Novel mechanisms regulating Notch signaling

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Novel mechanisms regulating Notch signaling; the importance of intracellular vesicles

“When Notch, a metal transporter, and an antimalarial drug meet...”

Judith Hounjet

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Novel mechanisms regulating Notch signaling; the importance of intracellular vesicles

“When Notch, a metal transporter, and an antimalarial drug meet...”

DISSERTATION

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CHAPTER I

Introduction

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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 (DII1) and Delta-like 4 (DII4) 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 unmask 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 (Tönnemann 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 or 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- κ B 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)

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In preparation

CHAPTER III

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

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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 μ M of γ -secretase inhibitor dibenzazepine (DBZ), 1 μ M of N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), 1 μ M of BMS-906024 (a kind gift from Bristol-Myers Squib) prepared in DMSO (Syncom, Groningen, The Netherlands), 15 μ M 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 N1ΔE-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 μ M 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 IgG1k was used as an

isotype control (Biolegend). After staining, cells were analyzed using a FACSCantoll 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 H₂O₂ for 15 min at 37°C in serum free medium prior to CellROX staining. For cell cycle, DNA damage, and cytoplasmic ROS analysis a FACSCantoll 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 γ H2AX and cytoplasmic ROS levels.

Transfection and N1ICD staining

U2OS cells were transfected with Polyethylenimine (Pei)(Polysciences, cat. #23966) to express Δ E-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 ($IC_{50} = 2.0$ nM) with or without 15 μ M 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 ($IC_{50} = 14.2$ μ M) 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 dibenzazepine, CQ: chloroquine. Data are representative of three independent experiments and values are expressed in mean $\pm$ 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, Δ PEST) (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 IC_{50} of 2.0

(± 0.1) nM of DBZ and 14.2 (± 0.2) μ M 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 μ M. Moreover, both the addition of CQ (15 μ M) 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 Δ E-RFP treated with DMSO, DBZ, CQ, and combined treatment for 24 hours, counterstained with Dapi. Scale bar: 10 μ m. 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 $\pm$ 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 (Δ E-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 Δ E-RFP expression exclusively in the nucleus (**Figure 2E**), while DBZ-treated cells only showed membrane/cytoplasmic Δ E-RFP, as expected. In CQ-treated cells we observed a reduction in nuclear Δ E-RFP and a cytoplasmic accumulation. When DBZ and CQ were combined, no nuclear localisation of the Δ E-RFP was observed and all Δ E-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 Δ E-RFP with the autophagosomal marker GFP-LC3 transfection to see whether Δ E-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 Δ E-RFP and GFP-LC3, however, Δ E-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 Δ E-RFP and stained for Val1744 (N1ICD). As expected, N1ICD accumulated in the nucleus in control-treated cells and co-localized with Δ E-RFP expression, which was undetectable in DBZ- or combined-treated cells (**Figure 3B**). Most interestingly, Δ E-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 dibenzazepine, CQ: chloroquine. Scale bar: 10 μ m. 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 γ H2AX (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 γ H2AX expression was further increased by CQ treatment and with the addition of DBZ, γ H2AX expression increased up to 3-fold compared to vehicle-treated cells. In HPB-ALL cells, γ H2AX protein expression was also significantly increased when treated with CQ (**Figure 4D**). DBZ treatment (200 nM) already induced a 2-fold increase in γ H2AX expression, which was not further increased by the combined treatment. Lowering the dose of DBZ (12.5 nM) did not show an increase in γ H2AX expression, however, when combined with CQ treatment, a significant increase in γ H2AX levels was obtained. GSI-resistant T-ALL cell lines did not show increased γ H2AX 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 lysosomotropic 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 heterodimerization domain are subject to regulated proteolysis (Malecki et al., 2006) by Zn²⁺ 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 autophagosomes 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
<i>Deltex</i>	5'-TGTGCCGCAAGACCAAGAAG-3'	5'-TCGTAGCCTGATGCTGTGAC-3'
<i>RPL13A</i>	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, γ -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 $\pm$ 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 $\pm$ 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 μ M 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 dibenzazepine, CQ:

chloroquine. Data are representative of three independent experiments and values are expressed in mean $\pm$ 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 γ H2AX 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 dibenzazepine, CQ: chloroquine, MFI: mean fluorescent intensity. Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM.

CHAPTER IV

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

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In preparation

CHAPTER V

Summary and General discussion

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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 strengthen 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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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.

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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, where 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

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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