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Regulation of pro-metastatic Notch3 receptor in melanoma

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Metastatic melanoma, which can form distant metastases through the lymphatic vessels, is the deadliest type of all skin cancer. Lymphatic endothelial cells facilitate metastasis of melanoma cells by activating the Notch3 signaling pathway in melanoma cells upon contact. The Notch signaling pathway regulates various fundamental functions of the cells, but overactivity of the pathway is associated with many cancers. However, it is not exactly known which Notch ligands can activate Notch3 receptors in melanoma cells. Notch3 signaling can be regulated in different ways. PIM kinases are known to affect Notch3 signaling in breast and prostate cancer cells in a tumorigenic manner, but it is not known whether PIM kinases can contribute to the regulation of Notch3 signaling in melanoma cells. The aim of this thesis was to examine how Notch3 signaling is regulated in melanoma cells. Melanoma cells were treated with immobilized Notch ligands fused with a Fc-fragment to address which Notch ligands activate Notch3 signaling. In addition, cells were treated with siRNAs targeting PIM 1-3 kinases to investigate whether PIM kinases contribute to the regulation of Notch3 signaling in melanoma cells. The activity of Notch3 signaling was determined by analyzing the mRNA and protein levels of its downstream targets, and the invasiveness of cells induced by Notch3 activation was examined using a 3D invasion assay. This study provided evidence that especially Notch ligands DLL4 and DLL1 can activate the tumorigenic Notch3 signaling in melanoma cells. Moreover, DLL4 treatment also promoted the invasion of metastatic melanoma cells in 3D fibrin, whereas the invasion of non-metastatic melanoma cells was not induced. Furthermore, PIM2 appeared to have a potentially oncogenic role in melanoma cells, since DLL4-mediated invasiveness of melanoma cells was reduced upon PIM2 silencing.

Key words: Melanoma, Notch signaling, Notch3, PIM kinases, Delta-like ligand 4 (DLL4), Delta-like ligand 1 (DLL1)

Contents

Abbreviations

BEC	Blood endothelial cell
CSL	CBF-1/RBP- Jκ, Su(H), Lag-1
DLL1	Delta-like ligand 1
DLL3	Delta-like ligand 3
DLL4	Delta-like ligand 4
ECM	Extracellular matrix
EMT	Epithelial Mesenchymal Transition
ESCRT	Endosomal sorting complex required for transport
HES	Hairy/enhancer of split
HEY	Hairy/enhancer of split related to YRPW motif
JAG1	Jagged 1
JAG2	Jagged 2
LEC	Lymphatic endothelial cell
MAML	Mastermind-like protein
MMP	Matrix metalloprotease
NECD	Notch extracellular domain
NEXT	Notch extracellular truncation
NICD	Notch intracellular domain
NLS	Nuclear localization sequence
NRR	Negative regulatory region
RAM	RBP-Jkappa-associated module
SNL	Sentinel lymph node
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Melanoma

Malignant melanoma is the most dangerous type of skin cancer due to its tendency to spread and metastasize (Sundararajan et al. 2022). Melanoma accounts for most of the skin cancer related deaths due to its aggressiveness although it is responsible for only 1% of all skin cancers (Eddy et al. 2020). The incidence of melanoma has increased over the years and therefore it is also important to diagnose the disease at an early stage because it may still be curable. Fortunately, melanoma is often diagnosed before it has formed distant metastases (Shah and Dronca 2014), which is important because the five-year survival rate for primary melanoma is approximately 93% (Siegel et al. 2022). However, relapse of melanoma is still a problem, and there is also a risk that the cancer will return at a more advanced state (Shah and Dronca 2014). Five-year survival for patients with metastasized melanoma is approximately 30% (Kestel et al. 2022; Siegel et al. 2022). In addition, metastatic melanoma usually develops resistance to treatments and therefore it is also essential to look for new approaches to treat melanoma patients (Paluncic et al. 2016).

Melanoma develops from the transformed melanocytes of the skin (Sundararajan et al. 2022). Melanocytes are located in places such as the epidermis of the skin, the eye and the meninges (Paluncic et al. 2016). Melanocytes are derived from neural crest cells and synthesize melanin pigments that give the skin its color and protect against ultraviolet radiation (van der Kooij et al. 2019). Melanoma cells resemble the precursors of melanocytes and may also use the same signaling pathways as their precursors (Liu et al. 2014). For example, Notch signaling pathway which is an important paracrine signaling system present in most animals, is required to maintain the precursor cells of melanocytes, and the inhibition of signaling causes defective pigmentation in mice (Moriyama et al. 2006). However, the pathway may also be dysregulated in melanoma cells, giving the cells oncogenic properties (Liu et al. 2014).

Melanoma can be classified into several subtypes based on the etiology and progression of the cancer (Sundararajan et al. 2022; Yeh and Bastian 2021). Melanoma develops from melanocytes in a multistep process in which the malignancy of cancer cells increases over time (Villanueva and Herlyn 2008). In the beginning, benign proliferations occur in melanocytes,

but eventually mutations can accumulate in melanoma cells and the cells may invade to the underlying tissue and send metastases (Shain and Bastian 2016). The stages of melanoma can be classified as 0, I, II, III and IV, of which IV represents metastatic melanoma (Eddy et al. 2020). However, the progression of melanoma is not so straightforward and the cancer can progress in different ways and even skip some stages of progression (Shain and Bastian 2016).

In melanoma, the mutational burden is high and some mutations probably drive the melanocytic transformation and tumor progression in the early stages of cancer (Eddy et al. 2020). These mutations can occur in several signaling pathways and differ between melanomas (Yeh and Bastian 2021). Mutations might affect proliferation, growth, cell cycle and apoptosis of the cells, and they accumulate in cancer cells when melanoma progresses (Shain and Bastian 2016). Although genetic alterations are needed for the development of melanoma, microenvironmental factors, such as other cell types and growth factors in the tumor environment, are also important for the process (Paluncic et al. 2016). In addition to these, also environmental factors, such as ultraviolet radiation, affect the progression of melanoma (Yeh and Bastian 2021). In sun-induced melanomas, the mutation burden is typically higher than in melanomas that are developed without chronic sun damage (Shain and Bastian 2016).

Melanoma cells can disseminate to anywhere in the body, such as lymph nodes, lungs or brain (Shain and Bastian 2016). Melanoma cells spread to distant sites via lymphatic vessels, and lymphatic invasion is known to be positively correlated with sentinel lymph node (SNL) metastasis (Moy et al. 2017). Peterson et al. (2009) have shown that lymphatic invasion decreases the overall survival of melanoma patients. Cancer cells can reach distal lymph nodes via the SLN and then enter the systemic circulation and metastasize to distal organs (Alitalo and Detmar 2012). Although SNL metastasis is an effective predictor of survival (Moy et al. 2017), it may rather indicate that the dissemination of melanoma cells has already happened (Shain and Bastian 2016). Furthermore, there are limitations in assessing which patients are at risk of developing SLN metastasis and who should undergo SLN biopsy (Moy et al. 2017).

1.1.1 Melanoma microenvironment

Nowadays, it has become clear that the progression of cancer is not only due to the accumulation of mutations, but also the surrounding microenvironment affects the malignant properties of cells (Lee and Herlyn 2007). The tumor is surrounded by stroma, which contains

extracellular matrix (ECM), non-neoplastic cells and microvessels, and growth factors and cytokines secreted by neoplastic or non-neoplastic cells (Ruiter et al. 2002). Stroma and its components are thought to play a role in tumor initiation, progression, as well as metastasis of cancer cells (Lee and Herlyn 2007). Melanoma cells interact with microenvironment through cell-cell and cell-matrix contacts, but also through soluble factors secreted by different cell types (Villanueva and Herlyn 2008). Both, the soluble components and the stromal cells can influence the metastatic properties of melanoma cells (Ruiter et al. 2002).

In addition to melanocytes, fibroblasts and keratinocytes are two other major cell types in the epithelium, and both interact with melanoma cells and can influence their properties (Lee and Herlyn 2007). Fibroblasts are stromal cells that produce ECM and maintain homeostasis in the epithelium (Lee and Herlyn 2007). Fibroblasts can secrete growth factors that stimulate the growth and proliferation of melanoma cell (Villanueva and Herlyn 2008; Lee and Herlyn 2007). In turn, melanoma cells can stimulate fibroblasts to secrete these growth factors, so there is an activation cycle between melanoma cells and fibroblasts that maintains a microenvironment favorable for tumor progression (Lee and Herlyn 2007). Keratinocytes regulate the proliferation of melanocytes under normal conditions, but in melanoma this control mechanism does not work (Villanueva and Herlyn 2008). Keratinocytes and melanocytes interact via E-cadherins, but the expression of E-cadherins is downregulated in melanoma cells and thus the adhesion between these two cell types is also reduced. Loss of adhesion may allow melanoma cells to disseminate from the tumor (Li et al. 2001). In addition, melanoma cells can switch to express N-cadherin and thus cancer cells might interact with other cells, such as endothelial cells, that also express N-cadherin (Villanueva and Herlyn 2008).

Melanoma cells can also secrete growth factors that can modulate their microenvironment by activating other cell types to induce angiogenesis or produce more stromal proteins (Villanueva and Herlyn 2008). For example, melanoma cells can produce vascular endothelial growth factor (VEGF), which stimulates the proliferation of endothelial cells and the formation of new blood vessels, which will then deliver oxygen and nutrients to the tumor (Eddy et al. 2020). Melanoma cells may also produce transforming growth factor-β (TGF-β), which can stimulate fibroblasts to produce more ECM components, which in turn can enhance the survival and metastasis of melanoma cells (Berking et al. 2001).

If melanoma cells begin to spread from the primary tumor, the cells must also survive during migration and in the new microenvironment, and thus the cells need to develop mechanisms to

grow successfully in the new location (Villanueva and Herlyn 2008). For example, in order to migrate to new locations, melanoma cells need to undergo Epithelial Mesenchymal Transition (EMT), a process in which epithelial cells become more migratory and invasive (Eddy et al. 2020). The authors also propose that tumor cell can actively intravasate via EMT, or -passively without EMT by shedding from the primary tumor, or by combining these two mechanisms.

1.1.2 Interaction of melanoma and lymphatic endothelial cells

Lymphatic endothelial cells (LECs) are one of the components in the tumor stroma. Melanoma cells metastasize via lymphatic vessels (Moy et al. 2017), but evidence is increasing that LECs can also facilitate the metastatic process of melanoma cells (Ubellacker et al. 2020; Pekkonen et al. 2018). Lymphangiogenesis, a process in which new lymphatic vessels grow in cancer or inflammation, is also an important process for melanoma progression (Alitalo and Detmar 2012). Tumor lymphangiogenesis has been shown to be increased in metastatic melanomas and it correlates with decreased overall survival of patients (Dadras et al. 2003). Lymphangiogenic growth factors such as VEGFC and VEGFD promote lymphangiogenesis (Stacker et al. 2014). *VEGFC* expression in cancer cells is associated with the increased metastasis of cancer cells and poor prognosis of patients (Lund et al. 2012). Dadras et al. (2003) suggest that in melanoma, cancer cells produce VEGFC, which is supplemented by VEGFC produced by stromal cells.

Cancer cells can adopt a dormant phenotype in the lymphatics before they metastasize to distant sites, or they may even proliferate in the lymphatics. In the dormant state, cancer cells are resistant to chemotherapies that target proliferative cells (Alitalo and Detmar 2012). Ubellacker et al. (2020) have shown that the survival of melanoma cells in the blood is increased after the cells have been exposed to the lymphatic circulation, which also protects melanoma cells from an intracellular iron-dependent form of cell death, ferroptosis. Chemokines produced by LECs might also attract cancer cells to the lymphatics and thus influence the metastasis of cancer cells. It is also possible that interaction with LECs induces immunity in cancer cells and thereby protects cancer cells from being attacked by immune cells (Stacker et al. 2014). Lund et al. (2012) have shown that LECs present cancer cell-specific antigens to immune cells and this increases the apoptosis of T cells specific to these antigens. Furthermore, Pekkonen et al. (2018) have shown that the metastatic properties and aggressiveness of melanoma cells are increased when melanoma cells interact with LECs in a co-culture setting. Interaction with LECs also

alters gene expression in melanoma cells and the expression of several genes such as *NOTCH3* is increased (Pekkonen et al. 2018).

1.1.3 Melanoma research models

Cancer cells are typically studied *in vitro* using two-dimensional (2D) cell culture methods (Lv et al. 2017). However, these 2D models are not an ideal way to study solid cancers because the 2D culture conditions do not reflect the actual tumor microenvironment, where cell-cell and cell-ECM contacts also influence the functions of the cells (Lv et al. 2017). Three-dimensional (3D) culture methods are a more promising way to study the behavior of melanoma cells under conditions that more closely mimic the *in vivo* situation without the need for animal models (Alve et al. 2021). The properties of cancer cells, such as differentiation, signal transduction, and drug resistance can be studied in 3D models in ways that better reflect the tumor microenvironment than 2D models (Lv et al. 2017).

Alve et al. (2021) have developed an approach to investigate the behavior of melanoma cells in a 3D cross-linked fibrin matrix. In this model, cancer cells can be studied alone or together with some other cell type under 3D culture conditions. The fibrin matrix provides melanoma cells an environment that represents their actual ECM, as fibrin is typically found in the melanoma microenvironment. The properties of melanoma cells, such as invasive potential, can be studied in the fibrin matrix by staining the cells with different antibodies or fluorescent markers and followed by imaging with a confocal microscope.

1.2 Notch signaling

The Notch signaling pathway was discovered in the early 1900s in *Drosophila Melanogaster,* in which the pathway has been studied a lot (Siebel and Lendahl 2017). The Notch signaling is a highly conserved signaling pathway in metazoans that is involved in the regulation of proliferation and differentiation of cells, and controls cell death and the determination of cell fates (Kopan and Ilagan 2009). It is active during development and participates in various processes such as the formation of organs (Zhou et al. 2022), but it also participates in the maintenance and regeneration of the tissues in adult organisms (Hosseini-Alghaderi and Baron 2020; Kopan and Ilagan 2009). Because it is needed for fundamental functions of this kind,

defective Notch signaling may cause a pathological state (Zhou et al. 2022; Fortini 2009). For example, the overactivity of Notch signaling is related to many cancers, including melanoma (Capaccione and Pine 2013). Notch signaling differs from other typical signaling pathways in several ways, one of which is that the ligand-presenting and receptor-expressing cells need to be in cell-cell contact (Bray 2016). The basic mechanism of Notch signaling is simple, as the intracellular, cleaved domain of the receptor acts as a transcription factor, but the outputs of signaling are still diverse and complex (Bray 2016) and the responses of signaling might be different even in the same tissue (Zhou et al. 2022).

Notch receptors are transmembrane proteins and in mammals there are four of them (Notch1-4). Notch precursors are post-translationally modified in the Golgi apparatus, where glycosyl groups necessary for the stability and proper function of the receptor are added to the precursor. After glycosylation, the precursor is enzymatically cut into two parts (S1 cleavage) and thus heterodimer is formed. The heterodimeric receptor is translocated to the cell membrane and is held together by non-covalent interactions. Epidermal growth factor (EGF)-like repeats are found in the extracellular domain of the receptor, some of which are required for ligand binding. The negative regulatory region (NRR) is located between the EGF repeats and the cell membrane. NRR is needed for the activation of the receptor (S2 cleavage), but it also prevents the activation of the receptor without ligand binding. The intracellular part of the receptor includes a region that interacts with other transcription factors (RBP-Jkappa-associated module, RAM) and a region that localizes Notch intracellular domain (NICD) to the nucleus. (Kopan and Ilagan 2009; Zhou et al. 2022.)

Humans have five known Notch ligands and these are the delta-like ligands (DLL) DLL1, DLL3 and DLL4, and Jagged (JAG) 1 and 2 (Zhou et al. 2022). The ligands are also transmembrane proteins, as are the receptors. Ligands are structurally similar to receptors and contain EGF-like repeats in their extracellular domain as well (Zhou et al. 2022). The N-terminus of the ligand also has a Delta/Serrate/LAG-2 (DSL) domain, which is responsible for the specific binding to the receptor, and the EGF domains assist this binding (Cordle et al. 2008). Because ligands and receptors are transmembrane proteins expressed on separate cells, Notch signaling depends on cell-cell contacts (Fortini 2009). The ligands guide different cell fates in the Notch-expressing cells that are activated by the ligands. Delta ligands act by a negative feedback mechanism, where Delta inhibits Delta expression and promotes Notch expression in the neighboring cell receiving the signal (Bocci et al. 2020). In turn, Jagged ligands act by a positive feedback mechanism when they activate the expression of Notch and

Jagged in the neighboring-cell receiving the signal (Bocci et al. 2020). These signaling mechanisms can also be called lateral inhibition and lateral induction mechanisms, respectively (Bocci et al. 2020; Sjöqvist and Andersson 2019). Due to these mechanisms, the activity of the signaling pathway also enhances Notch signaling in the receptor-expressing cell and can direct the signal-sending and the signal-receiving cells to adopt different cell fates (Fortini 2009).

1.2.1 Notch3

Notch3 is one of the Notch family receptors and it plays a role during development in processes such as the differentiation of pericytes and vascular smooth muscle cells, but also in the adult organism in terms of the proper function of vasculature (Hosseini-Alghaderi and Baron 2020). It has been shown in a mouse knockout study that Notch3 is not essential for embryonic development (Krebs et al. 2003). Different Notch receptors work in collaboration, but they also have more specific functions and the functions can even be opposite (Hosseini-Alghaderi and Baron 2020). Notch signaling is involved in the regulation of stem cell differentiation, and Notch3 signaling has been found to limit the proliferation of neural stem cells, whereas Notch1 signaling promotes it (Kawai et al. 2017). Similar results have been observed in the differentiation of other stem cells, such as muscle satellite cells (Kitamoto and Hanaoka 2010) and mammary luminal progenitor cells (Lafkas et al. 2013), whose proliferation is also regulated by Notch3 signaling.

Although the structure and function of different Notch receptors are quite similar, there are still some differences between the receptors. The NRR of Notch3 has been reported to be less stable than the NRR in Notch1 or Notch2, and this is associated with the increased ligand-independent activity of the receptor and may also play a role in pathogenesis (Xu et al. 2015). The function of Notch3 may also differ from the other Notch receptors, as it has been reported that NICD3 activates the expression of Notch target gene *HES1* poorly compared to NICD1, and NICD3 may even suppress NICD1-mediated *HES1* and *HES5* expression (Beatus et al. 1999).

Notch3 has oncogenic functions and it is known to be upregulated and overactivated in different types of cancers (Hosseini-Alghaderi and Baron 2020). *NOTCH3* upregulation has been found to be related with chemoresistance and poor survival in patients with ovarian serous carcinoma (Jung et al. 2010). In addition, the expression of *NOTCH3* is associated with recurrence of ovarian high-grade serous carcinoma (Park et al. 2010). Furukawa et al. (2013) have found that

Notch3 signaling is involved in cancer cell migration in colorectal cancer. Notch3 signaling has also been shown to promote the growth of basal and triple negative breast cancers (Choy et al. 2017; Leontovich et al. 2018). Choy et al. (2017) reported that this oncogenic Notch3 signaling is ligand-independent and constitutively active in basal breast cancer. It has also been shown that non-canonical Notch3 signaling via activating PI3K/Akt pathway maintains cholangiocarcinoma cell survival (Guest et al. 2016). Although Notch3 signaling is associated with tumorigenesis, mutations in *NOTCH3* are rare in common human cancers (Lee et al. 2007). However, activating *NOTCH3* mutations can be found, for example, in some T-cell acute lymphoblastic leukemia cell lines (Bernasconi-Elias et al. 2016).

It has also been reported that Notch3 signaling may have a tumor suppressive role in some lung cancers, while in other lung cancers it may promote tumor growth (Hassan et al. 2016). In addition, Notch3 has been observed to act as a tumor suppressor in medullary thyroid carcinoma, where *NOTCH3* expression is lower in cancer tissue than in normal tissue (Jaskula-Sztul et al. 2015). Cui et al. (2013) have reported that Notch3 signaling promotes cellular senescence through canonical Notch signaling, and this was also observed in some tumor cells.

1.2.2 Notch3 and melanoma

The significance of Notch3 signaling in melanoma growth and tumorigenesis has been much less studied than the role of Notch signaling in some other cancers. However, different Notch receptors have a different role in the function of melanoma cells (Liu et al. 2014). In uveal melanoma, Notch1 and Notch2 appear to induce cell growth (Asnaghi et al. 2012), while Notch4 promotes the proliferation of cells in aggressive melanoma cell lines (Hardy et al. 2010). However, Notch3 signaling appears to promote melanoma cell invasion but it does not appear to affect the growth of the melanoma cells (Liu et al. 2014). Pekkonen et al. (2018) have observed that the expression of *NOTCH3* is increased in melanoma cells co-cultured with LECs, and this interaction induces the invasiveness of melanoma cells in a Notch3-dependent manner. Howard et al. (2013) have reported that co-culture with blood endothelial cells also stimulates Notch3 signaling in melanoma cells. In addition, they showed that the overexpression of *NOTCH3* can enhance the migration of melanoma cells.

Normal melanocytes (Liu et al. 2014) or nonaggressive melanoma cells do not express *NOTCH3*, but malignant melanoma cells are strongly expressing *NOTCH3* (Hendrix et al.

2002). It has also been suggested that Notch3 expression in patient samples correlates with melanoma malignancy (Howard et al. 2013). In addition, it has been shown that Notch3 signaling is involved in the induction of stem-cell like properties of melanoma cells (Hsu et al. 2017). However, it has also been observed that Notch3 promotes cellular senescence and the expression of *NOTCH3* is reduced in melanoma samples compared to non-cancer tissue (Cui et al. 2013). They found out that when Notch3 was restored in the melanoma cells, the proliferation of cells was inhibited.

1.2.3 Canonical Notch signaling

Notch signaling can be classified into canonical and non-canonical signaling depending on the activation mechanism of signaling pathway. The canonical form of Notch signaling is activated when the ligand in the ligand-presenting cell binds to the extracellular part of the Notch receptor of the cell receiving the signal (Kopan and Ilagan 2009) as shown in Figure 1. The adhesion force between the ligand and the receptor is especially high, which promotes the initiation of the signaling cascade in the Notch-expressing cell (Ahimou et al. 2004). When the ligand binds to the receptor, endocytosis of the ligand back into the ligand-expressing cell is activated (Nichols et al. 2007). Meloty-Kapella et al. (2012) have proposed that endocytosis of the ligand generates a mechanical pulling force that induces a conformational change in Notch and this allows the subsequent proteolytical activation of Notch signaling to occur. Conformational change in the receptor reveals the site 2 (S2) for proteolytical cleavage and ADAM metalloproteases cleave the receptor near the membrane (Siebel and Lendahl 2017). Without ligand activation, the NRR domain covers the S2 cleavage site and thus the activation of receptor is inhibited (Zhou et al. 2022). After the S2 cleavage, NICD and transmembrane domains of the receptor, together called Notch extracellular truncation (NEXT), remain on the membrane (Zhou et al. 2022). The Notch extracellular domain (NECD) is endocytosed with the ligand into the ligand-presenting cell (Nichols et al. 2007). NEXT is further cleaved at the site 3 (S3) by γ-secretase, which releases NICD from the cell membrane into the cytoplasm (Kopan and Ilagan 2009). It is possible that the S3 cleavage may take place on the cell membrane or on the endosomal membrane (Tagami et al. 2008).

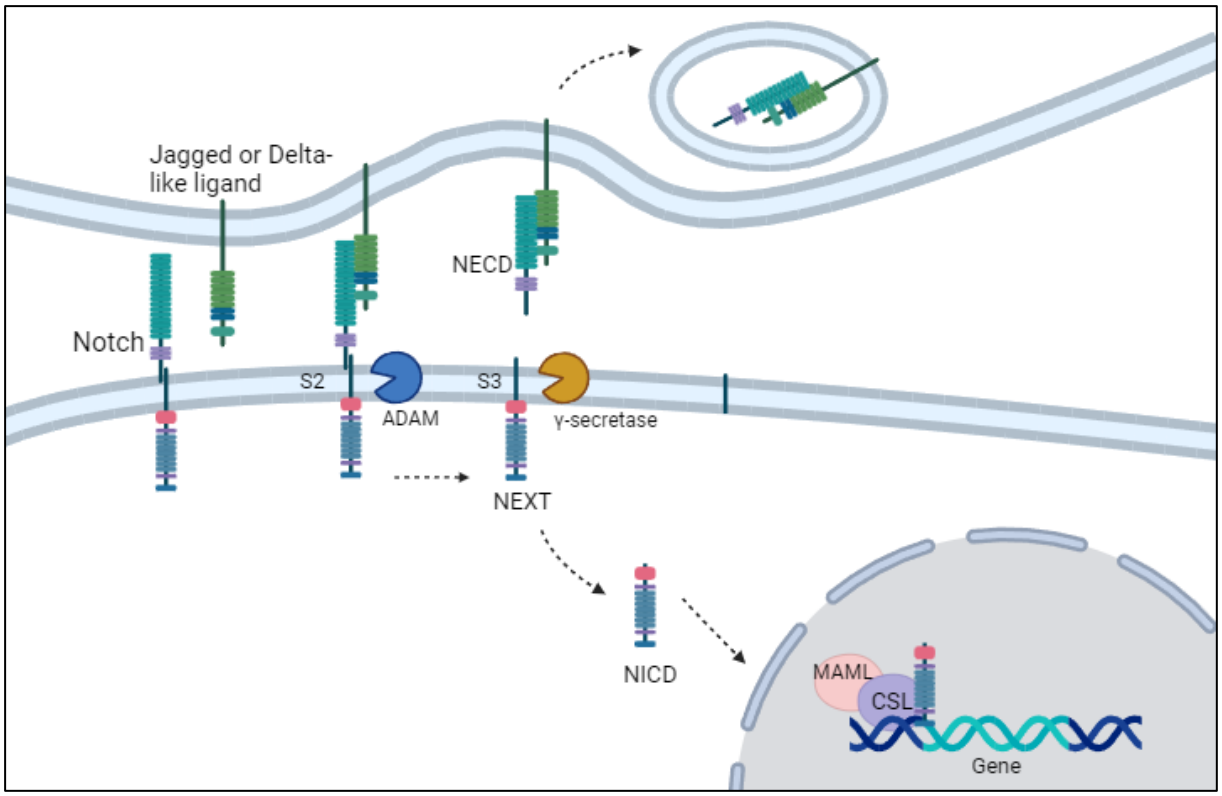

Figure 1. The canonical Notch signaling pathway. The ligand binds to the receptor, which causes endocytosis of the ligand. This induces a conformational change in the receptor and thus ADAM (S2 cleavage) and γ-secretase (S3 cleavage) can cleave the receptor. Proteolytical cleavages release NICD from the membrane and it can move into the nucleus and form a transcriptionally active complex with CSL and MAML. This complex will then activate the transcription of Notch signaling target genes. Abbreviations are explained in the main text. Picture created in BioRender.com (Kopan and Ilagan 2009).

After the S3 cleavage, NICD translocates to the nucleus via a mechanism which details are unclear (Zhou et al. 2022). However, the nuclear localization sequence of NICD and importins may be involved in this process (Huenniger et al. 2010). In the nucleus, NICD forms a complex through its RAM domain with CSL (CBF-1/RBP-Jκ, Su(H), Lag-1), a DNA-binding protein also called RBP-Jκ (Recombination Signal Binding protein for Immunoglobulin Kappa J Region) (Kopan and Ilagan 2009). CSL regulates the transcriptional activity of Notch target genes (Wilson and Kovall 2006). In the absence of NICD in the nucleus, CSL represses the transcriptional activation of Notch target genes, but the presence of NICD converts it into a transcriptional activator (Wilson and Kovall 2006). The binding of NICD to CSL also recruits other transcriptional activators, such as the Mastermind-like protein (MAML), to bind to the transactivation complex (Zhou et al. 2022). The binding of NICD and MAML to CSL changes the conformation of CSL and it becomes a transcriptional activator (Wilson and Kovall 2006). Other coactivators are also recruited into the complex, after which the transcription of Notch

target genes is activated (Kopan and Ilagan 2009). Since one NICD forms one transactivation complex, the activity of Notch signaling is determined by the number of ligand-activated receptors (Kopan 2012). Because NICD acts as a transcription factor itself, the pathway has no second messengers or other amplifying mechanisms that distinguish it from other typical signaling pathways (Fortini 2009).

The target genes of Notch signaling are, for example, hairy/enhancer of split (*HES*) and hairy/enhancer of split related to YRPW motif (*HEY*) -genes (Borggrefe and Oswald 2009). Hes and Hey proteins are also transcription factors and they act as suppressors of transcription (Borggrefe and Oswald 2009). Both of these transcription factors can act as homodimers, but they can also function as heterodimer if the cell expresses them together (Iso et al. 2003). Heterodimerization may increase the DNA binding efficiency of the dimer and thus Notch signaling may function more potentially through this mechanism in cells expressing both transcription factors (Iso et al. 2003).

1.2.4 Non-canonical Notch signaling

Notch signaling can also be activated in other ways than described above. In non-canonical Notch signaling, signaling is activated independently of CSL and can be independent or dependent on ligand binding to the receptor (Andersen et al. 2012). Ligand-independent Notch signaling typically results from a disruption in receptor endosomal sorting (Palmer and Deng 2015). Non-canonical form of Notch signaling has been much less studied than the canonical pathway (Ayaz and Osborne 2014). It is possible that non-canonical form of Notch signaling can be more susceptible to cause some pathological states such as cancer, than the canonical pathway, which may be more important in normal physiological processes (Ayaz and Osborne 2014).

Non-canonical Notch signaling can be activated after the binding of a Notch ligand to the receptor, but the activation of target genes is mediated without CSL (Sanalkumar et al. 2010). NICD is still cleaved from the membrane, as in canonical signaling, and it can then interact with other signaling pathways leading to activation of Notch target genes or other transcription factors (Sanalkumar et al. 2010). For example, non-canonical Notch signaling interacts with the JAK/STAT pathway and thereby activates the expression of IL-6 (Interleukin 6) through a mechanism regulated by the NF-kB and p53 pathways (Jin et al. 2013). It is also known that

Notch can interact with other signaling pathways, such as Wnt/β-catenin signaling (Andersen et al. 2012). Non-canonical Notch signaling has been shown to regulate the activity of β-catenin in *Drosophila,* and this also applies to vertebrates (Hayward et al. 2005). In addition, β-catenin can modulate Notch1 signaling and its transcriptional activity by enhancing it (Jin et al. 2009). It has been shown that NICD1 can also interact with HIF-1α (Hypoxia-inducible factor 1-alpha) in hypoxia, and this can activate Notch signaling of the cell (Gustafsson et al. 2005).

Another way for non-canonical Notch signaling is independent of the cleavage of NICD from the membrane by γ-secretase (Sanalkumar et al. 2010). For example, Notch signaling can activate the PI3K/Akt pathway and thus cytokine production independently of γ-secretase (Gentle et al. 2012). It is also possible that JAG1 may promote the tumorigenicity of cancer cells in a Notch receptor-independent manner (Pelullo et al. 2019). Other signaling pathways, such as Ras/MAPK signaling, may directly regulate the expression of Notch target genes without Notch activation (Stockhausen et al. 2005). Steinbuck et al. (2018) have shown that Notch signaling can be activated in T lymphocytes by TCR (T cell receptor) signaling in a Notch ligand-independent manner, where receptors are endocytosed and proteolytic cleavages are carried out in endosomes.

If the ligand does not activate the Notch receptor, it is targeted for degradation due to ubiquitination (Palmer and Deng 2015). Endosomal degradation of Notch receptors has been studied a lot with *Drosophila melanogaster* (Hori et al. 2004; 2011). It is possible that Notch signaling is activated without ligand binding in the endosomal compartment (Hori et al. 2004). Hori et al. (2004) have found that ubiquitin ligase Deltex can induce Notch to be internalized into late-endosomes, where it can prevent Notch degradation and promote the non-canonical Notch signaling. They reported that this Deltex-mediated activation of Notch signaling was independent of CSL (Su(H) in *Drosophila*). They further found that Deltex inhibits Notch degradation by antagonizing the effect of proteolysis mediated by the ESCRT (endosomal sorting complex required for transport) complex. They showed that Deltex was also able to activate Notch signaling at the membrane of an endosome in a ligand independent manner, but this still required γ-secretase and Su(H). Thus, it is possible that Deltex mediated non-canonical Notch signaling can be independent or dependent on CSL. It is not known in details how this endosomal activation of non-canonical signaling works, but it is possible that NRR is destabilized under lysosomal conditions and this promotes signaling activity of Notch (Bray 2016; Steinbuck and Winandy 2018). Another possible mechanism for endosomal activation of Notch is that NECD is proteolyzed under lysosomal conditions or it is dissociated in some other

way and thus NRR is also removed and γ-secretase can cleave NICD and release it into the cytosol with or without ADAM mediated S2 cleavage (Steinbuck and Winandy 2018).

It is possible that non-typical Notch ligands also have the ability to activate Notch signaling, and these are called non-canonical ligands (D'Souza et al. 2008). Some of these ligands may use CSL as an effector of Notch signaling, while some may use Deltex (D'Souza et al. 2008). For example, Rauen et al. (2009) have shown that YB-1 protein (Y-box binding protein 1) can bind to Notch3 and activate signaling through it. Similarly, DLK1 (Delta-like non-canonical Notch ligand 1) has been found to interact directly with Notch1 in mammals and it appears to regulate Notch signaling (Traustadóttir et al. 2016). In addition to these membrane-bound non-canonical ligands, some non-canonical ligands may exist in a secreted form (D'Souza et al. 2008). Miyamoto et al. (2006) have shown that microfibril-associated glycoproteins MAGP-1 and MAGP-2 can activate Notch1 signaling without the need for ADAM metalloproteases. However, it is not exactly known, how these non-canonical ligands mediate the activation of Notch signaling and it is possible that they act indirectly (Siebel and Lendahl 2017).

1.3 Regulation of Notch signaling

The activity of Notch signaling pathway is under thigh control, and the mechanisms that are involved in the regulation of Notch activity are diverse. This is important because dysregulation of Notch signaling may lead to various diseases such as cancer (Hosseini-Alghaderi and Baron 2020). However, the regulation differs from other typical signaling pathways because the cleaved receptor acts as a transcription factor thus reducing the possibilities of regulation of the pathway (Bray 2016). For example, there are no amplification or integration steps in the pathway, so the regulation has to be done in other ways (Siebel and Lendahl 2017). One important level of regulation is to regulate the number of different receptors, ligands and enzymes needed to activate the signaling pathway (Bray 2016).

First, the Notch receptor is post-translationally modified in the Golgi apparatus by glycosylation prior being trafficked to the cell membrane (Zhou et al. 2022). Glycosylation is essential for the proper function of the receptor, but it can also affect ligand binding affinities between the receptor and its ligands (Bray 2016; Fortini 2009). Glycosylation can also affect processing of the receptor. For example, glycosylation catalyzed by glycosyltransferase Rumi has been shown to be essential for S2 cleavage and thus signaling transduction after ligand binding to the

receptor in *Drosophila* (Acar et al. 2008). It is possible that this glycosylation is needed for the correct folding of Notch and thus lack of Rumi prevents normal S2 cleavage of the receptor (Acar et al. 2008). Three-step proteolytical cleavage ensures that Notch signaling is activated only when the receptor is properly synthesized, presented on the cell membrane, and activated by a suitable ligand (Fortini 2009). After S3 cleavage, two different types of NICDs can be formed, which have different stabilities and thus they might transmit signals with varying intensities, so proteolysis can also influence the outcomes of signaling (Tagami et al. 2008).

The activation of Notch is regulated in various ways in cells. Notch ligands in neighboring cells activate Notch signaling of the receptor-expressing cell (*trans*-activation), while ligands in the receptor-expressing cell repress ligand-dependent signaling (*cis*-inhibition) (Palmer and Deng 2015). It has also been found in *Drosophila* that *cis*-inhibition can attenuate endogenous ligand-independent Notch signaling (Palmer et al. 2014). They suggest that *cis*-inhibition is possibly a cellular mechanism that keeps Notch pathway ready for activation from outside of the cell. In addition to this, Ladi et al. (2005) have shown that instead of activating the Notch signaling pathway in cells, DLL3 inhibits signaling activated by other Notch ligands, thus acting as an antagonist of Notch. Geffers et al. (2007) propose that DLL3 functions primarily inside the cell at the Golgi apparatus, where DLL3 binds to Notch in a *cis* manner. In addition, the NRR region of receptor prevents inappropriate activation of the Notch in the absence of ligand and is thus an important autoinhibitory regulatory mechanism of signaling activity (Gordon et al. 2007).

The activity of both ligands and receptors is regulated by endocytosis. Endocytosis regulates the number of Notch receptors on the membrane, but endocytosed receptors may also be degraded, activated in the cytoplasm without ligand binding or recycled back to the membrane (Bray 2016; Zhou et al. 2022). Whether the receptor is degraded or activated under endosomal conditions depends on regulatory proteins, mainly E3 ubiquitin ligases, that are found in the cell (Palmer and Deng 2015). McGill et al. (2009) suggest that Numb is one of the negative regulatory proteins of Notch signaling. They have shown that Numb regulates intracellular Notch1 trafficking and degradation, and its overexpression correlates with Notch1 degradation, while lack of Numb correlates with Notch1 recycling back to the membrane. McGill et al. (2009) observed that Numb is not needed for Notch1 internalization, and Numb-mediated degradation of Notch1 needs ubiquitin ligases such as Itch (Suppressor of Deltex in *Drosophila*) to target Notch1 for degradation. Hori et al. (2011) have found that Shrub, a component of the ESCRT-III complex, promotes the lysosomal degradation of Notch. However, the synergy between Shrub and Deltex determines whether Notch is degraded in lysosomes or not, as Deltex

is a protein that promotes non-canonical ligand-independent signaling in endosomes rather than the degradation of Notch (Hori et al. 2011).

E3 ubiquitin ligases in the ligand-expressing cell are important for the activation of signaling. Ligand binding to Notch activates endocytosis of the ligand, which generates mechanical pulling force to the receptor causing its conformational change and thus the activation of signaling in the receptor-expressing cell (Meloty-Kapella et al. 2012). E3 ubiquitin ligases are needed for ligand ubiquitylation in the ligand-presenting cell, which is needed for endocytosis and generation of the mechanical pulling force (Meloty-Kapella et al. 2012). Hansson et al. (2010) have found that only JAG1, which can bind to Notch, can be ubiquitinylated and endocytosed after interaction with the receptor, whereas mutant JAG1 cannot. Interaction between the ligand and the Notch appears to promote ligand ubiquitylation as reported for JAG1 and DLL1 (Hansson et al. 2010; Meloty-Kapella et al. 2012). NECD is trans-endocytosed into the ligand-expressing cell with the ligand (Nichols et al. 2007) and trans-endocytosed NECD is degraded in lysosomes along with JAG1 after signaling activation (Hansson et al. 2010).

Different NICDs may also be post-translationally modified differently, and thus the output of signaling activity of different receptors may vary (Bray 2016). The NICD turnover is also important for controlling the duration and intensity of Notch signaling (Fortini 2009). NICDs can be acetylated, methylated, ubiquitinylated, phosphorylated and hydroxylated (Borggrefe et al. 2016). Hein et al. (2015) have shown that NICD1 can be methylated by CARM1 (coactivator-associated arginine methyltransferase 1), which enhances NICD1 signaling but decreases its stability. Similarly, Guarani et al. (2011) have observed that SIRT1 (sirtuin 1) can deacetylate NICD1, which also destabilizes it. Thus, acetylation and methylation seem to be important for the duration and amplitude of NICD1 signaling activity (Hein et al. 2015; Guarani et al. 2011). However, Palermo et al. (2012) have reported that acetylation of NICD3 by acetyltransferase p300 promotes its ubiquitination and degradation, which decreases the transcriptional activity of NICD3. NICD1 can also be ubiquitinylated by Sel-10, an F-box protein, in the nucleus and this reduces NICD1 transactivation (Oberg et al. 2001). In addition, NICD1 can be hydroxylated by factor-inhibiting HIF-1, which decrease the activity of Notch signaling (Zheng et al. 2008). Glycogen synthase kinase 3 (GSK3) has been shown to phosphorylate NICD2, which decreases its transcriptional activity (Espinosa et al. 2003), whereas the phosphorylation of NICD1 promotes its activity (Foltz et al. 2002). It has also recently been found that PIM kinases can phosphorylate the NICDs of Notch1 and Notch3 (Santio et al. 2016) with different signaling outputs (Landor et al. 2021).

Fryer et al. (2004) suggest that it is MAML that recruits kinases that hyperphosphorylate NICD, which promotes proteasomal degradation of NICD due to ubiquitylation. The degradation of NICD attenuates Notch signaling in the cell and thus limits the amount of signaling activity. The duration of signaling activity may also vary between Notch receptors. For example, the number of activated NICDs that reach the nucleus and the duration of transcriptional activity is different in Notch1 and Notch2 signaling, although their signaling works on the same principles and they are structurally related (Liu et al. 2015).

1.3.1 CSL and transcriptional regulation of Notch signaling

CSL proteins are highly conserved transcription factors important in regulating the canonical Notch signaling pathway (Siebel and Lendahl 2017). CSL acts as a link between DNA and other regulatory proteins of the pathway. CSL proteins have three structural domains which are N-terminal domain (NTD), beta-trefoil domain (BTD) and C-terminal domain (CTD) (Kovall and Hendrickson 2004). CSL interacts with DNA through its NTD and BTD domains as a monomer (Kovall and Hendrickson 2004). CSL, in turn, binds to the RAM domain and ankyrin repeats of NICD through its BTD and CTD domains (Wilson and Kovall 2006). The interaction between CSL and MAML is formed through the CTD and NTD domains of CSL, but MAML also interacts with the ankyrin repeats of NICD (Wilson and Kovall 2006). This CSL-NICD-MAML complex is called a ternary complex and the formation of the complex causes conformational change in CSL (Wilson and Kovall 2006). Wilson and Kovall (2006) propose that corepressors are displaced from CSL due to this conformational change or due to the binding of NICD to the BTD domain, or then these both contribute to displacement.

CSL act as both a repressor and an activator of Notch signaling. The interaction between CSL and co-operating repressors and enhancers of transcription determines the responses of Notch signaling (Siebel and Lendahl 2017). CSL is constitutively expressed in the cells and, in the absence of NICD, it is bound together with corepressors to the promoter regions of Notch target genes in DNA keeping them silent (Sanalkumar et al. 2010). The repressor complex presumably keeps the chromatin structure in silent form, and thus genes in this region cannot be transcribed (Wilson and Kovall 2006). The presence of NICD in the nucleus and its binding to CSL causes the dislocation of corepressors from the CSL complex the recruitment of MAML to the complex (Wilson and Kovall 2006). It has also been suggested that CSL-repressor and CSL-activator complexes compete for binding to DNA in the nucleus rather than replacing CSL-bound

corepressors with coactivators (Siebel and Lendahl 2017; Bray 2016). Altogether, CSL is important in the activation of Notch target genes because NICD cannot bind to DNA itself (Borggrefe and Oswald 2009).

It is also possible that the CSL can operate independently of the NICD. Some viral oncoproteins, such as EBNA2 (Epstein-Barr virus), RTA (Kaposi´s sarcoma-associated herpesvirus), and 13SE1A (adenovirus), can bind to CSL, transform it to an activator of transcription, and activate gene expression (Strobl et al. 1997; Liang et al. 2002; Ansieau et al. 2001). This allows the viruses to manipulate Notch signaling in the transfected cells and the viruses can trigger the cell to proliferate and differentiate (Borggrefe and Oswald 2009). It has also been shown that CSL can regulate angiogenesis in the adult heart independently of Notch signaling (Díaz-Trelles et al. 2016).

Mammals have only one type of CSL, although they have many different receptors and ligands of Notch signaling (Yuan et al. 2012). Yuan et al. (2012) have shown that mutations in CSL can affect differently Notch1 and Notch2 signaling, which reveals that Notch paralogs have potential differences in function due to the different binding energies between NICD and CSL. However, the study suggested that Notch signaling activity was reduced in cells with the CSL mutation. The phenotype of CSL knockout mice is lethal and the embryo cannot fully develop during gestation (Borggrefe and Oswald 2009).

1.3.2 PIM kinases

PIM (provirus integration site for Moloney leukemia virus) kinases are serine/threonine kinases and vertebrates have three isoforms of them, named PIM1, PIM2 and PIM3 (Bachmann and Möröy 2005). The structure and function of these three kinases overlap, and they seem to compensate the functions of each other's. The expression of PIM kinases is stimulated by cytokines, mitogens or growth factors (Narlik-Grassow et al. 2014). PIM1 and PIM2 are constitutively active, and thus the activity of these kinases is likely regulated by protein turnover rather than via post-translational modifications (Fox et al. 2003; Qian et al. 2005). PIM1 can be phosphorylated, but it is more likely to affect the stability of the kinase rather than its activity (Qian et al. 2005). Heat shock proteins regulate the proteasomal degradation of PIM1 (Shay et al. 2005), but it is possible that phosphorylation may also affect PIM kinase functions (Narlik-Grassow et al. 2014). A study on PIM mutant mice reveals that PIM kinases are not essential

for development, but the animal's body size is reduced and PIM deficiency also somehow affects the differentiation of hematopoietic cells (Mikkers et al. 2004).

PIM kinases have some tumorigenic potential, but the effect is not so straightforward and the activity of other oncogenes affects the oncogenicity of PIM kinases (Narlik-Grassow et al. 2014). However, elevated levels of PIM kinases are found in various solid and hematological cancers (Brault et al. 2010). The prevalence of different PIM kinases differs between cancer types, and in many cases the poor outcome of the patient can be connected to an increase in PIM levels (Santio and Koskinen 2017). However, PIM kinases are also reported to be associated with good prognosis in some solid cancers (Nawijn et al. 2011).

Phosphorylation by PIM kinases can either positively or negatively affect the activity of their substrate proteins, which in many cases leads to cell survival or proliferation (Santio and Koskinen 2017). PIM kinases can promote cancer cell growth by regulating proteins involved in cell cycle regulation (Brault et al. 2010). PIM1 and PIM2 can phosphorylate the anti-apoptotic Bad protein and thereby promote cell survival and prevent cells from apoptosis (Fox et al. 2003; Yan et al. 2003; Aho et al. 2004; Brasó-Maristany et al. 2016). This anti-apoptotic effect of PIM1 can be reversed by inhibiting PIM kinases (Santio et al. 2010). Santio et al. (2010) have also shown that the inhibition of PIM kinases reduces the motility of cancer cells *in vitro*. Brasó-Maristany et al. (2016) have also observed that PIM1 is essential for the malignancy of many breast cancer cell lines. The inhibition of PIM kinases can also reduce the tumorigenicity of melanoma cells by decreasing melanoma cell proliferation and invasion *in vitro* and tumor growth *in vivo* (Shannan et al. 2016). A study by Rang et al. (2016) has shown that tumor suppressive miR-542-3 can target PIM1 in melanoma and inhibit cell invasion. Genetic alterations in *PIM* genes are rare in melanoma, but this does not exclude the possibility of pathway deregulation due to the constitutively active nature of PIM kinases (Shannan et al. 2016).

PIM kinases can phosphorylate Notch1 and Notch3 NICDs. All PIM kinases can phosphorylate Notch1 at its nuclear localization sequence (NLS), which enhances NICD1 nuclear localization and transcriptional activity, and promotes prostate cancer cell migration. NICD3 is phosphorylated on its RAM domain, and this inhibits the interaction of NICD3 with CSL, thereby inhibiting CSL-dependent gene expression. However, it has been shown that phosphorylated NICD3 can still act in a tumorigenic manner independently of CSL, promoting estrogen-driven breast cancer cell growth. In breast cancer cells, phosphorylated NICD3

appears to have more oncogenic potential than non-phosphorylated NICD3 does. Also, *NOTCH3* and *PIM1* upregulation correlates with poor outcome of estrogen-positive breast cancer patients. *PIM1* expression can also be enhanced by Notch1 phosphorylation, and it is possible that there is a positive feedback loop that in turn enhances Notch1 signaling through increased PIM1 expression. In conclusion, PIM kinases play a regulatory role in Notch1 and Notch3 signaling and promote the tumorigenesis of some cancers, albeit through a different mechanism. (Santio et al. 2016; Landor et al. 2021.)

1.4 Aim of the research and research questions

Melanoma is the most dangerous skin cancer due to its invasive abilities. It has been found that Notch3 signaling can increase the invasiveness of melanoma cells after co-culture with lymphatic endothelial cells (LECs) (Pekkonen et al. 2018). Since it is not known which Notch ligands activate Notch3 signaling in melanoma cells, the aim of this study is to investigate how Notch3 is activated in melanoma cells. The goal is to reveal which Notch ligands can activate Notch3 in melanoma cells and thereby increase the invasiveness of melanoma cells. The hypothesis is that a Notch ligand expressed in LECs mediates the activation of Notch3 signaling in melanoma cells.

Another aim of this study is to examine how Notch3 is regulated in melanoma cells. The regulation of Notch signaling is a complex process where many cellular proteins and interactions are involved. PIM kinases have recently been identified as important regulators of Notch1 and Notch3 signaling in breast cancer (Santio et al. 2016) and thus one of the aims of this study is to examine whether they contribute to the regulation of Notch3 signaling in melanoma cells. The relationship between PIM kinases and Notch3 signaling in melanoma has not yet been studied. The role of PIM kinases in melanoma progression is poorly understood, but it is possible that they increase the tumorigenic potential of melanoma cells (Shannan et al. 2016). Therefore, one aim is to address whether PIM kinases promote tumorigenesis of melanoma cells. The hypothesis is that PIM kinases might regulate Notch3 signaling in melanoma cells and thereby affect the invasiveness and metastatic properties of the cells.

2 Materials and methods

2.1 Cell lines and cell cultures

Experiments were done using human melanoma cell lines WM852, WM165 and WM793 (Wistar Institute, Philadelphia, PA). WM852 and WM165 are metastatic melanoma cell lines, while WM793 cell line is derived from a primary tumor (Pekkonen et al. 2018). WM852 cells are obtained from abdomen metastases and are morphologically epithelial cells (Rockland 2023a). WM165 are from lymph-node metastases and have fibroblast kind of morphology (Rockland 2023b). The WM852 cells had been stably transduced with lentiviruses encoding eGFP and Luciferase (dual eGFP-luc ;pMX-Rgt, Invitrogen) and WM165 and WM793 cells with lentiviruses encoding eGFP (pLENTI6-eGFP, Invitrogen) to visualize cells.

Melanoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine (Lonza) and 1% penicillin-streptomycin (Lonza). Blasticidine S (Sigma, 10 µg/ml) was also added to the medium of WM165 and WM793 cells to maintain the GFP expression. For splitting the cells with trypsinization (Euroclone), cells were first washed with phosphate-buffered saline (1xPBS). DMEM was used to inactivate trypsin after cell detachment. An automated cell counter (tc20, Bio-Rad) was used to count cells. For centrifugation 500g for 4 min was used unless not otherwise stated. Cells were cultured in the incubator in 5% CO^2 at +37 °C.

2.2 siRNA transfection

WM852, WM165 and WM793 melanoma cells were plated on a 6-well plate at approximately 25% confluence one day before transfection and the plate was placed in the incubator. Cells were transfected with siRNAs targeting PIM1, PIM2 or PIM3 (ON-TARGET*plus*, Dharmacon, Chicago, IL) or a non-target control siRNA (Dharmacon, Chicago, IL) using Lipofectamine RNAiMAX (invitrogen). WM852 cells were also transfected with siRNA targeting siRBP-Jκ (Santa Cruz, later in the text called siCSL). For each transfection sample, siRNAs (final concentration 10 nM per well) were diluted in 150 µl of OptiMEM (Gibco), mixed and incubated for about 5 min at room temperature. In addition, 6 µl of RNAiMAX was diluted in 150 µl of OptiMEM, mixed and incubated for about 5 min at room temperature. These two

solutions were combined, vortexed and incubated for about 10 min at room temperature. Before adding the Lipofectamine-siRNA complexes to each 6-wells containing cells, culture medium was removed, and cells were washed with PBS. 700 µl of fresh DMEM medium was added to the wells and 300 µl of lipofectamine-siRNA solution was pipetted all around the wells as small droplets. The cells were incubated at 37°C in a CO_2 incubator for 24 hours. Later, the cells were used for qPCR and western blot and the siPIM transfected WM852 cells also for the Notch activation experiments.

2.3 Activation of Notch signaling

To activate Notch signaling, WM852, WM165 and WM793 melanoma cells were treated with different Fc-ligands of Notch receptors. The ligands used were DLL1-Fc (Biotechne), DLL3-Fc (ACRO Biosystems), DLL4-Fc (Sino Biological Inc.), JAG1-Fc (Sino Biological Inc.) and JAG2-Fc (R&D Systems). In addition, a control Fc-ligand (Jackson ImmunoResearch) was used. The ligands were used at a concentration of 10 µg/ml diluted in PBS with 200 µl needed for each well of 24-well plate. The plate was incubated for about 4.5 hours at room temperature to immobilize ligands on wells. The PBS-ligand solution was removed from the wells and the wells were washed with 200 µl of PBS to remove unattached ligands. Unbound ligands were washed because these could inhibited Notch signaling, as observed in study of Klose et al. (2015), in which soluble forms of Notch ligands antagonized Notch signaling in endothelial cells. 500 µl of cell suspension was added into each coated well. WM852 cells were added to the wells at a concentration of 1,5 x 10^5 /ml, WM165 cells at a concentration of 1,0 x 10^5/ml and WM793 cells at a concentration of 0,5 x 10^5/ml. The cells were incubated at 37°C in a CO_2 incubator for 48 hours.

2.4 Three-dimensional (3D) invasion assay

Melanoma cells treated with Notch ligands in the 24-well plate were collected, centrifuged, and resuspended into 20 µl of medium (WM852; 12000 cells and WM165 and WM793; 8000 cells). Fibrinogen (6 mg/ml, EMD Millipore) was diluted in Hank´s Balanced Salt Solution (HBSS, Gibco) and was incubated in a heat bath (+37 °C) for 15 min. The fibrinogen solution was filtered using a 0.22 µm PVDF Syringe filter (Merck Millipore). Cells in 20 µl were combined

with 100 µl of fibrinogen solution and 100 µl of HBSS containing 4 U/ml human thrombin (EMD Millipore) and 400 µg/ml aprotinin (Merck Millipore) The solution was pipetted as four 50 µl droplets on the bottom of a 6-well plate so that one droplet contained approximately 3000 (WM852) and 2000 (WM165 and WM793) cells. Fibrin droplets were incubated in the incubator in 5% CO^2 at +37 °C for 30 min, after which 3 ml of medium supplemented with aprotinin (44,4 µg/ml) was added to each well. The cells were allowed to grow in the incubator in 5% CO^2 at +37 °C for four days.

Fibrin droplets were fixed with 3 ml of 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature. The droplets were washed with 3 ml of PBS and another 3 ml of PBS was added to prevent drying of droplets. The droplets were transferred to a 48-well plate, where they were post-fixed with 500 µl of ice-cold acetone-methanol (1:1) for 1 min. The droplets were washed with 500 µl of PBS and another 500 µl of PBS was added to prevent drying of droplets. Droplets were blocked by incubation in 500 µl of blocking buffer (15% FBS + 0,3% TritonX in PBS) for 1 hour at room temperature. Blocking buffer was removed and 150 µl of Texas Red Phalloidin (Molecular Probes) diluted in blocking buffer (1:200) was added to the wells and incubated over night at +4 °C. The phalloidin solution was removed from the wells and the droplets were washed with 500 µl of washing buffer (0,3% TritonX in PBS) for 3x15 min. The fibrin droplets were incubated with 500 µl of Hoechst 33342 (Sigma-Aldrich) in PBS (1 µg/ml) for 20 min at room temperature and washed with 500 µl of washing buffer for 2x15 min and with 500 µl of PBS for 1x15 min. Droplets were rinsed with MQ-water and transferred to microscope glass slides. 200 µl of Mowiol was added on top of the droplets and glass objectives were placed on top.

The droplets were imaged with a Zeiss LSM780 confocal microscope with a z-stack imaging option using a 10x objective. Cell clusters in z-stack images were analyzed by thresholding the intensity of GFP or phalloidin fluorescence of the cells in Image J software. The Z-stack images of the droplets were quantified and analyzed with Image J software using the ´skeleton´ tool. The average length of branches of the cell clusters was used as a measure of cell sprouting in the fibrin matrix. The final sprouting index of each sample shows the average branch length value from 4-5 images per sample. The relative branch length of the cell clusters was obtained by normalizing the other ligand treatments to the Fc-control treatment. Experiments were repeated at least two times. One-way ANOVA and Tukey´s multiple comparison tests were used to calculate differences between different treatments using Graphpad Prism (9.5.0) software.

2.5 Real time quantitative PCR (qRT-PCR)

Real time quantitative PCR (qRT-PCR) was used to study the gene expression of melanoma cells after different treatments. Notch ligand treated or siRNA transfected WM852, WM165 and WM793 cells, or siRNA transfected WM852 cells treated with Notch ligands, were collected from the well plates (see 2.2 and 2.3), after which the cells were centrifuged, and pellets were stored at -80 °C for later use. RNA was isolated from frozen cells following RNA isolation protocol of the NucleoSpin RNA II kit (Machinery Nagel, protocol in appendix 2, step 1 of the protocol was not done). The RNA concentration of the samples was measured by using nanodrop (Thermo Fisher Scientific).

Two different cDNA synthesis kits were used to translate RNA into cDNA; however, the same kit was used for samples that belonged to the same experiment and were compared to each other. When using kit 1 (Taqman reverse transcription kit, N8080234, Applied Biosystems), 500 ng of RNA was diluted in 19,3 µl of nuclease free water. A reverse transcription (RT) reaction mix was prepared for the samples according to Table 1, and 30,7 µl of the solution was added to the diluted RNA samples. The RT reaction was done using a Thermocycler (Bio-Rad).

Table 1. Reverse transcription reaction setup for one RNA sample using Applied Biosystems kit

Reverse transcription reaction mix	**1x reaction volume**
10x buffer	5 µl
25 mM $MgCl_2$	11 µl
dNTP	10 µl
Oligo (T)	2,5 µl
RNAse inhibitor	1 µl
Multiscripe	1,25 µl
Priming 25°C 10 min Reverse transcription 48°C 30 min RT inactivation 95°C 5 min Optional step Hold at +4°C	

Using another cDNA synthesis kit (Bio-Rad, 1708891), 500 ng of RNA was diluted in 15 µl of nuclease-free water. The RT reaction mix was prepared according to Table 2 and 5 µl of the solution was added to the diluted RNA samples. The RT reaction was done using a Thermocycler (Bio-Rad).

Table 2. Reverse transcription reaction setup for one RNA sample using Bio-Rad kit

Reverse transcription reaction mix	**1x reaction volume**
5x iScript Reaction Mix	4 µl
iScript Reverse Transcriptase	1 µl
Priming 25°C 5 min Reverse transcription 46°C 20 min RT inactivation 95°C 1 min Optional step Hold at +4°C	

If the concentration of RNA in the samples did not reach 500 ng in 19,3 µl or in 15 µl, a slightly lower concentration of RNA was used in the RT reaction and the end concentration was adjusted to be the same in the samples being compared. Before qRT-PCR, for cDNA samples transcribed by Bio-Rad kit, 30 µl of nuclease-free water was added so that the end cDNA concentration was the same when using both kits.

The real time RT-PCR assay was carried out on a A Lightcycler 480 system (Roche, Basel, Switzerland) using the SYBR Green PCR mix (Fermentas) (Tables 3 and 4). SYBR Green binds to dsDNA allowing monitoring of levels of amplified PCR product. Primers used in RT-PCR were *NOTCH1* (Oligomer), *NOTCH2* (Oligomer), *NOTCH3* (Qiagen), *NOTCH4* (Oligomer), *HEY1* (Oligomer), *HES1* (Oligomer), *PIM1* (Metabion), *PIM2* (Metabion), *PIM3* (Metabion) and *RBP-Jκ* (Oligomer). *ACTIN* (Oligomer) housekeeping gene was used as an endogenous control for all samples. Sequences of the primers are found in Appendix 1.

Table 3. PCR reaction solution for one cDNA sample

PCR reaction solution	**1x reaction volume**
SYBR Green	5 µl
primer (for)	0,08 µl
primer (rev)	0,08 µl
H_20 (nuclease free)	2,84 µl

Table 4. PCR reaction solution for Notch3 primer (for one cDNA sample)

PCR reaction solution for Notch3 primer	1x reaction volume
SYBR Green	5 µl
Notch3 primer	1 µl
H2O (nuclease free)	2 µl

8 µl of the prepared SYBR Green PCR mix and 2 µl of cDNA was pipetted into the wells of a 384-well plate. Each cDNA sample was pipetted as triplicates. Nuclease free water was used as a negative control. The plate was centrifuged at 1000g for 1 min. The qRT-PCR (Lightcycler 480) was run using the follow program;

Initial denaturation	95°C 10 min	
Denaturation	95°C 15 s	⎫
Annealing	60°C 30 s	⎬ 50 cycles
Elongation	72°C 30 s	⎭

To determine the relative gene expression of samples from different treatments, the qRT-PCR data was statistically analyzed by converting logarithmic values to ddCt values (linear log2 scale values). The data obtained from cells treated with Notch ligands and/or siRNAs were normalized against an internal control, i.e., Fc-control or control siRNA treated cells, respectively. The significance of any changes was tested, depending on the experiment, with one-way or two-way ANOVA and Tukey´s multiple comparison test using Graphpad Prism (9.5.0) software. At least two independent experiments were done for each cell line and different treatment, except for the treatment of WM852 cells with siCSL, which was performed only once.

2.6 Western blot

siRNA transfected or Notch ligand treated WM852, WM165 and WM793 cells were collected from the well plates (see 2.2 and 2.3) after which cells were centrifuged and the pellets were stored at -80 °C for later use. Frozen cells were lysed for western blot using RIPA lysis buffer containing protease and inhibitor cocktails (Thermo Fisher Scientific). The pellets were resuspended in the buffer, incubated for 40 min, and mixed every 10 min by vortexing,

centrifuged at 12000g for 10 min at +4 °C and the supernatants were collected. Laemmli sample buffer (Bio-Rad) supplemented with β-mercaptoethanol was added to the supernatants at a ratio of 1:3 to the volume of supernatant. Samples were incubated at 95 °C heat block for 5 min. Samples and ladder (Thermo Fisher Scientific, #26619) were loaded on a 4-15% SDS-PAGE gel (Bio-Rad, 5671084) in volumes of 30 µl and 4 µl, respectively. The gel was run at 55mA for 50 min and proteins were transferred to a nitrocellulose membrane (Bio-Rad, 1704159) using High Molecular Weight Protein Transfer system of Trans-Blot Turbo (Bio-Rad). Membranes were blocked in 5% milk in 1xTBS 0,1% Tween for 30 min to prevent unspecific binding of the antibodies. The membrane for anti-PIM3 was blocked in 3% BSA in 0,05% Tween in PBS as described in the instructions of the manufacturer. Following blocking, primary antibodies (Table 5) were diluted in 5% milk in 1xTBS 0,1% Tween or in in 3% BSA in 0,05% Tween in PBS (the anti-PIM3 antibody) and incubated with nitrocellulose membrane overnight at +4 °C on a tube roller.

Table 5. Primary antibodies used in Western blot.

Antibody	Use	Host specie	Dilution	Manufacturer	Manufacturer and Product code
Notch3	siRNA treated samples	Rat	1:1000	Cell Signaling	Cell Signaling, 3446S
Notch3	Notch ligand treated samples	Rabbit	1:500	Santa Cruz	Santa Cruz, M-143
PIM2	siRNA treated WM852 cells	Rabbit	1:500	Cell Signaling	Cell Signaling, 4723S
PIM3	siRNA treated WM852 cells	Rabbit	1:1000	ABCEPTA	ABCEPTA, AP7171a
β-actin	endogenous control for all the samples	Mouse	1:1000	Sigma	Sigma, A1978

Following incubation with primary antibodies, membranes were washed with 1xTBS 0,1% Tween for about 3x10 min at room temperature on the tube roller. Membranes were then incubated with HRP-linked secondary antibodies (Table 6) diluted in 5% milk in 1xTBS Tween for about 4 hours at room temperature on the tube roller, and again washed with 1xTBS 0,1% Tween for about 3x10 min at room temperature. Proteins were detected by chemiluminescence

using substrate solution (WesternBright Sirius, Advansta) diluted 1:20 in PBS and visualized with Chemi-Doc (Bio-Rad). The protein amounts of the samples were determined related to the amount of endogenous actin using Image Lab software. Two independent experiments were done with each cell line using Notch3 antibody. Only one experiment was done with PIM antibodies.

Table 6. Secondary antibodies used in Western blot.

Antibody	Host	Dilution	Manufacturer	Manufacturer and Product code
Anti-Rat IgG, HRP-linked	Goat	1:1000	Cell Signaling	Cell Signaling, 7077S
Anti-Mouse IgG, HRP-linked	Horse	1:1000	Cell Signaling	Cell Signaling, 7076S
Anti-Rabbit IgG, HRP-linked	Goat	1:1000	Cell Signaling	Cell Signaling, 7074S

2.7 Cell viability assay

The purpose of the experiment was to determine whether siRNA transfection affects the viability of melanoma cells. WM852, WM165 and WM793 cells were transfected with siRNAs targeting PIM1, PIM2 and PIM3 as described in section 2.2. Transfected cells were collected from the 6-well plate in which transfection was performed and were plated on a 24-well plate as duplicates. WM852 and WM165 cells were plated at a concentration of 1,0 x 10^5/ml and WM793 cells at a concentration of 0,5 x 10^5/ml. The 24-well plates were placed in the incubator where the cells were growing for 24 or 48 hours, after which the cells were collected. The number of cells in the wells was counted with trypan blue. Two independent experiments were done for each cell line. The graphs were made with GraphPad Prism (9.5.0) software.

3 Results

3.1 DLL4 and DLL1 activate Notch3 signaling pathway in metastatic melanoma cells

Notch3 signaling has been shown to increase the invasiveness of melanoma cells (Pekkonen et al. 2018). To study which of the known Notch ligands activate Notch3 signaling in melanoma cells, the expression of *NOTCH3* and its known downstream targets were investigated in melanoma cells cultured with Fc-fusions of the Notch receptor ligands immobilized on plastic. Ligand immobilization mimics the activating pulling force from native cell-cell interaction by well-defined cell-material interactions. DLL1-Fc and DLL4-Fc ligands increased the mRNA expression of *NOTCH3* and its downstream target *HEY1* in metastatic WM852 and WM165 cells, but not in non-metastatic WM793 cells (Figure 2), which suggest a positive feedback loop of Notch3 signaling especially in metastatic melanoma. The results further demonstrate that Notch3 preferentially upregulates Hey1 but not Hes1 in these metastatic melanoma cell lines, as the expression of the Notch3 signaling downstream target *HES1* was not increased in any of the cell lines after treatment with any of the ligands (Figure 2).

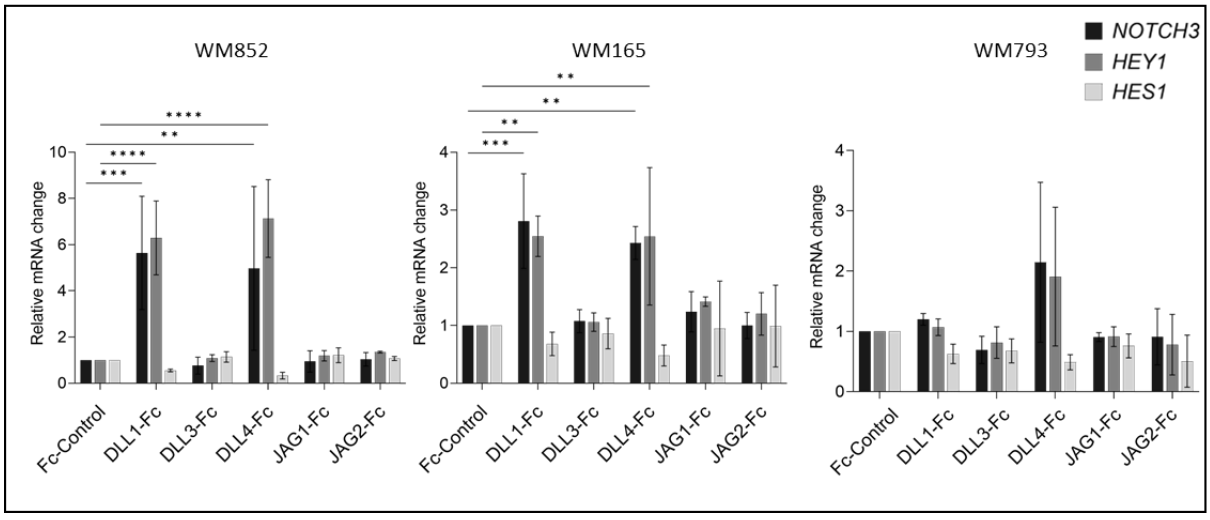

Figure 2. Treatments with DLL4-Fc and DLL1-Fc induce the Notch3 signaling pathway in WM852 and WM165 melanoma cell lines. The relative mRNA fold changes of the indicated targets in WM852, WM165 and WM793 cells treated with the indicated Fc-Notch ligands or Fc-control fragment (10 µg/ml) for 48 hours. Two-way ANOVA was used to measure statistical significances between the treatments. Graphs show the average of three independent experiment. Error bars represent SD. **: $p<0.01$; ***: $p<0.001$, ****: $p<0.0001$.

As Notch receptor activation releases NICD from the cell membrane, the amount of the NICD3 was evaluated in the cells. DLL1-Fc and DLL4-Fc treatments (48h) increased protein levels of both full length Notch3 and cleaved, active NICD3 in WM852 and WM165 cells (Figure 3). DLL1-Fc and DLL4-Fc treatments also slightly elevated protein levels of NICD3 in non-metastatic WM793 cells (Figure 3).

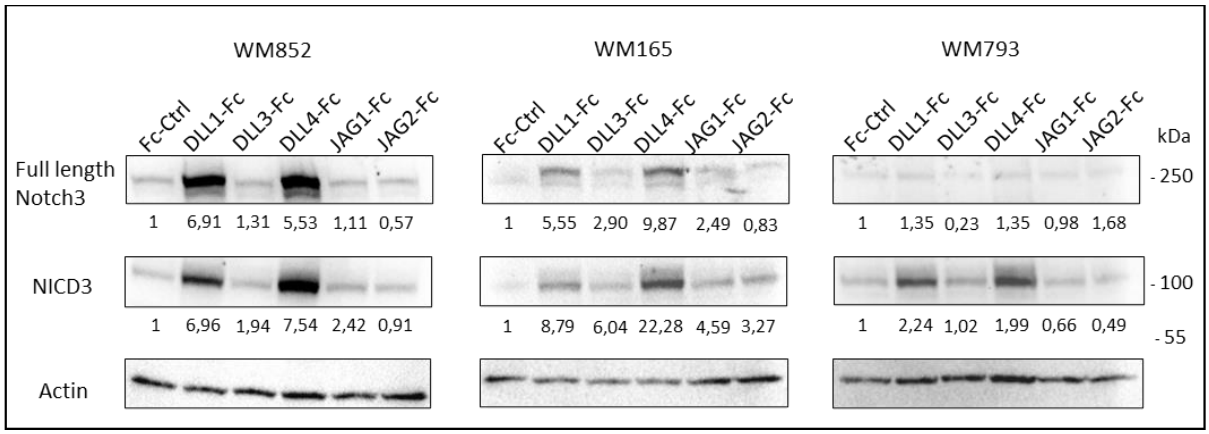

Figure 3. Notch3 signaling pathway is activated in WM852 and WM165 melanoma cells upon DLL4-Fc and DLL1-Fc treatments. Representative images of western blot of WM852 (left panel), WM165 (middle panel) and WM793 (right panel) cells treated with the indicated Fc-ligands of Notch receptors (10 µg/ml) for 48 hours. The amount of full length Notch3 and NICD3 are normalized to the amount of actin in the samples.

3.2 NOTCH3 is the only NOTCH receptor positively upregulated in melanoma cells upon stimulation by Notch ligands

To study whether other Notch receptors are positively regulated by Notch ligand stimulations, the basal mRNA expression levels of Notch receptors 1-4 were first investigated in melanoma cells treated with the Fc-control ligand. *NOTCH2* and *NOTCH3* were expressed at measurable levels in each cell line, whereas *NOTCH1* was expressed at very low levels (Figure 4a). *NOTCH4* expression is not shown due to its minimal levels in Figure 4a. To measure whether *NOTCH2* expression showed a pattern similar to that of *NOTCH3* upon stimulation with the Fc-Notch ligands, *NOTCH2* mRNA expression was measured from ligand treated cells. Although the expression levels of *NOTCH3* mRNA increased strongly after DLL4-Fc and DLL1-Fc stimulation in WM852 and WM165 cells (Figure 2), the expression level of *NOTCH2* remained unchanged upon Notch Fc-ligand stimulation in all melanoma cell lines studied (Figure 4b).

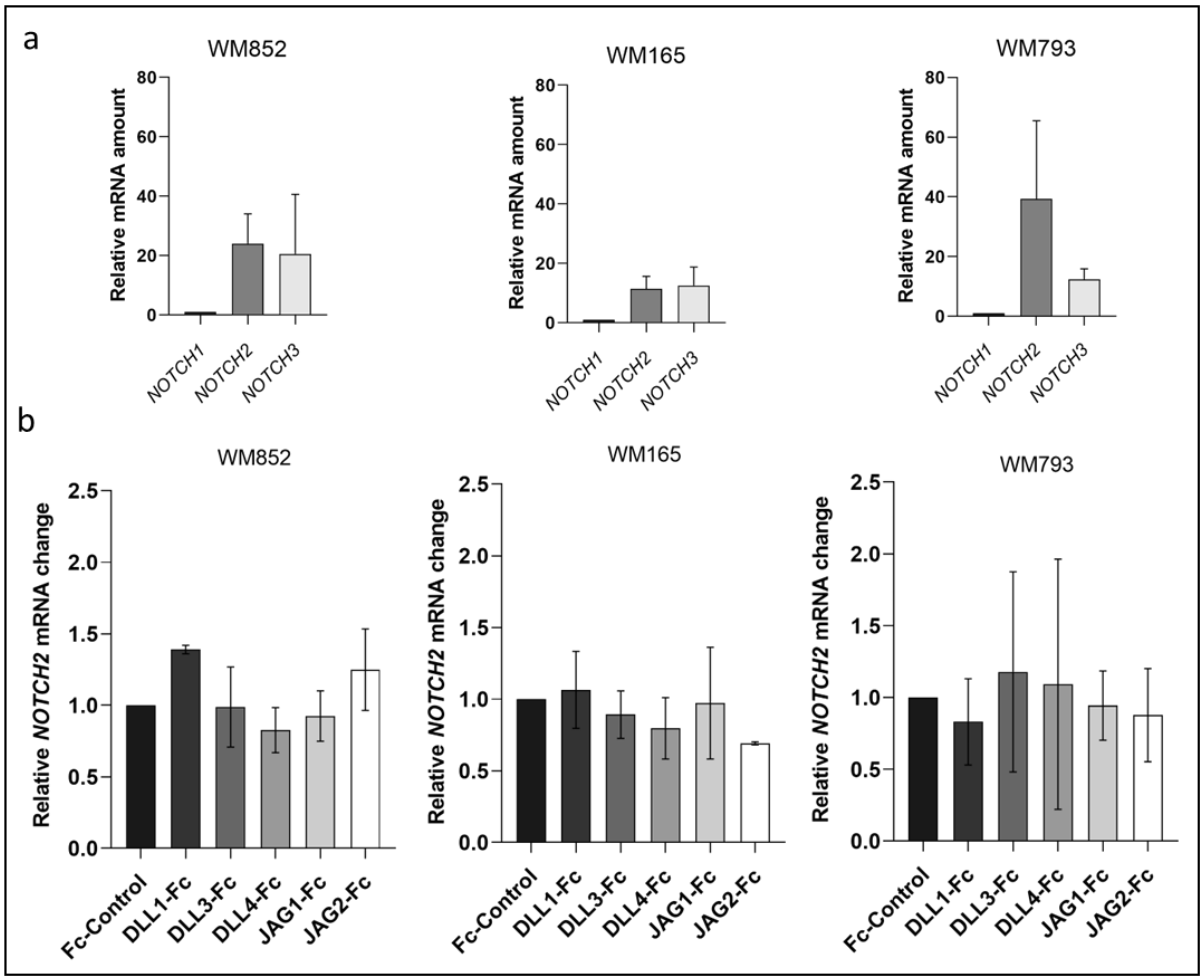

Figure 4. The expression of *NOTCH2* is not increased in WM852, WM165 or WM793 melanoma cells after treating cells with ligands of the Notch receptors. (a) The relative, basal expression levels of different Notch receptors in WM852, WM165 and WM793 cells treated with the Fc-control ligand. The expression of *NOTCH2* and *NOTCH3* were normalized to the expression of *NOTCH1* in the samples. *NOTCH4* is not shown due to very low expression level. Graph shows the average of two independent experiments. Error bars represent SD. (b) The relative mRNA fold change of *NOTCH2* in WM852, WM165 and WM793 cells treated with Notch Fc-ligands for 48 hours. The mRNA expression of the samples treated with the ligands were normalized to Fc-control treated sample of the cell line. One-way ANOVA was used to measure statistical significances between the treatments. Graphs show the average of two independent experiments. Error bars represent SD.

3.3 DLL4 treatment increases the invasion of WM852 and WM165 melanoma cells

Since Notch3 signaling activation can increase the invasion of melanoma cells (Pekkonen et al. 2018), the ability of different Notch ligands to induce the Notch3 dependent invasion in melanoma cells was next characterized. First, the melanoma cells were cultured with Fc-fusions of the Notch receptor ligands immobilized on plastic to mimic the activating pulling force on the receptor. The cells stimulated with the ligands were subsequently embedded into 3D fibrin matrix where cells were cultured for 96 hours. Melanoma cells treated with a Fc-only control ligand (Fc-control) formed round cell colonies in fibrin matrix (Figure 5a). However, the extent of string-like, invasive WM852 and WM165 cell colonies were markedly increased upon treatment with the immobilized DLL4-Fc ligand, as indicated with arrows in Figure 5a. The fibrin-invasive activity on WM852, but not WM165 cells was also increased after DLL1-Fc and lesser extent after DLL3-Fc and JAG1-Fc treatment (Figure 5b). These results indicate that the activation of Notch3 conferred the metastatic cell lines WM852 and WM165 with the ability to invade fibrin gels. However, this was not observed in WM793 cells derived from primary tumor, as there was no difference in morphology of the cell colonies between the treatments with Fc-control ligand or any of the Notch ligand in these cells (Figure 5a, b).

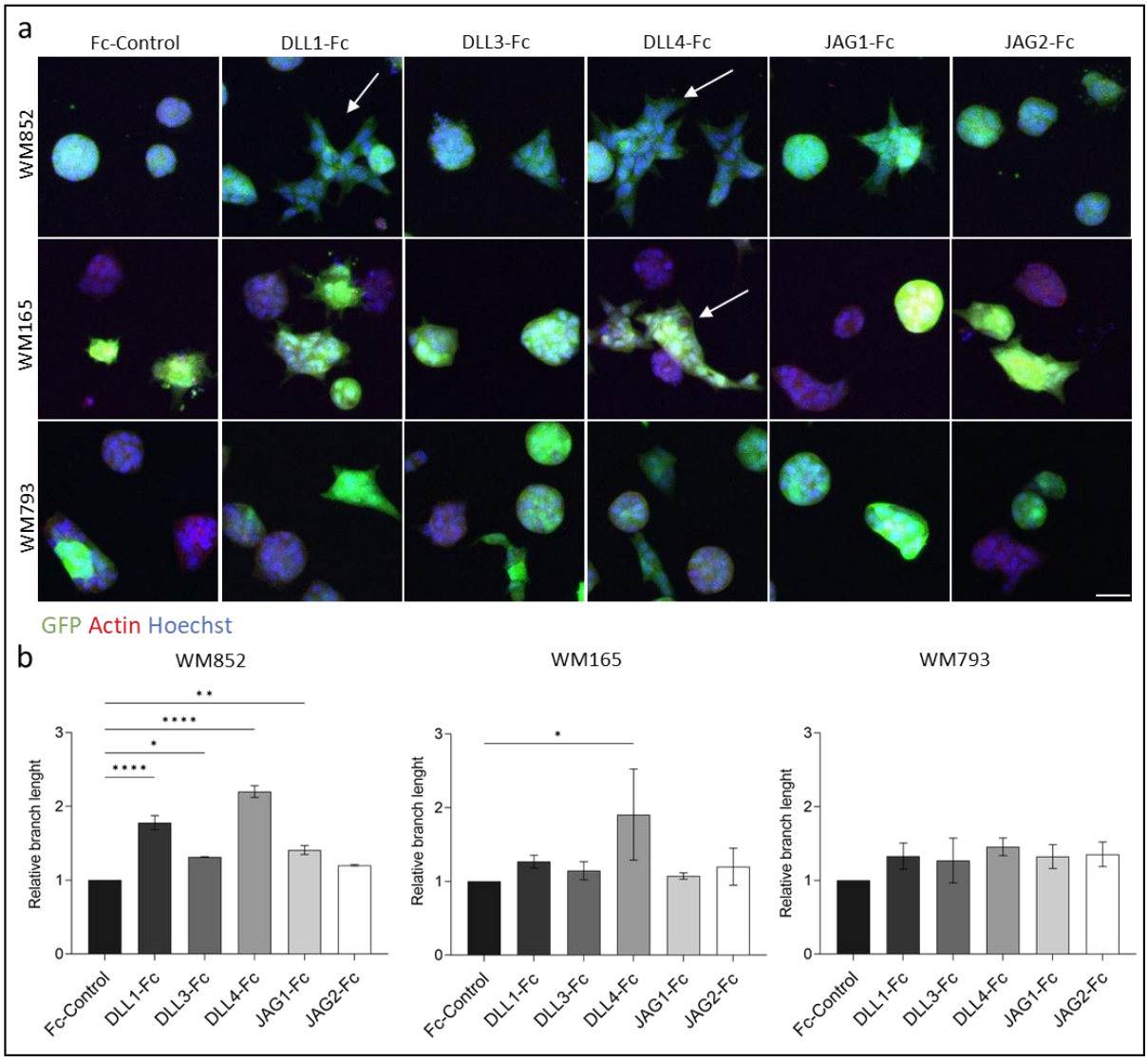

Figure 5. **DLL4-Fc and DLL1-Fc treatments increase the invasive properties of WM852 melanoma cells and DLL4-Fc treatment also the ones of WM165 but not WM793 melanoma cells.** (a) Representative confocal images of the 3D invasion assay of WM852 (top panel), WM165 (middle panel) and WM793 cells (bottom panel) treated with Fc-ligands of the Notch receptors. Cells expressed GFP (green), and actin filaments were stained with Texas Red phalloidin (red), and nuclei were counterstained with Hoechst 33342 (blue). Arrows represent examples of invasive phenotypes of cell colonies. Confocal stacks with maximum intensity Z-projections are shown. Scale bar = 20µm. (b) Quantification of the relative branch length of WM852, WM165 and WM793 cell clusters in the 3D fibrin matrix in (a). The relative branch length represents invasive ability of the cell colonies in the fibrin. The branch lengths of the samples treated with Notch ligands were normalized to samples treated with Fc-control. One-way ANOVA was used to assess the significance of the treatments. Graphs show the mean of at least four images per given treatment per two or three independent experiments. Error bars represent SD. *: $p<0.05$; ** $p<0.01$; ****: $p<0.0001$.

3.4 Silencing of PIM kinases in melanoma cells does not change the activation of Notch3 signaling pathway of the cells

PIM kinases were recently found to regulate Notch3 signaling in breast cancer cells in a tumorigenic manner (Landor et al. 2021), and thus their contribution to Notch3 signaling activation in melanoma cells was studied. First, the relative mRNA expressions of PIM kinases in WM852, WM165 and WM793 cells were assessed. Interestingly, the different PIM kinases showed variable expression patterns between different melanoma cell lines (Figure 6). In WM852 cells, *PIM2* and *PIM3* seemed to be highly expressed compared to *PIM1*, whereas the mRNA expression levels of PIM kinases were not dramatically different in WM165 cells (Figure 6). In non-metastatic WM793 cells, the expression level of *PIM2* mRNA was high compared to *PIM1* (Figure 6).

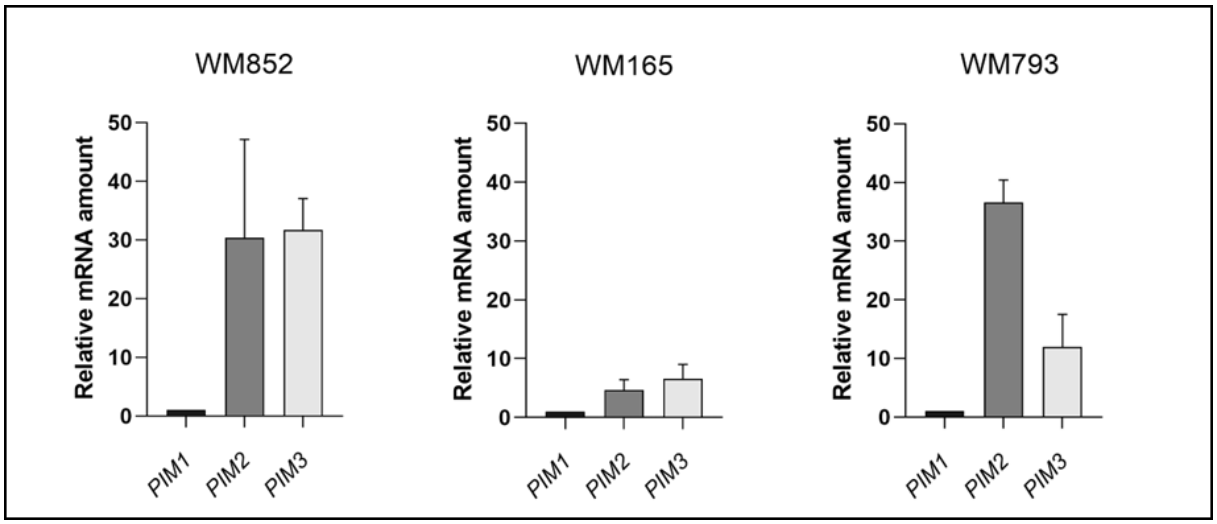

Figure 6. The relative mRNA expressions of PIM kinases in WM852, WM165 and WM793 cells treated with control siRNA (10 nM). The expressions of *PIM2* and *PIM3* are normalized to the expression of *PIM1* in the samples from each cell line. One-way ANOVA was used to assess statistical significance between treatments. Graph shows the average of two independent experiments. Error bars represent SD.

To investigate the role of PIM kinases in melanoma cells, PIM kinases were silenced in the cells using siRNAs. PIM2 and PIM3 silencing efficiency reached 75-90% in each cell line (Figure 7a). Also, the protein levels of PIM2 and PIM3 decreased by approximately 50% when their genes were silenced in WM852 cells (Figure 7b). PIM1 siRNA did not work in any of the cell lines (Figure 7a), possibly due to the low basal mRNA expresion of *PIM1* (Figure 6). The

depletion of PIM3 and PIM2 induced the upregulation of *PIM1* in each cell lines studied (Figure 7a).

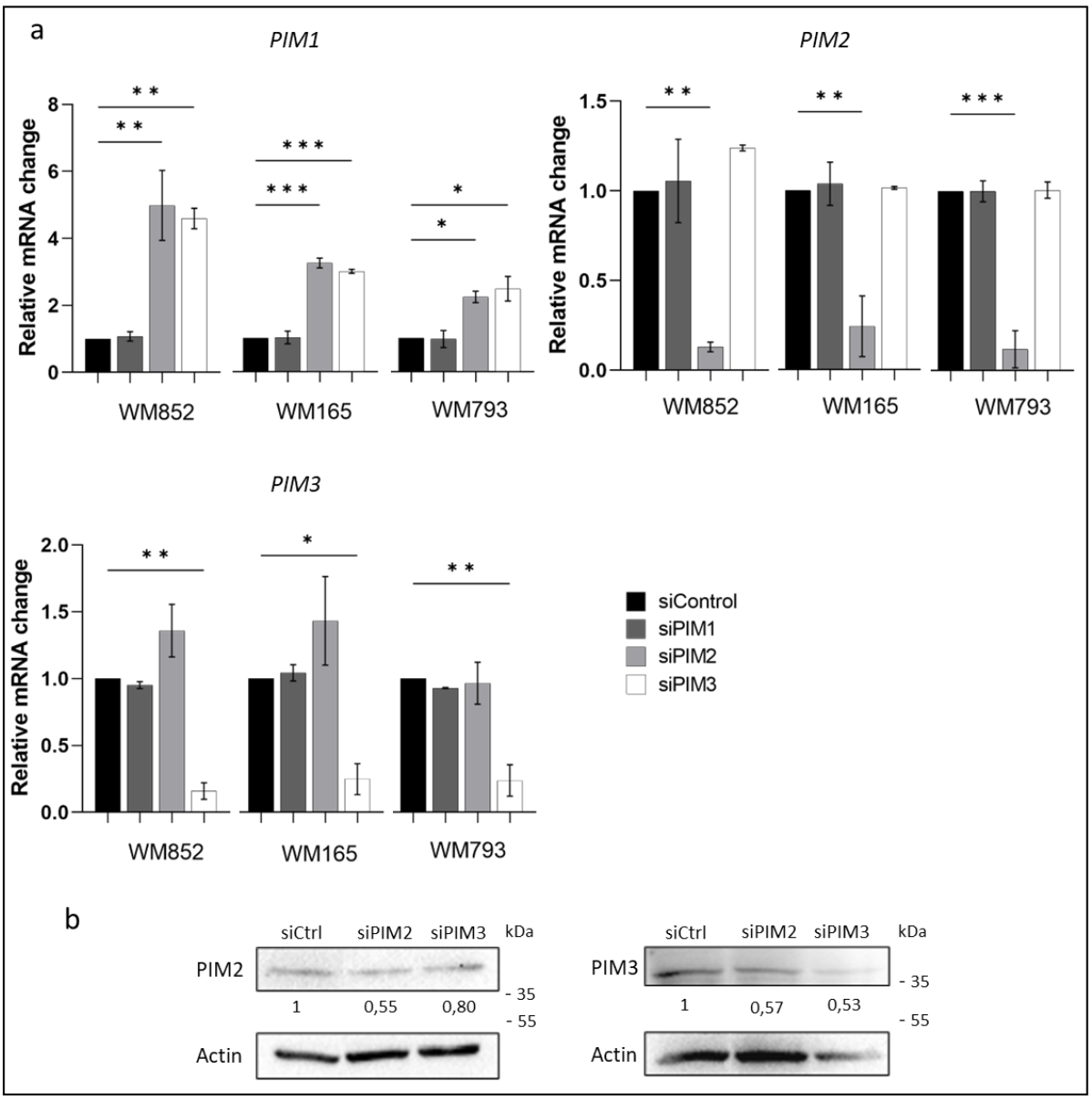

Figure 7. Efficiency of the siRNA treatments to deplete PIM kinases. (a) The relative mRNA fold change of *PIM1*, *PIM2* and *PIM3* in WM852, WM165 and WM793 cells treated with the indicated siRNAs for 24 hours. Samples treated with siRNAs targeting PIM kinases are normalized to the sample treated with control siRNA. One-way ANOVA was used to measure statistical significance between the treatments. Graphs show the average of two independent experiments. Error bars represent SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. (b) The relative protein levels of PIM2 (left panel) and PIM3 (right panel) in WM852 melanoma cells treated with the indicated siRNAs for 24 hours. The amounts of PIM kinases are normalized to actin in the samples.

In addition, it was investigated whether the siRNA treatment has an infuence on the survival of the melanoma cells. It was discovered that PIM1, PIM2 or PIM3 siRNA treatments did not change the viability of WM852, WM165 nor WM793 cells (Figure 8).

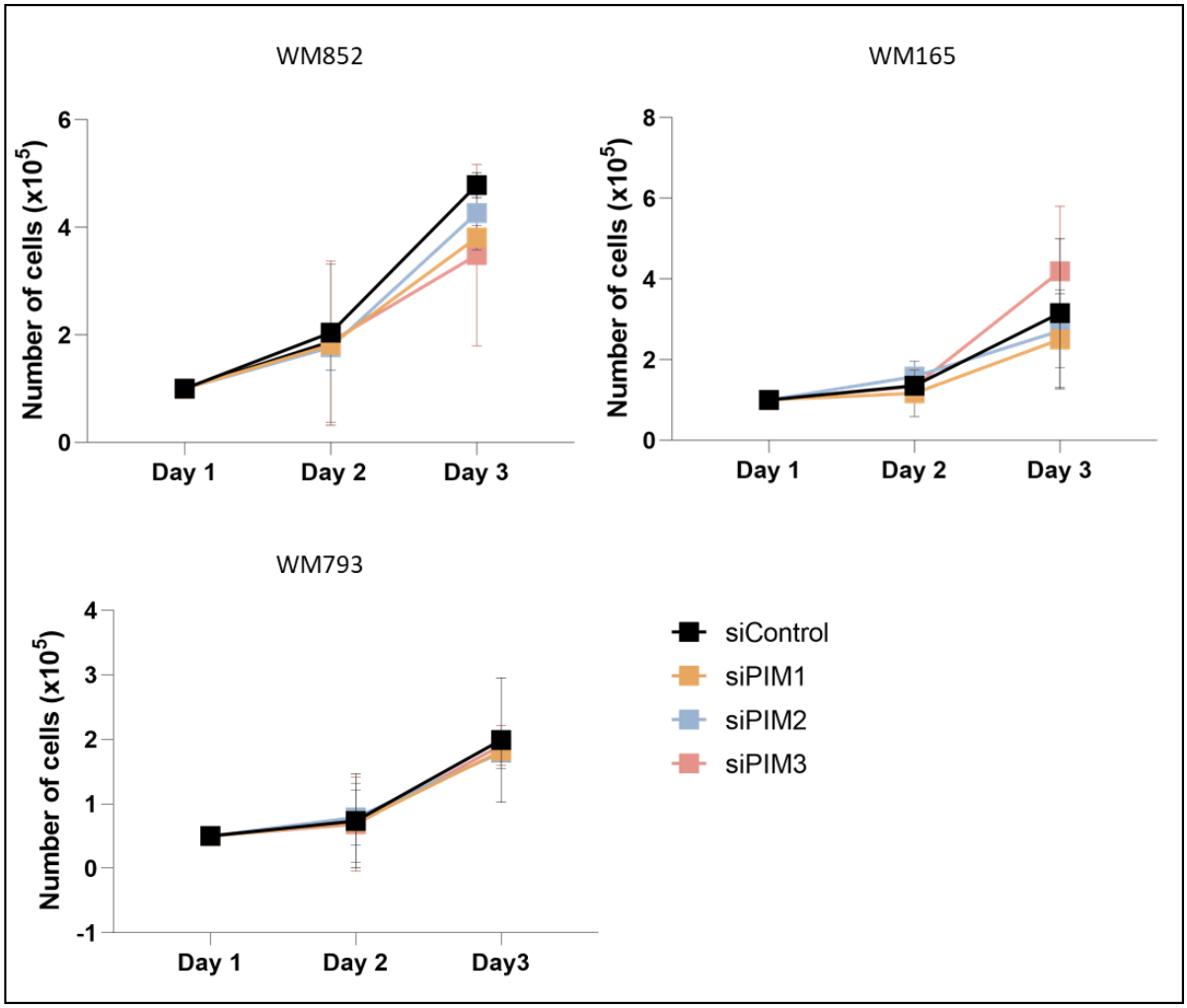

Figure 8. Treatment with the indicated siRNAs (10 nM) does not reduce the viability of WM852, WM165 or WM793 melanoma cells. Cells were collected and counted approximately 48 or 72 hours after siRNA transfection. Two-way ANOVA was used to measure statistical significance between the treatments. Graphs show the average of two independent experiments. Error bars represent SD.

Since PIM kinases can regulate Notch3 in breast cancer cells in a tumorigenic manner (Landor et al. 2021), their effect to the activity of the Notch3 signaling pathway in WM852, WM165 and WM793 melanoma cells was investigated. PIM1 silencing was not done in further experiments since it was not effective. Silencing of PIM kinases with siRNAs did not change the mRNA expression of *NOTCH3* in WM852, WM165 nor WM793 cells (Figure 9a). Protein levels of full length Notch3 or NICD3 were also unchanged after silencing of PIM kinases in

any melanoma cell line (Figure 9b), indicating that Notch3 signaling of the cells is not activated. However, Notch3 downstream target *HEY1* mRNA expression was slightly increased in WM852 and WM165 but not in WM793 cells after PIM2 silencing (Figure 9a).

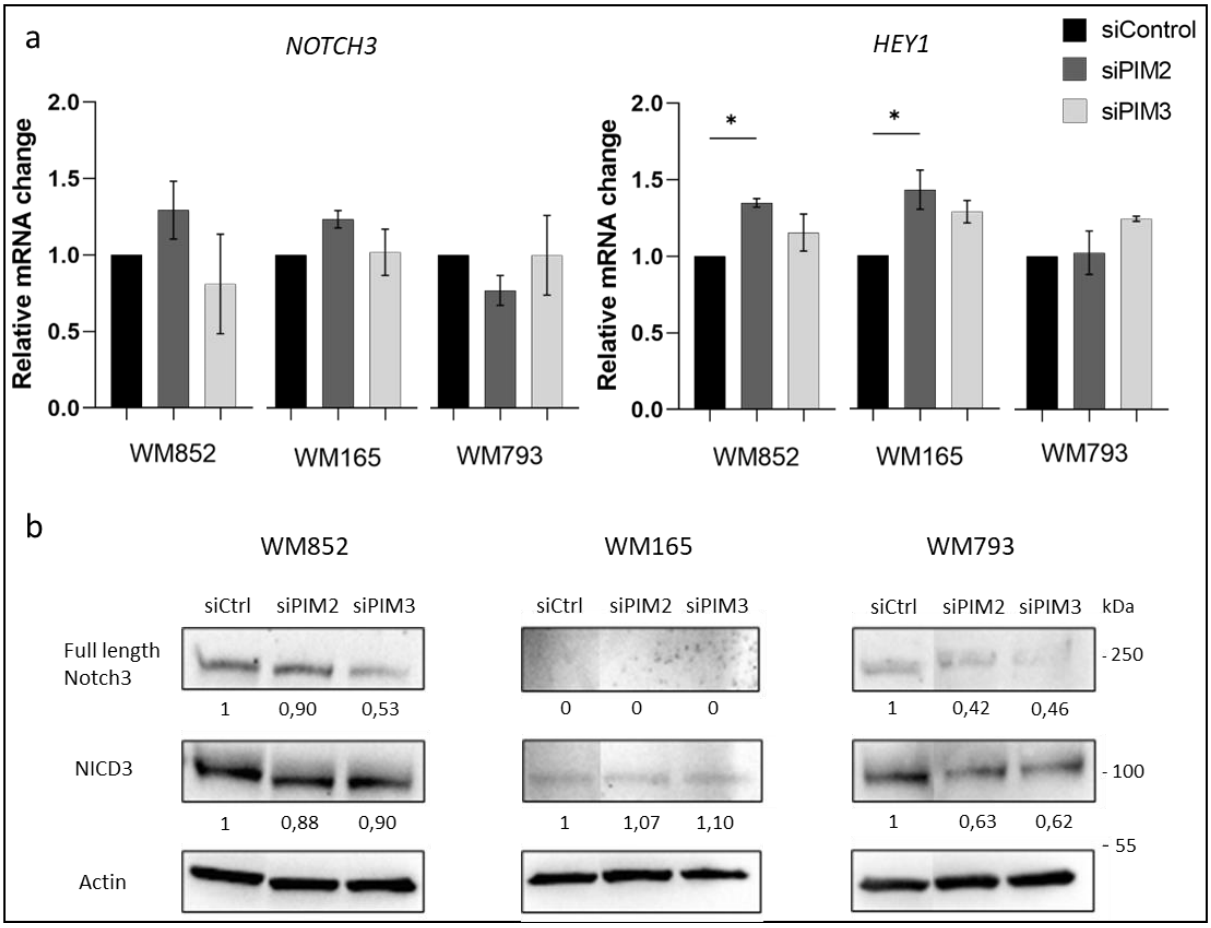

Figure 9. PIM2 or PIM3 depletion does not affect the activation of the Notch3 signaling pathway in WM852, WM165 or WM793 melanoma cells. (a) The relative mRNA fold change of *NOTCH3* and *HEY1* in WM852, WM165 and WM793 cells treated with the indicated siRNAs for 24 hours. One-way ANOVA was used to measure statistical significance between the treatments. Graphs show the average of two independent experiments. Error bars represent SD. *: $p<0.05$. (b) Representative images of western blot samples of WM852 (left panel), WM165 (middle panel) and WM793 (right panel) cells treated with the indicated siRNAs for 24 hours. The amount of full length Notch3 and NICD3 are normalized to the amount of actin in the samples. The relative amounts of full length Notch3 and NICD3 are shown.

3.5 Silencing of PIM2 decreases the invasive properties of DLL4 and DLL1 stimulated WM852 melanoma cells

Since it is known that PIM kinases can increase the tumorigenicity of cancer cells at least in breast cancer (Landor et al. 2021), their contribution to Notch3 dependent melanoma cell invasion was studied with WM852 melanoma cells. It was first aimed to determine whether Notch3 signaling could influence the expressions of PIM kinases. This was determined in cells treated with control siRNA and DLL4-Fc or DLL1-Fc ligand. Timeline of the control siRNA and ligand treatments is shown in Figure 10a. It was discovered that *PIM1* mRNA expression was increased when the cells were treated with DLL4-Fc (Figure 10b). However, DLL4-Fc treatment reduced the expression of PIM3 (Figure 10b). PIM2 mRNA expression was not changed upon DLL4-Fc or DLL1-Fc ligand treatment (Figure 10b).

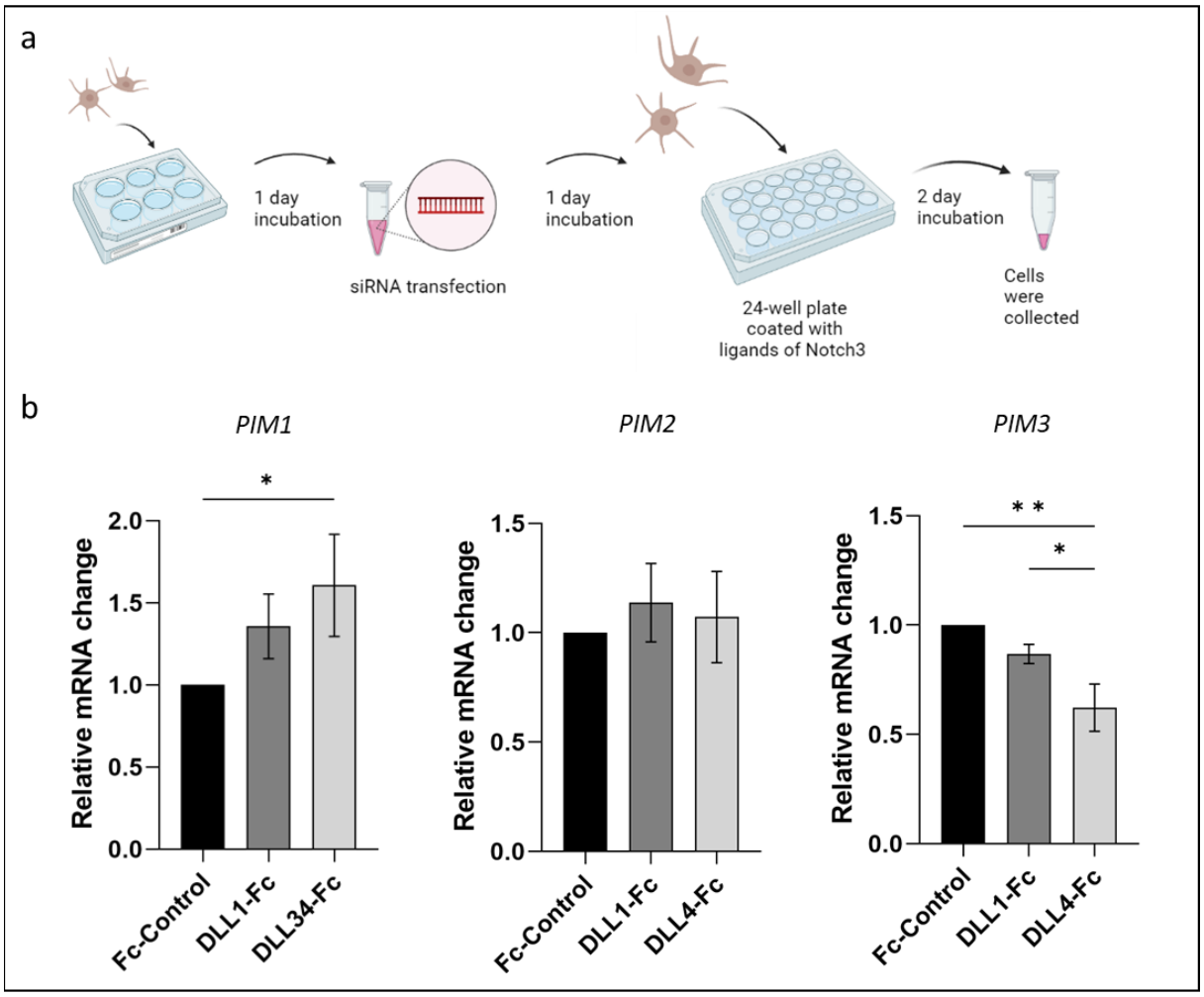

Figure 10. DLL4-Fc treatment decreases *PIM3* and increases *PIM1* mRNA expression in control siRNA treated WM852 melanoma cells. (a). Timeline of siRNA and ligand

treatments of WM852 cells. Picture created with BioRender.com. (b). The relative mRNA fold change of the indicated targets in WM852 cells treated with siControl for 72 hours are shown. 24 hours after control siRNA treatment, cells were plated on the wells coated with Fc-control, DLL1-Fc, or DLL-Fc, where the cells were allowed to grow for 48 hours. Samples were normalized to sample treated with Fc-control fragment. One-way ANOVA was used to measure statistical significance between the treatments. Graphs show the mean of three independent experiments. Error bars represent SD. *: $p<0.05$, **: $p<0.01$.

To study whether PIM kinases affect to the invasive properties of WM852 cells, the cells treated with siRNAs targeting PIM kinases and activated with Notch ligands DLL1-Fc or DLL4-Fc (Figure 10a) were embedded into a 3D fibrin matrix where the cells were growing for 96 hours. WM852 cells that were treated with the Fc-control and siRNAs for PIM kinases formed round colonies in fibrin matrix (Figure 11a). Again, cells that were treated with DLL1-Fc or DLL4-Fc formed invasive cell colonies as shown also in the Figure 5 (Figure 11a). However, treatment with siPIM2 decreased the invasive properties of the cells that were treated also with DLL4-Fc or DLL1-Fc (Figure 11a, b).

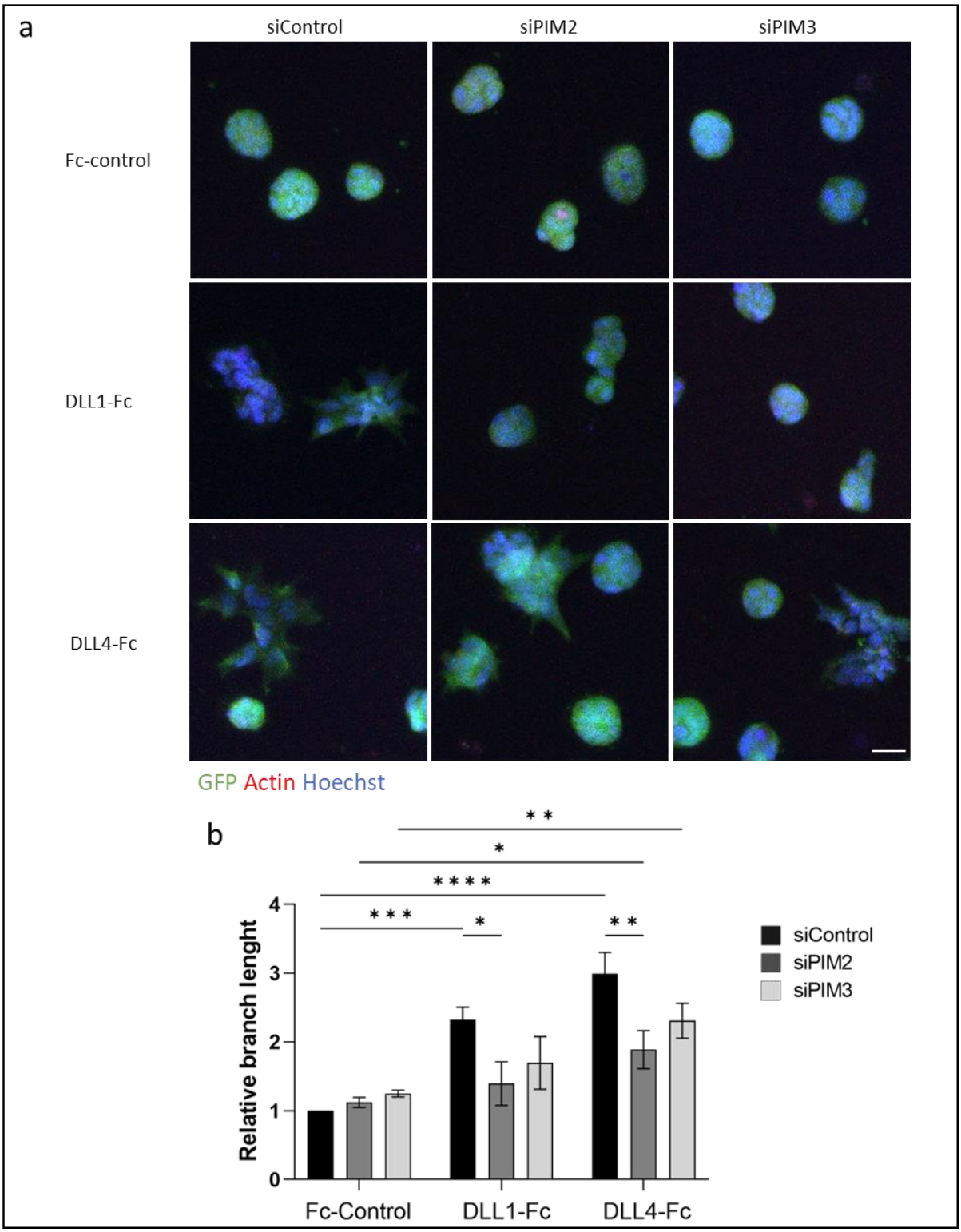

Figure 11. **Silencing PIM2 expression reduces the DLL4-Fc and DLL1-Fc induced invasive properties of WM852 melanoma cells.** (a) Representative confocal pictures of the 3D invasion assay of WM852 cells treated with the indicated siRNAs and Fc-control (top panel), DLL1-Fc (middle panel) and DLL4-Fc (bottom panel). Cells were expressing GFP (green), and actin filaments were stained with Texas Red phalloidin (red), and nuclei were counterstained with Hoechst 33342 (blue). Confocal stacks with maximum intensity Z-

projections are shown. Scale bar = 20µm. (b) Quantification of the relative branch length of cell clusters in the 3D fibrin matrix in (a). The branch length of the cell clusters was normalized to the branch length of the control siRNA and Fc-control treated sample (=1). Two-way ANOVA was used to assess statistical differences between treatments. Graphs show the mean of at least four images per given treatment per three independent experiments. Error bars represent SD. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$.

In addition to the invasive properties of WM852 cells, it was also investigated whether PIM kinases modify the activity of Notch3 signaling in WM852 cells treated with Notch ligands. Treatment with DLL4-Fc increased *NOTCH3* and *HEY1* mRNA expressions in cells but they remained unchanged upon treatments with any of the siRNAs (Figure 12). Treatment with DLL1-Fc increased *NOTCH3* mRNA expression in cells treated with control siRNA (Figure 12). Although not statistically significant, there was a trend of *NOTCH3* mRNA expression decreasing in the PIM2 silenced WM852 cells upon DLL4-Fc treatment (Figure 12). Treatment with siRNAs did not significantly alter *HEY1* mRNA expression in the Notch ligand treated cells (Figure 12).

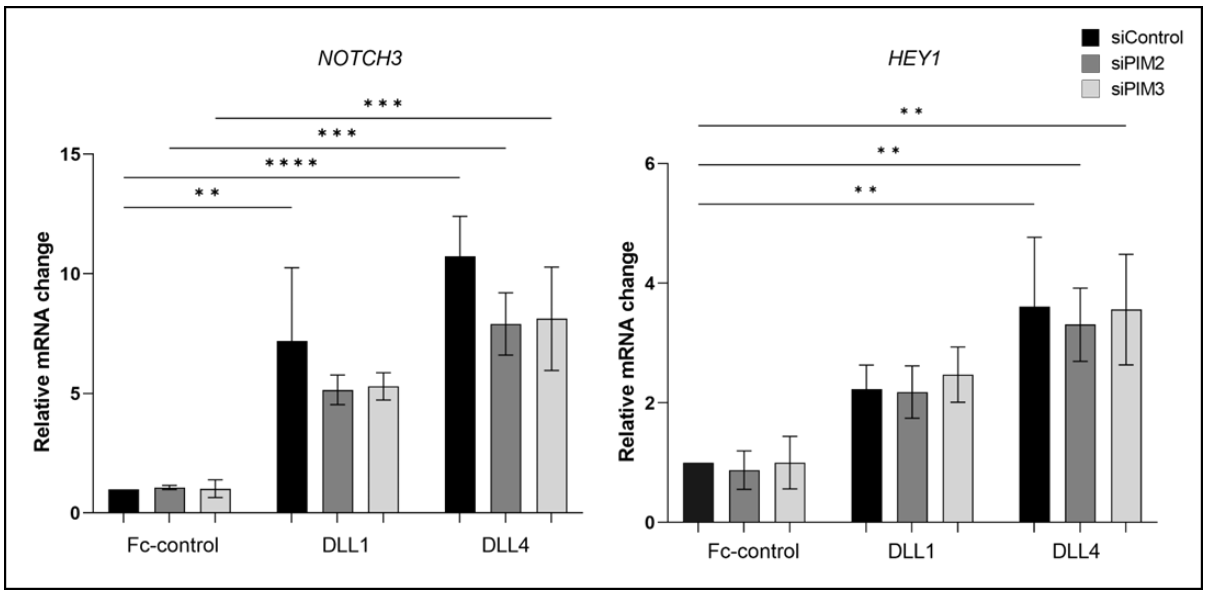

Figure 12. Depletion of PIM2 or PIM3 in the WM852 cells did not significantly alter the activation of the Notch3 signaling pathway in cells upon treatment with DLL1-Fc or DLL4-Fc. The relative mRNA fold changes of *NOTCH3* and *HEY1* are shown. Cells were treated with the indicated siRNAs for 72 hours. Two-way ANOVA was used to assess the differences between treatments. Graphs show the average of three independent experiments. Error bars represent SD. **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$.

3.6 Silencing CSL does not alter the activation of the Notch3 signaling pathway or its interacting proteins in WM852 cells

Since CSL (also called RBP-Jκ) acts as transcriptional suppressor of Notch target genes when the NICD is not presented in the nucleus (Wilson and Kovall 2006), it was investigated if silencing CSL would affect to Notch3 signaling activation in WM852 cells. First, CSL was silenced by treating cells with siCSL for 24 hours. siRNA mediated depletion reached around 80% efficacy (Figure 13a). Interestingly, *NOTCH3* or its downstream target *HEY1* mRNA expressions were not altered after the siRNA treatment (Figure 13a). Neither the protein amounts of full length Notch3 nor NICD3 were changed after treatment with siCSL (Figure 13b), indicating that Notch3 signaling was not activated in the cells. Also, the mRNA expressions of PIM kinases were investigated in siCSL treated cells, as it was observed previously that Notch ligand stimulation could possibly affect their expressions (Figure 10). The expressions of *PIM2* or *PIM3* mRNA were neither changed in siCSL treated cells, but the expression of *PIM1* mRNA possibly increased (Figure 13a). However, the experiment with mRNA and protein level changes was performed only once, so the results are very suggestive.

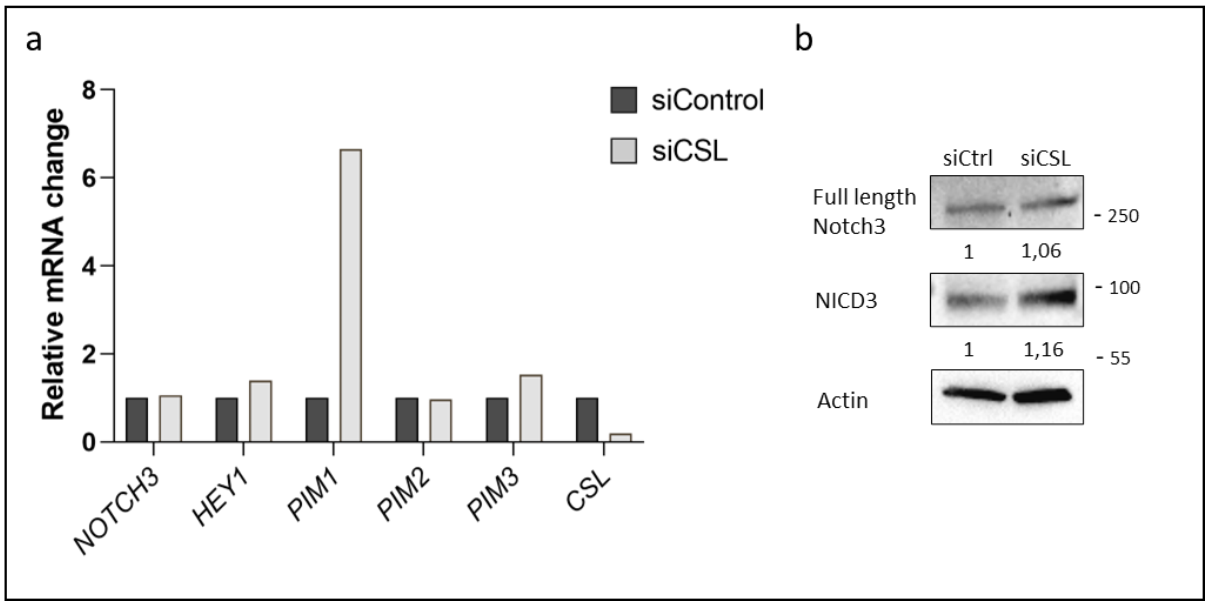

Figure 13. Silencing CSL did not change the activation of the Notch3 signaling pathway in WM852 melanoma cells. (a) The relative mRNA fold changes of the indicated targets in WM852 cells treated with siRNA targeting CSL for 24 hours are shown. The graph represents only one experiment. (b) The relative protein levels of full length Notch3 and NICD3 in WM852 cells treated with siRNA targeting CSL. The amounts of other proteins are normalized to the actin amount in the sample.

4 Discussion

4.1 DLL4 mediated Notch3 signaling in melanoma cell invasion

This study provides new insight into the mechanisms of how Notch3 signaling is activated in melanoma. It has previously been found that both lymphatic and blood endothelial cells (LECs and BECs, respectively) can stimulate the invasion of melanoma cells through Notch3 signaling (Pekkonen et al. 2018; Howard et al. 2013), but how this invasive Notch3 signaling is activated in melanoma has remained unknown. This study shows that the Notch ligand DLL4 stimulates the invasively sprouting growth of metastatic WM852 and WM165 melanoma cells in a 3D fibrin matrix. The invasion was mediated by Notch3 as reflected by increased amount of NICD3, the active form of Notch3, and increased expression of *NOTCH3* and its downstream target *HEY1*. Furthermore, DLL1-Fc treatment of WM852 and WM165 melanoma cells stimulated the expression and activation of Notch3, albeit the 3D growth phenotype was significantly observed to change only in WM852 cells. Matrix metalloproteinases (MMPs) contribute to the invasiveness of cells in fibrin (Hotary et al. 2002). Tatti et al. (2011) have found that MMP16 shows slightly different expression patterns in WM852 and WM165 cells upon culturing in 3D fibrin. On the other hand, Pekkonen et al. (2018) have shown that melanoma cell invasion in fibrin is a complex process that requires both MMP14 and Notch3 activity. Thus, it may be possible that Notch ligands stimulate the invasion of WM852 and WM165 cells in the fibrin differently due to the different MMP levels and activities in these cells, which would require further investigation.

The contribution of DLL4 or DLL1 to Notch3 signaling activation in melanoma cells is still poorly defined. However, DLL4 is known to stimulate the migration of colorectal tumor cells following the activation of Notch3 signaling (Furukawa et al. 2013). LECs and BECs are known to express DLL4 (Zheng et al. 2011; Shutter et al. 2000), suggesting that melanoma cell interaction with LECs or BECs could participate to the progression and metastasis of tumor through DLL4 activated Notch3 signaling. Moreover, it has also been suggested that DLL4 expressed by BECs can stimulate Notch3 signaling in T-cell acute lymphoblastic leukemia cells and colorectal cancer cells promoting the escape of these cells from dormancy (Indraccolo et al. 2009).

In this study, neither DLL4-Fc nor DLL1-Fc stimulated the invasion of WM793 cells that are originally derived from a primary tumor. The results are supported by the finding from the study of Pekkonen et al. (2018), where LECs were shown to stimulate Notch3 dependent invasion only in the metastatic melanoma cell lines, such as WM852 cells, but not in the non-metastatic, WM793 melanoma cells. The authors also observed that overexpression of NICD3 can stimulate the invasion of the otherwise non-metastatic WM793 cells. The finding that malignant, but not the non-aggressive melanomas highly express Notch3 (Hendrix et al. 2002), further supports the role of Notch3 signaling in melanoma cell metastasis. Taken together, this study and previous findings indicate that DLL4 can promote the metastasis of metastatic melanoma cells by activating Notch3 signaling in the cells, but it is unlikely to affect the invasion of non-metastatic melanoma cells that do not highly express Notch3.

Given that Delta ligands act by a mechanism, where Delta expression is inhibited and Notch expression is promoted in the cell receiving the signal (Bocci et al. 2020), it is not surprising that in this study, *NOTCH3* mRNA expression was elevated in metastatic WM852 and WM165 cells after DLL4-Fc and DLL1-Fc stimulation. This suggests the presence of a positive feedback loop resulting in the activation of the Notch signaling i.e., the activation of Notch3 stimulates more *NOTCH3* expression. Moreover, positive feedback loop was observed between the full length Notch3 and NICD3 levels in WM852 and WM165 cells after DLL4-Fc and DLL1-Fc stimulation. Upon ligand stimulation, Notch3 receptors of WM852 and WM165 cells were activated to release intracellular domain form NICD3 into the cytoplasm, resulting increased expression of *NOTCH3* and increased cleavage of Notch3 receptors to produce the active NICD3.

In this study, Notch signaling downstream target *HES1* was not upregulated after DLL4-Fc or DLL1-Fc stimulation in WM852 or WM165 cells. Previously, Howard et al. (2013) have found Notch downstream target *HEY1* expression to be consistently elevated in NICD3-overexpressing melanoma cells, whereas *HES1* expression was not elevated in all melanoma cell lines used. It has also been reported that Notch3 may activate Notch signaling target genes differently than other Notch receptors, as NICD3 has been found to weakly activate *HES1* expression compared to NICD1 (Beatus et al. 1999). These findings may explain why *HES1* was not upregulated in any of the investigated cell lines in this study after the Notch ligand stimulation.

It appears that Notch2 and Notch3 are the predominant Notch receptors expressed in the studied melanoma cell lines. However, only the expression of *NOTCH3* was increased in WM852 and WM165 cells after treatment with Notch ligands DLL4-Fc and DLL1-Fc. This trend is not observed in non-metastatic WM793 cells, where both *NOTCH2* and *NOTCH3* expressions are unchanged after Notch ligand treatments. It has also been previously found in co-culture studies with LECs and BECs that only *NOTCH3* expression, but not the expression of other Notch receptors, is increased in melanoma cells after co-culture (Pekkonen et al. 2018; Howard et al. 2013).

To summarize this part, the invasive ability of WM852 cells was greatly improved after Notch3 activation due to DLL4-Fc and DLL1-Fc stimulation, as was also the invasive ability of WM165 cells after DLL4-Fc stimulation. Moreover, the ability of WM165 cells to invade in the fibrin was not increased after DLL1-Fc treatment, suggesting a difference in the activation of key effectors required for the invasive phenotype of these metastatic melanoma cell lines. Importantly, non-metastatic WM793 cells did not respond to any Notch ligand, thus neither *NOTCH3* mRNA expression nor the amount of full length Notch3 were increased as in WM852 and WM165 cells. These results are in accordance with the study of Pekkonen et al. (2018), where they showed that N*OTCH3* expression was upregulated in WM852 and WM165 cells, but not in WM793 cells, after cells were co-cultured with LECs.

4.2 Role of PIM2 in the regulation of Notch3 signaling in melanoma cells

It is well established that PIM kinases contribute to the promotion of cell migration and invasion by maintaining cell survival (Mukaida et al. 2011; Yang et al. 2011). However, whether PIM kinases regulate melanoma cell metastasis is still not well known. This study provides new insight into the melanoma research by combining the study of Notch3 signaling and PIM kinases. The results show that PIM2 depletion decreased the DLL4 and DLL1 mediated invasiveness of WM852 melanoma cells to some extent. Thus, PIM2 may have an oncogenic role in melanoma, as previously shown for breast cancer cells, where all PIM kinases were shown to phosphorylate NICD3 and increase the estrogen-driven tumorigenicity of the cells (Landor et al. 2021). Although PIM2 silencing reduced the invasion of WM852 cells after DLL4-Fc and DLL1-Fc treatments, the invasive potential of the cells was still higher compared to cells treated with control siRNA and Fc-control. This indicates that there are likely other regulatory mechanisms that contribute to the Notch3 stimulated melanoma cell invasion that

need to be considered. In addition, it is possible that other PIM kinases, especially PIM3, compensate for PIM2 function and maintain the invasiveness of these cells, as the roles of PIM kinases are known to overlap (Narlik-Grassow et al. 2014).

The oncogenic role of PIM kinases in melanoma has also been observed previously. PIM3, which is commonly overexpressed in various tumors including melanoma, has been found to stimulate melanoma cell invasion via STAT3 phosphorylation (Liu et al. 2018). Furthermore, Shannan et al. (2016) have found PIM1 silencing to cause reduction in melanoma cell proliferation and invasion. However, the authors observed that PIM1 silencing was insufficient to entirely arrest the growth of melanoma cells and other PIM isoforms were also compensatory upregulated in some cases. Thus, as the authors stated, the role of all PIM kinases and the consequences of PIM kinase inhibition in other melanoma cell lines also remain to be investigated. In the present study, some compensatory expression between PIM kinases was observed after one of the kinases had been depleted with siRNA, as *PIM1* expression increased when any of the three cell lines used was treated with siPIM2 or siPIM3. However, it is still good to note that the expression level of *PIM1* was quite low in all cell lines used in this study, which may also contribute to the observed *PIM1* compensation. Unfortunately, in the present study, no further information was obtained for PIM1 role in melanoma or its possible effect to Notch3 signaling and cell invasiveness as PIM1 could not be silenced with siRNA. This may be due to the low expression of *PIM1* in the cells as protein levels of PIM1 were not detectable in WM852 cells and the mRNA level of *PIM1* was lower compared to *PIM2* and *PIM3* expressions in each cell line used. This is discrepancy with the study of Shannan et al. (2016) where the melanoma cells used expressed *PIM1* at significant levels since silencing PIM1 had an effect to cell proliferation. Santio et al. (2016) have reported that *PIM1* and *NOTCH3* are positively correlated in breast cancer cells but not in prostate cancer cells, so it is possible that not all cancer types express these proteins at the same manner. Perhaps it is possible that different cell lines of the same cancer also express *PIM1* and *NOTCH3* differently.

Although Notch3 phosphorylation by PIM kinases prevents NICD3 from interacting with CSL, NICD3 can still maintain the tumorigenicity of estrogen-driven breast cancer cells, indicating that PIM kinases can stimulate oncogenic Notch3 signaling through a CSL-independent mechanisms (Landor et al. 2021). CSL acts as a transcriptional activator in the canonical Notch signaling pathway, whereas non-canonical Notch signaling can function without CSL (Siebel and Lendahl 2017). In the present study, the phosphorylation of NICD3 was not addressed. However, silencing of PIM2 decreased the invasive properties of WM852 cells upon DLL4-Fc

and DLL1-Fc treatment, possibly due to reduced phosphorylation of NICD3 and the activity of non-canonical Notch3 signaling in cells, as non-phosphorylated NICD3 can interact with CSL (Landor et al. 2021). This may imply that non-canonical Notch3 signaling is responsible for the invasiveness of melanoma cells, as it has been observed that tumorigenicity of estrogen-driven breast cancer cells can be promoted by phosphorylated NICD3 (Landor et al. 2021). In addition, non-canonical Notch3 signaling is also found to maintain cancer cell survival in cholangiocarcinoma (Guest et al. 2016).

The potential oncogenic role of the non-canonical Notch3 signaling in cancer is also partially supported by the observation that *NOTCH3* expression was reduced to some extent, although not significantly, in siPIM2 treated cells upon DLL4-Fc stimulation in this study. It is possible, that significant reduction in *NOTCH3* or *HEY1* expression in siPIM2 and DLL4-Fc treated cells was not observed due to the activity of other PIM kinases which can still be active in the cells. In particular, the activity of PIM3 may still affect the phosphorylation of NICD3, since it was expressed in WM852 cells quite similar levels as *PIM2,* and all PIM kinases are known to be able to phosphorylate NICD3 (Landor et al. 2021). Afterall, the result may indicate that Notch3 signaling autoregulates itself positively through the non-canonical Notch3 signaling in melanoma cells instead of the canonical signaling, if Notch3 autoregulation is reduced in PIM2 depleted cells where the non-phosphorylated form of NICD3 could interact with CSL. In addition, this may indicate that PIM kinases have stimulatory role in Notch3 signaling in melanoma cells, as PIM2 silencing appears to reduce Notch3 autoregulation in siPIM2 treated WM852 cells upon DLL4-Fc stimulation. Previously, it has been observed that PIM kinases stimulate the Notch activity at least in breast cancer cells, where inhibition of all PIM kinases reduces the activity of Notch signaling (Santio et al. 2016). However, the study did not differentiate the activity of different Notch receptors or the form of Notch signaling, but it suggests a stimulatory role for PIM kinases in Notch3 signaling. As previously mentioned, the decrease of DLL4 stimulated *NOTCH3* expression in PIM2 depleted WM852 cells was not significant in this study, and thus this topic should be further investigated to make conclusions regarding melanoma.

NOTCH3 mRNA expression or protein levels of full length Notch3 or NICD3 were not altered in WM852, WM165 or WM793 cells treated with PIM siRNAs in the absence of simultaneous treatment with Notch ligands. This result is in contrast to a study of Santio et al. (2016) where the inhibition of PIM kinases was found to decrease the basal Notch activity of the breast cancer cells. In this study, the efficacy of siRNA treatment was confirmed at the mRNA and protein

levels, indicating that lack of changes in Notch3 signaling activity was not due to inefficient silencing of PIM2 or PIM3. It is possible that the incubation time with siRNAs was too short to cause changes in activation of Notch3 signaling in the cells. However, while PIM2 silencing appears to reduce the *NOTCH3* expression in the DLL4 treated but not in the Fc-control treated WM852 cells, where the incubation time with siRNAs was longer due to additional ligand treatment, it is possible that PIM kinases do not affect unstimulated Notch3 signaling in melanoma cells. Full length Notch3 and NICD3 protein levels were also examined by Western blot in WM852 cells treated with siPIMs and Notch ligands, but there was too much variability between different experiments, so no conclusions could have been drawn at the protein level. Therefore, the potential stimulatory role of PIM kinases in Notch3 signaling in melanoma cells should be further investigated.

In this study, Notch3 signaling activity also regulated the expression of PIM kinases, as DLL4-Fc treatment increased *PIM1* and reduced *PIM3* mRNA expression in WM852 cells treated with control siRNA. Previous studies have indicated that PIM kinases can modify the signaling output of Notch (Santio et al. 2016, Landor et al. 2021), but the effect of Notch3 signaling on PIM expression has not gained attention yet. Since PIM kinases regulate Notch3 signaling by phosphorylation (Landor et al. 2021), it is possible that Notch3 receptor activation may affect the expression of PIM kinases in some autoregulatory manner. Santio et al. (2016) have also observed that phosphorylated Notch1 can stimulate the expression of PIM kinases, which then further stimulates Notch1 activity. Thus, it is possible that PIM kinases and Notch3 have a similar relationship in melanoma cells, but this requires further examination.

4.3 Role of CSL in Notch3 signaling in melanoma cells

In this study, the effect of CSL (also called RBP-Jκ) depletion in WM852 cells was briefly investigated. CSL is an important mediator of the canonical Notch signaling (Siebel and Lendahl 2017), and it is suggested that mutations in CSL reduces Notch activity of cells (Yuan et al. 2012). Thus, it could be assumed that silencing of CSL suppresses Notch3 mediated transcription in melanoma cells. However, CSL depletion did not alter the gene expression of *NOTCH3* or its downstream target *HEY1,* nor the protein levels of full-length Notch3 or NICD3. These results may be due to a too short incubation time with siRNA targeting CSL, which was only 24 hours, and it would be interesting to investigate in the future how a longer incubation with siCSL could affect Notch3 signaling in melanoma cells. In addition, it is possible that

Notch signaling should first be stimulated in melanoma cells to observe any changes in Notch3 signaling responses after CSL silencing.

CSL is also known to act as a transcriptional suppressor of Notch target genes when Notch receptors are not activated (Wilson and Kovall 2006; Liefke et al. 2010), and depletion of CSL can also lead to Notch target gene expression (Kulic et al. 2015). It has been observed that downregulation of CSL is common in tumors, such as breast and non-small cell lung cancers (Kulic et al. 2015). The authors suggested that deficiency of CSL can promote tumor growth, indicating that non-canonical Notch signaling has tumorigenic potential. Similarly, Raafat et al. (2009) have found that the interaction between CSL and NICD is required for mammary gland development, whereas this interaction is not needed for mammary tumor development. Belyea et al. (2014) have also reported that deletion of CSL from B-lymphocyte progenitor cells in mice leads to the development of B-cell leukemia. Taken together, these findings indicate that CSL may have a tumor suppressive role because NICD can also mediate Notch signaling without CSL via the non-canonical Notch signaling pathway, which may be more oncogenic than the canonical one (Ayaz and Osborne 2014). In the present study, silencing of CSL appeared to induce *PIM1* expression in WM852 cells indicating that CSL might act as a suppressor of possibly PIM1 but no other investigated genes. But as mentioned, CSL silencing experiment was performed only once, and thus the results are also very preliminary, and the topic needs to be further investigated in melanoma cells.

4.4 Future study direction

This study supports the stimulatory role of Notch3 signaling in melanoma cell invasion and provides new insight into the mechanisms that activate Notch3 mediated invasion of melanoma cells. It has previously been reviewed that Notch3 signaling is more likely needed for the migration of cancer cells rather than for cancer cell proliferation (Liu et al. 2014). The finding that Notch3 signaling does not stimulate the invasion of non-metastatic WM793 cells but stimulates the invasion of metastatic WM852 and WM165 cells also supports a role of Notch3 in cancer cell dissemination. In the future, additional cell lines should be investigated to further confirm the role of DLL4 in the activation of invasive Notch3 signaling in metastatic melanoma cells. Also, it could be investigated whether Notch3 activation can affect the expression of Notch ligands in melanoma cells. Zhang et al. (2014) have found that DLL1 expressed by melanoma cells can increase the adhesion capacity of melanoma cells and thus promote cancer

cell metastasis. It is also known that Notch ligands in the receptor-expressing cell can suppress the ligand-dependent Notch signaling of the cell (Palmer and Deng 2015), which may also affect Notch3 signaling activity in melanoma cells. Therefore, it would be important to know how different Notch ligands are expressed in melanoma cells.

This study provides a new perspective on the regulatory mechanisms of Notch3 signaling in melanoma. It is an interesting observation that silencing PIM kinases, or at least PIM2, can reduce the DLL4 stimulated invasive Notch3 signaling in melanoma cells, because it is known that LECs and BECs can activate melanoma cell invasion (Pekkonen et al. 2018; Howard et al. 2013), and these cells express DLL4 (Zheng et al. 2011; Shutter et al. 2000). In the future, it would be important to further examine the possible role of PIM2 in oncogenic Notch3 signaling in melanoma cells. For example, it could be studied how PIM2 silencing may affect the expression of other Notch3 downstream targets than those investigated in this study. It would also be useful to examine how a longer incubation time with PIM silencing siRNAs would affect Notch3 signaling responses in melanoma cells. In addition, it was observed that the melanoma cells investigated in this study showed slightly different expression patterns of PIM kinases. Thus, the role of PIM kinases to the invasion of different melanoma cell lines could be examined. In addition, the influence of other potential targets of PIM kinases on the invasive properties of melanoma cells could be investigated, as it has previously been shown that PIM3 can stimulate melanoma cell invasion via STAT3 phosphorylation (Liu et al. 2018).

It is possible that PIM kinases can compensate for each other's expressions after one of them is silenced in melanoma cells (Shannan et al. 2016), which was also observed in this study. In addition, the ones that are not silenced are still functional in the cells. Thus, it would be useful to investigate how PIM inhibitors or silencing all the PIM kinases at once would affect Notch3 signaling activity in melanoma cells. In this way, a possible compensatory role of PIM kinases would be eliminated. It was found in this study that DLL4 and DLL1 mediated invasion, but not Notch3 signaling activity, was significantly decreased in siPIM2 treated WM852 cells. Thus, it is possible that PIM3, which was still expressed in the cells, has also affected Notch3 signaling activity of the cells and compensated the function of PIM2, as silencing of PIM3 also slightly reduced DLL4 mediated invasion of WM852 cells, but not significantly. Inhibition of all PIM kinases simultaneously would also reveal whether PIM kinases stimulate Notch3 signaling in melanoma cells, as PIM kinases have been found to stimulate Notch signaling at least in breast cancer cells (Santio et al. 2016). This could be done with and without

simultaneous Notch ligand treatment to reveal whether PIM kinases have an influence on unstimulated Notch3 signaling in melanoma cells.

The contribution of the canonical and non-canonical Notch3 signaling to melanoma cell invasion would also be valuable to investigate, as it appears that the non-canonical Notch3 signaling has tumorigenic potential, at least in estrogen-driven breast cancer and cholangiocarcinoma cells (Landor et al. 2021; Guest et al. 2016). Landor et al. (2021) also suggest that phosphorylated Notch3 act as oncogene while non-phosphorylated NICD3 is more like a tumor suppressor in luminal A breast cancer cells. The findings of this study also suggest that this could potentially apply to melanoma cells as well. In addition, Cui et al. (2013) have previously been found that canonical Notch3 signaling is at least partially involved in the induction of cellular senescence in melanoma and thus may have tumor suppressive role. Investigating the function of CSL with siRNAs could be one way to study the role of the canonical and non-canonical Notch3 signaling in melanoma cells. For this approach, a longer siCSL treatment would need to be tested, as well as the effect of CSL depletion on cell viability. In addition, it could be investigated whether siCSL affects Notch3 signaling of melanoma cells if they were also treated with DLL4 or DLL1.

Cancer cells need an ability to invasion to form distant metastasis and thus different *in vitro* invasion assays are important tools in cancer research. In this study, a 3D fibrin matrix was used to investigate the invasion of melanoma cells after Notch signaling activation of the cells. Thus, the properties of melanoma cells were investigated in an environment that better reflects the actual environment of the cells than the 2D culture methods (Alve et al. 2021). Also, the 3D invasion assays provide physiologically more relevant results than the 2D models. Next, *in vivo* research models could be used to confirm the results of this study. Although *in vivo* invasion animal models are ethically more concerning and the costs are much higher, they are needed to investigate the behavior of the cells in an actual physiological microenvironment where, for example, other cell types, soluble factors, and the extracellular matrix influence the behavior of cancer cells.

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Appendices

Appendix 1: Product information

Reagent type	Designation	Sourcor reference	Identifiers	Additional information
WM852		Wistar Institute Philadelphia		RRID:CVCL_6804
WM165		Wistar Institute Philadelphia		RRID:CVCL_L033
WM793		Wistar Institute Philadelphia		RRID:CVCL_8787
Antibody	Notch3	Cell Signaling	3446S	Used 1:1000
Antibody	β-actin	Sigma	A1978	Used 1:1000
Antibody	PIM1	Santacruz	13513	Used 1:200
Antibody	PIM2	Cell Signaling	4723S	Used 1:500
Antibody	PIM3	ABCEPTA.com	AP7171a	Used 1:1000
Antibody	HRP-linked anti-mouse IgG	Cell Signaling	7076S	Used 1:1000
Antibody	HRP-linked anti-rat IgG	Cell Signaling	7077S	Used 1:1000
Antibody	HRP-linked anti-rabbit IgG	Cell Signaling	7074S	Used 1:1000
Ladder	Thermo Scientific PageRuler Plus Prestained Protein Ladder	Thermo Fisher Scientific	26619	4 µl / well

Fluorescent stain	Texas Red Phalloidin	Molecular Probes	T7471	Used 1:200
Fluorescent stain	Hoechst 33342	Sigma-Aldrich		Used 1 µg/ml
siRNA	siControl	Dharmacon	D-001210	Used at 10nM
siRNA	siPIM1	Dharmacon	L-003923-00	Used at 10nM
siRNA	siPIM2	Dharmacon	L-005359-00	Used at 10nM
siRNA	siPIM3	Dharmacon	L-032287-00	Used at 10nM
siRNA	siRBP-Jκ	Santa Cruz	sc-38214	Used at 10nM
NOTCH3 RTqPCR primer	QuantiTect Primer Assay NOTCH3	Qiagen Science	QT00003374	Used 1:10
HEY1 RTqPCR primer (for, rev)	GTTCGGCTCTAGGTTCCATGT CGTCGGCGCTTCTCAATTATTC	Oligomer		Used at 160nM
HES1 RTqPCR primer (for, rev)	TCAACACGACACCGGATAAA TCAGCTGGCTCAGACTTTCA	Oligomer		Used at 160nM
PIM1 (RTqPCR primer (for, rev)	CGAGCATGACGAAGAGATCAT TCGAAGGTTGGCCTATCTGA	Metabion		Used at 160nM
PIM2 RTqPCR primer (for, rev)	GAACATCCTGATAGACCTACGC CATGGTACTGGTGCTGAGAG	Metabion		Used at 160nM

PIM3 RTqPCR primer (for, rev)	GACATCCCCTTCG AGCAG ATGGGCCGCAATC TGATC	Metabion		Used at 160nM
NOTCH1 RTqPCR primer (for, rev)	GAGGCGTGGCAGA CTATGC CTTGTACTCCGTCA GCGTGA	Oligomer		Used at 160nM
NOTCH2 RTqPCR primer (for, rev)	CCTGGGCTATACT GGGAGCTACTG ACACCCTGATAGC CTGGGACAC	Oligomer		Used at 160nM
NOTCH4 RTqPCR primer (for, rev)	GATGGGCTGGACA CCTACAC CACACGCAGRGAA AGCTACCA	Oligomer		Used at 160nM
ACTIN RTqPCR primer (for, rev)	TCACCCACACTGT GCCATCTACGA CAGCGGAACCGCT CATTGCCAATGG	Oligomer		Used at 160nM
RBP-Jκ RTqPCR primer (for, rev)	CGG TTT TAC CGT GCT TTC AT GGC TCC TGT TTT ATG GGA CA	Oligomer		Used at 160nM
Recombinant Protein	Fc-control	Jackson Immuno-Research	009-000-008	Used at 10 µg/ml
Recombinant protein	DLL1-Fc	Biotechne	10184-DL-050	Used at 10 µg/ml

Recombinant protein	DLL3-Fc	ACRO Biosystems	DL3-H5255	Used at 10 µg/ml
Recombinant protein	DLL4-Fc	Sino Biological Inc.	158-10171-H02H-100	Used at 10 µg/ml
Recombinant protein	JAG1-Fc	Sino Biological Inc.	158-11648-H02H	Used at 10 µg/ml
Recombinant protein	JAG2-Fc	R&D Systems	1726-JG-050	Used at 10 µg/ml
Antibiotic	Blasticidine S	Sigma	15205	10 µg/ml
Kit	NucleoSpin RNA II kit	Machinery Nagel	740955	
Kit	iScript cDNA Synthesis kit	Bio-Rad	1708891	
Kit	Taqman reverse transcription kit	Applied Biosystem	N8080234	
PCR reagent	SYBR Green PCR mix	Fermentas	4415440	
Transfection reagent	Lipofectamine RNAiMAX	Invitrogen	13778150	

Appendix 2: NucleoSpin RNA II kit protocol

5 NucleoSpin® RNA protocols

5.1 RNA purification from cultured cells and tissue

Before starting the preparation:

• Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize sample

Disrupt up to 30 mg of tissue (for sample amounts see section 2.2; for homogenization methods see section 2.3).

Up to 5 x 106 eukaryotic cultured cells can be collected by centrifugation and lysed by addition of Buffer RA1 directly.

2 Lyse cells

Add 350 µL Buffer RA1 and 3.5 µL ß-mercaptoethanol (ß-ME) to the cell pellet or to ground tissue and vortex vigorously.

For appropriate sample and lysis buffer amounts see section 2.2.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

Reduce viscosity and clear the lysate by filtration through NucleoSpin® Filter (violet ring): Place NucleoSpin® Filter in a Collection Tube (2 mL), apply the mixture, and centrifuge for 1 min at 11,000 x g.

The lysate may be passed alternatively ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

In case of visible pellet formation (depending on sampleamount and nature) transfer supernatant without any formed pellet to a new 1.5 mL microcentrifuge tube (not supplied).

Important: To process higher amounts of cells (> 1 x 106) or tissue (> 10 mg), the lysate should first be homogenized using the 0.9 mm needle (20 gauge), followed by filtration through NucleoSpin® Filters.

4 Adjust RNA binding conditions

Discard the NucleoSpin® Filter and add 350 µL ethanol (70 %) to the homogenized lysate and mix by pipetting up and down (5 times).

Alternatively, transfer flowthrough into a new 1.5 mL microcentrifuge tube (not provided), add 350 µL ethanol (70 %), and mix by vortexing (2 x 5 s).

After addition of ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to disaggregate any precipitate by mixing and load all of the precipitate on the column as described in step 5. Do not centrifuge the ethanolic lysate before loading it onto the column in order to avoid pelleting the precipitate.

5 Bind RNA

For each preparation take one NucleoSpin® RNA Column (light blue ring) placed in a Collection Tube. Pipette lysate up and down 2–3 times and load the lysate to the column. Centrifuge for 30 s at 11,000 x g. Place the column in a new Collection Tube (2 mL).

Maximal loading capacity of NucleoSpin® RNA Columns is 750 µL. Repeat the procedure if larger volumes are to be processed.

6 Desalt silica membrane

Add 350 µL MDB (Membrane Desalting Buffer) and centrifuge at 11,000 x g for 1 min to dry the membrane. Salt removal will make the following rDNase digest much more effective. If the column outlet has come into contact with the flowthrough for any reason, discard the flowthrough and centrifuge again for 30 s at 11,000 x g.

7 Digest DNA

Prepare DNase reaction mixture in a sterile 1.5 mL microcentrifuge tube (not provided): For each isolation, add 10 µL reconstituted rDNase (also see section 3) to 90 µL Reaction Buffer for rDNase. Mix by flicking the tube.

Apply 95 µL DNase reaction mixture directly onto the center of the silica membrane of the column. Incubate at room temperature for 15 min.

8 Wash and dry silica membrane

1st wash

Add 200 µL Buffer RAW2 to the NucleoSpin® RNA Column. Centrifuge for 30 s at 11,000 x g. Place the column into a new Collection Tube (2 mL). Buffer RAW2 will inactivate the rDNase.

2nd wash

Add 600 µL Buffer RA3 to the NucleoSpin® RNA Column. Centrifuge for 30 s at 11,000 x g. Discard flowthrough and place the column back into the Collection Tube.

Note: Make sure that residual buffer from the previous steps is washed away with Buffer RA3, especially if the lysate has been in contact with the inner rim of the column during loading of the lysate onto the column. For efficient washing of the inner rim flush it with Buffer RA3.

3rd wash

Add 250 µL Buffer RA3 to the NucleoSpin® RNA Column. Centrifuge for 2 min at 11,000 x g to dry the membrane completely. Place the column into a nucleasefree Collection Tube (1.5 mL, supplied).

If for any reason, the liquid level in the Collection Tube has reached the NucleoSpin® RNA Column after centrifugation, discard flowthrough, and centrifuge again.

9 Elute RNA

Elute the RNA in 60 µL RNase-free H2O, (supplied) and centrifuge at 11,000 x g for 1 min.

If higher RNA concentrations are desired, elution can be done with 40 µL. Overall yield, however, will decrease when using smaller volumes.