PhD degree in Molecular Medicine (Curriculum in Molecular Oncology)

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# Targeting Notch trafficking in human cancer cells:

a. Pharmacologic inhibition of the vacuolar $H^+$ ATPase reduces physiologic and oncogenic Notch signaling

b. High Content Screen for Novel modulators of the Notch pathway

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# List of abbreviations

ADAM: A Disintegrin And Metalloproteinase
ALL: Acute Lymphoblastic Leukemia
APP: Amyloid Precursor Protein
BafA1: Bafilomycin A1
BCC: Basal Cell Carcinoma
ConA: Concanamycin A
CSL: CBF1, Suppressor of hairless, Lag-1
DIC: Differential interference contrast
DAPT: *N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester
DLK-1: Delta-like 1
DNER: Delta/Notch-like EGF-related receptor
DN-MAML: Dominant Negative Mastermind Like
DSL: Delta/Serrate/Lag2
Dx: Deltex
EGTA: Ethylene Glycol Tetraacetic Acid
ESCRT: Endosomal Sorting Complexes Required for Transport
esiRNA: endoribonuclease-prepared siRNAs
ER: Endoplasmic Reticulum
GA: Golgi apparatus
GIT: Gastro Intestinal Tract
GPCR: G-Protein Couple Receptor
GSI: Gamma Secretase Inhibitor
HCS: High Content Screen
HES: Hairy Enhancer of Split
LNR: Lin12 Notch Repeats
LSDs: Lysosomal Storage Disorders
MMTV: Mouse Mammary Tumor Virus
NECD: Notch Extracellular Domain
NEXT: Notch Extracellular Truncation
NICD: Notch Intracellular Domain
NRR: Negative Regulatory Region
PTEN: Phosphatase and TENsin homolog
RER: Rough Endoplasmic Reticulum
S1: Notch cleavage Site-1
S2: Notch cleavage Site-2
SCC: Squamous Cell Carcinoma
siRNA: short interfering RNA
SOP Sensory Organ Precursor cell
TA: Transiently Amplifying
T-ALL: T-cell Acute Lymphoblastic Leukemia
TFEB: Transcription Factor EB
V-ATPase: Vacuolar $H^+$ ATPase

# Figures

# 1. Abstract

Notch signaling is prominently involved in cell fate decision and growth regulation in metazoan tissues. Because of this, Notch is often upregulated in cancer and current efforts point to developing drugs that block its activation. Notch receptor endocytosis towards acidic compartments is a recently appreciated determinant of signaling activation. The Vacuolar $H^+$ ATPase (V-ATPase) is responsible for acidification of endocytic organelles and recently it has been shown that mutants in V-ATPase subunit encoding genes in model organisms display loss of Notch signaling phenotypes. In the first part of my graduate studies, we aimed at discovering whether pharmacologic reduction of V-ATPase activity affected Notch signaling. We found that administration of BafilomycinA1 (BafA1), a highly specific V-ATPase inhibitor decreases Notch signaling during *Drosophila* and Zebrafish development, and in human cells in culture. In normal breast cells, we have found that BafA1 treatment leads to accumulation of Notch in the endo-lysosomal system, and reduces its processing and signaling activity. In Notch-addicted breast cancer cells, BafA1 treatment reduces growth in cells expressing membrane tethered forms of Notch, while sparing cells expressing cytoplasmic forms. In contrast, V-ATPase inhibition reduces growth of leukemia cells, without affecting Notch activating cleavage. However, consistent with the emerging roles of V-ATPase in controlling multiple signaling pathways, in these cells Akt activation is reduced, as it is also the case in BafA1-treated breast cancer cells. Our data support V-ATPase inhibition as a novel therapeutic approach to counteract tumor growth sustained by signaling pathways regulated at the endo-lysosomal level.

The functions of Notch throughout the life of an individual are varied and complex. This complexity is not sufficiently accounted for by the limited core of known Notch signaling

components and a growing body of evidence attributes it to additional factors that determine whether, when and how Notch functions within a given context. Considering this, in the second part of my graduate work, we sought to identify novel genes that might influence Notch. Thus, we performed a high content immunofluorescence-based RNA interference screen of a pharmacologically-relevant subset of the human genome. To this end, we monitored how knockdown of specific genes perturbs the localization of the Notch-1 receptor in human breast cells under resting and signaling conditions. Here we present the screen setup, the primary screen results and the candidate follow-up strategy.

# 2. Introduction

## 2.1 Overview of Notch signaling

Notch signaling is one of a handful signaling pathways that are conserved and extensively deployed in the regulation of tissue growth and fate specification in metazoans (Andersson et al. 2011). This evolutionarily conserved medium of cell-cell communication was first identified in *Drosophila melanogaster* and borrows its name from the notched wings observed in flies mutant for the Notch receptor gene (Morgan 1917). As an intermediary between adjacent cells, Notch signaling directs multiple and highly pleiotropic cellular functions. Since its discovery about a century ago it has been shown to influence multiple cell fate decisions, tissue differentiation, as well homeostasis in the developing embryo and adult organism (Koch et al. 2013). All metazoans are equipped with this ancient system of intercellular messaging. *Drosophila* has only one Notch receptor for its two Notch ligands, Delta and Serrate. In contrast, mammals possess four orthologues of the *Drosophila* Notch receptor, Notch 1-4 and five Notch ligands of the Delta-Serrate-Lag2 (DSL) type (Bray 2006).

Notch receptors and their ligands are single pass transmembrane proteins. The Notch receptor is synthesized in the Endoplasmic Reticulum (ER) as a single precursor protein. Soon after synthesis the receptor is transported to the Golgi where it undergoes a furin convertase mediated, Site-1 (S1) cleavage. This early biosynthetic cleavage generates a bipartite, mature Notch receptor that is then trafficked to cell surface membrane from where it may engage in Notch signaling processes (Blaumueller et al. 1997; Logeat et al. 1998; Gordon et al. 2009). In addition to this biosynthetic cleavage, it has been shown across species that the nascent receptor in the Golgi, undergoes sugar modifications on its EGF-like residues (Moloney et al. 2000; Moloney 2000), an event that is necessary for productive Notch signaling (Jafar-Nejad et

al. 2010; Stanley & Okajima 2010; Fernandez-Valdivia et al. 2011). However, some forms of glycosylation, particularly by fringe glycosyltransferases can negatively modulate signaling (Moloney et al. 2000; Yang 2004; Taylor et al. 2014). The four mammalian Notch receptors are structurally very similar (Figure 1).

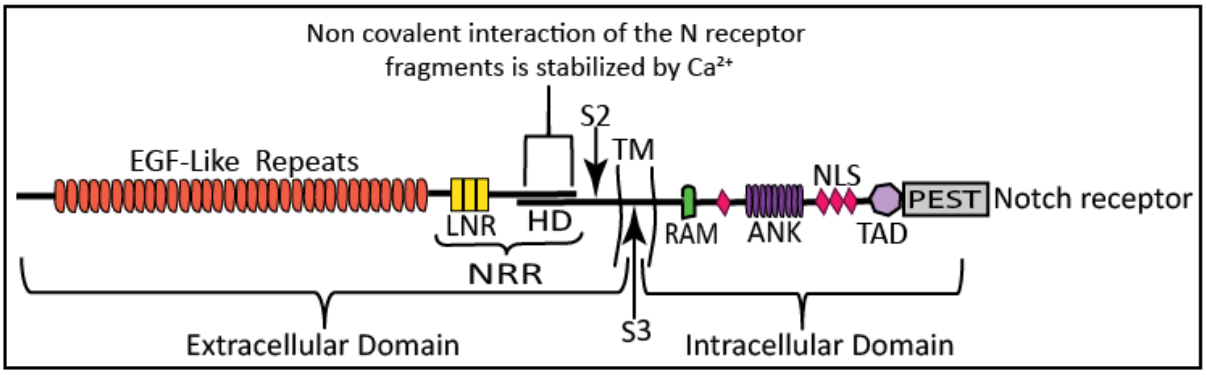


**Figure 1: General structure of the Notch receptor**

The extracellular part of the Notch receptor has a variable number of EGF-like repeats necessary for interaction with ligands. The two portions of the receptor are joined at the HD. The HD and LNR comprise the NRR and protect against ligand independent receptor activation. The intracellular domain bears the NLS and functions as a transcription factor. EGF- Epidermal Growth Factor, LNR- Lin12/Notch Repeats; HD- HeteroDimerization; NRR- Negative Regulatory Region; S2- Cleavage Site 2; S3- Cleavage Site 3; TM- Trans-Membrane; RAM- RBP-J-kappa-Associated Module; ANK- ANKyrin repeats; NLS- Nuclear Localization Signal; PEST- rich in Proline (P), glutamic acid (E), serine (S) and threonine (T); TAD- Transcription-Activation Domain, present in Notch-1 and 2 only.

Like their receptors, canonical Notch ligands are transmembrane proteins. On the basis of likeness to their *Drosophila* counterparts, the five mammalian Notch ligands are grouped into Delta-like (Dll) and Serrate-like Notch ligands. In mammals three Delta-like Notch ligands named Dll-1, Dll-3 and Dll-4 and two Serrate-like ligands named Jagged-1 and Jagged-2 are known (Bray 2006; Kopan & Ilagan 2009). Members of any of the two classes bear several structural similarities (Figure 2). Like Notch receptors, ligands have also been shown to

undergo sugar modification (Brückner et al. 2000; Panin et al. 2002; Jafar-Nejad et al. 2010), an event thought to mediate and strengthen ligand-receptor interaction.

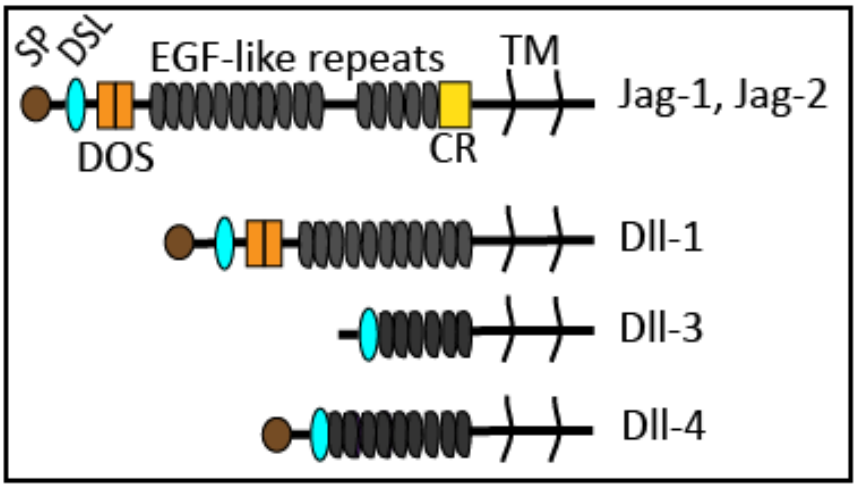


**Figure 2: Structure of the mammalian Notch ligands**
Notch ligands are multi-domain, transmembrane proteins. SP- Signal Peptide. DSL- Delta-Serrate-Lag2. EGF- Epidermal Growth Factor. CR- Cystein Rich domain. TM- Trans-Membrane. Jag- Jagged like. DOS- Delta and OSM-11. Dll- Delta like. (Adapted from 'the many facets of Notch ligands'; (D'Souza et al. 2008).

It is not clear why mammalian systems have evolved multiple Notch receptors and ligands. A possibility is that it facilitates a greater complexity by enabling tissue specific variations of Notch signaling. For instance, Notch-1 and to lesser extent Notch-2 function broadly during mammalian Notch signaling, operating in numerous cell types. While functions of Notch-3 and 4 are less extensive and largely restricted to the vascular and smooth muscle systems (Wu & Bresnick 2007). Also not fully understood, is how the expression of Notch receptors is governed and what factors direct the expression of a particular Notch receptor(s) within a specific tissue or cell type (Wu & Bresnick 2007).

### 2.1.1 The Canonical Notch signaling pathway

Physiological Notch signaling is a highly coordinated and tightly regulated process. Unlike many other cell signaling pathways, a single activated Notch receptor engages in a single episode of cell-cell communication, getting consumed on delivery of its message to the receiving cell. It does not engage second messengers, and the signal is not known to undergo any form of amplification (Andersson et al. 2011). The best characterized form of Notch signaling is termed the 'canonical Notch signaling pathway' and involves cell surface resident Notch receptors and ligands.

Notch receptors undergo a stepwise activation process. The extracellular and intracellular fragments of the Notch receptor are non-covalently linked at the HD domain, which along with the LNR, constitute the NRR. The NRR shields the receptor from ligand independent activation by concealing the cleavage Site-2 (S2) – making it inaccessible for cleavage (Figure 1). Canonical Notch signaling commences when a Notch ligand on a signal-sending cell binds to a Notch receptor on a receiving cell. This ligand-receptor interaction triggers a series of events that lead first, to a S2 cleavage of the receptor by metalloproteases of the ADAM family (van Tetering et al. 2009). Of the currently known ADAMs, ADAM-10 has been demonstrated to be the main processor of Notch receptors for subsequent cleavage by γ-secretase (Hartmann et al. 2002). As the receptor's NRR normally shields it from the S2 cleavage, ligand binding is required to make the S2 cleavage site accessible to the ADAMs. This accessibility is thought to be provided mechanically through ligand endocytosis. This 'pulling force' model posits that as the ligand is endocytosed into the signaling cell, it tugs on the extracellular portion of the receptor, causing a conformational change on the NRR and exposing the receptor's S2 for cleavage site (Stephenson & Avis 2012). Execution of the S2 cleavage generates a membrane tethered fragment of the Notch receptor called the Notch

Extracellular Truncation (NEXT), which is a substrate for the multi-subunit enzyme complex, γ-secretase. Eventually γ-secretase executes the final step of Notch signaling activation by cleaving NEXT at Site-3 (S3), releasing the Notch Intracellular Domain (NICD) into the signal receiving cell (Mumm et al. 2000). The liberated NICD swiftly translocates into the nucleus, where it drives target gene expression (Figure 3) (Borggrefe & Oswald 2009). Once in the nucleus, the NICD becomes a transcription factor. It expresses target genes by lifting the transcriptional repression imposed on them by CSL (CBF1/RBPjκ/Su(H)/Lag-1). In the absence of NICD, CSL acts in association with co-repressors to curb the expression of Notch target genes. NICD's interaction with CSL displaces the co-repressors and replaces them with Mastermind-like – a Notch signaling co-activator. This converts CSL into a signaling activator and triggers the expression of target genes, including the Hes (Hairy Enhancer of Split) family of transcription factors (Klein et al. 2000; Barolo et al. 2002). Thus, NICD function in the nucleus is essentially that of flipping on a transcriptional switch. In the nucleus, NICD is relatively short-lived as it undergoes ubiquitination within its PEST domain followed by rapid proteasomal degradation (Oberg et al. 2001) – flipping the transcriptional switch off.

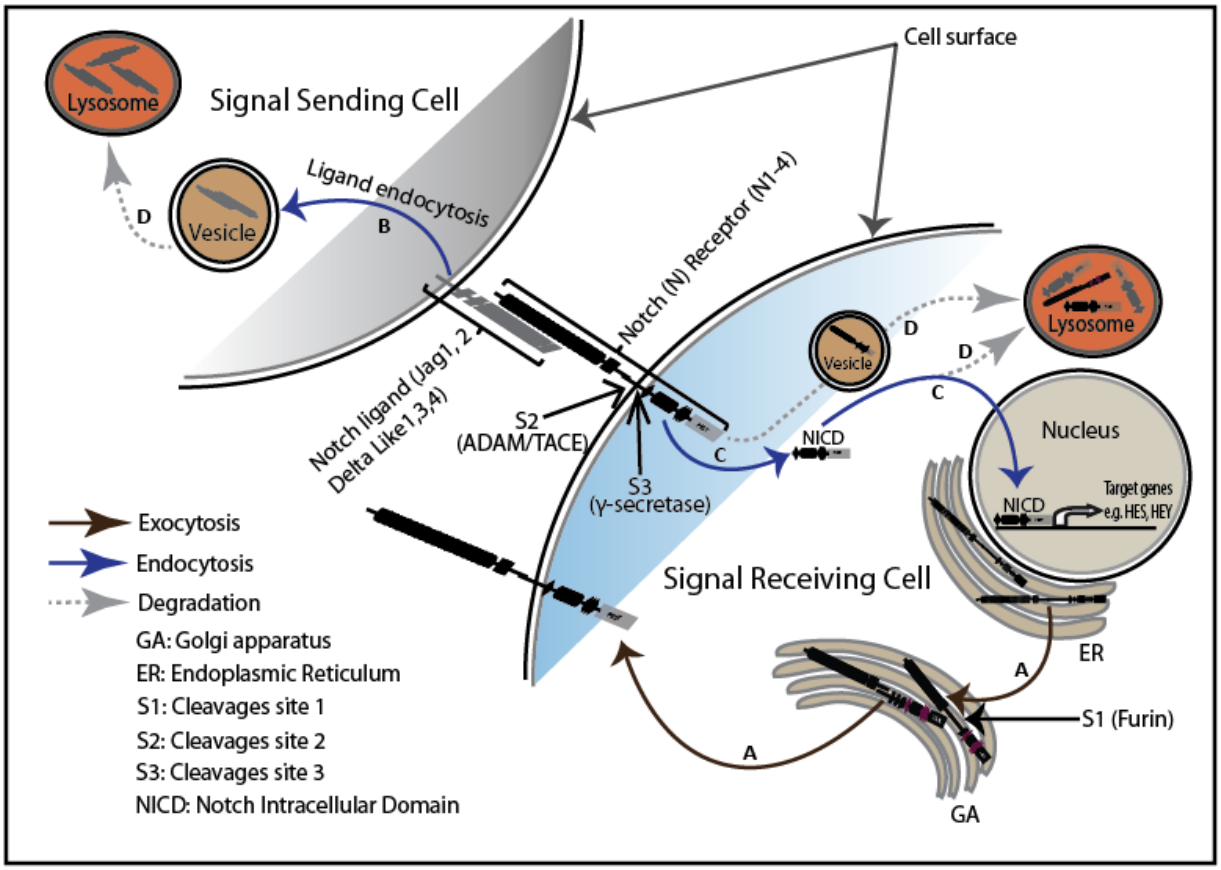


**Figure 3: The canonical Notch signaling pathway**

The Notch receptor is synthesized in the ER as a single precursor protein before transport to the GA where it is cleaved by furin and processed into a bi-partite Notch receptor; the mature receptor is transported to the cell surface (route A). At the cell surface it may engage with a Notch ligand which triggers a succession of cleavages by ADAM family metalloproteases and by γ-secretase to liberate NICD. NICD then translocates to the nucleus and drives target gene expression (route C). Receptor bound ligand endocytosis is thought to provide the pulling force necessary for receptor cleavage (route B). The levels of Notch receptors and ligands at the cell surface are checked by being targeted for lysosomal degradation (route D).

## 2.1 Non-canonical Notch signaling

Forms of Notch signaling that do not strictly abide to the principles described for canonical signaling are collectively termed non-canonical Notch signaling. Since such modes of Notch signaling are diverse and not as pervasively employed in cell communication as the canonical pathway, the mechanisms and components through which they function are not as intensively studied. They include CSL-independent Notch activity, regulation of Notch by non-

canonical ligands, and ligand independent Notch signaling (Sanalkumar et al. 2010; Andersen et al. 2012).

Notch signaling independently of the CSL has been demonstrated in various species, from *Drosophila* to humans and largely occurs via direct or indirect interaction of Notch with components of other signaling pathways – thereby modulating their activity (Shawber et al. 1996; Rusconi & Corbin 1998; Demehri et al. 2008; Kwon et al. 2011; Andersen et al. 2012). The majority of such instances have been described in the regulation of Wnt signaling by Notch (Andersen et al. 2012).

Modulation of Notch by non-canonical ligands has been shown to occur through proteins like DLK-1 (Delta-like 1) and DNER (Delta/Notch-like EGF-related receptor) that structurally resemble Notch ligands but lack a receptor binding domain. While DLK-1 has been reported to negatively influence Notch signaling (Baladrón et al. 2005; Nueda et al. 2007; Bray et al. 2008), DNER positively modulates signaling (Eiraku et al. 2005). Some proteins unrelated to Notch ligands have been shown interact with Notch receptors and trigger processing by γ-secretase (Hu et al. 2003; Cui et al. 2004; Guruharsha et al. 2012).

## 2.2 Intracellular trafficking in Notch signaling

Perhaps the best studied form of non-canonical Notch signaling is ligand independent Notch signaling, which is best characterized in the activation of Notch along the endolysosomal pathway. Intracellular trafficking is a chief regulator of cell signaling by virtue of the fact that majority of receptors and signal transducers are transmembrane or membrane-associated proteins. Notch signaling is intricately coupled to intracellular trafficking. Initial indicators of this came from observations that mutations affecting the *Drosophila* gene *shibire* (Dynamin in mammals), resulted phenotypes similar to those caused by Notch loss of function (Seugnet et

al. 1997). These observations prompted vigorous investigations into how Notch signaling is modulated through intracellular trafficking. In addition to the imperative exocytic transport of Notch receptors and their ligands to the cell surface, they both are subject to continuous endocytic transport (Fortini & Bilder 2009).

Following synthesis, Notch ligands are transported to the Golgi, where they may undergo glycosylation before proceeding on their outward journey to the cell surface (Brückner et al. 2000; Panin et al. 2002; Jafar-Nejad et al. 2010). Endocytosis of Notch ligands from the cell surface has been shown by different groups (Le Borgne & Schweisguth 2003; Itoh et al. 2003; Meloty-Kapella et al. 2012). This uptake of Notch ligands follows their ubiquitination and subsequent interaction with endocytic adaptors (Yamamoto et al. 2010). Ubiquitination is a well-known mediator of endocytosis and mutations in the E3 ligases Neuralized (Neur) and Mind bomb (Mib) impairs Notch ligand trafficking as well as signaling (Lai et al. 2001; Le Borgne, Remaud, et al. 2005; Lai et al. 2005; Song et al. 2006; Yamamoto et al. 2010). The exact role played by ligand endocytosis during Notch signaling is still debated and two compelling theories have been advanced. A) That upon interaction with the receptor, ligand endocytosis into the signaling cell generates a pulling mechanical force that prompts Notch receptor cleavage on the surface of the signal receiving cell (Nichols et al. 2007; Meloty-Kapella et al. 2012). B) That endocytosis is required for proper ligand maturation as it is incapable signaling in its nascent form. Through unclear mechanisms, endocytosis is thought incorporate the newly-made ligand into an intracellular compartment where it matures and acquires Notch activating potential before being recycled back to the cell surface. This notion is supported by observations that impaired ligand recycling suppresses Notch signaling in both *Drosophila* and mammalian cells (Jafar-Nejad et al. 2005; Emery et al. 2005; Rajan et al. 2009). Although the exact part played by ligand endocytosis in support of Notch signaling remains

unsettled, it is likely that both mechanisms play a part and the particular process executed by endocytosis might strongly depend on the context. The ligand-NECD (Notch Extracellular Domain) complex generated on receptor cleavage is postulated to undergo transendocytosis into the signaling cell, followed by trafficking to the lysosome for degradation. Ligands not engaged with Notch receptors may also undergo endocytosis into the signaling cell, an event that may inhibit Notch signaling by limiting the availability of Notch ligands at the cell surface.

Following synthesis Notch receptors are exocytosed to the cell surface from where they may interact with ligands. Newly made receptors are transported to the Golgi where they undergo a cleavage by furin (Blaumueller et al. 1997; Logeat et al. 1998; Gordon et al. 2009) and undergo glycosylation (Moloney et al. 2000; Moloney 2000; Stanley & Okajima 2010) before being transported to the cell surface (Kopan 2012). As with the signaling cell, endocytic trafficking in the signal receiving cell may influence Notch signaling either positively or negatively. By removing Notch receptors from the cell surface and targeting them for degradation, endocytosis may make them unavailable for activation by ligands – inhibiting signaling (Le Borgne, Bardin, et al. 2005; Fortini & Bilder 2009; Yamamoto et al. 2010). By recycling receptors destined for degradation back to the cell surface or mediating their activation along the endolysosomal pathway, trafficking may conversely, enhance Notch signaling (Vaccari et al. 2008; Fortini & Bilder 2009).

It is not clear how the delicate balance between enhancing or inhibiting Notch signaling is struck and maintained during endocytic trafficking of Notch receptors. It is becoming increasingly clear that this intricate system is orchestrated by a myriad other factors beyond the core components of the Notch pathway. A prominent example of a group of such factors, and how they may impact Notch signaling is provided by the ubiquitin system. Several members of the ubiquitin system are known to affect Notch signaling. For instance, both the

*Drosophila* and mammalian versions of the E3 ubiquitin ligase, dx (deltex), are modulators of Notch signaling (Xu & Artavanis-Tsakonas 1990; Diederich et al. 1994; Matsuno et al. 1995; Matsuno et al. 1998; Kishi et al. 2001; Hori et al. 2004). *Drosophila* dx has been demonstrated to promote Notch signaling by ubiquitinating the Notch receptor and promoting its activation in late endosomes independently of ligands (Hori et al. 2004; Wilkin et al. 2008; Shimizu et al. 2014). However, in some contexts it negatively regulates Notch signaling by driving receptor degradation in the lysosomes (Mukherjee et al. 2005; Wilkin et al. 2008). Another E3 ligase, Su(dx) (AIP4/Itch in mammals), opposes dx and negatively regulates Notch signaling by promoting its lysosomal degradation (Cornell et al. 1999; Wilkin et al. 2004; Qiu et al. 2000; Chastagner et al. 2008; Shimizu et al. 2014). It is thought that dx promotes Notch signaling by inducing receptor endocytosis and preventing it from accessing MVB lumens where it would be degraded and that Su(Dx) counters dx by recruiting Notch into MVBs for degradation (Wilkin et al. 2008; Shimizu et al. 2014). AIP4/Itch has been postulated to also target mammalian dx for lysosomal degradation (Chastagner et al. 2006). In *Drosophila*, factors like Lgd and components of the ESCRT machinery, which are necessary for endosomal sorting and MVB (MultiVesicular Body) formation also impact on Notch signaling. Ablation of Lgd, which is known to promote Notch degradation impairs endosomal sorting and leads to ligand independent activation of Notch (Childress et al. 2006; Jaekel & Klein 2006; Gallagher & Knoblich 2006; Schneider et al. 2013). Similarly, mutations affecting ESCRT or HOPS components impair endosome maturation and as a result Notch trafficking and degradation, leading to ectopic activation of Notch (Moberg et al. 2005; Vaccari & Bilder 2005; Thompson et al. 2005; Herz et al. 2006; Wilkin et al. 2008; Vaccari et al. 2009; Hori et al. 2011).

Once Notch receptors and ligands are endocytosed, they are transported along the endolysosomal route in membrane vesicles. As the vesicle travels from the cell surface toward

the lysosome at the perinuclear region, its luminal pH is progressively acidified by the V-ATPase (Vacuolar $H^+$ ATPase). This function of the V-ATPase is crucial for various cellular processes including membrane trafficking and fusion (Forgac 2007). More recently, the V-ATPase has been shown to modulate clathrin mediated endocytosis (Kozik et al. 2013). Thus acidification is bonafide mediator of intracellular trafficking. As discussed in subsequent sections, V-ATPase/acidification has important implications for Notch among other signaling pathways.

## 2.3 The V-ATPase pump

The vacuolar $H^+$ ATPase (V-ATPase) is a large evolutionarily conserved proton pump that serves a wide range of cellular functions (Forgac 2007). This versatile pump is built of several subunits that assemble into a membrane embedded $V_0$ sector and a soluble cytoplasmic $V_1$ sector (Figure 4). The $V_0$ sector of the pump consists of six subunits (a-e) and the $V_1$ sector of eight (A-H) (Forgac 2007). When assembled, the two sectors are capable of reversible association, to form the complete, functional V-ATPase complex (Kane 1995; Sumner et al. 1995; Lafourcade et al. 2008).

The primal function of the V-ATPase pump is to translocate protons across cell membranes, into the lumens of membrane bound intracellular compartments (Forgac 2007). In some specialized cells like those of the kidneys and bones, the translocated protons are extruded into the extracellular space as part of physiologic function by these organs. Thus, the pump makes an indispensable contribution in maintenance of the body's acid-base equilibrium and bone tissue homeostasis, respectively (Brown et al. 2009; Qin et al. 2012). To execute these functions, the pump's cytosolic $V_1$ sector hydrolyzes ATP as a source of the energy. While the membrane embedded $V_0$ sector forms a channel through which the protons translocate across membranes. Proton translocation and associated intracellular acidification is efficiently

blocked by plecomacrolide antibiotics. Members of this family of specific V-ATPase inhibitors specifically clutch on the $V_0C$ subunit of the pump and prevent the rotary motion of its $V_0$ sector, hence impairing proton translocation (Bowman & Bowman 2005).

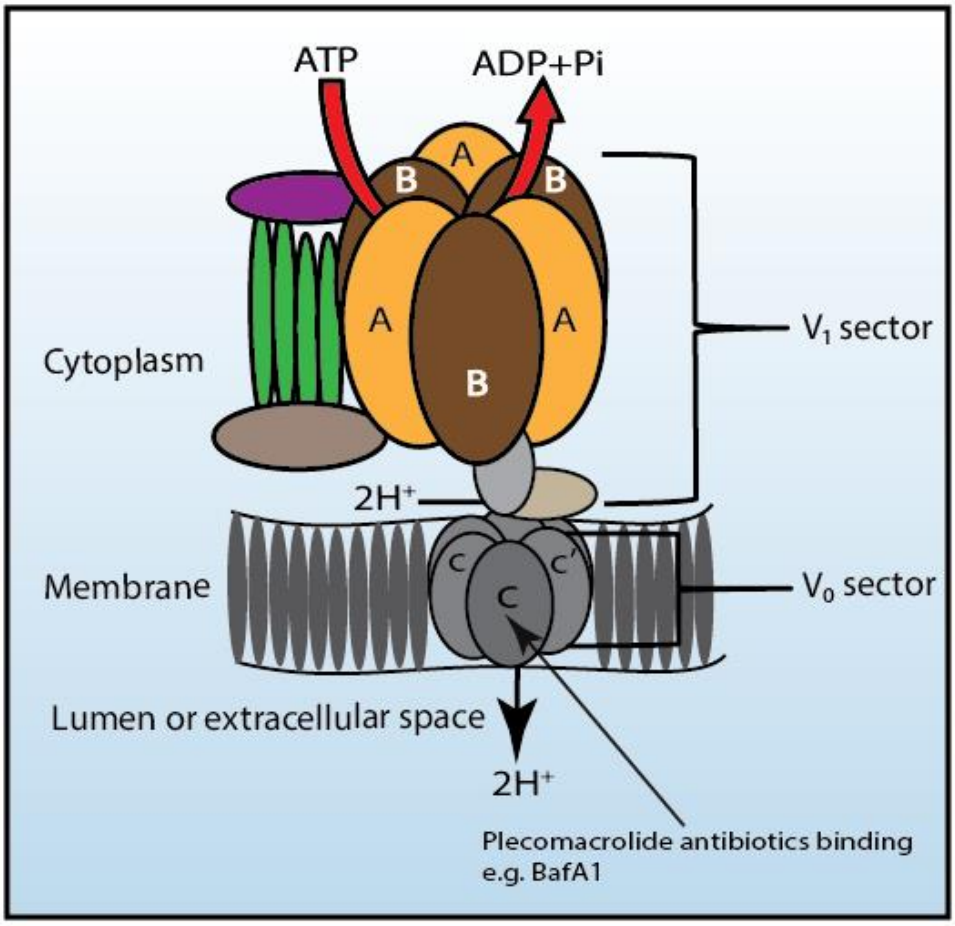


**Figure 4: General structure of the V-ATPase pump**

The V-ATPase is made up of multiple subunits that assemble into a cytoplasmic $V_1$ sector and a membrane embedded $V_0$ sector. The two individual sectors reversibly associate to make the complete, membrane residing enzyme complex. The primary function of this ancient proton pump is to acidify the cell's intracellular organelles or in specialized tissues, the extracellular space. V-ATPase's proton pumping activity can be specifically blocked with plecomacrolide family of antibiotics e.g. Concanamycin A (ConA) and Bafilomycin A1 (BafA1), that specifically bind to the C subunit of the $V_0$ sector, interrupting proton translocation.

Because of its well established role in intracellular acidification, the V-ATPase is best known as a mediator of lysosomal acidification and function. This notion is grounded on the fact that lysosomes are the most acidic organelles and that their low luminal pH is established and maintained by the V-ATPase. A lysosomal pH of around 5.0 is required for optimal function by lysosomal hydrolases which degrade unwanted cellular material – including proteins, lipids and nucleic acids (Cooper 2000). Also referred to as acid hydrolases, these enzymes cease to function at higher pH. Protracted stifling of their activity leads to lysosomal storage disorders, a group of diseases characterized by compromised removal of cellular waste (Cooper 2000).

Due to this entrenched view of the lysosome, it is commonly regarded the cell's 'dump site' and by association the V-ATPase is largely considered a facilitator of the lysosome's degradative function. However, backed by numerous recent findings, it is becoming increasingly evident that lysosomes are a not just cellular incinerators and that the V-ATPase is not simply their power supply. Indeed, apart from performing their traditional duties, these two execute additional critical tasks in very diverse cellular processes, including cell signaling (Appelqvist et al. 2013).

### 2.3.1 The V-ATPase in Cell signaling

The V-ATPase, along with the lysosome has so far been implicated in Notch signaling (Yan et al. 2009; Vaccari et al. 2010; Lange et al. 2011; Valapala et al. 2013), Wnt signaling (Cruciat et al. 2010; Buechling et al. 2010), mTOR signaling (Peña-Llopis et al. 2011; Zoncu et al. 2011; Settembre et al. 2012; Bar-Peled et al. 2012; Roczniak-Ferguson et al. 2012; Zhang et al. 2014), $Ca^{2+}$ signaling (Zhu et al. 2010; Medina et al. 2015) and AMPK signaling (Zhang et al. 2014). These reports, citing evidence from human cells as well as model organisms paint a picture of the V-ATPase and the lysosome as a well-coordinated cell signaling hub (Figure 5).

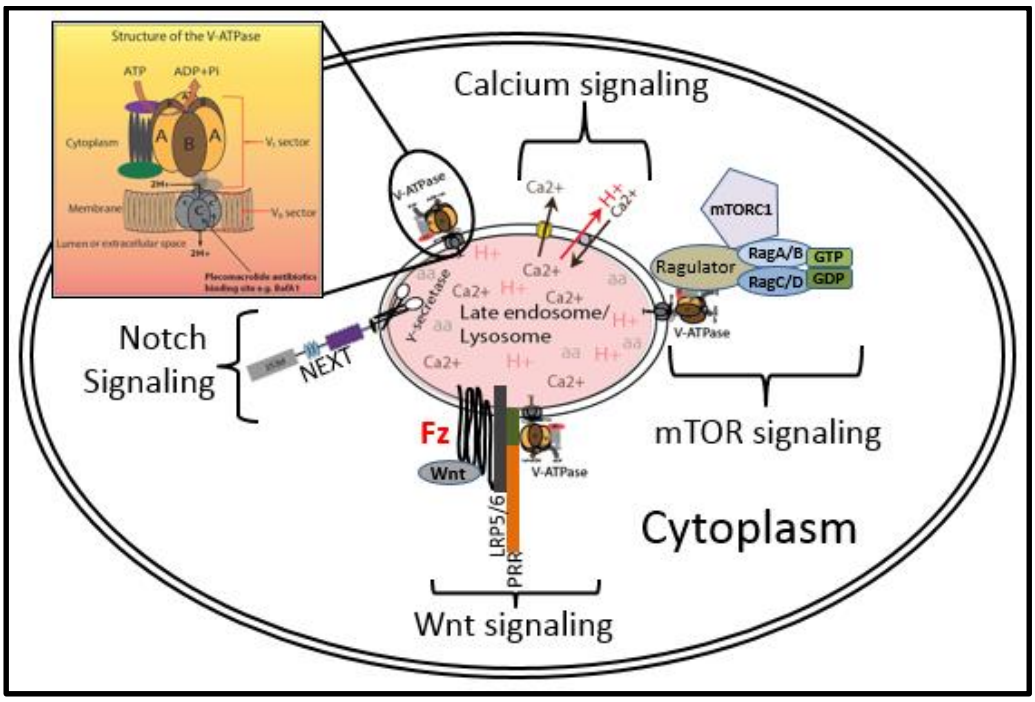


**Figure 5: The lysosome/V-ATPase as a signaling hub**

Recent data emerging from research on human and animal models cast the view that together, the lysosome and V-ATPase are at the center of numerous cell signaling pathways – including mTOR, Wnt and Notch signaling.

Together, the V-ATPase/lysosome, mTOR and the Transcription Factor EB (TFEB), form a homeostatic loop that regulates cellular energy. TFEB is a master regulator of lysosomal genes including V-ATPase subunits genes and therefore controls lysosome biogenesis (Sardiello et al. 2009; Settembre et al. 2011). Under normal circumstances, mTOR negatively regulates TFEB activity by phosphorylating and keeping it in the cytosol. When mTOR signaling is inhibited TFEB translocates to the nucleus, where it stimulates the expression of target genes (Settembre et al. 2011; Roczniak-Ferguson et al. 2012). However, for mTOR to restrain TFEB from translocating into the nucleus, it must establish whether or not, the nutritional status of the cell is adequate. It does this by moving to the lysosomal surface, where it interacts with the V-ATPase and in case of nutritional adequacy becomes active. Active mTOR can now phosphorylate TFEB and sequester it outside the nucleus. Inhibiting V-ATPase function

inactivates mTOR, making it incapable of phosphorylating TFEB and therefore inducing TFEB's translocation into the nucleus (Zoncu et al. 2011; Roczniak-Ferguson et al. 2012).

During Wnt signaling, the V-ATPase has been shown to be important for Wnt receptor activation. The V-ATPase is connected to Wnt signaling by prorenin (PRR), an accessory component of the V-ATPase also known as ATP6AP2. PRR is a transmembrane protein that interacts with the Wnt receptor complex at the cell surface. It is thought that following Wnt ligand binding, the Wnt signaling complex becomes internalized by the cell. PRR is postulated to mediate this endocytic event by acting as an adapter that physically links the Wnt signaling complex with the V-ATPase. On the endosomal surface and now attached to the Wnt receptor via PRR, the V-ATPase acidifies the vesicle's lumen as it travels along the endolysosomal path – generating a low pH that is required for Wnt receptor phosphorylation and signaling activation. This Wnt signaling activation is blocked by V-ATPase inhibitors. A role for the V-ATPase in Wnt signaling has been demonstrated in model organisms as well as in human cells, indicating an evolutionary conservation of the crucial role it plays during Wnt signaling (Cruciat et al. 2010; Buechling et al. 2010).

In recent years, our group has contributed to the body of evidence that points to the V-ATPase as a versatile mediator of cell signaling, particularly its involvement in the Notch signaling pathway (Vaccari et al. 2010). Prior to this, our group had demonstrated endosomal entry of Notch as a prerequisite for productive Notch signaling (Vaccari et al. 2008). Regarding the V-ATPase in the Notch pathway, we found that mutations affecting *Drosophila* V-ATPase subunits impair intracellular acidification and cause an accumulation of Notch in lysosomes. Flies carrying these mutation also display a significant impairment of Notch signaling. Similar independent observations were reported by others (Yan et al. 2009). They showed that *Drosophila* rabconnectins regulate V-ATPase activity and that rabconnectin mutations cause

phenotypes similar to those of impaired V-ATPase function – including lysosomal accumulation of Notch and reduced Notch signaling (Yan et al. 2009). A conserved role for the V-ATPase in Notch signaling was highlighted by different groups that using animal models, demonstrated a requirement for V-ATPase function in mammalian Notch signaling (Lange et al. 2011; Valapala et al. 2013). Using a dominant negative form of the V1e1 subunit of the V-ATPase to inhibit the pumps function, Lange and colleagues showed that the loss of V-ATPase activity impairs Notch signaling in the developing mouse brain (Lange et al. 2011). V-ATPase function in mammalian Notch signaling was also demonstrated by Valapala and colleagues, who showed that mutant βA3/A1-crystallin impairs V-ATPase function and Notch signaling during formation of the rat retina (Valapala et al. 2013). Together, these independent findings establish the V-ATPase as a conserved, bonafide regulator of Notch signaling. These data also propose the V-ATPase as a therapeutic target to counter out of control Notch signaling.

## 2.4 The role of Notch signaling during development

The consequences of Notch signaling are highly pleiotropic. During development signaling by Notch is a well-known determiner of cell fates through the processes of lateral inhibition, tissue pattern induction and binary cell fate choice (Lai 2004). In all of these processes, Notch signaling acts to specify or influence the identities assumed by daughter cells.

In the case Lateral inhibition, a cell within a group of equipotent cells (cells with an equal capacity to differentiate into a certain cell type) progressively acquires a Notch signal-sending status. It then employs Notch signaling to instruct neighboring cells not to become signal sending cells. Lateral inhibition is best exemplified by neurogenesis in *Drosophila*. During this process, a cell within a group of cells that are equally capable of becoming neurons, acquires Notch signal-sending capacity and uses it to compel its neighbors not to adopt a

neuronal fate and thus revert to an epidermal fate. Impaired Notch signaling causes the entire cluster to differentiate into neurons, giving rise to the telling neurogenic phenotype (Parks et al. 1997).

Unlike lateral inhibition, inductive patterning by Notch occurs between distinct cell populations. In such instances, signaling cells instruct their signal receiving neighbors to express genes necessary for the development of a specific tissue or organ. This is best demonstrated in the formation of the *Drosophila* wing margin. During this developmental process, the cells on the dorsal side of the wing imaginal disc communicate with those on the ventral side via Notch signaling to delineate the wing margin. Impaired Notch signaling in this context leads to a malformed wing margin with the characteristic wing notches (Lai 2004).

During binary cell fate decisions, a cell has to choose one of two possible outcomes. A fitting example is provided by the process of *Drosophila* bristle development. A mechanosensory fly bristle originates from a stem cell within a proneural field of cells called a sensory organ precursor cell (SOP). A SOP cell can divide into a pIIa and pIIb cell which can in turn divide into a hair and socket cell or a neuron and sheath cell respectively – eventually forming the bristle. During this process, Notch signaling pushes for a pIIa fate during division of the dual-potent SOP and in the next round of division it specifies a socket cell from the pIIa cell and a sheath cell from the pIIb cell. The remainder of the cells, that do not become SOPs and form bristles, become epidermal (Bardin et al. 2004).

## 2.5 The role of Notch signaling in adult tissue homeostasis

Notch controls a myriad of signaling events that are key for tissue development and homeostasis across species. Here we will focus only on Notch signaling in the regulation of adult tissue homeostasis to provide background for discussing the role of Notch signaling in

cancer. For a more comprehensive description of the roles of Notch signaling during tissue development and homeostasis please see (Koch et al. 2013).

In its capacity as a maintainer of tissue homeostasis, the functions of Notch signaling are varied and context dependent. It promotes proliferation and stem cell maintenance, directs binary cell fate choice outcomes and induces cell differentiation (Figure 6) (Dumortier et al. 2005). Thus depending on the context, improper signaling by Notch may lead to a broad range of developmental disorders and cancers (Penton et al. 2012; South et al. 2012).

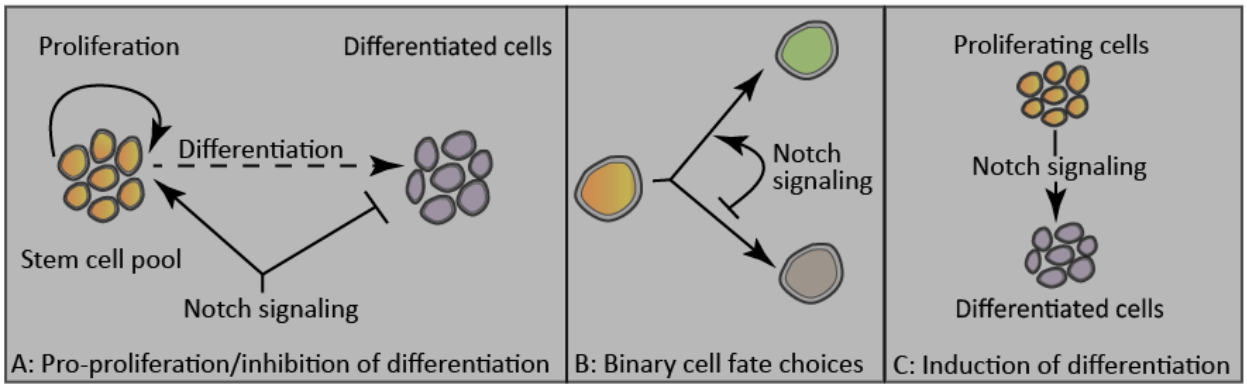


**Figure 6: Notch signaling in adult tissue physiology**
Depending on the context, Notch signaling may promote proliferation of stem cells and inhibit their differentiation (A), dictate the outcome of binary cell fate choices (B) or inhibit stem cell proliferation and induce their differentiation (C). Modified from (Wilson & Radtke 2006)

In keeping with its context dependent nature, varied functions for Notch signaling in adult tissue physiology have been demonstrated in various mammalian tissues and organs, including but not limited to the intestines, skin and blood (Sato et al. 2012). These three are highlighted as paradigmatic examples of the pervasive yet context dependent Notch signaling that occurs in adult mammalian tissues and its relevance to cancer as well as its implications for therapeutic intervention against Notch driven cancers.

### 2.5.1 The role of Notch signaling in the GIT

By nature of its function, the epithelial wall of the Gastrointestinal Tract (GIT) endures a constant stream of 'assault' that inflicts it massive cell death. It must therefore undergo constant rejuvenation to replace the shed cells. With a complete overhaul every 3-5 days, the intestinal epithelium's turnover rate is one of the highest and is supported by a high rate of cell proliferation. In the small intestines this rapid proliferation is maintained by a pool of stem cells that resides within the intestinal crypts. These cells have to replenish the huge population of cells that is constantly lost and maintain a stem cell reservoir to ensure constant supply. To achieve this, a stem cell divides into a self-renewing stem cell and a transiently amplifying (TA) cell. The TA cell briefly proliferates while migrating to the tip of the microvilli and eventually differentiates into an intestinal epithelial cell (Lin & Barker 2011; Noah & Shroyer 2013; Barker 2014). The TA cells can mature into multiple cell types. The bulk of the cells resulting from this process differentiate into absorptive cells (enterocytes) that take up nutrients; while a smaller proportion of them become secretory (goblet) cells that secrete mucus (Noah & Shroyer 2013). That Notch signaling is active in intestinal epithelia, is well-documented (Schröder & Gossler 2002; Sander & Powell 2004; Noah & Shroyer 2013). Here it controls binary cell fate choices and promotes the differentiation of precursor cells into absorptive cells at the expense of secretory cells. Impaired Notch signaling results in the expansion of the secretory cell compartment at the expense of absorptive cells (Fre et al. 2005; Stanger et al. 2005; Pellegrinet et al. 2011). This role of Notch in the maintenance of intestinal homeostasis is conserved across species including *Drosophila* and Zebrafish. *Drosophila* intestinal stem cells differ from their mammalian equivalents in that they do not form TA cells, but rather become enteroblasts that may directly differentiate into absorptive or secretory cells without further rounds of division (Micchelli & Perrimon 2006; Ohlstein & Spradling 2006). Through unclear mechanisms, some

of the intestinal stem cells acquire a signal receiving status and activate Notch signaling, which promotes their differentiation. Those that acquire signal sending capacity maintain stemness. As in mammals, impaired Notch signaling in *Drosophila* intestinal tract causes an increase in proliferating cells while the opposite causes a loss of stem cells (Micchelli & Perrimon 2006; Ohlstein & Spradling 2006). Similar observations have been made in Zebrafish (Crosnier et al. 2005; Yang et al. 2009). In mammals, pharmacological inhibition of γ-secretase suppresses Notch signaling, causing complications similar to those observed upon genetic interruption of Notch signaling in animal models (van Es et al. 2005). These observations underscore the importance of an optimally functioning Notch pathway for healthy intestinal function – a concept that is revisited in the section 'Notch as a therapeutic target'.

### 2.5.2 The role of Notch signaling in the skin

Like intestines, skins have to brave a perpetual onslaught of environmental insults. The skin forms the outer covering and shelters our internal organs from mechanical stress, injuries, infections, cold and dehydration. As a result, it undergoes massive shedding and therefore must sustain continuous self-regeneration in order to counter the losses incurred (Koster & Roop 2007; Okuyama et al. 2008). The skin is made up of an outermost layer called the epidermis and an underlying dermis. The epidermis is stratified and comprises of multiple differentiated outer layers and a single proliferative layer beneath them (Sotiropoulou & Blanpain 2012). Replacement of the perennially lost epidermal cells is driven by a stem cell pool that resides in the proliferative layer. Cells in this pool undergo asymmetric cell division to generate stem cells that continue to reside and divide within the proliferative layer and short-lived TA cells that proliferate for a short period before differentiating and moving to the upper layers, where they replace lost cells (Okuyama et al. 2008). During this process Notch

signaling induces the terminal differentiation of TA cells. Loss of Notch signaling in the skin impairs differentiation of the precursor cells, hence expanding the proliferating compartment, while increased Notch signaling induces their exit from the cell cycle (Rangarajan et al. 2001; Nicolas et al. 2003; Blanpain et al. 2006; Okuyama et al. 2008). Like in the GIT, Notch function in the skin has important implications for therapeutic interventions and will be revisited in the section 'Notch as a therapeutic target'.

Mammary glands are derivatives of the skin and the stem cells that give rise to them continue to proliferate until they differentiate during adulthood (Robinson 2007). Their development is subject to control by various signaling pathways including Notch (Robinson 2007; Guo et al. 2011). Unlike in the skin where it promotes differentiation, Notch signaling has been reported to promote mammary stem cell proliferation (Dontu et al. 2004). Constitutively active Notch signaling in animal models interferes with normal mammary gland formation by impairing differentiation and leading to tumor formation (Uyttendaele et al. 1998; Soriano et al. 2000; Guo et al. 2011). These reports present an important role for Notch in mammary gland homeostasis. Indeed, Notch receptor mutations leading to excessive Notch signaling have been found in human breast cancers (Robinson et al. 2011).

### 2.5.3 The role of Notch signaling in the hematopoietic system

The blood, whose cellular constituents are very short-lived, is another organ with a remarkable turnover rate and thus undergoes constant renewal throughout life. As with the GIT and the skin, this high turnover is maintained by a pool of stem cells. Hematopoietic stem cells (HSCs) reside in the bone marrow (Pietras et al. 2011). Notch signaling in the hematopoietic system is best characterized in the process of T-cell development. HSCs arriving to the thymus from the bone marrow are coaxed by Notch signaling into adopting a T-cell fate

at the expense of a B-cell outcome (Radtke et al. 2013). Impaired Notch signaling leads to an excessive expansion of the B-cell compartment with a concomitant shrinkage of the T-cell population (Radtke et al. 1999; Maillard et al. 2004). On the other hand increased Notch signaling induces an overrepresentation of T-cells and a shrinkage of the B-cell compartment (Radtke et al. 1999; Taghon et al. 2005).

Collectively, these data illustrating the pleiotropy of Notch function in adult tissue homeostasis, give us a glimpse into the obstacles facing the targeting of Notch for therapeutic benefit. Some of which are further mentioned in 'Notch signaling in cancer' and 'Notch as a therapeutic target'.

## 2.6 Notch signaling in cancer

Cancer is an age-old disease characterized by runaway cell growth and proliferation. Early records of cancer trace as far back as 1600 BC, in ancient Egypt (Sudhakar 2009). Through the millennia our understanding of cancer, its causes and management strategies has gradually but tremendously improved. From Socrates's humoral theory that cancer was caused by bodily fluid imbalances to the current consensus that cancer is the result of a normal cell's gene(s) regulation gone awry (Sudhakar 2009).

Genes that upon deregulation lead to cancer formation are broadly grouped into two categories: oncogenes and tumor suppressors. Oncogenes are typically pro-proliferation genes that when deregulated gain extra function and confer the affected cell the capacity to grow uncontrollably, leading to tumorous growth. Tumor suppressors on the hand, are genes that when functioning normally protect against rampant cell multiplication. Their loss of function leads to cell overgrowth and cancer development (Lodish et al. 2000).

As a result if its broad functions throughout the life of an individual, abnormal Notch signaling underlies numerous developmental diseases and cancers (Louvi & Artavanis-Tsakonas 2012). How Notch signaling contributes to disease development depends on its function in the context (organ) in which the particular disease occurs. Hence its mechanisms of contributing to pathogenesis vary with the disease type, and sometimes act in opposite direction. For instance, while some cancers and many congenital disorders are due to loss of Notch function (Rangarajan et al. 2001; Nicolas et al. 2003; Restivo et al. 2011; Klinakis et al. 2011), most Notch driven cancers result from excessive Notch activity (Capaccione & Pine 2013).

Cancer development as a result of insufficient Notch signaling results from the loss of the Notch pathway's tumor suppressive function. The tumor suppressive role of Notch signaling stems from its pro-differentiative function and is best characterized in the skin (Lowell et al. 2000; Rangarajan et al. 2001; Nicolas et al. 2003; Nguyen et al. 2006; Dotto 2008; Restivo et al. 2011). Ablation of Notch signaling in the skin induces BCC-like (Basal Cell Carcinoma-like) skin cancer in animal models (Nicolas et al. 2003) and in line with this, BCC patient samples have been reported to possess reduced levels of Notch signaling (Thelu et al. 2002). Disruption of Notch signaling using DN-MAML has been shown to cause SCC (Squamous Cell Carcinoma) in a mouse model (Proweller 2006). Almost 20% of head and neck SCCs have been attributed to mutational losses affecting various components of the Notch pathway, including mutations that truncate and disable the Notch-1 or Notch-2 receptor (Stransky et al. 2011; Agrawal et al. 2011; Wang et al. 2011).

Oncogenic Notch signaling occurs in the vast majority of Notch associated cancer. They include but are not limited to T-cell Acute Lymphoblastic Leukemias (T-ALL) and breast cancers

(Ellisen et al. 1991; Weng et al. 2004; Pece et al. 2004; Westhoff et al. 2009; Robinson et al. 2011; Van Vlierberghe & Ferrando 2012; Reedijk 2012), which are mentioned in further detail.

### 2.6.1 Oncogenic Notch signaling in T-ALLs

T-ALL is an aggressive form of Acute Lymphoblastic Leukemia (ALL) that accounts for about 15% of ALLs in children and up to 25% of the cases in adults. At diagnosis T-ALL patients present with immature T-cell infiltration of the bone marrow, unusually high numbers of white blood cells, mediastinal masses and an affected central nervous system (Van Vlierberghe & Ferrando 2012). In spite of recent improvements in survivability, prognosis in cases of resistant or disease relapse remains poor (Van Vlierberghe & Ferrando 2012).

The earliest indication that Notch function could be oncogenic came from observations that a small proportion of T-ALL patients harbored a translocation mutation that juxtaposed the intracellular portion of the Notch-1 receptor with the TCRβ locus (Ellisen et al. 1991), resulting in the production of an excessive amount of active Notch. Although this particular translocation was found to occur in less than 1% of the cases, it later emerged that more than half of T-ALL cases carry Notch-1 activating mutations of one form or another (Weng et al. 2004). Since then oncogenic Notch function in T-ALLs has been under intense scrutiny and its role in T-cell malignant transformation is now well-established. Excessive Notch signaling in T-ALLs principally follows mutations in the HD and/or the PEST domain of the Notch-1 receptor. HD mutations destabilize the receptor's NRR, rendering it susceptible to ligand independent cleavage by γ-secretase, while PEST mutations prolong signaling by stabilizing NICD (Weng et al. 2004). A functional Notch-1 PEST domain is recognized and ubiquitinated by FBW-7 (also FBXW7), an E3 ligase that marks NICD for proteasomal degradation (Oberg et al. 2001). Failure to efficiently degrade NICD inappropriately prolongs Notch signaling. In fact, FBW-7 function

is reported to be lost in approximately 20% of T-ALL cases (Malyukova et al. 2007; Mullighan 2009). Therefore, mutations that directly or indirectly promote ectopic Notch signaling may lead to T-ALL development – with the excess Notch activity driving pro-cancer processes like Inhibition of apoptosis, cell cycle progression & excessive growth (Figure 7). The actual contribution of Notch to T-ALL derives from its physiological function during T-cell development. Notch signaling is required for T-cell precursor cells to choose a T-cell outcome at the expense of a B-cell fate, a process that requires just the right amount of signaling by Notch. Constitutive Notch activity causes an expansion of the T-cell population over that of B-cells, hence the emergence of T-cell leukemia. The knowledge that uncontrolled Notch activity causes majority of T-ALLs, makes inhibition of its function an enticing therapeutic strategy.

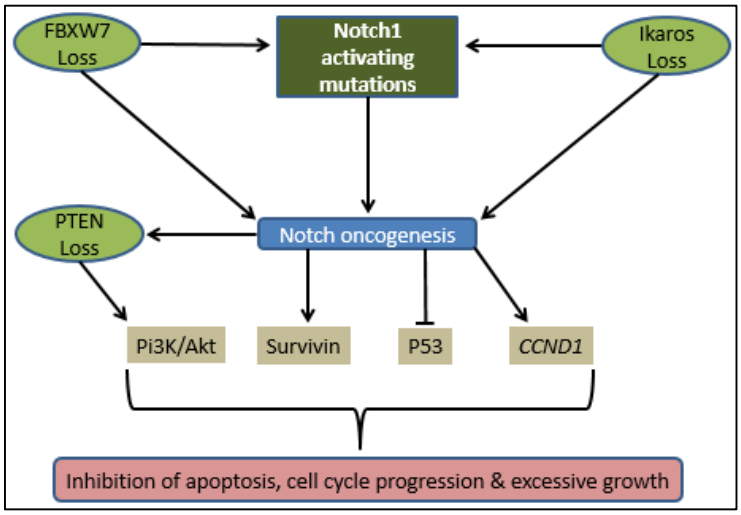


**Figure 7: Many avenues lead to Notch oncogenesis in T-ALLs**
Majority of T-ALLs are due to activating mutations in the Notch-1 receptor that leads to an out of control Notch signaling. Mutations affecting negative regulators of Notch, such as FBXW7 and Ikaros, may lead to or contribute to oncogenic Notch signaling in T-ALLs. The resultant excessive Notch signaling drives cancer by activating pro-proliferation pathways like Akt or by over expressing pro-survival genes like Survivin and CCND1 while inhibiting apoptotic ones, like p53.

### 2.6.2 Oncogenic Notch signaling in breast cancer

Breast cancer is the most prevalent cancer in women and the second most common type of cancer overall (Torre et al. 2015). Although largely viewed a 'female disease', cancers of the breasts also occur in men – albeit at a much lower frequency (Ly et al. 2013).

Early mentions of the Notch pathway's involvement in breast cancer were informed by studies on wild mice that carried the Mouse Mammary Tumor Virus (MMTV) and the observation that these animals sometime developed mammary gland tumors (Gallahan & Callahan 1987). Deeper investigations revealed that in some of the tumors, the MMTV genome had integrated within the Notch-4 locus, which at the time was unknown and referred to as int-3, causing an overexpression of its active form (Gallahan & Callahan 1987; Reedijk 2012). Similar analysis showed that insertion of the MMTV genome into the Notch-1 locus caused overexpression of its intracellular fragment and induced mammary tumor formation (Diévart et al. 1999; Reedijk 2012). This excessive Notch signaling has been demonstrated to cause mammary tumors by inhibiting differentiation and proper mammary gland development in the rodent (Jhappan et al. 1992; Smith et al. 1995).

Clues that Notch signaling might contribute to human breast cancer development came from observations that the Ras oncogene increased expression of the Notch ligand Dll-1 and also enhanced Notch-1 signaling (Weijzen et al. 2002). Since then numerous reports have demonstrated the role of oncogenic Notch signaling in human breast cancers (Mittal et al. 2009; Robinson et al. 2011; Bolós et al. 2013; Buckley et al. 2013). Excessive Notch signaling in breast cancer is often attributed to increased receptor and/or ligand expression (Reedijk 2012). However, mutations leading to constitutive activation of Notch, and similar to those previously characterized in T-ALLs have recently been identified in Notch driven breast cancers. They

include mutations in the HD and PEST as well as receptor translocation mutations (Lee et al. 2007; Robinson et al. 2011; Reedijk 2012).

Along with the earlier findings in T-ALLs, this evidence makes Notch signaling an alluring therapeutic target against cancers that thrive on oncogenic Notch.

## 2.7 Notch crosstalk with other signaling pathways

Once active, Notch functions as a transcription factor whose instructions to the cell are implemented by the proteins under its control. Through these effectors, Notch gains wider reach in the regulation of cellular functions, including the capacity to cooperate with or influence other signaling pathways. These effectors, employed by Notch for such functions are often under instruction from other signaling pathways. Thus, although frequently presented as stand-alone circuits, cell signaling pathways are in constant communication – engaging in a back and forth interaction that allows them to coordinate their instructions to the cell. As a result of this cell signaling integration, dysfunctional activity by one or more pathways may directly or indirectly affect another or other signaling pathways. Indeed, malignant transformation due to dysfunctional Notch is not solely dependent on Notch's aberrant activity. Rather, oncogenic Notch activity is often intertwined with the out of order signaling from other pathways. In cancer contexts, crosstalk has been documented between Notch and a number of other major signaling pathways, including but not limited to Wnt and PI3K/AKT (Ayyanan et al. 2006; Fre et al. 2009; Wong et al. 2012).

Cell signaling through Wnt has several parallels with Notch signaling. Like Notch, Wnt signaling is evolutionarily conserved and extremely pleiotropic. And since it functions extensively during development and in the adult organism, deregulated Wnt signaling is causative of multiple developmental disorders and cancers – including cancers of the breast

and the hematopoietic system (Staal 2007; Lamb et al. 2013; Ng et al. 2014). During Wnt signaling, β-catenin functions in a manner analogous to that of NICD. Wnt ligand binding to cell surface receptors causes stabilization and nuclear entry of β-catenin, which otherwise resides in the cytosol and possesses a very short half-life. Once in the nucleus β-catenin drives transcription of target genes to regulate diverse processes, including cell fate determination and cell proliferation (MacDonald et al. 2009). Like Notch, Wnt signaling is also critical for T-cell development (Staal & Clevers 2005; Staal 2007). Expression of constitutively active β-catenin in the mouse thymus during T-cell development causes invasive T-cell lymphoma, through a process thought to rely on a cooperation between excessive Notch and Wnt signaling (Staal 2007; Guo et al. 2007). Wnt signaling is also linked with breast cancer development through an association that traces back to the discovery of the first Wnt gene, Wnt-1 (Nusse & Varmus 1982). The identification of Wnt-1 came from observations that mice infected with the MMTV virus sometime developed mammary tumors. The animals that developed the tumors were found to have integrated the MMTV genome within the Int-1 locus, which was later renamed Wnt-1 (Nusse & Varmus 1982).

Crosstalk between Notch and Wnt, referred to as Wntch signaling, has been widely reported across species (Muñoz Descalzo & Martinez Arias 2012) and has largely been shown to occur through non-canonical modulation of Wnt signaling by Notch. During Wntch signaling, Notch acts in a non-transcriptional capacity to regulate the transcriptional activity of the Wnt pathway (Rusconi & Corbin 1998; Kwon et al. 2011; Acosta et al. 2011; Muñoz Descalzo & Martinez Arias 2012; Andersen et al. 2012). It is not clear whether the non-canonical form of Notch signaling is can drive cancer formation on its own but one may imagine that excessive Notch might cooperate with/drive Wnt toward oncogenic signaling. Nonetheless, Wnt and

Notch signaling have been shown to cooperate in breast cancer formation with ectopic Wnt activation inducing ectopic Notch signaling (Ayyanan et al. 2006; Collu & Brennan 2007).

As with Notch and Wnt signaling, PI3K/Akt signaling functions during T-cell development (Juntilla & Koretzky 2008). Crosstalk between Notch and PI3K/Akt signaling is well documented in T-ALL and is thought to go through Notch signaling mediated, Hes-1 regulation of PTEN (Phosphatase and TENsin homolog). PTEN is a tumor suppressor that normally checks the activity of the pro-proliferation PI3K/Akt signaling pathway (Song et al. 2012). Some of the Notch signaling pathway's proliferative effect is proposed to be through an indirect stimulation of PI3K/Akt signaling through the transcriptional suppression of PTEN by the Notch target, Hes-1 – a transcription factor that represses target gene expression (Wong et al. 2012). These evidences underscore the fact that cancer cells are often reliant on more than one oncogenic pathway and calls for a combinatorial approach to cancer therapy.

## 2.8 Notch as a therapeutic target

Various strategies, ranging from small molecule inhibitors, to RNA interference, to the use of monoclonal antibodies have been advanced for countering oncogenic Notch function (Figure 8) (Shih & Wang 2007; Moellering et al. 2009; Aste-Amézaga et al. 2010). Small molecule inhibitors of Notch signaling have been developed mainly against γ-secretase. In spite of early encouraging prospects that GSIs (γ-secretase inhibitors) would prove clinically beneficial, these hopes were dampened by the realization that these compounds have high toxicity in patients. Some of these blockers of γ-secretase's catalytic function were developed for the treatment of AD but were discontinued in the course of clinical trials due to associated severe gut toxicities (Aster & Blacklow 2012). The toxicity is believed to result from simultaneously blocking the activation of Notch-1 and 2 receptors (Wong et al. 2004; van Es et

al. 2005; Aster & Blacklow 2012). Another major obstacle to the clinical application of GSIs is the risk of triggering additional cancers. It is possible that while attempting to treat a Notch driven cancer, intervention with GSIs might in fact induce new cancers in other organs due to loss of Notch's tumor suppressive function. This fear is especially real for the skin – where Notch signaling protects against cancer. Indeed, a GSI in clinical trials was discontinued due to heightened risk of skin cancers in patients (Extance 2010; Aster & Blacklow 2012; Andersson & Lendahl 2014) and several GSIs have failed during clinical trials due to various safety concerns (Toyn & Ahlijanian 2014). Other challenges to the clinical use of GSIs include the fact that γ-secretase as an enzyme is extremely promiscuous and hence very difficult to target without detrimental side effects (Selkoe & Wolfe 2007). The concerns raised against the use of GSIs hold true for many of the strategies that have so far been suggested for countering excessive Notch function. The main concern being the inability to discriminate between the different Notch receptors. Even in situations where selectivity for a Notch receptor is achieved, for example with the use of monoclonal antibodies against specific Notch receptors or ligands (Andersson & Lendahl 2014), the pleiotropy of this pathway makes it a recalcitrant target (Liu et al. 2011; Ryeom 2011).

There are additional hurdles faced in pursuing Notch for therapeutic purposes, including a rapid acquisition of resistance to GSIs by Notch dependent cancers and their use of other signaling pathways. For instance, in addition to Notch, T-ALLs also depend on excessive Akt signaling for their proliferation. In such cases the transcriptional repression of PTEN by Notch through Hes-1, or the mutational loss of PTEN, drives Akt signaling. This co-operation between Notch and Akt signaling makes T-ALLs resistant to Notch signaling inhibition with GSIs (Palomero et al. 2007).

Given the available evidence, there is an urgent need for newer, safer and more efficacious ways of toning down excessive Notch signaling. A goal that is potentially achievable through the multi-pronged approach of: developing/identifying safer inhibitors, uncovering novel targets that when inhibited reduce Notch signaling subtly, hence causing milder side effects and combining two or more therapeutic strategies at lower individual strengths to minimize their respective adverse effects while pooling their benefits.

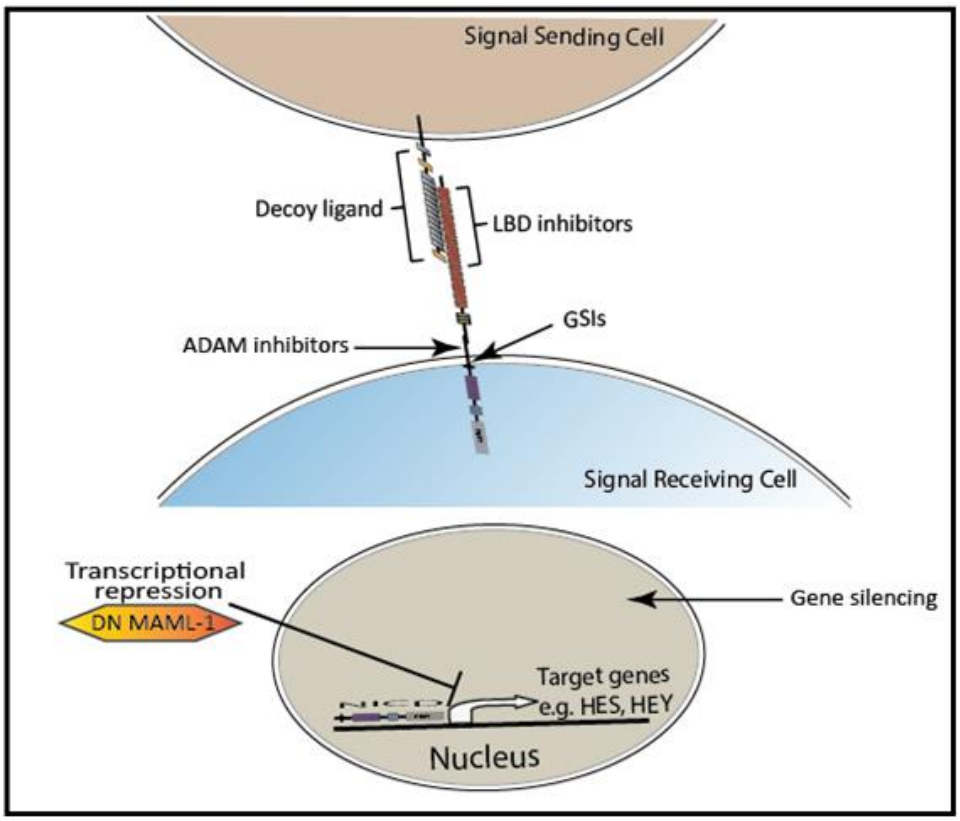


**Figure 8: Strategies for countering oncogenic Notch signaling**

Some of the approaches proposed for countering excessive Notch signaling are shown. Decoy ligands can occupy the receptor in place of actual ligands but lack the ability to activate the receptor. LBD antibody inhibitors render the receptor inaccessible to ligands, by occluding their binding regions. Chemical inhibitors can block receptor S2 cleavage by ADAM family metalloproteases. DN MAML-1 is capable of inhibiting Notch transcriptional activity. Conceptually gene silencing could downregulate Notch target gene transcripts. Of these suggestions, the most promising approach is the use GSIs to inhibit Notch receptor S3 cleavage by the γ-secretase. Modified from (Yin et al. 2010). LBD– Ligand Binding Domain. GSI– γ-secretase inhibitor. DN MAML-1– Dominant Negative MasterMind-Like-1.

# 3. Aims of the projects

## 3.1 V-ATPase inhibition reduces physiologic and oncogenic Notch signaling

Guided by recent findings that lysosomal acidification is required for physiologic and pathologic Notch signaling (Yan et al. 2009; Vaccari et al. 2010; Lange et al. 2011), we sought to examine whether pharmacologically inhibiting the V-ATPase would suffice to curtail excessive Notch signaling. In the first part of this dissertation, we used model organisms, normal human cells, Notch driven human cancer cells and specific V-ATPase inhibition to address this question. Our findings on the pharmacologic inhibition of the V-ATPase to counter oncogenic Notch signaling have been published in **Kobia et al. 2014**. Data on the regulation of Notch signaling by TFEB will appear in **Tognon et al. 2015**, which has been accepted for publication.

## 3.2 Screen for novel trafficking components in the Notch pathway

One major question in the field of Notch research is how such a seemingly simple and direct pathway attains so much sophistication in function. This has been attributed to the regulation of Notch signaling at different levels by a multitude of factors, including intracellular trafficking modulators and posttranslational modifiers (Bray 2006; Guruharsha et al. 2012). With the aim of identifying novel factors that regulate Notch signaling, we performed a siRNA screen in MCF10-A cells. We are currently validating candidates from the screen. In the second part of the thesis, I present the rationale for the screen, the screen setup, screen pilot and the results of the primary screen.

# 4. Materials and Methods

## 4.1 Drosophila strains

The following fly strains were used: Oregon R (OreR), E(spl)mb-lacZ (Nellesen et al. 1999) and UAS shrub::GFP (Sweeney et al. 2006). The temperature sensitive Rotund-Gal4-TubGal80ts (Smith-Bolton et al. 2009) driver, which is expressed in the wing pouch, was used to induce the expression of UAS shrub::GFP in larval wing imaginal discs. Flies were kept at 18°C to restrict the driver's activity. First instar larvae from rotund-Gal4, TubGal80ts/UAS shrub::GFP flies were collected at 18°C and then shifted to 29°C for 24 hours to allow driver activity and induce expression of shrub::GFP in the wing imaginal discs.

## 4.2 Treatment of flies with compounds

For drug treatment the following compounds were used: DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine butyl ester, Sigma), Leupeptin (Sigma), $NH_4Cl$ (Sigma), BafilomycinA1 (BafA1) (Calbiochem), ConcanamycinA (ConA) (Calbiochem) and Chloroquine (Sigma). Depending on the manufacturer's suggestions, the compounds were diluted in DMSO or ethanol and stock solutions for each compound added to 0.5 ml of liquid yeast to a final concentration of 1mM for DAPT and 2-4 µM for BafA1. Yeast containing the appropriate compound was added on top of a low-sugar fly food (Agarose 1%, Propionic Acid, 15% Sucrose, Tegosept, Ampicillin, water). Vials were stored overnight to ensure evaporation of residual solvent. 20 females and 10 males were introduced into each vial, and kept at 25°C for 5 days. Adults were removed, and the progeny allowed to remain in the presence of compound until eclosion. For experiments with rotund-Gal4, TubGal80ts/UAS shrub::GFP flies, 20 females and 10 males were kept at 18°C in vials containing the compounds until first instar

larvae appeared. Adults were then removed and vials containing first instar larvae shifted to 29°C. They were allowed to grow at 29°C until the 3rd instar stage.

## 4.3 Treatment of Zebrafish with compounds

Zebrafish lines were maintained and bred through standard procedures and in accordance with EU regulations on the use of laboratory animals. We used embryos from the Notch responsive reporter line *Tg(Tp1bglob:eGFP)^um14* (Parsons et al. 2009). To inhibit γ-secretase, zebrafish embryos at gastrulation (80-90% epiboly stage) were manually dechorionated. They were then placed in 2 ml petri dishes containing E3 medium (embryo water) and DAPT added to final concentrations of 50, 100 or 200mM ensuring that a DMSO did not exceed 0.4% of the water volume. The fish embryo were then incubated overnight, in the dark at 28.5°C. To specifically block the V-ATPase pump's activity, we incubated gastrulation, 1-somite or 18 somites stage zebrafish embryos in the presence of BafA1 (Sigma). BafA1, dissolved in DMSO was added into zebrafish E3 media to final concentrations of 50, 100 or 300nM, ensuring the solution did not exceed 0.4% of the water. For the Mock treated embryos, DMSO only, was added into the water at a final volume of 0.4% and the fish incubated in similar conditions as the compound treated ones. The embryos were left in the incubation medium at 28.5°C until the 26-28 hours post-fertilization stage. All recorded phenotypes were scored in at least 10 (n >10) embryos for each condition and repeated at least twice. Treated and control embryos were then mounted in 3% methylcellulose solution and observed under a Nikon fluorescence stereomicroscope.

## 4.4 Cell culture

MCF10-A cells (ATCC) were cultured in DMEM/F12 (1:1) supplemented with 5% Horse Serum (Invitrogen), 10 mg/ml Insulin, 0.5 mg/ml Hydrocortisone, 100 ng/ml cholera toxin (SIGMA) and freshly added 20 ng/ml EGF (Vinci-Biochem). HCC2218, HCC1187, and CCRFCEM cells (ATCC) were cultured in RPMI 1640 (Lonza) supplemented with 10% FBS and 1% L-glutamine. HCC1599 (ATCC) was cultured in RPMI 1640 (Lonza) supplemented with 1% L-glutamine and 20% FBS. DND-41 was cultured in RPMI 1640 (Lonza) supplemented with 15% FBS and 2 mM glutamine. HCC1599 and DND-41 cells require in our hands higher FBS concentrations, compared to the other cell lines, for optimal growth. All cells were cultured at 37°C and 5% CO2 in a humidified incubator.

## 4.5 Treatment of cells with compounds

To treat with compounds, the cells were diluted to appropriate densities and the drugs (dissolved in DMSO) added directly into the media at indicated final concentrations. As mock treatment, a volume of DMSO equal to that of respective compounds and their respective concentrations, was added directly into the medium. The amount of DMSO did not exceed 0.5% of the cell culture medium. The cells were then mixed by gently rocking the cell culture vessel before being seeded onto appropriate cell culture vessels and being placed in culture. All cells were cultured at 37°C and 5% CO2 in a humidified incubator. The following compounds were used on mammalian cells: GI 254023X (Tocris), DAPT (Sigma), BafA1 (Sigma), FCCP (Sigma), SH6 (Sigma) and Apicularen A (Api-A) (kindly offered by Prof. Angelika Vollmar, University of Munich). DIC (Differential Interference contrast) images of treated cells were acquired using an inverted EVOSfl Fluorescence Microscope (AMG).

## 4.6 Lysotracker and DQ-Red BSA assays

Wing discs from 3rd instar larvae were dissected in cold *Drosophila* cell culture medium (M3, Sigma) and then incubated in medium containing 1mM Lysotracker (DND-99, Molecular Probes) for 5 minutes at RT. The medium was then decanted and the wing discs rinsed with fresh medium before being mounted and imaged. To label organelles with low luminal pH in Zebrafish embryos, LysoTracker was directly added directly into the fishes' E3 medium at a final concentration of 0.08mM. In this experiment, *Tg(Tp1bglob:eGFP)^um14* embryos at the 28 hours post-fertilization stage were incubated for 60 min at 28.5°C in the presence of LysoTracker. They were then mounted on 1% low melting agarose dissolved in E3 medium before observation and confocal imaging. MCF10-A cells were seeded onto glass coverslips and cultured to approximately 70% confluence. They were then treated with DMSO, DAPT or BafA1 by introducing them directly into the well at the desired final concentrations. The medium was mixed and the cells placed under normal cell culture conditions for 3 hrs. To label acidified cellular compartments, lysotracker was added into the culture medium at a final concentration of 1uM. The cells were mixed well and placed in normal culture conditions for 30 minutes. They were then rinsed thrice with ice cold PBS and the nuclei stained with DAPI for 10 minutes. They were then immediately mounted on glycerol for confocal analysis. For DQ-BSA (LifeTechnologies) assays, the stock solution was added directly into the cell culture medium to a 1uM final concentration. The medium was mixed well and the cells placed in normal culture conditions for 3 hours. They were then rinsed thrice with ice-cold PBS-1X and fixed with 4% PFA. After nuclei staining with DAPI for 10 minutes, they were mounted on glycerol for confocal examination.

## 4.7 Notch translocation assay

50,000 MCF10-A cells were seeded onto glass coverslips placed into 24-well plate and grown to approximately 70-80% confluence. Notch cleavage and its translocation to the nuclear was induce by adding EGTA directly into the wells at a final concentration of 10mM. The cells were then put back under normal culture conditions for 30 min after which the medium was decanted and cells rinsed twice with ice cold PBS-1X. The cells were then fixed with 4% PFA for immunostaining. Where noted, MCF10-A cells were pre-treated for 3 hours by adding the drugs at the indicated final concentrations directly into the wells.

## 4.8 Immunostainings

For *Drosophila* experiments, wing imaginal discs were dissected and fixed in 4% paraformaldehyde (PFA) for 20 minutes. They were then rinsed thrice, 5 minutes per rinse in 0.1% triton-PBS 1X. Permeabilization was performed with 1% triton-PBS for 10 minutes. The samples were then blocked using 5% BSA in 0.1% triton-PBS (blocking solution) for 30 minutes. The samples were then incubated overnight with primary antibodies diluted in blocking solution at 4°C. After staining with primary antibody staining, samples were washed thrice with 0.1% triton-PBS 1X, 5 minutes per wash. They were then subjected to secondary antibody staining at room temperature for 2 hours. The secondary antibody was diluted in PBS 1X. Finally the samples were mounted on glycerol or moviol (Calbiochem). Where indicated, phalloidin was added to the secondary antibody solution at 1:100. The primary antibodies used were: mouse anti β-Gal [1:25; E7, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-N (1:50; C17.9C6 DHSB), rabbit anti-Ubiquitin (1:1000; FK2 1:1000; Biomol), rabbit anti-Avalanche (1:500; gift from D. Bilder).

For cell tissue culture experiments, cells were seeded onto glass coverslips and grown to the desired confluence before being fixed for 10 minutes with 4% PFA at room temperature. They were then rinsed thrice with PBS 1X, and then permeabilized for 10 minutes in 0.1% triton-PBS 1X. They were then incubated in 3% BSA dissolved in PBS 1X blocking solution for 30 minutes followed by incubation with primary antibody dissolved in blocking solution for 1 hour, at room. This was followed by 3 washes, 5 minutes each, using PBS 1X. Samples were then incubated with secondary antibody diluted in PBS 1X for 1 hour at room temperature followed by three washes using PBS 1X, 5 minutes per wash. The primary antibodies used were: mouse anti Lamp1/CD107a H4A3 (BD Pharmingen) at 1:1000, or rat anti-Full length Notch1, 5B5 monoclonal antibody at 1:300 (Sigma). For both *Drosophila* wing discs and cells, cortical actin was stained using Rodamine-Phalloidin (Sigma) at 1:100 and the nuclei were labeled with DAPI (Sigma) diluted at 1:1000. The samples then rinsed thrice with PBS 1X and mounted using on glycerol. They were then analyzed and imaged using a Leica TCS SL confocal system. Where noted, digital images were processed using the Photoshop and/or ImageJ softwares without biased manipulations.

## 4.9 Western blot assays

For western blot assays, cells were seeded and treated by adding the drugs at indicated doses directly into the cell culture medium. The cells were placed under normal culture conditions for 7 days after which they were harvested. For adherent cells, the medium was decanted and cells rinsed twice with ice cold PBS 1X. They were then collected by scraping them into 1ml ice cold PBS 1X. Where indicated, MCF10-A cells were stimulated with 10mM EGTA in the presence of the drugs for 30 minutes prior to collection. To collect cells growing in suspension, they were centrifuged at 1200 revolutions per minute (rpm) for 5 minutes. The

medium was then decanted and the pellet resuspended in 1ml ice cold PBS 1X. The cells were centrifuged at full speed, at 4°C for 5 minutes. The PBS was decanted and the pellets resuspended in appropriate volumes of RIPA buffer (prepared using standard recipe) freshly supplemented protease inhibitor cocktail set III (Calbiochem). The cells were next placed on ice for 30 minutes while vortexing them at 10 minutes intervals. The Cell lysates were cleared by centrifugation at full speed for 30 minutes, at 4°C. The supernatants were recovered and quantified using the BCA protein assay kit (LifeTechnologies) and by following the manufactures instructions. The relevant amounts of protein samples were denatured by adding β-mercaptoethanol containing laemmli buffer (prepared using standard recipe), briefly vortexing to mix and then heating for 5 minutes at 98°C. The samples were again briefly mixed by vortexing before being loaded and resolved on 8% polyacrylamide gels. Norvex Sharp prestained marker (LifeTechnologies) was used as band/protein molecular weight standard. The proteins were then transferred onto nitrocellulose membranes. Protein transfer was confirmed by reversibly staining with ponceau (prepared using standard recipe). The ponceau was washed off using 0.1% tween-20 in PBS 1X (0.1% PBS-T). The membranes were then blocked with 5% milk in 0.1% PBS-T for 2 hours. After blocking, membranes were incubated with the following antibodies, diluted in 0.1% PBS-T: anti-cleaved N-1 (Val1744, Cell Signaling) at 1:500, Rat anti full length N-1 (5B5 monoclonal antibody, Sigma) at 1:500, Rabbit anti phospho-S473-Akt (Cell Signaling) at 1:1000, Rabbit anti pan-Akt (Cell Signaling) at 1:1000, Rabbit anti-p70 S6K (Cell Signaling) at 1:1000, Rabbit anti phospho-p70-S6K (Cell Signaling) at 1:1000, Rabbit anti TFEB (Bethyl) at 1:1000 and Goat anti cathepsin D (C-20) (Santa Cruz Biotechnology). Normalization of protein loading was done by staining the membranes with mouse anti-Vinculin (Sigma) antibody at 1:4000 or mouse anti-tubulin (Sigma) at 1:10,000. The following HRP conjugated secondary antibodies were used: Goat anti-rabbit (Biorad), Goat

antimouse (Biorad), Goat anti-rat (GE Healthcare) and Rabbit anti-goat (Dako). Signal detection was done using Pierce ECL western blotting substrate (Thermo Scientific), developed and imaged using a Chemidoc molecular imager (Biorad). Western blot band intensities were quantified using imageJ image data analysis software.

## 4.10 Cell Proliferation assays

The cells were counted and resuspended so as to have the desired number of cells per 100µL of cell culture medium. They were then treated by adding the drugs directly into the medium to the desired final concentration. As mock treatment, a similar volume of DMSO was added into the cells. The cells were then seeded in triplicate onto 96-well plates by transferring 100µl of the cell suspension into each well. The cells were seeded at the following numbers per well: all breast cells at 3,000 and all leukemia cells at 5,000. As blank, 100µl of cell culture medium was seeded in triplicate. The cells were then placed under normal cell culture conditions for 7 days, after which cell proliferation was assessed using the WST-1 cell proliferation assay (Roche), following the manufacturer's instructions. WST-1 reagent is broken by the cells into a soluble, colored metabolite, whose concentration is then measured by absorbance reading at 450 nm. Absorbances were read using a Wallac 1420 VICTOR plate reader (Perkin Elmer).

## 4.11 RT-PCR

Total RNA from wing imaginal discs (40 discs per conditions) or human cells was extracted using TRIZOL Reagent (Invitrogen) and RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Concentration and purity of the RNA was determined by measuring optical density at 260 and 280nm with a Nanodrop 2000 spectrophotometer. 500ng

of Total RNA was reverse transcribed using SuperScript VILO cDNA Synthesis kit (Invitrogen) and in accordance with the manufacturer's instructions. 5ng of cDNA was amplified (in triplicate) in a reaction volume of 15uL containing the following reagents: 7.5uL of TaqMan PCR Mastermix (2X) No AmpErase UNG (Applied Biosystems), 0.75uL of TaqMan Gene expression assay 20X (Applied Biosystems), 300nM of primers and 100nM of Roche probes. RT-PCR was carried out on the ABI/Prism 7900 HT Sequence Detector System (Applied Biosystems), using a pre-PCR step of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The following primers (5'-3') were used: *Drosophila* E(Spl)-mβ: forward-gagtgcctgacccaggag, reverse- cggtcagctccaggatgt. *Drosophila* E(Spl)-m7: forward-agcgacaacgagtctctgct, reverse-ttaccagggacgccacac. *Drosophila* rpL32-RA: forward-cggatcgatatgctaagctgt, reverse- cgacgcactctgttgtcg. GFP: forward- gaagttcgagggcgacac, reverse- ccgtcctccttgaagtcg. For human genes the following Applied Biosystems' probes were used: Hes-1: Hs00172878_m1, Hes-2; Hs00219505_m1, Hes-5; Hs01387463_g1, Hey-1: Hs00232618_m1, Hey-2: Hs00232622_m1, Notch-1: Hs00413187-m1, Notch-2: Hs00225747-m1, Notch-3: Hs00166432_m1, Notch-4: Hs00270200_m1, Jag-1: Hs00164982-m1, Jag-2: Hs00171432_m1, Delta1:Hs00194509_m1, Delta-3: Hs00213561_m1, Delta-4: Hs00184092_m1, Numb: Hs00377772_m1, c-Myc: Hs00153408_m1, TFEB: hs01065085_m1, GNS: hs00157741_m1, MCOLN-1: hs00220937_m1, ATP6V0C: Hs007983308_sH, ATP6V1A: Hs01097169_m1, ATP6V1F: Hs00855096_g1, ADAM-10: Hs00153853_m1, PSENEN: Hs00708570_s1 and GADPH: Hs99999905_m1. Statistical analysis was done using Graphpad Prism and statistical significance calculated based on student's t-test. Results are flagged with two asterisks when the P-value is less than 0.01, and three asterisks when the P-value is less than 0.001.

## 4.12 siRNA knock-downs

For RNA interference mediated knock down of genes, siRNAs against the respective genes were reverse transfected into MCF10-A cells using the lipofectamine RNAi-Max transfection reagent (Life technologies) and using the manufacturer's instructions. Following transfection, the cells were placed under normal cell culture conditions for 72 hours after which RNA was extracted and processed for qPCR as described in section 4.10. siRNA duplexes against PSENEN (D-008057-01-0010), ATP6V0C (D 017620-03) ATP6V1A (D-017590-01) and ATP6V1F (D-011930-01) were purchased from GE Dharmacon.

## 4.13 γ-secretase assay

To perform the γ-secretase assay, 80% confluent MCF10A cells were pretreated for 3 hours with 3nM BafA1, 1uM FCCP, DMSO as negative control, or with 3uM DAPT as a positive control. The treated cells were then scraped in 1mL of ice cold fractionation buffer (1mM EGTA, 50mM sucrose, 20mM HEPES-pH 7.4) freshly supplemented with 5mM glucose, protease inhibitor cocktail at 1:200, and the respective compounds and concentrations as used for pre-treatment. While working on ice, the cells were homogenized by passing them through a 23 gauge needle 5 times. The homogenate was then centrifuged at 2000 g, 4°C to remove the nuclei, unbroken cells and large cellular debris. The post-nuclear supernatant was collected and centrifuged at maximum speed, 4°C, for 20 minutes to pellet the light endomembranes which were then resuspended in 1mL of fractionation buffer containing the respective compounds and pre-warmed to 37°C. 8uM γ-secretase fluorogenic substrate (Calbiochem) was then added to each light endomembrane suspension and mixed well. The γ-secretase fluorogenic substrate is internally quenched and only fluoresces when cleaved by γ-secretase. The light endomembrane suspensions were then transferred into black 96 multi-well plates in

triplicate (200uL per well) and incubated at 37°C for 12 hours before reading fluorescence. Fluorescence measurements were taken using a Wallac 1420 VICTOR plate reader (Perkin Elmer). To establish the presence of the endolysosomal compartments in the light endomembrane fraction, we performed a western blot assay and stained for LAMP-1.

# 5. Results

All *Drosophila* experiments presented below were performed by **Serena Duchi**, a postdoctoral fellow who had already initiated this project by the time I joined the lab. The Zebrafish experiments presented below were performed by **Gianluca Deflorian**, a Zebrafish expert in charge of IFOM's Zebrafish facility at the campus.

## 5.1 V-ATPase inhibition reduces physiologic Notch signaling in Drosophila

Mutating genes that encode for subunits of the V-ATPase disrupts proton pumping in *Drosophila* tissues. Previous reports by us and others have observed that this is accompanied by impaired Notch activation and function (Yan et al. 2009; Vaccari et al. 2010). We therefore sought to determine whether pharmacologically inhibiting the V-ATPase can inhibit physiologic or excessive Notch signaling in fly tissue. First, to assess whether pharmacologic inhibition of the pump was feasible, we fed *Drosophila* larvae with fresh yeast supplemented with the following compounds: BafA1 or ConA to block $H^+$ pumping and $NH_4Cl$ or Chloroquine to dissipate organellar luminal pH independently of V-ATPase function. BafA1 and ConA are members of the plecomacrolide family of V-ATPase inhibitors. This family of natural compounds exhibits very high specificity in binding to the $V_0C$ subunit of the enzyme, hence preventing $H^+$ translocation (Bowman & Bowman 2002; Huss & Wieczorek 2009). Chloroquine and $NH_4Cl$ which do not interact with the pump, are weak bases that gain entry into the cells by simple diffusion. They readily accumulate in acidified compartments where they get protonated, hence consuming protons and raising luminal pH. As negative controls, the animals were fed with: the Mock – vehicle in which the compounds were dissolved or, DAPT – a GSI frequently used to block Notch activation without affecting V-ATPase function or

organellar pH, and Leupetin – an inhibitor of lysosomal proteases that is not expected to alter lysosomal pH (Figure 9). To determine whether this method of compound administration was efficient, Serena Duchi, a postdoc in the lab assessed the effectiveness of the compounds in blocking endolysosomal acidification. This was done by dissecting the larvae's wing imaginal discs and staining them with LysoTracker, an acidophilic dye that only fluoresces when incorporated in acidic cellular environments. We then observed the cells of the wing discs to establish the extent of lysotracker retention. In the wing disc tissue of mock (A) treated animals, LysoTracker incorporates in acidified endosomes. In $NH_4Cl$ (B), Chloroquine (C), ConA (D), and BafA1 (E) fed animals, LysoTracker incorporation in the discs is markedly reduced, indicating decreased endolysosomal acidification. LysoTracker incorporation was not reduced in discs of DAPT (F) and Leupetin (G) fed animals. This indicates that endolysosomal acidification can be effectively blocked pharmacologically *in vivo*. As BafA1, is a highly specific inhibitor of V-ATPase (Bowman & Bowman 2002), we elected to use it for all further experiments.

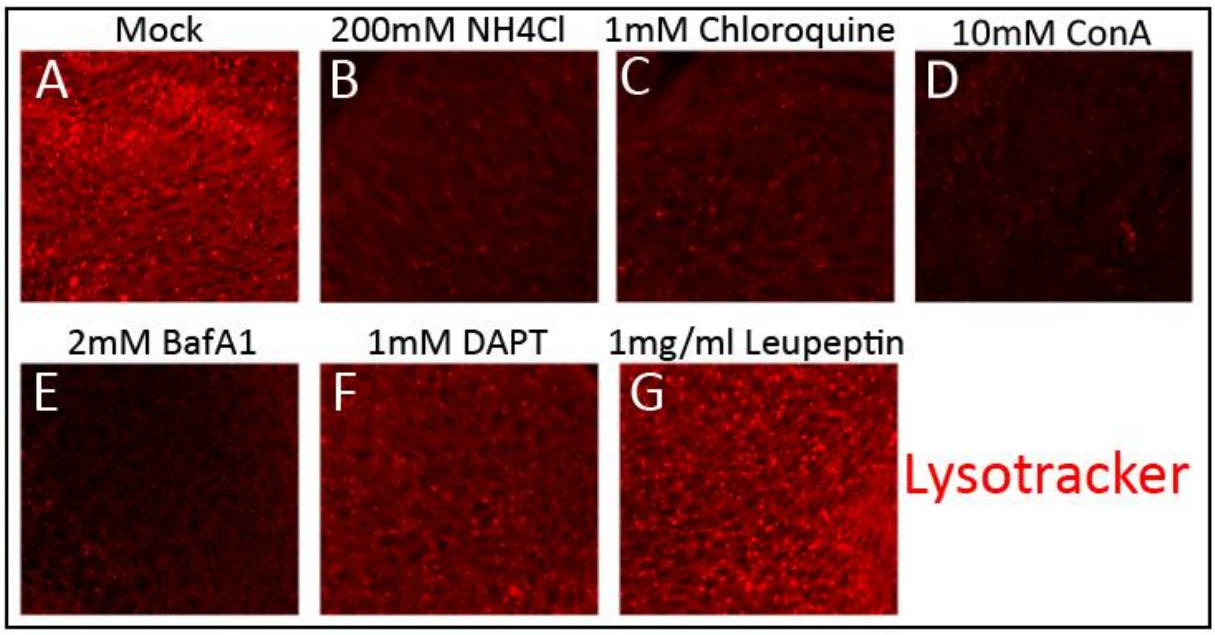


**Figure 9: V-ATPase inhibition diminishes intracellular acidification**
(A–G) Single confocal sections of the wing pouch portion of 3rd instar wing imaginal discs from Ore(R) flies. Flies were fed the respective compounds at the indicated doses before performing lysotracker assays on the wing discs. Acidification is strongly reduced in flies fed with $NH_4Cl$, Chloroquine, ConA and BafA1, but not with DAPT or Leupeptin, illustrating that drugs administered to the animals in food function effectively. ***Experiment by Serena Duchi.***

To test whether pharmacologic inhibition of the V-ATPase reduces Notch signaling *in vivo*, Serena fed larvae expressing the Notch reporter *E(spl)mβ-LacZ* with BafA1 and assessed the extent of β-gal expression in wing discs (Figure 10). *E(spl)mβ-LacZ* reports expression of the Notch target gene *E(spl)mβ* along the dorso-ventral boundary of the disc, which corresponds to the future wing margin, as well as in other parts of the discs whereby Notch signaling is active (A), (Nellesen et al. 1999). Compared to mock treated controls, discs from larvae fed with the DAPT exhibit a marked decrease of β-gal expression as determined through immunofluorescent staining (B). This results showed that reduction of Notch target gene expression is achievable through feeding an established Notch signaling inhibitor to the animals, confirming previous findings (Micchelli et al. 2003). Similarly, discs from BafA1-fed larvae show a decrease of β-gal expression (C). qPCR analysis of endogenous E(spl)mβ and

E(spl)m7 expression reveals a more than 50% reduction of mRNA expression following treatment with BafA1, an effect comparable to that of DAPT (D). This indicates that pharmacologic V-ATPase inhibition leads to reduction of physiologic Notch signaling activity *in vivo*, further supporting recent reports that the V-ATPase could be part of a multicomponent machinery that controls Notch activation on the endolysosomal track.

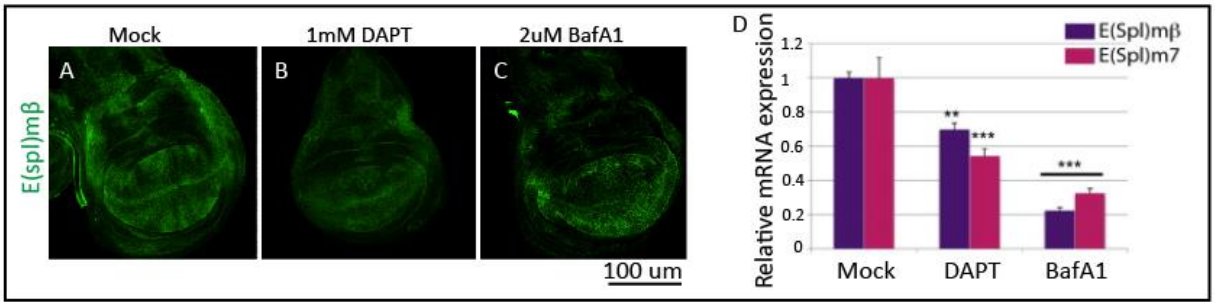


**Figure 10: Reduced Notch signaling in *Drosophila* wing imaginal disc upon V-ATPase inhibition**

(A–C) Single confocal sections of 3rd instar wing imaginal discs from larvae expressing the Notch signaling reporter E(spl)mβ-LacZ that have been fed the drugs as indicated. Anti-βGal staining was done to detect expression of the Notch target. Compared with discs from mock-fed animals (A), discs from animals fed with DAPT (B) and BafA1 (C) show a significant decrease of β-Gal expression. (D) Quantitative RT-PCR on mRNA extracted from 3rd instar wing imaginal discs from flies fed with the drugs as indicated. E(spl)mβ-LacZ and E(spl)m7 show a 30–40% decrease upon γ-secretase inhibition and more than 60% with V-ATPase inhibitor BafA1. ***Experiment by Serena Duchi.***

## 5.2 V-ATPase inhibition suppresses ectopic Notch signaling in *Drosophila*

Armed with the knowledge that we can effectively block V-ATPase activity, we wondered whether such inhibition is capable of curtailing the excessive Notch activation seen when Notch is trapped on the endolysosomal path. Chronically blocking endolysosomal degradation during development of wing discs is observed in fly mutants for ESCRT (Endosomal Sorting Required for Transport) genes. The products of these genes function at the endosomal membrane to coordinate the sorting of Notch as well as other cargoes destined for

degradation. ESCRTs mutant imaginal discs display ligand-independent but γ-secretase reliant Notch signaling activation (Herz et al. 2006; Moberg et al. 2005; Thompson et al. 2005; Vaccari & Bilder 2005; Hori et al. 2011). To block endolysosomal degradation in our experiments, Serena expressed a GFP tagged dominant negative version of VPS32 (Shrub::GFP), a component of the ESCRT III complex in the wing discs (Sweeney et al. 2006). Expression of Shrub::GFP for 24 hours during late wing disc development results in the accumulation of Notch along with ubiquitinated cargoes in endosomes, which is consistent with a block in endosomal sorting (Figure 11). This accumulation of Notch is accompanied by a greater than 2.5 fold increase in the expression of E(spl)mβ, as revealed by qPCR analysis of the wing discs RNA extract (I). Compared to mock-fed controls, DAPT feeding of larvae expressing Shrub::GFP lowers the excess Notch signaling to almost basal levels. We observed that upon V-ATPase inhibition with BafA1, Notch signaling was reduced to intermediate levels (I). These data indicate that like γ-secretase inhibition, V-ATPase inhibition reduces Notch signaling in a model of pathological Notch activation and signaling.

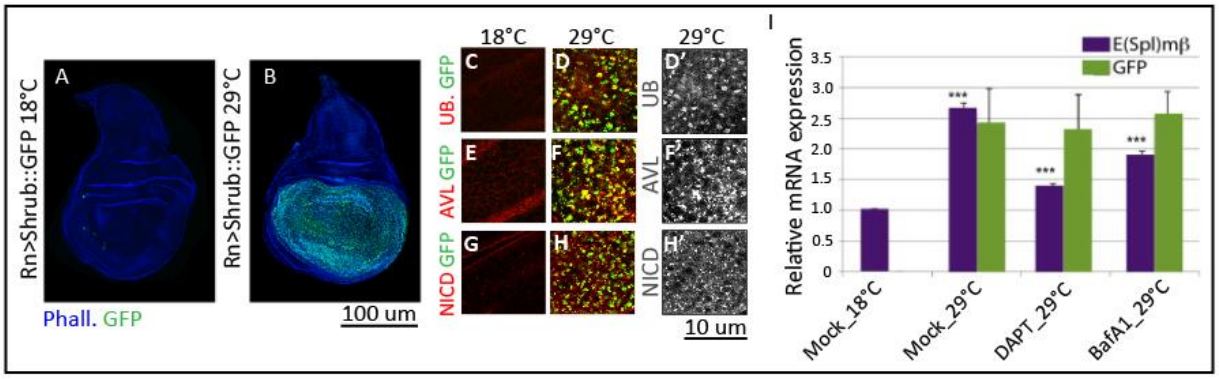


**Figure 11: V-ATPase inhibition reduces ectopic Notch signaling in *Drosophila***
(A–B) Single confocal section of discs not expressing (A) or expressing (B) Shrub::GFP under the control of RnGAL4, a wing pouch specific driver. (C–H) High magnification confocal sections of wing pouch cells not expressing (C, E, G) or expressing (D, F, H) Shrub::GFP. Discs have been stained to detect Ubiquitin (C–D), the endosomal marker Avl (E–F) or the Notch intracellular domain (NICD; G-H). In discs expressing Shrub::GFP, accumulation of ubiquitinated cargoes, including Notch, at endosomal sites is observed. Single channels for Ubiquitin, Avl and NICD are shown in D', F', H'. (I) Quantitative RT-PCR on mRNA extracted from wing imaginal discs of Shrub::GFP expressing flies that had been fed with the indicated drugs before temperature stimulation. E(spl)mβ expression is 30% reduced upon feeding with BafA1 and 50% upon γ-secretase inhibition. Comparable GFP expression levels indicate equal amounts of Shrub::GFP expressing cells in induced samples under different drug treatment. ***Experiments by Serena Duchi.***

## 5.3 V-ATPase inhibition reduces Notch signaling in Zebrafish

These observations raised the question of whether regulation of Notch signaling by the V-ATPase is exclusive to invertebrate systems or whether it is conserved in vertebrates. To test this, in collaboration with Gianluca Deflorian, we assessed whether BafA1 is capable of reducing Notch signaling in developing Zebrafish embryos. To visualize Notch activity, we used a transgenic fish line that carries a Notch signaling reporter *Tg(Tp1bglob:eGFP)^um14* in which EGFP expression is under the control of 12 Notch-responsive RBP-Jk binding sites (Figure 12) (Parsons et al. 2009). Compared to controls, embryos treated with 100uM DAPT from gastrulation display only minor developmental defects (B) and as previously reported, DAPT-treated fish embryo display reduced GFP expression, indicating reduced Notch signaling (B'

and B''; (Parsons et al. 2009). When the embryos are treated with BafA1 at 300nM, lysotracker incorporation is lost relative to the controls, indicating that V-ATPase function is impaired (C-D). However, compared with the controls, treatment of the fish at this dose of BafA1 causes obvious developmental defects, including tail and trunk shortening (E). These defects are consistent with those previously observed following loss of Zebrafish V-ATPase subunit genes (Amsterdam et al. 2004). Still, reduction of GFP expression is observed, indicating reduced Notch signaling (E'-E''). When the embryos are treated with BafA1 later in development, at the 1-somite stage, they present milder defects (F) while reduction in GFP expression persists at (F'-F''). Treating with BafA1 at an even later developmental stage, at the end of somitogenesis, further diminishes the defects (G) while maintaining reduced GFP expression at levels comparable to those attained with DAPT (G'-G").

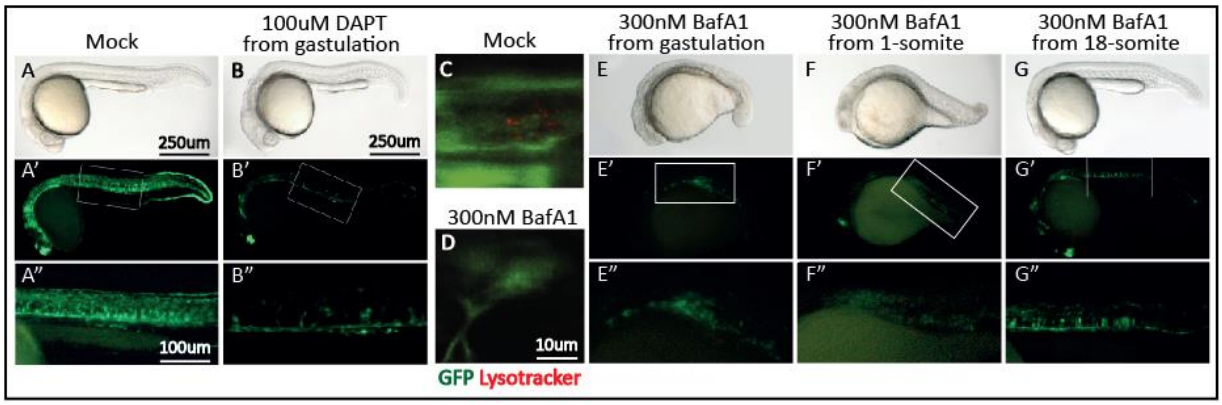


**Figure 12: Developmental defects and reduced Notch signaling in Zebrafish embryos upon V-ATPase inhibition**

Zebrafish embryos were treated as labeled, at the indicated developmental stages. (A-B & E-G) Lateral view bright field images reveal embryo morphology. Compared to mock-treated embryos (A), those given DAPT (B) show mild morphological alterations and reduced Notch signaling (B'-B''). A'' and B'' are higher magnifications of the trunk regions of A' and B' respectively. (C-D) High magnification confocal sections of the region of the intersomitic vessels of transgenic embryos treated with LysoTracker. Compared to mock-treated controls (C), embryos treated with 300nM BafA1 at somitogenesis (D), exhibit diminished lysotracker staining, indicating loss of V-ATPase function. (E-G) Embryos given BafA1 at early developmental stages show morphologic defects characterized by shorter body length along with tail and trunk defects (E and F). When BafA1 is administered post-somitogenesis, the defects are remarkably milder defects (G) and similar to those caused by DAPT (B). Treating the fish embryos with BafA1 results in a strong reduction of GFP expression (E'-G''), to levels similar to those seen upon administration of DAPT (B'). E''-G'' are higher magnifications of the trunk region of embryos E–G. ***Experiment by Gianluca Deflorian.***

Following these observations, we sought to determine whether the developmental defects observed upon BafA1 administration early in embryonic development can be alleviated by lowering the doses applied (Figure 13). Administering higher doses of DAPT, at 200uM does not cause major morphological defects (B) but leads to a very strong reduction in Notch signaling as seen from reduced GFP expression (B'). Treatment with DAPT at 50uM does not have any apparent morphological alterations (C) and still reduces Notch signaling – albeit to a lesser extent (C'). Using lower amounts of BafA1, at 100nM and 50nM during gastrulation, allows embryos to develop with almost normal morphologies (D-E) and still reduces Notch

signaling (D'-E') to levels comparable with those reached using a lower dose DAPT (C). Combining low dose BafA1 with low dose DAPT lowers Notch signaling in an additive manner (F'), to similar levels as when using the high dose of DAPT (B'). These data indicate that V-ATPase inhibition reduces Notch signaling activity in Zebrafish embryos, and that Notch signaling might rely on V-ATPase activity during vertebrate development. It also demonstrates that low doses of the compounds can be combined to obtain stronger efficacies than can be achieved with either drug individually.

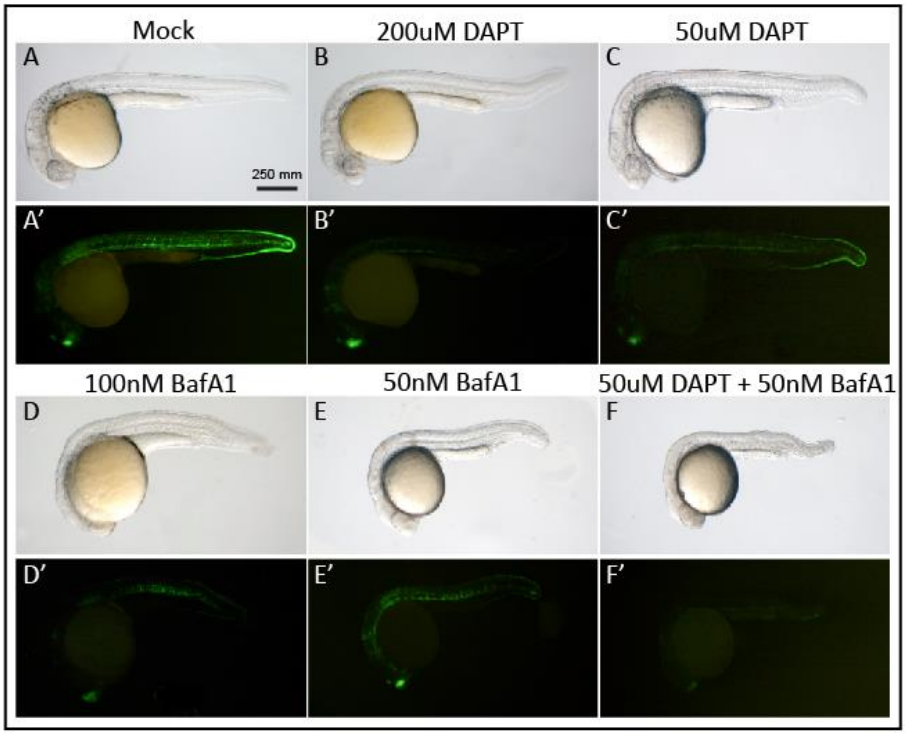


**Figure 13: Low dose V-ATPase reduces Notch signaling in Zebrafish embryos**
(A–F) Bright field lateral views of zebrafish embryo morphology. Representative images of embryos treated as indicated are shown. Compared to mock-treated embryo (A), embryos treated with varying doses of DAPT (B–C) or BafA1 (D–E) or both (F) have mild physical defects. (A'–F') GFP, which indicates the expression levels of Notch in the reporter line Tg(Tp1bglob:eGFP)ˆum14 is visualized. Treating the embryos with DAPT or BafA1 results in a dose-dependent reduction of the GFP signal (B'–F'). Note that combining both drugs at low doses attains a reduction in GFP expression that is comparable to treatment with DAPT at a high dose (compare B' and D' with F'). ***Experiment by Gianluca Deflorian.***

## 5.4 V-ATPase inhibition reduces physiologic Notch signaling in human cells

Given the observations that V-ATPase inhibition lowers Notch signaling in flies and Zebrafish, we decided to explore whether V-ATPase inhibition has similar effects in human cells. To perform such assessment on physiologic Notch signaling, we used MCF10-A cells. This is a non-transformed human breast epithelial cell line commonly used as a model of normal human epithelial cells (Soule et al. 1990). To assess whether BafA1 impairs V-ATPase function in MCF10-A, we treated them for 3 hours with BafA1 and compared the effect against that of the vehicle, or of DAPT (Figure 14). We then assayed V-ATPase function using lysotracker. Relative to Mock (A) and DAPT (D) treated cells, treatment of these cells with BafA1 leads to a dose-dependent loss of lysosomal acidification (B-C), indicating a dose-dependent blockade of V-ATPase activity. Since lysotracker is not indicative of lysosomal function, to assess lysosomal function we performed a DQ-RED BSA assay. DQ-RED BSA only fluoresces when cleaved by lysosomal hydrolases. Relative to Mock (A') and DAPT (D') treatment, BafA1 exhibits a dose dependent reduction in the capacity of the cells' to hydrolyze DQ-RED BSA (B'-C'), indicating reduced lysosomal function.

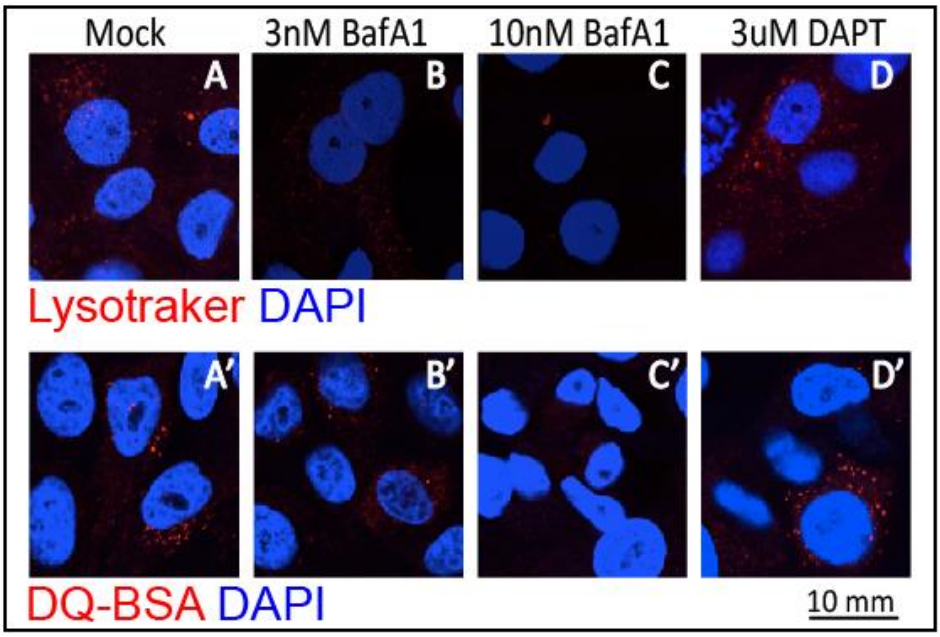


**Figure 14: BafA1 reduces lysosomal acidification**

Confocal images of MCF10-A cells that were treated as indicated for 3 hours, following which lysotracker (upper panel) and DQ-RED BSA (lower panel) assays were performed. Relative to Mock treatment (A), BafA1 impairs lysotracker retention by the cells in a dose dependent fashion (B-C). Treatment with DAPT does not affect lysotracker incorporation (D). The DQ-RED BSA assay reveals a similar pattern. Compared to the Mock (A'), BafA1 treatment impairs the capacity of the cells to hydrolyze the substrate in a dose dependent manner (B'-C') while DAPT has no effect on the function of lysosomal hydrolases. This indicates that BafA1 can effectively block V-ATPase function in cultured human cells.

As revealed through a qPCR analysis, MCF10-A cells endogenously express components of the Notch pathway, including receptors and ligands. They however engage in a low level of Notch signaling as seen from the basal expression of the Notch target, Hes-1 (Figure 15). This makes them an excellent cell line in which to investigate Notch signaling as it obviates the need for transfecting elements of the pathway into the cells.

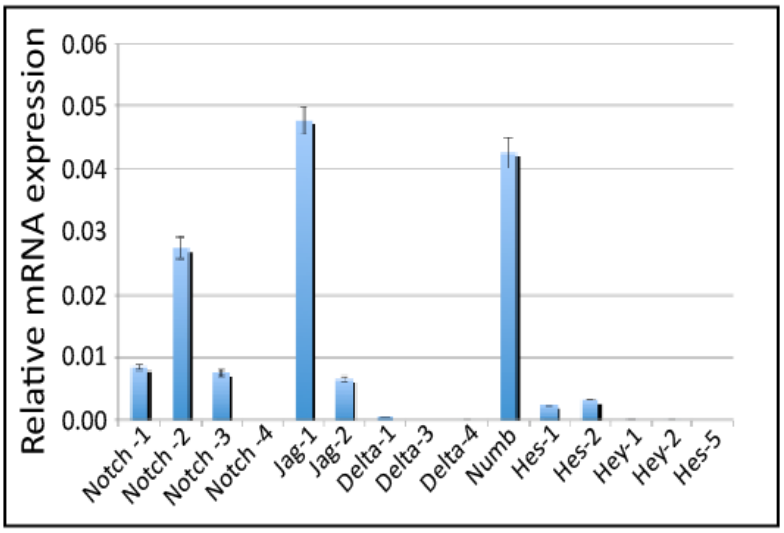


**Figure 15: MCF10-A cells endogenously express Notch pathway components**
MCF10-A cells possess the complete Notch pathway. It expresses the Notch receptors 1-3 and the ligands Jag-1 and 2. As seen from the expression of Notch target genes of the Hes family of transcription factors, these cells physiologically engage in basal levels of Notch signaling.

Endogenous Notch-1 in MCF10-A is readily detectable by IF (Figure 16). Immunostaining the cells with an anti-Notch-1 antibody shows that under physiological conditions, the receptor resides on the cell surface membrane (A). Relative to untreated cells, the presence of DAPT for 3 hours does not alter Notch-1 localization (B). When compared to both controls, treatment with BafA1 for 3 hours greatly causes Notch to accumulate intracellularly. A co-staining for Notch-1 and the late endosomal/lysosomal marker, Lamp-1, reveals that intracellularly accumulated Notch-1 localizes in late endosomes and lysosomes (C). This accumulation is attributable to impaired degradation of cellular material, including Notch and shows that endosomal trafficking of Notch and its high turnover rate along the endolysosomal system can be tracked efficiently in human cells.

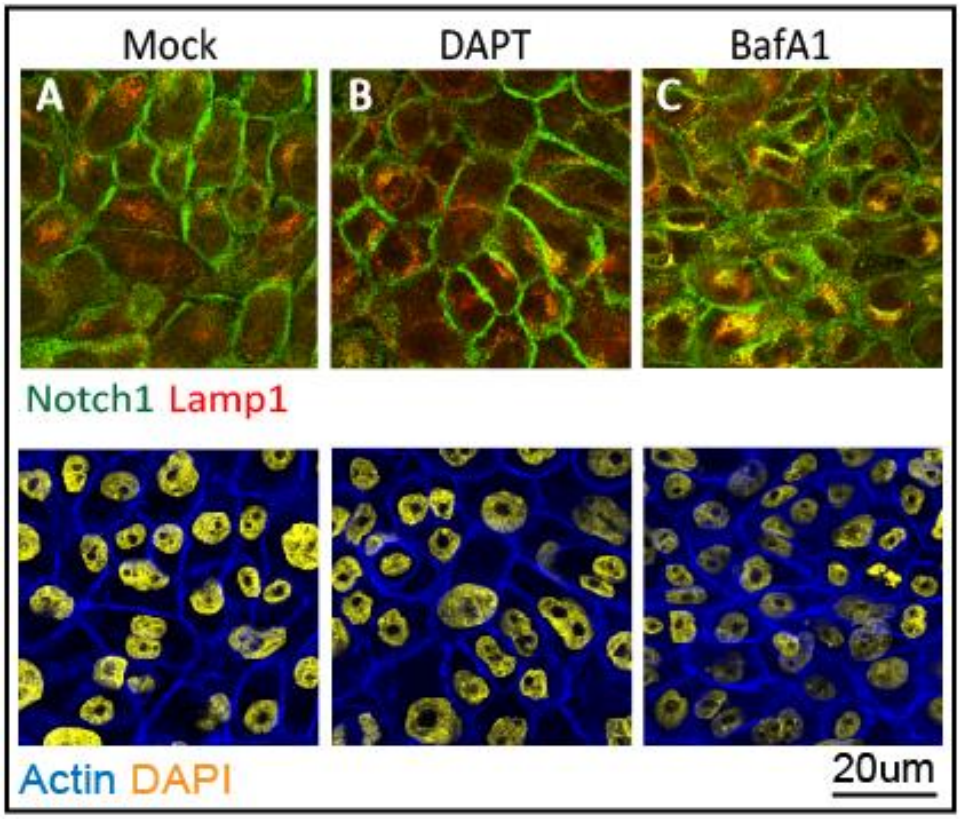


**Figure 16: Notch-1 resides on the cell surface of MCF10-A cells**
Staining Mock treated cells with an anti-Notch-1 antibody reveals its localization on the cells surface membrane (A). This localization of Notch-1 remains unchanged in cells treated with DAPT (B). Staining cells treated with BafA1 reveals accumulation of Notch-1 along the endolysosomal path, as told by co-localization of the Notch signal with the Lamp-1 signal (C). Lamp-1 is a late endosomal/lysosomal marker. The cells are counter stained with DAPI and phalloidin to highlight the nuclei and cell surface membranes respectively (lower panel).

Notch signaling activation can be induced in cultured cells through calcium depletion (Rand et al. 2000). Extracellular $Ca^{2+}$ chelation sheds the extracellular portion of the Notch receptor, triggering its ligand independent cleavage by γ-secretase and NICD translocation into the nucleus. To test whether BafA1 treatment interferes with Notch-1 translocation to the nucleus, we treated MCF10-A for 3 hours with DMSO, BafA1, and as positive control, with DAPT and then induced Notch cleavage with EGTA (Figure 17). Immunolabeling the cells with an antibody that recognizes both the full-length and cleaved forms of Notch-1 (cNICD-1), revealed that in mock treated cells, EGTA caused Notch-1 translocation into the nucleus (A). As

expected, this translocation was completely blocked by DAPT, which keeps Notch on the cell surface membrane in the presence of EGTA (B). Adding EGTA to cells treated with BafA1, reveals a partial, dose-dependent reduction of relocation to the nucleus (C-D; quantification in E).

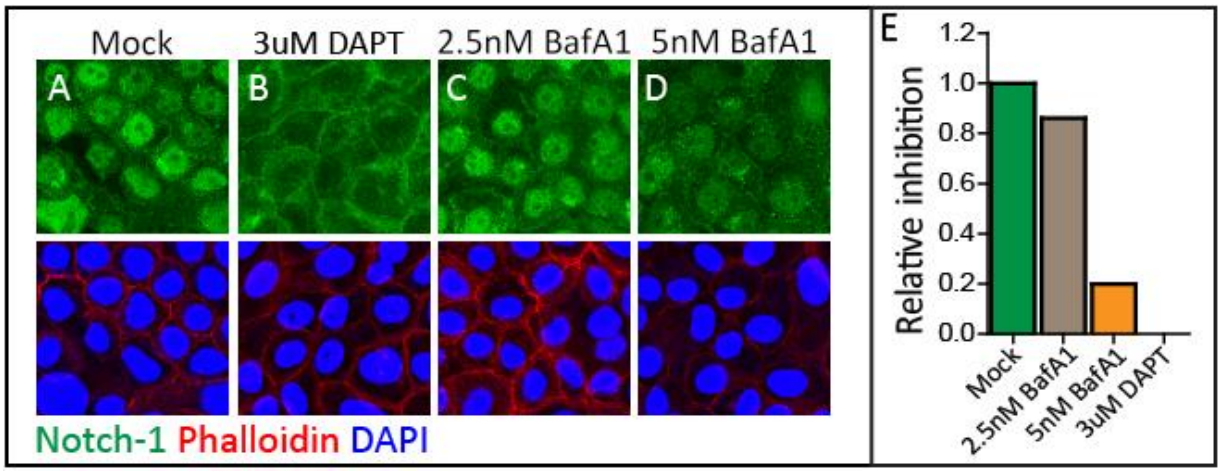


**Figure 17: V-ATPase inhibition reduces Notch activation in human breast cells**
(A–D) Confocal images showing the cellular localization of endogenous Notch-1 in MCF10A cells. The cells were treated as indicated for 3 hours before Notch cleavage was stimulated with EGTA. Immunostaining was performed with an anti-Notch-1 antibody that recognizes both the full-length and cleaved receptor (A-D). Phalloidin and DAPI were used to mark the cell surface membrane and nuclei respectively (lower panel). Relative to Mock treated cells (A), Notch-1 localization in the nucleus is completely prevented by DAPT (B) and significantly reduced by BafA1 treatment in a dose responsive manner (C-D). (E) Nuclear pixel intensity quantifications of A–D is shown.

To establish whether BafA1 prevents Notch cleavage and signaling, we treated MCF10-A cells for 7 days with 3nM BafA1, 3uM DAPT as positive control and DMSO as negative control. These doses were selected as we found that the cells can withstand low dose BafA1 for extended periods (Figure 18). A treatment period of 7 days was selected so as to provide sufficient doubling time for the cells so as to capture differences in cell proliferation between in treated vs untreated cells.

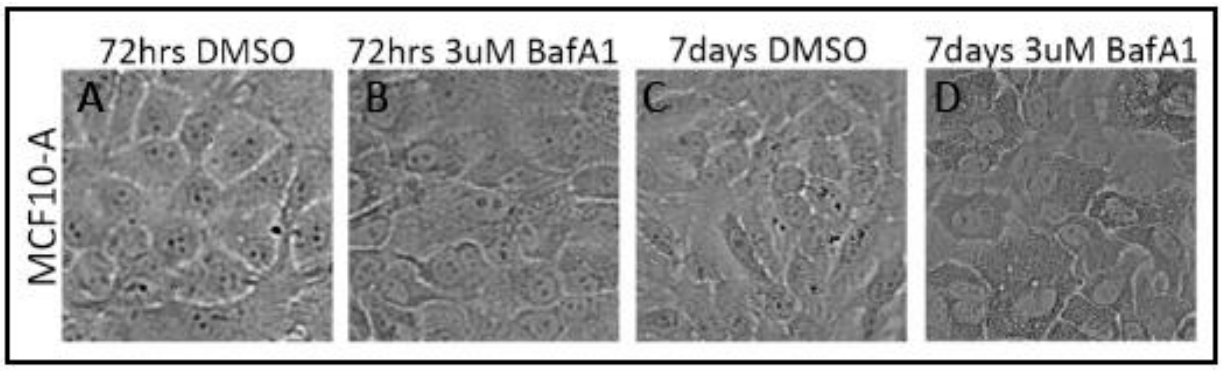


**Figure 18: MCF10-A cells tolerate low doses of BafA1**
DIC images of cells treated as shown for the indicated periods. The cells remain viable for up to 7 days, the longest period of time for which we treated them.

At the end of 7 days treatment, Notch cleavage was stimulated with EGTA (Figure 19). EGTA induction of Notch cleavage generates a strong amount of cNICD-1, which is detectable by western blot using an antibody specific to the cleaved form of the receptor. The NICD produced upon EGTA triggered Notch cleavage is accompanied by a steep increase in Hes-1 expression, a Notch signaling target (Borggrefe & Oswald 2009). Relative to negative control treated cells, in the presence of EGTA BafA1-treated cells display reduced Notch cleavage (A) and no spike in Hes-1 expression, similar to treating the cells with DAPT (B). Treating the cells with Api-A, a second V-ATPase inhibitor unrelated to BafA1 (Osteresch et al. 2012) also inhibited cNICD-1 production upon stimulation with EGTA, indicating that the observed results are due to V-ATPase inhibition and not off target effects (C). Moreover, knocking down non-redundant V-ATPase subunit genes for 72 hours also reduced the spike of Hes-1 levels upon stimulation with EGTA, as did knock down of PSENEN, a component of the γ-secretase complex (D). A qPCR assay reveals strongly reduced mRNA levels upon knockdown of the respective genes (E). Overall, these data indicated that the V-ATPase controls Notch signaling activation in a model of normal human epithelial cells.

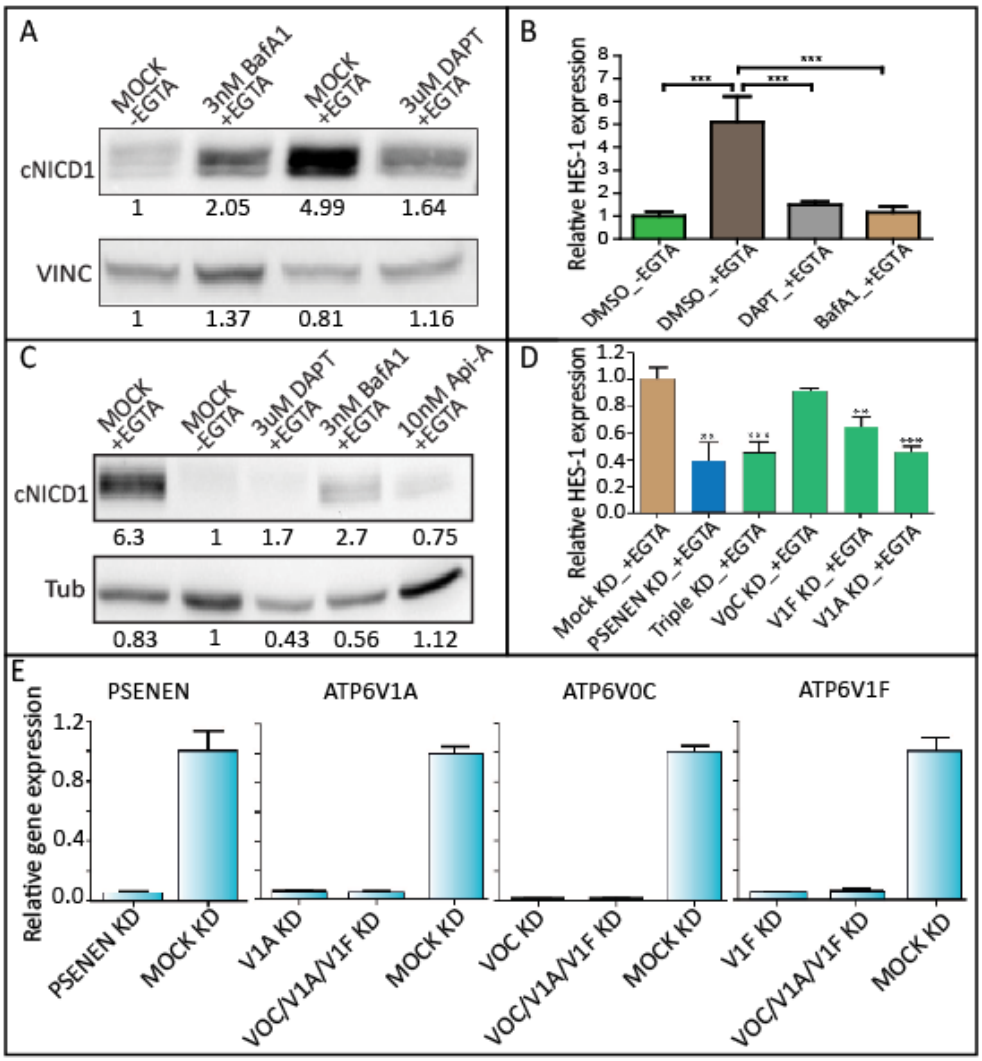


**Figure 19: V-ATPase inhibition reduces Notch cleavage and signaling in MCF10-A**

(A-C) MCF10-A cells were treated with the mentioned drugs at the indicated doses for 7 days before being stimulated (or not) with EGTA for 30 minutes. Compared to unstimulated cells, addition of EGTA triggers strong generation of cNICD-1. Relative to Mock, BafA1 treatment inhibited EGTA driven cNICD-1 production to levels similar to those achieved with DAPT (A). A qPCR analysis of similarly treated cells reveals the spike in Hes-1 expression in EGTA stimulated, Mock treated cells when compared to unstimulated ones. Both BafA1 and DAPT strongly suppress this EGTA triggered spike in Hes-1 – indicating inhibited Notch signaling (B). A western blot analysis of cells treated with API-A, a V-ATPase inhibitor unrelated to BafA1, has an effect similar to that of BafA1 – suggesting that the observed effects are indeed due to V-ATPase inhibition (C). (D) Knocking down genes encoding subunits of the V-ATPase or a γ-secretase component, PSENEN, suppresses the spike in Hes-1 upon EGTA treatment – indicating that V-ATPase inhibition reduces Notch signaling. (E) qPCR analysis shows significant reduction in the expression of the indicated genes upon their knockdown. Western blot oading differences between the samples were equalized using vinculin or tubulin and the corrected cNICD-1 band intensities normalized to obtain a fold cNICD-1 band intensity relative to the Mock_-EGTA sample.

## 5.5 V-ATPase inhibition suppresses Notch signaling in breast cancer cells

Having established that pharmacologic inhibition of V-ATPase reduces physiologic Notch signaling, we wondered whether it could mitigate excessive Notch signaling in cancer cells. Oncogenic Notch signaling has been observed in several cancers including those of the breast and hematopoietic system (Weng et al. 2004; Stylianou et al. 2006; Robinson et al. 2011). As such, oncogenic Notch signaling has been the focus of intense interest for therapeutic purposes. However, owing to a number of issues, targeting Notch signaling in disease has so far been difficult (Andersson & Lendahl 2014). We therefore wondered whether pharmacologic inhibition of the V-ATPase could inhibit oncogenic Notch signaling in Notch driven breast cancers. To test this, we used the breast cancer cell lines HCC1599 and HCC2218, which harbor translocation mutations in the Notch-1 gene that have caused them to express membrane-tethered forms of the receptor (Robinson et al. 2011). This truncated receptor is substrate for cleavage by γ-secretase independently of ligand interaction, generating cNICD-1 and constitutively activating the Notch pathway. HCC1599 and HCC2218 express both Notch receptors and ligands, and consistent with a constitutively active Notch pathway, they express high levels of the Notch target Hes-1 (Figure 20).

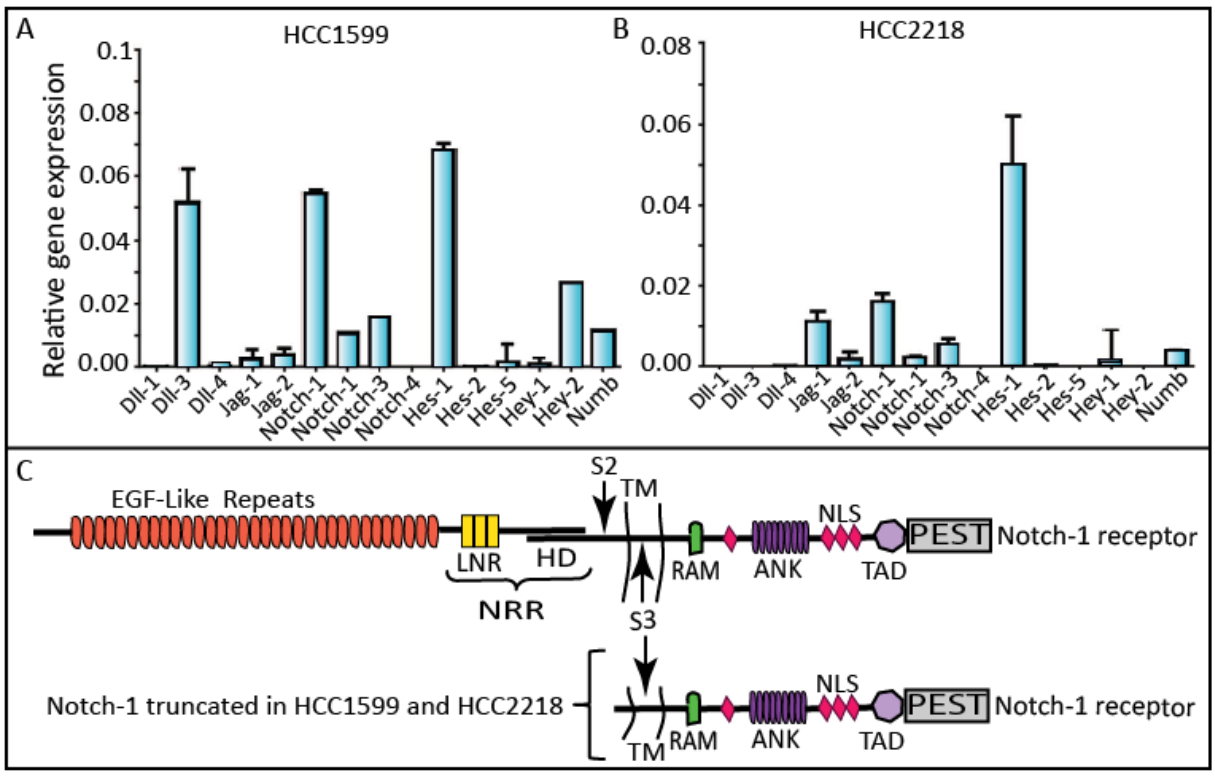


**Figure 20: Constitutive Notch signaling in human breast cancer cell lines**
(A-B) A qPCR analysis of human breast cancer cell lines known to possess a constitutively active Notch pathway shows that they both express Notch ligands and receptors. HCC1599 (A) and HCC2218 (B) possess translocation mutations in their Notch-1 receptors (C). These truncated forms of the Notch-1 receptor are subject to ligand independent cleavage by γ-secretase. As a consequence of a constantly 'on' Notch signaling, the cells express high levels of the Notch target gene Hes-1 (A-B).

Since the membrane-tethered stubs of Notch-1 expressed by HCC1599 and HCC2218 require processing by γ-secretase, Notch cleavage can be blocked by GSI (Robinson et al. 2011). To assess whether V-ATPase inhibition can block constitutive cleavage of Notch-1 in these cells we treated them with BafA1 and assessed the extent of cNICD-1 generation by western blot (Figure 21). This assay revealed that both DAPT and BafA1 reduce the level of c-NICD-1 when compared to mock treated HCC1599 and HCC2218 (A-B). A qPCR analysis of the Notch target HES-1 reveals that both the GSI and BafA1 significantly inhibit Notch signaling (C).

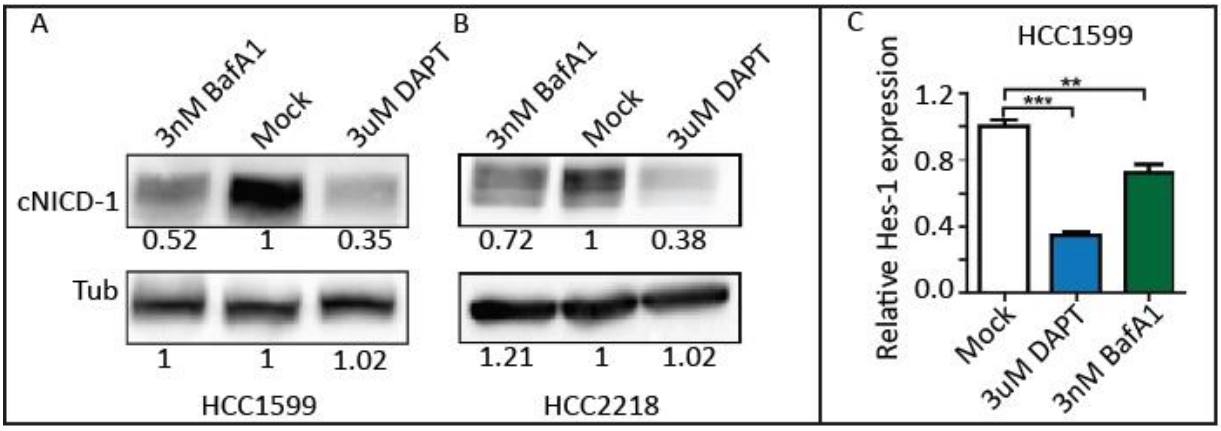


**Figure 21: V-ATPase inhibition suppresses Notch cleavage in Notch reliant breast cancer cells**
(A-B) Western blot analysis on extracts from HCC1599 and HCC2218 treated as indicated for 7 days. cNICD-1 production is strongly reduced by both BafA1 and DAPT when compared to the negative control. (C) qPCR assay to detect Hes-1 expression levels in HCC1599 following treatment as shown. Upon BafA1 pretreatment we observe a 25% reduction of Hes-1 expression, confirming reduced Notch signaling. Western blot loading differences between the samples were equalized using tubulin and the corrected cNICD-1 band intensities normalized to obtain fold cNICD-1 band intensities relative to the Mock treated sample.

The constitutive activation of the Notch signaling pathway sustains the proliferation of HCC1599 and HCC2218, which is reduced upon Notch signaling inhibition with GSI (Robinson et al. 2011). We therefore wondered whether the reduced Notch activation and signaling observed upon V-ATPase inhibition corresponds with a reduction of cell proliferation. Another breast cancer cell line, HCC1187, bears a truncating mutation in Notch-2. Unlike HCC1599 and HCC2218, this mutation generates a fragment of the receptor that lacks the entire extracellular portion of Notch-2, including the S3 cleavage site, resulting in constitutive Notch signaling that is both ligand and γ-secretase independent (Robinson et al. 2011). Since this cell line directly produces Notch-2 NICD, its Notch signaling and associated proliferation is unresponsive to γ-secretase inhibition (Robinson et al. 2011). We therefore treated the cells with DAPT and assessed their proliferation and survival after 7 days (Figure 22). We observed that in the GSI insensitive HCC1187, growth and proliferation is not affected by DAPT when compared to the

negative control treated cells (B). A similar observation is made in similarly treated MCF10-A cells, whose growth and proliferation is not reliant on excessive Notch signaling (A). On the other hand, treating the GSI sensitive HCC2218 and HCC1599 with DAPT causes a strong dose-dependent reduction in cell proliferation when compared to control treated cells (C-D). These results confirmed previous evidence (Robinson et al. 2011) and permitted us to test the effect of BafA1 on the Notch addicted breast cancer cells. We observed that V-ATPase inhibition reduces the proliferation of HCC1599 and HCC2218 (C-D) to levels comparable with those attained by γ-secretase inhibition. At a similar concentration of BafA1, proliferation of HCC1187 was minimally affected while that of MCF10-A cells remained unaltered relative to Mock treated cells (A-B). In addition, we found that the effect of BafA1 on the proliferation of HCC2218 and HCC1599 is dose-dependent and can be potentiated by co-treating the cells with DAPT and BafA1 (C-D). All experiments from this point on, were performed by treating the cells with 3nM BafA1.

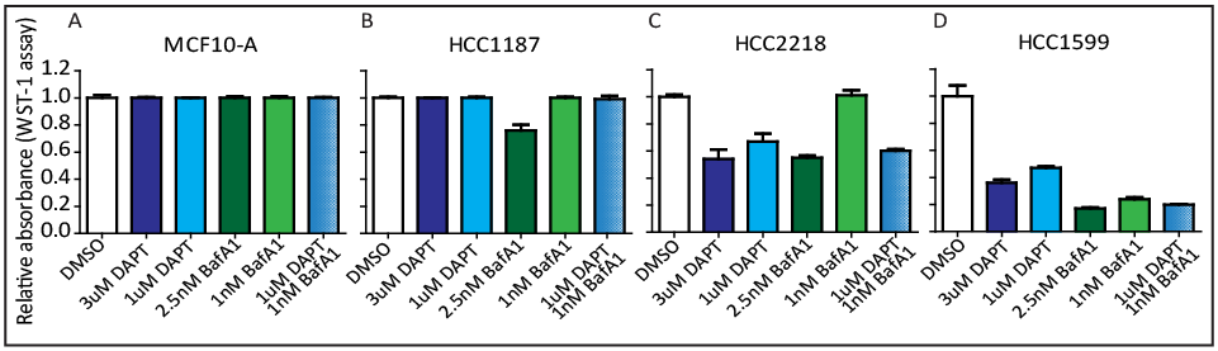


**Figure 22: V-ATPase inhibition reduces growth of Notch addicted breast cancer cells**
(A–D) Cells were treated as indicated for 7 days before a WST-1 assay was performed to assess cell growth. WST-1 is metabolized by the cells, which then release a soluble product that is quantified by absorbance, which directly correlates with the number and viability of the cells. Growth of normal breast MCF10A cells and breast cancer HCC1187 cells, which express constitutively active Notch-2, is not affected by the drugs. In contrast, growth of the breast cancer cells HCC2218 and HCC1599, which express activated but membrane tethered forms of Notch-1, is sensitive to both DAPT and BafA1 in a dose-sensitive fashion.

On the whole, these data indicate that V-ATPase inhibition reduces Notch dependent growth and survival in breast cancer cells, and that combined treatment with BafA1 along with a GSI permits the use of both compounds at very low doses while preserving efficacy.

## 5.6 V-ATPase inhibition suppresses proliferation of GSI sensitive T-ALL cells

The role of excessive Notch signaling in T-ALLs is well established and T-ALL cell lines that rely on oncogenic Notch signaling are well characterized. Many of these cells possess mutations in the NRR region of the Notch-1 receptor, making it prone to ligand independent Notch activation, hence constitutive signaling (Weng et al. 2004). A qPCR analysis of two representative T-ALL cell lines revealed that they express components of the Notch signaling pathway, including Notch ligands and receptors. They also express the Notch targets Hes-1 and Myc (Figure 23).

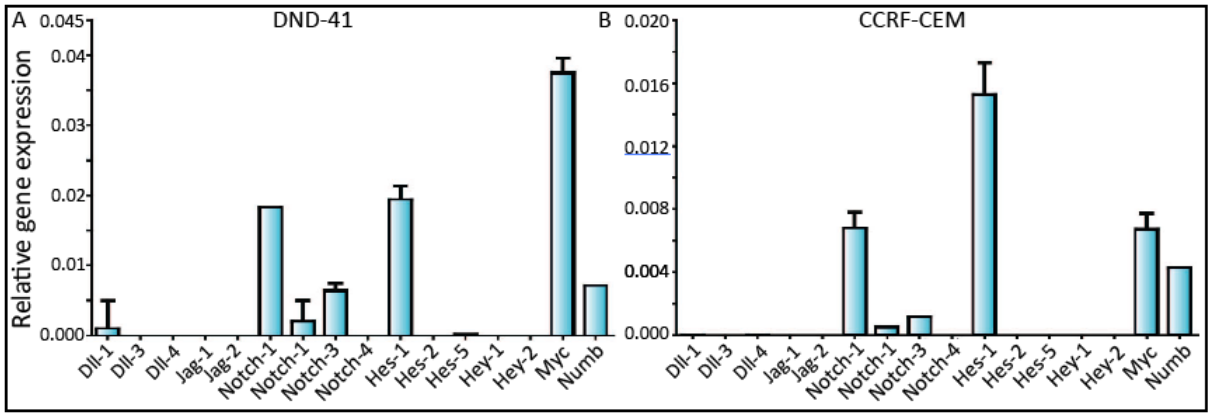


**Figure 23: T-ALL cell lines express Notch signaling pathway components**
(A-B) A qPCR analysis of some Notch pathway genes in the T-ALL cell lines DND-41 (A) and CCRF-CEM (B) reveals that they express relative levels of Notch receptors (Notch-1-4), ligands (Jag1-2, Delta1,3-4), targets (Hes-1, 2, 5, Hey-1, 2, Myc) as well as the Notch inhibitor Numb.

Several of these Notch dependent T-ALL cell lines, for example DND-41, are responsive to γ-secretase inhibition. Because DND-41 represents an established model of T-ALL cells with ligand-independent, constitutive Notch signaling, we tested whether its Notch signaling activity could be inhibited by pharmacologic inhibition of the V-ATPase (Figure 24). Treating DND-41 cells for 7 days with low doses of DAPT reduces their proliferation in a dose dependent manner (A). As is the case of DAPT treatment, low doses of BafA1 also reduce the cells' proliferation dose dependently (A). In addition, we observe that co-treatment with both compounds at low dose, is more efficient than using either singular treatment at high dose (A). However, western blot analysis showed minimal reduction of cNICD-1 generation upon BafA1 treatment (B). These results imply that inhibition of pathways other than Notch might account for the considerable reduction in proliferation observed in DND-41 cells.

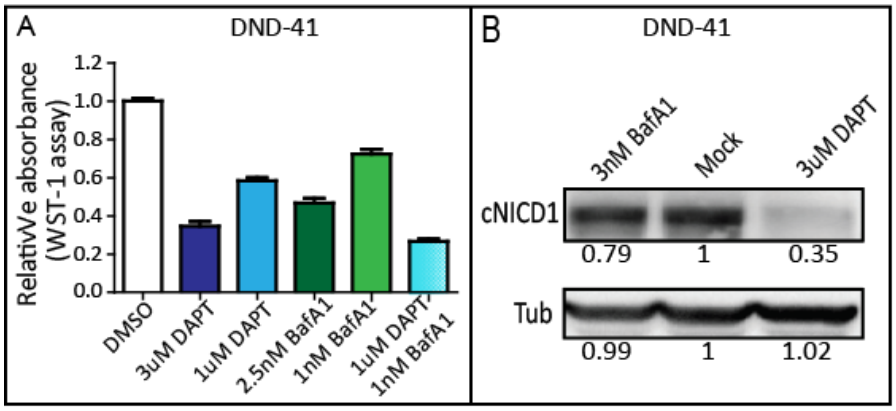


**Figure 24: V-ATPase inhibition reduces proliferation of DND-41 T-ALL cells**

(A-B) DND-41 cells were treated with the indicated doses of BafA1 or DAPT for 7 days before cell proliferation or western blot assays were performed. Relative to Mock treated cells, DAPT significantly inhibits cell growth in a dose specific manner. V-ATPase inhibition also reduces their proliferation to levels nearing those of DAPT. Co-treating with low concentrations of both compounds has an additive effect in reducing cell proliferation (A). A western blot analysis on similarly treated cells indicates that unlike DAPT, which as expected abolishes cNICD-1 production, the V-ATPase inhibitor only modestly reduces cNICD-1 generation (B). Western blot loading differences between the samples were equalized using tubulin and the corrected cNICD-1 band intensities normalized to obtain fold cNICD-1 band intensities relative to the Mock treated sample.

Because Akt/mTOR signaling also contributes to growth of T-cell leukemia lines (Palomero et al. 2007; Guo et al. 2009; Shepherd et al. 2013), we assessed Akt signaling activation with an anti-phospho-Akt antibody upon treatment of the cells for 7 days (Figure 25). This western blot assay revealed a reduction in Akt phosphorylation following treatment with both DAPT and BafA1 (A), indicating suppressed Akt signaling. Consistent with a possible inhibition of Akt signaling upon V-ATPase inhibition, additive growth reduction was observed when DND-41 cells were co-treated with either BafA1+SH6 or DAPT+SH6. SH6 is a selective inhibitor of Akt signaling that does not affect other kinases upstream or downstream of Akt (Kozikowski et al. 2003). Breast cells treated with BafA1 also display reduced Akt phosphorylation (C-E), indicating reduced Akt signaling. These data suggest that BafA1 is likely to reduce DND-41 proliferation mostly by inhibition of Akt signaling. Reduced Akt signaling upon V-ATPase inhibition is could contribute to the anti-proliferative effect of BafA1 both breast cancer and T-ALL cells.

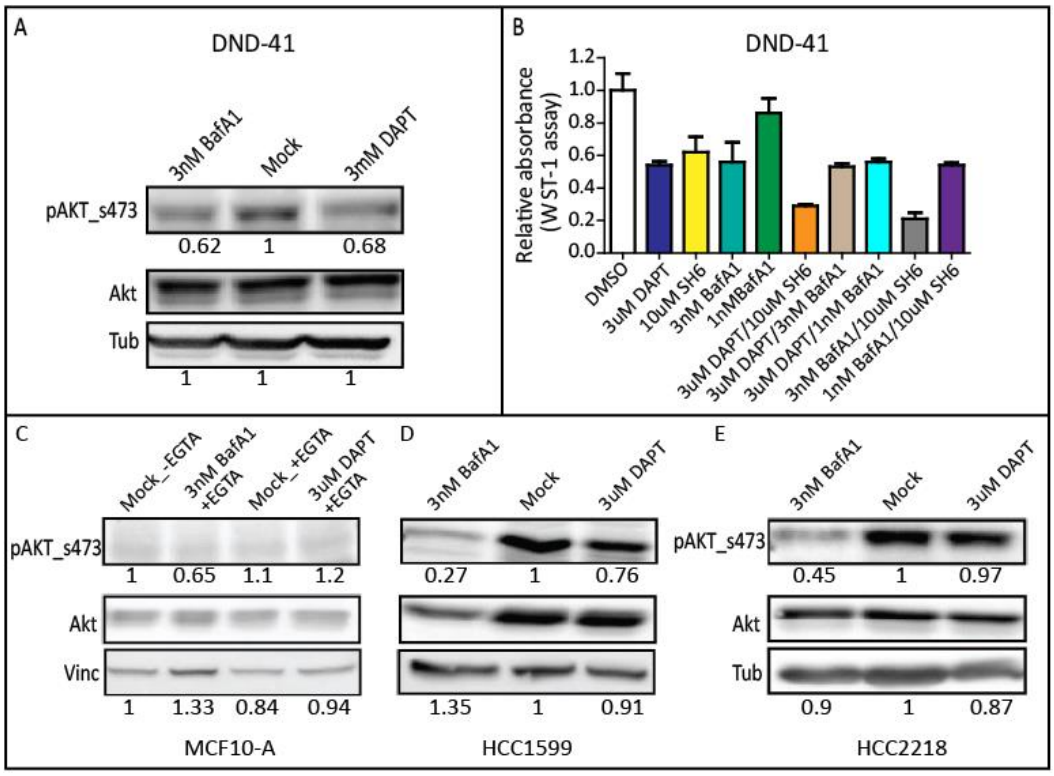


**Figure 25: V-ATPase inhibition reduces Akt signaling in leukemia and breast cancer cells**

(A-E) Cells were cultured for 7 days before western blot and proliferation assay were performed. Relative to Mock treated cells, both γ-secretase and V-ATPase inhibited cells exhibit suppressed levels Akt signaling as seen through a western blot assay for activated Akt (A). Growth of DND-41 cells is sensitive to both DAPT and BafA1 (B). Akt signaling is not altered upon similar treatment of MCF10-A cells, which do not appear to engage in high level Akt signaling (C). Similarly treated Notch driven breast cancer cells display significant reduction in Akt signaling (D-E). This indicates that V-ATPase inhibition might simultaneously suppress both Notch and Akt signaling. Western blot loading differences between the samples were equalized using vinculin or tubulin and the corrected cNICD-1 band intensities normalized to obtain fold cNICD-1 band intensities relative to the Mock treated sample.

## 5.7 V-ATPase inhibition reduces proliferation of GSI-insensitive T-ALL cells independently of Notch signaling

A major hindrance to the use of GSIs against Notch dependent leukemias is the rapid emergence of resistance. In some T-ALLs the resistance has been linked to a reliance on oncogenic Akt signaling on top of the excessive Notch signaling. The T-ALL cell line, CRF-CEMM harbors activating mutations in Notch-1 and possesses constitutive Notch signaling as seen

from elevated expression of Notch targets (Figure 23 B). In addition, these cells have lost PTEN, a negative modulator of Akt signaling, hence they possess excessive Akt activity. It is their use of Akt for secondary oncogenic signaling that makes them resistant to GSIs (Palomero et al. 2007). In a bid to establish whether V-ATPase inhibitions might be of therapeutic value against Notch dependent, GSI resistant T-ALL cells, we treated CCRF-CEM for 7 days and assessed the status of Notch cleavage (Figure 26). Treating CCRF-CEM with DAPT blocks cNICD-1 generation (A) but does not inhibit their proliferation (C), reiterating earlier findings (Palomero et al. 2007). V-ATPase inhibition reduces growth of CCRF-CEM dose dependently, alone or in combination with Akt inhibitors (C). As with DND-41, no reduction of Notch cleavage is observed in BafA1 treated CCRF-CEM when compared to the controls (A). In CCRF-CEM phospho-Akt levels are very high and contrary to what we see in DND-41, neither drug significantly inhibits activation of Akt signaling in relation to the negative control (B).

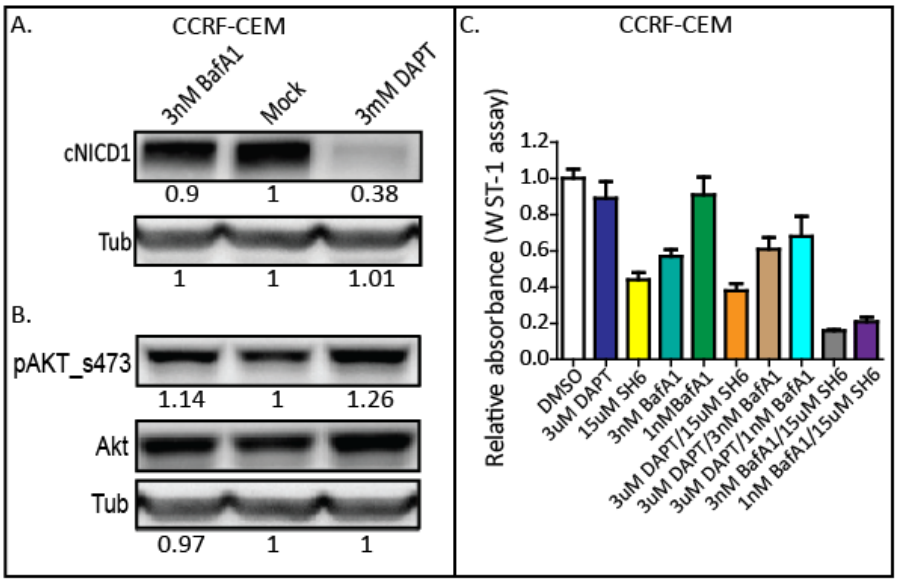


**Figure 26: V-ATPase inhibition reduces the proliferation of CCRF-CEM**

CCRF-CEM is a GSI resistant, Akt addicted T-ALL cell line. (A-C) The cells were treated as indicated for 7 days before performing western blot and proliferation assays. As expected, DAPT blocked cNICD-1 generation but not cell proliferation (A-C). V-ATPase inhibition reduces proliferation but not cNICD-1 production (A-C). Unlike the GSI sensitive DND-41 and breast cancer cells, none of the compounds reduces Akt signaling in CCRF-CEM (B), suggesting that the observed growth reduction upon V-ATPase suppression is due to inhibition of a another pathway(s), outside of Notch and Akt. CCRF-CEM is sensitive to specific Akt inhibition. Note the strongly additive effect of combining low dose BafA1 and SH6 (C). Western blot loading differences between the samples were equalized using tubulin and the corrected cNICD-1 band intensities normalized to obtain fold cNICD-1 band intensities relative to the Mock treated sample.

Our findings suggest that in T-ALL lines displaying ectopic Notch and Akt signaling, V-ATPase inhibition might reduce growth in part by affecting Akt signaling, and in part by affecting additional proliferative signaling pathways, as is the case in CCRF-CEM cells. Since V-ATPase has been reported to regulate Wnt and mTOR signaling (Hermle et al. 2010; Buechling et al. 2010; Cruciat et al. 2010; Zoncu et al. 2011), we examined whether treatment with the drugs affected these two pathways. To test the status of mTOR signaling, we performed a western blot assay on lysates from treated cells and assessed the level of phosphorylated S6K (Figure 27), an established readout of mTOR signaling (Fingar et al. 2002). Despite differences

in levels of phospho-S6K among the T-ALL cell lines, we did not observe major change in its activation upon drug treatments (A-B). This could be due to the fact that we grew the cells in rich media, and suggests that V-ATPase inhibition is unlikely to affect mTOR activation in conditions of high nutrition. To test if at least part of the effects observed following BafA1 treatment might be due to inhibition of Wnt signaling, we finally treated T-ALL cells with ICG-001 and IWR-1, both Wnt signaling inhibitors (Emami et al. 2004; Chen et al. 2009). We observed that at inhibitor doses that do not affect growth of MCF10-A (D), there is a significant reduction in the growth of DND-41 cells but not CCRF-CEM (C). Suggesting that in CCRF-CEM V-ATPase inhibition is likely to affect an additional, yet to be identified growth pathway.

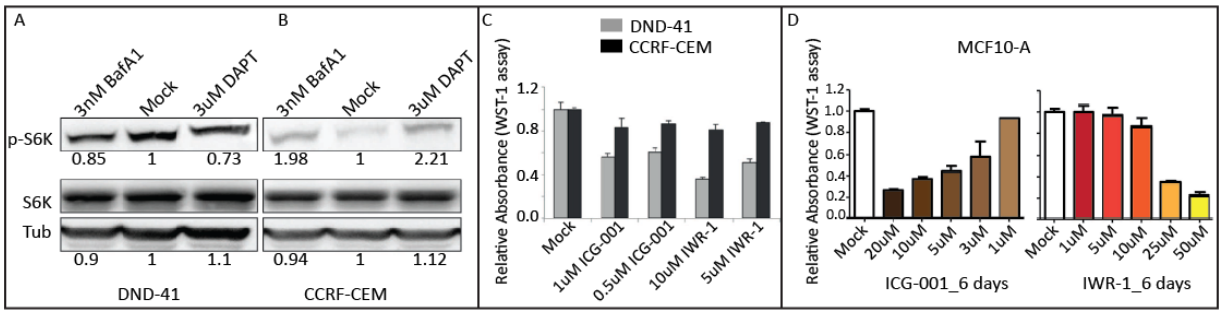


**Figure 27: V-ATPase inhibition does not affect mTOR signaling in T-ALL cell lines**

(A-B) DND-41 and CCRF-CEM were treated for 7 days as indicated before a western blot assessment of the status mTOR signaling. None of the compounds inhibits mTOR in our experimental setup (A-B). (C) The cells were treated with two inhibitors of Wnt signaling at doses that do not affect MCF10-A (D). While proliferation of DND-41 is reduced after 7 days of treatment, that of CCRF-CEM is unaffected (C). Western blot loading differences between the samples were equalized using tubulin and the corrected cNICD-1 band intensities normalized to obtain fold cNICD-1 band intensities relative to the Mock treated sample.

## 5.8 The V-ATPase may regulate Notch signaling by modulating γ-secretase activity

How exactly, the V-ATPase pump and intracellular acidification influence Notch signaling remains mysterious. One possibility is that the V-ATPase, through its $H^+$ pumping role creates and maintains an environment favorable for γ-secretase function. In fact, γ-secretase is membrane embedded and has been shown to require low pH for optimal function (Pasternak et al. 2003). To assess whether the reduced Notch signaling observed after V-ATPase inhibition could be due to reduced γ-secretase activity, we performed a γ-secretase assay *in-vitro*. Such an assay relies on a quenched substrate that fluoresces when processed by γ-secretase (Farmery et al. 2003). The intensity of fluorescence is directly proportional to the extent of cleavage, which can be equated to enzymatic efficiency. MCF10-A cells were treated with BafA1 to inhibit the V-ATPase or as positive control, with DAPT to block γ-secretase function, or finally, with FCCP an ionophore that dissipates lysosomal pH independently of V-ATPase function (Steinberg et al. 2010), (Figure 28). A viability assay on cells treated with the compounds revealed that they tolerate all the compounds (B). A lysotracker analysis of treated cells revealed, as expected, impaired lysotracker incorporation in the presence of BafA1 and FCCP relative to the controls (C). Indicating that both approaches effectively block intracellular acidification. To perform the γ-secretase assay, we lysed the cells and prepared a lysosome enriched fraction. By western blotting for Lamp-1, an established lysosomal marker, we confirmed the presence and enrichment of lysosomes in the fractionated extract used for the γ-secretase assay (D). Compared to the negative control (DMSO), samples treated with DAPT displayed low fluorescence intensity (D), consistent with reduced γ-secretase activity. Relative to the controls, both BafA1 and FCCP reduced γ-secretase efficiency to intermediate levels (D). These observations suggest that the reduced Notch signaling seen on inhibition of the V-

ATPase correlates with reduced γ-secretase activity and that it is due not to V-ATPase inhibition per se, but rather to impaired lysosomal acidification.

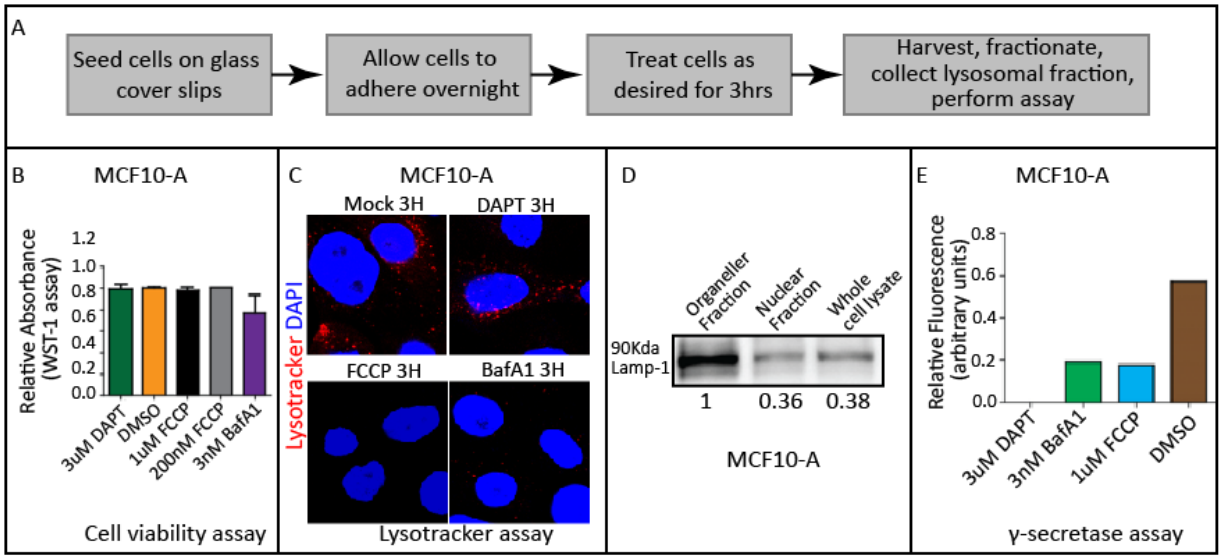


**Figure 28: V-ATPase inhibition might inhibit γ-secretase efficiency**

(A-E) To assess whether V-ATPase inhibition might reduce the efficiency of γ -secretase, an *in vitro* γ-secretase assay was performed on MCF10A (A). MCF10-A cells were treated with the indicated compounds and concentrations for 3 hours and a viability assay performed. None of the drugs were toxic (B). A lysotracker assay performed on similarly treated cells, revealed that unlike the controls, both FCCP and BafA1 impair intracellular acidification. For the γ-secretase assay, the cells were lysed and a lysosome enriched fraction prepared. The presence of lysosomal membranes in this preparation was established by western blotting for LAMP-1 (D). A late endolysosomal fraction was incubated with the γ-secretase fluorogenic substrate. Relative to the negative control and DAPT treated samples, FCCP and BafA1 reduce fluorescence to intermediate levels (E), implying that they reduce the ability of γ-secretase to cleave it substrates. Western blot band intensities were normalized to obtain fold LAMP-1 band intensities relative to the organellar fraction LAMP-1 intensity. Analysis

## 5.9 V-ATPase inhibition may repress Notch receptor expression

In our experiments we have inhibited the V-ATPase pump for varying lengths of time: acutely (3-hour inhibition), semi-chronically (3-day inhibition) and chronically (7-day inhibition). To ensure full inhibition of the pump upon acute treatment, we used 10nM BafA1. For the semi-chronic and chronic treatments we used 3nM BafA1, as this concentration is well

tolerated by the cells over extended periods. To examine whether V-ATPase inhibition affects Notch signaling activation similarly in the three experimental conditions, we stimulated MCF10-A cells that had been treated as described with EGTA. We then performed western blot analysis to measure Notch activation and compared the efficiency of cNICD-1 generation across the treatments (Figure 29). This analysis revealed that in all three conditions of V-ATPase suppression, Notch cleavage was reduced relative to the negative controls, with the strength of Notch cleavage inhibition increasing over time. Stimulation of the cells with EGTA following acute treatment resulted in the generation of about 77% cNICD-1 relative to the amount generated in Mock treated cells (A). Stimulation of Notch cleavage with EGTA following semi-chronic and chronic treatments yielded 56% and 26% cNICD-1 respectively, when compared to cNICD-1 generation in Mock treated cells (B and C). cNICD-1 levels did not change under γ-secretase inhibition as DAPT consistently inhibited Notch-1 cleavage across all treatment conditions. No cNICD-1 generation was observed in non-stimulated cells across all treatment conditions using either compound (A-C).

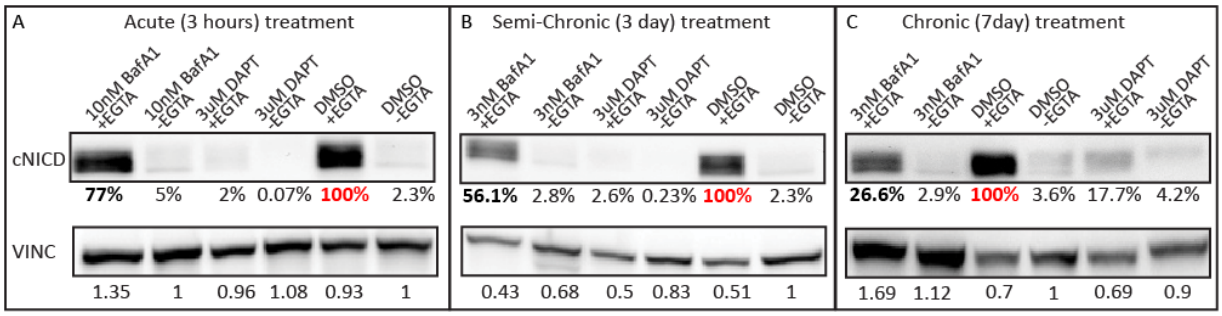


**Figure 29: Suppression of Notch activation increases with length of V-ATPase inhibition**

(A-C) MCF10-A cells were treated as indicated before stimulation (or not) with EGTA and western blot analysis of the extent of Notch cleavage. Loading differences between the samples were normalized using vinculin and the corrected band intensities used to compare the levels of cNICD-1 generated under the three treatment conditions. Following acute inhibition of the V-ATPase and EGTA treatment, the cNICD-1 generated was **77%** of that generated in Mock (**100%**) treated cells – indicating a 23% drop in Notch activation (A). Similar analysis upon semi-chronic and chronic treatment show cNICD-1 generation at **56%** and **26%** relative to Mock (**100%**) treated cells respectively, corresponding with 44 and 74% drops in activated Notch, respectively (B and C). cNICD-1 levels do not change in conditions of γ-secretase inhibition, or in the absence of EGTA (A-C).

These observations raised the question why inhibition of Notch cleavage following suppression of V-ATPase function gets more potent with time. One possibility is that chronic suppression of the V-ATPase affects Notch receptor expression levels. To test whether this is the case, we used qPCR to measure the expression levels of Notch-1, 2 and 3 following V-ATPase inhibition acutely, semi-chronically and chronically (Figure 30). With the exception of Notch-3 which appears more sensitive to V-ATPase inhibition, expression of the receptors is not affected by acute treatment when compared to Mock treated cells. However, Notch receptor expression begins to fall after 72 hours and is lowest after 7 days of treatment.

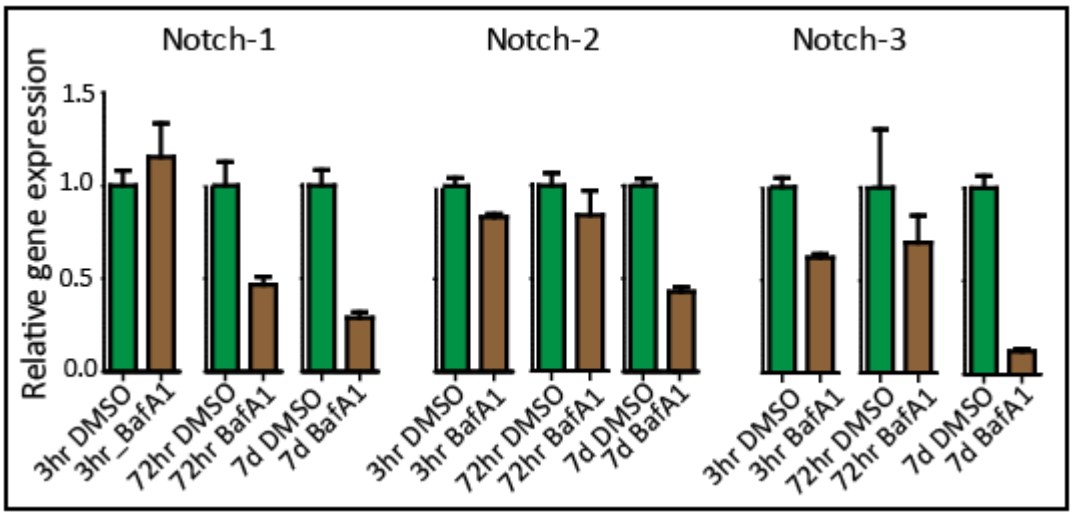


**Figure 30: Chronic inhibition of the V-ATPase reduces Notch receptor expression**
MCF10-A cells were treated with BafA1 acutely, semi-chronically or chronically before qPCR analysis was done for Notch 1, 2 and 3. Except for the Notch-3 receptor, receptor mRNA levels are not changed upon acute treatment. Chronic treatment strongly reduces Notch receptor expression. These observations imply that V-ATPase inhibition may affect Notch signaling in two ways; a) acutely, it may affect Notch cleavage and b) chronically, it may reduce Notch receptor synthesis in addition to impaired Notch cleavage.

In parallel, we analyzed localization of Notch-1 by immunostaining (Figure 31). Notch-1 levels in acutely treated cells are comparable to those of mock treated cells (A-B) although relative to Mock treated cells Notch-1 is slightly accumulated in intracellular vesicles of the treated cells (B). As shown previously, the amount of cNICD-1 in the nucleus is reduced in EGTA-stimulated, acutely treated cells when compared to Mock treated ones (B'). Relative to Mock and acute treatment, semi-chronic treatment results in a strong intracellular accumulation of Notch-1 and a lower amount of Notch-1 at the cell surface membrane (C-D). In contrast to acutely or Mock treated cells, Notch does not translocate into the nucleus upon stimulation of semi-chronically treated cells with EGTA (C'-D'). Similar observations are made upon chronic V-ATPase inhibition (E-F and E'-F').

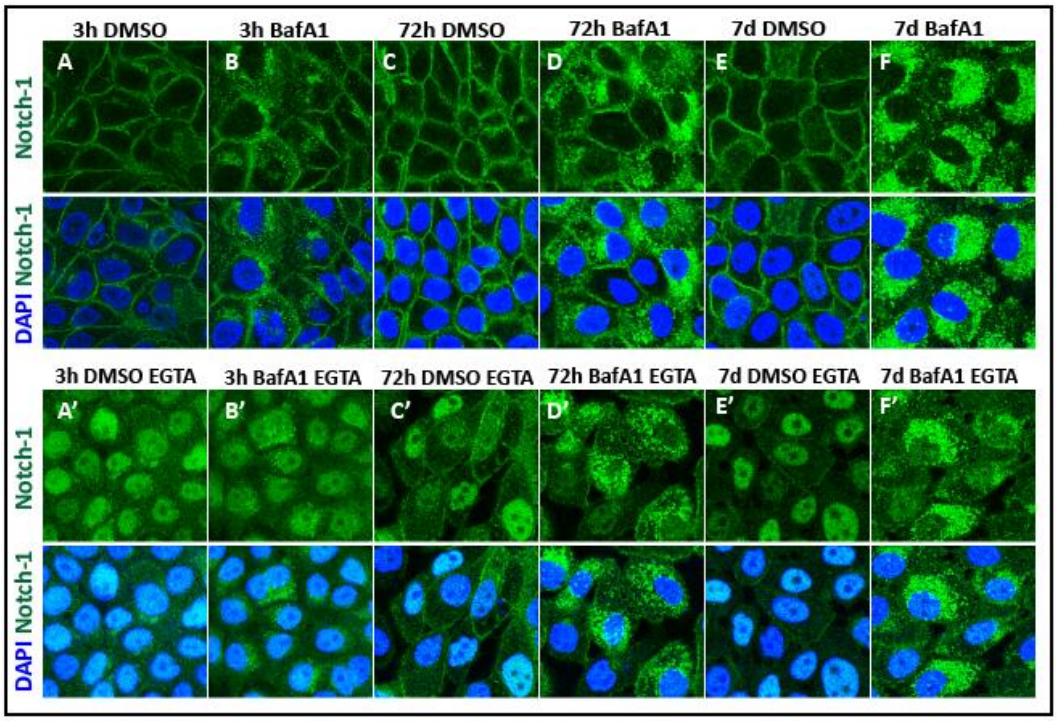


**Figure 31: Extended V-ATPase inhibition appears to reduce Notch-1 levels**

(A-F; A'-F') The cells were treated acutely, semi-chronically or chronically before EGTA stimulation (or not) and staining for Notch-1. On acute treatment (B), Notch-1 levels are similar to those in Mock treated cells (A). EGTA stimulation sends cNICD-1 to the nucleus (A'-B'), but the amount of nuclear Notch is reduced in treated cells. Semi-chronically treated cells (D) strongly accumulate Notch intracellularly relative to control cells (C). EGTA does not induce a strong presence of cNICD-1 in the nucleus when compared to Mock treated cells (C'-D'). Chronically treated cells (F), exhibit high amounts of cytosolic Notch and lesser amounts of Notch when observed against control cells (E). Compared to control cells, these cells have much less nuclear Notch upon EGTA treatment (E'-F').

To establish whether this effect was specifically due to pump inhibition and not compound specific, we used siRNA to chronically knock down the expression of ATP6V0C, the pump's subunit to which BafA1 binds. We observed an even stronger relocalization of Notch intracellularly compared to chronic inhibition of the pump with BafA1 (Figure 32).

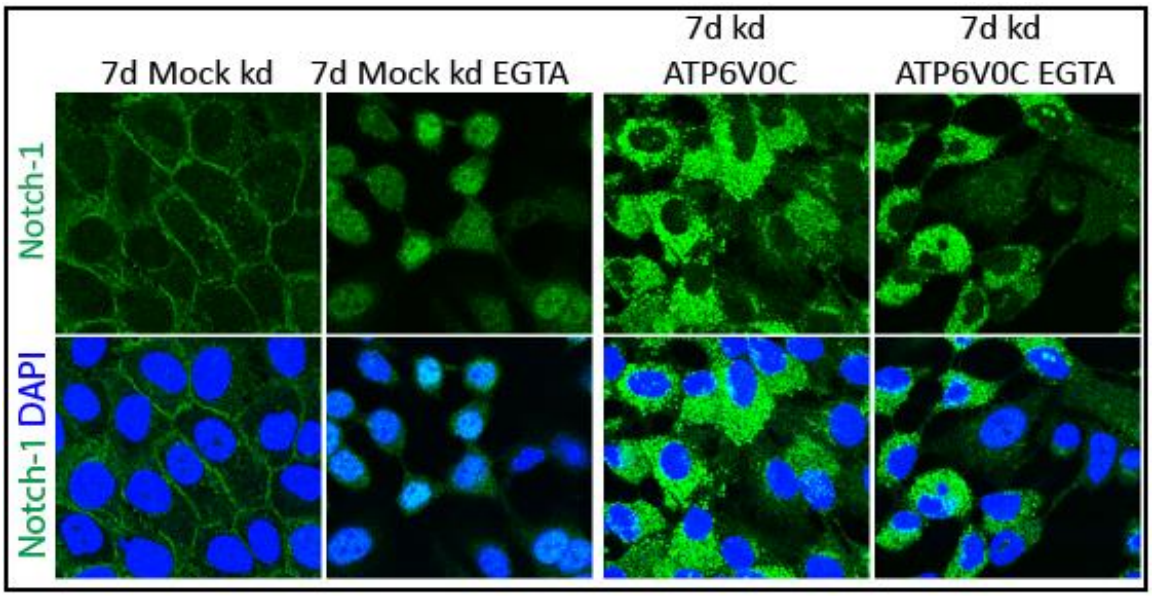


**Figure 32: Chronic Knockdown of V-ATPase genes resembles chronic V-ATPase inhibition**
Knocking down the V-ATPase subunit ATP6V0C, BafA1's target phenocopies chronic inhibition with the compound.

Collectively, these data hint at multiple modes of V-ATPase mediated regulation of Notch signaling. A) Under acute inhibition γ-secretase's efficiency to process its substrates is reduced. B) Under chronic inhibition expression of Notch receptors is reduced. C) Notch1 is relocalized to intracellular compartments (most likely defective endo-lysosomes), and this might affect ligand dependent activation, which is mimicked by EGTA treatment.

## 5.10 V-ATPase inhibition activates TFEB signaling

TFEB has recently emerged as the master regulator of lysosomal biogenesis (Sardiello et al. 2009). Inhibition of lysosomal function causes TFEB dephosphorylation and translocation from the cytosol into the nucleus (Roczniak-Ferguson et al. 2012), where TFEB upregulates lysosomal genes expression, including components of the V-ATPase. We therefore wondered whether in our experimental conditions in which we inhibit V-ATPase, TFEB translocates into the nucleus and whether the endolysosomal system is affected as a result. To answer the first

question, we chronically treated MCF10-A cells with BafA1 and then immunostained them for TFEB (Figure 33). Relative to the negative control, we observed increased presence of TFEB in the nucleus after 7 days of V-ATPase inhibition (A). This observation raised the question of whether the nuclear resident TFEB was transcriptionally active. As TFEB is expected to induce lysosomal biogenesis, we performed a staining for the lysosomal resident protein Lamp-1 to assess whether there was a change in the lysosomal population in chronically treated cells (B). This assay revealed a strong Lamp-1 staining in chronically treated cells when compared to the negative control, indicating a strong expansion of the lysosomal compartment. A similar pattern was observed in cells knocked down for the $V_0C$ subunit of the pump (C) or when treated cells are subjected to lysotracker assay (D-E). These observations are similar to those made upon overexpression of TFEB, which induces lysosomal biogenesis (Sardiello et al. 2009). In all, these data indicate that chronic inhibition of the V-ATPase leads to a striking expansion of the endolysosomal compartment. Since we observe that reduced V-ATPase function leads to nuclear presence of TFEB, this expansion might be due to increased and sustained transcriptional activity by TFEB. A qPCR analysis shows that chronically knocking down the V-ATPase's V1F subunit gene raises the expression of some target genes (F).

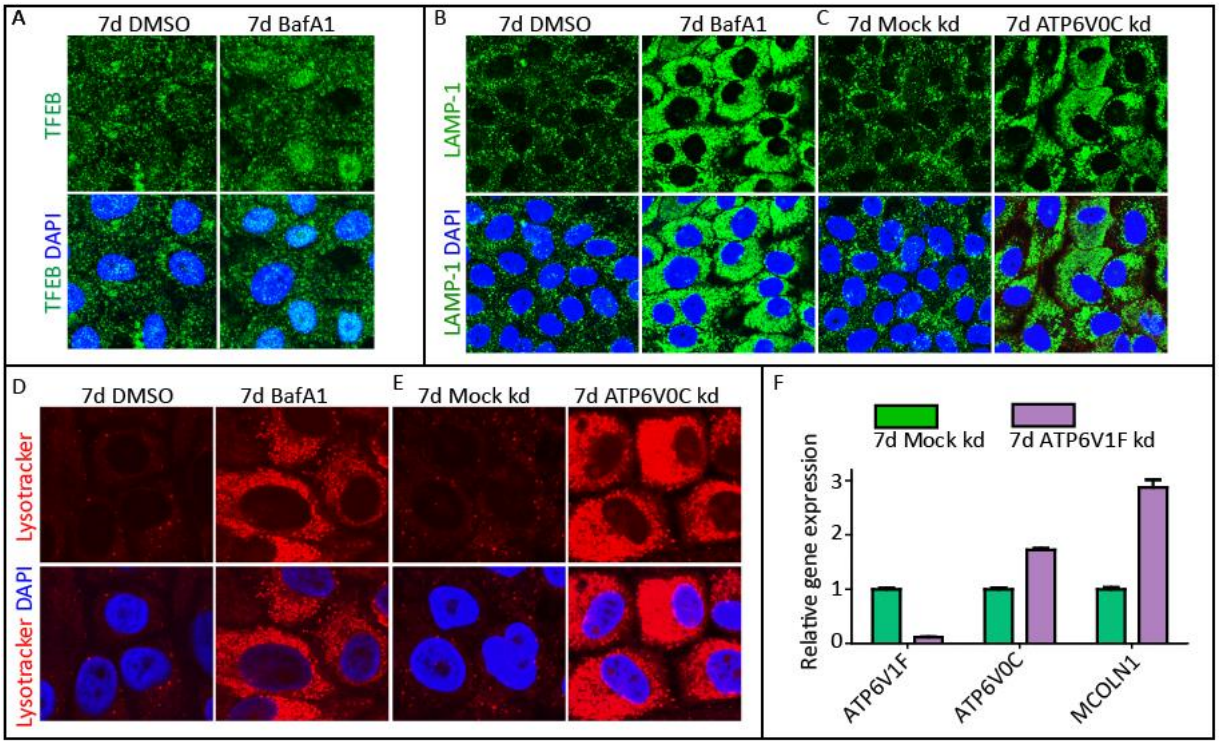


**Figure 33: Chronic V-ATPase inhibition expands the lysosomal compartment**

(A-F) MCF10-A cells were chronically treated as shown or chronically knocked down for the indicated genes. Immunostaining with a TFEB antibody reveals its translocation into the nucleus upon treatment when compared to Mock treated cells (A). A LAMP-1 staining of similarly treated cells shows a strong presence of endolysosomal compartments in V-ATPase inhibited cells as opposed to the control cells (B). This effect is reproduced by chronic knock down of the pump's V0C subunit (C). A lysotracker analysis of cells treated in this way shows a remarkable increment in lysotracker positive compartments in treated cells when compared to untreated ones (D) an outcome that is replicated by chronic knockdown of the V0C subunit of the pump (E). A qPCR analysis of chronically treated cells reveals an upregulation of some TFEB target genes (F).

These results expose a circuit in which 'tuning down' the V-ATPase forces the cells to attempt coping by 'turning up' lysosomal gene expression. The dramatic expansion that we see is explained by the fact that we do not withdraw the drug, essentially keeping lysosome biogenesis 'turned on'. To test whether such an expanded endolysosomal compartment means enhanced lysosomal function, we performed a western blot analysis for Cathepsin D. Mature, 32kDa cathepsin D develops from a 53kDa precursor (pro-cathepsin D) through a process that

requires optimal lysosomal function (Gieselmann et al. 1983; De Luca et al. 2014). Conditions that impair lysosomal activity lead to an accumulation of pro-cathepsin D. When compared to negative controls, maturation of cathepsin D ranges from 'normal' in acutely treated cells, to 'moderately impaired' in semi-chronic conditions, to 'strongly impaired' in chronically treated cells (Figure 34). Thus, despite an apparent expansion of the lysosomal compartment, continued inhibitory pressure on the V-ATPase does not permit for normal lysosomal activity.

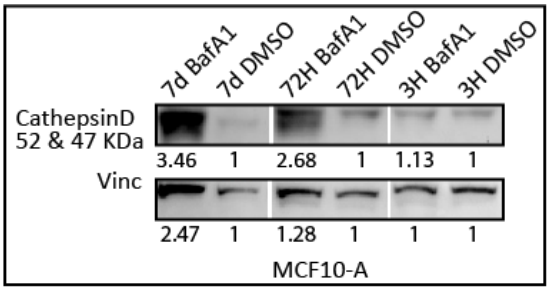


**Figure 34: Chronic suppression of the V-ATPase reduces lysosomal function**
MCF10-A cells were treated with BafA1 acutely, semi-chronically or chronically before western blot analysis for cathepsin D maturation. Relative to the acutely treated cells, semi-chronically and chronically treated cells display accumulation of pro-cathepsin D, indicating reduced lysosomal function. Western blot loading differences between the samples were equalized using vinculin and the corrected cathepsin D band intensities normalized to obtain fold band intensities relative to the mock treated sample.

## 5.11 TFEB may regulate basal Notch signaling in human cells

Finally, we wondered whether a link might exist between TFEB and Notch signaling in human cells. To address this, we knocked down TFEB in MCF10-A cells and performed a qPCR analysis of Hes-1 expression as a measure of Notch signaling. As controls, we knocked down components of the Notch pathway (Figure 35). A qPCR analysis shows that lowering TFEB mRNA, results in reduced expression of the $V_0C$ subunit of the V-ATPase, as would be expected upon reduction of TFEB activity. In this condition, basal Notch signaling, as reported by Hes-1 expression levels, was reduced when compared to the negative control (A). This level of

reduction in Notch signaling is comparable to that obtained by knocking down PSENEN (B) or the Notch-1 receptor (C). Knocking down ADAM-10, which mediates S2 cleavage of the Notch receptor does not affect Notch signaling (D). Since ligand-receptor interaction is an absolute requirement for Notch processing by ADAM-10, this observations suggests that the Notch signaling affected by reducing TFEB, is mostly ligand-independent and is likely to occur along the endolysosomal route.

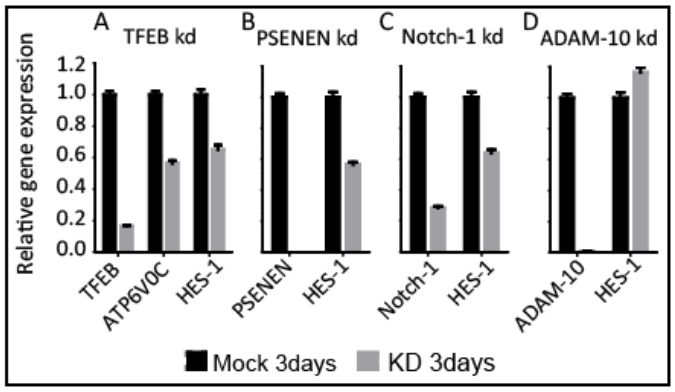


**Figure 35: TFEB knockdown reduces basal Notch signaling**
(A-D) The indicated genes were knocked down in MCF10-A cells for 72 hours before a qPCR assay was done to assess the level of basal Notch signaling activity. Knocking down TFEB results in the downregulation of its target, V0C subunit of the V-ATPase (A) and is accompanied by reduced mRNA levels of the Notch target Hes-1, indicating reduced Notch signaling (A). This reduction in Notch signaling is comparable to that attained by knocking down PSENEN, a component of the γ-secretase complex (B) or the Notch-1 receptor (C). Knocking down ADAM-10, which is needed for ligand triggered Notch cleavage, does not affect basal Notch signaling (D).

Together, these observations indicate that TFEB might support Notch signaling in mammalian cells. Such a hypothesis is within reason if one considers that reducing TFEB levels suppresses V-ATPase function, as expression of V-ATPase subunits is controlled by TFEB. Hence removal of TFEB is analogous to pharmacologic inhibition of the V-ATPase. The mechanistic aspects of how this might occur will be of future interest to the lab.

# 6. Discussion

## 6.1 Involvement of the V-ATPase in Notch signaling

To date, no drug capable of blocking ectopic Notch activation has been deemed safe for clinical applications. This is a major problem considering that the vast majority of T-ALLs possess oncogenic mutations in the Notch-1 receptor (Weng et al. 2004) and that a rising number of solid cancers, including breast, lung, melanoma, medulloblastoma, colon, and ovarian cancers, possess excessive Notch signaling (Roy et al. 2007). Here we find that V-ATPase inhibition using the highly-specific but structurally unrelated inhibitors, BafA1 and Api-A (Bowman & Bowman 2002; Osteresch et al. 2012), or by RNAi against V-ATPase subunit genes, phenocopies the effect of a GSI in curtailing oncogenic Notch signaling.

Using genetic analysis in *Drosophila*, we and others have previously proposed the V-ATPase as a positive modulator of Notch signaling. This proposition is based on observations that mutations which directly or indirectly affect proton pumping, impair Notch signaling in the fly (Vaccari et al. 2010; Yan et al. 2009). Given the evolutionary conservation of both the V-ATPase and the Notch signaling pathway, it is not surprising that crippling the pump's function impairs Notch signaling in mammalian systems as well (Sethi et al. 2010; Lange et al. 2011; Valapala et al. 2013; Kobia et al. 2014). This, coupled with the fact that gain of oncogenic Notch function is a major contributor to the development of multiple cancer types, makes the V-ATPase an attractive alternative target to γ-secretase in countering excessive Notch signaling.

Despite the apparent involvement of the V-ATPase in Notch signaling, the mechanism(s) through which the pump directly or indirectly modulates the pathway have remained elusive. Reports have shown that γ-secretase cleaves its other famous substrate, the

APP (Amyloid Precursor Protein), along the endolysosomal route (Koo & Squazzo 1994; Koo et al. 1996; Yamazaki et al. 1996). This observation implies that in addition to acting at the cell surface, γ-secretase catalytically processes its membrane bound substrates as they are trafficked intracellularly. Further support for this notion is provided by findings that the γ-secretase complex resides on the membranes of intracellular vesicles. It has been shown that intracellular γ-secretase is particularly enriched on lysosomal membranes, and that the low lysosomal pH sustains its function (Pasternak et al. 2003). Indeed, our *in vitro* γ-secretase assay shows that both the specific V-ATPase inhibitor, BafA1 and an ionophore that dissipates lysosomal pH without affecting pump activity, FCCP, inhibit the capacity of γ-secretase to process its substrates. Besides its more established role as an acidifier of intracellular compartments, the V-ATPase also mediates intracellular vesicular trafficking and has recently been proposed to modulate clathrin mediated endocytosis (Hurtado-Lorenzo et al. 2006; Kozik et al. 2013). Therefore by simultaneously regulating both vesicular acidification and trafficking, this versatile pump may conduce toward efficient γ-secretase function in two ways; a) by generating the low luminal pH necessary for its efficient catalytic activity, and b) by ensuring that γ-secretase substrates, including Notch, are spatially accessible to the enzyme. The view that γ-secretase processes its substrates during their trafficking is bolstered by findings that like APP (Koo & Squazzo 1994), *Drosophila* Notch is activated in the endo-lysosome. Such Notch activation is independent of ligand stimulation (Hori et al. 2004; Mukherjee et al. 2005; Moberg et al. 2005; Thompson et al. 2005; Vaccari & Bilder 2005; Vaccari et al. 2008; Wilkin et al. 2008).

## 6.2 Effect of V-ATPase inhibition on physiologic Notch signaling

Building on evidence that impairing the V-ATPase by genetic means diminishes Notch signaling in model organisms (Yan et al. 2009; Vaccari et al. 2010; Lange et al. 2011; Valapala et al. 2013), we sought to establish whether pharmacologically inhibiting the pump would have similar effects. We found that specific V-ATPase inhibitors reduce physiologic Notch signaling in *Drosophila*, Zebrafish and the non-transformed human breast epithelial cell line, MCF10-A. We attribute the reduction in Notch signaling to inhibition of Notch receptor processing by γ-secretase as a result of suppressed V-ATPase activity. This finding is in line with an earlier report that pharmacologic inhibition of the V-ATPase lowers Notch signaling (Sethi et al. 2010). That report however, largely focused on HaCaT, a keratinocyte cell line, and therefore on a tumor suppressive context of Notch signaling (Rangarajan et al. 2001; Nicolas et al. 2003; Dotto 2008). In this study, we have focused on the V-ATPase and Notch signaling in contexts of oncogenic activity. In addition we find that chronic inhibition of the V-ATPase reduces Notch expression levels of Notch receptors. Collectively, our findings point to a dual effect of V-ATPase inhibition on the Notch pathway. A) Upon acute inhibition, it reduces γ-secretase's catalytic efficiency and B), upon chronic inhibition it suppresses expression of Notch receptors.

Since the V-ATPase has been reported to be important for clathrin mediated endocytosis (Kozik et al. 2013), we cannot rule out repressed ligand trafficking as a contributing factor to the reduction in Notch signaling observed upon V-ATPase inhibition. We however contend that the contribution of impaired ligand trafficking to reduced Notch signaling following V-ATPase suppression is minimal. This is because we observe that inhibiting the V-ATPase reduces Notch signaling in numerous instances of ligand independent Notch signaling. Such as in fly wing imaginal discs when endosomal sorting is blocked and ligand independent Notch activation is prominent, or in cases of ligand-independent Notch receptor activating

mutations. In this work, we did not address how V-ATPase inhibition affects Notch ligands, a question that deserves further scrutiny. In fact, the bulk of reports regarding the role of the V-ATPase in Notch signaling have focused on the receptor. Only one previous report suggests an effect of the V-ATPase on Notch ligands. In this, it is reported that pharmacologically inhibiting the V-ATPase in breast cancer cells increases expression of Jagged-1 (Pamarthy & Beaman 2014), lending support to the view that reduced Notch signaling upon V-ATPase inhibition is due to effects on the Notch receptor.

## 6.3 The V-ATPase as a potential therapeutic target in Notch driven malignancies

Majority of the efforts proposed for curtailing oncogenic Notch signaling have relied on GSIs. However, most of the early promise for efficacious GSI-based therapy has been dampened by their high toxicities in patients (Aster & Blacklow 2012; Andersson & Lendahl 2014). The findings that inhibiting the V-ATPase reduces Notch signaling and that it very likely does so by affecting the receptor, opens up the possibility of a therapeutic benefit in Notch dependent cancers. Here we evaluated the capacity of V-ATPase inhibition to counter oncogenic Notch signaling in Notch driven breast cancer cells. V-ATPase inhibition diminishes Notch cleavage as well as Notch signaling and associated proliferation of the GSI sensitive HCC1599 and HCC2218, but not the GSI insensitive HCC1187. Taken together these observations imply that the effect of V-ATPase inhibition on Notch is due to subdued γ-secretase activity. Probably as a result of cell line specific responses, reduced Notch cleavage by the V-ATPase inhibitor is more pronounced in HCC1599 than in HCC2218. This is reflected in proliferation assays, which show a correspondingly stronger growth reduction of HCC1599 than HCC2218. HCC1599 appears to have more cleaved Notch-1 than HCC2218. It therefore is possible that V-ATPase activity is more critically required by HCC1599 to sustain the high levels

of ligand independent Notch-1 cleavage. This could also explain the higher sensitivity by HCC1599 to V-ATPase inhibition.

Unlike in the breast cells, V-ATPase inhibition in the T-ALL cell lines achieved very modest reductions in Notch-1 cleavage. This observation raises a prominent question: how is it that treating Notch signaling reliant breast cancer cells with the V-ATPase inhibitor reduces cleaved Notch-1 levels, while similar treatment of T-ALL cells fails to do so? And yet both types of cells rely on γ-secretase for elevated Notch cleavage? One possibility that requires further examination is that T-ALL cells are less reliant on the endolysosomal system for cleavage of mutant Notch-1. Upon V-ATPase inhibition, we observe reduced proliferation of T-ALL cells to levels almost comparable to those attained with GSI. This might result from modest inhibition of Notch signaling and/or suppression of another pathway(s).

## 6.4 V-ATPase inhibition suppresses multiple cells signaling pathways

In recent years, there have been numerous reports implicating the V-ATPase in various growth and proliferation signaling pathways, including Wnt and mTOR. Both pathways are known to be suppressed by V-ATPase inhibition (Cruciat et al. 2010; Buechling et al. 2010; Zoncu et al. 2011; Roczniak-Ferguson et al. 2012). Indeed, when we treat Zebrafish embryos with BafA1, they exhibit developmental defects similar to those reported in frog embryos lacking Wnt signaling due to V-ATPase inhibition (Cruciat et al. 2010). This implies that in addition to the reduced Notch signaling that we observe on treating the fish with BafA1, we also upset Wnt signaling. This is important when taken in the context of crosstalk between Notch and Wnt signaling in metazoans (Sanders et al. 2009; Collu et al. 2014), especially considering the cooperation between the two pathways in the development of some solid tumors and leukemias (Balint et al. 2005; Fre et al. 2005; van Es et al. 2005; Ayyanan et al.

2006; Staal 2007; Guo et al. 2007). Additional work is necessary to determine whether in our setup, the two pathways are simultaneously blocked upon V-ATPase inhibition. Nonetheless, within the context of crosstalk between Wnt and Notch, our observations suggest that V-ATPase inhibition could be of therapeutic benefit against cancer types that thrive on an active Wnt/Notch axis.

Some T-ALL cell lines, including CCRF-CEM, are resistant to GSIs. This resistance has been ascribed to the cells activating a secondary oncogenic pathway, Akt/mTOR signaling (Palomero et al. 2007). After treating Notch reliant T-ALL cells with the V-ATPase inhibitor, we observed only modest reduction in Notch activation in spite of a significant reduction in cell proliferation, suggesting suppression of additional pathways. An analysis of the status of Akt signaling revealed that V-ATPase inhibition impedes Akt activation in the GSI sensitive DND-41 cells but not in the GSI resistant CCRF-CEM. Akt signaling was also inhibited upon V-ATPase suppression in the Notch dependent breast cancer cells, HCC1599 and HCC2218. This shows that at least in these three cell lines, part of the growth reduction upon V-ATPase inhibition is due to reduced Akt signaling. We postulate that this growth reduction might involve a factor(s) downstream of Akt, such as FOXO; a pro-apoptotic transcription factor that is active in conditions of inhibited Akt signaling (Zhang et al. 2011). This observation proposes V-ATPase inhibition as a means of countering excessive Akt signaling in cancers. The finding that Akt signaling is not hampered in similarly treated CCRF-CEM, suggests the involvement of another/other signaling pathways. However, we could not observe any change in mTOR signaling. Since proliferation of CCRF-CEM is unaffected by a Wnt inhibitor, it is likely that yet another pathway(s) that it relies upon for proliferation is inhibited by V-ATPase inhibition. In fact both BafA1 and an Akt inhibitor reduce proliferation of CCRF-CEM by approximately 50%. Combining low doses of the two compounds results in a remarkably additive reduction in

proliferation of these cells. The nature of this mutual potentiation by the two inhibitors requires a more focused examination and could help elucidate the pathway responsible for the reduction in CCRF-CEM's proliferation in the presence of BafA1. The identification of such a pathway would potentially unveil a new therapeutic avenue against Notch dependent, GSI resistant T-ALLs and possibly elucidate a novel avenue for the emergence GSI resistance in T-ALLs.

## 6.5 The V-ATPase as a suitable therapeutic target in cancer

Surprisingly, we find that very low dose BafA1 (3nM or less) is well tolerated by cells in culture over extended periods and curtails oncogenic signaling through Notch, Akt and potentially other signaling pathways yet to be identified. Raising organellar pH will inevitably have wide ranging pleiotropic effects, including impairment of lysosomal function and associated compensatory signaling like activation of TFEB. Still, lysosomotropic drugs like chloroquine, which alkalinize acidic environments are well tolerated by patients and have been extensively prescribed as antimalarial agents. Commonly used over the counter proton pump inhibitors like omeprazole, which is used to control gastric acid have also been shown to inhibit V-ATPase function (Luciani et al. 2004). Such medicines have recently been gaining traction as potential anti-cancer agents (Luciani et al. 2004; Fan et al. 2006; Solomon & Lee 2009; Sasaki et al. 2010; Kimura et al. 2013; Choi et al. 2014). This interest has been spawned by the realization that tumors, particularly solid ones, are often faced with an 'acid challenge'. This low pH problem comes from the fact that the tumor environment is poorly vascularized and hypoxic, forcing tumor cells into anaerobic glycolysis and resulting in acid build-up. These cancer cells are thought to escape the harmful effects of low intracellular pH by increasing their rate of extruding protons into the extracellular space. This eventually reverses the tumor

cells pH gradient, making the extracellular space more acidic. These events have been associated with numerous cancer promoting processes including metastasis, increased proliferation, evasion of apoptosis and multi-drug resistance (Webb et al. 2011; Kato et al. 2013; Daniel et al. 2013). As a result, pharmacologically modulating the regulators of intracellular pH, including the V-ATPase, has emerged as an appealing therapeutic prospect for cancer treatment (McCarty & Whitaker 2010). Our data suggest that reduction of oncogenic cell signaling might be an additional benefit to considering V-ATPase inhibition as an anticancer intervention.

Both chloroquine and BafA1 are modulators of intracellular pH, albeit through different mechanisms. Both appear to possess anticancer properties which have been attributed to induction of apoptosis (Ohta et al. 1998; Nakashima 2003; Fan et al. 2006; Maclean et al. 2008; Wu et al. 2009; Sasaki et al. 2010). We posit that by affecting intracellular pH, their anticancer properties could be through inhibition of one or several of the signaling pathways that rely on efficient V-ATPase/lysosomal function. These may include Notch, Akt/mTOR, Wnt signaling or even AMPK signaling (Cruciat et al. 2010; Zoncu et al. 2011; Kobia et al. 2014; Zhang et al. 2014). The pathway(s) specifically affected might depend on the cancer type and how critically it relies on that particular pathway(s) for growth and proliferation.

To minimize the adverse effects of drugs, clinical oncology is moving towards development of low-dose, multi-drug regimens (Real et al. 2009). We observe that compared to the effect of using any of the compounds separately, combining low doses of BafA1 with low doses of GSI allows significant growth inhibition in an additive manner. We therefore envisage that V-ATPase inhibitors could be combined with GSIs and/or other specific Notch inhibitors to curtail GSI toxicity while preserving or even enhancing efficacy. Because Notch functions along with other signaling pathways, a persistent pharmacologic inhibition of Notch

might induce development of resistance and addiction of the tumor cells to the other unsuppressed growth pathways (Palomero et al. 2007). Thus, multi-drug regimens are being proposed for inhibition of multiple pathways (Shepherd et al. 2013). Pharmacologic V-ATPase inhibition allows simultaneous reduction of Notch, Akt, maybe Wnt and at least another unidentified pathway. As more signaling pathways that converge at the V-ATPase/lysosomal signaling hub are identified, the profile of the V-ATPase as a possible therapeutic target rises. This is because inhibiting the V-ATPase using a single compound, can inhibit multiple pathways, mimicking combination therapy. Thus V-ATPase inhibitors are promising prospects for use alone or in combination with existing inhibitors of other pathways.

## 6.6 Implications of chronic V-ATPase inhibition

Curing cell signaling dysfunction in cancer will require a deep understanding of how signals are controlled, integrated and made to interact with each other. For instance, we note that chronically suppressing V-ATPase function with low amounts of inhibitor reduces the expression of Notch receptors. This hints at a direct or indirect function of the V-ATPase at the transcriptional level. Such a function, though initially unexpected, is not entirely surprising considering that the signaling pathways influenced by V-ATPase have wide ranging transcriptional outputs. For example TFEB, the only transcriptional factor so far known to respond to V-ATPase manipulation, is an indirect target of V-ATPase inhibitors and becomes transcriptionally active upon V-ATPase suppression, leading to the expression of large group of genes (Sardiello et al. 2009; Roczniak-Ferguson et al. 2012). This paints a complex picture as such changes in gene expression will undoubtedly affect the output of multiple signaling pathways. In our conditions of chronic V-ATPase inhibition, we observe that some signaling pathways including Notch are inhibited. Also, the expanded endolysosomal system observed

upon chronic inhibition of the pump functions sub-optimally, and yet the cells are viable. This raises the question of how the cells adjust to such protracted pressure and whether there are signaling pathways that get upregulated in such settings.

## 6.7 Potential of chronic V-ATPase inhibition in studying LSDs

The fact that we can inhibit the V-ATPase with low nM doses of inhibitor without killing the cells, suggests that this approach can be used to mimic and study lysosomal storage disorders (LSDs) *in vitro*. LSDs are a group of disorders originating from mutations that impair lysosomal functions and are characterized by accumulation of undegraded cellular material (Ballabio & Gieselmann 2009). Although chronic V-ATPase inhibition would not model specific LSDs, it would help address some pending but important general questions surrounding LSDs. For instance how cell signaling is altered in the event of impaired clearance of unnecessary receptors and ligands. Impaired lysosomal clearance potentially decreases lysosomal function. This system can thus serve as a model to study how LSD affected cells cope with sub-par lysosomal function. Finally, experimental approaches that restore lysosomal function in conditions of chronic V-ATPase inhibition might offer clues into ways of intervening in LSDs. This could include interventions to upregulate expression or activity of TFEB. One of the ways to achieve this is protracted treatment with very low doses of BafA1, which stimulates TFEB activity and lysosomal biogenesis. To minimize the reduction of lysosomal activity associated with such treatment, treatment could be pulsated, or V-ATPase inhibitor dosage could be further reduced. The endolysosomal system is emerging as a major signaling control and coordination hub for multiple cancer-relevant signaling pathways that rely on membrane-associated factors. This work represents a step in the direction of effective pharmacological modulation of endocytic events to counter oncogenic cell growth.

# 7. Appendix: High Content Screen for Novel Notch signaling components

## 7.1 Introduction

The Notch signaling pathway is extremely complex. Yet it possesses a rather small core signaling unit that consists of Notch receptors, Notch ligands, ADAM metalloproteases, the γ-secretase complex and the CSL transcriptional complex (Bray 2006; Andersson et al. 2011). Of these factors, the ligands, ADAMs and γ-secretase are required for receptor activation, leaving the intracellular portion of the receptor and the CSL complex, as the direct effectors of signaling. Given how widely Notch signaling is used throughout the life of an individual and how diverse its effects are in different tissues and organs (Wilson & Radtke 2006), one outstanding question is how is such complexity is attained.

Part of the pathway's complexity can be explained by posttranslational modifications of the main components of the pathway. Glycosylation of Notch receptors and ligands is thought to modulate the strength of their interactions (Stanley & Okajima 2010). Phosphorylation of the nuclear portion of the receptor is known to promote or inhibit Notch signaling depending on the Notch receptor targeted and/or kinase employed (Andersson et al. 2011). Acetylation of the nuclear portion of Notch-1 protects it from ubiquitination and subsequent degradation, an event countered by deacetylation. Hence acetylation/deacetylation events modulate the strength and duration of Notch signaling (Kitajewski 2011; Guarani et al. 2011). The best characterized modulators of the strength and duration of Notch signaling are members of the ubiquitin system. As discussed in section 2.6.1, ubiquitination limits NICD's half-life in the nucleus (Oberg et al. 2001) and as discussed in section 2.2, it modulates productive Notch signaling by controlling trafficking of both ligands and receptors.

A peculiar characteristic of Notch signaling, is its strong sensitivity to gene dosage. Whether incremental or decremental, slight variations in Notch signaling are sufficient to produce loss or gain of Notch function phenotypes (Andersson et al. 2011; Guruharsha et al. 2012). As implied by modulators of intracellular trafficking (Le Borgne, Bardin, et al. 2005; Vaccari et al. 2008; Fortini & Bilder 2010), the remarkable complexity of this pathway might rely on a multitude of factors that depending on the context affect the strength and/or duration of signaling or even whether signaling occurs at all. The probable existence of such unidentified factors implies that numerous compartment-specific points might control Notch signaling and possibly, they could be pharmacologically modulated in pathological settings.

Aiming to identify novel modulators of Notch signaling we performed a high content siRNA screen in collaboration with Dr. Mark Wade's iit@SEMM (Italian Institute of Technology at the European School of Molecular Medicine) screening unit.

# 8. Specific aims of the screen

The general aim of this HCS project is to uncover novel human genes regulating Notch signaling. Specifically, we aim to identify factors that might influence Notch signaling by affecting:

1. Notch trafficking and subcellular localization
2. Notch receptor stability/degradation
3. Notch receptor synthesis
4. Notch receptor cleavage/processing
5. Notch signaling transcriptional output

Since this is a subgenomic, medium-throughput and high content screen, aims 1-4 have been addressed simultaneously in the primary screen data analysis. Candidates selected as possible modulators of aims 1-4 will be subjected to a primary validation screen. Aim 5 will be pursued in a secondary validation that will focus on the factors that pass initial validation. The implementation of these aims will enable us to select leading candidates that we will study further to:

6. Characterize the mechanism through which they affect Notch signaling in mammalian cells and if applicable, *in vivo* using *Drosophila* as a model system.
7. Assess their potential as therapeutic targets in Notch driven cancers as the gene libraries screened belong to the human druggable genome.

# 9. Materials and methods

## 9.1 Screen setup

The Notch translocation assay on MCF10-A cells was performed as described in section 4.7. To inhibit Notch activation upon EGTA addition, the cells were treated with 3uM DAPT (Sigma) or as negative control, with DMSO for 3hrs. Immunostaining with the Notch-1 antibody and image acquisition was done as described in section 4.8. Reverse transfection to knockdown components of the γ-secretase complex was done as outlined in section 4.12. esiRNA targeting PSENEN (EHU220611), PSEN-1 (EHU073361), PSEN-2 (EHU070541), APH1A (EHU059921) and firefly luciferase (EHUFLUC) were purchased from sigma. PSENEN (D-008057-01-0010) siRNA was purchased from GE Dharmacon.

## 9.2 Pilot screen

Before embarking on the primary screen, we first performed a pilot screen. For the pilot screen, we knocked down genes belonging to two custom sub-library plates: HUSIGENSUB07 and HUSIGENSUB08. Together, the two sub-genomic libraries contained siGenome smartpools targeting 560 genes. Genes represented in the two libraries were knocked down in 384-well format along with the following controls: a transfection positive control siRNA for monitoring transfection efficiency (PLK-1 SmartPool siGenome), an assay specific positive control siRNA (PSENEN SmartPool siGenome), siRNA targeting Renilla Luciferase (RLUC siGenome siRNA) as negative control and as a second negative control, non-targeting OTP (On Target Plus). These control siRNA were purchased from GE Dharmacon. This execution of the pilot screen was essential to identify the pitfalls in our procedures and to modify the pipeline to be used in the primary screen. For instance, in the pilot's image acquisition stage, we acquired 6 fields of

view per well using a 10X objective. Based on the quality of images, this was changed to 8 fields of view per well, using a 20X objective. In the pilot screen, we placed more emphasis on the EGTA stimulated condition. In the analysis of the pilot screen it also became apparent that the dynamic range of the signal is larger for non-stimulated condition, so we decided to eventually consider a much lower number of hits from the EGTA stimulated plate. Finally, in the pilot phase we did not have a marker for the cell surface membrane and this fact prevented optimal automatic segmentation of the images. Thus, the protocol was revised to include phalloidin staining in the primary screen phase. These modifications necessitated revision of the image data analysis pipeline. To avoid repetition, only the protocols used in the primary screen are described here as they are modifications of the ones applied in the pilot screen.

## 9.3 siRNA knock-downs

In the primary screen 2,749 genes belonging to 10 sub-genomic library plates were assayed. The gene knockdowns were performed in 384-well plate format, with each well containing a pool of four distinct siRNA oligos against different sequences of the same target transcript. Each library plate was knocked down in 6 replicates. 3 of the replicates were subjected to EGTA stimulation while the other 3 were left unstimulated. Cell culture medium and conditions for growing MCF10-A cells were same as described in section 4.4. For siRNA transfection, cells were always maintained at less than 6 passages and trypsinized for transfection at 60-80% confluence. All siRNA transfections were done in black 384-well, tissue culture treated optical plates (Corning), using the reverse method. Briefly, 25nM of the desired siRNA diluted in 20ul of Optimem (Invitrogen) was allowed to complex with 0.12uL of RNAiMax (Invitrogen), for 20 minutes at room temperature. Where indicated esiRNA was used at a concentration of 50nM. 700 cells suspended in 20uL of 2X medium were added to each well.

2X medium was prepared by using each component of the medium at 2X the concentration indicated in section 4.4. 2X the concentration of EGF shown in section 4.4 was freshly added into the cells before they were added into the wells. The cells were then cultured for 72 hours under the conditions described in section 4.4, in a SteriStore automated incubator (HighRes Biosolutions). All solutions were dispensed using the Freedom EVO automated liquid handler system (Tecan).

## 9.4 Stimulation of Notch translocation

At the end of the 72 hours of gene knockdown, 3 plates from each library were treated with EGTA for 2 hours to stimulate Notch cleavage. To do this, 10uL of fresh medium containing 12.5mM EGTA was directly added into the wells to have a final concentration of 2.5mM EGTA per/well, and a final volume of 50uL/well. 10uL of sterile water was added into the NoEGTA stimulated plates. The plates were then incubated for 2hrs at 37°C and then fixed with 2% PFA for 15 minutes at room temperature. Fixation was done by adding 50uL of 4% PFA directly into the wells. All solutions were dispensed into the wells using a Thermo Scientific Multidrop dispenser.

## 9.5 Immunofluorescence

Automated immunostaining was performed using a Biotek EL406 Washer/Dispenser equipped with a 192-tube aspiration manifold. The solution was removed from the wells and the cells rinsed once with 1X PBS. Cells were permeabilized with 0.05% triton in 1% BSA blocking solution for 1 hour at RT followed by a single wash with 1X PBS. Cells were then incubated for 1 hour at room temperature with anti-Notch-1 primary antibody (Sigma), diluted at 1:350 in 1% BSA blocking solution. The primary antibody solution was removed and the

plates washed twice with 1X PBS. Next, the cells were incubated for 1 hour with 1% BSA blocking solution containing DAPI (SIGMA) at 1:4,500, phalloidin at 1:350 and Alexa Fluor 488 anti-rat secondary antibody (Life Technologies) at 1:400. They were then washed thrice with 1X PBS before imaging. When imaging could not be performed immediately, the cells were kept at 4°C for not longer than 48 hours prior to image acquisition.

## 9.6 Image acquisition

For image acquisition, the 384-well plates were scanned using an automated Olympus Scan^R (Tokyo, Japan) microscope equipped with a Hamilton arm for plate handling. 8 fields of view (FOV) and three emission fluorescent channels (for DAPI, phalloidin and Alexa 488), were acquired for each well using a 20X objective. The image data was annotated and transferred to the Isilon infrastructure and network software, where it was indexed by plate barcodes for image data storage. In the annotation each well was assigned information regarding the date of the experiment, gene knocked down, the sub-genomic library it belongs to and the treatment (EGTA vs NoEGTA). Upon annotation, the images were uploaded to the Columbus server (PerkinElmer) from where they can be accessed for visual inspection and automated analysis.

## 9.7 Image Analysis

Annotated images were used for automated image analysis. For the analysis, an in-house Acapella (PerkinElmer) image analysis script was developed and used to batch analyze the images and to quantitatively describe a set of phenotypic features. All analyzed images were first subjected to background correction and exclusion of uneven illumination. The background correction was performed for each channel separately. DAPI, which was used to

mask the nuclei and phalloidin, which labeled cell surfaces were used for cell segmentation. Segmentation was performed using a modified version of the watershed algorithm. This modification allowed the inversion of phalloidin channel images so as to display high pixel intensities in the cell and low intensities along the cell membrane; allowing application of the watershed approach to identify cell boundaries. This algorithm also detects and excludes regions of the image fields not occupied by cells. Once the cells, their membranes and corresponding nuclei were detected, each cell was segmented into the nucleus based on DAPI staining, the membrane based on phalloidin and the cytosol (region between the nuclei and cell surface membrane).

Depending on the gene targeted, RNAi may alter cellular morphology and as a result, cell segmentation is not always accurate. To ensure that only properly segmented cells are analyzed, a series of restrictions were imposed: a) Cells on the image borders were excluded as they are incomplete, b) Size restrictions were imposed so as to define the maximum and minimum areas (in pixels) in order to exclude objects segmented as cells, nuclei or cytosplasmic regions but were either too large or too small, c) objects with saturated intensities for Notch-1 and DAPI staining were excluded, and d) poorly segmented cells were excluded from the analysis by use of the cellular compactness parameter that assesses cell shape.

The occurrence of out-of-focus images is unavoidable in HCS campaigns due to the automated mode of image acquisition. The position of the objective during imaging is optimized for plate type (supplier) and batch. To exclude out of focus images from the analysis, we used boxplot statistics on the distribution of intensity contrast values (on the DAPI channel) of all nuclei detected on the entire well. Using the first and the third quantiles of this distribution, we estimated the Lower Inferior Fence (LIF) using the 95% confidence interval. We then established for each field of view, the number of nuclei presenting a contrast lower

than the LIF. Fields were considered out of focus if more than 50% of the nuclei failed to cross the LIF threshold.

Analysis of the EGTA stimulated plates, was performed independently of the corresponding non-stimulated plates. The quantification of each parameter, in each well (gene knockdown) was reported as a z-score value. For instance, Notch pixel intensities in a particular compartment were obtained for all cells in the wells, including the control wells. Averaging the intensities for the cells analyzed per well, gave single values descriptive of the effect of a gene's knockdown on the parameter. These values (for each parameter) were used to calculate the z-scores, which are statistical measures of how many standard deviations each quantification value falls relative to the population mean for the respective parameter (Birmingham et al. 2009). In calculating the z-scores, it is assumed that majority of the genes knocked down will have no effect on the parameter under interrogation and will have a z-score close to or equal to 0. Factors with positive scores enhance the parameter and those with negative ones inhibit it. Since each gene was knocked down in triplicate (3 plates), 3 z-scores were obtained for each parameter. The median z-score was taken as the effect of a gene's knockdown on the parameter in question. In cases where data from one of the wells was unavailable e.g. out of focus wells, the average z-score between the remaining two was taken. If images from more than one of the 3 wells were unusable, it (the well, candidate) was excluded from the analysis.

## 9.8 Candidate selection for validation

For each library plate, candidates from EGTA and NoEGTA conditions were considered to affect respective parameters if upon knockdown, they shift the z-score positively or negatively. The bigger the shift, the stronger the phenotype. The main parameters quantified were: a) cell viability (QC1_NoOfAnalysedCells), b) Overall Notch intensity in the cell

(N4_CellNotch), c) nuclear Notch intensity (N1_NucNotch), d) cell surface membrane Notch intensity (N3_MembNotch) and, e) Notch accumulation in intracellular compartments (N5_PercentOfCellsWithSpots & N9_NoOfSpotsPerCell). To select candidates for subsequent validation, we applied filters on the z-scores and grouped the candidates into separate categories on the basis of whether, and how they affected the parameters, while taking into account the negative controls. A primary filter was applied on the parameter QC1_NoOfAnalysedCells in order to exclude candidates that had too few cells. A secondary filter on the parameter N4_CellNotch separated candidates with very low levels of Notch. An upper filter on N4_CellNotch identified candidates whose knock down increased overall Notch levels. A series of filters were sequentially imposed on the remaining candidates in order to group them by how they affected the remaining main parameters. The threshold at which to establish filters for respective parameters was determined by the following criteria:

a) Average z-score was determined for respective parameters in the negative controls.
b) Average z-score was determined for respective parameters in the samples (excluding the controls).
c) Phenotypes of candidates with average z-scores for respective parameters (b) were visually compared with the phenotypes for the same parameters in negative controls (a).
d) Depending on whether it was enhancers or suppressors of a respective parameter being sought, phenotypes of candidates with lower or higher than average z-scores for respective parameters were compared with phenotype for the same parameter in negative controls.

For instance, the negative controls have an average z-score of +2.4 for the parameter QC1_NoOfAnalyzedCells and the samples an average of z-score of 0. By visually verifying the

images, samples with a z-score of 0 have much fewer cells relative to negative controls. Samples with z-scores < -0.5 have strongly reduced cell numbers relative to the negative controls, hence were considered to be too few for further analysis.

# 10. Results

All primary screening experiments were performed in collaboration with Mark Wade's IIT@SEMM screening unit. Cell culture and siRNA transfections were performed by **Michela Mattioli**. Immunofluorescence and imaging were performed by **Fernanda Ricci**. Image and statistical analysis were performed by **Adrian Andronache**. Candidate sorting and selection were performed with the help of **Victor Alfred**, a PhD student in our group.

## 10.1 Rationale for the screen

To identify novel factors that modulate the Notch signaling pathway, we performed a HCS in MCF10-A cells. From previous work we knew that these cells express Notch signaling pathway components including the Notch-1 receptor and that they are amenable to induction of Notch receptor cleavage through $Ca^{2+}$ chelation (Figure 36) (Rand et al. 2000; Kobia et al. 2014). EGTA induced translocation of cNICD-1 into the nucleus is verifiable by staining the cells with an antibody that recognizes both the full-length and cleaved forms of Notch-1. This antibody reveals the presence of Notch-1 at the cell surface membrane of the cells (B) and that it relocalizes to the nucleus upon addition of EGTA (C). Notch cleavage and nuclear translocation is blocked by treating the cells with the γ-secretase inhibitor, DAPT (D-E) or the ADAM-10 metalloprotease inhibitor, GI 254023X (F-G) indicating that EGTA stimulation mimics ligand mediated Notch cleavage. Treatment of the cells with BafA1 causes Notch-1 to accumulate in intracellularly (H-I).

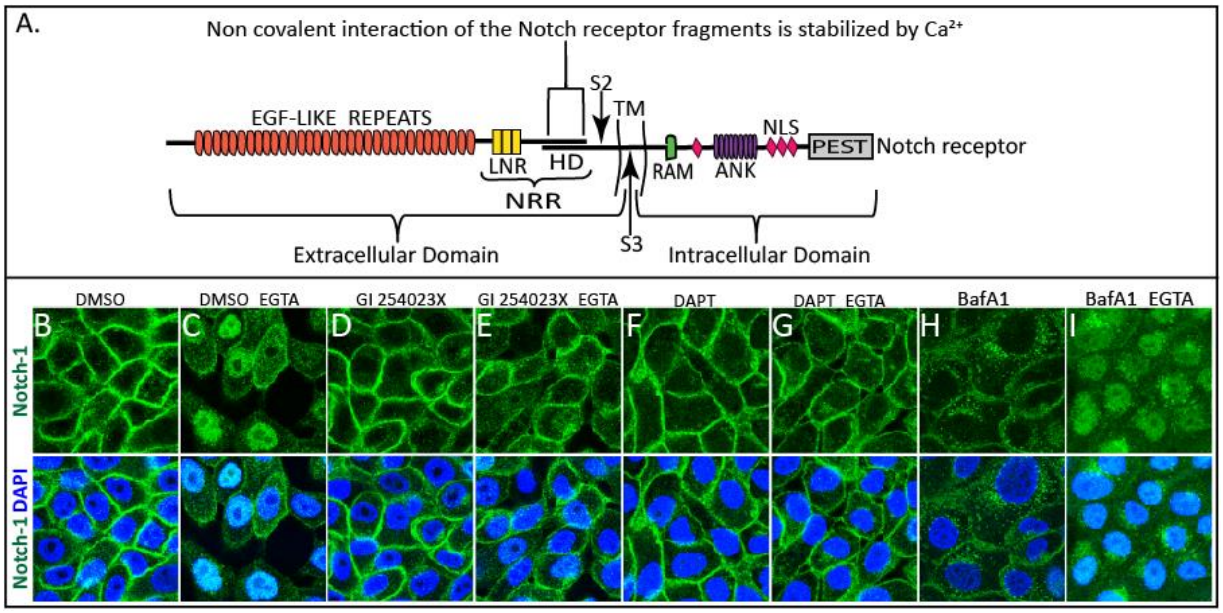


**Figure 36: Basis of the HCS in MCF10-A cells**

The intracellular and extracellular portions of Notch receptors are non-covalently glued together by $Ca^{2+}$, whose depletion causes ADAM-10 and γ-secretase to sequentially cleave the receptor at S2 and S3 respectively (A). The intracellular localization of Notch-1 can be monitored using an antibody that recognizes both the cleaved and the whole form of the receptor (B-I). In MCF10-A cells, Notch-1 resides on the cell surface membrane (B) but relocates to the nucleus upon stimulation with EGTA (C). Treating the cells with GI 254023X (D), prevents cleavage and relocation of cNICD-1 into the nucleus upon EGTA addition (E); as does treatment with DAPT (F-G). Inhibition of the V-ATPase pump causes Notch to accumulate in intracellular vesicles (H-I). These experiments indicate that we can mimic physiologic, ligand induced Notch activation and that we can utilize antibody staining to track the receptor intracellularly.

## 10.2 Positive controls for inhibition of Notch cleavage

With the aim of establishing a positive control for impaired Notch cleavage, we knocked down PSENEN (Figure 37), a component of the γ-secretase complex. A qPCR assay of the level of PSENEN expression after 72 hours of knockdown reveals a strong reduction of its mRNA levels (A). At the end of 72 hours, Notch cleavage was stimulated with 10mM EGTA for 30 minutes, followed by immunostaining for Notch-1 and confocal imaging. Relative to the mock knockdown cells (B-C), cells knocked down for PSENEN displayed an intermediate reduction in Notch cleavage and cNICD-1 presence in the nucleus (D-E).

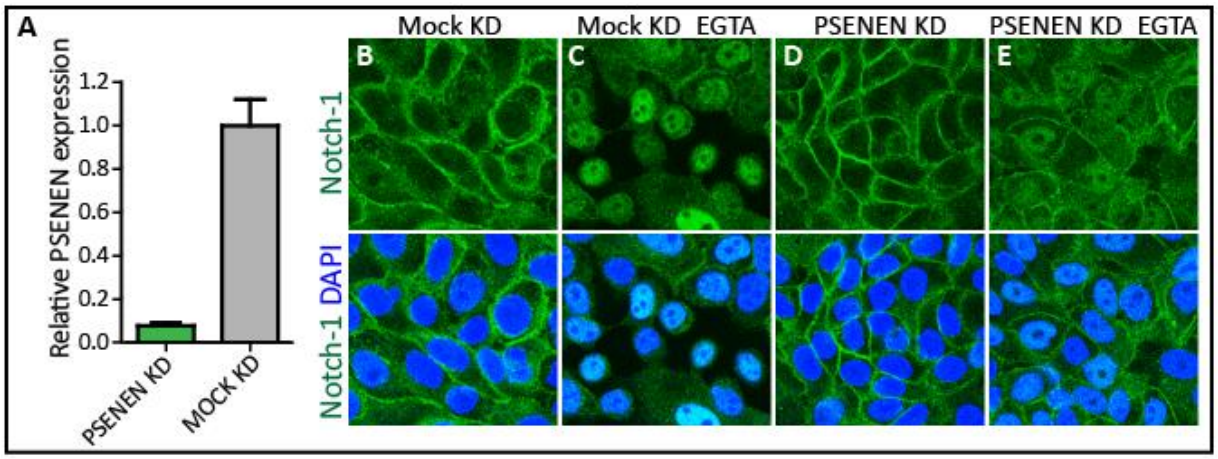


**Figure 37: PSENEN KD reduces Notch cleavage and nuclear translocation**

PSENEN was knocked down with siRNA for 72 hours in MCF10-A cells (A-E). A qPCR analysis shows that PSENEN mRNA levels are significantly reduced (A). Upon knockdown, the cells were stimulated with 10mM EGTA for 30 minutes and immunostained for Notch-1 (B-E). Relative to mock knockdown (B-C), reducing PSENEN mRNA levels intermediately inhibits Notch cleavage and nuclear translocation (D-E). Confocal images (B-E).

Based on these observations, we elected to knockdown PSENEN as a positive control for suppressed Notch cleavage and translocation into the nucleus. In the pilot screen, we knocked down genes for 72 hours and then stimulated Notch cleavage with 2.5mM EGTA for 2 hours, followed by immunostaining and wide-field fluorescent imaging. We however noticed during pilot image analysis that knocking down PSENEN, reduced overall Notch-1 levels and did not appear to inhibit its cleavage and nuclear translocation as expected (Figure 38).

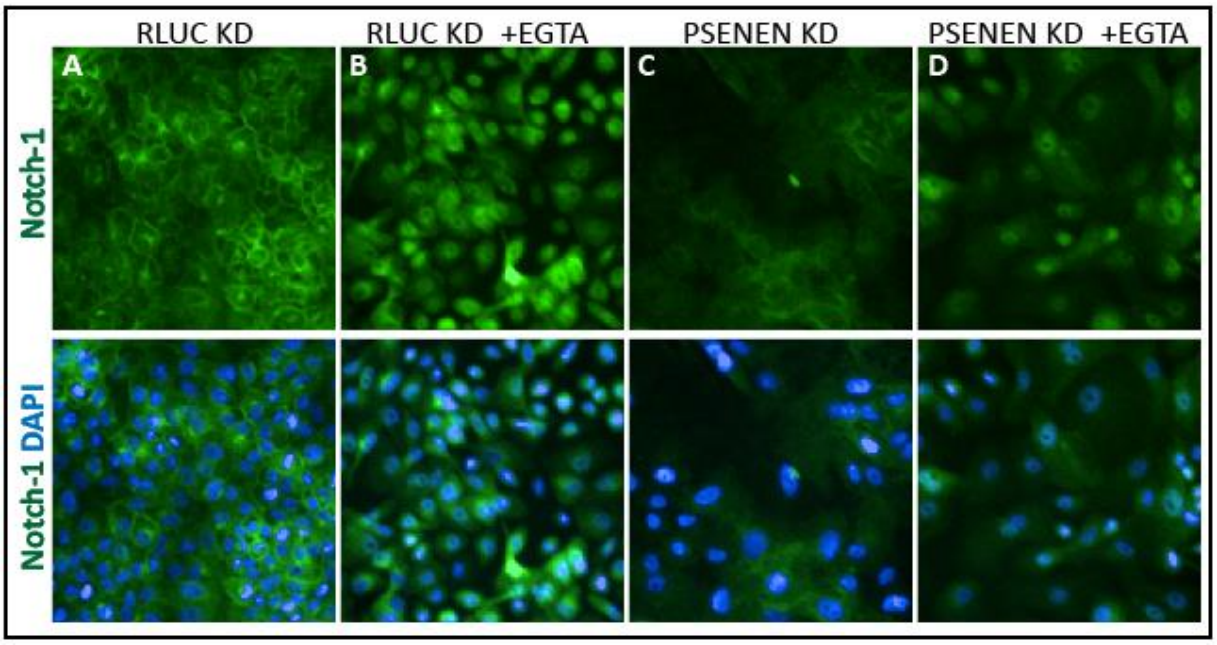


**Figure 38: siRNA mediated KD of PSENEN appears to reduce the overall levels of Notch-1**
Wide field fluorescence images of MCF10-A cells immunostained for the Notch-1 receptor (A-D). siRNA was used to target the indicated genes for 72 hours after which Notch cleavage was stimulated (or not) with 2.5mM EGTA for 2 hours. In RLUC (mock) knockdown cells, ample amounts Notch localize on cell membranes (A) before moving to the nucleus upon stimulation with EGTA (B). Relative to mock knockdowns, PSENEN knockdown cells display a reduced intensity of Notch-1 staining on their cell surface (C) and in their nuclei upon EGTA addition (D). This is contrary to expectation as we do not see a retention of Notch at the cell surfaces upon EGTA stimulation of PSENEN knockdown cells (D).

These knockdowns were performed using GE Dharmacon's siRNA and we wondered whether the unexpected observations might have been due to off-targeting. To test this, we knocked down PSENEN using Sigma's esiRNA for 72 hours and then stimulated the cells with EGTA before immunostaining for Notch-1. As with the siRNA we observed by confocal imaging that relative to mock knockdown, knocking down PSENEN partially impaired Notch cleavage and translocation into the nucleus (figure 39).

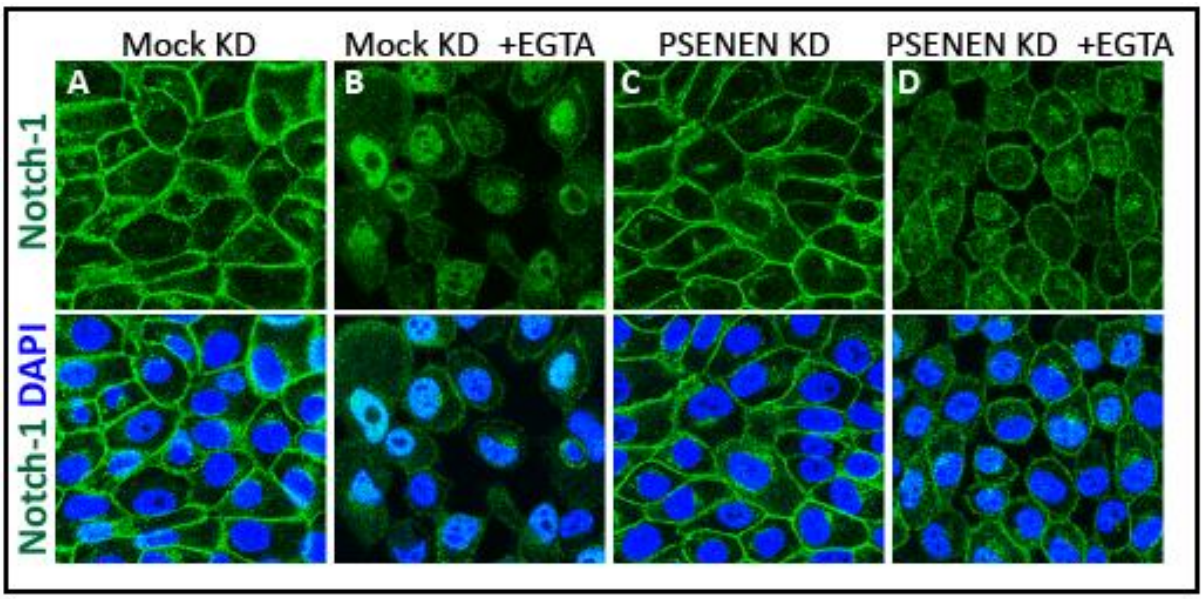


**Figure 39: PSENEN knock down with esiRNA partially blocks Notch cleavage**
Confocal images of MCF10-A cells knocked down for PSENEN for 72 hours (A-D). The cells were stimulated with 10mM EGTA for 30 minutes before immunostaining for Notch-1. Relative to mock knockdown cells (A-B), PSENEN knockdown with esiRNA reduces Notch cleavage and translocation into the nucleus (C-D).

We therefore decided to include both the siRNA and esiRNA as 'positive' controls in the primary screen. It was noticed during primary screen image analysis that the esiRNA too, did not have the expected retention of Notch at the cell surface upon EGTA addition. Its effects on the Notch receptor and its nuclear localization upon EGTA stimulation were identical to those of siRNA, albeit milder (Figure 40).

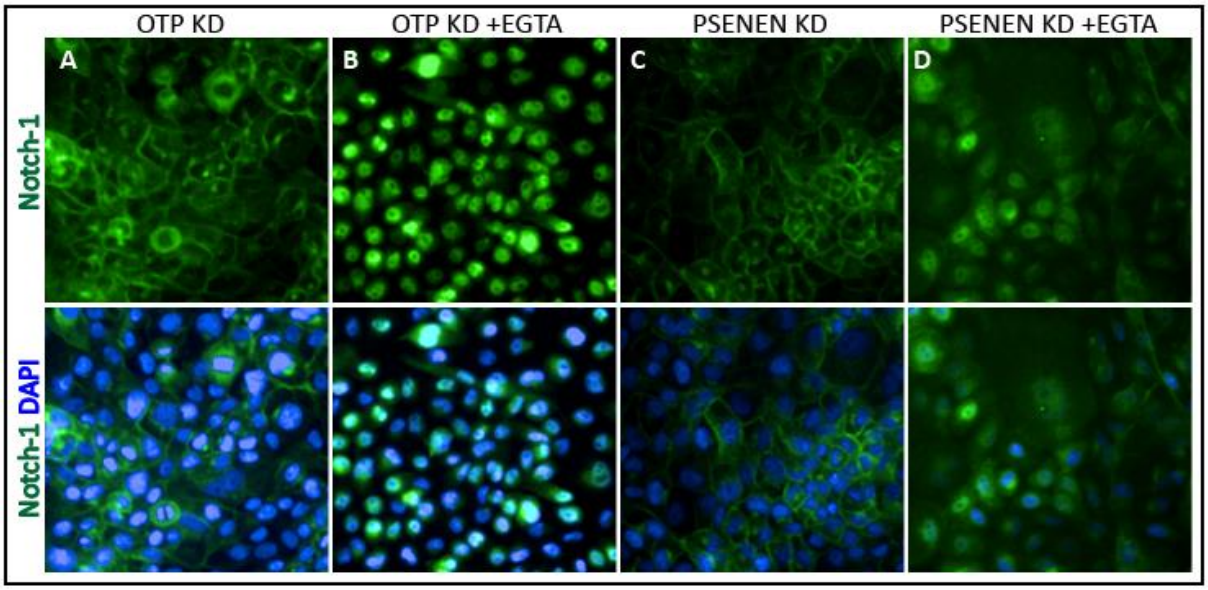


**Figure 40: Knocking down PSENEN with esiRNA phenocopies siRNA**

Wide field fluorescence images of MCF10-A cells immunostained for the Notch-1 receptor (A-D). esiRNA was used to knock down the indicated genes for 72 hours after which Notch cleavage was stimulated (or not) with 2.5mM EGTA for 2 hours. In OTP (mock) knockdown cells, ample amounts of Notch localize on cell membranes (A) and move to the nucleus upon EGTA treatment (B). Relative to mock knockdown, PSENEN knockdown cells display a lower amount of Notch-1 on the cells (C) and in their nuclei upon EGTA addition (D). Although milder, these effects on the Notch receptor are similar to those obtained with Dharmacon's siRNA against PSENEN.

Because PSENEN down-modulation was sub-optimal as a strong positive control for image data analysis, we decided to apply z-score normalization. This is a commonly used approach in screening data analysis whereby phenotypes are quantified and scored on the basis of how far they deviate from the population's mean score for the respective phenotype (Birmingham et al. 2009). This approach has previously been taken in high content, multi-parametric screening data analysis (Collinet et al. 2010)

## 10.3 Primary HCS approach

In the primary screen we targeted 2,749 human genes belonging a 10 library subset of the human genome. The 10 libraries contain members of various protein families including kinases, phosphatases, GPCRs, peptidases, ion channels and ubiquitin ligases. The transfections, EGTA stimulations, immunostainings and image analysis were performed as described in materials and methods. An outline of the primary screen approach is shown on figure 41. Cell segmentation, which relied on DAPI and phalloidin partitioned the cells into 3 compartments that Notch occupies at various stages of its cycle. These are: a) the cell membrane – where it resides before activation; b) the cytosol – which Notch traverses on its way to the nucleus upon activation, crosses on its way to the cell surface upon synthesis or momentarily occupies before degradation or recycling to the cell surface; and c), the nucleus – where the Notch cycle terminates upon signaling activation.

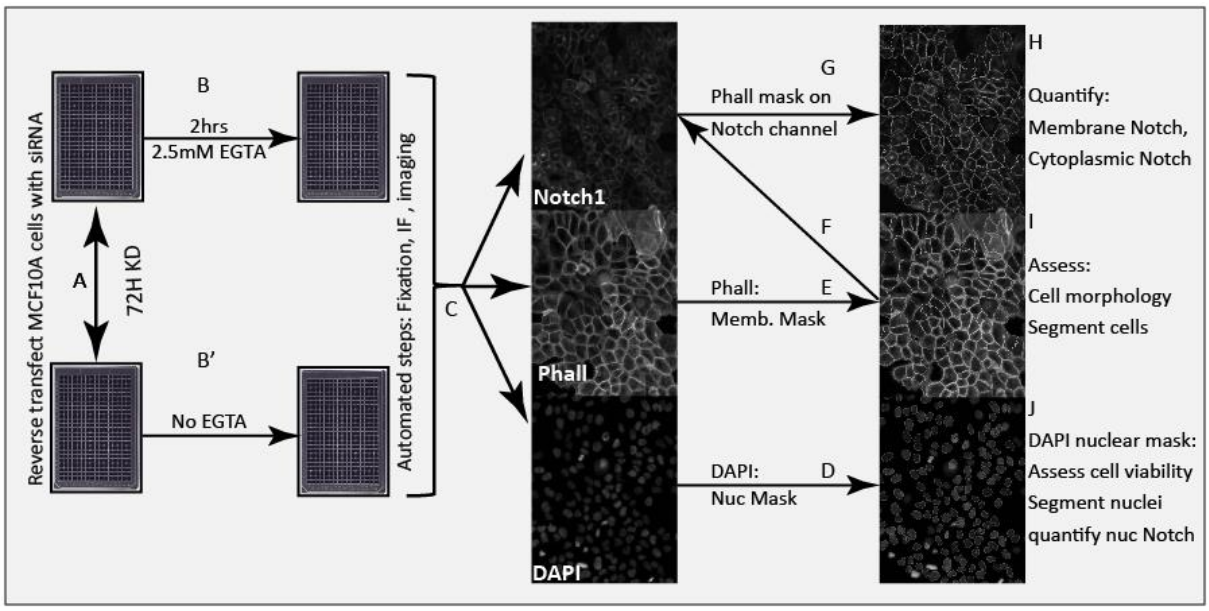


**Figure 41: Schematic of the HCS pipeline and image analysis strategy**

The primary screen was performed on MCF10-A cells on a 384-well format (A-J). Genes belonging to a subset of the human genome (grouped in 10 libraries), were knocked down for 72 hours by RNAi (A). The knockdown was performed in 6 replicates for each of the 10 libraries. 3 of the 6 replicates (B) were stimulated with EGTA and the other 3 (B') left unstimulated. After 2 hours they were subjected to automated IF, image acquisition and finally image analysis (C). An in-house built script was used for image analysis. The nuclei were masked using DAPI staining (D) and a cell surface mask applied using phalloidin staining (E). The phalloidin mask was overlaid on the Notch channel (F) to allow quantification of cell surface membrane Notch (G-H). The phalloidin channel, together with the DAPI channel enabled cell segmentation and identification of the cytoplasmic region (H), where cytoplasmic Notch was quantified. The DAPI channel was used for identification of cells, assessment of cell viability, cell segmentation as well as quantification of nuclear resident Notch (J). This allowed each gene knockdown to be defined by how it affects Notch levels and presence at various stations within the cell.

Various image parameters were analyzed and their quantifications reported as z-scores. The main parameters analyzed are:

- QC1_NoOfAnalysedCells: a measure of cell viability. Low z-scores indicate low cell viability upon a gene's knock down.
- N1_NucNotch: a measure of nuclear Notch intensity. Low z-scores indicate reduced levels of Notch in the nucleus. In the EGTA treated cells, this suggests inhibited

trafficking of Notch into the nucleus. It could also be due to a general reduction in cellular Notch levels.

- N2_CytoNotch: a measure of cytoplasmic Notch intensity. High z-scores indicate cytoplasmic accumulation of Notch, suggesting impaired trafficking and/or degradation.
- N3_MembNotch: a measure of cell surface membrane Notch levels. Low z-scores indicate low amounts of Notch at the cell surface. In NoEGTA conditions, this could be due to a general reduction in cellular Notch, increased Notch activation or impaired outward trafficking of Notch. High z-scores suggest increased Notch synthesis or impaired endocytic trafficking of Notch from the cell surface. High z-scores in EGTA conditions would suggest impaired Notch cleavage.
- N4_CellNotch: a measure of total cellular Notch levels. Low z-scores indicate overall low levels of cellular Notch and suggest reduced Notch biosynthesis. High z-scores indicate overall high levels of Notch. This could be due to increased Notch synthesis or accumulation on Notch in the cell.
- N9_NoOfSpotsPerCell: a measure of Notch accumulation in intracellular vesicles. High z-scores imply impaired Notch trafficking either inwards from the cell surface or outward to the cell surface.
- N5_PercentOfCellsWithSpots: A redundant measure of Notch accumulation in intracellular vesicles. Used in concert with N9_NoOfSpotsPerCell.

## 10.4 Candidate sorting

In order to select hits for primary validation, the candidates were sorted into groups on the basis of their effect on respective parameters (Figure 42). Z-scores directly inform on how knocking down a gene affects each parameter, with a negative score indicating a negative effect and vice versa. The lower or higher the z-score, the stronger the effect knocking down a gene has on the parameter in question. Genes represented in each library plate were knocked down in 6 replicates, half of which were treated with EGTA. Thus for each gene knocked down, z-scores were obtained for each parameter in the presence and absence of induced Notch cleavage. The two conditions were handled as independent experiments.

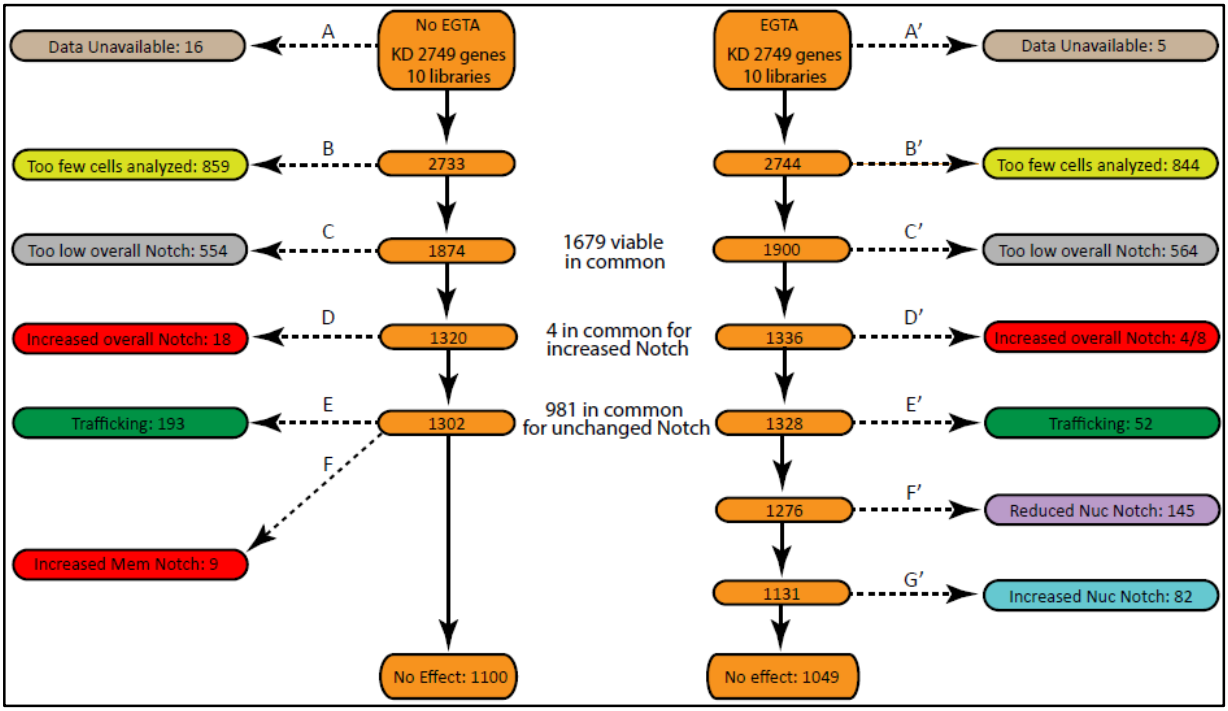


**Figure 42: Steps in primary screen candidate classification**

The primary screen was run on 2,749 genes with or without EGTA stimulation. Fluorescence images were analyzed and the quantified parameters reported as z-score values. A stepwise approach based on the z-scores was taken to categorize the genes into various classes (A-F, A'-G'). Candidates whose knock down affected cells in a relatively identical manner were grouped together. Broken-line arrows indicate candidate groups subtracted from the main list at each sorting step. Solid-line arrows indicate the remainders after each sorting step.

We first filtered out candidates with low cell viability by excluding those that had z-scores < -0.5 for QC1_NoOfAnalysedCells. These candidates were labeled 'cytotoxic' and their knockdown was considered to be strongly anti-proliferative (Figure 43). As expected, PLK-1 knockdown belongs to this class. Candidates with z-score values > -0.5 for this parameter were labeled 'viable'. Both positive controls (Dharmacon's siRNA and Sigma's esiRNA targeting PSENEN) and the negative controls fall in the viable class.

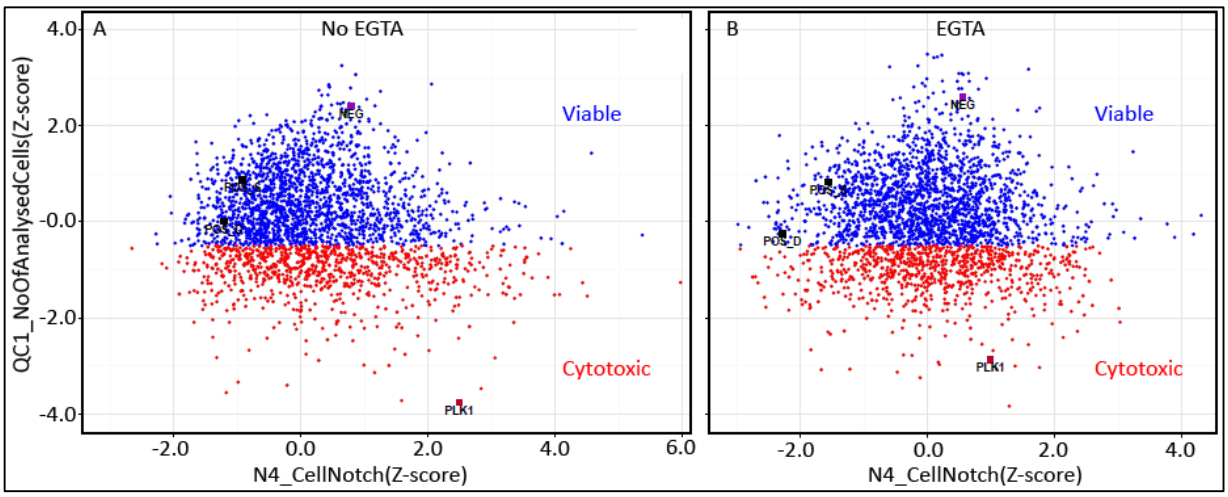


**Figure 43: Excluding anti-proliferative candidates**

Candidates with very few analyzed cells were excluded from further analysis as their knockdown is anti-proliferative (A-B). The same genes were knocked down in A and B and EGTA used to stimulate Notch cleavage in B. Each point represents a candidate's z-score for QC_NoOfAnalysedCells (y-axis) against its z-score for N4_CellNotch (x-axis). Candidates with z-scores < -0.5 for the former parameter were considered 'cytotoxic' (red points) and excluded from further analysis. The positive control for transfection efficiency, PLK-1, falls in this class. Candidates with values > -0.5 were considered 'viable' (blue points). Both positive (POS_D and POS_S) and the negative controls (NEG) fall into this class. Median z-scores are shown for the controls. Note that the negative controls have among the highest scores for number of cells analyzed.

Some of the genes, when knocked down cause a general reduction in the cellular levels of Notch-1. To group such candidates together, we filtered out factors with z-scores < -0.5 for the parameter N4_CellNotch, which quantifies the overall levels of Notch-1 in the cell. Factors with z-scores below this cutoff were labeled 'NotchReduced' and their knockdown was considered to impede Notch production. Both positive controls fall in this class. Other factors may cause an increase in overall Notch levels. Such factors were isolated by filtering for candidates with a z-score > 3.0 for N4_CellNotch and were labeled 'NotchIncreased'. Candidates with z-scores ranging from -0.5 to 3.0 were labeled 'NothcUnchanged'. This category was considered not to affect the overall levels of cellular Notch (Figure 44). Candidates in this category possess a spectrum of cellular Notch levels ranging from 'low' in factors with z-scores close to -0.5 and 'high' for those with z-scores approaching 3.0. The 'NothcUnchanged' category was carried forward for further scrutiny.

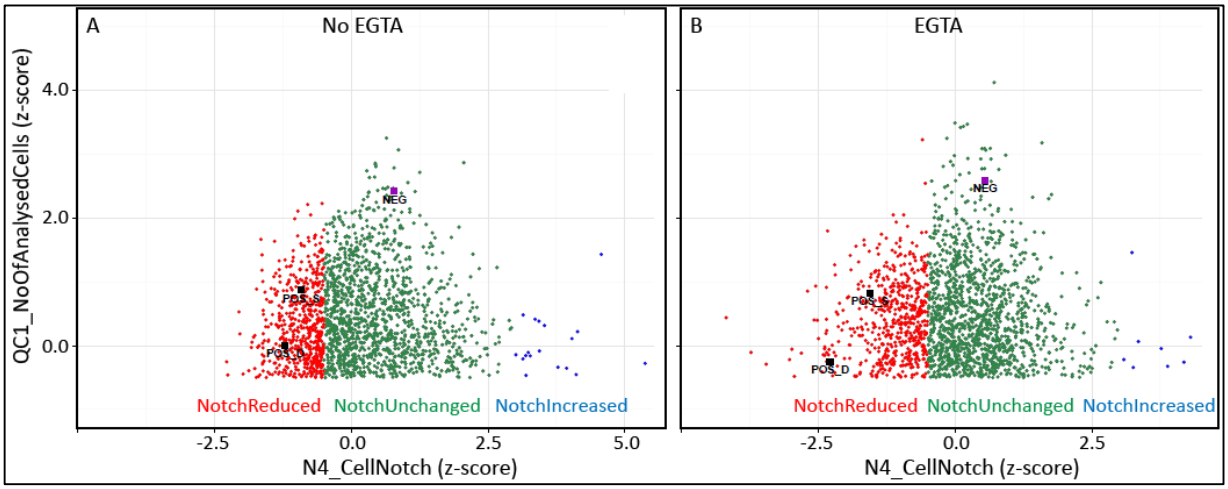


**Figure 44: Excluding candidates with low Notch levels**

This step was performed on the 'viable' class of candidates (see figure 42 and 43). Those with too low or too high levels of Notch were separated from the rest of the candidates (A-B). Each point represents a candidate's z-score for N4_CellNotch (x-axis) against its z-score for QC_NoOfAnalysedCells (y-axis). Candidates with z-scores < -0.5 for N4_CellNotch (red points) were considered to have too low levels of cellular Notch. Those with scores > 3.0 (blue points) were considered have high amounts of overall cellular Notch. Candidates with scores falling between the lower and upper thresholds were considered to have 'unchanged' levels of Notch in the cells (green points). As observed during the screen setup, both Dharmacon's siRNA and Sigma's esiRNA reduce the overall Notch levels in the cells, in both EGTA stimulated and non-stimulated cells (A-B). The negative controls' Notch levels fall in the middle of the 'NotchUnchanged' distribution.

To identify candidates that affect Notch trafficking we relied on the parameters 'N9_NoOfSpotsPerCell' and 'N5_PercentOfCellsWithSpots'. These parameters are based on the fact that impairing Notch trafficking traps it in intracellular vesicles, where it appears as 'Notch spots' upon immunostaining. The former parameter is an average of the number of Notch spots identified per cell. The latter is a quantification of the number of cells that contains Notch spots out of the total analyzed for each candidate. Although the two parameters are redundant, 'N5_PercentOfCellsWithSpots' allows the identification of factors that might escape 'N9_NoOfSpotsPerCell'. We first set a filter on the parameter 'N9_NoOfSpotsPerCell' and classified candidates as affecting Notch trafficking if they had z-scores > 1.0 in NoEGTA or

> 2.0 in EGTA conditions. The remaining candidates were analyzed on the basis of 'N5_PercentOfCellsWithSpots'. Candidates were considered to affect trafficking of Notch if they had scores > 1.7 in NoEGTA conditions or > 1.3 under EGTA stimulation. The candidates emerging from this process were labeled 'Trafficking' (Figure 45). However, some candidates in this class might affect Notch degradation as the knock down of such factors also causes intracellular Notch spots. Hence the term 'trafficking' is loosely applied here to mean candidates that cause intracellular Notch accumulation. Higher cutoff thresholds were applied in EGTA conditions in order to limit spurious identification of candidates as affecting trafficking. This is necessitated by the observation that EGTA causes a 'shrunken' cell morphology characterized by a small cytoplasmic region and high nuclear Notch intensity. As such, some of the nuclear signal might appear cytoplasmic.

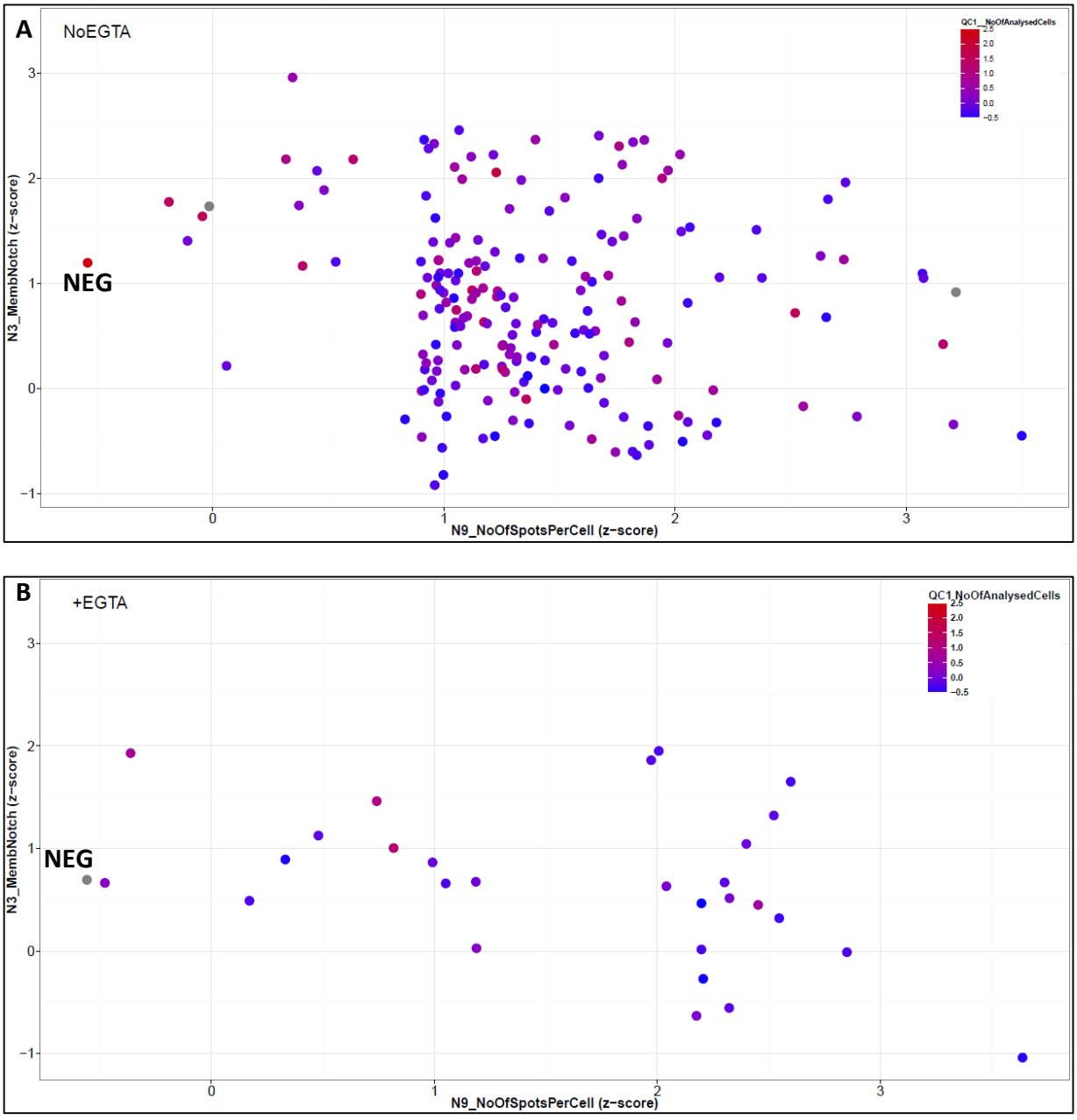


**Figure 45: Identification of Notch trafficking candidates**

Factors affecting Notch trafficking were identified by the parameters 'N9_NoOfSpotsPerCell' and 'N5_PercentOfCellsWithSpots' (A-B). Each point represents a candidate's z-score for the NoOfSpotsPerCell (x-axis) against the MembraneNotch intensity (y-axis). Z-scores for the NoOfCellsAnalyzed are color coded on a spectrum of blue to red, blue being the least and red the highest. Relative to negative controls (NEG), these candidates exhibit high intracellular Notch. A candidate's effect on Notch trafficking can be inferred from these plots. For instance factors with high z-scores on the x-axis and low ones on the y-axis, may imply sub-optimal Notch trafficking to the cell surface. The shorter list from EGTA treated cells (B) is due to the application of higher z-score cutoffs and exclusion of candidates already present in the NoEGTA (A) list of trafficking candidates.

To identify candidates that increase membrane levels of Notch in NoEGTA conditions, we relied on the parameter 'N3_MembNotch'. MCF10-A cells have a substantial amount of cell surface Notch, making it difficult to detect subtle changes. Therefore a high cutoff threshold was applied on this parameter and candidates considered to have increased 'N3_MembNotch' only if they had z-scores > 3.0 (Figure 46). This analysis was not deemed necessary in EGTA conditions as Notch is stripped from the cell surface membranes.

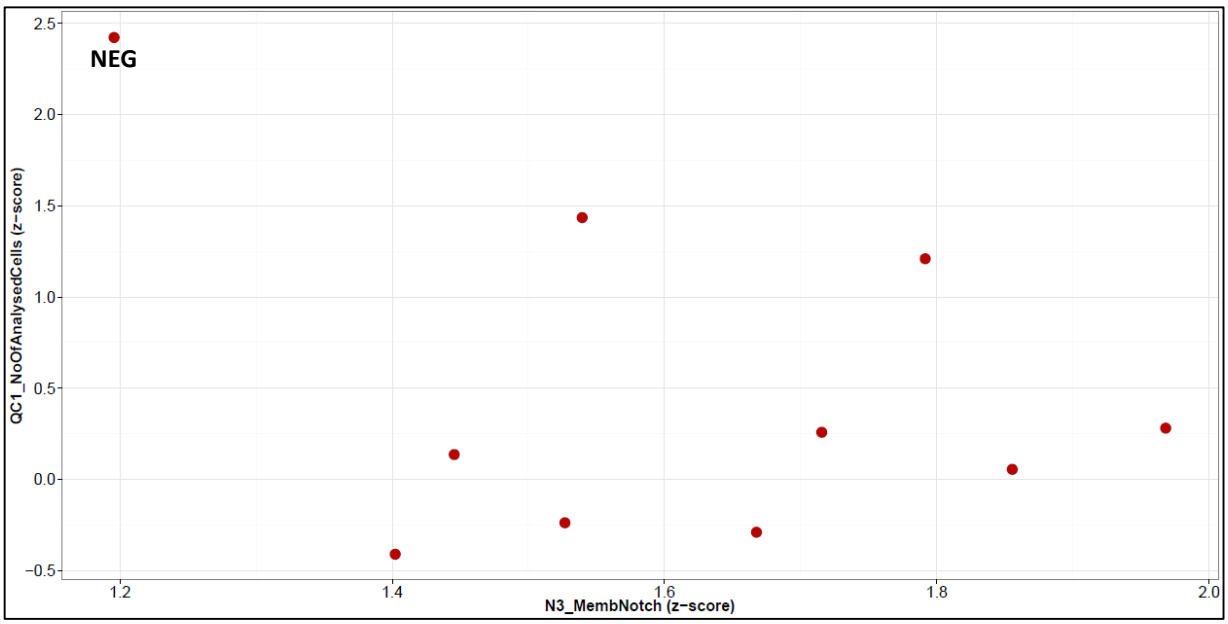


**Figure 46: Identification of candidates with increased cell surface Notch.**
Factors with increased Notch levels on the cell surface membrane were identified by the parameter 'N3_MembNotch'. Each point represents a candidate's z-score for 'N3_MembNotch' (x-axis) against its z-score for QC1_NoOfCellsAnalyzed. Relative to the negative controls (NEG), these candidates possess high levels of Notch on their cell surfaces. This analysis was performed in the NoEGTA condition.

The main purpose for incorporating EGTA stimulation into the screen, was to identify factors that when knocked down prevent (or reduce) Notch cleavage and translocation into the nucleus. If present in our libraries, such candidates would have reduced amounts of nuclear Notch and hence low z-scores for the parameter N1_NucNotch. Low z-scores for this

parameter might also be caused by trafficking factors as they may hinder Notch from getting into the nucleus. To ensure that we were not considering factors with reduced overall Notch levels, this analysis was performed on the 'NotchUnchanged' class of candidates (Figure 44 B). To identify candidates with reduced levels of nuclear Notch, we filtered for factors with z-score values < -0.5 in N1_NucNotch (Figure 47 A). To identify candidates with high levels of nuclear Notch under EGTA stimulation we filtered for those with z-scores > 1.5 in N1_NucNotch (Figure 47 B). Such an increase in nuclear Notch upon EGTA treatment might be due to increased membrane Notch levels, increased efficiency of Notch activation upon knock down of respective genes or increased cNICD-1 stability. This might also be caused by impaired Notch entry into the nucleus as perinuclear Notch may appear to be nuclear.

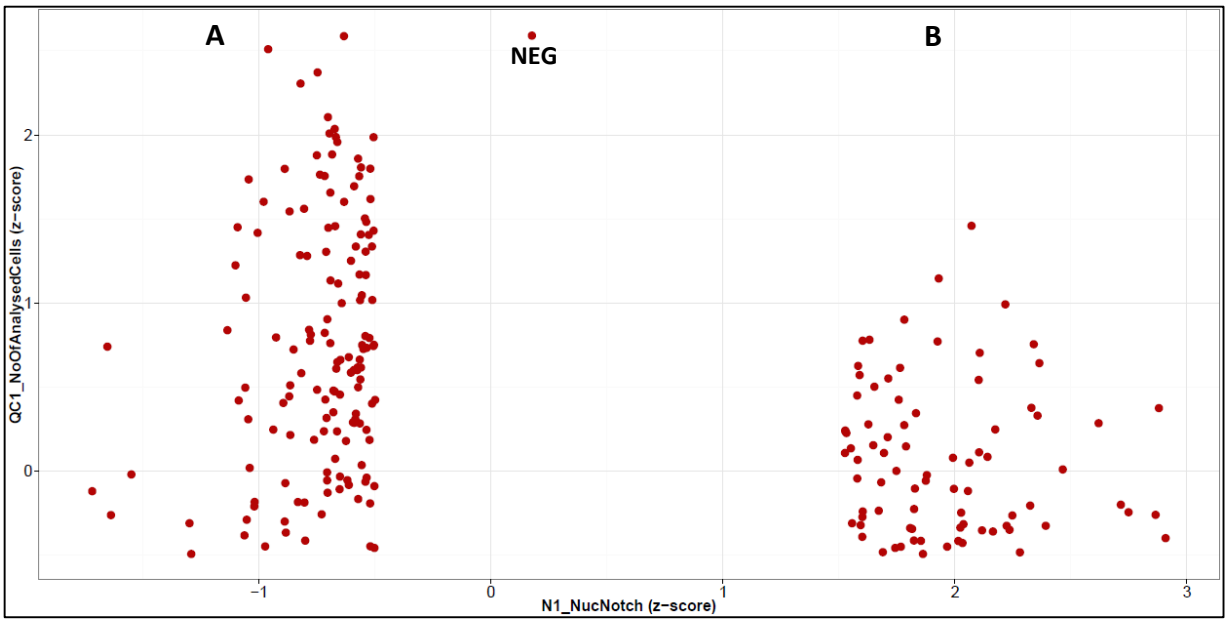


**Figure 47: Candidates affecting nuclear Notch levels in EGTA conditions**

To identify candidates that affect levels of nuclear Notch under EGTA treatment, the 'NotchUnchanged' list (figure 44 B) was analyzed on the basis of 'N1_NucNotch' (A-B). Z-score cutoffs were established so as to identify factors with reduced nuclear Notch (A) or increased nuclear Notch (B), in relation to the negative controls (NEG). Each point represents candidate's z-score for nuclear Notch intensity against its z-score for the number of analyzed cells. Some of the candidates in both A and B might upon knockdown, affect Nuclear Notch by impeding its trafficking.

To identify factors that might affect Notch cleavage at the cell surface, we plotted 'N1_NucNotch' against 'N3_MembNotch' for the genes identified (on Figure 47 A) as having reduced nuclear Notch (Figure 48). We then visually inspected the candidates that had low z-scores for 'N1_NucNotch' and high ones for 'N3_MembNotch'. Such candidates were likely to have more Notch-1 on their cell surfaces and less in their nuclei relative to negative controls, implying reduced Notch cleavage. We however could not verify with confidence, any candidates as presenting this phenotype.

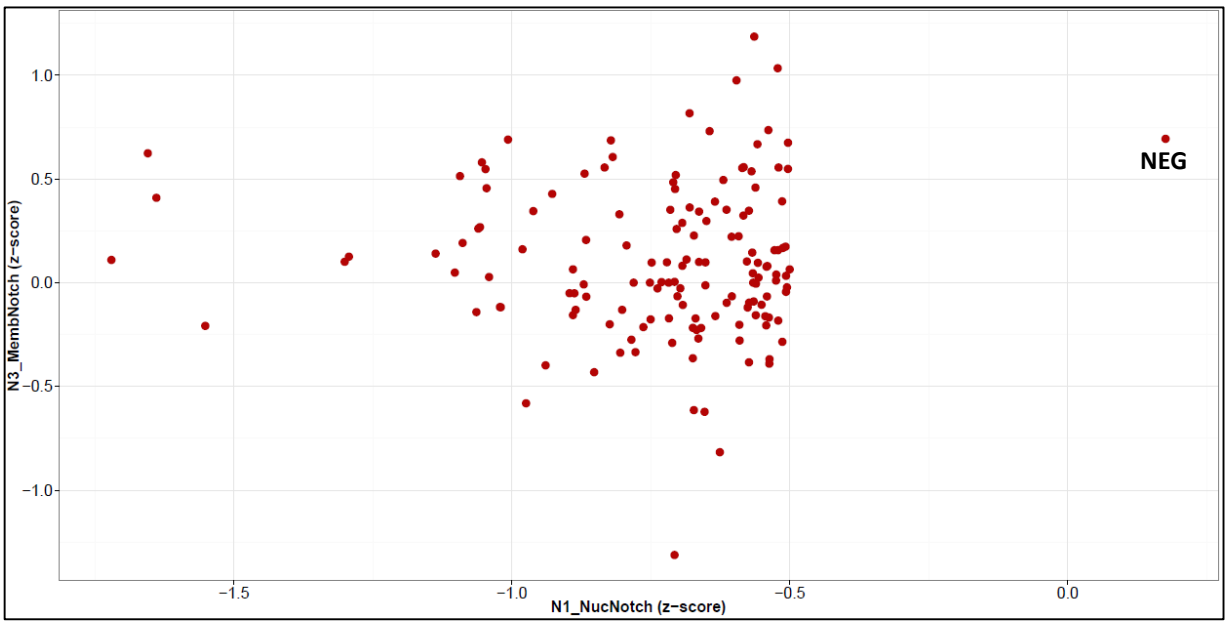


**Figure 48: Possible inhibitors of Notch cleavage upon EGTA stimulation**

To identify candidates with inhibited Notch cleavage, factors with reduced nuclear Notch under EGTA conditions were analyzed on the basis of 'N1_NucNotch' and 'N3_MembNotch'. Each point represents a candidate's z-score for nuclear Notch (x-axis) against its z-score for membrane Notch (y-axis). Relative to the negative controls (NEG), this group of candidates possesses low nuclear Notch. Low nuclear Notch levels might be due impaired Notch trafficking into the nucleus or reduced Notch cleavage. Upon visual inspection of the candidate's images we could not confidently identify factors with the latter effect.

Candidates that did not satisfy the criteria for grouping into any of the described categories, whether in the NoEGTA or EGTA conditions, were labeled 'No effect' (Figure 43). Members of this list were regarded as not to having an effect on the Notch receptor upon knock down.

## 10.5 Candidate selection for validation

In the primary screen each gene was targeted for knock with a pool of 4 siRNA. This approach is aimed at minimizing off-targeting while maximizing the on-target effects (Jackson & Linsley 2010). However, this strategy does not rule out off-targeting and therefore necessitates a deconvolution step to tease apart which (and how many) of the 4 siRNA present in the pool are responsible for the observations made. Alternatively, one may perform the validation using a reagent that is unrelated to the one used for the primary screen. The latter approach is more feasible in terms of effort and costs. To generate a list of candidate genes for validation, we selected the best ranking candidates from each of the categories outlined on figure 42. This process yielded 231 candidates for validation using Sigma's esiRNA (Table 1). The selected candidates were inspected to verify presence of the relevant phenotypes as reported by automated image analysis.

| | Phenotypic class | Candidates |
|---|---|---|
| a | Increased overall Notch EGTA | 4 |
| b | Increased overall Notch NoEGTA | 12 |
| c | In common between trafficking NoEGTA and trafficking EGTA | 24 |
| d | Increased Nuclear Notch in common with trafficking NoEGTA genes | 19 |
| e | Unique to trafficking EGTA | 28 |
| f | Increased Membrane Notch NoEGTA | 9 |
| g | Unique to increased Nuclear Notch | 43 |
| h | Reduced Notch overall | 6 |
| i | Putative inhibitors of Notch translocation | 4 |
| j | No effect | 5 |
| k | Best from the remaining trafficking NoEGTA candidates | 77 |
| l | total | 231 |

**Table 1: Representation of candidate classes in the validation list**

The validation list was generated with representatives of the phenotypic classes shown on figure 43 except the 'cytotoxic' one. Candidates were prioritized by strength phenotype. Where only a few candidates were present, they were all included as long as the phenotype was confirmed by visual inspection of the respective images. Since some candidates emerged in more than one group, the various categories were compared and the candidates in common given first priority. For instance candidates in common between 'trafficking EGTA' and 'trafficking NoEGTA' were considered first priority trafficking candidates. The phenotypes of 231 selected candidates were visually confirmed from the images. Those not confirmed were excluded and replaced with lower priority candidates of the same phenotypic class.

Because their lists were short, all candidates belonging to the 'increased overall Notch' categories in NoEGTA and EGTA conditions were selected for validation. Out of the 8 candidates identified under EGTA conditions, 4 were also present in the list from NoEGTA conditions, leaving 4 candidates for 'increased overall Notch EGTA'. Out of the 16 candidates classified as causing increased 'increased overall Notch NoEGTA', the 12 confirmed by visual inspection of corresponding images were taken.

Since some trafficking candidates are bound to be present in the 'trafficking NoEGTA' and 'trafficking EGTA' classes, the lists were compared. The 24 candidates in common between them, were considered 'high confidence' mediators of Notch trafficking. The remaining 28

candidates ‘Unique to trafficking EGTA’ were included on the validation list as affecting Notch trafficking ‘only’ in the presence of EGTA.

Depleting extracellular $Ca^{2+}$ causes a ‘shrunken’ cell morphology. The cells separate from one another due to disruption of the $Ca^{2+}$ stabilized cell junctions (Hirano et al. 1987) and appear rounded with a very small cytoplasmic area. Hence cytoplasmic Notch might in some instances be quantified as nuclear Notch. Because of this ‘shrunken’ morphology trafficking candidates might be grouped in the ‘increased nuclear Notch’ class under EGTA conditions. By comparing the ‘increased nuclear Notch EGTA’ list with the remaining ‘trafficking NoEGTA’ candidates, 19 candidates were found in common. These 19 were considered ‘high ranking’ trafficking candidates. The remaining 43 candidates unique to the ‘increased nuclear Notch EGTA’ group were considered to somehow increase the efficiency of Notch cleavage upon EGTA stimulation and were included to the list for validation.

All candidates belonging to the ‘Increased Membrane Notch NoEGTA’ class were selected for validation.

From the data analysis we observed that 554 and 564 candidates reduced overall levels of Notch in NoEGTA and EGTA conditions respectively. Candidates in this class possibly affect Notch synthesis and might be general regulators of transcription. We selected representative candidates for validation from this class by taking the highest ranking 6 in common between the NoEGTA and EGTA conditions.

Candidates that inhibit Notch cleavage upon EGTA stimulation are expected to have low nuclear Notch intensity and high levels of membrane Notch relative to candidates that do not. To select representative candidates from this class, we ranked those with reduced nuclear Notch by the amount of membrane Notch (Figure 48). Candidates that had the least amount of nuclear Notch and highest amounts of membrane Notch were visually inspected to verify

this phenotype. However none of them were convincingly found to possess this phenotype and the best 4 were selected to represent this category.

From the list of candidates classified as having no effect on the Notch receptor, the best 5 were selected on the basis of their phenotypic semblance to non siRNA transfected negative controls. These were included in the list for validation.

Finally, the remaining 'trafficking NoEGTA' candidates were ranked by strength of phenotype and the highest ranking ones selected to bring the list of validation candidates to 231. Images representing the different phenotypic classes are shown (Figure 49 A-L).

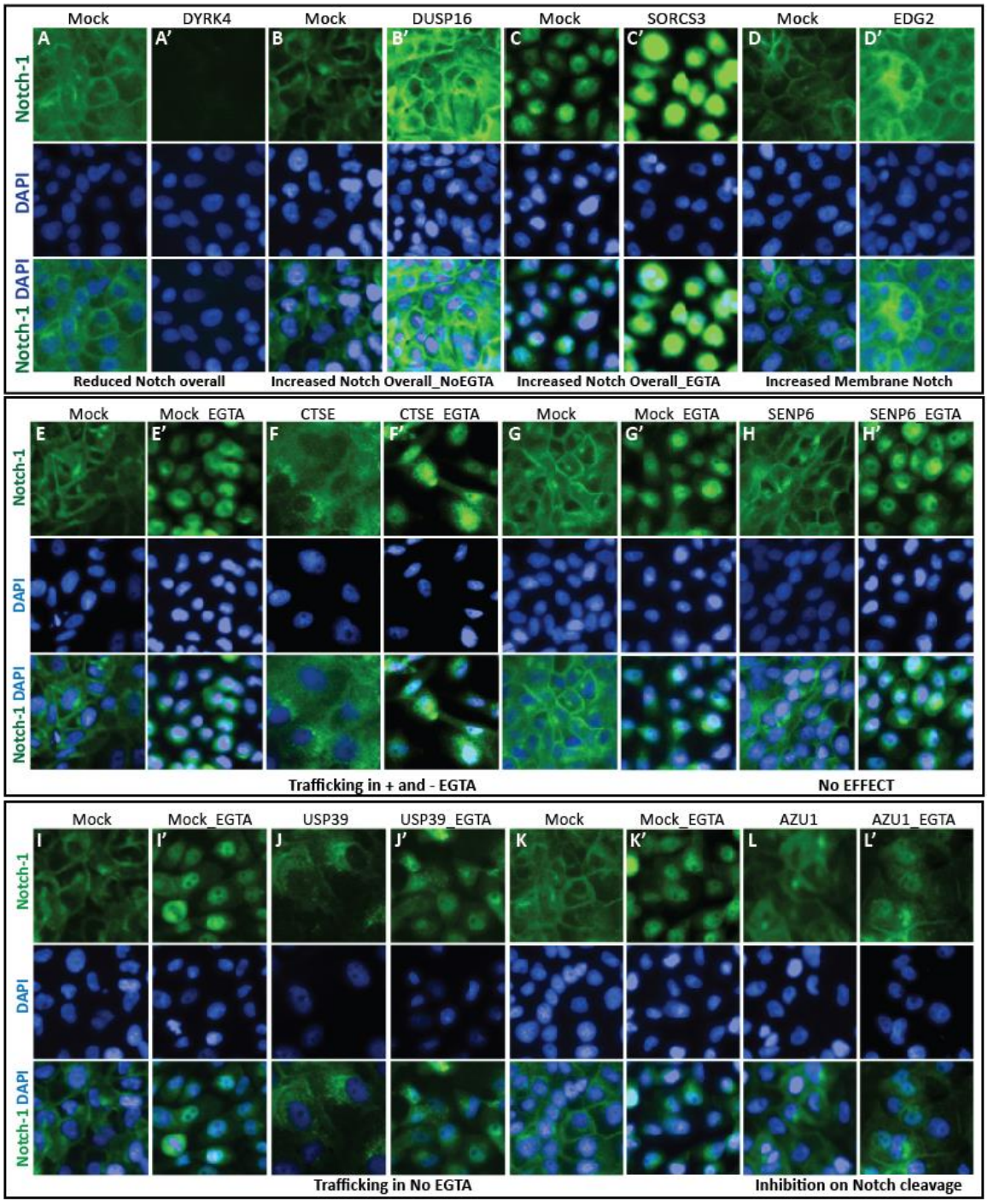


**Figure 49: Representative images of the Notch phenotypic categories**

Wide field fluorescence images of MCF10-A cells immunostained for the Notch-1 receptor (panels I-III). The indicated genes were knocked in 384 well format for 72 hours before immunostaining. Where shown, EGTA was added for 2 hours prior to immunostaining. Following image analysis, the candidates were sorted into groups depending on how they affected the Notch-1 receptor. Relative to respective negative controls these phenotypic classes include: reduced Notch overall (A-A'), increased Notch overall (B-B'), Increased Notch overall in EGTA conditions (C-C'), Increased membrane Notch (D-D'), trafficking (F-F' and J-J'), No effect (H-H') and probable inhibition of Notch cleavage (L-L').

# 11. Discussion

## 11.1 Screen design

To uncover novel components of the Notch pathway, we coupled siRNA and immunofluorescence to screen for factors that affect the Notch receptor during any of the major events fundamental for productive Notch signaling; i.e. synthesis, trafficking, degradation, cleavage and nuclear translocation. Most of our knowledge of the Notch pathway comes from screens in *Drosophila* and *C. elegans* (Guruharsha et al. 2012) but screens in human cells have also been reported (Moretti et al. 2010; Krämer et al. 2013; Roti et al. 2013; Izrailit et al. 2013). Compared to previous screens, ours has the following strengths: a) being performed in human cells, it allows identification of factors that affect mammalian Notch signaling, including those without orthologues in lower organisms; b) MCF10-A cells are non-transformed, allowing identification of modulators of basal Notch signaling; c) MCF10-A endogenously express Notch pathway components and obviate the need to transfect constructs; an important factor in light of gene dosage sensitivity to Notch (Guruharsha et al. 2012); d) Since the only manipulation is gene knockdown, phenotypes can be directly attributed to target genes.

An unexpected shortcoming of our approach is the inability to confidently detect regulators Notch cleavage. This is due to altered cell morphology and lack of a positive control for impaired Notch cleavage. Physiologic NICD levels are hard to detect by immunofluorescence (Schroeter et al. 1998), necessitating EGTA stimulation. However, $Ca^{2+}$ depletion disrupts cell-cell adhesion (Hirano et al. 1987), making it difficult to accurately segment the cells for membrane Notch quantification. Knocking down PSENEN as a potential positive control for impaired Notch cleavage caused reduced Notch levels. PSENEN loss is

reported to cause ER $Ca^{2+}$ leakage (Tu et al. 2006; Bezprozvanny 2013), an event that impairs protein synthesis (Brostrom & Brostrom 2003). The possibility that loss of PSENEN reduces Notch synthesis thus deserves further attention.

The human genes that we screened are either demonstrated or anticipated to interact with known drugs (Hopkins & Groom 2002; Russ & Lampel 2005). This has attractive implications: a) Inhibitors of promising candidates can make excellent investigative tools and accelerate hit characterization and b), identification of novel modulators of the Notch pathway against which inhibitors exist, reciprocally identifies potential drugs against Notch associated pathologies.

## 11.2 Candidate classification and possible implications

The 231 candidates belong to the different Notch phenotypic classes. The power of this approach in identifying factors that affect intracellular localization of Notch-1 and possibly its signaling output is exemplified by the identification cathepsin E (CTSE) and SORCS3. In CTSE knockdown cells Notch accumulates intracellularly. This is expected as loss of CTSE has been reported to impair lysosomal function, causing intracellular accumulation of undegraded material (Tsukuba et al. 2013). In SORCS3 knockdown cells, a strong increase in NICD is observed upon EGTA stimulation, implying increased Notch cleavage by γ-secretase upon SORCS3 knockdown in relation to mock knockdown cells. This is in line with reported findings that SORCS3 is a negative modulator of γ -secretase function (Reitz et al. 2013).

The majority of the 231 candidates fall in the trafficking class. This is expected since we broadly define trafficking to cover all candidates that present cytosolic Notch accumulation. Such factors might affect diverse processes like Notch endocytosis, exocytosis, recycling,

degradation, maturation and nuclear import. While it is premature to make predictions or conclusions, such candidates, if modulating Notch trafficking, may present a therapeutic potential (Krämer et al. 2013; Roti et al. 2013).

Some candidates appear to affect Notch trafficking in the presence of EGTA but not in Non-EGTA conditions, implying that they only affect trafficking of cleaved Notch. Such candidates would present the prospect of blocking nuclear translocation of NICD when ectopically generated, as happens in T-ALLs and some breast cancers (Weng et al. 2004; Robinson et al. 2011), while sparing wild type Notch.

A tenth of the candidates, appear to cause cell death only conditions of EGTA stimulation. This suggests that the absence of such genes in the presence of excessive Notch activity may induce cell toxicity, an attractive prospect for selectively killing cells that possess oncogenic Notch function.

A third of the candidates that do not cause cell toxicity display low Notch levels. This could be due to a general suppression of protein production or a transcriptional repression of Notch synthesis. Other candidates display strongly increased Notch levels. This could be due to impaired Notch turnover or increased transcriptional Notch production. Little is currently known about how Notch receptors are transcriptionally regulated (Wu & Bresnick 2007) and it will be curious to see whether such candidates act on Notch transcriptionally. Candidates that cause overall Notch reduction might have important implications for cancers caused by loss of Notch function (Rangarajan et al. 2001; Nicolas et al. 2003; Nguyen et al. 2006; Dotto 2008) while those causing increased Notch levels might have oncogenic Notch roles (Weijzen et al. 2002).

Following EGTA stimulation, some candidates present increased nuclear Notch relative to negative controls. Such candidates might modulate NICD stability in the nucleus, similarly to FBXW7 (Oberg et al. 2001).

It is difficult to identify candidates whose knockdown affects Notch cleavage at the cell surface. Such candidates are expected to have low levels of nuclear Notch and high levels of Notch at the cell surface following EGTA treatment. However, considering the tight regulation of physiological Notch signaling, only a small number of proteins are expected to affect Notch cleavage by γ-secretase at the cell surface. With a coverage of less than a $5^{th}$ of the genome, this likelihood is even lower in our screen.

## 11.3 Candidate validation and hit follow-up

In order to exclude off-targets, the 231 candidates will be retested with esiRNA. Since this assay does not reveal the status of Notch signaling, we will use the Notch signal transduction reporter, RBP-Jκ-Luc, to identify factors that affect Notch transcriptional output. The validated list of novel putative regulators of Notch signaling will be analyzed to identify those that have clear orthologues in *Drosophila.* These will be knocked down *in vivo*, in larval wing imaginal discs, pupal nota, and adult follicle cells and then scored for well-characterized Notch associated phenotypes. To this end, we will use the inducible GAL4/UAS system to drive tissues specific expression of RNAi hairpins from available transgenic fly stocks. We will also evaluate the effects of knockdowns on Notch signaling by using established Notch reporters. In addition, we will immunostain *Drosophila* Notch to determine whether intracellular localization is perturbed as predicted by the screen. Validation in flies will provide initial evaluation of the importance of the identified genes in tissue and organ physiology.

# 12.Acknowledgements

I would like to express my sincerest gratitude to all those that directly or indirectly, wittingly or unwittingly, in big or in small ways helped and urged me on to the completion of this dissertation. I am especially thankful to my boss **Thomas Vaccari** for taking me on as a student when my knowledge of science and experience in research were a lot more modest than they currently are. My gratefulness for your patience, support and availability over the past four years cannot be overstated. My internal supervisor Dr. Diego Pasini and my external supervisor Dr. Gian-Paolo Dotto, thank you for having the time; your time reading and evaluating my project reports as well as your suggestions were of immense value. My PhD thesis committee of Dr. Stefano Casola and Dr. Martin Baron, thank you for the questions, suggestions and for the extensive discussion of my thesis. **Dr. Mark Wade** and the entire iit@SEMM screening unit, thank you for bearing with me during every step of the screen. **The whole of Vaccarilab**, I couldn't have asked for a better crop of colleagues; thank you so much for the joy and laughter, for support during those tough periods we are all so familiar with, for your engaging and very insightful discussions especially during our very legendary lab meetings (I believe I still hold the record for the longest one, at almost 4hrs of lab meeting marathon) and for putting up with me especially during those 'days of the month'. To family, thank you so much for probably being the most unique and uniquely supportive family. Your backing has been a gentle driving force on this arduous trip.

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