretion via exosomes?

Recently, Notch ligands were shown to localize to exosomes secreted by endothelial cells (Sheldon et al., 2010), mouse embryonic stem cells (Cruz et al., 2018), and mesenchymal cells (Wang et al., 2019). In addition, Notch receptors (Notch1 and Notch2), Adam10, and Itch have been reported to be secreted in arrestin domain-containing protein 1 (ARRDC1)–expressing extracellular vesicles (Wang and Lu, 2017). Interestingly, they report that cleaved Notch2 receptors, encompassing the S2- and S3-cleavage sites, but not full length Notch2 was detected in exosomes. Moreover, knockdown of Itch or Adam10 resulted in loss of Notch2 in exosomes. Transferred Notch2 receptors from exosomes increased the expression of Notch target genes in recipient cells, which was diminished upon γ-secretase inhibition. These data imply that Notch ligands, Notch receptors, and Notch activity can be exchanged via exosomes.

Nuclear translocation of NICD and Notch signaling activation

Although nuclear import of the NICD is essential for Notch signaling activation, the exact mechanism of nuclear translocation of NICD remains unknown. In *Drosophila,* importin-α3 directly binds to NICD and stimulates the nuclear import of NICD via the importin-α3/β transport system (Sachan et al., 2013). In mammals, importin-α3 also binds directly to one of the nuclear localization signals in the NICD and imports the NICD into the nucleus. However, also importin-α4 and importin-α7 were shown to be involved in the nuclear translocation of the NICD in mammals. Upon silencing of the expression of importin-α3, α4, and α7 nuclear import of NICD was significantly reduced *in vivo* (Huenniger et al., 2010). Together, these data show that the nuclear translocation of the NICD is regulated by the importin-α3/β signaling in both *Drosophila* and mammals. In mammals additional importins are involved in the nuclear import of the NICD, preceding Notch signaling activation.

Altogether, these data show that also in mammals both ligand-dependent and independent Notch signaling requires endocytic trafficking and interfering with the

different steps of intracellular trafficking has distinct effects on Notch signaling. Does ligand-independent signaling rely more on intracellular trafficking compared to physiological Notch signaling?

Ligand-independent Notch signaling in cancer

Gain-of-function mutations in *NOTCH1* are hallmark driver mutations in T-cell acute lymphoblastic leukemia (T-ALL) leading to ligand-independent NOTCH1 signaling (Weng et al., 2004). These activating mutations are found in more than 50% of all human T-ALL and localize to the heterodimerization (HD) and the PEST domain of *NOTCH1.* In 27% of T-ALL, mutations in the HD and PEST domain can be found in cis in the same allele emphasizing the selection for high sustained *NOTCH* activation in T-ALL progression. Due to the high frequency of NOTCH1 mutations in T-ALL, γ-secretase inhibitors (GSI) have been investigated to target the NOTCH1 pathway in patients. Unfortunately, GSI have shown limited anti-leukemic activity in patients due to dose-limiting toxicities in normal tissues, including severe goblet cell metaplasia in the intestine and other side-effects as observed in animal models (Milano et al., 2004; Wong et al., 2004). In addition, a selective group of T-ALL acquired mutations or deletions in the *F-box and WD repeat domain-containing* (*FBXW7/cdc4*) gene, which encodes for an E3 ubiquitin ligase with many substrates, including cyclin E and Myc, and also regulating NICD turnover (O'Neil et al., 2007; Thompson et al., 2007). These alterations in the *FBXW7* gene result in a constitutively active NICD, similar to PEST mutations and define a subclass of tumors with ectopic Notch activation that are resistant to GSI, but remain ligand-dependent.

Notch signaling has also been linked to other types of leukemia in which *NOTCH1* and *NOTCH2* gain-of-function mutations are also reported (Aster et al., 2017). Similar gain-of-function PEST domain mutations in *NOTCH1* have been observed in chronic lymphocytic leukemia (CLL) (Puente et al., 2011) and in different subtypes of B-cell lymphoma (Arcaini et al., 2015; Martinez et al., 2016; Rossi et al., 2012). In addition, aberrant Notch signaling is also observed in acute myeloid leukemia (AML)(Thiel et al., 2017) and mantle cell lymphoma (MCL)(Beà et al., 2013; Kridel et al., 2012). Typically, with the exception of T-ALL, HD mutations are

infrequent in other diseases and other Notch receptors; these mutations occur mostly in the PEST domain. Our recent work described that HD mutations in *NOTCH1* found in T-ALL engineered into human NOTCH2 expression constructs are not ligand-independent and also resistant to EDTA activation. We found that the NRR domain of NOTCH2 is more tightly packed and more difficult to 'open' compared to Notch1 and Notch3, where NRR mutations cause ligand-independent Notch signaling activation (Habets et al., 2015).

Activating *NOTCH* mutations have also been reported in breast cancer. Different rearrangements in *NOTCH1* and *NOTCH2* were found in a subset of breast cancer patients and cell lines, mostly triple negative breast cancers (Robinson et al., 2011). These rearrangements result in membrane-tethered truncated NOTCH1 proteins, which no longer have a S2-cleavage site and therefore are only regulated by γ-secretase activity. The rearrangement in *NOTCH2* leads to a truncated, cytoplasmic NICD, which no longer requires γ-secretase cleavage to become transcriptionally active. Moreover, high expression of NOTCH1 was associated with a poor patient prognosis, while high levels of NOTCH2 are correlated with a better patient outcome (Mittal et al., 2014; Parr et al., 2004). Besides mutations in *NOTCH1* and *NOTCH2*, approximately 50% of all human breast cancers show loss of NUMB signaling, due to ubiquitination and degradation of NUMB, which act as a tumor suppressor (Pece et al., 2004). Besides direct regulation of NOTCH activity and degradation, NUMB also regulates MDM2, which is a E3 ligase and master regulator of TP53 stability. Therefore, loss of NUMB in breast cancer also results in loss of TP53 (Colaluca et al., 2008). In addition, loss of Numb expression or reduced expression of NUMB in primary breast tumors correlates with poor disease free survival and a higher risk of developing metastases (Rennstam et al., 2010) and is mainly found in triple negative breast cancers.

In melanoma no activating *NOTCH1* mutations have been found, although up-regulation of NOTCH receptors and ligands has been reported (Balint et al., 2005; Massi et al., 2006). Moreover, *FBXW7* mutations have been reported in melanoma patients, leading to the accumulation of active NOTCH1 (Aydin et al., 2014). Recently, NOTCH1 signaling has been shown to inhibit anti-tumor immunity by up-

regulating TGF-β1, resulting in an inhibition of infiltration of cytotoxic T-cell and NK cells to the tumor in immune-competent mice promoting tumor growth (Yang et al., 2018). Alterations in the Notch pathway have also been found in lung cancer. In approximately 30% of all human non-small lung cancers (NSCLC) *NUMB* expression is also lost leading to the activation of NOTCH1 signaling. In addition to loss of NUMB, *NOTCH1* gain-of-function mutations are found in approximately 10% of all NSCLC patients, which localize to the heterodimerization, transactivation, and PEST domain of *NOTCH1* (Westhoff et al., 2009). In contrast, another study showed loss-of-function mutations in all NOTCH receptors, although mutations in *NOTCH1* were most frequent (25% of human small-cell lung cancer (SCLC))(George et al., 2015). The loss-of-function mutations in *NOTCH1* located mainly to the extracellular domain with no specific domain preference and show that NOTCH signaling acts as a tumor suppressor in SCLC.

Altogether, these data show that Notch signaling is deregulated in a broad range of human malignancies either by mutations in *NOTCH* (only in a limited number of cancers) or by loss of negative regulators leading to (mainly) ligand-independent, constitutively active NOTCH signaling. However, does ligand-independent NOTCH signaling only occur in cancers due to genomic mutations and rearrangements or is there also a physiological role for ligand-independent NOTCH signaling without the need for activating mutations?

Physiological ligand-independent signaling in mammals?

Physiological ligand-independent signaling has been reported in crystal cells in *Drosophila.* Crystal cells are platelet-like cells that are part of the flies immune system. These cells require Notch activity for their survival. This Notch signaling acts independently of ligand but requires endosomal trafficking of Notch (Mukherjee et al., 2011). Mechanistically, crystal cells express high levels of nitric oxide synthase 1 (NOS1) under normoxic conditions, which increases nitric oxide levels. Nitric oxide stabilizes HIF-α under normal oxygen levels, which lead to the accumulation of full-length Notch receptors in early endocytic vesicles, without activating hypoxia response targets.

HIF-α mediated Notch signaling activation was shown to act ligand-independently and required γ-secretase activity. A plausible explanation for this endogenous need of ligand-independent Notch activation may be the lack of ligand present. If there is no guaranteed source of Notch ligand while active Notch signaling is required for survival, as is the case for circulating blood cells, an alternative activation of Notch signaling, independent of ligand is essential. However, is endogenous ligand-independent Notch signaling only restricted to *Drosophila* or is the phenomena also present in mammalian cells?

In mammals, ligand-independent Notch1 signaling has been reported during T-cell development in mice (Gómez-del Arco et al., 2010). This ligand-independent Notch1 signaling is the result of an alternative use of the *Notch1* promoter leading to the expression of alternative transcripts downstream of the canonical promoter (**Figure 3A**). These transcripts encode a truncated, membrane-tethered Notch1 variant. Ikaros, which is required for proper lymphoid differentiation of early hematopoietic progenitors, directly regulates the epigenetic state of the canonical and alternative promoters of *Notch1*, where it acts as a negative regulator of Notch1

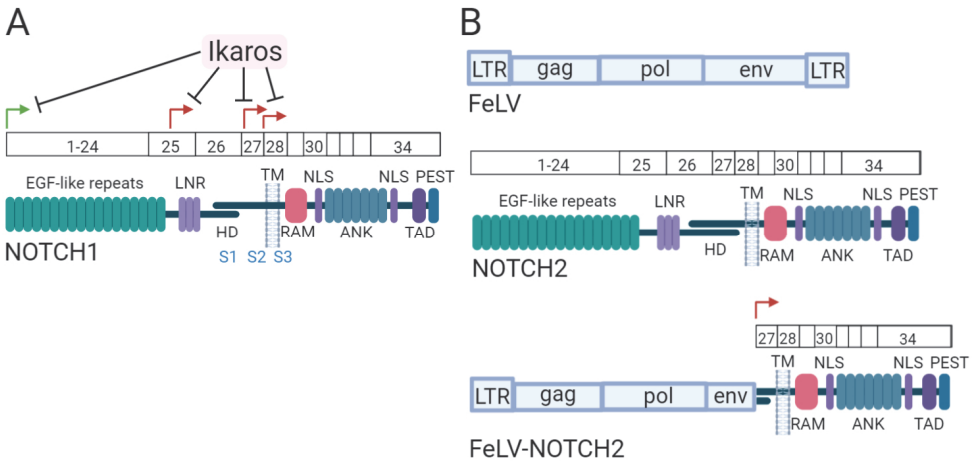


Figure 3. Endogenous ligand-independent Notch signaling in mammals. A. *Ikaros regulates the physiological expression of Notch1 of both canonical and alternative transcripts (Gómez-del Arco et al., 2010).* ***B.*** *A tumor-derived FeLV provirus expresses a truncated active form of Notch2 (Lauring and Overbaugh, 2000).*

signaling. Loss of Ikaros during T-cell development leads to aberrant Notch1 activation and rapid development of T-ALL. In addition, a constitutively active nuclear isoform of Notch2 can be induced by an internal ribosome entry site (IRES) within the Notch2 ectodomain (Lauring and Overbaugh, 2000)(**Figure 3B**). However, whether this mechanism can also occur with endogenous *Notch2* genes remains unknown.

Together these data prove that ligand-independent Notch signaling does not only drive carcinogenesis, but can also occur during physiological signaling in mammals. Whether ligand-independent Notch signaling is used during additional steps of mammalian development or homeostasis during adult life remains elusive.

Mechanisms of ligand-independent Notch signaling

First, what are the similarities and differences of ligand-dependent and independent Notch signaling? Both ligand-dependent and ligand-independent signaling require S3-cleavage by γ-secretase to become active (De Strooper et al., 1999; Schroeter et al., 1998; Struhl and Adachi, 2000; Struhl and Greenwald, 2001). Although, deletions in the *NOTCH2* receptor gene reported in breast cancer, leading to the expression of a truncated Notch receptor (NICD-like), no longer require S3-cleavage. A second requirement for the activation of both ligand-dependent and ligand-independent Notch signaling are O-fucosyltransferase-1 POFUT1 (McMillan et al., 2017) and O-glucosyltransferase RUMI (Chammaa et al., 2018), which are two proteins involved in the glycosylation of the EGF-like repeats of the Notch receptor that enable and regulate ligand binding. Loss of POFUT1 showed no Notch activity in both ligand-dependent and ligand-independent Notch1 (T-ALL mutants) expressing cells (McMillan et al., 2017). Loss of RUMI suppressed ligand-independent Notch signaling in *Drosophila* (Leonardi et al., 2011) and RUMI expression is also found to be highly up-regulated in NSCLC patients and is a predictive marker of poor patient prognosis and survival (Chammaa et al., 2018).

Ligand binding leads to conformational changes in the NRR of Notch, which result in the unmasking of the S2-cleavage site for cleavage by Adam proteases. Moreover, endocytosis of the Notch ligand bound to the Notch receptor makes sure

the extracellular domain (ECD) of the Notch receptor is internalized and removed after S2 cleavage preparing the receptor for further processing by γ-secretase (Gordon et al., 2015; Nichols et al., 2007). Accumulating evidence shows that S2-cleavage and removal of the NRR is essential for Notch signaling activation (Mumm et al., 2000; Pan and Rubin, 1997; van Tetering et al., 2009). Therefore, without ligand binding an alternative mechanism of shedding of the ECD of the Notch receptor is required and/or conformational changes to make the S2-cleavage site accessible for cleavage. In addition to ligand binding, ligand-dependent Notch signaling requires Adam10 activity for endogenous Notch activation, which is not essential for a ligand-independent signal (Bozkulak and Weinmaster, 2009; Sulis et al., 2011; van Tetering et al., 2009). Notably, Notch cleavage at Val1711 upon ligand-stimulation was reduced, however, not absent in Adam10 KO cells (Bozkulak and Weinmaster, 2009; van Tetering et al., 2009) and was lost upon metalloprotease inhibition (Bozkulak and Weinmaster, 2009). Moreover, residual ligand-independent Notch activity, induced by T-ALL mutations, is observed upon loss of Adam10 and Adam17, which may be processed by other proteases yet to be identified (Bozkulak and Weinmaster, 2009; Sulis et al., 2011; van Tetering et al., 2009). In addition, ligand-independent Notch1 S2 cleavage was detected, although Val1711 levels were absent, indicating that additional proteases may perform S2-cleavage at a different cleavage site. However, how does Notch become active without ligand-binding and Adam10 cleavage? As ligand-independent Notch signaling requires no ligand binding, the effect of ligand binding; unfolding and removal of the ECD of Notch, has to be managed by a different mechanism. Several models explain this ligand-independent activation of Notch signaling (**Figure 4**).

Mutations in the Notch receptor

Mutations in the *Notch* receptor localizing to the heterodimerization domain, which are found almost exclusively in T-cell leukemia's and mostly affect Notch1, are one of the mechanisms of ligand-independent Notch signaling (Robinson et al., 2011;

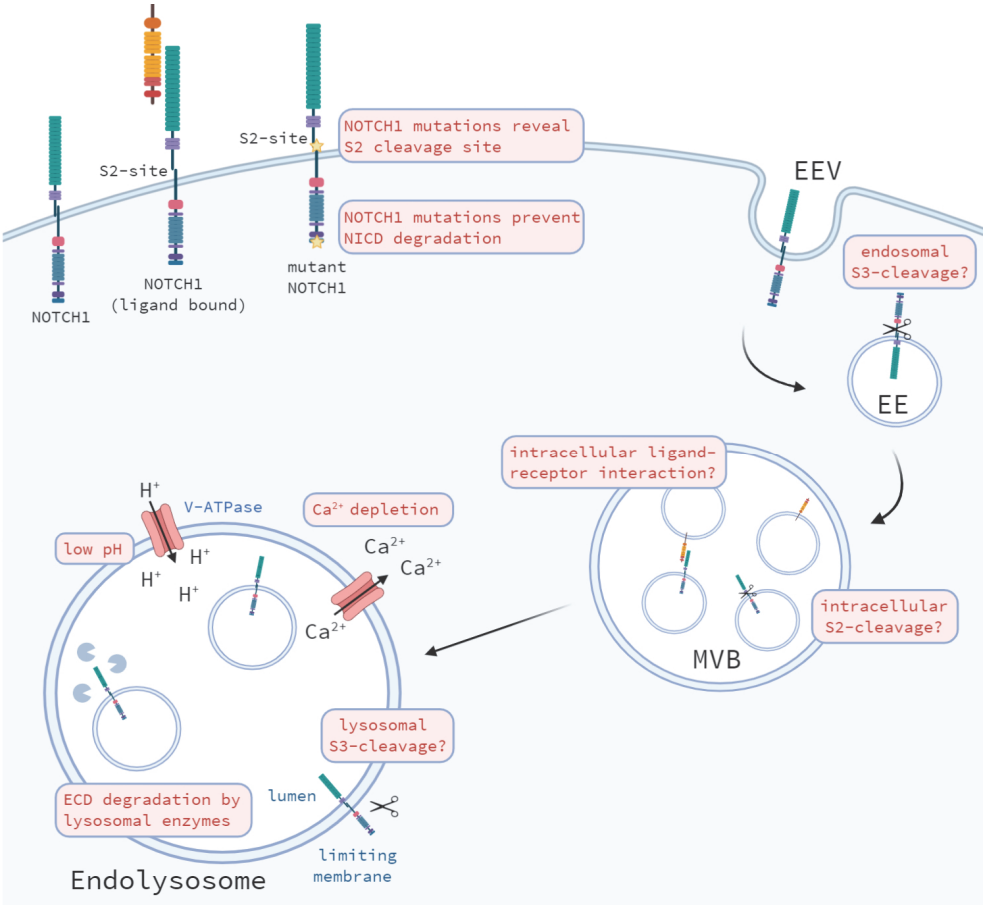


Figure 4. Possible mechanisms of ligand-independent Notch signaling. *Ligand-independent Notch signaling can be elicited by mutations in the Notch receptor. Other mechanisms of intracellular ligand-independent Notch activation have been proposed, including: low pH, Ca^{2+} depletion, lysosomal activation by degradation of the ECD of the Notch receptor, and intracellular S2- and S3-cleavage of the Notch receptor.*

Weng et al., 2004). Due to the mutations in the heterodimerization (HD) domain, the Notch receptor undergoes a conformational change. Mutations in the HD domain can be divided into two classes: *1)* mutations which destabilize Notch1 heterodimers or *2)* mutations which do not affect heterodimer stability, but enhance the access of metalloproteases to the S2-site which both increase Notch1 signaling activity ligand-independently (Malecki et al., 2006). In addition, mutations in the PEST domain of

the Notch receptor, which are also found in T-ALL and often in combination with a HD mutation, prevent rapid degradation of the active NICD, leading to a constitutively cleaved and activated Notch receptor which degradation is partially inhibited. However, human NOTCH2 activity is not affected by NRR disruption and ligand-independent activation by Adam17, which may explain the low frequency of NOTCH2 mutations (Habets et al., 2015). Moreover, auto-inhibition by the NRR of the Notch3 receptor is less tightly associated compared to the NRR of Notch1, leading to increased basal ligand-independent Notch3 activity (Xu et al., 2015). The mechanism of Notch4 activation has not been resolved, but Notch1-3 receptors all undergo the paradigm of sequential cleavage by a metalloprotease and subsequent cleavage by γ-secretase to produce NICD (Groot et al., 2014).

Activation during endocytic and lysosomal trafficking of the Notch receptor

Besides mutations in the Notch receptor that induce the spontaneous unfolding of the ECD of Notch, alternative shedding mechanisms have been proposed. Upon endosomal trafficking the Notch receptor can be transported towards the lysosomes for degradation. Therefore, another possible mechanism of ECD shedding could be the degradation of the ECD by hydrolases, to which also Adam metalloproteases belong, and subsequent γ-secretase cleavage, which are both present in lysosomes, resulting in the active form of NICD, which may be released and translocated towards the nucleus (Schneider et al., 2013). Notably, during the incorporation of the Notch receptor into endosomes, the Notch receptor is located to the limiting membrane on the late endosome, resulting in the intracellular domain of Notch protruding into the cytosol (Hori et al., 2011; Schneider et al., 2013; Wilkin et al., 2008). During this intracellular trafficking, γ-secretase may cleave the receptor at the free S3-site, releasing the active NICD into the cytosol which can subsequently travel towards the nucleus and activate its downstream targets. However, excessive shortening of the extracellular domain of Notch may be still required for γ-secretase cleavage, since γ-secretase cleavage increases upon a shorter Notch receptor substrate (Struhl and Adachi, 2000).

Furthermore, defects in genes involved in the ubiquitination and lysosomal degradation of the Notch receptor may also, in a ligand-independent manner, activate Notch signaling. For example, loss of Numb decreases Notch localization towards late endosomes and leads to an accumulation of Notch at the cell surface, due to increased recycling of the Notch receptor, which results in reduced degradation of the Notch receptor (Johnson et al., 2016). Interestingly, blocking the fusion between late endosomes and lysosomes or by reducing their acidification by Chloroquine, Bafilomycin A1, or NH_4Cl results in an accumulation of Notch in late endosomes and lysosomes, down-regulating ligand-independent Notch signaling (Fortini and Bilder, 2009; Hounjet et al., 2019; Kobia et al., 2014; Vaccari et al., 2010; Yan et al., 2009).

These data suggest that endosomal fusion is required for ligand-independent Notch signaling. Moreover, additional fusions of intracellular vesicles may be affected by these treatments leading to different blockage steps in endocytic trafficking. Together these data show that intracellular trafficking is important for ligand-independent Notch activation and that defects at different steps of trafficking can either support or inhibit ligand-independent signal transduction.

Changes in the endosomal-lysosomal environment

During intracellular trafficking changes in different ion-concentrations occur in the endosomal-lysosomal microenvironment (**Figure 5**) (Scott and Gruenberg, 2011). During endocytic trafficking the pH of endosomal compartments becomes gradually more acidic (Marshansky and Futai, 2008), due to increased H^+-ion transport towards these vesicles by V-ATPase activity (Yan et al., 2009). Changes in pH affect protein-protein interactions, enzymatic activity and protein degradation and may affect the activity of the enzymes regulating the proteolytic cleavage of the Notch receptor. Therefore, the acidic environment in endosomes and lysosomes may promote the dissociation the ECD of the Notch receptor and result in ligand-independent activation of Notch (Wilkin et al., 2008). Besides the gradual increase in acidity of intracellular vesicles, also Ca^{2+}-ion concentrations have been shown to change during endocytic transport. Calcium concentrations are high at the plasma

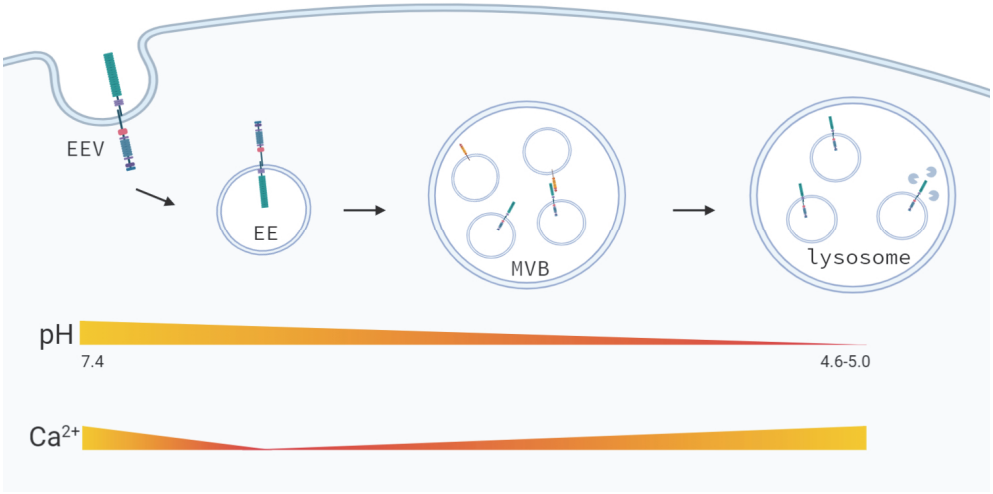


Figure 5. Changes in the ion-concentrations during intracellular trafficking. *The vesicular pH decreases upon endocytic trafficking due to import of H+-ions leading to gradient acidification. Ca^{2+}-ion concentrations initially decrease during early endosomal trafficking and gradually increase during late endosomal trafficking reaching the highest Ca^{2+}-ion concentration in the lysosomes. Adjusted from Scott and Gruenberg (Scott and Gruenberg, 2011).*

membrane and rapidly decrease upon internalization and formation of early endosomes, from which a calcium gradient builds-up towards lysosomal trafficking (Christensen et al., 2002; Gerasimenko et al., 1998). This high concentration of calcium in the lysosomes is required to induce a calcium efflux from the lysosomes to ensure fusion of lysosomes with late endosomes and autophagosomes (Tian et al., 2015). The calcium depletion in early endosomes may, however, decrease the stability of the Notch receptor, especially since the LNR repeats in the NRR require Ca^{2+} for their folding and stabilization (Gordon et al., 2007). This is consistent with the fact that Ca^{2+} chelators (i.e. EDTA) are potent activators of Notch signaling (Rand et al., 2000; Vardar et al., 2003) resulting in unmasking of the Notch1 S2-cleavage site, leading to Notch activation (van Tetering et al., 2009).

Alternative S2 cleavage?

As mentioned before, ligand-independent Notch signaling does not require Adam10 for its activation, however, can be cleaved by Adam10 at Val1711. Van Tetering et al showed that both genetic and pharmacologic inhibition of metalloprotease activity blocks ligand-independent Notch receptor cleavage at Val1711, however, does not block general S2-cleavage and activation of the Notch receptor (van Tetering et al., 2009). While the majority of Val1711-cleaved Notch receptors occurred at the plasma membrane, a fraction of Val1711-cleaved Notch receptors also localized to cytoplasmic vesicles. However, the identity of these intracellular vesicles has not yet been revealed and the exact site(s) of alternative S2-cleavage of the Notch receptor and the protease(s) involved have not been elucidated. Taking a similar approach, Hounjet et al identified that Val1744-cleaved NICD can accumulate in vesicles when cells are treated with chloroquine in LC3B+ auto-phagosomal vesicles (Hounjet et al., 2019). Notably a large fraction of Val1744+ vesicles did not co-localize with LC3B-GFP and remain unidentified to date.

The hypothesis that S2-cleavage occurs at the plasma membrane is supported by data showing that: (1) S2-cleaved Notch remains at the plasma membrane in γ-secretase mutant fly cells (López-Schier and St Johnston, 2002), (2) S2-cleaved Notch, which harbors a mutated ubiquitination site required for internalization of the receptor, can still be cleaved by γ-secretase into active NICD (López-Schier and St Johnston, 2002), and (3) Adam10 and Adam17 are predominantly expressed at the cell surface (Ebsen et al., 2013). Others show that Adam10 is expressed at the cell membrane, but that the majority of the pro-enzyme is expressed in the Golgi (Lammich et al., 1999). In addition, Dornier et al showed that tetraspanins regulate Adam10 trafficking from the ER to the cell surface and that tetraspanins promote ER exit and surface expression of Adam10 thereby stimulating Notch activation (Dornier et al., 2012; Saint-Pol et al., 2017). In contrast, there are also reports showing that S2-cleavage can occur in intracellular vesicles (Chastagner et al., 2017; Skovronsky et al., 2000). Chastagner et al showed that ligand-bound Notch receptors are internalized by ubiquitination induced by E3-ligase Dtx4, which

results in Rab5-expressing early endosomes containing Notch1 and Dtx4, which recruit Adam10 (Chastagner et al., 2017).

In addition, S2-cleavage may even be circumvented during ligand-independent Notch signaling as γ-secretase cleavage does not depend on the recognition of a particular target sequence in Notch1 (Struhl and Adachi, 2000). Moreover, alternative ECD shedding may result in a short ECD of Notch1, short enough (<300aa) to be recognized by γ-secretase as γ-secretase activity increases when the extracellular domain of Notch is decreased in size. Interestingly, ligand-independent Notch signaling induced by HD mutations showed enhanced S2-cleavage at Val1711, however, when this cleavage site is mutated, proteases cleave at other sites or cleavage by γ-secretase may even occur in the absence of S2-cleavage due to the dissociation of the HD domain (van Tetering et al., 2009). However, whether γ-secretase may even bypass S2-cleavage remains unproven.

Alternative S3 cleavage?

Currently, the role of intracellular trafficking of the Notch receptor in γ-secretase cleavage is understudied. First, a mutation in the mono-ubiquitination site at Lys1749 of a NEXT-like fragment results in a loss of γ-secretase cleavage and the accumulation of Notch in the endosomes (Gupta-Rossi et al., 2004). These data suggest a requirement of endocytic trafficking following S2-cleavage of Notch. Furthermore, endocytosis was required prior to γ-secretase cleavage since inhibition of endocytosis prevented S3-cleavage and that mono-ubiquitination at the plasma membrane or during early endocytosis of the Notch receptor is essential prior to γ-secretase cleavage.

Moreover, the γ-secretase complex is not only expressed at the plasma membrane but also on endocytic compartments, lysosomes, ER, and Golgi apparatus (Pasternak et al., 2003; Small and Gandy, 2006). In addition, PSEN1 or PSEN2, the catalytic components of the γ-secretase complex, show distinct subcellular localization and activity (Sannerud et al., 2016). While the majority of PSEN1-expressing γ-secretase complexes localize to the plasma membrane, PSEN2-expressing γ-secretase complexes are restricted to the late endosomes and

lysosomes, via targeting through an Acidic-Dileucine sorting motif. Disruption of this sorting motif results in a translocation of PSEN2-containing γ-secretase activity to the plasma membrane.

In addition to the intracellular localization of the γ-secretase complex, its activity has also been reported to be affected by changes in pH. During endocytic trafficking cargo protein experience a reducing pH gradient towards the lysosomes. Indeed, γ-secretase activity was shown to be more efficient in acidic environments, especially in the endocytic compartments, where the pH is lower, compared to the plasma membrane (Pasternak et al., 2003). Furthermore, the differences in pH also affect γ-secretase cleavage sites in the Notch transmembrane domain (Fukumori et al., 2006). Tagami and colleagues showed for the first time that γ-secretase is able to cleave the Notch receptor at two different amino acid sites in mammalian cells for both ligand-dependent and independent Notch signaling (Tagami et al., 2008). These two different cleavages by γ-secretase result in the release of the NICD-V (Gly1743-Val1744) or the novel NICD-S (Leu1746-Ser1747), from which the latter is more produced (especially when the internalization rate is high), less stable, and predominantly expressed in endosomes. Blockage of the acidification of endosomes and lysosomes by Bafilomycin A1 treatment results in a shift in NICD cleavage in which the NICD-V becomes the major site of cleavage. Thus, vesicular pH and trafficking has a major impact on the fate of Notch molecules *in vitro*.

Besides endopeptidase activity leading to alternative S3-cleavages, γ-secretase also possesses carboxy-peptidase activity (Takami et al., 2009). Okochi and colleagues showed that the Notch transmembrane is cleaved twice; first at the S3-cleavage site which depends on endopeptidase activity (S3ε) and secondly at the S4-cleavage site (Ala1731-Ala1732) which depends on the carboxy-peptidase activity of γ-secretase (S4γ)(Okochi et al., 2006; Okochi et al., 2002). Importantly, γ-secretase cleavage at the cytoplasmic leaflet (S3) is required prior to S4-cleavage (Chandu et al., 2006). This dual intra-membrane cleavage by γ-secretase results in the release of a S4-Notch peptide, which is secreted and does not accumulate intracellularly. The significance of this peptide has not been investigated but perhaps

acts as a mechanism to clear processed remnants of the Notch receptor from the cell.

Together these data show that there are multiple possible mechanisms for ligand-independent Notch signaling and that intracellular trafficking plays an important role in all proposed mechanisms.

Therapeutic targeting of ligand-independent Notch signaling

Ectopic activation of Notch signaling is a driver in many human malignancies, including T-ALL, breast cancer, and lung cancer, hence Notch inhibition is a high priority clinical target. However, inhibition of the Notch pathway as a therapeutic approach faces challenges due to its essential function in the maintenance of adult tissues. The lack of predictive biomarkers for patient selection (Val1744 is not a good marker for Notch activity as Val1744 levels do not necessarily correlate with NOTCH1 activity (Hounjet et al., 2019)), the sparse availability of robust pharmacological markers to monitor on-target activity (Krop et al., 2012), and the pleiotropic effect of current pan-Notch inhibitors targeting multiple Notch family members (both with GSI, as well as, ligand neutralization) lead to low anti-tumor activity and dose-limiting toxicities in normal tissues.

However, toxicity due to γ-secretase inhibition depends on the schedule of the treatment. Intermittent scheduling results in strong modulation of a Notch gene signature, including down-regulation of *NOTCH1* and its target genes, evidence of anti-tumor activity and low toxicity in phase I clinical trials (Krop et al., 2012; Tolcher et al., 2012). Moreover, the recent availability of a NOTCH1 receptor specific monoclonal antibody showed promising results in preclinical studies, including inhibition of tumor growth, deregulation of angiogenesis, and reduced intestinal toxicity compared to pan-Notch inhibition in preclinical models (Wu et al., 2010). Other monoclonal antibodies that specifically recognize activated Notch receptors have also shown promising results in preclinical studies and efficacy results in phase II clinical studies are awaited (Li et al., 2008; Tiyanont et al., 2013). Besides, Tarextumab, a monoclonal antibody targeting Notch2 and Notch3, was well tolerated using intermittent scheduling and showed inhibition of Notch signaling in a phase I

trial (Smith et al., 2019). Unfortunately, the addition of Tarextumab to chemotherapy as a therapeutic strategy in pancreatic adenocarcinoma failed to improve patient outcome (Hu et al., 2019).

Therefore, it is essential to obtain a better understanding in the mechanisms behind this ectopic, ligand-independent activation of Notch signaling, especially in the differences between ligand-dependent and independent signaling. Moreover, ligand-dependent Notch signaling may also be activated in human malignancies due to mutation-independent defects, for example defects in endocytosis, and may therefore also differ from physiological Notch signaling. If such differences could be exploited this would allow targeting of oncogenic ligand-independent Notch signaling without affecting physiologic Notch signaling in adult tissues.

Recently, we published a repurposed drug use approach in which T-ALL cell lines were treated with chloroquine in combination with γ-secretase inhibition (Hounjet et al., 2019). Chloroquine sensitized oncogenic NOTCH1 driven human T-ALL to GSI resulting in decreased T-ALL cell viability and proliferation. In these NOTCH1 driven T-ALL cells chloroquine interfered with the intracellular trafficking and processing of ligand-independent NOTCH1 receptors, leading to the accumulation of full length but also S2- and S3-cleaved NOTCH1 and down-regulation Notch target gene expression. Importantly, this reduction in NOTCH1 target gene expression was not observed in GSI-resistant T-ALL cells with wild-type Notch signaling. Moreover, while GSI treatment revealed an accumulation of oncogenic NOTCH1 receptors at the plasma membrane, when combined with chloroquine, NOTCH1 accumulated in LC3B+ autophagosomes but also in other intracellular vesicles, yet to be identified. In addition, Notch receptor expression at the plasma membrane was decreased upon chloroquine treatment, indicating a defect in receptor internalization or recycling. However, the efficacy of chloroquine in the treatment of T-ALL (in combination with GSI) in preclinical studies remains to be studied.

In contrast, Maes and colleagues showed that chloroquine increases endogenous Notch1 signaling in endothelial cells, inducing tumor vessel normalization in tumors (Maes et al., 2014). They also report that Notch1 receptors

accumulate in late endosomal vesicles upon chloroquine treatment and show a slow and sustained increase in NEXT and NICD levels, which may be due to reduced lysosomal degradation, while ligand-induced activation leads to an acute and transient increase in NICD levels. However, Kobia et al showed that V-ATPase inhibition reduces ligand-dependent Notch signaling in human breast cells (Kobia et al., 2014). Although we show that inhibition of the lysosomal fusion of endosomes, by changing the intracellular pH by chloroquine, inhibits ligand-independent Notch signaling, its remain controversial whether ligand-dependent Notch signaling is also effected by chloroquine treatment and may depend on the cellular background and might be tissue specific.

Conclusion

To conclude we reviewed the role of intracellular trafficking in ligand-dependent and independent Notch signaling. We conclude that intracellular trafficking is an important regulator of Notch signaling activation in *Drosophila.* In *Drosophila* inhibition of early endosomal trafficking and endosomal-lysosomal fusion inhibits Notch signaling activation, while accumulation of Notch in maturing endosomes results in Notch signaling activation. In mammals, intracellular trafficking seems to play a similar role in the regulation of Notch signaling, although the expression of several orthologs of endosomal regulation may induce redundancy and makes studying the effects of loss-of-function more complex. Importantly, deregulation of the pH in intracellular vesicles in mammals results in an accumulation of (cleaved) Notch receptors in late endosomes and lysosomes, leading to decreased ligand-independent signaling. In addition, the release of Notch ligands, receptors, and regulators in extracellular vesicles and internalization and Notch activation in recipient cells in mammals adds another layer of complexity to the role and importance of intracellular trafficking in the activation of mammalian Notch signaling.

Moreover, we summarized the potential mechanisms of ligand-independent Notch signaling. While mutations, rearrangements, and fusions of *NOTCH* leading to Notch activation are well defined, the Notch activation mechanisms in other malignancies remains unclear. The recruitment of Adam10 and γ-secretase

complexes towards intracellular vesicles strengthens the model of intracellular Notch receptor processing and activation. Intracellular pH and ion-concentrations (i.e. Ca^{2+}, Zn^{2+}) may play a critical role in the regulation of Notch activation either by directly affecting the stability of the receptor itself or indirectly by affecting the activity and/or cleavage site precision of proteolytic enzymes required for ligand-independent Notch activation.

Overall, the exact differences between ligand-dependent and independent Notch signaling in mammals and how these are regulated remains inconclusive. Undoubtedly, additional regulators of Notch cleavage and activation exist that control or drive these distinct modes of activation. It will be interesting to see under what developmental programs Notch can be activated in the absence of ligand in mammalian cells and how this mechanism prevents inappropriate Notch signaling during development and in adult tissues. Such regulators are prime candidates for curtailing aberrant NOTCH signaling in cancer with limited effects on physiological Notch signaling in adult tissues. Current knowledge on the distinct functions of the different Notch receptors depending on the tissue type, is guiding us towards a more delicate approach, keeping an open future for the targeting of Notch signaling in cancer.

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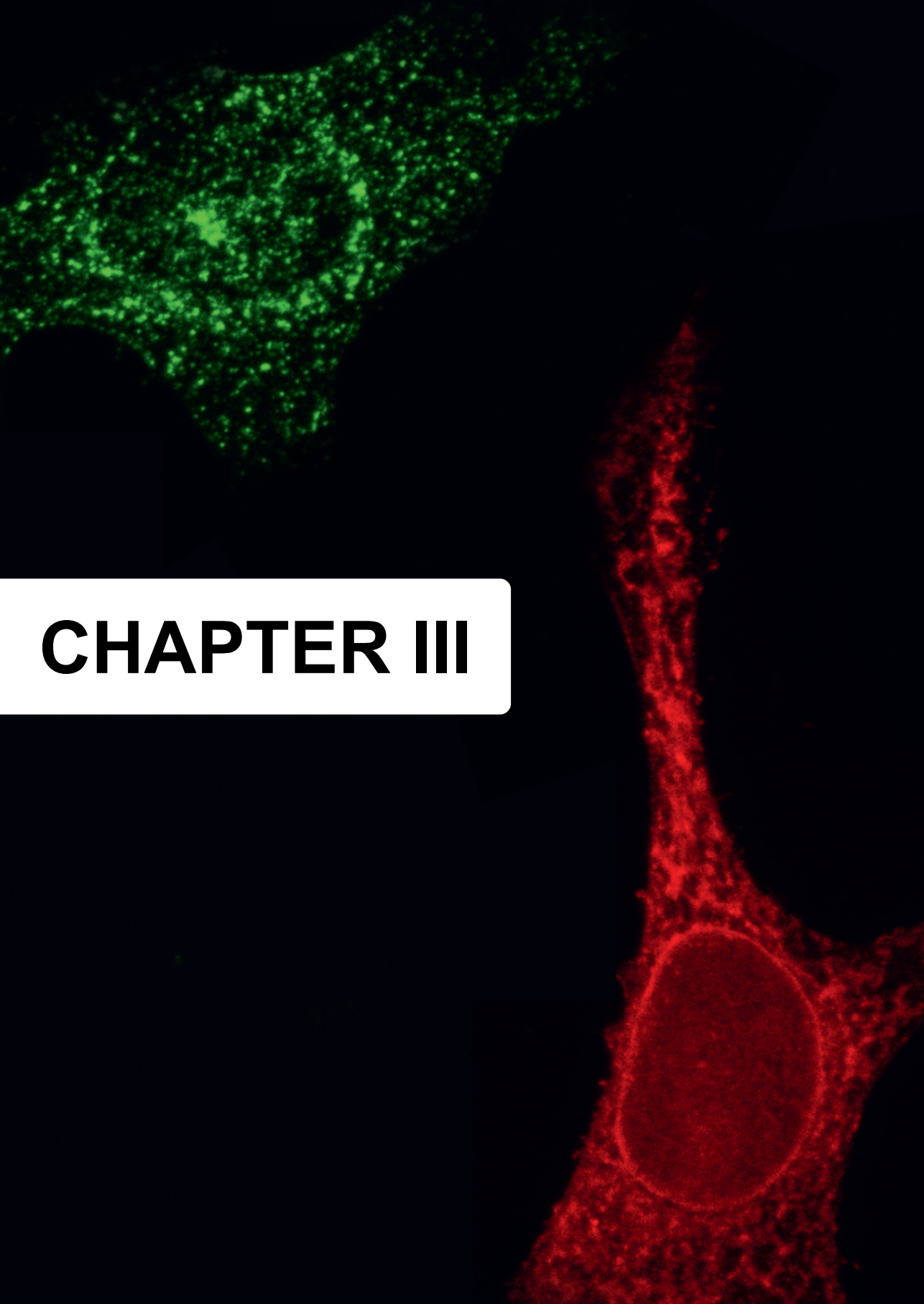
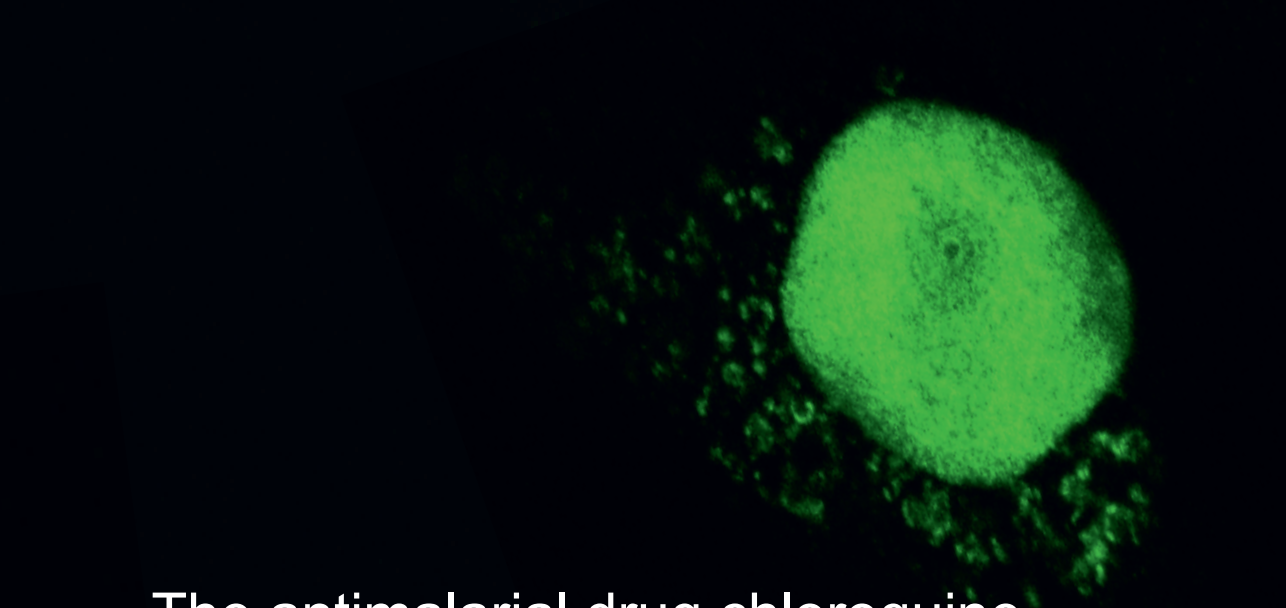


CHAPTER III



The antimalarial drug chloroquine sensitizes oncogenic NOTCH1 driven human T-ALL to γ-secretase inhibition

Judith Hounjet[1,2], Roger Habets[1], Marco B. Schaaf[1], Tessa C. Hendrickx[1], Lydie M.O. Barbeau[1], Sanaz Yahyanejad[1], Kasper M. Rouschop[1], Arjan J. Groot[1], and Marc Vooijs[1,2#]

[1]Department of Radiation Oncology (Maastro), GROW School for Oncology, Maastricht University Medical Centre+, Maastricht, the Netherlands
[2]Maastro clinic, Maastricht, The Netherlands

[#]corresponding author: marc.vooijs@maastrichtuniversity.nl, Tel: +31 (0)433882912

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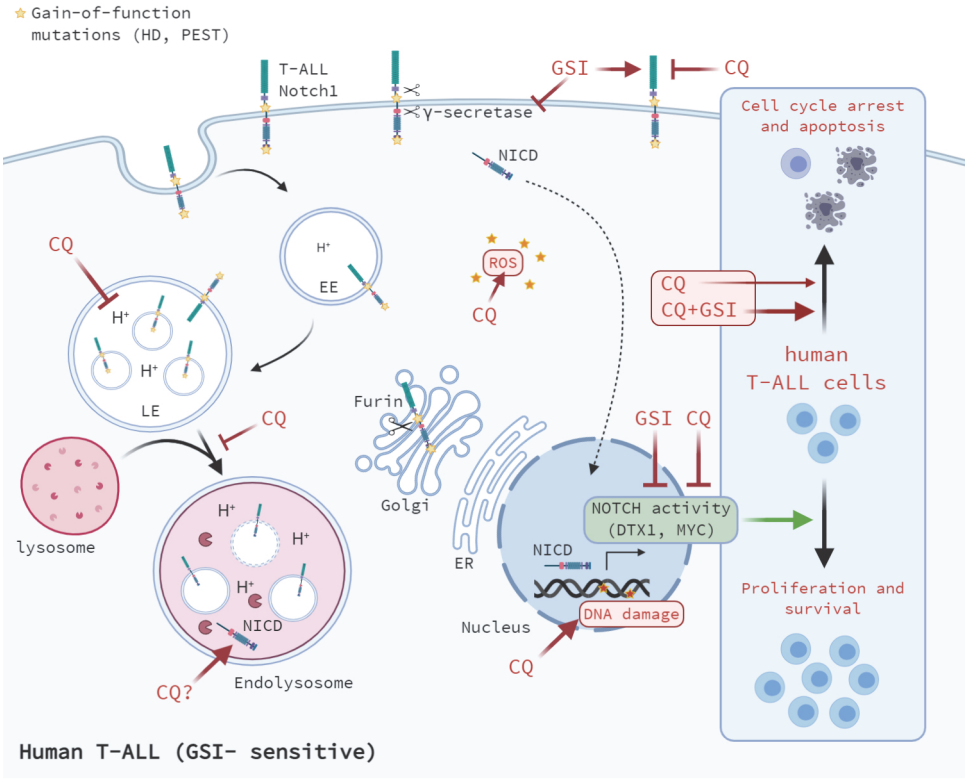
Graphical abstract



Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive cancer arising from T-cell progenitors. Although current treatments, including chemotherapy and glucocorticoids, have significantly improved survival, T-ALL remains a fatal disease and new treatment options are needed. Since more than 60% of T-ALL cases bear oncogenic NOTCH1 mutations, small molecule inhibitors of NOTCH1 signalling; γ-secretase inhibitors (GSI), are being actively investigated for the treatment of T-ALL. Unfortunately, GSI have shown limited clinical efficacy and dose-limiting toxicities. We hypothesized that by combining known drugs, blocking NOTCH activity through another mechanism, may synergize with GSI enabling equal efficacy at a lower concentration. Here, we show that the clinically used antimalarial drug chloroquine (CQ), an inhibitor of lysosomal function and autophagy, decreases T-ALL cell viability and proliferation. This effect of CQ was not observed in GSI-resistant T-ALL cell lines. Mechanistically, CQ impairs the redox balance, induces ds DNA breaks, and activates the DNA damage response. CQ also interferes with intracellular trafficking and processing of oncogenic NOTCH1. Interestingly, we show for the first time that the addition of CQ to γ-secretase inhibition has a synergistic therapeutic effect on T-ALL and reduces the concentration of GSI required to obtain a reduction in cell viability and a block of proliferation. Overall, our results suggest that CQ may be a promising repurposed drug in the treatment of T-ALL, as a single treatment or in combination with GSI, increasing the therapeutic ratio.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a very aggressive, acute cancer that accounts for 15% of pediatric and 50% of adult leukemia's. Standard treatment consists of chemotherapy combined with glucocorticoids. While about 80% of children and 50% of adults can be cured, a significant percentage is resistant to primary treatment or remains at risk for relapse. Thus, although current treatments have significantly improved survival, T-ALL still remains a fatal disease and new treatment options are required (Marks et al., 2009).

In T-ALL, specific molecular classes exist based on mutations and translocations in multiple T-cell oncogenes (Ferrando et al., 2002). The most common alterations found in T-ALL are mutations in *NOTCH1*, leading to ligand-independent activation of NOTCH1 driving proliferation and survival in up to 60% of T-ALL cases (Mansour et al., 2006; Weng et al., 2004). NOTCH1 has essential roles in cell fate decisions within the hematopoietic system and is required to adopt T-cell fates from lymphoid progenitors, explaining the strong selection for oncogenic NOTCH1 mutations in T-ALL (Radtke et al., 1999). The frequent involvement of NOTCH1 in T-ALL resulted in the development of small molecule inhibitors targeting the NOTCH1 receptor pathway. The most widely used are γ-secretase inhibitors (GSI) that prevent proteolytic cleavage of NOTCH and the downstream activation of target genes. Unfortunately, clinical trials have shown limited anti-leukemic activity of γ-secretase inhibition and dose-limiting toxicities in normal tissues, most notably causing goblet cell metaplasia in the intestine resulting in severe diarrhoea (Milano et al., 2004; Wong et al., 2004).

Recent evidence indicates that γ-secretase activity not only occurs at the cell surface, but also in the acidic environment of the lysosomes and endosomes (Kaether et al., 2006; Pasternak et al., 2003; Tagami et al., 2008). Ligand-dependent NOTCH signalling requires V-ATPase activity, suggesting that acidification is essential for NOTCH signalling (Sethi et al., 2010; Vaccari et al., 2010). In addition, it was shown that a defective endosomal pathway, regulating NOTCH degradation, results in ligand-independent activation within the lysosomes due to accumulation of NOTCH (Childress et al., 2006; Jaekel and Klein, 2006; Maes et al., 2014; Vaccari

et al., 2010). Interestingly, depletion of Rab7, required for the fusion between the late endosome and the lysosome, suppressed ligand-independent, but not ligand-dependent signalling (Schneider et al., 2013). Altogether, these findings suggest that endosomes play a key role in the NOTCH signal relay and that fusion with the lysosomes is a prerequisite for ligand-independent signalling. Therefore, a potent intervention to block ligand-independent NOTCH signalling may be to inhibit lysosomal function by increasing the pH of the lysosomal lumen.

An FDA-approved drug that has such a mode of action is chloroquine (CQ), which is an extensively clinically used antimalarial drug with an acceptable toxicity profile. Accumulating data indicate that CQ has anti-cancer activity and has been widely investigated as a sensitizer of radio- and chemotherapy (Ding et al., 2011; Liang et al., 2014; Ratikan et al., 2013; Rouschop et al., 2010; Verbaanderd et al., 2017). Therefore, CQ has recently been included in several clinical trials to investigate safety and efficacy in a broad range of malignancies (Shi et al., 2017).

The anti-cancer activity of CQ was previously mostly attributed to its effect of blocking autophagy (Degenhardt et al., 2006), a major vesicular pathway to recycle damaged/obsolete cytoplasmic components via lysosomes. However, blockade of lysosomal acidification generates additional, autophagy-independent effects as well (Eng et al., 2016). Interestingly, CQ has been shown to phosphorylate ataxia-telangiectasia mutated (ATM), one of the upstream DNA damage response (DDR) kinases, in the absence of detectable DNA damage, leading to activation of the DDR. Subsequent activation of p53 and its downstream target $p21^{CIP1}$ have been shown to result in a cell cycle arrest in several cancer types (Bakkenist and Kastan, 2003; Hu et al., 2016; Loehberg et al., 2007; Maclean et al., 2008). Moreover, recent evidence showed that CQ up-regulates p53 expression by reactive oxygen species (ROS) accumulation induced by the loss of lysosomal and mitochondrial membrane potential in colorectal cancer cell lines (Chen et al., 2017). Furthermore, it has been demonstrated that autophagy is up-regulated upon p53 activation, enabling tumour cells to escape from apoptosis in a Myc-induced lymphoma model (Amaravadi et al., 2007). Together these data suggest a complex interaction between autophagy, DNA

damage response, and redox balance in tumour cell survival creating a therapeutic opportunity for CQ treatment.

Therefore, we questioned whether CQ would suppress the viability and proliferation of T-ALL cells that rely on ligand-independent NOTCH1 signalling for their survival and whether combined treatment of GSI and CQ would have an additional effect on T-ALL survival as compared to GSI alone. Here, we show for the first time that CQ decreases T-ALL cell viability and proliferation, which was not observed in GSI-resistant T-ALL cell lines. Moreover, CQ treatment decreases the concentration of GSI required to reduce T-ALL cell proliferation and shows an additional effect when combined with γ-secretase inhibitors. Mechanistically, CQ impairs the redox balance and induces DNA damage with a subsequent activation of the DNA damage response (DDR). When CQ was combined with GSI treatment, a major cell cycle arrest was shown with a synergistic increase in apoptosis. Furthermore, CQ also interferes with the intracellular trafficking and processing of oncogenic NOTCH1. Together, our findings provide new insights in the mechanisms by which CQ impacts on cancer cell survival and identify opportunities to enhance treatment effects in NOTCH1 driven T-ALL.

Materials and Methods

Cell lines, compounds and constructs

All T-ALL cell lines used were kind gifts from J Meijerink (Erasmus MC Rotterdam, The Netherlands). GSI-sensitive (ALL-SIL, HPB-ALL, and DND41) and GSI-resistant (Jurkat and CCRF-CEM) T-ALL cell lines were maintained in RPMI-1640 containing 10% FBS, 2 mM GlutaMAX™ (Thermofisher) and 50U/mL Penicillin/Streptomycin. U2OS cells were maintained in DMEM (Dulbecco's Modified Eagle's medium) containing 10% FBS and 50U/mL Penicillin/Streptomycin. All cell lines were regularly tested for mycoplasma contamination. Cells were once treated with dimethyl sulfoxide (DMSO), 0.2 μM of γ-secretase inhibitor dibenzazepine (DBZ), 1 μM of N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), 1 μM of BMS-906024 (a kind gift from Bristol-Myers Squib) prepared in DMSO (Syncom, Groningen, The Netherlands), 15 μM of Chloroquine, 1 nM of Bafilomycin A1 (Sigma)

or a combined treatment at day 0. After this single treatment, cells were followed up for 4 or 7 days, as indicated. Chloroquine diphosphate salt (Sigma Aldrich) was dissolved in deionized water. PCR amplified fragment from monomeric RFP (Campbell et al., 2002) was Mlu1-Xba1 cloned into the murine N1∆E-Myc (van Tetering et al., 2009), replacing the Myc tag with a monomeric RFP tag. The pEGFP-LC3 construct (Kabeya et al., 2000) was a kind gift from N. Mizushima, from which GFP-LC3 was cloned into a pQCXIP vector using AgeI/EcoRI restriction sites.

Cell viability, proliferation and apoptosis assay

Trypan blue (Sigma Aldrich) exclusion was used to analyse GSI-sensitive and GSI-resistant T-ALL cell viability and living cells using a TC20™ Automated Cell Counter (Biorad). To determine cell viability and IC50 values CellTiter-Glo Cell Viability assay (Promega, G9682) was used according to the manufacturer's protocol after T-ALL cells were treated with a dilution range of DBZ or CQ. In short, at 7 days post-treatment CellTiter-Glo was added to the medium (1:1). Cells were lysed for 5 min on an orbital shaker and subsequently incubated for 25 min at room temperature. Luminescence was measured with an integration time of 0.5s using a filter-based multi-mode microplate reader (FLUOstar Omega, BMG Labtech). IC50 values of DBZ and CQ were estimated with the curve of the log (inhibitor) vs. response (Variable slope). To measure apoptosis a Pacific Blue™ Annexin V/SYTOX™ AADvanced™ Apoptosis Kit for flow cytometry (Thermofisher) was used according to the manufacturer's protocol. As a positive control ALL-SIL cells were treated with 10 µM of etoposide for 24 hours to induce apoptosis (data not shown). The number of cells in early and late apoptosis was analysed using a FACSCantoII cytometer with BD FACSDiva 6.1.1 software. Using FLowV10.1 doublets and cellular debris were excluded.

Immunoblotting

Cell lysates were prepared in 1x Laemlli loading buffer. Proteins were separated on Tris-HCL SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked in 5% dried skimmed milk (Marvel) and 0.05% Tween20 in TBS.

Protein detection was performed with subsequent primary antibodies: rabbit anti-cleaved caspase-3 Asp175 (5A1E)(Cell Signaling, cat. #9664, 1:1 000), rabbit anti-cleaved-PARP Asp214 (Cell Signaling, cat. #9541, 1:1 000), rabbit anti-LC3 (MBL International, cat. #PM036, 1:1 000), rabbit anti-Notch1 (D1E11) XP (Cell Signaling, cat. #3608 1:1 000), cleaved Notch1 (Val 1744)(D3B8)(Cell Signaling, #4147S, 1:1 000), rabbit anti-phospho-ATM (EP1890Y)(Abcam, cat. # ab81292, 1:1 000), mouse anti-ATM (Sigma Aldrich, cat. #A1106, 1:1 000), rabbit anti-phospho-Chk2 (T68) (C13C1)(Cell Signaling, cat. #2197S, 1:1 000), rabbit anti-phospho-Chk1 (Ser345)(133D3) (Cell Signaling, cat. #2348S, 1:1 000), mouse anti-P53 D0-7 (Santa Cruz, cat. #sc-47698, 1:1 000), mouse anti-actin clone C4 (MP Biomedicals, cat. # 691001, 1:20 000) and mouse anti-P62 (BD Biosciences, cat. # 610832, 1:1 000). Secondary antibodies used were anti-mouse (Cell Signaling, cat. #7076S, 1:5 000) or rabbit IgG-horseradish peroxidase (Cell Signaling, cat. #7074S, 1:5 000). Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) was used for visualization as described by the manufacturer.

Quantitative PCR

Total RNA was isolated using NucleoSpin RNA (Macherey-Nagel) from cells treated with DMSO, DBZ, CQ or combinational treatment at 4 days post-treatment according to the manufacturer's protocol. cDNA was obtained using Iscript cDNA synthesis kit (Biorad) followed by SYBR-green based reverse transcription quantitative PCR (RT-PCR) using SensiMix SYBR high-ROX kit (GC Biotech). mRNA expression was analysed using 3 µM of forward and reverse primers **(Supplementary Table 1)**. Cycle threshold (Ct) values were analysed with CFX Connect Real Time System (Biorad) and RPL13A was used as a housekeeping gene.

NOTCH receptor flow cytometry

NOTCH receptor availability on the cell surface was analysed by flow cytometry. ALL-SIL cells were fixed in 4% PFA and stained with a PE labelled mouse anti-human NOTCH1 antibody targeting the extracellular domain of NOTCH1 (Biolegend, cat. #352105) at 4 days post-treatment. A PE labelled mouse IgG1κ was used as an

isotype control (Biolegend). After staining, cells were analyzed using a FACSCantoII cytometer with BD FACSDiva 6.1.1 software. Using FLowV10.1 doublets and cellular debris were excluded. Mean fluorescent intensity (MFI) was determined and normalized to the control to obtain the Fold-Change in extracellular NOTCH1 receptor expression.

Cell cycle, DNA damage and cytoplasmic ROS analysis

Cell cycle analysis was performed using Click-iT™ Plus EdU Pacific Blue™ Flow Cytometry Assay Kit (Thermofisher) in combination with Propidium Iodide (PI) staining. Edu was incorporated for 1 hour at 37°C in cell culture conditions. The Click-iT reaction was performed according to the manufacturer´s protocol. For PI staining cells were incubated with 1 µg/ml of PI, 100 µg/ml of RNAse A and 0.1% TritonX-100 in PBS for 30 min at RT. An unstained, PI only, Edu only, and Click-iT only sample were used for compensation and correction for background and auto-fluorescence.

DNA damage was analysed by γH2AX expression. In short, cells treated with DMSO, DBZ, CQ, and the combinational treatment were fixed in 1% PFA and stored in 70% ethanol at -20°C overnight at 4 and 7 days post-treatment. Cells were stained overnight at 4°C with 1 µg of FITC conjugated mouse anti-phospho-Histone H2A.X (Ser139)(JBW301) (Merck, cat. #16-202A) in 1% BSA and 0.2% Triton X100 in PBS. As a control, ALL-SIL cells were irradiated with 2 Gy and stained for γH2AX at 1.5 hours post-irradiation. An unstained sample was used to correct for auto fluorescence.

Cytoplasmic ROS levels were analysed by incubating ALL-SIL cells treated with DMSO, DBZ, CQ, and the combinational treatment with 5 µM of CellROX Deep Red Reagent (Thermofisher) in serum free medium at 37°C for 30 min. As controls, ALL-SIL cells were (pre-incubated with 10 mM of N-Acetylcysteine (Sigma Aldrich) overnight and) incubated with 250 µM of H_2O_2 for 15 min at 37°C in serum free medium prior to CellROX staining. For cell cycle, DNA damage, and cytoplasmic ROS analysis a FACSCantoII cytometer with BD FACSDiva 6.1.1 software was used. FlowJo V10.1 was used to: exclude doublets and cellular debris, determine the distribution of cells within G0-G1, S, and G2-M phase, and to analyze the mean

fluorescent intensity (MFI). MFI was normalized to the DMSO control to obtain the Fold-Change in γH2AX and cytoplasmic ROS levels.

Transfection and N1ICD staining

U2OS cells were transfected with Polyethylenimine (Pei)(Polysciences, cat. #23966) to express ΔE-RFP (and GFP-LC3). At 24h hours post-transfection cells were treated once with DMSO, DBZ, CQ, or combined treatment. Cells were fixed in 4% paraformaldehyde, incubated in 50 mM of Glycine, and nuclei were stained with Dapi at 24 hours post-treatment. For N1ICD staining, cells were permeabilized with 0.1% triton X in PBS after Glycine incubation and blocked in 1% BSA for 30 min at RT. Cells were incubated with rabbit anti-cleaved Notch1 (Val 1744)(D3B8)(Cell Signaling, cat. #4147S, 1:100) for 1 hour at RT, washed in PBS, and incubated with goat anti-Rabbit IgG- Alexa Fluor 488 (Invitrogen, cat. #A11008, 1: 500) for 30 minutes at RT. Microscopic analysis was performed using an inverted Leica SPE confocal microscope and Leica LAS AF Lite software.

Statistical analysis

Data are presented as mean including the standard error of the mean (SEM) of three independent experiments. Statistical analyses were performed using GraphPad Prism 5 and statistical significance was defined as p-value<0.05.

Results

Chloroquine inhibits T-ALL cell proliferation and induces apoptosis synergistically when combined with γ-secretase inhibition *in vitro*

Because recent evidence indicates that γ-secretase activity not only occurs at the cell surface, but also in vesicles, we addressed whether blocking vesicle function with CQ would attenuate the survival of T-ALL cells requiring ligand-independent NOTCH1 signalling for their survival. To answer this question, GSI-sensitive T-ALL cell lines (ALL-SIL, DND41, and HPB-ALL) were treated once with DMSO, the γ-secretase inhibitor DBZ, CQ, or a combination of DBZ and CQ. Indeed, DBZ treatment significantly reduced the number of living ALL-SIL cells as compared to

controls **(Figure 1A)**, while the effect on cell viability was only minor at 7 days post-treatment **(Figure 1B)**. Interestingly, ALL-SIL cells treated with CQ showed a significant decrease in cell viability that was accompanied with a significant lower number of living cells compared to vehicle treated cells. Combining DBZ and CQ showed additional inhibitory effects on ALL-SIL cell viability at 7 days post-treatment compared to single treatments. Similar observations were made in other GSI-sensitive T-ALL cell lines (HPB-ALL and DND41) in which γ-secretase inhibition significantly reduced both cell viability and proliferation **(Figure S1A, S1B)**. Consistent with these findings, combination treatment of DBZ and CQ in HPB-ALL and DND41 cells had a stronger effect on cell viability compared to single treatments. Interestingly, CQ treatment showed a similar effect in HPB-ALL cells, whereas in DND41 cells the number of living cells was reduced at 7 days post-treatment without a significant effect on cell viability. We obtained similar results using a structurally distinct γ-secretase inhibitor, DAPT, as single treatment or when combined with CQ **(Figure S1C)**, which also effectively blocked Val1744 N1ICD cleavage at 7 days post-treatment **(Figure S1D).** As expected, γ-secretase inhibition had no effect (Palomero et al., 2007) on cell viability in GSI-resistant T-ALL cell lines (CCRF-CEM and Jurkat) **(Figure S2A, S2B)**, despite the fact that DBZ was effective in blocking NOTCH1 Val1744 cleavage and N1ICD formation **(Figure S2C)**. In Jurkat cells, but not in CCRF-CEM, GSI reduced cell proliferation. Neither CQ nor combined treatment had an effect on GSI-resistant T-ALL cell viability, while both treatments showed a significant reduction in GSI-resistant T-ALL cell proliferation.

To test whether the effect of DBZ could be extrapolated to γ-secretase inhibition in general, ALL-SIL cells were treated with DAPT or BMS906024 as a single treatment and combined with CQ. Both structurally different γ-secretase inhibitors showed similar results on cell viability and proliferation compared to DBZ treatment **(Figure S3A, S3B)**. Bafilomycin A1(BAFA), a V-ATPase inhibitor which, like CQ, inhibits lysosomal and endosomal fusion (Yoshimori et al., 1991), also significantly decreased ALL-SIL cell proliferation, and reduced cell viability albeit not significantly. However, BAFA treatment combined with GSI did significantly decrease

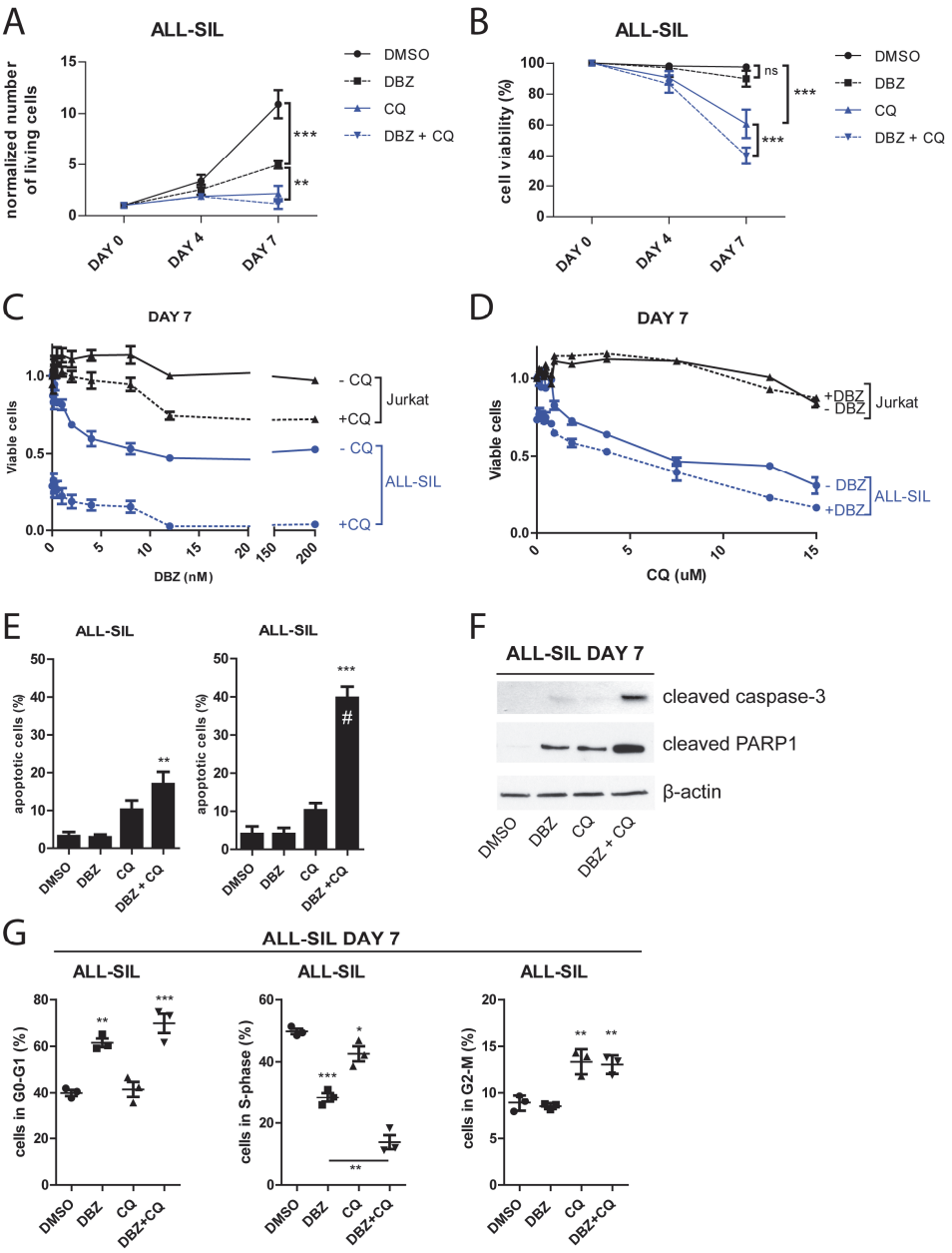


Figure 1. Chloroquine inhibits T-ALL cell viability and survival when combined with GSI. A) *Time course of the number of living ALL-SIL cells treated once with DMSO, DBZ, CQ, or combined treatment followed until 7 days post-treatment (2-Way ANOVA, **P<0.01, ***P<0.001).* ***B)*** *Cell viability time course of ALL-SIL cells treated once with DMSO, DBZ, CQ,*

*or combined treatment followed until 7 days post-treatment (2-Way ANOVA, ns: non-significant, ***P<0.001). C) Assessment of viable T-ALL cells, both GSI-sensitive (ALL-SIL) and GSI-resistant (Jurkat), using CellTiter Glo cell viability assay. T-ALL cells were treated once with a dilution range of DBZ (IC50 =2.0 nM) with or without 15 μM of CQ and cell viability was determined at 7 days post-treatment.* ***D)*** *GSI-sensitive (ALL-SIL) and GSI-resistant (Jurkat) T-ALL cells were treated once with a dilution range of CQ (IC50 = 14.2 μM) with or without 2 nM of DBZ. CellTiter Glo cell viability assay was used to determine cell viability at 7 days post-treatment.* ***E)*** *Quantification of the total percentage of early and late apoptotic ALL-SIL cells based on Annexin V and SYTOX AADvanced flow cytometry at 4 (left) and 7 (right) days post-treatment (1-Way ANOVA (Tukey comparison), **P<0.01, ***P<0.001, significant compared to DMSO). #Synergy was determined (2-Way ANOVA, ***P<0.001).* ***F)*** *Immunoblot analysis of cleaved-PARP1, cleaved-caspase-3 and β-actin (loading control) protein levels at 7 days post-treatment in ALL-SIL cells.* ***G)*** *Quantitative overview of the percentages of cells in G0-G1, S-, and G2-M phase in ALL-SIL cells at 7 days post-treatment assessed by Edu incorporation and Propidium Iodide (PI) staining (1-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01, ***P<0.001, significant compared to DMSO). DBZ: γ-secretase inhibitor dibenzoazepine, CQ: chloroquine. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

ALL-SIL cell viability and proliferation, although this effect was not as strong as observed with DBZ and CQ. All three GSI's (DBZ, DAPT, and BMS) blocked NOTCH1 Val1744 cleavage **(Figure S3C)**. In contrast, both CQ and Bafilomycin treatment showed an accumulation of N1ICD-Val1744. CQ treatment resulted in the accumulation of two TransMembrane/IntraCellular fragments (TMIC) NOTCH1 proteins derived from wild type and mutated allele (L1594P, ΔPEST) (Weng et al., 2004), and the membrane bound (S2) cleaved form, Notch Extracellular Truncation (NEXT). Interestingly, we detected a Val1744 fragment of higher molecular weight in CQ-treated ALL-SIL cells. The accumulation of NOTCH1 proteins was only observed with CQ and not observed with BAFA treatment.

Furthermore, we tested whether there was a dose dependent effect of DBZ and CQ on cell viability using a CellTiter Glo, which measures ATP producing (viable) cells. Both DBZ **(Figure 1C)** and CQ **(Figure 1D)** treatment showed a dose-dependent decrease in ALL-SIL viability at 7 days post-treatment with an IC50 of 2.0

(± 0.1) nM of DBZ and 14.2 (± 0.2) μM of CQ. Notably, a dose of 12.5 nM of DBZ showed a similar decrease in the number of living cells compared to a dose of 200 nM of DBZ. GSI-resistant Jurkat cells did not show a dose-dependent effect of DBZ or CQ, although CQ showed a small inhibitory effect on Jurkat cell viability at 15 μM. Moreover, both the addition of CQ (15 μM) to the DBZ dilution range and the addition of DBZ (2 nM) to the CQ dilution range further decreased ALL-SIL cell viability. These data indicate that a lower dose of GSI can be used to obtain a similar decrease in T-ALL cell viability when combined with CQ. A similar dose-dependent effect on cell viability by DBZ treatment was also shown in HPB-ALL cells **(Figure S3D),** which was further reduced by the addition of CQ. However, HPB-ALL cells showed to be significantly more sensitive to GSI inhibition compared to ALL-SIL cells **(Figure S3E).**

Next, we investigated if apoptosis was the mechanism by which single and combined treatment reduced cell viability in T-ALL by using Annexin V/AAD staining. In ALL-SIL cells, the total percentage of early and late apoptotic cells was not affected by γ-secretase inhibition at 4 and 7 days post-treatment (in accordance with a main effect on proliferation rather than survival as observed previously), but CQ increased the number of Annexin V+ and AAD+ cells by 10% (*p-value = 0.09*) at 4 days post-treatment **(Figure 1E)**. This effect was significantly enhanced in cells treated with both DBZ and CQ at 4 and 7 days post-treatment resulting in 17% and 40% of apoptotic cells, respectively, compared to 5% in vehicle-treated controls. These findings were supported by increased protein levels of cleaved caspase-3 and cleaved PARP1 at 7 days post-treatment **(Figure 1F)**. These data are in line with our cell viability results and show that CQ attenuates T-ALL cell viability and induces apoptosis, especially in combination with γ-secretase inhibition, which showed a synergistic effect. In HPB-ALL cells combined treatment also showed a synergistic increase in the number of apoptotic cells (80%) at 7 days post-treatment **(Figure S3F)**. Interestingly, DBZ treatment showed also a high number (20%) of early apoptotic cells, which combined with late apoptotic cells increased to 40% of HPB-ALL cells in apoptosis. Moreover, when a DBZ dose of 12.5 nM (16 fold lower) was added, which effectively blocked N1ICD formation **(Figure S4A)**, we could still obtain a synergistic effect with 20% of apoptotic cells in the combined treatment. GSI-

resistant cell lines did not show high numbers of apoptotic cells when combined treatment was applied **(Figure S4B)**.

Chloroquine enhances the cell cycle block exerted by GSI treatment

As indicated before, there may be a link between CQ treatment and cell cycle arrest due to p53 activation. Therefore, we first investigated whether CQ was able to induce alterations in cell cycle progression, as single and combined treatment with DBZ, by assessing EdU incorporation and PI for labelling DNA content by flow cytometry. In ALL-SIL cells, DBZ treatment increased the number of cells in G0/G1 phase with a concomitant reduction of cells in S-phase at 4 days post-treatment (data not shown). This effect was strongly enhanced at 7 days post-treatment where DBZ caused a 20% increase in G0/G1 and a two-fold reduction in S-phase cells **(Figure 1G)**. While CQ as single treatment showed only minor effects, it augmented the effect of DBZ by reducing the proportion the cells in S-phase from 28% (DBZ alone) to 14% (combination treatment) with 70% of cells arresting in G0/G1 phase at 7 days post-treatment. Furthermore, CQ treatment showed a mild, but significant, increase in the percentage of cells in G2-M. HPB-ALL cells were more sensitive to GSI treatment and already at 4 days post-treatment the percentage of cells in S-phase reduced from 55% to 13% (data not shown), which was not further enhanced with CQ. At 7 days post-treatment, there was a complete loss of cells in S-phase in DBZ-treated cells. Taken together, these data indicate that CQ enhances the G0/G1 cell cycle block induced by DBZ treatment in T-ALL, but the extent of this cell cycle block differs between T-ALL cell lines.

Chloroquine affects NOTCH1 trafficking, signalling, and turnover in T-ALL

Since combined treatment of DBZ and CQ showed synergistic effects on T-ALL survival compared to DBZ treatment alone and NOTCH1 mutated T-ALL cells rely on ligand-independent NOTCH signalling, we questioned whether CQ directly interfered with NOTCH1 signalling. As expected, NOTCH1 cleavage at Val1744, resulting in the release of NOTCH1 intracellular domain (N1ICD) and activation of NOTCH1, was blocked at 7 days post-DBZ treatment in ALL-SIL **(Figure 2A)**, HPB-

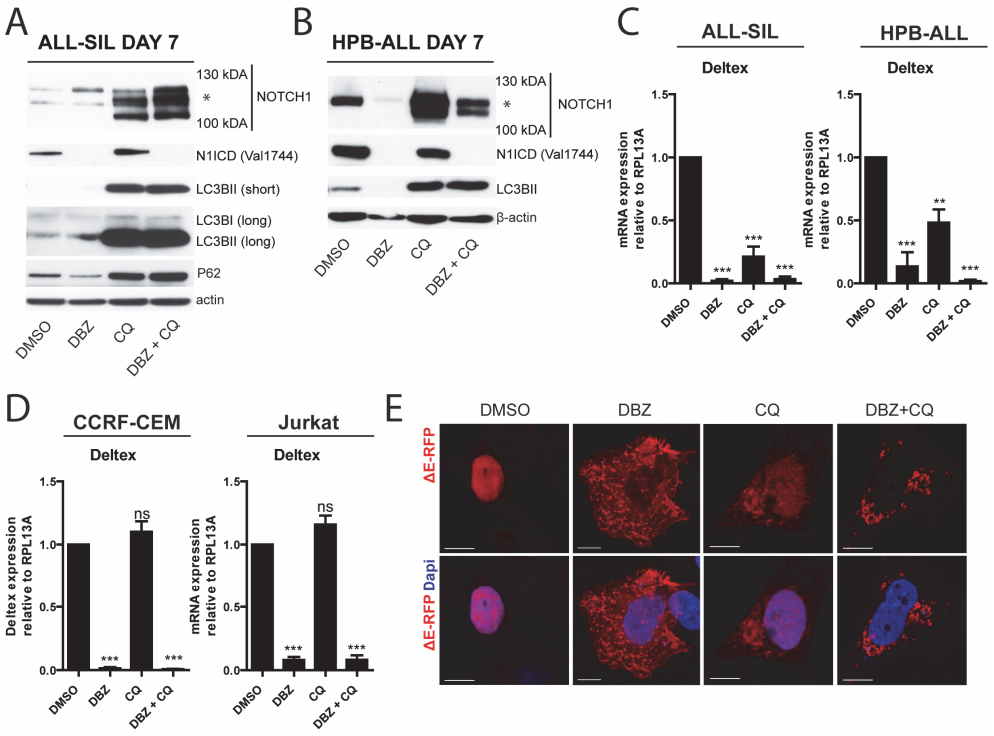


Figure 2. Chloroquine affects NOTCH1 trafficking, localisation, and turnover. A) *Immunoblot analysis of LC3BI and II, P62, cleaved NOTCH1, N1ICD (Val1744) and β-actin protein levels in ALL-SIL cells at 7 days post-treatment. *Asterisk indicates the accumulation of TMIC, NEXT, and NICD NOTCH1 cleaved fragments.* ***B)*** *Immunoblot analysis of LC3B-II, cleaved NOTCH1, N1ICD (Val1744) and β-actin protein levels in HPB-ALL cells at 7 days post-treatment. *Asterisk indicates the accumulation of TMIC, NEXT, and NICD NOTCH1 cleaved fragments.* ***C)*** *RT-PCR for NOTCH1 target gene Deltex at 4 days post-treatment in GSI-sensitive (ALL-SIL and HPB-ALL) cells. RPL13A was used as housekeeping gene (1-way ANOVA (Tukey comparison), **P<0.01, ***P<0.001, significant compared to DMSO).* ***D)*** *RT-PCR for NOTCH1 target gene Deltex at 4 days post-treatment in GSI-resistant (CCRF-CEM and Jurkat) cells and RPL13A (housekeeping gene) (1-way ANOVA (Tukey comparison), ns: non-significant, ***P<0.001, significant compared to DMSO).* ***E)*** *U2OS cells transfected with ΔE-RFP treated with DMSO, DBZ, CQ, and combined treatment for 24 hours, counterstained with Dapi. Scale bar: 10 μm. LC3B: membrane associated microtubule-associated protein 1 light chain 3, N1ICD: NOTCH1 intracellular domain. DBZ: γ-secretase inhibitor dibenzoazepine, CQ: chloroquine. Data and images are representative of three independent experiments and values are expressed in mean ± SEM.*

ALL **(Figure 2B),** CCRF-CEM, and Jurkat **(Figure S2C)** cells. In CQ treated samples N1ICD-Val1744 levels increased in ALL-SIL cells and we also observed a strong increase in membrane-bound NOTCH1 receptor fragments in CQ-treated and combination treated ALL-SIL, HBP-ALL, Jurkat, and CCRF-CEM cells **(Figure 2A, 2B, S2C and S3C)**. In both GSI-sensitive **(Figure 2C)** and GSI-resistant **(Figure 2D)** T-ALL cells expression of the NOTCH1 target gene *Deltex* was inhibited by DBZ. CQ treatment also reduced *Deltex* expression but only in GSI-sensitive T-ALL despite high levels of N1ICD **(Figure 2C)**. Notably, in GSI resistant T-ALL cell lines, with only wild-type NOTCH1 signalling, CQ treatment did not affect NOTCH1 target gene expression (**Figure 2D**), indicating a key difference in the effect of CQ on wild-type and mutant NOTCH1 signalling.

Since the integrity of the endo/lysosomal compartment can regulate NOTCH1 signalling (Man et al., 2017), we determined if CQ affected NOTCH1 signalling by attenuating endo/lysosomal function in T-ALL. As expected, CQ inhibited lysosomal breakdown of autophagy-related vesicles (autophagosomes), as indicated by accumulation of membrane associated microtubule-associated protein 1 light chain 3 (Atg8) LC3B-II, the golden standard for autophagic flux at 7 days post-treatment **(Figure 2A).** In addition to LC3B-II, the levels of the autophagosome cargo protein P62 also accumulated in CQ-treated ALL-SIL cells. Similar observations were made in HPB-ALL cells **(Figure 2B).**

We hypothesized that inhibition of the endosomal and lysosomal pathway by CQ led to deregulated NOTCH1 trafficking. Therefore, the expression of NOTCH1 at the cell surface was assessed by flow cytometry, using an antibody specific for the extracellular domain of NOTCH1. NOTCH1 receptor availability at the cell surface was significantly increased in ALL-SIL cells treated with GSI compared to DMSO treated cells at 4 days post-treatment **(Figure S4C)**. In contrast, a significant decrease in NOTCH1 extracellular domain expression at the cell surface was found in ALL-SIL cells treated with CQ. When GSI-treatment was combined with CQ the effect of GSI was abolished resulting in a similar NOTCH1 extracellular domain expression compared to vehicle-treated cells. Next, we transfected U2OS cells with a delta-E-RFP (ΔE-RFP) construct, in which the complete extracellular domain of

NOTCH1 is deleted, but the S2-cleavage site is intact and the PEST domain is exchanged for RFP, which signals ligand-independently, similar to mutated NOTCH1 in leukemic cells. At 24 hours post-treatment, control treated cells showed ΔE-RFP expression exclusively in the nucleus **(Figure 2E)**, while DBZ-treated cells only showed membrane/cytoplasmic ΔE-RFP, as expected. In CQ-treated cells we observed a reduction in nuclear ΔE-RFP and a cytoplasmic accumulation. When DBZ and CQ were combined, no nuclear localisation of the ΔE-RFP was observed and all ΔE-RFP localized to cytoplasmic vesicle-like structures. Together these data suggest that CQ treatment leads to NOTCH1 accumulation in the cytoplasm and this accumulation is enhanced when CQ is combined with DBZ treatment, indicating that CQ has an effect on NOTCH1 receptor localisation, trafficking, and turnover.

Ligand-independent NOTCH1 trafficking, localisation, and signalling are disturbed by chloroquine

To further elucidate the effect of CQ on ligand-independent NOTCH1 trafficking and signalling, we combined ΔE-RFP with the autophagosomal marker GFP-LC3 transfection to see whether ΔE-RFP co-localized with LC3-positive vesicles. Indeed, GFP-LC3 puncta strongly accumulated in CQ and combination treated U2OS cells **(Figure 3A)**, while no accumulation was observed in control and DBZ-treated cells. Both CQ and combined treatment showed partial co-localisation of ΔE-RFP and GFP-LC3, however, ΔE-RFP was also detected in LC3-negative vesicles. These data indicate that ligand-independent NOTCH1 trafficking is disturbed by CQ treatment and leads to accumulation of NOTCH1 in autophagosomes, but also in LC3-negative vesicles.

Next, we hypothesized that also N1ICD may accumulate outside of the nucleus in CQ treated cells, as we previously showed high N1ICD protein levels and reduced NOTCH target gene activation in CQ-treated T-ALL cells. Therefore, we transfected U2OS cells with ΔE-RFP and stained for Val1744 (N1ICD). As expected, N1ICD accumulated in the nucleus in control-treated cells and co-localized with ΔE-RFP expression, which was undetectable in DBZ- or combined-treated cells **(Figure 3B)**. Most interestingly, ΔE-RFP and N1ICD expression did not only co-localize in

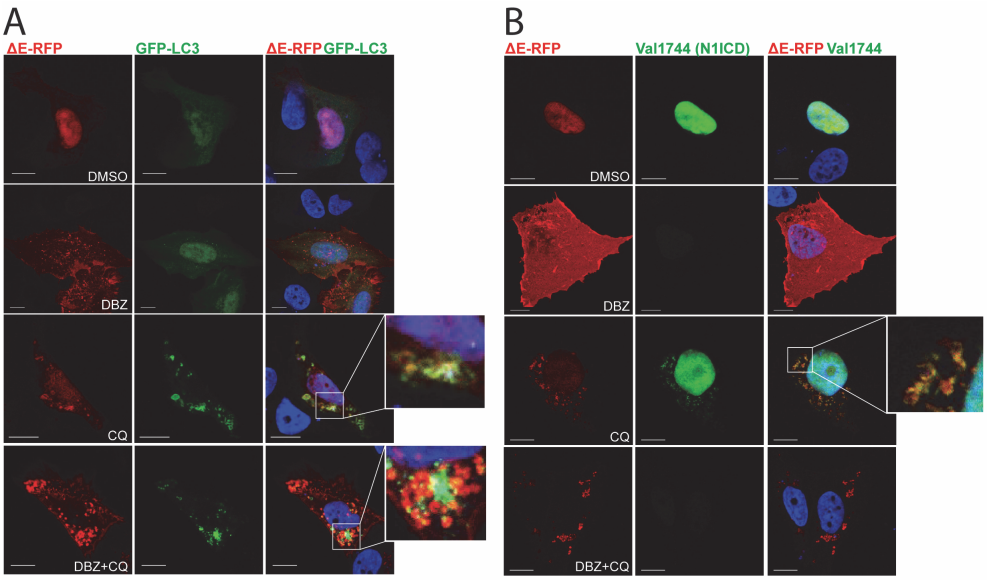


Figure 3. Ligand-independent NOTCH1 trafficking, localisation, and signalling are disturbed by CQ. A) *U2OS cells transfected with ΔE-RFP and GFP-LC3 treated with DMSO, DBZ, CQ, and combined treatment for 24 hours, counterstained with Dapi. Magnification is used to show co-localization of ΔE-RFP and GFP-LC3 indicated by the white square.* ***B)*** *U2OS cells transfected with ΔE-RFP treated with DMSO, DBZ, CQ, and combined treatment for 24 hours, stained for Val1744 (N1ICD), and counterstained with Dapi. Magnification is used to show co-localization of ΔE-RFP and Val1744 (N1ICD) indicated by the white square. LC3B: membrane associated microtubule-associated protein 1 light chain 3, N1ICD: NOTCH1 intracellular domain. DBZ: γ-secretase inhibitor dibenzoazepine, CQ: chloroquine. Scale bar: 10 μm. Images are representative of three independent experiments.*

the nucleus but also in the cytoplasm of CQ-treated cells. Altogether, our data suggest that CQ affects both ligand-independent NOTCH1 receptor and N1ICD trafficking and localisation.

Chloroquine induces an accumulation of ROS in T-ALL

Redox imbalance due to high levels of ROS is associated with DNA damage and cell cycle arrest. CQ has been shown to increase cellular ROS levels in solid cancer cells with defective autophagy-mediated degradation of mitochondria (mitophagy)

(Rouschop et al., 2009). Flow cytometry analysis showed increased cytoplasmic ROS levels in ALL-SIL cells treated with CQ compared to vehicle treated cells at 4 days post-treatment **(Figure 4A)**. Interestingly, at 7 days post-treatment also DBZ-treated cells showed increased levels of cytoplasmic ROS, albeit to a smaller extent than with CQ. Furthermore, combined treatment showed the highest accumulation of cytoplasmic ROS levels. Similar results were obtained in HPB-ALL cells **(Figure 4B)**. CQ treatment alone also induced a 2-fold increase in cytoplasmic ROS levels, similar to ALL-SIL cells. A high dose of DBZ and combined treatment induced a massive ROS accumulation at 7 days post-treatment. Even at a 16-fold lower dose of DBZ significant interaction with CQ in the induction of cytoplasmic ROS levels was obtained. In GSI-resistant cells lines DBZ treatment did not increase cytoplasmic ROS levels **(Figure S4D)**. However, in Jurkat cells CQ treatment significantly increased ROS levels, which was not observed in CCRF-CEM cells.

Chloroquine induces DNA damage and a subsequent activation of the DNA damage response

High levels of ROS cause activation of the DNA damage response (DDR) and can lead to cell cycle arrest. Flow cytometry analysis of CQ-treated ALL-SIL cells showed increased γH2AX (a marker of dsDNA breaks) expression by 2-fold at 4 days post-treatment, which was similar when combined with DBZ **(Figure 4C)**. At 7 days post-treatment γH2AX expression was further increased by CQ treatment and with the addition of DBZ, γH2AX expression increased up to 3-fold compared to vehicle-treated cells. In HPB-ALL cells, γH2AX protein expression was also significantly increased when treated with CQ **(Figure 4D)**. DBZ treatment (200 nM) already induced a 2-fold increase in γH2AX expression, which was not further increased by the combined treatment. Lowering the dose of DBZ (12.5 nM) did not show an increase in γH2AX expression, however, when combined with CQ treatment, a significant increase in γH2AX levels was obtained. GSI-resistant T-ALL cell lines did not show increased γH2AX expression **(Figure S4E)**. Since we demonstrated elevated levels of ds DNA breaks, we assessed the DDR in CQ-treated cells **(Figure 4E)**. Consistent with the increase in DNA damage, increased levels of

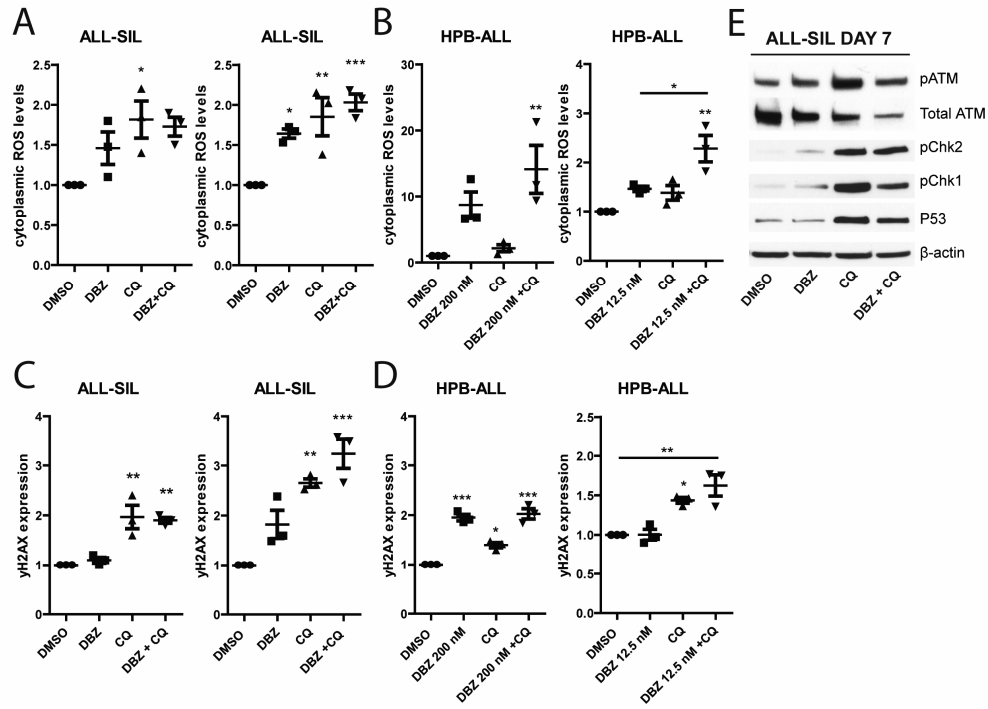


Figure 4. Chloroquine treatment elevates ROS levels and activates the DNA damage response. A) *Quantification of cytoplasmic ROS levels in ALL-SIL cells at 4 (left) and 7 (right) days post-treatment analysed by CellROX labelling by flow cytometry (1-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01, ***P<0.001, significant compared to DMSO).* ***B)*** *Flow cytometry analysis of cytoplasmic ROS levels in HPB-ALL cells at 7 days post-treatment using a high dose (200 nM, left) and low dose (12.5 nM, right) of DBZ (1-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01, significant compared to DMSO).* ***C)*** *Flow cytometry analysis of γH2AX expression at 4 (left) and 7 (right) days post-treatment in ALL-SIL cells (1-Way ANOVA (Tukey comparison), **P<0.01, ***P<0.001, significant compared to DMSO).* ***D)*** *Quantification of γH2AX expression in HPB-ALL cells at 7 days post-treatment using a high dose (200 nM, left) and low dose (12.5 nM, right) of DBZ (1-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01, ***P<0.001, significant compared to DMSO).* ***E)*** *DNA damage response protein expression analysis by immunoblot in ALL-SIL cells at 7 days post-treatment. β-actin was used as loading control. DBZ: γ-secretase inhibitor dibenzoazepine, CQ: chloroquine, ATM: Ataxia telangiectasia mutated kinase, Chk1: Checkpoint kinase 1, Chk2: Checkpoint kinase 2. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

phosphorylated ATM kinase (pATM) and downstream cascades pCHK1, pCHK2, and p53 induction were observed in CQ- and combination-treated ALL-SIL cells at 7 days post-treatment. DBZ treatment also showed a slight increase in pATM. Taken together, these data indicate that the DDR is activated, due to induction of DNA damage by CQ treatment, explaining a persistent cell cycle arrest observed in T-ALL cells.

Discussion

Here, we show for the first time that the clinically used lysosomotrophic drug CQ decreases T-ALL cell viability and proliferation. We demonstrate that γ-secretase inhibition induces a potent G0-G1 cell cycle arrest, which is more pronounced upon CQ addition. Whereas GSI treatment predominantly blocks T-ALL proliferation, CQ treatment induces apoptosis, which is synergistic in the combination treatment. The anti-proliferative effect of DBZ is dose-dependent and a general effect of γ-secretase enzyme inhibition, since three structurally different GSI's show a similar effect. Furthermore, CQ also shows a dose-dependent decrease in cell viability in GSI-sensitive T-ALL. When using Bafilomycin, which also blocks lysosomal and endosomal fusion, instead of CQ, we obtained comparable effects on T-ALL cell viability and proliferation, albeit less strong. Above-mentioned effects of DBZ and CQ are not observed in GSI-resistant T-ALL cell lines. Interestingly, our *in vitro* results demonstrate that the addition of CQ to GSI treatment reduces the concentration of GSI required to obtain reduced cell proliferation and viability. Importantly, the interaction between GSI and CQ is synergistic with respect to apoptosis induction and allowed a reduction of 16-fold of the dose of GSI. This strategy may be applied to increase the therapeutic ratio of GSI in T-ALL treatment by reducing GSI-induced gastro-intestinal toxicity while achieving a synergistic anti-proliferative and pro-apoptotic effect in leukemic cells.

Mechanistically, we show that CQ treatment increases cytoplasmic levels of ROS, implicating a redox imbalance, which might result from an accumulation of mitochondrial mass, indicative of blocking mitophagy, resulting in the accumulation of (damaged) mitochondria, previously shown by our lab in solid tumours (Rouschop

et al., 2009), but not in T-ALL. Notably, also γ-secretase inhibition showed increased levels of cytoplasmic ROS in T-ALL, which may result from inhibition of the ROS responsive (Nrf2/Keap1) pathway. This pathway normally neutralizes ROS and is stimulated by NOTCH signalling (Wakabayashi et al., 2014). Increased mitochondrial mass and cytoplasmic ROS levels resulting from CQ treatment were also found in other tumour types, including: glioma and cholangiocarcinoma (Hori et al., 2015; Qu et al., 2017) and induced cytotoxicity in cancer stem cells (Zhang et al., 2015). Together with our data, these results suggest a general dependency of tumour cells on autophagy to maintain their redox balance. Furthermore, DBZ treatment showed a minor increase in DDR activation as expected since NOTCH1 has been shown to be a negative regulator of the DNA damage response (Kim et al., 2007; Vermezovic et al., 2015). In CQ- and dual-treated T-ALL, we observed increased levels of DNA damage and activation of the DDR that can be responsible for the cell cycle arrest we observed.

Previously, CQ has been shown to activate ATM, in the absence of any DNA damage, resulting in p53-induced cell death in a lymphoma mouse model (Maclean et al., 2008). Indeed, in GSI-sensitive T-ALL cells we observed that CQ treatment increased p53 levels and also increased ds DNA breaks and apoptosis. This is consistent with CQ enhanced p53-induced apoptosis shown in a Myc-induced model of lymphoma (Amaravadi et al., 2007) and CQ-induced secretion of tumour suppressor protein PAR-4 via p53 in mice and cancer patients leading to apoptosis (Burikhanov et al., 2017). Although likely we have not directly demonstrated that ROS are responsible for the increased DNA damage, others have shown that CQ up-regulates p53 expression via ROS in which p53 was in turn also able to stimulate ROS formation (Chen et al., 2017). Together, our data are consistent with an important role for p53 activation induced by CQ treatment and a complex relationship between p53, autophagy and redox balance in several tumour types.

Besides the promising effects of CQ on GSI-sensitive T-ALL cells observed in this study, CQ was already proposed to have anti-cancer activity in various different tumour types (Maclean et al., 2008; Rouschop et al., 2010; Sasaki et al., 2010) and is being investigated in many clinical trials, to evaluate the safety and

efficacy of CQ in different types of cancer, including: breast cancer, melanoma, prostate cancer, lung cancer, and colorectal cancer, but not in T-ALL (Shi et al., 2017). Interestingly, CQ was shown to normalize tumour vasculature, thereby increasing perfusion, reducing tumour hypoxia, and increasing the response to treatment (Maes et al., 2014). This vasculature normalization was shown to be dependent on NOTCH1 activation specifically within endothelial cells. Interestingly, in these experiments the action of CQ seemed to be autophagy independent. While we show that CQ affects autophagic flux, we did not prove that inhibition of autophagy was required for its anti-leukemic effects.

Previous studies showed that NOTCH1 activation by γ-secretase processing occurs both on the cell surface and in endosomes (Tagami et al., 2008). NOTCH heterodimers at the cell surface expressing T-ALL mutations in the hetero-dimerization domain are subject to regulated proteolysis (Malecki et al., 2006) by Zn+ dependent ADAM metalloproteases and internalized to intracellular vesicles (van Tetering et al., 2009). Moreover, vesicle acidification was shown to be important, both for physiological ligand-dependent and ligand-independent NOTCH signalling (Vaccari et al., 2010). Therefore, we hypothesized that CQ treatment, which abrogates the fusion between endosomes, autophagosomes, and lysosomes by increasing the intra-vesicular pH, could effectively suppress oncogenic NOTCH1 signalling and therefore decrease cell viability of NOTCH-dependent T-ALL. We demonstrate that CQ treatment results in high levels of both nuclear and cytoplasmic Val1744 cleaved N1ICD. We note that Val1744 cleaved N1ICD co-localized with LC3 in autophagosomes but also in LC3-negative vesicles possibly in endosomes, where γ-secretase has been shown to be more active due to the low pH environments (Valapala et al., 2013). Moreover, we did observe a decrease in the expression of the downstream target gene *Deltex* of NOTCH1. This suggests that, together with our finding that CQ only attenuates Notch target gene expression in GSI-sensitive T-ALL cell lines, intracellular trafficking of NOTCH mutated proteins is an important contributor to Notch activity and a therapeutic vulnerability for GSI-sensitive T-ALL. Besides, we also demonstrated increased total levels of the TMIC fragment of NOTCH1 after CQ treatment, from which S2 cleavage has also been shown to be

pH dependent (Mathews et al., 2010). Interestingly, Bafilomycin also showed accumulation of cleaved N1ICD, but no accumulation of total cleaved NOTCH1 levels, indicating that the effect of CQ on NOTCH1 receptor turnover might be not specific to blocking endo-lysosomal fusion in general. Of interest to our work here is that the Ca^{2+} pump SERCA, previously identified as a therapeutic target for mutated NOTCH proteins (Roti et al., 2013), is also a substrate for Bafilomycin A (Mauvezin et al., 2015). Thus, while CQ and Bafilomycin both block NOTCH signaling they most probably do so in a different manner, which may have resulted in the observed differences in T-ALL cell viability. The exact contribution of each of these mechanisms to blocking NOTCH needs further investigation but illustrates that there may be multiple distinct intervention points that could be combined for targeting intracellular mutated NOTCH proteins.

Of special interest is the appearance and accumulation of an additional higher molecular Val1744-cleaved NOTCH1 fragment. We speculate that this fragment, which shows a shift of approximately 15 kDa, might be due to mono-ubiquitination. It has been shown previously that mono-ubiquitination of N1ICD is necessary preceding receptor endocytosis (Gupta-Rossi et al., 2004). Together our data suggest that CQ affects NOTCH1 receptor turnover and recycling, trapping the NOTCH1 receptor and its cleaved forms in intracellular vesicles including auto-phagosomes but also other unidentified vesicles, likely endosomes, and blocking lysosomal degradation of γ-secretase cleaved NOTCH proteins.

Thus, our results support the notion that autophagy may be a central survival mechanism in T-ALL and a therapeutic vulnerability when NOTCH signalling is blocked simultaneously. Recent evidence showed that γ-secretase inhibition in T-ALL leads to a metabolic shutdown, triggering autophagy as a rescue mechanism to support leukemic cell metabolism and proliferation (Herranz et al., 2015). Mice harbouring Atg7 deleted isogenic leukemia's treated with GSI induced a marked anti-leukemic response *in vivo* with improved survival. We indeed show that inhibition of autophagy by blockage of lysosomal function has an additional effect on T-ALL cell viability when combined with NOTCH inhibition. We confirm that autophagy inhibition using CQ in PTEN-negative T-ALL (i.e. Jurkat) does not sensitize GSI-resistant T-

ALL. This autophagy independence can be explained by activation of AKT signalling (Herranz et al., 2015; Palomero et al., 2007). In addition, in glioblastoma neurospheres GSI inhibition resulted in elevated levels of autophagy and simultaneous inhibition of NOTCH and autophagy led to decreased cell growth, proliferation, and clonogenicity with increased apoptosis *in vitro* (Natsumeda et al., 2016). Together, these results suggest that tumours with wild-type PTEN are particularly sensitive to small molecule inhibitors of the NOTCH and autophagy pathways.

Overall, our results reveal a novel application for small molecule inhibitors targeting intracellular vesicles, such as CQ and BAFA, to synergistically enhance the effect of GSI on blocking NOTCH signalling in leukemic cells with NOTCH mutations. While this needs further study one testable hypothesis is that this could be applicable to other NOTCH addicted cancers that are wild-type for PTEN as well. The repurposing of drugs, such as CQ, is of interest as developing new drugs is costly and time-consuming, while CQ, an already clinically approved drug with minor side effects, may widen the therapeutic window in cancer with activated NOTCH signalling.

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Conflict of interest

The authors declare no conflict of interest.

Author contributorship information

J.H., R.H., M.S., T.H. and L.B. performed the measurements and analysed the experimental data. J.H. drafted the manuscript and designed the figures under supervision of A.G. and M.V.

A.G., M.V., S.Y. and K.R. contributed to the design and implementation of the research. All authors discussed the results and commented on the manuscript.

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Supplementary information

Supplementary table 1. RT-PCR primer sequences.

Gene	Forward	Reverse
Deltex	5'-TGTGCCGCAAGACCAAGAAG-3'	5'-TCGTAGCCTGATGCTGTGAC-3'
RPL13A	5'-CCGGGTTGGCTGGAAGTACC-3'	5'-CTTCTCGGCCTGTTTCCGTAG-3'

Supplementary figures

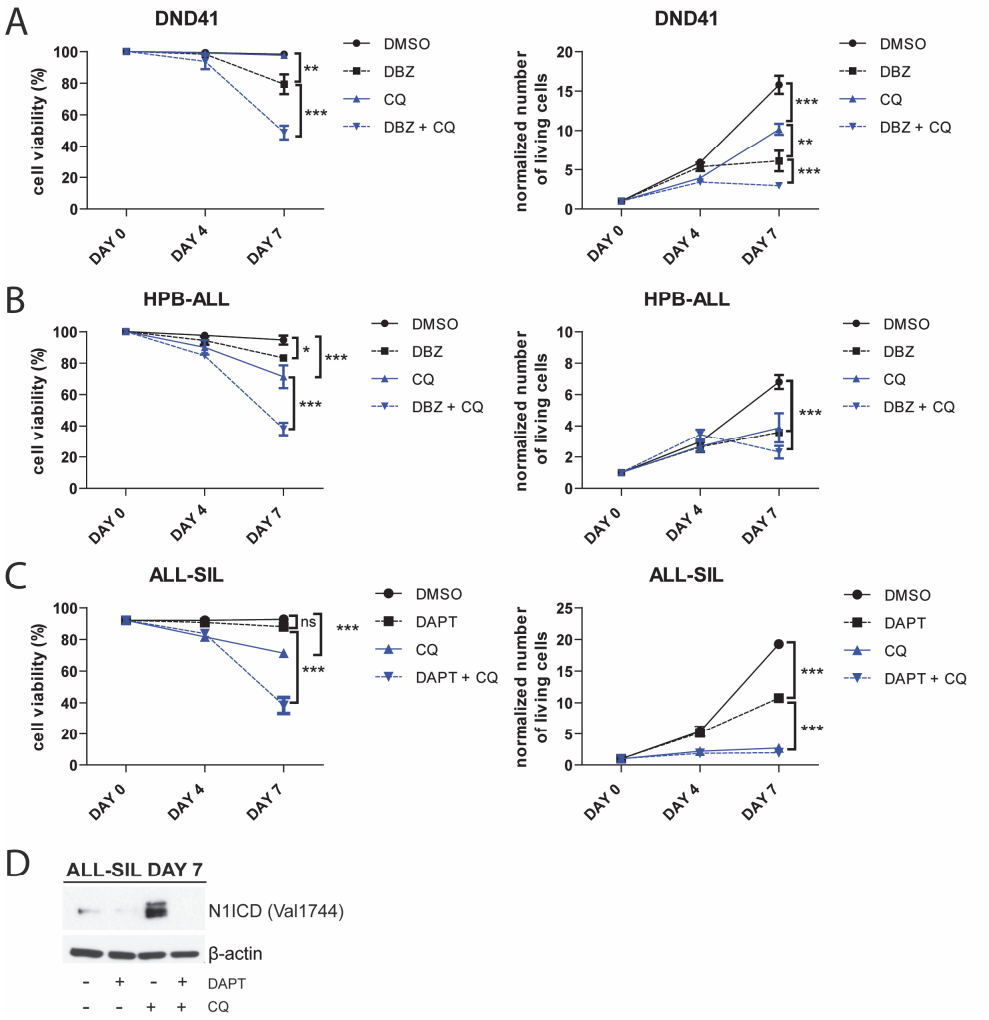


Figure S1. Chloroquine decreases cell viability in GSI-sensitive T-ALL in vitro. A) *Time course of GSI-sensitive DND41 cell viability and the number of living cells until 7 days post-treatment (2-Way ANOVA, **P<0.01, ***P<0.001).* ***B)*** *Time course of GSI-sensitive HPB-ALL cell viability and the number of living cells until 7 days post-treatment (2-Way ANOVA, *P<0.05, ***P<0.001).* ***C)*** *Time course of ALL-SIL cell viability and the number of living cells treated with DMSO, y-secretase inhibitor DAPT, CQ or combined treatment until 7 days post-treatment (2-Way ANOVA, ns: non-significant, ***P<0.001).* ***D)*** *Immunoblot analysis of N1ICD*

(Val1744) and β-actin protein levels in ALL-SIL cells at 7 days post-treatment. DBZ: γ-secretase inhibitor dibenzoazepine, CQ: chloroquine. Data are representative of three independent experiments and values are expressed in mean ± SEM.

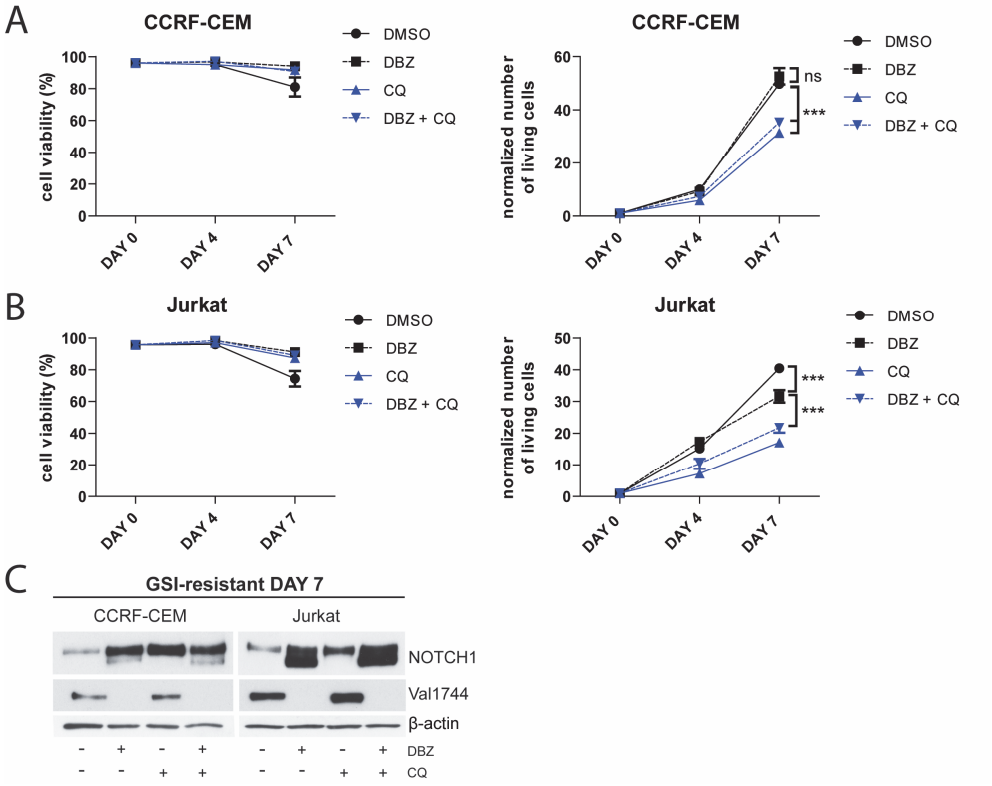


Figure S2. Chloroquine decreases GSI-resistant T-ALL cell proliferation and has no effect cell viability in vitro. A) *Time course of GSI-resistant CCRF-CEM cell viability and proliferation until 7 days post-treatment (2-Way ANOVA, ns: non-significant, ***P<0.001).* ***B)*** *Time course of GSI-resistant Jurkat cell viability and proliferation until 7 days post-treatment (2-Way ANOVA, ***P<0.001).* ***C)*** *Immunoblot analysis of cleaved NOTCH1 fragments, N1ICD (Val1744), and β-actin protein levels in GSI-resistant T-ALL cells at 7 days post-treatment. DBZ: γ-secretase inhibitor dibenzoazepine, CQ: chloroquine. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

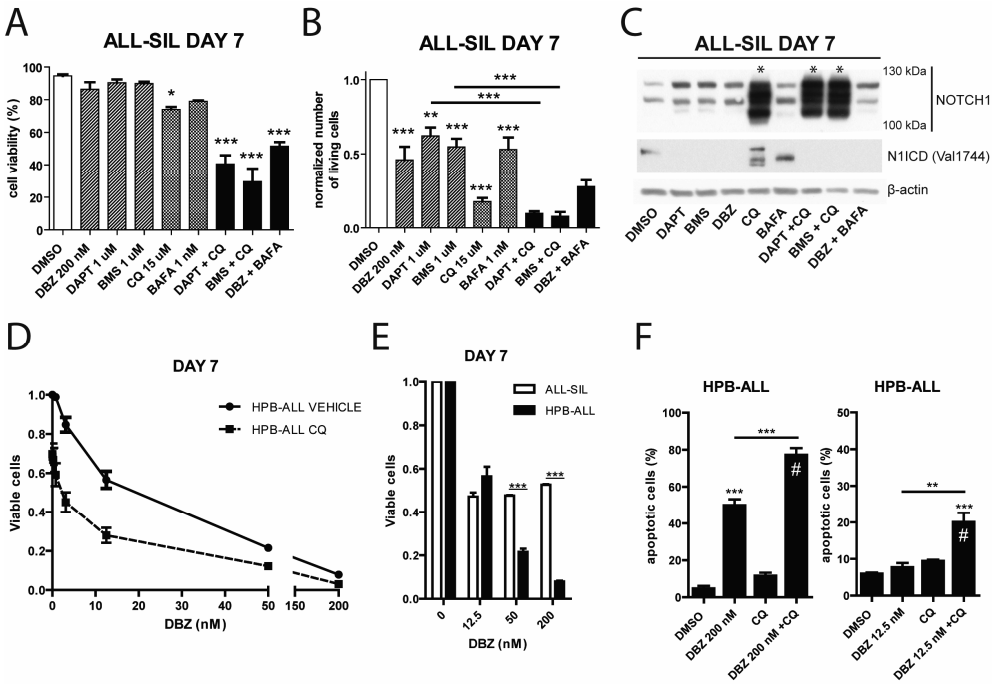


Figure S3. Structurally distinct γ-secretase inhibitors show similar effects when combined with Chloroquine or Bafilomycin and HPB-ALL cells are more sensitive to GSI in vitro. A) *ALL-SIL cell viability treated with DMSO, structurally different γ-secretase inhibitors (DBZ, DAPT and BMS), CQ, Bafilomycin or combined treatment at 7 days post-treatment (1-Way ANOVA (Tukey comparison), *P<0.05, ***P<0.001).* ***B)*** *The number of living ALL-SIL cells at 7 days post-treatment (1-Way ANOVA (Tukey comparison), **P<0.01, ***P<0.001).* ***C)*** *Immunoblot analysis of cleaved NOTCH1 fragments, N1ICD (Val1744) and β-actin protein levels in ALL-SIL cells at 7 days post-treatment. *Asterisks indicate the accumulation of TMIC, NEXT, and NICD NOTCH1 cleaved fragments.* ***D)*** *Assessment of viable HPB-ALL cells using CellTiter Glo cell viability assay. HPB-ALL cells were treated once with a dilution range of DBZ with or without 15 μM of CQ and cell viability was determined at 7 days post-treatment.* ***E)*** *Quantification of the number of viable ALL-SIL and HPB-ALL cells treated with different doses of DBZ (1-Way ANOVA (Tukey comparison), ***P<0.001).* ***F)*** *Quantification of the total percentage of early and late apoptotic HPB-ALL cells based on Annexin V and SYTOX AADvanced flow cytometry at 7 days post-treatment using a high dose (200 nM, left) and low dose (12.5 nM, right) of DBZ (1-Way ANOVA (Tukey comparison), ***P<0.001). #Synergy was determined for both the high (2-Way ANOVA, **P<0.01) and low dose of DBZ (2-Way ANOVA, *P<0.05). DBZ: γ-secretase inhibitor dibenzoazepine, CQ:*

chloroquine. Data are representative of three independent experiments and values are expressed in mean ± SEM.

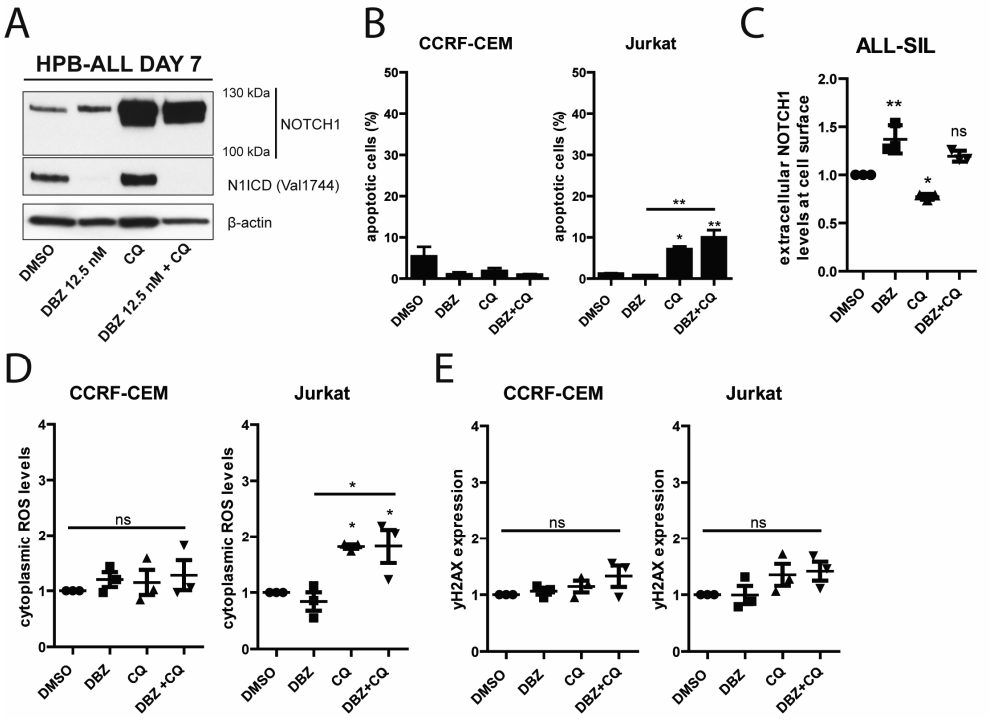
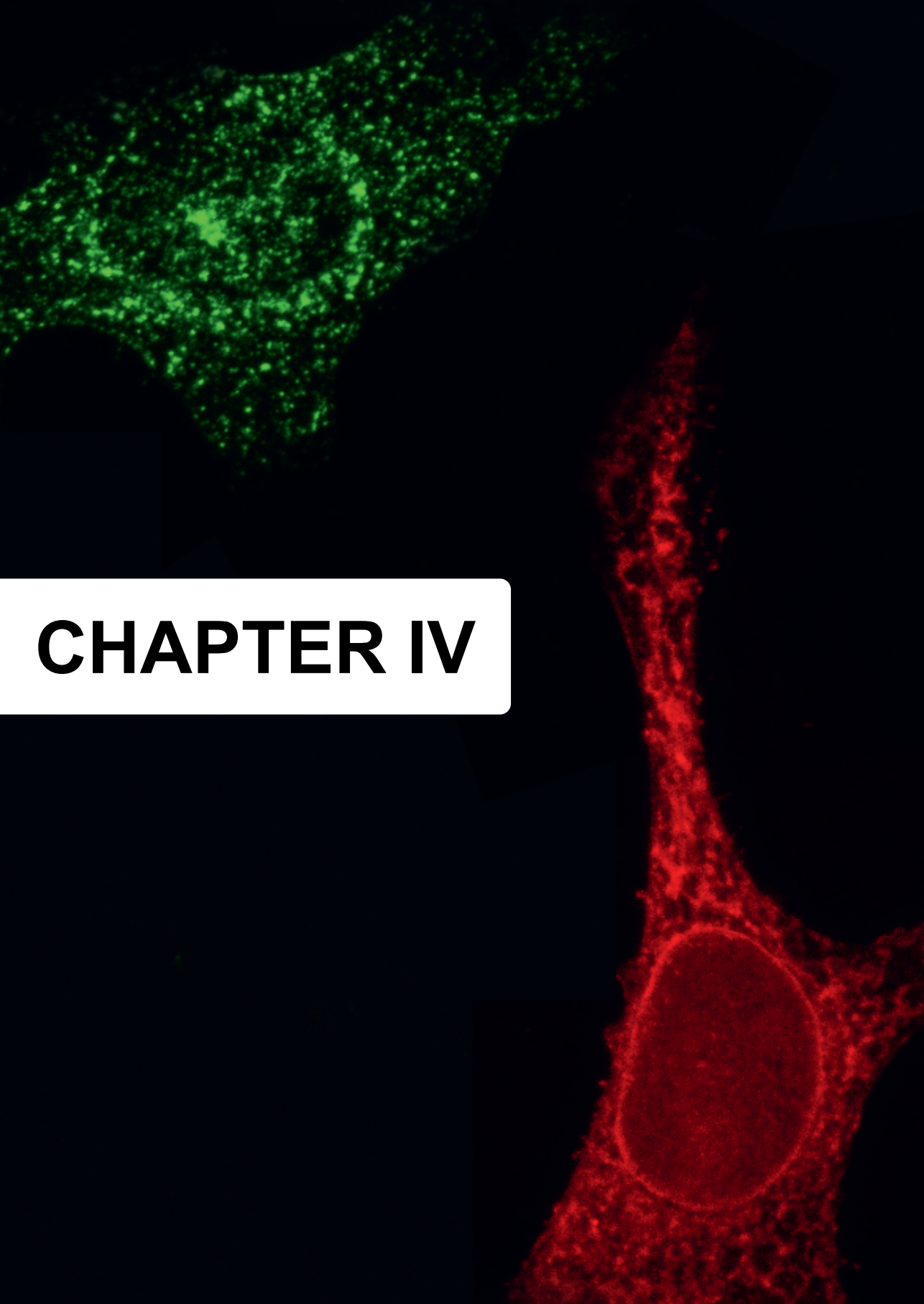
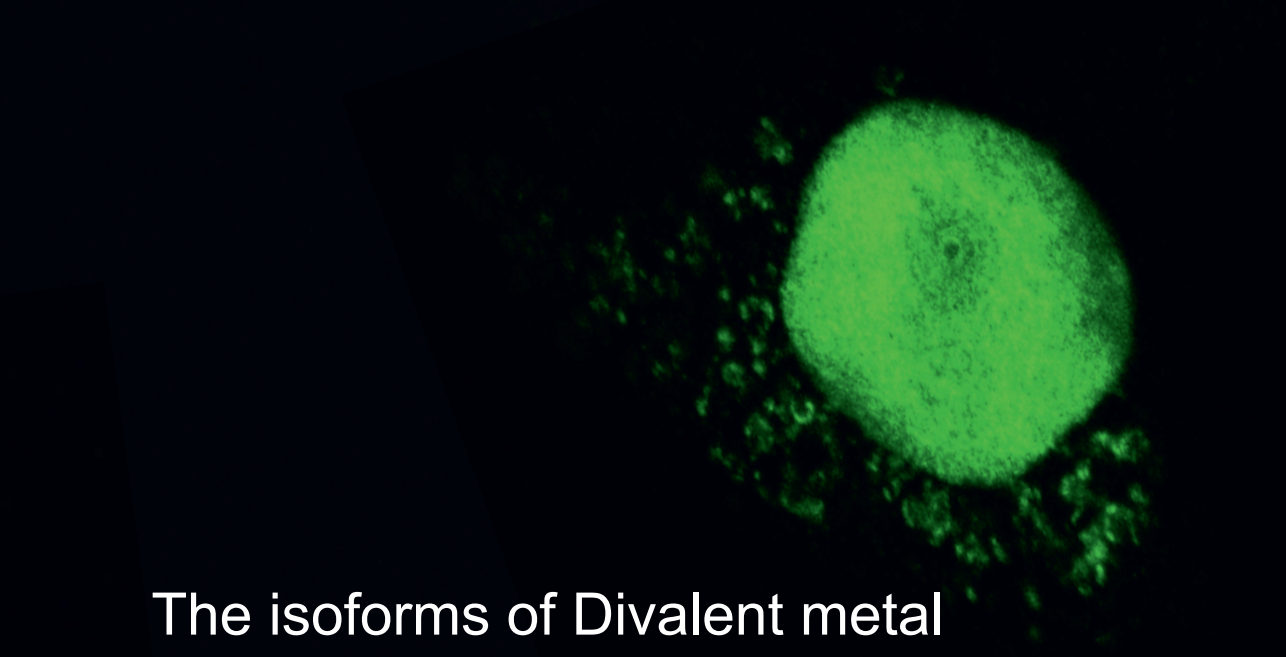


Figure S4. Chloroquine and combined treatment induce no apoptosis and ds breaks in GSI-resistant T-ALL cell lines. A) *Immunoblot analysis of cleaved NOTCH1, N1ICD (Val1744) and β-actin protein levels in HPB-ALL cells treated with a low dose of DBZ (12.5 nM) at 7 days post-treatment.* ***B)*** *Flow cytometry analysis of the total apoptotic GSI-resistant T-ALL cells at 7 days post-treatment (1-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01, significant compared to DMSO).* ***C)*** *Quantification of the mean fluorescent intensity (MFI) normalized to DMSO of NOTCH extracellular domain levels at the cell surface at 4 days post-treatment analysed by flow cytometry (1-Way ANOVA (Tukey comparison), ns: non-significant, *P<0.05, **P<0.01).* ***D)*** *Quantification of cytoplasmic ROS levels in GSI-resistant cell lines at 7 days post-treatment (1-Way ANOVA (Tukey comparison), ns: non-significant, *P<0.05, significant compared to DMSO).* ***E)*** *Flow cytometry analysis of γH2AX expression in GSI-resistant T-ALL cell lines at 7 days post-treatment (1-Way ANOVA (Tukey comparison), ns: non-significant). DBZ: γ-secretase inhibitor dibenzoazepine, CQ: chloroquine, MFI: mean fluorescent intensity. Data are representative of three independent experiments and values are expressed in mean ± SEM.*



CHAPTER IV



The isoforms of Divalent metal transporter 1 (Dmt1) differentially control Notch-mediated cell fate decisions

Judith Hounjet[1,2*], Arjan J. Groot[1*], Jolanda Piepers[1,2], Francesca Rapino[1], Kim R. Kampen[1], and Marc Vooijs[1,2#]

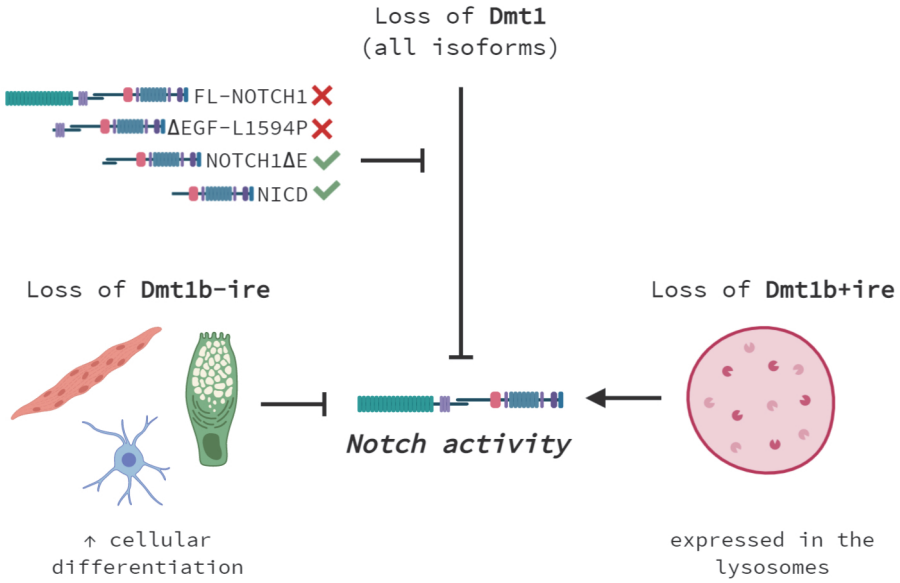
[1]Department of Radiation Oncology (Maastro), GROW School for Oncology, Maastricht University Medical Centre+, Maastricht, The Netherlands
[2]Maastro clinic, Maastricht, The Netherlands

**These authors share first authorship.*

[#]corresponding author: marc.vooijs@maastrichtuniversity.nl, Tel: +31 (0)433882912

In preparation

Graphical abstract



Abstract

Consecutive cleavage of Notch by Adam metallo-proteases and γ-secretase are essential rate-limiting steps in the activation of physiological Notch signaling. In cells in which Adam10 and Adam17 proteases are lacking, physiological Notch signaling is blocked. Nevertheless, ligand-independent Notch signaling, which remains processed by γ-secretase but becomes strongly dependent on endosomal and lysosomal function, stays active. Recently, we demonstrated that inhibition of endosomal and lysosomal vesicles strongly inhibited ligand-independent NOTCH signaling in T-ALL. To identify the novel rate-limiting steps in ligand-independent Notch1 signaling, we performed a shRNA screen in Adam 10/17 protease-deficient mouse embryonic fibroblasts (MEFs). We identified the Divalent metal transporter 1 (Dmt1), which strongly affected Notch1 activity, depending on the expression of the four different Dmt1 isoforms (Dmt1a and Dmt1b with or without an iron responsive element (ire)).

Interestingly, we show for the first time that the different isoforms of Dmt1 play an opposite role in Notch1 signaling activation. While knockdown of *Dmt1-ire* leads to a suppression of Notch1 activation, silencing of *Dmt1+ire* results in hyper-activation of Notch1 signaling in Adam10$^{-/-}$17$^{-/-}$ MEFs. Consistently, in both C2C12 muscle and Neuro2A neuronal cell models, silencing of *Dmt1-ire* resembles a Notch loss-of-function differentiation phenotype, which can be overruled by constitutive expression of active Notch1, while knockdown of *Dmt1+ire* blocks differentiation and mimics Notch1 gain-of-function. In the cancer context, a similar Notch1 loss-of-function phenotype is observed in human colorectal adenocarcinoma cells (LS174T), where silencing of *DMT1-IRE* induces spontaneous goblet cell differentiation. A complete loss of Dmt1 in Dmt1 knockout (KO) MEFs shows a strong inhibition of ligand-induced Notch target gene activation, which is rescued by constitutively active Notch1. Interestingly, Dmt1 KO MEFs show increased levels of Val1744-cleaved Notch and disturbed intracellular trafficking, including: increased EEA1, LAMP1 and Rab5 protein levels and increased basal autophagic flux indicative of active autophagy/lysophagy of damaged intracellular vesicles.

Our study identifies Dmt1 as a new major regulator of Notch activity, which in an isoform-specific manner, determines the fate of Notch molecules and outcome, upstream of transcriptional activation but downstream of γ-secretase cleavage. We propose that Dmt1 containing intracellular vesicles identify an important intracellular node, through which Notch signaling traverses and a vulnerability that can be modulated with small molecule inhibitors of Dmt1.

Introduction

NOTCH signaling is a highly conserved pathway involved in multiple cell differentiation processes during embryonic development and in adult life. In adults, NOTCH controls cell fate decisions within the hematopoietic system, gut and skin homeostasis, neurogenesis, and skeletal muscle regeneration (Siebel and Lendahl, 2017). NOTCH receptors are transmembrane-anchored transcriptional regulators, which are activated by ligand induced cleavage. Upon transcription of NOTCH1 in the nucleus, the receptor matures in the Golgi apparatus and is cleaved at the S1-site by Furin-like convertases after which the receptor (TMIC) travels to the cell membrane, where it is present in a “proteolysis-resistant state”. Upon ligand binding, the receptor unfolds and undergoes cleavage by Adam10 protease at the S2-site (NEXT). Subsequent cleavage at the S3-site by γ-secretase releases the NOTCH intracellular domain (NICD), which activates NOTCH downstream target genes (Kopan and Ilagan, 2009). This signaling paradigm is similar for physiological ligand induced activation of NOTCH1, NOTCH2, and NOTCH3 receptors (Groot et al., 2014; Saxena et al., 2001).

Aberrant (oncogenic) NOTCH signaling has been found in a broad range of human malignancies, including: T-ALL, breast cancer, colorectal cancer, ovarian cancer, and non-small cell lung cancer (Aster et al., 2017), which is not surprising since NOTCH signaling is a key regulator of cell fate decisions promoting cell proliferation and inhibiting differentiation in many adult tissues. Similar to canonical ligand- and Adam10-dependent NOTCH signaling, ligand-independent (oncogenic) NOTCH signaling requires γ-secretase activity but is independent of Adam10 (Groot et al., 2013; Groot et al., 2014; Sulis et al., 2011; van Tetering et al., 2009). It is well

described that γ-secretase cleavage of NOTCH not only occurs at the cell surface, but also in the endosomes and lysosomes (Kaether et al., 2006; Pasternak et al., 2003) and that this endosomal γ-secretase cleavage differentially regulates NOTCH stability and transcriptional activity (Tagami et al., 2008). Recently, we demonstrated that PSEN2-containing γ-secretase complexes localize to endosomes (Sannerud et al., 2016) and we and others showed that increasing endosomal and lysosomal pH strongly inhibits oncogenic NOTCH signaling in flies and diverse cell types (Hounjet et al., 2019; Kobia et al., 2014; Schneider et al., 2013; Sethi et al., 2010; Vaccari et al., 2010). Targeting endosomal and lysosome function in T-ALL strongly synergizes with γ-secretase inhibitors in blocking the survival of NOTCH-dependent T-ALL cells (Hounjet et al., 2019). Taken together, these findings indicate that endosomal and lysosomal trafficking play an important role in the ligand-independent processing of NOTCH1.

In this chapter, we show for the first time that ligand-independent Notch signaling does not require Adam10 or Adam17 for its activation. However, the molecular mechanisms regulating this signaling pathway remain poorly known. To obtain a better understanding of the mechanisms of ligand-independent Notch activation, we performed a shRNA silencing screen in Adam10 and Adam17 protease-deficient mouse embryonic fibroblasts (MEFs) to identify rate-limiting steps in the Notch activation cascade. We identified Divalent metal transporter 1 (Dmt1) as a novel Notch regulator.

Dmt1 is a proton-coupled transmembrane transport protein, also known as natural resistance-associated macrophage protein 2 (Nramp2) or divalent cation transporter 1 (Dct1), which is responsible for the transport of iron (Fe^{2+}) and other divalent metals (Fleming et al., 1997). Dmt1 is essential for intestinal iron absorption and transport as rodents harbouring a spontaneous missense mutation in *Dmt1* are highly anaemic (Fleming et al., 1998; Fleming et al., 1997; Su et al., 1998; Touret et al., 2004). Dmt1 is well known for its role in the absorption of iron in the duodenum. Once absorbed and bound to transferrin, iron is internalized by the transferrin receptor via endocytosis into an early endosome. Next, iron is released from

transferrin and transported across the endosomal membrane into the cytosol by Dmt1 (Morgan, 1996).

Dmt1 is encoded by the *Slc11A2* (solute carrier family 11, member 2) gene, which can be transcribed into four isoforms. These include *Dmt1a* and *Dmt1b*, due to alternative promoter usage (*a* or *b*), with or without an iron responsive element (ire), as a result of variations in the 3'UTR of the mRNA, which lead to the presence or absence of the iron responsive element (*+ire*, *-ire*)(Hubert and Hentze, 2002; Lee et al., 1998; Mackenzie et al., 2007). Recent evidence suggests that the four different isoforms of *Dmt1* have distinct functional properties, however, all four isoforms show a similar iron transport efficiency (Garrick et al., 2006; Mackenzie et al., 2007). Nevertheless, the *Dmt1* isoforms present organ-specific expression since *Dmt1a* is only expressed in the duodenum and kidney, whereas *Dmt1b* is ubiquitously expressed (Hubert and Hentze, 2002). Moreover, Dmt1+ire isoforms are localized to the cell membrane, whereas the Dmt1-ire variants show intracellular punctate labelling (Garrick et al., 2006; Mackenzie et al., 2007). *Dmt1+ire* and *Dmt1a* mRNA levels are regulated in a complex manner by iron response protein (IRPs) that control mRNA stability and protein translation as part of iron homeostasis (Galy et al., 2013). Together these data indicate that there may be different functionalities of the *Dmt1* isoforms, due to distinct localization and degradation routes, yet to be discovered.

In this study, we identified Dmt1 as a novel key regulator of Notch signaling and challenged to delineate the contribution of Dmt1 isoforms to ligand-dependent and independent activation of the Notch signaling cascade. Interestingly, silencing of *Dmt1-ire* leads to a suppression of both ligand-dependent and independent Notch activation and induces Notch-mediated differentiation of muscle, neural, and intestinal epithelial cells. In contrast, knockdown of *Dmt1+ire* leads to a hyper-activation of Notch. Our study shades light on a novel Dmt1-isoform dependent regulation of Notch signaling that purposively affects the intracellular vesicular transport through which Notch signaling traverses.

Material and Methods

Compounds

Cells were treated with dimethyl sulfoxide (DMSO), 0.2 μM of γ-secretase inhibitor dibenzazepine (DBZ) (Syncom, Groningen, The Netherlands) dissolved in DMSO, 30 μM of 2-(3-carbamimidoyl-sulfanylmethyl-benzyl)-Isothiourea (CISMBI) dissolved in DMSO (Sigma, cat. #s302678), or 1 μg/mL of Doxycycline (Sigma, cat. #D9891) dissolved in water, as indicated, unless stated otherwise. Neuro2A cells were stimulated with 0.3 mM of dibutytyl-cAMP (Bio connect, cat. #sc-201567A) dissolved in DMSO. Chloroquine diphosphate salt (Sigma Aldrich) and Bafilomycin A1 (Sigma) were dissolved in deionized water and cells were treated with 15 μM of chloroquine or 1 nM of Bafilomycin A1 for 24 hours.

shRNA constructs

The pLKO.1-TRC cloning vector, a gift from David Root (Addgene, cat. #10878) (Moffat et al., 2006), was used to generate our different shRNA constructs according to Addgene's pLKO.1 protocol. Primers: pLKOHygroF: 5'-CGGGATCCGCCGCCA CCATGCCTGAACTCACCGCGACGTCTG-3' and pLKOHygroR: 5'-CGGGTA CCGCTATTCCTTTGCCCTCGGACG-3' were used to PCR amplify the hygromycin gene from a pcDNA5FRT vector used as a template in order to replace the puromycin gene from our pLKO.1-puro vector with the hygromycin resistance gene using the BamHI-KpnI restriction sites in the TRC cloning (stuffer) vector. Functional hairpins were sub-cloned from their puromycin to the hygromycin vector with either a BbvCI-BamHI digest to swop the hairpins or a BamHI-KpnI digest to swop the resistance cassettes in the backbones. The pLKO.1 vector scramble shRNA was a gift from David Sabatini (Addgene, cat. #1864) (Sarbassov et al., 2005). Enzymes used were purchased from New England BioLabs. All vectors used were verified by sequencing. Targeting sequences are listed (**Supplementary table 1**).

Viral vectors

pGL4.24 12xCSL Luc2P-[minP] (Groot et al., 2014) was digested with NheI-EcoRV, a fragment was isolated and ligated into a SpeI digested pGreenFire1 vector (SBI) that was blunted with Klenow (in addition of dNTPs) sequentially digested with XbaI, purified from a gel and CIP treated. This resulted in the lenti-viral Notch reporter plasmid 12xCSL-GFP-Luc. From PCS2+ ΔEGF-Notch1-L1594P-6xMYC (van Tetering et al., 2009) was retrieved by a BamHI-XbaI (blunted) digest a fragment, which was sub-cloned by ligation into the a BamHI-HincII, digested retro-viral pBABE-Puro vector generating pBABE-ΔEGF-Notch1-L1594P-6xMYC. pBABE-puro JAGGED2-3xMYC is described previously (Groot et al., 2014).

pCW57-MCS1-P2A-MCS2 (Blast) was a gift from Adam Karpf (Addgene, cat. #80921) (Barger et al., 2019). In this lenti-viral vector the NOTCH1ΔE-RFP cDNA was sub-cloned in the NheI site of this vector. The cDNA was retrieved with an SpeI and XbaI digest from a construct, recently published by our lab (Hounjet et al., 2019), to express an inducible NOTCH1ΔE-RFP (Tet-On). In this backbone the N-terminus was replaced by ligation with a PmeI-BclI fragment, from an unpublished vector pcDNA/FRT/TO-3xFLAG-mNICD, that we generated using the 3XFlagNICD plasmid a gift from Raphael Kopan (Addgene, cat. #20183) (Ong et al., 2006), after digestion with SrfI-BclI, generating a lenti-viral vector 3xFLAG-NICD1-RFP (Tet-On). These two backbone vectors were used to replace the RFP at the C-terminus with a MluI flanked gBlock encoding a fusion of mCherry with GFP based on the vector sequence of pBABE-puro mCherry-EGFP-LC3B (Addgene, cat. #22418) after digestion with MluI, generating lenti-viral vectors NOTCH1ΔE-mCherry-GFP (Tet-On) and 3xFLAG-NICD1-mCherry-GFP (Tet-On), respectively. Lenti-viral vector FUCGW-hNICD-FLAG was a kind gift of David Spencer (Shahi et al., 2011). This vector was digested with EcoRI removing hNICD-FLAG and back ligated to generate the FUCGW empty vector.

The pDEST30-mDmt1bnonire FLAG vector was a kind gift of Michael Garrick (Garrick et al., 2012). The cDNA including the 5'prime UTR was retrieved from this vector by an EcoRV digest and the fragment was sub-cloned in the mung bean blunted, sequentially CIP treated, PacI site digested into the retro-viral pQCXIH

vector. A gBlock with an AgeI site followed by the 5'prime UTR of the Dmt1a isoform running up to the unique internal BclI site in the cDNA of all Dmt1 isoforms was used to generate the Dmt1a isoforms. In order to generate the +ire isoforms of Dmt1, a gBlock encoding from the BclI site, the N-terminal sequence of these cDNAs followed by an MluI flanked HA-tag, stop-codon and flanking XhoI site was generated and sub-cloned in the pQCXIH and pQCXIN by use of the XhoI and ApaI sites in these vectors. The digested and CIP treated MluI site in the plasmid was used to replace the HA tag by FLAG annealing and ligating with phosphorylated oligo's MLUflagPMEsense: 5'-CGCGTGTTTAAACTCACTTGTCGTCATCGTCTTTGTAGT CAGTA-3' and MLUflagPME-sense: 5'-CGCGTACTGACTACAAAGACGATGAC GACAAGTGAGTTTAAACA -3' containing a unique PmeI site for analysis of the isolated plasmids. This resulted in generation of all four C-terminal tagged Dmt1 isoform encoding cDNAs in different selectable retro-viral vectors: pQCXIN-Dmt1a-ire-HA, pQCXIN-Dmt1a+ire-FLAG, pQCXIH-Dmt1b-ire-FLAG and pQCXIH-Dmt1b+ire-HA. Enzymes used were purchased from New England BioLabs. All vectors used were verified by sequencing. Lenti-viral packaging vectors were derived from the Trono (Dull et al., 1998) and Weinberg (Stewart et al., 2003) labs and pseudo- or lenti-viral production were described previously (Groot et al., 2014).

Generation of the Notch reporter (screening) cell line

Adam10$^{-/-}$17$^{-/-}$ MEFs (Groot et al., 2014), devoid of physiological ligand-dependent Notch signaling, were transduced with a 12xCSL-GFP-Luc reporter and ΔEGF-Notch1-L1594P-6xMYC vector with a puromycin selectable. Monoclonal cell lines were generated by single cell seeding and selecting for GFP-positive clones (an indication for Notch activity) for expansion. Next, a clone was selected that showed high luciferase counts, which were significantly inhibited after γ-secretase inhibition (GSI)(**Figure S1A, S1B**) and is referred to as screening cell line (U9).

Genetic screen

Lenti-viral shRNA libraries were kindly provided and developed by Cellecta (Mountain View, USA) based on NIH-funded research grant support 44RR024095

and 44HG003355. We screened the Cellecta DECIPHER shRNA Library Disease-Associated Targets (Mouse Module 2) against 4,520 Targets containing 27,500 hairpins in the pRSI12 vector, expressing TagRFP (Cellecta, cat. #DMDAC-M2V2-P). A transfection mix of 120 µg of Module 2 plasmid DNA, 120 µg of psPAX2 (a gift from Didier Trono) (Addgene, cat. #12260) and 120 µg of pCMV-VSV-G (a gift from Bob Weinberg) (Addgene, #8454)(Stewart et al., 2003) plasmids in DMEM (Dulbecco's Modified Eagle's medium, Sigma) without additives was prepared. Poly(ethylenimine) (P-PEI, 25kDa, pH 5.0) was added and the transfection mix was incubated for 15 minutes at room temperature. The transfection mix (38 µg of plasmid mix) was applied to sub-confluent 293FT cells and incubated for 24 hours at 37°C. After 24 hours, cells showed red fluorescence and the medium was replaced. At 48 hours post-transfection virus containing medium was harvested through a 0.45um filter. The presence of virus was checked by using a GoStick (Clontech) and was found positive. Cells from our screening cell line were transduced in the presence of polybrene (1ug/ml) at a MOI of 0.7 in which 50% of the cells should become TagRFP positive with one hairpin per cell. At 48 hours post-infection TagRFP expression showed that 51.5% of the transduced cells were RFP positive.

Transduced cells were FACS sorted (BD FACSAria Cell-Sorting system, BD Biosciences) for modest TagRFP (shRNA) expression shRNA and total loss of GFP expression (suppression of NOTCH activity) and cell populations were expanded. Next, DNA was extracted and subjected to HT barcode sequencing by Cellecta, in triplicate. Enriched genes were identified based on a unique barcode present for each hairpin.

Generation of Dmt1 KO MEFs

mNramp2(Dmt1)fl/fl MEFs were a kind gift from Sara Cherry. For genetic typing primer combinations F1-R1 and F1-R9 were used as described previously (Rose et al., 2011). Dmt1 KO clones were generated by transduction with Adeno-Cre or Adeno-dsRED as a control ($5.0*10^{12}$ particles/ml, 1:1 000, VDR). The medium was replaced at 48 hours post-transduction. Three days post-transduction, single cell suspensions were generated and expanded to obtain single cell derived clones. First,

mNramp2fl/fl MEFs were transduced with overexpression constructs and monoclonal cell lines were generated and analysed before Dmt1 KO MEFs were derived from these clones, as described above.

Cell lines

Screening cell line (U9) MEFs (Adam10$^{-/-}$17$^{-/-}$ MEFs, ΔEGF-Notch1-L1594P-6xMYC, 12xCSL-GFP-Luc), mNramp2fl/fl MEFs, Neuro2A cells (a kind gift from Pilar Martinez-Martinez), LS174T cells (a kind gift from Marc van de Wetering), and U2OS/JAGGED2 cells (Fiddes et al., 2018) were maintained in DMEM (Dulbecco's Modified Eagle's medium, Sigma) containing 10% FBS and 50U/mL of Penicillin/Streptomycin. C2C12 cells (a kind gift from Ramon Langen) were maintained in DMEM (Dulbecco's Modified Eagle's medium, high glucose, Gibco) containing 10% FBS and 50U/mL of Penicillin/Streptomycin (growth medium). All cell lines were maintained under cell culture conditions (37°C, 5%CO_2) and regularly tested for mycoplasma contamination.

Calcein quenching assay

Dmt1 transport activity was detected as described previously (Foot et al., 2008). In short, Dmt1 transport activity was measured by loading cells with 0.25 μM of calcein-AM (Invitrogen, cat. # C3100MP) for 20 min at 37°C in DMEM supplemented with 20 mM of Hepes. Cells were washed with 1XPBS and resuspended in transport buffer (150 mM NaCl, 20mM of MES, pH 6.0). Fluorescence (excitation 485 nm, emission 520 nm) was recorded using a FLUOstar Omega multi-mode microplate reader (BMG Labtech). Baseline fluorescence was measured for 20 seconds (every 2 seconds), 100 μM of $CoCl_2$ or Ammonium iron(II) sulfate hexahydrate (FAS)(Sigma, cat. #F2262) was added to the cells and quenching of fluorescence was measured every 0.5s for 150 seconds. Fluorescent counts were corrected for baseline fluorescence.

Notch activity reporter assay

Cells were seeded and treated for 24 hours with DMSO, 800 nM of DBZ, 15 μM of chloroquine, or 1 nM of Bafilomycin A1. After 24 hours, luciferase activity was measured using dual-luciferase reporter assay system (Promega, cat. #E1910) according to the manufacturer's protocol or GFP fluorescence was measured by flow cytometry using a FACSCantoII cytometer with BD FACSDiva 6.1.1 software. For luciferase measurements, cells were lysed in 1xPassive Lysis Buffer and incubated for 20 min on a shaker at room temperature. Luciferase activity was measured in cell lysates by adding 1:1 firefly luciferin substrate using a FLUOstar Omega multi-mode microplate reader (BMG Labtech).

Notch stimulation by ligand

Delta-like 4 (Dll4) stimulation was performed using recombinant human Dll4 (R&D systems, cat. #1506-D4-050/CF). Well plates were coated with 5 μg/mL of Dll4 in 0.2% gelatin 0.1% BSA in 1XPBS for 24 hours at 4°C. Control well plates were coated with 0.2% gelatin 0.1% BSA in 1XPBS without Dll4. Cells were seeded on coated plates and incubated for 24 hours at 37°C before further analysis. JAGGED2 stimulation was performed seeding U2OS cells constitutively expressing JAGGED2 in 6-wells plates. U2OS cells were used as a control. After 24 hours, cells were fixed in 4% PFA in 1XPBS for 15 min at room temperature. U2OS cells were washed with 1XPBS and cells were added on top for 24 hours at 37°C for ligand stimulation. Dmt1 wild-type (WT) and knockout (KO) MEFs stably expressing JAGGED2-3xMYC were seeded sub-confluent treated with DMSO or DBZ for 24 hours at 37°C. After 24 hours, Dmt1 WT or KO MEFs stably expressing JAGGED2-3xMyc were seeded on top, treated with DMSO or DBZ, and incubated for another 24 hours, to ensure Notch activation by ligand-binding.

C2C12 differentiation

C2C12 cells were grown at medium passage number (10-20) maintained in growth medium. Myogenic differentiation was induced according to a previously published protocol (Langen et al., 2003). In short, Matrigel (Corning, cat. #356230) was diluted

1:50 in DMEM (high glucose) without any additives and incubated for 30 minutes at 37°C to coat the wells or coverslips for C2C12 differentiation. After 30 minutes, the Matrigel dilution was removed and cells in growth medium were added on top of the Matrigel coating and were treated with DMSO or 800nM of DBZ. After 24 hours, cells were washed with 1XPBS and differentiation medium (DMEM, high glucose, Gibco, containing 0.5% heat-inactivated FBS and 50U/mL of Penicillin/Streptomycin) was added. Undifferentiated and differentiated cells up to 6 days post-differentiation initiation were analysed for myotube formation and expression of differentiation markers.

Neuro2A differentiation

Neuro2A cells were passed through a cell strainer and sparsely seeded in DMEM containing 10% FBS and 50U/mL of Penicillin/Streptomycin and treatments with DMSO or 800nM of DBZ were added. After 24 hours, pictures were taken from undifferentiated Neuro2A cells, cells were washed with 1XPBS, and DMEM without any additives containing 0.3 mM of dibutytyl-cAMP (Bio connect, cat. #sc-201567A) was added to the cells for 4 hours at 37°C. After 4 hours, pictures were taken from differentiated Neuro2A cells and differentiation was analysed by counting the neurites/cell and neurite length using ImageJ.

Immunofluorescence

For *Myc staining* in U9 cells with knockdown of *Dmt1* or Dmt1 isoform overexpression, cells were fixed in methanol for 15 minutes at -20°C. Cells were washed with 1XPBS, blocked and permeabilized in 0.3% triton-X 1% BSA in 1XPBS for one hour at room temperature. After blocking, cells were incubated with mouse anti-Myc (9e10)(1:150, hybridoma, 3 mg/ml) in 0.3% triton 1% BSA in 1XPBS at 4°C overnight, washed with 1XPBS, and incubated for 2 hours at room temperature with goat anti-mouse Alexa488 in 0.3% triton 1% BSA in 1XPBS. MEFs overexpressing the Dmt1 isoforms were also stained with rabbit-anti-HA (1:400, Sigma, cat #H6908) or mouse-anti-FLAG M2 (1:500, Sigma, cat. #F3165). For combined Myc and Flag staining (both antibodies generated in mice) first anti-Myc primary and secondary

antibodies were incubated. Next, after one hour of blocking (5% NGS 0.3% triton-X 1% BSA in 1XPBS) at room temperature, primary and secondary antibodies against FLAG were added. HA- or FLAG tags were visualized by using secondary goat-anti-mouse/rabbit Alexa594 antibodies (Invitrogen).

For *Lamp1 staining* in U9 cells with Dmt1 isoform overexpression, cells were fixed in methanol for 15 minutes at -20°C. Cells were washed with 1XPBS, blocked and permeabilized in 5% NGS 0.2% triton-X 1% BSA in 1XPBS (blocking solution) for 30 minutes at room temperature. After blocking, cells were incubated with rat anti-Lamp1 (1:200, Abcam, cat. #ab24170) in blocking solution at 4°C overnight, washed with 1XPBS, and incubated for one hour at room temperature with anti-rat Alex594 (1:500, Invitrogen, cat #A11007). MEFs overexpressing the Dmt1 isoforms were also stained with rabbit anti-HA (1:400, Sigma, cat #H6908) or mouse anti-FLAG M2 (1:500, Sigma, cat. #F3165). For combined Lamp-1 and Flag staining (both antibodies generated in closely related species) first anti-LAMP-1 primary and secondary were incubated. Next, after one hour of blocking (5% NGS 0.2% triton X 1% BSA in 1XPBS) at room temperature, primary and secondary antibodies against FLAG were added. HA- or FLAG tags were visualized by using secondary goat-anti-rabbit or mouse Alexa488 antibodies (Invitrogen), respectively.

For *Myotube staining* after C2C12 differentiation, cells were fixed in 4% PFA in 1XPBS, incubated in 50 mM of Glycine, and permeabilized with 0.1% triton-X in 1XPBS, each for 15 min at room temperature. Cells were blocked in 1% BSA in 1xPBS for 30 minutes at room temperature. After blocking, cells were incubated with mouse anti-skeletal myosin FAST (1:200, Sigma, cat. #M4276) in 1% BSA in 1XPBS for 60 minutes at room temperature, washed in 1XPBS and incubated for 30 minutes with goat anti-mouse Alexa488 in 1% BSA in 1XPBS at room temperature.

For *Val1744 staining* cells were seeded on coverslips and treated for 24 hours with DMSO or DBZ. After 24 hours, cells were fixed in 4% PFA in 1XPBS, incubated in 50 mM of Glycine, and permeabilized with 0.1% triton-X in 1XPBS, each for 15 min at room temperature. Val1744 staining was performed using a Alexa488-Tyramide SuperBoost™ Kit (goat anti-rabbit IgG) (Thermo Fisher Scientific, cat. #B40943) according to the manufacturer's instructions. As a primary antibody rabbit

anti-Val1744 (1:100, Cell Signaling, cat. #4147) was used diluted in 0.1% triton-X 10% NGS in 1XPBS for 60 minutes at room temperature. MEFs constitutively expressing NICD or NOTCH1ΔE tagged with mCherry-GFP were fixed in 4% PFA in 1XPBS and incubated in 50 mM of Glycine for 15 minutes at room temperature.

All fluorescently labelled cells were counterstained with Dapi and analysed using an inverted Leica SPE confocal microscope and Leica LAS AF Lite software.

Immunohistochemistry

For *Periodic Acid Schiff (PAS) staining* LS174T cells with stable knockdown of *DMT1-IRE* or SCRAMBLED (SCR) were seeded on Poly-L-lysine coated coverslips. As a control, shSCR cells were treated with DMSO or DBZ for 7 days. After 7 days, cells were rinsed with 1XPBS and fixed in 4% PFA in 1XPBS for 15 minutes at room temperature. After fixation, cells were incubated with periodic acid solution (Sigma, cat. #3951) for 5 minutes at room temperature. Cells were rinsed with MilliQ and stained with Schiff reagent (Sigma, cat. #3952) for 15 min at room temperature. Cells were rinsed with tab water, dehydrated using 80% ethanol, and mounted on glass slides using DPX mounting medium.

Immunoblotting

Cell lysates were prepared in 1xLaemlli loading buffer. Proteins were separated on Tris-HCL SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked in 5% dried skimmed milk (Marvel) and 0.05% Tween20 in 1XTBS. Protein detection was performed with subsequent primary antibodies: rabbit anti-cleaved Notch1 (Val 1744)(D3B8) (Cell Signaling, cat. #4147S, 1:1 000), rabbit anti-lamin A (C-term) (Sigma, cat. #L1293, 1:1 000), mouse anti-actin clone C4 (MP Biomedicals, cat. # 691001, 1:20 000), mouse anti-Myc (9e10) (hybridoma, 3 mg/ml, 1:1 000), mouse anti-skeletal myosin FAST (Sigma, cat. #M4276, 1: 1 000), rabbit anti-NOTCH1 (C-20) (Santa Cruz, cat. #sc-6014-R, 1:1 000), rabbit anti-GFP (Abcam, cat. #ab290, 1:1 000), rabbit anti-LC3B (MBL, cat. #PM036, 1:1 000), mouse anti-Rab5A (Cell signaling, cat. #46449, 1:1 000), rabbit anti-EEA1 (Abcam, cat. #ab2900, 1:1 000), mouse anti-vinculin (Sigma, cat. #V9131, 1: 5 000), mouse

anti-FLAG M2 (Sigma, cat. #F3165, 1:1 000), rabbit anti-HA (Sigma, cat. #H6908, 1: 1 000), rabbit anti-Dmt1 (Novus Biologicals, cat. #NBP2-30045, 1: 1 000), and rabbit anti-LAMP1 (Abcam, cat. #ab24170, 1:1 000). Secondary antibodies used were anti-mouse (Cell Signaling, cat. #7076S, 1:5 000) or anti-rabbit IgG-horseradish peroxidase (Cell Signaling, cat. #7074S, 1:5 000). Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) was used for visualization as described by the manufacturer.

Quantitative RT-PCR

Total RNA was isolated using NucleoSpin RNA (Macherey-Nagel) from cells according to the manufacturer's protocol. cDNA was obtained using Iscript cDNA synthesis kit (Biorad) followed by SYBR-green based reverse transcription quantitative PCR (qRT-PCR) using SensiMix SYBR high-ROX kit (GC Biotech). mRNA expression was analysed using of forward and reverse primers (**Supplementary table 2 and 3**). Cycle threshold (Ct) values were analysed with CFX Connect Real Time System (Biorad) and Csnk2a2 (mouse) and GAPDH (human) were used as housekeeping genes.

Notch1 receptor flow cytometry

Notch1 receptor availability at the cell surface was analysed by flow cytometry. Dmt1 WT and KO MEFs were fixed in 4% PFA in 1XPBS and stained with a PE labelled mouse anti-Notch1 antibody targeting the extracellular domain of Notch1 (1:40, Biolegend, cat. #352105) for 15 minutes at 4°C. For total Notch1 receptor expression, Dmt1 WT and KO MEFs were permeabilized with saponin-based permeabilization reagent (Thermo Fisher Scientific, cat. #C10418) prior to the staining. A PE-labelled mouse IgG1κ was used as an isotype control (Biolegend). After staining, cells were analyzed using a FACSCantoII cytometer with BD FACSDiva 6.1.1 software. Using FLowV10.1 doublets and cellular debris were excluded. Mean fluorescent intensity (MFI) was determined and normalized to the control to obtain the Fold-Change in extracellular NOTCH1 receptor expression.

Transferrin and dextran uptake assay

Cells were serum starved for 30 min at 37°C. After serum starvation, cells were incubated with 10 µg/mL of mouse FITC-labelled transferrin (Jackson Immunoresearch, cat. #015-090-050) for 10 min at 37°C, or 40 µg/mL of Alexa488-labelled dextran (Thermo Fisher Scientific, cat. #D22910) for 60 min at 37°C, or 0.5 mg/ml Lysosensor yellow/blue dextran (Thermo Fisher Scientific, cat. #L22460) for 24 hours at 37°C. For a pulse-and-chase assay, FITC-transferrin was removed after a pulse of 10 minutes, washed with 1XPBS, and chased for 50 minutes at 37°C. Cells were washed with 1XPBS, trypsinized, and analysed by flow cytometry using a FACSCantoII cytometer with BD FACSDiva 6.1.1 software. Mean fluorescent intensity (MFI) was determined, corrected for background, and normalized to the control to obtain the Fold-Change in transferrin or dextran fluorescence.

Electron microscopy

Cells were seeded in a 6-wells plate at 90% confluence and left to attach overnight. Next, cells were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at room temperature for 1 hour. After the first fixation, fresh fixative was added and the cells were stored at 4 degrees for 24 hours. Next, the cells were washed in 0.1M cacodylate buffer and post-fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide in cacodylate buffer for 1 hour at 4 degrees. The fixed cells were dehydrated in ethanol, infiltrated with Epon resin, embedded in resin and polymerized at 60 degrees for 72 hours. Ultrathin sections were obtained using Ultracut UCT ultramicrotome (Leica Microsystems, Vienna) and mounted on Formvat-coated copper grids. These grids were stained with 2% uranyl acetate in 50% ethanol and lead citrate. Electron microscopic images were obtained using a Tecnai T12 electron microscope equipped with an Eagle 4kx4k CCD camera (Thermo Fisher Scientific, The Netherlands).

Statistical analysis

Data are presented as mean including the standard error of the mean (SEM) of three independent experiments. Statistical analyses were performed using GraphPad

Prism 5 and statistical significance was defined as p-value<0.05. One-way ANOVA and a Tukey post-test were used for statistical analysis (unless stated otherwise).

Results

Dmt1 isoforms differentially regulate ligand-independent Notch signaling

Our lab and others have previously shown that ligand-dependent Notch signaling requires Adam10 (van Tetering et al., 2009). Next, we asked if ligand-independent signaling through Notch1 receptors carrying gain-of-function mutations also requires Adam10 and the related Adam17 proteases. We transduced *Adam10* and *Adam17* knockout (Adam10$^{-/-}$/17$^{-/-}$) MEFs with an expression vector encoding for a truncated murine Notch1 lacking the extracellular domain, containing the 36 ligand binding EGF-like repeats and carrying a common gain-of-function mutation found in T cell leukemia's (ΔEGF-Notch1-L1594P-6xMYC). These cells also expressed a Notch transcriptional reporter (12xCSL-GFP-Luciferase) and is referred to as screening cell line (U9). We found that even in the absence of both Adam10 and Adam17, Notch1 was still transcriptionally active analyzed by luciferase reporter activity (**Figure S1A**) and cleaved at the Val1744-cleavage site (**Figure S1B**). Notch transcriptional activity and cleavage was blocked by γ-secretase inhibition (DBZ) as shown by the appearance of S2-cleaved Notch1 (Next), due to inhibition of Nicd1 formation. Consistent with our previous work, we found that Notch1 activity in U9 cells was also completely blocked by vesicle targeting agents chloroquine and Bafilomycin A1 (**Figure S1A, S1B**)(Hounjet et al., 2019). These data show that the absence of Adam10 and Adam17 proteolysis does not impair ligand-independent Notch signaling and suggest the presence of essential proteins that endow cells with Notch cleavage and activity, which requires intracellular transport by vesicles.

Next, in order to identify these essential regulators of Notch signaling, we conducted a RNAi silencing screen with a targeted library encoding shRNA vectors labelled with RFP targeting >4,500 disease-associated mouse genes (**Figure S1C**). After viral transduction and expansion, RFP^{moderate}/GFP^{null} cells (Notch1-OFF) were

recovered by fluorescence activated cell sorting (FACS) (**Figure S1D**). Barcode next generation sequencing identified the corresponding shRNA targeted genes (**Supplementary table 4,** link supplied on request). Hairpins that showed >2800 reads were considered significant as they fall in the top 5% analyzed by distribution analysis (**Figure 1A**). From this screen we obtained >100 significant hits, however, two genes showed most reads (>40.000) per gene, including *Cntnap1* and *Slc11a2* (**Figure 1B**). Pathway analysis indicated a highly significant enrichment for (ion) transmembrane transporter and transition metal ion binding (**Figure S1E**). Since *Cntnap1* has already been validated as a γ-secretase associated protein regulating Notch processing and activity (Hur et al., 2012; Wu et al., 2017), we focus on the *Scl11a2* gene encoding Divalent metal transporter 1 (Dmt1) in this chapter, which has not been linked to Notch signaling before.

To validate our findings, we measured the expression of *Dmt1* mRNA isoforms by qRT-PCR in U9 cells. Both *Dmt1b-ire* and *Dmt1b*+ire were expressed, however, no measurable mRNA levels of *Dmt1a* isoforms were detected (**Figure S1F**). Next, we stably transduced U9 cells with shRNAs against *Dmt1-ire* (identified in the shRNA screen) and *Dmt1+ire*, resulting in ire-specific mRNA knockdown of 88% and 72%, respectively (**Figure S1G**).

Silencing of *Dmt1-ire* in U9 cells caused a reduction in Notch1 transcriptional reporter activity (**Figure 1C**), decreased Notch1 target mRNA expression of *Hey1* and *Hes1* Notch target genes (**Figure 1D**), and reduced Val1744-cleaved Nicd1 levels (**Figure 1E**). Surprisingly, silencing of *Dmt1+ire* had the opposite effect, causing increased Notch1 activity, target gene expression, and elevated levels of Nicd1, which could be prevented by GSI treatment. Importantly, the observed isoform specific effects of *Dmt1* silencing on Notch1 activity were not caused by overall changes in the expression of Notch1 (**Figure 1E**). Next, we tested the effect of pharmacological Dmt1 inhibition using a commercially available Dmt1 inhibitor (CISMBI) (Montalbetti et al., 2015). We observed that CISMBI, similar to *Dmt1+ire* silencing, increased Notch1 reporter activity in a dose-dependent manner and this increase was blocked by GSI (**Figure S1H**). CISMBI treatment resulted in a slight decrease in Nicd1 (Val1744) levels and increased levels of non-furin-cleaved

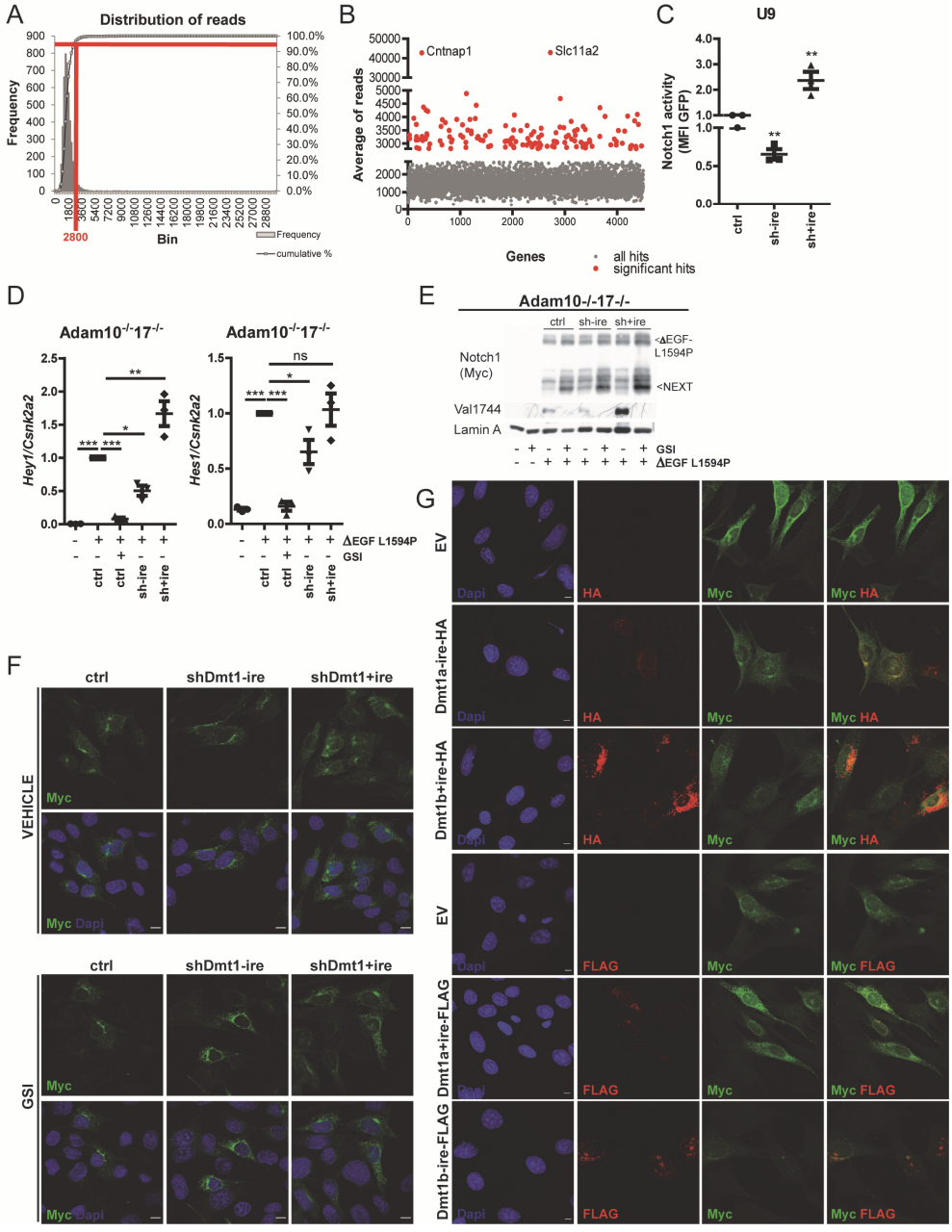


Figure 1. Dmt1 isoforms differentially regulate ligand-independent Notch signaling. A) *Distribution analysis of reads from shRNA screen.* ***B)*** *Representation of genes which were included in the shRNA screen. Non-significant (grey dot) and significant (red dot) genes are*

shown according to the average number of reads from the screen. ***C)*** *Flow cytometry analysis of Notch1 reporter activity measured by GFP expression in U9 cells stably transduced with empty vector, shDmt1-ire, and shDmt1+ire (Student t-test, **P<0.01, significant compared to empty vector control).* ***D)*** *Notch target gene expression for Hey1 and Hes1 in U9 cells with stable expression of empty vector, shDmt1-ire, and shDmt1+ire treated with DMSO or DBZ (GSI) for 24 hours measured by qRT-PCR. Csnk2a2 mRNA expression was used as a house keeping control (One-Way ANOVA (Tukey comparison), ns = non-significant, *P<0.05, **P<0.01, ***P<0.001).* ***E)*** *Immunoblot analysis of Myc (ΔEGF-Notch1-L1594P), Val1744, and lamin A (loading control) protein levels in empty vector and Dmt1 knockdown cells treated with DMSO and DBZ for 24 hours. GSI: γ-secretase inhibitor dibenzoazepine, NEXT: Notch1 extracellular truncation.* ***F)*** *Immunofluorescent staining for ΔEGF-Notch1-L1594P-6xMYC expression in U9 cells with stable knockdown for empty vector, Dmt1-ire, and Dmt1+ire treated with DMSO or DBZ for 24 hours. Scale bar: 10 µm.* ***G)*** *Immunofluorescence co-staining for HA- and FLAG-tagged Dmt1 isoforms (red) and Myc (ΔEGF-Notch1-L1594P, green) expression in U9 cells. Cells were counterstained with Dapi. EV: empty vector. Scale bar: 10 µm. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

mature mNotch1ΔEGF-L1594P (**Figure S1I**). The experiments above identify Dmt1 as a novel rate-limiting regulator of Notch signaling, in which the Dmt1 isoforms function in an opposite manner to regulate Notch activity. Because the differences in total Notch expression could not explain isoform specific effects of Dmt1 on Notch activity, we tested whether the subcellular localization of Notch1 was affected by Dmt1 isoform silencing. Anti-Myc immunofluorescence staining showed dispersed cellular Notch1 (ΔEGF-L1594P-6xMYC) expression demonstrating cell surface, cytoplasm, perinuclear and nuclear localization in U9 cells overexpressing an empty vector control (**Figure 1F**). As expected, GSI treatment blocked nuclear Notch1 and caused perinuclear accumulation of Notch1. No increase in plasma membrane accumulation was observed in Adam10$^{-/-}$/17$^{-/-}$ MEFs, as is normally the case in wild-type GSI-treated cells demonstrating that most Notch receptors are localized intracellularly and have a defect in cell surface maturation. In *Dmt1-ire* knockdown MEFs, a slight decrease in nuclear Notch1 was apparent compared to Dmt1 wild-type cells, which was blocked by GSI treatment. Notch1 localization was similar in

Dmt1+ire knockdown MEFs although nuclear staining appeared more intense even in the presence of GSI.

Constitutive overexpression of Dmt1 isoforms shows a differential localization without affecting Notch activity

Next, we determined Dmt1 isoform localization to gain more insight into whether the four different isoforms of Dmt1 may affect the Notch trafficking system. Single Dmt1 isoforms, carrying either C-terminal HA- or FLAG-tags, were transduced in U9 cells. Dmt1 overexpression was confirmed by qRT-PCR (**Figure S2A**), HA- or FLAG-tag immunofluorescence staining (**Figure 1G**), and western blot analysis (**Figure S2B**). All four isoforms showed different expression patterns and localization (**Figure 1G**). Dmt1a-ire, Dmt1a+ire, and Dmt1b-ire were expressed in small cytoplasmic speckles, while Dmt1b+ire overexpression marked large cytoplasmic speckles. However, Dmt1a+ire also presented a ring-shaped expression around the nucleus, while Dmt1b-ire showed (peri-)nuclear accumulation. To obtain a better understanding in the subcellular localization of the Dmt1 isoforms, a co-staining with Lysosomal-Associated Membrane Protein 1 (LAMP1) was performed (**Figure S2C**). Dmt1b+ire presented an almost complete co-localization with LAMP1, indicating that Dmt1b+ire is almost exclusively localized to the lysosomes. Dmt1a-ire and Dmt1b-ire showed only minor LAMP1 co-localization in U9 cells.

Subsequently, we investigated if Dmt1 overexpression had an effect on Notch1 cleavage and activity. Dmt1 overexpression did not significantly affect Notch1 reporter activity (**Figure S2D**) nor target gene expression (**Figure S2E**). Despite a slight decrease in NICD1 (Val1744) levels upon overexpression of both Dmt1b isoforms no changes in expression of full length or furin-cleaved (Tmic) Notch1 were observed (**Figure S2B**). Moreover, anti-Myc staining showed similar Notch1 expression patterns between the overexpression of the different Dmt1 isoforms (**Figure 1G**). All Dmt1 isoforms showed co-localization with ΔEGF-Notch1-L1594P, at least to some extent. Together, these data show that overexpression of the different Dmt1 isoforms shows similar localization as Notch1, although Notch1 activity is not directly affected. Residual endogenous Dmt1 expression might be

masking the contribution of Dmt1 isoform overexpression to regulate Notch activity, however, these data indicate that lysosomal trafficking might contribute to the Notch1 phenotypes resulting from Dmt1 isoform inhibition.

Dmt1 controls Notch1 regulated myoblast differentiation

Since we show that Dmt1 controls ligand-independent Notch1 signaling, we questioned whether Dmt1 also regulates ligand-dependent Notch1 signaling. To this end, we performed knockdown of the different isoforms of Dmt1 in murine myoblasts (C2C12 cells). C2C12 cells differentiate into myotubes upon serum starvation and differentiation is blocked by Notch1 activity and accelerated by Notch inhibition (Nofziger et al., 1999). First, *Dmt1* levels were measured using qRT-PCR. *Dmt1b-ire* and *Dmt1b+ire* but not *Dmt1a* isoforms were expressed in C2C12 cells (**Figure S3A**). Upon stable transduction with shRNAs, *Dmt1-ire* and *Dmt1+ire* knockdown was obtained, resulting in a knockdown of 79% and 76%, respectively (**Figure S3B**). Notably, a significant increase in *Dmt1-ire* expression was detected upon *Dmt1+ire* knockdown but not vice versa.

Next, C2C12 cells were treated with vehicle or GSI and left to differentiate for 6 days, characterized by increased levels of differentiation marker skeletal myosin FAST (Msf) (**Figure S3C**). From 3 days of differentiation onwards an accumulation of Nicd1 was observed, which was blocked by GSI, and resulted in increased Msf levels compared to vehicle-treated cells (**Figure S3C, 2A**). After 4 days of treatment the levels of three additional myogenic markers; myoblast determination protein 1 (MyoD), myogenin (MyoG) and myogenic factor 5 (Myf5) (Sabourin and Rudnicki, 2000), were also (slightly) elevated in GSI-treated cultures (**Figure S3D, 2B**). Increased *Myf5* levels were already present at the start of differentiation. Expression levels of Notch1 target gene *Hey1* were decreased at 4 and 6 days of differentiation, confirming the Notch-dependent myoblast differentiation. *Dmt1-ire* knockdown cells were characterized by an increase in the formation of myotubes (**Figure 2A**) and earlier and higher expression of Msf during differentiation (**Figure 2C**). Moreover, *Dmt1-ire* knockdown resulted in elevated mRNA expression levels of *Myf5*, *MyoD,* and *MyoG* at 4 and 6 days of differentiation and decreased expression levels

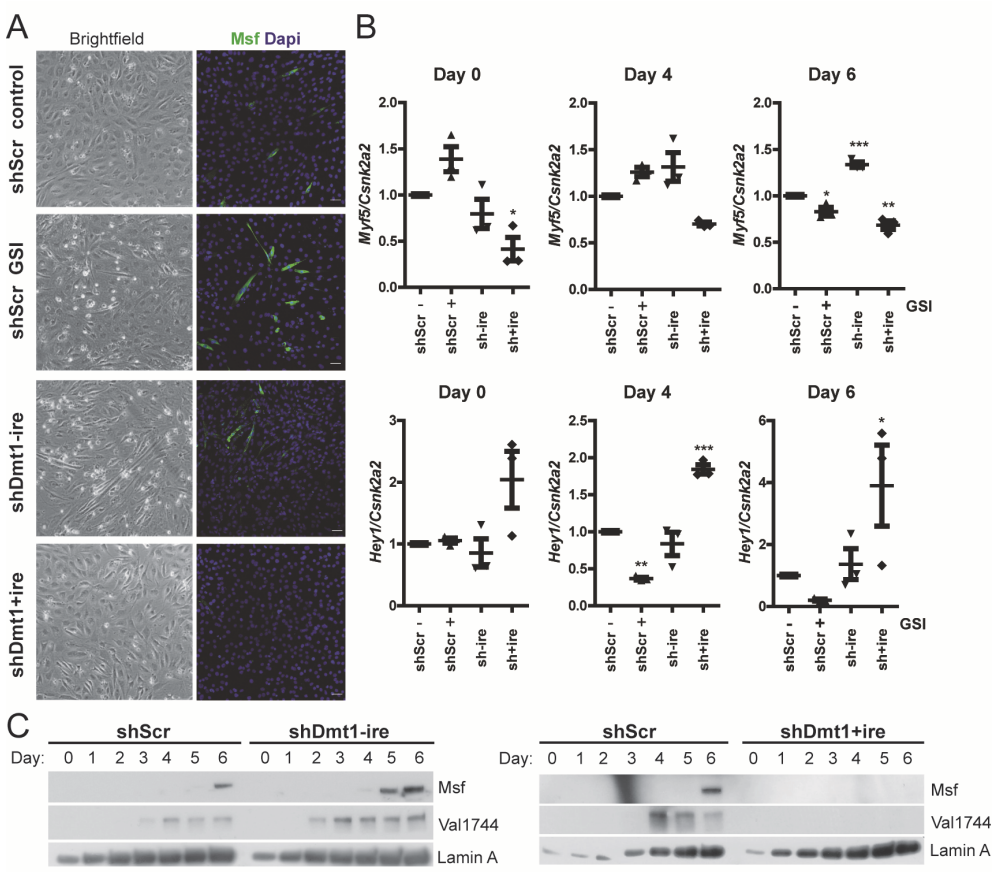


Figure 2. Dmt1 controls Notch1-mediated myoblast differentiation. A) *Bright field representative images and immunofluorescence staining for Msf counterstained with Dapi of C2C12 cells differentiated for 6 days. Scale bar: 100 µm.* ***B)*** *qRT-PCR for the mRNA expression of differentiation markers Myf5 and Notch target gene Hey1 at 0, 4, and 6 days post-differentiation initiation in C2C12 cells with knockdown of scrambled treated with DMSO or DBZ, Dmt1-ire, or Dmt1+ire. Csnk2a2 mRNA expression was used as a housekeeping control (One-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01, ***P<0.001, significant compared to the DMSO-treated scrambled control). DBZ: γ-secretase inhibitor dibenzoazepine.* ***C)*** *Msf, Val1744, and lamin A (loading control) protein levels in C2C12 cells with knockdown of scrambled, Dmt1-ire (left), and Dmt1+ire (right) during 6 days of differentiation. GSI: γ-secretase inhibitor dibenzoazepine. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

of *Hey1* at 4 days of differentiation (**Figure 2B, S3D**). In contrast, *Dmt1+ire* knockdown showed decreased levels of differentiation, including: reduced myotube formation, decreased *MyoD*, *MyoG* and *Myf5* mRNA expression levels at 4 and 6 days, and diminished Msf expression. Moreover, *Dmt1+ire* knockdown showed increased *Hey1* mRNA expression levels, while Val1744 (Nicd1) protein expression was diminished. All together these data show that silencing of *Dmt1-ire* drives premature myoblast differentiation into myotubes through inhibition of endogenous Notch1 signaling. In contrast, *Dmt1+ire* inhibition stimulates Notch1 signaling and inhibits muscle cell differentiation by increasing Notch1 activity.

Neuronal differentiation is regulated by Dmt1

In follow-up, we investigated the role of Dmt1 in an additional Notch-dependent model of cellular differentiation: neuronal differentiation of Neuro2A cells (Franklin et al., 1999). First, *Dmt1* mRNA expression was measured by qRT-PCR (**Figure S4A**). *Dmt1b* was expressed and *Dmt1a* was undetectable. Silencing of *Dmt1* using shRNA interference resulted in 90% silencing of *Dmt1-ire* and 65% reduced mRNA expression of *Dmt1+ire* (**Figure S4B**). Scrambled control cells were treated with vehicle or GSI and subsequent neuronal differentiation was induced under conditions of serum depletion and cAMP addition (**Figure 3A**). GSI treatment resulted in both an increased number and length of neurites upon cAMP treatment compared to the control (**Figure 3B, 3C**). Interestingly, under basal conditions *Dmt1-ire* knockdown showed an increased number of neurites, which was further magnified under differentiation conditions, comparable to GSI-treated control cells. Knockdown of the *Dmt1+ire* isoforms showed only a slight trend of reduced neuronal differentiation.

To test whether the increased neurite formation of *Dmt1-ire* knockdown could be rescued by Notch1 activation, we co-transduced Neuro2A cells with stable knockdown of *Dmt1-ire* with a constitutively active NICD1 construct or empty vector (EV) control expressing GFP and subjected both to cAMP induced differentiation (**Figure S4C**). Notch activation strongly reduced neurite formation (**Figure 3D**) and length (**Figure 3E**) in both basal and differentiated conditions. These findings illustrate how constitutive NICD1 expression is sufficient to block neuronal

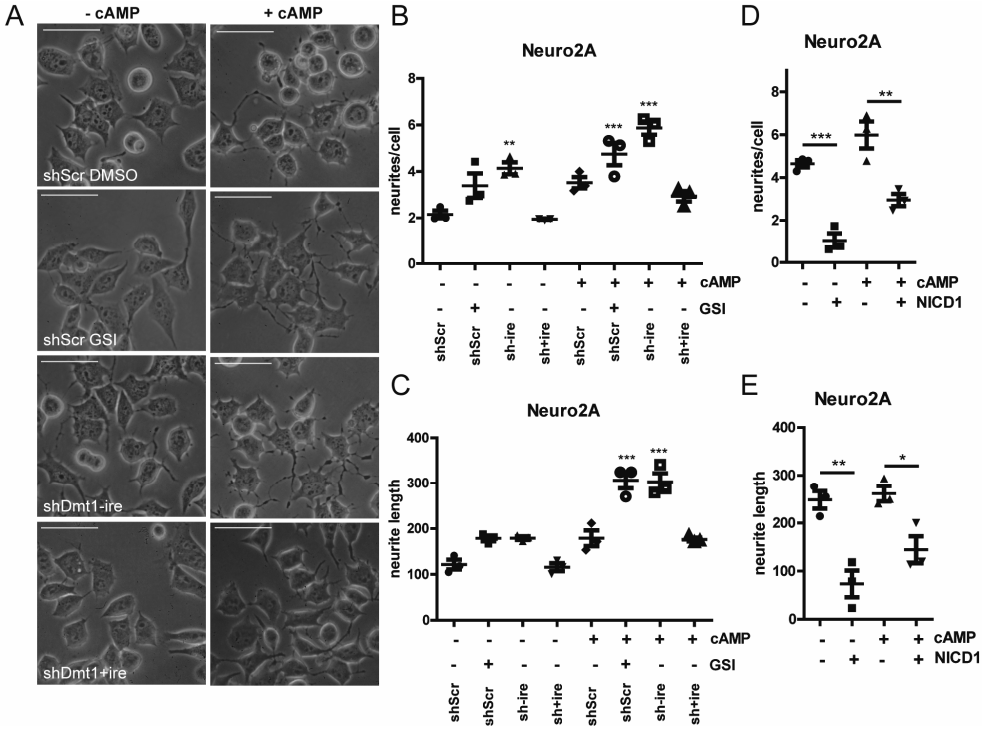


Figure 3. Notch-mediated neuronal differentiation is regulated by Dmt1. A) *Bright field representative images of basal (left) and differentiated (right) Neuro2A cells with knockdown of scrambled (treated with DMSO or DBZ), Dmt1-ire, or Dmt1+ire. Scale bar: 50 µm.* ***B-C)*** *Quantification of the number of neurites per cell and neurite length under basal and differentiated conditions (One-Way ANOVA (Tukey comparison), **P<0.01, ***P<0.001, significant compared to the DMSO-treated scrambled control).* ***D-E)*** *Quantification of the number of neurites per cell and neurite length in Neuro2A cells with overexpression of empty vector or NICD1 and knockdown of Dmt1-ire (One-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01, ***P<0.001, significant compared to the control). GSI: γ-secretase inhibitor dibenzoazepine. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

differentiation induced by the loss of *Dmt1-ire* expression. Taken together, we showed in multiple Notch-dependent differentiation models that *Dmt1-ire* knockdown inhibits endogenous Notch activity associated with differentiation.

Endogenous Notch1 signaling is controlled by DMT1 in colorectal cancer cells. To investigate if regulation of NOTCH1 signaling by DMT1 is a general feature in both murine and human cells, we assessed the effects of *DMT1* knockdown in human colorectal adenocarcinoma (LS174T) cells. Intestinal crypt progenitors, as well as LS174T colorectal cancer cells, differentiate into secretory cells including goblet cells upon NOTCH inhibition (Milano et al., 2004; Yang et al., 2011). LS174T colorectal cancer cells, only expressing the *DMT1B* isoforms (**Figure S5A**), were transduced with shRNAs against *DMT1-IRE* and *DMT1+IRE*. *DMT1-IRE* (3.3) knockdown of 83% in LS174T cells (**Figure S5B)** modulated cellular morphological changes characterized by decreased colonized growth and adhesive flattening features (**Figure S5C**). *DMT1+IRE* knockdown associated with lethality was observed in multiple colorectal adenocarcinoma cell lines, including: LS174T, Caco-2, and DLD-1 cells. LS174T colorectal cancer cells were treated with vehicle or GSI for 7 days. Periodic acid-Schiff (PAS) staining revealed an increased number of goblet cells in GSI-treated cells (**Figure 4A**) and elevated mRNA levels of *MUC5AC*, a mucin produced by goblet cells, compared to the control (**Figure 4B**). Increased *MUC5AC* expression levels were only observed shortly after *DMT1-IRE* silencing and disappeared during serial-passaging (**Figure 4C**). Expression of NOTCH target genes *HES1* and *HES4* was reduced comparably by GSI treatment and by *DMT1-IRE* knockdown (**Figure 4D**). This was confirmed using an independent shRNA targeting a different region of *DMT1-IRE* (4.8). As expected, GSI treatment diminished NICD1 and increased S2-cleaved NOTCH1 (NEXT) levels compared to the control (**Figure 4E**). Remarkably, *DMT1-IRE* silencing did not change S2-cleavage of NOTCH1 but induced robust NICD1 accumulation (top panel, lower fragment) and Val1744-cleaved Notch1, despite being inactive. Collectively, these data indicate that DMT1 acts in the NOTCH signaling cascade between γ-secretase cleavage and NICD1 translocation to the nucleus.

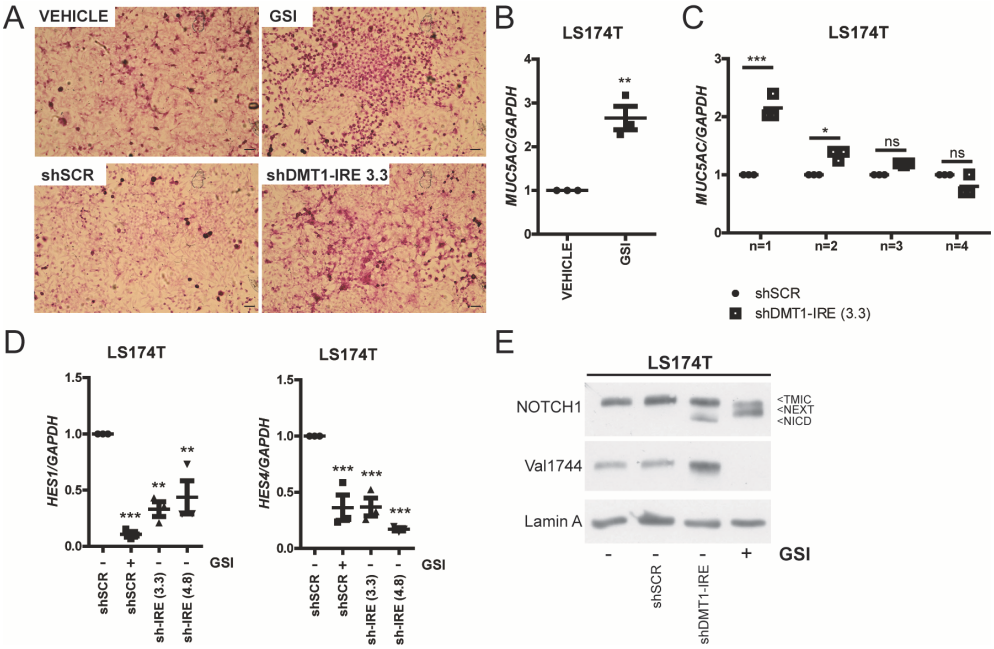


Figure 4. Endogenous Notch1 signaling is controlled by DMT1 in colorectal cancer cells. A) *Periodic Acid Schiff (PAS) staining in LS174T cells treated with DMSO or DBZ for 7 days or LS174T cells with stable knockdown of SCRAMBLED or DMT1-IRE.* ***B)*** *qRT-PCR for the mRNA levels of MUC5AC in LS174T cells treated with DMSO or DBZ for 7 days. GAPDH was used as a house-keeping control (One-Way ANOVA (Tukey comparison), **P<0.01, significant compared to the DMSO control).* ***C)*** *MUC5AC mRNA expression in LS174T cells with knockdown of DMT1-IRE during serial passaging measured by qRT-PCR. GAPDH was used as a house-keeping control.* ***D)*** *qRT-PCR for Notch target gene (HES1, HES4) mRNA expression in LS174T cells with knockdown of SCRAMBLED (treated with DMSO or DBZ for 7 days) or DMT1-IRE (two independent hairpins). GAPDH was used as a house-keeping control.* ***E)*** *Immunoblot analysis of protein levels of NOTCH1 (C-20), Val1744, and lamin A (loading control) in LS174T cells with knockdown of SCRAMBLED (treated with DMSO or DBZ for 7 days) or DMT1-IRE. GSI: γ-secretase inhibitor dibenzoazepine. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

Loss of Dmt1 results in diminished endogenous Notch1 signaling which is rescued by Notch1 activation

Our previous results show that the different isoforms of Dmt1 control Notch1-mediated differentiation of different cell types. As a next step, we tested the effect on

Notch1 signaling in the complete absence of Dmt1. We generated Dmt1 knockout (KO) MEFs. Dmt1 WT MEFs express both *Dmt1b* isoforms, while *Dmt1a* was undetectable (**Figure S6A**). Dmt1 KO MEFs were established after Adeno-Cre mediated recombination and subsequent loss of exon 6 to 8 (Rose et al., 2011) (**Figure S6B**). To functionally validate our model, we determined if Fe^{2+} and Co^{2+} transport was affected by Dmt1 loss. As expected, Dmt1 KO MEFs were impaired in Fe^{2+} and Co^{2+} transport, as defined by a calcein quenching assay (**Figure S6C**), showed diminished Dmt1 protein expression (**Figure S6D**) and demonstrated reduced uptake of labeled-transferrin as compared to Dmt1 wild-type (WT) MEFs (**Figure S6E**). No differences in Tf receptor (TfR) expression were observed between Dmt1 WT and KO MEFs (**Figure S6D)**.

To assess whether loss of Dmt1 affected endogenous Notch1 signaling, Dmt1 wild-type (WT) and KO MEFs were stimulated with Notch ligand (Dll4) and stained for Nicd1 (Val1744) (**Figure 5A**). We found that Dmt1 WT MEFs showed nuclear expression of Nicd1 upon Dll4 stimulation and potent transcriptional activation of *Hey1* and *Hey2*, which was blocked upon GSI treatment (**Figure 5B**). In contrast, in Dll4 stimulated Dmt1 KO MEFs no nuclear Nicd1 nor transcriptional activation of *Hey1* and *Hey2* was observed. Similarly, JAGGED2 induced *Hey1* and *Hey2* mRNA expression was also significantly reduced in Dmt1 KO MEFs (**Figure S6F**). Of note, the observed differences could not be attributed to changes in total *Notch1* expression (**Figure S6G**). Altogether, our data show that endogenous Notch1 signaling is significantly reduced when Dmt1 expression is lost and this maybe the result of defective Notch1 receptor internalization and/or recycling upon ligand binding.

To further investigate the defect in Notch1 activation and signaling in Dmt1 KO MEFs, we overexpressed a doxycycline-inducible NICD1 tagged with mCherry-GFP. Notch target gene expression was reduced in Dmt1 KO MEFs compared to Dmt1 WT MEFs in the absence of doxycycline upon JAGGED2 stimulation (**Figure S7A**). Doxycycline treatment induced NICD1 expression (**Figure S7B**), nuclear NICD1 localization (**Figure 5C**), and *Hey1* mRNA expression (**Figure 5D**) to a similar extent in both Dmt1 WT and KO MEFs. Ectopic expression of NOTCH1ΔE (**Figure**

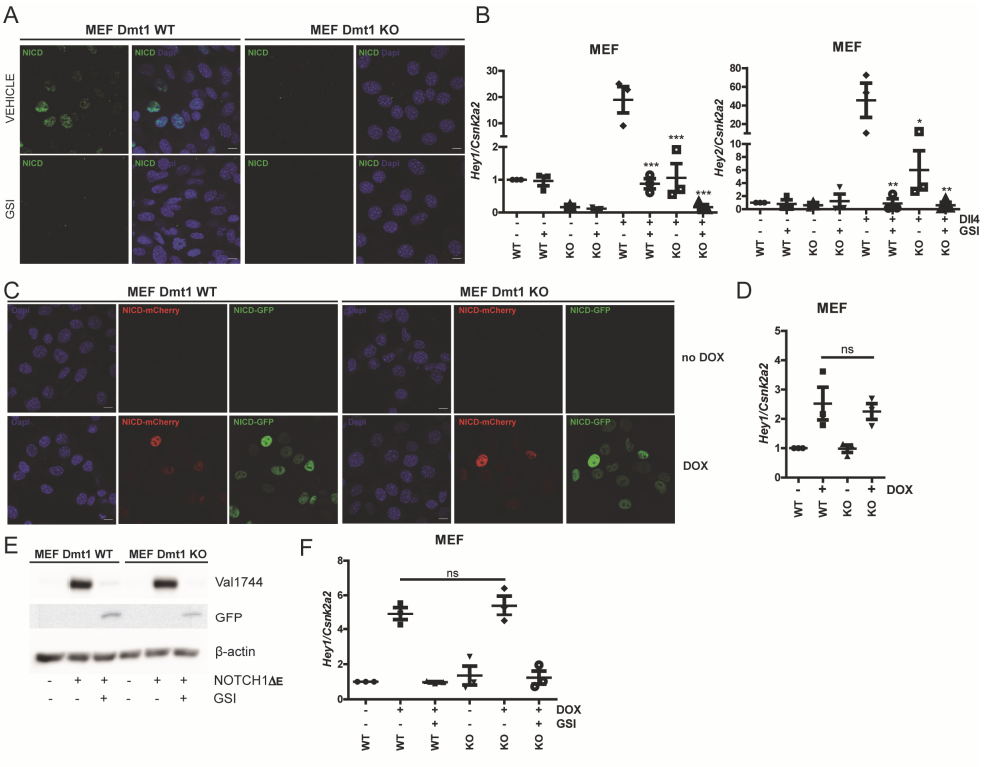


Figure 5. Loss of Dmt1 results in diminished endogenous Notch1 signaling which is rescued by Notch1 activation. A) *Immunofluorescence staining for Val1744 in Dmt1 wild-type (WT) and knockout (KO) cells stimulated with Dll4 and treated with DMSO or DBZ for 24 hours, which were counterstained with Dapi.* ***B)*** *mRNA expression levels of Notch target genes Hey1 and Hey2 in Dmt1 WT and KO MEFs stimulated with Dll4 and treated with DMSO or DBZ for 24 hours measured by qRT-PCR. Csnk2a2 mRNA expression was used as a house-keeping control (One-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01, ***P<0.001, significant compared to Dmt1 WT cells stimulated with Dll4).* ***C)*** *Fluorescent ectopic expression of NICD1-mCherryGFP in Dmt1 WT and KO cells upon doxycycline (DOX)-induction of NICD1-mCherryGFP expression.* ***D)*** *Notch target gene Hey1 mRNA expression in Dmt1 WT and KO cells upon induction of NICD expression by doxycycline (DOX) measured by qRT-PCR (One-Way ANOVA (Tukey comparison), ns = non-significant).* ***E)*** *Immunoblot analysis of Val1744, GFP (NOTCH1ΔE-mCherryGFP), and β-actin (loading control) protein levels in Dmt1 WT and KO MEFs treated with DMSO or DBZ for 24 hours.* ***F)*** *qRT-PCR for Hey1 expression in Dmt1 WT and KO MEFs with doxycycline-inducible*

NOTCH1ΔE-mCherryGFP expression, treated with DMSO or DBZ for 24 hours (One-Way ANOVA (Tukey comparison), ns = non-significant). GSI: γ-secretase inhibitor dibenzoazepine. Data are representative of three independent experiments and values are expressed in mean ± SEM.

5E, S7C), which requires S2- and S3-cleavage to become active, also rescued Notch1 signaling in Dmt1 KO MEFs as shown by *Hey1* mRNA expression, which was comparable between Dmt1 WT and KO MEFs (**Figure 5F**). This rescue by activation of Notch1 signaling was reversed by GSI treatment. Altogether, our data confirm that both NOTCH1ΔE and NICD1 overexpression rescue the loss of Notch1 signaling in the absence of Dmt1.

Accumulation of cleaved Notch1 and disturbed vesicular trafficking upon loss of Dmt1

To gain more insights in the mechanism of diminished Notch signaling upon loss of Dmt1, we co-expressed JAGGED2 in Dmt1 WT and KO MEFs. Validation of this model confirmed equal JAGGED2 expression **(Figure 6A, S7D)** and again showed that Dmt1 KO MEFs display reduced *Hey1* mRNA expression levels as compared to Dmt1 WT MEFs **(Figure S7E).** Surprisingly, increased levels of Val1744-cleaved Notch1 were observed in Dmt1 KO MEFs compared to Dmt1 WT MEFs **(Figure 6A)** despite diminished *Hey1* mRNA expression. In addition, also full length, furin-cleaved Tmic, and S2-cleaved Next Notch1 fragments were increased in Dmt1 KO MEFs compared to WT MEFs. Total and cell surface staining for Notch1 by flow cytometry showed that Dmt1 KO MEFs have a two-fold increase of Notch1 receptor levels albeit the surface/total ratio expression of Notch1 is unchanged **(Figure 6B)**.

Because of the high levels of cleaved Notch1, we hypothesized that loss of Dmt1 disturbs intracellular trafficking of Notch1 and might trap cleaved Notch1 fragments within intracellular vesicles. To test this, we measured internalization of fluorescently-labelled dextran in Dmt1 WT and KO MEFs. Within 60 minutes Dmt1 KO MEFs showed about a 2.5 fold increase in dextran uptake compared to Dmt1 WT MEFs (**Figure S7F**). After 24 hours, Dmt1 KO MEFs showed a 3 fold increase in dextran uptake, which was not affected by blocking autophagy with chloroquine

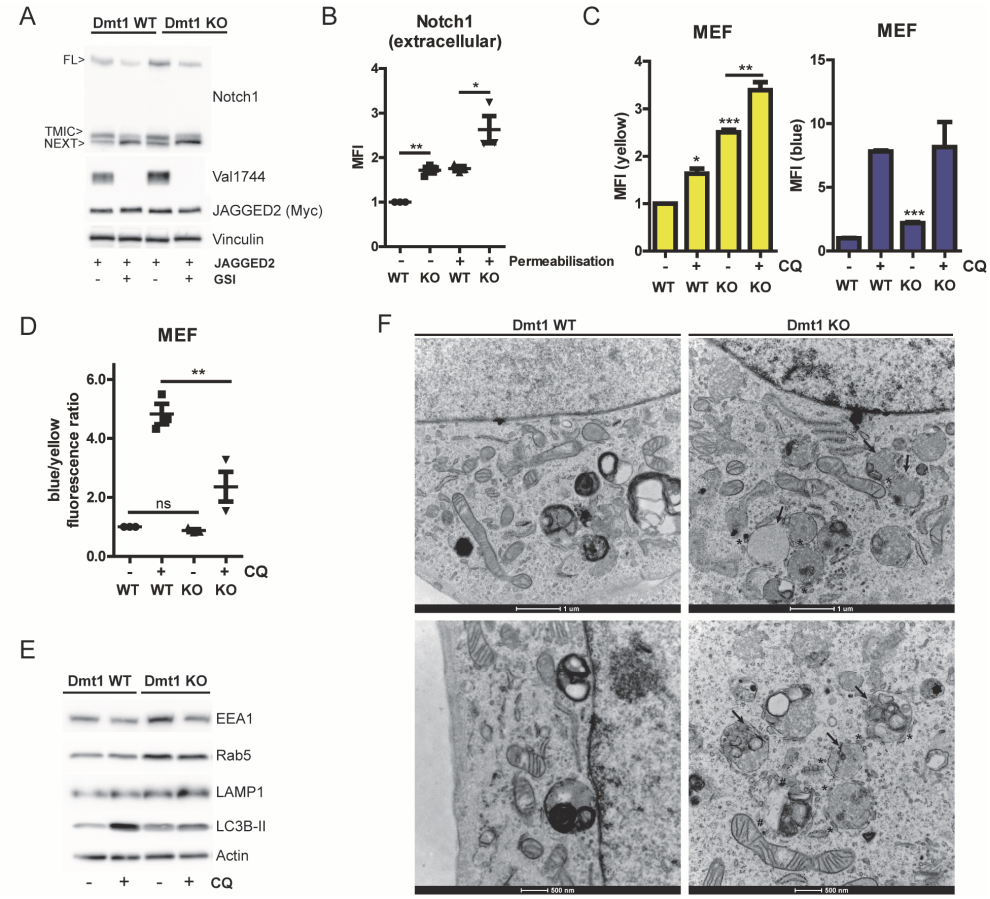


Figure 6. Loss of Dmt1 results in increased Notch1 cleavage and disturbed intracellular trafficking. A) *Immunoblot analysis of Notch1 (C-20), Val1744, Myc (JAGGED2), and Vinculin (loading control) protein levels in Dmt1 WT and KO MEFs treated with DMSO or DBZ for 24 hours.* ***B)*** *Flow cytometry analysis of extracellular Notch1 receptor expression at the plasma membrane (unpermeabilized) and total expression (permeabilized) in Dmt1 WT and KO MEFs (One-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01).* ***C)*** *pH-dependent fluorescence of Lysosensor-labelled dextran in Dmt1 WT and KO MEFs, pre-treated with or without chloroquine for 24 hours, after 24 hours of dextran uptake measured by flow cytometry. Yellow bars indicate yellow fluorescence and blue bars indicate blue fluorescence (One-Way ANOVA (Tukey comparison), *P<0.05, **P<0.01, ***P<0.001, significant compared to untreated Dmt1 WT cells).* ***D)*** *Blue/yellow fluorescence ratio of Dmt1 WT and KO MEFs, pre-treated with or without chloroquine for 24 hours, after 24 hours of Lysosensor-*

*labelled dextran uptake (One-Way ANOVA (Tukey comparison), ns= non-significant, **P<0.01, significant compared to untreated Dmt1 WT MEFs).* ***E)*** *Immunoblot analysis of EEA1, Rab5, LAMP1, LC3B-II, and actin (loading control) protein levels in Dmt1 WT and KO MEFs treated with or without chloroquine for 24 hours.* ***F)*** *Representative electron microscopy images of Dmt1 WT and KO MEFs. Dmt1 KO MEFs show damaged intracellular vesicles, displaying non-intact membranes (black arrows), missing membranes (#) and formation of isolation membranes (phagophores, asterisks). MFI: median fluorescent intensity. GSI: γ-secretase inhibitor dibenzoazepine. CQ: chloroquine. EEA1: early endosome antigen-1, LAMP1: lysosomal associated membrane protein 1, LC3B-II: membrane associated microtubule-associated protein 1 light chain 3-II. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

treatment (**Figure S7G**). Since Dmt1 is a proton-coupled transporter, we asked if loss of Dmt1 would alter the pH, due of an accumulation of protons in intracellular vesicles, leading to disturbed endocytosis. To test this, Dmt1 WT and KO MEFs (pre-treated with chloroquine) were incubated for 24 hours with pH-dependent fluorescently-labelled dextran, which shows yellow fluorescence in acid environments and blue fluorescence in pH neutral environments. Dmt1 KO MEFs showed increased yellow and blue fluorescence compared to Dmt1 WT MEFs (**Figure 6C**). As expected, chloroquine treatment strongly increased blue fluorescence and to a lesser extent yellow fluorescence, in both Dmt1 WT and KO MEFs. Although we did not observe a significant decrease in blue/yellow fluorescence ratio in Dmt1 KO MEFs (**Figure 6D**), Dmt1 KO MEFs showed a significantly lower blue/yellow fluorescence ratio after chloroquine treatment, indicating that intracellular vesicles in Dmt1 KO MEFs may have a more acidic pH compared to Dmt1 WT MEFs upon chloroquine treatment.

Besides increased internalization of dextran, Dmt1 KO MEFs also showed increased levels of early endosomal marker 1 (EEA1), Rab5, lysosomal associated membrane protein 1 (LAMP1) and LC3B-II compared to Dmt1 WT MEFs (**Figure 6E**). In addition, chloroquine treatment showed decreased accumulation of LC3B-II in Dmt1 KO MEFs upon chloroquine treatment, which accumulates as chloroquine prevents the fusion of lysosomes with endosomes and autophagosomes. In line with

these data, electron microscopic analysis of Dmt1 KO MEFs showed damaged intracellular vesicles, showing disrupted membranes, no membranes and formation of isolation membranes **(Figure 6F)**. These data suggest that autophagy/lysophagy may be activated due to damaged membranes of endosomes/lysosomes as a result of Dmt1 loss.

Discussion

Here, we identify Dmt1 as an essential regulator of physiological ligand-dependent and independent Notch signaling. Notably, we discovered a first and unique feature that *Dmt1-ire* and *Dmt1+ire* isoform knockdown have opposing functions on Notch activity, namely suppressing or hyper-activating Notch1 activity, respectively. While many screens have been performed to the best of our knowledge, this study for the first time identifies the conserved iron transporter Dmt1 as a novel key regulator of the Notch pathway (Gutierrez et al., 2014; Jia et al., 2015; Roti et al., 2013; Saj et al., 2010). Our results suggest that this may in part be due to the use of different gain of function Notch constructs because ΔEGF-Notch1-L1594P signaling requires intracellular transport by vesicles and hence Dmt1 function for its activation, while Notch1ΔE, which only has 12aa of the extracellular domain, may not require vesicle transport and overcomes a Dmt1 loss-of-function on Notch signaling.

Consistently, in two murine differentiation models silencing of Dmt1-ire mimics a Notch loss-of-function phenotype showing an increased and accelerated formation of myotubes and neurites, which is rescued by constitutive overexpression of Nicd1. In contrast, silencing of Dmt1+ire blocks differentiation and increases Notch signaling. In addition, we observed a similar loss-of-function phenotype in human colorectal cancer cells upon silencing of DMT1-IRE resulting in reduced NOTCH activity and promoting goblet cell differentiation. Prolonged silencing of DMT1-IRE led to a depletion of differentiated goblet cells strongly supporting the notion that Dmt1 loss mimics a Notch1 loss-of-function to accelerate terminal differentiation of epithelial cells (van Es et al., 2005; Vooijs et al., 2007). Knockdown of DMT1+IRE is not viable in colorectal adenocarcinoma cells, which might result from high NOTCH activity or divalent metal transport deficiency.

Interestingly, we observed high levels of Val1744-cleaved NICD1 when DMT1-IRE was silenced in LS174T cells, while NOTCH target genes were down-regulated. In a previous report we show a similar phenomenon, showing high levels of Val1744-cleaved NICD1 of oncogenic NOTCH1 in T-ALL cells, while NOTCH1 target genes are inactive (Hounjet et al., 2019). In addition, we show reduced levels of Val1744-cleaved Nicd1 and increased Notch1 activity upon Dmt1 inhibition by CISMBI in U9 cells or Dmt1+ire silencing in C2C12 cells. Furthermore, we observed increased levels of Val1744-cleaved Notch1 in Dmt1 KO cells with diminished Notch activity. These findings indicate that the Val1744 antibody only recognizes a subset of cleaved NICD1 species and does not always correlate with Notch activity (Hounjet et al., 2019; Vooijs et al., 2004). Our work emphasizes that Val1744-NICD1 expression is not sufficient for Notch transcriptional activity and that monitoring Val1744 as a biomarker for Notch activity in tumors should be avoided in the absence of other evidence.

A complete loss of Dmt1 showed a strong inhibition of ligand-induced Notch signaling, an accumulation of the Notch1 at the plasma membrane and total Notch1 expression, and decreased Val1744-cleaved Notch1 protein levels in the nucleus, although increased levels of Val1744 were observed by immunoblot analysis, indicating that Notch1 signaling from the membrane to the nucleus may be blocked and Val1744-cleaved Notch1 may be trapped in intracellular vesicles (**Figure 7**). While overexpression of both NICD1 or NOTCH1ΔE recued these effects, ΔEGF-Notch1-L1594P in an Adam10$^{-/-}$17$^{-/-}$ background was not able to restore Notch1 activity upon silencing of Dmt1-ire. We suggest that since we did not observe an accumulation of ΔEGF-Notch1-L1594P at the plasma membrane upon GSI treatment that this Notch1 signal may require activation in endocytic compartments, while NICD or NOTCH1ΔE do not.

Loss of Dmt1 showed disrupted intravesicular trafficking, including increased internalization of dextran, increased protein levels of EEA1, Rab5 and LAMP1, and increased basal autophagic flux. As EEA1-positive endosomes were shown to transport surface proteins directly from the cell surface to the nucleus (Chaumet et al., 2015), increased levels of EEA1 upon loss of Dmt1 may reflect a block of

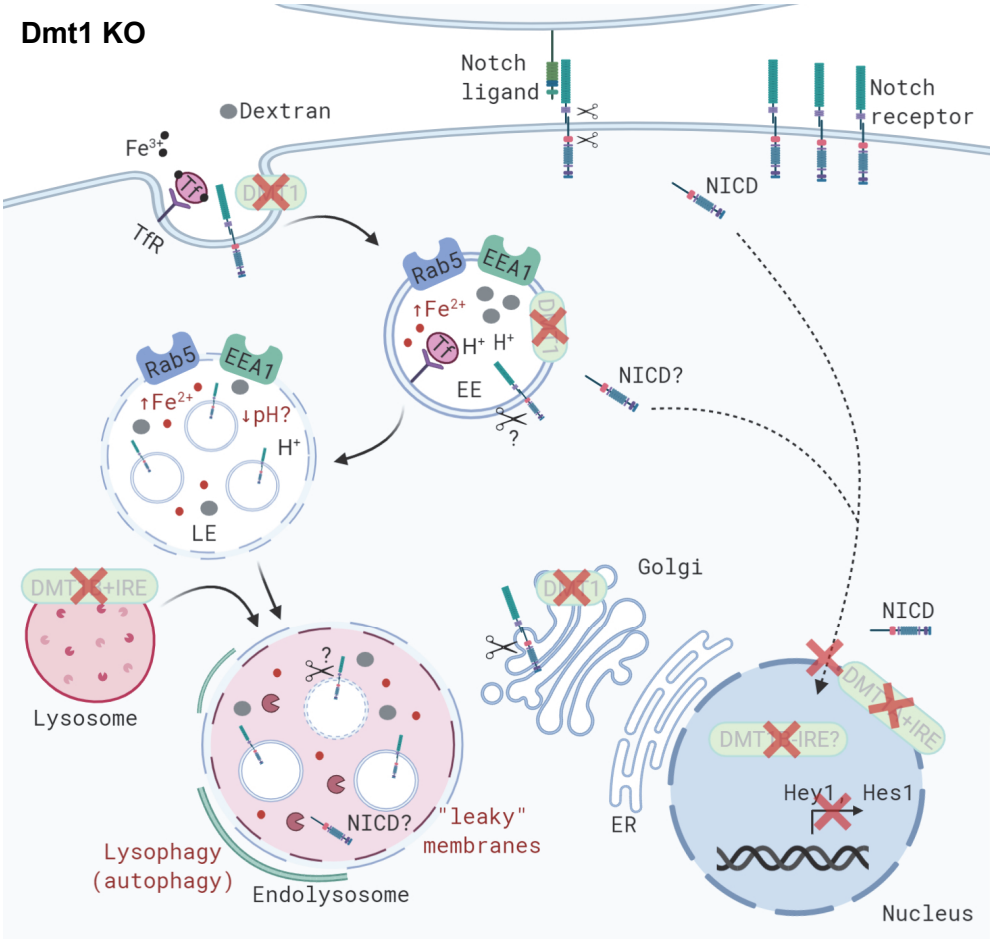


Figure 7. Graphical hypothesis of Notch1 inhibition in Dmt1 KO MEFs. *Dmt1 KO MEFS show diminished Notch signaling activation while (cleaved) Notch1 expression is increased both at the cell surface and in total. Loss of Dmt1 results in disturbed intracellular trafficking, including: increased dextran uptake, increased protein levels of EEA1, Rab5, and LAMP1 and increased basal autophagic flux. We hypothesize that loss of Dmt1 results in an accumulation of iron (Fe^{2+}) in intracellular vesicles, leading to the formation of ROS and subsequent oxidative stress, resulting in lysosomal membrane peroxidation. This lysosomal membrane damage may activate lysophagy, the selective autophagy of damaged lysosomes. In addition, accumulation of protons in intracellular vesicles may decrease the vesicular pH, which might also affect Notch trafficking and activation. We also observed distinct localization of the Dmt1 isoforms, which may reflect on their different functions. Therefore, we*

hypothesize that the Dmt1 isoforms differentially affect Notch1 signaling by regulating intracellular trafficking and activation of the Notch1 receptor. EE: early endosome, LE: late endosome, Tf: transferrin, TfR: transferrin receptor, NICD: Notch1 intracellular domain, ER: Endoplasmic reticulum.

vesicular transport which may lead to impaired Notch transport and activation. In addition, loss of Dmt1 resulted in damaged intracellular vesicles with disrupted/missing membranes and the formation of isolation membranes, which is characteristic of the formation of phagophores, an early step of autophagy/lysophagy (Papadopoulos and Meyer, 2017). Lysosomal membrane damage may occur in Dmt1 KO MEFs due to the accumulation of iron in the lysosomes leading to the formation of ROS and oxidative stress resulting in lysosomal membrane peroxidation (Kurz et al., 2008) (**Figure 7**). This lysosomal damage is sensed by cathepsins and cleared via lysophagy, the selective autophagy of lysosomes, which confirms the elevated basal autophagy flux we observed in Dmt1 KO MEFs (Papadopoulos and Meyer, 2017).

Moreover, we showed that loss of Dmt1 results in a decreased vesicular pH upon chloroquine treatment (**Figure 7**), which may lead to a decreased activation of Notch1 in these compartments, which we also showed upon increasing of the vesicular pH using chloroquine and BAFA1 (Hounjet et al., 2019). The luminal pH of the endosomal compartment has been shown to become more acidic along the endocytic trafficking process (Marshansky and Futai, 2008). The γ-secretase complex, which is present on the plasma membrane, endocytic compartments, and lysosomes (Sannerud et al., 2016; Small and Gandy, 2006), shows more efficient S3-cleavage in endocytic compartments, due to this lower pH (Pasternak et al., 2003). In addition, NICD1 was shown to be cleaved at a different amino acid position in endosomes, resulting in a less stable and different NICD (NICD-S) (Tagami et al., 2008). Together with the finding that defects in the vacuolar-ATPase reduce Notch signaling (Yan et al., 2009), these data support the notion that disturbed vesicular trafficking and pH upon loss of Dmt1 result in an accumulation of (cleaved) Notch1 in vesicles and decreased Notch activity.

Important to note is that we did not detect expression of the endogenous Dmt1a isoform in any model, in-line with previous data showing that Dmt1a is mainly expressed in the duodenum, while Dmt1b is widely expressed (Hubert and Hentze, 2002). Here, we show distinct localization of all four different Dmt1 isoforms. We show that Dmt1a-ire, Dmt1a+ire, and Dmt1b-ire are expressed in small intracellular vesicles with minor co-localization to the lysosomes, while Dmt1b+ire is almost exclusively located to the lysosomes. In line with our data, Dmt1-ire was shown to be expressed in early, sorting, and recycling endosomes, where its functionally bound to the transferrin receptor (Lam-Yuk-Tseung and Gros, 2006; Tabuchi et al., 2002; Tabuchi et al., 2000). Therefore, we hypothesize that silencing of *Dmt1-ire* decreases Notch1 signaling due to impaired intracellular trafficking, and receptor processing in early, sorting, and recycling endosomes since endosomal trafficking is important in the activation of Notch1 signaling (Yamamoto et al., 2010). In contrast, Dmt1+ire is localized to the plasma membrane and targeted to late endosomes and lysosomes (Lam-Yuk-Tseung and Gros, 2006; Tabuchi et al., 2002; Tabuchi et al., 2000). Thus the differential subcellular localization and function shown here supports our finding that inhibition of Dmt1-ire isoforms suppress Notch signaling while inhibition of Dmt1+ire isoforms inhibit Notch degradation in the lysosomes and thereby enhance Notch1 activity. Notably, we also report a ring of Dmt1a+ire expression around the nucleus, while Dmt1b-ire shows perinuclear expression and a dense accumulation in the nucleus. Previous studies also detected Dmt1-ire the nucleus, while Dmt1+ire showed no nuclear localization (Roth et al., 2000). The function of these Dmt1 isoforms in the nucleus remains unknown.

Due to the distinct expression patterns of the Dmt1 isoforms and the different effects of Dmt1-ire and Dmt1+ire inhibition on Notch signaling, we hypothesize that the Dmt1 isoforms may have different functions besides the regulation of iron homeostasis. This hypothesis is supported by the data of several studies, including (1) Dmt1a is strongly regulated by iron, while Dmt1b-ire is poorly regulated by iron (Hubert and Hentze, 2002), although all Dmt1 isoforms transport iron and do so with comparable efficiency (Mackenzie et al., 2007), (2) loss of iron responsive proteins (IRPs) only elevates *Dmt1a* and *Dmt1+ire* levels, while *Dmt1-ire* and *Dmt1b* mRNA

levels are not affected (Galy et al., 2013), (3) Dmt1 isoforms are degraded by different E3-ligases and Parkin has been shown to specifically target both Dmt1b isoforms to the proteasome (Garrick et al., 2012; Roth et al., 2010), (4) NF-kB, which is activated in Parkinson's disease, binds to the *Dmt1b* promoter and increases the expression of *Dmt1b*, without affecting *Dmt1a* expression (Paradkar and Roth, 2006) and *Dmt1b+ire* expression is positively correlated with neuro-degeneration in Parkinson's disease (Salazar et al., 2008), (5) *Dmt1a* is specifically up-regulated by HIF-2a upon hypoxia (Lis et al., 2005; Mastrogiannaki et al., 2009). Altogether these data suggest a different function of each of the isoforms of Dmt1 although the exact functions and differences require further investigation.

In addition to the similar localization and intracellular trafficking routes of Dmt1 and Notch, recent evidence also suggests that Dmt1 and Notch1 are degraded by the proteasome requiring similar adaptor proteins. Dmt1 is ubiquitinated by E3-ligases for which adaptor proteins Ndfip1 and Ndfip2 are required (Foot et al., 2008; Foot et al., 2016; Garrick et al., 2012; Howitt et al., 2009). *Drosophila* Ndfip, the functional homolog of mammalian Ndfip 1 and Ndfip2, also negatively regulates Notch1 signaling by augmenting Nedd4 and Su (dx) in Notch1 degradation (Dalton et al., 2011).

Although we show for the first time that Dmt1 regulates Notch1 signaling, a relation between iron and Notch signaling has already been reported. Iron drives the proliferation of Notch-induced T-ALL in mice (Khwaja et al., 2010), breast cancer cells up-regulate Dmt1 to satisfy their increased demand for iron (Jiang et al., 2010), and iron deficiency has been shown to promote growth and metastasis of breast cancer by stimulation of epithelial to mesenchymal transition (EMT) induced by activation of Notch signaling. Interestingly, a high iron diet showed decreased Notch signaling (Jian et al., 2013). However, Dmt1 transports other divalent metals beyond iron, including Zn^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+}, which are known cofactors for many different enzymes. Of special interest are the zinc-binding motif in the active site of Adam metallo-proteases (Düsterhöft et al., 2014; Stawikowska et al., 2013) and the modulation of γ-secretase activity by zinc and copper (Gerber et al., 2017), which may also play an important role in the regulation of Notch signaling by Dmt1.

To conclude, we identified Dmt1, a “simple” iron transporter, as a major regulator of Notch activity, which in an isoform-specific manner determines the fate of the Notch signaling cascade between γ-secretase cleavage and downstream transcriptional activation. We propose that Dmt1 containing intracellular vesicles identify an important intracellular node, through which Notch signaling traverses and amendable to small molecule inhibition that may be therapeutically exploited to modulate Notch signaling in disease and regenerative applications.

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Conflict of interest

The authors declare no conflict of interest.

Author contributorship information

J.H., A.J.G., F.R., K.R.K, and J.P. performed the experiments and analysed the experimental data. J.H. drafted the manuscript and designed the figures under supervision of A.J.G. and M.V. J.H., A.J.G., M.V., and K.R.K. contributed to the design and implementation of the research. All authors discussed the results and commented on the manuscript.

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Supplementary information

Supplementary table 1. Targeting sequences of shRNAs.

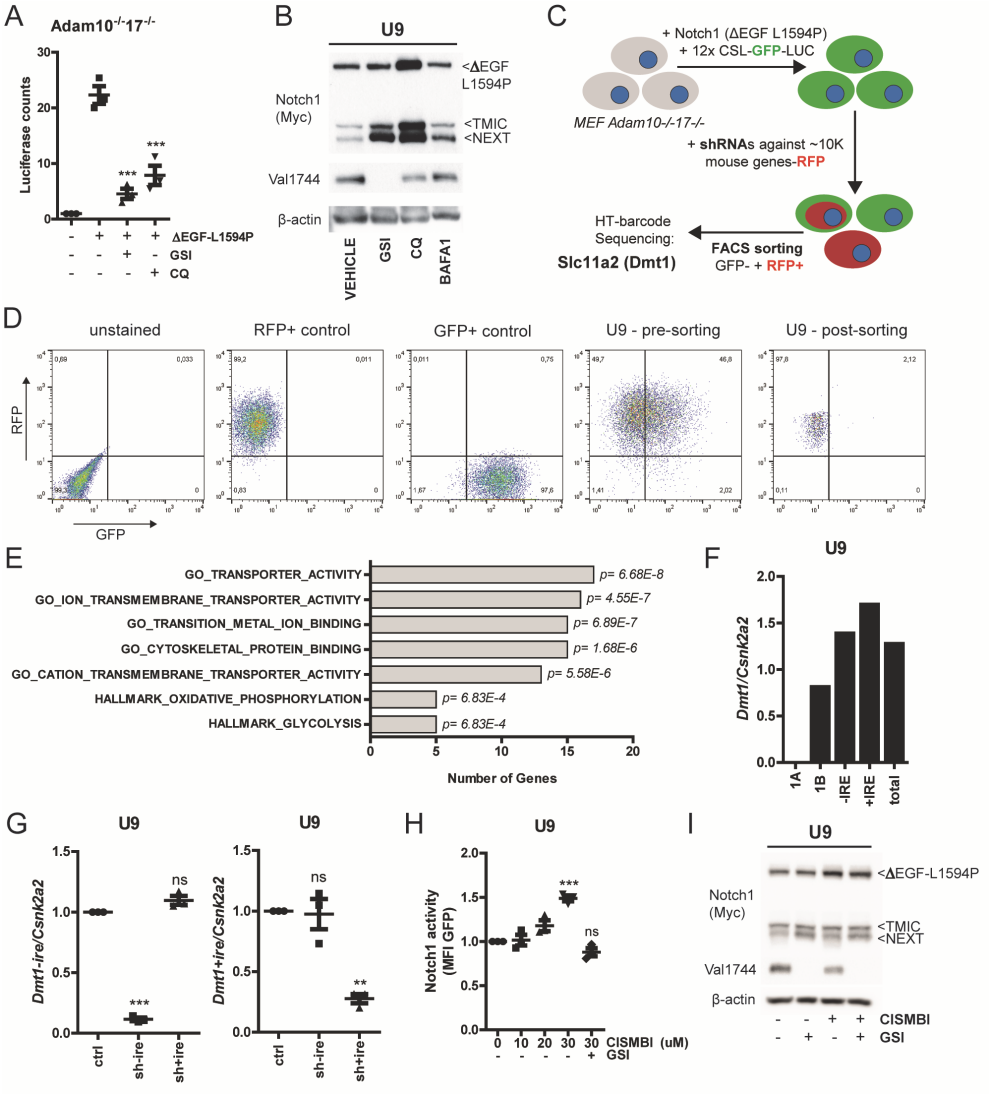
shRNA Target	Species	Sequence	Targeting Location
DMT1-IRE	Mouse	CCAGGAGACCTTAAGAACACT	Exon 17, 3'-UTR
DMT1-IRE 3.3	Human	GGCATTGCCAAAGAGCTTTAA	Exon 17, 3'-UTR
DMT1-IRE 4.8	Human	AGGTCCAAAGTCTATTTCTAG	Exon 17, 3'-UTR
DMT1+IRE	Mouse, Human	TGGTTTACTGTGTGAACATAG	Exon 16, 3'-UTR

Supplementary table 2. Mouse qRT-PCR primer sequences.

Gene	Forward	Reverse
Dmt1-ire	5'-CTCAGGTCTTCCTGGACAGC-3'	5'-CGCGTAGAGTGGGAAGAAA-3'
Dmt1+ire	5'-CACAGAGATCCTCCTGCCTC-3'	5'-AGGTGAGCCATCATCTGTCC-3'
Dmt1a	5'-CTCCCAACTGTGAGCTAAAATCC-3'	5'-GATGAGTTGCTGTAGGCAGGG-3'
Dmt1b	5'-GCGGAGCCGAATCCTATTCTA-3'	5'-CTGTAGGCAGGGTTGATGGC-3'
Dmt1 total	5'-GATTTAAGCTGCTCTGGGTGC-3'	5'-GATTGCCAACTCCACCATCA-3
Hes1	5′-TCCTAACGCAGTGTCACCTTCCAG-3′	5′-CCAAGTTCGTTTTTAGTGTCCGTC-3′
Hey1	5'-CAGGAGGGAAAGGTTATTTTGACG-3'	5'-TAGTTGTTGAGATGGGAGACCAGGCG-3'
Hey2	5'-AAGCGCCCTTGTGAGGAAAC-3'	5'-GGTAGTTGTCGGTGAATTGGAC-3'
Notch1	5'-ACACGTGGCTCCTGTATATG-3'	5'-ACAACAACGAGTGTGAGTCC-3'
Notch1 (ICD)	5'- GCAATCTCAAGTCTGCCACA-3'	5'- GCTTCCTTGCTACCACAAGC -3'
MyoD	5'-GGCCGTGGCAGCGAG-3'	5'-CGCTGTAATCCATCATGCCAT-3'
MyoG	5'-CCCATGGTGCCCAGTGAA-3'	5'-GCAGATTGTGGGCGTCTGTA-3'
Myf5	5'-GAACAGCAGCTTTGACAGCAT-3'	5'-AATGCTGGACAAGCATCCAA-3'
Csnk2a2	5'-CCACATAGACCTAGATCCACACT-3'	5'-CGCAGGAGCTTGTCAAGAAGA-3'

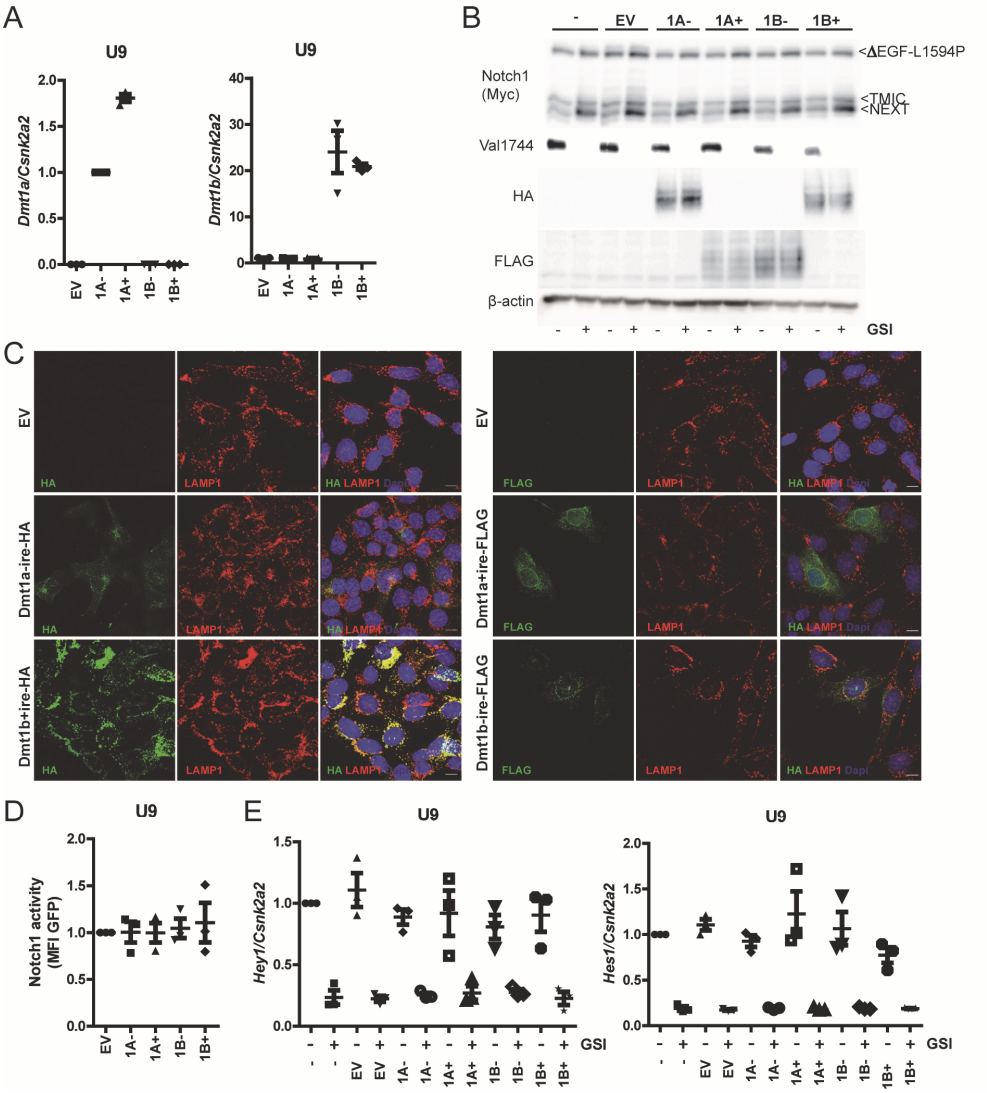
Supplementary table 3. Human qRT-PCR primer sequences.

Gene	Forward	Reverse
DMT1-IRE	5'-TCACCCACCTTCCTAACCAG-3'	5'-TGGAATCCCAGCTATTCAGG-3'
DMT1+IRE	5'-TTTGGAGCTTTCCTTCCAGA-3'	5'-AGACCATCCATCCAGTCTGC-3'
DMT1A	5'-GGAGCTGGCATTGGGAAAGTC-3'	5'-GGAGATCTTCTCATTAAAGTAAG-3'
DMT1B	5'-GTTGCGGAGCTGGTAAGAATC-3'	5'-GGAGATCTTCTCATTAAAGTAAG-3'
MUC5AC	5'-TGCCATCACCCATCTGCC-3'	5'-ACCACATCCAGGTCCGTCA-3'
HES1	5'-AGGCGGACATTCTGGAAATG-3'	5'-CGGTACTTCCCCAGCACACTT-3'
HES4	5'- CACCGCAAGTCCTCCAAG-3'	5'-TCACCTCCGCCAGACACT-3'
NOTCH1	5'-AGGACCTCATCAACTCACACGC-3'	5'-TCTTTGTTAGCCCCGTTCTTCAG-3'
JAGGED2	5'-GTCGTCATCCCCTTCCAGT-3'	5'-CTCCTCATTCGGGGTGGTAT-3'
GAPDH	5'-ACACTCAGACCCCCACCACA-3'	5'-CATAGGCCCCTCCCCTCTT-3'



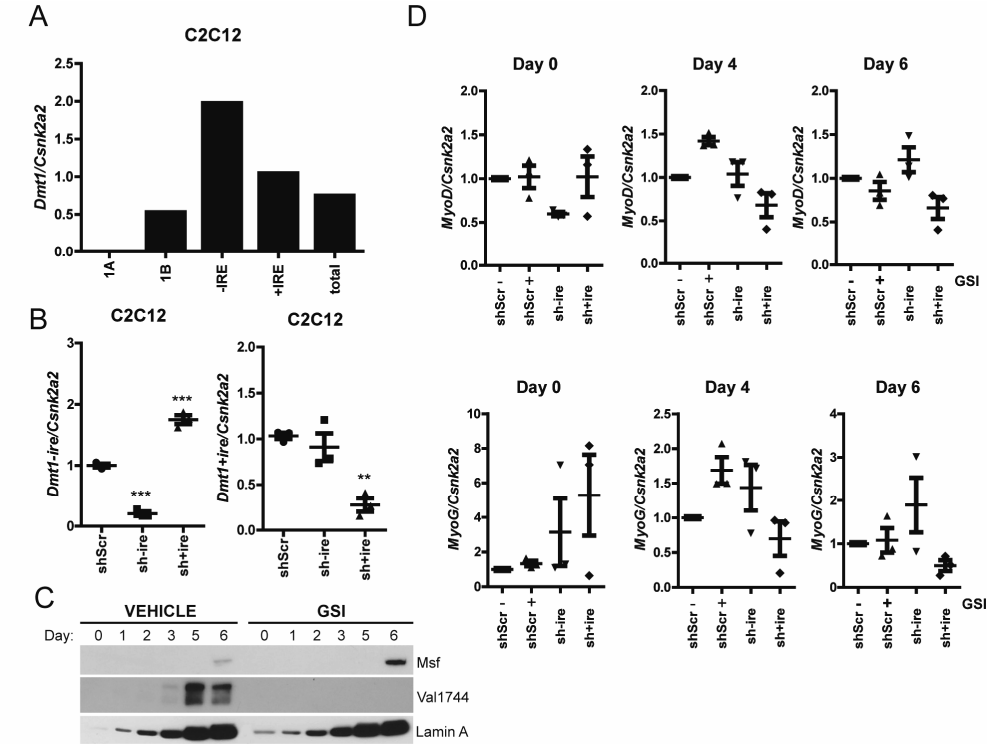
Supplementary figure 1. Dmt1 isoforms are novel regulators of mutant Notch signaling. ***A)*** *Notch1 activity in screening cell line (U9) upon ΔEGF-Notch1-L1594P expression and DMSO, GSI, or chloroquine (CQ) treatment for 24 hours measured by luciferase counts (One-Way ANOVA (Tukey comparison), ***P<0.001, significant compared to control).* ***B)*** *Immunoblot analysis of (cleaved) Notch1 (Myc), Val1744, and β-actin (loading control) in U9 cells treated with DMSO, DBZ, chloroquine (CQ), or Bafilomycin A1 (BAFA1) for 24 hours.* ***C)*** *shRNA screen in Adam10$^{-/-}$17$^{-/-}$ deficient MEFs expressing active ligand-independent Notch1 and Notch1 reporter. RFP-positive and GFP-null expressing cells were sorting and HT-*

barcode sequencing revealed the Slc11a2 gene, encoding Dmt1, as a novel regulator of Notch signaling. ***D)*** *Fluorescent activated cell sorting (FACS) gates of shRNA screening population.* ***E)*** *Pathway analysis of the shRNA screen reporting the number of genes included in each of the pathways.* ***F)*** *Dmt1 isoform mRNA expression in U9 cells measured by qRT-PCR. Csnk2a2 mRNA expression was used as a house-keeping control.* ***G)*** *qRT-PCR for Dmt1-ire and Dmt1+ire mRNA levels in U9 cells with Dmt1-ire and Dmt1+ire knockdown. Csnk2a2 mRNA expression was used as a house-keeping control (One-Way ANOVA (Tukey comparison), ns= non-significant, **P<0.01, ***P<0.001, significant compared to empty vector control).* ***H)*** *Flow cytometry analysis of Notch activity (GFP expression) in U9 cells treated with Dmt1 inhibitor CISMBI and DBZ for 72 hours (One-Way ANOVA (Tukey comparison), ns= non-significant, ***P<0.001, significant compared to DMSO control).* ***I)*** *Immunoblot analysis of ΔEGF-Notch1-L1594P (Myc), Val1744, and β-actin (loading control) protein levels in U9 cells treated with CISMBI and DBZ for 72 hours. TMIC: transmembrane/intracellular fragment. NEXT: Notch1 extracellular truncation. GSI: γ-secretase inhibitor dibenzoazepine. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

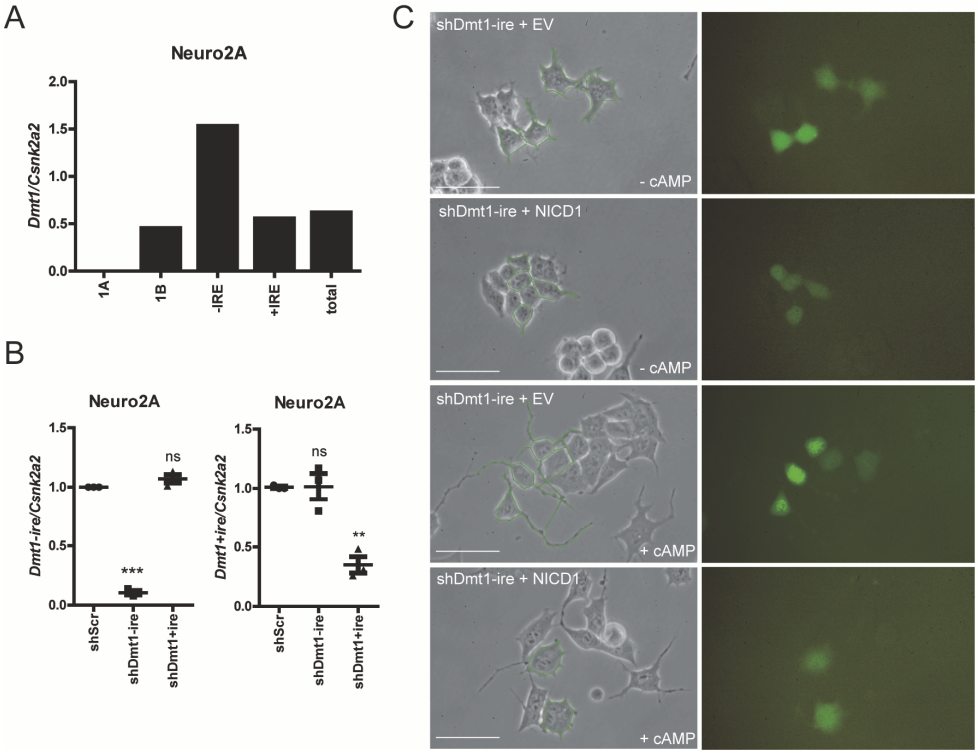


Supplementary figure 2. Ectopic expression of Dmt1b+ire localizes to the lysosomes and overexpression of Dmt1 isoforms has no effect on ligand-independent Notch1 activity. A) *Dmt1a and Dmt1b mRNA expression levels in U9 cells with stable overexpression of empty vector (EV), Dmt1a-ire (1A-), Dmt1a+ire (1A+), Dmt1b-ire (1B-), or Dmt1b+ire (1B+). Csnk2a2 mRNA expression was used as a house-keeping control.* ***B)*** *Immunoblot analysis of (cleaved) ΔEGF-Notch1-L1594P (Myc), Val1744, HA, FLAG, and β-actin (loading control) protein levels in U9 cells with stable overexpression of empty vector (EV) or the different Dmt1 isoforms treated with DMSO or DBZ for 24 hours.* ***C)*** *Immunofluorescence co-staining for HA-*

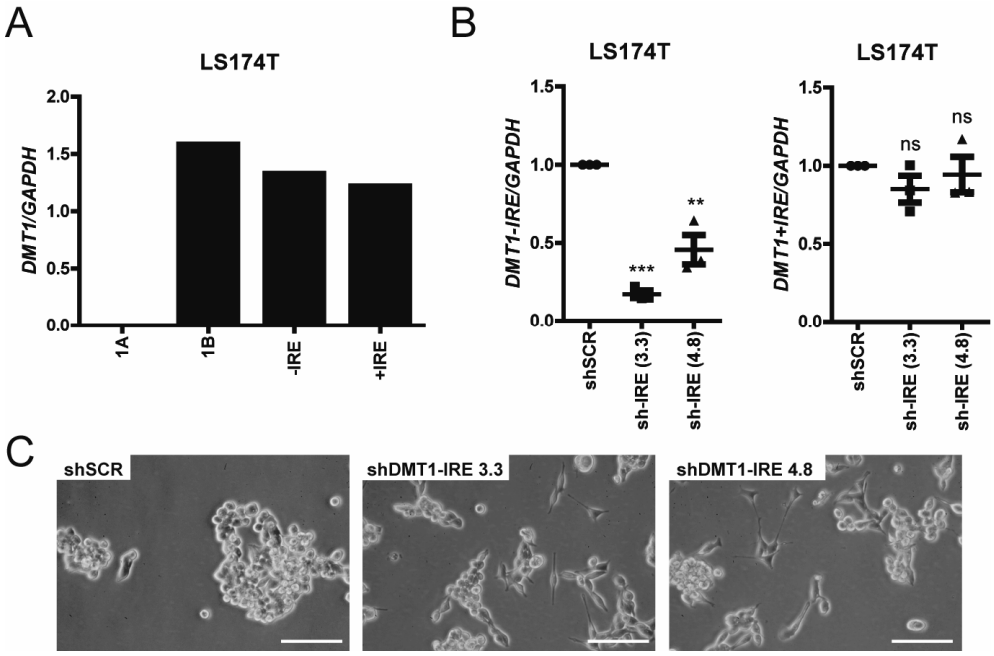
or FLAG-tagged Dmt1 isoforms (green) and LAMP1 (red) in U9 cells with stable overexpression of empty vector (EV) or the different Dmt1 isoforms. Scale bar: 10 µm. ***D)*** *Flow cytometry analysis of Notch1 reporter activity in U9 cells with stable overexpression of empty vector (EV) or the different Dmt1 isoforms measured by GFP expression.* ***E)*** *Notch1 target gene expression (Hey1, Hes1) in U9 cells with stable overexpression of empty vector (EV) or the different Dmt1 isoforms treated with DMSO or DBZ for 24 hours measured by qRT-PCR. Csnk2a2 mRNA expression was used as a house-keeping control. TMIC: transmembrane/intracellular fragment. NEXT: Notch1 extracellular truncation. GSI: γ-secretase inhibitor dibenzoazepine. LAMP1: Lysosomal-Associated Membrane Protein 1. MFI: mean fluorescent intensity. Data are representative of three independent experiments and values are expressed in mean ± SEM.*



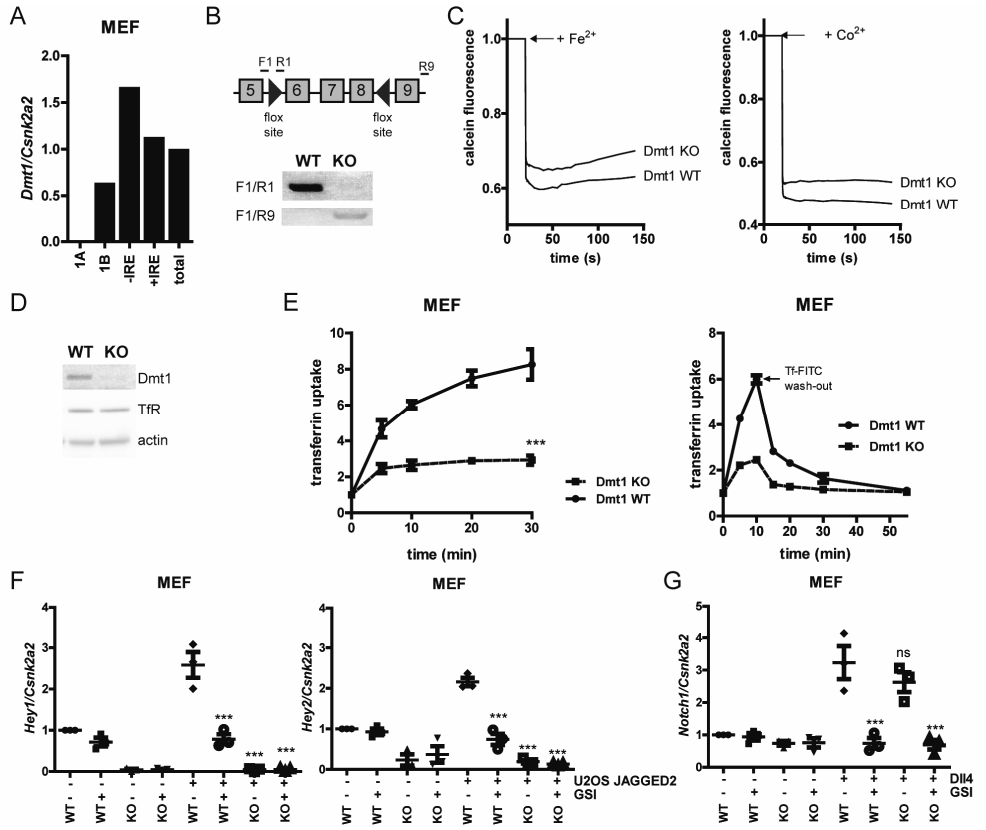
Supplementary figure 3. Dmt1 knockdown in a Notch-dependent myoblast differentiation model. A) *qRT-PCR for endogenous Dmt1 isoform expression in C2C12 cells. Csnk2a2 mRNA expression was used as a house-keeping control.* ***B)*** *Dmt1-ire and Dmt1+ire mRNA expression in C2C12 cells with stable knockdown of scrambled, Dmt1-ire, or Dmt1+ire analyzed by qRT-PCR (One-Way ANOVA (Tukey comparison), **P<0.01, ***P<0.001, significant compared to scrambled control. Csnk2a2 mRNA expression was used as a house-keeping control.* ***C)*** *Immunoblot analysis of Msf, Val1744, and lamin A (loading control) protein levels in undifferentiated (day 0) and differentiated (day 1 to 6) C2C12 cells treated with DMSO or DBZ.* ***D)*** *MyoD and MyoG mRNA expression in differentiating C2C12 cells with stable knockdown of scrambled (treated with DMSO or DBZ), Dmt1-ire, or Dmt1+ire at day 0, 4, and 6 of differentiation measured by qRT-PCR. Csnk2a2 mRNA expression was used as a house-keeping control. Msf: Myosin skeletal FAST. GSI: γ-secretase inhibitor dibenzoazepine. MyoD: myoblast determination protein 1. MyoG: myogenin. Data are representative of three independent experiments and values are expressed in mean ± SEM.*



Supplementary figure 4. Dmt1 knockdown in Neuro2A cells. A) *qRT-PCR for endogenous Dmt1 isoform mRNA expression in Neuro2A cells. Csnk2a2 mRNA expression was used as a house-keeping control.* ***B)*** *Dmt1-ire and Dmt1+ire mRNA expression in Neuro2A cells with stable knockdown of scrambled, Dmt1-ire, or Dmt1+ire measured by qRT-PCR. Csnk2a2 mRNA expression was used as a house-keeping control (One-Way ANOVA (Tukey comparison), ns= non-significant, **P<0.01, ***P<0.001, significant compared to scrambled control.* ***C)*** *Bright-field and fluorescent images of basal (-cAMP) and differentiated (+cAMP) Neuro2A cells with stable Dmt1-ire knockdown and overexpression of empty vector (GFP) or NICD1 (GFP). Scale bar: 50 µm. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

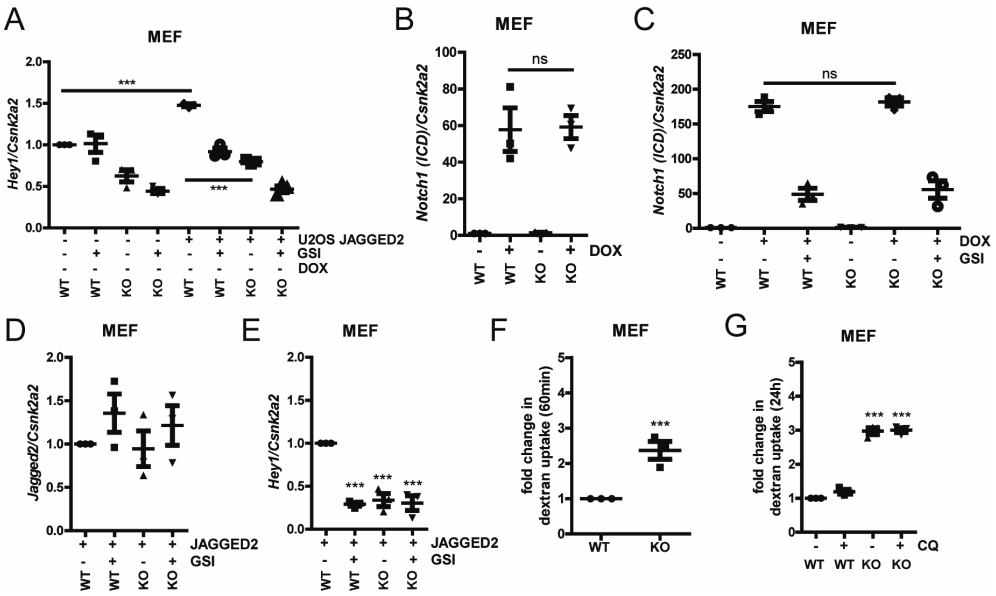


Supplementary figure 5. Dmt1 knockdown in colorectal adenocarcinoma cells. A) *Endogenous Dmt1 isoform mRNA expression in LS174T cells measured by qRT-PCR. GAPDH mRNA expression was used as a house-keeping control.* ***B)*** *qRT-PCR for DMT1-IRE and DMT1+IRE mRNA expression in LS174T cells with stable knockdown of scrambled (shSCR) or DMT1-IRE (sh-IRE3.3). An independent hairpin targeting another sequence of DMT1-IRE was used as a control (sh-IRE 4.8) (One-Way ANOVA (Tukey comparison), ns= non-significant, **P<0.01, ***P<0.001, significant compared to scrambled control).* ***C)*** *Bright-field images of LS174T cells with stable knockdown of scrambled, DMT1-IRE (3.3), or DMT1-IRE (4.8). Scale bar: 100 µm. Data are representative of three independent experiments and values are expressed in mean ± SEM.*



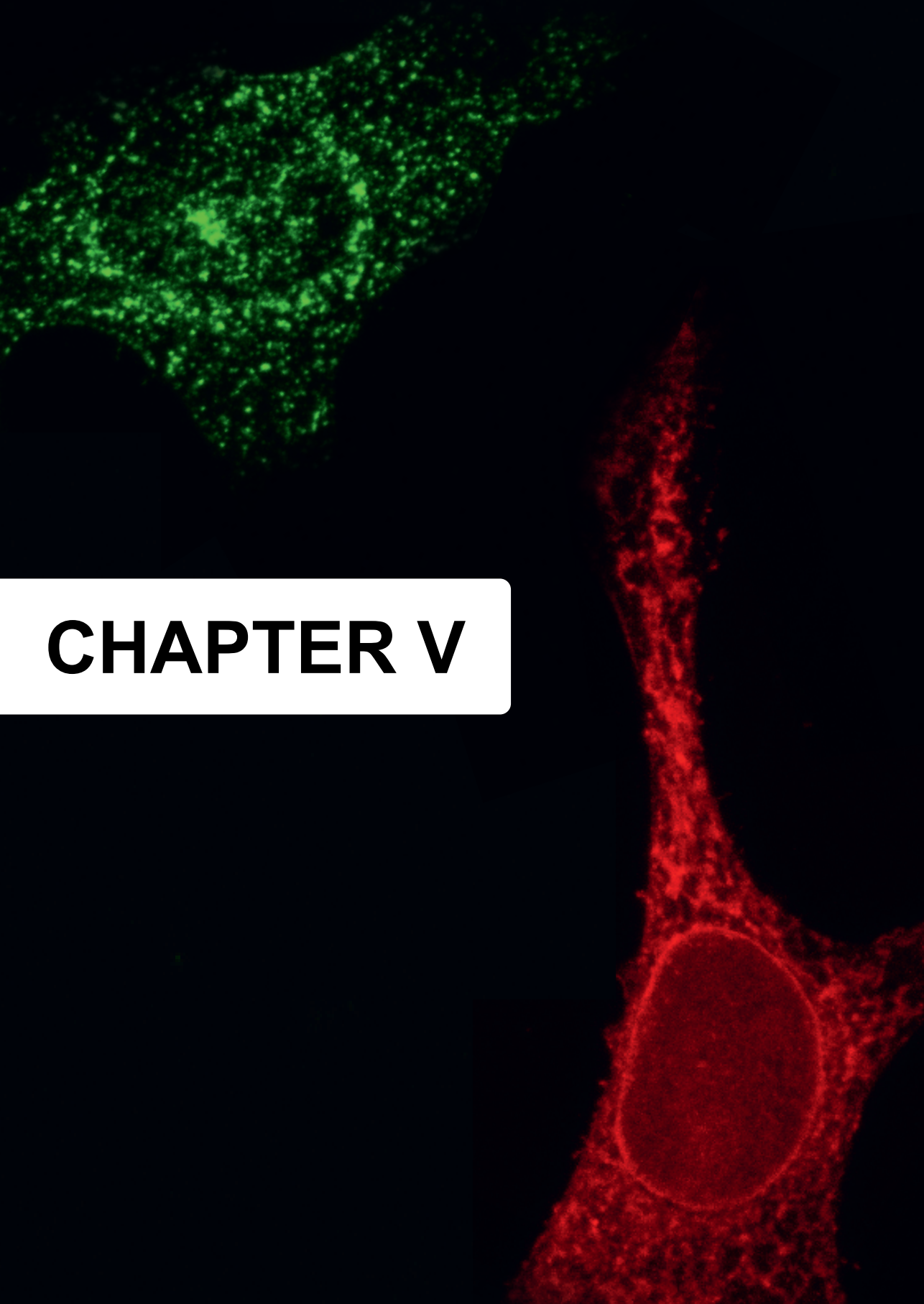
Supplementary figure 6. Endogenous Notch signaling is diminished upon loss of Dmt1. ***A)*** *qRT-PCR for Dmt1 isoform mRNA expression in mNramp2 (Dmt1) fl/fl MEFs. Csnk2a2 mRNA expression was used as a house-keeping control.* ***B)*** *Flox-sites in Dmt1 fl/fl MEFs, including exon 6 to 8 of the Slc11a2 gene, encoding Dmt1. Specific Dmt1 wild-type (WT) and Dmt1 KO PCR products are shown generated by the Dmt1 WT primer set (F1/R1) and the Dmt1 KO primer set (F1/R9).* ***C)*** *Dmt1 transport activity measured by iron (Fe^{2+}) and cobalt (Co^{2+}) induced fluorescent quenching of calcein in Dmt1 WT and KO MEFs.* ***D)*** *Immunoblot analysis of Dmt1, transferrin receptor (TfR) and β-actin (loading control) protein levels in Dmt1 WT and KO MEFs.* ***E)*** *Fluorescently labelled-transferrin uptake in Dmt1 WT and KO MEFs measured by flow cytometry (Student t-test, ***P<0.001, significant compared to Dmt1 WT control)(left panel). Pulse- and chase assay of fluorescently labelled-transferrin in Dmt1 WT and KO MEFs analysed by flow cytometry (right panel).* ***F)*** *Notch target gene expression (Hey1, Hey2) in Dmt1 WT and KO MEFs stimulated with JAGGED2 and treated with DMSO or DBZ for 24 hours measured by qRT-PCR. Csnk2a2 mRNA expression was used as a*

*house-keeping control (One-Way ANOVA (Tukey comparison), ***P<0.001, significant compared to Dmt1 WT MEFs stimulated with JAGGED2).* ***G)*** *qRT-PCR for Notch1 mRNA expression in Dmt1 WT and KO cells stimulated with Dll4 and treated with DMSO or DBZ for 24 hours. Csnk2a2 mRNA expression was used as a house-keeping control (One-Way ANOVA (Tukey comparison), ns= non-significant, ***P<0.001, significant compared to Dmt1 WT MEFs stimulated with Dll4). GSI: γ-secretase inhibitor dibenzoazepine. Data are representative of three independent experiments and values are expressed in mean ± SEM.*

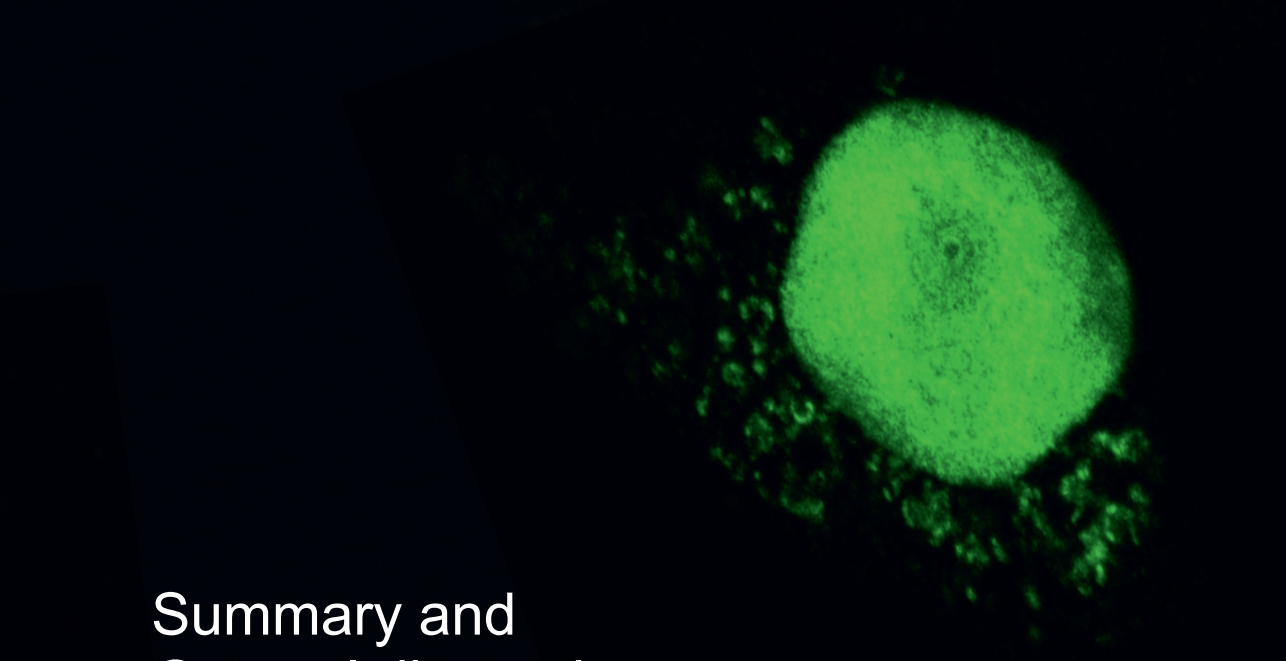


Supplementary figure 7. Ectopic activation of Notch1 rescues loss of Notch signaling in Dmt1 KO MEFs. A) *Notch target gene Hey1 mRNA expression in Dmt1 WT and KO MEFs (with doxycycline-inducible NICD1 expression) stimulated with JAGGED2 and treated with DMSO or DBZ for 24 hours without doxycycline treatment measured by qRT-PCR. Csnk2a2 mRNA expression was used as a house-keeping control (One-Way ANOVA (Tukey comparison), ***P<0.001).* ***B)*** *Ectopic NICD1 mRNA expression upon doxycycline treatment in Dmt1 WT and KO MEFs with doxycycline-inducible NICD1-mCherry-GFP expression analyzed by qRT-PCR. Csnk2a2 mRNA expression was used as a house-keeping control (One-Way ANOVA (Tukey comparison), ns= non-significant).* ***C)*** *Ectopic Notch1ΔE mRNA expression upon doxycycline treatment in Dmt1 WT and KO MEFs with doxycycline inducible Notch1ΔE-mCherry-GFP expression treated with DMSO or DBZ for 24 hours analyzed by qRT-PCR. Csnk2a2 mRNA expression was used as a house-keeping control (One-Way*

ANOVA (Tukey comparison), ns= non-significant). ***D)*** *qRT-PCR for human Jagged2 expression in Dmt1 WT and KO MEFs with stable overexpression of JAGGED2 treated with DMSO or DBZ for 24 hours. Csnk2a2 mRNA expression was used as a house-keeping control.* ***E)*** *Notch target gene Hey1 mRNA expression in Dmt1 WT and KO MEFs with stable overexpression of JAGGED2 treated with DMSO or DBZ for 24 hours. Csnk2a2 mRNA expression was used as a house-keeping control (One-Way ANOVA (Tukey comparison), ***P<0.001, significant compared to DMSO-treated Dmt1 WT MEFs).* ***F)*** *Fluorescently labelled dextran uptake within 60 minutes in Dmt1 WT and KO MEFs measured by flow cytometry (Student t-test, ***P<0.001, significant compared to Dmt1 WT MEFs).* ***G)*** *Fluorescently labelled dextran uptake within 24 hours in Dmt1 WT and KO MEFs, pre-treated with or without chloroquine for 24 hours, measured by flow cytometry (One-Way ANOVA (Tukey comparison), ***P<0.001, significant compared to untreated Dmt1 WT MEFs). GSI: γ-secretase inhibitor dibenzoazepine. Data are representative of three independent experiments and values are expressed in mean ± SEM.*



CHAPTER V



Summary and General discussion

Judith Hounjet

Department of Radiation Oncology (Maastro), GROW School for Oncology, Maastricht University Medical Centre+, Maastricht, The Netherlands

Maastro clinic, Maastricht, The Netherlands

Aberrant Notch signaling in cancer

Although tumor biology has been studied for decades, cancer remains one of the major causes of morbidity and mortality worldwide. However, during these last decades cancer research has revealed extensive fundamental insights in tumor initiation and progression and underlined the molecular complexity of human malignancies. Cancer can occur in almost each tissue of the body and different cancers can arise within specific tissues and express general cancer hallmarks. Targeting these cancer hallmarks is complicated, since cancer cells adapt endogenous signaling pathways that operate in healthy tissues to provide in tumor growth and progression, which are normally required during embryonic development and homeostasis throughout adult life. Therefore, “smart” cancer therapeutics are required, which specifically target cancer cells without damaging normal tissues.

One of these therapeutic windows may be aberrant Notch signaling, which has been reported in a broad range of human malignancies. Gain-of-function mutations in NOTCH1 have been observed in T-cell acute lymphoblastic leukemia (T-ALL), breast cancer, and non-small cell lung cancer (Robinson et al., 2011; Weng et al., 2004; Westhoff et al., 2009). However, the majority of elevated Notch signaling observed in tumors is not related to direct mutations in the *NOTCH* gene, but results from aberrant activation of ligand-dependent Notch signaling or the loss of function of negative regulators of Notch signaling, including NUMB and FBXW7 (O'Neil et al., 2007; Pece et al., 2004; Westhoff et al., 2009). As a result, γ-secretase inhibitors (GSI) have been used to target NOTCH1 signaling, however, failed due to dose-limiting toxicities, although this may be circumvented by altered GSI scheduling (Krop et al., 2012; Tolcher et al., 2012). In addition to pan-γ-secretase inhibitors, novel approaches of NOTCH inhibition have been developed, including monoclonal antibodies targeting Notch ligands and (activated) receptors, which are currently tested in clinical trials. Importantly, these therapeutic strategies of NOTCH targeting in cancer are not tumor specific and will effect NOTCH signaling in both tumor and normal cells. Therefore, **in this thesis our aim** was to identify new vulnerabilities in the NOTCH signaling pathway involved in human malignancies, sparing normal NOTCH signaling. Using this approach, NOTCH signaling in tumor cells in cancer

patients may be targeted more specifically, resulting in a higher tolerated treatment dose and longer treatment periods, leading to normal tissue sparing, a higher chance of cure, and more tolerable therapies.

Intravesicular activation of the Notch receptor

In this thesis, we emphasized on the role of intracellular trafficking of the Notch receptor in Notch signaling. We showed that intracellular trafficking of the Notch receptor, in both ligand-dependent and independent signaling is important for a proper signal cascade. In **chapter II**, we reviewed the current knowledge on intracellular trafficking of the Notch receptor in both *Drosophila* and mammals. Intracellular trafficking of the Notch receptor is an important regulator of Notch activity in both *Drosophila* and mammals and applies an additional layer of complexity to the Notch signaling cascade. In *Drosophila* inhibition of early endosomal trafficking results in decreased Notch signaling activation (Chapman et al., 2016; Vaccari et al., 2008), which is also reported upon inhibition of endosomal and lysosomal fusion (Kobia et al., 2014; Vaccari et al., 2010). However, blocking Notch receptor trafficking in maturing and late endosomes results in Notch signaling activation (Childress et al., 2006; Vaccari et al., 2008). Therefore, tight regulation of vesicular transport of the Notch receptor is essential. Inhibition of intracellular trafficking showed similar effects on mammalian Notch receptor activation. In addition, the mammalian genome often encodes several orthologs of the endosomal compartment machinery, thus making studying their function in mammals more complex. Whether aberrant Notch signaling in tumor cells relies more on intracellular trafficking for their activation remains understudied. In **chapter III**, we show that ligand-independent Notch signaling in human T cell leukemia (T-ALL) depends more on intracellular trafficking, because chloroquine treatment did not affect wild-type Notch signaling in human T-ALL cells, however, this remains to be confirmed *in vivo.*

In **chapter II**, we also reviewed the potential mechanisms of ligand-independent Notch activation and the role of intracellular vesicles herein. Accumulating evidence shows that intracellular vesicles play an essential role in ligand-independent Notch activation. First, the enzymes required for Notch cleavage

and activation, including Adam10 and γ-secretase are expressed on the plasma membrane, but also in intracellular vesicles (Chastagner et al., 2017; Pasternak et al., 2003; Sannerud et al., 2016; Skovronsky et al., 2000). Second, previous work from our laboratory and others showed that cleaved Notch proteins localize to intracellular vesicles (van Tetering et al., 2009). Third, the Notch extracellular domain (ECD) maybe degraded by lysosomal hydrolases or dissociate in intracellular vesicles during trafficking due to the decreasing pH or lack of Ca^{2+} (Marshansky and Futai, 2008). Fourth, the decreasing pH in intracellular vesicles during endocytic trafficking may also increase γ-secretase activity (Pasternak et al., 2003) and cleavage precision (Tagami et al., 2008) resulting in increased Notch processing and pathway activity.

In line with this hypothesis, increasing the pH in intracellular vesicles showed increased localization of Notch receptors to late endosomes and lysosomes and blocked ligand-independent signaling activation (Kobia et al., 2014; Vaccari et al., 2010; Yan et al., 2009). In **chapter III**, we confirmed these findings in human T-ALL cells, as we showed decreased ligand-independent NOTCH signaling in GSI-sensitive T-ALL cells upon chloroquine treatment, which increases the intravesicular pH, with concomitant elevated levels of (cleaved) NOTCH1. Our finding challenges the paradigm that cleaved Notch receptors are always active, since Notch target gene expression is blocked following chloroquine treatment. This has important consequences for using Val1744-cleaved Notch1 antibodies as a biomarker for patients selected for GSI. Combined treatment of chloroquine and GSI showed synergic effects on T-ALL cell proliferation and viability, leading to the accumulation of (all cleaved fragments of) NOTCH1 in intracellular vesicles, yet to be identified. Moreover, we showed that in GSI-resistant T-ALL cell lines, which express wild-type Notch signaling, chloroquine treatment does not affect NOTCH activity. These findings suggest that deregulation of intracellular trafficking by chloroquine treatment only affects ligand-independent Notch signaling. However, others showed that chloroquine increases endogenous Notch1 signaling in endothelial cells by accumulation of endogenous Notch1 receptors in late endosomal vesicles, inducing tumor vessel normalization (Maes et al., 2014). This discrepancy in endogenous

Notch signaling activation may arise from the differences in cellular background and may be tissue specific. These findings suggest that oncogenic Notch signaling in T-ALL cells, and may be cancer cells in general, may depend more on intracellular trafficking for its activation, compared to normal tissues. If chloroquine indeed inhibits Notch signaling in cancer cells, while increasing or not affecting Notch signaling in normal tissues, it may be a promising treatment for Notch-addicted tumors.

Together these data suggest that intracellular trafficking of the ligand-independently activated NOTCH receptors may be a therapeutic vulnerability in T-ALL and might be similar in other human malignancies. Furthermore, it may be possible to improve tissue tolerance against Notch inhibitors by combining GSI with drugs like chloroquine that can synergize with Notch inhibitors but only in cells with hyper-activated Notch. Our unpublished data showed that combined treatment of chloroquine and GSI *in vivo* did not lead to increased gastrointestinal toxicity using a GSI dose that was therapeutically effective. Secondly, Notch addicted tumors may be more sensitive to pharmacological reduction of Notch levels than wild-type cells and it may be easier to tip the balance towards a therapeutic effect in tumor cells versus a toxic effect in normal cells when the correct patients are selected.

The metal transporter Dmt1 is a novel regulator of Notch signaling

Our observations that cleaved Notch receptors also occur in intracellular vesicles and Notch signaling is effectively blocked by inhibitors of vesicle function, prompted us to identify regulators of vesicular trafficking, which are essential for Notch signaling activation. Using a genetic loss-of-function screen, we identified a high enrichment for genes that encode vesicle-associated receptors and transmembrane (ion) transport proteins, indicating an important role for these proteins in Notch signal transduction. In **chapter IV** of this thesis, we showed for the first time that inhibition of the different isoforms of Divalent metal transporter 1 (Dmt1) differentially affect both ligand-dependent and independent Notch signaling. We confirmed in different cell models that silencing of *Dmt1-ire* expression results in decreased ligand-dependent Notch signaling, while inhibition of *Dmt1+ire* expression results in

enhanced ligand-dependent Notch activation. These effects of Dmt1 silencing were also observed in ligand-independent Notch signaling.

Moreover, we showed that the intracellular localization upon ectopic expression of the different isoforms of Dmt1 is distinct. We observed almost exclusive expression of Dmt1b+ire in the lysosomes, indicating that Dmt1b+ire may be involved in metal transport in lysosomes, however, might also regulate protein degradation, including the degradation of (cleaved) Notch receptors. Dmt1a-ire, Dmt1a+ire, and Dmt1b-ire showed diffuse intracellular expression in small vesicular structures, yet to be identified, and minor co-localization with lysosomal marker LAMP1. Interestingly, Dmt1a+ire showed a ring-shaped expression around the nucleus and Dmt1b-ire showed nuclear expression. These different expression patterns of the Dmt1 isoforms may reflect on their function, which may be metal transport at different cell sites, however, may also include other functions. Upon complete loss of Dmt1, we showed a deregulation of intracellular transport, including: (1) increased uptake of dextran, which may reflect increased internalization and/or decreased degradation or recycling, (2) increased Rab5 protein levels, (3) decreased LC3B-II levels upon chloroquine treatment, and (4) decreased elevation of intravesicular pH upon chloroquine treatment. Disturbed intracellular pH levels may arise from changes in intracellular proton transport, as Dmt1 is a proton-coupled receptor, which upon metal transport co-transports protons into the cytoplasm. Therefore, loss of Dmt1 may result in an accumulation of protons in intracellular vesicles, resulting in more acidic vesicles. However, further research is required to study the exact mechanism. We also hypothesize that the different isoforms of Dmt1 might function as chaperones, regulating intracellular transport of proteins, including the Notch receptor, at different cellular sites including nuclear transport.

Both iron and Notch1 signaling have been linked to ROS. While iron uptake produces ROS via the Fenton reaction, which leads to the internalization of Dmt1 into the endosome and is inhibited by antioxidant treatment, Notch1 activation protects against ROS induction (Esparza et al., 2015). Moreover, oxidative stress induced by iron, due to up-regulation of Dmt1, is related to neurotoxicity in animal models of both Parkinson's and Alzheimer's disease (Núñez et al., 2012; Salazar et

al., 2008), where inhibition of both Dmt1-ire and Dmt1+ire protects against neuronal toxicity (Zheng et al., 2009). In response to oxidative stress, the oxidative response (Keap1-Nrf2) pathway is activated. Nrf2 has been shown to activate Notch1 signaling as Nrf2 regulates the expression of antioxidant response elements (ARE) containing genes, including Notch1 (Paul et al., 2014; Wakabayashi et al., 2014). Conversely, Notch1 signaling has been shown to induce Nrf2 and its target gene expression (Wakabayashi et al., 2014). Recently, inhibition of Dmt1 was shown to specifically kill cancer stem cells, that are dependent on a high iron metabolism, by retaining ROS in the lysosomes, leading to increased lysosomal ROS and cell death via ferroptosis (Turcu et al., 2020). Our lab also showed that both Notch inhibition and chloroquine treatment in NOTCH1-driven T-ALL result in an accumulation of ROS, oxidative stress, and subsequent DNA damage (Hounjet et al., 2019)(**chapter III**). Together these data show that Dmt1 and Notch1 are closely involved in the regulation of ROS and the subsequent oxidative stress response.

Although we show for the first time that Dmt1 regulates Notch1 signaling, a relation between iron and Notch signaling was already reported. Notch1 activation in murine models of T-ALL increases the expression of HIV-1 Reb-binding protein (Hrb), which increases uptake of iron, leading to increased T-ALL survival (Khwaja et al., 2010). Moreover, breast cancer cells up-regulate Dmt1 to satisfy their increased demand for iron (Jiang et al., 2010) and iron deficiency has been shown to promote growth and metastasis of breast cancer by stimulation of epithelial to mesenchymal transition (EMT) induced by the activation of Notch signaling. Interestingly, a high iron diet showed decreased Notch signaling (Jian et al., 2013).

An additional link between Notch signaling, Dmt1, and iron may be hypoxia-inducible factor 1α (HIF1α) signaling. In normoxic conditions, HIF1α is degraded by prolyl-hydroxylases (PHDs) and factor inhibiting hypoxia-inducible factor 1 (FIH), which require iron (Cho et al., 2013). Iron chaperones poly (rC) binding protein 1 (PCBP1) and PCBP2 sequester iron from the cytoplasm and deliver it to ferritin for iron storage. Loss of these iron chaperones results in decreased PHD activity, leading to reduced hydroxylation, and degradation of HIF1α (Nandal et al., 2011). An accumulation of HIF1α was shown to increase Notch target gene expression by

directly binding to NICD and decreasing NICD degradation, targeting NICD to Notch-responsive promoters to induce their expression (Gustafsson et al., 2005). In addition, NICD increases HIFα target gene expression.

FIH hydroxylates both HIF1α and NICD and reduces Notch activity (Wilkins et al., 2009; Zheng et al., 2008). Moreover, silencing of Notch1 inhibits HIF1α signaling by increasing FIH activity (Lawton et al., 2010). In contrast, high levels of HIF1α and HIF2α decrease Notch ligand expression and Notch activation during erythropoiesis (Myllymäki et al., 2017) and HIF2α induces Dmt1 expression to increase iron uptake in colorectal tumor cells (Xue et al., 2016). Together these findings show that changes in iron metabolism may affect Notch signaling directly or indirectly via HIF1-signaling and may be tissue specific.

Can chloroquine target intracellular trafficking of Notch in cancer patients?

In this thesis, we showed that silencing of *Dmt1-ire* and chloroquine or Bafilomycin A1 treatment reduce ligand-independent Notch signaling and result in disturbed intracellular trafficking and an accumulation of Val1744-cleaved NICD, without being active.

We showed in **chapter III** that chloroquine sensitizes oncogenic NOTCH1 driven human T-ALL cells to γ-secretase inhibition (GSI), by decreasing NOTCH1 signaling and by inducing DNA damage and oxidative stress. As a result, the DNA damage response is activated and induces a cell cycle arrest and a subsequent block in proliferation and a synergistic effect on the induction of apoptosis. Moreover, we showed that the addition of chloroquine to GSI treatment required a decreased GSI concentration to obtain similar effects on proliferation and cell viability compared to GSI treatment alone. These promising findings suggest that chloroquine may be suitable for the treatment of human T-ALL and may reduce GSI-toxicity, including intestinal toxicity, by requiring a lower dose of GSI. However, *in vivo* experiments need to be performed to confirm the anti-leukemic effects of chloroquine and its synergistic effects when combined with GSI treatment, before clinical trials can be set-up to test its safety and efficacy in the treatment of human T-ALL.

Chemotherapy and radiotherapy often lead to treatment resistance, as tumor cells up-regulate autophagy as a pro-survival mechanism. Therefore, chloroquine, which inhibits autophagy by blocking the fusion of autophagosomes with lysosomes, has been combined with conventional treatments to sensitize tumor cells to these treatments, which showed increased therapeutic responses (Verbaanderd et al., 2017). However, additional anti-tumor effects have been observed upon chloroquine treatment, which act independently of autophagy inhibition, including: inhibition of NFκβ-signaling, inhibition of chemokine signaling, activation of p53-signaling, normalization of tumor vasculature, and immunomodulation. These autophagy-independent effects of chloroquine may also contribute to the sensitization of tumor cells to conventional treatments.

Since chloroquine showed promising anti-tumor effects in different types of cancer in preclinical studies, a broad range of clinical trials to test the safety and efficacy of chloroquine, as a mono-treatment or combined with other anti-cancer agents, in various cancer types are ongoing. Several clinical trials showed positive or partial therapeutic responses in glioblastoma patients when chloroquine was combined with chemotherapy or radiotherapy (Briceño et al., 2003; Sotelo et al., 2006) and improved brain metastases control from solid tumors when combined with whole-brain irradiation (Rojas-Puentes et al., 2013). In multiple myeloma, chloroquine treatment in combination with anti-cancer therapies only showed minor anti-tumor effects (Kyle et al., 1975; Montanari et al., 2014). Overall, chloroquine may have beneficial effects for cancer patients, however, whether the anti-tumor effects of chloroquine rely on Notch inhibition in these patients remains elusive and may require proper patient selection. To our knowledge, chloroquine treatment has not been tested in human T-ALL, however, may have beneficial effects for patients as a single treatment or combined with conventional systemic therapies of Notch inhibition.

Can Dmt1 isoform inhibition target Notch signaling in cancer patients?

Inhibition of Dmt1 in preclinical studies showed increased death of cancer stem cells due to increased iron and ROS levels in the lysosomes (Turcu et al., 2020).

Moreover, Dmt1 is highly expressed in colorectal cancer patients (Xue et al., 2016). In *in vivo* studies, iron was shown to induce JAK1/STAT3 signaling, sustaining tumor progression and Dmt1 inhibition reduced colorectal tumor growth (Xue et al., 2016). In addition, iron-targeting therapies are under clinical investigation based on (1) iron depletion; since cancer cells are more dependent on iron for their survival compared to normal cells and (2) iron overload; which promotes ROS induction, resulting in oxidative stress and cell death by iron-induced ferroptosis. Iron depletion using iron chelators or antibodies against the transferrin receptor showed modest therapeutic responses and toxicity in clinical trials, and are currently optimized in additional *in vivo* studies (Daniels-Wells et al., 2020). Iron overload can be induced by iron chelators, which bind iron but also promote iron-induced ROS formation. This approach also showed promising results in preclinical trials. For example, Erastin, which elevates oxidative stress by deregulating mitochondrial function resulting in ferroptosis, showed reduced tumor growth and metastases in a murine model of ovarian cancer (Basuli et al., 2017).

Since all isoforms of Dmt1 have been shown to transport metals with a similar efficiency (Garrick et al., 2006; Mackenzie et al., 2007), developing Dmt1b-ire specific inhibitors may provide a new therapeutic vulnerability in the treatment of Notch-addicted tumors, without affecting metal-transport. However, first these data need to be verified *in vivo*. Moreover, preclinical studies are required to test whether upon loss of Dmt1b-ire isoforms, Dmt1b+ire isoforms are able to efficiently support iron homeostasis. Furthermore, we show that inhibition of Dmt1b-ire isoforms not only reduces ligand-independent Notch signaling, but also physiological ligand-dependent signaling. Therefore, specific inhibition of Dmt1b-ire isoforms may result in Notch-inhibition related gastrointestinal toxicity, however, this may be circumvented by intermitted scheduling.

Moreover, we observed no expression of *Dmt1a* in our cell lines and could therefore not study the endogenous effects of Dmt1a-ire or Dmt1a+ire inhibition on ligand-dependent and independent Notch signaling. This is in-line with a previous study on Dmt1 isoform expression, which showed ubiquitous expression of Dmt1b isoforms and Dmt1a isoform expression in the duodenum and kidney (Hubert and

Hentze, 2002). Therefore, studying the role of Dmt1a in duodenum cell lines or duodenum organoids will provide novel insights in the role of Dmt1a in endogenous Notch signaling. To conclude, additional research is required for studying the exact functions of the different Dmt1 isoforms and their effects on both ligand-dependent and independent Notch signaling, which may reveal therapeutic vulnerabilities in the treatment of aberrant Notch signaling in cancer patients.

Fundamental research or/and repurposed-use of drugs?

Overall, we used two different approaches in this thesis to obtain a better understanding in the importance of intracellular trafficking in NOTCH activation. First, we conducted a shRNA screen to identify novel regulators of Notch activation and we discovered that Dmt1 is a novel regulator of both ligand-dependent and independent Notch signaling by deregulation of intracellular trafficking. Notably, changing Dmt1 isoforms expression levels can both activate and inhibit Notch signaling. These novel findings provide us with a better understanding of the intracellular activation of Notch signaling and may help finding novel vulnerabilities for the treatment of different types of human malignancies expressing aberrant NOTCH signaling (both gain- and loss-of-function). They also highlight the importance of fundamental research, which is often neglected in research and research funding as it may not directly lead to new therapeutics. However, fundamental research is most essential for understanding and gaining new insights in molecular processes in finding a cure for all different types of cancer leading to new therapeutic opportunities. Cancers are complex diseases and simple “trial and error” in finding a cure will probably not work. First, we have to understand and identify which pathways tumors use for their survival, to specifically target these, and to win the fight against cancer.

The second approach we used was to repurpose chloroquine, a FDA-approved drug, in the treatment of NOTCH1-driven T-ALL. Our rationale came from the observation that Notch signaling also required vesicle function and that blocking NOTCH1 cleavage and localization might enhance the therapeutic ratio of Notch targeting, in favor for cancer cell derived NOTCH versus physiological NOTCH

signaling. We indeed show that inhibiting vesicle transport reduced oncogenic NOTCH activity, while chloroquine treatment did not affect wild-type NOTCH signaling in GSI-resistant T-ALL cells. However, additional evidence is required to confirm the increased dependence of intravesicular transport of aberrant NOTCH signaling in T-ALL, which may also exist in solid tumors. The development of new drugs may take decades to implement in the clinic, while FDA-approved drugs may be more quickly approved for other diseases. Moreover, toxicity profiles and tolerated doses of these repurposed drugs are known.

In conclusion, fundamental research is still essential in finding a cure for most cancers. Basic research increases our understanding of the differences in cell biology between tumor and normal cells, which we can use to specifically kill tumor cells, without affecting normal tissues. Combining fundamental research with repurposing drugs may lead to fast, effective, and cheap cancer treatments and might be one of the best strategies to find a cure for all different types of cancer, for those that have easy but also for those with more difficult access to the best treatment.

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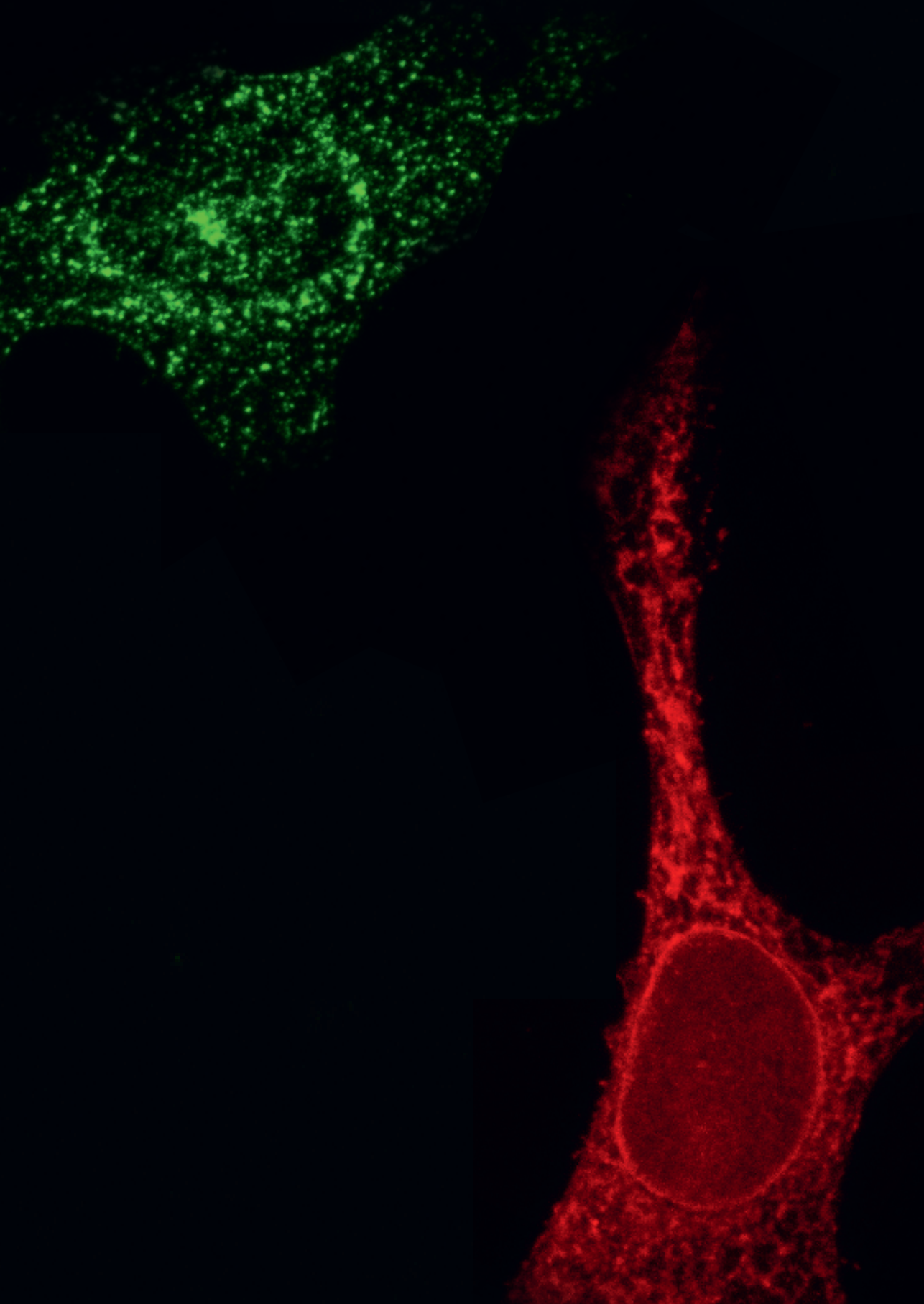
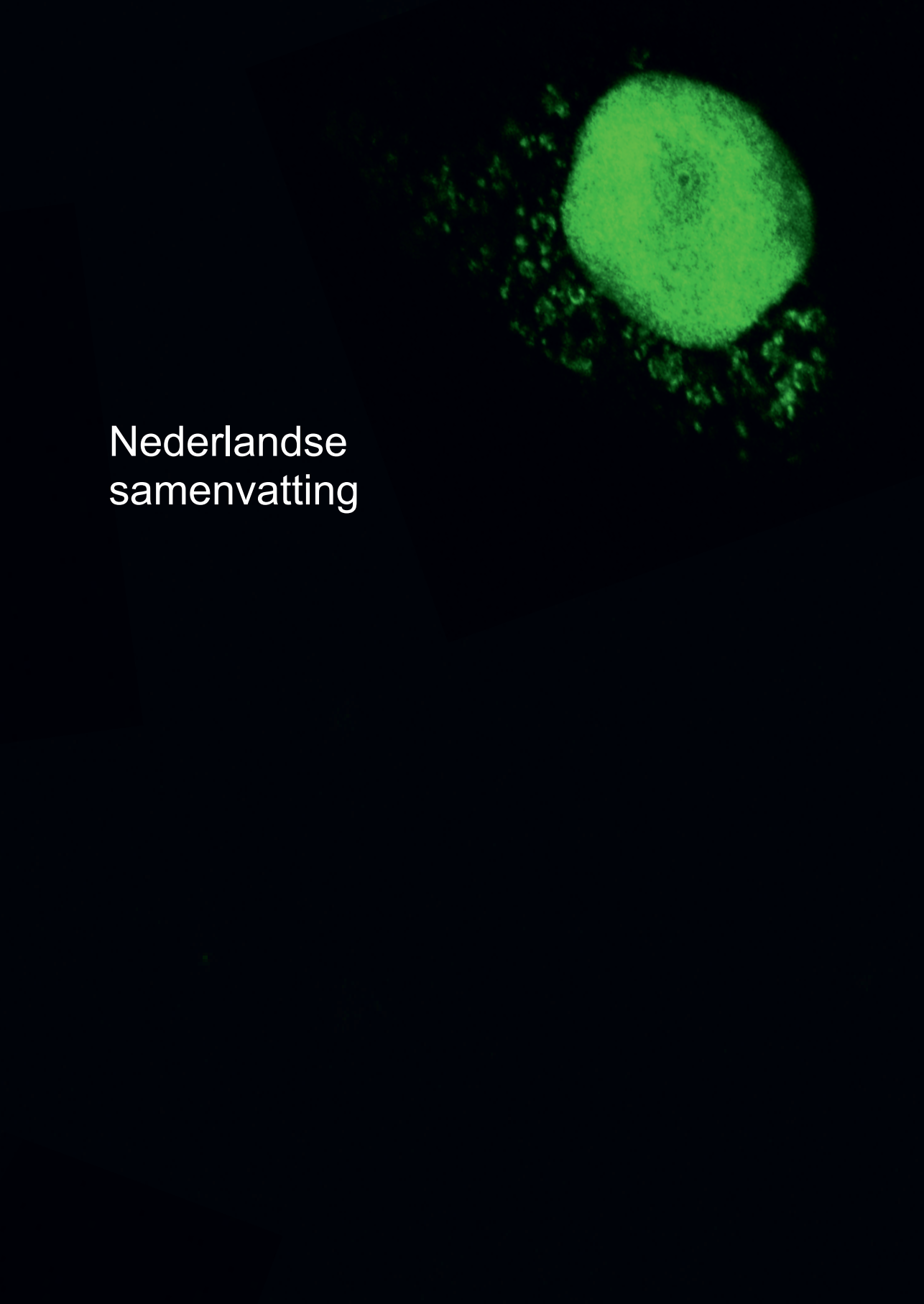
V





Nederlandse samenvatting

Kanker is een van de belangrijkste doodsoorzaken wereldwijd. Kanker ontstaat door een ongecontroleerde celgroei die veroorzaakt wordt door mutaties in het DNA. Door deze mutaties worden eiwitten die groei stimuleren meer aangemaakt en actiever, terwijl eiwitten die groei normaalgesproken onderdrukken, worden geremd. Hierdoor ontstaat een disbalans, waardoor cellen ongeremd kunnen delen en er een tumor kan vormen, groeien en uitzaaien. Notch signalering is een communicatie mechanisme tussen twee cellen dat celgroei, cel differentiatie en celdood reguleert tijdens de ontwikkeling en in bijna alle volwassen weefsels. Cellen in deze organen dienen continu vernieuwd te worden en hebben dus celgroei signalen nodig voor het correct uitvoeren van hun functie.

Notch is een receptor eiwit dat aan het cel oppervlak zit. Om actief te worden, moet de Notch receptor binden aan een ligand eiwit op een naastgelegen cel. Vervolgens wordt de Notch receptor door enzymen in meerdere fragmenten geknipt. Dit zorgt ervoor dat het actieve deel van de Notch receptor vrijkomt van het cel oppervlak en via het cytoplasma van de cel zich verplaatst naar de celkern, waar het celgroei aanzet. Recent onderzoek wijst echter uit dat Notch activiteit ook wordt beïnvloed door de locatie van de (actieve) Notch receptor fragmenten in de cel. Deze fragmenten van de Notch receptor worden in de cel getransporteerd in kleine blaasjes, zogenaamde “vesicles”. Afhankelijk van het soort vesicle, waarin de Notch fragmenten zich bevinden, is Notch actief of inactief. Dit zal uiteindelijk bepalend zijn voor het gedrag van de cel en welke verandering deze ondergaat.

Kankeronderzoek wijst uit dat de Notch signaal route vaak ontregeld en actief is in kankercellen en dus de groei van deze kanker cellen stimuleert. Hoge activiteit van Notch komt in verschillende soorten kanker voor, onder andere in: leukemie, borstkanker en longkanker. Het merendeel van de beschreven mutaties in de Notch signaal route komen voor in een specifiek soort leukemie van de T-cellen. In andere soorten kanker komen mutaties in het Notch eiwit sporadisch voor, hoewel Notch wel heel actief is. Waarom de activiteit van de Notch signaal route zo hoog is in veel

soorten kanker is nog onduidelijk. Doordat veel tumoren hoge Notch activiteit laten zien, en deze activiteit afhankelijk is van de enzymen die Notch knippen, zijn remmers van deze enzymen getest als potentiële anti-kanker behandelingen. Hoewel deze Notch remmers effectief zijn in het bestrijden van kankercellen, laten ze ook nadelige effecten zien in normale weefsels. Dit komt doordat Notch remmers niet alleen Notch activiteit blokkeren in kankercellen, maar ook in de normale weefsels. Hierdoor worden Notch remmers (tot op heden) niet gebruikt in de behandeling van kankerpatiënten.

Het **doel van dit promotie onderzoek** was het bestuderen van activiteit van het Notch eiwit in kanker en normale cellen en het vinden van de verschillen tussen beiden. Deze verschillen zouden we kunnen benutten om nieuwe kankerbehandelingen te ontwikkelen die meer specifiek tumor cellen aanpakken zonder de normale weefsels aan te tasten. Deze kankerbehandelingen zouden dus effectiever kunnen zijn in het bestrijden van de kankercellen met minder schadelijke bijwerkingen.

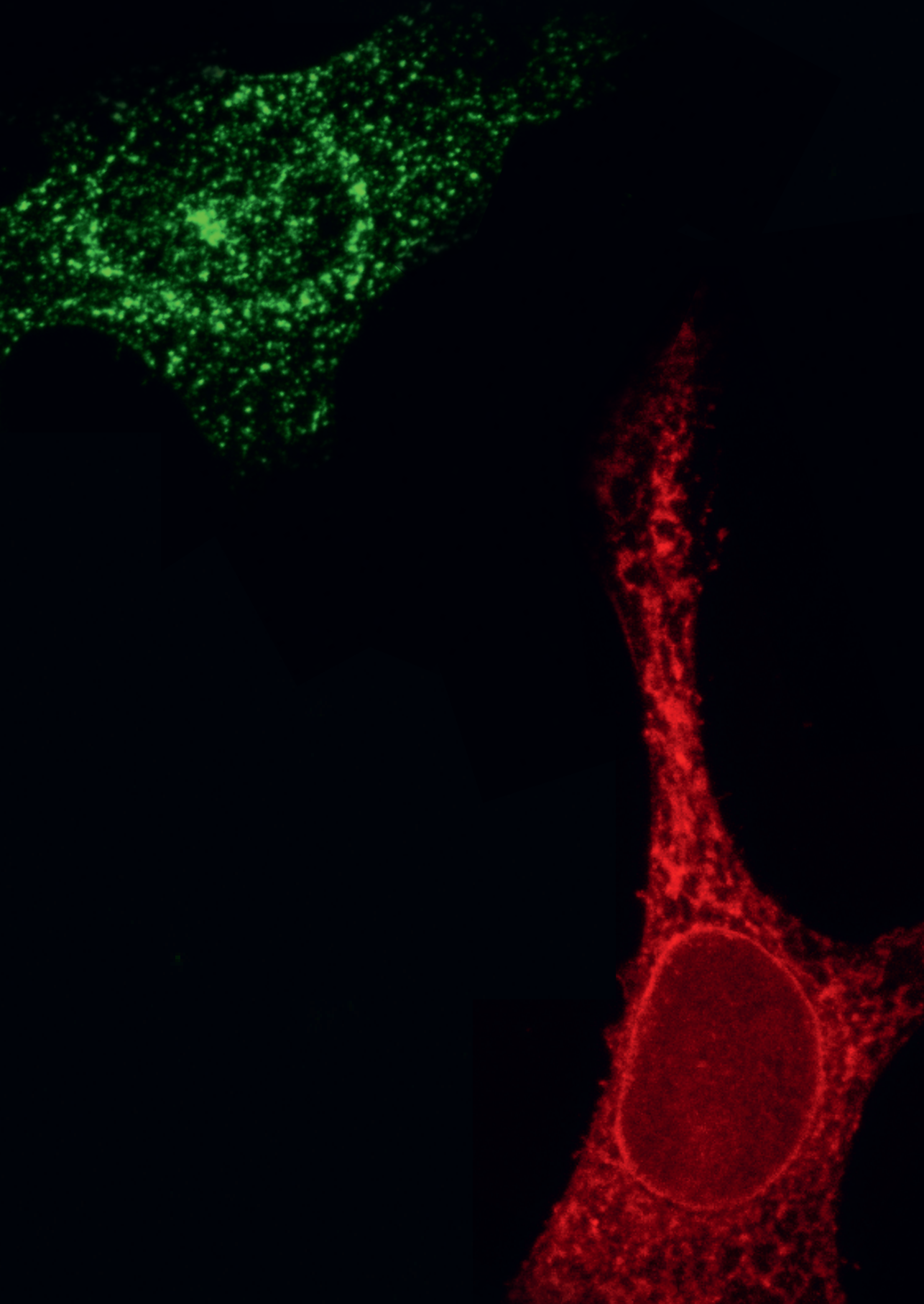
In **hoofdstuk II** van dit proefschrift geven we een overzicht van de actuele kennis op het gebied van Notch activiteit in de fruitvlieg (waarin de Notch signaal route oorspronkelijk is ontdekt en waarin veel van de huidige kennis over Notch activiteit is verkregen) en in zoogdieren. In deze review vatten we samen waarom de regulatie van Notch activiteit door andere eiwitten in vesicles belangrijk is in de Notch signaal route. Afhankelijk van het soort vesicle waarin Notch zich bevindt, kan Notch activiteit worden geactiveerd of geremd. Er zijn aanwijzingen dat deze manier van Notch activatie meer wordt gebruikt door kankercellen ten opzichte van normale cellen. Er is echter meer onderzoek nodig om deze hypothese te bewijzen en hiervoor een gerichte therapeutische aanpak te ontwikkelen. In de toekomst zouden met meer kennis geneesmiddelen, die de activatie van Notch in vesicles verstoren, mogelijk Notch activiteit nog specifieker kunnen remmen in kankercellen.

In **hoofdstuk III** van dit proefschrift beschrijven we een studie gedaan op leukemie cellen, die hoge Notch activiteit vertonen, met chloroquine (in combinatie met Notch remmers). Chloroquine is een geneesmiddel dat wordt gebruikt in het voorkomen en behandelen van malaria, maar wordt ook in steeds meer in klinische studies getest in de behandeling van verschillende soorten kanker. Chloroquine verstoort namelijk het transport via vesicles in de cel. We laten zien dat de combinatie van chloroquine en Notch remmers zorgt voor een afname van groei en een toename van celdood van de leukemie cellen, doordat chloroquine DNA schade veroorzaakt, die de leukemie cellen niet meer kunnen repareren. Daarnaast zorgt chloroquine ervoor dat het actieve Notch deel ophoopt in vesicles in de cel, maar niet meer actief is. Tenslotte laten we zien dat door het combineren van chloroquine met Notch remmers een lagere dosis van Notch remmers nodig is om hetzelfde anti-leukemie effect te krijgen ten opzichte van een behandeling met alleen Notch remmers. In theorie zou dit de bijwerkingen van deze Notch remmers in de behandeling leukemiepatiënten sterk kunnen verminderen.

In **hoofdstuk IV** van dit proefschrift hebben we Dmt1, een eiwit dat verschillende metalen waaronder ijzer transporteert, geïdentificeerd als een nieuwe regulator van Notch activiteit. Dmt1 kan in vier verschillende vormen in de cel voorkomen: Dmt1a-, Dmt1a+, Dmt1b- en Dmt1b+. We laten zien dat het remmen van de Dmt1- vormen Notch activiteit remt, terwijl het remmen van de Dmt1+ vormen Notch activiteit juist stimuleert. Als alle vormen van Dmt1 inactief zijn in een cel wordt Notch ook inactief. Hoewel het mechanisme hierachter nog niet helemaal duidelijk is, laten we zien dat verlies van Dmt1 leidt tot een verstoord transport van vesicles en dat dit hoogstwaarschijnlijk ook verantwoordelijk is voor de inactiviteit van de Notch signaal route.

In **hoofdstuk V** van dit proefschrift geven we een samenvatting van onze bevindingen en bediscussiëren we de eventuele mechanismen die Notch, chloroquine en Dmt1 samenbrengen. Daarnaast veronderstellen we hoe deze

nieuwe kennis toegepast kan worden in de behandelingen van kankerpatiënten en welke uitdagingen we nog dienen te overbruggen met toekomstig kankeronderzoek om te komen tot meer kanker specifieke behandelingen, die effectiever zijn met minder bijwerkingen.



Reflection

Scientific and social impact

Scientific impact

Cancer is one of the leading causes of death world-wide and cancer research gained extensive insights in tumor biology over the last decades. Cancer research showed that cancer cells make “smart” use of common mechanisms normal cells use for their homeostasis to sustain their growth and survival. Therefore, the main struggle in fighting cancer is to not damage normal cells while killing cancer cells. Although these mechanisms used for cell growth by cancer and normal cells seem similar, cancer cells show some modifications in these mechanisms, that maybe exploited to achieve a higher tumor selectivity of treatments. Anti-cancer drugs targeting these differences may kill cancer cells specifically, with less normal tissue damage.

Notch is a protein, which is highly active throughout mammalian development and in adult tissues, and frequently deregulated in a broad range of human cancers, sustaining cancer stem cell survival, tumor progression, and treatment resistance. Therefore, the use of Notch inhibitors appears to be a promising anti-cancer treatment. Unfortunately, Notch inhibitors also target Notch signaling in normal tissues and therefore have shown limited anti-cancer effects due to dose-limiting toxicities in normal tissues, including the intestine and skin. To achieve effective targeting of Notch in cancer tissues only, differences in Notch signaling in cancer and normal cells need to be studied to obtain a better understanding in the vulnerabilities of cancer cells regarding to Notch activity.

In this thesis, we provide novel insights in the regulation of Notch activity, which will support future cancer research in developing novel Notch targeting therapeutic strategies for anti-cancer treatments. First, we have shown that Notch in cancer cells uses intracellular trafficking by vesicles as a major source of its activity, while normal cells are not dependent to the same extent on this activation mode. Therefore, targeting the intracellular routing of Notch and thus limiting its activation in cancer cells, may result in specific cancer cell killing, without affecting Notch activity in normal tissues.

In addition, we discovered that Dmt1, a general metal transporter, is a novel regulator of Notch activity. We hypothesize that Dmt1 also affects Notch signaling by disrupting its activation in intracellular vesicles. Notch and iron addiction are common

features of various cancers. Therefore, the development of isoform specific inhibitors of Dmt1 may provide a double edged sword in cancer treatment. First, inhibition of Dmt1 may block Notch activity and secondly reduce iron uptake, which cancer cells are more dependent on to sustain their growth and tumor progression compared to normal cells. In addition, various types of cancer may benefit from this therapeutic approach. However, more research is required to demonstrate the safety and efficacy of isoform specific Dmt1 inhibitors. Furthermore, proper patient selection will be required since not all cancer cells are driven by high levels of Notch activity and iron. Unfortunately, bringing novel drugs towards clinical use in cancer patients may require several decades of research. First, the safety and efficacy of the novel treatment needs to be proven *in vivo* and subsequently in clinical trials, which will be also expensive.

An effective anti-cancer treatment which may be faster, cheaper, and show low toxicity may be repurposing existing drugs. In this approach, drugs that are already approved for the treatment of other human diseases find other off-label-applications. In this thesis, we show that chloroquine, a FDA-approved drug, which is used for the prevention and treatment of malaria, is effective in killing leukemic cells, which are ‘addicted’ to Notch activity. Moreover, we show that when combining chloroquine with Notch inhibitors this requires a lower dose of Notch inhibitors to gain the same anti-leukemic efficacy *in vitro* compared to either monotherapy. Although these data need to be confirmed in *in vivo* studies, combining Notch inhibitors with chloroquine in the treatment of leukemia may result in an increased anti-leukemic efficacy with decreased toxicity, due to the requirement of a lower dose of Notch inhibitors.

Future cancer research may benefit of combining fundamental research with repurposing drugs. Fundamental research will discover novel vulnerabilities of cancer cells, which do not affect normal cells. Using repurposed drugs, which target these vulnerabilities, instead of developing new drugs, may lead to faster and cheaper testing of the efficacy and safety of these drugs in cancer patients and may take less time to be implemented as approved anti-cancer treatments.

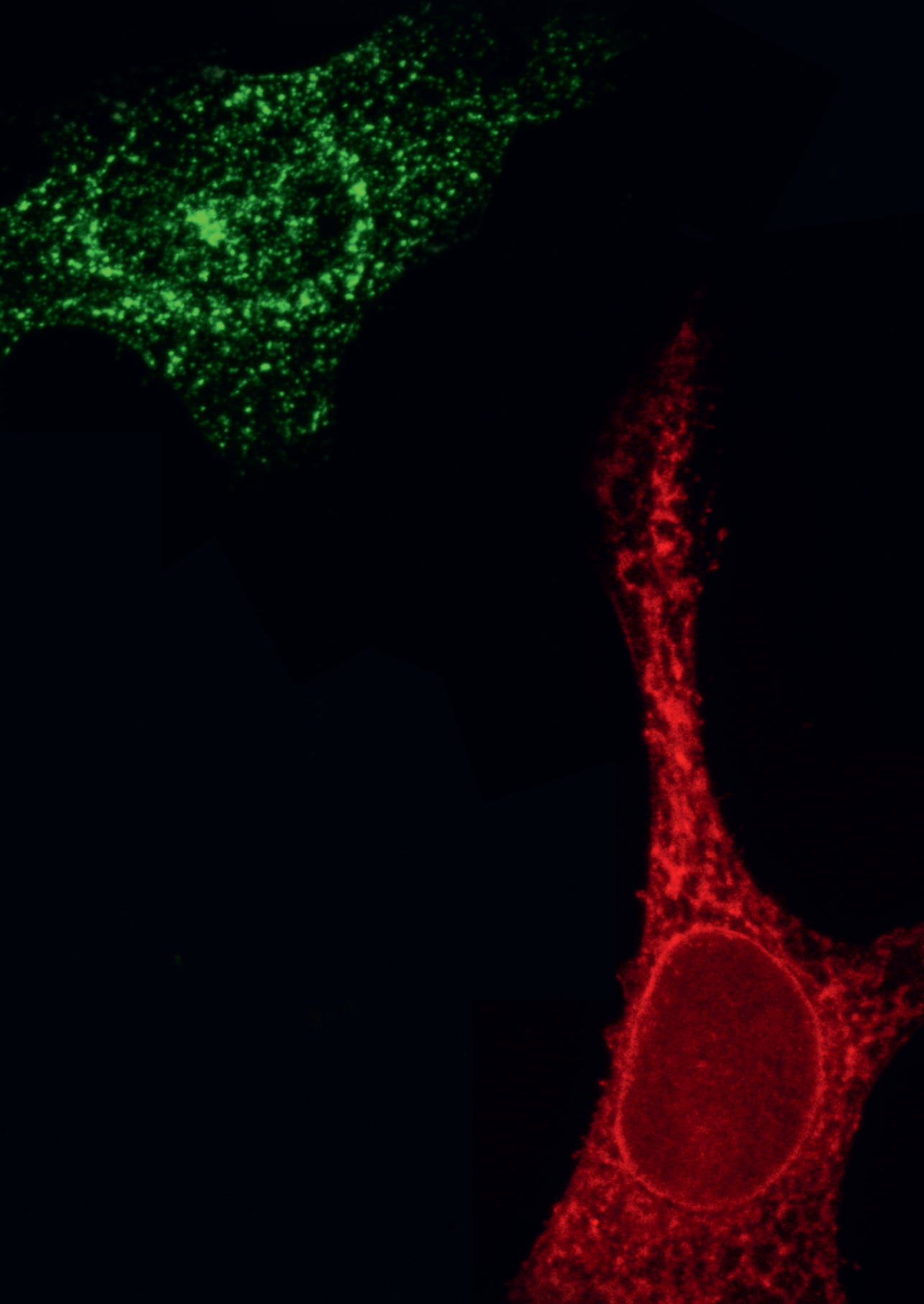
Social impact

In addition to the benefit of our findings for future research, this knowledge may also benefit cancer patients. In this thesis, we highlight the importance of fundamental research, which will strengthens our insights in cancer biology and supports the development of more specific anti-cancer therapies. Patients will benefit from treatments, which target cancer cells more specifically, as these treatments will probably lead to increased anti-cancer activity and low toxicity. Therefore, these treatments will provide a high chance of more durable responses and increased patient survival with enhanced quality of life. In addition, repurposing drugs, which are currently used to treat other human diseases, will be probably faster implemented as anti-cancer treatments for patients compared to developing new drugs, which is expensive and may take decades to be implemented in the clinic. Less expensive anti-cancer treatments may be more accessible for minorities and developing countries increasing the equality of healthcare globally.

Over the last decades drug repurposing has been successful in several cases. The most well-known repurposed drug maybe sildenafil, marketed as Viagra, which was originally developed for the treatment of angina, a pain in the chest induced by oxygen shortage in the heart muscle, however, became the leading drug in the treatment of erectile dysfunction. A more recent success story of repurposing drugs is aspirin. Aspirin is a commonly used pain killer, however, showed to be also effective in the prevention of cardiovascular disease and colorectal cancer. Currently, several drugs are being tested as repurposed drugs in clinical trials to obtain better, faster, and less expensive treatments.

Although cancer is one of the leading causes of death world-wide, we are currently facing an additional threat to the survival of the human species: the coronavirus (COVID-19 or SARS-CoV-2) pandemic. Severe cases of COVID-19 infection result in acute respiratory syndrome and systemic organ failure, which is currently increasing mortality world-wide. Therefore, there is an unmet demand for effective treatments, however, developing novel drugs or vaccines is very expensive and most importantly time-consuming. Therefore, currently a broad range of FDA-approved drugs are tested for their anti-viral activity against COVID-19 in clinical

trials, including: chloroquine, dexamethasone, and others. Although chloroquine treatment in COVID-19 patients is currently not recommended, dexamethasone treatment shows promising results. A large randomized controlled clinical trial in the UK showed that dexamethasone was effective in increasing survival in patients with severe COVID-19 disease. Currently, the main hypothesis on the cause of severe COVID-19 disease is an inappropriate immunological response. Dexamethasone is a corticosteroid, which is currently used to suppress inflammation in several diseases. This example shows the importance of finding effective treatments fast by combining fundamental research with repurposed drugs, which are currently improving COVID-19 patient survival and reducing their symptoms. Thus, this approach may also benefit both cancer research and patients in the future.



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Dear **Anaís, Joel, Èlia, Annemarie, Ying and Yanchao,** I wish you all good luck with you PhD projects! Do not only work hard but also try to enjoy it!

Ook wil ik graag de PhD studenten die gepromoveerd zijn tijdens mijn PhD traject bedanken. Beste **Raymon**, het was altijd gezellig met jou op het PhD kantoor. Bedankt voor al je humor en gezelligheid, ook tijdens onze bezoekjes bij elkaar thuis. Ik wens jou en Maaike het allerbeste voor de toekomst en nog heel veel gezonde, gelukkige en liefdevolle jaren samen en natuurlijk ook voor Henk, Hai en Huub ;)

Dear **Eloy, Venus** and **Veronica**, it was a real pleasure working with you at Maastrolab. I really enjoyed all our talks and the fun we had. I wish you all the best!

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Lieve **Kim Kampen**, wat ben jij een toponderzoeker! Hoewel we in het begin een beetje aan elkaar moesten wennen, hadden we al snel in de gaten dat wij een topteam vormen! Kim, wat heb ik genoten van onze brainstormsessies! Wat kan ik lekker met jou theorieën verzinnen over hoe dat nu zit met die Dmt1 isovormen. Hoewel je eigenlijk pas aan het einde van mijn PhD project bij ons in het lab aan het

werk ging, ben ik je zeer gaan waarderen als slimme en lieve collega. Lieve Kim, ik wens jou en Juul het allerbeste voor de toekomst.

Dear **Franscesca**, although we only worked together for a few months I really appreciate all the effort you made in analyzing the data of the screen, all your database searches on Dmt1, your help with generating the organoid data and your feedback on the Dmt1 paper. Thank you very much for your interest our projects. I wish you all the best!

I would also like to thank the **Microscopy CORE Lab** of the FHML/M4I Institute of Maastricht University for their scientific and technical support in the electron microscopy work. Dear **Carmen, Kevin, Willine** and **Hans**, thank you for all your help, support, enthusiasm and interest in our projects. I really enjoyed our collaboration.

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Lieve Fleur, wat ken ik jou al lang en wat ben jij een lieve vriendin! Vroeger speelden we nog met de barbies en de Playmobil. Tegenwoordig gaan we gezellig lunchen, lekker een ijsje eten in Ulvend, een avondje uiteten of gezellig bloemetjes plukken in de plukweide. Je denkt aan alles en bent een super lieve, knappe en gezellige meid! Ik verheug me altijd weer op onze dates die eigenlijk wel altijd een paar uur in beslag nemen aangezien we altijd zoveel hebben om over te kletsen. Toen ik druk was met het schrijven van mijn proefschrift stond jij als een echte cheerleader voor me klaar en werden onze gezellige dates even uitgesteld. Je bent een schat! Ik hou van jou lieve Fleur en ik weet zeker dat ergens die lieve, knappe, gezellige en humorvolle prins op het witte paard op jou wacht. Ik wens je het allerbeste voor de toekomst!

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bezoek, ook al zijn die vieze Parijse metro's echt niets voor jou. Met je lieve kaartjes, cadeautjes en bezoekjes sta je altijd voor me klaar en heb je ook een fantastisch vrijgezellenfeest georganiseerd en was je een top ceremoniemeester op onze bruiloft. Ook als Romeo en ik vier katten adopteren sta je voor ons klaar en kom je met alle liefde onze vier schatjes verzorgen, zodat Romeo en ik toch een weekje op vakantie kunnen gaan naar ons geliefde Fuerteventura. Lieve Esther, ik bof maar met zo een zus als jou! Ik wens jou, Twan, Mitch en ome Dokus het allerbeste voor de toekomst en dat jullie nog heel veel jaren van elkaar mogen genieten in goede gezondheid en met veel liefde.

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opvang op het eiland in verband met de quarantaine, waarbij een paar dagen voor haar vlucht blijkt dat ze uiteindelijk moeder wordt. Maar een moederkat en haar drie kittens overlaten vliegen van Fuerteventura naar Kelmond was zeker een van onze beste beslissingen! Poesjes, met jullie in huis is er altijd iets te doen en we kunnen altijd weer lachen om jullie gekke streken.

Dan blijft er tenslotte natuurlijk nog de belangrijkste person in mijn leven over: **mijn lieve Romeo**. Als ik jou zou willen bedanken voor alles wat je voor me hebt gedaan kan ik wel een heel boek schrijven. We hebben elkaar 11 jaar geleden ontmoet en wat is er in die tijd veel gebeurd. Je was een wildvreemde persoon voor mij toen ik je 11 jaar geleden in de Peppermill ontmoette en dat was toch wel een beetje eng. Maar na een paar weken inclusief vele uren op msn, afspraakjes en gekkigheid was het al snel duidelijk: jij bent het voor mij! Jij bent mijn beste vriend, mijn maatje, mijn medekomiek, mijn gekke Romeo, mijn alles! Samen met jou kan ik alles aan, je staat altijd achter me, jij accepteert mijn gekke buien en doet gewoon lekker mee, laat de rest van de wereld maar denken wat zijn dat voor een twee gekken, wij zijn zielsgelukkig met elkaar! Jij maakt me iedere dag aan het lachen om iets stoms of iets kleins. Misschien vinden mensen je wel stil in een groep en kijk je liever de kat uit de boom, voor mij ben je perfect! En bij mij ben je dat helemaal niet, haha, soms klets je me zelfs de oren van de kop af!

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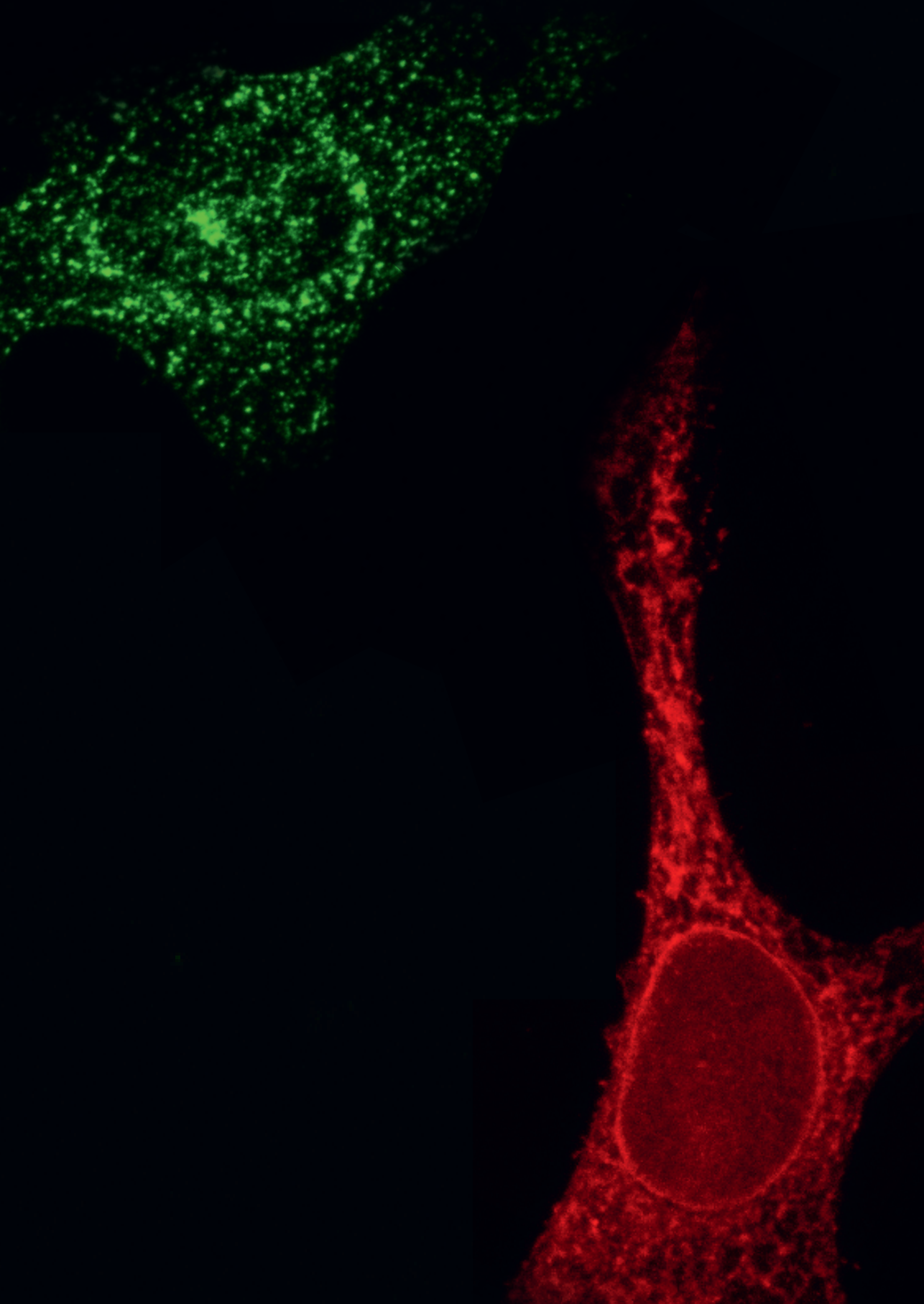
Naast de liefde voor elkaar is er natuurlijk ook onze liefde voor de poesjes. Terwijl andere mensen heerlijk genieten van hun welverdiende vakantie regelen wij de adoptie van een toch wel heel snoezig straatkatje op Fuerteventura, die dan ook nog bevalt van drie kittens. “Och dan nemen we ze toch gewoon alle vier”, zegt Romeo dan. Wat was dat een goede beslissing. Nu genieten we elke dag van elkaar en onze vier schatjes. Al 11 jaar samen en ik had er geen seconde van willen missen, lieve Romeo. Mijn Romeo, ik hou van jou en dat zal ook altijd zo blijven …

If I lay here, if I just lay here, would you lie with me and just forget the world? ♥



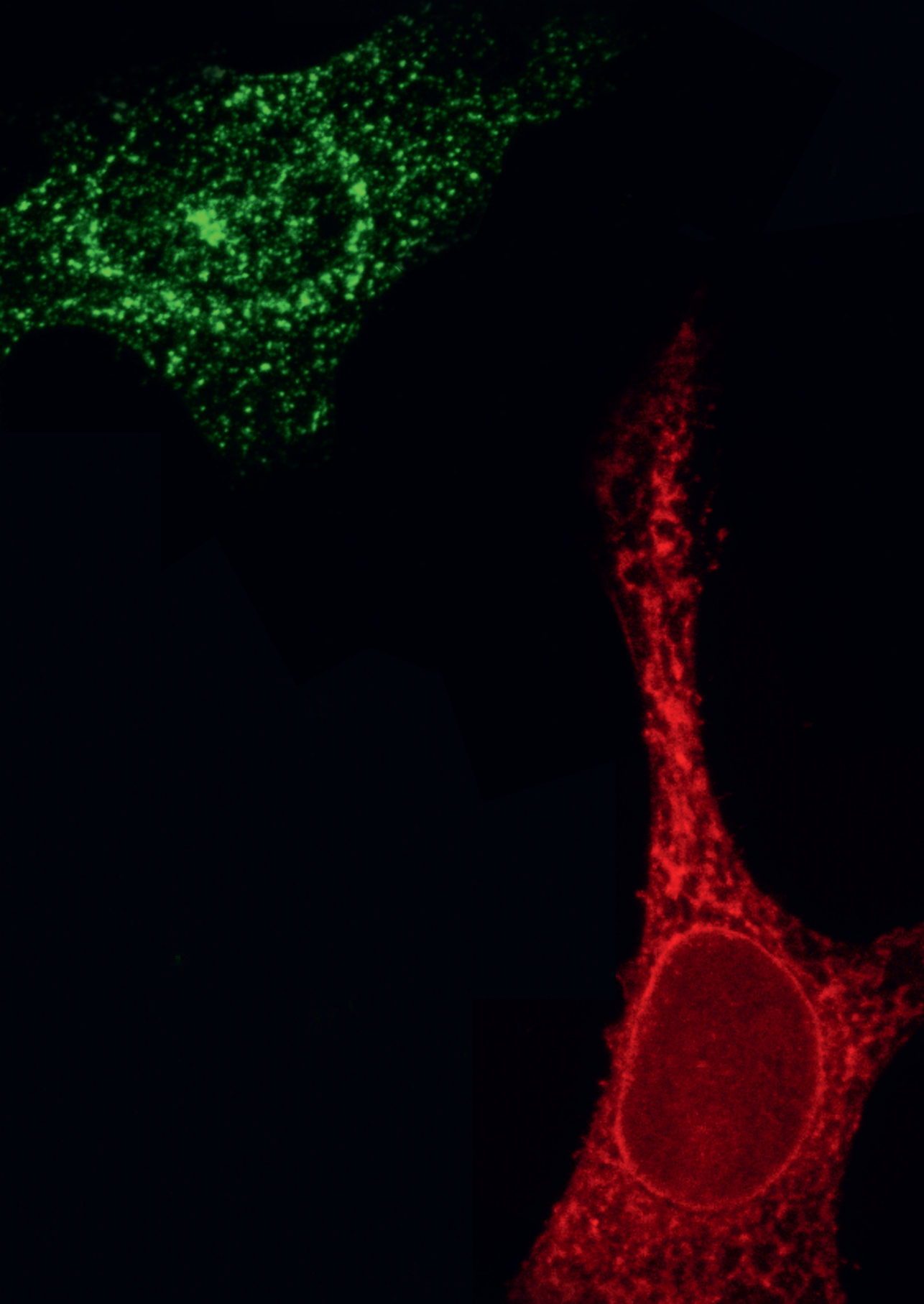
Curriculum Vitae

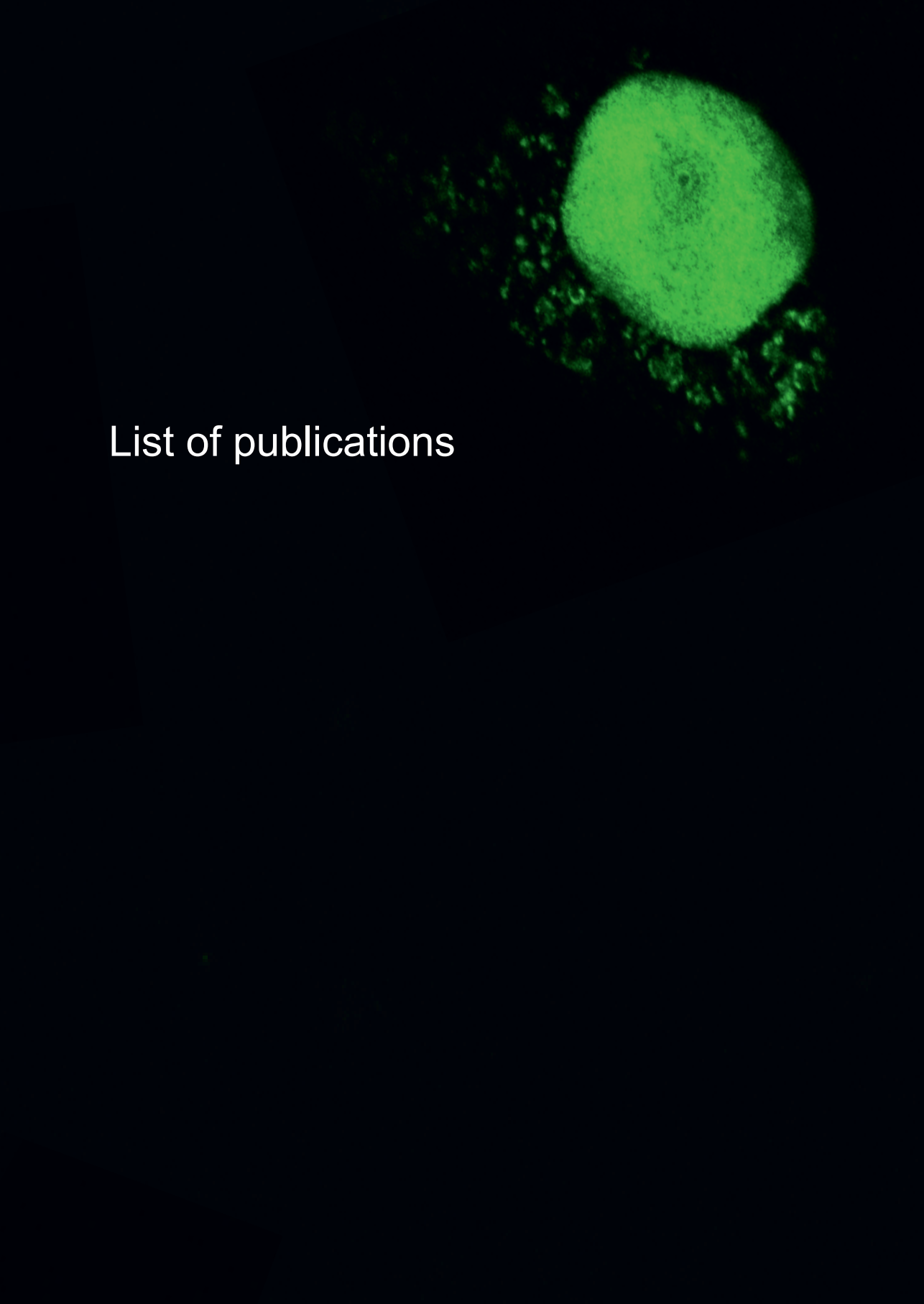
Judith Hounjet was born on February 10th 1993 in Kerkrade, the Netherlands. In 2011, she obtained her secondary school degree at Sophianum SG in Gulpen, the Netherlands. In 2011, she started her bachelor degree in Molecular Life Sciences at Maastricht University, the Netherlands. During her bachelor studies she was member of the 10% best performing students of all bachelor programs in the Netherlands (Socrates International Honour Society). In addition, she joined a Maastricht Research based learning for excellence (Marble+) program, which focused on developing competences and presentation skills. She performed her bachelor internship under the supervision of Dr. M.R. Losen at the Department of Psychiatry and Neuropsychology (Maastricht University, the Netherlands) where she studied the generation of antibodies from immortalized B cells in Myasthenia Gravis. In addition, she performed a voluntary internship during the summer holidays to extent her lab skills and completed her bachelor degree with cum laude in 2014.

She continued her academic studies starting a master in Biomedical Sciences at the Transnational University Limburg (a collaboration between Hasselt University and Maastricht University) in 2014. During her first year she performed her junior internship under the supervision of Prof. Dr. M.A.G.G. Vooijs at the Department of Radiotherapy at Maastricht University. She studied the role of Divalent metal transporter 1 (Dmt1) on Notch signaling and extended her internship voluntarily during the summer holidays. She obtained an Erasmus fellowship to complete her Master studies with an one-year exchange with the Université Pierre et Marie Curie (UPMC) in Paris, France, were she belonged to the top 3 students of her class. During this one-year exchange she performed two extracurricular courses, including the International Developmental Biology course at the UPMC and the Development of Marine Organisms course at the Marine station of Villefranche-sur-Mer (Sorbonne Université) in France. She completed her senior research laboratory internship under the supervision of Dr. A. J. Bardin at the Department of Stem cells and Tissue Homeostasis at the institute Curie in Paris, performing a molecular analysis of

spontaneous intestinal stem cell neoplasia in aged male *Drosophila*. She completed her Master degree in 2016.

In 2016, Judith was granted the Kootstra Talent Fellowship to start her PhD research under the supervision of Prof. Dr. M.A.G.G. Vooijs at the Department of Radiotherapy at Maastricht University. During her PhD she studied the effect of combined treatment of chloroquine and Notch inhibitors in human T-ALL cells and investigated the role of the isoforms of Dmt1 on Notch trafficking and signaling. During her PhD she supervised students in the lab, taught as a tutor the course Growth and Development II (2nd year bachelor of Medicine, Maastricht University), gave poster presentations at the EACR25 and KWF Cancer Biology meeting in 2018, and an oral presentation at the Notch targeting in Cancer conference in Cyprus.





List of publications

Publications

Giuranno L, Wansleeben C, Iannone R, Arathoon L, **Hounjet J**, Groot AJ, Vooijs M. NOTCH signaling promotes the survival of irradiated basal airway stem cells. Am J Physiol Lung Cell Mol Physiol. 2019 Sep 1;317(3):L414-L423. doi: 10.1152/ajplung.00197.2019. Epub 2019 Jul 19.

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Nogales-Gadea G, Saxena A, Hoffmann C, **Hounjet J**, Coenen D, Molenaar P, Losen M, Martinez-Martinez P. Generation of Recombinant Human IgG Monoclonal Antibodies from Immortalized Sorted B Cells. J Vis Exp. 2015 Jun 5;(100):e52830. doi: 10.3791/52830.

Hounjet, JSJM. Myasthenia gravis: a pathologic approach. Biomedical and health sciences research: FHML MaRBLe Research Papers. 2014;3:306-18.

In preparation

Hounjet J, Groot, AJ, Piepers J, Rapino F, Kampen KR, Vooijs M. The isoforms of Divalent metal transporter 1 (Dmt1) differentially control Notch-mediated cell fate decisions.

Hounjet J, Vooijs M. The role of intracellular trafficking of Notch receptors in Notch signaling activation (review)

Presentations

28/06/2019	Notch targeting in cancer meeting, Konnos Bay, Cyprus (oral)
22/11/2018	GROW Science Day, Maastricht University, The Netherlands (poster)
19/11/2018	KWF Cancer Biology meeting, Lunteren, The Netherlands (poster)
01/07/2018	European Association for Cancer Research (EACR25), Amsterdam, The Netherlands (poster)
28/05/2018	GROW Pizza meeting, Maastricht University, The Netherlands (oral)
17/11/2017	GROW Science Day, Maastricht University, The Netherlands (poster)

Awards

Kootstra talent fellowship (2016)
First year of funding to start PhD program

Erasmus fellowship (2015)
One-year exchange with Université Pierre et Marie Curie (UPMC) in Paris, France

Member of Socrates International Honour Society (2014)
10% of best performing students of all bachelor programs in the Netherlands