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# Characterizing the role of dependence receptor Notch3 in tumour angiogenesis

Shuheng Lin

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**Characterizing the role of dependence receptor  
Notch3 in tumour angiogenesis**

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## Summary

The Notch signalling is a highly conserved signalling which mediates numerous biological processes, including embryonic development and tissue homeostasis via its role in cell fate decisions. Widely implicated in tumorigenesis, the Notch signalling is also a key regulator of developmental angiogenesis and tumour angiogenesis. Therefore, various efforts have been made to inhibit the canonical Notch pathway. Notch3, mainly expressed in the vascular system, was implicated in various vascular pathologies such as CADASIL, ischemic stroke and hypertension. Nevertheless, its role in tumour angiogenesis has never been studied. Therefore, the objectif of my thesis was to study the role of Notch3 during tumour angiogenesis. We showed that Notch3 is aberrantly expressed in tumour endothelial cells where it presents an unexpected pro-apoptotic effect. By silencing Noth3 in the tumour microenvironment, we showed that Notch3 limits tumour angiogenesis via its pro-apoptotic function in tumour endothelial cells which results in inhibition of tumour growth. In addition, we found that JAG1 is up-regulated in a fraction of human cancers. Furthermore, tumour derived JAG1 facilitates the survival of tumour endothelial cells by inhibiting the pro-apoptotic effect of Notch3. We thus present here that Notch3 behaves as a dependence receptor inducing apoptosis in tumour endothelial cells which is blocked by JAG1. Consequently, we show that the well-documented anti-tumour effect mediated by gama-secretase inhibition is at least in part dependent on the apoptosis triggered by Notch3 in endothelial cells.

## Résumé

La voie de signalisation Notch est une signalisation fortement conservée chez les métazoaires. Elle régule de nombreux processus biologiques, y compris le développement embryonnaire, l'homéostasie tissulaire en régulant les mécanismes de différenciation cellulaire. Largement décrite dans la tumorigénèse, la voie Notch est aussi un régulateur clé dans l'angiogénèse. De plus en plus d'études montrent que la voie Notch joue un rôle important dans la régulation de l'angiogénèse tumorale. Par conséquent, de nombreux efforts ont été faits pour inhiber la voie canonique de Notch. Notch3 est exprimé principalement au niveau du système vasculaire et impliqué dans de nombreuses maladies vasculaires, par exemple la CADASIL, l'AVC ischémique et aussi l'hypertension. En revanche il n'a jamais été étudié dans le contexte de l'angiogénèse tumorale. Par conséquent, l'objectif de ma thèse a été d'étudier le rôle de Notch3 dans l'angiogénèse tumorale. Nous avons montré que l'expression de Notch3 est augmentée au niveau des cellules endothéliales tumorales où il présente un effet inattendu. En inhibant Notch3 dans le microenvironnement de la tumeur, nous avons montré que Notch3 limite l'angiogénèse tumorale par sa fonction pro-apoptotique dans les cellules endothéliales tumorales, entraînant l'inhibition de la croissance tumorale. De plus, nous avons constaté que l'expression de Jagged1 est augmentée dans une fraction des cancers humains. En outre, Jagged1 exprimée par les cellules tumorales favorise la survie des cellules endothéliales tumorales en inhibant l'effet pro-apoptotique de Notch3. Nous démontrons donc que Notch3 est un récepteur à dépendance induisant l'apoptose dans les cellules endothéliales tumorales qui est bloquée par Jagged1. Finalement, nous montrons que l'effet antitumoral bien documenté médié par l'inhibition de la  $\gamma$ -sécrétase dépend au moins en partie de l'apoptose induite par Notch3 dans les cellules endothéliales.

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## ABBREVIATION LIST

|               |  |
|---------------|--|
| $\alpha$ -SMA | $\alpha$ smooth muscle actin                                   |
| Apaf-1        | Apoptotic protease-activating factor 1                         |
| AT            | Adipose tissue   |
| BAT           | Brown adipose tissue   |
| BID           | BH3-interacting domain death agonist                           |
| tBID          | Truncated BID  |
| CAAs          | Cancer associated adipocytes                                   |
| CADASIL       | Arteriopathy with subcortical infarcts and leukoencephalopathy |
| CAFs          | Cancer associated fibroblasts                                  |
| CARD          | Caspase recruitment domain                                     |
| CCL5          | C-C motif chemokine ligand 5                                   |
| CDON          | Cell-adhesion molecule-related/down-regulated by Oncogenes     |
| CLL           | Chronic lymphocytic leukaemia                                  |
| CSF           | Colony-stimulating factor                                      |
| CSL           | CBF1/RBPjk/Su(H)/Lag-1   |
| CTGF          | Connective tissue growth factor                                |
| CXCL          | C-X-C motif chemokine ligand                                   |
| DCC           | Deleted in Colorectal Cancer                                   |
| DD            | Death domain   |
| DED           | Death effector domain  |
| DISC          | Death-inducing signalling complex                              |
| DLBCL         | Diffuse large B cell lymphoma                                  |

|              |  |
|--------------|--|
| DRs          | Dependence Receptors                         |
| ECM          | Extra cellular matrix                        |
| EDA-FN       | Extradomain A-fibronectin                    |
| EGFR         | Epithelial growth factor receptor            |
| ER           | Endoplasmic reticulum                        |
| FAF          | Fibrosis associated fibroblast               |
| FAP          | Fibroblast activation protein                |
| FGF          | Fibroblast growth factor                     |
| GDNF         | Glia cell line-derived neurotrophic factor   |
| HGF          | Hepatocyte growth factor                     |
| IAP          | Inhibitors of apoptosis proteins             |
| ICAM1        | Intracellular adhesion molecule 1            |
| IFN $\gamma$ | Interferon- $\gamma$                         |
| IL           | Interleukin                                  |
| LECs         | Lymphatic endothelial cells                  |
| LOX          | Lysyl oxidase                                |
| MDSCs        | Myeloid-derived suppressor cells             |
| MMP          | Matrix metalloproteinase                     |
| MOMP         | Mitochondria outer membrane permeabilization |
| MSCs         | Mesenchymal stem cells                       |
| NAFs         | Normal activated fibroblasts                 |
| NEXT         | Notch extracellular truncation               |
| NICD         | Notch intracellular domain                   |

|                  |   |
|------------------|---|
| NF-κB            | Nuclear factor-κB                       |
| NK               | Natural killer                          |
| PDAC             | Pancreatic ductal adenocarcinoma        |
| PDGF             | Platelet derived growth factor          |
| PGE <sub>2</sub> | Prostaglandin E <sub>2</sub>            |
| PLGF             | Placental growth factor                 |
| RET              | Rearranged during transfection          |
| RGM              | Repulsive Guidance Molecule             |
| ROS              | Reactive oxygen species                 |
| SDF1             | Stromal cell-derived factor 1           |
| SHH              | Sonic Hedgehog                          |
| SMZL             | Splenic marginal zone lymphoma          |
| SQCC             | Squamous cell carcinoma                 |
| SVF              | Stromal vascular fraction               |
| TAF              | Tumour-associated fibroblast            |
| T-ALL            | T cell acute lymphocyte leukaemia       |
| TAM              | Tumour associated macrophage            |
| TASCs            | Tumour associated stromal cells         |
| TGFβ             | Transforming growth factor β            |
| TIMPs            | Tissue inhibitors of metalloproteinases |
| TME              | Tumour microenvironment                 |
| TNF              | Tumour necrosis factor                  |
| TRAIL            | TNF-related apoptosis-inducing ligand   |

|       |                                    |
|-------|------------------------------------|
| TSP-1 | Thrombospondin-1                   |
| VCAM1 | Vascular adhesion molecule 1       |
| VEGF  | Vascular endothelial growth factor |
| WAT   | White adipose tissue               |

# I. Introduction

During my thesis, I was particularly interested in understanding the crosstalk between the tumour microenvironment and the epithelial tumour compartment, with an emphasis on understanding how does the tumour angiogenesis influence the tumour progression. During tumorigenesis, modification of the tumour vasculature is only one of the numerous modifications of the tumour microenvironment to regulate tumorigenesis. In addition, crosstalk of the different components of the tumour microenvironment is also important for the tumour growth. Even my thesis is mainly focused on the role of Notch3 in tumour angiogenesis, I found that it is important to have a global vision of the different components of the tumour microenvironment. That's why I made a general presentation of the tumour microenvironment in the first part of my introduction, then followed by a summarization of the Notch signalling in cancer and in angiogenesis, and finished by a general presentation of apoptosis and dependence receptor in cancer.

## **1. Tumour microenvironment**

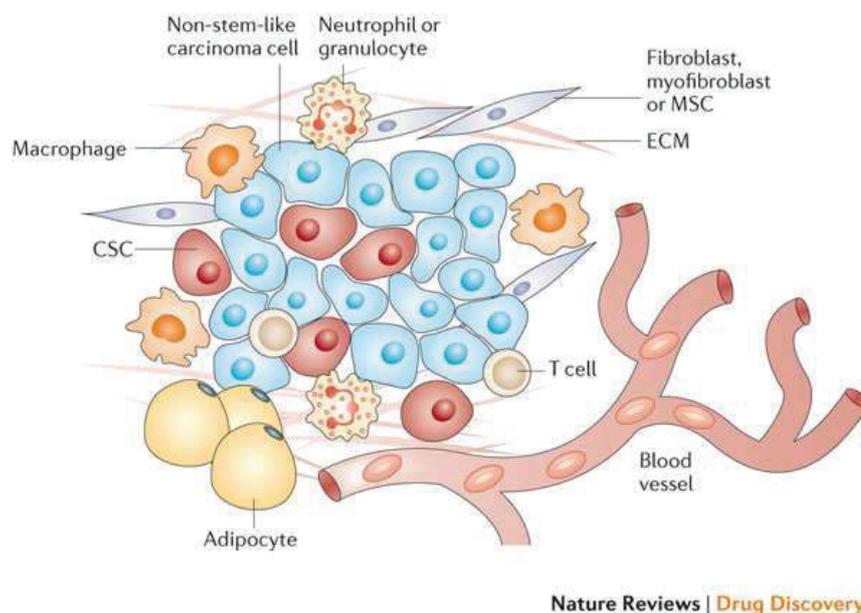
### **1.1 General consideration on tumour microenvironment**

The tumour progression was thought to be restricted to accumulation of mutations in cancer cells for a long time, and contributions of the microenvironment to the tumour malignancy had been largely ignored. Even the hypothesis of “seed and soil” was proposed by Steven Paget early in 1889, considerable efforts investigating the tumour microenvironment have been only engaged in the last twenty years.

Tumour is a highly complex organ which do not simply consist of epithelial tumour cells but also of a variety of stromal cells present in the tumour microenvironment (Figure 1). These stromal cells are recruited inside or in the surrounding environment of the tumour bulk along the tumorigenesis and these can not only enhance the primary tumour growth but also facilitate metastasis to distant organs and colonize new sites. The microenvironment has a critical influence during the tumour progression. Even if cells harbour oncogenic mutations, they couldn't initiate neither progress the disease without a proper microenvironment. Furthermore, increasing evidences show that tumour cells can stay dormant for years without triggering the disease, and that a little perturbation of the microenvironment is sufficient to launch the tumour growth. On the other hand, during wound healing and inflammation diseases, local stromal cells can be recruited and change their phenotype to become active stromal cells which in turn improve the process (Bussard et al., 2016). In line with this, tumour cells can send oncogenic signals to educate their microenvironment and then create a proper niche to facilitate the tumour initiation, progression and even metastasis. They recruit local host stromal cells and activate them. These reactive stromal cells will then change their phenotype and transform into tumour associated stromal cells (TASCs).

The most abundant TASCs found in solid tumours can be classified into three main groups according to their origins: the hematopoietic origin group, the mesenchymal origin group, and the non-cellular component group. The hematopoietic group consist of cells arising from bone marrow and they can be sub-divided into two sub-groups according to their lineage: lymphoid lineage and myeloid lineage. The lymphoid lineage consists of T cells, B cells and Natural killer (NK) cells and the myeloid lineage includes macrophage, neutrophils, and myeloid-derived

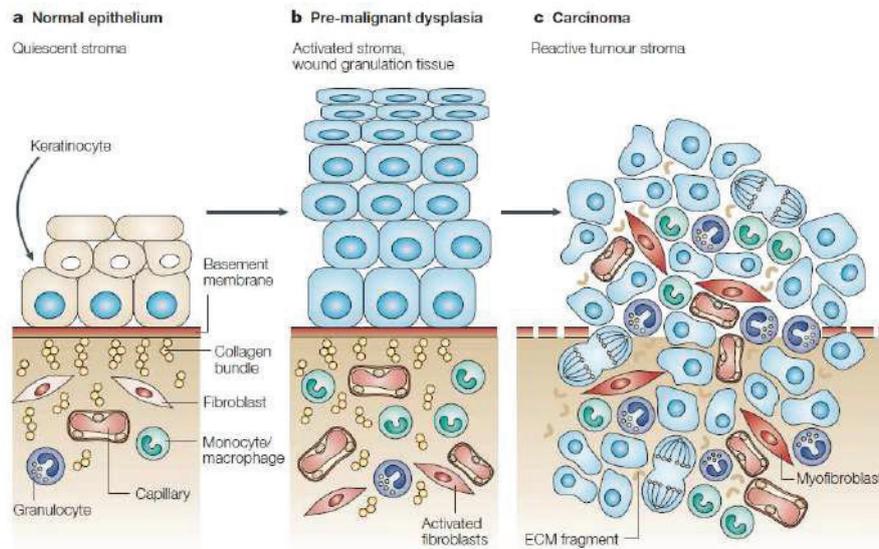
suppressor cells (MDSCs). Cells from these two groups can both have pro and anti-tumour effect. That's why understanding their function is of primary importance for immune therapy. In the mesenchymal group, cells derive from mesenchyme and include endothelial cells, adipocytes, fibroblast, myofibroblast and mesenchymal stem cells (MSCs). Many studies have shown that MSCs are an important support for cancer stem cells niche (Quante et al., 2011). Cancer associated fibroblasts (CAFs) are very well documented, which can have critical influence on tumour progression, especially in the case of pancreatic ductal adenocarcinoma (PDAC). Endothelial cells and pericytes are the main components of the tumour vascular network and hyperproliferation of tumour endothelial cells is usually found in high angiogenic tumours. The major non-cellular component of the tumour microenvironment is the extra cellular matrix (ECM) (Lu et al., 2011; Pattabiraman and Weinberg, 2014). ECM is composed of collagens, laminin, fibronectin etc. More and more studies have demonstrated that an abnormal ECM is favourable to tumour progression and tumour angiogenesis (Pattabiraman and Weinberg, 2014). Taken together it is necessary to better understand the dynamics between tumour cells and their microenvironment, which could help us to find out new diagnostic markers and therapeutic targets.



**Figure 1. Tumour microenvironment is composed of various stromal cells including immune cells, cancer associated fibroblasts, tumour vasculature and adipocytes, extra cellular matrice etc (Nature Reviews Drug Discovery 2014).**

## 1.2 Common abundant components of tumour stroma

Tumour stroma includes all the supporting tissues inside or surrounding the tumour. They consist of various cell types. The most common abundant stromal cell populations found in solid tumours are fibroblasts, immune cells, blood vessel cells, and adipocytes. Alongside the tumour progression, different stromal cells can escape the quiescent status and turn into active stage. Furthermore, following the cross-talk signalisation with tumour cells, some tumour stromal cells can change their phenotype and form a reactive stroma at late tumorigenesis stage (Figure 2). It has been shown that cancer cells can modulate their stromal microenvironment by secreting stroma-modulating growth factors such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor receptor (EGFR) ligands, interleukins, colony-stimulating factor (CSF), transforming growth factor- $\beta$  (TGF $\beta$ ) and protease (Mueller and Fusenig, 2004). These stimulating factors can disrupt the normal tissue homeostasis like during the wound healing process and they act in a paracrine manner to induce stromal reactions such as angiogenesis and inflammatory response. Consistent with this, tumour cells can also modulate the microenvironment by producing different proteolytic enzymes to degrade the ECM and destabilize the basement membrane creating a pro-migratory and pro-invasive environment. Nevertheless, the tumour stromal cells also vary a lot in term of amount and composition between tumours and they don't correlate totally to the tumour grade (Mueller and Fusenig, 2004).



**Figure 2. Tumour stages depend on stroma activation. a Normal well-differentiated epithelium, made of keratinocytes and well separated by a well-delineated basement membrane from the stromal compartment. b During transition to pre-malignant dysplasia, epithelial cells become proliferative resulting in hyperplastic epithelium. The basement membrane remains intact, but stroma become reactive. c Carcinoma is associated with proliferation of epithelial cells along with an activated tumour stroma and the degradation of ECM (Nature Reviews Cancer, 2004).**

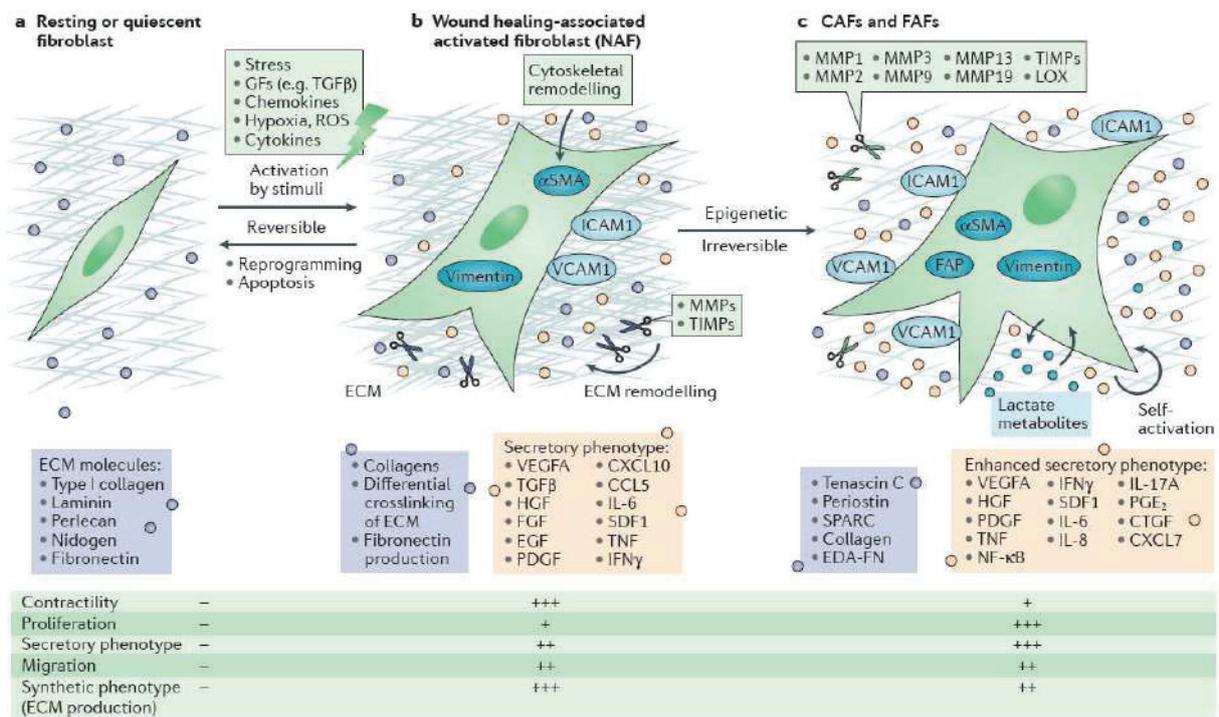
### 1.2.1 Cancer associated fibroblasts

As the most abundant cells types in connective tissue, fibroblasts remain normally in a quiescent state and they become activated when wound healing takes place. Their major function is to synthesise the ECM and maintain the structural framework of animal tissue. Fibroblasts are the dominant component of the tumour stroma and they are associated with all stages of disease progression. Fibroblasts associated with cancer are named as cancer-associated fibroblasts (CAF) or tumour-associated fibroblasts (TAF).

In healthy physiological conditions, fibroblasts stay inactive. Inactive fibroblasts are usually in spindle-shaped and smaller than activated fibroblasts. They have a negligible metabolic and transcriptomic activity. However, in response to tissue injury and other stimuli, the quiescent fibroblasts are reversibly activated and gain the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and vimentin (Kalluri, 2016). They present a more cruciform or stellate shape and they have a very active metabolism and secrete various growth factors. Moreover, they synthesis more

ECM and become more migratory. At this stage, activated fibroblasts can reverse to quiescent status through reprogramming or going into apoptosis and they are named normal activated fibroblast (NAF) (Figure 3) (Table 1). Besides wound healing, resting fibroblasts can also be activated in acute and chronic inflammation and tissue fibrosis (Desmouliere et al., 2003) or in culture *in vitro* by adding growth factors such as TGF $\beta$ , PDGF, bone morphogenic proteins (BMPs), FGFs, sonic hedgehog (SHH) and IL-6 (Kalluri, 2016; Kalluri and Zeisberg, 2006).

Furthermore, activated fibroblast can gain a more advanced phenotype which is characterized by increase of proliferation, remodelling of the ECM, robust autocrine activation, and dynamic modulation of immune cells. This process is usually irreversible and associated with the development of cancer lesion. That's why tumours are considered as 'wounds that do not heal' (Figure 3) (Kalluri, 2016). Compared to NAF, CAF or TAF are more proliferative and invasive. They can enhance tumorigenesis when they are co-cultured with cancer cells, which has been shown by inoculating the mixture of Simian virus 40 (SV40)-transformed prostate epithelial cells and NAF or CAF in mice. Only the mixture with CAF could initiate the tumorigenesis in mice (Olumi et al., 1999; Orimo et al., 2005). CAFs also facilitate the tumour progression and metastasis by enhancing tumour angiogenesis, modulation of tumour immune cells phenotypes (Takahashi et al., 2017; Zhang et al., 2017), ECM modification, and inhibition of cancer cells death (Martinez-Outschoorn et al., 2010; Wang et al., 2017). Moreover, CAFs are reported to participate in the generation and the maintenance of the cancer stem cell niche (Albini et al., 2015). In colon cancer, cancer stem cells with elevated WNT signalling are found to be proximal to CAFs (Vermeulen et al., 2010). In line with this, the paracrine signals induced by CAFs trigger the expression of insulin-like growth factor II (IGF2) and IGF1 receptor (IGF1R) signalling in lung cancer stem cells, inducing Nanog expression and stemness-like phenotype in lung cancer cells (Chen et al., 2014). Besides the direct communication between CAFs and cancer cells, CAFs can also promote tumour progression in an indirect manner by a metabolic competition with other stromal cells that may result in T cell hyporesponsiveness in tumours. It has been shown that CAFs have a high glycolysis metabolic program resulting in nutrient restriction in T cells which in turn inhibits IFN- $\gamma$  production (Chang et al., 2015).



**Figure 3. Multi-step activation of fibroblasts.** Quiescent fibroblasts are spindle-shaped like, embedded in physiological ECM (a). In response to tissue injury and the associated stimuli, fibroblasts are activated and become NAF with a gain of expression of  $\alpha$ SMA and vimentin, and with an enhanced production of ECM and a synthetic secretory (b). Activated fibroblast become CAF or FAF by gaining a further secretory phenotype and ECM remodelling ability (c) (The biology and function of fibroblast in cancer-*Nature Reviews Cancer*, 2016).

| Quiescent or resting fibroblasts                               | Activated fibroblasts   |
|--|---|
| Morphologically bland (spindle shaped)                         | Morphologically active (cruciform or stellate shaped)                         |
| Metabolically indolent   | Metabolically active  |
| G0/G1 arrest or slow cycling self-renewal                      | Proliferative   |
| Activated by growth factors                                    | Further activated by growth factors   |
| FSP1 <sup>+</sup> , $\alpha$ 1 $\beta$ 1 integrin <sup>+</sup> | $\alpha$ SMA <sup>+</sup> , PDGFR $\beta$ <sup>+</sup> , FAP <sup>+</sup>     |
| Non-migratory  | Migratory   |
| No ECM production  | ECM production and synthetic phenotype  |
| No active secretome  | Active and dynamic secretome  |
| Epigenetically stable  | Epigenetically modified (e.g. RASAL1 hypermethylation)                        |
| Precursor for activated fibroblasts                            | Precursor for iPSCs, chondrocytes, adipocytes, myocytes and endothelial cells |

$\alpha$ SMA,  $\alpha$ -smooth muscle actin; ECM, extracellular matrix; iPSCs, induced pluripotent stem cells; PDGFR $\beta$ , platelet-derived growth factor receptor- $\beta$ ; RASAL1, RAS protein activator like 1.

**Table 1. Characterization of quiescent versus activated fibroblasts (The biology and function of fibroblast in cancer-*Nature Reviews Cancer*, 2016).**

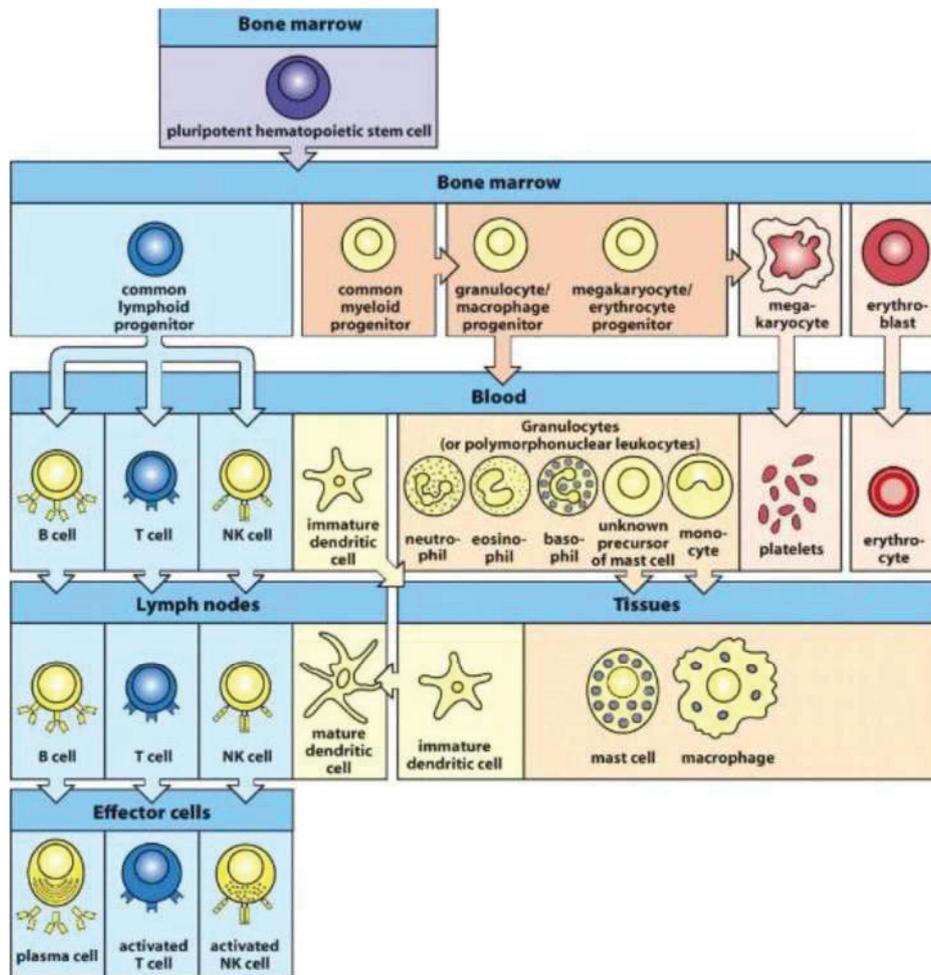
### 1.2.2 Immune cells

Immune cells represent an important component of the tumour microenvironment. To better understand and describe the relationship between immune system and tumour cells during tumour progression, the concept of cancer immunoediting was proposed at the early 2000 (Dunn et al., 2002; Dunn et al., 2004; Shankaran et al., 2001). Cancer immunoediting consists of three phases: elimination, equilibrium, and escape. In the elimination phase, tumour cells are destroyed by innate and adaptive immunity before they become clinically apparent. However, if cancer cells are not destroyed in the elimination phase, they will enter equilibrium phase, in which the tumour immunogenicity is edited by the adaptive immunity. In the escape phase, tumour cells that have acquired the ability to circumvent immune recognition and/or destruction emerge as progressively growing, visible tumours (Mittal et al., 2014).

Different immune cell types present in the tumour microenvironment can play two opposite roles: pro-tumour progression or anti-tumour response. One of the most direct evidence that an abnormal immune microenvironment affects tumorigenesis is that tissues exposed to chronic inflammation generally show higher cancer incidence (Grivennikov et al., 2010). For instance, patients who have liver cirrhosis or bowel disease present higher risk to develop liver cancer or colorectal cancer. However, it has also been shown that impaired immune responses can correlate with elevated cancer incidence. For example, AIDS patients have elevated incidence of skin, lung, or central nervous system cancers (Barcellos-Hoff et al., 2013). Consistent with this, similar retrospective studies (Vajdic and van Leeuwen, 2009) indicated that adequate immune response may have a protective effect against certain cancers. Furthermore, these paradoxical effects of immune response along tumorigenesis highlight the importance of the context. Therefore, the opposite functions of immune cells in cancers should be considered in the therapeutic strategies adapted in clinical trials. In addition, in the past twenty years, immunotherapy for cancer treatment become a very hot topic, and the successes of antibodies which target immune checkpoints in clinic trials is very encouraging (Kyi and Postow, 2014). However, mechanisms under these anti-tumour responses or pro tumour progression are still not clear, and identifying new targets to lower the pro-tumour effect and increase the anti-tumour response will be necessary.

Arising from hematopoietic stem cells, immune cells can be divided into two lineages: myeloid and lymphoid. The different populations derived from myeloid progenitor cells are monocytes,

macrophages, neutrophils, basophils, eosinophils, erythrocytes, dendritic cells (DC), and megakaryocytes (or platelets) and the population derived from lymphoid progenitor cells are T and B lymphocytes, Natural killer cells (NK) and other innate lymphoid cells (Figure 4).



**Figure 4: All the cellular elements of blood, including the lymphocytes of the adaptive immune system, arise from hematopoietic stem cells in the bone marrow (*Janeway's immunobiology, 8ed, Garland science 2012*).**

These different immune cell populations in the tumour microenvironment present distinct functions (Table 2). Cells involved in anti-tumour responses include innate lymphocytes (NK, NKT,  $\gamma\delta$  T cells), antigen presenting cells (APCs) including DC and macrophages type 1, and adaptive lymphocytes (CD4, CD8 T and B cells). The mediators include molecules such as: cytokines (IFN- $\gamma$ , IFN- $\alpha/\beta$ , IL-2, TNF $\alpha$ ...), recognition receptors (NKG2D, CD1d, MHC1) and cytotoxic molecules such as Perforin and Trail. By contrast, cells that can acquire pro-

tumorigenic functions are tumour associated macrophages (TAM) also called macrophage type II, myeloid-derived suppressive cells (MDSC) and T<sub>reg</sub> cells. They support the tumour progression by various mechanisms. For instance, TAMs promote tumour growth by facilitating tumour angiogenesis, secreting pro-tumorigenic proteases, cytokines, and growth factors. As tumour grow, MDSC and Treg become mobilized into the circulation in response to cytokines axes and infiltrate into tumour and then disrupt immune surveillance by disruption of antigen presentation by DCs, inhibition of T and B cell proliferation and activation or inhibition of NK cell cytotoxicity (Quail and Joyce, 2013).

| Cell type              | Markers (human)  | Markers (mouse)   | Function   |
|------------------------|--|---|--|
| <b>Myeloid lineage</b> |  |   |  |
| TAM                    | CD11b <sup>+</sup><br>CD68 <sup>+</sup><br>CSF1R <sup>+</sup><br>CD163 <sup>+</sup><br>EMR1 <sup>+</sup>   | CD11b <sup>+</sup> GR1 <sup>-</sup><br>CD68 <sup>+</sup> CSF1R <sup>+</sup><br>F4/80 <sup>+</sup>   | Classically activated M1 macrophages are proinflammatory and anti-tumorigenic and secrete T <sub>H</sub> 1 cytokines. Alternatively activated M2 macrophages are anti-inflammatory and pro-tumorigenic and secrete T <sub>H</sub> 2 cytokines. TAMs frequently exhibit an M2 phenotype; their presence in tumors supports angiogenesis and invasion. |
| DC                     | CD11c <sup>+</sup> CD83 <sup>+</sup><br>CD123 <sup>+</sup>   | CD11c <sup>+</sup> CD83 <sup>+</sup><br>CD123 <sup>+</sup>  | DCs are monocytic antigen-presenting cells that are derived from the bone marrow. DCs presenting tumor-specific antigens are being developed as vaccines to induce both innate and adaptive immune responses to regress tumors and prevent relapse.  |
| TEM                    | CD11b <sup>+</sup><br>SCA1 <sup>+</sup><br>TIE2 <sup>+</sup><br>CD14 <sup>+</sup><br>CD16 <sup>+</sup>   | CD11b <sup>+</sup><br>GR1 <sup>-</sup> SCA1 <sup>+</sup><br>TIE2 <sup>+</sup>   | TIE2 is a receptor for the angiogenic growth factor, angiopoietin. TIE2-expressing monocytes (TEMs) have a role during tumor angiogenesis through a paracrine signaling loop with angiopoietin-expressing endothelial cells.   |
| Neutrophil             | CD11b <sup>+</sup> CD66b <sup>+</sup><br>CD63 <sup>+</sup>   | CD11b <sup>+</sup><br>GR1 <sup>+</sup><br>7/4 <sup>+</sup>  | Neutrophils are the most abundant circulating leukocyte in humans and are phenotypically plastic. Similar to TAMs, neutrophils have been shown to have opposing functions in regulating cancer progression and metastasis, indicating that they have context-dependent roles within the TME.   |
| Mast cell              | CD11b <sup>-</sup><br>CD49d <sup>+</sup><br>CD117 <sup>+</sup><br>CD203c <sup>+</sup>  | CD11b <sup>-</sup> CD49d <sup>+</sup><br>CD117 <sup>+</sup><br>CD203c <sup>+</sup>  | Mast cells are best known for their role during allergies and autoimmunity. Mast cells are recruited to tumors, where they release factors that enhance proliferation of endothelial cells to promote tumor angiogenesis.  |
| MDSC                   | CD11b <sup>+</sup><br>CD33 <sup>+</sup><br>HLA-DR <sup>-</sup><br>CD14 <sup>+</sup> (monocytic)<br>CD14 <sup>-</sup><br>CD15 <sup>+</sup> (granulocytic) | CD11b <sup>+</sup><br>GR1 <sup>+</sup><br>Ly6G <sup>-</sup> Ly6C <sup>+</sup> (monocytic)<br>Ly6G <sup>+</sup> Ly6C <sup>-</sup> (granulocytic) | MDSCs are immunosuppressive precursors of dendritic cells, macrophages and granulocytes. In cancer, their main function is to disrupt tumor immunosurveillance by interfering with T cell activation, cytotoxic activity, antigen presentation and cell polarization.  |

|                         |  |  |   |
|-------------------------|--|--|---|
| <b>Lymphoid lineage</b> |  |  |   |
| NK cell                 | CD56 <sup>+</sup> CD16 <sup>+</sup>  | CD335 <sup>+</sup> NK1.1 <sup>+</sup>  | NK cells are cytotoxic lymphocytes that can kill stressed cells in the absence of antigen presentation. NK cells detect and kill tumor cells through 'missing-self' activation (loss of healthy cell markers) or 'stress-induced' activation (gain of stressed cell markers).   |
| T <sub>H</sub> cell     | CD3 <sup>+</sup> CD4 <sup>+</sup>  | CD3 <sup>+</sup> CD4 <sup>+</sup>  | CD4 <sup>+</sup> T <sub>H</sub> cells can be divided into T <sub>H</sub> 1 and T <sub>H</sub> 2 lineages. T <sub>H</sub> 1 cells secrete proinflammatory cytokines and can be anti-tumorigenic. T <sub>H</sub> 2 cells secrete anti-inflammatory cytokines and can be pro-tumorigenic. The ratio of T <sub>H</sub> 1 to T <sub>H</sub> 2 cells in cancer correlates with tumor stage and grade. |
| T <sub>reg</sub> cell   | CD4 <sup>+</sup> CD25 <sup>+</sup><br>FOXP3 <sup>+</sup><br>CTLA-4 <sup>+</sup><br>CD45RA <sup>+</sup> | CD4 <sup>+</sup> CD25 <sup>+</sup><br>FOXP3 <sup>+</sup> CTLA-4 <sup>+</sup><br>CD103 <sup>+</sup> | T <sub>reg</sub> cells have primarily pro-tumorigenic roles by suppressing immunosurveillance; however, their presence in tumors is positively correlated with overall survival in certain cancer types. These divergent roles may be attributed to context-dependent functions or distinct subpopulations that are challenging to identify at present using conventional markers.              |
| T <sub>C</sub> cell     | CD3 <sup>+</sup> CD8 <sup>+</sup>  | CD3 <sup>+</sup> CD8 <sup>+</sup>  | CD8 <sup>+</sup> cytotoxic T (T <sub>C</sub> ) cells are effector cells of the adaptive immune system. They specifically recognize and destroy cancer cells through perforin- and granzyme-mediated apoptosis.  |
| B cell                  | CD19 <sup>+</sup> CD20 <sup>+</sup>  | B220 <sup>+</sup> CD19 <sup>+</sup> CD22 <sup>+</sup>  | B lymphocytes are important mediators of humoral immunity. In cancer, they can promote disease progression by secreting pro-tumorigenic cytokines and altering T <sub>H</sub> 1-to-T <sub>H</sub> 2 ratios. Their importance in supporting tumor growth is evident in B cell-deficient mice, which exhibit resistance to engraftment of certain syngeneic tumors.                               |

**Table 2 Immune cell populations in the tumour microenvironment have distinct functions during tumorigenesis (Targeted Cancer therapies, *Nature medicine* 2013).**

In addition, immune infiltrates are heterogeneous between tumour types. Patients classified into the same stage of the same cancer have been found to present different immune infiltrates patterns resulting in different clinical outcome (Fridman et al., 2012). Furthermore, detailed intra-tumour analysis illustrates that these immune infiltrates are not randomly distributed. Consequently, the immunoscore, an alternative tumour classification method compared to the traditional histological analysis has been developed to better predict the clinical outcome and the disease recurrence (Galon et al., 2012). According to their CD3/CD8 ratio, patients are attributed an immunoscore from 0 to 4. Two large independent cohorts analyse showed that patients that have less recurrence and better outcome are strongly associated with higher immunoscore (Galon et al., 2014).

### 1.2.3 Tumour vasculature

As one of the hallmarks of cancer (Hanahan and Weinberg, 2011) (Figure 5), tumour angiogenesis is important for tumour growth by providing tumour with oxygen and nutrients as well as evacuating metabolic wastes and carbon dioxide. The tumour vasculature is constituted of multiple cell types including vascular endothelial cells, pericytes, and bone marrow precursor cells. They co-operate to ensure initiation of tumour angiogenesis, endothelial adhesion, vessel integrity, vessel coverage and maturation which are orchestrated by hypoxia (Du et al., 2008; Semenza, 2013). In addition to cell types constituting blood vessels, the tumour vasculature is also modulated by other tumour microenvironment (TME) cells, including TAMs, MSCs and CAFs that secrete various pro-angiogenic factors including endothelin-2, VEGF, FGF-2, IL-8, and TGF- $\beta$  to stimulate proliferation and migration of tumour endothelial cells and activate proteases degrading the local ECM and basement membrane (Weis and Cheresh, 2011).

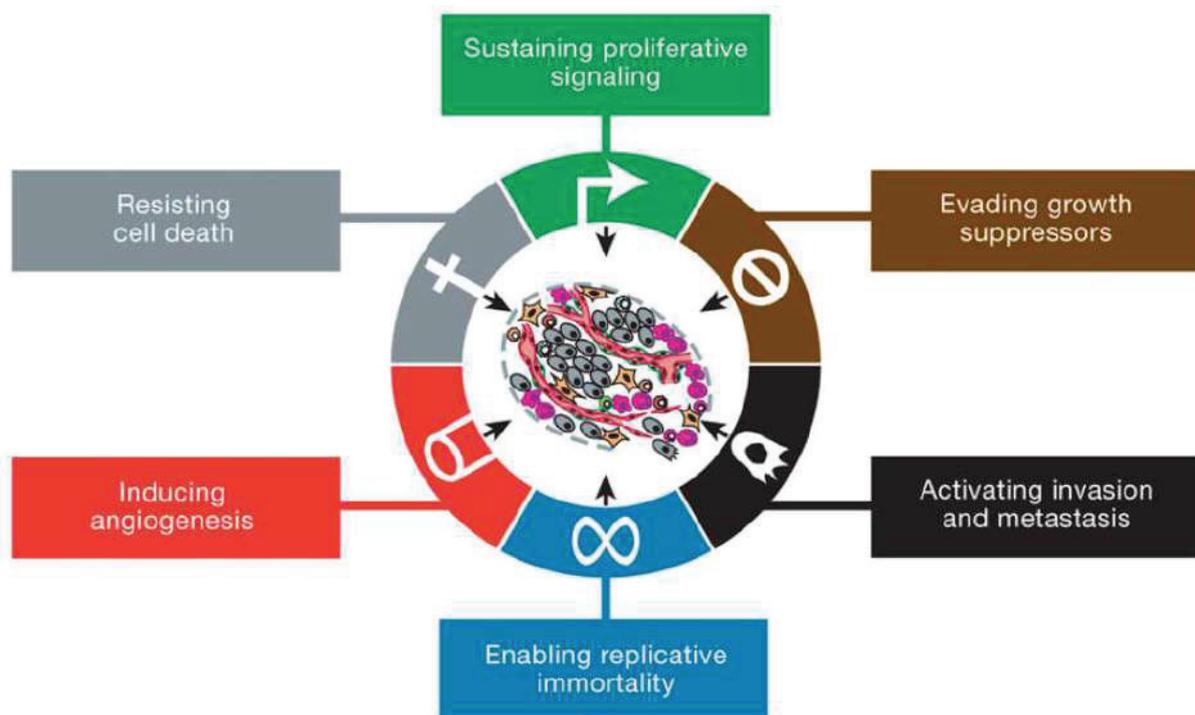
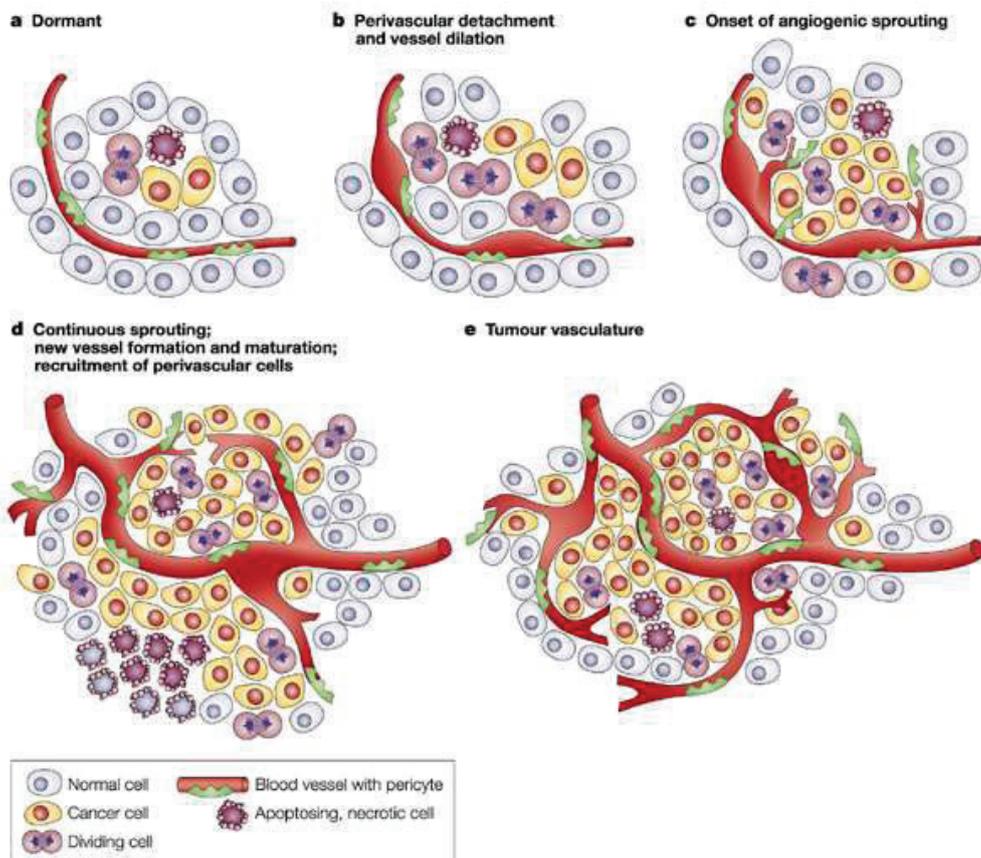


Figure 5 The hallmarks of cancers (Hallmarks of cancers: the next generation. *Cell* 2011).

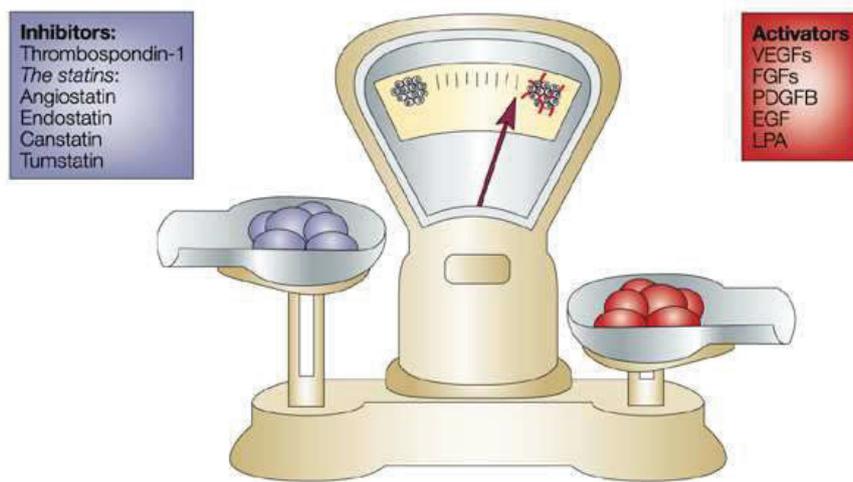
### 1.2.3a Sprouting angiogenesis

The neovascularization of solid tumour is mediated by various mechanisms. The most known is the sprouting angiogenesis which is characterized by the growth of new blood vessels based on the pre-existing ones. Tumour angiogenesis is a multi-steps process (Figure 6) which begins with the perivascular cells detachment and vessel dilation followed by angiogenic sprouting (Bergers and Benjamin, 2003). At this stage, endothelial cells are activated by the binding of specific growth factors including FGF-2, VEGF, angioprotein, PDGF, EGF etc on their receptors which in turn accelerates their proliferation and migration. This phenomenon is accompanied by the degradation of ECM and basement membrane to allow tumour endothelial cells invade into the surrounding matrix (Bergers and Benjamin, 2003). By polarization of the migrating endothelial cells, a lumen is created and the new immature vessel is formed. To establish the new born vessel, vascular mural cells such as pericytes and vascular smooth muscle cells are recruited to the coverage of vessel following by the generation of a new basement membrane and ECM.



**Figure 6. Steps of tumour angiogenesis. In response to pro-angiogenic stimuli, dormant vessels (a) initiate the angiogenic switch with perivascular detachment and vessel dilation (b), followed by angiogenic sprouting (c), new vessel formation and maturation, and the recruitment of perivascular cells (d). Blood-vessel formation will continue as long as the tumour grows, and the blood vessels specifically feed hypoxic and necrotic areas of the tumour to provide it with essential nutrients and oxygen (e) (Tumorigenesis and angiogenic switch. *Nature Reviews Cancer* 2003).**

Nevertheless, the angiogenic switch is usually considered as the disruption of the balance between anti-angiogenic factors and pro-angiogenic factors (Figure 7). Among those angiogenesis inhibitors, Thrombospondin-1 (TSP-1) is the most well-known. TSP-1, a matrix-bound adhesive glycoprotein, is expressed by fibroblasts as well as endothelial cells (Kazerounian et al., 2008). It is an endogenous inhibitor of angiogenesis which functions by binding to CD36, CD40 or LRP on endothelial cells. As a consequence, endothelial cells become insensitive to VEGF stimuli (Lawler, 2002; Taraboletti et al., 2010). In another way, TSP-1 also binds to and inactivates MMP-9, which enables the release of VEGF from the ECM (Bergers et al., 2000). It has been reported that TSP-1 is virtually silenced in several human breast cancer cell lines and its repression is mediated by the PI3-kinase/Rho GTPase/ROCK/Myc pathway in a phosphorylation dependent manner (Watnick et al., 2003). On the other hand, among these angiogenic activators, VEGFs, FGFs, and EGFs are usually found to be up-regulated in cancers (Zhang et al., 2013; Zhao et al., 2011). As the most potent pro-angiogenic proteins, VEGF is secreted by various cell types such as tumour cells, TAMs, CAFs etc. VEGFs is a general activator of endothelial cell proliferation and mobility. The VEGF pathway is mediated by 6 ligands: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor (PLGF) and three receptors: VEGFR1, VEGFR2 and VEGFR3. The VEGF-A/VEGFR2 is the major regulator during tumour sprouting angiogenesis. By contrast, VEGFR1 reacts as a negative regulator of endothelial cells during development and does not affect the functionality of physiological vessel (Carmeliet et al., 2001).



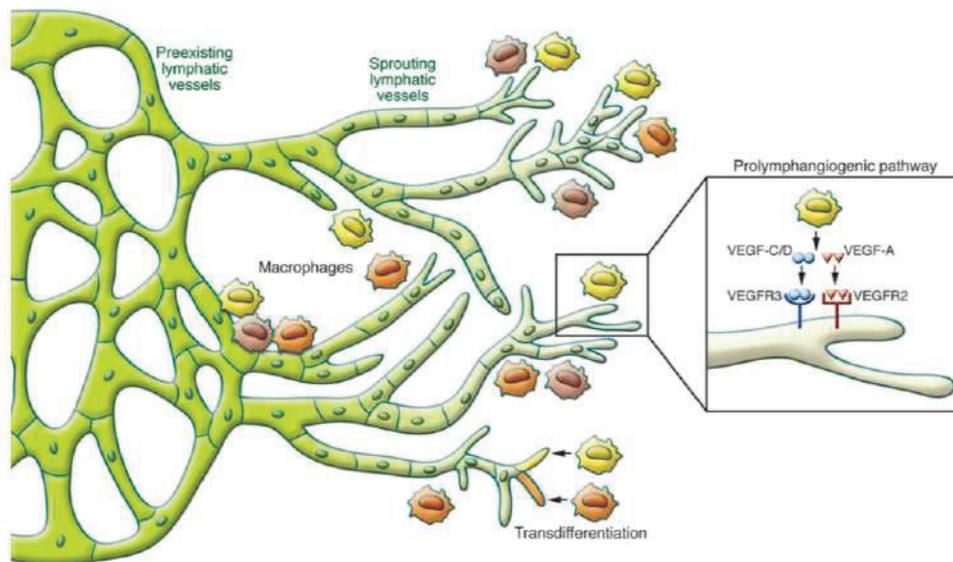
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**Figure 7** Angiogenesis is orchestrated by a variety of activators and inhibitors (The classical angiogenic switch. *Nature Reviews Cancer* 2013).

### 1.2.3b lymphangiogenesis

Lymphangiogenesis is another mechanism of vascularization in tumours. Accumulating evidences show that the lymphatic system participates to the tumour progression especially in tumour metastasis (Alishekevitz et al., 2016; Hirakawa et al., 2007; Paduch, 2016; Skobe et al., 2001). The lymphatic vessel is a blind-ended system, owing to the thin walled lymphatic capillaries, macro molecules such as proteins or antigens are collected from peripheric tissues, and immune cells such as DCs are carried into lymph nodes (Christiansen and Detmar, 2011). The lymphatic vasculature is important for immune function and tissue fluid homeostasis (Chung and Iwakiri, 2013). Unlike blood vascular system, lymphatic system provides the unidirectional traffic way which lack of a central driving force, consequently the shear stress is minimal in lymphatic fluid and the survival of cells inside is optimal. Physiological lymphangiogenesis takes place during embryonic development. Once the development is finished, the lymphatic endothelial cells (LECs) reside in quiescent state until they receive stimuli signals such as inflammation, tissue injury, and tumour growth (Kim et al., 2014). Lymphogenic growth factors are usually secreted by activated macrophages in the case of inflammation. LECs are activated by lymphangiogenic growth factors such as VEGF-C/D which

bind to their receptors VEGFR2/VEGFR3 expressed on the LECs surface. Lymphangiogenesis can also be triggered by transdifferentiation of passive LEC to active LEC (Figure 8).



**Figure 8. Schematic lymphangiogenesis under acute inflammation. In response to inflammatory stimuli, a local sprouting at the preexisting lymphatic vessels actively occurs in response to macrophage-secreted VEGF. As a result, the lymphatic network grows and expands. And this process is regulated by VEGF-C/VEGFR-3 and VEGF-A/VEGF-R2 signaling pathways (Inflammation-associated lymphangiogenesis: a double-edged sword? *JCI* 2014).**

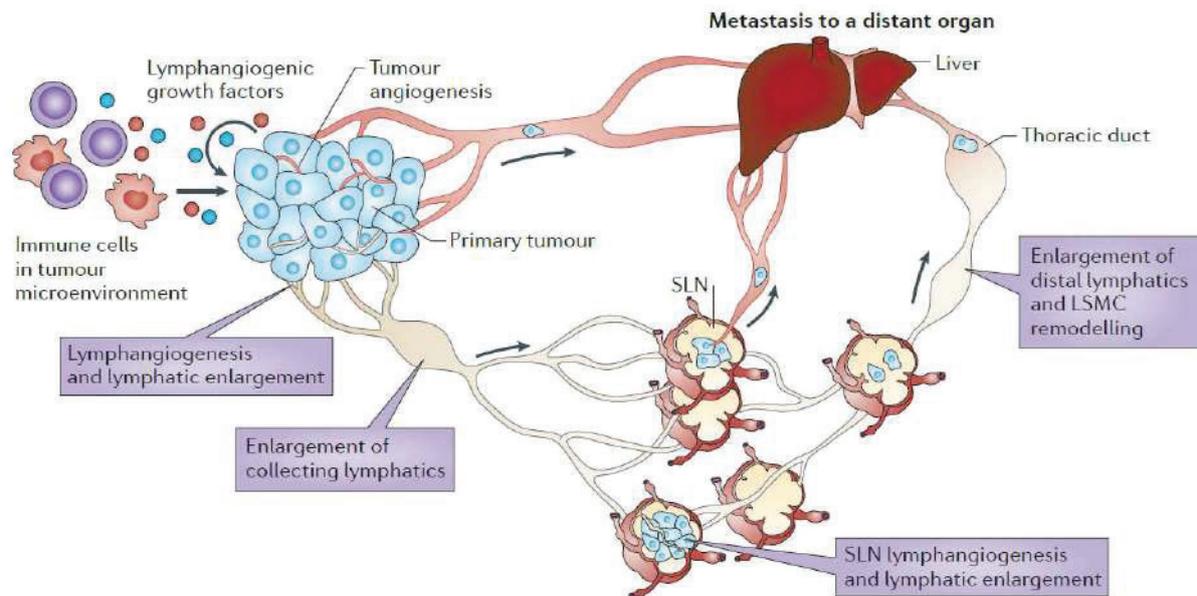
Lymphatic vasculature was considered to play a passive role in tumour progression, however accumulating clinicopathological and experimental studies showed that lymphatic vessels can be changed along tumour progression to facilitate the metastasis. The presence of tumour cells at the proximity to sentinel lymph nodes (SLNs) is a poor prognostic marker in human breast cancer (Tuttle, 2004). Moreover, many clinical studies showed that lymphangiogenesis is correlated to the incidence of lymph node metastasis and a poor outcome for patients (Li et al., 2011; Renyi-Vamos et al., 2005) (Table 3).

| Cancer type       | Expression of lymphangiogenic growth factors and/or receptors  | Tumoural lymphatics   | Lymphatic invasion   |
|-------------------|--|---|--|
| Melanoma          | VEGFC expression predicted shorter DFS and OS <sup>149</sup> , and it is correlated with lymph node metastasis <sup>150</sup>  | Intratumoural lymphatics are associated with distant metastasis <sup>151</sup> and poor DFS <sup>151</sup> ; lymphatic vessel area is associated with poor OS <sup>151</sup> and is prognostic for SLN metastasis <sup>150</sup>  | Associated with SLN metastasis <sup>152</sup> and is a prognostic indicator of metastasis <sup>152</sup>   |
| Breast cancer     | VEGFC expression is associated with lymph node <sup>152</sup> and distant metastasis and shorter OS <sup>154,155</sup> ; no such correlation is observed in other studies <sup>156,157</sup>                                     | Lymphatic vessel density is associated with lymph node metastasis, and with worse DFS and OS <sup>158</sup> ; various studies report few intratumoural lymphatics <sup>120,159</sup> or that peritumoural lymphatics might be more important for tumour spread <sup>160,161</sup> (reviewed in REF. 122)  | Correlates with lymph node metastasis and is an indicator of reduced survival <sup>162-164</sup>   |
| Colorectal cancer | VEGFD expression is prognostic for DFS and OS <sup>165</sup> , and it is associated with lymphatic involvement <sup>165</sup> ; VEGFC is an independent risk factor for lymph node metastasis <sup>166</sup>                     | High lymphatic vessel density predicts disease recurrence and is related to lymph node and distant metastasis <sup>120,167,168</sup> ; lymphatic vessel density at the site of deepest penetration is a predictor of lymph node metastasis <sup>169</sup> ; high peritumoural lymphatic density is associated with disease progression <sup>170</sup> | Correlates with lymphangiogenesis <sup>171</sup> , lymph node metastasis <sup>160</sup> and disease stage; prognostic after curative resection <sup>172</sup> ; prognostic for DFS and OS <sup>173,174</sup> |
| Lung cancer       | Positive staining of tumour cells for VEGFC and endothelial cells for VEGFR3 is an independent prognostic factor in T1 lung adenocarcinoma <sup>175</sup> ; tumoural VEGFC status is a prognostic factor in NSCLC <sup>176</sup> | Lymphatic vessel density is associated with tumour status, stage and lymphatic invasion in NSCLC, and it is an independent predictor of lymph node metastasis <sup>177</sup> ; high lymphatic vessel density is a good indicator of lymphatic vessel invasion and lymph node metastasis <sup>178</sup> and correlates with reduced OS <sup>179</sup>  | Associated with increased risk of regional lymph node involvement in NSCLC, and prognostic for distant metastasis, DFS and long-term survival <sup>100-102</sup>   |

DFS, disease-free survival; NSCLC, non-small-cell lung cancer; OS, overall survival; SLN, sentinel lymph node; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

**Table 3 The relationship between tumour lymphatic parameters and patient outcome (Lymphangiogenesis and lymphatic vessel remodelling cancer *Nature Reviews-Cancer* 2014).**

How does the lymphatic vessel remodelling contribute to metastasis? Cancer cells and other stromal cells secrete lymphangiogenic growth factors including VEGF-C, VEGF-D which bind to VEGFR2 and VEGFR3 on LECs to trigger the lymphangiogenesis. This induces lymphatic enlargement through elevated proliferation of LECs and lymphatic hyperplasia. This lymphatic enlargement is thought to facilitate the uptake of tumour cells into the lymphatic system where they can be transported to lymph nodes or distant organs such as liver, spleen etc (Figure 9).



**Figure 9 Remodelling of lymphatic vessels in cancer and its contribution to metastasis.** In cancer, in response to lymphangiogenic growth factors secreted by immune cells, these pre-existing lymphatics undergo various types of remodelling, including lymphangiogenesis (resulting in the generation of new lymphatics) and lymphatic enlargement. Lymphatic enlargement does not generate new vessels and can involve proliferative or non-proliferative mechanisms. (**Lymphangiogenesis and lymphatic vessel remodelling cancer *Nature Reviews-Cancer 2014***).

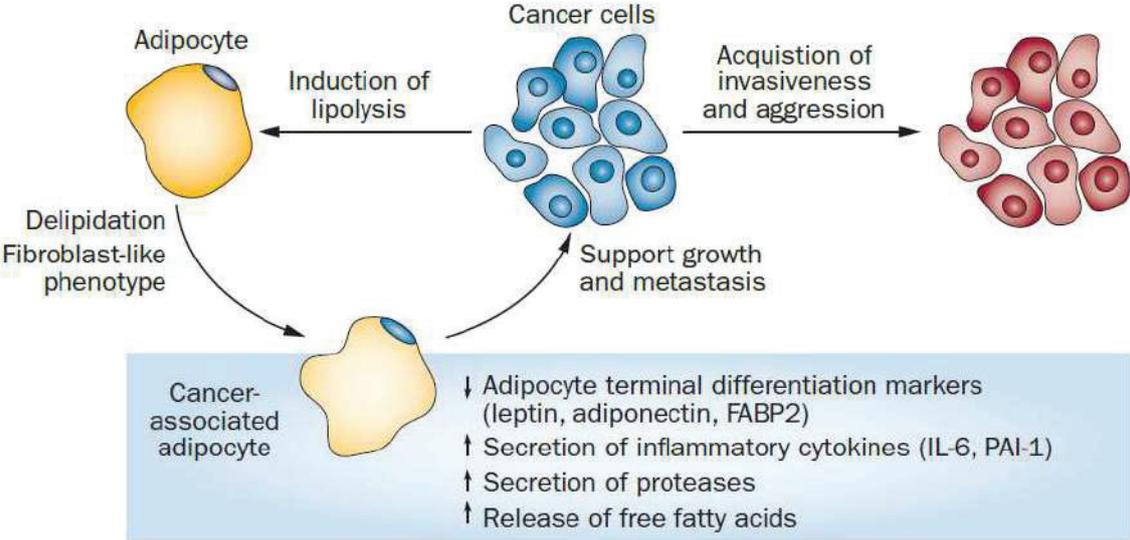
Besides angiogenesis and lymphangiogenesis, there are also other alternative mechanisms involved in tumour vascularization. For instance, the recruitment of EC progenitors from the bone marrow (Simons et al., 2011; Yu et al., 2010), transdifferentiation of MSCs and cancer stem cells (CSCs) even fibroblasts into VECs (Chen and Wu, 2016; Sayed et al., 2015; Soda et al., 2011).

#### 1.2.4 Adipocytes

Adipose tissue (AT) is one of the major components of human body which represents 18-25% in man and 25-31% in woman (Duong et al., 2017). AT, also called fat, is a loose connective tissue mainly constituted of adipocytes. In addition to adipocytes, AT also contains stromal vascular fraction (SVF) including vascular endothelial cells, fibroblasts, preadipocytes and numerous immune cells such as macrophages. AT can be divided into two groups according to its morphology: white adipose tissue (WAT) which stores energy and brown adipose tissue (BAT) which produces heat. The major function of AT is to store energy in form of lipid, nevertheless it can also have a cushion effect in case of mechanical shock. Furthermore, AT is considered to be an important endocrine organ which is characterized by its production of different hormones such as leptin, estrogen, resistin and TNF $\alpha$  (Duong et al., 2017). AT accumulation causes obesity which has been shown to have a direct link to cancer incidence, morbidity and mortality (Park et al., 2011). In line with this, growing evidences show that the obesity can increase the risk of cancer occurrence (Parekh et al., 2012). Many cancers are associated with obesity including breast cancer, reproductive cancer both in men and women, renal, gastrointestinal cancer etc (Laurent et al., 2016; Nieman et al., 2011).

The crosstalk between adipocytes and cancer cells can modulate the phenotype and the function of adipocyte which turn over to support tumour progression and metastasis by secreting a variety of growth factors and the production of ECM (Nieman et al., 2011). Moreover, adipose tissue can also influence the metabolism of tumour cells (Diedrich et al., 2015; Giles et al., 2012). The connection between obesity and cancer relies on three main axes: the insulin-IGF-1 axis, sex hormones and adipocytes-derived cytokines (Bussard et al., 2016). The direct contact between adipocytes and cancer cells induces considerable morphological and functional changes in adipocytes. In the presence of cancer cells, especially at the tumour invasive front, cancer associated adipocytes (CAAs) undergo delipidation, lose mature adipocyte markers such as adiponectin, leptin and become fibroblast-like. They then secrete inflammatory cytokines and pro-angiogenic growth factors inducing cancer cell proliferation and metastasis (Bussard et al., 2016) (Figure 10). Moreover, a recent study showed that leukaemia patients with obesity are more resistant to chemotherapy, owing to the increase of CSCs present in the AT. Adipocytes thus can play a role in the maintenance of CSCs (Ye et al., 2016). AT is also the major reservoir of fatty acids which are used by cancer cells as an

energy source by facilitating  $\beta$ -oxidation (Nieman et al., 2011). Therefore, it is worth to note that, AT should be taken into account in the therapeutic strategies and adequate exercises may provide good benefits for cancer patients (Duong et al., 2017).



**Figure 10 Heterotypic crosstalk between adipocytes and cancer cells.** Cancer cells stimulate lipolysis in adipocytes, which leads to delipidation and acquisition of a fibroblast-like phenotype in adipocytes. Cancer-associated adipocytes are associated with functional changes in the cells, such as loss of adipogenic markers, increased secretion of inflammatory cytokines and proteases, and increased release of free fatty acids, all of which support aggressive tumour growth and invasiveness (Obesity and cancer—mechanisms underlying tumour progression and recurrence *Nature Reviews-Endocrinology* 2014).

### 1.2.5 Extracellular matrix

Extracellular matrix (ECM) is a complex network which is composed by more than 300 macromolecules including fibronectin, collagen type IV, proteoglycans, laminin, and thrombospondin etc. The major function of ECM is to maintain the cell structure, cell adhesion, and cell communication (Kalluri, 2003). In normal tissue, ECM is well organised and tightly controlled. ECM is often found to be degraded and disorganized in tumour cells. Furthermore, an excessive ECM deposition, also named fibrosis, has been found in solid tumours (Bataller and Brenner, 2005). In line with this, increased expression of genes encoding proteins that mediate ECM remodelling is associated with increased mortality in patients with breast, lung, and gastric cancers (Chang et al., 2005; Chang et al., 2004).

ECM is the only non-cellular component of the tumour stroma and its composition is very variable and context-dependent (Bergamaschi et al., 2008). In line with this, its role is also variable along the tumour progression. The ECM can limit the cancer initiation and tumour progression at the early stage. For instance, TSP-1, an ECM protein, functions as an inhibitor of angiogenesis (Kazerounian et al., 2008). Moreover, patients with high expression of protease inhibitors in the ECM of the tumours are associated with good prognosis. Nevertheless, ECM is described to facilitate tumour cell proliferation and metastasis (Qin et al., 2017; Sahoo et al., 2017) at the later stage of disease through degradation of ECM by MMPs and increasing tumour angiogenesis (Campbell et al., 2010; Romero-Lopez et al., 2017). In addition to this, high expression of integrins and MMPs correlates to poor prognosis and high risk of disease recurrence (Bergamaschi et al., 2008; Giussani et al., 2015). As the most abundant ECM component, the deposition of collagen is the most well-recognized ECM alteration in tumours. Collagen type I deposition has been shown to be strongly associated to cancer metastasis (Shen et al., 2012). For example, Mice expressing a collagenase-resistant  $\alpha 1$  chain of type I collagen (Col1a1<sup>tm1jae</sup> mice) showed a threefold increase in the tumour incidence and metastasis compared to WT in a breast cancer model (Liu et al., 1995).

### 1.3 Therapies targeting tumour microenvironment

With accumulating clinical evidences and experimental studies, the importance of tumour microenvironment for tumorigenesis has been pointed out and widely accepted. Increasing efforts have been made in the past twenty years to target the tumour stromal compartment. Different strategies have been adapted in order to target one or various of stromal components with or without chemotherapy. To date, clinical trials focus on three axes: targeting stromal fibroblast, targeting vasculature and targeting immune cells through different small molecules or antibodies (Table 4) (Junttila and de Sauvage, 2013).

| Molecule               | Target  | Molecule type        | Company                  | Status (reference)   |
|------------------------|---|----------------------|--------------------------|--|
| <b>ECM/fibroblasts</b> |   |                      |                          |  |
| Marimastat             | MMP — broad spectrum  | Small molecule       | British Biotech          | Phase III negative for NSCLC, SCLC and breast cancer (NCT00002911, NCT00003010, NCT00003011)               |
| Prinomastat            | MMP 2, 3, 9, 13 and 14  | Small molecule       | Agouron/Pfizer           | Phase III negative for NSCLC and prostate cancer (NCT00004199, NCT00003343)                                |
| Tanomastat             | MMP 2, 3 and 9  | Small molecule       | Bayer                    | Phase III terminated (NCIC-CTG trial OV12)   |
| Neovastat              | VEGFR2, MMP 2, 9, 12  | Small molecule       | AEterna Laboratories     | Phase III negative for NSCLC (NCT00005838)   |
| Rebimastat             | MMP 1, 2, 8, 9 and 14   | Small molecule       | Bristol-Myers Squibb     | Phase III negative for NSCLC (NCT00006229)   |
| Vismodegib             | SMO   | Small molecule       | Genentech/Roche          | Phase II negative for CRC and ovarian cancer and phase II for PDAC (NCT00636610, NCT00739661, NCT01064622) |
| Saridegib              | SMO   | Small molecule       | Infinity Pharmaceuticals | Phase II terminated for PDAC (NCT01130142, NCT01310816)  |
| Sonidegib              | SMO   | Small molecule       | Novartis                 | Phase III (NCT01708174)  |
| <b>Vasculature</b>     |   |                      |                          |  |
| Bevacizumab            | VEGFA   | Antibody             | Genentech/Roche          | FDA-approved ((BLA) 125085)  |
| Vandetanib             | VEGFRs, PDGFRs, EGFR  | Small molecule       | AstraZeneca              | FDA-approved ((NDA) 022405)  |
| Sunitinib              | VEGFRs, PDGFRs, FLT3, CSF1R   | Small molecule       | Pfizer                   | FDA-approved ((NDA) 021938)  |
| Axitinib               | VEGFRs, PDGFRs, KIT   | Small molecule       | Pfizer                   | FDA-approved ((NDA) 202324)  |
| Sorafenib              | VEGFRs, RAF, PDGFRs, KIT  | Small molecule       | Bayer                    | FDA-approved ((NDA) 021923)  |
| Pazopanib              | VEGFRs, PDGFRs, KIT   | Small molecule       | GlaxoSmithKline          | FDA-approved ((NDA) 022465)  |
| Cabozantinib           | VEGFR2, RET, MET  | Small molecule       | Exelixis                 | FDA-approved ((NDA) 203756)  |
| Ziv-aflibercept        | VEGFA, VEGFB, PIGF  | Receptor-Fc fusion   | Regeneron                | FDA-approved ((BLA) 125418)  |
| Cilengitide            | Integrins $\alpha_v\beta_3$ , $\alpha_v\beta_5$ and $\alpha_5\beta_1$ | Small molecule       | Merck Serono             | Phase III negative for GBM (NCT00689221)   |
| AMG386                 | ANG2  | RP-Fc fusion protein | Amgen                    | Phase III (NCT01281254)  |
| Parsatuzumab           | EGFL-7  | Antibody             | Genentech/Roche          | Phase II (NCT01399684, NCT01366131)  |
| Enoticumab             | DLL4  | Antibody             | Regeneron                | Phase I (NCT00871559)  |
| Demcizumab             | DLL4  | Antibody             | OncoMed                  | Phase I (NCT00744562, NCT01189968, NCT01189942, NCT01189929)   |
| Nesvacumab             | ANG2  | Antibody             | Regeneron                | Phase I (NCT01688960, NCT01271972)   |

| Immune           |                        |                  |                               |  |
|------------------|------------------------|------------------|-------------------------------|--|
| Ipilimumab       | CTLA-4                 | Antibody         | Bristol-Myers Squibb          | FDA-approved ((BLA) 125377)  |
| Sipuleucel-T     | PAP                    | DC vaccine       | Dendreon                      | FDA-approved ((BLA) 125197)  |
| Aldesleukin      | IL-2                   | RP               | Prometheus                    | FDA-approved ((BLA) 103293)  |
| IFN $\alpha$ -2b | IFN- $\alpha$ receptor | RP               | Merck                         | FDA-approved ((BLA) 103132)  |
| MK-3475          | PD1                    | Antibody         | Merck                         | Phase III (NCT01866319)  |
| Nivolumab        | PD1                    | Antibody         | Bristol-Myers Squibb          | Phase III (NCT01668784, NCT01673867, NCT01642004, NCT01721772, NCT01721746, NCT01844505) |
| Nivolumab        | OX40                   | Antibody         | Bristol-Myers Squibb and PPMC | Phase III (NCT01668784, NCT01642004, NCT01673867, NCT01721772, NCT01721746, NCT01844505) |
| MPDL3280A        | PDL1                   | Antibody         | Genentech/Roche               | Phase II (NCT01846416)   |
| PLX3397          | KIT, CSF1R, FLT3       | Small molecule   | Plexxikon                     | Phase II (NCT01349036)   |
| BMS-663513       | CD137 (4-1BB)          | Antibody         | Bristol-Myers Squibb          | Phase II (NCT00612664)   |
| Blinatumomab     | CD3 and CD19           | Bi-specific scFv | Amgen                         | Phase II (NCT01741792, NCT01466179, NCT01207388, NCT01471782, NCT00560794, NCT01209286)  |
| AMG 820          | CSF1R                  | Antibody         | Amgen                         | Phase I (NCT01444404)  |
| AMP-224          | PD1                    | Antibody         | GlaxoSmithKline               | Phase I (NCT01352884)  |
| TRX-518          | GITR                   | Antibody         | GITR, Inc.                    | Phase I (NCT01239134)  |
| IMC-CS4          | CSF1R                  | Antibody         | ImClone/Eli Lilly             | Phase I (NCT01346358)  |

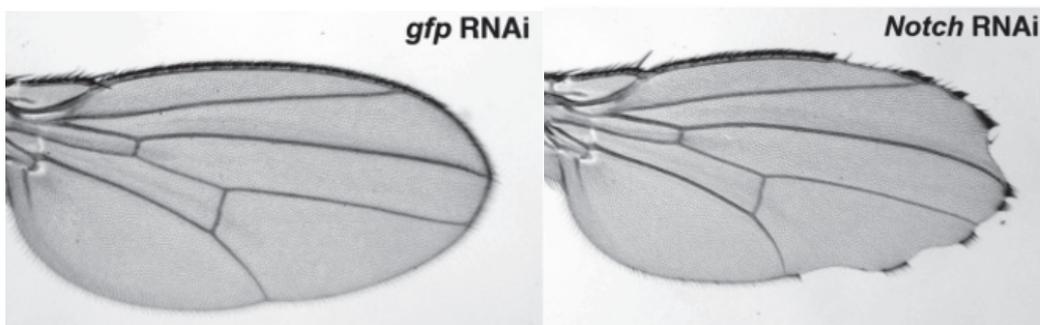
**Table 4 Examples of therapies that target the tumour stroma listed by compartment (Influence of tumour micro-environment heterogeneity on therapeutic *response-Reviews Nature 2013*).**

Although numerous clinical trials have shown promising effects, drug resistances and disease recurrence are still the significant challenges to overcome. For instance, one of the matrix-metalloproteinase inhibitors: tanomastat failed in small-cell lung cancer patients (Coussens et al., 2002; Michael et al., 1999); anti-VEGF antibodies showed few effect in pancreatic cancer (Kindler et al., 2010). Better understanding the mechanisms used by different stromal components to contribute the tumour progression, the crosstalk of different signalling pathways and the dynamics of tumour microenvironment before and after treatment will be necessary for future drug development. Furthermore, efforts should also be made to discover more diagnostic biomarkers and generate more complex genetically engineered models.

## 2. Notch signalling pathway

### 2.1 General consideration on Notch signalling pathway

Notch signalling is a highly conserved signalling pathway which governs various aspects of metazoan development and tissue homeostasis. The notch phenotype, characterized by the notches at the margins on the wing blades of fruit fly, was firstly described by Thomas Hunt Morgan and his colleagues in 1910 (Morgan, 1911). This notched wing phenotype is due to a partial loss of the notch gene in drosophila (Figure 11).



**Figure 11 The notch wing phenotype in adult drosophila ( adapted from a targeted in vivo RNAi screen reveals deubiquitinases as new regulators of Notch signalling-G3 2012).**

However it was not until the mid eighties that the notch gene was cloned (Artavanis-Tsakonas et al., 1983; Kidd et al., 1986; Wharton et al., 1985). In drosophila, the notch gene encodes a type I transmembrane receptor which is activated by two different ligands: Delta and Serrate (Figure 12a). In mammals, there are four notch receptors Notch 1-4 and five ligands also named as DSL ligands because of their Delta/Serrate/LAG-2 motif in the N-terminal domain which is involved in receptor binding: Delta-like 1, 3 and 4, and Jagged 1 and Jagged 2. They are classified into Jagged/Serrate or Delta family depending on the presence or absence of a cysteine-rich domain which is between the transmembrane domain and the EGF repeats (Figure 12b).

### Drosophila

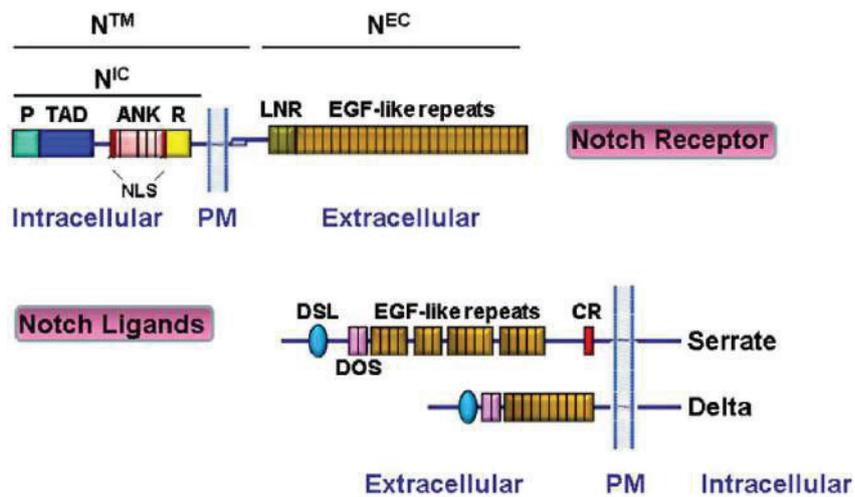


Figure 12a Notch receptors and ligands in drosophila (Notch inhibitors for cancer treatment-*Pharmacology & Therapeutics 2013* ).

### Mammals

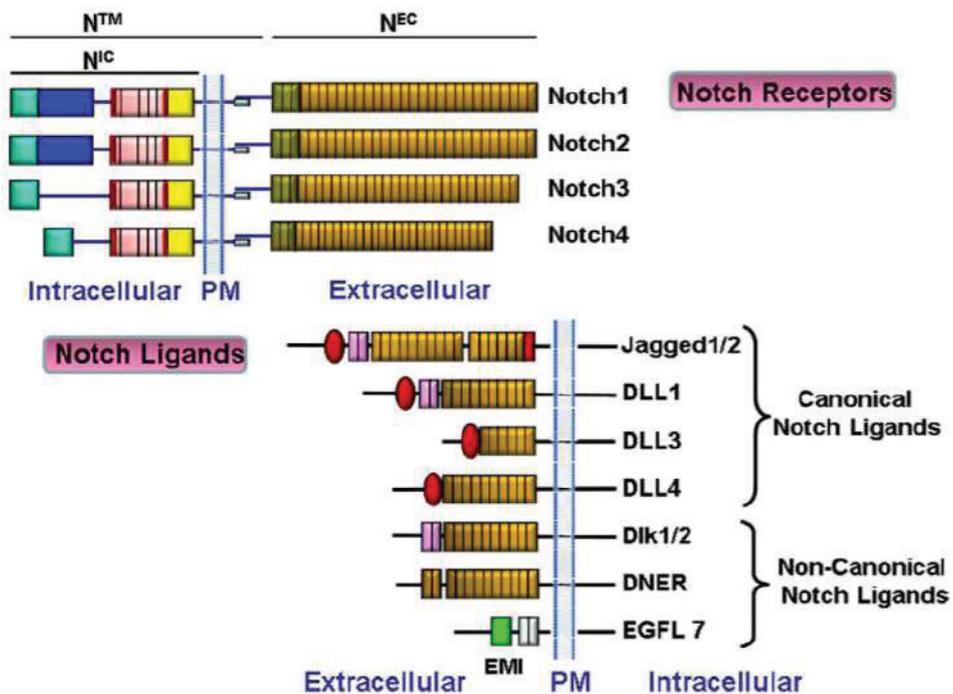


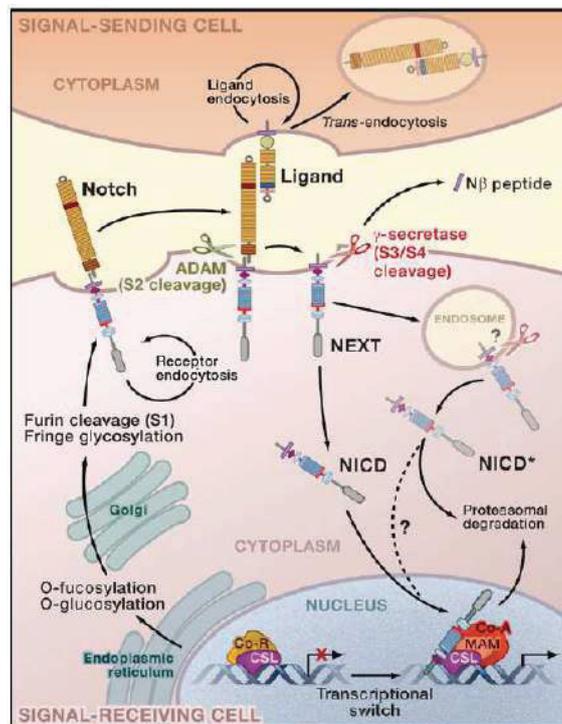
Figure 12b Notch receptors and ligands in mammals. Notch receptors are constituted of an extracellular domain ( $N^{EC}$ ) which is rich in EGF repeats (orange rectangle), a transmembrane domain ( $N^{TM}$ ), and an intracellular domain ( $N^{IC}$ ). Five Notch ligands: the Delta and the Jag family which are also rich in EGF repeats on their extracellular part (Notch inhibitors for cancer treatment-*Pharmacology & Therapeutics 2013* ).

The newly synthesized Notch receptors undergo their first cleavage in the Golgi, in which the Notch receptors are proteolytically cleaved at the S1 site by a furin-like protease. Besides the furin processing, Notch receptors are also modified by glycosylation by Rumi and fucosylation by O-Fut1 on their EGF repeat. These modifications are responsible for discriminating the interaction between Delta ligand or Serrate ligand. After the S1 cleavage, a heterodimer form of the receptor is generated and transported to the cell surface where it is held by noncovalent interactions. The receptor is constituted of an extracellular subunit ( $N^{EC}$ ) and the second subunit which contains the transmembrane domain and intracellular domain ( $N^{TMIC}$ ). The  $N^{EC}$  is rich in EGF repeat which mediates the interaction with its ligands and it is followed by a negative regulatory region (NRR) which plays a critical role in preventing receptor activation in the absence of ligands (Kopan and Ilagan, 2009).

### **2.1.1 The Canonical Notch pathway**

The canonical Notch signalling is induced at cell-cell contact which allows the ligand-receptor interaction, followed by two proteolytic cleavages. The first one is at the S2 site located at 12-13 amino acids from the transmembrane domain, mediated by ADAM metalloprotease family including ADAM 17/TACE (tumour necrosis factor  $\alpha$  converting enzyme) and the Kuzbanian/ADAM10/Sup-17 which is the principal mediator of the Notch signalling (Deuss et al., 2008). The S2 cleavage allows the exposure of the cleavage site for  $\gamma$ -secretase, a multiprotein complex.  $\gamma$ -secretase cleaves the receptor at S3 site, located in the transmembrane domain. The S3 cleavage leads to the liberation of the intracellular domain of the Notch receptor (NICD) which is subsequently translocated into the nucleus and interacts with the transcription factor CSL (CBF1 in human/RBPjk in mouse/Su(H) in *Drosophila*/Lag-1 in *Caenorhabditis elegans*). CSL is constitutively located on the promoters of Notch target genes. In the absence of NICD, the transcriptional co-repressors are recruited thereby inhibiting the Notch transcriptional activities. Once NICD binds to CSL, the transcriptional co-repressors are displaced and the transcription co-activators such as Mastermind/lag3 is recruited to the transcription activation complex, thereby triggering the upregulation of Notch target genes (Kopan and Ilagan, 2009; Kovall, 2008) (Figure 13) such as Hairy and enhancer of split (*Hes*) or Hairy related (*Hey* or *Hrt*). *Hes* genes are the principal target genes of Notch signalling and are expressed ubiquitously. Other tissue-specific targets such as brain lipid binding protein (BLBP), Modular Arithmetic Secure Hash (Mash1) and NeuroD have also been reported (Anthony et

al., 2005; Cau et al., 1997; Taylor et al., 2015). Furthermore, increasing data suggest that the Notch pathway can crosstalk with other signalling pathways including TGF- $\beta$ , NF- $\kappa$ B, and hypoxia (Poellinger and Lendahl, 2008; Samon et al., 2008), hence the spectrum of the Notch target genes is enlarged.



**Figure 13 The core Notch Signalling Pathway is mediated by regulated proteolysis ( The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism- *Review Cell, Kopan et al, 2009*).**

### 2.1.2 The Non-Canonical Notch pathway

Although the ligand-CSL canonical signalling is involved in diverse biological processes, non-canonical Notch signalling including ligand and transcription independent have also been widely reported (Andersen et al., 2012; Hayashi et al., 2016; Sanalkumar et al., 2010a). The early evidences of non-canonical Notch signalling were mostly reported in studies using drosophila (Andersen et al., 2012; Bray, 2006; Rusconi and Corbin, 1998). Various mutants of both Notch receptors and CSL were generated to study the non-canonical pathway (Martinez Arias et al., 2002; Rusconi and Corbin, 1998) which provide compelling evidence that non-canonical Notch pathway is involved during development. Since then, ligand/CSL-independent functions have been widely reported in many systems across species (Table 5).

| Species | Cell type  | System                              | Independence | Function                                  | Interacting molecule/signaling (direct or indirect) | Refs       |
|---------|--|-------------------------------------|--------------|---|---|------------|
| Human   | Stem cells (hESCs), Cancer                         | <i>in vitro</i>                     | Ligand, CSL  | Negative regulation of Wnt signaling      | Active $\beta$ -catenin/Wnt signaling               | [5]        |
| Rodent  | Stem cells (mESCs, NSCs, MSCs), Progenitors (CPCs) | <i>in vivo</i> ,<br><i>in vitro</i> | Ligand, CSL  | Negative regulation of Wnt signaling      | Active $\beta$ -catenin/Wnt signaling               | [5]        |
|         | T cells  | <i>in vitro</i>                     | CSL          | Notch-1 stimulates NF- $\kappa$ B         | NF- $\kappa$ B pathway                              | [28]       |
|         | Primary embryonic cells                            | <i>in vitro</i>                     | PS, Ligand   | HES1 activation and MCK inhibition        | HES1 and MCK  | [6]        |
|         | Skin progenitors                                   | <i>in vivo</i>                      | CSL          | Leukocytosis, longevity                   | nd  | [7]        |
|         | Muscle stem cells (C2C12)                          | <i>in vitro</i>                     | CSL          | Inhibition of muscle cell differentiation | nd  | [8-10]     |
|         | Fibroblasts (3T3)                                  | <i>in vitro</i>                     | CSL          | Inhibition of E47                         | E47   | [11]       |
|         | CHO cell line                                      | <i>in vitro</i>                     | CSL          | b1 integrin activation                    | R-Ras   | [12]       |
| Avian   | Neural crest (stem cells)                          | <i>in vivo</i>                      | CSL          | Slug expression                           | Slug  | [13,14]    |
| Frog    | Embryo   | <i>in vivo</i>                      | CSL          | Negative regulation of Wnt signaling      | $\beta$ -catenin/Wnt signaling                      | [15]       |
| Fly     | Wing primordium                                    | <i>in vivo</i>                      | Ligand, CSL  | Negative regulation of Wnt signaling      | Active $\beta$ -catenin/Wnt signaling               | [16,17,27] |
|         | Muscle progenitors                                 | <i>in vivo</i>                      | Ligand, CSL  | Muscle precursor selection                | Wnt signaling                                       | [18,19]    |
|         | Neural progenitors                                 | <i>in vivo</i>                      | Ligand, CSL  | Neuronal Cell (MP2) selection             | nd  | [20]       |
|         | Blood cells  | <i>in vivo</i>                      | Ligand       | Hemocyte survival                         | Hif-a   | [21]       |
|         | Wing primordium                                    | <i>in vivo</i> ,<br><i>in vitro</i> | CSL          | Inhibition of ligand function             | Serrate   | [22]       |
|         | Embryo   | <i>in vivo</i>                      | CSL          | Dorsal epidermis patterning (closure)     | JNK pathway   | [23]       |
|         | Visceral mesoderm progenitors                      | <i>in vivo</i>                      | CSL          | Inhibition of Wnt signaling               | Ubx   | [24]       |
|         | Neural precursors                                  | <i>in vivo</i>                      | CSL          | Repression of neural fate                 | Wnt signaling                                       | [25,26]    |

Abbreviations: hESC, human embryonic stem cell; mESC, mouse embryonic stem cells; NSCs, neural stem cells; MSCs, mesenchymal stem cells; CPCs, cardiac progenitor cells; PS, presenilin; nd, not determined.

**Table 5. Evidence of CSL/ligand-independent Notch signalling (Non-canonical Notch signalling: emerging role and mechanism-Review Cell Press 2012).**

The definition of the non-canonical Notch signalling is broad and it can be classified into : DSL-independent activation, interactions with non-DSL ligands, CSL-independent signalling, signal transduction without cleavage, differential posttranslational modifications (D'Souza et al., 2010). Nevertheless, two major types of non-canonical pathways have been well studied. Type I which requires ligands to trigger the receptor cleavage to release the NICD which translocates into the nucleus and interacts with tissue-specific co-activators (Co-A) and other uncleared co-factors to induce CBF1-independent transcription (Figure 14a). By contrast, the type II non-canonical Notch signalling is completely ligand-mediated NICD-release or CBF1 independent. In this case, the transcription of Notch target genes is mediated alternatively. It has been shown that, downstream effectors such as JNK, Shh and Wnt pathways can activate the expression of Hes-1/Hairy-1 (Figure 14b) (Sanalkumar et al., 2010b; Wall et al., 2009).

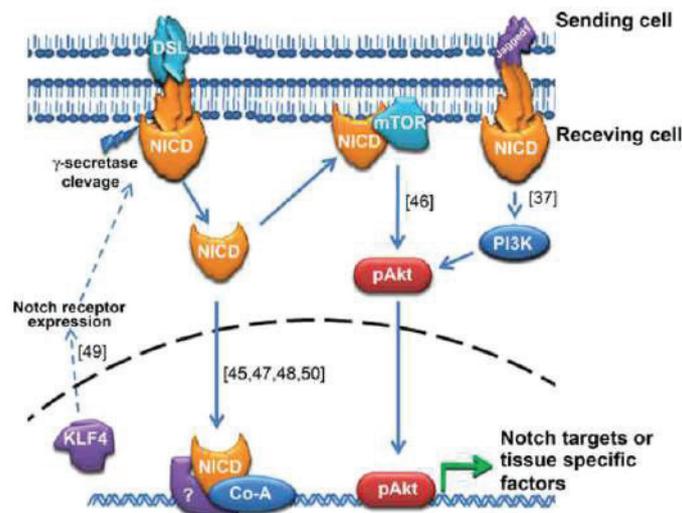


Figure 14a. Schematic of non-canonical Notch signalling/target gene activation, type I-CBF1 independent (Non-canonical activation of Notch signalling/target genes in vertebrates-*Review Cellular and Molecular Life Sciences 2010*).

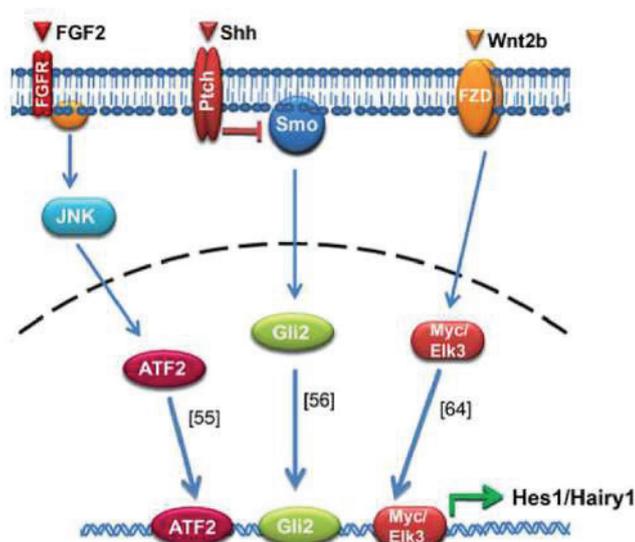


Figure 14b. Schematic of non-canonical Notch signalling/target gene activation, type II-Ligand and CBF1 independent (Non-canonical activation of Notch signalling/target genes in vertebrates-*Review Cellular and Molecular Life Sciences 2010*).

Notch target genes activation through the non-canonical Notch signalling in vertebrates is involved in many biological processes including proliferation of lineage-restricted progenitors, cell fate specific differentiation, and tumorigenesis (Andersen et al., 2012). NICD-independent/ CBF1-independent notch signalling has been reported to regulate the lineage-restricted progenitors such as neuronal progenitors (Sanalkumar et al., 2010b), retinal progenitor (Wall et al., 2009), hematopoietic stem cells (Maillard et al., 2008). Moreover, it also participates in the tumorigenesis in different aspects such as cancer cells proliferation

(Raafat et al., 2009), inhibition of apoptosis (Perumalsamy et al., 2009), and neoplastic transformation (Dumont et al., 2000; Jeffries and Capobianco, 2000). Therefore, the non-canonical Notch signalling should be taken into account and may provide alternative strategies for cancer treatment.

## 2.2 Notch pathway in tumorigenesis

Since the Notch pathway is important for adult tissue homeostasis, it is not surprising that the Notch signalling pathway is also implicated in tumorigenesis. The first evidence of the involvement of Notch pathway in tumorigenesis is the Notch1 constitutive activation in T cell acute lymphoblastic leukaemia patients, which is due to a chromosomal translocation (Ellisen et al., 1991). It has been shown later that 50% of T-ALL patients harbour activating mutations of *NOTCH1* which lead to Notch1 constitutive activation in ligand independent manner or an increase stability of NICD1 in T cells (Weng et al., 2004). Besides its implication in hematological malignancies, the Notch pathway has also been widely reported to be implicated in solid tumours initiation and progression. The first evidence that Notch signalling is involved in solid tumour comes from studies that identified a frequent insertion site of the mouse mammary tumour virus (MMTV) (Gallahan et al., 1996). This region of integration was named *int-3*, and subsequently identified as the *Notch4* locus. Transgenic mice that express this truncated *int-3* gene, a 2.4-kb transcript encoding the intracellular domain of the Notch4 receptor, developed mammary and salivary-gland adenocarcinomas within 7 months (Gallahan et al., 1996). Afterwards, increasing studies have shown the involvement of Notch signalling in solid tumours, however little evidence of genetic alterations of the pathway has been shown (Rizzo et al., 2008).

### 2.2.1 Mechanisms by which Notch signalling regulates tumorigenesis

Oncogenic Notch signalling regulates tumorigenesis mainly via its aberrant activation in cancer cells, which results in increase proliferation, inhibition of apoptosis and induction of EMT in cancer cells (Leong and Karsan, 2006). Aberrant activation of the Notch signalling is mainly due to the deregulation of different Notch pathway members, such as mutations or truncation of Notch receptors which induce constitutive Notch activation; deregulated expression of wild type Notch receptors or ligands; deregulated expression of Notch target genes; cross-talk with other oncogenes; epigenetic regulation; posttranslational modification, especially receptor and ligand fucosylation (Fortini, 2009; Lee et al., 2015; Pakkiriswami et al., 2016). Aberrant Notch signalling is implicated in a wide panel of tumours which vary from hematopoietic lineage origin tumours including T cell acute lymphocyte leukaemia (T-ALL), chronic lymphocytic leukaemia (CLL), splenic marginal zone lymphoma (SMZC), diffuse large B cell lymphoma (DLBCL), squamous cell carcinoma (SQCC), and various solid tumours. Even the Notch receptors was firstly considered as an oncogene, increasing data showed that they can also be a tumour suppressor in a context dependent manner. The tumour suppressor role of Notch receptor was firstly showed in skin cancer, in which Notch1 activation is important for cell differentiation and suppression of Wnt and Sonic-hedgehog pathway (Demehri and Kopan, 2009; Lowell et al., 2000; Rangarajan et al., 2001). Afterward, the tumour suppressor role of different Notch receptors was widely reported in different cancers (Table 6).

| Tumor Type                                  | Oncogene or Tumor Suppressor | Mutations (%) and Noteworthy Observations                                  | References                                      |
|---|------------------------------|--|---|
| T cell acute lymphoblastic leukemia (T-ALL) | oncogene                     | 50%–60% NOTCH1, 30% FBXW7<br>role in cancer initiation and maintenance     | (Malyukova et al., 2007)                        |
| Chronic lymphocytic leukemia (CLL)          | oncogene                     | 5%–12% NOTCH1<br>role in cancer initiation and survival                    | (Fabbri et al., 2011)<br>(Puente et al., 2011)  |
| Melanoma                                    | oncogenic                    | ~50% NOTCH1 overexpression in human samples<br>possible role in metastasis | (Balint et al., 2005)<br>(Bedogni et al., 2008) |

|   |                  |   |   |
|---|------------------|---|---|
| Cholangiocarcinoma (CCC)                | oncogenic        | 35% FBXW7~<br>Notch1 promotes tumor initiation and maintenance  | (Akhoondi et al., 2007)<br>(Zender et al., 2013)                            |
| Colorectal cancer                       | oncogenic        | 8%–9% FBXW7<br>crosstalk with Wnt and Hippo signaling   | (Miyaki et al., 2009)<br>(Akhoondi et al., 2007)                            |
| Lung adenocarcinoma                     | oncogenic        | 10% NOTCH1<br>role in initiation and maintenance (Notch1), and metastasis (Jagged2)<br>specific role for Notch3 in tumor propagation  | (Licciulli et al., 2013)<br>(Westhoff et al., 2009)<br>(Zheng et al., 2013) |
| Glioblastoma                            | oncogenic        | role in tumor propagation and radioresistance   | (Chu et al., 2013)<br>(Wang et al., 2010)                                   |
| Renal cell carcinoma                    | oncogenic        | role in progression and maintenance   | (Sjolund et al., 2008)  |
| Ovarian cancer                          | oncogenic        | role in maintenance and therapy response  | (McAuliffe et al., 2012)  |
| Prostate                                | oncogenic        | activation of the pathway associated with tumor progression, metastasis, and recurrence   | (Marignol et al., 2013)<br>(Santagata et al., 2004b)                        |
| Breast cancer                           | mostly oncogenic | NOTCH1 and NOTCH4 fusions<br>potential NOTCH2 dominant-negative truncated mutant<br>other alterations activate Notch signaling, but hyperactive Notch signaling may inhibit cancer growth | (Fu et al., 2010)<br>(Imatani and Callahan, 2000)                           |
| Pancreatic ductal adenocarcinoma (PDAC) | mostly oncogenic | Notch2 loss inhibits progression and maintenance<br>overexpression of ligands (Jagged2 [90%], Dll4 [50%], but Notch1 loss may promote tumor initiation                                    | (Hanlon et al., 2010)<br>(Mazur et al., 2010)<br>(Mullendore et al., 2009)  |
| Cervical cancer                         | mostly oncogenic | pathway activation in human tumors, but dosedependent effects<br>possible role in tumor-propagating cells   | (Bajaj et al., 2011)<br>(Maliekal et al., 2008)<br>(Zagouras et al., 1995)  |

|  |                  |  |                    |
|--|------------------|--|--------------------|
| Head and neck squamous cell carcinomas (HNSCC) | mostly oncogenic | possible bimodal pattern of Notch pathway alterations with a small subset of tumors with inactivating NOTCH1 mutations, but a larger group with pathway activation | (Sun et al., 2014) |
|--|------------------|--|--------------------|

| Tumor Type                                  | Oncogene or Tumor Suppressor    | Mutations (%) and Noteworthy Observations  | References                                     |
|---|---------------------------------|--|--|
| Hepatocellular carcinoma (HCC)              | oncogenic and tumor suppressive | context-dependent effects that may be related to various molecular subtypes                            | (Qi et al., 2003)<br>(Villanueva et al., 2012) |
| Medulloblastoma                             | oncogenic and tumor suppressive | opposite roles for Notch1 and Notch2   | (Fan et al., 2004)                             |
| B cell acute lymphoblastic leukemia (B-ALL) | tumor suppressive               | no mutations<br>role in maintenance (activation induces growth arrest and death)                       | (Zweidler-McKay et al., 2005)                  |
| Acute myeloid leukemia (AML)                | tumor suppressive               | Notch1 and Notch2 are expressed, but the pathway is not active   | (Kannan et al., 2013)                          |
| Small cell lung carcinoma (SCLC)            | tumor suppressive               | no mutations<br>inhibits tumor maintenance (possible similar role in other neuroendocrine tumor types) | (Sriuranpong et al., 2001)                     |
| Lung squamous cell carcinoma (SqCC)         | tumor suppressor                | 5%–12.5% NOTCH1 and NOTCH2   | (Wang et al., 2011)                            |
| Cutaneous squamous cell carcinoma (SqCC)    | tumor suppressor                | 60%–75% NOTCH1 and NOTCH2  | (Wang et al., 2011)                            |
| Chronic myelomonocytic leukemia (CMML)      | tumor suppressor                | 12% various pathway genes (NCSTN, APH1, MAML1, and NOTCH2)<br>role in cancer initiation                | (Klinakis et al., 2011)                        |

**Table 6. Oncogenic and Tumor-Suppressive Roles of Notch Signaling in Human Cancers (Adapted by From *Fly Wings to Targeted Cancer Therapies: A centennial for Notch Signaling-Cancer Cell 2014* ).**

### 2.2.2 Notch3 in cancer

Even though most studies concerning the implication of Notch signalling in tumorigenesis were done on Notch1 and Notch2, increasing data unveil the fact that Notch3 is also implicated in tumorigenesis. As the other Notch receptors, Notch3 can be oncogene or tumour suppressor in a tissue dependent manner. Notch3 was firstly reported to be an oncogene in ovarian cancer, in which over expression of Notch3 were both found in RNA and protein level, as a consequences of *Notch3* gene amplification at the amplicon 19p13 (Park et al., 2006). Moreover, mice with overexpression of N3ICD under the control of the MMTV promoter develop mammary gland tumours (Hu et al., 2006). Nevertheless, Notch3 can also behave as a tumour suppressor. In certain tissues, Notch3 can play opposite roles in different subtypes of cancers, such as in lung cancer, in which Notch3 is considered as an oncogene in non-small cell lung carcinoma (NSCLC) but a tumour suppressor in small cell lung carcinoma (SCLC) (Hassan et al., 2016; Yi et al., 2013). As Notch3 is expressed in mammary gland luminal progenitors, its activation has been reported to be important for the maintenance of the non-proliferative state of these cells (Lafkas et al., 2013). Moreover, in breast cancer, over expression of N3ICD in MDA-MB-231 breast cancer cell line causes arrest of the cell cycle at G0/G1 (Chen et al., 2016), and this ectopic expression also inhibit the EMT in cancer cells (Zhang et al., 2016). However, Notch3 has been shown to be important for cancer cell proliferation in ErbB2-negative human breast cancer cells (Yamaguchi et al., 2008). Furthermore, Notch3 is reported to be a tumour suppressor by inducing senescence in breast cancer as well (Cui et al., 2013). Taken together, the role of Noth3 in tumorigenesis is not clear as simply black and white. Tissue dependent or even cell type dependent may determine the role of Notch3 during tumorigenesis. It could be also possible that in the same tissue even in the same cell type, presence of different elements such as the availability of different ligands in the microenvironment could influence Notch3 into one side rather than the other one.

In this manuscript, we study the role of Notch3 in tumour angiogenesis in a lung cancer setting. Notch3 is implicated not only in lung development but also in lung cancers. Constitutive expression of Notch3 in the peripheral epithelium in the developing lung results in altered lung morphology and delayed development which is due to the inhibition of terminal epithelial differentiation in the lung (Dang et al., 2003). Moreover, Notch3 is detected in many NSCLC

cell lines and human lung cancer tissue but not in the SCLC (Galluzzo and Bocchetta, 2011; Hassan et al., 2016). Inhibition of Notch3 results in tumour apoptosis and regression of tumour growth by modulating the pro-apoptotic protein Bim (Konishi et al., 2010). In addition, it has been shown that a stem-like property in NSCLC is Notch3 dependent, indicating that Notch3 could also modulate the lung cancer progression via regulating the cancer stemness (Ali et al., 2016; Ma et al., 2016). However, most studies of Notch3 and its implication in lung cancers are mainly focused on the epithelial cancer cell compartment, tumour microenvironment especially the tumour vasculature has been never studied. Whether Notch3 can regulate the tumorigenesis by its role in other cell populations is still unclear. We thus decided to study the role of Notch3 in the tumour vasculature to investigate how could Notch3 regulate the tumorigenesis beyond the epithelial tumour compartment.

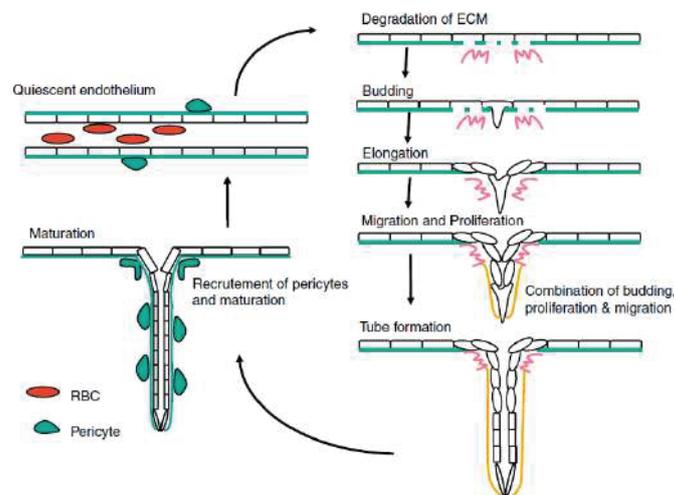
## **2.3 Notch signalling in the vasculature**

### **2.3.1 Notch pathway in vascular development**

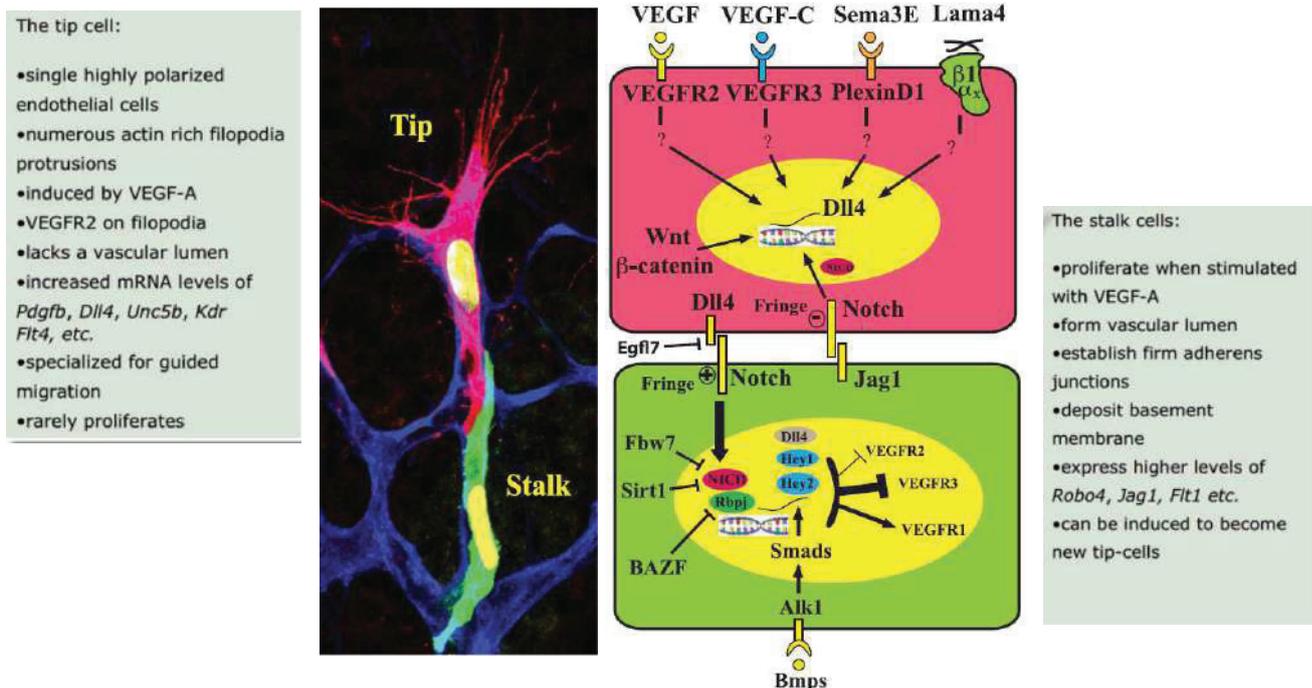
The Notch signalling pathway plays a critical role for the formation and morphogenesis of the vascular system during the embryonic development (Krebs et al., 2000). In mammals, two Notch receptors: Notch1 and Notch4, three ligands: Jagged1, Dll1 and Dll4 are predominantly expressed in vascular endothelial cells (Claxton and Fruttiger, 2004; Favre et al., 2003; Hofmann and Iruela-Arispe, 2007; Reaume et al., 1992). Homozygous loss of Notch1 is embryonic lethal at E9.5 (Huppert et al., 2000), however embryos with loss of Notch4 are viable and fertile. Surprisingly ~50% of Notch1 and Notch4 double homozygous knockout embryos display more severe defects in angiogenic vascular remodelling than Notch1 alone, which indicates that *Notch1* and *Notch4* genes play partially redundant role during embryogenesis (Krebs et al., 2000). Concerning ligands, *Jag1*<sup>-/-</sup> or *Dll1*<sup>-/-</sup> embryos die from vascular defects and haemorrhaging at approximately E10.5 (Hrabe de Angelis et al., 1997; Xue et al., 1999). In addition, Dll4 restricted to arterial endothelial cells is essential for the arterial differentiation (Duarte et al., 2004; Krebs et al., 2004; Villa et al., 2001). All these data confirm that Notch pathway is essential for the proper vascular development in mammals.

### 2.3.2 Notch pathway in developmental angiogenesis

The Notch pathway is also important for developmental angiogenesis. Angiogenesis is the biological process of generating new blood vessels from the pre-existing one. The process is composed of a series of events in endothelial cells in response to angiogenic stimulation: ECM degradation, budding, proliferation, migration, tube formation, maturation, and maintenance of quiescent endothelium (Figure 15). Together with different components of VEGF family, the Notch pathway mediates the sprouting angiogenesis by tip cell selection and proliferation of stalk cells (Kofler et al., 2011). In terms of morphology, tip cells have numerous actin rich long filopodia, are highly polarized, and positioned at the angiogenic front. In response to angiogenic growth factor such as VEGFA, tip cells are stimulated through the activation of VEGFR2. Tip cells are followed by stalk cells which produce less filopodia and are more proliferative when they are stimulated by VEGFA. They form the vascular lumen and establish tight junction and basement membrane (Figure 15).

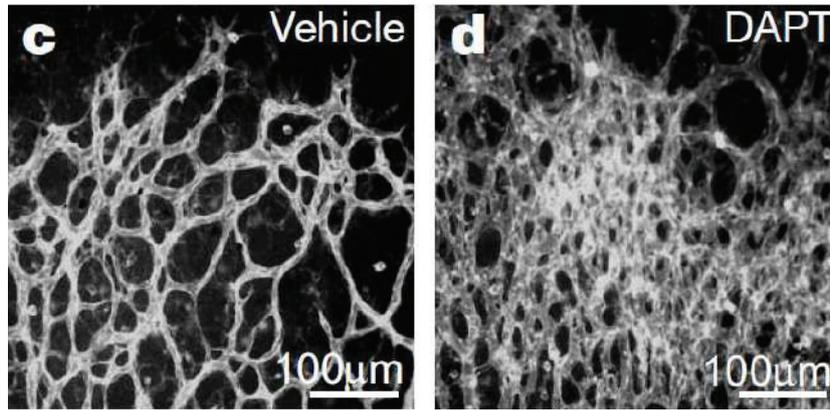


**Figure 15. Schematic of angiogenic progression including degradation of ECM, followed by endothelial cell activation, proliferation, migration and finally the recruitment of smooth muscle cell to complete the maturation (Notch signalling regulates tumor angiogenesis by diverse mechanisms-Review *Oncogene* 2008).**



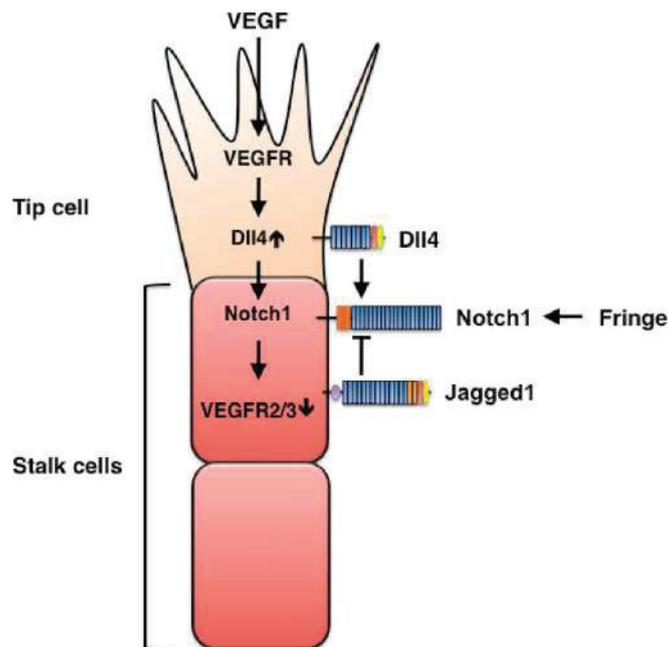
**Figure 16. Phenotypic and Molecular differences between endothelial tip cell and stalk cell (Adapted from *Angiogenesis: A Team Effort Coordinated by Notch-Review, Developmental Cell 2009* and *Notch as a hub for signalling in angiogenesis-Review, ELSEVIER 2013*).**

DLL4, PDGFR $\beta$  and VEGFR2 are used as tip cell molecular markers due to their high expression in these cells (Blanco and Gerhardt, 2013). Moreover, tip cells have low Notch signalling. By contrast, Notch pathway is highly activated in stalk cells which is mainly mediated by the DLL4/Notch1 (Hellstrom et al., 2007). As a consequence, expression of VEGFR2 and VEGFR3 are inhibited, which lead to inhibition of tip cell phenotype in stalk cells (Phng and Gerhardt, 2009). As the most important Notch component to regulate the tip cell phenotype, Dll4 is mainly modulated by the response of VEGFR2 to VEGFA (Liu et al., 2003). In addition to VEGFA, VEGFC can also modulate the Dll4 expression via VEGFR3 which depend on the Notch pathway in tip cells (Benedito et al., 2012). Moreover, Semaphorin3E/PlexinD1 and laminin 4/ $\beta$ 1 integrin can also induce Dll4 expression in tip cells, however, the mechanisms are still unknown (Stenzel et al., 2011) (Figure 16). Genetic inactivation or pharmacological inhibition of either Notch1 or Dll4 signalling leads to hypersprouting which means an increase of tip cells at the angiogenic front, resulting in the formation of hyper dense but not functional vascular network (Hellstrom et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007) (Figure 17).



**Figure 17. Inhibition of the Notch pathway by gamma secretase inhibitor DAPT induces hypersprouting in mouse retina during postnatal angiogenesis (DLL4 signalling through Notch1 regulates formation of tip cells during angiogenesis-*Nature 2007*).**

On the other hand, activation of the Notch pathway leads to a decrease of the number of tip cells and a less dense vascular network (Hellstrom et al., 2007). But surprisingly, Jagged1 plays an opposite role as Dll4 during sprouting angiogenesis in mouse retina (Benedito et al., 2009). Jagged1 expressed in stalk cells or by other cell populations behaves as an antagonist to DLL4 to activate the Notch pathway in stalk cells. This antagonistic effect may be controlled by Fringe-dependent modulation of Notch signalling which increases the tip cell numbers, and enhances vessel sprouting (Figure 18).



**Figure 18. Opposing effects of DLL4 and Jagged1 on sprouting angiogenesis. Jagged1 antagonizes DLL4-mediated Notch activation in stalk cells and increases tip cell numbers. The antagonistic effect of the two ligands could be mediated by Fringe-dependent modulation (Adapted from Novel insights into the differential functions of Notch ligands in vascular formation-J Angiogenesis Res 2009).**

In addition to tip cell selection and proliferation of stalk cells, to generate a functional new blood vessel still need a proper vessel maturation, which means the deposit of the basement membrane and the recruitment of pericytes and mural cells on the new born vessel. These processes are also mediated by Notch signalling. For instance, endothelial Notch signaling is crucial for the maturation and quiescence of retinal blood vessels (Ehling et al., 2013). Moreover, endothelial cells isolated from mouse embryos overexpressing DLL4 show an increase of transcription of *fibronectin*, *laminin*, and *collagen* (Scehnet et al., 2007; Trindade et al., 2008) which indicates that Notch signalling regulates the expression of ECM molecules and the maturation of the blood vessel. Furthermore, studies have shown that endothelial cells expressing Jagged1 can activate Notch signalling via Notch3 in neighbour cells which subsequently promotes and maintains the differentiated phenotype of mural cells including vascular smooth muscle cells on big vessel and pericytes in microvasculature (Liu et al., 2009; Liu et al., 2010).

### **2.3.3 Notch pathway in pathological angiogenesis-tumour angiogenesis**

Angiogenesis is essential for solid tumour growth and metastasis. Notch signalling can mediate angiogenesis by its role in different cell populations including tumour endothelium, tumour cells and inflammatory cells (Dufraigne et al., 2008). Cell-cell contact between tumour endothelial cells and tumour cells or other cell populations present in the tumour microenvironment such as macrophages activates the Notch signalling in the tumour endothelium which in turn to trigger the tumour angiogenesis (Murakami et al., 2008). Consistent with its role in the developmental angiogenesis, the Notch signalling also regulates the tumour angiogenesis by regulating angiogenic sprouting.

Two key Notch ligands Dll4 and Jagged1 have been reported to be implicated in tumour angiogenesis. Dll4, an endothelial specific Notch ligand is robustly expressed in tumour endothelial cells of murine and human tumours, comparing with normal tissue vessels

(Mailhos et al., 2001). During tumour angiogenesis, expression of Dll4 is mainly regulated by the VEGF pathway. In response to VEGF secreted by tumour cells and tumour stromal cells such as fibroblasts, macrophages, and endothelial cells, Dll4 expression is upregulated in tumour endothelial cells and it is usually correlated with VEGF levels (Lobov et al., 2007; Patel et al., 2006; Patel et al., 2005). Paradoxically, Dll4 plays a negative role during the vascular sprouting and branching. Pharmacological inhibition of Dll4 in tumour vasculature leads to increase tumour angiogenesis but regression of tumour growth because of the non-functional production of tumour blood vessels (Jia et al., 2016; Thurston et al., 2007). On the other hand, Jagged 1, another Notch ligand also plays an important role during tumour angiogenesis. Endothelium-specific deletion of Jagged1 leads to embryonic lethal and severe cardiovascular defects because of striking deficit in VSMCs (High et al., 2008; Tattersall et al., 2016). Nevertheless, Jagged 1 expression is not restricted in tumour endothelium. Jagged1 is widely upregulated in a large panel of solid tumours including breast cancer, various squamous cell carcinomas (SQCC) such as head and neck, lung, and skin. Tumour-derived Jagged1 is dependent on the activation of MAPK pathway (Zeng et al., 2005) which signals to tumour endothelium and promotes tumour angiogenesis. Moreover, tumour-derived Jagged1 may also promote tumour growth through modulating the Notch signalling in their neighbouring tumour cells, stromal or inflammatory cells (Tattersall et al., 2016; Zeng et al., 2005). In addition, a recent study showed that endothelial specific activation of Notch1 induced endothelial cells senescence and promoted metastasis (Wieland et al., 2017). Taken together Notch signalling is highly implicated in tumour angiogenesis via different aspects, efforts should be made to investigate other Notch receptors such as Notch4 and Notch3 since they are important for the vascular development as well as in tumorigenesis.

#### **2.3.4 Notch3 in the vasculature**

Besides Notch1 and Notch4, Notch3 also plays an important role in vascular system. Notch3 is mainly expressed in the vascular mural cells, such as pericytes and vascular smooth muscle cells. The role of Notch3 in vascular system was investigated in different *in vivo* models. In zebrafish, Notch3 has been shown to be essential for the oligodendrocyte vascular integrity (Zaucker et al., 2013). Notch3 homozygous knockout mice are viable and fertile and they do not present major defaults in vascular development (Krebs et al., 2003). However, Notch3 is required to generate functional arteries in mice by regulating arterial differentiation and maturation of vascular smooth muscle cells (vSMC) (Domenga et al., 2004). Moreover, Notch3 was reported to be important for the maintenance but not for the recruitment of mural cells in *Notch3*<sup>-/-</sup> mouse models (Domenga et al., 2004; Liu et al., 2010). These observations may explain why Notch3 is implicated in many vascular diseases. For instance, Mutations of Notch3 is responsible for cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) which causes stroke and dementia (Joutel et al., 1996). Recently, a new antibody which targets the Notch3 signalling was developed to prevent mural cells loss in CADASIL (Machuca-Parra et al., 2017). Moreover, Notch3 homozygous knockout mice are more susceptible to ischemic stroke and hypertension (Arboleda-Velasquez et al., 2008; Li et al., 2009). Taken together, these results prompt us to ask whether Notch3 could be also implicated in tumour angiogenesis.

#### **2.3.5 Targeting Notch signalling in anti-angiogenic therapies**

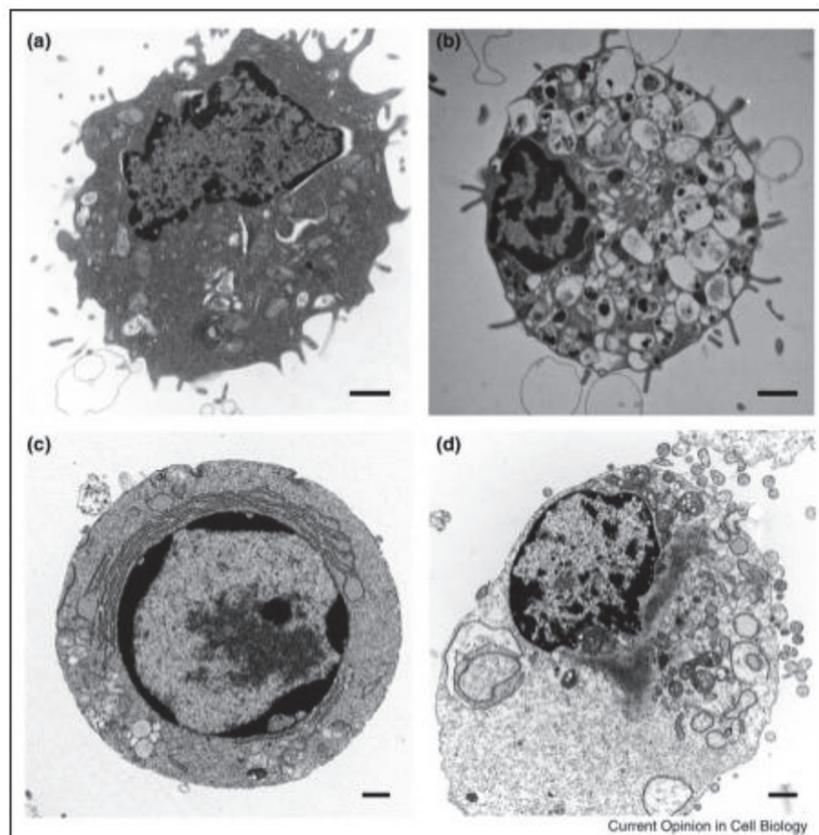
As VEGF is the most potent pro-angiogenic factor, many efforts have been made to develop anti-VEGF therapies to target the tumour angiogenesis. Blocking the VEGF/VEGFR2 signalling is the most abundant ones in the clinical field of anti-angiogenic therapy (Jain et al., 2006). VEGF inhibitors, including Food and Drug Administration (FDA)–approved bevacizumab (Avastin), have been shown to reduce tumor angiogenesis and tumor growth and are now validated for cancer therapy (Jain et al., 2006). However, not all the cancers are susceptible to the anti VEGF treatment, moreover resistance and recurrence are usually observed in patients

(Lu and Bergers, 2013). That's why other anti-angiogenic strategies should be developed. Since increasing evidences showed that Notch signalling is not only implicated in tumorigenesis but also in tumour angiogenesis, targeting the Notch pathway become an interesting strategy for anti-cancer therapy. Inhibition of DLL4 by anti-DLL4 antibody has been shown to increase non-functional tumour vasculature but decrease tumour growth (Noguera-Troise et al., 2006; Ridgway et al., 2006; Sclafani et al., 2007; Thurston et al., 2007). These studies unveiled an alternative concept that the tumour growth can be adversely affected by the 'abnormalization' strategies. In addition, Jagged1 which has been demonstrated as a pro-angiogenic factor in developmental angiogenesis, is usually associated with tumour vessels in many cancers such as brain, ovarian, lung, and head and neck squamous cell carcinoma (Chang et al., 2016; Jubb et al., 2012; Lu et al., 2007; Zeng et al., 2005). Disruption of Jag1/ Notch signalling could be also a good strategy for the anti-angiogenic therapy (Chen et al., 2015; Oon et al., 2017; Steg et al., 2011). Moreover, a recent study showed that Jagged1 can mediate the cell plasticity via the Notch pathway in adult lung (Lafkas et al., 2015) which indicates again that targeting Jagged1 could benefit cancer patients. Besides targeting Notch ligands, interruption of Notch signalling by targeting specifically Notch receptor has also shown promising anti-angiogenic effect in cancers. The soluble form of the Notch 1 (Notch1 decoy) functioned as an antagonist to inhibit the Notch signalling stimulated by Dll1, Dll4, Jagged1 and the expression of VEGF, which results in inhibition of tumour angiogenesis and tumour growth (Funahashi et al., 2008). Moreover, general inhibition of the Notch signalling by  $\gamma$ -secretase inhibitors induces tumour endothelial cell death (Cook et al., 2012; Paris et al., 2005a). Besides the canonical Notch signalling, increasing non-canonical ligands and target genes are unveiled, which could also to be potential targets to inhibit tumour angiogenesis (Rodriguez et al., 2012).

### 3. Apoptosis and Dependence Receptors

#### 3.1 Apoptosis

Since 1842, the first report of discovering cell death by Carl Vogt, numerous observations and studies have been done to characterize cell death during development and in pathologies. Afterward, various types of cell death have been discovered including apoptosis, necroptosis, mitotic catastrophe, ferroptosis, and autophagic cell death. Apoptosis is the first kind of cell death which has been discovered and has been most studied. It is a major cell death pathway which is involved in immune reaction and different pathologies such as cancer. In 1973, Schweibel and Merker proposed a classification of cell death in three categories: type I cell death: apoptosis, type II cell death: autophagy, type III cell death: necrosis (Schweichel and Merker, 1973) (Figure 19).



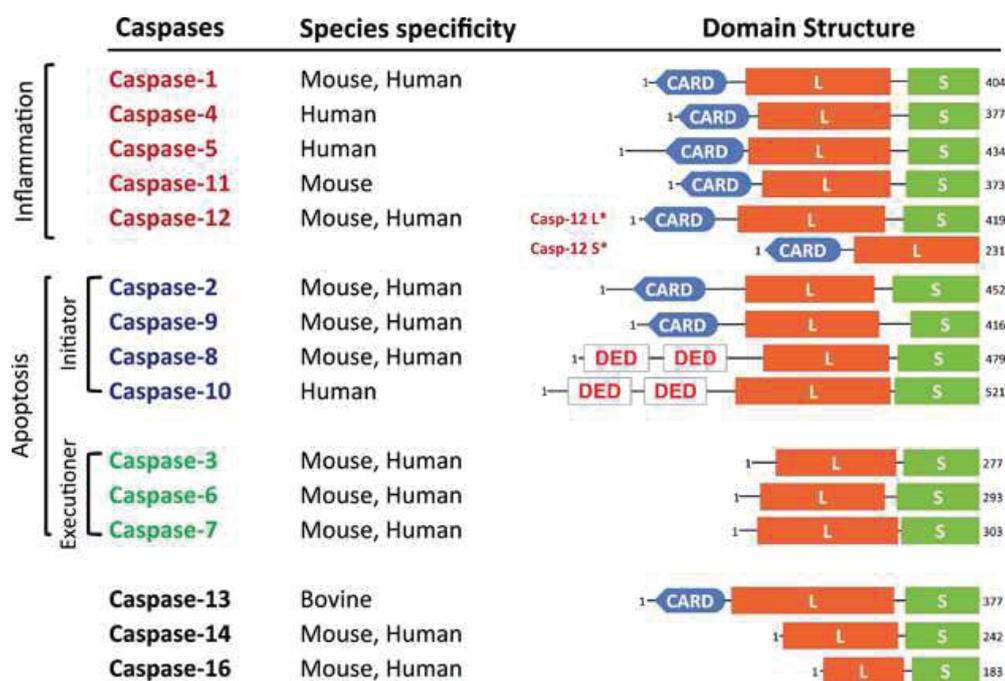
**Figure 19. Morphology of different types of cell death. Normal cell (a), cell in autophagy (b), cell in apoptosis (c), cell in necrosis (d) (Death by design: apoptosis, necrosis and autophagy (Death by design: apoptosis, necrosis and autophagy-Curr Opin Cell Biol 2004).**

In term of morphology, apoptotic cells have various changes during this process. During the early phase, cell shrinkage is visible under light microscopy which makes cells smaller in size. Moreover, pyknosis is also observed and it is the most important characteristic of apoptotic cells which is the result of chromatin condensation. A process called budding is just following because of the separation of cell fragments into apoptotic bodies, structures enclosed within plasma membrane. Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without nuclear fragments. And these apoptotic bodies are subsequently phagocytosed by macrophages, parenchymal cells and are degraded within phagolysosomes. As opposed to what is observed following necrosis, there is essentially no inflammatory reaction during apoptosis owing to the removal of apoptotic cells. Therefore, no cellular constituents are released and there is no production of inflammatory cytokines (Kurosaka et al., 2003; Savill and Fadok, 2000). This process is mediated by intrinsic or extrinsic pathways and both pathways are mediated by a class of proteases called caspase.

### **3.1.1 Caspases**

Apoptosis is highly conserved and can be triggered both intrinsically (for example, by DNA damage) and extrinsically (for example, by growth factor withdraw, steroid hormones or ligation of death receptors). During this process, accumulation and activation of caspases are observed in cells. Caspase is a class of cysteine-aspartic proteases which are expressed as inactive zymogens in cells (Thornberry and Lazebnik, 1998). Activation of caspase can often activate other procaspases which can afterward trigger a proteolytic cascade. Hence, apoptotic signal is amplified in the cell and thus lead to rapid cell death. Caspases are varied in different species, in *C. elegans* we can find 4 caspases, whereas flies and mammals contain 7 and 13 caspases respectively, which indicates that higher complexity of organism matches with a greater number of caspases (Shalini et al., 2015). Moreover, some caspases are not involved in apoptosis, but in immune response and some are in differentiation or other mechanisms (Yi and Yuan, 2009) (Figure 20). To date, caspases can be classified into two main groups according to their functions. The group1 consists of caspase 1, 4, 5, 11, 12 which are implicated in inflammation during immune responses. The group 2 mediating apoptosis includes caspase 2, 9, 8 and 10 which are initiators and caspase 3, 6, and 7 which are effectors (Shalini et al., 2015). The initiator caspases comprise a long amino-terminal pro-domain: death

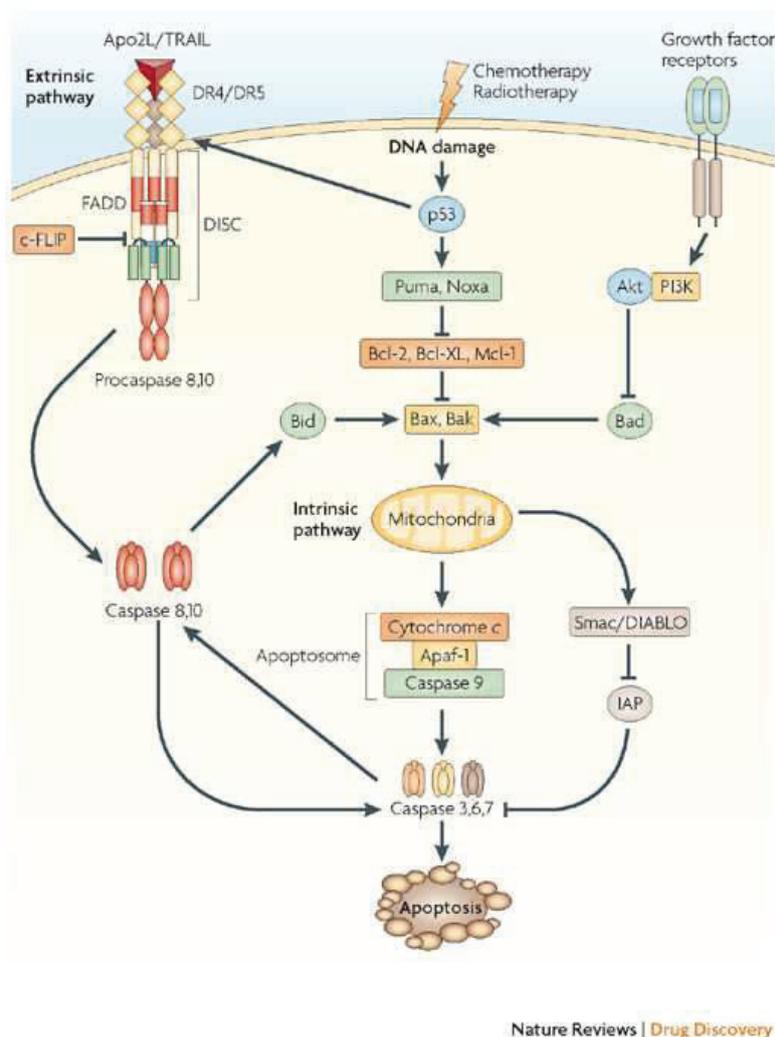
effector domain (DED; caspase8 and 10) or caspase recruitment domains (CARD; caspase2, 9, 1 and 11) which facilitate the formation of protein platform by regulating the dimerization of caspases and /or recruitment into larger complexes to trigger their activation. To date, there are two main pathways to induce apoptosis: the intrinsic pathway also called the mitochondrial pathway and the extrinsic pathway also called the death receptor pathway. Besides these two main pathways, there is an additional pathway can induce apoptosis called the granzyme B pathway which is involved in the T-cell mediated cytotoxicity and perforin-granzyme dependent killing of the cell. Despite the initiation of the apoptosis is different between different pathways, they converge finally to the same terminal, or execution pathway which is triggered by the cleavage of caspase-3. Afterward, apoptotic cell will undergo a series of events including DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Hengartner, 2000).



**Figure 21. Caspase family.** CARD, caspase recruitment domain; DED, death effector domain; L, large subunit; S, small subunit; S\*, short form; L\*, long form (Old, new and emerging functions of caspases-Cell death and differentiation 2015).

### 3.1.2 The intrinsic pathway of apoptosis

The intrinsic apoptotic pathway is also called the mitochondrial pathway and it is activated in response to a large variety of intracellular stress such as cytotoxic stimuli, DNA damage, unfolded protein in the endoplasmic reticulum (ER), cytosolic  $Ca^{2+}$ . This pathway is mainly regulated by Bcl-2 family proteins (Schuler and Green, 2001). This process is initiated at mitochondria and mediated by various pro-apoptotic effectors including BAX and BAK, which induce mitochondrial outer membrane permeabilization (MOMP) and cytochrome C release. MOMP is a decisive point to determine whether the cell commits to apoptosis. MOMP can be due to an opening of mitochondrial permeability transition (MPT) pore in the inner membrane of mitochondria or a formation of pore by BAX and BAK (Antignani and Youle, 2006). Afterward, mitochondrial transmembrane potential is lost and two main groups of pro-apoptotic proteins are released into the cytosol. The first group contains cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi (Cai et al., 1998; Saelens et al., 2004). Cytochrome C associates with Apaf-1 (apoptotic protease-activating factor 1) to form a large complex called apoptosome to activate caspase-9 and then caspase-3 (Chinnaiyan, 1999; Hill et al., 2004). Smac/DIABLO and HtrA2/Omi promote apoptosis by inhibiting IAP (inhibitors of apoptosis proteins) activity. The second group of pro-apoptotic proteins are AIF, endonuclease G and CAD which are released at the late stage of apoptosis when the cell has committed to die (Elmore, 2007). The intrinsic pathway is highly regulated by the Bcl-2 family of proteins (Bouillet et al., 2002) which contains anti-apoptotic proteins such as Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and pro-apoptotic proteins including Bcl-10, BAX, BAK, Bid, Bim, Bik, and Blk. It is thought that the main mechanism to mediate cell apoptosis is through the interplay of Bcl-2 family proteins meaning that the ratio between pro-apoptotic and anti-apoptotic proteins determines whether the cell undergoes apoptosis. And these proteins are regulated by the tumour suppressor p53 (Figure 21).

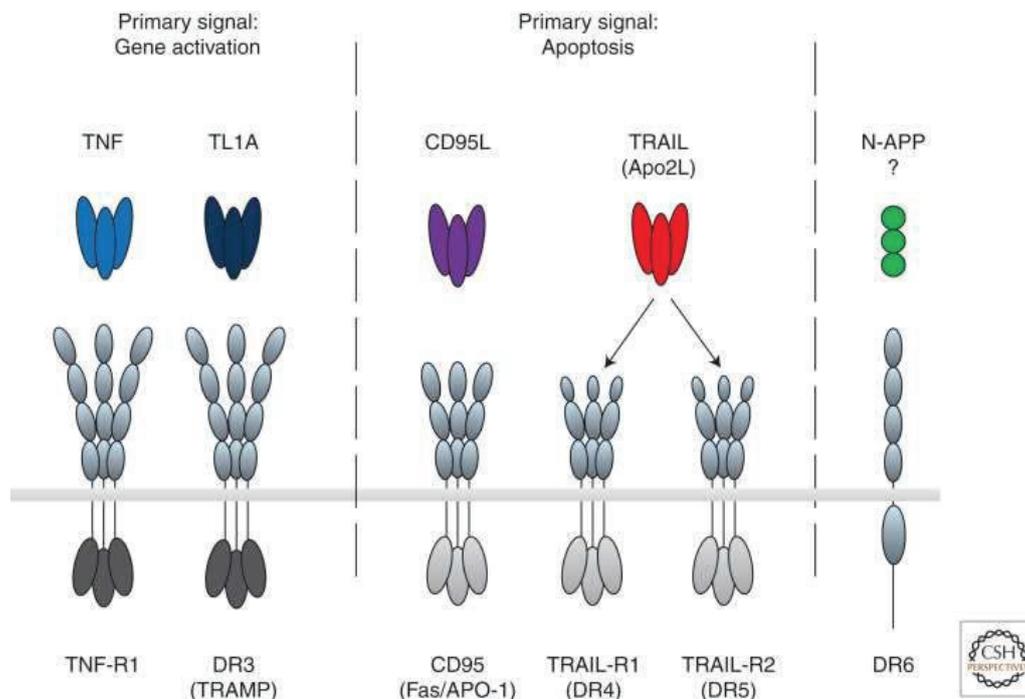


**Figure 21. Key steps to induce apoptosis: intrinsic and extrinsic pathways (Directing cancer cells to self-destruct with pro-apoptotic receptor agonists-Nature reviews. Drug discovery 2008).**

### 3.1.3 The extrinsic pathway of apoptosis

The extrinsic pathway, which was generally used to describe the cell death initiated by extracellular signals, usually refers to cell death induced by cytokines belonging to the TNF $\alpha$  superfamily such as FAS/CD95 ligand, TNF $\alpha$  or the TNF-related apoptosis inducing ligand (TRAIL) (Siegel, 2006). These cytokines bind to their specific death receptors and then transmit the death signal inside the cells. The common points shared by these death receptors are that they contain 2-4 cysteine-rich repeats in their extracellular domain, required for ligand binding and a 'death domain' (DD) about 80 amino acids (Ashkenazi and Dixit, 1998) which is essential

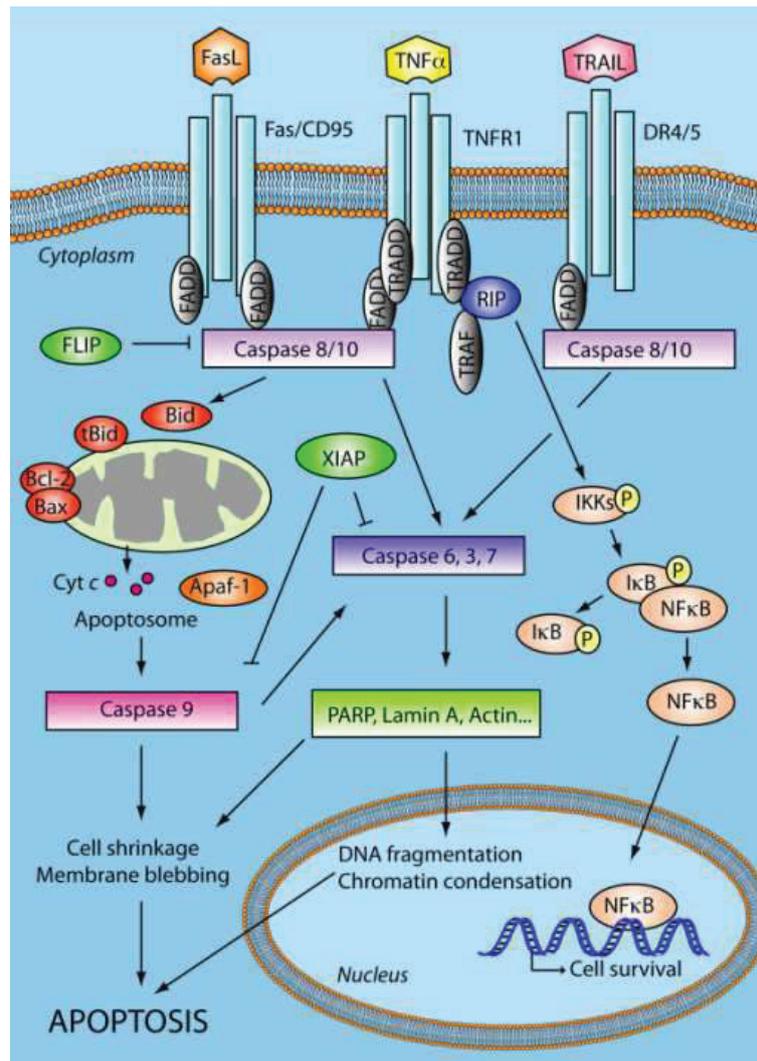
to transmit the death signal from the cell surface to the intracellular signalling pathways via recruitment of specific adaptators. To date, the best-characterized ligands/death receptors include FasL/FasR, TNF $\alpha$ /TNFR1 or TNFR2, TRAIL/DR4, DR5, TL1A/DR3, and the DR6 with its uncleared ligand (Figure 22).



**Figure 22. Death receptors and their ligands. Death receptors trigger two main signals. TNF-R1 and DR3 induce gene activation as their primary signalling output, whereas CD95, TRAIL-R1, and TRAIL-R2 induce apoptosis as their primary signal. The DR6 pathway is still unclear (Death Receptor–Ligand Systems in Cancer, Cell Death, and Inflammation-*Cold Spring Harb Perspect Biol* 2013).**

The best characterized example of apoptosis triggered by extrinsic pathway is the FasL/FasR and the TNF $\alpha$ /TNFR1 models which both involve ligand binding, clustering of receptors and recruitment of adapter proteins in the cytoplasm. The binding of Fas ligand to Fas receptor results in the binding of FADD whereas the binding of TNF ligand to TNF receptor engage the binding of TRADD with recruitment of RIP and FADD (Schulze-Osthoff et al., 1998; Wajant, 2002). Following the recruitment of FADD, the death domain of the receptors dimerize, which induces the association of procaspase-8 or 10 with FADD to form the death-inducing signalling complex (DISC), resulting in the auto-catalytic activation of procaspase-8 or 10 (Schneider and Tschopp, 2000) (Figure 23). The execution phase of the extrinsic pathway of apoptosis is cell

type dependent. In type I cells, for example lymphocytes, activation of procaspase-8 triggers directly the catalytic caspase cascade which activates the caspase effector caspase-3 and then further engages the apoptosis in a mitochondria independent manner. Whereas, in type II cells such as pancreatic  $\beta$  cells, the activated caspase-8 or 10 cleaves BID (BH3-interacting domain death agonist) to a truncated form (tBID) which engages the mitochondria pathway to amplify the apoptotic signal.



**Figure 23** The extrinsic apoptotic pathway is activated upon ligand binding to their death receptors to form the DISC and then triggers auto-catalytic activation of procaspase-8. This results in amplification of pro-apoptotic signal via crosstalk with intrinsic apoptosis pathway or degradation of nuclear substrates to commit the cell to apoptosis (Survival signalling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics -*Mol. Cancer* 2010).

### 3.1.4 Apoptosis in Cancer

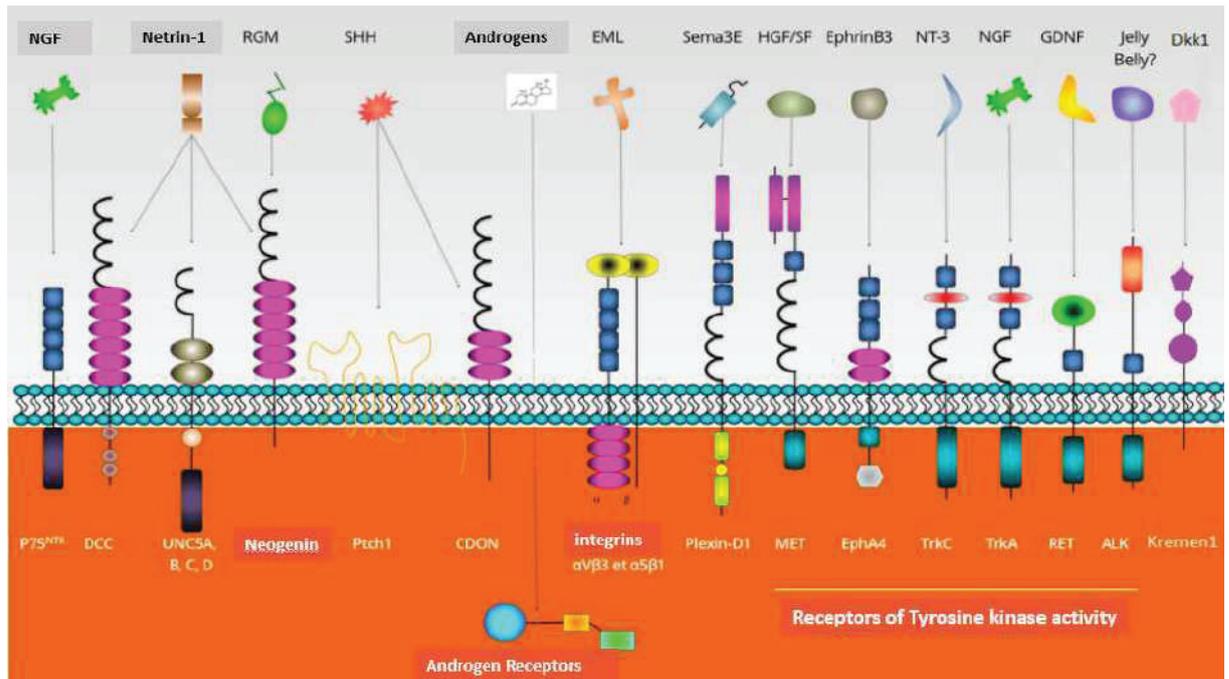
As an important safeguard of tissue homeostasis, numerous alterations have been found in the apoptotic signalling in cancer. Apoptosis is inhibited or altered via different mechanisms during tumorigenesis. Increasing data showed that apoptosis is inhibited via overexpression of anti-apoptotic proteins or loss of pro-apoptotic proteins in different types of cancers. One of the first evidence was the overexpression of Bcl-2 in lymphomas due to the translocation t(14;18) (Tsujiimoto et al., 1984). Afterward, overexpression of Bcl-2 has been widely reported in a variety of cancers including neuroblastomas, lung cancer, prostate cancer, and colorectal cancer (Bonkhoff et al., 1998; Ikeda et al., 1995; Ikegaki et al., 1994; Kaklamanis et al., 1998). Besides Bcl-2, alterations of other pro-apoptotic proteins such as Bax, BH3-only proteins, Bim, and Puma have been also found in cancers (Czabotar et al., 2011; Ng et al., 2012). Mutations or loss of pro-apoptotic proteins with BH-3 domain such as Bim and Puma facilitate the tumour progression (Shamas-Din et al., 2011; Zhou et al., 2012). In line with this, Mcl-1, an anti-apoptotic protein, is also up-regulated in leukaemia and colorectal cancer via the loss of expression of miR-29 which modulates its expression (Mott et al., 2007). Moreover, another critical pro-apoptotic protein Bax is also found to be lost or mutated in colon cancer (Ionov et al., 2000).

In addition, alteration of the extrinsic apoptosis pathway also contributes the tumorigenesis. Mutations of death receptors including TRAIL-R1 and TRAIL-R2 are reported in breast cancer (Shin et al., 2001). C-FLIP, a master anti-apoptotic regulator, which inhibits apoptosis via interaction with FADD and caspase-8 or 10 to form the apoptosis inhibitory complex (AIC) is found to be over-expressed in melanoma and lung cancers (Hassan et al., 2014). Moreover, the potent tumour suppressor P53 which is important for the intrinsic as well as the extrinsic apoptosis pathway is mutated in more than 50% of cancer (Andrews et al., 2004; Fridman and Lowe, 2003; Lee et al., 2008) and it mediates apoptosis via modulation of apoptotic regulators transcriptionally.

## 3.2 Dependence Receptors

### 3.2.1 General consideration of dependence receptors

The first dependence receptors (DRs) was discovered by Dr. Dale Bredesen in 1993 when he showed that while neural cells are cultured in absence of NGF, the unbound receptor p75NGFR does not stay inactive but rather induces cell death actively (Rabizadeh et al., 1993). Five years later, the concept of DRs was proposed by Patrick Mehlen in the study that characterized the cell death induced by Deleted in Colorectal Cancer (DCC) (Mehlen et al., 1998). To date, more than twenty dependence receptors have been discovered including DCC (Deleted in Colorectal Cancer) (Mehlen et al., 1998), UNC5 A-D (Llambi et al., 2001), Neogenin (Repulsive Guidance Molecule) (Matsunaga et al., 2004), RET (REarranged during Transfection) (Bordeaux et al., 2000; Canibano et al., 2007), Ptch1, CDON (Cell-adhesion molecule-related / down-regulated by Oncogenes) (Delloye-Bourgeois et al., 2013; Thibert et al., 2003), Integrin  $\alpha\beta3$  and  $\alpha\beta1$ / ECM (Stupack et al., 2001), TrkC/NT-3 (neurotrophin 3)(Tauszig-Delamasure et al., 2007) etc. (Figure 24). Recently, Notch3 has joined the family: indeed, Notch 3 receptor presents a pro-apoptotic role in tumour endothelial cells which is inhibited by tumour derived Jagged1 (Lin et al., 2017). Even though all these dependence receptors do not share homologies in their structure, they have two common distinct cellular functions: in the presence of their ligands, these receptors activate their classic signalling which results in cell survival, proliferation, migration, and differentiation; however, in the absence of their ligands, they do not stay inactive but induce a negative signalling that leads cells to apoptosis. Therefore, cells expressing this kind of receptor are dependent on the presence of ligand to survive (Goldschneider and Mehlen, 2010) (Figure 25).



- Domaine riche en cystéine
- Motif P
- Domaine SAM (*Sterile Alpha Motif*)
- Domaine de mort
- Répétition polyQ
- Répétitions riches en leucine
- Domaine Fibronectine Type III
- Site de liaison aux hormones
- Domaine apparenté aux cadhérines
- Domaine Immunoglobuline-like
- Site de liaison à IADN
- Domaine riche en glycine
- Domaine Thrombospondine Type I
- Motif MIDAS (*Metal ion dependent adhesion domain*)
- Domaine Sema
- Domaine ZU5
- Domaine tyrosine kinase

Figure 24. The Dependence Receptor Family (Adapted of Dependence receptors: a new paradigm in cell signaling and cancer therapy-Oncogene Review 2010).

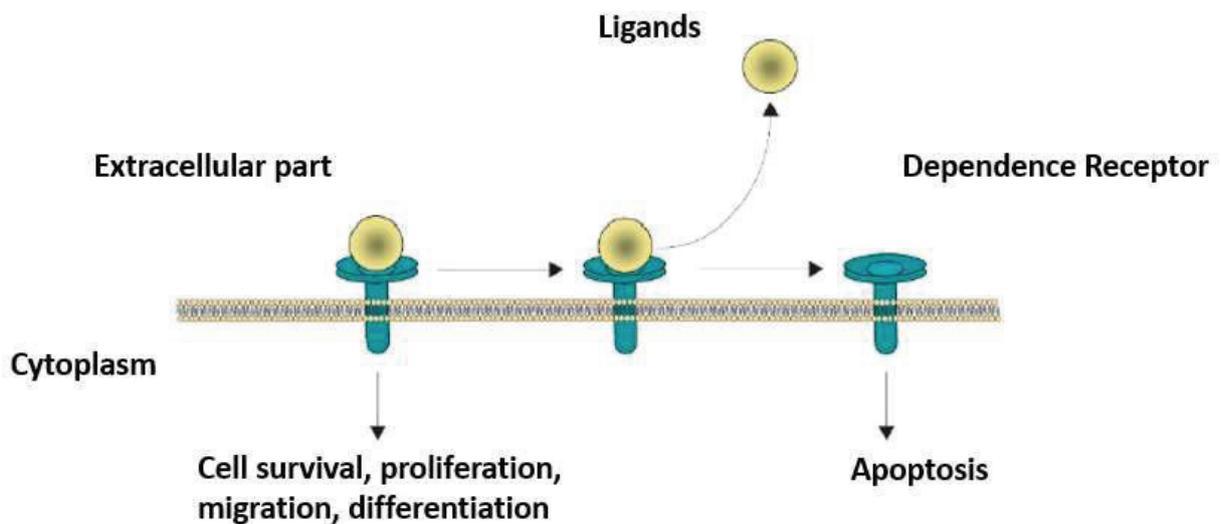
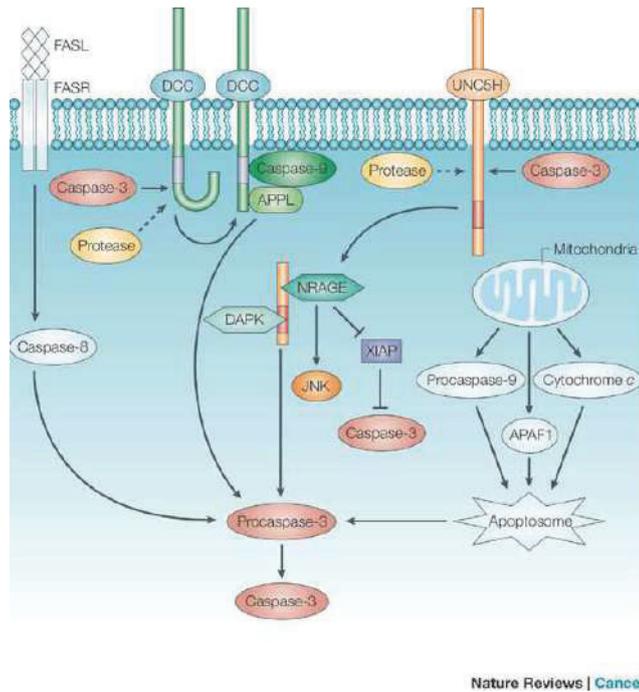


Figure 25. The Dependence Receptor Model (Adapted of Dependence receptors: a new paradigm in cell signaling and cancer therapy-Oncogene Review 2010).

### 3.2.2 The signalling pathway of dependence receptor

Dependence receptors can trigger negative signalling pathway in the absence of ligands. The mechanisms by which these receptors engage the cell to undergo apoptosis are distinct. Different dependence receptors are cleaved in their intracellular part by caspases. This cleavage enables the receptor to unveil the pro-apoptotic domain of DCC, UNC5 family, Neogenin, Patched, ALK, and Ephrin-A4 (Goldschneider and Mehlen, 2010). Following this caspase cleavage, the dependence receptor can therefore engage other caspase activation or the recruitment of adapter proteins and other pro-apoptotic partners to amplify the apoptotic signal.

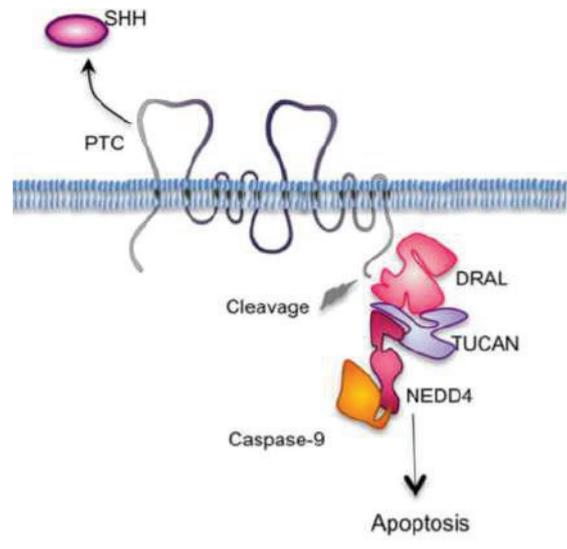
In absence of its ligand Netrin-1, DCC undergoes a conformational change in its intracellular part, followed by a cleavage by caspase-3 or other unknown caspases on Asp1290 which leads to the formation of a caspase activation complex. This cleavage might enable the receptor to be a scaffold and then facilitate the recruitment of caspase-9 and other adapter proteins to engage apoptosis which is independent on the intrinsic as well as on the extrinsic apoptosis pathways (Forcet et al., 2001). Similar to DCC, in the absence of Netrin-1, UNC5H is cleaved by caspases at Asp412, resulting in a release of a peptide which interacts with death-associated protein kinase (DAPK), thereby inducing apoptosis via the activation of caspase-9 and caspase-3 (Figure 26).



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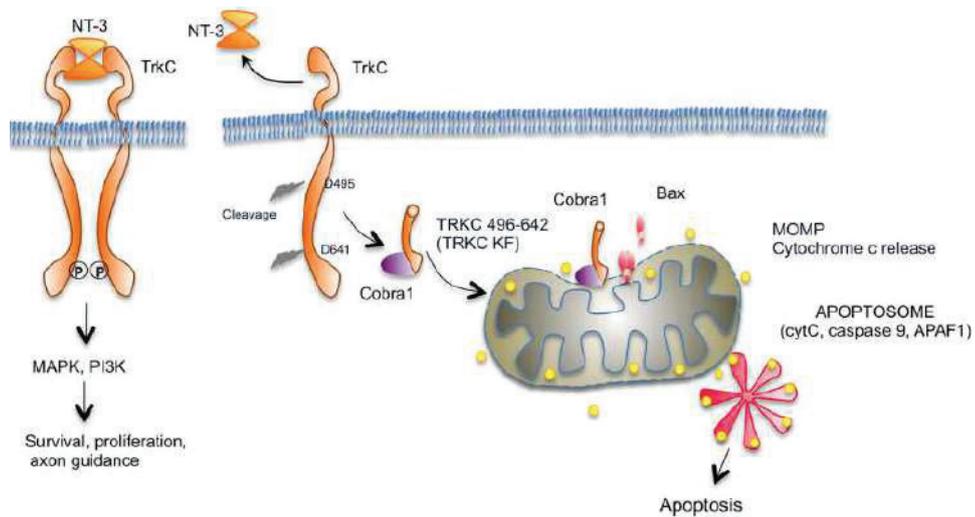
**Figure 26. Pro-apoptotic signalling from the netrin-receptors (Netrin-1 and its receptors in tumorigenesis-Nature Review Cancer 2004).**

Concerning Patched 1, the absence of its ligand Shh enables the receptor to interact with Downregulated rhabdomyosarcoma LIM-domain protein (DRAL), thereby recruiting the adapter protein TUCAN which will interact with caspase-9 via the CARD domain (Mille et al., 2009). The activation of caspase-9 is done by poly-ubiquitination via the E3-ubiquitin ligase NEDD4 (Fombonne et al., 2012) (Figure 27).



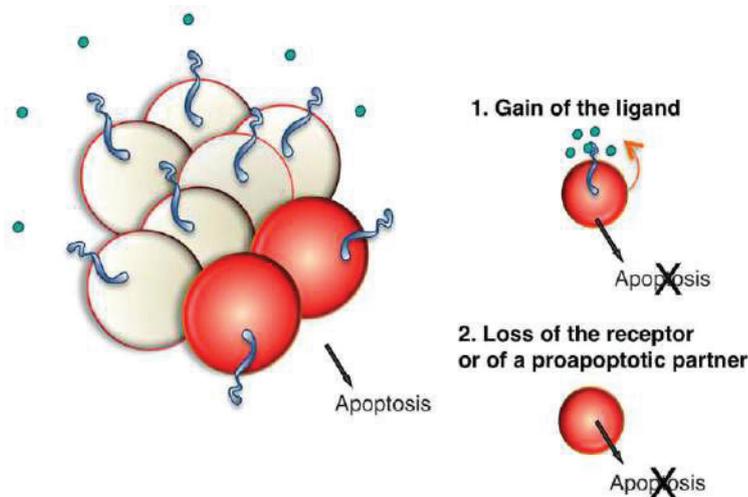
**Figure 27. Pro-apoptotic signalling of dependence receptor Patched 1 (Dependence Receptor and Colorectal Cancer-Gut 2014).**

Even though most of the dependence receptors induce apoptosis independently on the traditional apoptotic pathways, crosstalk with the mitochondrial pathway is observed in the pro-apoptotic pathway of dependence receptor TrkC. In the absence of NT3, TrkC is double-cleaved by caspase in two sites: D495 and D642. The released killer fragment (KF) interacts with Cobra1 and translocates to the mitochondria where it activates Bax and induces MOMP to trigger apoptosis via the intrinsic pathway (Figure 28).



**Figure 28. Pro-apoptotic signalling of dependence receptor TrkC (Dependence Receptor and Colorectal Cancer-*Gut* 2014).**

### 3.2.3 Dependence Receptor in tumorigenesis



**Figure 29. Anti-tumour mechanism of dependence receptors. (Dependence Receptor and Colorectal Cancer-Gut 2014).**

Because of the pro-apoptotic function of dependence receptors, they play an important role in mediating tissue homeostasis during development and tumorigenesis. In line with this, many studies have shown that dependence receptors behave as tumour suppressors in various cancers, in which they are lost or mutated (Goldschneider and Mehlen, 2010; Mehlen and Tauszig-Delamasure, 2014). To date, dependence receptors are reported to regulate the tumorigenesis via three main mechanisms: the gain of ligands, the loss or mutation of the receptor, the loss or mutations of their pro-apoptotic partners (Figure 29). For instance, DCC is reported to be lost not only in colon cancer but also in prostate cancer, breast cancer, ovarian cancer, and neuroblastoma (Mehlen and Fearon, 2004). Mutation of the caspase cleavage site of DCC is sufficient to induce neoplasia in intestine and increase the tumour incidence in an adenomatous polyposis coli (APC) mutated mouse model (Castets et al., 2011). In addition, the UNC5 A-C are also found to be lost and behave as tumour suppressors in numerous cancers (Thiebault et al., 2003). Not only the loss of receptor itself, but also pro-apoptotic partners facilitate the tumour progression. DAPK1, a pro-apoptotic partner of the UNC5 family is lost in breast cancer and lung cancer via the methylation of its promoter (Grandin et al., 2016a; Grandin et al., 2016b). In neuroblastoma, TrkA is reported to be a good prognostic marker and its expression is negatively correlated to the up-regulation of MycN and the tumour grade (Eggert et al.,

2000; Nakagawara, 1998). In line with this, expression of TrkC facilitates the survival of neuroblastoma patients (Brodeur et al., 2009) and its expression is lost in colon cancer via hypermethylation of its promoter (Genevois et al., 2013).

Besides the modification of receptors, up-regulation of ligands including Netrin-1, NT3, and Semaphorin 3E are also usually observed in different type of cancers and their expression could be correlated to metastasis (Fitamant et al., 2008; Luchino et al., 2013; Mancino et al., 2011). This makes these ligands to be putative biomarkers for the prognostics and potential therapeutic targets. Therefore, antibodies neutralizing and antagonizing specifically the interaction between ligand and receptor have been adopted as therapeutic strategy and widely developed in the past fifteen years (Broutier et al., 2016; Grandin et al., 2016b). However, increasing specificity of antibodies, identifying more biomarkers to better select patients, are still big challenges for the transition from therapeutic concept to clinical practice.

## **II. Results**



#### **4.1 Article 1: Non-canonical NOTCH3 signalling limits tumour angiogenesis**

## **Non-canonical NOTCH3 signalling limits tumour angiogenesis**

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## **ABSTRACT**

**Notch signalling is a causal determinant of cancer and efforts have been made to develop targeted therapies to inhibit the so-called canonical pathway. We describe here an unexpected pro-apoptotic role of Notch3 in regulating tumour angiogenesis independently of the Notch canonical pathway: The Notch3 ligand Jagged-1 is up-regulated in a fraction of human cancer and our data support the view that Jagged-1, produced by cancer cells, is inhibiting the apoptosis induced by the aberrant Notch3 expression in tumour vasculature. We thus present Notch3 as a novel dependence receptor inducing endothelial cell death while this pro-apoptotic activity is blocked by Jagged-1. Along this line, using Notch3 mutant mice, we demonstrate that tumour growth and angiogenesis are increased when Notch3 is silenced in the stroma. Consequently, we show that the well-documented anti-tumour effect mediated by  $\gamma$ -secretase inhibition is at least in part dependent on the apoptosis triggered by Notch3 in endothelial cells.**

## INTRODUCTION

Tumour angiogenesis has been considered as an attractive target for cancer therapy for more than forty years. However, clinical results using drugs targeting tumour angiogenesis are inconsistent and often disappointing (Jayson et al., 2012). Most anti-angiogenic therapies target the vascular endothelial growth factors (VEGFs) signalling pathways, in which VEGFs activate VEGF receptors (VEGFRs) on endothelial cells in order to regulate vascular growth in both developing tissues and growing tumours. Notch signalling is a major regulator of these processes. Four Notch receptors (Notch1-4) have been described in mammals. Notch receptors are single-pass type I transmembrane non-covalently linked heterodimer coded by a single precursor, which is cleaved by furins. The Notch pathway activation follows the binding of the transmembrane ligands of the Delta/Serrate/LAG-2 (DSL) family, Delta-like and Jagged to Notch receptors. In mammals, three Delta-like ligands (Dll1, Dll3 and Dll4) and two Jagged ligands (Jag-1 and Jag-2) have been identified. The well-described so-called “canonical pathway” depends on a strictly controlled proteolytic cascade induced by ligand binding: an S2 cleavage by metalloproteases followed by an S3 cleavage mediated by a presenilin- $\gamma$ -secretase complex. These proteolytic cleavages release the intracellular domain of the Notch receptor (NICD) which then translocates into the nucleus to mediate target genes activation (Kopan and Ilagan, 2009).

Notch signalling has been implicated in cancer, with observed genetic alterations in a large number of hematopoietic and solid tumours (Ntziachristos et al., 2014). As the presenilin- $\gamma$ -secretase complex activity is necessary for the activation of the canonical signalling pathway,  $\gamma$ -secretase inhibitors such as DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) derivatives have been proposed as targeted therapies for treatment of pathologies such as T-cell acute

lymphoblastic leukemia. However, such therapeutic approaches have so far been limited due to intestinal toxicity (van Es et al., 2005). Other approaches to inhibit the Notch canonical pathway are thus in development with strategies including antibodies raised specifically against individual Notch receptors (Li et al., 2008; Wu et al., 2010).

Notch signalling is also a major regulator of angiogenesis as Dll4-mediated Notch activation controls the expression of the VEGFRs and therefore limits endothelial cells sprouting and proliferation (Benedito and Hellstrom, 2013; Lobov et al., 2007). However, whereas the role of Notch signalling is well described in developmental angiogenesis, its role in tumour angiogenesis is not clearly understood. *In vitro*, Notch inhibition has been shown to induce endothelial cell death (Patel et al., 2005) as well as vascular sprouting (Hellstrom et al., 2007). *In vivo*, Notch inhibition using chemical inhibitors or Notch1 ectodomain is generally associated with endothelial cell death and reduced vascularisation (Cook et al., 2012; Funahashi et al., 2008; Paris et al., 2005b). In contrast, anti-ligand approaches such as anti-Dll4 treatments produces non-productive angiogenesis through increased endothelial cells sprouting (Noguera-Troise et al., 2006). These paradoxical observations could suggest that the role of Notch in tumour angiogenesis cannot be completely explained by canonical Notch signalling. In contrast to other Notch receptors, Notch3 expression is restricted to the vasculature in physiological condition. Notch3 mutations are associated with CADASIL (Chabriat et al., 2009) (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) and Notch3 knock-out mice are more susceptible to ischemic stroke (Arboleda-Velasquez et al., 2008) whereas they are less susceptible to pulmonary hypertension (Li et al., 2009). These studies show that even if *Notch3* mutant mice have no major phenotype in developmental angiogenesis, Notch3 is involved in pathological angiogenesis.

However, its role in tumour angiogenesis has never been studied. In the disorganised tumour vasculature, tumour endothelial cells show a different phenotype than normal endothelial cells (St Croix et al., 2000). Interestingly, Notch3 has been shown to be upregulated in human lung cancer-associated endothelial cells (Herbert 2013) and this led us to evaluate the role of Notch3 in endothelial cell in cancer development. While analysing the importance of Notch3 in the stroma during tumour progression, we observed an unexpected pro-apoptotic activity of Notch3. We describe Notch3 as a novel dependence receptor in endothelial cells. Such receptors that include the netrin-1 receptors DCC and UNC5H (Mehlen et al., 2011) or the Hedgehog receptors Ptc and CDON (Delloye-Bourgeois et al., 2013; Thibert et al., 2003) share the ability to actively transduce a death signal in settings of ligand limitation, thus creating a state of cellular dependence to the presence of ligand for cell survival. This pro-apoptotic activity has been proposed to act as a negative constrain for tumour progression by controlling cancer cell death (Castets et al., 2012; Krimpenfort et al., 2012). We propose here that Notch3 by acting as a dependence receptor in endothelial cells regulate tumour angiogenesis by regulating endothelial cell death.

## RESULTS

### Notch3 is expressed in tumour associated endothelial cells

We first investigated Notch3 expression in a small panel of human lung cancers by immunohistochemistry. In all the studied samples (11 adenocarcinoma (ADC) and 10 squamous cell carcinoma (SCC)), the expression of Notch3 was very strong in the vasculature (Supplementary Fig.1a). Conversely, the cancer cell expression of Notch3 was very heterogeneous between patients but also within the same patient (Supplementary Fig.1a). SCC showed the strongest Notch3 expression in the cancer cells, however, only a small fraction of patients showed nuclear expression (4/10 for SCC and 2/11 for ADC) (Supplementary Fig. 1a; Supplementary Fig.1b). The role of Notch signalling and in particular Notch3 in the epithelial compartment of tumours and more specifically of non-small cell lung cancers has been extensively studied (Ye et al., 2013; Zheng et al., 2013). However, Notch3 implication in tumour vasculature has not been addressed. We thus focused on the vascular expression of Notch3 in these patients. In the patients for whom we could observe histological normal peritumoural tissue, we noticed that the expression of Notch3 was localized, as described previously (Li et al., 2009), in the vascular smooth muscle cells or in the mural cells of smaller vessels (Fig. 1a). However, in the malignant part, we could observe Notch3 expression in the endothelial cells (EC) (Fig. 1a). This prompted us to investigate a possible role of this aberrant expression of Notch3 in tumour endothelial cells. To study this role, we first assessed whether this aberrant expression was also observed in mouse model of lung cancers. We first purified EC from lung adenomas in the *Kras*<sup>+G12D</sup> hit and run mice model characterized previously (Johnson et al., 2001). Endothelial cell fraction was verified on flow cytometry for CD45 negative staining and CD31/CD105 co-staining (not shown). Whereas in wild-type mice or in the healthy part of lung from

*Kras*<sup>+/*G12D*</sup> mice, no or little expression of Notch3 was detected in EC-enriched fraction, we observed an over-expression of Notch3 in the EC-enriched fraction from the tumour nodules (Fig. 1b). We next used the LacZ reporter to monitor Notch3 expression in the Notch3 LacZ knock-in mice described previously (Arboleda-Velasquez et al., 2008). We confirmed, in this model, that the Notch3/LacZ mRNA fusion was expressed to a similar amount than the wild-type allele (Supplementary Fig. 2a). We also used an anti-β-galactosidase antibody to check the staining of the LacZ enzymatic reaction and confirmed expression in the smooth muscle cells in healthy lungs (Supplementary Fig. 2b). As described by others, we observed that the expression of Notch3 was restricted to mural cells and Notch3 was absent in endothelial cells in normal vasculature. Notch3 was indeed mostly associated to α-smooth-muscle actin (αSMA) expressing cells surrounding big vessels and to a lesser extend to NG2 (neural/glial antigen 2) expressing mural cells in smaller vessels as seen in lungs from *Notch3*<sup>+/*LacZ*</sup> mice (data not shown). We then looked at Notch3 expression in the adenocarcinoma from *Kras*<sup>+/*G12D*</sup>-*Notch3*<sup>LacZ/+</sup> mice. We confirmed the data obtained by purifying tumour associated endothelial cells. Indeed, the LacZ staining is detected in the tumour and in healthy lung, the LacZ staining is not associated with ERG staining –i.e. ERG staining was reported to be strongly specific for EC (Miettinen et al., 2011). However, in the peri-tumoural part, we observed ERG staining in LacZ positive cells (red arrow) (Fig. 1c). We next injected LLC1 syngeneic lung cancers cells in *Notch3*<sup>+/*LacZ*</sup> to assess expression of Notch3 in subcutaneous graft. We confirmed that Notch3 is expressed in the vasculature of the grafted tumours and as observed in the tumour nodules from the *Kras*<sup>+/*G12D*</sup> mice, we observed an aberrant expression of Notch3 in tumour-associated EC (Fig. 1d and e): Notch3 co-localizes with CD31 (endothelial cell marker platelet/endothelial cell adhesion molecule, PECAM-1) but not with mural cell markers

$\alpha$ SMA or NG2 (Fig. 1d). We further confirmed the up-regulation of Notch3 mRNA in purified tumour associated endothelial cells from subcutaneous injected LLC1 in Ve-Cadherin-tomato mice allowing FACS sorting of tumour-associated endothelial cells (Fig. 1e). The LLC1 model thus provides a good model to study the functional impact of Notch3 aberrant expression on tumour vasculature. Furthermore, co-culturing LLC1 cells with HUVEC was sufficient to induce an upregulation of Notch3 in the endothelial cells, showing that the epithelial cancer cells are sufficient to induce Notch3 expression in endothelial cells (Fig. 1f).

### **Stroma specific Notch3 silencing promotes tumour angiogenesis**

We next assessed the role of this aberrant expression of Notch3 in tumour vasculature by establishing a model in which Notch3 is silenced only in the stroma but not in the tumour cells. As we started with observations in human lung carcinomas, we chose the murine lung carcinoma LLC1 syngeneic grafts in wild-type and in *Notch3<sup>LacZ/LacZ</sup>* mice. As shown in Figure 2a, the absence of stromal Notch3 was associated with an increase of tumour growth. This suggests that the endothelial expression of Notch3 limits tumour angiogenesis. This observation was also true in another model of syngenic graft, the E0771 mammary gland tumour model, although to a lesser extent (Supplementary Fig. 3a). In line with a role of Notch3 in tumour associated endothelial cells, we observed an increase in CD31 and DLL4 expression in tumours from *Notch3<sup>LacZ/LacZ</sup>* mice (Fig. 1b and supplementary Fig. 3b), but no change in  $\alpha$ SMA or PDGFR $\beta$  (Beta-type platelet-derived growth factor receptor) two pericyte markers (Fig. 2b). As Notch3 has been reported to be expressed in certain immune cells (Fung et al., 2007), we looked for the expression of CD11b and CD45

that remained unchanged (Fig. 2b). We next looked at the vascularisation of tumours grown in the absence of stromal Notch3 expression. CD31 staining of tumours grown in the wild-type mice or in the *Notch3<sup>LacZ/LacZ</sup>* mice showed an increased vascularisation in the latter (Fig. 2c). Furthermore, expression of  $\alpha$ SMA in these tumours was unchanged (Fig. 2c). This suggests that the aberrant expression of Notch3 in tumour endothelial cells could limit tumour angiogenesis whereas the absence of Notch3 in vascular smooth muscle cells has no effect. Furthermore, this effect seems to be independent of the normal role of Notch3 in smooth-muscle cells.

### **Notch3 behaves as a dependence receptor**

*In order to understand how the absence of Notch3 would impact the tumour vascularisation, we studied in vitro human umbilical vein endothelial cells (HUVEC). As described previously (Patel et al., 2005), these cells express a low level of Notch3 which is almost entirely cleaved into N3ICD as treatment with DAPT completely abolished the presence of a 75kDa band recognized by a C-terminal antibody (Supplementary Fig. 4a). We then asked what would be the consequence of an up-regulation of Notch3 in these cells that would mimic the aberrant expression of Notch3 observed in lung cancers-associated endothelial cells. We first used electroporation in HUVEC cells (with 80% electroporation efficiency (Supplementary Fig. 4b)). As shown in Figure 3, Notch3 forced expression in HUVECs triggered cell death as evidenced by an increase of the sub-G1 cell population (Fig. 3a) and annexin-V positive cell population (Fig. 3b). This cell death is probably, at least in part, apoptosis as it is inhibited by general caspase inhibitor z-VAD-fmk (Fig. 3c). At this stage we cannot however exclude that Notch3-induced cell death is not only apoptosis as inhibition of cell death by caspase inhibitors is not complete. Interestingly, N3ICD did not induce*

cell death (Supplementary Fig. 4c). Furthermore, although Notch3 level is low in HUVEC under normal condition, knocking-down Notch3 in a setting of network formation in matrigel was sufficient to inhibit significantly apoptosis during network regression (Supplementary Fig. 4d). We further used the S1-Cter Notch3 construct (a truncated version of Notch3 (S1-Cter Notch3; aa1573 [furin cleavage site] to the C-terminus, Supplementary Fig. 4e) as it mimics the absence of ligands and also helps bypassing the possible effect of varying levels of ligand expression in different cellular models. S1-Cter Notch3 induced very low Notch transcriptional activity in comparison to N3ICD (Supplementary Fig. 4f). Whereas S1-CterN3 expression induced caspase-3 cleavage (Supplementary Fig. 4g), electroporation of N3ICD, or of CBF1-VP16 (which both activates canonical Notch signalling in HUVEC (Supplementary Fig. 4f)), or of DNMAHL (Dominant negative Mastermind-like, which inhibits endogenous Notch signalling (Supplementary Fig. 4f)) had no effect on induction of cell death (Supplementary Fig. 4g and h), suggesting that canonical Notch signalling is not involved in this process. While S1-Cter Notch3 triggers apoptosis, this was not observed with both Notch1 and Notch2 (not shown). Of interest, S1-Cter Notch3 mutant, that fails to interact with the CBF1 transcription factor (S1-Cter WFP-LAA), is still able to induce caspase-3 cleavage (Supplementary Fig. 4h) supporting the view that the canonical Notch3 signalling pathway is not involved here. Such ability of a transmembrane receptor to trigger apoptosis in a setting of absence of ligand, recalls the behaviour of dependence receptors (Mehlen and Bredesen, 2011). Such receptors that include the netrin-1 receptors DCC and UNC5H (Mehlen and Mazelin, 2003) or the Hedgehog receptors Ptc and CDON (Delloye-Bourgeois et al., 2013; Thibert et al., 2003) share the ability to actively transduce a death signal in settings of ligand limitation, thus creating a state of cellular dependence to the presence of ligand for cell

survival. Most of these dependence receptors share the trait of being cleaved by caspase (Mehlen and Bredesen, 2011). We thus looked whether Notch3 could similarly be cleaved by caspases. Expression of S1-Cter Notch3 or an S2-Cter Notch3 (aa1631 to the C-terminus) in HEK293T cells allows the identification of a 60-65kD N-terminal fragment and a lower size 25-30kD Notch3 C-terminal reactive fragment (Supplementary Fig. 4i and j). These fragments were no longer detected upon incubation with z-VAD-fmk and more specifically with initiator caspase inhibitors IETD-fmk and LEHD-fmk, supporting the view that a Notch3 fragment is released upon a caspase-like dependent cleavage (Supplementary Fig. 4i). To map more precisely the caspase-cleavage site in Notch3, systematic mutations of aspartic acid residues were performed. The specific mutations of the aspartic acid residues 2104 and 2107 into asparagine residues (D2104N-D2107N) fully blocked the detection of the Notch3 fragment without affecting canonical Notch signalling (Supplementary Fig.4k). Thus, Notch3 is cleaved by a caspase-like protease at DSLD (2104-2107). Interestingly, this cleavage site is not present in other Notch receptors (not shown) but is conserved in Notch3 receptors (Supplementary Fig. 4i). Therefore, expression of Notch3 *in vitro* induces cell death of EC, and Notch3 is cleaved by caspase-like proteases. Another frequent characteristic of dependence receptors is their ability to recruit and activate the initiator caspase-9 (Forcet, 2001, Mille 2009, Fombonne 2012). We first observed that caspase-9 might be required for Notch-3-induced cell death as treatment with z-LEHD-fmk significantly inhibited cell death induced by Notch3 over-expression (Fig. 3c). We further confirm the importance of caspase-9 by analysing Notch3-induced cell death upon silencing of caspase-9. As shown in Figure 3d, silencing of caspase-9 strongly inhibits cell death induced by Notch3 (Fig. 3d). We then asked whether Notch3 could interact with caspase-9. Interestingly, we observed that S1-Cter Notch3, but not

S1-Cter Notch1 or S1-Cter Notch2, was able to interact with caspase-9 when both Notch proteins and caspase-9 were ectopically expressed (Fig. 3e). We confirmed the interaction between Notch3 and caspase-9 by immunoprecipitation of endogenous caspase-9 (Fig. 3f). Interestingly N3ICD did not interact with caspase-9 under the same condition, suggesting that the interaction with caspase-9 needs the anchorage of Notch3 to the membrane. We also performed Proximity Ligation Assay (PLA) with endogenous caspase-9 upon Notch3 overexpression in HEK293T cells. We observed a clear interaction between Notch3 and caspase-9 whereas no interaction was observed with caspase-8 (Fig. 3g). Moreover, to explore whether the recruited caspase-9 could be activated, we performed caspase-9 activity assessment on Notch3 pull-down. As shown in Figure 3h, Notch3, but not N3ICD, is pulling down caspase-9 activity, supporting the view that Notch3 could trigger cell death similarly to other dependence receptors.

These observations prompted us to further investigate whether Notch3 could be a dependence receptor for tumour EC aberrantly expressing Notch3. As a dependence receptor, it is expected that Notch3 ligand blocks Notch3 induced endothelial cell death. As Jag-1, a Notch3 ligand, has been shown to be associated with increased tumour angiogenesis (Zeng et al., 2005), we looked for the effect of Jag-1 expression on Notch3-induced cell death in tumour. For this purpose, HUVEC were co-cultured with two lung carcinoma cells expressing low or high levels of Jag-1, murine LLC1 cells and human H358 cells respectively (Supplementary Fig. 5a). We observed that over-expression of Jag-1 in LLC1 cells reduced endothelial cell apoptosis and therefore induced stabilisation of the endothelial network (Fig. 4a and b). Conversely, silencing Jag-1 in H358 cells led to an increase in endothelial cell apoptosis and earlier destabilization of the endothelial network (Fig. 4a and b). To confirm *in vivo* that

tumour-derived expression of Jag-1 could increase angiogenesis, we established graft of LLC1 overexpressing Jag-1. As shown in Figure 4c, overexpression of Jag-1 in LLC1 cells induced a dramatic increase in angiogenic markers CD31 as seen both on mRNA level and on protein staining by immunohistochemistry (Fig. 4c). To go further and prove that Notch3 behaves as a dependence receptor, we then over-expressed Notch3 in HUVEC cells and co-cultured them with LLC1 cells expressing or not high level of Jag-1. Overexpression of Jag-1 in LLC1 cells rescued the HUVEC death induced by Notch3 (Fig. 4d). We also showed that in co-culture conditions, neither N3ICD nor DNMAML was able to induce cell death (Supplementary Fig. 5b). This further supports the view that Notch3 induces endothelial cell death independently of Notch canonical signalling pathway, and that the expression of Jag-1 by cancer cells can cell non-autonomously rescue endothelial cell death. Jag-1 is also frequently over-expressed in epithelial cancer cells (Santagata et al., 2004a; Sethi et al., 2011). We observed that Jag-1 was over-expressed in a fraction of human lung cancers using the GSE7670 dataset (Fig. 4e) and confirmed Jag-1 over-expression in human clear cell renal cell carcinomas (Supplementary Fig. 5c). Of interest, Jag-1 expression was only poorly correlated with HES1, HEY1 and HEYL Notch target genes expression in this dataset as well as in the GSE10245 dataset (Supplementary Fig.5d). This observation supports the hypothesis that Jag-1 could have a different role in the tumour than activating Notch canonical signalling. As Jag-1 was shown to have a paradoxical pro-angiogenic role regarding Notch activation (Benedito et al., 2009), we compared the expression of Jag-1 with the expression of CD31 among tumours that over-express Jag-1 in the GSE7670 dataset. In these patients, we observed a strong correlation with CD31 expression (Fig. 4e). We observed the same correlation in clear cell renal cell carcinoma (Supplementary Fig.5c). By carrying out non-supervised clustering using

the GSE10245 dataset, we observed a population in which Jag-1 and CD31 clustered together whereas Jag-1 did not cluster with Notch target genes (Supplementary Fig. 5e). In this population, we observed a strong correlation between Jag-1 and CD31 but not with Notch target genes (Supplementary Fig. 5f). Taken together these data support the view that Notch3 behaves as a dependence receptor in endothelial cells and that Jagged-1 expression in tumour may act as a pro-angiogenic mechanism by limiting Notch3 induced apoptosis in endothelial tumour cells.

### **Notch3 is required for $\gamma$ -secretase-induced tumour regression**

We then hypothesized that  $\gamma$ -secretase inhibitors, by blocking the N3ICD formation may mimic the absence of Notch3 ligand and thus induce Notch3-dependent tumour associated-endothelial cell death. The general view for the mode of action of  $\gamma$ -secretase inhibitors as anticancer agents is the inhibition of cancer cell proliferation. However,  $\gamma$ -secretase inhibitors treatments have been paradoxically associated with decreased angiogenesis (Funahashi et al., 2008; Paris et al., 2005b) and endothelial cell death (Cook et al., 2012) as opposed to anti-Dll4 antibody treatment which induces increase in non-productive angiogenesis (Noguera-Troise et al., 2006). We first observed that, *in vitro*, DAPT treatment induced HUVEC cell death (Fig. 5a). Of interest, this cell death was rescued by silencing Notch3 (Fig. 5b) but not by silencing Notch1 or Notch2 which had no effect on DAPT induced cell death (Supplementary Fig. 6a). We confirmed that tumour-associated endothelial cells were sensitive to DAPT treatment by purifying endothelial cells from tumours of *Kras*<sup>G12D/+</sup> mice (Fig. 5c). Further confirming the role of Notch3 in DAPT-induced cell death, we also showed that tumour-associated endothelial cells of tumours purified from *Notch3*<sup>LacZ/LacZ</sup>; *Kras*<sup>G12D/+</sup> mice were not sensitive to DAPT treatment (Fig.5c). We then asked whether this effect could also be seen *in vivo*. We therefore treated wild-type mice bearing LLC1 tumours

with DAPT. Whereas DAPT had no effect on LLC1 cells *in vitro* (Supplementary Fig. 6b), DAPT treatment in wild-type mice was associated with tumour growth inhibition (Supplementary Fig. 6c). As described by others (Cook et al., 2012; Funahashi et al., 2008; Paris et al., 2005b), this reduction was associated with a regression of the tumour vasculature as seen here by a decrease of CD31 staining and of the collagen IV/CD31 co-staining which shows a regression of pre-existing vessels (Supplementary Fig. 6d). This tumour growth inhibition induced by DAPT treatment would classically be attributed to canonical Notch signalling inhibition. However, we report here that the tumour growth inhibitory effect of DAPT treatment was no longer observed in *Notch3* mutant mice (Fig. 5d). Because *Notch3* is only silenced in stromal cells, this phenotypic rescue can only point out an effect of DAPT treatment on the stroma and cannot be easily explained by a difference in the canonical pathway (i.e. if DAPT is inhibiting tumour angiogenesis by blocking the canonical pathway induced by Notch receptors, knocking down *Notch3* should only add more tumour angiogenesis inhibition). In line with this, *HeyL* mRNA expression was not affected in both wild type and *Notch3* mutant mice in presence of DAPT (Supplementary Fig. 6e). We further purified tumour-associated endothelial cells and treated these cells with DAPT. In this setting, we saw no significant down-regulation of Notch target genes *HeyL*, *Hes1* and *Hey1* (Supplementary Fig. 6f). In agreement with an effect on vasculature, we observed an increase in necrotic area in wild-type mice treated with DAPT but not in *Notch3* mutant mice (Fig. 5d). Confirming Cook et al. (Cook et al., 2012) data obtained in a different model, we observed increased endothelial cell death in wild-type mice treated with DAPT (Fig. 5e). In contrast, no effect was seen in *Notch3* mutant mice (Fig. 5e). This indicates that the apoptotic pathway mediated by *Notch3* accounts, at least in part, for the regression of the tumour vasculature following DAPT treatment.

## DISCUSSION

We uncovered here an unexpected function of Notch3 expression in tumour vasculature. Whereas Notch3 is normally expressed in smooth-muscle cells surrounding large vessels, Notch3 is up-regulated in tumour endothelial cells. We have observed this ectopic expression in human lung cancer samples regardless of the expression of Notch3 in the cancer cells (Fig. 1 and Supplementary Fig.1). This expression was also observed in mice predisposed to develop lung cancers (Kras<sup>+G12D</sup>) as well as in lung cancer cells grafted subcutaneously (Fig. 1). These results are in line with the transcriptomic analysis data obtained by others (Herbert 2013). Interestingly, although Notch3 has been shown to be involved in different pathological settings affecting the vasculature, its role in tumour vasculature has never been addressed. Here, we showed that Notch3 behaves as a novel dependence receptor, regulating tumour angiogenesis. As for the other dependence receptors, this new function is independent on the canonical Notch signalling pathway. Indeed, activation or inhibition of the canonical Notch signalling by expression of a dominant version of Mastermind-like or a constitutive active CBF-1 do not induce cell death as does Notch3. Furthermore, mutating the residues necessary for the interaction between N3ICD and CBF-1 did not abrogate the ability of Notch3 to induce cell death. Interestingly, we showed here that Notch3 was the only receptor of the Notch family to present this function. This is also described for other dependence receptors: for example, TrkA and TrkC behave as dependence receptors whereas TrkB does not (Nikoletopoulou et al., 2010; Tauszig-Delamasure et al., 2007). Interestingly, Notch3 has been shown to arise from the second duplication of Notch1 (Theodosiou et al., 2009). As hypothesized for other dependence receptors, the dependence receptor function of Notch3 is thus probably a late acquisition during evolution. In line with this, the caspase

cleavage site, present only in Notch3, has likely appeared during Notch3 differentiation after duplication from Notch1. Notch4 has been proposed to derive from Notch3 (Kortschak et al., 2001), however, this has been questioned more recently (Theodosiou et al., 2009) and due to its rapid evolution, it is not clear from which Notch gene it actually derives.

This new function appears in a context in which Notch3 is aberrantly expressed in the pathological tumour vascularisation where Notch3 limits tumour angiogenesis through an unexpected pro-apoptotic activity. Of note, tumour associated endothelial cells have been described to have an aberrant expression of DR5 which render them more susceptible to apoptosis induced by TRAIL (Wilson et al., 2012). It would be of interest to study whether this function is conserved in other pathological situations where Notch3 is aberrantly expressed in non-endothelial cells, for example in cancer cells in which Notch3 and its ligands have been shown to be expressed.

We also observed that Notch3 was, at least in part, responsible for the anti-angiogenic effect of  $\gamma$ -secretase inhibitors described by others (Cook et al., 2012). Indeed DAPT treatment induced a reduced vascularisation associated with a reduced tumour growth. Importantly, this effect of DAPT is not because of inhibition of the canonical Notch signalling pathway as the effect of DAPT can be reversed by deletion of Notch3. If the effect of DAPT was a consequence of inhibition of Notch signalling, Notch3 deletion should either not have any effect or exasperate the effect of DAPT). Furthermore, inhibition of the canonical Notch pathway, would lead to a hypersprouting of endothelial cells as observed upon anti-Dll4 treatment which could be associated with decreased growth but not decrease vascularization. In contrast, Notch3-induced apoptosis in tumour-associated endothelial cells following DAPT treatment could explain at least partly the anti-angiogenic effect followed by tumour growth inhibition.

In *Notch3* mutant setting, DAPT cannot trigger Notch3-induced apoptosis and thus angiogenic effect. It may thus be of interest to take this unexpected function of Notch3 into account when evaluating the anti-tumour efficacy of  $\gamma$ -secretase inhibitors. This new function of Notch3 is not in contradiction with the well-described oncogenic canonical Notch3 signalling in epithelial cells (Hu et al., 2006; Zheng et al., 2013). In fact, as other dependence receptor, the availability of ligands would impact on the role of Notch3. We showed here that Jag-1 expression by cancer cells is important to limit the dependence receptor function of Notch3. Furthermore, the function we describe here in tumour angiogenesis could account for some paradoxical observations regarding Notch3. In fact, while it may play a role in the epithelial tumour cells as an oncogene through its canonical signalling, it may also represent a constraint for tumour progression by acting as a cellular sentinel for endothelial cell death. The Notch3 receptor may therefore act as a regulator of tumour angiogenesis depending on the context such as the heterotypic interactions between the tumour and the stroma or the availability of the ligands in the tumours. Jag-1 has been shown to be very important in signalling from the endothelium to the cancer cells (Cao et al., 2014; Lu et al., 2013). Together with the present data, it shows how reciprocal interactions between the tumour vasculature and the tumour are important. The data presented here also raises the question of targeting Notch to regulate tumour angiogenesis. We propose that targeting Jag-1 in tumour angiogenesis might therefore be an original approach and targeting more specifically the Notch3-Jag-1 interaction could be advantageous allowing targeting of both the canonical Notch signalling in epithelial cells and Notch3-induced apoptosis in endothelial cells.

## EXPERIMENTAL PROCEDURES

### Mice experiments

*Notch3* mutant mice have been characterized previously (Arboleda-Velasquez et al., 2008). Mice were constantly bred into C57Bl/6 mice and experiments have been conducted in agreement with the local ethic comity (CECCAPP, Comité d'Evaluation Commun au PBES, à AniCan, au laboratoire P4, à l'animalerie de transit de l'ENS, à l'animalerie de l'IGFL, au PRECI, à l'animalerie du Cours Albert Thomas, au CARRTEL INRA Thonon-les-Bains et à l'animalerie de transit de l'IBCP). LLC1 cells were purchased from ATCC and were tested for mycoplasmas and murine viruses (Murine essential panel, Charles River) before being implanted in mice. For sub-cutaneous engraftment,  $5 \times 10^5$  LLC1 cells were implanted into the left flank of wild-type C57Bl/6 mice or *Notch3*<sup>LacZ/LacZ</sup> C57Bl/6 littermate. Standard variation was established in control experiment before establishing groups of 4-12 animals with homogenous tumour size were selected to obtain equal variance between genotype. No randomization method was applied. Tumour size was measured every day from day 10 when the tumours are palpable until day 14 or 21 by two different persons for each measure without knowing the genotype of animals. Animal showing prostration or obvious sign of suffering were excluded. Sub-cutaneous engraftment with E0771 cells was performed as described previously. The measurements of tumours were begun from day 14 to day 25. When the measures were too different, the point could be excluded. Measurement of the tumours was carried out without knowing the genotype of the animals. Mice were sacrificed before the end of the experiment if necessary according to animal care guidelines. For DAPT treatment, DAPT was diluted in Corn Oil/Ethanol (9/1) at 1mg/ml. 10 $\mu$ l/g was injected intraperitoneally to reach a 10mg/kg concentration. Experiments were all conducted on male and female littermate of 4-7

weeks of age. Animals were treated according to their identification number (even = untreated; odd=treated, this was arbitrary chosen for each experiment). Tumour dissection, fixation, and immunochemistry analysis were performed simultaneously.

### **Cell Culture and cell transfection**

Human umbilical vein endothelial cells (HUVEC) were obtained from Promocell (Heidelberg, Germany) and maintained in Endothelial Cell Growth Medium 2, supplemented with Endothelial Cell Growth Medium 2 Supplement Mix and 1% penicillin/streptomycin. H358 and LLC-1 were obtained from the ATCC maintained in RPMI Medium 1640 (1x)+ GlutaMAX™-I, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) and in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% PS, respectively. E0771 cells were obtained from our lab and culture in DMEM as described previously.

For electroporation,  $1 \times 10^6$  HUVEC cells were harvested by trypsinization and electroporated either with 10nM siRNA (si Notch3, Sigma SASI\_Hs01\_00101286, Sigma SASI\_hs01\_00100441, si negative control Sigma #SIC001) or 5µg DNA plasmids with Neon kit (Invitrogen). 24 hours later, transfection efficiency was verified by RT quantitative PCR. LLC-1 or H358 cells were seeded at  $0.25 \times 10^6$  cells in 6 wells-plates one day before transfection. Transfections were performed with lipofectamine TM reagent (Invitrogen) following the manufacturer's instructions.

For caspase inhibitors treatment, HUVEC cells were pre-incubated 2 hours with 5µM caspase inhibitors (BioVision, Caspase-9 Inhibitor Z-LEHD-FMK, MerckMillipore, Z-VAD-FMK ) or DMSO for 2 hours. Cells were then transfected with empty vector or Notch3 and incubated for 24h with 5µM caspase inhibitors or DMSO.

### **Endothelial cells purification**

Lung from 16 weeks-old KrasG12D mice were dissected and tumour nodules extracted under a binocular before being digested in 1mg/ml collagenase Type 1 (Invitrogen) for 1 hour. Cell suspension was then incubated with magnetic beads (Dynabeads® Sheep Anti-Rat IgG, Invitrogen) incubated overnight with CD31 antibody (clone MEC13.3, Pharmingen).

### **β-galactosidase staining**

After dissection, organs from *Notch3<sup>LacZ/+</sup>* mice were fixed for 20 min before being washed three times in 0.2% NP-40, 0.01% NaDOC, 2mM MgCl<sub>2</sub> in PBS. Organs were then incubated for 1 hour in 25mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 25mM K<sub>4</sub>Fe(CN)<sub>6</sub> Wash Buffer. X-gal reaction was then performed in 25mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 25mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 1mg/ml Xgal in Wash Buffer at 37°C.

### **Co-culture experiments**

HUVEC were incubated with a CellTracker™ Green CMFDA at 1.25µg/ml (Molecular probes, Life technologies, C7025) for 30 minutes. Afterwards, cells were washed two times with PBS. 60µL of Basement Membrane Matrix (Matrigel, BD Bioscience) was added to a 96-wells plate, followed by 30 minutes incubation at 37°C. HUVEC were harvested by trypsinization and 15x10<sup>3</sup> HUVEC were added into each Matrigel coated well and incubated at 37°C for two hours. LLC1 or H358 transfected one day before were then added to wells containing HUVEC. Each condition was carried out in triplicate. After 9h of co-culture, cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature and rinsed 3 times with PBS for 10 minutes at room temperature. Fixed cell culture plates were stocked at 4°C.

### **Immunofluorescence staining**

Immunofluorescence analysis was performed on tumours obtained from littermates. Fixation and staining were performed simultaneously. Paraffin embedded tissue samples were deparaffinized and heat-induced antigen retrieval was performed. Cells or tissue samples fixed with 4% PFA were permeabilized with PBS-0.2% Triton X-100 (TX-100) for 30 minutes at room temperature. Samples were then washed 3 times with PBS for 5 minutes. Samples were blocked in PBS with 4% bovine serum albumin (BSA), 2% normal donkey serum and 0.2% TX-100 for one hour. Samples were then washed 3 times with PBS for 5 minutes. Primary antibodies were diluted in the blocking solution: 1:500 dilution for anti-cleaved caspase 3 (Cell signaling Asp175 5A1E Rabbit mAb), 1:100 dilution for anti-CD31 (Abcam, anti-CD31 ab28364), 1:100 dilution for anti-collagen IV (Abcam anti-collagen IV ab19808). Alexa-conjugated secondary antibodies (Alexa555-donkey anti rabbit, Alexa488-donkey anti rabbit) were used at 1:1000 dilution. DAPI (0.5µg/ml) was added at the end to stain nuclei. Images were acquired with Zeiss Axio fluorescence microscopy, NIS element AR 4.20.01 Nikon fluorescence microscopy and confocal microscopy.

### **Proximity ligation assay**

10<sup>5</sup> HEK293T cells (stably selected to carry a Doxycycline-inducible plasmid for Notch3 or N3ICD) were seeded in lab-tek chamber (Thermo scientific, 4-well, 177399). After 24 hours, cells were treated with 1µg/ml Doxycycline. After 24 hours induction, cells were fixed with 4% PFA and PLA assay (Sigma, Duolink® In Situ PLA) was performed according to the manufacturer's instructions. Anti-HA (sigma, H6908) and anti-caspase 9 (Santa cruz, sc-73548) were used for primary antibodies.

### **Cell death assay**

TUNEL assay: Detection of DNA fragmentation, a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed by following the protocol of the TUNEL assay kit (Roche). Briefly, fixed cells or tissue samples were permeabilized with 0.2% TX-100 in PBS (30 minutes at room temperature), washed with PBS, incubated with 300U/ml TUNEL enzyme and 6 $\mu$ mol/L biotinylated deoxyuridine triphosphate (Roche Diagnostics, Mannheim, Germany). The extremities of the biotin coupled DNA were revealed by using Cy-3-coupled streptavidin (1:1000 in PBS, Jackson ImmunoResearch). The slides were washed with PBS, DAPI- stained, then washed with PBS and finally, mounted with Fluoromount G (SouthernBiothec). Images were acquired with Zeiss Axiovision fluorescence microscopy and NIS element AR 4.20.01 Nikon fluorescence microscopy. Caspase 3 activity assay: Cells were first harvested by scraping. Cell pellets were obtained by centrifugation at 4°C and lysed. The caspase 3 activity assay was performed according to the manufacturer's instructions (Biovision caspase-3 colorimetric assay kit). Total protein concentrations were measured with the BCA assay kit using BSA as a standard (Pierce Biotechnology, Rockford, IL, USA). Absorbance readings were done on a TECAN infinite F500.

### **Immunohistochemistry analysis**

Immunohistochemistry was performed on 4- $\mu$ m-thick sections of formalin-fixed, paraffin-embedded and heat-treated (for antigen retrieval) tissues (DakoCytomation). Sections were stained with hematoxylin-eosin-safran and tumour endothelium was stained with an anti-CD31 antibody (1:50 Abcam, ab28364). Diaminobenzidine was used as chromogen. Images were acquired with a Zeiss Axiovert. The whole slide was

scanned automatically with the Histolab 6.2.0 MICROVISION Instrument system. Necrotic and CD31 positive areas were quantified by Histolab 6.2.0 or ImageJ angiogenesis plugin. Staining of human sample was performed with the cell signaling anti-Notch3 antibody (D11B8).

### **Co-culture images analysis**

Images of co-culture experiment were acquired with Zeiss Axiovision fluorescence microscopy. 8-12 images were acquired for each well at 5X magnification. Images were analyzed by Image J angiogenesis plugin (Gilles Carpentier, Faculté des Sciences et Technologie, Université Paris Est Creteil Val-de-Marne, France). Briefly, channels were split automatically. On the GFP channel, total tube length, branches number and total length of branches were acquired by Analysis HUVEC Fluo program. Each condition was carried out in triplicate.

### **Tumour section CD31 fluorescent images Analysis**

Whole slides were scanned to acquired total images at 10x magnification with a Zeiss Axiovision fluoresces microscopy. CD31 expression areas were analysis by Image J. Briefly, The image was converted into RGB stack format. CD31 staining was quantified by choosing threshold program and adjusting the threshold parameters. Once the threshold parameter was adjusted, it was always the same for each image and CD31 expression areas were measured automatically. TUNEL positive and CD31 positive cells were counted manually. For blinded quantification, images were organized in folder identified by letters by one person and quantified by another.

### **Western Blot**

Cells were lysed in SDS buffer (2% SDS, 150mM NaCl, 50mM Tris-HCl, pH 7.4). Cell extract was next centrifuged at 2,500g for 5 minutes. Protein concentration was measured with the BCA assay kit using BSA (?) as a standard (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instructions. The following antibodies were used: anti-Notch3 (1:1000 dilution, Cell Signaling #2889), anti-Jagged1 (1:1000 dilution, Santa Cruz C-20 sc-6011), anti-cleaved caspase 3 (1:1000 dilution, Cell signaling Asp175 5A1E Rabbit mAb), anti-CBF-1 (1:1000 dilution, Cell signaling #5313P), anti-GFP (1:2000 dilution, Molecular probes A11122), anti-Myc (1:2000 dilution, sigma, M5546) and anti-HA (1:5000 dilution, Sigma H4908).

### **Flow cytometry analysis of Sub-G1 and Annexin V staining**

For the Sub-G1 experiment, cells were harvested cells by trypsinization and counted. Cells were first washed once with PBS followed by the addition of cold 70% ethanol, vortexed, and then resuspended at 4°C for 30 minutes. Samples were stocked at -20°C. Ethanol was removed and the pellet washed with PBS. Staining solution (40µg/ml propidium iodide, 2mg/ml RNase in PBS) was added.

For the Annexin V experiments, cells were harvested by trypsinization and counted. Afterwards, 100µl of a  $1 \times 10^6$  cells solution was incubated with 5µl Annexin V allophycocyanin conjugated (Life technologies, A35110) and 2µl Propidium Iodide (Life technologies, V13242, for 15 minutes at room temperature.

For the CD31/CD105 staining, cells were detached in PBS/EDTA (5mM) and  $10^6$  cells were re-suspended for 20 min on ice in 100µl PBS with anti-mouse-CD31-FITC (Ebioscience) and Anti-CD105 antibody [MJ7/18] (Phycoerythrin) (Abcam) before analysing with the flow cytometer. Data acquisition and analysis were performed on a FACSCalibur using CellQuestPro software (BD Bioscience, San Jose, USA).

## **Statistical analysis**

For tumour growth analysis, a two-way ANOVA was realized to test for effect of time and treatment. For analysis of *in vitro* experiments, a normality test was realized when number of samples was sufficient (Shapiro-Wilk or KS Normality test). Similarity of variance was tested before application of any statistical test using graphpad. If samples followed a Gaussian distribution, a t-test was applied, either paired-ratio or unpaired depending on the experimental data. When samples did not pass the normality test, non-parametric test was applied (Mann-Whitney for unpaired samples and Wilcoxon signed-rank test for paired samples). \* :  $p < 0,05$ ; \*\* :  $p < 0,01$ ; \*\*\* :  $p < 0,001$ .

## **Quantitative RT PCR**

mRNA were extracted with the NucleoSpin RNA kit (Machery-Nagel) according to manufacturer's instructions. cDNA were generated with the iScript cDNA Synthesis kit (BIO-RAD) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed using a LightCycler 480 (Roche Applied Science) and the FastStart TaqMan<sup>®</sup> Probe Master Mix (Roche Applied Science).

Primers and taqman probes used were the following:

|          | Forward primer         | Reverse primer        | Taqman probes |
|----------|------------------------|-----------------------|---------------|
|          | Mouse primers          |                       |               |
| HPRT     | tcctcctcagaccgcttt     | cctggttcatcatcgctaac  | 95            |
| CD 31    | gctgggtgctctatgcaagc   | atggatgctgttgatgggga  | 64            |
| DII-4    | aggtgccacttcggttacac   | gggagagcaaattggctgata | 106           |
| SMA      | ctctctccagccatctttcat  | tataggtggtttcgtggatgc | 58            |
| VEGFR 2  | cagtggactggcagctagaa   | acaagcatacgggcttggtt  | 68            |
| VEGF A   | ttaaacgaacgtactgcaga   | agaggtctggtcccga      | 4             |
|          | Human primers          |                       |               |
| HPRT     | tgacactggcaaaacaatgca  | gctcctttcaccagcaagct  | 73            |
| Jagged-1 | caggacctggtaacggattt   | gcctcacattgcatc       | 48            |
| Notch3   | gccaagcggctaaaggta     | cactgacggcaatccaca    | 30            |
| CD31     | gcaacacagtccagatagtcgt | gacctcaaactgggcatcat  | 14            |

## **Immunoprecipitation**

5.10<sup>6</sup> HEK293T cells (stably selected to carry a Doxycycline-inducible plasmid for Notch3 or N3ICD) were treated with 1µg/ml Doxycycline for 24 hours. Cells were harvested and lysed in lysis buffer (HEPES 50mM, NaCl 150mM, EDTA 5mM, NP40 0.1%, PH7.6) for one hour at 4°C and then sonicated. 1ml of lysate was then incubated with 10µl Anti-HA (sigma, H6908-.5ML) over night at 4°C. 100µl of protein A sepharose beads (sigma, P3391-1G) were added into the lysate and incubated at 4°C for one hour. Beads were then washed for 3 times with lysis buffer at 4°C. Beads were harvested and incubated with caspase 9 assay kit (Promega, Caspase-Glo® 9 Assay) for 30 minutes. Luminescence was measured by TECAN infinite F500.

## **Data availability**

All raw data corresponding to *in vivo* or *in vitro* data, are available from the authors.

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## FIGURES LEGENDS

### **Figure 1: Notch3 is aberrantly expressed in tumour endothelial cells**

**a**, Notch3 immunohistochemistry on peritumoural and tumour part of three sections from non-small cell lung cancers patients. **b**, Quantitative RT PCR was performed to measure Notch3 mRNA expression in endothelial cell enriched fraction (EC, CD31-expressing purified cell population) or non-endothelial cell (NEC) purified from lung dissected from wild type mice, the healthy part of tumour bearing lung dissected from Kras mice or from the nodules dissected from the lung of Kras mice (n=6 WT lungs, n=5 Kras lungs, mean +/- SEM, ordinary one-way ANOVA, multiple comparisons). **c**, **d**,  $\beta$ -galactosidase staining was performed on lungs or LLC1 tumour whole mount from Kras<sup>G12D/+</sup> (c) or WT mice (d) mice before inclusion in paraffin and immunohistochemistry staining for ERG, CD31, SMA, NG2 as indicated. **e**, Quantitative RT PCR was performed to measure Notch3 mRNA expression in endothelial cell FACS-sorted from lung or tumour dissected from a VE-Cadherin-Tomato mice (n=3 tumours, mean+/-SEM, unpaired t-test). **f**, HUVEC cells were co-cultured for 48 hours with LLC1 cells stably expressing GFP before being FACS sorted. DAPI (alive cells) GFP negative (HUVEC) cells were used to prepare mRNA and Notch3 expression was measured by quantitative RT-PCR (n= 3 independent experiments, paired ratio t-test).

**Figure 2: Notch3 limits tumour growth and vascularization *in vivo*.** **a**,  $5 \times 10^5$  LLC1 cells were implanted into the left flank of wild-type C57Bl/6 mice (N3<sup>+/+</sup>, n=4) or of Notch3 LacZ homozygous Knock-in C57Bl/6 littermates (N3<sup>LacZ/LacZ</sup>, n=4). Tumour growth was monitored from day 16 until day 24 when mice were sacrificed. Two-Way ANOVA was performed to assess Time and Genotype effect on tumour growth (Interaction: p=0,013; Time: p<0,0001; Genotype: p=0,0015). **b**, mRNA was extracted

from tumours dissected after 14 days of growth from wild-type C57Bl/6 mice ( $N3^{+/+}$ ,  $n=6$ ) or *Notch3* mutant mice  $N3^{LacZ/LacZ}$  C57Bl/6 littermates ( $N3^{LacZ/LacZ}$ ,  $n=5$ ). Quantitative RT-PCR was performed to measure CD31, DLL4, PDGFR $\beta$ ,  $\alpha$ -SMA, VEGFR2, VEGFA, CD11b and CD45 expression (means  $\pm$  SD, unpaired t-test was applied). **c**, Immunohistochemistry for CD31 and  $\alpha$ -SMA was performed on tumours dissected from wild-type mice ( $N3^{+/+}$ ,  $n=15$ ) or *Notch3* mutant ( $Notch3^{LacZ/LacZ}$ ) mice littermates ( $n=9$ ) on day 14. Images are representative of four different sections from each tumour. Quantification was done using ImageJ angiogenesis plug-in on four different images from each tumour. (mean  $\pm$  SEM for quantification, unpaired t-test).

**Figure 3: Notch3 induces endothelial cell death *in vitro*.** **a**, Sub-G1 analysis of HUVEC electroporated with a control plasmid (Control), or a plasmid expressing the full-length version of Notch3 for 24h. Quantification was made for three independent experiments and a paired-ratio t-test was applied. **b**, AnnexinV/Propidium iodide was performed on HUVEC cells electroporated with a control plasmid or a plasmid expressing Notch3. Quantification was made on three independent experiments and a paired ratio student t-test was applied. **c**, Sub-G1 quantification was made after electroporation of HUVEC cells with a control plasmid or a plasmid expressing Notch3 after 48 hours of treatment with DMSO or z-LEHD-fmk or z-VAD-fmk pan-caspase inhibitors. Quantification was made on three independent experiments and a paired ratio student t-test was applied. **d**, HUVEC were electroporated with a control siRNA (control) or a siRNA targeting caspase-9 (C9). 48 hours later, cells were electroporated with a control plasmid or a plasmid expressing Notch3 and 24 hours later, Sub-G1 analysis was made. Quantification was made on three independent experiments and a paired ratio student t-test was applied. **e**, Immunoprecipitation of Myc-tagged S1-

Cter Notch1 (S1-CterN1), S1-Cter Notch2 (S1-CterN2) and S1-Cter Notch3 (S1-CterN3) constructs in HEK293t cells together with a control plasmid (-) or a plasmid expressing an HA-tagged dominant negative version of caspase-9 (C9). **f**, Lysates from HEK293t cells carrying Doxycycline (DOX)-inducible HA-tagged Notch3 (piN3) or DOX-inducible HA-tagged N3ICD (piN3ICD) plasmids were immunoprecipitated for endogenous caspase-9 and western blot were done to analyse Notch3 (HA) or caspase-9 in total lysates or IP. **g**, Proximity Ligation Assay was performed for endogenous caspase-9 or endogenous caspase-8 and doxycycline-induced Notch3 in HEK293 cells. Quantification was made on 5 images containing 100-150 cells each. **h**, Caspase-9 activity was measured from Notch3 immunoprecipitated lysates from HEK293t cells carrying inducible Notch3 (piN3) or inducible N3ICD (piN3ICD) plasmids.

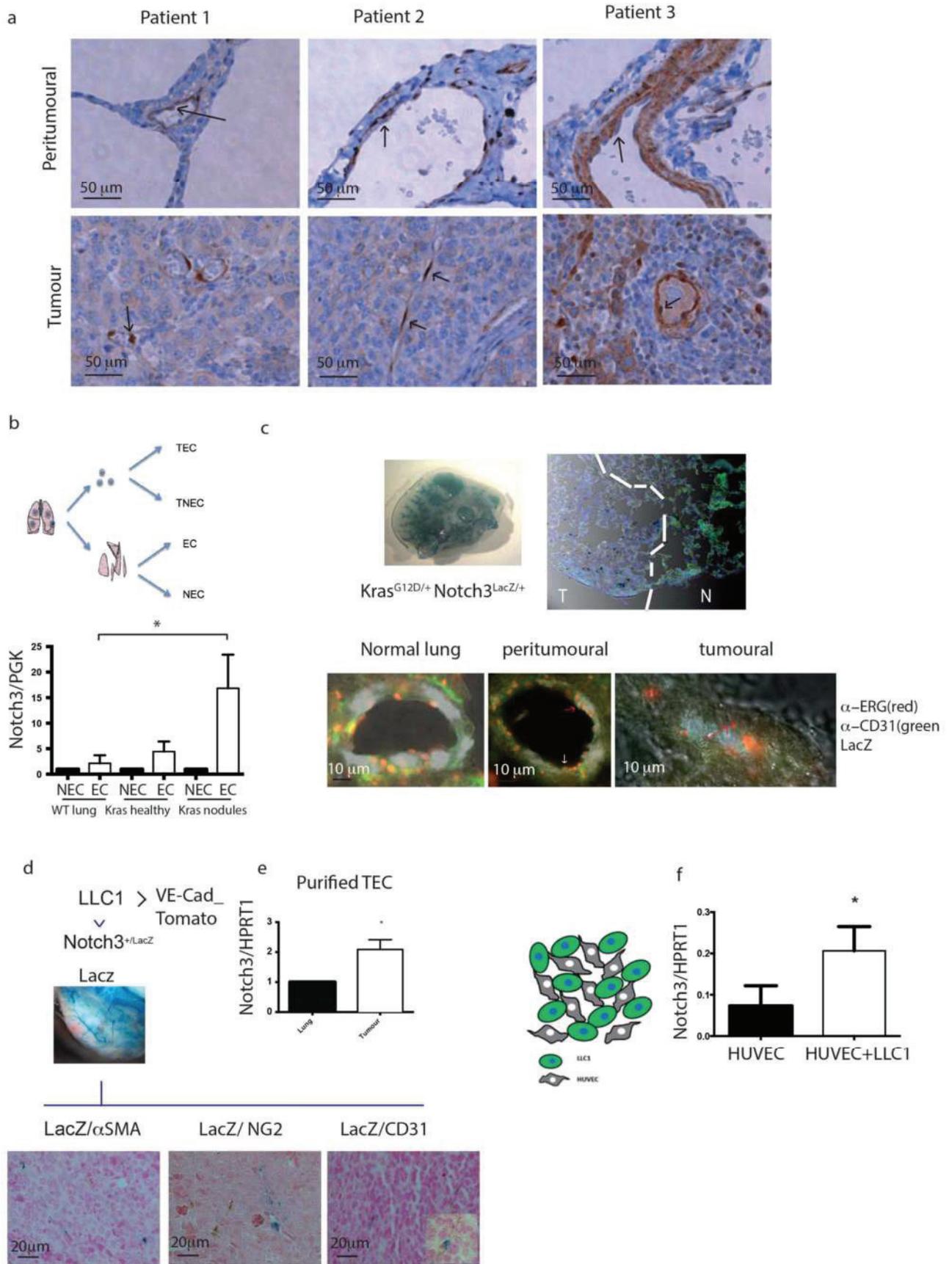
**Figure 4: Jag-1 rescues Notch3-induced endothelial cell death.** **a**, HUVEC were stained with the cellTRacker green CMFDA. Cleaved-caspase-3 staining of HUVEC co-cultured with 1) LLC1 expressing or not Jag-1 (LLC1-Jag-1 and LLC1-Control, respectively) or 2) H358 cells transfected with a siRNA control (sicontrol) or a siRNA targeting Jag-1 (siJag-1). Co-cultures were maintained in matrigel for 9 hours before being fixed and stained. Images are representative of three independent experiments, each performed in triplicate. **b**, Quantification of HUVEC networks presented in **a** (n=3, means +/- SD, t-test was applied). Jag-1 expression was verified on western blot before the cells were added to HUVEC in Matrigel. **c**, Immunohistochemistry staining and quantification of CD31 on tumour section from tumours obtained from LLC1 cells or LLC1 cells overexpressing Jag-1 (n=5 tumours, quantification on 4 images/tumours, unpaired t-test). **d**, Co-culture of HUVEC electroporated (Notch3) or not (Control) with Notch3 and LLC1 cells transfected (LLC1-Jag-1) or not (LLC1 control) with Jag-1 were

stained with Annexin V APC and studied by flow cytometry. HUVEC were gated (M1) according to FL1 staining (cellTracker green CMFDA), and Annexin V (FL4) positive cells were quantified among HUVEC. Number of Annexin V positive HUVEC cells is specified in each condition. **e**, Tumour/Normal tissue ratio of Jag-1 mRNA in patients with non-small-cell lung adenocarcinomas from the GSE7670 dataset and Correlation between Jag-1 and Pecam-1 expression in patients overexpressing Jag-1 in the GSE7670 dataset.

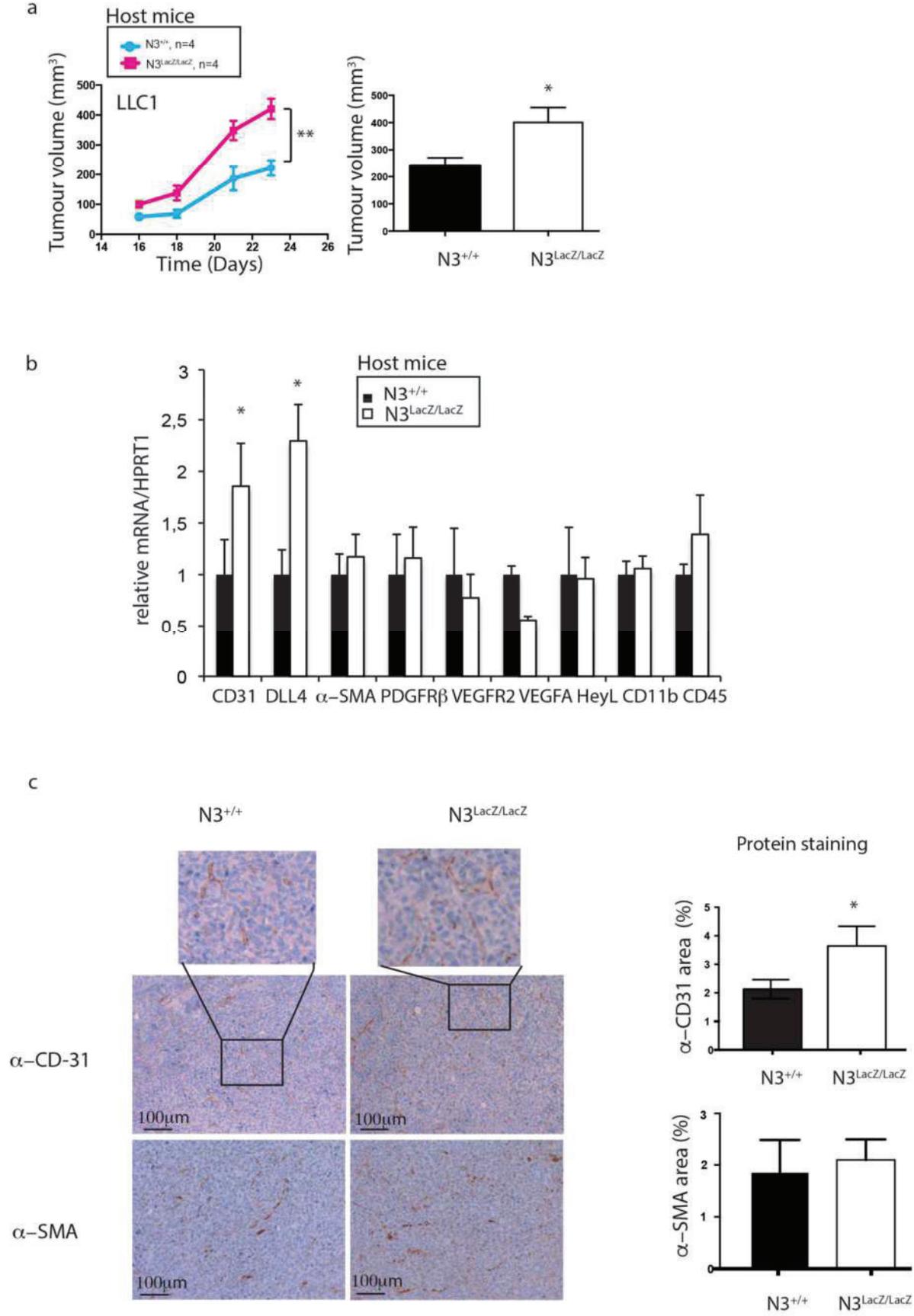
**Figure 5: Notch3 is required for  $\gamma$ -secretase-induced tumour regression.** **a**, Caspase-3 activity determined in HUVEC treated with DAPT (4 $\mu$ M) for 48h (n=7, means +/- SD, paired t-test was applied). **b**, Caspase-3 activity in HUVEC electroporated with siRNA control (sicontrol) or a siRNA targeting *Notch3* (siNotch3) and treated (+) or not (-) with DAPT (4 $\mu$ M) (n=7, mean +/- SD, paired t-test was applied). Effect of DAPT treatment on Notch3 cleavage was monitored by western blot. **c**, Caspase-3 activity was determined in lysates from purified tumour-associated endothelial cells from tumours dissected from *Kras*<sup>G12D/+</sup>; *Notch3*<sup>+/+</sup>(WT) or *Kras*<sup>G12D/+</sup>; *Notch3*<sup>LacZ/LacZ</sup> (*Notch3*<sup>LacZ/LacZ</sup>) mice and treated *in vitro* or not with 4  $\mu$ M of DAPT. **d**, 5x10<sup>5</sup> LLC1 cells were implanted into the left flank of wild-type C57Bl/6 mice or of *Notch3* knock-out C57Bl/6 littermates, and injected intraperitoneally with 10 $\mu$ l/g of ethanol-corn oil (1/9) (*N3*<sup>+/+</sup> OIL, n=10, *N3*<sup>LacZ/LacZ</sup> OIL, n=4) or 10 $\mu$ l/g of 1mg/ml DAPT diluted in ethanol-corn oil (1/9) (*N3*<sup>+/+</sup> DAPT, n=8 ; *N3*<sup>LacZ/LacZ</sup> DAPT, n=4) on days 12,13,14 and 15. Mice were sacrificed on day 16 (two way ANOVA was performed to test for significance). Necrosis area was quantified automatically on whole sections with HistoLab software settings parameters on hematoxylin staining

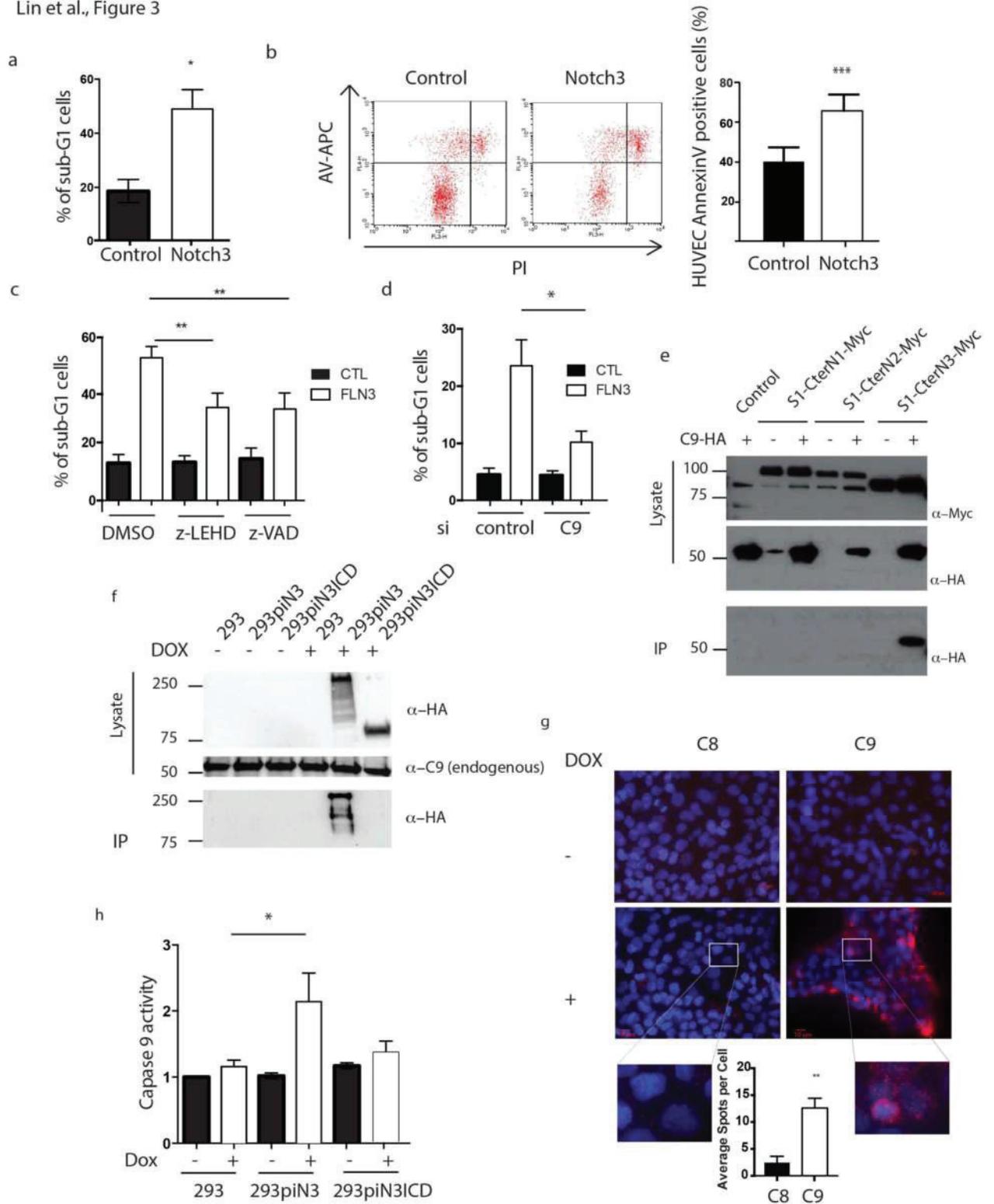
intensity. **e**, Immunofluorescence and TUNEL staining were performed on LLC1 tumour sections from wild-type mice or *Notch3* knock-out littermates treated (DAPT, *N3<sup>+/+</sup>* DAPT, n=5 ; *N3<sup>LacZ/LacZ</sup>* DAPT, n= 3) or not (OIL, *N3<sup>+/+</sup>* OIL, n=5, ; *N3<sup>LacZ/LacZ</sup>*, n=3) with DAPT as previously described. For each tumour, the entire section was imaged (8-12 images per tumour), and the number of CD31 positive TUNEL positive cells was quantified. Number of TUNEL positive cell was then normalized on CD31 area that was quantified on each image using the ImageJ angiogenesis plugin (mean +/- SEM, unpaired t-test).

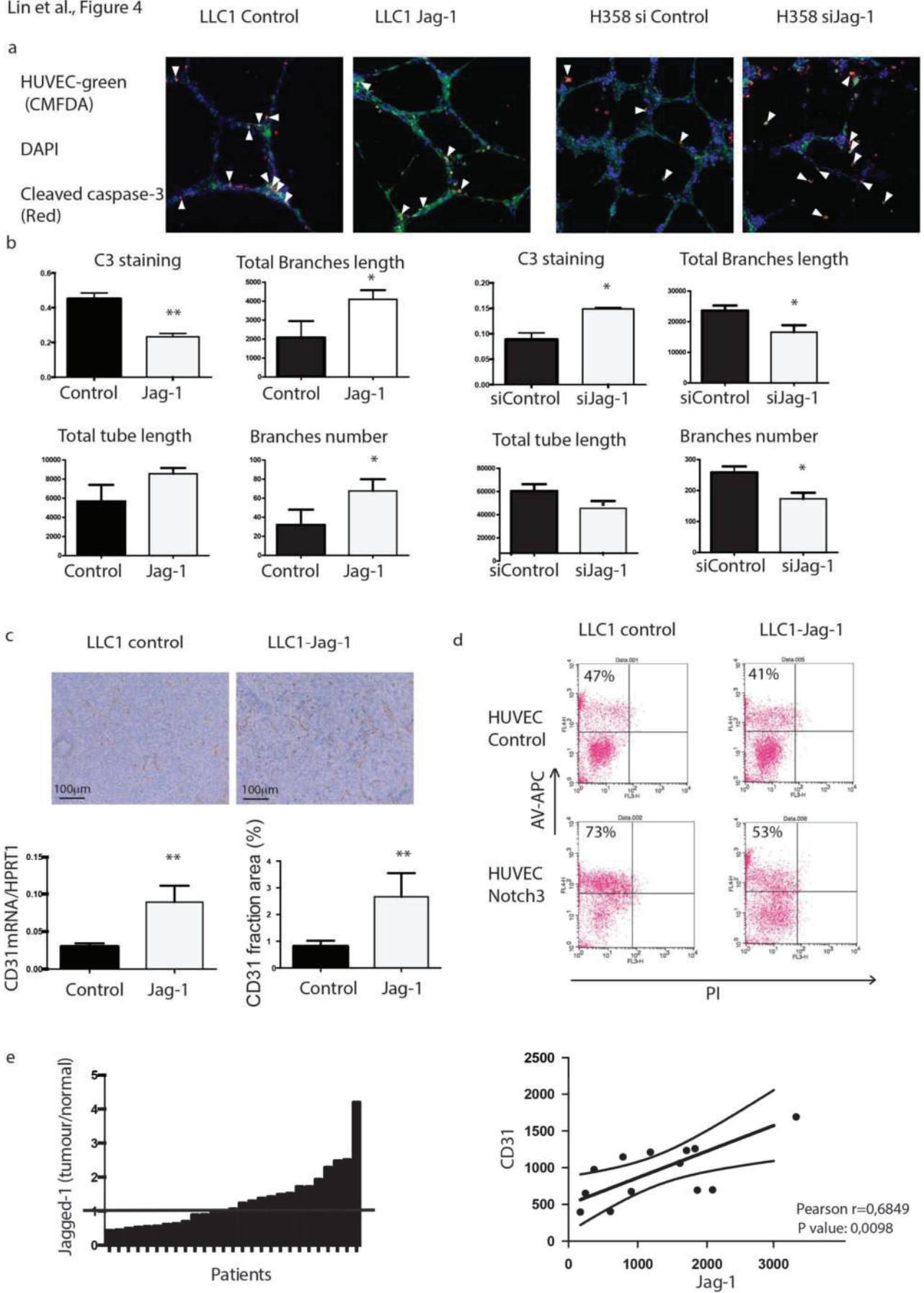
Lin et al., Figure 1



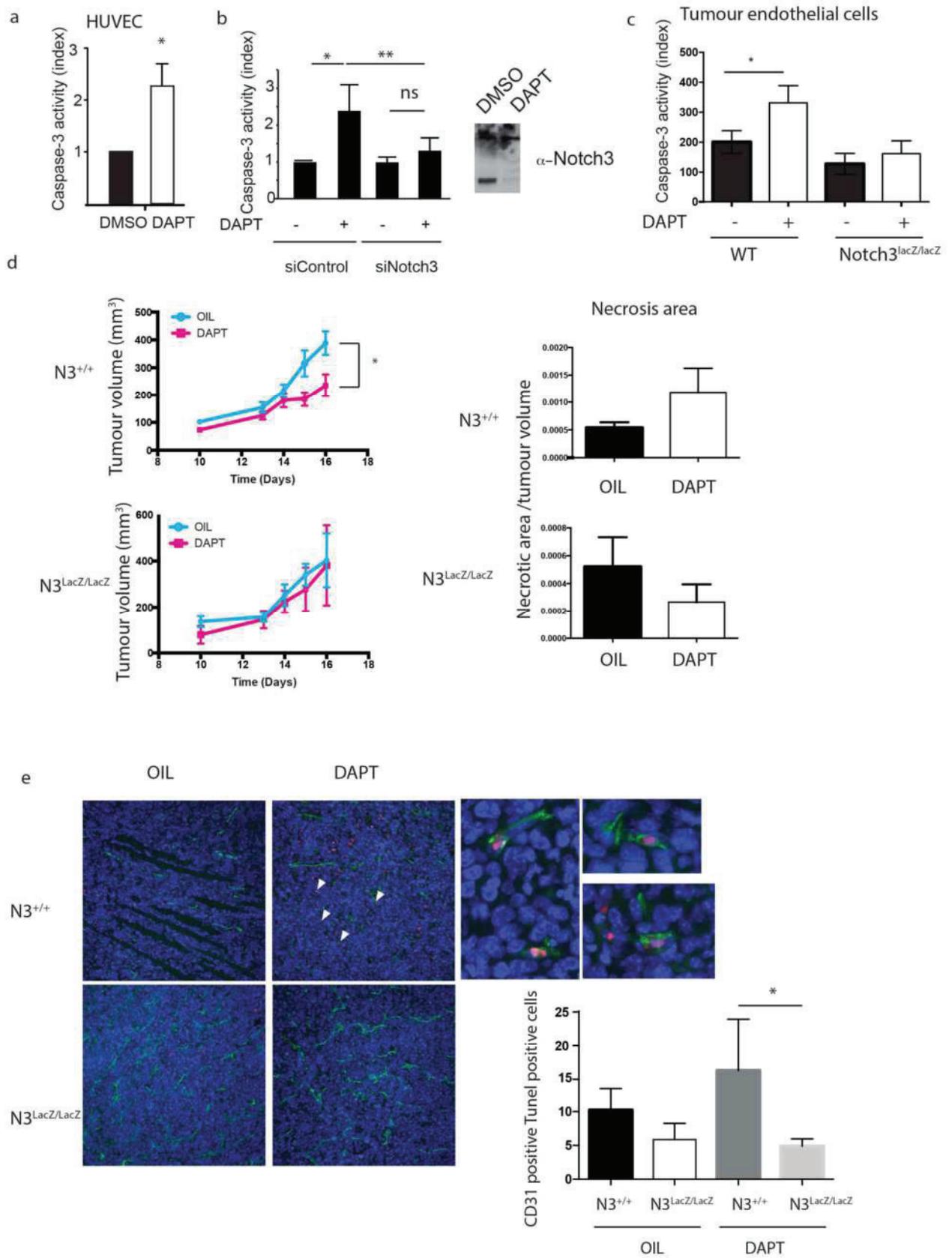
Lin et al., Figure 2







Lin et al., Figure 5

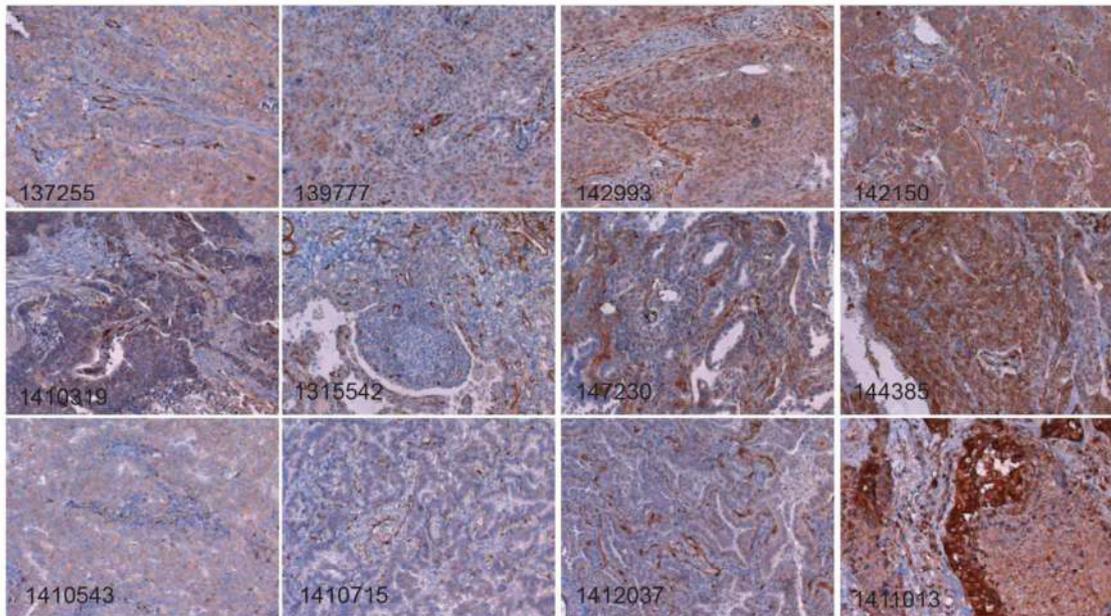


# **Non-canonical NOTCH3 signalling limits tumour angiogenesis**

Shuheng Lin et al.

**SUPPLEMENTARY FIGURES 1-6**

a



b

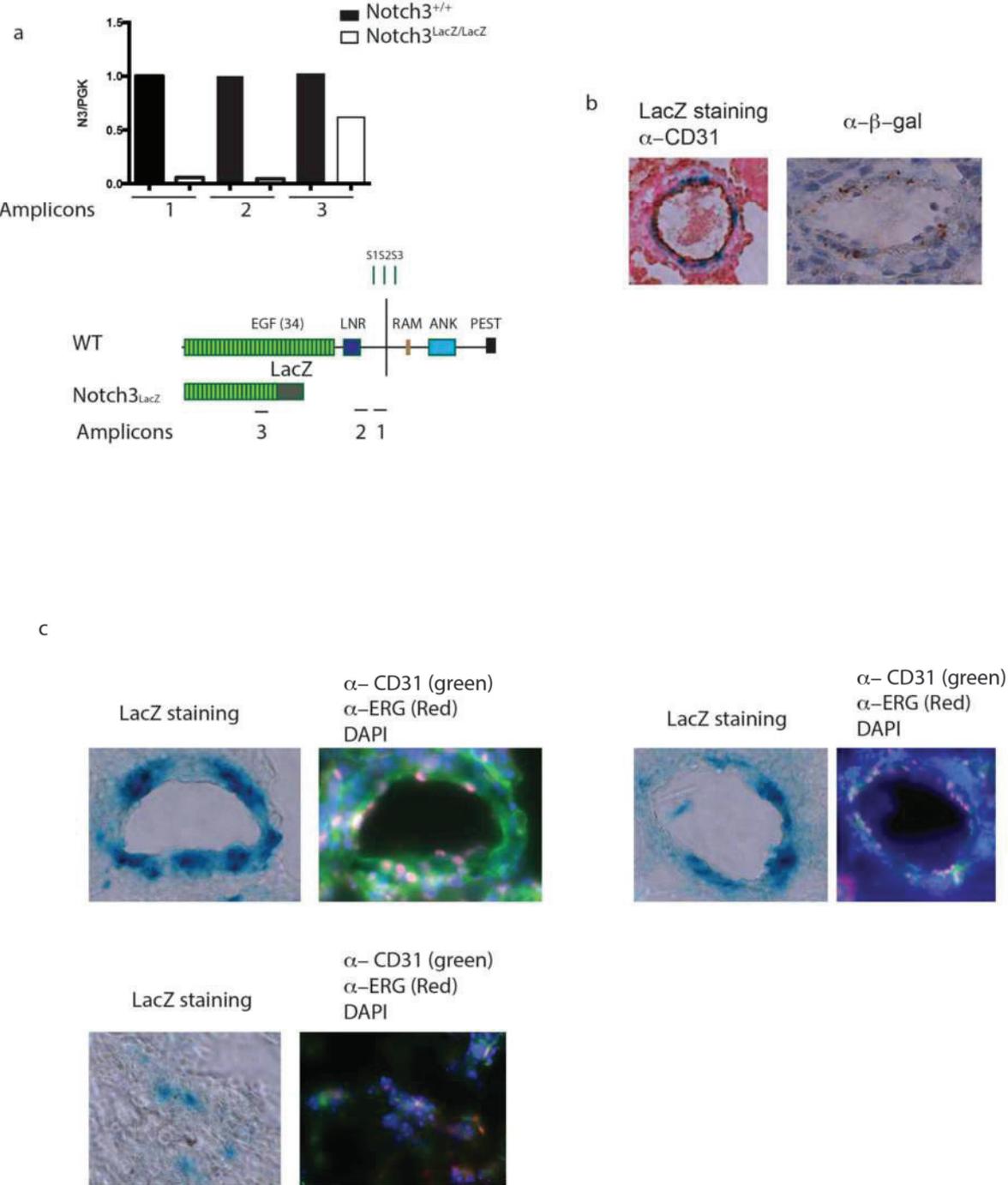
| Histology | Tumor tissue |           |           |           |          |           | Vasculature |
|-----------|--------------|-----------|-----------|-----------|----------|-----------|-------------|
|           | Nucleus      |           | Cytoplasm |           | Membrane |           |             |
|           | %            | Intensity | %         | Intensity | %        | Intensity |             |
| SCC       | 0            | 0         | 50        | ++        | 40       | ++        | +++         |
| SCC       | 50           | 0         | 70        | +         | 70       | +         | +++         |
| SCC       | 0            | 0         | 90        | ++        | 30       | ++        | +++         |
| SCC       | 0            | 0         | 60        | ++        | 10       | ++        | +++         |
| SCC       | 80           | +         | 20        | +         | 10       | ++        | +++         |
| SCC       | 0            | 0         | 90        | ++        | 0        | 0         | +++         |
| SCC       | 70           | +         | 50        | ++        | 5        | ++        | +++         |
| SCC       | 80           | ++        | 90        | ++        | 40       | ++        | +++         |
| SCC       | 70           | ++        | 100       | ++        | 10       | +         | +++         |
| SCC       | 10           | +         | 40        | ++        | 60       | +         | +++         |
| ADC       | 0            | 0         | 0         | 0         | 0        | 0         | ++          |
| ADC       | 0            | 0         | 40        | +         | 0        | 0         | ++          |
| ADC       | 0            | 0         | 50        | +         | 0        | 0         | +++         |
| ADC       | 0            | 0         | 70        | +++       | 30       | +         | +++         |
| ADC       | 60           | +         | 0         | 0         | 0        | 0         | +++         |
| ADC       | 30           | +         | 30        | +         | 0        | 0         | +++         |
| ADC       | 10           | +         | 30        | +         | 20       | +         | +++         |
| ADC       | 0            | 0         | 0         | 0         | 60       | ++        | +++         |
| ADC       | 0            | 0         | 10        | +         | 10       | +         | +++         |
| ADC       | 10           | +         | 5         | +         | 5        | +         | +++         |
| ADC       | 10           | +         | 40        | +         | 0        | 0         | +++         |

**Supplementary Figure 1: Notch3 expression in NSCLC patients.**

a, Representative images from Notch3 immunohistochemistry performed on tumour section from NSCLC lung cancers patients showing the diversity of Notch3 expression in the tumour compartment and the constant staining of Notch3 in the tumour

vasculature. **b**, Quantification of the expression of Notch3 in 10 squamous cell carcinomas and 11 adenocarcinomas from NSCLC patients.

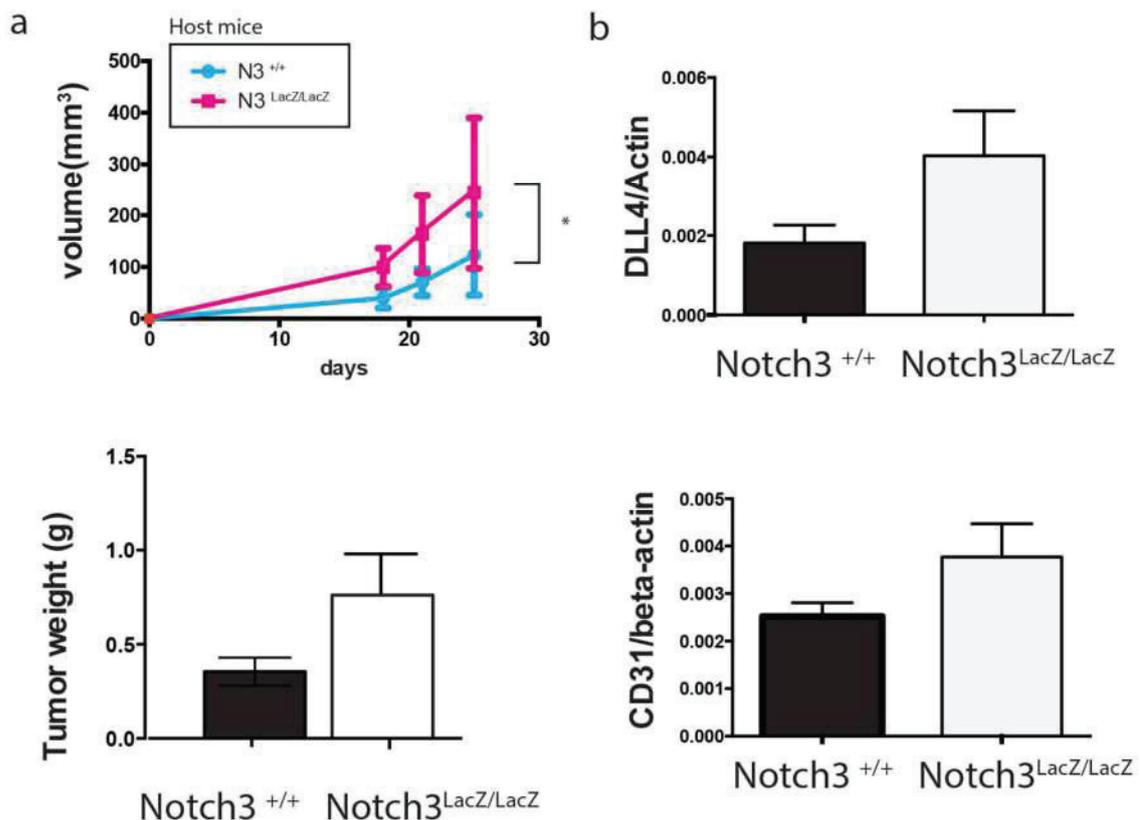
Lin et al., Supplementary Figure 2



**Supplementary Figure 2: Notch3 expression in the Notch3LacZ/+ mice.**

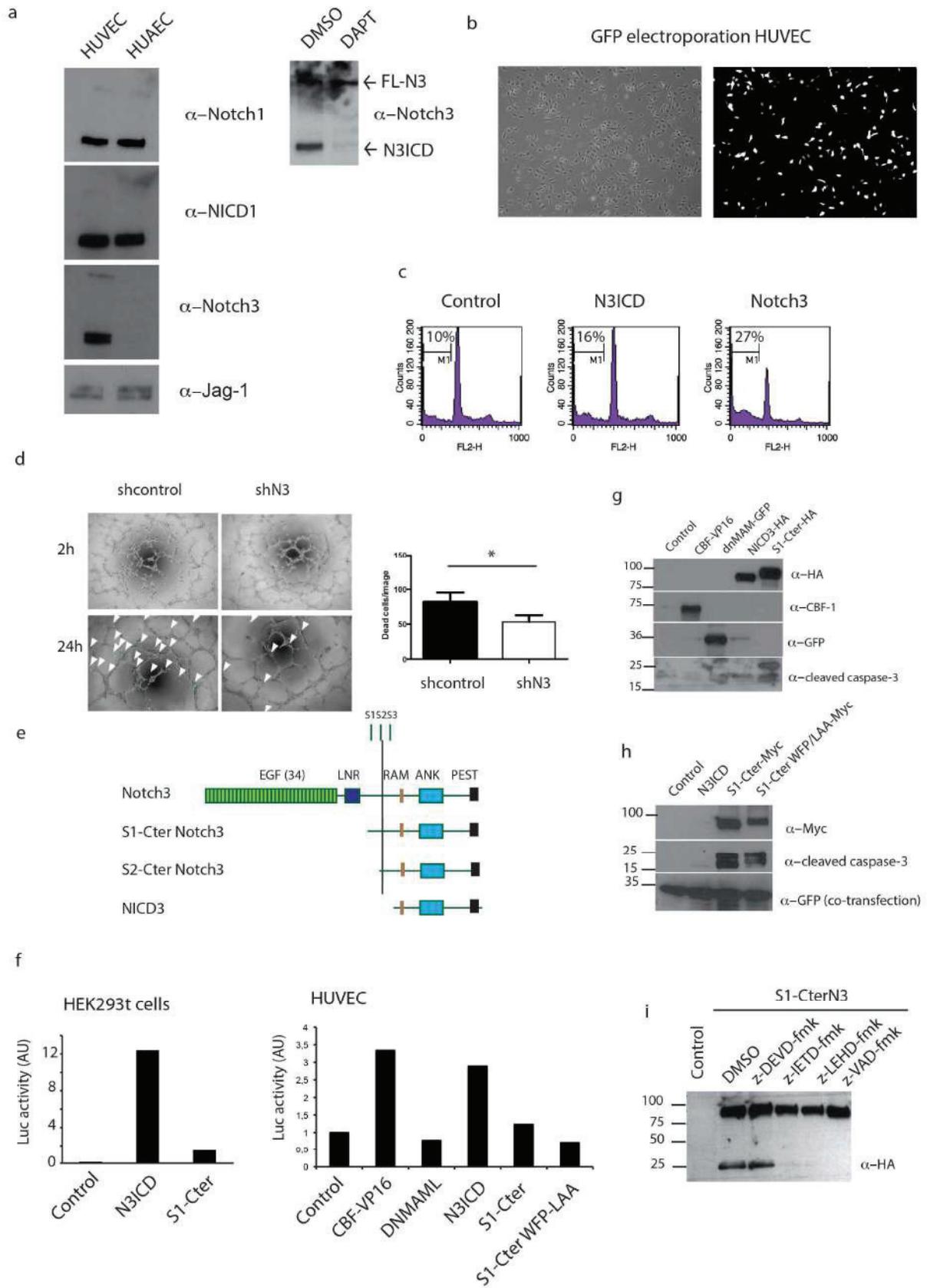
**a**, Notch3 mRNA expression determined with 3 different primer pairs amplifying three amplicons on the Notch3 mRNA in wild type mice or Notch3lacZ/LacZ mice. **b**, Comparison of the LacZ staining with the immunohistochemistry staining with anti- $\beta$ -galactosidase antibody showing the specificity of the  $\beta$ -galactosidase enzymatic reaction. **c**, Raw images from LacZ staining and immunofluorescence corresponding to Fig. 1c.

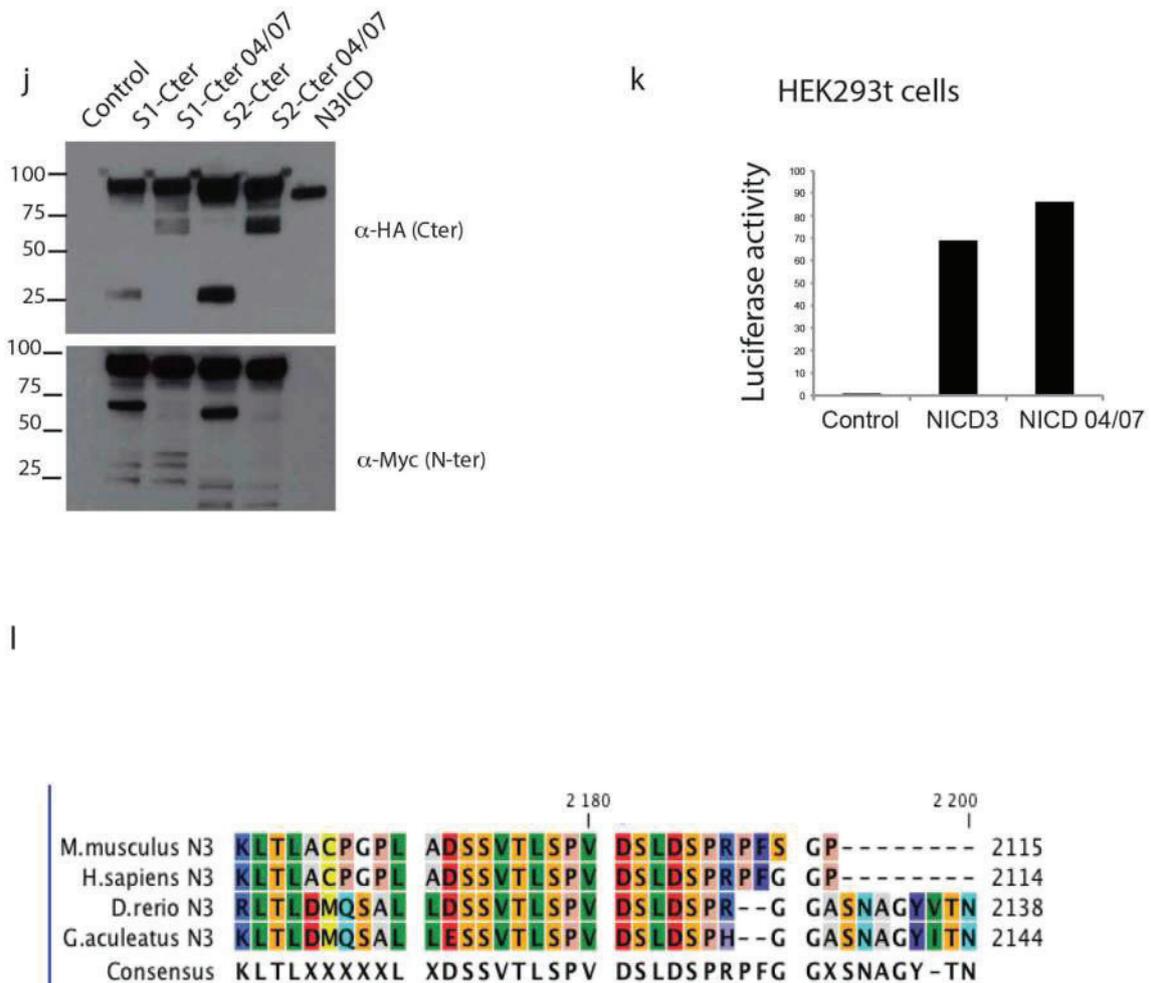
Lin et al., Supplementary figure 3



**Supplementary Figure 3: Notch3 limits tumour growth and vascularization *in vivo*.**

**a**, 5.105 EO771 cells were implanted into the left flank of wild-type C57Bl/6 mice (N3<sup>+/+</sup>, n=4) or of Notch3 LacZ homozygous Knock-in C57Bl/6 littermates (N3LacZ/LacZ, n=4). Tumour growth was monitored from day 16 until day 24 when mice were sacrificed. Two-Way ANOVA was performed to assess Time and Genotype effect on tumour growth (Time:  $p < 0,0001$ ; Genotype:  $p = 0,0028$ ). **b**, mRNA was extracted from tumours dissected after 25 days of growth from wild-type C57Bl/6 mice (N3<sup>+/+</sup>, n=4) or Notch3 mutant mice N3LacZ/LacZ C57Bl/6 littermates (N3LacZ/LacZ, n=4). Quantitative RT-PCR was performed to measure CD31, DLL4, expression (means  $\pm$  SD).



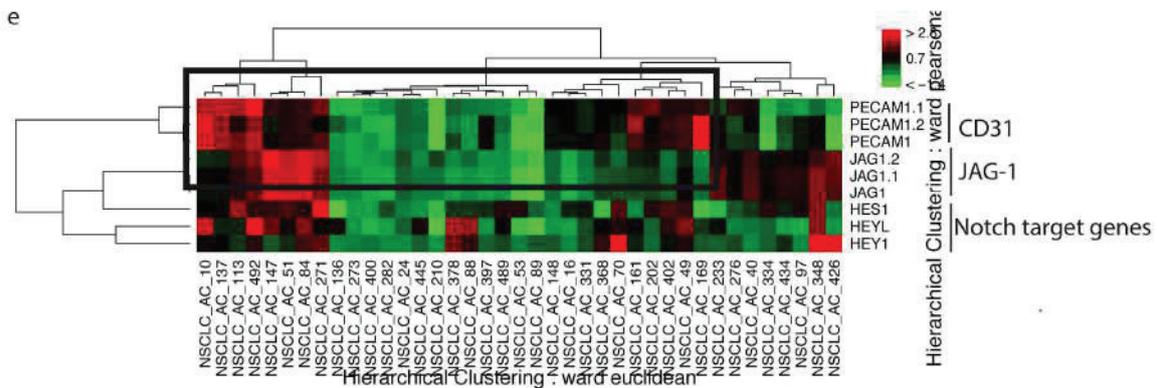
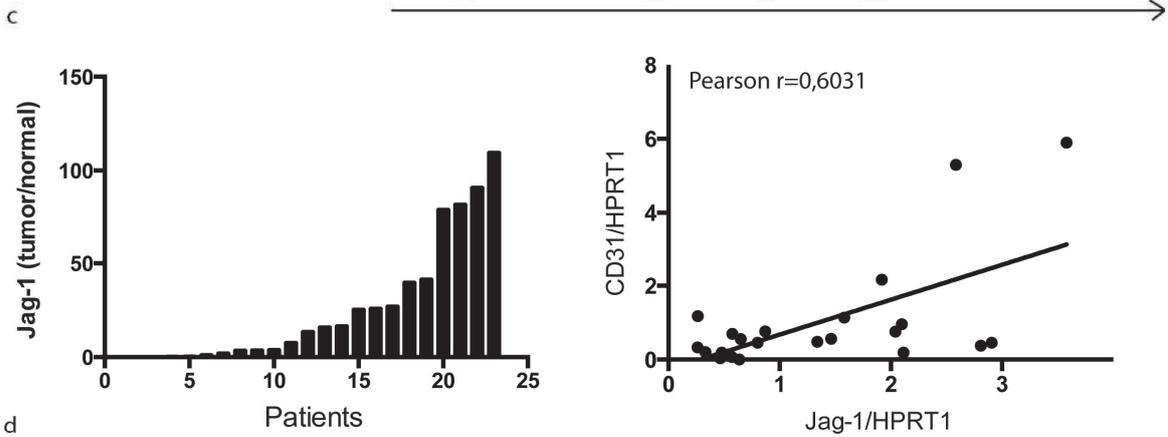
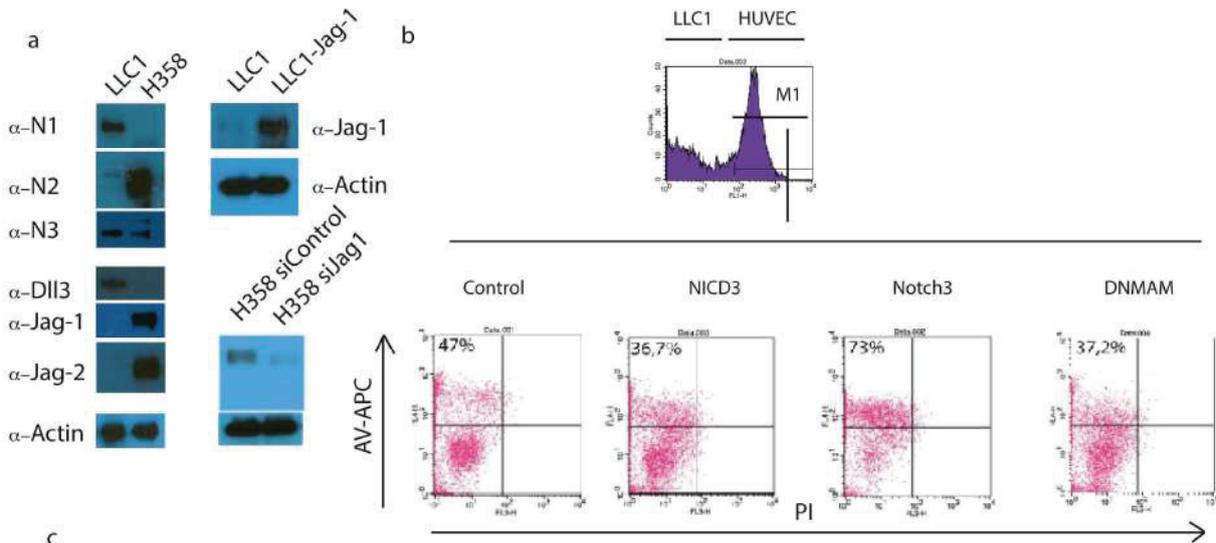


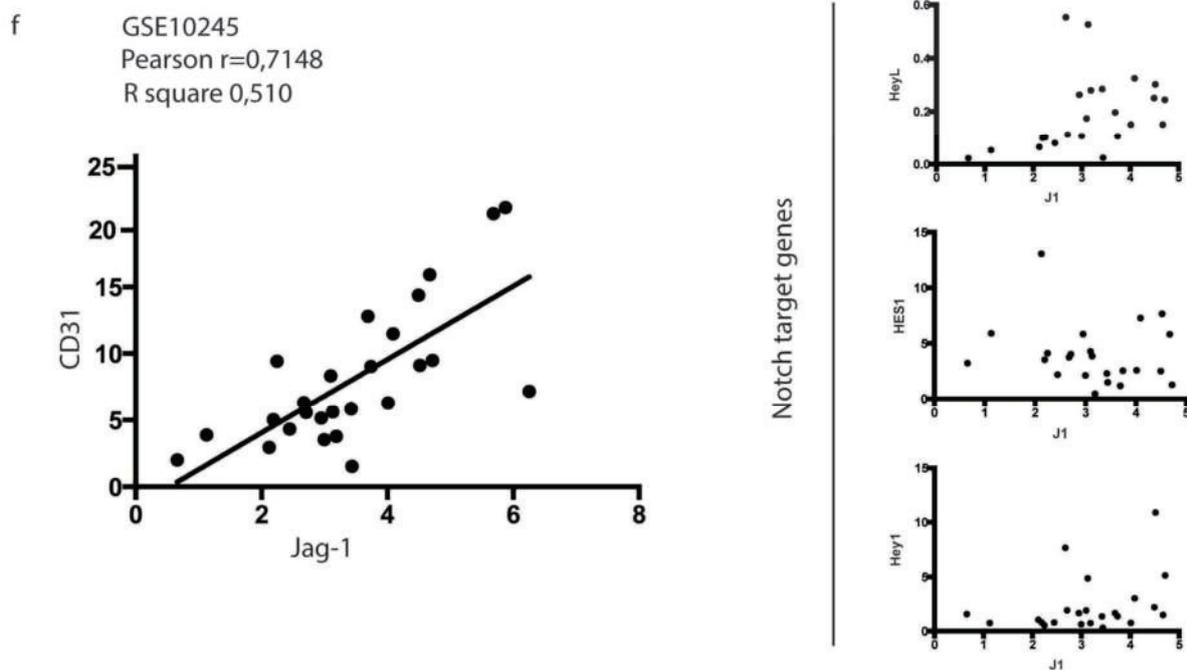
**Supplementary Figure 4: Notch canonical signalling is not required for Notch3-induced cell death.**

**a**, Western blot analysis of Notch1, Notch3 and Jag-1 expression of HUVEC and HUAEC cells and of Notch3 in HUVEC cells treated with DAPT (4 $\mu$ M). **b**, HUVEC cells were electroporated with a GFP expressing plasmid for 24h before being imaged. **c**, Sub-G1 analysis of HUVEC electroporated with a control plasmid (Control), or a plasmid expressing the full-length version of Notch3 or N3ICD for 24h. **d**, HUVEC were electroporated with plasmids expressing a shRNA control (sh control) or a shRNA targeting Notch3 (shNotch3) for 24 hours. Cells were then trypsinized and put in

matrigel. YO-PRO®-1 iodide was added 9 hours later to each well 30 min before imaging. Quantification of three independent experiments, mean +/- SD, unpaired t-test). **e**, Scheme representing the different versions of Notch3 used in the figure 3. S1-Cter (aa1573-Cterminus), S2-Cter (1631-Cterminus) and N3ICD (1664-Cterminus) are represented along with Notch3. **f**, HEK293t cells were transfected with the indicated constructs together with a pGL3-Renilla construct and a pGL3-CBF1-Firefly constructs for 48 hours before being assessed for the luciferase expression. **f**, Luciferase activity of HEK293t cells transfected with a pGL3-Renilla construct and a pGL3-CBF-1-Firefly construct together with a plasmid expressing N3ICD or S1-Cter construct and HUVEC electroporated with a pGL3-Renilla construct and a pGL3-CBF1-Firefly construct together with an empty vector, N3ICD, DNMA1L, CBF-VP16, S1-CterN3 or an S1-Cter WFP/LAA mutant construct for 48h. **g** and **h**, Western blot performed on HUVEC cells electroporated with the indicated constructs for 24 hours. **i and j**, S1-Cter Notch3 WT (S1-CterN3) or an S1-Cter version of Notch3 in which aspartic acids 2104 and 2107 have been mutated to asparagines (2104/2107) or a S2-Cter Notch3 construct with the same mutation or not were transfected in HEK293t cells treated with an inhibitor of caspase-3 (DEVDfmk, 5µM), an inhibitor of caspase-9 (LEHD-fmk, 5µM), an inhibitor of caspase-8 (IETD-fmk, 5µM) or a pan-caspase inhibitor (Z-VAD-fmk, 5µM) and analyzed by Western Blot for HA (C terminal tag). **k**, HEK293T cells were transfected with a plasmid expressing the intracellular domain of Notch3 (N3ICD) or a N3ICD version mutated for aspartic acids 2104 and 2107 (N3ICD 04/07) together with a pGL3-Renilla construct and a pGL3-CBF1-Firefly construct for 48 hours before being assessed for the luciferase activity. **l**, Alignment of the Notch3 receptor from mouse (M.musculus), Human (H.sapiens), zebrafish (D. rerio) and stickleback (G.aculeatus).

Lin et al., Supplementary Figure 5



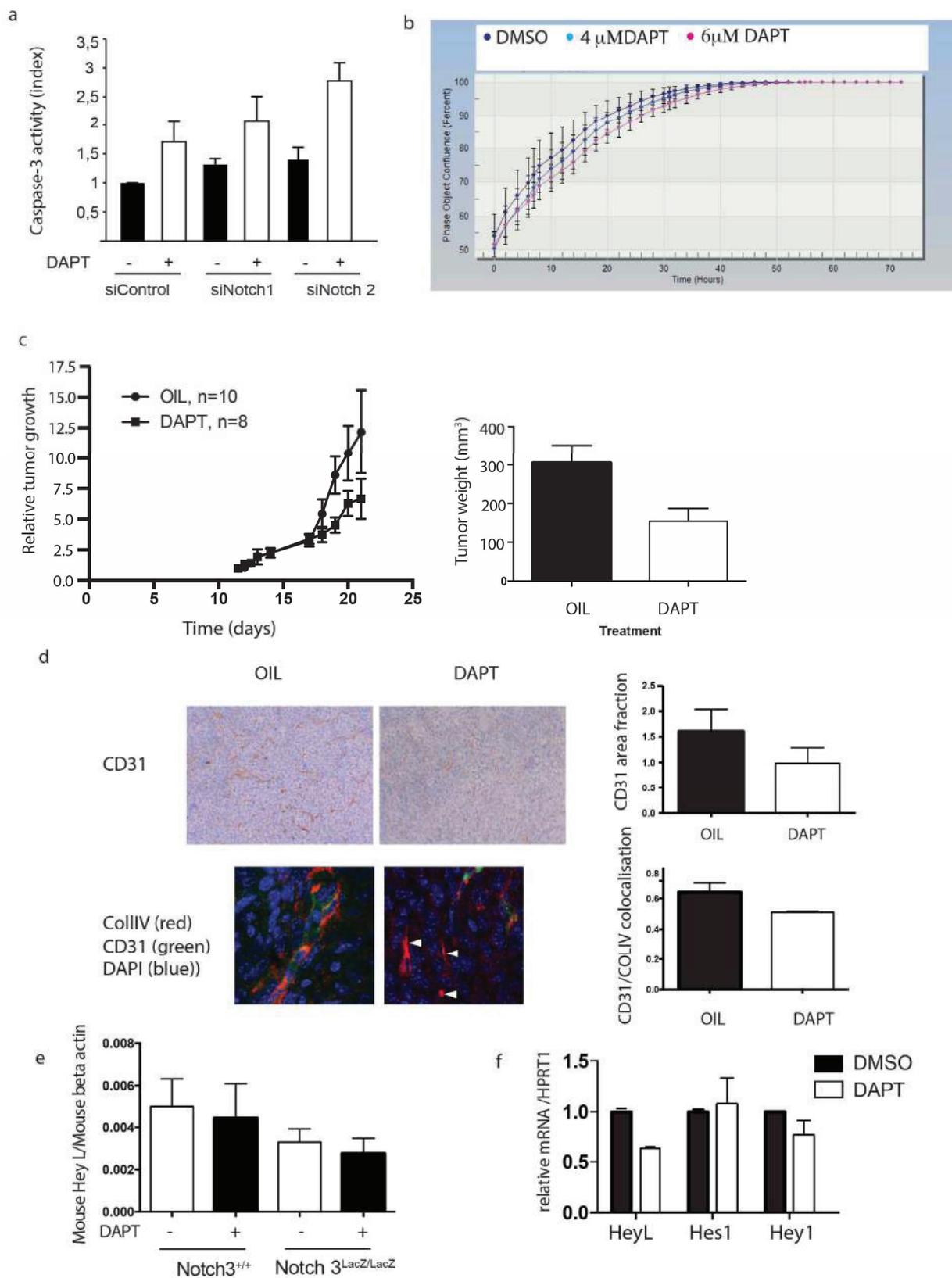


**Supplementary Figure 5: Jag-1 rescues Notch3-induced endothelial cells.**

**a**, Western blot analysis of LLC1 and H358 cells for Notch1, Notch2, Notch3, Dll3, Jag-1 and Jag-2 expression and of Jag-1 expression in LLC1 transfected or not with Jag-1 expressing construct or H358 transfected with siRNA control or with a siRNA targeting Jag-1. **b**, Co-culture of CMFDA cellTracker green stained HUVEC electroporated with the indicated plasmids and LLC1 were stained with Annexin V APC and studied by flow cytometry. HUVEC were gated (M1) according to FL1 staining and Annexin V (FL4) positive cells were quantified among HUVEC. Number of Annexin V positive HUVEC cells is specified in each condition. **c**, Tumour/normal tissue ratio of 23 Clear Cell Renal Cell Carcinoma of Jag-1 expression and correlation of PECAM-1 (CD31) mRNA expression with Jag-1 mRNA expression. **d**, Pearson correlation coefficient between Jag-1 and Notch target genes Heyl, Hey1 and Hes1 in non-small cell lung adenocarcinoma from the GSE7670 and GSE10245 datasets. **e**, Expression pattern of the three CD31 (PECAM1) probes and the three Jag-1 probes and from Notch target

genes HES1, HEY1 and HEYL probes were extracted from the GSE10245 dataset. R package EMA was used to establish the non-supervised clustering on gcRMA-calculated Signal intensity provided for each probe. **f**, Correlation between Jag-1 and CD31 or Notch target genes HeyL, Hey1 and Hes1 from patients represented in the black box in **e**.

Lin et al., Supplementary Figure 6



**Supplementary Figure 6: DAPT treatment induces regression of tumour vascularization.**

**a**, Caspase-3 activity assay of HUVEC electroporated with control siRNA (siControl), Notch1 siRNA (siNotch1) or Notch2 siRNA (siNotch2) for 48 hours and treated with DAPT (4 $\mu$ M). **b**, Growth curve of LLC1 cells treated with DMSO (1/1000) or 4 or 6  $\mu$ M DAPT was measured every two hours with the Incucyte Zoom. **c**, 5x10<sup>5</sup> LLC1 cells were implanted into the left flank of Wild Type C57Bl/6 mice and injected intraperitoneally with 10 $\mu$ l/g of ethanol-corn oil (1/9) or 10 $\mu$ l/g of 1mg/ml DAPT (n=8) on days 11,12,13,14 and 17,18,19,20. Mice were sacrificed on day 21. **d**, Immunohistochemistry for CD31 expression or immunofluorescence for CD31/collagen IV co-staining was performed on tumour sections from wild-type mice treated (DAPT, n=8) or not (OIL, n=10) with 10mg/kg of DAPT. CD31 expression and CD31/Collagen IV co-localization was quantified using imageJ. **e**, mRNA was extracted from tumours dissected after 21 days of growth from wild-type C57Bl/6 mice (N3+/, n=6) or Notch3 mutant mice N3LacZ/LacZ C57Bl/6 littermates (N3LacZ/LacZ, n=5) treated or not with DAPT. Quantitative RT-PCR was performed to measure HeyL mRNA expression (means +/- SD). **f**, mRNA was extracted from purified tumour-associated endothelial cells from nodules dissected from Kras+/G12D mice.

## **4.2 Article 2: Notch3 is a putative tumour suppressor in Breast Cancers**

## **Notch3 is a putative tumour suppressor in Breast Cancers**

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## ABSTRACT

Notch signalling is a conserved signalling pathway that has been involved in many aspects of mammary gland biology. Among the four Notch receptors, Notch3 is expressed in the vascular system and in the mammary luminal progenitors. Paradoxical data describe the involvement of Notch3 in human breast cancer and in mouse mammary gland tumours. While an activated form of Notch3 induces mammary gland tumour in mice, Notch3 expression is lost in human breast cancers and can induce senescence. We decided to study this dual role of Notch3 in the mammary gland. Immunohistochemistry analysis showed that Notch3 is lost human breast cancers epithelial cells when compared to healthy adjacent tissue, while being maintained in the tumour vasculature. We also showed in a TMA of 120 patients that Notch3 expression at the membrane was of good prognostic value whereas nuclear Notch3 had no prognostic value. Furthermore, by combining *in vitro* and *in silico* analysis, we showed that Notch3 promoter methylation correlates with Notch3 mRNA expression in human breast cancer tissue and cell lines. In line with this, by using demethylating agent, we could restore Notch3 expression in cells that do not express Notch3. We further show that Notch3 limits growth in soft agar of basal-like breast cancer cell lines MDA-MB-231.

## INTRODUCTION

Notch signalling is a highly conserved signalling pathway involved in development and in tumourigenesis (Ntziachristos et al., 2014). Although Notch signalling has first been associated with oncogenic properties it is now well established that Notch receptors can act as tumour suppressors in different contexts (Klinakis et al., 2011). Another complexity of Notch signalling consists in the existence of four paralogs in mammals, Notch1 to 4, which may have different effects on tumourigenesis. For example in mice model of pancreatic cancers, Notch1 behaves as a tumour suppressor (Hanlon et al., 2010) whereas Notch2 is required for progression (Mazur et al., 2010). In the mammary gland, Notch3 has recently been shown to be expressed in the luminal progenitors of the mammary gland (Lafkas et al., 2013). With regards to mammary tumours, the identification of the Mouse Mammary Tumour Integration site has revealed an oncogenic role of Notch signalling in mouse mammary tumours that has been confirmed in human breast cancers (Stylianou et al., 2006). Nevertheless, Notch

canonical signalling may not be necessary for Notch induced mammary tumours in mice (Raafat et al., 2009). Thus Notch signalling role in mammary gland tumorigenesis is still unclear. In particular, regarding Notch3, it has been described to be oncogenic (by overexpressing N3ICD under the control of the MMTV promoter) (Hu et al., 2006). But this view has been more recently challenged by describing that Notch3 is able to induce senescence and that Notch3 mRNA is decreased in some breast cancer datasets (Cui et al., 2013). Notch3 has also been shown to be a good prognostic marker in breast cancers (Xu et al., 2015). However, Notch3 has also been shown to collaborate with ErbB2 both *in vitro* and *in vivo* (Pradeep et al., 2012; Yamaguchi et al., 2008). More recently, Notch3 has also been shown to be involved in maintenance of CD133 stem cells and resistance to endocrine therapy (Sansone et al., 2016). We recently showed that Notch3 could in some contexts, behave as a dependence receptor (Lin et al., under revision). We indeed showed that in absence of its ligands, Notch3 could induce cell death in tumour-associated endothelial cells. We therefore asked, if the paradoxical data concerning Notch3 as an oncogene or as a tumour suppressor in Breast cancers could be at least in part explained by a dependence receptor function in this context as well. We indeed confirm by immunohistochemistry that Notch3 was lost in breast cancers. More interestingly, we showed in TMAs that Notch3 membrane expression was a good prognostic marker. We further show that Notch3 is lost by methylation of its promoter and that Notch3 expression inhibits growth in soft agar, especially when the interaction with ligands is abrogated by specific mutations. We are currently investigating this putative tumour suppressor function for Notch3 *in vivo*.

## RESULTS

As Notch3 has been suggested to be downregulated in breast cancers (Cui et al., 2013) at mRNA level, we wanted to assess expression of NOTCH3 protein by immunohistochemistry. We looked in a small cohort of 21 infiltrating adenocarcinoma for which we had paired samples with normal adjacent tissue and cancer tissue. We could observe a clear downregulation of Notch3 in the epithelial compartment whereas Notch3 was still strongly expressed in vascular system Figure 1A. This show that assessing Notch3 expression on mRNA from bulk tumours probably underestimate the loss of Notch3 in the tumour compartment since the stroma can still contribute to a strong Notch3 mRNA expression. We next looked at Notch3 expression in a TMA of

114 patients. We quantified expression of Notch3 at the subcellular localization. Interestingly, we could show that Notch3 expression at the membrane was associated with a good prognosis whereas Notch3 expression in the nucleus had no prognostic value (Figure 1B). We further looked in the TCGA breast cancer datasets. We looked for paired samples of normal and tumor tissue and observed a normal distribution of Notch3 in these samples (Supplementary Figure 1A). This is slightly in contradiction with the GSE3165 dataset in which, the expression of Notch3 mRNA is decreased in tumour samples when compared with pooled normal samples (Cui et al., 2013) and also when compared with classification of patients according to PAM50 classification (Supplementary Figure 1B). Notch3 was significantly downregulated in each subgroup when comparing to normal but there was no significant difference in between the different subgroups. However, the TCGA dataset has the advantage to contain paired samples, which is not the case in the GSE3165 dataset. We also looked in the TCGA data set for expression of Notch3 in the PAM50 classification. Notch3 was slightly more expressed in basal-like tumours (Supplementary Figure 1C). In order to understand how Notch3 is lost in Breast Cancers we first looked at methylation of the Notch3 promoter in breast cancer cell lines. We used the GSE44837 and GSE44836 datasets that combine Methylation array and expression array dataset to assess correlation between Notch3 promoter methylation and Notch3 mRNA expression. We identified CpG probes in Notch3 promoter and looked for methylation in breast cancer cell lines. We could observe a clear correlation between Notch3 promoter methylation in most of these probes and Notch expression (Supplementary Figure 2A). We then confirmed these data in the TCGA breast cancer patients. Indeed we observed a clear correlation between Notch3 methylation and Notch3 expression (Figure 2A). Furthermore, when we looked in paired sample, we could observe an increase in Notch3 methylation in the tumour part when compared with the normal tissue for the same patients (Figure 2B). We also looked in the different clusters published by the TCGA consortium for methylation status (Cancer Genome Atlas, 2012). We observed that Notch3 was higher in the cluster 5, cluster in which methylation is low further reinforcing the correlation between methylation and Notch3 expression (Figure 2C). Furthermore, even if this data set we saw no diminution of Notch3 expression in paired samples (Supplementary Figure 1A), we saw that methylation was increased in patients in which expression was decreased (Figure 2D). We then looked for the methylation of Notch3 promoter by sequencing of bisulfite treated DNA of two cell lines that expresses high

level of Notch3 (MCF7 and T47D) and two cell lines that express low level of Notch3 (MDAMB-231 and HS578T). We could identify methylated CpG sites in MDA-MB-231 and T47D that were not methylated in MCF7 and HS578T (Figure 2E, data showed for MDA-MB-231 cells and for MCF7 cells). Furthermore, treatment with decitabine induced a demethylation of these sites (Figure 2E) together with increased expression of Notch3 (Figure 2F). As we have recently shown that Notch3 could induce apoptosis of tumour associated endothelial cells in absence of ligands (Lin et al., under revision), we reasoned that if Notch3 would have a tumour suppressor function, it would be in absence of its ligands. We therefore assessed the prognostic value of Notch3 in the TCGA dataset in the population in which ligands were low. It was interesting to note that Notch3 was of good prognosis in the population of patients that had a low expression of Jagged-2 (Figure 3A). In order to study the effect of Notch3 in relation with ligands, we mutated Notch3 on a site that would abrogate interaction with Jagged ligands and we engineered another mutant that abrogates the interaction with Delta-like ligands (Figure 3B). We mutated the valine 304, which correspond to the valine 361 of *Drosophila* Notch that has been shown to discriminate between Delta and Serrate binding (Yamamoto et al., 2012). This mutant is supposed not to interact with Jagged ligands. We also mutated threonine 446 (Homolog of threonine 466 in Notch1), which prevents the O-Fucosylation and therefore impairs Delta-mediated activation (Ge and Stanley, 2008). We next engineered a stable cell line expressing inducible version of the different mutants of Notch3 in MDA-MB-231 cells. We saw no clear effect in induction of cell death by expression of the different Notch3. Interestingly, however, we saw an inhibition of soft-agar colony formation, which was higher for both mutants, showing that either Delta-like or Jagged mutant could be involved in the inhibitory effect of Notch3 (Figure 3C). We next sought to confirm the tumour suppressor function of Notch3 *in vivo*. In order to reveal the dependence receptor function of Notch3 we looked for a model in which Notch ligand Jagged-2 was weakly expressed. We looked for transcriptomic comparison in mouse models of mammary gland. As the MMTV-Neu was among the model expressing the less Jagged-2 (Pfefferle et al., 2013), we decided to look for the effect of knocking-down Notch3 in this model. We used the LacZ knock-in model for Notch3 loss of function that has previously been described (Arboleda-Velasquez et al., 2008) and MMTV-Neu mice in a Balb/C genetic background (Boggio et al., 1998). We first observed that Notch3 was indeed expressed in a sub-population of luminal mammary gland cells in (Figure 4) confirming previously published lineage

tracing experiments (Lafkas et al., 2013). We could observe the expression of Notch3 in more cells than what has been published in the previous mentioned study as in some terminal end buds we saw expression of Notch3 in almost all cells, that may therefore be clonal (Figure 4A). We next looked at expression of Notch3 in MMTVNeu tumour from Notch3<sup>LacZ/+</sup>. We observed that Notch3 was not expressed anymore. This is interesting to not that Notch3 has been described to be expressed in hyperplasia in the same mouse model (Pradeep et al., 2012). However, in this study, expression of Notch3 was not assessed in the tumours. We are currently, the putative tumour suppressive function of Notch3 by looking at tumour-free survival in MMTV-Neu mice expressing or not Notch3 (MMTVneu/Notch3<sup>+/+</sup> versus MMTVNeu/Notch3<sup>LacZ/LacZ</sup>) (Figure 4B).

## DISCUSSION

Notch3 has first been thought to behave as an oncogene in mammary gland tumour. Indeed, expression of the N3ICD transcriptionally active fragment under the control of the MMTV promoter, as does expression of the N1ICD fragment, induces mammary gland tumours in mice (Hu et al., 2006). However, expression of N3ICD under the control of the Notch3 promoter has not any effect on tumour formation, but instead limit luminal progenitor expansion (Lafkas et al., 2013). Interestingly, we observed in a TMA that Notch3 expression at the membrane was associated with a good prognosis whereas Notch3 expression in the nucleus has no impact (Figure 1). This shows that the transgenic model expressing N3ICD cannot account for some of the effect of Notch3 in mammary gland tumour. Furthermore, we observed that Notch3 expression was of good prognosis in patients that express a low level of Jagged-2 ligand. This would point out to a signalisation of Notch3 in absence of ligands that would limit tumour progression. We recently described a pro-apoptotic function of Notch3 in tumour-associated endothelial cells (Lin et al., under revision). In this study, we showed that Notch3 could induce cell death in endothelial cells under aberrant expression. Jagged-1 expressed by tumour cells was shown to inhibit cell death induced by Notch3. This shows that Notch3 behaves as a dependence receptor in the context of aberrant expression of Notch3 in endothelial cells. However, we observed no significant induction of cell death upon expression of Notch3 in MDA-MB-231 cells. MDA-MB-231 cells express no Jagged-2 but express Jagged-1. We have not seen any benefit for Notch3 prognosis value in Jagged-1 low

patients as we did for Jagged-1. Still, we could explain, this absence of cell death induction by the presence of Jagged-1 in this cell line. We therefore produced mutants of Notch3 receptor that cannot bind either Jagged ligands, either Delta-like ligands. These mutations have no effect on the absence of cell death induced by Notch3. However, these mutations increase the inhibition of Notch3 of soft agar colony formation (Figure 3). This observation point toward a possible tumour suppressive function of Notch3 in absence of ligands. We are currently investigating how Notch3 could limit growth in soft agar, by performing anoikis experiments. Regarding the molecular mechanism we also saw no major differences between mutants for Jagged ligands and mutants for Delta-like ligands. It is therefore possible that all Notch ligands can inhibit the dependence receptor function of Notch3. Although we focused on Jagged-1 in our previous study (Lin et al., under revision), it is highly possible that all Notch ligands are involved in inhibiting Notch3 induced cell death. An aspect we still need to assess, is the effect of cis-ligand in such signalisation.

In order to study the *in vivo* relevance of Notch3 dependence receptor function, we decided to use the MMTV-HER2 (Neu) mouse model, previously characterised (Boggio et al., 1998). Crosstalk between Notch signalling and Her2 signalling is controversial in published data. Notch3 has been suggested to be necessary for HER2-induced growth of MCF10A non-transformed cells in soft agar (Pradeep et al., 2012). However, Her2 signalling has been shown to inhibit Notch signalling (Osipo et al., 2008), pointing toward clinical interest to inhibit both Notch and Her2 signalling (Pandya et al., 2011). In contrast to a role of Notch in MCF10A transformation, Notch3 has also been shown *in vitro* to be more relevant in HER2 negative cells (Yamaguchi et al., 2008). Pradeep and colleagues observed that Notch3 was expressed in Hyperplasia in MMTV-Neu mice. Surprisingly, we observed that Notch3 was not expressed in MMTV-Neu tumour in mice (Figure 4). This is in agreement with our observation that Notch3 is lost in human breast cancer (in HER2 positive as in other group of breast cancers). This raises the question whether Notch3 is lost in the transition between hyperplasia and breast cancers. In order to answer this question, we crossed MMTVNeu mice with Notch3 LacZ knock-in mice previously described (Arboleda-Velasquez et al., 2008). To our knowledge, there is no published data on mammary gland tumours in mice knock-out for Notch receptors or ligands. We have also recently shown that Notch3 plays an important role in controlling tumour

angiogenesis (Lin et al., under revision). It would be interesting in the MMTVNeu model to assess the relative contribution of Notch3 expressed in the stroma and Notch3 loss in the epithelial compartment. Indeed, in this model, we suppress Notch3 expression both in the vascularisation and in the tumour cells.

## **MATERIAL AND METHODS**

### **Cell culture**

The human MDA-MB-231 cell line is derived from pleural effusion metastatic site of a breast cancer. MDA-MB-231 cell line were stably transfected using the Tet-pITR puro GFP plasmid empty as a control or containing the full length of Notch3-WT and mutated Notch3-T446V or Notch3-V304M which encode for Notch3-HA-tag fused proteins. This plasmid allows the establishment of stable cell lines through the sleeping beauty transposase mediated insertion of the cassette containing the tetresponsive promoter, the puromycin resistance gene, the GFP under the control of a constitutive promoter (PGK) and the tet repressor sequence. Cell lines were maintained in Dulbecco's modified Eagle's media (DMEM media) complemented with 10% heat inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) and 4% gentamycin. Cells are cultured at 37°C and 5% CO<sub>2</sub>. Notch3 and mutated Notch3 expression were induced 24 hours after the plating by Doxycycline (Dox) at 0.1, 0.5, 1, 2 and 5 µg/mL concentration. A total of 200 000 cells and 5 000 cells were respectively plated in 6-wells plate and 96-wells plate. MDA-MB-231 cell pellets were harvested by centrifugation at 4°C.

### **TCGA data analysis**

TCGA datasets were analysed using R software and TCGA2STAT packages. RNAseqV2 data were used and RPKM value was imported. The Tumor/Normal match function was used to study matched normal and tumour samples.

### **Soft agar assay**

A single-cell suspension of 30.000 MDA-MB-231-pitr1-Kremen1 cells selected for high expression of GFP by flow cytometry in 1,5 ml 0.45% agarose (Sea Plaquar Agarose Lonza, 50100, lot 0000287875) was seeded into 6-well plates containing a 0.9% agarose base. Treatments were added in the cell suspension and at every medium

change. Medium was thereafter changed every 3-4 days. After incubation for 8 weeks, the media was removed and after a PBS wash, the colonies were fixed in 4%PFA and 0,005% crystal blue for an hour. The samples were washed 3 times in PBS for 10 minutes each and visualized by microscopy. Images were acquired with Zeiss Axio microscopy and colonies were counted.

### **Bisulfite assay**

DNA extraction from cells was done with the DNA kit (Machery-Nagel) according to manufacturer's instruction. The bisulfite reaction was done with the EpiTect Bisulfite kit (Qiagen) according to manufacturer's instruction. A PCR was done in a highly enriched region of Notch3 promoter and its sequencing was analysed.

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## FIGURE LEGENDS

### Figure 1. Notch3 is lost in Breast Cancers

A. Notch3 expression was assessed by immunohistochemistry using the Notch3 D11B8 Cell Signaling antibody. Expression in cytosol or nucleus was assessed by a qualified pathologist. B. Survival of 114 patients was plot as a Kaplan-Meier plot depending on Notch3 expression localisation.

### Figure 2. Notch3 promoter is methylated in breast cancers

A. Correlation between global methylation of Notch3 and Notch3 expression (RNAseq) in the TCGA BRCA dataset. B. Notch3 methylation of probe x in patients from the TCGA dataset with paired samples: normal (blue) and tumeur (green). C. Notch3 expression (RNAseqV2) in the different methylation clusters from the TCGA BRCA (Breast carcinoma) dataset. D. Methylation of Notch3 probe x in patients in which Notch3 is down-regulated in tumours and in patients in which Notch3 is not downregulated in tumours. E. PCR sequence of bisulfite-treated DNA from the indicated cell lines. MDA-MB-231 or MCF7 cell lines were treated with decitabine (5-Aza) or not (non treated). F. Notch3 expression was assessed by qPCR in MDA-MB-231 cells after treatment with decitabine or not.

### Figure 3. Notch3 behaves as a tumour suppressor in Breast cancers

A. Survival of patients from the TCGA BRCA dataset was assessed in patients in which Jagged-2 level was low. B. Scheme showing the effect of the different Notch3 mutations. C. Quantification of soft agar colony formed by MDA-MB-231 cells carrying

the inducible Notch3 pITR plasmid. Doxycyclin (DOX) was added every three days at the indicated concentration.

**Figure 4. Notch3 is expressed in luminal cells of the mammary gland and lost in MMTV-Neu driven tumours.**

A. LacZ staining was realised in mammary gland of a Notch3<sup>LacZ/+</sup> mice and in tumour dissected from a MMTV-Neu/Notch3<sup>+/-LacZ</sup> mice. B. Disease-free survival of MMTVNeu mice.

**Supplementary Figure 1. Notch3 expression in breast cancers**

A. Notch3 mRNA Tumour/Normal ratio in paired samples from the TCGA dataset. B. Notch3 expression in the different subgroups of breast cancers in the GSE3165 dataset. C. Notch3 mRNA expression in the different breast cancer subgroups of the TCGA dataset.

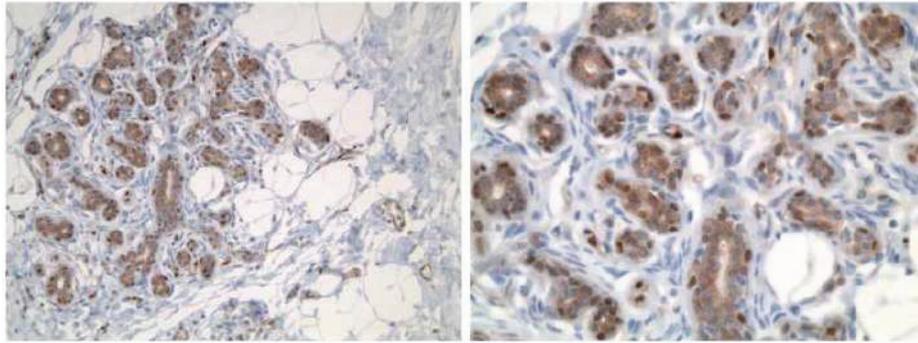
**Supplementary Figure 2. Notch3 methylation in breast cancer cell lines.**

A. Notch3 promoter CpG island with probes from the 450K chip. Correlation between methylation of the indicated probes and Notch3 expression in different breast cancer cell lines. B. Methylation at the indicated chromosomal location in normal and tumour tissue from breast cancer patients.

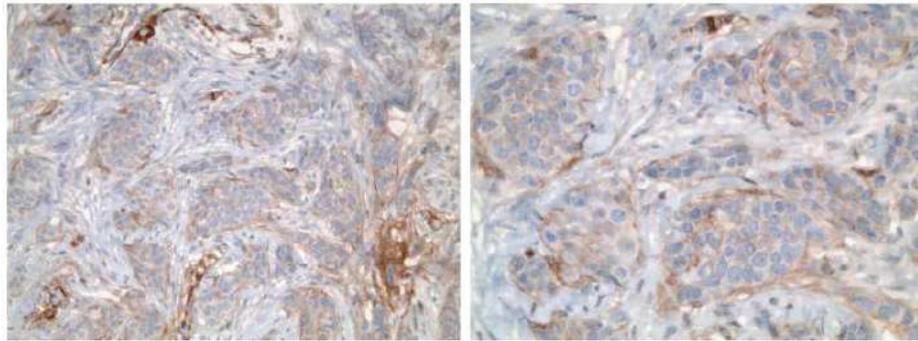
Negulescu et al., Figure 1

A

Normal



Tumour



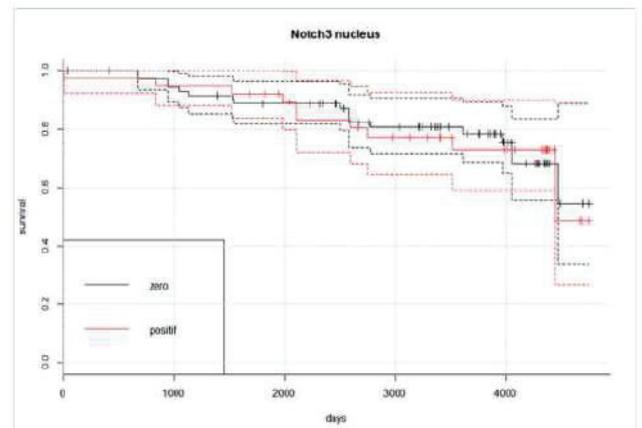
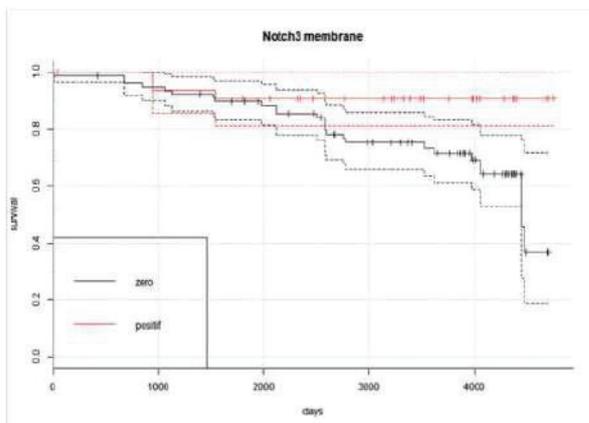
Cytoplasmic staining

| Normal      |             |    |              |  | Tumour       |             |               |               |  |
|-------------|-------------|----|--------------|--|--------------|-------------|---------------|---------------|--|
| ND          | 0           | 1  | 2            |  | ND           | 0           | 1             | 2             |  |
| 15 % (3/20) | 10 % (2/20) | 0% | 75 % (15/20) |  | 9,5 % (2/21) | 19 % (4/21) | 42,9 % (9/21) | 28,6 % (6/21) |  |

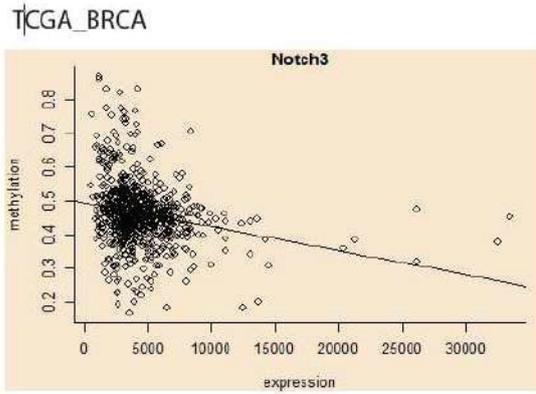
Nuclear staining

| Normal      |             |            |             |  | Tumour       |               |             |   |  |
|-------------|-------------|------------|-------------|--|--------------|---------------|-------------|---|--|
| ND          | 0           | 1          | 2           |  | ND           | 0             | 1           | 2 |  |
| 15 % (3/20) | 15 % (3/20) | 25 (5/20)% | 45 % (9/20) |  | 9,5 % (2/21) | 61,9% (13/21) | 28,6 (6/21) | 0 |  |

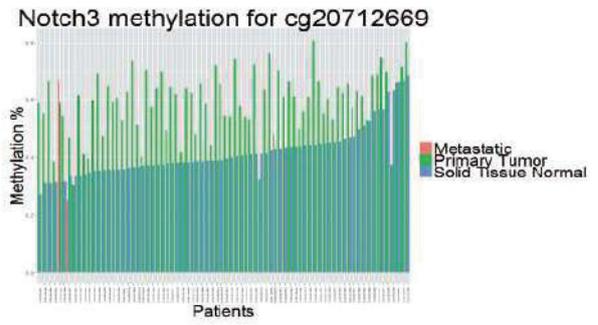
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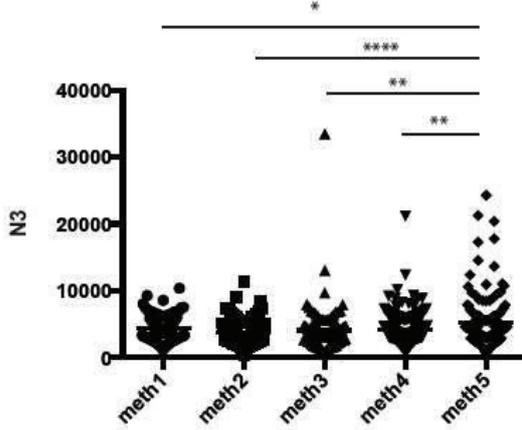
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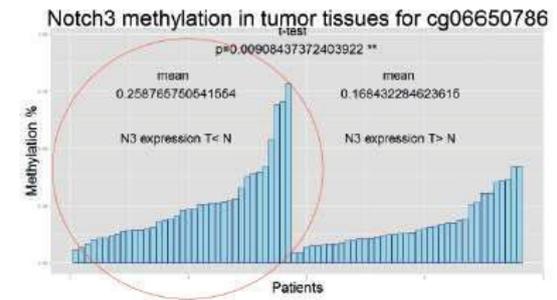
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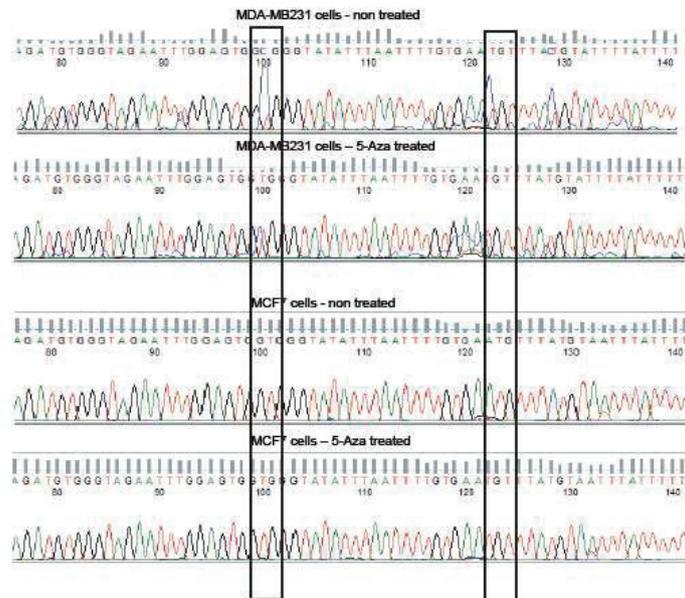
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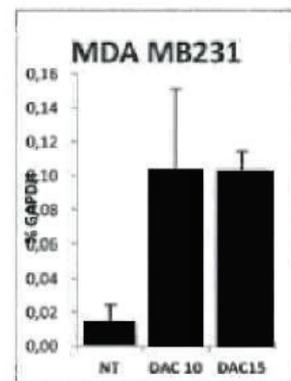
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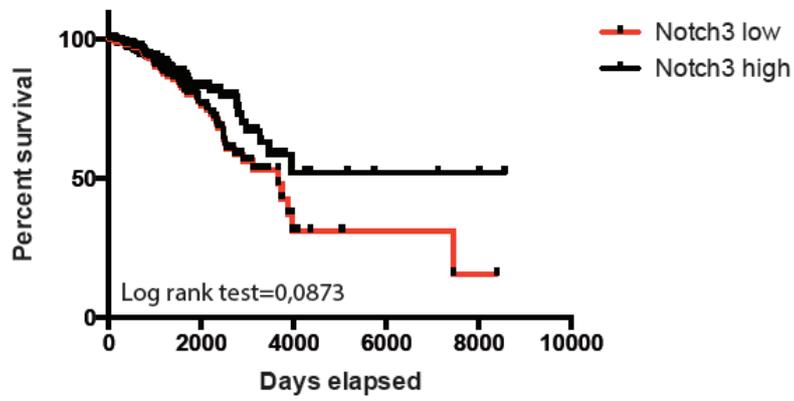
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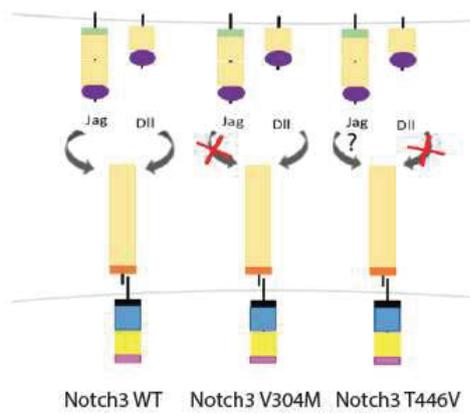
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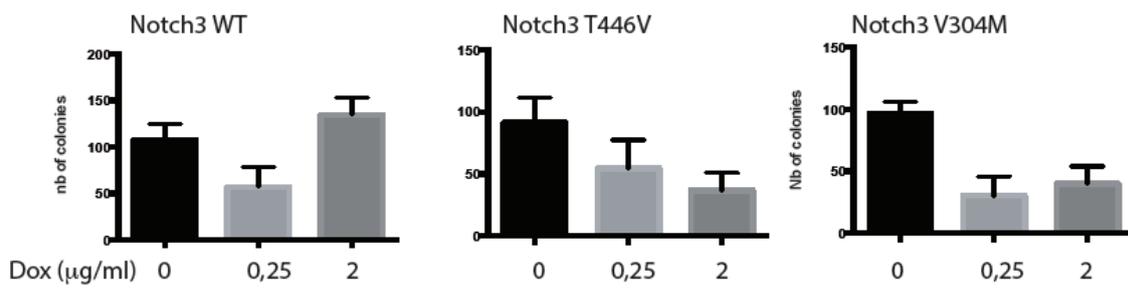
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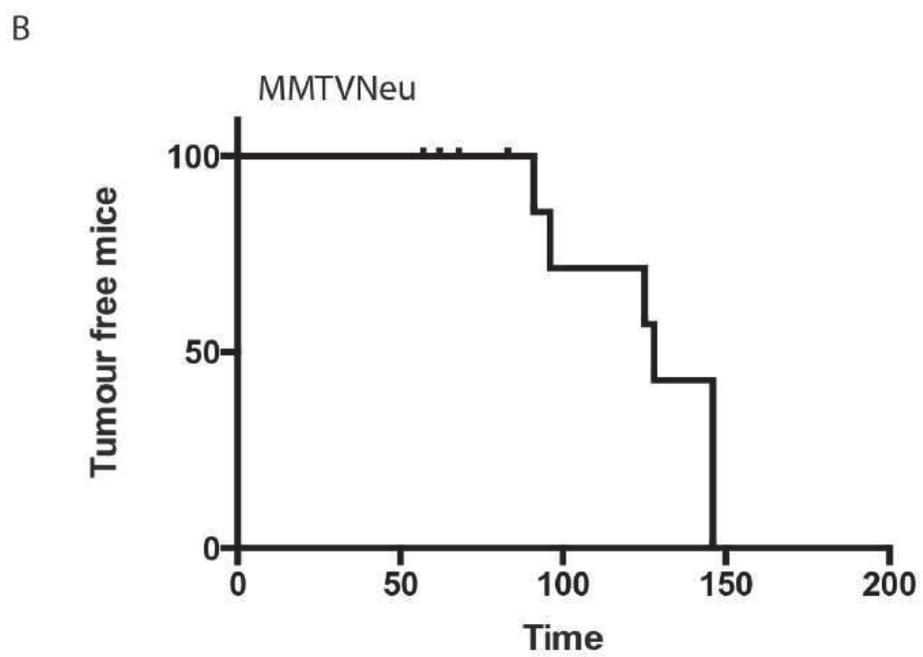
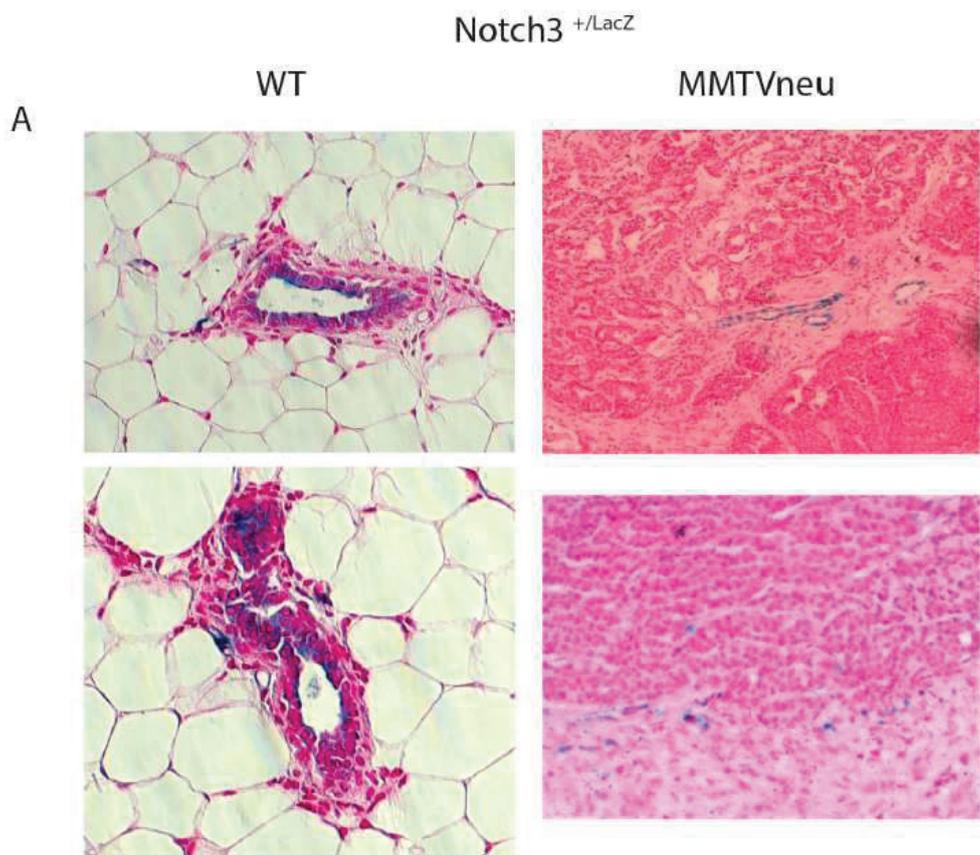
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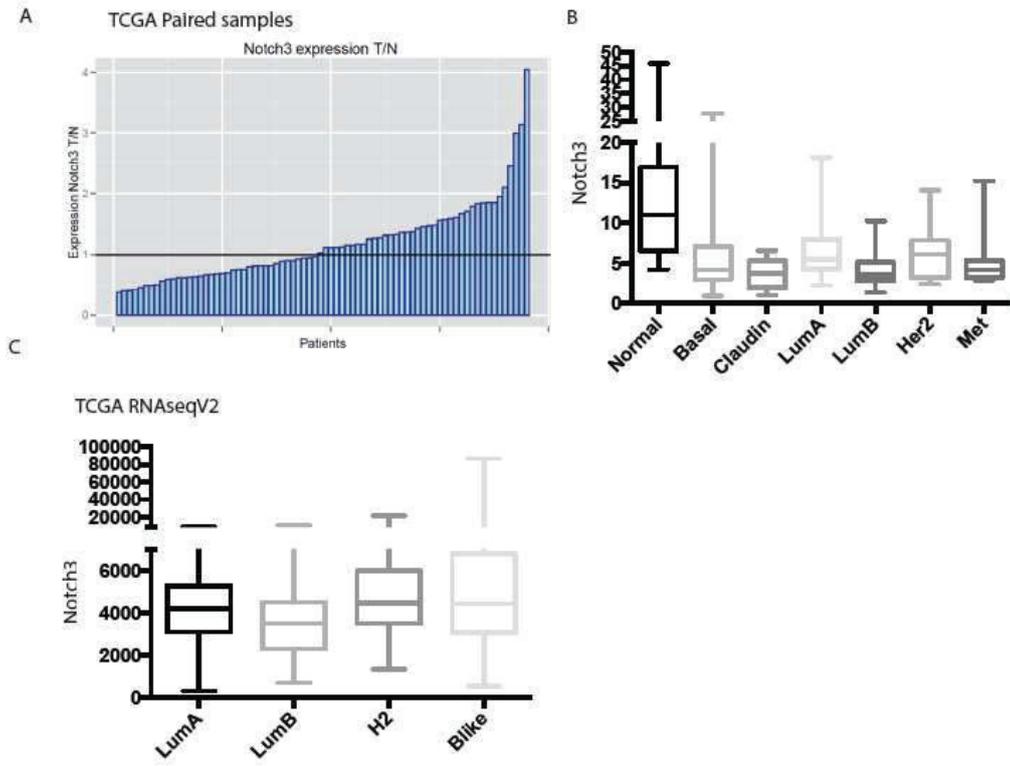


C

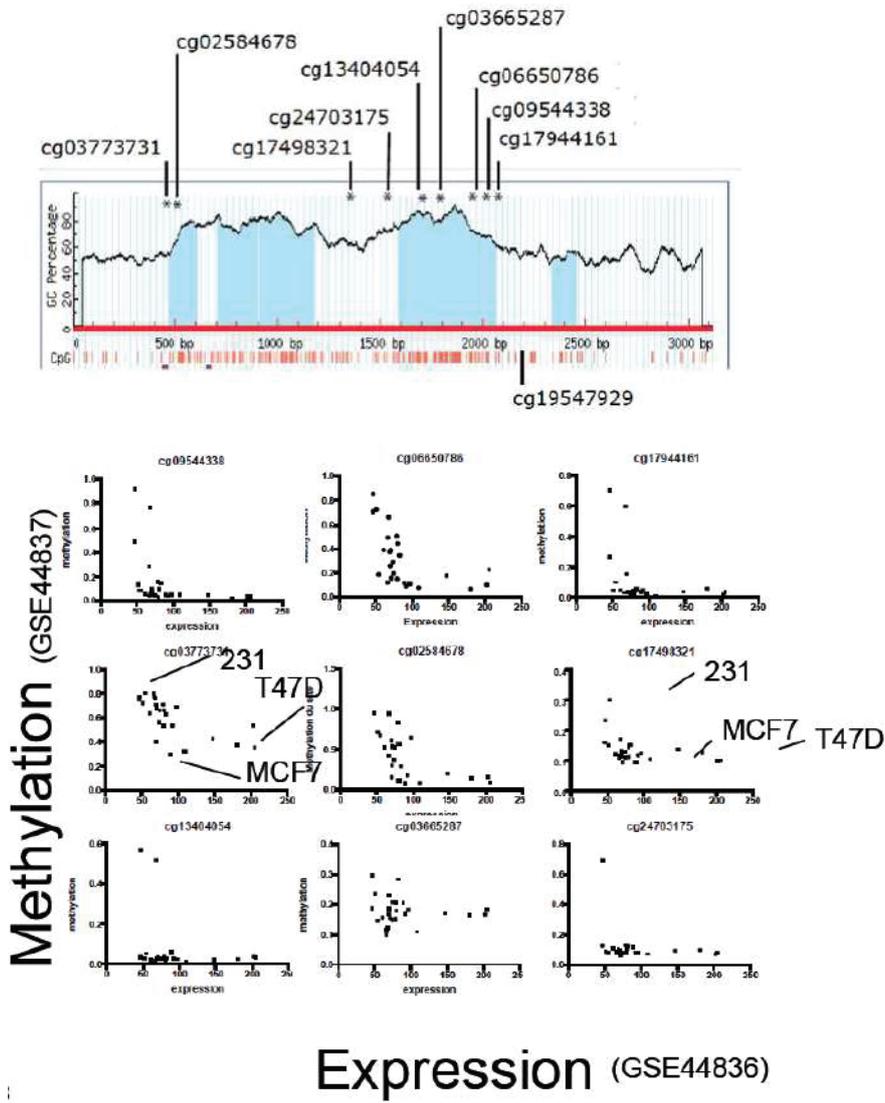


Negulescu et al., Figure 4



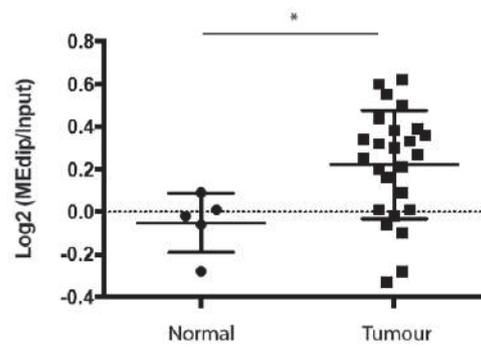


A



B

GSE43095



19:15310781/15310836

## **III. Discussion and perspectives**



In this study, we showed that Notch3 is aberrantly expressed in tumour associated endothelial cells and that it plays a pro-apoptotic role which limits tumour angiogenesis independently on the canonical Notch signalling. Indeed, Notch3 induces cell death in tumour endothelial cells and tumour-derived Jagged1 facilitates the survival of endothelial cells in co-culture system *in vitro* and in subcutaneous grafted tumour model *in vivo*. Thus, Notch3 behaves as a dependence receptor. Moreover, we showed that the anti-tumour effect induced by inhibitor  $\gamma$ -secretase is at least partly dependent on the apoptosis induced by Notch3 in endothelial cells.

This study raises numerous questions and brings new insights in the complexity of Notch signalling in tumorigenesis and angiogenesis, especially for Notch3. The concept of 'anti-angiogenic therapy' has been arisen and applied in pre-clinical study for the last twenty years. However, the principle strategy, targeting the VEGF pathway, didn't get a big success in clinical trial. Therefore, many efforts have been done on targeting other alternative pathways to inhibit tumour angiogenesis, such as the Notch pathway. However, contradictory results were observed in tumour angiogenesis studies when Notch pathway is inhibited. Moreover, opposite roles of Notch3 were also observed in tumorigenesis. Notch3 was reported to be oncogene or tumour suppressor in different context. The dependence receptor role of Notch3 reported in this study prompts us to ask whether the new discovered role of Notch3 can explain these paradoxical observations and its potential application in cancer treatment? Hence, we will discuss here different aspects regarding to the pro-apoptotic function of Notch3.

## **5.1 Is Notch3 an oncogene or a tumour suppressor?**

### **5.1.1 Does the dependence receptor function of Notch3 distinguish its oncogene and tumour suppressor role**

In this manuscript, we showed that Notch3 limits tumour neovascularization via its pro-apoptotic function in tumour associated endothelial cells. We therefore wonder whether this pro-apoptotic function also exist in other cell types. Indeed, Notch3 was reported to behave as an oncogene in many studies (Dang et al., 2000; Park et al., 2006; Ye et al., 2013). Nevertheless, it was also reported to be a tumour suppressor via controlling cellular senescence (Cui et al., 2013). Indeed, these controversial observations could be due to different Notch3 signalling. In most studies, when Notch3 is considered as an oncogene, the canonical Notch3 signalling is aberrantly activated, which is due to the over expression of ligands or receptor (Park et al., 2010; Reedijk et al., 2005). Consequently, the tumour progression is strongly enhanced. For instance, mice overexpressing N3ICD under the control of the MMTV promoter have a block of mammary gland development and develop mammary gland tumours (Hu et al., 2006). Moreover, Jagged1 and Jagged2 are reported to be up-regulated in serous human ovarian cancer (Choi et al., 2008; Euer et al., 2005). In contrast, in absence of ligands, the canonical Notch3 pathway is inhibited, which provides the possibility to trigger the negative pathway of Notch3 and impair the tumour growth. Preliminary results in the lab showed that its expression is lost in some human breast cancers. Membrane staining of Notch3 in human breast cancer samples is correlated to a good prognosis when Jagged2 expression is low. These data suggest that Notch3 could be a putative tumour suppressor via its dependence receptor function. In line with this, the pro-apoptotic function of Notch3 we present here is independent on the canonical Notch signalling. Indeed, activation or inhibition of the canonical Notch pathway didn't impair the cell death induced by Notch3. Furthermore, mutating the residues necessary for the interaction between N3ICD and CBF-1 did not abrogate the ability of Notch3 to induce cell death. Therefore, it is not as simple as black and white to define Notch3 as an oncogene or tumour suppressor. The new role of Notch3 as dependence receptor could explain its duality in breast cancer. In presence of ligand, Notch3 behaves as an oncogene, however, in absence of ligand, it behaves as a tumour suppressor.

### 5.1.2 Pro-apoptotic function of Notch3 in other cell populations besides tumour associated endothelial cells during tumorigenesis?

Notch3 expression is mainly restricted to the vascular system, however its expression is also found in other cell populations. A recent study showed that Notch3 is also expressed in luminal progenitor cell of mouse mammary gland (Lafkas et al., 2013). In addition, Notch3 expression has also been observed in some pathological settings, such as in cancer cells or cancer associated fibroblasts (Kayamori et al., 2016). Therefore, better determination of Notch3 expression pattern during tumorigenesis would be very helpful to complete the puzzle of how Notch3 limits tumour angiogenesis and tumour growth. To monitor Notch3 expression during tumorigenesis, it would be interesting to cross the Notch3-CreERT2<sup>SAT</sup>/R26<sup>mTmG</sup> mice (Fre et al., 2011; Lafkas et al., 2013) with predisposed cancer mice models such as MMTVneu mice or *Kras*<sup>G12D</sup> mice. Sorting cell via GFP expression would allow us to characterize the expression pattern of Notch3 in different stages of tumorigenesis.

In our study, Notch3 invalidation is not restricted to endothelial cells. The pro-apoptotic function of Notch3 could also be presented in other stromal cells besides tumour endothelial cells. Whether these cells can inhibit the neovascularization in tumour or contribute directly to inhibit tumour growth is unclear. To investigate that, comparing Notch3 expression in different stromal cells such as fibroblasts and macrophages from healthy mice lungs and *Kras*<sup>G12D</sup> mice lungs may give us a clearer answer. Moreover, treating these isolated stromal cells with DAPT *in vitro* will let us know whether the anti-tumour effect of DAPT is also due to the induction of cell death in other tumour stromal cells besides tumour endothelial cells. Moreover, it will be also interesting to generate a Notch3 conditional knockout transgenic mouse model which does not exist so far. By crossing the Notch3 flox mouse with the Cdh5-cre/ERT2 mouse, we can therefore invalid the Notch3 expression specifically in endothelial cells. We can also investigate the role of Notch3 in tumour endothelial cells in a more physiological condition by crossing the Cdh5-cre/ERT2, *Notch3*<sup>flox/flox</sup> mouse with another mouse model which can develop tumour spontaneously, such as the *Kras*<sup>G12D</sup> model and the MMTVneu model to monitor the tumour growth and tumour angiogenesis.

Besides the tumour microenvironment, whether the pro-apoptotic function of Notch3 is also functional in cancer cells still need to be confirmed. Preliminary results regarding the expression of Notch3 in human breast cancer samples showed that, Notch3 could be a

putative tumour suppressor owing to its dependence receptor function. Ectopic expression of Notch3 in human breast cancer cell line MDA-MB-231 failed to induce significant cell death as in HUVEC. This observation indicates that cancer cells might develop some strategies to inhibit the pro-apoptotic function of Notch3 conserved in normal cells. Therefore, additional cancer cell lines should be tested to verify this hypothesis. To better investigate this question, invalidation of Notch3 in MMTVneu mouse model which is ongoing in the laboratory will be a good way to verify the dependence receptor function of Notch3 not only in the stroma but also in tumour cells for its putative tumour suppressor role.

## **5.2 Why only Notch3 but not the other Notch receptors behaves as dependence receptor?**

In this study, we showed that only Notch3 but not the other Notch receptors behaves as a dependence receptor. Similar observation is also found in other dependence receptors, such as Trka and Trkc which behave as dependence receptors but not TrkB. Indeed, only Notch3 but not Notch1 neither Notch2 is necessary for the cell death induced by DAPT in HUVEC, which indicates that Notch3 plays a different role in endothelial cell death comparing to other Notch receptors. Furthermore, a common transmembrane domain presenting  $\alpha$  helices structure, the Double dependence receptor-Associated Receptor Transmembrane (DART) motif is found in all the dependence receptors (del Rio et al., 2007; Delcros and Mehlen, 2013) (Table 7). And only Notch3 presents this DART motif but not the other Notch receptors. This motif enables the caspase to cleave on its intracellular domain. Furthermore, Notch3 is the only Notch receptor to present the caspase cleavage motif identified in the laboratory (Figure 29). Moreover, Notch3 has been shown to arise from the second duplication of Notch1 which suggests that the dependence receptor function of Notch3 might be probably acquired lately during evolution.

Table 3. 16 human proteins found to display the DART motif.

| Entry Name | Accession # | Protein Name  | TM Location | DART Location | Subcellular Location         | Function (per Swiss-Prot)   |
|------------|-------------|---|-------------|---------------|------------------------------|---|
| APLP2      | Q06481      | Amyloid-like protein 2 [Precursor]                    | 693-716     | 698-716       | Type I membrane protein (MP) | May play a role in the regulation of hemostasis   |
| CS001      | O15165      | Protein C18orf1                                       | 65-85       | 67-85         | Type Ib MP                   | May confer susceptibility to schizophrenia  |
| BM88       | Q8N111      | BM88 antigen  | 126-146     | 126-144       | Type IV MP                   | Involved in neuroblastoma cell differentiation (by similarity)                                      |
| NOTC3      | Q9UM47      | Neurogenic locus notch homolog protein 3              | 1644-1664   | 1646-1664     | Type I MP                    | Receptor for membrane-bound ligands Jagged1, Jagged2 and Delta1 to regulate cell-fate determination |
| EPHB3      | P54753      | Ephrin type-B receptor 3                              | 560-580     | 559-577       | Type I MP                    | Receptor for members of the ephrin-B family   |
| TR10B      | O14763      | Tumor necrosis factor receptor superfamily member 10B | 211-231     | 213-231       | Type I MP                    | Receptor for TNFSF10/TRAIL  |
| IL3R1      | P78552      | Interleukin-13 receptor alpha-1 chain                 | 344-367     | 349-367       | Type I MP                    | Binds IL13 with a low affinity  |
| TF         | P13726      | Tissue factor   | 252-274     | 254-272       | Type I MP                    | Initiates blood coagulation by forming a complex with circulating factor VII or VIIa                |
| TR10D      | Q9UBN6      | Tumor necrosis factor receptor superfamily member 10D | 212-232     | 212-230       | Type I MP                    | Receptor for TRAIL  |
| LU         | P50895      | Lutheran blood group glycoprotein                     | 548-568     | 551-569       | Type I MP                    | Probable receptor. May mediate intracellular signaling. Member of the immunoglobulin superfamily IG |
| STX3       | Q13277      | Syntaxin-3  | 264-284     | 264-282       | Type IV MP                   | Potentially involved in docking of synaptic vesicles at presynaptic active zones                    |
| VAMP5      | O95183      | Vesicle-associated membrane protein 5                 | 73-93       | 75-93         | Type IV MP                   | May participate in trafficking events that are associated with myogenesis                           |
| COMT       | P21964      | Catechol O-methyltransferase                          | 7-26        | 7-25          | Type II MP                   | Catalyzes the O-methylation of catecholamine neurotransmitters and catechol hormones                |
| LEUK       | P16150      | Leukosialin   | 254-276     | 255-273       | Type I MP                    | Physicochemical properties of the T-cell surface and lectin binding                                 |
| NEO1       | Q92859      | Neogenin  | 1106-1126   | 1104-1122     | Type I MP                    | Receptor for repulsive guidance molecule  |
| TACD2      | P09758      | Tumor-associated calcium signal transducer 2          | 275-297     | 277-295       | Type I MP                    | May function as growth factor receptor  |

Sixteen human proteins were discovered that display high-scoring matches for the DART motif when the Swiss-Prot database was searched using the MAST software program. Table 3 shows the transmembrane location and the location of the DART motif. Data taken from the Swiss-Prot database. All accession numbers are from Swiss-Prot.

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Table 7. Sixteen human proteins display the DART motif in their transmembrane domain (A Novel Motif Identified in Dependence Receptors- Plos One, 2007).

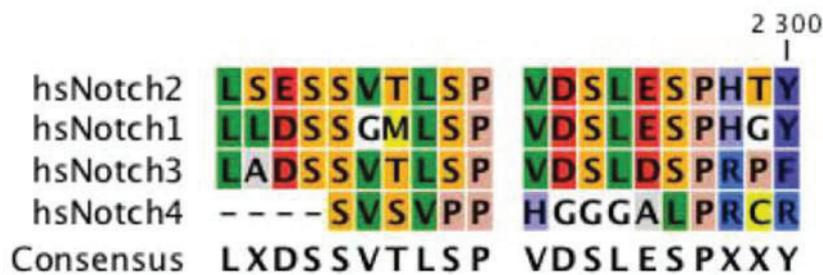


Figure 29. Only Notch3 but not other Notch Receptors contains the consensus of the caspase cleavage site in its intracellular domain.

### **5.3 Could other ligands besides Jagged1 inhibit the pro-apoptotic function of Notch3?**

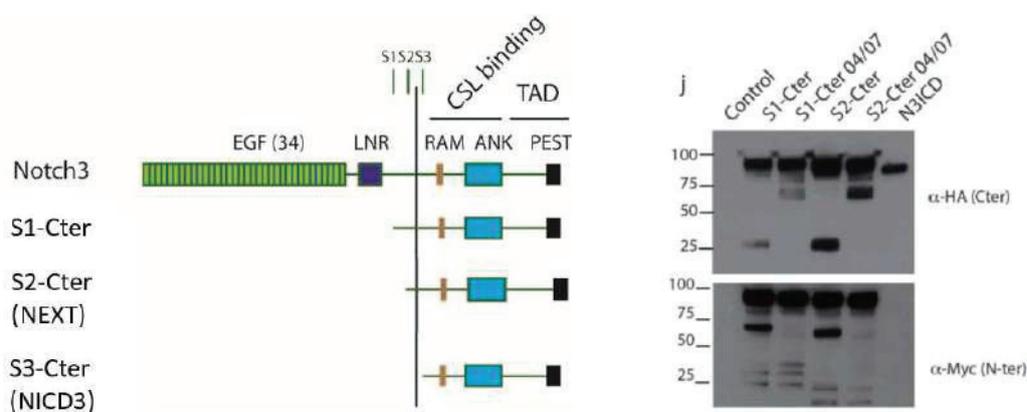
In this study, we showed that tumour-derived Jagged1 facilitates the survival of endothelial cells via inhibiting the pro-apoptotic function of Notch3 *in vitro* and promotes tumour angiogenesis *in vivo*. However, the role of other Notch ligands such as Dll4 and Dll1, which are also implicated in angiogenesis have not been investigated here. Endothelial overexpression of Dll4 is reported to reduce vascular response and inhibit tumour growth (Trindade et al., 2017). Nevertheless, impact of tumour-derived Dll4 on tumour angiogenesis has not yet been addressed. Moreover, Dll4 expression is also observed in neoplastic cells and it is up-regulated in some human cancers and it is associated with poor outcome in patients (Drouillard et al., 2016; Jubb et al., 2009; Miao et al., 2017). Therefore, it will be interesting to test whether tumour-derived Dll4 also has an impact on the pro-apoptotic function of Notch3 in tumour endothelial cells. To do that we can co-culture HUVEC with cancer cells with overexpressed or knock-down of DLL4 *in vitro*. We can also afterward do subcutaneous implantation with these cell lines on Notch3 WT and KO mice. Similar experiments could be also performed to test other Notch ligands, such as Jagged2, which is up-regulated in some human breast cancers (Negulescu et al.).

### **5.4 Identification of Notch3 pro-apoptotic pathway partners**

In this study, we showed that only Notch3 but neither Notch1 nor Notch2 can interact with caspase-9 to trigger apoptosis in 293T cell line in the absence of ligands. Moreover, ectopic expression of full length Notch3 but not N3ICD in 293T cells induces caspase-9 activity *in vitro*. It would be interesting to confirm these results in isolated mouse tumour endothelial cells especially in the caspase-9 knockout condition. However, it is still unclear which part of Notch3 is necessary for the interaction with caspase 9 and how caspase 9 is activated. Since Notch3 triggers apoptosis in a similar way to Ptc, it will be logical to test whether Notch3 can also interact with TUCAN which contains a CARD domain to recruit caspase 9 (Mille et al., 2009). Moreover, Crispr/cas9 library screening will be a potent tool to identify other pro-apoptotic partners of the caspase-activating complex induced by Notch3. To do that, it will be necessary to generate non-transformed normal cell lines which stably express Notch3 under the control

of doxycycline. Infecting these cell lines with a Crispr/Cas9 library will allow us to isolate and identify some potential partners of the Notch3 pro-apoptotic pathway.

As we mentioned previously, Notch3 is also cleaved by caspase. A putative caspase cleavage site is identified at DSLD (2104-2107) of the intracellular part of the receptor. Ectopic expression of S1-CterN3 and S2-CterN3 which can mimic the absence of ligand induces the cleavage by caspase and generates a small fragment of about 25kd in 293T cells. Furthermore, mutation of this cleavage site can no longer generate this small fragment (Figure 30). This observation resembles to the case of DCC. However, so far, we have not confirmed yet whether this cleavage is necessary to the apoptosis triggered by Notch3.

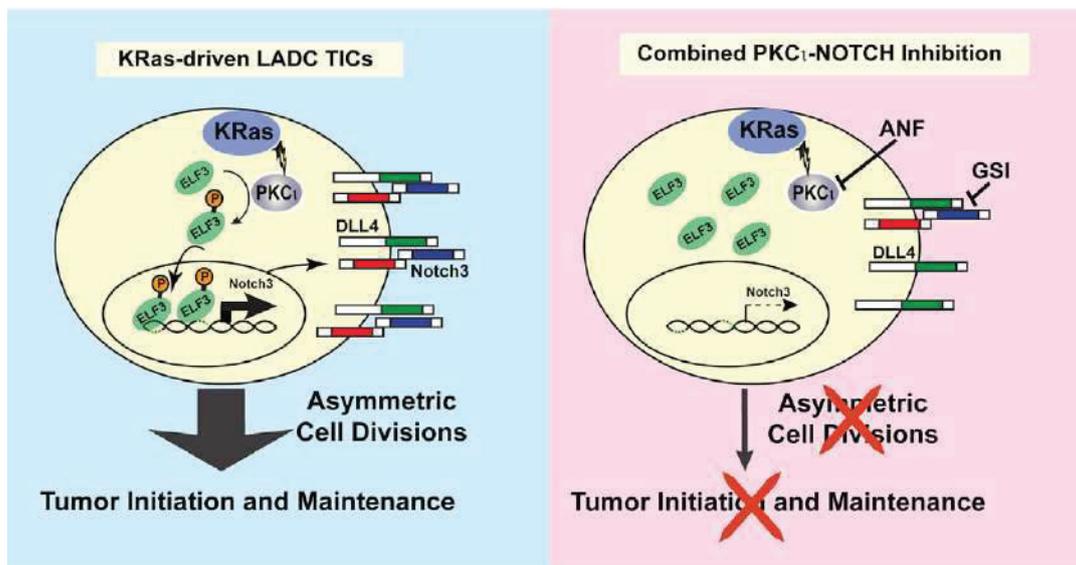


**Figure 30. Scheme representing the different versions of Notch3: S1-Cter (aa1573-Cterminus), S2-Cter (1631-Cterminus) and N3ICD (1664-Cterminus) are represented along with Notch3; Ectopic expression of S1-Cter Notch3 WT (S1-CterN3) or an S1-Cter version of Notch3 in which aspartic acids 2104 and 2107 have been mutated to asparagines (2104/2107) or a S2-Cter Notch3 construct with the same mutation or not in 293T cells.**

To investigate this question, it would be interesting to confirm this *in vivo*. To do that, we can generate a caspase cleavage site mutation knock-in mouse model like what has been done for DCC (Castets et al., 2011). Crossing this mouse with another mouse model which develops tumour spontaneously, can allow us to verify whether this caspase cleavage site is important for the pro-apoptotic function of Notch3 during tumorigenesis.

## 5.5 Identification of the mechanisms by which Notch3 is aberrantly expressed in tumour associated endothelial cells.

According to the literature, Notch3 expression is restricted to the vascular system, especially in vascular mural cells such as VSMC and pericytes. Its expression is also observed in the luminal progenitor cells of mammary gland (Lafkas et al., 2013). Whereas in this study, we discovered an unexpected expression of Notch3 in tumour associated endothelial cells firstly in human lung cancer samples which was confirmed afterward in  $Kras^{G12D/+}$  mutated lung tumours and in subcutaneous tumours of lung cancer cell line. This observation is indeed in line with an endothelial cell transcriptomic analysis done by a group in the university of Birmingham in 2013 (Herbert et al., 2013). However, the mechanism by which Notch3 is up-regulated in tumour endothelial cells is unclear. Some recent studies indicate that Notch3 expression is up-regulated in a pro-inflammatory setting in which Notch3 is regulated transcriptionally via the Stat3/Notch3/CD133. Blockade of IL-6 impairs Notch3 expression in breast cancer cells (Sansone et al., 2016). In addition, another study showed that in a *kras* mutated setting, protein kinase C $\alpha$  (PKC $\alpha$ ) phosphorylates ELF3 which in turn facilitates the recruitment of ELF3 on the promoter of Notch3 thereby activating its transcription (Figure 31) (Ali et al., 2016).

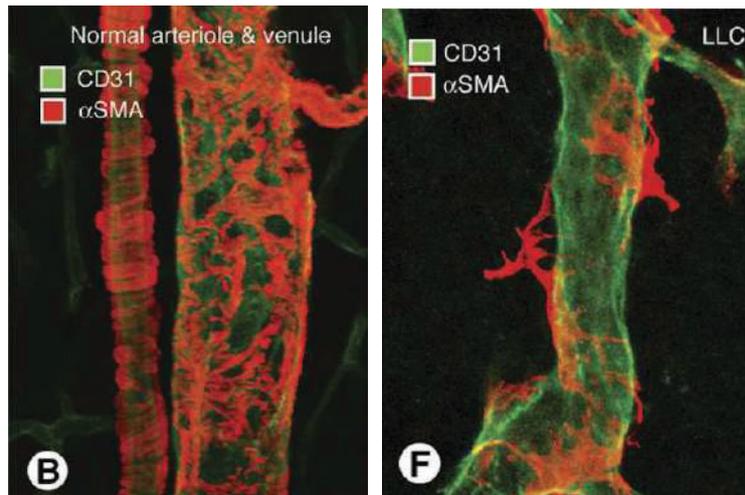


**Figure 31. PKC $\alpha$ -ELF3-NOTCH3 signaling: protein kinase C $\alpha$  (PKC $\alpha$ ) oncogene controls expression of NOTCH3 in *KRAS*-mediated lung adenocarcinoma (LADC). PKC $\alpha$  activates NOTCH3 expression by phosphorylating the ELF3 transcription factor and driving ELF3 occupancy on the *NOTCH3* promoter.**

Besides transcriptional modification, we found in the lab that Notch3 is lost in human breast cancers and this loss of expression could be due to the hypermethylation of the Notch3 promoter (Negulescu et al.). In addition, preliminary results in the lab showed that treating breast cancer cell lines with demethylating agent restores Notch3 expression. Therefore, it is important to understand how Notch3 is up-regulated in tumour endothelial cells. First of all, it would be interesting to verify whether there is a modification of IL-6 expression in our mouse tumour samples. If so, blockade of IL-6 might inhibit Notch3 expression in tumour endothelial cells which in turn impair the apoptosis induced by DAPT. On the other hand, preliminary data in the lab showed that treating HUVEC with demethylating agent can up-regulate Notch3 expression. Thus, it would also be interesting to verify whether the promoter of Notch3 is methylated in healthy endothelial cells comparing to tumour endothelial cells. If so, treating Notch3 WT mice bearing LLC1 subcutaneous grafted tumour with combination of demethylating agent and inhibitors of  $\gamma$ -secretase might have an enhanced anti-tumour effect. Taken together, identification of the mechanism through which Notch3 is aberrantly expressed in tumour endothelial cells will be necessary to better understand the role of Notch3 in tumour microenvironment which may in turn let us better target the Notch pathway during tumorigenesis.

## **5.6 Genomic and transcriptomic analysis of tumour associated endothelial cells**

During tumour progression, tumour neovascularization facilitates the tumour growth by providing oxygen and nutrient. Generated in a pathological condition, usually associated with inflammation and interaction with oncogenic mutation in cancer cells, the tumour vessels are phenotypically as well as genotypically different compared to normal blood vessels. For instance, it has been shown that the well studied death receptor DR5 is expressed in tumour endothelial cells but not in normal endothelial cells (Wilson et al., 2012). Moreover, many studies have shown that tumour vessels are much less organized hierarchically than normal vessels as they present less adherent junction between endothelial cells, less coverage of mural cells on the endothelium to support the vessel, the permeability is usually increased (Goel et al., 2011) (Figure 32). In line with this, the instability of the tumour endothelium genome and the reciprocal interaction with cancer cells make the cancer treatment more complicated (Palumbo et al., 2015).



**Figure 32. Tumour vessels are less supported. Left: endothelium (green) of normal vessel are well covered by mural cells such as vascular smooth muscle cell (red); Right: endothelium of tumour vessel are poorly supported by VSMA.**

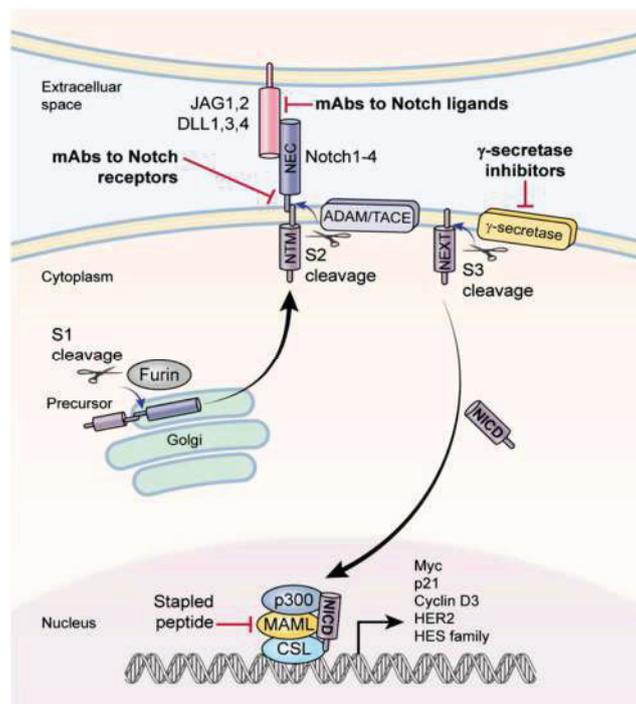
However, to date, few studies have been done on large genomic and transcriptomic analyse of tumour endothelium. To better understand how the crosstalk between the tumour endothelium and tumour progression and identify new targets for cancer therapy, genomic and transcriptomic sequencing of isolated tumour endothelial cells from human cancer samples or spontaneous mouse tumours will be a powerful tool. We can afterward retest these putative targets by generating knockout mice *in vivo*. Moreover, not only cancer cells, but also tumour stroma cells present a high heterogeneity. Therefore single cell DNA or RNA sequencing of tumour endothelial cells may explain why some patients are resistant to the anti-angiogenic therapy and can also help us to establish a signature profile or even some dignostic markers.

## 5.7 Reconsideration and new insights on targeting Notch pathway for cancer treatments.

Notch signalling is widely implicated in tumorigenesis, not only in cancer cells proliferation, but also in tumour angiogenesis and the maintenance of cancer stem cells. Consistent with this, the Notch signalling is reported to be highly deregulated in human hematopoietic malignancies and solid tumours. Besides Notch1 in T-ALL, few mutations of the Notch family are reported in solid tumours (Egloff and Grandis, 2012). Nevertheless, deregulated expression of WT Notch receptors and Notch ligands, aberrant activation of the Notch pathway have been found in an increasing number of human solid tumours including pancreatic, breast, prostate, lung, glioblastoma multiforme (GBM), sarcomas, cervical, melanoma, head and neck, renal cancers, and gastrointestinal tumours. Therefore, many efforts have been made to target the Notch pathway for cancer treatment. To date, molecules targeting the Notch pathway are classified into two main categories:  $\gamma$  secretase inhibitors (GSI) and mAbs to Notch receptors or ligands (Figure 33) (Table 8).

GSIs which inhibit the Notch signalling in a general way, have shown a promising anti-tumour effect in patients with advanced or metastatic solid tumours (Takebe et al., 2014). However, GSI is not specific to Notch receptors, moreover, side effects of GSI, especially the gastrointestinal toxicity has slowed down its clinical application (Olsauskas-Kuprys et al., 2013). Thus, a more adequate dose and a better intermittent schedule should be adapted to increase the life quality and lifespan of patients. To target more specifically the Notch pathway, mAbs against Notch receptors and Notch ligands have been developed for clinical trial. The Notch1/Dll4 pathway was particularly targeted in tumour angiogenesis. Anti-Dll4 mAb treatment increased sprouting angiogenesis but resulted in reduced tumour growth due to the non-functional angiogenesis in mouse model. However, continuous dosing with anti-DLL4 mAbs has resulted in new vessels formation and angioma in rats (Dufraine et al., 2008; Yan et al., 2010). This effect has been shown to be decreased when patients received VEGF inhibitors, which resulted in more anti-tumour effect. In line with this, Notch1 decoy N1<sub>10-24</sub> targeting EGF repeat 10-24, which block the Jagged1/Jagged2-mediated Notch1 signalling results in decreasing tumour angiogenesis and tumour growth. On the other hand, N1<sub>1-13</sub> targeting EGF repeat 1-13, by blocking the Dll1/DLL4-mediated Notch1 signalling, it promotes a dysfunctional tumour angiogenesis and inhibits tumour growth (Kangsamaksin et al., 2015).

These results are very encouraging, providing us the possibility to target specifically not only one of the Notch receptors but also a specific receptor/ligand interaction. In our study, we showed that tumour-derived Jagged1 increases tumour endothelial cell survival via inhibiting the pro-apoptotic function of Notch3. In line with this, expression of Jagged1 is elevated in many cancers which facilitates tumour progression (Li et al., 2014). Therefore, according to the dependence receptor function of Notch3, decoy or mAb specific to Notch3/Jagged1 could have a potent anti-tumour effect via not only interrupting the tumour angiogenesis but also inhibiting the canonical Notch signalling in tumour cells.



**Figure 33. Agents in clinical development targeting Notch signalling pathway. Two main categories of Notch inhibitors are currently in early clinical development:  $\gamma$ -secretase inhibitors (GSI) and monoclonal antibodies against Notch receptor and ligands. (Targeting Notch signaling pathway in cancer: Clinical development advances and challenges- *Pharmacology & Therapeutics*, 2014).**

Drugs in clinical development.

| Class                           | Drug(s)                               | Target                  | Phase        | Enrollment or estimated | Primary endpoint; subjects                                   | Trial status  |                               |
|---------------------------------|---------------------------------------|-------------------------|--------------|-------------------------|--|---|-------------------------------|
| γ-Secretase inhibitors          | MK0752                                | γ-Secretase             | I            | 50                      | Safety/efficacy; relapsed or refractory T-ALL/T-LL           | Terminated  |                               |
|                                 | MK0752                                |                         | I            | 103                     | Safety/MTD; advanced breast cancer/advanced solid tumors     | Completed   |                               |
|                                 | MK0752                                |                         | I            | 33                      | Safety; recurrent or refractory CNS malignancies             | Terminated  |                               |
|                                 | MK0752 + Docetaxel                    |                         | I/II         | 30                      | DLT; locally advanced or metastatic breast cancer            | Completed   |                               |
|                                 | MK0752 + Gemcitabine                  |                         | I/IIa        | 60                      | Safety/MTD; stage IV pancreatic cancer                       | Recruiting  |                               |
|                                 | MK0752                                |                         | I            | 30                      | Notch response signature; healthy young adults               | Completed   |                               |
|                                 | MK0752                                |                         | Pre-surgical | 20                      | Safety/tolerability; early stage ER-positive breast cancer   | Unknown   |                               |
|                                 | RO4929097 <sup>b</sup>                |                         | -            | -                       | -  | -   | Terminated                    |
|                                 | PH3084014                             |                         | I            | 60                      | Safety/DLT; advanced solid Tumors/T-ALL/T-LL                 | Ongoing   |                               |
|                                 | BMS-906024                            |                         | I            | 95                      | Safety/tolerability; advanced solid tumors                   | Recruiting  |                               |
|                                 | BMS-906024                            |                         | I            | 42                      | Safety/tolerability; T-ALL/T-LL                              | Recruiting  |                               |
|                                 | BMS906024 + chemotherapy <sup>c</sup> |                         | Ib           | 60                      | Safety; advanced or metastatic solid tumors                  | Recruiting  |                               |
|                                 | LY3039478                             |                         | I            | 80                      | DLTs; advanced or metastatic cancer                          | Recruiting  |                               |
|                                 | mAbs to Notch receptors or ligands    |                         | OMP-59R5     | Notch2/3                | I  | 44  | Safety; advanced solid tumors |
| OMP-59R5 + Nab-P & Gemcitabine  |                                       | Notch2/3                | Ib/II        | 154                     | DLT/MTD; first line for stage IV pancreatic cancer           | Recruiting  |                               |
| OMP21M18                        |                                       | DLL4                    | I            | 30                      | Safety; advanced solid tumors                                | Recruiting  |                               |
| OMP21M18 + FOLFIRI <sup>c</sup> |                                       | DLL4                    | Ib           | 32                      | MTD; first or second line for advanced colorectal cancer     | Recruiting  |                               |
| OMP21M18 + CP <sup>c</sup>      |                                       | DLL4                    | Ib           | 32                      | MTD; first line for advanced non-squamous NSCLC              | Recruiting  |                               |
| OMP21M18 + Gemcitabine          |                                       | DLL4                    | Ib           | 40                      | MTD; first line for advanced or metastatic pancreatic cancer | Recruiting  |                               |
| REGN421                         |                                       | DLL4                    | I            | 80                      | Safety/tolerability; advanced solid tumors                   | Recruiting  |                               |
| OMP52M51                        |                                       | Notch1                  | I            | 33                      | Safety; relapsed or refractory solid tumors                  | Recruiting  |                               |
| OMP52M51                        |                                       | Notch1                  | I            | 53                      | Safety; relapsed or refractory lymphoid malignancies         | Recruiting  |                               |
| γ-Secretase modifier            |                                       | MPC-7869/R-flurbiprofen | γ-Secretase  | I/II                    | Unknown  | TTP <sup>c</sup> ; localized prostate cancer at risk of recurrence following radiation therapy and/or prostatectomy | Unknown                       |

**Table 8. Drugs targeting Notch Pathway in clinical development.** <sup>a</sup>, Clinical trial registration and information at [www.clinicaltrials.gov](http://www.clinicaltrials.gov). <sup>b</sup> Clinical trials with RO4929097 are not shown due to the termination of its development. <sup>c</sup> Chemotherapy, weekly paclitaxel, FOLFIRI or carboplatin plus paclitaxel; FOLFIRI, FOLolinic acid (leucovorin), 5-Fluorouracil (5-FU) plus IRInotecan (irinotecan) or carboplatin plus paclitaxel; CP, carboplatin and pemetrexed; Nab-P, Nab-paclitaxel; TTP, time to progression (Targeting Notch signaling pathway in cancer: Clinical development advances and challenges- *Pharmacology & Therapeutics*, 2014).



## **IV. Annexe**



ARTICLE

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OPEN

# Non-canonical NOTCH3 signalling limits tumour angiogenesis

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Notch signalling is a causal determinant of cancer and efforts have been made to develop targeted therapies to inhibit the so-called canonical pathway. Here we describe an unexpected pro-apoptotic role of Notch3 in regulating tumour angiogenesis independently of the Notch canonical pathway. The Notch3 ligand Jagged-1 is upregulated in a fraction of human cancer and our data support the view that Jagged-1, produced by cancer cells, is inhibiting the apoptosis induced by the aberrant Notch3 expression in tumour vasculature. We thus present Notch3 as a dependence receptor inducing endothelial cell death while this pro-apoptotic activity is blocked by Jagged-1. Along this line, using Notch3 mutant mice, we demonstrate that tumour growth and angiogenesis are increased when Notch3 is silenced in the stroma. Consequently, we show that the well-documented anti-tumour effect mediated by  $\gamma$ -secretase inhibition is at least in part dependent on the apoptosis triggered by Notch3 in endothelial cells.

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**T**umour angiogenesis has been considered as an attractive target for cancer therapy for more than forty years. However, clinical results using drugs targeting tumour angiogenesis are inconsistent and often disappointing<sup>1</sup>. Most anti-angiogenic therapies target the vascular endothelial growth factors (VEGFs) signalling pathways, in which VEGFs activate VEGF receptors (VEGFRs) on endothelial cells to regulate vascular growth in both developing tissues and growing tumours. Notch signalling is a major regulator of these processes. Four Notch receptors (Notch1-4) have been described in mammals. Notch receptors are single-pass type I transmembrane non-covalently linked heterodimer coded by a single precursor, which is cleaved by furins. The Notch pathway activation follows the binding of the transmembrane ligands of the Delta/Serrate/LAG-2 (DSL) family, Delta-like and Jagged to Notch receptors. In mammals, three Delta-like ligands (Dll1, Dll3 and Dll4) and two Jagged ligands (Jag-1 and Jag-2) have been identified. The well-described so-called ‘canonical pathway’ depends on a strictly controlled proteolytic cascade induced by ligand binding: an S2 cleavage by metalloproteases followed by an S3 cleavage mediated by a presenilin- $\gamma$ -secretase complex. These proteolytic cleavages release the intracellular domain of the Notch receptor (NICD), which then translocates into the nucleus to mediate target genes activation<sup>2</sup>.

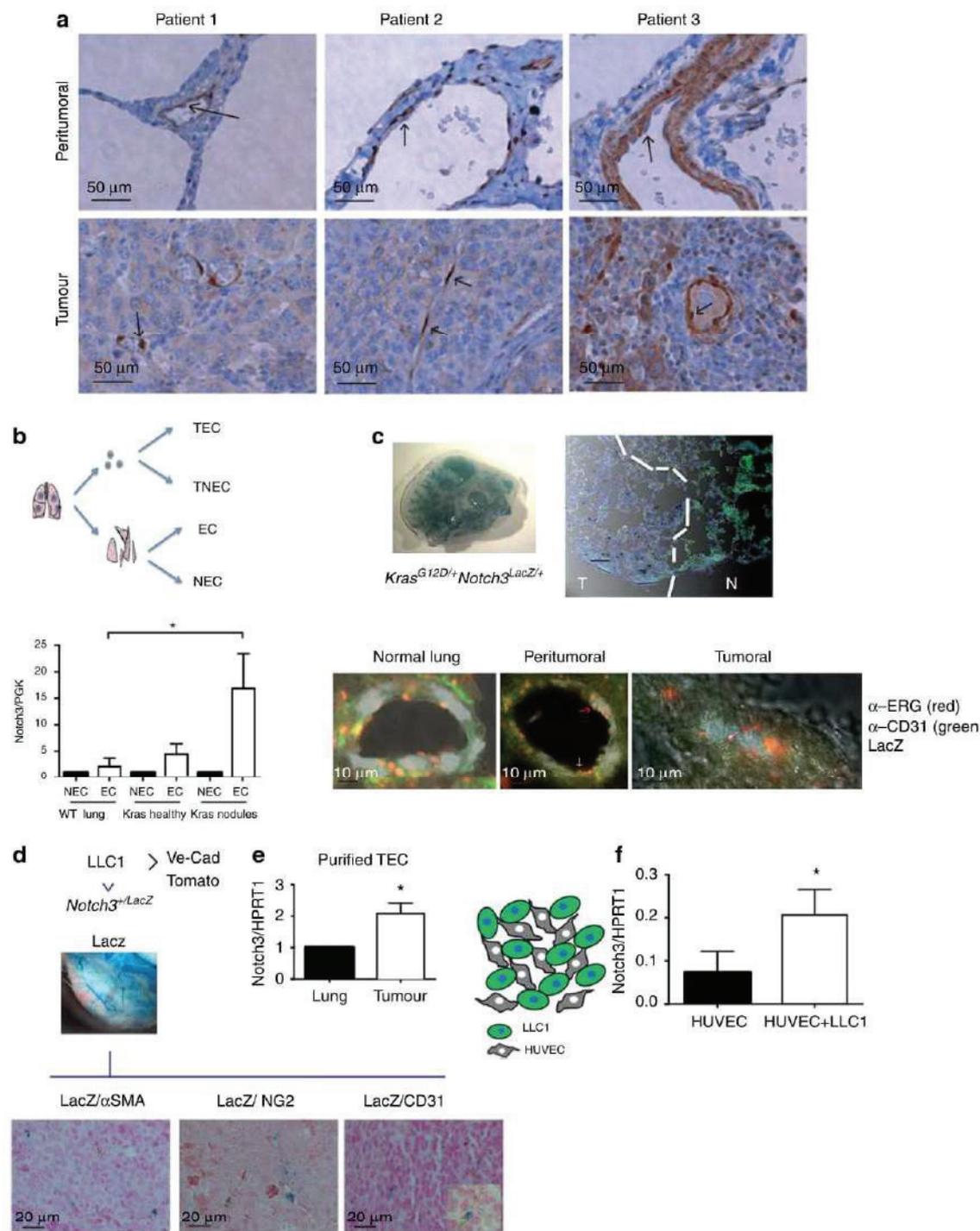
Notch signalling has been implicated in cancer, with observed genetic alterations in a large number of hematopoietic and solid tumours<sup>3</sup>. As the presenilin- $\gamma$ -secretase complex activity is necessary for the activation of the canonical signalling pathway,  $\gamma$ -secretase inhibitors such as DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester) derivatives have been proposed as targeted therapies for treatment of pathologies such as T-cell acute lymphoblastic leukemia. However, such therapeutic approaches have so far been limited due to intestinal toxicity<sup>4</sup>. Other approaches to inhibit the Notch canonical pathway are thus in development with strategies including antibodies raised specifically against individual Notch receptors<sup>5,6</sup>.

Notch signalling is also a major regulator of angiogenesis as Dll4-mediated Notch activation controls the expression of the VEGFRs and therefore limits endothelial cells sprouting and proliferation<sup>7,8</sup>. However, whereas the role of Notch signalling is well described in developmental angiogenesis, its role in tumour angiogenesis is not clearly understood. *In vitro*, Notch inhibition has been shown to induce endothelial cell death<sup>9</sup> as well as vascular sprouting<sup>10</sup>. *In vivo*, Notch inhibition using chemical inhibitors or Notch1 ectodomain is generally associated with endothelial cell death and reduced vascularization<sup>11–13</sup>. In contrast, anti-ligand approaches such as anti-Dll4 treatments produces non-productive angiogenesis through increased endothelial cells sprouting<sup>14</sup>. These paradoxical observations could suggest that the role of Notch in tumour angiogenesis cannot be completely explained by canonical Notch signalling. In contrast to other Notch receptors, Notch3 expression is restricted to the vasculature in physiological condition. Notch3 mutations are associated with CADASIL<sup>15</sup> (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) and Notch3 knockout mice are more susceptible to ischemic stroke<sup>16</sup> whereas they are less susceptible to pulmonary hypertension<sup>17</sup>. These studies show that even if *Notch3* mutant mice have no major phenotype in developmental angiogenesis, Notch3 is involved in pathological angiogenesis. However, its role in tumour angiogenesis has never been studied. In the disorganized tumour vasculature, tumour endothelial cells show a different phenotype than normal endothelial cells<sup>18</sup>. Interestingly, Notch3 has been shown to be upregulated in human lung cancer-associated endothelial cells<sup>19</sup> and this led us to evaluate the role of Notch3 in endothelial cell in cancer development. While analysing the importance of Notch3 in

the stroma during tumour progression, we observed an unexpected pro-apoptotic activity of Notch3. We describe Notch3 as a dependence receptor in endothelial cells. Such receptors that include the netrin-1 receptors DCC and UNC5H (ref. 20) or the Hedgehog receptors Ptc and CDON<sup>21,22</sup> share the ability to actively transduce a death signal in settings of ligand limitation, thus creating a state of cellular dependence to the presence of ligand for cell survival. This pro-apoptotic activity has been proposed to act as a negative constrain for tumour progression by controlling cancer cell death<sup>23,24</sup>. We propose here that Notch3 by acting as a dependence receptor in endothelial cells regulate tumour angiogenesis by regulating endothelial cell death.

## Results

**Notch3 is expressed in tumour associated endothelial cells.** We first investigated Notch3 expression in a small panel of human lung cancers by immunohistochemistry. In all the studied samples (11 adenocarcinoma (ADC) and 10 squamous cell carcinoma (SCC)), the expression of Notch3 was very strong in the vasculature (Supplementary Fig. 1a). Conversely, the cancer cell expression of Notch3 was very heterogeneous between patients but also within the same patient (Supplementary Fig. 1a). SCC showed the strongest Notch3 expression in the cancer cells, however, only a small fraction of patients showed nuclear expression (4/10 for SCC and 2/11 for ADC) (Supplementary Fig. 1a,b). The role of Notch signalling and in particular Notch3 in the epithelial compartment of tumours and more specifically of non-small cell lung cancers has been extensively studied<sup>25,26</sup>. However, Notch3 implication in tumour vasculature has not been addressed. We thus focused on the vascular expression of Notch3 in these patients. In the patients for whom we could observe histological normal peritumoral tissue, we noticed that the expression of Notch3 was localized, as described previously<sup>17</sup>, in the vascular smooth muscle cells or in the mural cells of smaller vessels (Fig. 1a). However, in the malignant part, we could observe Notch3 expression in the endothelial cells (EC) (Fig. 1a). This prompted us to investigate a possible role of this aberrant expression of Notch3 in tumour endothelial cells. To study this role, we first assessed whether this aberrant expression was also observed in mouse model of lung cancers. We first purified EC from lung adenomas in the *Kras*<sup>+/G12D</sup> hit and run mice model characterized previously<sup>27</sup>. Whereas in wild-type mice or in the healthy part of lung from *Kras*<sup>+/G12D</sup> mice, no or little expression of Notch3 was detected in EC-enriched fraction, we observed an over-expression of Notch3 in the EC-enriched fraction from the tumour nodules (Fig. 1b). We next used the LacZ reporter to monitor Notch3 expression in the Notch3 LacZ knock-in mice described previously<sup>16</sup>. We confirmed, in this model, that the Notch3/LacZ mRNA fusion was expressed to a similar amount than the wild-type allele (Supplementary Fig. 2a). We also used an anti- $\beta$ -galactosidase antibody to check the staining of the LacZ enzymatic reaction and confirmed expression in the smooth muscle cells in healthy lungs (Supplementary Fig. 2b). As described by others, we observed that the expression of Notch3 was restricted to mural cells and Notch3 was absent in endothelial cells in normal vasculature. Notch3 was indeed mostly associated to  $\alpha$ -smooth-muscle actin ( $\alpha$ SMA) expressing cells surrounding big vessels and to a lesser extend to NG2 (neural/glial antigen 2) expressing mural cells in smaller vessels as seen in lungs from *Notch3*<sup>+/LacZ</sup> mice. We then looked at Notch3 expression in the adenocarcinoma from *Kras*<sup>+/G12D</sup>-*Notch3*<sup>LacZ/+</sup> mice. We confirmed the data obtained by purifying tumour-associated endothelial cells. Indeed, the LacZ staining is detected in the tumour and in healthy lung, the LacZ staining is not associated with ERG staining—that is, ERG



**Figure 1 | Notch3 is aberrantly expressed in tumour endothelial cells.** (a) Notch3 immunohistochemistry on peritumoral and tumour part of three sections from non-small cell lung cancers patients. (b) Quantitative RT-PCR was performed to measure Notch3 mRNA expression in endothelial cell enriched fraction (EC, CD31-expressing purified cell population) or non endothelial cell (NEC) purified from lung dissected from wild-type mice, the healthy part of tumour-bearing lung dissected from *Kras* mice or from the nodules dissected from the lung of *Kras* mice ( $n=6$  WT lungs,  $n=5$  *Kras* lungs, mean  $\pm$  s.e.m., ordinary one-way ANOVA, multiple comparisons). (c,d)  $\beta$ -galactosidase staining was performed on lungs or LLC1 tumour whole mount from *Kras*<sup>G12D/+</sup> (c) or WT mice (d) mice before inclusion in paraffin and immunohistochemistry staining for ERG, CD31, SMA, NG2 as indicated. (e) Quantitative RT-PCR was performed to measure Notch3 mRNA expression in endothelial cell FACS-sorted from lung or tumour dissected from *Cdh5:Cre*<sup>ERT2</sup> $\times$ Tomato (Ve-Cad Tomato) mice ( $n=3$  tumours, mean  $\pm$  s.e.m., unpaired *t*-test). (f) HUVEC cells were co-cultured for 48 h with LLC1 cells stably expressing GFP before being FACS sorted. DAPI (alive cells) GFP negative (HUVEC) cells were used to prepare mRNA and Notch3 expression was measured by quantitative RT-PCR ( $n=3$  independent experiments, paired ratio *t*-test).

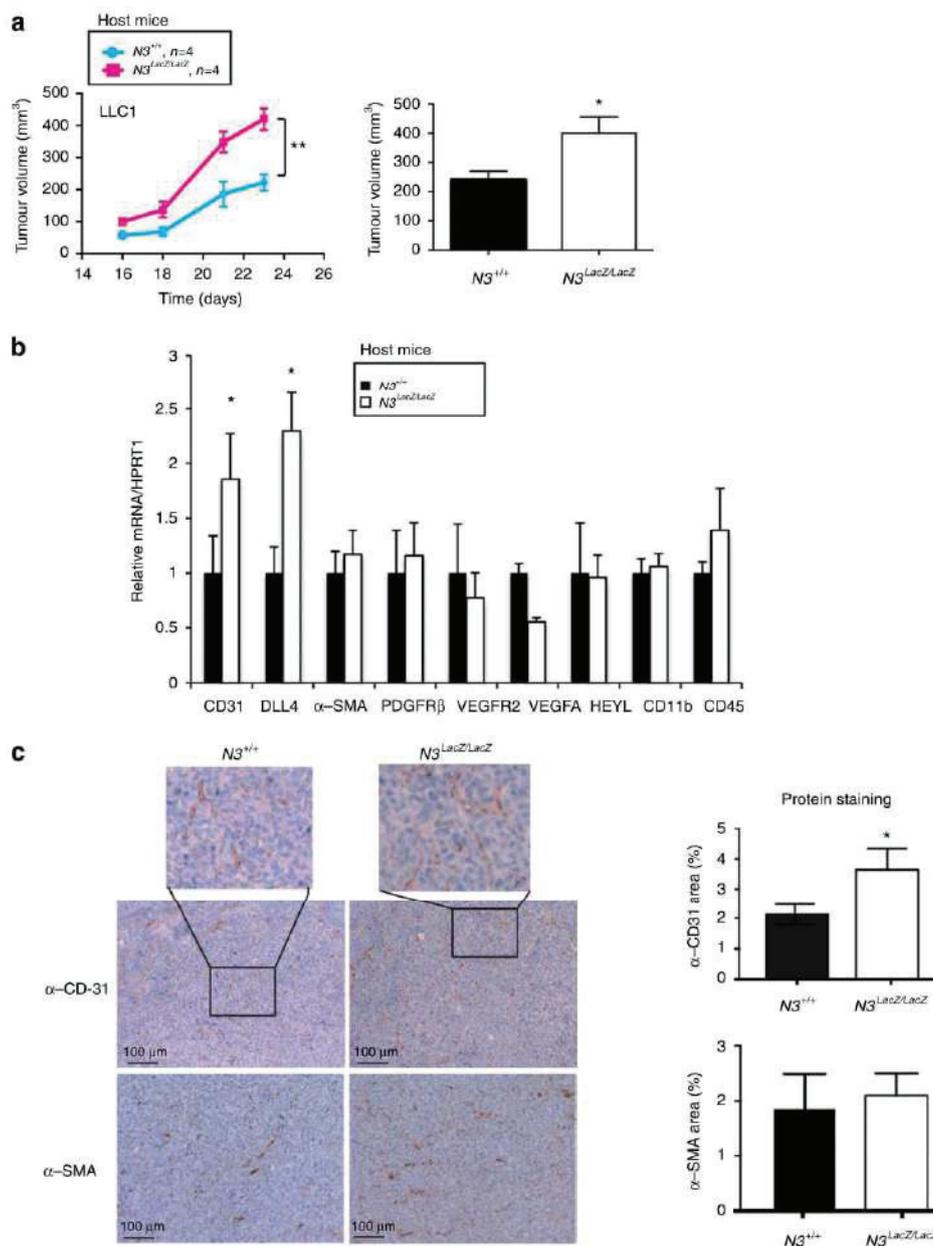
staining was reported to be strongly specific for EC<sup>28</sup>. However, in the peritumoral part, we observed ERG staining in LacZ positive cells (red arrow) (Fig. 1c). We next injected LLC1 syngeneic lung cancers cells in *Notch3*<sup>+/LacZ</sup> to assess expression of Notch3 in subcutaneous graft. We confirmed that Notch3 is expressed in the vasculature of the grafted tumours and as observed in the tumour nodules from the *Kras*<sup>+/G12D</sup> mice, we observed an aberrant expression of Notch3 in tumour-associated EC (Fig. 1d,e): Notch3 co-localizes with CD31 (endothelial cell marker platelet/endothelial cell adhesion molecule, PECAM-1) but not with mural cell markers  $\alpha$ SMA or NG2 (Fig. 1d). We further confirmed the up-regulation of Notch3 mRNA in purified tumour-associated endothelial cells from subcutaneous injected LLC1 in *Cdh5:Cre*<sup>ERT2</sup> xTomato mice allowing FACS sorting of tumour-associated endothelial cells (Fig. 1e). The LLC1 model thus provides a good model to study the functional impact of Notch3 aberrant expression on tumour vasculature. Furthermore, co-culturing LLC1 cells with HUVEC was sufficient to induce an upregulation of Notch3 in the endothelial cells, showing that the epithelial cancer cells are sufficient to induce Notch3 expression in endothelial cells (Fig. 1f).

#### Stroma specific Notch3 silencing promotes tumour angiogenesis.

We next assessed the role of this aberrant expression of Notch3 in tumour vasculature by establishing a model in which Notch3 is silenced only in the stroma but not in the tumour cells. As we started with observations in human lung carcinomas, we chose the murine lung carcinoma LLC1 syngeneic grafts in wild-type and in *Notch3*<sup>LacZ/LacZ</sup> mice. As shown in Fig. 2a, the absence of stromal Notch3 was associated with an increase of tumour growth. This suggests that the endothelial expression of Notch3 limits tumour angiogenesis. This observation was also true in another model of syngeneic graft, the E0771 mammary gland tumour model, although to a lesser extent (Supplementary Fig. 3a). In line with a role of Notch3 in tumour associated endothelial cells, we observed an increase in CD31 and DLL4 expression in tumours from *Notch3*<sup>LacZ/LacZ</sup> mice (Fig. 1b and supplementary Fig. 3b), but no change in  $\alpha$ SMA or PDGFR $\beta$  (Beta-type platelet-derived growth factor receptor), two pericyte markers (Fig. 2b). As Notch3 has been reported to be expressed in certain immune cells<sup>29</sup>, we looked for the expression of CD11b and CD45 that remained unchanged (Fig. 2b). We next looked at the vascularization of tumours grown in the absence of stromal Notch3 expression. CD31 staining of tumours grown in the wild-type mice or in the *Notch3*<sup>LacZ/LacZ</sup> mice showed an increased vascularization in the latter (Fig. 2c). Furthermore expression of  $\alpha$ SMA in these tumours was unchanged (Fig. 2c). This suggests that the aberrant expression of Notch3 in tumour endothelial cells could limit tumour angiogenesis whereas the absence of Notch3 in vascular smooth muscle cells has no effect. Furthermore this effect seems to be independent of the normal role of Notch3 in smooth-muscle cells.

**Notch3 behaves as a dependence receptor.** To understand how the absence of Notch3 would impact the tumour vascularization, we studied *in vitro* human umbilical vein endothelial cells (HUVEC). As described previously<sup>9</sup>, these cells express a low level of Notch3 which is almost entirely cleaved into N3ICD as treatment with DAPT completely abolished the presence of a 75 kDa band recognized by a C-terminal antibody (Supplementary Fig. 4a). We then asked what would be the consequence of an upregulation of Notch3 in these cells that would mimic the aberrant expression of Notch3 observed in lung cancers-associated endothelial cells. We first used electroporation in HUVEC cells (with 80% electroporation efficiency

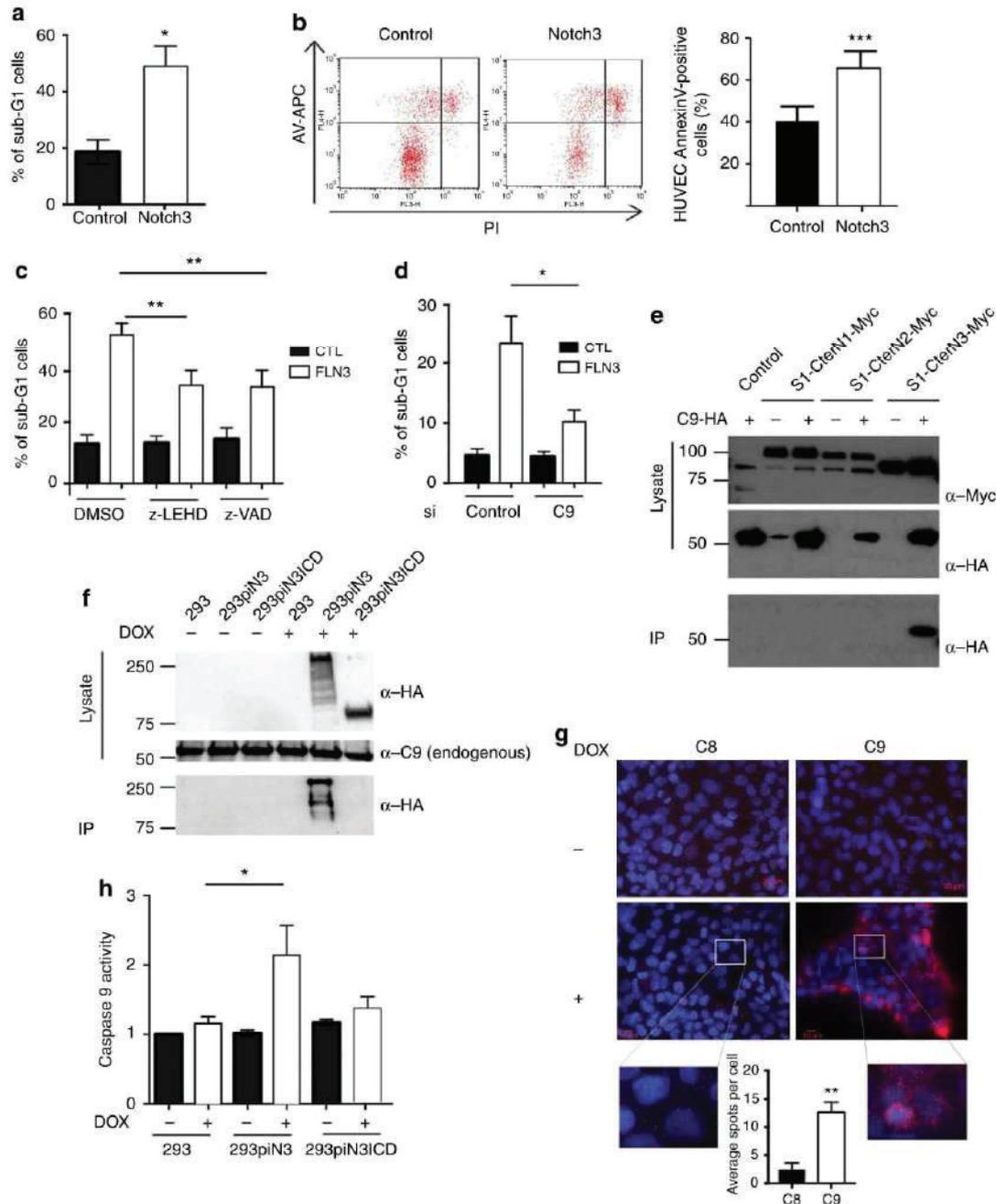
(Supplementary Fig. 4b)). As shown in Fig. 3, Notch3 forced expression in HUVECs triggered cell death as evidenced by an increase of the sub-G1 cell population (Fig. 3a) and Annexin-V-positive cell population (Fig. 3b). This cell death is probably, at least in part, apoptosis as it is inhibited by general caspase inhibitor z-VAD-fmk (Fig. 3c). At this stage we cannot however exclude that Notch3-induced cell death is not only apoptosis as inhibition of cell death by caspase inhibitors is not complete. Interestingly, N3ICD did not induce cell death (Supplementary Fig. 4c). Furthermore, although Notch3 level is low in HUVEC under normal condition, knocking-down Notch3 in a setting of network formation in matrigel was sufficient to inhibit significantly apoptosis during network regression (Supplementary Fig. 4d). We further used the S1-Cter Notch3 construct (a truncated version of Notch3 (S1-Cter Notch3; aa1573 (furin cleavage site) to the C terminus, Supplementary Fig. 4e) as it mimics the absence of ligands and also helps bypassing the possible effect of varying levels of ligand expression in different cellular models. S1-Cter Notch3 induced very low Notch transcriptional activity in comparison to N3ICD (Supplementary Fig. 4f). Whereas S1-CterN3 expression induced caspase-3 cleavage (Supplementary Fig. 4g), electroporation of N3ICD, or of CBF1-VP16 (which both activates canonical Notch signalling in HUVEC (Supplementary Fig. 4f)), or of DNMM1 (Dominant negative Mastermind-like, which inhibits endogenous Notch signalling (Supplementary Fig. 4f)) had no effect on induction of cell death (Supplementary Fig. 4g,h), suggesting that canonical Notch signalling is not involved in this process. Of interest, S1-Cter Notch3 mutant, that fails to interact with the CBF1 transcription factor (S1-Cter WFP-LAA), is still able to induce caspase-3 cleavage (Supplementary Fig. 4h) supporting the view that the canonical Notch3 signalling pathway is not involved here. Such ability of a transmembrane receptor to trigger apoptosis in a setting of absence of ligand, recalls the behaviour of dependence receptors<sup>30</sup>. Such receptors that include the netrin-1 receptors DCC and UNC5H (ref. 31) or the Hedgehog receptors Ptc and CDON<sup>21,22</sup> share the ability to actively transduce a death signal in settings of ligand limitation, thus creating a state of cellular dependence to the presence of ligand for cell survival. Most of these dependence receptors share the trait of being cleaved by caspase<sup>30</sup>. We thus looked whether Notch3 could similarly be cleaved by caspases. Expression of S1-Cter Notch3 or an S2-Cter Notch3 (aa1631 to the C terminus) in HEK293T cells allows the identification of a 60–65 kD N-terminal fragment and a lower size 25–30 kD Notch3 C-terminal reactive fragment (Supplementary Fig. 4i,j). These fragments were no longer detected upon incubation with z-VAD-fmk and more specifically with initiator caspase inhibitors IETD-fmk and LEHD-fmk, supporting the view that a Notch3 fragment is released upon a caspase-like dependent cleavage (Supplementary Fig. 4i). To map more precisely the caspase-cleavage site in Notch3, systematic mutations of aspartic acid residues were performed. The specific mutations of the aspartic acid residues 2104 and 2107 into asparagine residues (D2104N-D2107N) fully blocked the detection of the Notch3 fragment (Supplementary Fig. 4j) without affecting canonical Notch signalling (Supplementary Fig. 4k). Thus, Notch3 is cleaved by a caspase-like protease at DSLD (2104–2107). Interestingly, this cleavage site is not present in other Notch receptors but is conserved in Notch3 receptors (Supplementary Fig. 4l). Therefore, expression of Notch3 *in vitro* induces cell death of EC, and Notch3 is cleaved by caspase-like proteases. Another frequent characteristic of dependence receptors is their ability to recruit and activate the initiator caspase-9 (ref. 30). We first observed that caspase-9 might be required for Notch3-induced cell death as treatment with z-LEHD-fmk significantly inhibited cell death induced by Notch3 over-expression (Fig. 3c). We further confirm the importance of caspase-9 by analysing Notch3-induced



**Figure 2 | Notch3 limits tumour growth and vascularization *in vivo*.** (a)  $5 \times 10^5$  LLC1 cells were implanted into the left flank of wild-type C57Bl/6 mice ( $N3^{+/+}$ ,  $n = 4$ ) or of Notch3 LacZ homozygous Knock-in C57Bl/6 littermates ( $N3^{LacZ/LacZ}$ ,  $n = 4$ ). Tumour growth was monitored from day 16 until day 24 when mice were sacrificed. Two-way ANOVA was performed to assess time and genotype effect on tumour growth (Interaction:  $P = 0,013$ ; Time:  $P < 0,0001$ ; Genotype:  $P = 0,0015$ ). (b) mRNA was extracted from tumours dissected after 14 days of growth from wild-type C57Bl/6 mice ( $N3^{+/+}$ ,  $n = 6$ ) or Notch3 mutant mice  $N3^{LacZ/LacZ}$  C57Bl/6 littermates ( $N3^{LacZ/LacZ}$ ,  $n = 5$ ). Quantitative RT-PCR was performed to measure CD31, DLL4, PDGFR $\beta$ ,  $\alpha$ -SMA, VEGFR2, VEGFA, CD11b and CD45 expression (means  $\pm$  s.d., unpaired t-test was applied). (c) Immunohistochemistry for CD31 and  $\alpha$ -SMA was performed on tumours dissected from wild-type mice ( $N3^{+/+}$ ,  $n = 15$ ) or Notch3 mutant ( $Notch3^{LacZ/LacZ}$ ) mice littermates ( $n = 9$ ) on day 14. Images are representative of four different sections from each tumour. Quantification was done using ImageJ angiogenesis plug-in on four different images from each tumour (mean  $\pm$  s.e.m. for quantification, unpaired t-test).

cell death upon silencing of caspase-9. As shown in Fig. 3d, silencing of caspase-9 strongly inhibits cell death induced by Notch3 (Fig. 3d). We then asked whether Notch3 could interact with caspase-9. Interestingly, we observed that S1-Cter Notch3, but not S1-Cter Notch1 or S1-Cter Notch2, was able to interact with caspase-9 when both Notch proteins and caspase-9 were ectopically expressed (Fig. 3e). We confirmed the interaction between Notch3 and caspase-9 by immunoprecipitation of endogenous caspase-9 (Fig. 3f). Interestingly, N3ICD did not interact with caspase-9

under the same condition, suggesting that the interaction with caspase-9 needs the anchorage of Notch3 to the membrane. We also performed Proximity Ligation Assay (PLA) with endogenous caspase-9 upon Notch3 overexpression in HEK293T cells. We observed a clear interaction between Notch3 and caspase-9 whereas no interaction was observed with caspase-8 (Fig. 3g). Moreover to explore whether the recruited caspase-9 could be activated, we performed caspase-9 activity assessment on Notch3 pull-down. As shown in Fig. 3h, Notch3, but not N3ICD, was pulling down



**Figure 3 | Notch3 induces endothelial cell death *in vitro*.** (a) Sub-G1 analysis of HUVEC electroporated with a control plasmid (Control), or a plasmid expressing the full-length version of Notch3 for 24 h. Quantification was made for three independent experiments and a paired-ratio *t*-test was applied. (b) AnnexinV/Propidium iodide was performed on HUVEC cells electroporated with a control plasmid or a plasmid expressing Notch3. Quantification was made on three independent experiments and a paired ratio student *t*-test was applied. (c) Sub-G1 quantification was made after electroporation of HUVEC cells with a control plasmid or a plasmid expressing Notch3 after 48 h of treatment with DMSO or z-LEHD-fmk or z-VAD-fmk pan-caspase inhibitors. Quantification was made on three independent experiments and a paired ratio student *t*-test was applied. (d) HUVEC were electroporated with a control siRNA (control) or a siRNA targeting caspase-9 (C9). 48 h later, cells were electroporated with a control plasmid or a plasmid expressing Notch3 and 24 h later, sub-G1 analysis was made. Quantification was made on three independent experiments and a paired ratio student *t*-test was applied. (e) Immunoprecipitation of Myc-tagged S1-Cter Notch1 (S1-CterN1), S1-Cter Notch2 (S1-CterN2) and S1-Cter Notch3 (S1-CterN3) constructs in HEK293t cells together with a control plasmid (–) or a plasmid expressing a HA-tagged dominant-negative version of caspase-9 (C9). (f) Lysates from HEK293t cells carrying Doxycycline (DOX)-inducible HA-tagged Notch3 (piN3) or DOX-inducible HA-tagged N3ICD (piN3ICD) plasmids were immunoprecipitated for endogenous caspase-9 and western blot were done to analyse Notch3 (HA) or caspase-9 in total lysates or IP. (g) Proximity Ligation Assay was performed for endogenous caspase-9 or endogenous caspase-8 and doxycycline-induced Notch3 in HEK293 cells. Quantification was made on five images containing 100–150 cells each. (h) Caspase-9 activity was measured from Notch3 immunoprecipitated lysates from HEK293t cells carrying inducible Notch3 (piN3) or inducible N3ICD (piN3ICD) plasmids.

caspace-9 activity, supporting the view that Notch3 could trigger cell death similarly to other dependence receptors.

These observations prompted us to further investigate whether Notch3 could be a dependence receptor for tumour EC aberrantly expressing Notch3. As a dependence receptor, it is expected that Notch3 ligand blocks Notch3 induced endothelial cell death. As Jag-1, a Notch3 ligand, has been shown to be associated with increased tumour angiogenesis<sup>32</sup>, we looked for the effect of Jag-1 expression on Notch3-induced cell death in tumour. For this purpose, HUVEC were co-cultured with two lung carcinoma cell lines expressing low or high levels of Jag-1, murine LLC1 cells and human H358 cells, respectively (Supplementary Fig. 5a). We observed that over-expression of Jag-1 in LLC1 cells reduced endothelial cell apoptosis and therefore induced stabilization of the endothelial network (Fig. 4a,b). Conversely, silencing Jag-1 in H358 cells led to an increase in endothelial cell apoptosis and earlier destabilization of the endothelial network (Fig. 4a,b). To confirm *in vivo* that tumour-derived expression of Jag-1 could increase angiogenesis, we established graft of LLC1 overexpressing Jag-1. As shown in Fig. 4c, overexpression of Jag-1 in LLC1 cells induced a dramatic increase in angiogenic markers CD31 as seen both on mRNA level and on protein staining by immunohistochemistry (Fig. 4c). To go further and prove that Notch3 behaves as a dependence receptor, we then over-expressed Notch3 in HUVEC cells and co-cultured them with LLC1 cells expressing or not high level of Jag-1. Overexpression of Jag-1 in LLC1 cells rescued the HUVEC death induced by Notch3 (Fig. 4d). We also showed that in co-culture conditions, neither N3ICD nor DNMA1 was able to induce cell death (Supplementary Fig. 5b). This further supports the view that Notch3 induces endothelial cell death independently of Notch canonical signalling pathway, and that the expression of Jag-1 by cancer cells can cell non-autonomously rescue endothelial cell death. Jag-1 is also frequently over-expressed in epithelial cancer cells<sup>33,34</sup>. We observed that Jag-1 was overexpressed in a fraction of human lung cancers using the GSE7670 data set (Fig. 4e) and confirmed Jag-1 over-expression in human clear cell renal cell carcinomas (Supplementary Fig. 5c). Of interest, Jag-1 expression was only poorly correlated with HES1, HEY1 and HEYL Notch target genes expression in this data set as well as in the GSE10245 dataset (Supplementary Fig. 5d). This observation supports the hypothesis that Jag-1 could have a different role in the tumour than activating Notch canonical signalling. As Jag-1 was shown to have a paradoxical pro-angiogenic role regarding Notch activation<sup>35</sup>, we compared the expression of Jag-1 with the expression of CD31 among tumours that over-express Jag-1 in the GSE7670 data set. In these patients, we observed a strong correlation with CD31 expression (Fig. 4e). We observed the same correlation in clear cell renal cell carcinoma (Supplementary Fig. 5c). By carrying out non-supervised clustering using the GSE10245 dataset, we observed a population in which Jag-1 and CD31 clustered together whereas Jag-1 did not cluster with Notch target genes (Supplementary Fig. 5e). In this population we observed a strong correlation between Jag-1 and CD31 but not with Notch target genes (Supplementary Fig. 5f). Taken together these data support the view that Notch3 behaves as a dependence receptor in endothelial cells and that Jagged-1 expression in tumour may act as a pro-angiogenic mechanism by limiting Notch3 induced apoptosis in endothelial tumour cells.

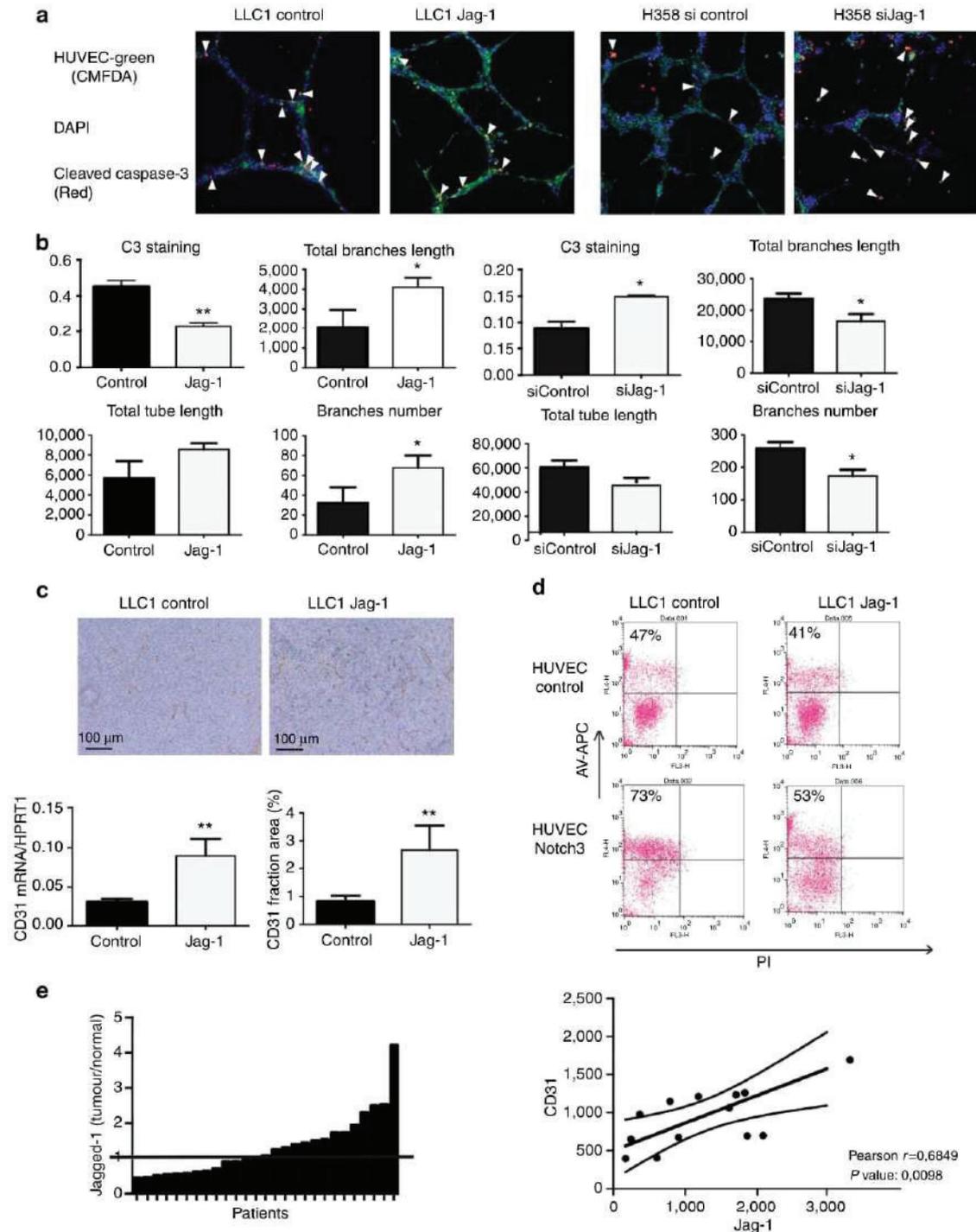
### Notch3 is required for $\gamma$ -secretase-induced tumour regression.

We then hypothesized that  $\gamma$ -secretase inhibitors, by blocking the N3ICD formation may mimic the absence of Notch3 ligand and thus induce Notch3-dependent tumour-associated

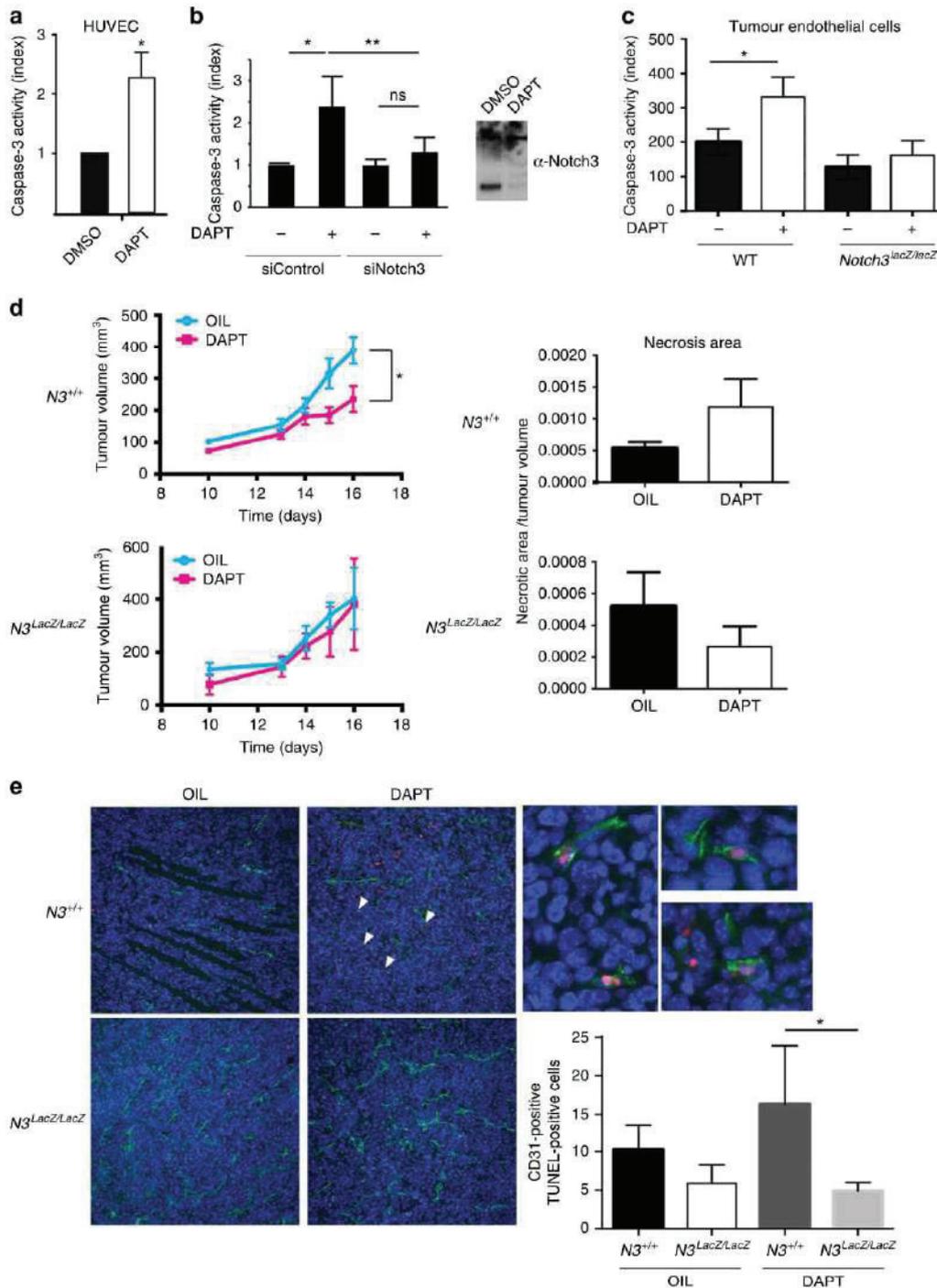
endothelial cell death. The general view for the mode of action of  $\gamma$ -secretase inhibitors as anticancer agents is the inhibition of cancer cell proliferation. However,  $\gamma$ -secretase inhibitors treatments have been paradoxically associated with decreased angiogenesis<sup>12,13</sup> and endothelial cell death<sup>11</sup> as opposed to anti-Dll4 antibody treatment which induces increase in non-productive angiogenesis<sup>14</sup>. We first observed that, *in vitro*, DAPT treatment induced HUVEC cell death (Fig. 5a). Of interest, this cell death was rescued by silencing Notch3 (Fig. 5b) but not by silencing Notch1 or Notch2 which had no effect on DAPT induced cell death (Supplementary Fig. 6a). We confirmed that tumour-associated endothelial cells were sensitive to DAPT treatment by purifying endothelial cells from tumours of *Kras*<sup>G12D/+</sup> mice (Fig. 5c). Further confirming the role of Notch3 in DAPT-induced cell death, we also showed that tumour-associated endothelial cells of tumours purified from *Notch3*<sup>LacZ/LacZ</sup>; *Kras*<sup>G12D/+</sup> mice were not sensitive to DAPT treatment (Fig. 5c). We then asked whether this effect could also be seen *in vivo*. We therefore treated wild-type mice bearing LLC1 tumours with DAPT. Whereas DAPT had no effect on LLC1 cells *in vitro* (Supplementary Fig. 6b), DAPT treatment in wild-type mice was associated with tumour growth inhibition (Supplementary Fig. 6c). As described by others<sup>11–13</sup>, this reduction was associated with a regression of the tumour vasculature as seen here by a decrease of CD31 staining and of the collagen IV/CD31 co-staining, which shows a regression of pre-existing vessels (Supplementary Fig. 6d). This tumour growth inhibition induced by DAPT treatment would classically be attributed to canonical Notch signalling inhibition. However, we report here that the tumour growth inhibitory effect of DAPT treatment was no longer observed in *Notch3* mutant mice (Fig. 5d). Because Notch3 is only silenced in stromal cells, this phenotypic rescue can only point out an effect of DAPT treatment on the stroma and cannot be easily explained by a difference in the canonical pathway (that is, if DAPT is inhibiting tumour angiogenesis by blocking the canonical pathway induced by Notch receptors, knocking down Notch3 should only add more tumour angiogenesis inhibition). In line with this, HeyL mRNA expression was not affected in both wild type and *Notch3* mutant mice in presence of DAPT (Supplementary Fig. 6e). We further purified tumour-associated endothelial cells and treated these cells with DAPT. In this setting, we saw no significant downregulation of Notch target genes HeyL, Hes1 and Hey1 (Supplementary Fig. 6f). In agreement with an effect on vasculature, we observed an increase in necrotic area in wild-type mice treated with DAPT but not in *Notch3* mutant mice (Fig. 5d). Confirming Cook *et al.*<sup>11</sup> data obtained in a different model, we observed increased endothelial cell death in wild-type mice treated with DAPT (Fig. 5e). In contrast, no effect was seen in *Notch3* mutant mice (Fig. 5e). This indicates that the apoptotic pathway mediated by Notch3 accounts, at least in part, for the regression of the tumour vasculature following DAPT treatment.

### Discussion

We uncovered here an unexpected function of Notch3 expression in tumour vasculature. Whereas Notch3 is normally expressed in smooth-muscle cells surrounding large vessels, we observed that Notch3 was upregulated in tumour endothelial cells. We have observed this ectopic expression in human lung cancer samples regardless of the expression of Notch3 in the cancer cells (Fig. 1; Supplementary Fig. 1). This expression was also observed in mice predisposed to develop lung cancers (*Kras*<sup>+G12D</sup>) as well as in lung cancer cells grafted subcutaneously (Fig. 1). These results are in line with the transcriptomic analysis data obtained by others<sup>19</sup>. Interestingly, although Notch3 has been



**Figure 4 | Jag-1 rescues Notch3-induced endothelial cell death.** (a) HUVEC were stained with the CellTracker green CMFDA. Cleaved-caspase-3 staining of HUVEC co-cultured with 1) LLC1 expressing or not Jag-1 (LLC1-Jag-1 and LLC1-Control, respectively) or 2) H358 cells transfected with a siRNA control (siControl) or a siRNA targeting Jag-1 (siJag-1). Co-cultures were maintained in matrigel for 9 h before being fixed and stained. Images are representative of three independent experiments, each performed in triplicate. (b) Quantification of HUVEC networks presented in a ( $n=3$ , means  $\pm$  s.d.,  $t$ -test was applied). Jag-1 expression was verified on western blot before the cells were added to HUVEC in Matrigel. (c) Immunohistochemistry staining and quantification of CD31 on tumour section from tumours obtained from LLC1 cells or LLC1 cells overexpressing Jag-1 ( $n=5$  tumours, quantification on 4 images/tumours, unpaired  $t$ -test). (d) Co-culture of HUVEC electroporated (Notch3) or not (Control) with Notch3 and LLC1 cells transfected (LLC1-Jag-1) or not (LLC1 control) with Jag-1 were stained with Annexin V APC and studied by flow cytometry. HUVEC were gated (M1) according to FL1 staining (cellTracker green CMFDA), and Annexin V (FL4)-positive cells were quantified among HUVEC. Number of Annexin V positive HUVEC cells is specified in each condition. (e) Tumour/normal tissue ratio of Jagged-1 mRNA in patients with non-small-cell lung adenocarcinomas from the GSE7670 data set and Correlation between Jagged-1 and Pecam-1 expression in patients overexpressing Jag-1 in the GSE7670 data set.



**Figure 5 | Notch3 is required for  $\gamma$ -secretase-induced tumour regression.** (a) Caspase-3 activity determined in HUVEC treated with DAPT (4  $\mu$ M) for 48 h ( $n = 7$ , means  $\pm$  s.d., paired  $t$ -test was applied). (b) Caspase-3 activity in HUVEC electroporated with siRNA control (siControl) or a siRNA targeting *Notch3* (siNotch3) and treated (+) or not (-) with DAPT (4  $\mu$ M) ( $n = 7$ , mean  $\pm$  s.d., paired  $t$ -test was applied). Effect of DAPT treatment on Notch3 cleavage was monitored by western blot. (c) Caspase-3 activity was determined in lysates from purified tumour-associated endothelial cells from tumours dissected from *Kras<sup>G12D/+</sup>;Notch3<sup>+/+</sup>* (WT) or *Kras<sup>G12D/+</sup>;Notch3<sup>LacZ/LacZ</sup>* (*Notch3<sup>LacZ/LacZ</sup>*) mice and treated *in vitro* (DAPT) or not with 4  $\mu$ M of DAPT. (d)  $5 \times 10^5$  LLC1 cells were implanted into the left flank of wild-type C57Bl/6 mice or of *Notch3<sup>LacZ/LacZ</sup>* C57Bl/6 littermates, and injected intraperitoneally with  $10 \mu\text{g l}^{-1}$  of ethanol-corn oil (1/9) (*N3<sup>+/+</sup>* OIL,  $n = 10$ , *N3<sup>LacZ/LacZ</sup>* OIL,  $n = 4$ ) or  $10 \mu\text{g l}^{-1}$  of 1 mg ml<sup>-1</sup> DAPT diluted in ethanol-corn oil (1/9; *N3<sup>+/+</sup>* DAPT,  $n = 8$ ; *N3<sup>LacZ/LacZ</sup>* DAPT,  $n = 4$ ) on days 12,13,14 and 15. Mice were killed on day 16 (two-way ANOVA was performed to test for significance). Necrosis area was quantified automatically on whole sections with HistoLab software settings parameters on hematoxylin staining intensity. (e) Immunofluorescence and TUNEL staining were performed on LLC1 tumour sections from wild-type mice or *Notch3<sup>LacZ/LacZ</sup>* littermates treated (DAPT, *N3<sup>+/+</sup>* DAPT,  $n = 5$ ; *N3<sup>LacZ/LacZ</sup>* DAPT,  $n = 3$ ) or not (OIL, *N3<sup>+/+</sup>* OIL,  $n = 5$ ; *N3<sup>LacZ/LacZ</sup>* OIL,  $n = 3$ ) with DAPT as previously described. For each tumour, the entire section was imaged (8–12 images per tumour), and the number of CD31-positive TUNEL-positive cells was quantified. Number of TUNEL-positive cell was then normalized on CD31 area that was quantified on each image using the ImageJ angiogenesis plugin (mean  $\pm$  s.e.m., unpaired  $t$ -test).

shown to be involved in different pathological settings affecting the vasculature, its role in tumour vasculature has never been addressed. Here we showed that Notch3 behaves as a dependence receptor, regulating tumour angiogenesis. As for the other dependence receptors, this new function is independent on the canonical Notch signalling pathway. Indeed, activation or inhibition of the canonical Notch signalling by expression of a dominant version of Mastermind-like or a constitutive active CBF-1 do not induce cell death as does Notch3. Furthermore, mutating the residues necessary for the interaction between N3ICD and CBF-1 did not abrogate the ability of Notch3 to induce cell death. Interestingly, we showed here that Notch3 was the only receptor of the Notch family to present this function. This is also described for other dependence receptors: for example TrkA and TrkC behave as dependence receptors whereas TrkB does not<sup>36,37</sup>. Interestingly, Notch3 has been shown to arise from the second duplication of Notch1 (ref. 38). As hypothesized for other dependence receptors, the dependence receptor function of Notch3 is thus probably a late acquisition during evolution. In line with this, the caspase cleavage site, present only in Notch3, has likely appeared during Notch3 differentiation after duplication from Notch1. Notch4 has been proposed to derive from Notch3 (ref. 39), however, this has been questioned more recently<sup>38</sup> and due to its rapid evolution, it is not clear from which Notch gene it actually derives.

This function of Notch3 appears in a context in which Notch3 is aberrantly expressed in the pathological tumour vascularization where Notch3 limits tumour angiogenesis through an unexpected pro-apoptotic activity. Of note, tumour associated endothelial cells have been described to have an aberrant expression of DR5 which render them more susceptible to apoptosis induced by TRAIL<sup>40</sup>. It would be of interest to study whether this function is conserved in other pathological situations where Notch3 is aberrantly expressed in non-endothelial cells, for example in cancer cells in which Notch3 and its ligands have been shown to be expressed.

We also observed that Notch3 was, at least in part, responsible for the anti-angiogenic effect of  $\gamma$ -secretase inhibitors described by others<sup>11</sup>. Indeed DAPT treatment induced a reduced vascularization associated with a reduced tumour growth. Importantly, this effect of DAPT was not due to inhibition of the canonical Notch signalling pathway as the effect of DAPT could be reversed by deletion of Notch3. If the effect of DAPT was a consequence of inhibition of Notch signalling, Notch3 deletion should either not have any effect or exasperate the effect of DAPT.

Furthermore, inhibition of the canonical Notch pathway, would lead to a hypersprouting of endothelial cells as observed upon anti-Dll4 treatment which could be associated with decreased growth but not decrease vascularization. In contrast, Notch3-induced apoptosis in tumour-associated endothelial cells following DAPT treatment could explain at least partly the anti-angiogenic effect followed by tumour growth inhibition. In *Notch3* mutant setting, DAPT cannot trigger Notch3-induced apoptosis and thus angiogenic effect. It may thus be of interest to take this unexpected function of Notch3 into account when evaluating the anti-tumour efficacy of  $\gamma$ -secretase inhibitors. This function of Notch3 is not in contradiction with the well-described oncogenic canonical Notch3 signalling in epithelial cells<sup>26,41</sup>. In fact, as other dependence receptor, the availability of ligands would impact on the role of Notch3. We showed here that Jag-1 expression by cancer cells was important to limit the dependence receptor function of Notch3. Furthermore, the function we describe here in tumour angiogenesis could account for some paradoxical observations regarding Notch3. In fact, while it may play a role in the epithelial tumour cells as an oncogene through

its canonical signalling, it may also represent a constraint for tumour progression by acting as a cellular sentinel for endothelial cell death. The Notch3 receptor may therefore act as a regulator of tumour angiogenesis depending on the context such as the heterotypic interactions between the tumour and the stroma or the availability of the ligands in the tumours. Jag-1 has been shown to be very important in signalling from the endothelium to the cancer cells<sup>42,43</sup>. Together with the present data, it shows how reciprocal interactions between the tumour vasculature and the tumour are important. The data presented here also raise the question of targeting Notch to regulate tumour angiogenesis. We propose that targeting Jag-1 in tumour angiogenesis might therefore be an interesting approach and targeting more specifically the Notch3-Jag-1 interaction could be advantageous allowing targeting of both the canonical Notch signalling in epithelial cells and Notch3-induced apoptosis in endothelial cells.

## Methods

**Mice experiments.** *Notch3* mutant mice have been characterized previously<sup>16</sup>. Cdh5:Cre<sup>ERT2</sup> mouse line was generated by Ralph Adams at Cancer Research UK. Mice were constantly bred into C57Bl/6 mice and experiments have been conducted in agreement with the local ethic comity (CECCAPP, Comité d'Evaluation Commun au PBES, à AniCan, au laboratoire P4, à l'animalerie de transit de l'ENS, à l'animalerie de l'IGFL, au PRECI, à l'animalerie du Cours Albert Thomas, au CARRTEL INRA Thonon-les-Bains et à l'animalerie de transit de l'IBCP). LLC1 cells were purchased from ATCC and were tested for mycoplasmas and murine viruses (Murine essential panel, Charles River) before being implanted in mice. For sub-cutaneous engraftment,  $5 \times 10^5$  LLC1 cells were implanted into the left flank of wild-type C57Bl/6 mice or *Notch3*<sup>LacZ/LacZ</sup> C57Bl/6 littermate. Standard variation was established in control experiment. Groups of 4–12 animals with homogenous tumour size were selected to obtain equal variance before treatment. No randomization method was applied. Tumour size was measured every day from day 10 when the tumours are palpable until day 14 or 21 by two different persons for each measure without knowing the genotype of animals. Animal showing prostration or obvious sign of suffering were excluded. Sub-cutaneous engraftment with E0771 cells was performed as described previously. Tumours were measured from day 14 to day 25. When the measures were too different, the point could be excluded. Measurement of the tumours was carried out without knowing the genotype of the animals. Mice were sacrificed before the end of the experiment if necessary according to animal care guidelines. For DAPT treatment, DAPT was diluted in Corn Oil/Ethanol (9/1) at  $1 \text{ mg ml}^{-1}$ .  $10 \mu\text{g g}^{-1}$  was injected intraperitoneally to reach a  $10 \text{ mg kg}^{-1}$  concentration. Experiments were all conducted on male and female littermate of 4–7 weeks of age. Animals were treated according to their identification number (even = untreated; odd = treated, this was arbitrary chosen for each experiment). Tumour dissection, fixation, and immunochemistry analysis were performed simultaneously.

**Cell culture and cell transfection.** Human umbilical vein endothelial cells (HUVEC) were obtained from Promocell (Heidelberg, Germany) and maintained in Endothelial Cell Growth Medium 2, supplemented with Endothelial Cell Growth Medium 2 Supplement Mix and 1% penicillin/streptomycin. H358 and LLC-1 were obtained from the ATCC maintained in RPMI Medium 1640 ( $1 \times$ ) + GlutaMAX-1, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) and in DMEM supplemented with 10% FBS and 1% PS, respectively. E0771 cells were obtained from our lab and culture in DMEM as described previously.

For electroporation,  $1 \times 10^6$  HUVEC cells were collected by trypsinization and electroporated either with 10 nM siRNA (si Notch3, Sigma SASI\_Hs01\_00101286, Sigma SASI\_hs01\_00100441, si negative control Sigma #SIC001) or 5  $\mu\text{g}$  DNA plasmids with Neon kit (Invitrogen). Twenty four hours later, transfection efficiency was verified by RT-quantitative PCR. LLC-1 or H358 cells were seeded at  $0.25 \times 10^6$  cells in 6 wells plates one day before transfection. Transfections were performed with lipofectamine TM reagent (Invitrogen) following the manufacturer's instructions.

For caspase inhibitors treatment, HUVEC cells were pre-incubated 2 h with 5  $\mu\text{M}$  caspase inhibitors (BioVision, Caspase-9 Inhibitor Z-LEHD-FMK, MerckMillipore, Z-VAD-FMK) or DMSO for 2 h. Cells were then transfected with empty vector or Notch3 and incubated for 24 h with 5  $\mu\text{M}$  caspase inhibitors or DMSO.

**Endothelial cells purification.** Lung from 16 weeks-old KrasG12D mice were dissected and tumour nodules extracted under a binocular before being digested in  $1 \text{ mg/ml}$  collagenase Type 1 (Invitrogen) for 1 h. Cell suspension was then incubated with magnetic beads (Dynabeads Sheep Anti-Rat IgG, Invitrogen) incubated overnight with CD31 antibody (clone MEC13.3, Pharmingen).

**$\beta$ -galactosidase staining.** After dissection, organs from *Notch3<sup>LacZ/+</sup>* mice were fixed for 20 min before being washed three times in 0.2% NP-40, 0.01% NaDOC, 2 mM MgCl<sub>2</sub> in PBS. Organs were then incubated for 1 h in 25 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 25 mM K<sub>4</sub>Fe(CN)<sub>6</sub> Wash Buffer. Xgal reaction was then performed in 25 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 25 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 1 mg ml<sup>-1</sup> Xgal in Wash Buffer at 37 °C.

**Co-culture experiments.** HUVEC were incubated with a CellTracker Green CMFDA at 1.25  $\mu$ g ml<sup>-1</sup> (Molecular probes, Life technologies, C7025) for 30 min. Afterwards, cells were washed two times with PBS. 60  $\mu$ l of Basement Membrane Matrix (Matrigel, BD Bioscience) was added to a 96-wells plate, followed by 30 min incubation at 37 °C. HUVEC were collected by trypsinization and  $15 \times 10^3$  HUVEC were added into each Matrigel coated well and incubated at 37 °C for two hours. LLC1 or H358 transfected one day before were then added to wells containing HUVEC. Each condition was carried out in triplicate. After 9 h of co-culture, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and rinsed three times with PBS for 10 min at room temperature. Fixed cell culture plates were stocked at 4 °C.

**Immunofluorescence staining.** Immunofluorescence analysis was performed on tumours obtained from littermates. Fixation and staining were performed simultaneously. Paraffin embedded tissue samples were deparaffinized and heat-induced antigen retrieval was performed. Cells or tissue samples fixed with 4% PFA were permeabilized with PBS-0.2% Triton X-100 (TX-100) for 30 min at room temperature. Samples were then washed three times with PBS for 5 min. Samples were blocked in PBS with 4% bovine serum albumin (BSA), 2% normal donkey serum and 0.2% TX-100 for one hour. Samples were then washed three times with PBS for 5 min. Primary antibodies were diluted in the blocking solution: 1:500 dilution for anti-cleaved caspase 3 (Cell Signaling Asp175 5A1E Rabbit mAb), 1:100 dilution for anti-CD31 (Abcam, anti-CD31 ab28364), 1:100 dilution for anti-collagen IV (Abcam anti-collagen IV ab19808). Alexa-conjugated secondary antibodies (Alexa555-donkey anti rabbit, Alexa488-donkey anti rabbit) were used at 1:1,000 dilution. DAPI (0.5  $\mu$ g ml<sup>-1</sup>) was added at the end to stain nuclei. Images were acquired with Zeiss Axio fluorescence microscopy, NIS element AR 4.20.01 Nikon fluorescence microscopy and confocal microscopy.

**Proximity ligation assay.** HEK293T cells ( $10^5$ ; stably selected to carry a Doxycycline-inducible plasmid for Notch3 or N3ICD) were seeded in lab-tek chamber (Thermo scientific, 4-well, 177399). After 24 h, cells were treated with 1  $\mu$ g ml<sup>-1</sup> Doxycycline. After 24 h induction, cells were fixed with 4% PFA and PLA assay (Sigma, Duolink *In Situ* PLA) was performed according to the manufacturer's instructions. Anti-HA (sigma, H6908) and anti-caspase 9 (Santa Cruz, sc-73548) were used for primary antibodies.

**Cell death assay.** TUNEL assay: Detection of DNA fragmentation, a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed by following the protocol of the TUNEL assay kit (Roche). Briefly, fixed cells or tissue samples were permeabilized with 0.2% TX-100 in PBS (30 min at room temperature), washed with PBS, incubated with 300 U ml<sup>-1</sup> TUNEL enzyme and 6  $\mu$ mol l<sup>-1</sup> biotinylated deoxyuridine triphosphate (Roche Diagnostics, Mannheim, Germany). The extremities of the biotin coupled DNA were revealed by using Cy-3-coupled streptavidin (1:1,000 in PBS, Jackson Immunoresearch). The slides were washed with PBS, DAPI-stained, then washed with PBS and finally, mounted with Fluoromount G (SouthernBiotech). Images were acquired with Zeiss Axiovision fluorescence microscopy and NIS element AR 4.20.01 Nikon fluorescence microscopy. Caspase-3 activity assay: Cells were first harvested by scraping. Cell pellets were obtained by centrifugation at 4 °C and lysed. The caspase 3 activity assay was performed according to the manufacturer's instructions (Biovision caspase-3 colorimetric assay kit). Total protein concentrations were measured with the BCA assay kit using BSA as a standard (Pierce Biotechnology, Rockford, IL, USA). Absorbance readings were done on a TECAN infinite F500.

**Immunohistochemistry analysis.** Immunohistochemistry was performed on 4- $\mu$ m-thick sections of formalin-fixed, paraffin-embedded and heat-treated (for antigen retrieval) tissues (DakoCytomation). Sections were stained with hematoxylin-eosin-safran and tumour endothelium was stained with an anti-CD31 antibody (1:50 Abcam, ab28364). Diaminobenzidine was used as chromogen. Images were acquired with a Zeiss Axiovert. The whole slide was scanned automatically with the Histolab 6.2.0 MICROVISION Instrument system. Necrotic and CD31 positive areas were quantified by Histolab 6.2.0 or ImageJ angiogenesis plugin. Staining of human sample was performed with the cell signaling anti-Notch3 antibody (D11B8).

**Co-culture images analysis.** Images of co-culture experiment were acquired with Zeiss Axiovision fluorescence microscopy. 8–12 images were acquired for each well at  $\times 5$  magnification. Images were analysed by Image J angiogenesis plugin (Gilles Carpentier, Faculté des Sciences et Technologie, Université Paris Est Creteil

Val-de-Marne, France). Briefly, channels were split automatically. On the GFP channel, total tube length, branches number and total length of branches were acquired by Analysis HUVEC Fluo program. Each condition was carried out in triplicate.

**Tumour section CD31 fluorescent images analysis.** Whole slides were scanned to acquired total images at  $\times 10$  magnification with a Zeiss Axiovision fluorescence microscopy. CD31 expression areas were analysis by Image J. Briefly, The image was converted into RGB stack format. CD31 staining was quantified by choosing threshold program and adjusting the threshold parameters. Once the threshold parameter was adjusted, it was always the same for each image and CD31 expression areas were measured automatically. TUNEL positive and CD31-positive cells were counted manually. For blinded quantification, images were organized in folder identified by letters by one person and quantified by another.

**Western blot.** Cells were lysed in SDS buffer (2% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4). Cell extract was next centrifuged at 2,500g for 5 min. Protein concentration was measured with the BCA assay kit (Pierce Biotechnology, Rockford, IL, USA) using Bovine Serum Albumine (BSA) as a standard according to manufacturer's instructions. The following antibodies were used: anti-Notch3 (1:1,000 dilution, Cell Signaling #2889), anti-Jagged1 (1:1,000 dilution, Santa Cruz C-20 sc-6011), anti-cleaved caspase 3 (1:1,000 dilution, Cell signaling Asp175 5A1E Rabbit mAb), anti-CBF-1 (1:1,000 dilution, Cell signaling #5313P), anti-GFP (1:2,000 dilution, Molecular probes A11122), anti-Myc (1:2,000 dilution, sigma, M5546) and anti-HA (1:5,000 dilution, Sigma H4908).

**Flow cytometry analysis of sub-G1 and Annexin V staining.** For the sub-G1 experiment, cells were harvested cells by trypsinization and counted. Cells were first washed once with PBS followed by the addition of cold 70% ethanol, vortexed, and then resuspended at 4 °C for 30 min. Samples were stocked at -20 °C. Ethanol was removed and the pellet washed with PBS. Staining solution (40  $\mu$ g ml<sup>-1</sup> propidium iodide, 2 mg ml<sup>-1</sup> RNase in PBS) was added.

For the Annexin V experiments, cells were collected by trypsinization and counted. Afterwards, 100  $\mu$ l of a  $1 \times 10^6$  cells solution was incubated with 5  $\mu$ l Annexin V allophycocyanin conjugated (Life technologies, A35110) and 2  $\mu$ l Propidium Iodide (Life technologies, V13242), for 15 min at room temperature.

For the CD31/CD105 staining, cells were detached in PBS/EDTA (5 mM) and  $10^6$  cells were re-suspended for 20 min on ice in 100  $\mu$ l PBS with anti-mouse-CD31-FITC (Ebioscience) and Anti-CD105 antibody ([M7/18] Phycocerythrin; Abcam) before analysis with the flow cytometer. Data acquisition and analysis were performed on a FACSCalibur using CellQuestPro software (BD Bioscience, San Jose, USA).

**Statistical analysis.** For tumour growth analysis, a two-way ANOVA was realized to test for effect of time and treatment. For analysis of *in vitro* experiments a normality test was realized when number of samples was sufficient (Shapiro-Wilk or KS Normality test). Similarity of variance was tested before application of any statistical test using graphpad. If samples followed a Gaussian distribution, a *t*-test was applied, either paired-ratio or unpaired depending on the experimental data. When samples did not pass the normality test, non-parametric test was applied (Mann-Whitney for unpaired samples and Wilcoxon signed-rank test for paired samples). \**P* < 0,05; \*\**P* < 0,01; \*\*\**P* < 0,001.

**Quantitative RT-PCR.** mRNA were extracted with the NucleoSpin RNA kit (Machery-Nagel) according to manufacturer's instructions. cDNA were generated with the iScript cDNA Synthesis kit (BIO-RAD) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed using a LightCycler 480 (Roche Applied Science) and the FastStart TaqMan Probe Master Mix (Roche Applied Science). Primer sequences are provided in Supplementary Table 1.

**Immunoprecipitation.**  $5.10^6$  HEK293T cells (stably selected to carry a Doxycycline-inducible plasmid for Notch3 or N3ICD) were treated with 1  $\mu$ g ml<sup>-1</sup> Doxycycline for 24 h. Cells were collected and lysed in lysis buffer (HEPES 50 mM, NaCl 150 mM, EDTA 5 mM, NP40 0.1%, PH7.6) for one hour at 4 °C and then sonicated. One millilitre of lysate was then incubated with 10  $\mu$ l Anti-HA (Sigma, H6908-.5ML) over night at 4 °C. Hundred microlitre of protein A sepharose beads (Sigma, P3391-1G) were added into the lysate and incubated at 4 °C for one hour. Beads were then washed three times with lysis buffer at 4 °C. Beads were collected and incubated with caspase 9 assay kit (Promega, Caspase-Glo 9 Assay) for 30 min. Luminescence was measured by TECAN infinite F500.

**Data availability.** All data are available within the Article and Supplementary Files, or available from the authors upon request.

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## Author contributions

S.L. and A.N. designed and conducted most experiments. S.B. helped in conducting experiments. N.R., B.G., B.D., N.G. and J.-G.D. helped in designing experiments and provided critical advices. P.S. selected human lung cancer samples. I.T. analysed the staining of human lung cancer samples. O.M. designed and conducted experiments, analysed the data and together with P.M. designed the research plan and wrote the manuscript.

## Additional information

**Supplementary Information** accompanies this paper at <http://www.nature.com/naturecommunications>

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# V. References



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