Manipulating the hypoxic tumour microenvironment to study therapy resistance

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CHAPTER 1

Introduction

Jonathan Ient

Cancer

Cancer is a group of related diseases with more than 200 types, arising from different cell types within the body. Cancer is the second leading cause of death globally with an estimated 9.6 million deaths in 2018 (WHO, 2020). Cancer cells are able to grow and divide unchecked altering tissue architecture and invading distant sites, eventually leading to a partial or total loss of function of the affected tissues and organs resulting in death. The majority of cancer-related deaths are a result of metastases from the primary tumour. There are many risk factors for developing cancer, some of which are inherent such as age and genetics, but a person's lifestyle choices such as smoking, alcohol consumption and diet strongly affect risk by increasing the risk of spontaneous errors in DNA replication and repair. Exposure to carcinogens increases the DNA damage caused to cells within the body, increasing the chance of mutations which can ultimately lead to cancer. There are a number of hallmarks that cancers possess which are, sustaining proliferative signalling, evading growth suppressors, deregulating cellular energetics, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis and avoiding immune destruction (Hanahan & Weinberg, 2011). These have been coined the hallmarks of cancer and are common to all types of cancer. Genome instability and mutation, and tumour-promoting inflammation have also been described as 'enabling characteristics' and facilitate the acquisition of the hallmarks of cancer. The surrounding tumour micro-environment containing the normal non-cancerous cells has also been found to play a pivotal role in cancer progression and treatment response.

Hypoxia

The high proliferation rate of cancer cells requires a steady supply of oxygen, however the high proliferation rate can force blood vessels apart reducing the vasculature density within the tumour creating a population of cells that become more distant from the blood vessels (Thomlinson & Gray, 1955). This problem is exacerbated by poor vascular architecture (Sevick & Jain, 1991), irregular blood flow (Chaplin *et al*., 1987; Dewhirst *et al*., 1998) and cancer cell-induced compression of blood & lymphatic vessels (Padera *et al*., 2004). This leads to areas of hypoxia (low oxygen conditions) which is a feature of many solid tumours (Brahimi-Horn *et al*., 2007; Thomlinson & Gray, 1955). There are two main types of hypoxia. The first is chronic or diffusion-limited hypoxia which is caused by the limited distance that oxygen is able to diffuse from the vasculature. Areas far from the tumour vasculature experience chronic hypoxia and those areas furthest from vessels become necrotic due to extended oxygen deprivation. The second type is acute or cycling hypoxia which is caused by the temporary occlusion of the vasculature stopping the supply of oxygen for a period of time before it is restored and the tumour bed area is reoxygenated (Dewhirst, 2009; Salem *et al*., 2018).

This combination of acute and chronic hypoxia makes the oxygenation of the tumour dynamic and leads to a very heterogeneous oxygenation profile of the tumour which is constantly changing (Peter Vaupel *et al*., 2004).

The main cellular response to hypoxia is the stabilisation of the oxygen regulated HIF-α proteins HIF-1α, HIF-2α and HIF-3α (Figure 1). The HIF-α proteins contain basic helix loop helix domains for DNA binding, PAS domains which facilitate dimerization with the HIF-β protein, transactivation domains (N-TAD & C-TAD) which mediate transcriptional activity and an oxygen-dependent degradation (ODD) domain which regulates the stability of the protein in an oxygen-dependent manner. The HIF-α proteins are constitutively expressed and are principally regulated post-translationally. Oxygen-dependent prolyl and asparagine hydroxylases, hydroxylate specific asparagine and proline residues in the ODD domain of HIF-α. Hydroxylation of asparagine (N^{851}) inhibits binding of p300, a transcriptional coactivator. Hydroxylation of 2 proline residues (Pro^{402} & Pro^{564}) promotes interaction with von Hippel-Lindau (pVHL) protein, which recruits E3 ubiquitin ligases, targeting the protein for proteasomal degradation (Ivan *et al*., 2001; Lando *et al*., 2002). Under hypoxic conditions, the function of prolyl hydroxylases (PHDs) is attenuated allowing the accumulation of HIF-α which can then translocate to the nucleus and bind to the constitutively expressed HIF-1β and form a complex with the co-activator p300. This complex can then bind to a core consensus sequence 5'-(A/G)CGTG-3' within hypoxia response elements (HREs) which are found in the regulatory elements of target genes (Wenger *et al*., 2005). In addition to the HRE, promoters of several HIF-1α target genes possess a reversed imperfect repeat of the consensus sequence (5'-CAGGT-3') known as the HIF ancillary sequence (HAS) (Kimura *et al*., 2001).

HIF target genes play a role in an abundance of pathways many of which tailor the cellular response to hypoxia by reducing the demand for oxygen while also inducing systems to increase the oxygen supply. Examples include many genes in the glycolytic pathway such as PGK and GLUT-1 which alter the cellular metabolism to favour glycolysis even in aerobic conditions known as the Warburg effect. This increased glycolytic activity leads to an increase of intracellular lactate and CO_2 which increases the intracellular acidity. To combat this hypoxic cells upregulate lactate transporters such as monocarboxylate transporter 4 (MCT-4) which export lactate leading to acidification of the tumour microenvironment (Damaghi *et al*., 2013). CAIX upregulation in hypoxia also controls pH by catalysing the conversion of CO_2 to HCO_3^- and H+ leading to intracellular alkalisation and extracellular acidification (Sedlakova *et al*., 2014). HIF-α also promotes increased oxygen supply by inducing angiogenesis via vascular endothelial growth factor (VEGF) and increased erythropoiesis via the release of erythropoietin (EPO). Other pathways that are upregulated include those involved in cell proliferation (TGF-α), Cell adhesion (MIC2), cell invasion and EMT (LOX, LOXL2) among others (LaGory & Giaccia, 2016; Muz *et al*., 2015; Schietke *et al*., 2010; Wilson & Hay, 2011). The DNA damage response is also affected by hypoxia with downregulation of both homologous recombination and base excision

repair pathway genes (Kaplan & Glazer, 2020; Leszczynska *et al*., 2016). Downregulation of these pathways is linked to genomic instability and an increased mutation rate.

Hypoxia also affects epigenetics adding another layer of complexity. Hypoxic tumour cells display a distinct epigenetic profile which affects the transcription of many genes including upregulation of Histone deacetylase 1 (HDAC1) which was linked to decreased expression of the tumour suppressors TP53 & VHL (Kim *et al*., 2001; Ramachandran *et al*., 2015). Hypoxia also epigenetically downregulates DICER expression, suppressing miRNA processing which has been shown to promote a stem cell phenotype and poor prognosis in breast cancer patients (van den Beucken *et al*., 2014).

Hypoxia has been shown to inhibit proliferation in many cell types including cancer cells, with HIF overexpression alone being able to induce cell cycle arrest (Hackenbeck *et al*., 2009; Hubbi *et al*., 2013). The mechanism is both transcriptional through HIFs regulation of CDK, p21 & p27 and non-transcriptional through HIFs direct effects on the pre-replication complex (Hubbi & Semenza, 2015). There are many conflicting reports on HIFs role in apoptosis with HIF-1α and HIF-2α reported to have opposing effects and for these effects to be dependent on cell type. HIFα have been shown to regulate a host of proteins involved in apoptosis (BNIP, Noxa, Bcl-2, Bid, Bad, Bax MCL-1) giving rise to both pro and anti-apoptotic effects (Sendoel & Hengartner, 2014). HIFs are also known to interact with the tumour suppressor TP53. A number of mechanisms have been postulated including the direct interaction between the two proteins (An *et al*., 1998), indirect regulation through the ubiquitin ligase Mdm2 (D. Chen *et al*., 2003), competition for the coactivator p300 (Schmid *et al*., 2004) and regulation by HIF target genes such as nucleophosmin which directly interacts with p53 regulating its activation by inhibiting p53 phosphorylation at serine 15. (J. Li *et al*., 2004). The net effect of HIF stabilisation on apoptosis are context-dependent. In hepatoma HepG2 cells, hypoxia inhibited etoposide-induced apoptosis by decreasing p53 activity. While in the MCF-7 cell line, hypoxia promoted apoptosis by increasing p53 abundance and in A549 cells hypoxia did not affect etoposide-induced apoptosis (Cosse *et al*., 2007). High levels of HIF in cancer are associated with and correlate with more malignant tumours, immunosuppression, metastasis, therapy resistance and poor prognosis in a number of cancers (Giatromanolaki *et al*., 2001; Ioannou *et al*., 2009; LaGory & Giaccia, 2016; Ren *et al*., 2016; Roig *et al*., 2018; S.-S. Zheng *et al*., 2013).

The tumour microenvironment

The tumour microenvironment (TME) is the environment around the cancer cells within a tumour and includes blood vessels, immune cells, fibroblasts, secreted proteins and the extracellular matrix (ECM), and plays an important role in cancer with much cross-talk with the cancer cells and the rest of the tumour ecosystem.

The ECM consists of approximately 300 proteins that regulate homeostasis, organ development, inflammation and disease (Naba *et al*., 2012). The majority of these are fibrous proteins and proteoglycans that are excreted locally and organized into a mesh which forms the structural framework for most tissues (Frantz *et al*., 2010). The most abundant fibrous ECM proteins are collagens, laminin, fibronectin, elastins and tenascin, while the proteoglycans fill up the majority of the extracellular interstitial space within the tissue in the form of a hydrated gel.

Tumours are able to alter the ECM and change the expression of the components, this along with the increased expression of remodelling proteins such as MMP1 and MMP12, promotes tumour progression, metastasis and worse outcome (Chang *et al*., 2005; Finak *et al*., 2008; Naba *et al*., 2012; Ramaswamy *et al*., 2003). These studies as well as histological studies reveal that solid tumours show excessive ECM deposition (fibrosis) (Bataller & Brenner, 2005; Boyd *et al*., 2000). The most well-recognised alteration that occurs in the tumour ECM is increased collagen deposition (Colpaert *et al*., 2003; Hasebe *et al*., 1997; Huijbers *et al*., 2010). Collagens make-up to 90% of the ECM and provide structural integrity and tensile strength as well as regulating the physical and biochemical properties of the tumour microenvironment, modulating cancer cell polarity, migration and signalling (Fraley *et al*., 2010; Levental *et al*., 2009). Murine studies using a model of increased stromal collagen in mammary tissue found an increased incidence of tumour formation and metastasis compared with wild type controls (Provenzano *et al*., 2008). Moreover, histological studies have shown that fibrosis is localised to areas of hypoxia within the tumour and correlates with CAIX expression with the most fibrotic tumours also containing the highest CAIX immune reactivity, which can also predict patient relapse and is a predictor of shorter disease survival (Colpaert *et al*., 2003; Hasebe *et al*., 1997; Trastour *et al*., 2007).

Fibroblasts and myofibroblasts within the primary tumour are believed to be the mediators of tumour fibrosis. There is also evidence that hypoxia drives the recruitment of fibroblasts to the tumour via VEGF upregulation which induces microvascular permeability, which in turn mediates an influx of fibroblasts as well as inflammatory and endothelial cells (ECs) (Brown *et al*., 1999). Cancer cells secrete proteins such as transforming growth factor-β (TGFβ) which activate fibroblasts stimulating the synthesis of the ECM proteins such as connective tissue growth factor (CTGF), periostin and multiple MMPs. These altered fibroblasts are termed cancer-associated fibroblasts and secrete higher levels of normal ECM constituents especially in areas of hypoxia, and proliferate more than their normal counterparts. Under conditions of hypoxia, there is evidence that cancer cells, as well as fibroblasts, contribute to fibrosis (Eisinger-Mathason *et al*., 2013; Gilkes *et al*., 2013). HIF-1 also regulates the expression of collagen modifying enzymes that increase collagen crosslinking, stability, and stiffness (Cox *et al*., 2013). High ECM stiffness enhanced mechanical and molecular reprogramming of cancer cells by enhancing growth factor and integrin signalling (Levental *et al*., 2009), invadopodia activity (Alexander *et al*., 2008),

metastasis (Wei *et al*., 2017). Expression of the HIF regulated LOX family of enzymes has also been shown in mice, to re-model existing collagen structures in the lung to establish a pre-metastatic niche facilitating metastasis (Janine T Erler *et al*., 2009). Reducing the levels of HIF-1α or some of these remodelling enzymes decreased fibrosis and stiffness as well as inhibiting spontaneous metastasis of breast cancer cells by reducing collagen fibres which are required for cancer cell adhesion and invasion (Gilkes *et al*., 2013; Xiong *et al*., 2014). HIF1 and HIF2 also regulate the expression of enzymes that degrade the ECM such as the matrix metalloproteinases. As well as increased ECM degradation, hypoxic cancer cells show increased proteolytic activity via induction of urokinase plasminogen activator surface receptor which promotes cell invasion by altering the interaction between the ECM and integrins (Graham *et al*., 1999).

In conclusion, HIFs are able to promote the degradation of the basement membrane while simultaneously increasing the synthesis of fibrillar collagens, facilitating tumour invasion.

Immune cells are also an important component of the tumour microenvironment. Hypoxia-induced growth factor secretion promotes the recruitment of macrophages whose gene expression is changed in response to the hypoxic conditions promoting the conversion to and immunosuppressive activity of M2 macrophages while repressing M1 macrophages as well as reducing their antigen expression (Multhoff & Vaupel, 2020). These immunosuppressive macrophages are often termed tumour-associated macrophages and they can stimulate fibrosis through the production of growth factors such as TGFα, TGFβ1, TNFα, FGF, interleukin-1 (IL-1) and IL-8 which attract additional macrophages, fibroblasts and ECs and can also further activate stromal cells (Brown *et al*., 1999). Macrophages also promote cancer cell intravasation into nearby blood vessels (Condeelis & Pollard, 2006). On top of this, macrophages secrete matrix metalloproteases (MMPs) which contribute to ECM degradation giving macrophages a role in both ECM promotion and degradation affecting the level of tumour fibrosis.(Chi *et al*., 2006).

Cancer stem cells (CSCs) are a subpopulation of tumour cells that demonstrate properties similar to that of normal stem cells. They are cells within the tumour that possess the capability to self-renew and can initiate new tumours and produce the heterogeneous lineages of cancer cells that make up the tumour (Clarke *et al*., 2006). It has been demonstrated that it is these cells that mainly contribute to cancer initiation and growth. Two separate studies have shown that a small subpopulation of cancer cells were able to generate a tumour following xenotransplantation into immunosuppressed mice (Hope *et al*., 2004; O'Brien *et al*., 2007). There is increasing evidence that hypoxia regulates the subpopulations of CSCs, with CSCs preferentially located in areas of hypoxia in several brain tumours. HIFs were also shown to be able to increase stemness features and markers of CSCs in a number of cancers including glioblastoma, leukaemia and lung cancer (Deynoux *et al*., 2016; Shi *et al*., 2015; Sun *et al*., 2015). The HIFs are also able to inhibit the

differentiation of glioblastoma stem cells while inducing their self-renewal capacity (Lee *et al*., 2016). The impact of hypoxia is believed to be partially mediated through induction of the Hippo signalling pathway in breast CSCs, through the HIF-1α target TAZ (Samanta *et al*., 2014; Semenza, 2015). *Oct-4,* a transcription factor essential for maintaining stem cell pluripotency, has also been found to be a target of HIF-2α (Covello *et al*., 2006). Knockdown of HIF-1α or HIF2-α in glioblastoma stem cells inhibited neurosphere formation *in vitro* as well as attenuating tumour-initiating potential *in vivo* (Z. Li *et al*., 2009).

The tumour microenvironment contains a large variety of cells which have complex functions and interactions. Hypoxia influences many aspects of the tumour microenvironment and its impact is profound yet still not fully understood.

Hypoxia and Metastasis

Tumour metastasis is the dissemination of cancer cells from the primary tumour to distant organs where they grow and form secondary tumours. 90% of cancer deaths can be attributed to metastasis, however, treatments that prevent or cure metastasis remain elusive. Patients whose primary tumour is poorly oxygenated have an increased risk of metastasis and poorer prognosis (Bos *et al*., 2003; P Vaupel *et al*., 2007; Peter Vaupel *et al*., 2004). Hypoxia is strongly linked to metastasis and in a mouse model of melanoma, inactivation of HIF-1α and HIF-2α abrogated metastasis (Hanna *et al*., 2013). However, many of the mechanisms by which metastasis occur and hypoxia's influence on the process remain to be elucidated. Much of this is due to the complexity of the *in vivo* environment which cannot be effectively mimicked *in vitro*.

The metastatic process is complex and contains a series of steps. First is an epithelial-mesenchymal transition (EMT) where cancer cells lose cell to cell contact and gain motility. The next step is local tissue invasion which is aided by the degradation of the ECM facilitating cell movement. This is followed by intravasation into the blood. The cells must then survive in the blood and reach a distant site in a process called homing. Once at the distant site the cells extravasate out of the blood into the tissue where they survive proliferate and form secondary tumours.

EMT is characterised by cellular changes that include the loss of cell to cell adhesion. HIF-1 promotes EMT through upregulation of EMT associated transcription factors and repressors, activating the EMT associated pathways as well as modulating EMT associated inflammatory cytokines and affecting epigenetic regulators. The regulation of EMT by HIF is complex but involves multiple direct effects. HIFs control the transcription of a number of proteins in this process including the direct HIF targets LOX and LOXL2 which repress the cell adhesion molecule E-cadherin (Schietke *et al*., 2010). Further to this the HIF target genes, Snail, TCF3, ZFHX1A and ZFHX1B have also been shown to repress E-Cadherin expression promoting EMT (Krishnamachary *et al*., 2006; G. H. Zhu *et al*., 2013). Also,

the HIF target genes TWIST1 induces dramatic transcriptional changes in extracellular compartment and cell-matrix adhesion genes including E-cadherin, which have been shown to induce the dissemination of cells (Gort *et al*., 2008; Shamir *et al*., 2014). HIF-1 may also regulate EMT through regulation of non-coding RNAs and calcium signalling to promote cancer cell invasion (Davis *et al*., 2014; Matouk *et al*., 2014).

HIF-1 also mediates invasion. Several mechanisms of ECM remodelling and degradation have been mentioned earlier such as expression of MMPs, and these proteins promote invasion and motility of cancer cells. Further to this, when tumour size increases collagen fibres straighten and align, which facilitates cancer cell invasion (Provenzano *et al*., 2006). Hypoxia also regulates proteins associated with re-modelling of the extracellular matrix, for example, LOX upregulation correlates with poor distant metastasis-free overall survival in breast and head and neck tumours and LOX blockade was able to decrease the frequency of metastasis (Janine T. Erler & Giaccia, 2006). Hypoxia also regulates the expression of a specific variant of the scaffold protein A-kinase anchor protein 12 which regulates protein kinase A (PKA) mediated phosphorylation events promoting, tumour cell invasion and migration *in vitro* and metastasis *in vivo* (Finger *et al*., 2015). *In vivo* data suggests that there are two phenotypes of cell migration. Slow migratory cells which display increased invadopodia, matrix degradative ability and intravasation over time (Gligorijevic *et al*., 2014), and fast migratory cells which comigrate with macrophages along collagen fibres (Sidani *et al*., 2006). Both phenotypes are required for metastatic dissemination.

Intravasation occurs throughout the tumour and not just at the invasive front. Intravasation is hypothesised to occur at sites called tumour microenvironment of metastasis (TMEM) which is composed of a tumour cell, macrophage and an EC all in direct contact. TMEM act as doorways for tumour cell dissemination into the blood and their number has been shown to be prognostic for distant metastatic recurrence in breast cancer (Harney *et al*., 2015; Karagiannis *et al*., 2016). However, only a fraction of cancer cells that enter the blood are able to survive the harsh conditions and form metastases (Fidler, 1970). Godet *et al*. were able to show *in vivo* through lineage tracing, that post-hypoxic tumour cells maintain a ROS-resistant phenotype. This provides a survival advantage in the bloodstream, promoting their ability to form distant metastases (Godet *et al*., 2019).

To extravasate from the blood, a cancer cell must adhere to ECs and then disrupt the tight interactions between ECs to permeate out of the blood vessel. This extravasation requires invadopodia which extend through the endothelium into the extravascular stroma prior to their extravasation at endothelial junctions (H. S. Leong *et al*., 2014). Inhibition of HIF in breast cancer cells inhibited metastasis to the lungs by blocking the expression of L1CAM and ANGPTL4 which facilitate binding to ECs and inhibit EC-EC interaction (Huang *et al*., 2011; Zhang *et al*., 2012).

Once extravasated from the blood the cancer cell must survive and grow in its new environment.

HIF-1 has been shown in a number of studies to regulate pre-metastatic niche formation at distant organs prior to cancer cell arrival (Janine T Erler *et al*., 2009; Wong *et al*., 2011). The LOX family of enzymes have been shown to be able to remodel the ECM at distant sites as well as the primary tumour. LOX secreted by the primary tumour enters circulation causing collagen crosslinking in metastatic tissues. This facilitates the recruitment of bone marrow-derived cells which form clusters and form the pre-metastatic niche facilitating tumour cell colonisation (Janine T Erler *et al*., 2009; Wong *et al*., 2011; Xing *et al*., 2011).

Tumour hypoxia and therapy resistance

Tumour hypoxia has been found to have a strong association with worse outcome in a plethora of different cancers irrespective of treatment (P Vaupel & Mayer, 2007). Radiotherapy is known to have reduced efficacy in areas of hypoxia owing to the fact that DNA damage produced is not 'fixed' by oxygen (Azzam *et al*., 2012; Peter Vaupel *et al*., 2001). Radiosensitivity increases with increasing oxygen concentration, with anoxic cells roughly 3 times more resistant to radiation than well-oxygenated cells (Gray *et al*., 1953). Evidence also suggests that the cellular hypoxic response increases the levels of heat-shock proteins, decreasing the apoptotic potential while increasing the proliferation potential of selected clones which has been linked to radioresistance (Samali & Cotter, 1996; Zhivotovsky *et al*., 1999). This is in addition to the reduced DNA repair under hypoxia and increased genomic instability of hypoxic cancer cells which along with a reduced proliferation rate granting more time for repair allows them to avoid programmed cell death. This hypoxia conferred radiation protection leads to tumour recurrence as the hypoxic population of cells is able to survive radiotherapy and repopulate the tumour. Direct O_2 measurements in clinical studies of neck squamous cell carcinoma, prostate cancer and cervix cancer treated with radiotherapy show that hypoxia is strongly associated with local-regional control (Fyles *et al*., 1998; Milosevic *et al*., 2012; Nordsmark *et al*., 2005).

Many chemotherapeutics also have reduced efficacy in areas of hypoxia due to a number of factors. The larger distances that the drug must penetrate as well as irregular blood flow and compressed blood and lymphatic vessels limit the amount of drug that can reach the target cells. On top of this, the structure and composition of the extracellular matrix (ECM) can further limit the delivery of the drug (Minchinton & Tannock, 2006). Further to this hypoxia is known to upregulate the expression of multidrug resistance protein (MDR1) and multidrug resistance-associated protein (MRP1). These proteins pump chemotherapeutic drugs out of the cell decreasing their intracellular concentrations, conferring increased resistance as has been reported in a number of different cancer types (Ding *et al*., 2010; Kruh & Belinsky, 2003; Liu *et al*., 2008). Hypoxia-induced downregulation of pro-apoptotic proteins such as Bax and Bid has been shown in a colon xenograft model to contribute to drug resistance (J. T. Erler *et al*., 2004). Downregulation of the pro-apoptotic

proteins, Bim and Bmf in normal breast epithelial cells has been shown to result in ductal carcinoma in situ-like phenotype (Whelan *et al*., 2010). Survivin, an anti-apoptotic protein is also regulated by HIF-1α and high levels correlated with doxorubicin resistance in a panel of organotypic human breast tumours (Faversani *et al*., 2014).

Hypoxia has been shown to induce the DDR including both ATM and ATR in the absence of detectable DNA damage in a background of repressed DNA repair. ATR has been shown to mediate p53 accumulation and cell cycle arrest in S phase cells, in hypoxia through phosphorylation, with ATR inhibition leading to a reduction in p53 accumulation (E. M. Hammond *et al*., 2002; Ester M. Hammond & Giaccia, 2005) ATM has also been shown to phosphorylate and stabilise HIF-1α in milder hypoxic conditions (0.2-1% O_2) leading to a reduction of mTORC1 signalling which coordinates cell growth and metabolism (Cam *et al*., 2010). ATR also has an effect on HIF-1α. ATR inhibition was shown to delay HIF-1α stabilisation and slow down hypoxia-induced cell motility in a HIF-1α dependent manner (Olcina & Hammond, 2014). ATR has also been shown to positively regulate HIF-1α translation at 0.1% O_2 (Fallone *et al*., 2013). Hypoxia-induced DDR can act as a mechanism to protect against genomic instability, however, this induction has been shown to be transient and is repressed following exposure to chronic hypoxia (Pires, Bencokova, McGurk, *et al*., 2010; Pires, Bencokova, Milani, *et al*., 2010). Genetic instability is also promoted under hypoxic conditions. Aberrant DNA replication (Young *et al*., 1988), gene amplification, (Rice *et al*., 1986) and base substitutions and DNA deletions have all been linked to the hypoxic microenvironment (Reynolds *et al*., 1996). Defects in double-strand break sensing have also been implicated, with irradiated cells under chronic hypoxia or anoxia showing increased residual double-strand breaks and chromosomal aberrations (Kumareswaran *et al*., 2012). Moreover, hypoxia leads to the repression of a number of DNA repair pathways, driving genomic instability (Klein & Glazer, 2010). This genomic instability allows cells that have been targeted by DNA damaging chemotherapeutics to survive and can result in more heterogeneous and aggressive tumours. However, downregulation of DNA repair proteins opens up the possibility of targeting the hypoxic regions through synthetic lethality for example through PARP inhibition in Homologous recombination deficient hypoxic cells (Chan *et al*., 2010)

Additionally the acidic pH in the tumour microenvironment can limit the capacity of small molecules to pass the cellular membrane or cause localisation to unfavourable cellular compartments due to their altered pH. This phenomenon is called 'ion trapping' and reduces the intracellular concentration or concentration in the desired location of the drug. If a drug requires active transport to enter a cell, the abnormal pH environment can also lead to reduced efficiency of these transport proteins limiting the transport of the drugs into the cell. As well as impaired transport, the activity of some of these drugs may be pH-dependent as well (Stubbs *et al*., 2000). Many chemotherapeutics have been shown to be less effective under hypoxic conditions including bleomycin, procarbazine, streptonigrin, actinomycin D, vincristine, Sorafenib, Irinotecan and docetaxel (Strese *et al*.,

2013; Teicher *et al*., 1981). The combination of reduced uptake and the upregulation of survival pathways and reduced proliferation leads to many drugs being less effective in hypoxic tumour cells.

Hypoxia also plays an important role in reducing the effectiveness of immunotherapy. Anaerobic glycolysis by tumour cells results in increased production and secretion of adenosine into the extracellular matrix which is a potent suppressor of NK and T-cells (Facciabene *et al*., 2011; Sitkovsky *et al*., 2014). Moreover, HIF-1α has been shown to be inhibitory to the cells of the adaptive immune system (Hatfield & Sitkovsky, 2016). Areas of hypoxia within the tumour have been shown to be infiltrated by high levels of immunosuppressive cells including myeloid-derived suppressor cells (MDSCs), tumour associated macrophages and T-regulatory cells (Tregs) (Noman *et al*., 2015). Hypoxic cells within the tumour express stromal cell-derived factor 1α which binds to IL-6 and IL-8, directly regulating the function and differentiation of MDSCs within the tumour microenvironment which show increased immunosuppressive function (Alfaro *et al*., 2011; Hong *et al*., 2015). Accumulation of Tregs in areas of hypoxia suppresses the anti-tumour response while also promoting neo-angiogenesis (Facciabene *et al*., 2011). HIF-1α can also promote immune evasion through upregulation of immune checkpoint proteins such as CD47, PD-L1 and HLA-G. On top of this, the hypoxia-induced acidic tumour microenvironment is believed to hamper the anti-tumour effects of the immune system and aid in immune evasion (Y. Li *et al*., 2018).

Many of the drug resistance mechanisms described above are found in the CSC subpopulation. CSCs have high expression of drug efflux proteins and anti-apoptotic factors. They also often maintain a quiescent state to avoid the induction of apoptosis when they receive DNA damage (Gottesman *et al*., 2002). This combined with their localisation in areas of hypoxia protecting them from radiotherapy as well as increased activation of the DDR (Bao *et al*., 2006) implicates them in the recurrence of tumours after treatment as they have the innate capacity to repopulate the tumour. Altogether this data shows the negative impact that hypoxia has on treatment efficacy and shows its pivotal role in treatment resistance and recurrence.

Overcoming hypoxia-induced therapy resistance

Several approaches have been taken to overcome tumour hypoxia, one is the development of hypoxia-activated prodrugs (HAPs). These are drugs which are inert until they reach areas of hypoxia where they are reduced usually by one or two-electron reductases producing the cytotoxic form of the drug (Y. Chen & Hu, 2009). This spares normal tissues while targeting the hypoxic population of tumours. Once activated the drug can also diffuse into the more well-oxygenated areas of the tumour in a bystander effect. Another method which uses the same mechanisms is the delivery of drugs to areas of hypoxia in

micro/nanocapsules. They use drug delivery nanocarriers that are susceptible to reduction by the increased expression of reducing agents such as glutathione in hypoxia releasing their cargo of drugs in the hypoxic environment, however, they are still in the design and research stages of development (Meng *et al*., 2009; Zeng *et al*., 2018). Although many HAPs such as evofosfamide have been promising in pre-clinical studies this has not translated into the clinic. Evofosfamide was studied in a phase III clinical trial and was found to improve progression-free survival and had a higher objective response rate, it failed because it did not significantly improve the overall survival time which was the primary endpoint (Van Cutsem *et al*., 2016).

A second approach to overcoming hypoxia is to increase the oxygen concentrations in the tumour itself, either by supplying more oxygen or reducing the oxygen consumption of tumours. Strategies include hyperbaric oxygen therapy which involves inhalation of pure oxygen under elevated pressure to systemically increase oxygen tension (Daruwalla & Christophi, 2006). Hyperthermia has also been tested to locally increase the temperature, leading to vasodilation and increased blood and therefore oxygen supply (Song *et al*., 2001). Accelerated radiotherapy with carbogen and nicotinamide (ARCON) which involves breathing carbogen (95% oxygen 5% carbon dioxide) in combination with nicotinamide, a vasodilator, to improve tumour oxygenation has also been tested. In a phase III clinical trial, ARCON showed limited success, with improved 5-year regional control, specifically in patients with hypoxic tumours, however no improvement in overall or disease-free survival was found (Janssens *et al*., 2012). Metformin, an inhibitor of mitochondrial complex I has been used to decrease oxygen consumption in tumours and is currently in phase II clinical trial in cervix cancer in combination with cisplatin and radiation (NTC02394652). A systematic review of 10,108 patients in 86 clinical trials designed to modify tumour hypoxia in patients receiving primary radiotherapy alone showed that overall modification of tumour hypoxia significantly improved the effect of radiotherapy but had no effect on metastasis (Overgaard, 2007). While many clinical trials have been unsuccessful, many have failed due to underpowered studies, dose-limiting toxicity in tissues that experience mild hypoxia, lack of hypoxia biomarkers to stratify patients and differences between murine and human tolerances (Spiegelberg *et al*., 2019). As well as this, a greater understanding of tumour hypoxia and the tumour microenvironment are needed to aid in drug development and other therapies for overcoming tumour hypoxia.

Hypoxia Biomarkers & models

Differences observed between *in vitro* cell line responses to hypoxia and the responses seen *in vivo* and primary human tumours have also been part of the problem that has lead to hypoxia modification and targeting strategies failing. This is due to the dynamic nature of hypoxia being hard to recapitulate *in vitro* as well as the complexity of the tumour

microenvironment being lost. One study compared the hypoxia transcriptome differences in bladder cancer, between the cell line EJ28 and 39 bladder tumour specimens. Of the 6000 genes measured 32 were induced in EJ28 while only eight of these 32 were found to be upregulated in more than 5 of the 39 tumour samples (Ord *et al*., 2005).

Another study in breast cancer compared the hypoxic signatures and their prognostic value of *in vitro* cell lines and *in vivo* models compared to patient material. The *in vitro* signature of upregulated genes showed little prognostic power, however the repressed *in vitro* genes and *in vivo* signatures did show prognostic value (Starmans *et al*., 2012). Toustrup *et al*. developed a 15 gene signature in head and neck cancer from 58 patient biopsies to predict the impact of hypoxia on radiotherapy outcome (Toustrup *et al*., 2011). They found that the hypoxic signature was able to predict higher 5-year locoregional failure of 'more' hypoxic patients from the radiotherapy only arm of the DAHANCA 5 trial, compared with the 'less' hypoxic group. In the trial arm receiving nimorazole as well as radiotherapy they were able to predict a benefit of nimorazole on 5-year locoregional failure, but only in patients whose signature classified 'more' hypoxic, showing the utility of their signature to stratify patients and as a prognostic and predictive tool. This signature was then used by Sørensen *et al*. to see if it could be applied to prostate, colon and oesophageal cancers (Sørensen *et al*., 2015). They used cell lines from each of these different cancers to see if the signature was conserved. They found that while there was definite overlap in the genes that were upregulated in hypoxia, some genes from the signature were not upregulated in the other cancer types tested. This is an example of the differential hypoxic transcriptome observed in cancers of different tissues.

Another study used RNA sequencing of 31 breast cancer cell lines representative of different sub-types or normal mammary epithelial cells exposed to normoxia or 1% O_2 (Ye *et al*., 2018). They found a 42 gene signature out of over 1000 hypoxia-regulated genes, that had a conserved response to hypoxia among all cell lines used. They found that lower expression of this hypoxic signature in the basal subtype correlated with a better prognosis than high expression in two METABRIC sample cohorts. On the other hand, one study in bladder cancer patients found that only 2 out of 13 published hypoxia signatures (Lendahl *et al*., 2009; Riester *et al*., 2012) had prognostic and predictive significance in a cohort of a phase III randomised trial of radiotherapy alone or with carbogen and nicotinamide (BCON) (Yang *et al*., 2017). They go on to create their own 24 gene hypoxic signature, of which high expression was significantly associated with poor prognosis in 4 out of 6 independent bladder cancer patient cohorts with the other two cohorts showing similar trends but no significance. Their signature also predicted poorer local progression-free survival of hypoxic patients in the BCON trial receiving either radiotherapy alone or BCON.

Interestingly when they combined their signature with that of Riester *et al*. (Riester *et al*., 2012) it improved the prognostic value in patients only receiving radiotherapy and increased predictive significance in patients with both high hypoxia (based on the 24

gene signature) and high Riester signature scores. Adding the Lendahl signature (Lendahl *et al*., 2009) to the 24 gene signature did not improve its prognostic significance but did improve its predictive power. The 24 gene signature showed little overlap with any of the other hypoxia signatures tested likely due to the large proportion of the genome that is transcriptionally responsive to changes in oxygenation. Janssens *et al*. found in a phase III clinical trial that Accelerated radiotherapy with carbogen and nicotinamide (ARCON) improved 5-year regional control specifically in patients with hypoxic tumours as measured with pimonidazole highlighting the need for reliable hypoxia biomarkers.

These studies show that data obtained from *in vitro* studies is not always consistent with what is seen in the clinic and should be confirmed using *in vivo* models that tend to produce data that is much more clinically relevant. They also show that one universal hypoxia signature is unlikely and that a signature may be required for each type of cancer to have the greatest predictive and prognostic capabilities.

Within the scope of *in vivo* models, there are also differences that affect its similarity to the patient. For instance, orthotopic or spontaneous tumour models better mimic the patient situation than Xenograft models, because the cancer grows in the same organ-specific tissue micro-environment as it would in a patient. This exposes it to the same factors such as hormones, tissue architecture, blood supply etc. as is found in the human equivalent. On top of this, it has been shown that the phenotypic properties of metastatic cells are governed by genes that are regulated in large part by the interaction with the relevant organ environment (Killion *et al*., 1999; Kubota, 1994). One example of this is the study by Naito *et al*. which compared the phenotypic differences between renal carcinoma cells implanted in the kidney versus subcutaneously in nude mice (Naito *et al*., 1987). They found that implantation into the kidney produced faster-growing tumours that produced more systemic metastases than subcutaneous tumours. They also observed that the kidney tumours were large, invasive, highly vascularised, non-encapsulated and contained minimal central necrosis. In contrast, the subcutaneous tumours highly encapsulated, with peripheral vascularisation and extensive areas of necrosis, showing the large phenotypic differences between xenograft and orthotopic models. On top of this, the immune system's role in cancer and therapy efficacy is becoming more and more evident and so the use of immunocompetent models is desirable.

Notch signalling

Notch proteins (Notch1-4) and their ligands (Delta/Jagged) are transmembrane proteins that provide a cell-cell communication system. The expression of Notch receptors and their ligands is tissue and context-specific. Notch receptors undergo a number of post-translational modifications in the Golgi/Endoplasmic reticulum that can alter their activity and stability as well as maturing by cleaving Notch creating a non-covalently associated

heterodimer before transport to the plasma membrane (Bray, 2016). The canonical Notch pathway occurs through the interaction of a Notch ligand on the "signal-sending cell" and the receptor on the "signal receiving cell". This interaction leads to two sequential proteolytic cleavage events by ADAM 10/17 and the gamma-secretase complex, which releases the Notch intracellular domain (NICD). The NICD then translocates to the nucleus where it forms a complex with CSL and MAML, allowing it to induce transcription of Notch downstream targets (Kopan & Ilagan, 2009). NICD is further regulated through post-translational modifications such as methylation, hydroxylation and acetylation as well as being influenced by other signalling pathways (Antfolk *et al*., 2019). There are a number of non-canonical pathways downstream of Notch that have been described including transcriptional activation of NF-kB, the PI3K-AKT-mTOR pathway and mitochondrial metabolism (Hales *et al*., 2014; Landor *et al*., 2011; L. Li *et al*., 2014; Perumalsamy *et al*., 2010).

Notch in cancer

Notch signalling is known to be deregulated in a number of cancers. Oncogenic Notch signalling alters the developmental state maintaining the cells in a proliferative or undifferentiated state while inhibiting apoptosis and promoting cell survival. This is seen in a number of cancers including T-ALL, breast cancer and lung cancer. However, Notch can also act as a tumour suppressor in some cell types including keratinocytes (Das & Teoh, 2017). Sequencing of cancer genomes has revealed 3 main patterns of mutation in the Notch gene, which are thought to be context-specific based on a particular selective pressure.

The first is a mutation that removes or alters the negative regulatory region (NRR) in some way, either through truncation of the gene or point mutations within the NRR or within the promoter leading to a gain of function Notch mutation. These mutated Notch proteins undergo ligand-independent proteolysis, releasing the NICD, this was first discovered in T-ALL and subsequently found in triple-negative breast cancer among others (Ashworth *et al*., 2010; Robinson *et al*., 2011). T-ALL and triple-negative breast cancers have also been shown to contain nonsense or frameshift mutations that lead to loss of the C- terminal PEST domain of Notch often in the same allele as mutations in the NRR. This leads to even higher levels of NICD because of reduced degradation. This lends evidence to the ongoing selective pressure that these cells experience to accumulate ever-increasing levels of Notch activation (K. Wang *et al*., 2015; Weng *et al*., 2004).

The second pattern is characterised by mutations which affect the PEST domains without any alterations to the NRR domain, leading to the NICD having an increased half-life. This is mainly seen in B cell tumours such as leukaemias and lymphomas (Kiel *et al*., 2012; Lohr *et al*., 2012; Puente *et al*., 2011). These alterations do not lead to constitutive

Notch signalling presumably because there is no selective pressure. This is likely because the levels of Notch that provide a selective benefit is lower than that of pattern 1 and they are in a tumour microenvironment that supplies sufficient activation from adjacent cells (Kluk *et al*., 2013).

The third pattern is described by mutations in the N-terminal portion of Notch, producing a loss of function. Some mutations lead to a failure to produce the protein while others produce a dominant-negative Notch receptor with a non-functional or deleted intracellular domain. These types of mutation are prevalent in cutaneous squamous cell carcinomas, oesophageal and lung cancers as well as others (Agrawal *et al*., 2012; N. J. Wang *et al*., 2011). This type of mutation was shown to increase the incidence of skin tumours of mice exposed to carcinogens suggesting that Notch plays a role as a tumour suppressor in these tissues (Nicolas *et al*., 2003).

Another mutation commonly found in cancer directly affecting Notch activity is the Fbwx7/cdc4 mutation, disrupting a ubiquitin ligase targeting Cyclin E Myc but also Notch (O'Neil *et al*., 2007; Thompson *et al*., 2007).

The most well characterised oncogenic function of Notch is to turn on the expression of genes that promote cell growth such as cMyc, a global regulator of pro-growth metabolism (Palomero *et al*., 2006). Notch is also able to enhance PI3K-Akt signalling in T-ALL as well as downregulating the tumour suppressor *PTEN* which normally functions to suppress PI3K-AKT signalling (Palomero *et al*., 2007; Trimarchi *et al*., 2014). AKT has a number of pro-oncogenic activities including upregulation of glucose transporters and promoting cell survival via various downstream mechanisms. There is also evidence for co-regulation of genes by Notch and HIF transcription factors promoting tumour cell migration, invasion and tumour cell metabolism. However, whether these observations are generally relevant to cancers with Notch alterations or are only relevant in particular contexts are still to be elucidated (Gustafsson *et al*., 2005; Sahlgren *et al*., 2008; X. Zheng *et al*., 2008). Notch has also been implicated in maintaining cancer stem cell populations in solid tumours including glioblastoma, ovarian and breast cancer (D'Angelo *et al*., 2015; McAuliffe *et al*., 2012; T. S. Zhu *et al*., 2011). Notch has also been implicated in promoting tumour metastasis as well as resistance to chemotherapy in a number of studies through a number of different mechanisms (Chanrion *et al*., 2014; K. G. Leong *et al*., 2007; Z. Wang *et al*., 2010; Xie *et al*., 2013).

These are just a few of the effects that Notch has in cancer, Many more have been characterised and many are still to be fully understood especially in context with other oncogenic mutations and shows the broad variety of effects that Notch has in cancer biology (Aster *et al*., 2017).

Studying gene function and cell fate

Elucidating the role of genes can be difficult, with so many direct and indirect interactions as well as effects from downstream targets, compensation mechanisms and genetic redundancy obscuring the effects of the gene. A common way to study the effects of genes is to use gene overexpression in concert with gene disruption or knockout (KO) models. Overexpression can be achieved in a few ways. The gene can be introduced through viral transduction under the control of a ubiquitous promoter such as ROSA26 or EF1α. tissue-specific gene regulatory elements/promoters can also be used e.g. Villin to target gene expression to absorptive cells specifically, or GFAP to direct expression to astrocytes. Alternatively, the active form of the protein can be expressed or regulatory domains/amino acids deleted or mutated, such as expression of the NICD alone. Fluorescent proteins can also be added to these inserted genes either with a fusion protein of the two or with the use of an internal ribosome entry site so that both the fluorescent protein and the gene of interest are expressed separately from the same mRNA. This can aid in the visualisation of the localisation of the protein when a fusion protein is used, and to confirm in which cells there is overexpression.

Knockout of a gene is used to study the other extreme. This can be done in a number of ways but CRISPR/Cas9 is the current standard. CRISPR/Cas9 allows for insertion of DNA into a specific site or deletion/mutation of a specific stretch of DNA through the use of guide RNAs. The combination of an overexpression model and a KO model can help to understand the effects that individual genes have.

One caveat of using overexpression or gene deletion is that from the moment the gene affects cell functionality gene lineage may change and if this happens early during development the observed phenotypes may be a consequence of something else rather than a causal effect on that cell type. For studying gene KO or overexpression as well as cell fate *in vivo* it is useful to use lineage tracing.

Lineage tracing is the method by which a specific cell or population of cells is marked so that they and their progeny can be traced and identified at a later timepoint. Lineage tracing has been useful in developmental biology to determine from which progenitor cells, organs and tissues are derived. An advantage of lineage tracing is that it only marks gene activity it does not interfere with gene activity and therefore also does not alter lineages such as with overexpression or gene disruption. There are a number of lineage tracing strategies but the most widely used method involves site-specific genetic recombination, a popular example of this is Cre-Lox.

In this method, a reporter gene such as a fluorescent protein is preceded by a stop cassette or secondary marker flanked by loxP sites. This reporter is introduced into the genome of a cell line or animal under the control of a ubiquitous promoter. Cre-recombinase (Cre) is then expressed in the cells of choice via cell-type or tissue-specific promoters, or through another strategy that only labels the cells of choice. The Cre can

then recombine and delete the DNA between the loxP sites initiating expression of the reporter.

Over time this strategy has been built on to allow more control over Cre induced recombination such as the addition of tamoxifen-inducible oestrogen receptor (ERT^2). The ERT^2 domain sequesters Cre in the cytoplasm until tamoxifen is added allowing temporal control of labelling as well. The use of fluorescent markers also allows live imaging *in vivo* with the advent of methods such as intravital imaging. This technique is also utilised in cancer research to study a specific subpopulation of cells within the tumour such as Notch1 expressing cells in murine intestinal tumours or to elucidate the initiation of tumorigenesis (Frumkin *et al*., 2008; Mourao *et al*., 2019; Vooijs *et al*., 2007).

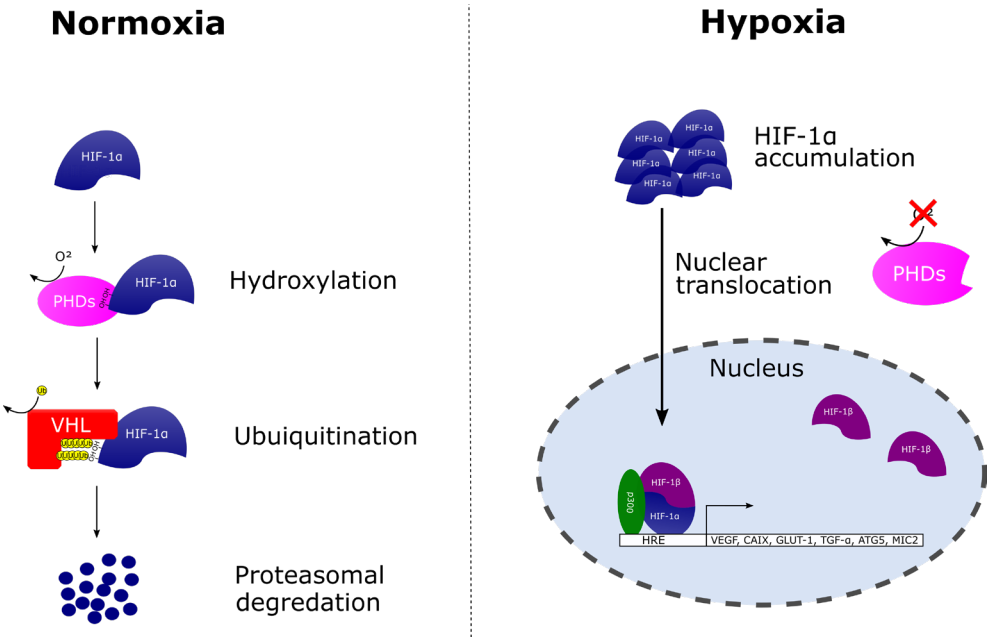

Figure 1: Overview of the oxygen-dependent regulation of HIF-1α.

Thesis outline

There is a need to better understand the mechanisms that promote tumour progression and therapy resistance. Notch signalling is known to be frequently deregulated in cancer and is involved in treatment resistance. Hypoxia and high levels of HIF in cancer are associated with and correlate with more malignant tumours, therapy resistance, immunosuppression, metastasis and poor prognosis. Notch and HIFs also co-regulate the transcription of genes promoting tumour cell migration, invasion and tumour cell metabolism. The aim of this thesis was to investigate the role of Notch and hypoxia in tumour progression and resistance to therapy.

In **chapter 2** we reviewed the role of Notch in breast cancer and targeting strategies to enhance current treatments and combat treatment resistance. We find that treatment-resistant cancers can be re-sensitised by Notch inhibition, providing a strong rationale for a combination of Notch inhibition and current breast cancer treatment modalities in patients with Notch to overcome treatment resistance. This combination would require stratification of patients based on Notch activity due to only a subset of cancers showing aberrant Notch signalling. Furthermore, Notch signalling may not become clinically evident until treatment initiation as a resistance mechanism.

In **chapter 3** we investigate the effect of hypoxia on a cell contact independent mechanism of Notch activity stimulation. We use conditioned medium from H460 ΔE-GFP lung cancer cells cultured in different oxygen concentrations and with a Notch gamma-secretase inhibitor to study the effect on Notch activity on receptor cells. We find that conditioned medium from hypoxic or anoxic cells induces Notch activity in 5XCSL luciferase reporter cells and upregulation of the *hes1* Notch target gene. This suggests that that H460 Notch overexpressing cells when cultured under hypoxia secrete soluble factors in the medium that can stimulate notch signalling in receiver cells at a distance.

In **chapter 4** we created and utilised a novel hypoxia lineage tracing strategy termed MARCer. We inserted this system into the H1299 lung cancer cell line and characterised the lineage tracing properties of the system. *In vitro,* we tested the labelling characteristics of the cell line at different oxygen and 4OHT concentrations. We find that labelling is restricted to hypoxic conditions when 4OHT is present. We continue with the cell line *in vivo* using a tumour xenograft model with intravital imaging. We demonstrate its use as a valuable tool to study the behaviour of post-hypoxic cells both *in vivo* and *ex vivo* at single-cell resolution.

In **chapter 5** we build on this lineage tracing strategy with the addition of the diphtheria toxin receptor to the reporter allowing selective ablation of labelled cells to study the role of hypoxic cells on treatment resistance. We investigate this system in the metastatic 4T1 murine mammary carcinoma cell line. We start by characterising it *in vitro* finding it to be regulated in an oxygen and 4OHT dependent manner, labelling only hypoxic cells in the presence of 4OHT. We then used the cell line in an *in vivo* orthotopic

model to study the effect of hypoxic cells on radiotherapy treatment, and their behaviour within the primary tumour with and without treatment.

In **chapter 6** we created a transgenic mouse model which contains the hypoxic lineage tracing construct inserted into the Hif-1α locus. We test the ES cells used to create the mouse *in vitro* to characterise the lineage tracing properties. We look at the stabilisation of the MARCer protein in normoxia and when treated with the hypoxia mimetic DFO. We go on to test the lineage tracing properties by inserting a reporter. We test it in normoxia and hypoxia and find that it only labels cells in hypoxic conditions in the presence of 4OHT. From this, we conclude that the generated mouse should be able to label hypoxic cells in a tamoxifen dependent manner and be a useful tool to study the fate of hypoxic cells.

In **chapter 7** we discuss the findings from the previous chapters and outline the remaining challenges to be addressed in future research, and the potential for the hypoxic lineage tracing in this research.

References

1. Agrawal, N., Jiao, Y., Bettegowda, C., Hutess, S. M., Wang, Y., David, S., Cheng, Y., Twaddell, W. S., Latt, N. L., Shin, E. J., Wang, L. D., Wang, L., Yang, W., Velculescu, V. E., Vogelstein, B., Papadopoulos, N., Kinzler, K. W., & Meltzer, S. J. (2012). Comparative genomic analysis of esophageal adenocarcinoma and squamous cell carcinoma. *Cancer Discovery*, *2*(10), 899–905. https://doi.org/10.1158/2159-8290.CD-12-0189
2. Alexander, N. R., Branch, K. M., Parekh, A., Clark, E. S., Iwueke, I. C., Guelcher, S. A., & Weaver, A. M. (2008). Extracellular matrix rigidity promotes invadopodia activity. *Current Biology : CB*, *18*(17), 1295–1299. https://doi.org/10.1016/j.cub.2008.07.090
3. Alfaro, C., Suárez, N., Martínez-Forero, I., Palazón, A., Rouzaut, A., Solano, S., Feijoo, E., Gúrpide, A., Bolaños, E., Erro, L., Dubrot, J., Hervás-Stubbs, S., Gonzalez, A., Perez-Gracia, J. L., & Melero, I. (2011). Carcinoma-derived Interleukin-8 disorients dendritic cell migration without impairing T-cell stimulation. *PLoS ONE*, *6*(3). https://doi.org/10.1371/journal.pone.0017922
4. An, W. G., Kanekal, M., & Blagosklonny, M. V. (1998). *Stabilization of wild-type p53 by hypoxia-inducible factor 1 ␣*. *392*(March), 7416–7419.
5. Antfolk, D., Antila, C., Kemppainen, K., Landor, S. K. J., & Sahlgren, C. (2019). Decoding the PTM-switchboard of Notch. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1866*(12), 118507. https://doi.org/10.1016/j.bbamcr.2019.07.002
6. Ashworth, T. D., Pear, W. S., Chiang, M. Y., Blacklow, S. C., Mastio, J., Xu, L., Kelliher, M., Kastner, P., Chan, S., & Aster, J. C. (2010). Deletion-based mechanisms of Notch1 activation in T-ALL: Key roles for RAG recombinase and a conserved internal translational start site in Notch1. *Blood*, *116*(25), 5455–5464. https://doi.org/10.1182/blood-2010-05-286328
7. Aster, J. C., Pear, W. S., & Blacklow, S. C. (2017). The Varied Roles of Notch in Cancer. *Annual Review of Pathology*, *12*(4), 245–275. https://doi.org/10.1146/annurev-pathol-052016-100127
8. Azzam, E. I., Jay-Gerin, J.-P., & Pain, D. (2012). Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. *Cancer Letters*, *327*(1–2), 48–60. https://doi.org/10.1016/j.canlet.2011.12.012
9. Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D., & Rich, J. N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature*, *444*(7120), 756–760. https://doi.org/10.1038/nature05236
10. Bataller, R., & Brenner, D. a. (2005). Liver fibrosis. *The Journal of Clinical Investigation*, *115*(2), 209–218. https://doi.org/10.1172/JCI24282
11. Bos, R., Van der Groep, P., Greijer, A. E., Shvarts, A., Meijer, S., Pinedo, H. M., Semenza, G. L., Van Diest, P. J., & Van der Wall, E. (2003). Levels of hypoxia-inducible factor-1?? independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*, *97*(6), 1573–1581. https://doi.org/10.1002/cncr.11246

12. Boyd, N. F., Jensen, H. M., Cooke, G., Han, H. L., Lockwood, G. A., & Miller, A. B. (2000). Mammographic densities and the prevalence and incidence of histological types of benign breast disease. Reference Pathologists of the Canadian National Breast Screening Study. *European Journal of Cancer Prevention : The Official Journal of the European Cancer Prevention Organisation (ECP)*, *9*(1), 15–24. https://doi.org/10.1097/00008469-200002000-00003
13. Brahimi-Horn, M. C., Chiche, J., & Pouysségur, J. (2007). Hypoxia and cancer. *Journal of Molecular Medicine*, *85*(12), 1301–1307. https://doi.org/10.1007/s00109-007-0281-3
14. Bray, S. J. (2016). Notch signalling in context. *Nature Reviews. Molecular Cell Biology*, *17*(11), 722–735. https://doi.org/10.1038/nrm.2016.94
15. Brown, L. F., Guidi, A. J., Schnitt, S. J., Van De Water, L., Iruela-Arispe, M. L., Yeo, T. K., Tognazzi, K., & Dvorak, H. F. (1999). Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *5*(5), 1041–1056. https://doi.org/10.1055/s-0030-1255354
16. Cam, H., Easton, J. B., High, A., & Houghton, P. J. (2010). mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1α. *Molecular Cell*, *40*(4), 509–520. https://doi.org/10.1016/j.molcel.2010.10.030
17. Chan, N., Pires, I. M., Bencokova, Z., Coackley, C., Luoto, K. R., Bhogal, N., Lakshman, M., Gottipati, P., Oliver, F. J., Helleday, T., Hammond, E. M., & Bristow, R. G. (2010). Contextual synthetic lethality of cancer cell kill based on the tumor microenvironment. *Cancer Research*, *70*(20), 8045–8054. https://doi.org/10.1158/0008-5472.CAN-10-2352
18. Chang, H. Y., Nuyten, D. S. A., Sneddon, J. B., Hastie, T., Tibshirani, R., Sørlie, T., Dai, H., He, Y. D., Van, L. J., Bartelink, H., Rijn, M. Van De, & Brown, P. O. (2005). *Robustness , scalability , and integration of a wound-response gene expression signature in predicting breast cancer survival*.
19. Chanrion, M., Kuperstein, I., Barrière, C., El Marjou, F., Cohen, D., Vignjevic, D., Stimmer, L., Paul-Gilloteaux, P., Bièche, I., Tavares, S. D. R., Boccia, G. F., Cacheux, W., Meseure, D., Fre, S., Martignetti, L., Legoix-Né, P., Girard, E., Fetler, L., Barillot, E., … Robine, S. (2014). Concomitant Notch activation and p53 deletion trigger epithelial-to-mesenchymal transition and metastasis in mouse gut. *Nature Communications*, *5*. https://doi.org/10.1038/ncomms6005
20. Chaplin, D. J., Olive, P. L., & Durand, R. E. (1987). Intermittent Blood Flow in a Murine Tumor: Radiobiological Effects. *Cancer Research*, *47*(2), 597–601.
21. Chen, D., Li, M., Luo, J., & Gu, W. (2003). Direct interactions between HIF-1 alpha and Mdm2 modulate p53 function. *The Journal of Biological Chemistry*, *278*(16), 13595–13598. https://doi.org/10.1074/jbc.C200694200
22. Chen, Y., & Hu, L. (2009). Design of anticancer prodrugs for reductive activation. *Medicinal Research Reviews*, *29*(1), 29–64. https://doi.org/10.1002/med.20137
23. Chi, J.-T., Wang, Z., Nuyten, D. S. A., Rodriguez, E. H., Schaner, M. E., Salim, A., Wang, Y., Kristensen, G. B., Helland, A., Børresen-Dale, A.-L., Giaccia, A., Longaker, M. T., Hastie, T., Yang, G. P., van de Vijver, M. J., & Brown, P. O. (2006). Gene expression programs in response to hypoxia: cell type specificity and prognostic significance in human cancers. *PLoS Medicine*, *3*(3), e47. https://doi.org/10.1371/journal.pmed.0030047

24. Clarke, M. F., Dick, J. E., Dirks, P. B., Eaves, C. J., Jamieson, C. H. M., Jones, D. L., Visvader, J., Weissman, I. L., & Wahl, G. M. (2006). Cancer stem cells - Perspectives on current status and future directions: AACR workshop on cancer stem cells. *Cancer Research*, *66*(19), 9339–9344. https://doi.org/10.1158/0008-5472.CAN-06-3126
25. Colpaert, C. G., Vermeulen, P. B., Fox, S. B., Harris, A. L., Dirix, L. Y., & Marck, E. A. Van. (2003). *The presence of a fibrotic focus in invasive breast carcinoma correlates with the expression of carbonic anhydrase IX and is a marker of hypoxia and poor prognosis*. 137–147.
26. Condeelis, J., & Pollard, J. W. (2006). Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis. *Cell*, *124*(2), 263–266. https://doi.org/10.1016/j.cell.2006.01.007
27. Cosse, J. P., Sermeus, A., Vannuvel, K., Ninane, N., Raes, M., & Michiels, C. (2007). Differential effects of hypoxia on etoposide-induced apoptosis according to the cancer cell lines. *Molecular Cancer*, *6*, 1–16. https://doi.org/10.1186/1476-4598-6-61
28. Covello, K. L., Kehler, J., Yu, H., Gordan, J. D., Arsham, A. M., Hu, C., Labosky, P. A., Simon, M. C., & Keith, B. (2006). HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes & Development*, *20*(5), 557–570. https://doi.org/10.1101/gad.1399906
29. Cox, T. R., Bird, D., Baker, A. M., Barker, H. E., Ho, M. W. Y., Lang, G., & Erler, J. T. (2013). LOX-mediated collagen crosslinking is responsible for fibrosis-enhanced metastasis. *Cancer Research*, *73*(6), 1721–1732. https://doi.org/10.1158/0008-5472.CAN-12-2233
30. D'Angelo, R. C., Ouzounova, M., Davis, A., Choi, D., Tchuenkam, S. M., Kim, G., Luther, T., Quraishi, A. A., Senbabaoglu, Y., Conley, S. J., Clouthier, S. G., Hassan, K. A., Wicha, M. S., & Korkaya, H. (2015). Notch reporter activity in breast cancer cell lines identifies a subset of cells with stem cell activity. *Molecular Cancer Therapeutics*, *14*(3), 779–787. https://doi.org/10.1158/1535-7163.MCT-14-0228
31. Damaghi, M., Wojtkowiak, J. W., & Gillies, R. J. (2013). pH sensing and regulation in cancer. *Frontiers in Physiology*, *4 DEC*(December), 1–10. https://doi.org/10.3389/fphys.2013.00370
32. Daruwalla, J., & Christophi, C. (2006). Hyperbaric oxygen therapy for malignancy: A review. *World Journal of Surgery*, *30*(12), 2112–2131. https://doi.org/10.1007/s00268-006-0190-6
33. Das, S., & Teoh, S. (2017). Notch signalling pathways and their importance in the treatment of cancers. *Current Drug Targets*, *18*(999), 1–1. https://doi.org/10.2174/1389450118666170309143419
34. Davis, F. M., Azimi, I., Faville, R. A., Peters, A. A., Jalink, K., Putney, J. W., Goodhill, G. J., Thompson, E. W., Roberts-Thomson, S. J., & Monteith, G. R. (2014). Induction of epithelial-mesenchymal transition (EMT) in breast cancer cells is calcium signal dependent. *Oncogene*, *33*(18), 2307–2316. https://doi.org/10.1038/onc.2013.187
35. Dewhirst, M. W. (2009). Relationships between cycling hypoxia, HIF-1, angiogenesis and oxidative stress. *Radiation Research*, *172*(6), 653–665. https://doi.org/10.1667/RR1926.1
36. Dewhirst, M. W., Braun, R. D., & Lanzen, J. L. (1998). Temporal changes in pO2 of R3230AC tumors in Fischer-344 rats. *International Journal of Radiation Oncology Biology Physics*. https://doi.org/10.1016/S0360-3016(98)00304-6
37. Deynoux, M., Sunter, N., Hérault, O., & Mazurier, F. (2016). Hypoxia and hypoxia-inducible factors in leukemias. *Frontiers in Oncology*, *6*(FEB), 1–9. https://doi.org/10.3389/fonc.2016.00041

38. Ding, Z., Yang, L., Xie, X., Xie, F., Pan, F., Li, J., He, J., & Liang, H. (2010). Expression and significance of hypoxia-inducible factor-1 alpha and MDR1/P-glycoprotein in human colon carcinoma tissue and cells. *Journal of Cancer Research and Clinical Oncology*, *136*(11), 1697–1707. https://doi.org/10.1007/s00432-010-0828-5
39. Eisinger-Mathason, T. S. K., Zhang, M., Qiu, Q., Skuli, N., Nakazawa, M. S., Karakasheva, T., Mucaj, V., Shay, J. E. S., Stangenberg, L., Sadri, N., Puré, E., Yoon, S. S., Kirsch, D. G., & Simon, M. C. (2013). Hypoxia-dependent modification of collagen networks promotes sarcoma metastasis. *Cancer Discovery*, *3*(10), 1190–1205. https://doi.org/10.1158/2159-8290.CD-13-0118
40. Erler, J. T., Cawthorne, C. J., Williams, K. J., Koritzinsky, M., Wouters, B. G., Wilson, C., Miller, C., Demonacos, C., Stratford, I. J., & Dive, C. (2004). Hypoxia-Mediated Down-Regulation of Bid and Bax in Tumors Occurs via Hypoxia-Inducible Factor 1-Dependent and -Independent Mechanisms and Contributes to Drug Resistance. *Molecular and Cellular Biology*, *24*(7), 2875–2889. https://doi.org/10.1128/mcb.24.7.2875-2889.2004
41. Erler, Janine T., & Giaccia, A. J. (2006). Lysyl oxidase mediates hypoxic control of metastasis. *Cancer Research*, *66*(21), 10238–10241. https://doi.org/10.1158/0008-5472.CAN-06-3197
42. Erler, Janine T, Bennewith, K. L., Cox, T. R., Lang, G., Bird, D., Koong, A., Le, Q.-T., & Giaccia, A. J. (2009). Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell*, *15*(1), 35–44. https://doi.org/10.1016/j.ccr.2008.11.012
43. Facciabene, A., Peng, X., Hagemann, I. S., Balint, K., Barchetti, A., Wang, L. P., Gimotty, P. A., Gilks, C. B., Lal, P., Zhang, L., & Coukos, G. (2011). Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T reg cells. *Nature*, *475*(7355), 226–230. https://doi.org/10.1038/nature10169
44. Fallone, F., Britton, S., Nieto, L., Salles, B., & Muller, C. (2013). ATR controls cellular adaptation to hypoxia through positive regulation of hypoxia-inducible factor 1 (HIF-1) expression. *Oncogene*, *32*(37), 4387–4396. https://doi.org/10.1038/onc.2012.462
45. Faversani, A., Vaira, V., Moro, G. P., Tosi, D., Lopergolo, A., Schultz, D. C., Rivadeneira, D., Altieri, D. C., & Bosari, S. (2014). Survivin family proteins as novel molecular determinants of doxorubicin resistance in organotypic human breast tumors. *Breast Cancer Research*, *16*(3), 1–13. https://doi.org/10.1186/bcr3666
46. Fidler, I. J. (1970). Metastasis: quantitative analysis of distribution and fate of tumor emboli labeled with 125 I-5-iodo-2'-deoxyuridine. *Journal of the National Cancer Institute*, *45*(4), 773–782. http://www.ncbi.nlm.nih.gov/pubmed/5513503
47. Finak, G., Bertos, N., Pepin, F., Sadekova, S., Souleimanova, M., Zhao, H., Chen, H., Omeroglu, G., Meterissian, S., Omeroglu, A., Hallett, M., & Park, M. (2008). Stromal gene expression predicts clinical outcome in breast cancer. *Nature Medicine*, *14*(5), 518–527. https://doi.org/10.1038/nm1764
48. Finger, E. C., Castellini, L., Rankin, E. B., Vilalta, M., Krieg, A. J., Jiang, D., & Banh, A. (2015). *Hypoxic induction of AKAP12 variant 2 shifts PKA-mediated protein phosphorylation to enhance migration and metastasis of melanoma cells*. *112*(14), 4441–4446. https://doi.org/10.1073/pnas.1418164112
49. Fraley, S. I., Feng, Y., Krishnamurthy, R., Kim, D. H., Celedon, A., Longmore, G. D., & Wirtz, D. (2010). A distinctive role for focal adhesion proteins in three-dimensional cell motility. *Nature Cell Biology*, *12*(6), 598–604. https://doi.org/10.1038/ncb2062

50. Frantz, C., Stewart, K. M., & Weaver, V. M. (2010). The extracellular matrix at a glance. *Journal of Cell Science*, *123*(24), 4195–4200. https://doi.org/10.1242/jcs.023820
51. Frumkin, D., Wasserstrom, A., Itzkovitz, S., Stern, T., Harmelin, A., Eilam, R., Rechavi, G., & Shapiro, E. (2008). Cell lineage analysis of a mouse tumor. *Cancer Research*, *68*(14), 5924–5931. https://doi.org/10.1158/0008-5472.CAN-07-6216
52. Fyles, A. W., Milosevic, M., Wong, R., Kavanagh, M. C., Pintilie, M., Sun, A., Chapman, W., Levin, W., Manchul, L., Keane, T. J., & Hill, R. P. (1998). Oxygenation predicts radiation response and survival in patients with cervix cancer. *Radiotherapy and Oncology*, *48*(2), 149–156. https://doi.org/10.1016/S0167-8140(98)00044-9
53. Giatromanolaki, A., Koukourakis, M. I., Sivridis, E., Turley, H., Talks, K., Pezzella, F., Gatter, K. C., & Harris, A. L. (2001). Relation of hypoxia inducible factor 1α and 2α in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. *British Journal of Cancer*, *85*(6), 881–890. https://doi.org/10.1054/bjoc.2001.2018
54. Gilkes, D. M., Bajpai, S., Wong, C. C., Chaturvedi, P., Hubbi, M. E., Wirtz, D., & Semenza, G. L. (2013). Procollagen lysyl hydroxylase 2 is essential for hypoxia-induced breast cancer metastasis. *Molecular Cancer Research : MCR*, *11*(5), 456–466. https://doi.org/10.1158/1541-7786.MCR-12-0629
55. Gligorijevic, B., Bergman, A., & Condeelis, J. (2014). Multiparametric Classification Links Tumor Microenvironments with Tumor Cell Phenotype. *PLoS Biology*, *12*(11). https://doi.org/10.1371/journal.pbio.1001995
56. Godet, I., Shin, Y. J., Ju, J. A., Ye, I. C., Wang, G., & Gilkes, D. M. (2019). Fate-mapping post-hypoxic tumor cells reveals a ROS-resistant phenotype that promotes metastasis. *Nature Communications*, *10*(1), 1–18. https://doi.org/10.1038/s41467-019-12412-1
57. Gort, E. H., Van Haaften, G., Verlaan, I., Groot, A. J., Plasterk, R. H. A., Shvarts, A., Suijkerbuijk, K. P. M., Van Laar, T., Van Der Wall, E., Raman, V., Van Diest, P. J., Tijsterman, M., & Vooijs, M. (2008). The TWIST1 oncogene is a direct target of hypoxia-inducible factor-2α. *Oncogene*, *27*(11), 1501–1510. https://doi.org/10.1038/sj.onc.1210795
58. Gottesman, M. M., Fojo, T., & Bates, S. E. (2002). Multidrug resistance in cancer: Role of ATP-dependent transporters. *Nature Reviews Cancer*, *2*(1), 48–58. https://doi.org/10.1038/nrc706
59. Graham, C. H., Forsdike, J., Fitzgerald, C. J., & Macdonald-Goodfellow, S. (1999). Hypoxia-mediated stimulation of carcinoma cell invasiveness via upregulation of urokinase receptor expression. *International Journal of Cancer*, *80*(4), 617–623. https://doi.org/10.1002/(SICI)1097-0215(19990209)80:4<617::AID-IJC22>3.0.CO;2-C
60. Gray, L. H., Conger, A. D., Ebert, M., Hornsey, S., & Scott, O. C. (1953). The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *The British Journal of Radiology*, *26*(312), 638–648. https://doi.org/10.1259/0007-1285-26-312-638
61. Gustafsson, M. V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U., & Bondesson, M. (2005). Hypoxia requires Notch signaling to maintain the undifferentiated cell state. *Developmental Cell*, *9*(5), 617–628. https://doi.org/10.1016/j.devcel.2005.09.010

62. Hackenbeck, T., Knaup, K. X., Schietke, R., Schödel, J., Willam, C., Wu, X., Warnecke, C., Eckardt, K. U., & Wiesener, M. S. (2009). HIF-1 or HIF-2 induction is sufficient to achieve cell cycle arrest in NIH3T3 mouse fibroblasts independent from hypoxia. *Cell Cycle*, *8*(9), 1386–1395. https://doi.org/10.4161/cc.8.9.8306
63. Hales, E. C., Taub, J. W., & Matherly, L. H. (2014). New insights into Notch1 regulation of the PI3K-AKT-mTOR1 signaling axis: targeted therapy of γ-secretase inhibitor resistant T-cell acute lymphoblastic leukemia. *Cellular Signalling*, *26*(1), 149–161. https://doi.org/10.1016/j.cellsig.2013.09.021
64. Hammond, E. M., Denko, N. C., Dorie, M. J., Abraham, R. T., & Giaccia, A. J. (2002). Hypoxia Links ATR and p53 through Replication Arrest. *Molecular and Cellular Biology*, *22*(6), 1834–1843. https://doi.org/10.1128/mcb.22.6.1834-1843.2002
65. Hammond, Ester M., & Giaccia, A. J. (2005). The role of p53 in hypoxia-induced apoptosis. *Biochemical and Biophysical Research Communications*, *331*(3), 718–725. https://doi.org/10.1016/j.bbrc.2005.03.154
66. Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013
67. Hanna, S. C., Krishnan, B., Bailey, S. T., Moschos, S. J., Kuan, P. F., Shimamura, T., Osborne, L. D., Siegel, M. B., Duncan, L. M., O'Brien, E. T., Superfine, R., Miller, C. R., Simon, M. C., Wong, K. K., & Kim, W. Y. (2013). HIF1α and HIF2α independently activate SRC to promote melanoma metastases. *Journal of Clinical Investigation*, *123*(5), 2078–2093. https://doi.org/10.1172/JCI66715
68. Harney, A. S., Arwert, E. N., Entenberg, D., Wang, Y., Guo, P., Qian, B., Oktay, M. H., Pollard, J. W., Jones, J. G., & Condeelis, J. S. (2015). Real-Time Imaging Reveals Local, Transient Vascular Permeability, and Tumor Cell Intravasation Stimulated by TIE2hi Macrophage-Derived VEGFA. *Cancer Discovery*, *5*(9), 932–943. https://doi.org/10.1158/2159-8290.CD-15-0012
69. Hasebe, T., Tsuda, H., Tsubono, Y., Imoto, S., & Mukai, K. (1997). Fibrotic Focus in Invasive Ductal Carcinoma of the Breast: A Histopathological Prognostic Parameter for Tumor Recurrence and Tumor Death within Three Years after the Initial Operation. *Japanese Journal of Cancer Research*, *88*(6), 590–599. https://doi.org/10.1111/j.1349-7006.1997.tb00423.x
70. Hatfield, S. M., & Sitkovsky, M. (2016). A2A adenosine receptor antagonists to weaken the hypoxia-HIF-1α driven immunosuppression and improve immunotherapies of cancer. *Current Opinion in Pharmacology*, *29*, 90–96. https://doi.org/10.1016/j.coph.2016.06.009
71. Hong, J., Tobin, N. P., Rundqvist, H., Li, T., Lavergne, M., García-Ibáñez, Y., Qin, H., Paulsson, J., Zeitelhofer, M., Adzemovic, M. Z., Nilsson, I., Roswall, P., Hartman, J., Johnson, R. S., Östman, A., Bergh, J., Poljakovic, M., & Genové, G. (2015). Role of Tumor Pericytes in the Recruitment of Myeloid-Derived Suppressor Cells. *Journal of the National Cancer Institute*, *107*(10), 1–25. https://doi.org/10.1093/jnci/djv209
72. Hope, K. J., Jin, L., & Dick, J. E. (2004). Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nature Immunology*, *5*(7), 738–743. https://doi.org/10.1038/ni1080

73 Huang, R.-L., Teo, Z., Chong, H. C., Zhu, P., Tan, M. J., Tan, C. K., Lam, C. R. I., Sng, M. K., Leong, D. T. W., Tan, S. M., Kersten, S., Ding, J. L., Li, H. Y., & Tan, N. S. (2011). ANGPTL4 modulates vascular junction integrity by integrin signaling and disruption of intercellular VE-cadherin and claudin-5 clusters. *Blood*, *118*(14), 3990–4002. https://doi.org/10.1182/blood-2011-01-328716

74. Hubbi, M. E., Kshitiz, Gilkes, D. M., Rey, S., Wong, C. C., Luo, W., Kim, D. H., Dang, C. V., Levchenko, A., & Semenza, G. L. (2013). A nontranscriptional role for HIF-1α as a direct inhibitor of DNA replication. *Science Signaling*, *6*(262), 1–22. https://doi.org/10.1126/scisignal.2003417

75. Hubbi, M. E., & Semenza, G. L. (2015). Regulation of cell proliferation by hypoxia-inducible factors. *American Journal of Physiology - Cell Physiology*, *309*(12), C775–C782. https://doi.org/10.1152/ajpcell.00279.2015

76. Huijbers, I. J., Iravani, M., Popov, S., Robertson, D., Al-sarraj, S., Jones, C., & Clare, M. (2010). *A Role for Fibrillar Collagen Deposition and the Collagen Internalization Receptor Endo180 in Glioma Invasion*. *5*(3), 1–12. https://doi.org/10.1371/journal.pone.0009808

77. Ioannou, M., Papamichali, R., Kouvaras, E., Mylonis, I., Vageli, D., Kerenidou, T., Barbanis, S., Daponte, A., Simos, G., Gourgoulianis, K., & Koukoulis, G. K. (2009). Hypoxia inducible factor-1alpha and vascular endothelial growth factor in biopsies of small cell lung carcinoma. *Lung*, *187*(5), 321–329. https://doi.org/10.1007/s00408-009-9169-z

78. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., Kaelin, W. G., & Kaelin, J. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: Implications for O2 sensing. *Science*, *292*(5516), 464–468. https://doi.org/10.1126/science.1059817

79. Janssens, G. O., Rademakers, S. E., Terhaard, C. H., Doornaert, P. A., Bijl, H. P., Van Ende, P. Den, Chin, A., Marres, H. A., De Bree, R., Van Der Kogel, A. J., Hoogsteen, I. J., Bussink, J., Span, P. N., & Kaanders, J. H. (2012). Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: Results of a phase III randomized trial. *Journal of Clinical Oncology*, *30*(15), 1777–1783. https://doi.org/10.1200/JCO.2011.35.9315

80. Kaplan, A. R., & Glazer, P. M. (2020). Impact of hypoxia on DNA repair and genome integrity. *Mutagenesis*, *35*(1), 61–68. https://doi.org/10.1093/mutage/gez019

81. Karagiannis, G. S., Goswami, S., Jones, J. G., Oktay, M. H., & Condeelis, J. S. (2016). Signatures of breast cancer metastasis at a glance. *Journal of Cell Science*, *129*(9), 1751–1758. https://doi.org/10.1242/jcs.183129

82. Kiel, M. J., Velusamy, T., Betz, B. L., Zhao, L., Weigelin, H. G., Chiang, M. Y., Huebner-Chan, D. R., Bailey, N. G., Yang, D. T., Bhagat, G., Miranda, R. N., Bahler, D. W., Jeffrey Medeiros, L., Lim, M. S., & Elenitoba-Johnson, K. S. J. (2012). Whole-genome sequencing identifies recurrent somatic NOTCH2 mutations in splenic marginal zone lymphoma. *Journal of Experimental Medicine*, *209*(9), 1553–1565. https://doi.org/10.1084/jem.20120910

83. Kim, M. S., Kwon, H. J., Lee, Y. M., Baek, J. H., Jang, J. E., Lee, S. W., Moon, E. J., Kim, H. S., Lee, S. K., Chung, H. Y., Kim, C. W., & Kim, K. W. (2001). Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nature Medicine*, *7*(4), 437–443. https://doi.org/10.1038/86507

84. Kimura, H., Weisz, A., Ogura, T., Hitomi, Y., Kurashima, Y., Hashimoto, K., D'Acquisto, F., Makuuchi, M., & Esumi, H. (2001). Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. *Journal of Biological Chemistry*, *276*(3), 2292–2298. https://doi.org/10.1074/jbc.M008398200
85. Klein, T. J., & Glazer, P. M. (2010). The tumor microenvironment and DNA repair. *Seminars in Radiation Oncology*, *20*(4), 282–287. https://doi.org/10.1016/j.semradonc.2010.05.006
86. Kluk, M. J., Ashworth, T., Wang, H., Knoechel, B., Mason, E. F., Morgan, E. A., Dorfman, D., Pinkus, G., Weigert, O., Hornick, J. L., Chirieac, L. R., Hirsch, M., Oh, D. J., South, A. P., Leigh, I. M., Pourreyron, C., Cassidy, A. J., DeAngelo, D. J., Weinstock, D. M., … Aster, J. C. (2013). Gauging NOTCH1 Activation in Cancer Using Immunohistochemistry. *PLoS ONE*, *8*(6), 1–14. https://doi.org/10.1371/journal.pone.0067306
87. Kopan, R., & Ilagan, M. X. G. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*, *137*(2), 216–233. https://doi.org/10.1016/j.cell.2009.03.045
88. Krishnamachary, B., Zagzag, D., Nagasawa, H., Rainey, K., Okuyama, H., Baek, J. H., & Semenza, G. L. (2006). Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. *Cancer Research*, *66*(5), 2725–2731. https://doi.org/10.1158/0008-5472.CAN-05-3719
89. Kruh, G. D., & Belinsky, M. G. (2003). The MRP family of drug efflux pumps. *Oncogene*, *22*(47 REV. ISS. 6), 7537–7552. https://doi.org/10.1038/sj.onc.1206953
90. Kumareswaran, R., Ludkovski, O., Meng, A., Sykes, J., Pintilie, M., & Bristow, R. G. (2012). Chronic hypoxia compromises repair of DNA double-strand breaks to drive genetic instability. *J Cell Sci*, *125*(1477-9137 (Electronic)), 189–199. https://doi.org/10.1242/jcs.092262
91. LaGory, E. L., & Giaccia, A. J. (2016). The ever-expanding role of HIF in tumour and stromal biology. *Nature Cell Biology*, *18*(4), 356–365. https://doi.org/10.1038/ncb3330
92. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., & Whitelaw, M. L. (2002). Asparagine hydroxylation of the HIF transactivation domain: A hypoxic switch. *Science*, *295*(5556), 858–861. https://doi.org/10.1126/science.1068592
93. Landor, S. K. J., Mutvei, A. P., Mamaeva, V., Jin, S., Busk, M., Borra, R., Grönroos, T. J., Kronqvist, P., Lendahl, U., & Sahlgren, C. M. (2011). Hypo- and hyperactivated Notch signaling induce a glycolytic switch through distinct mechanisms. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(46), 18814–18819. https://doi.org/10.1073/pnas.1104943108
94. Lee, G., Auffinger, B., Guo, D., Hasan, T., Deheeger, M., Tobias, A. L., Kim, J. Y., Atashi, F., Zhang, L., Lesniak, M. S., James, C. D., & Ahmed, A. U. (2016). Dedifferentiation of Glioma Cells to Glioma Stem-like Cells By Therapeutic Stress-induced HIF Signaling in the Recurrent GBM Model. *Molecular Cancer Therapeutics*, *15*(12), 3064–3076. https://doi.org/10.1158/1535-7163.MCT-15-0675
95. Leong, H. S., Robertson, A. E., Stoletov, K., Leith, S. J., Chin, C. A., Chien, A. E., Hague, M. N., Ablack, A., Carmine-Simmen, K., McPherson, V. A., Postenka, C. O., Turley, E. A., Courtneidge, S. A., Chambers, A. F., & Lewis, J. D. (2014). Invadopodia Are Required for Cancer Cell Extravasation and Are a Therapeutic Target for Metastasis. *Cell Reports*, *8*(5), 1558–1570. https://doi.org/10.1016/j.celrep.2014.07.050

96. Leong, K. G., Niessen, K., Kulic, I., Raouf, A., Eaves, C., Pollet, I., & Karsan, A. (2007). Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. *Journal of Experimental Medicine*, *204*(12), 2935–2948. https://doi.org/10.1084/jem.20071082
97. Leszczynska, K. B., Göttgens, E.-L., Biasoli, D., Olcina, M. M., Ient, J., Anbalagan, S., Bernhardt, S., Giaccia, A. J., & Hammond, E. M. (2016). Mechanisms and consequences of ATMIN repression in hypoxic conditions: Roles for p53 and HIF-1. *Scientific Reports*, *6*. https://doi.org/10.1038/srep21698
98. Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., Fong, S. F. T., Csiszar, K., Giaccia, A., Weninger, W., Yamauchi, M., Gasser, D. L., & Weaver, V. M. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*, *139*(5), 891–906. http://www.ncbi.nlm.nih.gov/pubmed/19931152
99. Li, J., Zhang, X., Sejas, D. P., Bagby, G. C., & Pang, Q. (2004). Hypoxia-induced nucleophosmin protects cell death through inhibition of p53. *Journal of Biological Chemistry*, *279*(40), 41275–41279. https://doi.org/10.1074/jbc.C400297200
100. Li, L., Zhao, F., Lu, J., Li, T., Yang, H., Wu, C., & Liu, Y. (2014). Notch-1 signaling promotes the malignant features of human breast cancer through NF-κB activation. *PLoS ONE*, *9*(4), 1–12. https://doi.org/10.1371/journal.pone.0095912
101. Li, Y., Patel, S. P., Roszik, J., & Qin, Y. (2018). Hypoxia-driven immunosuppressive metabolites in the tumor microenvironment: New approaches for combinational immunotherapy. *Frontiers in Immunology*, *9*(JUL). https://doi.org/10.3389/fimmu.2018.01591
102. Li, Z., Bao, S., Wu, Q., Wang, H., Eyler, C., Sathornsumetee, S., Shi, Q., Cao, Y., Lathia, J., Mclendon, R. E., Hjelmeland, A. B., & Rich, J. N. (2009). Hypoxia-Inducible Factors Regulate Tumorigenic Capacity of Glioma Stem Cells cancer stem cell specific molecules involved in neoangiogenesis, including HIF2α and its regulated factors. *Cancer Cell*, *15*(6), 501–513. https://doi.org/10.1016/j.ccr.2009.03.018
103. Liu, L., Ning, X., Sun, L., Zhang, H., Shi, Y., Guo, C., Han, S., Liu, J., Sun, S., Han, Z., Wu, K., & Fan, D. (2008). Hypoxia-inducible factor-1α contributes to hypoxia-induced chemoresistance in gastric cancer. *Cancer Science*, *99*(1), 121–128. https://doi.org/10.1111/j.1349-7006.2007.00643.x
104. Lohr, J. G., Stojanov, P., Lawrence, M. S., Auclair, D., Chapuy, B., Sougnez, C., Cruz-Gordillo, P., Knoechel, B., Asmann, Y. W., Slager, S. L., Novak, A. J., Dogan, A., Ansell, S. M., Link, B. K., Zou, L., Gould, J., Saksena, G., Stransky, N., Rangel-Escareño, C., … Golub, T. R. (2012). Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(10), 3879–3884. https://doi.org/10.1073/pnas.1121343109
105. Matouk, I. J., Raveh, E., Abu-lail, R., Mezan, S., Gilon, M., Gershtain, E., Birman, T., Gallula, J., Schneider, T., Barkali, M., Richler, C., Fellig, Y., Sorin, V., Hubert, A., Hochberg, A., & Czerniak, A. (2014). Oncofetal H19 RNA promotes tumor metastasis. *Biochimica et Biophysica Acta - Molecular Cell Research*. https://doi.org/10.1016/j.bbamcr.2014.03.023

106. McAuliffe, S. M., Morgan, S. L., Wyant, G. A., Tran, L. T., Muto, K. W., Chen, Y. S., Chin, K. T., Partridge, J. C., Poole, B. B., Cheng, K. H., Daggett, J., Cullen, K., Kantoff, E., Hasselbatt, K., Berkowitz, J., Muto, M. G., Berkowitz, R. S., Aster, J. C., Matulonis, U. A., & Dinulescu, D. M. (2012). Targeting Notch, a key pathway for ovarian cancer stem cells, sensitizes tumors to platinum therapy. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(43). https://doi.org/10.1073/pnas.1206400109
107. Meng, F., Hennink, W. E., & Zhong, Z. (2009). Reduction-sensitive polymers and bioconjugates for biomedical applications. *Biomaterials*, *30*(12), 2180–2198. https://doi.org/10.1016/j.biomaterials.2009.01.026
108. Milosevic, M., Warde, P., Meńard, C., Chung, P., Toi, A., Ishkanian, A., McLean, M., Pintilie, M., Sykes, J., Gospodarowicz, M., Catton, C., Hill, R. P., & Bristow, R. (2012). Tumor hypoxia predicts biochemical failure following radiotherapy for clinically localized prostate cancer. *Clinical Cancer Research*, *18*(7), 2108–2114. https://doi.org/10.1158/1078-0432.CCR-11-2711
109. Minchinton, A. I., & Tannock, I. F. (2006). Drug penetration in solid tumours. *Nature Reviews Cancer*, *6*(8), 583–592. https://doi.org/10.1038/nrc1893
110. Mourao, L., Jacquemin, G., Huyghe, M., Nawrocki, W. J., Menssouri, N., Servant, N., & Fre, S. (2019). Lineage tracing of Notch1-expressing cells in intestinal tumours reveals a distinct population of cancer stem cells. *Scientific Reports*, *9*(1), 1–14. https://doi.org/10.1038/s41598-018-37301-3
111. Multhoff, G., & Vaupel, P. (2020). Hypoxia compromises anti-cancer immune responses. *Advances in Experimental Medicine and Biology*, *1232*, 131–143. https://doi.org/10.1007/978-3-030-34461-0_18
112. Muz, B., de la Puente, P., Azab, F., & Azab, A. K. (2015). The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia*, 83. https://doi.org/10.2147/hp.s93413
113. Naba, A., Clauser, K. R., Hoersch, S., Liu, H., Carr, S. A., & Hynes, R. O. (2012). The matrisome: In silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. *Molecular and Cellular Proteomics*, *11*(4), 1–18. https://doi.org/10.1074/mcp.M111.014647
114. Nicolas, M., Wolfer, A., Raj, K., Kummer, J. A., Mill, P., Van Noort, M., Hui, C. chung, Clevers, H., Dotto, G. P., & Radtke, F. (2003). Notch1 functions as a tumor suppressor in mouse skin. *Nature Genetics*, *33*(3), 416–421. https://doi.org/10.1038/ng1099
115. Noman, M. Z., Hasmim, M., Messai, Y., Terry, S., Kieda, C., Janji, B., & Chouaib, S. (2015). Hypoxia: A key player in antitumor immune response. A review in the theme: Cellular responses to hypoxia. *American Journal of Physiology - Cell Physiology*, *309*(9), C569–C579. https://doi.org/10.1152/ajpcell.00207.2015
116. Nordsmark, M., Bentzen, S. M., Rudat, V., Brizel, D., Lartigau, E., Stadler, P., Becker, A., Adam, M., Molls, M., Dunst, J., Terris, D. J., & Overgaard, J. (2005). Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. *Radiotherapy and Oncology*, *77*(1), 18–24. https://doi.org/10.1016/j.radonc.2005.06.038
117. O'Brien, C. A., Pollett, A., Gallinger, S., & Dick, J. E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*, *445*(7123), 106–110. https://doi.org/10.1038/nature05372

118. O'Neil, J., Grim, J., Strack, P., Rao, S., Tibbitts, D., Winter, C., Hardwick, J., Welcker, M., Meijerink, J. P., Pieters, R., Draetta, G., Sears, R., Clurman, B. E., & Look, A. T. (2007). FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to γ-secretase inhibitors. *Journal of Experimental Medicine*, *204*(8), 1813–1824. https://doi.org/10.1084/jem.20070876
119. Olcina, M. M., & Hammond, E. M. (2014). Hypoxia and the DNA Damage Response. In G. Melillo (Ed.), *Cancer and Metastasis Reviews* (Vol. 26, Issue 2, pp. 21–41). Springer New York. https://doi.org/10.1007/978-1-4614-9167-5_2
120. Organization, W. H. (2020). *www.who.int*. https://www.who.int/news-room/fact-sheets/detail/cancer
121. Overgaard, J. (2007). Hypoxic radiosensitization: Adored and ignored. *Journal of Clinical Oncology*, *25*(26), 4066–4074. https://doi.org/10.1200/JCO.2007.12.7878
122. Padera, T. P., Stoll, B. R., Tooredman, J. B., Capen, D., Di Tomaso, E., & Jain, R. K. (2004). Cancer cells compress intratumour vessels. *Nature*, *427*(6976), 695. https://doi.org/10.1038/427695a
123. Palomero, T., Sulis, M. L., Cortina, M., Real, P. J., Barnes, K., Ciofani, M., Caparros, E., Buteau, J., Brown, K., Perkins, S. L., Bhagat, G., Agarwal, A. M., Basso, G., Castillo, M., Nagase, S., Cordon-Cardo, C., Parsons, R., Zúñiga-Pflücker, J. C., Dominguez, M., & Ferrando, A. A. (2007). Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nature Medicine*, *13*(10), 1203–1210. https://doi.org/10.1038/nm1636
124. Palomero, T., Wei, K. L., Odom, D. T., Sulis, M. L., Real, P. J., Margolin, A., Barnes, K. C., O'Neil, J., Neuberg, D., Weng, A. P., Aster, J. C., Sigaux, F., Soulier, J., Look, A. T., Young, R. A., Califano, A., & Ferrando, A. A. (2006). NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(48), 18261–18266. https://doi.org/10.1073/pnas.0606108103
125. Perumalsamy, L. R., Nagala, M., & Sarin, A. (2010). Notch-activated signaling cascade interacts with mitochondrial remodeling proteins to regulate cell survival. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(15), 6882–6887. https://doi.org/10.1073/pnas.0910060107
126. Pires, I. M., Bencokova, Z., McGurk, C., & Hammond, E. M. (2010). Exposure to acute hypoxia induces a transient DNA damage response which includes Chk1 and TLK1. *Cell Cycle*, *9*(13), 2502–2507. https://doi.org/10.4161/cc.9.13.12059
127. Pires, I. M., Bencokova, Z., Milani, M., Folkes, L. K., Li, J. A., Stratford, M. R., Harris, A. L., & Hammond, E. M. (2010). Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. *Cancer Research*, *70*(3), 925–935. https://doi.org/10.1158/0008-5472.CAN-09-2715
128. Provenzano, P. P., Eliceiri, K. W., Campbell, J. M., Inman, D. R., White, J. G., & Keely, P. J. (2006). Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Medicine*, *4*, 1–15. https://doi.org/10.1186/1741-7015-4-38
129. Provenzano, P. P., Inman, D. R., Eliceiri, K. W., Knittel, J. G., Yan, L., Rueden, C. T., White, J. G., & Keely, P. J. (2008). Collagen density promotes mammary tumor initiation and progression. *BMC Medicine*, *6*, 1–15. https://doi.org/10.1186/1741-7015-6-11

130. Puente, X. S., Pinyol, M., Quesada, V., Conde, L., Ordóñez, G. R., Villamor, N., Escaramis, G., Jares, P., Beá, S., González-Díaz, M., Bassaganyas, L., Baumann, T., Juan, M., López-Guerra, M., Colomer, D., Tubío, J. M. C., López, C., Navarro, A., Tornador, C., … Campo, E. (2011). Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature*, *475*(7354), 101–105. https://doi.org/10.1038/nature10113

131. Ramachandran, S., Ient, J., Göttgen, E.-L., Krieg, A. J. A., Hammond, E. M. E., Göttgens, E.-L., Krieg, A. J. A., Hammond, E. M. E., Göttgen, E.-L., Krieg, A. J. A., & Hammond, E. M. E. (2015). Epigenetic Therapy for Solid Tumors: Highlighting the Impact of Tumor Hypoxia. *Genes*, *6*(4), 935–956. https://doi.org/10.3390/genes6040935

132. Ramaswamy, S., Ross, K. N., Lander, E. S., & Golub, T. R. (2003). A molecular signature of metastasis in primary solid tumors. *Nature Genetics*, *33*(1), 49–54. https://doi.org/10.1038/ng1060

133. Ren, H. Y., Zhang, Y. H., Li, H. Y., Xie, T., Sun, L. L., Zhu, T., Wang, S. D., & Ye, Z. M. (2016). Prognostic role of hypoxia-inducible factor-1 alpha expression in osteosarcoma: A meta-analysis. *OncoTargets and Therapy*, *9*, 1477–1487. https://doi.org/10.2147/OTT.S95490

134. Reynolds, T. Y., Rockwell, S., & Glazer, P. M. (1996). Genetic instability induced by the tumor microenvironment. *Cancer Research*, *56*(24), 5754–5757. https://doi.org/10.1016/s0360-3016(97)80581-0

135. Rice, G. C., Hoy, C., & Schimke, R. T. (1986). Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(16), 5978–5982. https://doi.org/10.1073/pnas.83.16.5978

136. Robinson, D. R., Kalyana-Sundaram, S., Wu, Y. M., Shankar, S., Cao, X., Ateeq, B., Asangani, I. A., Iyer, M., Maher, C. A., Grasso, C. S., Lonigro, R. J., Quist, M., Siddiqui, J., Mehra, R., Jing, X., Giordano, T. J., Sabel, M. S., Kleer, C. G., Palanisamy, N., … Chinnaiyan, A. M. (2011). Functionally recurrent rearrangements of the MAST kinase and Notch gene families in breast cancer. *Nature Medicine*, *17*(12), 1646–1651. https://doi.org/10.1038/nm.2580

137. Roig, E. M., Yaromina, A., Houben, R., Groot, A. J., Dubois, L., & Vooijs, M. (2018). Prognostic role of hypoxia-inducible factor-2α tumor cell expression in cancer patients: A meta-analysis. *Frontiers in Oncology*, *8*(JUN). https://doi.org/10.3389/fonc.2018.00224

138. Sahlgren, C., Gustafsson, M. V., Jin, S., Poellinger, L., & Lendahl, U. (2008). Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(17), 6392–6397. https://doi.org/10.1073/pnas.0802047105

139. Salem, A., Asselin, M. C., Reymen, B., Jackson, A., Lambin, P., West, C. M. L., O'Connor, J. P. B., & Faivre-Finn, C. (2018). Targeting hypoxia to improve non–small cell lung cancer outcome. *Journal of the National Cancer Institute*, *110*(1), 14–30. https://doi.org/10.1093/jnci/djx160

140. Samali, A., & Cotter, T. G. (1996). Heat shock proteins increase resistance to apoptosis. *Experimental Cell Research*, *223*(1), 163–170. https://doi.org/10.1006/excr.1996.0070

141. Samanta, D., Gilkes, D. M., Chaturvedi, P., Xiang, L., & Semenza, G. L. (2014). Hypoxia-inducible factors are required for chemotherapy resistance of breast cancer stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(50), E5429-38. https://doi.org/10.1073/pnas.1421438111

142. Schietke, R., Warnecke, C., Wacker, I., Schödel, J., Mole, D. R., Campean, V., Amann, K., Goppelt-Struebe, M., Behrens, J., Eckardt, K. U., & Wiesener, M. S. (2010). The lysyl oxidases LOX and LOXL2 are necessary and sufficient to repress E-cadherin in Hypoxia: Insights into cellular transformation processes mediated by HIF-1. *Journal of Biological Chemistry*, *285*(9), 6658–6669. https://doi.org/10.1074/jbc.M109.042424
143. Schmid, T., Zhou, J., Köhl, R., & Brüne, B. (2004). p300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (HIF-1). *Biochemical Journal*, *380*(1), 289–295. https://doi.org/10.1042/BJ20031299
144. Sedlakova, O., Svastova, E., Takacova, M., Kopacek, J., Pastorek, J., & Pastorekova, S. (2014). Carbonic anhydrase IX, a hypoxia-induced catalytic component of the pH regulating machinery in tumors. *Frontiers in Physiology*, *4 JAN*(January), 1–14. https://doi.org/10.3389/fphys.2013.00400
145. Semenza, G. L. (2015). Regulation of the breast cancer stem cell phenotype by hypoxia-inducible factors. *Clinical Science*, *129*(12), 1037–1045. https://doi.org/10.1042/CS20150451
146. Sendoel, A., & Hengartner, M. O. (2014). Apoptotic cell death under hypoxia. *Physiology*, *29*(3), 168–176. https://doi.org/10.1152/physiol.00016.2013
147. Sevick, E. M., & Jain, R. K. (1991). Microvascular Architecture in a Mammary Carcinoma: Branching Patterns and Vessel Dimensions. *Cancer Research*, *51*(1), 265–273.
148. Shamir, E. R., Pappalardo, E., Jorgens, D. M., Coutinho, K., Tsai, W. T., Aziz, K., Auer, M., Tran, P. T., Bader, J. S., & Ewald, A. J. (2014). Twist1-induced dissemination preserves epithelial identity and requires E-cadherin. *Journal of Cell Biology*, *204*(5), 839–856. https://doi.org/10.1083/jcb.201306088
149. Shi, Q. Y., Zhang, S. J., Liu, L., Chen, Q. S., Yu, L. N., Zhang, F. J., & Yan, M. (2015). Sevoflurane promotes the expansion of glioma stem cells through activation of hypoxia-inducible factors in vitro. *British Journal of Anaesthesia*. https://doi.org/10.1093/bja/aeu402
150. Sidani, M., Wyckoff, J., Xue, C., Segall, J. E., & Condeelis, J. (2006). Probing the microenvironment of mammary tumors using multiphoton microscopy. *Journal of Mammary Gland Biology and Neoplasia*, *11*(2), 151–163. https://doi.org/10.1007/s10911-006-9021-5
151. Sitkovsky, M. V, Hatfield, S., Abbott, R., Belikoff, B., Lukashev, D., & Ohta, A. (2014). Hostile, hypoxia-A2-adenosinergic tumor biology as the next barrier to overcome for tumor immunologists. *Cancer Immunology Research*, *2*(7), 598–605. https://doi.org/10.1158/2326-6066.CIR-14-0075
152. Song, C. W., Park, H., & Griffin, R. J. (2001). Improvement of Tumor Oxygenation by Mild Hyperthermia. *Radiation Research*, *155*(4), 515–528. https://doi.org/10.1667/0033-7587(2001)155[0515:iotobm]2.0.co;2
153. Spiegelberg, L., Houben, R., Niemans, R., de Ruysscher, D., Yaromina, A., Theys, J., Guise, C. P., Smaill, J. B., Patterson, A. V., Lambin, P., & Dubois, L. J. (2019). Hypoxia-activated prodrugs and (lack of) clinical progress: The need for hypoxia-based biomarker patient selection in phase III clinical trials. *Clinical and Translational Radiation Oncology*, *15*, 62–69. https://doi.org/10.1016/j.ctro.2019.01.005
154. Strese, S., Fryknäs, M., Larsson, R., & Gullbo, J. (2013). Effects of hypoxia on human cancer cell line chemosensitivity. *BMC Cancer*, *13*. https://doi.org/10.1186/1471-2407-13-331

155. Stubbs, M., McSheehy, P. M. J., Griffiths, J. R., & Bashford, C. L. (2000). Causes and consequences of tumour acidity and implications for treatment. In *Molecular Medicine Today*. https://doi.org/10.1016/S1357-4310(99)01615-9

156. Sun, J. C., He, F., Yi, W., Wan, M. H., Li, R., Wei, X., Wu, R., & Niu, D. L. (2015). High expression of HIF-2α and its anti-radiotherapy effect in lung cancer stem cells. *Genetics and Molecular Research*, *14*(4), 18110–18120. https://doi.org/10.4238/2015.December.22.37

157. Teicher, B. A., Lazo, J. S., & Sartorelli, A. C. (1981). Classification of Antineoplastic Agents by their Selective Toxicities toward Oxygenated and Hypoxic Tumor Cells. *Cancer Research*, *41*(1), 73–81.

158. Thomlinson, R. H., & Gray, L. H. (1955). The Histological Structure of Some Human Lung Cancers and the Possible Implications for Radiotherapy. *British Journal of Cancer*, *9*, 539–549. https://doi.org/10.1038/bjc.1955.55

159. Thompson, B. J., Buonamici, S., Sulis, M. L., Palomero, T., Vilimas, T., Basso, G., Ferrando, A., & Aifantis, I. (2007). The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. *Journal of Experimental Medicine*, *204*(8), 1825–1835. https://doi.org/10.1084/jem.20070872

160. Trastour, C., Benizri, E., Ettore, F., Ramaioli, A., Chamorey, E., Pouysségur, J., & Berra, E. (2007). HIF-1α and CA IX staining in invasive breast carcinomas: Prognosis and treatment outcome. *International Journal of Cancer*, *120*(7), 1451–1458. https://doi.org/10.1002/ijc.22436

161. Trimarchi, T., Bilal, E., Ntziachristos, P., Fabbri, G., Dalla-Favera, R., Tsirigos, A., & Aifantis, I. (2014). Genome-wide mapping and characterization of Notch-regulated long noncoding RNAs in acute leukemia. *Cell*, *158*(3), 593–606. https://doi.org/10.1016/j.cell.2014.05.049

162. Van Cutsem, E., Lenz, H.-J., Furuse, J., Tabernero, J., Heinemann, V., Ioka, T., Bazin, I., Ueno, M., Csõszi, T., Wasan, H., Melichar, B., Karasek, P., Macarulla, T. M., Guillen, C., Kalinka-Warzocha, E., Horvath, Z., Prenen, H., Schlichting, M., Ibrahim, A., & Bendell, J. C. (2016). MAESTRO: A randomized, double-blind phase III study of evofosfamide (Evo) in combination with gemcitabine (Gem) in previously untreated patients (pts) with metastatic or locally advanced unresectable pancreatic ductal adenocarcinoma (PDAC). *Journal of Clinical Oncology*, *34*(15_suppl), 4007. https://doi.org/10.1200/JCO.2016.34.15_suppl.4007

163. van den Beucken, T., Koch, E., Chu, K., Rupaimoole, R., Prickaerts, P., Adriaens, M., Voncken, J. W., Harris, A. L., Buffa, F. M., Haider, S., Starmans, M. H. W., Yao, C. Q., Ivan, M., Ivan, C., Pecot, C. V, Boutros, P. C., Sood, A. K., Koritzinsky, M., & Wouters, B. G. (2014). Hypoxia promotes stem cell phenotypes and poor prognosis through epigenetic regulation of DICER. *Nature Communications*, *5*, 5203. https://doi.org/10.1038/ncomms6203

164. Vaupel, P, Hockel, M., & Mayer, A. (2007). Detection and characterization of tumor hypoxia using pO2 histography. *Antioxid Redox Signal*, *9*(8), 1221–1235. https://doi.org/10.1089/ars.2007.1628

165. Vaupel, P, & Mayer, A. (2007). Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev*, *26*(2), 225–239. https://doi.org/10.1007/s10555-007-9055-1

166. Vaupel, Peter, Mayer, A., & Höckel, M. (2004). Tumor hypoxia and malignant progression. *Methods in Enzymology*, *381*(2), 335–354. https://doi.org/10.1016/S0076-6879(04)81023-1

167. Vaupel, Peter, Thews, O., & Hoeckel, M. (2001). Treatment resistance of solid tumors: Role of hypoxia and anemia. *Medical Oncology*, *18*(4), 243–259. https://doi.org/10.1385/MO:18:4:243

168. Vooijs, M., Ong, C.-T., Hadland, B., Huppert, S., Liu, Z., Korving, J., van den Born, M., Stappenbeck, T., Wu, Y., Clevers, H., & Kopan, R. (2007). Mapping the consequence of Notch1 proteolysis in vivo with NIP-CRE. *Development (Cambridge, England)*, *134*(3), 535–544. https://doi.org/10.1242/dev.02733
169. Wang, K., Zhang, Q., Li, D., Ching, K., Zhang, C., Zheng, X., Ozeck, M., Shi, S., Li, X., Wang, H., Rejto, P., Christensen, J., & Olson, P. (2015). PEST domain mutations in Notch receptors comprise an oncogenic driver segment in triple-negative breast cancer sensitive to a γ-secretase inhibitor. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *21*(6), 1487–1496. https://doi.org/10.1158/1078-0432.CCR-14-1348
170. Wang, N. J., Sanborn, Z., Arnett, K. L., Bayston, L. J., Liao, W., Proby, C. M., Leigh, I. M., Collisson, E. A., Gordon, P. B., Jakkula, L., Pennypacker, S., Zou, Y., Sharma, M., North, J. P., Vemula, S. S., Mauro, T. M., Neuhaus, I. M., LeBoit, P. E., Hur, J. S., … Cho, R. J. (2011). Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(43), 17761–17766. https://doi.org/10.1073/pnas.1114669108
171. Wang, Z., Li, Y., Ahmad, A., Azmi, A. S., Banerjee, S., Kong, D., & Sarkar, F. H. (2010). Targeting Notch signaling pathway to overcome drug resistance for cancer therapy. *Biochimica et Biophysica Acta - Reviews on Cancer*, *1806*(2), 258–267. https://doi.org/10.1016/j.bbcan.2010.06.001
172. Wei, B., Zhou, X., Liang, C., Zheng, X., Lei, P., Fang, J., Han, X., Wang, L., Qi, C., & Wei, H. (2017). Human colorectal cancer progression correlates with LOX-induced ECM stiffening. *International Journal of Biological Sciences*, *13*(11), 1450–1457. https://doi.org/10.7150/ijbs.21230
173. Weng, A. P., Ferrando, A. A., Lee, W., Morris IV, J. P., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T., & Aster, J. C. (2004). Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*, *306*(5694), 269–271. https://doi.org/10.1126/science.1102160
174. Wenger, R. H., Stiehl, D. P., & Camenisch, G. (2005). Integration of oxygen signaling at the consensus HRE. *Science's STKE : Signal Transduction Knowledge Environment*, *2005*(306). https://doi.org/10.1126/stke.3062005re12
175. Whelan, K. A., Caldwell, S. A., Shahriari, K. S., Jackson, S. R., Franchetti, L. D., Johannes, G. J., & Reginato, M. J. (2010). Hypoxia suppression of Bim and Bmf blocks anoikis and luminal clearing during mammary morphogenesis. *Molecular Biology of the Cell*, *21*(22), 3829–3837. https://doi.org/10.1091/mbc.E10-04-0353
176. Wilson, W. R., & Hay, M. P. (2011). Targeting hypoxia in cancer therapy. *Nat Rev Cancer*, *11*(6), 393–410. https://doi.org/10.1038/nrc3064
177. Wong, C. C. L., Gilkes, D. M., Zhang, H., Chen, J., Wei, H., Chaturvedi, P., Fraley, S. I., Wong, C. M., Khoo, U. S., Ng, I. O. L., Wirtz, D., & Semenza, G. L. (2011). Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(39), 16369–16374. https://doi.org/10.1073/pnas.1113483108
178. Xie, M., He, C. S., Wei, S. H., & Zhang, L. (2013). Notch-1 contributes to epidermal growth factor receptor tyrosine kinase inhibitor acquired resistance in non-small cell lung cancer in vitro and in vivo. *European Journal of Cancer*, *49*(16), 3559–3572. https://doi.org/10.1016/j.ejca.2013.07.007

179. Xing, F., Okuda, H., Watabe, M., Kobayashi, A., Pai, S. K., Liu, W., Pandey, P. R., Fukuda, K., Hirota, S., Sugai, T., Wakabayshi, G., Koeda, K., Kashiwaba, M., Suzuki, K., Chiba, T., Endo, M., Mo, Y.-Y., & Watabe, K. (2011). Hypoxia-induced Jagged2 promotes breast cancer metastasis and self-renewal of cancer stem-like cells. *Oncogene*, *30*(39), 4075–4086. https://doi.org/10.1038/onc.2011.122

180. Xiong, G., Deng, L., Zhu, J., Rychahou, P. G., & Xu, R. (2014). Prolyl-4-hydroxylase α subunit 2 promotes breast cancer progression and metastasis by regulating collagen deposition. *BMC Cancer*, *14*(1), 1–12. https://doi.org/10.1186/1471-2407-14-1

181. Young, S. D., Marshall, R. S., & Hill, R. P. (1988). Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(24), 9533–9537. https://doi.org/10.1073/pnas.85.24.9533

182. Zeng, Y., Ma, J., Zhan, Y., Xu, X., Zeng, Q., Liang, J., & Chen, X. (2018). Hypoxia-activated prodrugs and redox-responsive nanocarriers. *International Journal of Nanomedicine*, *13*, 6551–6574. https://doi.org/10.2147/IJN.S173431

183. Zhang, H., Wong, C. C. L., Wei, H., Gilkes, D. M., Korangath, P., Chaturvedi, P., Schito, L., Chen, J., Krishnamachary, B., Winnard, P. T., Raman, V., Zhen, L., Mitzner, W. A., Sukumar, S., & Semenza, G. L. (2012). HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs. *Oncogene*, *31*(14), 1757–1770. https://doi.org/10.1038/onc.2011.365

184. Zheng, S.-S., Chen, X.-H., Yin, X., & Zhang, B.-H. (2013). Prognostic significance of HIF-1α expression in hepatocellular carcinoma: a meta-analysis. *PloS One*, *8*(6), e65753. https://doi.org/10.1371/journal.pone.0065753

185. Zheng, X., Linke, S., Dias, J. M., Zheng, X., Gradin, K., Wallis, T. P., Hamilton, B. R., Gustafsson, M., Ruas, J. L., Wilkins, S., Bilton, R. L., Brismar, K., Whitelaw, M. L., Pereira, T., Gorman, J. J., Ericson, J., Peet, D. J., Lendahl, U., & Poellinger, L. (2008). Interaction with factor inhibiting HIF-1 defines an additional mode of cross-coupling between the Notch and hypoxia signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(9), 3368–3373. https://doi.org/10.1073/pnas.0711591105

186. Zhivotovsky, B., Joseph, B., & Orrenius, S. (1999). Tumor radiosensitivity and apoptosis. *Experimental Cell Research*, *248*(1), 10–17. https://doi.org/10.1006/excr.1999.4452

187. Zhu, G. H., Huang, C., Feng, Z. Z., Lv, X. H., & Qiu, Z. J. (2013). Hypoxia-induced snail expression through transcriptional regulation by HIF-1α in pancreatic cancer cells. *Digestive Diseases and Sciences*, *58*(12), 3503–3515. https://doi.org/10.1007/s10620-013-2841-4

188. Zhu, T. S., Costello, M. A., Talsma, C. E., Flack, C. G., Crowley, J. G., Hamm, L. L., He, X., Hervey-Jumper, S. L., Heth, J. A., Muraszko, K. M., DiMeco, F., Vescovi, A. L., & Fan, X. (2011). Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH ligands that nurture self-renewal of cancer stem-like cells. *Cancer Research*, *71*(18), 6061–6072. https://doi.org/10.1158/0008-5472.CAN-10-4269

CHAPTER 2

2

Moving Breast Cancer Therapy up a Notch

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Breast cancer is the second most common malignancy, worldwide. Treatment decisions are based on tumor stage, histological subtype, and receptor expression and include combinations of surgery, radiotherapy, and systemic treatment. These, together with earlier diagnosis, have resulted in increased survival. However, initial treatment efficacy cannot be guaranteed upfront, and these treatments may come with (long-term) serious adverse effects, negatively affecting a patient's quality of life. Gene expression-based tests can accurately estimate the risk of recurrence in early stage breast cancers. Disease recurrence correlates with treatment resistance, creating a major need to resensitize tumors to treatment. Notch signaling is frequently deregulated in cancer and is involved in treatment resistance. Preclinical research has already identified many combinatory therapeutic options where Notch involvement enhances the effectiveness of radiotherapy, chemotherapy, or targeted therapies for breast cancer. However, the benefit of targeting Notch has remained clinically inconclusive. In this review, we summarize the current knowledge on targeting the Notch pathway to enhance current treatments for breast cancer and to combat treatment resistance. Furthermore, we propose mechanisms to further exploit Notch-based therapeutics in the treatment of breast cancer.

Abstract

Introduction

Breast cancer

Breast cancer is the second most common malignancy, worldwide (1). Breast cancer screening and early detection has increased, leading to better outcome. Furthermore, a number of treatment options, have improved survival (2). First line therapies include surgery, radiotherapy, and systemic treatment (including: chemotherapy, endocrine therapy, and targeted therapy). Treatment options for breast cancer exist in the neo-adjuvant (prior to surgery) and adjuvant setting (after surgery). There is strong evidence that tumors that respond to neo-adjuvant chemotherapy with a pathological complete remission (pCR) have improved long-term prognosis (3). Conversely, tumors that do not respond to neo-adjuvant chemotherapy have a higher chance of recurrence. Adjuvant therapy targets remaining (micro-metastatic) cancerous cells, thereby preventing recurrent disease.

Clinical choices for the type of systemic treatment are guided by expression of estrogen receptor (ER), progesterone receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2) in tumor biopsies, in concurrence with TNM classification, tumor grade, and age. However, it is widely accepted that breast cancer is a heterogeneous disease, from primary tumor to metastatic sites (4). Gene expression profiling (involving hierarchical clustering) has had a significant impact on classification of breast malignancies. Molecular breast cancer subtypes revealed different clinical behaviors and retain distinct differences in biological mechanisms **(Table 1)**—associated with tumor aggressiveness, metastasis, and response (8, 10–18, 22). Based on gene expression profiling, the MINDACT trial has shown that tumors with genomic "low risk" features do not require chemotherapy. This included some node-positive tumors irrespective of molecular subtype (23). Similarly, the TAILORx study has recently shown that early stage, node-negative breast cancers with low or intermediate recurrence scores do not benefit from adjuvant chemotherapy and can be treated with endocrine therapy alone (24). Additionally, gene expression analysis showed a sub-classification in Triple Negative Breast Cancer (TNBC)into at least 4 molecular subgroups (12, 19, 25) with observed differences in response to chemotherapy (20), by providing more detailed information inter-tumor heterogeneity (26). The combination of histological and genetic classification of each tumor will further guide therapy selection and disease outcome (26, 27) and, ultimately, form the basis for personalized precision medicine.

Table 1. Intrinsic subtype classification of breast cancer

TABLE 1.	Normal Breast-Like	ERBB2+	Basal-Like	Luminal Subtype A	Luminal Subtype B	Luminal Subtype C
Specific Gene Signature	• Low to absent gene expression of the ER • Adipose tissue and other nonepithelial cell type gene expression • Strong expression of basal epithelial genes and low expression of luminal epithelial genes	• Low to absent ER gene expression • High ERBB2 pathway genes expression including; ERBB2 and GRB7 • Intermediate expression of luminal-related genes and proteins (e.g. ESR1 and PR) and low expression of basal-related genes and proteins (e.g. keratin 5 and FOXC1) • Enriched with high frequency of APOBEC3B-associated mutations • 1 out of 5 patients of HER2+/HR+ tumours will be identified as non-luminal	• Low to absent gene expression of the ER-related genes, intermediate expression of HER2- related genes • High expression of proliferation, suppression of apoptosis, cell migration and/or invasion genes • High expression of (HMW) keratins 5 ,6, 14 and 17, laminin, and FABP7 • BRCA1 mutations associated, none of the BRCA1 tumors showed evidence of ERBB2 amplification • Unique entitiy in breast cancer, more similarities with other cancer types • "stem/progenitor" cell phenotype • Multiple basal (TNBC) subtypes and mixed clinical response;. • TNBC often includes Claudin-low subtype.	• Highest expression (of luminal subgroups) of the ER α gene, X-box binding protein 1, trefoil factor 3, hepatocyte nuclear factor 3 a, and estrogen-regulated LIV-1 • vs. Luminal B: lower grade, lower number of mutations across the genome, lower number of chromosomal copy-number changes (e.g. lower rates of CCND1 amplification), less TP53, higher PIK3CA and MAP3K1, higher/similar GATA binding protein 3 • Subgroup shows HER2 amplification/overexpression	• Low to moderate expression of the luminal specific ER-related genes • vs. Luminal A: higher expression of proliferation/cell cycle-related genes or proteins – lower expression of several luminal related genes or proteins (e.g. lower progesterone) • Subgroup of Luminal B is found hypermethylated. • Subgroup shows HER2 amplification/overexpression	• Low to moderate expression of the luminal specific genes including the ER cluster • Separated from luminal A/B due to a subset of genes with unknown coordinated function, shared with Basal-Like and ERBB2+ tumors
TP53 **PIK3CA**	33% -	70% 39%	80% 9%	10-15% 45%	30-40% 29%	80% -
Similarities		• Highest mutational loads (Basal-Like > ERBB2+ > Luminal)		• Expression of the estrogen receptor separates luminal from non-luminal • Luminal expression signature: ER1, GATA3, FOXA1, XBP1 and cMYB. • Proliferation/cell cycle-related (e.g. RB1 and Cyclin D1) and luminal/hormone-regulated pathways • More diverse mutational profile		
			• Basoluminal subtype; distinguishable subtype based on heterogeneous CK5/14 expression, Laakso et al. 2006/ Haughian et al. 2012.			
Clinical outcome		• Worst/poor prognosis • Shorter disease free survival, earlier development of distant metastases • Shortest survival • Suggested not to benefit much from endocrine therapy • HR status shows predictive value for pCR • HR– increased 5yrs relapse	• Worst/poor prognosis • Shorter survival • Similar to Luminal B at 10yrs. • Suggested not to benefit much from endocrine therapy • Poly-chemotherapy is only effective treatment – variable between subtypes	• Best prognosis • Lowest rate of local or regional relapse. • vs. Luminal B: less chemo-sensitive (multi agent)	• Intermediate prognosis	• Worst prognosis luminal subtypes

Representation of gene-expression analysis and clinical outcome of the multiple intrinsic subtypes of breast cancer. TP53/ PIK3CA; percentage mutated cases. *Data from:* Sorlie *et al*. 2001 (5); Sorlie *et al*. 2003 (6); Laakso *et al*. 2006 (7); Cheang *et al*. 2008 (8); Prat *et al*. 2010 (9); Voduc *et al*. 2010 (10); The Cancer Genome Atlas 2012 (11); Prat *et al*. 2015 (12); Rakha *et al*. 2008 (13); Perou *et al*. 2000 (15); Lehman *et al*. 2011 (18); and Masuda *et al*. 2013 (20). *Abbreviations: HMW; High Molecular Weight, ER; estrogen receptor, HR; hormone receptor, PR; progesterone receptor, TN; triple negative, and TNBC; triple negative breast cancer.*

Intra-Tumor Heterogeneity and Tumor Stem Cells

Regardless of clinical or molecular subtype, intra-tumor heterogeneity is a common feature of all human solid tumors (28), and is a major determinant of treatment outcome in breast cancer (15, 29). Tumor growth is thought to be driven by small populations of cancer cells with self-renewal and multipotential properties (30), coined cancer stem cells (CSC) (31). These CSCs are involved in malignant behavior (invasion and metastasis) and resistance to treatment (32). Thus, CSCs are of high clinical importance, and targeting CSC self-renewal appears necessary for obtaining a durable response. Furthermore, intratumor heterogeneity can be driven by mutation or deregulation of stem cell signaling pathways such as Notch, Wnt, Shh, and others as well as through the tumormicroenvironment; including nutrient-, oxygen levels, and paracrine interactions with other cell types (fibroblasts, blood vessels, and immune cells) (33). Herein, Notch has shown interesting targeting opportunities in cancer (34).

Notch

Notch Signaling

Notch signaling (Figure 1) is a cell-to-cell communication system of type I single-pass transmembrane Notch receptors (Notch 1-4) and transmembrane ligands (Delta/Jagged (JAG)). Notch receptor maturation starts in the Golgi/Endoplasmic reticulum. Glycosylation of Notch proteins in the Golgi and ER is known to play a role in the regulation of Notch activity (35). Fringe proteins can both positively and negatively regulate Notch ligands however the full scope of their roles in breast cancer are unclear (36). Furin-like convertases cleave the non-covalently associated Notch heterodimer, which is transported to the plasma membrane (Figure 2A).

The extracellular domain consists of epidermal growth factor (EGF)-like repeats, followed by a negative regulatory region (NRR) which includes 3 LNRs (Lin12-Notch repeats) and a heterodimerization domain which prevents receptor activation in the absence of ligand(37) The intracellular portion of Notch (NICD) also contains multiple regions and domains, the RBPjk association module (RAM), Ankyrin repeats (ANK domain), and the TAD domain—which consists of Nuclear Localization Sequences (NLS) and the PEST domain (regulates receptor degradation) (38) (Figure 2B). Notch 1-4 have relatively short lifespans and undergo degradation through the ubiquitin proteasome and lysosomal pathways. The PEST domain contained in the NICD is likely to play a role in E3 ubiquitin mediated turnover. In fact, mutations in the PEST domain of Notch 1-3 in TNBCs, have been shown to increase Notch half-life and lead to increases in Notch downstream targets. Promisingly TNBCs with these mutations have been shown to be sensitive to GSIs (39). Similarly, alterations in the tumor suppressor and ubiquitin ligase Fbwx7/cdc4 target the PEST domain of Notch (40).

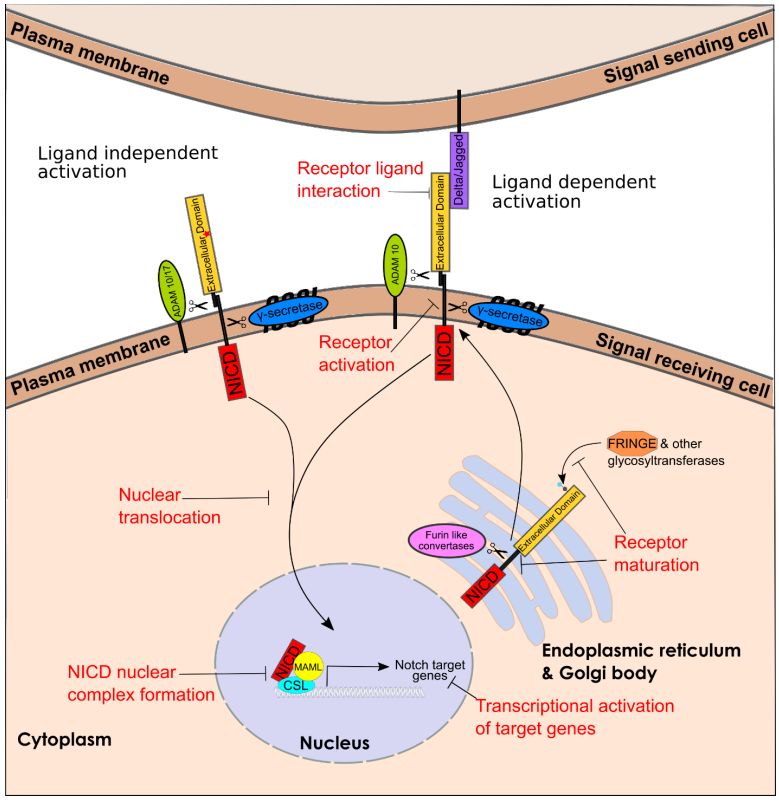

Figure 1: Notch receptor maturation and pathway activation, targetable options, and receptor functionality.

Notch receptor maturation and pathway activation, targetable options, and receptor functionality. (A) Stepwise representation of the process of Notch receptor maturation until receptor activation, followed by transcriptional output (not shown), and possibilities in targeting the Notch receptor pathway. (B) Notch receptor functional domains and corresponding functions ANK, Ankyrin repeats; LNR, Lin12-Notch Repeats and RAM, RBP-jk association module.

The E3 ligase MDM2 has been shown to contribute to the degradation of Numb and through ubiquitination leading to activation of Notch in breast cancer. Treatment of MCF7 cells with drugs targeting the acidic domain of MDM2 showed a reduction in Notch signaling (41). Furthermore, ubiquitination of Notch1 by MDM2 has been shown to activate Notch rather than leading to degradation (42). MDM2 has also been shown to regulate p53 degradation through ubiquitination, which, along with its role in Notch regulation makes it an attractive target for drug discovery along with other E1-3 ligases and interacting proteins. Knockdown of the E2-conjugating enzyme UBC9 and inhibition of the E1 activating complex SAE1/UBA2 has also been shown to impair the growth of Notch1-activated breast epithelial cells (43). Pevonedistat (MLN4924), is an inhibitor of NEDD8, a ubiquitin like protein that can neddylate E3 ligases. Pevonedistat has been

shown to induce apoptosis in MCF-7 & SKBR-3 cells in combination with 2-deoxyglucose (44), and to sensitize breast cancer cells to radiation in vivo (45). Bortezomib, an FDA approved proteasome inhibitor has been shown to inhibit multiple genes associated with poor prognosis in ERα breast cancer (46), however several clinical studies have shown contradictory results in advanced/metastatic breast cancer (47–49).

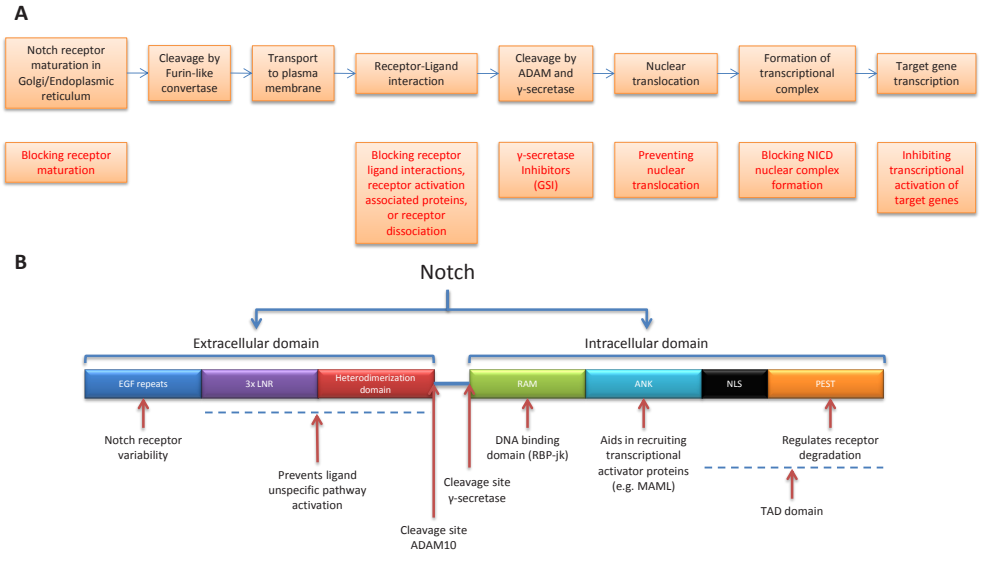

Figure 2: Notch receptor maturation and pathway activation, targetable options, and receptor functionality.

A. Stepwise representation of the process of Notch receptor maturation until receptor activation, followed by transcriptional output (not shown), and possibilities in targeting the Notch receptor pathway. **B.** Notch receptor functional domains and corresponding functions *Abbreviations: Ankyrin repeats (ANK), Lin12-Notch Repeats (LNR), and RBP-jk association module (RAM).*

A number of components of post-translational modifications pathway have been implicated in regulating Notch stability including Fbw7, Itch, β-arrestin, Fe65 and Numb (50–53). Numb negative breast cancers have increased Notch signaling which can be reverted to basal levels with overexpression of Numb and vice versa knockdown of Numb in Numb positive breast cancers leads to upregulation of Notch signaling (54). Further research into the mechanisms of Notch post-translational modifications and degradation may provide novel therapeutic targets as well as for other malignant diseases.

Signal transduction occurs through the Notch ligand on the "signal-sending cell" interacting with the Notch receptor on the "signal-receiving cell." This interaction process involves two sequential proteolytic cleavage events – first by the ADAM10 metalloprotease which sheds the extracellular domain, leading to the release of the NICD. The g-secretase complex is composed of 4 polytopic transmembrane proteins including a catalytic subunit

the aspartyl protease presenilin (55) The sequential proteolysis activation mechanism is essentially the same for Notch1-3 receptors (56). The activation mechanism for Notch4 -although likely similar to the other Notch family members- has not been reported yet. The NICD then translocates to the nucleus where it forms a protein complex with CSL (Cbf-1/RBP-jk in mammals, Su(H) in Drosophila and Lag-1 in C. elegans) and MAML (Mastermind-like) and induces transcription of multiple Notch downstream target genes (38) (Figure 2B). Additionally, a number of non-canonical pathways have been described downstream of Notch, including transcriptional activation of ERα-dependent genes (57), and NF-kβ (58), activation of the PI3K-AKT-mTOR pathway (59), and activation of mitochondrial metabolism (60, 61). Mammalian cells express four Notch receptors and five ligands in a highly tissue specific and content dependent manner (38). Activation levels of specific pathways within the global notch signaling pathway has been found to differ within mammary epithelial cells and this can lead to different phenotypic responses (62). Notch can also be phosphorylated which can have contradictory effects depending on the number of cleavage steps it has undergone and the specific kinase involved in phosphorylation (63). Phosphorylation of Notch by glycogen synthase kinase 3 (GSK-3) can reduce Notch transcriptional activity & protein levels (64, 65) and may be a target for possible therapies. Site specific methylation of NICD1 has been shown to make it less stable than a methylation defective mutant (66) indicating other possible post-translational targets (67).

Targeting Notch in Cancer

In many solid tumors the Notch signaling pathway is deregulated or mutated (68), affecting most hallmarks of cancer (69). Notch gene expression is frequently deregulated in breast cancer (70) and shows extensive crosstalk with several major signaling pathways. Further, there is ample evidence for the diverse role of Notch signaling in tumor formation, progression, and resistance to treatment in breast cancer (71).

Due to the multi-step activation process, several Notch pathway interventions are being explored at the level of: I. blocking receptor maturation, II. receptor–ligand interactions, III. receptor activation associated proteins, IV. nuclear translocation, V. NICD nuclear complex formation, and VI. transcriptional activation of target genes (72) (Figure 2A). This includes, but is not limited to antibodies, small molecule inhibitors, and inhibitors of γ-secretase (GSI) (72, 73).

Despite the increased evidence for deregulated Notch in numerous malignancies and resensitization opportunities (34, 74–81), many clinical studies investigating Notch targeting are on hold or have been terminated. Notably, most of these trials were conducted in recurrent, heavily pre-treated chemo resistant cancers and used dose-limiting non-selective pan-Notch/GSIs. Additionally, because of a lack of biomarkers predicting outcome to Notch therapies, potential responders were thus not effectively selected [reviewed in (71)]. As a result, this has not led to effective interventions using

Notch inhibitors combined with standard of care. Therefore, in this review, we have focused on the possible role of Notch in enhancing the efficacy of breast cancer treatment.

Notch and Breast Cancer

Role of Notch in breast development.

The normal mammary gland experiences a period of rapid growth and development at puberty. Thereafter, and until menopause, it undergoes cycles of expansion and regression with each estrous cycle, pregnancy, lactation, and involution (82). This homeostasis requires stem cells and their existence was first demonstrated using transplantation experiments to reconstitute a functional mammary gland in rodents (83). Dontu *et al*. demonstrated the presence of early progenitor/stem cells capable of differentiating along all three mammary epithelial lineages (myoepithelial, ductal-, and alveolar epithelial). Gene expression analysis revealed similarities with progenitor and stem cell associated pathways, thereby identifying mammary stem cells (MaSCs) in 3D culture systems (84). More recently, *in vivo* imaging has identified bi-potent basal stem cells in the mammary gland, yielding both myoepithelial and luminal cells (85) and Notch plays a role in this process (86–89). Bouras *et al*., have performed extensive research on the role of Notch in MaSCs. In MaSCs, Notch1 is differentially expressed between subtypes (90), and its expression is higher in the luminal type cells (90, 91). Furthermore, Notch1/3 mark the luminal progenitor cells in mammary gland development (89, 91). Downregulation of Cbf-1/RBP-jk resulted in increased proliferation of MaSCs, thereby influencing absolute stem cell numbers. However, this proliferation resulted in increased and disorganized side branching, with increased number of end buds and basal cells in these end buds. Therefore, RBP-jk downregulation regulates the formation of a more basal cell phenotype. Additionally, overexpression of the endocytic protein NUMB, a negative regulator of Notch, produced the same effects. This shows that reduced Notch signaling is important in proliferation of the basal cell population and MaSCs. Conversely, increased levels of Notch1 in the luminal cells showed that constitutive Notch activation is important for commitment to the luminal cell lineage (High Keratin8/18, Stat5, and p63 downregulation) (90). Moreover, it has been reported that Notch4 is involved in promoting stem cell renewal of mammary epithelial cells (mammospheres) *in vitro* (92, 93), and is involved in stem cell activity (94)–possibly through JAG1 signaling (95) and PKCa-Notch4 interaction (96). Furthermore, Notch and p63 signaling guide the establishment of basal and luminal epithelial cells (97) and PTEN/JAG1 play an important role in mammary epithelial stem cells (98).

Role of Notch in breast cancer development and metastasis.

The role of different Notch pathway components in breast cancer development has been extensively researched. Stylianou *et al*. showed that in many breast cancer cell lines Notch ligands, receptors, and target genes are aberrantly expressed (99). Charafe-Jauffret

et al. identified a 413-gene CSC profile (including Notch2) using normal and malignant mammary tissue (100), identifying breast cancer stem cells (BCSCs) through $ALDH^+$ (101). $ALDH^+$ cells were capable of self-renewal, differentiation, tumor formation in mice, and showed increased metastatic potential. $ALDH^-$ cells hardly generated tumors. Results from a Meta–analysis involving 3867 patients showed that Notch1 expression positively correlates with breast cancer progression and that higher expression is associated with a transition from ductal carcinoma *in situ* to invasive cancer. Furthermore notch1 overexpression was correlated with significantly worse overall and recurrence-free survival. The data further suggested that Notch inhibitors may be useful in blocking early progression of ductal carcinoma *in situ* (5).

Aberrant activation of the Notch signaling pathway has been shown to promote an aggressive phenotype partially through NF-κB, whereas de-activation of Notch signaling abrogates this aggressive phenotype (58). Furthermore, in TNBC, tumor cells activated NF-κB upregulates Jagged-1, which stimulates Notch signaling in CSCs (102).Tumor derived Jagged1 has been shown to be an important mediator of bone metastasis in breast cancer. Jagged1 activates stromal Notch signaling which in turn induces IL-6 secretion from osteoblasts stimulating tumor growth. Notch signaling also directly stimulates maturation of osteoclasts exacerbating bone metastasis. Destruction of bone matrices releases TGF-β upregulating Jagged1 in the tumor giving a positive feedback loop. GSIs treatment in turn reduces bone metastasis by targeting stromal Notch signaling (103).

In vivo studies using TNBC and $ERα^+$ cell lines showed an association between Notch3 expression and distant metastases which was diminished in Notch3 null cells. This finding was corroborated using TNBC cells from a patient-derived brain metastasis (104). An *in vivo* study using a more metastatic variant of the HER2+ MDA-MB435 isolated from *in vivo* brain metastasis showed activation of the Notch signaling pathway. Inhibition of Notch using the γ-secretase inhibitor DAPT or knockdown using RNAi against Notch and Jagged2 resulted in inhibition of the migratory and invasive phenotype (105). Furthermore fewer brain micrometastases were found when Notch1 was silenced in an MDA-MB-231 model (106). Breast tumor cells in the brain highly express IL-1β which leads to surrounding astrocytes expressing Jagged1 which stimulates Notch signaling in CSCs (107). Oskarsson *et al*. showed that breast cancer cells that metastasize to the lungs enhance their ability to survive through expression of the extracellular matrix protein tenascin C. Tenascin C is associated with aggressiveness and pulmonary metastasis and enhances stem signaling components including Notch (108).

Notch1. Notch1 is aberrantly expressed in breast cancer (99) and high Notch1/4 mRNA expression and activity are associated with worse prognosis (30). In Ductal Carcinoma *in situ* (DCIS), Notch1 signaling is active and associated with the development of breast cancer (109). Both Notch1 and Notch4 are identified as common sites of proviral integration in mammary mouse tumors (110, 111), and induce mammary (MMTV)-

tumors when overexpressed in transgenic mice (112–114). Larger studies have shown that expression of Notch1/4 and JAG1 is associated with poor prognosis in breast cancer (115). Moreover, JAG1 expression is an independent predictor of poor outcome in node-negative disease (116) and higher NICD1 expression correlated with sentinel lymph-node positive patients (117). Notch1 levels were progressively associated with the transition from DCIS to invasive basal cancer (5).

In human breast cancer, a meta-analysis including approximately 4000 cases showed that elevated Notch signaling is associated with increased disease recurrence (118). Pathway and network analysis revealed that altered Notch1 signaling occurred in $ER^+/PR^+/HER2^{+/-}$ breast cancers (119), whereby Notch1 mutations are more prevalent in $HER2^-$ than $HER2^+$ tumors (120). JAG1-Notch signaling leads to Cyclin-D1 induction (121), a gene that is essential for normal breast development in mice (122) and frequently deregulated or amplified in human breast cancer (123, 124). k Notch1 activating mutations/rearrangements have also been observed in TNBC (in EGF repeats and NRR) (125) and in the basal-like phenotype (116). Additionally, Notch1 promotes stem cell maintenance through c-Jun signaling (126). Further, Reedijk *et al*. revealed that JAG1 is an independent predictor of poor outcome in multivariate-analysis (115) with other well-known outcome predictors (nodal metastases, patient age, tumor size, node status, ER positivity, and tumor grade) (5, 115, 127). Higher NICD1 expression correlated with sentinel lymph-node positive patients—strengthening Notch1's role in the metastatic process (117).

Leong *et al*. provided data that JAG1 and Notch1 are involved in epithelial-mesenchymal transition (EMT) through SLUG and E-cadherin. They showed that SLUG facilitated E-cadherin repression (through Notch1 inhibition) and inhibition of HEYL blocked tumor growth and metastasis, showing JAG1-Notch1-SLUG dependency (128). Furthermore, NICD1 expression negatively correlated with E-cadherin and showed increased invasive capacity of Notch1, (129). This was also the case under hypoxia with differences observed in high/low Notch signaling cell lines (130). Mechanistically, hypoxia-induced EMT is mediated through SLUG and SNAIL (131). A JAG2-EMT relationship has been shown too (through Notch1), revealing a broader spectrum of Notch1 activation and involvement in hypoxia and metastatic potential of CSCs (132). Additionally, high Notch1 and HIF predict a worse prognosis (133). These results show that Notch1 signaling is important for EMT and downregulation of E-cadherin, ultimately creating a more invasive phenotype. Furthermore, as described above, the invasiveness of the tumor and hypoxia induced EMT requires Notch1 signaling, demonstrating a hypoxia/Notch1/EMT axis. Thus, inhibition of Notch1 can be tumor suppressive by removing the inhibition on E-cadherin expression, regardless of hypoxia.

Downregulation of JAG1 or blocking Notch with GSI in a metastatic breast cancer model (MDA-231) attenuates bone metastasis by reducing osteolysis in the bone microenvironment. Conversely, overexpression of JAG1 is sufficient to induce bone metastasis in this model (103). Others have demonstrated a role for Notch1 of tumor dormancy in the bone marrow microenvironment, instigating metastases, through a

Notch1/STAT3/LIFR signaling axis (134). Furthermore, circulating tumor cells "primed" for breast cancer brain metastases have a specific gene signature ($HER2^+$/$EGFR^+$/$HPSE^+$/$Notch1^+$) (135, 136). These CTCs could either be derived from the primary tumor or from metastatic lesions. Importantly, these CTCs were $EPCAM^-$. This would make them undetectable by the only FDA approved clinical test for CTCs, which is based on an $EPCAM^+$ profile (136).

Notch2. Notch2 can act as a transcriptional and functional regulator of Notch1 and Notch3 (137) and has been shown to be involved in specific mammary epithelial lineages affecting luminal cellular hierarchy (138). Mutations in Notch2 show increased incidence in breast cancer, and in addition to the TCGA database new mutations have been found (139). Notch2 is positively correlated with HER2 (140), low-grade tumors and improved outcome (141), and increased apoptosis (142). In the basal subtype, JAG1 and DLL4-induced Notch2 activation under the influence of FYN/STAT5 maintained the mesenchymal-phenotype. Notch2 siRNA decreased the EMT markers VIM, SNAI1, SNAI2 (SLUG), TWIST, and ZEB1 (143). Notch2/3 inhibition (Tarextumab) decreased CSC numbers in the UM-PE13 breast cancer cells (144). Furthermore, mutations in Notch2 could facilitate development of liver metastasis (145). However, other studies showed that, Notch2 mutations do not unequivocally associate with better prognosis and therapy efficacy (144, 146).

Notch3. Expression of oncogenic Notch3 in mice leads to mammary cancer (111), and is involved in: hormone-receptor positive breast cancer (120), the proliferation of $HER2^-$ breast cancer (147) and $HER2^+$ DCIS (148), and TNBC (149). Notch3 is involved in $HER2^+$ DCIS through transcriptional upregulation of the Notch pathway by HER2–whereby Notch3 upregulates the formation of luminal cells and increases proliferation through Cyclin-D1, c-MYC, and AKT (148). Furthermore, Notch3 signaling has been proposed to be an important regulator of the process whereby bipotent progenitors commit to the luminal lineage (93). Additionally, evidence from nonsense and missense mutations in multiple cancers, including breast cancer, showed tumor suppressor capabilities of Notch3 through controlling of the cellular senescence pathway (150). Interestingly, no significant change in Notch1, Notch2, or Notch4 expression was observed in these studies.

In TNBC, ectopic NICD3 (over)expression facilitated the inhibition of EMT through upregulation of the HIPPO pathway and E-cadherin in a RBP-jk dependent manner, whereby knockdown of Notch3 abrogated this effect (151). Furthermore, a correlation was shown between Notch3 and p21, a well-known senescence-involved protein. A significant decrease in Notch3 was observed in primary breast cancer, compared with normal tissue, suggesting a protective mechanism against Notch3-initiated cellular senescence. Re-introduction of Notch3 resulted in growth inhibition and activation of cellular senescence, suggesting that loss of Notch3 expression facilitates senescence induction and could play a critical role in tumor progression. Notch3 silencing has recently been shown to sensitize

TNBC cells to the EGFR inhibitor gefitinib by promoting EGFR tyrosine dephosphorylation and internalization (152). Notch3/4 were shown to have increased expression in low-burden metastatic cells relative to the primary tumor (153).

Notch4. The oncogenic function of Notch4 was first demonstrated by retroviral insertion in MMTV-induced mammary tumors (110). Additionally, Notch4 is highly expressed, and gain of function mutations have been identified, in mouse mammary cancer models [reviewed in (154)]. Expression of activated Notch4 in mammary epithelial cells lead to transformation (155) and rapid development of poorly differentiated adenocarcinoma in transgenic mice (110, 156). Additionally, truncated human Notch4/Int3 (activated Notch4) instigated mammary tumors (112), through transcription of RBP-jk (157) and ANK repeats (158). Interestingly, transgenic expression of Notch4 NICD caused mammary tumors in the absence of RBP-jk in mice harboring conditional knockout of RBP-jk (157). This suggests that non-canonical pathways may participate in the oncogenic activity of Notch4.

PEST domain. The PEST domain is a degradation domain that regulates the stability of all NICDs through ubiquitination and proteasomal degradation (39). Nonsense mutations are common in T-ALL (159, 160) and have been observed in Notch1/2/3 receptors in TNBC (39). Furthermore, Notch pathway and target genes, including Notch1/3, HES1, HEY2, HES4, MYC, Cyclin-D1, and NRARP, were highly overexpressed in TNBC (39, 125). Notch mutation-activated dependency was shown using GSI, as wild type tumors showed little to no response (39).

Notch pathway-associated proteins

Fringe. Fringe is an important regulator of the Notch receptor-ligand interaction (161) through modification (glycosylation) of EGF repeats in the extracellular domains of Notch receptors (162). Fringe enzymes add N-acetyl glucosamine to fructose residues in the extracellular domains of Notch receptors. More glycosylated receptors retain high affinity for Delta ligands but have reduced affinity for Serrate/JAG ligands. Hence, loss of Fringe glycosylation enhances Notch affinity for Serrate/JAG ligands. There are three Fringe genes in mammals: Lunatic Fringe (LF), Manic Fringe (MF), and Radical Fringe (RF). In breast stem or progenitor cells, and especially the terminal end bud cap cells termed "leader cells" (163), LF is highly expressed (93). Conversely, the majority of basal tumors and a subset of claudin-low tumors show reduced LF expression (164). In MMTV-driven tumors, absence of LF exclusively caused triple negative tumors. Furthermore, deletion of LF was enough to cause Notch-driven (Notch1-4) basal-like tumors via enhanced stem/progenitor cell proliferation (163). These tumors resembled "claudin-low" (mesenchymal) subtype of TNBC. p53 loss of function in these tumors resulted in a clear EMT profile (Vimentin, TWIST, E-cadherin) (165). Cells showed increased levels of Vimentin and E-cadherin and decreased expression of cytokeratin 8/14–this coincided with decreased

differentiation, increased levels of proliferation, and stem cells. Co-deletion of LF and p53 resulted in upregulated NICD3 and HES5, and downregulation of HES1. These data connect expression of LF, Notch (signaling), and p53 to impaired luminal differentiation.

In contrast to LF, MF is highly expressed in the claudin-low subtype of breast cancer and is associated with Notch4 (166). Deletion of MF shifted the tumor resemblance to a less claudin-low like, more luminal subtype—through increased levels of the luminal marker CK8 and basal marker CK14, and decreased levels of stem cell marker ALDH1. Furthermore, MF was shown to be able to regulate cancer stem cells and their migration in a spheroid model by increasing NICD1 expression and PIK3CG (encoding the g catalytic subunit of PIK3-γ) (166). These data show that Fringe is involved in a Notch-dependent manner in breast cancer with different roles observed for different Fringes (no data has been reported yet on RF)—causing a Fringe-dependent subtype switch (basal-luminal).

NUMB. NUMB is a cell fate determinant and endocytic protein that acts as a negative regulator of the Notch signaling pathway (54, 167, 168). NUMB is frequently down-regulated in breast cancer and suppresses the growth of breast cancer cells *in vitro* (169, 170) often involving the attenuation of the p53 tumor suppressor pathway (168). NUMB can drive Notch toward endocytic degradation. Additionally, NUMB inhibits ubiquitin ligase MDM2, which targets p53 for degradation. Hence Loss of NUMB results in a high-Notch, low p53 phenotype. Mechanistically, NUMB forms a ternary complex with MDM2 and TP53 and inhibits the activity of MDM2 (168, 171). In a cohort of breast cancer patients receiving adjuvant chemotherapy, NUMB, and indirectly Notch activation, were inversely correlated with clinical and pathological parameters indicative aggressive disease progression (168). In NUMB-deficient cells, p53 is ubiquitinated and degraded, resulting in chemoresistance and high Notch activity. MDM2 also ubiquitinates NUMB, which results in nuclear translocation and degradation (172). Thus, NUMB connects the MDM2/p53 pathway, the most frequent mutated pathway in human cancers, with Notch signaling.

MAST. In many breast cancers, gene translocations and fusions have been described. Recurrent gene arrangements involve MAST and Notch family members (Notch1/2), both showing phenotypic effects in breast cancer (e.g., greater proliferation). Notch fusions were found, almost exclusively, in ER^- breast carcinomas. All the fusion transcripts retained the exons that encode for the NICD. Furthermore, higher Notch responsive transcriptional activity was seen in breast cancer cell lines carrying MAST-Notch fusions, and showed dependence on Notch signaling for proliferation and survival (173). The discovery of these Notch fusions warrants further investigation and may identify a biomarker for Notch based therapeutics.

Nicastrin. Nicastrin is an essential component of the γ-secretase complex; it encodes an integral membrane protein which associates with the catalytic subunit of γ-secretase,

Presenilin (174). Nicastrin is crucial for maturation of Presenilin and cells that lack Presenilin are γ-secretase and Notch-deficient (175, 176). In breast cancer, high Nicastrin is mainly observed in the ER+ subtypes. Nicastrin expression correlates with age and tumor grade–and predicts worse tumor survival (177). Additionally, a set of 22 genes (located on chromosome 1) has been co-identified with Nicastrin amplification and breast cancer (178), however, these genes showed no clear Notch signature. Furthermore, Nicastrin seems to play a role in EMT (177, 179). Targeting of Nicastrin affects breast cancer stem cells and inhibits tumor formation *in vivo* (179). Inhibiting Nicastrin in TNBC, using monoclonal antibodies, showed anti-tumor activity (180). Thus, aiming at Nicastrin provides another opportunity to target the involvement of Notch in breast cancer.

These data suggest that (deregulated) Notch receptor/ligand signaling influences cell renewal in the mammary gland and reaches far beyond mammary development, as it possesses the ability to influence the pre-malignant lesions, primary tumors, the metastatic potential of tumors, and therapy resistance.

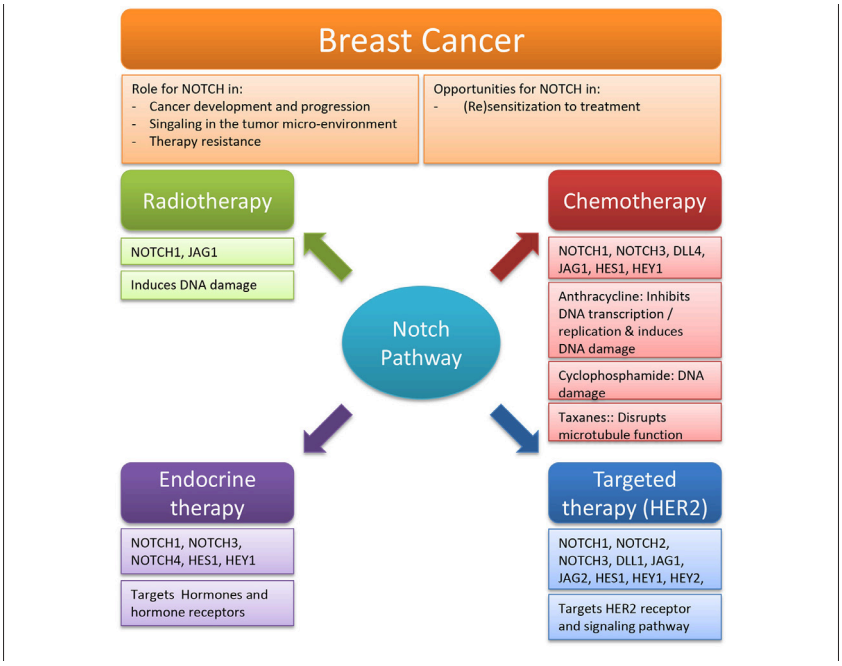

Figure 3: Overview of the role and opportunities for Notch in breast cancer therapy.

Summary of the 4 fields of breast cancer therapy [radiotherapy, chemotherapy, endocrine therapy, and targeted therapy (HER2)] in which Notch targeting can play a significant role.

Notch Signaling in the Tumor Microenvironment

The breast microenvironment consists of a number of cell types including fibroblasts, adipocytes, endothelial and immune cells as well as extracellular matrix.

Cancer associated fibroblasts (CAFs) have been shown to induce Notch activation in breast cancer cell lines through secretion of IL-6 (181). There is also evidence supporting a role for fibroblast-derived microvesicles in endocrine resistance. Cancer-Associated-Fibroblast (CAF)-derived microvesicles, containing oncomiR-221 promoted de novo endocrine resistance—as overexpression of oncomiR-221/222 in luminal breast cancer cells reduces ER expression (182) Furthermore CAFs can promote the cancer stem cell phenotype by secreting CCL2, inducing Notch1 (183). Stromal cells including fibroblasts have also been shown to promote therapy resistance in breast cancer cells through expression of Jagged1 and exosomal transfer leading to Notch3 and STAT1 signaling in cancer cells (184). GPER signaling from both CAFs and cancer cells has been shown to upregulate Notch signaling. 17β-estradiol and GPER ligand G-1 induces γ-secretase-dependent activation of Notch1. Furthermore, the 17β-estradiol and GPER induced migration of breast cancer cells and CAFs is attenuated with GSI treatment (185).

17β-estradiol also promoted increased Jagged1 as well as Notch1 expression in MCF7 cells and was similarly found in endothelial cells. The endothelial cells formed cord-like structures in matrigel in contrast to cells expressing a dominant negative form of Notch1. 17β-estradiol treatment was also able to increase tumor microvessels *in vivo*, which correlated with Notch1 expression (186). Clinical data has shown higher Notch1 activation in tumor endothelial cells compared to non-malignant tissue. A correlation between the rate of NICD1-positve vs negative tumor endothelial cells was higher in patients with positive sentinel lymph nodes (117) Co-culture *in vitro* and *in vivo* has demonstrated upregulation of notch ligands in endothelial cells after contact with breast cancer cells. Proliferation and survival was significantly reduced along with a reduction in the stem-cell population when co-cultures were treated with GSI. Knockdown of Jagged1 in endothelial cells reduced the survival ability of breast cancer cells under starvation conditions. Knockdown also reduced tumor cell proliferation but did not reduce survival of knockdown epithelial cells (187). Wnt signaling is known to be up-regulated in breast cancer. Aberrant wnt signaling has been shown to give a tumorigenic phenotype to primary epithelial cells. This conversion is in part caused by up-regulation of the Notch ligands Dll1, Dll3 & Dll4 which are required for the tumorigenic phenotype (188).

Mammospheres enriched with stem/progenitor cells from node invasive breast carcinoma tissue expressed more IL-6 than matched non-neoplastic mammary glands. Il-6 was only detected in basal-like breast carcinoma tissue which contained stem cell features. Il-6 upregulated Jagged1 and lead to growth and a hypoxia-resistant/invasive phenotype through Notch3 dependent expression of CAIX (189).

Adipocytes within the tumor microenvironment secrete leptin and IL-6. Leptin and IL-6 signaling in breast cancer cells adjacent to adipocytes upregulate multiple pathways including Notch promoting a stem-like phenotype as well as epithelial-mesenchymal transition (190). Leptin is able to induce Notch 1,3 & 4 however Notch3 appears to be cell dependent. The leptin-Notch signaling axis is involved in proliferation and migration and

leads to higher incidence and aggressiveness in obese patients. Leptin inhibitors were able to reduce Notch receptor, ligand and target expression (191).

Dll4 and Jag1 have opposite effects on regulating angiogenesis. Jag1 induces maturation of blood vessels, while Dll4/Notch regulates sprouting angiogenesis (192). Thus targeting Dll4 or Jag1 will have different effects. Targeting Dll4 using antibodies promotes non-productive angiogenesis (193). GSI treatment however targets both and leads to a decrease in angiogenesis (194). These differences in targeting may explain the contrasting in angiogenesis seen in pre-clinical models treated with GSI or Dll4 antibodies. In a phase I clinical trial, enoticumab, a Dll4 monoclonal antibody targeting the tumor vasculature, showed stable disease as best response in 2 of the 6 breast cancer patients enrolled. The antibody also gave a number of side effects, seen with previous Notch targeting therapeutics, as well as ventricular dysfunction and pulmonary hypertension (195).

Notch and the immune response

The role of Notch signaling in the immune response to tumors is complex and is dependent on the tumor type and microenvironment factors. Notch signaling is a key regulator of hematopoietic development and controls self-renewal, lineage commitment and terminal differentiation of the innate and adaptive immune system including B cells, T cells, myeloid cells, dendritic cells and natural killer cells (196, 197) Notch signaling, both canonical and non-canonical, also plays a role in tumor induced immuno-suppression.

It has been established that most stages of the tumor development from initiation to malignant conversion, invasion, metastasis, therapy resistance and relapse involve the inflammatory response (198). The interaction between tumor cells and immune cells in the tumor microenvironment controls the overall immune surveillance and response to therapies and patient outcome. The role of Notch signaling in the immune response to tumors is complex and is dependent on the tumor type and microenvironment factors Notch as well as regulating many aspects of the immune system regulates many components of the tumor microenvironment (199, 200).

There is a strong causal relationship between endocrine resistance and Jagged NOTCH signaling in breast cancer which promotes macrophage differentiation toward tumor-associated macrophages (TAMs), the most common immune cell found in the breast tumor microenvironment (200). TAMs can be pro or anti-inflammatory depending on micro environmental factors, which in most breast cancers develop the anti-inflammatory phenotype (200, 201). The anti-inflammatory phenotype in breast cancer plays a role in suppressing immune surveillance as well as promoting proliferation, angiogenesis and tissue remodeling (198). In a model of basal-like breast cancer, tumor cells secrete the CCL2 & IL-1β cytokines in a Notch dependent manner, which work to recruit monocytes (202). Within the tumor microenvironment monocytes differentiate into TAMs with a pro tumor phenotype supporting tumor growth and metastasis (203). TAMs also interact

with cancer cells via TGFβ, promoting Jagged 1 expression, causing a feedback loop that amplifies cytokine/chemokine secretion.

Myeloid-derived suppressor cells (MDSCs) promote tumor progression through a variety of mechanisms including immune suppression and enhancing angiogenesis and metastasis. MDSCs have been shown to have lower Notch activity in conditioned media from breast cancer cell lines through an inhibitory phosphorylation of NICD by casein kinase 2, disrupting NICD/ CSL interaction (204). MDSCs in breast cancer have also been shown to induce Notch signaling in cancer cells and promote CSC capacity through IL6/ STAT3 & Nitric Oxide/Notch cross talk signaling (205, 206). Cancer cells also increase Jagged-1 & Jagged-2 expression in MDSCs leading to a positive feedback loop between cancer cells, immune cells and CSCs.

Notch has been shown to be important in the regulation of Tregs, a subtype of T cells, which is important in peripheral self-tolerance and plays a role in tumor immunosuppression (207). Tregs promote evasion of immune surveillance and are linked to tumor invasiveness and poor prognosis. Notch-1-TGF-β signaling directly induces peripheral Tregs through upregulation of Foxp3 (208). Both Jagged-1 and Jagged-2 increase the generation of Tregs (209) and are highly expressed in TNBC, CSCs and treatment resistant populations (95, 132).

On the other hand $CD8^+$ cytotoxic T cells, which have been shown to have anti-tumor function, require Notch to become activated (210), and Notch2 has been shown to be required for the anti-tumor effect of cytotoxic T lymphocytes (211). Furthermore, selective activation of the Notch pathway in hematopoietic environments enhances T-cell activation and infiltration, inhibiting tumor growth in mouse models.

Research into targeting the immune response and the tumor microenvironment is ongoing and detailed reviews strategies and treatments can be found here (212, 213). GSI treatment has been shown to reduce the numbers of TAMs, MDSCs and TRegs, however it can't be excluded that this was in part due to inhibiting tumor growth (214). More research is needed to fully elucidate the complex interplay between Notch, tumor microenvironment and the immune system in breast cancer and to develop strategies that enhance the anti-tumorigenic effect but do not suppress the anti-tumor immune response.

Notch in Breast Cancer Therapies

Radiotherapy

For breast cancer, radiotherapy is mainly implemented in the adjuvant setting and involves the targeting of remaining tumor cells, with the aim to prevent recurrence of residual disease. Gene signatures (IGKC, RGS1, ADH1B, DNALI1) in primary breast cancers predict low and high risk groups for local regional recurrence after Radiotherapy (215, 216). Generally, cancer stem cells are often radiation resistant (217, 218). Radiotherapy resistance could

be intrinsic or acquired through changes in gene expression profiles and radiotherapy-resistant CSCs have been observed in breast cancer (219, 220). More specifically, BCSCs ($CD44^+/CD24^{-/low}$) were shown to be resistant to radiation (compared to non- $CD44^+/CD24^{-/low}$ mono-layer cultures), and contributed to tumor recurrence after fractionated radiation. In a clinically more relevant culture system (mammospheres) higher radiation resistance was observed correlating with lower levels of ROS compared to monolayer cultures. Consistently, mammosphere cultures showed higher radiation resistance than irradiated single cell suspensions. Thus, during fractionated radiation, repopulation derives from the more resistant subpopulation of CSCs. Increased levels of Notch1/JAG1 signaling could stimulate the more resistant phenotype of $CD44^+/CD24^{-/low}$ CSCs (219). Lagadec *et al*. showed that radiotherapy-exposed cancer cells have increased mammosphere formation, increased tumorigenicity, and (re)expressed stemness-related genes (transcription factors Oct4, Y-box 2, Nanog, and Klf4). Interestingly, both NICD1 and JAG1 expression were upregulated only in response to fractionated radiation (5 × 3Gy) and not after a single dose (10Gy) (221). Additionally, other research showed that a singular dose of 3Gy did upregulate NICD1 and JAG1 (222). Thus, induction of Notch pathway genes is radiation (multi)dose-dependent (222). Furthermore, targeting of Notch using siRNA (221) or GSI (222) decreased the induced BCSCs population after irradiation of non-tumorigenic cells. These data indicate that Notch is involved in the induction of radiation-induced CSCs from partially differentiated tumor cells. Recently it has been shown that, that Notch1 directly regulates the DNA damage response, through physical interaction and suppression of phosphorylation of ATM kinase (223). A plausible hypothesis is that after repeated irradiation, Notch1 could suppress apoptosis-inducing signals from the activated DNA damage response.

Chemotherapy

Chemotherapy is an important component of standard cancer treatment and includes anthracyclines, cyclophosphamide, and taxanes. Resistance to chemotherapy is the main cause of treatment failure in 90% of the patients with metastatic cancers (224). Importantly, chemo-resistance accompanies endocrine resistance, so that ER-positive recurrent tumors that are resistant to endocrine therapy are also almost invariably chemo-resistant. One of the main underlying causes for treatment failure is intra-tumor heterogeneity, a process affected by the presence of CSCs (168, 225).

Anthracycline/Cyclophosphamide

A role of Notch in doxorubicin sensitivity and resistance has been reported by Zang *et al*. (226). They showed that Notch1 inhibition (RNAi) and doxorubicin treatment led to a 50 and 70% growth inhibition, and increased apoptosis, compared to chemotherapy alone– in the MCF7 and MDA-MB-231 cell lines respectively. Li *et al*. showed that the efficacy of

doxorubicin could be increased when used in combination with a GSI (227). Additionally, chemotherapy increases the percentage of treatment resistant $CD44^+/CD24^{low}$ breast cancer cells in patients. In tumor xenografts combination treatment with GSI and doxorubicin led to better tumor control–by reducing $CD44^+/CD24^{low}$ population (168).

Interestingly, ALDH expression has been shown to inactivate chemotherapeutics such as doxorubicin and cyclophosphamide (228–230). In addition, Suman *et al*. (231) showed that Notch inhibition was effective in both $ALDH^-$ and $ALDH^+$ cells, though $ALDH^-$ cells were more sensitive. Additionally, they showed that Notch1 downregulation (using Psoralidin) and silencing resulted in inhibition of cell viability and proliferation, and a downregulation of EMT factors SLUG and TWIST.

In ER+ cell lines (MCF7 and T47D) Notch target genes HES1 and HEY1 were induced by doxorubicin, and could be inhibited using a GSI–suggesting a Notch signaling dependent effect. Furthermore, expression of Notch was associated with expression of multi drug resistance protein 1 (MRP1), a potential predictor of chemotherapy response and clinical outcome, in a dose-dependent manner (232). Importantly, in patients treated with neoadjuvant chemotherapy (anthracyclines ± taxanes), pre-treatment NICD1 levels were very low or absent, while post-therapy NICD1 was significantly upregulated (232).

In a doxorubicin resistant engineered-cell line, MCF7-AMD, Notch3 was shown to be downregulated in chemo-resistant cells, and EMT was activated. Furthermore, in ER^+ patients, low Notch3 predicted distant relapse-free survival, with Fos-related antigen 1 (Fra1) being negatively regulated by Notch3 (233).

Taxanes

The two most common used taxanes for breast cancer treatment are docetaxel and paclitaxel (234, 235). Qiu *et al*. showed that docetaxel treatment resulted in increased primary mammosphere formation. Notch1 inhibition increased chemotherapy efficacy in TNBC BCSCs ($CD44^+/CD24^{-/low}$ population) *in vitro* and in a patient-derived xenograft breast cancer model (236). In line with this, Zhang *et al*. reported similar findings, using a GSI in multiple xenograft models (237). "Tumor debulking" by docetaxel resulted in an increased BCSC population, quantified using $ALDH^+/CD133^+/CD44^+$. Interestingly, the $CD44^+/CD24^{-/low}$ population was not altered, however, this might be due to differential targeting methods (Qiu *et al*. (236): mAb vs. Zhang *et al*. (237): GSI). Docetaxel-treated tumors showed increased NICD1. Combination of GSI with docetaxel showed significant improved effect compared to docetaxel alone. Mechanistically, treatment with docetaxel caused an increase in survivin (inhibitor of apoptosis) and drug transporters, which could be inhibited by GSI. Furthermore, decreased expression of NUMB was observed in docetaxel treated tumors but not after dual treatment with GSI. Docetaxel treatment increased EMT markers SNAIL, SLUG and N-cadherin, which could be blocked by Notch inhibition. These findings indicate that Notch1 is involved in the resistance mechanisms of docetaxel treated tumors and that dual treatment could block enrichment of the BCSC population and increase therapy efficacy.

Schott *et al*. showed a residual BCSC subpopulation to be insensitive to docetaxel alone (238). However, in tumor-derived xenografts treatment with GSI (MK-0752) reduced the BCSC population; this resulted in reduced mammosphere formation and decreased NICD and HES1 expression. A concurrent clinical study, including 30 patients with recurrent disease after anthracycline treatment, showed that repeated cycles of GSI resulted in partial response in 11 patients and evidence for a reduction in $CD44^{+}/CD24^{-/low}$ and $ALDH^{+}$ cells. Repeated biopsies showed an initial increase in BCSC populations until after the 1st treatment cycle, after which it declined—this is consistent with the ability of GSIs to decrease BCSCs. However, additional treatment cycles where needed to additionally reduce BCSCs and tumor burden. An additive effect of Notch inhibitors and docetaxel has been recently observed in a phase 1b trial in TNBC, whereby docetaxel and GSI (PF-03084014) showed 4 partial responses and 9 had stable disease out of 25 patients, with a manageable safety profile (by dose reduction) (239). All in all, the combination of docetaxel and Notch1 targeting showed synergy, with a manageable toxicity profile (238, 239).

In TNBC cells treated with the microtubule stabilizing agent paclitaxel, surviving breast cancers cells expressed Notch1, Sox2, Oct3/4, c-Myc, c-SRC, c-MET, Nanog, and E-cadherin, and were highly tumorigenic. Surviving cells also became resistant to the BCR-Abl/Src family kinase inhibitor dasatinib (240). In parental MDA-231 cells, dasatinib reduced NICD1 and Cyclin-D1 levels, but in paclitaxel resistant clones NICD1 levels were not affected. Dasatinib resistant MDA-231 clones were not cross-resistant to doxorubicin or docetaxel. Targeting Notch1 signaling in TNBC (using GSI) was additive to paclitaxel treatment, as Notch wildtype tumors showed no additive effect (125). These results support a protective mechanism whereby Notch1 is upregulated to protect the survival of paclitaxel-treated TNBC cells. In the TNBC UM-PE13 xenograft, blockage of DLL-4, decreasing Notch1 signaling, resulted in delayed tumor regrowth after paclitaxel treatment, with additionally decreasing the CSC frequency (241). Paclitaxel is capable of preventing breast cancer bone metastases. However, resistance emerges over time through induction of osteoblast JAG1 expression. Hence, metastatic seeding could be prevented using a JAG1 antibody (15D11). Synergistic effects (100x, compared to IgG) were observed when used in combination with paclitaxel (242).

All together, these data indicate that Notch inhibition may sensitize breast cancer to chemotherapeutics and that this involves a treatment-resistant BCSC population characterized by $CD44^{+}/CD24^{-/low}$ cells. Further, chemotherapy resistant cell lines may be resensitized after treatment with Notch inhibitors.

Endocrine Therapy

In ER^{+} breast cancers, estrogen receptor signaling plays a pivotal role in tumor development and progression (243). Treatments that target the ER include blocking of receptor

with an antagonist (e.g., selective estrogen receptor modulators such as tamoxifen or selective estrogen receptor disruptors such as fulvestrant) or depriving the tumor of estrogen (aromatase inhibitors). This mainly targets the tumor bulk, however, important implications have been made for hormone receptor-positive stem cells (244, 245). Despite similar expression of hormone receptors, some tumors are more sensitive to endocrine therapy than others, resulting in inter- and intra-patient differences. Additionally, differences in clinical outcome are observed based on breast cancer subtype (Table 1). Notably, expression of ER/PR is not universal in both tumor and metastases (246), and this does affect tumor prognosis (247). Receptor conversion and intra-tumor heterogeneity of ER expression in primary and metastatic tumors are therefore still a barrier to effective endocrine therapy. Point mutations in the ESR1 gene, encoding ERα, have been shown to arise during endocrine therapy and lead to endocrine resistance (248, 249).

Notch, Estrogen Receptor Interactions, and Therapy Sensitivity/ Resistance

It has been suggested that in endocrine-resistant tumors, the ER is not the main survival pathway of breast cancer cells. Additionally, ER-targeting treatment resistance mechanisms are already in place (250, 251), and these resistance mechanisms show potential activating crosstalk with Notch (57). Endocrine resistant breast cancers show increased BCSCs numbers (9, 252) with Notch3/4 expression (94, 252, 253). Interestingly, in BCSCs paracrine EGFR and Notch signaling (under the influence of estrogen), is capable of activating estrogen signaling in ER^- BCSCs (253).

Estradiol inhibits the activation of Notch1/4, causing membrane accumulation of uncleaved receptors (254), and upon estrogen deprivation or anti-estrogen drugs increased Notch signaling was observed (254). Luminal breast cancers with Notch1 remain hormone responsive (9). Hence, decreasing Notch signaling using GSI in cell lines and xenografts resulted in G_2 growth arrest (254). Additionally, estrogen deprivation of luminal ER^+ cells (MCF-7) inhibits tumor growth. Conversely, in the engineered $HER2^+$ MCF-7 cell line, tamoxifen stimulated growth, even in combination with estrogen deprivation. This was accompanied by molecular crosstalk between ER and HER2 (255). Furthermore, involvement of the Akt and MAPK pathways were observed, with possible roles for Notch in this resistance (59, 256, 257). These experiments indicate that HER2 expression plays an important role in endocrine therapy resistance mechanism; however luminal cells are still dependent on estrogen receptor activation.

Interestingly, when grown orthotopically, original $ER^+/PR^+/CK5^-$ tumors showed an increased population of $ER^-/PR^-/CK5^+$ "luminobasal cells," this population further increased when estrogen was withdrawn, revealing receptor conversion when exposed to a new environmental niche (9). However, others have stated that this $ER^-/PR^-/CK5^+$ population doesn't increase over time, is under the influence of progesterone signaling, and is capable of surviving extensive ER-targeting (258). Many Notch1 pathway genes were

included in this new so-called luminobasal gene signature—involving TWIST1 and SLUG upregulation. These luminobasal cells resemble a more TNBC basal-like phenotype ($CK5^+$) while retaining their luminal origin, expand (at higher rates) within luminal tumors when deprived of estrogen signaling due to their independence of the estrogen receptor, and showed sensitivity to Notch1 silencing. These data suggest an important link between ovarian endocrine sensitivity (both progesterone and estrogen) and Notch1, and support a luminal origin of basal-like cells (9, 258).

Elevated Notch1/3 signaling upregulates IL6 and activates the JAK/STAT pathway, however, dependent on p53/IKKa/IKKb status, and through a non-canonical mechanism. Furthermore, Notch signaling upregulation resulted in different Notch target genes in different molecular subtypes of breast cancer (basal vs. luminal B) (259). This growth promoting effect can also be instigated by fibroblasts secreting IL6, in relation with Notch3 and JAG1 (181). Pioneering research by Sansone *et al*. showed that Notch3-IL6 signaling is under indirect control of hypoxia and that it promotes self-renewal and survival in mammary gland stem cells (260, 261). $CD133^{high}$ cells express low levels of ER, but high levels of Notch3 (252), are endocrine resistant and promote metastases. This process is regulated through IL6-Notch3 signaling (261). IL6 expression could be induced either by Tamoxifen or HER2. $CD133^{high}$ expressing cells could be resensitized to endocrine therapy through IL6R blockade, which reduced Notch3, STAT3, and CD133. Knockdown of STAT3 resulted in reduced Notch3 mRNA levels and re-expression of ERα, without changes in CD133 expression. Notch3 thus, indirectly, plays an important role in endocrine resistance observed in metastatic breast cancer by influencing stem cell behavior (260, 261).

As described earlier fibroblast-derived microvesicles containing oncomiR-221 promoted *de novo* endocrine resistance—as overexpression of oncomiR-221/222 in luminal breast cancer cells reduces ER expression (182). These microvesicles were capable of blocking endocrine therapy Notch3 down regulation and causing an estrogen-independent phenotype in breast cancer cells (96, 262). This was also observed in endocrine resistant luminal breast cancers whereby blockage of Notch3 abrogated the growth of these ER-resistant cells (262).

Moreover, Notch4 is a crucial mediator of endocrine therapy resistance in models of luminal breast cancers (95, 261–263). BCSC induced by endocrine treatment are characterized by upregulation of Notch target genes [and additionally induces an EMT phenotype (263)], and endocrine resistance in BCSC is driven through JAG1/Notch4 signaling (95). This could be inhibited through targeting of Notch4 using GSI RO4929097. Notch4 inhibition reduced HES1 and HEY1 expression, reversed EMT, decreased CSC populations, thereby attenuating proliferation and invasion. Notch4 thus promotes estrogen-independent, endocrine therapy resistant growth of breast cancer cell lines (95, 263) possibly through a Notch4/STAT3/EMT regulated axis (264). Very recent evidence shows that mutations in the ligand binding domain of ERα, which occur in patients and

are associated with endocrine therapy resistance, promote a stem-cell-like phenotype through activation of Notch4 (265).

Targeted Therapy (HER2)

HER2, a family member of the ERBB transmembrane receptor tyrosine kinases (ERBB1-4 or also known as EGFR and HER2-4) is a well-known target in HER2-amplified breast cancer therapy for both primary tumors (266, 267) and metastases (268–270). However, it is still unclear whether $HER2^+$ cells are truly addicted to oncogenic HER2 signaling as other EGFR members can compensate after HER2 blockade (266). Moreover, a single copy of HER2 (in the absence of genomic amplification) can elicit an expression signature associated with HER2 dependence. Thus, HER2-non-amplified tumors may in some cases benefit from HER2 targeted therapy. Yet, such tumors are currently not being selected for treatment (267, 271). $HER2^+$ breast cancer is mainly treated with combinations including taxane-based chemotherapy plus trastuzumab (272), pertuzumab (273), the tyrosine kinase inhibitor lapatinib (274, 275), or combinations thereof (266, 276). Many trials have shown remarkable response rates (277–281), and therefore HER2-targeted therapy is standard of care. However, intrinsic and acquired resistance may still result in relapse and progression of $HER2^+$ disease. This resistance can occur on many levels, including activation of the downstream signaling pathways, constitutively activated HER2, and crosstalk of HER2 with other growth factor receptors such as other EGFR-members and IGF (282–285).

HER2 is a direct Notch target gene and bidirectional crosstalk between Notch and HER2 has been extensively reviewed (286). Under trastuzumab treatment, Notch activation occurs and contributes to trastuzumab resistance (284, 287). Trastuzumab-resistant cells (treated with trastuzumab for 6 months) expressed higher levels of Notch pathway genes, and this could be reversed by Notch inhibition (siRNA). GSIs decreased proliferation (288). In HER2+ xenograft experiments, GSI MK0752 alone did not affect tumor volume, while trastuzumab alone caused complete regression of tumors. However, trastuzumab-treated tumors recurred in approximately 50% of the cases. When trastuzumab was combined with GSI MK0752, complete cures were obtained with no observed recurrences. This suggests that the combination trastuzumab/GSI targeted stem-like cells responsible for recurrent disease. Notch inhibition resulted in HER2 down regulation (under the influence of Notch/RBP-jk binding sites in HER2 promotor sequences), followed by decreased mammosphere formation (286, 289).

Furthermore, Notch signaling is upregulated after treatment with lapatinib, a clinically active small molecule EGFR/HER2 inhibitor. Blockade of HER2 signaling in HER2-dependent primary tumor cells led to upregulation of Notch signaling [NICD1, HEY1, and HEY2 (266)]. The feedback signaling between these pathways was confirmed by the ability of HER2 to represses Notch signaling through HES1 and NRARP. In a HER2-inducible mouse model,

Notch1 gain-of-function constructs identified Notch dependency in tumors recurring after suppression of HER2 expression in an HER2 inducible mouse model. After HER2 removal, the rate of recurrence was much higher in primary tumors that overexpressed NICD1, and this could be blocked using GSIs (118). The GSI sensitivity of these tumors suggests that other wild-type Notch paralogs (e.g., Notch3) induced by NICD1, may play a role. Moreover, a meta-analysis (17 studies, including 4,463 patients) revealed increased Notch activity in a subset of breast cancers associated with poor clinical outcomes (including basal-like tumors). These data suggest that Notch is positively associated with tumor recurrence in breast cancer patients and implicate that Notch targeting might prevent recurrent disease by targeting the dormant residual tumor cells.

Interestingly, HER2 expression can be heterogeneous both in bulk tumor cells (290) and BCSCs (291), and shows plasticity (291). $ER^+/HER2^-$ and TNBC acquire a $HER2^+$ subpopulation following therapy exposure (267, 291). Cultured BCSCs from $ER^+/HER2^-$ patients retained $HER2^{+/-}$ subpopulations and switching between these HER2 states is dependent on environmental stimuli (291). Notch was inversely correlated with HER2 expression and $HER2^-$ cells were sensitive to Notch inhibition. $HER2^+$ cells showed higher proliferation but were not addicted to HER2 oncogenic signaling. Following these sub-profiles, a proliferative state/niche favored the $HER2^+$ phenotype, whereas oxidative stress or chemotherapy selected for, or initiated transition to, $HER2^-$ BCSCs. Thus, Notch might mediate a protective mechanism by functioning in the switch between proliferative and survival-prone phenotypes of $HER2^{+/-}$ BCSCs.

Besides a direct link between Notch and HER2, Notch also interacts with downstream or parallel HER2 signaling pathways. Co-suppressing the activation of these pathways upon resistance (267) might bypass these resistance mechanisms. Alternative mechanisms to activate signaling pathways such as PI3K/AKT and/or MAPK can be triggered in response to trastuzumab or through constitutive activation of HER2. These pathways may mediate treatment resistance in selected clones. The communication between Notch and PI3k/AKT has been shown extensively in hematological cancers (59, 292) and to lesser extent in breast cancer (293). Bidirectional MAPK-Notch interactions have been described (256, 257). Additionally, when the HER2 receptor is inhibited, signaling might still occur due to dimerization with IGF1-R (285) and Notch interaction (294), possibly resulting in therapy resistance.

Discussion, Conclusion, and Future Perspectives

There is overwhelming evidence for a role of the Notch signaling pathway in breast cancer development and progression through upregulation of Notch receptors, ligands, and regulators. Overall, high Notch pathway activity is associated with more aggressive disease and poor outcomes. Only a limited number of breast cancers harbor Notch gain of

function mutations, but in many breast cancers Notch is expressed, active, and crosstalks with other oncogenic pathways. Further, many studies support an important role for Notch in the response to radiation, chemotherapy, hormonal therapy, and targeted therapies. Importantly, there is compelling evidence that treatment-resistant breast cancer and other malignancies can be resensitized by Notch inhibition (77, 78, 295–297). Taken together, this provides a strong rationale for studies combining Notch inhibitors with current breast cancer treatment modalities.

However, an important and complicating feature of Notch signaling is its receptor-ligand specific and context dependent signaling in different cancer subtypes. Furthermore, the optimal timing to initiate treatment to achieve therapeutic efficacy must be carefully considered. In treatment-naïve tumors, Notch activation might not become clinically evident until treatment initiation, as a resistance mechanism triggered by treatment, or after occurrence of metastases with different mutational profiles compared to primary tumors (4). Selecting patients most likely to benefit from Notch inhibition will require molecular profiling and screening to show possible co-targeting options (298). The identification of predictive biomarkers is of paramount importance.

In this review, we have highlighted several opportunities for Notch targeting in the context of first line breast cancer treatment and resistance. Additionally, we have discussed its extensive communication with many other pathways (59, 256, 257, 292, 293), its role in recurrent disease and involvement in the metastatic process (103, 134, 136, 145), and its association with clinically relevant hallmarks in breast cancer (69).

Research in the past decade has focused on preventing or treating tumor recurrence by targeting CSCs. Multiple different stem-like cell populations have been proposed within tumors, based on the expression of $CD44^{high}/CD24^{-}$, $ALDH^{+}$, CD133, $CD29^{high}/CD61^{+}$, $CD49f^{+}$, and CD90 (299–302). These cells showed increased levels of therapy resistance and distinctive gene expression patterns, irrespective of their potential origin (e.g., from transformation of mammary stem cells or from de-differentiation of non-stem-like tumor cells)—as stem cell plasticity occurs within tumors (32, 303). Notch signaling plays an important role in mammary stem cells as well as breast cancer stem cells (BCSCs) (84, 92, 304)—well documented for triple-negative breast cancer (94, 109, 153, 244, 253, 304–307). Furthermore, Notch4 has been shown to maintain the BCSC population (94, 307). Notch-PTEN signaling is important in the expansion of these stem-like cells (98, 308). PTEN/PIK3CA mutations are often observed in breast cancer and loss of PTEN decreases radiation sensitivity (309). In the future, combining radiotherapy and small molecule targeting in BCSC may improve the efficacy of radiation therapy and forestall radiation resistance. However, the timing and sequencing of treatments should be carefully optimized in order to achieve maximum efficacy. Radiotherapy dose scheduling might be easily adapted from the current schedule standards (310–312).

The effects of chemo-, radio- and targeted therapy on Notch signaling require further investigation. Observations have been made for Notch and tumor vascularization under

the influence of both anthracycline and taxane-based chemotherapy (313). Taxane (paclitaxel) therapy resistance coincides with the development of metastatic bone lesions, preventable by targeting JAG1 in osteoblasts (242).

BCSCs in ER+ tumors show responsiveness to hormone signaling/targeting despite often lacking ER and PR (244, 245). This may be mediated by paracrine crosstalk with ER^{+}/PR^{+} bulk tumor cells. Many endocrine therapy resistance mechanisms have been revealed (314–317). This has guided research toward the development of new therapeutic regimens (318), such as CDK4/6 inhibitors (319)—which have been clinically implemented. Notch inhibition could play a significant role in combinations targeting these resistance mechanisms. For instance, Notch inhibition could reverse ER-targeted-treatment resistance and improve the efficacy of CDK4/6 inhibitors through decreasing Cyclin-D1 (121).

Notch has been shown to crosstalk with the HER2 receptor (289) and development of breast cancer metastases is affected by HER2 (268–270, 320) and progesterone (268). Interestingly, plasticity of HER2 expression has been observed in circulating tumor cells—with a distinctive role for Notch1 (291). Thus, Notch is involved in the heterogeneity and plasticity observed in $HER2^{-/+}$ breast cancer, and the development of distant metastases. Combining CDK4/6 inhibitors (321, 322) and Notch inhibitors, it may be possible to simultaneously attenuate two main drivers in breast cancer, HER2 and Cyclin-D, promoting local control and preventing distant relapse.

A step forward, for individualized patient care, could be the use of patient-representative culture models, such as organoids, to capture information on individual tumor drug sensitivity *ex-vivo* (323). In general, organoids can provide rapid insight into individual treatment combinations and relationships between Notch signaling and breast cancer treatment (resistance), before the start of treatment. These models more closely represent individual tumors, and may enable us to rationally investigate the context-dependence of Notch signaling in each tumor. Breast cancer organoids have recently been developed, but to what extent they will be strong predictors of treatment response and their use as prospective platforms for individualized precision treatment remains to be established (324).

This review summarizes the evidence supporting the hypothesis that targeting Notch could a promising option in re-sensitizing breast cancer to current standard of care treatments (Figure 3). When biomarker quantification and patient stratification allow Notch targeting to live up to its potential, this strategy may be applicable to other cancers as well, targeted with concurrent chemo-radiation or targeted inactivation of other growth promoting pathways. However, clinical evidence in solid tumors showed that therapy timing is highly important to reach maximum effectivity (325). Thus, additional clinical and translational research will be required to determine the exact role of Notch in each disease- and treatment-specific context and fine-tune the use of Notch targeting agents to prevent or treat or acquired resistance. With the benefit of sufficient mechanistic

knowledge, we propose that in some cancer patients targeting Notch can be a major part of an effective strategy to address therapy resistance.

Author Contributions

EM, JI, MS, and MV made substantial contributions to conception and design of the review, and analysis and interpretation of articles. All authors have been involved in drafting the manuscript or revising it critically for important intellectual content. All authors have given final approval of the version to be published. EM, JI, MS, and MV have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

(B)CSC, Breast Cancer Stem Cell; ANK, Ankyrin Repeats; CAF, Cancer Associated Fibroblast; DCIS, Ductal Carcinoma *in situ*; EGF, Epidermal Growth Factor; EMT, Epithelial-Mesenchymal Transition; ER, Estrogen Receptor; GSI, Y-Secretase Inhibitor; HER2, Human Epidermal Growth Factor Receptor 2; JAG, Jagged; LF, Lunatic Fringe; LNR, Lin12-Notch Repeats; LRR, Loco-Regional Recurrence; MAML, Mastermind-Like; MaSCs, Mammary Stem Cells; MDSC, Myeloid-derived suppressor cell; MET, Mesenchymal-Epithelial Transition; MF, Manic Fringe; MMTV, Mouse Mammary Tumor Virus; MRP1, Multi Drug Resistance Protein 1; NICD, Notch Intracellular Domain; NLS, Nuclear Localization Sequences; NRR, Negative Regulatory Region; pCR, Pathological Complete Remission; PR, Progesteron Receptor; RAM, RBP-Jk Association Module; RF, Radical Fringe; TAM, Tumor associate macrophage; TCGA, The Cancer Genome Atlas; TN(BC), Triple Negative (Breast Cancer).

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, *et al*. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* (2015) 136:E359–86. doi: 10.1002/ijc.29210
2. Torre LA, Siegel RL, Ward EM, Jemal A. Global Cancer Incidence and Mortality Rates and Trends–An Update. *Cancer Epidemiol Biomarkers Prev*. (2016) 25:16–27. doi: 10.1158/1055-9965.EPI-15-0578
3. Rastogi P, Anderson SJ, Bear HD, Geyer CE, Kahlenberg MS, Robidoux A, *et al*. Preoperative chemotherapy: updates of national surgical adjuvant breast and bowel project Protocols B-18 and B-27. *J Clin Oncol*. (2008) 26:778–85. doi: 10.1200/JCO.2007.15.0235
4. Yates LR, Knappskog S, Wedge D, Farmery JHR, Gonzalez S, Martincorena I, *et al*. Genomic evolution of breast cancer metastasis and relapse. *Cancer Cell.* (2017) 32:169–84 e7. doi: 10.1016/j.ccell.2017.07.005
5. Yuan X, Zhang M, Wu H, Xu H, Han N, Chu Q, *et al*. Expression of Notch1 correlates with breast cancer progression and prognosis. *PLoS ONE* (2015) 10:e0131689. doi: 10.1371/journal.pone.0131689
6. Speiser J, Foreman K, Drinka E, Godellas C, Perez C, Salhadar A, *et al*. Notch-1 and Notch-4 biomarker expression in triple-negative breast cancer. *Int J Surg Pathol*. (2011) 20:137–43. doi: 10.1177/1066896911427035
7. Dou X-W, Liang Y-K, Lin H-Y, Wei X-L, Zhang Y-Q, Bai J-W, *et al*. Notch3 Maintains luminal phenotype and suppresses tumorigenesis and metastasis of breast cancer via trans-activating estrogen receptor-α. *Theranostics* (2017) 7:4041–56. doi: 10.7150/thno.19989
8. Laakso M, Tanner M, Nilsson J, Wiklund T, Erikstein B, Kellokumpu-Lehtinen P, *et al*. Basoluminal carcinoma: a new biologically and prognostically distinct entity between basal and luminal breast cancer. *Clin Cancer Res.* (2006) 12(14 Pt 1):4185–91. doi: 10.1158/1078-0432.CCR-06-0353
9. Haughian JM, Pinto MP, Harrell JC, Bliesner BS, Joensuu KM, Dye WW, *et al*. Maintenance of hormone responsiveness in luminal breast cancers by suppression of Notch. *Proc Natl Acad Sci USA*. (2012) 109:2742–7. doi: 10.1073/pnas.1106509108
10. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, *et al*. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*. (2001) 98:10869–74. doi: 10.1073/pnas.191367098
11. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, *et al*. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA*. (2003) 100:8418–23. doi: 10.1073/pnas.0932692100
12. Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, *et al*. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin Cancer Res.* (2008) 14:1368–76. doi: 10.1158/1078-0432.CCR-07-1658
13. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, *et al*. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res*. (2010) 12:R68. doi: 10.1186/bcr2635

14. Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, Kennecke H. Breast cancer subtypes and the risk of local and regional relapse. *J Clin Oncol.* (2010) 28:1684–91. doi: 10.1200/JCO.2009.24.9284
15. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. *Nature* (2012) 490:61–70. doi: 10.1038/nature11412
16. Prat A, Pineda E, Adamo B, Galvan P, Fernandez A, Gaba L, *et al*. Clinical implications of the intrinsic molecular subtypes of breast cancer. *Breast* (2015) 24(Suppl. 26):2568–81. doi: 10.1016/j.breast.2015.07.008
17. Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. *J Clin Oncol.* (2008) 26:2568–81. doi: 10.1200/JCO.2007.13.1748
18. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, *et al*. Molecular portraits of human breast tumours. *Nature* (2000) 406:747–52. doi: 10.1038/35021093
19. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, *et al*. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. (2011) 121:2750–67. doi: 10.1172/JCI45014
20. Masuda H, Baggerly KA, Wang Y, Zhang Y, Gonzalez-Angulo AM, Meric-Bernstam F, *et al*. Differential response to neoadjuvant chemotherapy among 7 triple-negative breast cancer molecular subtypes. *Clin Cancer Res*. (2013) 19:5533–40. doi: 10.1158/1078-0432.CCR-13-0799
21. Touplikioti P. Expression of Notch receptors in primary breast cancer and correlation with pathological features. *Clin Exp Pharmacol.* (2012) 2:1–8. doi: 10.4172/2161-1459.1000109
22. Kennecke H, Yerushalmi R, Woods R, Cheang MC, Voduc D, Speers CH, *et al*. Metastatic behavior of breast cancer subtypes. *J Clin Oncol.* (2010) 28:3271–7. doi: 10.1200/JCO.2009.25.9820
23. Cardoso F, van't Veer LJ, Bogaerts J, Slaets L, Viale G, Delaloge S, *et al*. 70-Gene signature as an aid to treatment decisions in early-stage breast cancer. *N Engl J Med*. (2016) 375:717–29. doi: 10.1056/NEJMoa1602253
24. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF, *et al*. Adjuvant Chemotherapy Guided by a 21-Gene Expression Assay in Breast Cancer. *N Engl J Med.* (2018) 379:111–21. doi: 10.1056/NEJMoa1804710
25. Le Du F, Eckhardt BL, Lim B, Litton JK, Moulder S, Meric-Bernstam F, *et al*. Is the future of personalized therapy in triple-negative breast cancer based on molecular subtype? *Oncotarget* (2015) 6:12890–908. doi: 10.18632/oncotarget.3849
26. Filho JS, Pusztai L. Gene expression profiling in breast cancer: classification, prognostication, and prediction. *Lancet* (2011) 378:1812–23. doi: 10.1016/S0140-6736(11)61539-0
27. Curigliano G, Burstein HJ, Winer EP, Gnant M, Dubsky P, Loibl S, *et al*. De-escalating and escalating treatments for early-stage breast cancer: the St. Gallen International Expert Consensus Conference on the Primary Therapy of Early Breast Cancer 2017. *Ann Oncol.* (2018) 28:1700–12. doi: 10.1093/annonc/mdx806
28. Tu SM, Lin SH, Logothetis CJ. Stem-cell origin of metastasis and heterogeneity in solid tumours. *Lancet Oncol*. (2002) 3:508–13. doi: 10.1016/S1470-2045(02)00820-3
29. Zardavas D, Irrthum A, Swanton C, Piccart M. Clinical management of breast cancer heterogeneity. *Nat Rev Clin Oncol.* (2015) 12:381–94. doi: 10.1038/nrclinonc.2015.73

30. Ercan C, van Diest PJ, Vooijs M. Mammary development and breast cancer: the role of stem cells. *Curr Mol Med*. (2011) 11:270–85. doi: 10.2174/156652411795678007
31. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA*. (2003) 100:3983–8. doi: 10.1073/pnas.0530291100
32. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* (2010) 29:4741–51. doi: 10.1038/onc.2010.215
33. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* (2013) 501:346. doi: 10.1038/nature12626
34. Pannuti A, Foreman K, Rizzo P, Osipo C, Golde T, Osborne B, *et al*. Targeting Notch to target cancer stem cells. *Clin Cancer Res*. (2010) 16:3141–52. doi: 10.1158/1078-0432.CCR-09-2823
35. Takeuchi H, Haltiwanger RS. Significance of glycosylation in Notch signaling. *Biochem Biophys Res Commun*. (2014) 453:235–42. doi: 10.1016/j.bbrc.2014.05.115
36. Moloney DJ, Panin VM, Johnston SH, Chen J, Shao L, Wilson R, *et al*. Fringe is a glycosyltransferase that modifies Notch. *Nature* (2000) 406:369–75. doi: 10.1038/35019000
37. Mumm JS, Schroeter EH, Saxena MT, Griesemer A, Tian X, Pan DJ, *et al*. A Ligand-Induced Extracellular Cleavage Regulates γ-Secretase-like Proteolytic Activation of Notch1. *Mol Cell* (2000) 5:197–206. doi: 10.1016/S1097-2765(00)80416-5
38. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* (2009) 137:216–33. doi: 10.1016/j.cell.2009.03.045
39. Wang K, Zhang Q, Li D, Ching K, Zhang C, Zheng X, *et al*. PEST domain mutations in Notch receptors comprise an oncogenic driver segment in triple-negative breast cancer sensitive to a gamma-secretase inhibitor. *Clin Cancer Res*. (2015) 21:1487–96. doi: 10.1158/1078-0432.CCR-14-1348
40. O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C, *et al*. FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. The Journal of experimental medicine. (2007) 204:1813–24. doi: 10.1084/jem.20070876
41. Sczaniecka M, Gladstone K, Pettersson S, McLaren L, Huart AS, Wallace M. MDM2 protein-mediated ubiquitination of NUMB protein: identification of a second physiological substrate of MDM2 that employs a dual-site docking mechanism. *J. Biol. Chem*. (2012) 287:14052–68. doi: 10.1074/jbc.M111.303875
42. Pettersson S, Sczaniecka M, McLaren L, Russell F, Gladstone K, Hupp T, *et al*. Non-degradative ubiquitination of the Notch1 receptor by the E3 ligase MDM2 activates the Notch signalling pathway. *Biochem J*. (2013) 450:523–36. doi: 10.1042/BJ20121249
43. Licciardello MP, Müllner MK, Dürnberger G, Kerzendorfer C, Boidol B, Trefzer C, *et al*. NOTCH1 activation in breast cancer confers sensitivity to inhibition of SUMOylation. *Oncogene* (2015) 34:3780–90. doi: 10.1038/onc.2014.319
44. Oladghaffari M, Islamian JP, Baradaran B, Monfared AS, Farajollahi A, Shanehbandi D, *et al*. High Efficiency Apoptosis Induction in Breast Cancer Cell Lines by MLN4924/2DG Co-Treatment. *Asian Pac J Cancer Prev*. (2015) 16:5471–6. doi: 10.7314/APJCP.2015.16.13.5471

45. Yang D, Tan M, Wang G, Sun Y. The p21-dependent radiosensitization of human breast cancer cells by MLN4924, an investigational inhibitor of NEDD8 activating enzyme. *PLoS ONE* (2012) 7:e34079-e. doi: 10.1371/journal.pone.0034079
46. Thaler S, Thiede G, Hengstler JG, Schad A, Schmidt M, Sleeman JP. The proteasome inhibitor Bortezomib (Velcade) as potential inhibitor of estrogen receptor-positive breast cancer. *Int J Cancer*. (2015) 137:686–97. doi: 10.1002/ijc.29404
47. Engel RH, Brown JA, Von Roenn JH, O'Regan RM, Bergan R, Badve S, *et al*. A phase II study of single agent bortezomib in patients with metastatic breast cancer: a single institution experience. *Cancer Invest*. (2007) 25:733–7. doi: 10.1080/07357900701506573
48. Schmid P, Kühnhardt D, Kiewe P, Lehenbauer-Dehm S, Schippinger W, Greil R, *et al*. A phase I/II study of bortezomib and capecitabine in patients with metastatic breast cancer previously treated with taxanes and/or anthracyclines. *Ann Oncol*.. (2008) 19:871–6. doi: 10.1093/annonc/mdm569
49. Trinh XB, Sas L, Van Laere SJ, Prové A, Deleu I, Rasschaert M, *et al*. A phase II study of the combination of endocrine treatment and bortezomib in patients with endocrine-resistant metastatic breast cancer. *Oncol Reports* (2012) 27:657–63. doi: 10.3892/or.2011.1562
50. Brou C. Intracellular trafficking of Notch receptors and ligands. *Exper Cell Res.* (2009) 315:1549–55. doi: 10.1016/j.yexcr.2008.09.010
51. Welcker M, Clurman BE. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nature Rev Cancer.* (2008) 8:83–93. doi: 10.1038/nrc2290
52. Chastagner P, Israël A, Brou C. AIP4/Itch regulates notch receptor degradation in the absence of ligand. *PLoS ONE* (2008) 3:e2735. doi: 10.1371/journal.pone.0002735
53. Kim MY, Mo JS, Ann EJ, Yoon JH, Park HS. Dual regulation of Notch1 signaling pathway by adaptor protein Fe65. *J Biol Chem*. (2012) 287:4690–701. doi: 10.1074/jbc.M111.289637
54. Pece S, Serresi M, Santolini E, Capra M, Hulleman E, Galimberti V, *et al*. Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. *J Cell Biol.* (2004) 167:215–21. doi: 10.1083/jcb.200406140
55. Takasugi N, Tomita T, Hayashi I, Tsuruoka M, Niimura M, Takahashi Y, *et al*. The role of presenilin cofactors in the γ-secretase complex. *Nature* (2003) 422:438. doi: 10.1038/nature01506
56. Groot AJ, Habets R, Yahyanejad S, Hodin CM, Reiss K, Saftig P, *et al*. Regulated Proteolysis of NOTCH2 and NOTCH3 Receptors by ADAM10 and Presenilins. *Mol Cell Biol*. (2014) 34:2822. doi: 10.1128/MCB.00206-14
57. Hao L, Rizzo P, Osipo C, Pannuti A, Wyatt D, Cheung LW, *et al*. Notch-1 activates estrogen receptor-alpha-dependent transcription via IKKalpha in breast cancer cells. *Oncogene* (2010) 29:201–13. doi: 10.1038/onc.2009.323
58. Li L, Zhao F, Lu J, Li T, Yang H, Wu C, *et al*. Notch-1 Signaling Promotes the Malignant Features of Human Breast Cancer through NF-κB Activation. *PLoS ONE* (2014) 9:e95912. doi: 10.1371/journal.pone.009591
59. Hales EC, Taub JW, Matherly LH. New insights into Notch1 regulation of the PI3K-AKT-mTOR1 signaling axis: targeted therapy of gamma-secretase inhibitor resistant T-cell acute lymphoblastic leukemia. *Cell Signal*. (2014) 26:149–61. doi: 10.1016/j.cellsig.2013.09.021

60. Landor SKJ, Mutvei AP, Mamaeva V, Jin S, Busk M, Borra R, *et al*. Hypo- and hyperactivated Notch signaling induce a glycolytic switch through distinct mechanisms. *Proc Natl Acad Sci USA*. (2011) 108:18814. doi: 10.1073/pnas.1104943108
61. Perumalsamy LR, Nagala M, Sarin A. Notch-activated signaling cascade interacts with mitochondrial remodeling proteins to regulate cell survival. *Proc Natl. Acad Sci USA*. (2010) 107:6882. doi: 10.1073/pnas.0910060107
62. Mazzone M, Selfors LM, Albeck J, Overholtzer M, Sale S, Carroll DL, *et al*. Dose-dependent induction of distinct phenotypic responses to Notch pathway activation in mammary epithelial cells. *Proc Natl. Acad Sci USA*. (2010) 107:5012–7. doi: 10.1073/pnas.1000896107
63. Flores AN, McDermott N, Meunier A, Marignol L. NUMB inhibition of NOTCH signalling as a therapeutic target in prostate cancer. *Nat Rev Urol*. (2014) 11:499–507. doi: 10.1038/nrurol.2014.195
64. Espinosa L, Inglés-Esteve J, Aguilera C, Bigas A. Phosphorylation by Glycogen Synthase Kinase-3β Down-regulates Notch Activity, a Link for Notch and Wnt Pathways. *J Biol Chem*. (2003) 278:32227–35. doi: 10.1074/jbc.M304001200
65. Jin YH, Kim H, Oh M, Ki H, Kim K. Regulation of Notch1/NICD and Hes1 expressions by GSK-3α/β. *Mol Cells* (2009) 27:15–9. doi: 10.1007/s10059-009-0001-7
66. Hein K, Mittler G, Cizelsky W, Kühl M, Ferrante F, Liefke R, *et al*. Site-specific methylation of Notch1 controls the amplitude and duration of the Notch1 response. *Sci Signal*. (2015) 8:ra30. doi: 10.1126/scisignal.2005892
67. Fryer CJ, White JB, Jones KA. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol Cell*. (2004) 16:509–20. doi: 10.1016/j.molcel.2004.10.014
68. Ranganathan P, Weaver KL, Capobianco AJ. Notch signalling in solid tumours: a little bit of everything but not all the time. *Nat Rev Cancer*. (2011) 11:338–51. doi: 10.1038/nrc3035
69. Aster JC, Pear WS, Blacklow SC. The Varied Roles of Notch in Cancer. *Annu Rev Pathol*. (2017) 12:245–75. doi: 10.1146/annurev-pathol-052016-100127
70. Guo S, Liu M, Gonzalez-Perez RR. Role of Notch and its oncogenic signaling crosstalk in breast cancer. *Biochim Biophys Acta* (2011) 1815:197–213. doi: 10.1016/j.bbcan.2010.12.002
71. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, *et al*. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. *Nat Rev Clin Oncol*. (2015) 12:445–64. doi: 10.1038/nrclinonc.2015.61
72. Andersson ER, Lendahl U. Therapeutic modulation of Notch signalling–are we there yet? *Nat Rev Drug Discov*. (2014) 13:357–78. doi: 10.1038/nrd4252
73. Ran Y, Hossain F, Pannuti A, Lessard CB, Ladd GZ, Jung JI, *et al*. γ-Secretase inhibitors in cancer clinical trials are pharmacologically and functionally distinct. *EMBO Mol Med*. (2017) 9:950. doi: 10.15252/emmm.201607265
74. Espinoza I, Pochampally R, Xing F, Watabe K, Miele L. Notch signaling: targeting cancer stem cells and epithelial-to-mesenchymal transition. *Onco Targets Ther*. (2013) 6:1249–59. doi: 10.2147/OTT.S36162
75. Espinoza I, Miele L. Deadly crosstalk: Notch signaling at the intersection of EMT and cancer stem cells. *Cancer Lett*. (2013) 341:41–5. doi: 10.1016/j.canlet.2013.08.027

76. Kim SJ, Lee HW, Baek JH, Cho YH, Kang HG, Jeong JS, *et al*. Activation of nuclear PTEN by inhibition of Notch signaling induces G2/M cell cycle arrest in gastric cancer. *Oncogene* (2016) 35:251–60. doi: 10.1038/onc.2015.80
77. McAuliffe SM, Morgan SL, Wyant GA, Tran LT, Muto KW, Chen YS, *et al*. Targeting Notch, a key pathway for ovarian cancer stem cells, sensitizes tumors to platinum therapy. *Proc Natl Acad Sci USA*. (2012) 109:E2939–48. doi: 10.1073/pnas.1206400109
78. Domingo-Domenech J, Vidal SJ, Rodriguez-Bravo V, Castillo-Martin M, Quinn SA, Rodriguez-Barrueco R, *et al*. Suppression of acquired docetaxel resistance in prostate cancer through depletion of notch- and hedgehog-dependent tumor-initiating cells. *Cancer Cell*. (2012) 22:373–88. doi: 10.1016/j.ccr.2012.07.016
79. Xie M, Zhang L, He CS, Xu F, Liu JL, Hu ZH, *et al*. Activation of Notch-1 enhances epithelial-mesenchymal transition in gefitinib-acquired resistant lung cancer cells. *J Cell Biochem.* (2012) 113:1501–13. doi: 10.1002/jcb.24019
80. Wang Z, Li Y, Ahmad A, Azmi AS, Banerjee S, Kong D, *et al*. Targeting Notch signaling pathway to overcome drug resistance for cancer therapy. *Biochim Biophys Acta* (2010) 1806:258–67. doi: 10.1016/j.bbcan.2010.06.001
81. Wang Z, Ahmad A, Li Y, Azmi AS, Miele L, Sarkar FH. Targeting notch to eradicate pancreatic cancer stem cells for cancer therapy. *Anticancer Res*. (2011) 31:1105–13.
82. Russo J, Russo IH. Development of the human breast. *Maturitas* (2004) 49:2–15. doi: 10.1016/j.maturitas.2004.04.011
83. Kordon EC, Smith GH. An entire functional mammary gland may comprise the progeny from a single cell. *Development* (1998) 125:1921–30.
84. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, *et al*. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev*. (2003) 17:1253–70. doi: 10.1101/gad.1061803
85. Rios AC, Fu NY, Lindeman GJ, Visvader JE. *In situ* identification of bipotent stem cells in the mammary gland. *Nature* (2014) 506:322–7. doi: 10.1038/nature12948
86. Tiede B, Kang Y. From milk to malignancy: the role of mammary stem cells in development, pregnancy and breast cancer. *Cell Res*. (2011) 21:245–57. doi: 10.1038/cr.2011.11
87. Junankar S, Baker LA, Roden DL, Nair R, Elsworth B, Gallego-Ortega D, *et al*. ID4 controls mammary stem cells and marks breast cancers with a stem cell-like phenotype. *Nat Commun*. (2015) 6:6548. doi: 10.1038/ncomms7548
88. Pamarthy S, Mao L, Katara GK, Fleetwood S, Kulshreshta A, Gilman-Sachs A, *et al*. The V-ATPase a2 isoform controls mammary gland development through Notch and TGF-beta signaling. *Cell Death Dis.* (2016) 7:e2443. doi: 10.1038/cddis.2016.347
89. Rodilla V, Dasti A, Huyghe M, Lafkas D, Laurent C, Reyal F, *et al*. Luminal progenitors restrict their lineage potential during mammary gland development. *PLoS Biol.* (2015) 13:e1002069. doi: 10.1371/journal.pbio.1002069

90. Bouras T, Pal B, Vaillant F, Harburg G, Asselin-Labat ML, Oakes SR, *et al*. Notch signaling regulates mammary stem cell function and luminal cell-fate commitment. *Cell Stem Cell* (2008) 3:429–41. doi: 10.1016/j.stem.2008.08.001
91. Lafkas D, Rodilla V, Huyghe M, Mourao L, Kiaris H, Fre S. Notch3 marks clonogenic mammary luminal progenitor cells *in vivo*. *J Cell Biol*. (2013) 203:47. doi: 10.1083/jcb.201307046
92. Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res*. (2004) 6:R605–15. doi: 10.1186/bcr920
93. Raouf A, Zhao Y, To K, Stingl J, Delaney A, Barbara M, *et al*. Transcriptome analysis of the normal human mammary cell commitment and differentiation process. *Cell Stem Cell.* (2008) 3:109–18. doi: 10.1016/j.stem.2008.05.018
94. Harrison H, Farnie G, Howell SJ, Rock RE, Stylianou S, Brennan KR, *et al*. Regulation of breast cancer stem cell activity by signaling through the Notch4 receptor. *Cancer Res*. (2010) 70:709–18. doi: 10.1158/0008-5472.CAN-09-1681
95. Simoes BM, O'Brien CS, Eyre R, Silva A, Yu L, Sarmiento-Castro A, *et al*. Anti-estrogen Resistance in Human Breast Tumors Is Driven by JAG1-NOTCH4-Dependent Cancer Stem Cell Activity. *Cell Rep.* (2015) 12:1968–77. doi: 10.1016/j.celrep.2015.08.050
96. Yun J, Pannuti A, Espinoza I, Zhu H, Hicks C, Zhu X, *et al*. Crosstalk between PKCalpha and Notch-4 in endocrine-resistant breast cancer cells. *Oncogenesis* (2013) 2:e60. doi: 10.1038/oncsis.2013.26
97. Yalcin-Ozuysal O, Fiche M, Guitierrez M, Wagner KU, Raffoul W, Brisken C. Antagonistic roles of Notch and p63 in controlling mammary epithelial cell fates. *Cell Death Differ*. (2010) 17:1600–12. doi: 10.1038/cdd.2010.37
98. Sizemore GM, Balakrishnan S, Hammer AM, Thies KA, Trimboli AJ, Wallace JA, *et al*. Stromal PTEN inhibits the expansion of mammary epithelial stem cells through Jagged-1. *Oncogene* (2017) 36:6658. doi: 10.1038/onc.2017.347
99. Stylianou S, Clarke RB, Brennan K. Aberrant activation of notch signaling in human breast cancer. *Cancer Res*. (2006) 66:1517–25. doi: 10.1158/0008-5472.CAN-05-3054
100. Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, *et al*. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res*. (2009) 69:1302–13. doi: 10.1158/0008-5472.CAN-08-2741
101. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, *et al*. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* (2007) 1:555–67. doi: 10.1016/j.stem.2007.08.014
102. Yamamoto M, Taguchi Y, Ito-Kureha T, Semba K, Yamaguchi N, Inoue J-i. NF-κB non-cell-autonomously regulates cancer stem cell populations in the basal-like breast cancer subtype. *Nature Commun.* (2013) 4:2299. doi: 10.1038/ncomms3299
103. Sethi N, Dai X, Winter CG, Kang Y. Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. *Cancer Cell* (2011) 19:192–205. doi: 10.1016/j.ccr.2010.12.022

104. Leontovich AA, Jalalirad M, Salisbury JL, Mills L, Haddox C, Schroeder M, *et al*. NOTCH3 expression is linked to breast cancer seeding and distant metastasis. *Breast Cancer Res*. (2018) 20:105. doi: 10.1186/s13058-018-1020-0
105. Nam D-H, Jeon H-M, Kim S, Kim MH, Lee Y-J, Lee MS, *et al*. Activation of Notch Signaling in a Xenograft Model of Brain Metastasis. *Clin Cancer Res.* (2008) 14:4059–66. doi: 10.1158/1078-0432.CCR-07-4039
106. McGowan PM, Simedrea C, Ribot EJ, Foster PJ, Palmieri D, Steeg PS, *et al*. Notch1 Inhibition Alters the CD44hi/CD24lo population and reduces the formation of brain metastases from breast cancer. *Mol Cancer Res.* (2011) 9:834. doi: 10.1158/1541-7786.MCR-10-0457
107. Xing F, Kobayashi A, Okuda H, Watabe M, Pai SK, Pandey PR, *et al*. Reactive astrocytes promote the metastatic growth of breast cancer stem-like cells by activating Notch signalling in brain. *EMBO Mol Med.* (2013) 5:384. doi: 10.1002/emmm.201201623
108. Oskarsson T, Acharyya S, Zhang XHF, Vanharanta S, Tavazoie SF, Morris PG, *et al*. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nature Med.* (2011) 17:867. doi: 10.1038/nm.2379
109. Farnie G, Clarke RB, Spence K, Pinnock N, Brennan K, Anderson NG, *et al*. Novel cell culture technique for primary ductal carcinoma in situ: role of Notch and epidermal growth factor receptor signaling pathways. *J Natl Cancer Inst*. (2007) 99:616–27. doi: 10.1093/jnci/djk133
110. Gallahan D, Callahan R. The mouse mammary tumor associated gene INT3 is a unique member of the NOTCH gene family (NOTCH4). *Oncogene* (1997) 14:1883–90. doi: 10.1038/sj.onc.1201035
111. Dievart A, Beaulieu N, Jolicoeur P. Involvement of Notch1 in the development of mouse mammary tumors. *Oncogene* (1999) 18:5973–81. doi: 10.1038/sj.onc.1202991
112. Raafat A, Bargo S, Anver MR, Callahan R. Mammary development and tumorigenesis in mice expressing a truncated human Notch4/Int3 intracellular domain (h-Int3sh). *Oncogene* (2004) 23:9401–7. doi: 10.1038/sj.onc.1208187
113. Kiaris H, Politi K, Grimm LM, Szabolcs M, Fisher P, Efstratiadis A, *et al*. Modulation of Notch Signaling Elicits Signature Tumors and Inhibits Hras1-Induced Oncogenesis in the Mouse Mammary Epithelium. *Am J Pathol*. (2004) 165:695–705. doi: 10.1016/S0002-9440(10)63333-0
114. Hu C, Diévart A, Lupien M, Calvo E, Tremblay G, Jolicoeur P. Overexpression of Activated Murine Notch1 and Notch3 in transgenic mice blocks mammary gland development and induces mammary tumors. *Am J Pathol.* (2006) 168:973–90. doi: 10.2353/ajpath.2006.050416
115. Reedijk M, Odorcic S, Chang L, Zhang H, Miller N, McCready DR, *et al*. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Res*. (2005) 65:8530–7. doi: 10.1158/0008-5472.CAN-05-1069
116. Reedijk M, Pinnaduwage D, Dickson BC, Mulligan AM, Zhang H, Bull SB, *et al*. JAG1 expression is associated with a basal phenotype and recurrence in lymph node-negative breast cancer. *Breast Cancer Res Treat*. (2008) 111:439–48. doi: 10.1007/s10549-007-9805-3
117. Wieland E, Rodriguez-Vita J, Liebler SS, Mogler C, Moll I, Herberich SE, *et al*. Endothelial Notch1 Activity Facilitates Metastasis. *Cancer Cell* (2017) 31:355–67. doi: 10.1016/j.ccell.2017.01.007

118. Abravanel DL, Belka GK, Pan TC, Pant DK, Collins MA, Sterner CJ, *et al*. Notch promotes recurrence of dormant tumor cells following HER2/neu-targeted therapy. *J Clin Invest*. (2015) 125:2484–96. doi: 10.1172/JCI74883
119. Dai X, Li Y, Bai Z, Tang XQ. Molecular portraits revealing the heterogeneity of breast tumor subtypes defined using immunohistochemistry markers. *Sci Rep*. (2015) 5:14499. doi: 10.1038/srep14499
120. Yi Z, Ma F, Li C, Chen R, Yuan L, Sun X, *et al*. Landscape of somatic mutations in different subtypes of advanced breast cancer with circulating tumor DNA analysis. *Sci Rep.* (2017) 7:5995. doi: 10.1038/s41598-017-06327-4
121. Cohen B, Shimizu M, Izrailit J, Ng NF, Buchman Y, Pan JG, *et al*. Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. *Breast Cancer Res Treat*. (2010) 123:113–24. doi: 10.1007/s10549-009-0621-9
122. Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H, *et al*. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* (1995) 82:621–30. doi: 10.1016/0092-8674(95)90034-9
123. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, *et al*. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res.* (1994) 54:1812–7.
124. Ling H, Sylvestre JR, Jolicoeur P. Notch1-induced mammary tumor development is cyclin D1-dependent and correlates with expansion of pre-malignant multipotent duct-limited progenitors. *Oncogene* (2010) 29:4543–54. doi: 10.1038/onc.2010.186
125. Stoeck A, Lejnine S, Truong A, Pan L, Wang H, Zang C, *et al*. Discovery of biomarkers predictive of GSI response in triple-negative breast cancer and adenoid cystic carcinoma. *Cancer Discov*. (2014) 4:1154–67. doi: 10.1158/2159-8290.CD-13-0830
126. Xie X, Kaoud TS, Edupuganti R, Zhang T, Kogawa T, Zhao Y, *et al*. c-Jun N-terminal kinase promotes stem cell phenotype in triple-negative breast cancer through upregulation of Notch1 via activation of c-Jun. *Oncogene* (2017) 36:2599–608. doi: 10.1038/onc.2016.417
127. Dickson BC, Mulligan AM, Zhang H, Lockwood G, O'Malley FP, Egan SE, *et al*. High-level JAG1 mRNA and protein predict poor outcome in breast cancer. *Mod Pathol*. (2007) 20:685–93. doi: 10.1038/modpathol.3800785
128. Leong KG, Niessen K, Kulic I, Raouf A, Eaves C, Pollet I, *et al*. Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. *J Exp Med.* (2007) 204:2935–48. doi: 10.1084/jem.20071082
129. Bolos V, Mira E, Martinez-Poveda B, Luxan G, Canamero M, Martinez AC, *et al*. Notch activation stimulates migration of breast cancer cells and promotes tumor growth. *Breast Cancer Res*. (2013) 15:R54. doi: 10.1186/bcr3447
130. Sahlgren C, Gustafsson MV, Jin S, Poellinger L, Lendahl U. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc Natl Acad Sci USA.* (2008) 105:6392–7. doi: 10.1073/pnas.0802047105
131. Chen J, Imanaka N, Chen J, Griffin JD. Hypoxia potentiates Notch signaling in breast cancer leading to decreased E-cadherin expression and increased cell migration and invasion. *Br J Cancer.* (2010) 102:351–60. doi: 10.1038/sj.bjc.6605486

132. Xing F, Okuda H, Watabe M, Kobayashi A, Pai SK, Liu W, *et al*. Hypoxia-induced Jagged2 promotes breast cancer metastasis and self-renewal of cancer stem-like cells. *Oncogene* (2011) 30:4075–86. doi: 10.1038/onc.2011.122
133. Ercan C, Vermeulen JF, Hoefnagel L, Bult P, van der Groep P, van der Wall E, *et al*. HIF-1α and NOTCH signaling in ductal and lobular carcinomas of the breast. *Cell Oncol*. (2012) 35:435–42. doi: 10.1007/s13402-012-0102-8
134. Johnson RW, Finger EC, Olcina MM, Vilalta M, Aguilera T, Miao Y, *et al*. Induction of LIFR confers a dormancy phenotype in breast cancer cells disseminated to the bone marrow. *Nat Cell Biol.* (2016) 18:1078–89. doi: 10.1038/ncb3408
135. Zhang L, Ridgway LD, Wetzel MD, Ngo J, Yin W, Kumar D, *et al*. The identification and characterization of breast cancer CTCs competent for brain metastasis. *Sci Transl Med.* (2013) 5:180ra48. doi: 10.1126/scitranslmed.3005109
136. Boral D, Vishnoi M, Liu HN, Yin W, Sprouse ML, Scamardo A, *et al*. Molecular characterization of breast cancer CTCs associated with brain metastasis. *Nat Commun.* (2017) 8:196. doi: 10.1038/s41467-017-00196-1
137. Shimizu K, Chiba S, Saito T, Kumano K, Hamada Y, Hirai H. Functional diversity among Notch1, Notch2, and Notch3 receptors. *Biochem Biophys Res Commun*. (2002) 291:775–9. doi: 10.1006/bbrc.2002.6528
138. Sale S, Lafkas D, Artavanis-Tsakonas S. Notch2 genetic fate mapping reveals two previously unrecognized mammary epithelial lineages. *Nat Cell Biol.* (2013) 15:451–60. doi: 10.1038/ncb2725
139. Lee JH, Zhao XM, Yoon I, Lee JY, Kwon NH, Wang YY, *et al*. Integrative analysis of mutational and transcriptional profiles reveals driver mutations of metastatic breast cancers. *Cell Discov*. (2016) 2:16025. doi: 10.1038/celldisc.2016.25
140. Florena AM, Tripodo C, Guarnotta C, Ingrao S, Porcasi R, Martorana A, *et al*. Associations between Notch-2, Akt-1 and HER2/neu expression in invasive human breast cancer: a tissue microarray immunophenotypic analysis on 98 patients. *Pathobiology* (2007) 74:317–22. doi: 10.1159/000110024
141. Parr C, Watkins G, Jiang WG. The possible correlation of Notch-1 and Notch-2 with clinical outcome and tumour clinicopathological parameters in human breast cancer. *Int J Mol Med.* (2004) 14:779–86. doi: 10.3892/ijmm.14.5.779
142. O'Neill CF, Urs S, Cinelli C, Lincoln A, Nadeau RJ, Leon R, *et al*. Notch2 signaling induces apoptosis and inhibits human MDA-MB-231 xenograft growth. *Am J Pathol.* (2007) 171:1023–36. doi: 10.2353/ajpath.2007.061029
143. Lee G-H, Yoo K-C, An Y, Lee H-J, Lee M, Uddin N, *et al*. FYN promotes mesenchymal phenotypes of basal type breast cancer cells through STAT5/NOTCH2 signaling node. *Oncogene* (2018) 37:1857–68. doi: 10.1038/s41388-017-0114-y
144. Yen WC, Fischer MM, Axelrod F, Bond C, Cain J, Cancilla B, *et al*. Targeting Notch signaling with a Notch2/Notch3 antagonist (tarextumab) inhibits tumor growth and decreases tumor-initiating cell frequency. *Clin Cancer Res.* (2015) 21:2084-95. doi: 10.1158/1078-0432.CCR-14-2808

145. Anjanappa M, Hao Y, Simpson ER, Bhat-Nakshatri P, Nelson JB, Tersey SA, *et al*. A system for detecting high impact-low frequency mutations in primary tumors and metastases. *Oncogene* (2018) 37:185–96. doi: 10.1038/onc.2017.32211.
146. Kim SH, Hahm ER, Arlotti JA, Samanta SK, Moura MB, Thorne SH, *et al*. Withaferin A inhibits in vivo growth of breast cancer cells accelerated by Notch2 knockdown. *Breast Cancer Res Treat.* (2016) 157:41–54. doi: 10.1007/s10549-016-3795-y
147. Yamaguchi N, Oyama T, Ito E, Satoh H, Azuma S, Hayashi M, *et al*. NOTCH3 signaling pathway plays crucial roles in the proliferation of ErbB2-negative human breast cancer cells. *Cancer Res.* (2008) 68:1881–8. doi: 10.1158/0008-5472.CAN-07-1597
148. Pradeep CR, Kostler WJ, Lauriola M, Granit RZ, Zhang F, Jacob-Hirsch J, *et al*. Modeling ductal carcinoma in situ: a HER2-Notch3 collaboration enables luminal filling. *Oncogene* (2012) 31:907–17. doi: 10.1038/onc.2011.279
149. Turner N, Lambros MB, Horlings HM, Pearson A, Sharpe R, Natrajan R, *et al*. Integrative molecular profiling of triple negative breast cancers identifies amplicon drivers and potential therapeutic targets. *Oncogene* (2010) 29:2013–23. doi: 10.1038/onc.2009.489
150. Cui H, Kong Y, Xu M, Zhang H. Notch3 functions as a tumor suppressor by controlling cellular senescence. *Cancer Res*. (2013) 73:3451–9. doi: 10.1158/0008-5472.CAN-12-3902
151. Zhang X, Liu X, Luo J, Xiao W, Ye X, Chen M, *et al*. Notch3 inhibits epithelial-mesenchymal transition by activating Kibra-mediated Hippo/YAP signaling in breast cancer epithelial cells. *Oncogenesis* (2016) 5:e269. doi: 10.1038/oncsis.2016.67
152. Diluvio G, Del Gaudio F, Giuli MV, Franciosa G, Giuliani E, Palermo R, *et al*. NOTCH3 inactivation increases triple negative breast cancer sensitivity to gefitinib by promoting EGFR tyrosine dephosphorylation and its intracellular arrest. *Oncogenesis* (2018) 7:42. doi: 10.1038/s41389-018-0051-9
153. Lawson DA, Bhakta NR, Kessenbrock K, Prummel KD, Yu Y, Takai K, *et al*. Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells. *Nature* (2015) 526:131–5. doi: 10.1038/nature15260
154. Politi K, Feirt N, Kitajewski J. Notch in mammary gland development and breast cancer. *Semin Cancer Biol.* (2004) 14:341–7. doi: 10.1016/j.semcancer.2004.04.013
155. Robbins J, Blondel BJ, Gallahan D, Callahan R. Mouse mammary tumor gene int-3: a member of the notch gene family transforms mammary epithelial cells. *J Virol*. (1992) 66:2594–9.
156. Jhappan C, Gallahan D, Stahle C, Chu E, Smith GH, Merlino G, *et al*. Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Dev.* (1992) 6:345–55. doi: 10.1101/gad.6.3.345
157. Raafat A, Lawson S, Bargo S, Klauzinska M, Strizzi L, Goldhar AS, *et al*. Rbpj conditional knockout reveals distinct functions of Notch4/Int3 in mammary gland development and tumorigenesis. *Oncogene* (2009) 28:219–30. doi: 10.1038/onc.2008.379
158. Raafat A, Bargo S, McCurdy D, Callahan R. The ANK repeats of Notch-4/Int3 activate NF-kappaB canonical pathway in the absence of Rbpj and causes mammary tumorigenesis. *Sci Rep.* (2017) 7:13690. doi: 10.1038/s41598-017-13989-7

159. Weng AP, Ferrando AA, Lee W, Morris JP, Silverman LB, Sanchez-Irizarry C, *et al*. Activating Mutations of *NOTCH1* in Human T Cell Acute Lymphoblastic Leukemia. *Science* (2004) 306:269. doi: 10.1126/science.1102160
160. Aifantis I, Raetz E, Buonamici S. Molecular pathogenesis of T-cell leukaemia and lymphoma. *Nat Rev Immunol*. (2008) 8:380–90. doi: 10.1038/nri2304
161. Panin VM, Papayannopoulos V, Wilson R, Irvine KD. Fringe modulates Notch-ligand interactions. *Nature* (1997) 387:908–12. doi: 10.1038/43191
162. Haines N, Irvine KD. Glycosylation regulates Notch signalling. *Nat Rev Mol Cell Biol.* (2003) 4:786–97. doi: 10.1038/nrm1228
163. Xu K, Usary J, Kousis PC, Prat A, Wang DY, Adams JR, *et al*. Lunatic fringe deficiency cooperates with the Met/Caveolin gene amplicon to induce basal-like breast cancer. *Cancer Cell* (2012) 21:626–41. doi: 10.1016/j.ccr.2012.03.041
164. Zhang S, Chung WC, Miele L, Xu K. Targeting Met and Notch in the Lfng-deficient, Met-amplified triple-negative breast cancer. *Cancer Biol Ther*. (2014) 15:633–42. doi: 10.4161/cbt.28180
165. Chung WC, Zhang S, Challagundla L, Zhou Y, Xu K. Lunatic Fringe and p53 Cooperatively Suppress Mesenchymal Stem-Like Breast Cancer. *Neoplasia* (2017) 19:885–95. doi: 10.1016/j.neo.2017.08.006
166. Zhang S, Chung WC, Wu G, Egan SE, Miele L, Xu K. Manic fringe promotes a claudin-low breast cancer phenotype through notch-mediated PIK3CG induction. *Cancer Res*. (2015) 75:1936–43. doi: 10.1158/0008-5472.CAN-14-3303
167. Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, *et al*. Alterations of the Notch pathway in lung cancer. *Proc Natl Acad Sci USA*. (2009) 106:22293–8. doi: 10.1073/pnas.0907781106
168. Colaluca IN, Tosoni D, Nuciforo P, Senic-Matuglia F, Galimberti V, Viale G, *et al*. NUMB controls p53 tumour suppressor activity. *Nature* (2008) 451:76–80. doi: 10.1038/nature06412
169. Tosoni D, Pambianco S, Ekalle Soppo B, Zecchini S, Bertalot G, Pruneri G, *et al*. Pre-clinical validation of a selective anti-cancer stem cell therapy for Numb-deficient human breast cancers. *EMBO Mol Med.* (2017) 9:655–71. doi: 10.15252/emmm.201606940
170. Pece S, Confalonieri S, P RR, Di Fiore PP. NUMB-ing down cancer by more than just a NOTCH. Biochim Biophys Acta. (2011) 1815:26-43. doi: 10.1016/j.bbcan.2010.10.001
171. Juven-Gershon T, Shifman O, Unger T, Elkeles A, Haupt Y, Oren M. The Mdm2 oncoprotein interacts with the cell fate regulator Numb. *Mol Cell Biol.* (1998) 18:3974–82. doi: 10.1128/MCB.18.7.3974
172. Yogosawa S, Miyauchi Y, Honda R, Tanaka H, Yasuda H. Mammalian Numb is a target protein of Mdm2, ubiquitin ligase. *Biochem Biophys Res Commun.* (2003) 302:869–72. doi: 10.1016/S0006-291X(03)00282-1
173. Robinson DR, Kalyana-Sundaram S, Wu YM, Shankar S, Cao X, Ateeq B, *et al*. Functionally recurrent rearrangements of the MAST kinase and Notch gene families in breast cancer. *Nat Med*. (2011) 17:1646–51. doi: 10.1038/nm.2580
174. Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L, Tandon A, *et al*. Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and βAPP processing. *Nature* (2000) 407:48. doi: 10.1038/35024009

175. Yu G, Chen F, Nishimura M, Steiner H, Tandon A, Kawarai T, *et al*. Mutation of conserved aspartates affect maturation of both aspartate-mutant and endogenous presenilin 1 and presenilin 2 complexes. *J Biol Chem*. (2000) 275:27348–53. doi: 10.1074/jbc.M002982200

176. Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ. Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity. *Nature* (1999) 398:513. doi: 10.1038/19077

177. Filipović A, Gronau JH, Green AR, Wang J, Vallath S, Shao D, *et al*. Biological and clinical implications of nicastrin expression in invasive breast cancer. *Breast Cancer Res Treat*. (2011) 125:43–53. doi: 10.1007/s10549-010-0823-1

178. Sarajlić A, Filipović A, Janjić V, Coombes RC, PrŽulj N. The role of genes co-amplified with nicastrin in breast invasive carcinoma. *Breast Cancer Res Treat*. (2014) 143:393–401. doi: 10.1007/s10549-013-2805-6

179. Lombardo Y, Filipović A, Molyneux G, Periyasamy M, Giamas G, Hu Y, *et al*. Nicastrin regulates breast cancer stem cell properties and tumor growth *in vitro* and *in vivo*. *Proc Natl Acad Sci USA*. (2012) 109:16558. doi: 10.1073/pnas.1206268109

180. Filipović A, Lombardo Y, Fronato M, Abrahams J, Aboagye E, Nguyen Q-D, *et al*. Anti-nicastrin monoclonal antibodies elicit pleiotropic anti-tumour pharmacological effects in invasive breast cancer cells. *Breast Cancer Res Treat*. (2014) 148:455–62. doi: 10.1007/s10549-014-3119-z

181. Studebaker AW, Storci G, Werbeck JL, Sansone P, Sasser AK, Tavolari S, *et al*. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer Res*. (2008) 68:9087–95. doi: 10.1158/0008-5472.CAN-08-0400

182. Rao X, Di Leva G, Li M, Fang F, Devlin C, Hartman-Frey C, *et al*. MicroRNA-221/222 confers breast cancer fulvestrant resistance by regulating multiple signaling pathways. *Oncogene* (2011) 30:1082–97. doi: 10.1038/onc.2010.487

183. Tsuyada A, Chow A, Wu J, Somlo G, Chu P, Loera S, *et al*. CCL2 mediates crosstalk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. *Cancer Res*. 72, 2768–79. doi: 10.1158/0008-5472.CAN-11-3567

184. Boelens MC, Wu TJ, Nabet BY, Xu B, Qiu Y, Yoon T, *et al*. (2014). Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. *Cell* (2012) 159:499–513. doi: 10.1016/j.cell.2014.09.051

185. Pupo M, Pisano A, Abonante S, Maggiolini M, Musti AM. GPER activates Notch signaling in breast cancer cells and cancer-associated fibroblasts (CAFs). *Int J Biochem Cell Biol*. (2014) 46:56–67. doi: 10.1016/j.biocel.2013.11.011

186. Soares R, Balogh G, Guo S, Gärtner F, Russo J, Schmitt F. Evidence for the Notch Signaling Pathway on the Role of Estrogen in Angiogenesis. Molecular Endocrinology. (2004) 18:2333-43. doi: 10.1210/me.2003-0362

187. Ghiabi P, Jiang J, Pasquier J, Maleki M, Abu-Kaoud N, Rafii S, *et al*. Endothelial cells provide a Notch-dependent pro-tumoral niche for enhancing breast cancer survival, stemness and pro-metastatic properties. *PLoS ONE* (2014) 9:e112424. doi: 10.1371/journal.pone.0112424

188. Ayyanan A, Civenni G, Ciarloni L, Morel C, Mueller N, Lefort K, *et al*. Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism. *Proc Natl. Acad Sci USA*. (2006) 103:3799. doi: 10.1073/pnas.0600065103
189. Sansone P, Storci G, Tavolari S, Guarnieri T, Giovannini C, Taffurelli M, *et al*. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J Clin Investig*. (2007) 117:3988–4002. doi: 10.1172/JCI32533
190. Wolfson B, Eades G, Zhou Q. Adipocyte activation of cancer stem cell signaling in breast cancer. *World J Biol Chem*. (2015) 6:39–47. doi: 10.4331/wjbc.v6.i2.39
191. Battle M, Gillespie C, Quarshie A, Lanier V, Harmon T, Wilson K, *et al*. Obesity Induced a Leptin-Notch Signaling Axis in Breast Cancer. *Int J Cancer J Int du Cancer* (2014) 134:1605–16. doi: 10.1002/ijc.28496
192. Pedrosa AR, Trindade A, Fernandes AC, Carvalho C, Gigante J, Tavares AT, *et al*. Endothelial jagged1 antagonizes Dll4 regulation of endothelial branching and promotes vascular maturation downstream of Dll4/Notch1. *Arterioscler Thrombos Vasc Biol*. (2015) 35:1134–46. doi: 10.1161/ATVBAHA.114.304741
193. Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, *et al*. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* (2006) 444:1032–7. doi: 10.1038/nature05355
194. Paris D, Quadros A, Patel N, DelleDonne A, Humphrey J, Mullan M. Inhibition of angiogenesis and tumor growth by β and γ-secretase inhibitors. *Eur J Pharmacol*. (2005) 514:1–15. doi: 10.1016/j.ejphar.2005.02.050
195. Chiorean EG, LoRusso P, Strother RM, Diamond JR, Younger A, Messersmith WA, *et al*. A phase I first-in-human study of enoticumab (REGN421), a fully human delta-like ligand 4 (Dll4) monoclonal antibody in patients with advanced solid tumors. *Clin Cancer Res*. (2015) 21:2695–703. doi: 10.1158/1078-0432.CCR-14-2797
196. Janghorban M, Xin L, Rosen JM, Zhang XHF. Notch signaling as a regulator of the tumor immune response: to target or not to target? *Front Immunol*. (2018) 9:1649. doi: 10.3389/fimmu.2018.01649
197. Radtke F, MacDonald HR, Tacchini-Cottier F. Regulation of innate and adaptive immunity by Notch. *Nature Rev Immunol*. (2013) 13:427–37. doi: 10.1038/nri3445
198. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* (2010) 140:883–99. doi: 10.1016/j.cell.2010.01.025
199. Grazioli P, Felli MP, Screpanti I, Campese AF. The mazy case of Notch and immunoregulatory cells. *J leukocyte Biol*. (2017) 102:361–8. doi: 10.1189/jlb.1VMR1216-505R
200. Liu H, Wang J, Zhang M, Xuan Q, Wang Z, Lian X, *et al*. Jagged1 promotes aromatase inhibitor resistance by modulating tumor-associated macrophage differentiation in breast cancer patients. *Breast Cancer Res Treat*. (2017) 166:95–107. doi: 10.1007/s10549-017-4394-2
201. Sousa S, Brion R, Lintunen M, Kronqvist P, Sandholm J, Mönkkönen J, *et al*. Human breast cancer cells educate macrophages toward the M2 activation status. *Breast Cancer Res*. (2015) 17:101. doi: 10.1186/s13058-015-0621-0

202. Shen Q, Cohen B, Zheng W, Rahbar R, Martin B, Murakami K, *et al*. Notch Shapes the Innate Immunophenotype in Breast Cancer. *Cancer Disc*. (2017) 7:1320–35. doi: 10.1158/2159-8290.CD-17-0037
203. Biswas SK, Allavena P, Mantovani A. Tumor-associated macrophages: functional diversity, clinical significance, and open questions. *Semin Immunopathol*. (2013) 35:585–600. doi: 10.1007/s00281-013-0367-7
204. Cheng P, Kumar V, Liu H, Youn J-I, Fishman M, Sherman S, *et al*. Effects of Notch signaling on regulation of myeloid cell differentiation in cancer. *Cancer Res*. (2014) 74:141 LP-52. doi: 10.1158/0008-5472.CAN-13-1686
205. Welte T, Kim IS, Tian L, Gao X, Wang H, Li J, *et al*. Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation. *Nature Cell Biol*. (2016) 18:632–44. doi: 10.1038/ncb3355
206. Peng D, Tanikawa T, Li W, Zhao L, Vatan L, Szeliga W, *et al*. Myeloid-Derived suppressor cells endow stem-like qualities to breast cancer cells through IL6/STAT3 and NO/NOTCH Cross-talk Signaling. *Cancer Res*. (2016) 76:3156–65. doi: 10.1158/0008-5472.CAN-15-2528
207. Shevach EM. Biological functions of regulatory T cells. *Adv Immunol*. (2011) 112:137–76. doi: 10.1016/B978-0-12-387827-4.00004-8
208. Samon JB, Champhekar A, Minter LM, Telfer JC, Miele L, Fauq A, *et al*. Notch1 and TGFbeta1 cooperatively regulate Foxp3 expression and the maintenance of peripheral regulatory T cells. *Blood* (2008) 112:1813–21. doi: 10.1182/blood-2008-03-144980
209. Kared H, Adle-Biassette H, Foïs E, Masson A, Bach J-F, Chatenoud L, *et al*. Jagged2-expressing hematopoietic progenitors promote regulatory T cell expansion in the periphery through notch signaling. *Immunity* (2006) 25:823–34. doi: 10.1016/j.immuni.2006.09.008
210. Cho OH, Shin HM, Miele L, Golde TE, Fauq A, Minter LM, *et al*. Notch Regulates Cytolytic Effector Function in CD8+ T Cells. *J Immunol*. (2009) 182:3380 LP-9. doi: 10.4049/jimmunol.0802598
211. Sugimoto K, Maekawa Y, Kitamura A, Nishida J, Koyanagi A, Yagita H, *et al*. Notch2 signaling is required for potent antitumor immunity *in vivo*. *J Immunol*. (2010) 184:4673–8. doi: 10.4049/jimmunol.0903661
212. Meurette O, Mehlen P. Notch signaling in the tumor microenvironment. *Cancer Cell*. (2018) 34:536–48. doi: 10.1016/j.ccell.2018.07.009
213. Nakasone ES, Hurvitz SA, McCann KE. Harnessing the immune system in the battle against breast cancer. *Drugs Context*. (2018) 7:212520. doi: 10.7573/dic.212520
214. Mao L, Zhao Z-L, Yu G-T, Wu L, Deng W-W, Li Y-C, *et al*. γ-Secretase inhibitor reduces immunosuppressive cells and enhances tumour immunity in head and neck squamous cell carcinoma. *Int J Cancer* (2017) 142:999–1009. doi: 10.1002/ijc.31115
215. Speers C, Zhao S, Liu M, Bartelink H, Pierce LJ, Feng FY. Development and Validation of a Novel Radiosensitivity Signature in Human Breast Cancer. *Clin Cancer Res*. (2015) 21:3667. doi: 10.1158/1078-0432.CCR-14-2898

216. Tramm T, Mohammed H, Myhre S, Kyndi M, Alsner J, Børresen-Dale A-L, *et al*. Development and Validation of a Gene Profile Predicting Benefit of Postmastectomy Radiotherapy in Patients with High-Risk Breast Cancer: a study of gene expression in the DBCG82bc Cohort. *Clin Cancer Res.* (2014) 20:5272. doi: 10.1158/1078-0432.CCR-14-0458
217. Pajonk F, Vlashi E, McBride WH. Radiation resistance of cancer stem cells: the 4 R's of radiobiology revisited. *Stem Cells* (2010) 28:639–48. doi: 10.1002/stem.318
218. Rich JN. Cancer stem cells in radiation resistance. *Cancer Res*. (2007) 67:8980–4. doi: 10.1158/0008-5472.CAN-07-0895
219. Phillips TM, McBride WH, Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. *J Natl Cancer Inst*. (2006) 98:1777–85. doi: 10.1093/jnci/djj495
220. Woodward WA, Chen MS, Behbod F, Alfaro MP, Buchholz TA, Rosen JM. WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. *Proc Natl Acad Sci USA*. (2007) 104:618–23. doi: 10.1073/pnas.0606599104
221. Lagadec C, Vlashi E, Della Donna L, Dekmezian C, Pajonk F. Radiation-induced reprogramming of breast cancer cells. *Stem Cells*. (2012) 30:833–44. doi: 10.1002/stem.1058
222. Lagadec C, Vlashi E, Alhiyari Y, Phillips TM, Dratver MB, Pajonk F. Radiation-Induced Notch Signaling in Breast Cancer Stem Cells. *Int J Radiat Oncol Biol Phys.* (2013) 87:609–18. doi: 10.1016/j.ijrobp.2013.06.2064
223. Vermezovic J, Adamowicz M, Santarpia L, Rustighi A, Forcato M, Lucano C, *et al*. Notch is a direct negative regulator of the DNA-damage response. *Nature Struc Mol Biol.* (2015) 22:417. doi: 10.1038/nsmb.3013
224. Longley DB, Johnston PG. Molecular mechanisms of drug resistance. *J Pathol.* (2005) 205:275–92. doi: 10.1002/path.1706
225. Abdullah LN, Chow EK. Mechanisms of chemoresistance in cancer stem cells. *Clin Transl Med.* (2013) 2:3. doi: 10.1186/2001-1326-2-3
226. Zang S, Chen F, Dai J, Guo D, Tse W, Qu X, *et al*. RNAi-mediated knockdown of Notch-1 leads to cell growth inhibition and enhanced chemosensitivity in human breast cancer. *Oncol Rep.* (2010) 23:893–9. doi: 10.3892/or_00000712
227. Li ZL, Chen C, Yang Y, Wang C, Yang T, Yang X, *et al*. Gamma secretase inhibitor enhances sensitivity to doxorubicin in MDA-MB-231 cells. *Int J Clin Exp Pathol.* (2015) 8:4378–87.
228. Mirkes PE, Ellison A, Little SA. Role of aldehyde dehydrogenase (ALDH) in the detoxication of cyclophosphamide (CP) in rat embryos. *Adv Exp Med Biol*. (1991) 284:85-95. doi: 10.1007/978-1-4684-5901-2_11
229. Liu S, Wicha MS. Targeting breast cancer stem cells. *J Clin Oncol*. (2010) 28:4006–12. doi: 10.1200/JCO.2009.27.5388
230. Chuthapisith S, Eremin J, El-Sheemey M, Eremin O. Breast cancer chemoresistance: emerging importance of cancer stem cells. *Surg Oncol.* (2010) 19:27–32. doi: 10.1016/j.suronc.2009.01.004
231. Suman S, Das TP, Damodaran C. Silencing NOTCH signaling causes growth arrest in both breast cancer stem cells and breast cancer cells. *Br J Cancer* (2013) 109:2587–96. doi: 10.1038/bjc.2013.642

232. Kim B, Stephen SL, Hanby AM, Horgan K, Perry SL, Richardson J, *et al*. Chemotherapy induces Notch1-dependent MRP1 up-regulation, inhibition of which sensitizes breast cancer cells to chemotherapy. *BMC Cancer* (2015) 15:634. doi: 10.1186/s12885-015-1625-y
233. Gu X, Lu C, He D, Lu Y, Jin J, Liu D, *et al*. Notch3 negatively regulates chemoresistance in breast cancers. *Tumour Biol.* (2016) 37:15825–33. doi: 10.1007/s13277-016-5412-4
234. de Weger VA, Beijnen JH, Schellens JH. Cellular and clinical pharmacology of the taxanes docetaxel and paclitaxel–a review. *Anticancer Drugs* (2014) 25:488–94. doi: 10.1097/CAD.0000000000000093
235. Gradishar WJ. Taxanes for the treatment of metastatic breast cancer. *Breast Cancer (Auckl)*. (2012) 6:159–71. doi: 10.4137/BCBCR.S8205
236. Qiu M, Peng Q, Jiang I, Carroll C, Han G, Rymer I, *et al*. Specific inhibition of Notch1 signaling enhances the antitumor efficacy of chemotherapy in triple negative breast cancer through reduction of cancer stem cells. *Cancer Lett*. (2013) 328:261–70. doi: 10.1016/j.canlet.2012.09.023
237. Zhang CC, Yan Z, Zong Q, Fang DD, Painter C, Zhang Q, *et al*. Synergistic effect of the gamma-secretase inhibitor PF-03084014 and docetaxel in breast cancer models. *Stem Cells Transl Med.* (2013) 2:233–42. doi: 10.5966/sctm.2012-0096
238. Schott AF, Landis MD, Dontu G, Griffith KA, Layman RM, Krop I, *et al*. Preclinical and clinical studies of gamma secretase inhibitors with docetaxel on human breast tumors. *Clin Cancer Res*. (2013) 19:1512–24. doi: 10.1158/1078-0432.CCR-11-3326
239. Locatelli MA, Aftimos P, Dees EC, LoRusso PM, Pegram MD, Awada A, *et al*. Phase I study of the gamma secretase inhibitor PF-03084014 in combination with docetaxel in patients with advanced triple-negative breast cancer. *Oncotarget* (2017) 8:2320–8. doi: 10.18632/oncotarget.13727
240. Jeong YJ, Kang JS, Lee SI, So DM, Yun J, Baek JY, *et al*. Breast cancer cells evade paclitaxel-induced cell death by developing resistance to dasatinib. *Oncol Lett*. (2016) 12:2153–8. doi: 10.3892/ol.2016.4852
241. Hoey T, Yen WC, Axelrod F, Basi J, Donigian L, Dylla S, *et al*. DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. *Cell Stem Cell.* (2009) 5:168–77. doi: 10.1016/j.stem.2009.05.019
242. Zheng H, Bae Y, Kasimir-Bauer S, Tang R, Chen J, Ren G, *et al*. Therapeutic Antibody Targeting Tumor- and Osteoblastic Niche-Derived Jagged1 Sensitizes Bone Metastasis to Chemotherapy. *Cancer Cell* (2017) 32:731–47 e6. doi: 10.1016/j.ccell.2017.11.002
243. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology* (2003) 144:4562–74. doi: 10.1210/en.2003-0567
244. Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, *et al*. Progesterone induces adult mammary stem cell expansion. *Nature* (2010) 465:803–7. doi: 10.1038/nature09091
245. Asselin-Labat ML, Vaillant F, Sheridan JM, Pal B, Wu D, Simpson ER, *et al*. Control of mammary stem cell function by steroid hormone signalling. *Nature* (2010) 465:798–802. doi: 10.1038/nature09027
246. Hoefnagel LD, van de Vijver MJ, van Slooten HJ, Wesseling P, Wesseling J, Westenend PJ, *et al*. Receptor conversion in distant breast cancer metastases. *Breast Cancer Res.* (2010) 12:R75. doi: 10.1186/bcr2645

247. Hoefnagel LD, Moelans CB, Meijer SL, van Slooten HJ, Wesseling P, Wesseling J, *et al*. Prognostic value of estrogen receptor alpha and progesterone receptor conversion in distant breast cancer metastases. *Cancer* (2012) 118:4929–35. doi: 10.1002/cncr.27518
248. Fuqua SAW, Gu G, Rechoum Y. Estrogen Receptor (ER) Mutations in breast cancer: hidden in plain sight. *Breast Cancer Res Treat*. (2014) 144:11–9. doi: 10.1007/s10549-014-2847-4
249. Jeselsohn R, Buchwalter G, De Angelis C, Brown M, Schiff R. ESR1 mutations as a mechanism for acquired endocrine resistance in breast cancer. *Nature Rev Clin Oncol*. (2015) 12:573–83. doi: 10.1038/nrclinonc.2015.117
250. Musgrove EA, Sutherland RL. Biological determinants of endocrine resistance in breast cancer. *Nat Rev Cancer* (2009) 9:631–43. doi: 10.1038/nrc2713
251. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med.* (2011) 62:233–47. doi: 10.1146/annurev-med-070909-182917
252. Sansone P, Berishaj M, Rajasekhar VK, Ceccarelli C, Chang Q, Strillacci A, *et al*. Evolution of cancer stem-like cells in endocrine-resistant metastatic breast cancers is mediated by stromal microvesicles. *Cancer Res*. (2017) 77:1927–41. doi: 10.1158/0008-5472.CAN-16-2129
253. Harrison H, Simoes BM, Rogerson L, Howell SJ, Landberg G, Clarke RB. Oestrogen increases the activity of oestrogen receptor negative breast cancer stem cells through paracrine EGFR and Notch signalling. *Breast Cancer Res*. (2013) 15:R21. doi: 10.1186/bcr3396
254. Rizzo P, Miao H, D'Souza G, Osipo C, Song LL, Yun J, *et al*. Cross-talk between notch and the estrogen receptor in breast cancer suggests novel therapeutic approaches. *Cancer Res*. (2008) 68:5226–35. doi: 10.1158/0008-5472.CAN-07-5744
255. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, *et al*. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst*. (2004) 96:926–35. doi: 10.1093/jnci/djh166
256. Izrailit J, Berman HK, Datti A, Wrana JL, Reedijk M. High throughput kinase inhibitor screens reveal TRB3 and MAPK-ERK/TGFbeta pathways as fundamental Notch regulators in breast cancer. *Proc Natl Acad Sci USA*. (2013) 110:1714–9. doi: 10.1073/pnas.1214014110
257. Mittal S, Subramanyam D, Dey D, Kumar RV, Rangarajan A. Cooperation of Notch and Ras/MAPK signaling pathways in human breast carcinogenesis. *Mol Cancer*. (2009) 8:128. doi: 10.1186/1476-4598-8-128
258. Horwitz KB, Dye WW, Harrell JC, Kabos P, Sartorius CA. Rare steroid receptor-negative basal-like tumorigenic cells in luminal subtype human breast cancer xenografts. *Proc Natl Acad Sci USA*. (2008) 105:5774–9. doi: 10.1073/pnas.0706216105
259. Jin S, Mutvei AP, Chivukula IV, Andersson ER, Ramskold D, Sandberg R, *et al*. Non-canonical Notch signaling activates IL-6/JAK/STAT signaling in breast tumor cells and is controlled by p53 and IKKalpha/IKKbeta. *Oncogene* (2013) 32:4892–902. doi: 10.1038/onc.2012.517
260. Sansone P, Storci G, Giovannini C, Pandolfi S, Pianetti S, Taffurelli M, *et al*. p66Shc/Notch-3 interplay controls self-renewal and hypoxia survival in human stem/progenitor cells of the mammary gland expanded *in vitro* as mammospheres. *Stem Cells* (2007) 25:807–15. doi: 10.1634/stemcells.2006-0442

261. Sansone P, Ceccarelli C, Berishaj M, Chang Q, Rajasekhar VK, Perna F, *et al*. Self-renewal of CD133(hi) cells by IL6/Notch3 signalling regulates endocrine resistance in metastatic breast cancer. *Nat Commun*. (2016) 7:10442. doi: 10.1038/ncomms10442
262. Magnani L, Stoeck A, Zhang X, Lanczky A, Mirabella AC, Wang TL, *et al*. Genome-wide reprogramming of the chromatin landscape underlies endocrine therapy resistance in breast cancer. *Proc Natl Acad Sci USA*. (2013) 110:E1490–9. doi: 10.1073/pnas.1219992110
263. Lombardo Y, Faronato M, Filipovic A, Vircillo V, Magnani L, Coombes RC. Nicastrin and Notch4 drive endocrine therapy resistance and epithelial to mesenchymal transition in MCF7 breast cancer cells. *Breast Cancer Res.* (2014) 16:R62. doi: 10.1186/bcr3675
264. Bui QT, Im JH, Jeong SB, Kim YM, Lim SC, Kim B, *et al*. Essential role of Notch4/STAT3 signaling in epithelial-mesenchymal transition of tamoxifen-resistant human breast cancer. *Cancer Lett*. (2017) 390:115–25. doi: 10.1016/j.canlet.2017.01.014
265. Gelsomino L, Panza S, Giordano C, Barone I, Gu G, Spina E, *et al*. Mutations in the estrogen receptor alpha hormone binding domain promote stem cell phenotype through notch activation in breast cancer cell lines. *Cancer Letts.* (2018) 428:12–20. doi: 10.1016/j.canlet.2018.04.023
266. Arteaga CL, Sliwkowski MX, Osborne CK, Perez EA, Puglisi F, Gianni L. Treatment of HER2-positive breast cancer: current status and future perspectives. *Nat Rev Clin Oncol.* (2011) 9:16–32. doi: 10.1038/nrclinonc.2011.177
267. Arteaga CL, Engelman JA. ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. *Cancer Cell*. (2014) 25:282–303. doi: 10.1016/j.ccr.2014.02.025
268. Hosseini H, Obradovic MM, Hoffmann M, Harper KL, Sosa MS, Werner-Klein M, *et al*. Early dissemination seeds metastasis in breast cancer. *Nature* (2016) 540:552–8. doi: 10.1038/nature20785
269. Harper KL, Sosa MS, Entenberg D, Hosseini H, Cheung JF, Nobre R, *et al*. Mechanism of early dissemination and metastasis in Her2+ mammary cancer. *Nature* (2016) 540:588–92. doi: 10.1038/nature20609
270. Schwartz RS, Erban JK. Timing of metastasis in breast cancer. *N Engl J Med*. (2017) 376:2486–8. doi: 10.1056/NEJMcibr1701388
271. Ciriello G, Gatza ML, Beck AH, Wilkerson MD, Rhie SK, Pastore A, *et al*. Comprehensive molecular portraits of invasive lobular breast cancer. *Cell* (2015) 163:506–19. doi: 10.1016/j.cell.2015.09.033
272. Balduzzi S, Mantarro S, Guarneri V, Tagliabue L, Pistotti V, Moja L, *et al*. Trastuzumab-containing regimens for metastatic breast cancer. *Cochrane Database Syst Rev*. (2014) 12:CD006242. doi: 10.1002/14651858.CD006242.pub2
273. Boix-Perales H, Borregaard J, Jensen KB, Ersboll J, Galluzzo S, Giuliani R, *et al*. The European Medicines Agency Review of Pertuzumab for the treatment of adult patients with HER2-positive metastatic or locally recurrent unresectable breast cancer: summary of the scientific assessment of the committee for medicinal products for human use. *Oncologist*. (2014) 19:766–73. doi: 10.1634/theoncologist.2013-0348
274. Blackwell KL, Burstein HJ, Storniolo AM, Rugo H, Sledge G, Koehler M, *et al*. Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. *J Clin Oncol.* (2010) 28:1124–30. doi: 10.1200/JCO.2008.21.4437

275. Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, Rahmeh M, *et al*. Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. *Cancer Res*. (2006) 66:1630–9. doi: 10.1158/0008-5472.CAN-05-1182
276. Kumler I, Tuxen MK, Nielsen DL. A systematic review of dual targeting in HER2-positive breast cancer. *Cancer Treat Rev*. (2014) 40:259–70. doi: 10.1016/j.ctrv.2013.09.002
277. Gianni L, Pienkowski T, Im YH, Roman L, Tseng LM, Liu MC, *et al*. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. *Lancet Oncol*. (2012) 13:25–32. doi: 10.1016/S1470-2045(11)70336-9
278. Gianni L, Pienkowski T, Im YH, Tseng LM, Liu MC, Lluch A, *et al*. 5-year analysis of neoadjuvant pertuzumab and trastuzumab in patients with locally advanced, inflammatory, or early-stage HER2-positive breast cancer (NeoSphere): a multicentre, open-label, phase 2 randomised trial. *Lancet Oncol.* (2016) 17:791–800. doi: 10.1016/S1470-2045(16)00163-7
279. Baselga J, Bradbury I, Eidtmann H, Di Cosimo S, de Azambuja E, Aura C, *et al*. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet* (2012) 379:633–40. doi: 10.1016/S0140-6736(11)61847-3
280. de Azambuja E, Holmes AP, Piccart-Gebhart M, Holmes E, Di Cosimo S, Swaby RF, *et al*. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. *Lancet Oncol.* (2014) 15:1137–46. doi: 10.1016/S1470-2045(14)70320-1
281. Baselga J, Gelmon KA, Verma S, Wardley A, Conte P, Miles D, *et al*. Phase II trial of pertuzumab and trastuzumab in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer that progressed during prior trastuzumab therapy. *J Clin Oncol.* (2010) 28:1138–44. doi: 10.1200/JCO.2009.24.2024
282. Gajria D, Chandarlapaty S. HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies. *Expert Rev Anticancer Ther.* (2011) 11:263–75. doi: 10.1586/era.10.226
283. Koninki K, Barok M, Tanner M, Staff S, Pitkanen J, Hemmila P, *et al*. Multiple molecular mechanisms underlying trastuzumab and lapatinib resistance in JIMT-1 breast cancer cells. *Cancer Lett*. (2010) 294:211–9. doi: 10.1016/j.canlet.2010.02.002
284. Mehta K, Osipo C. Trastuzumab resistance: role for Notch signaling. *ScientificWorldJournal* (2009) 9:1438–48. doi: 10.1100/tsw.2009.166
285. Huang X, Gao L, Wang S, McManaman JL, Thor AD, Yang X, *et al*. Heterotrimerization of the growth factor receptors erbB2, erbB3, and insulin-like growth factor-i receptor in breast cancer cells resistant to herceptin. *Cancer Res.* (2010) 70:1204–14. doi: 10.1158/0008-5472.CAN-09-3321
286. Baker AT, Zlobin A, Osipo C. Notch-EGFR/HER2 Bidirectional Crosstalk in Breast Cancer. *Front Oncol*. (2014) 4:360. doi: 10.3389/fonc.2014.00360
287. Pandya K, Meeke K, Clementz AG, Rogowski A, Roberts J, Miele L, *et al*. Targeting both Notch and ErbB-2 signalling pathways is required for prevention of ErbB-2-positive breast tumour recurrence. *Br J Cancer* (2011) 105:796–806. doi: 10.1038/bjc.2011.321

288. Osipo C, Patel P, Rizzo P, Clementz AG, Hao L, Golde TE, *et al*. ErbB-2 inhibition activates Notch-1 and sensitizes breast cancer cells to a gamma-secretase inhibitor. *Oncogene* (2008) 27:5019–32. doi: 10.1038/onc.2008.149

289. Korkaya H, Wicha MS. HER-2, notch, and breast cancer stem cells: targeting an axis of evil. *Clin Cancer Res.* (2009) 15:1845–7. doi: 10.1158/1078-0432.CCR-08-3087

290. Seol H, Lee HJ, Choi Y, Lee HE, Kim YJ, Kim JH, *et al*. Intratumoral heterogeneity of HER2 gene amplification in breast cancer: its clinicopathological significance. *Mod Pathol.* (2012) 25:938–48. doi: 10.1038/modpathol.2012.36

291. Jordan NV, Bardia A, Wittner BS, Benes C, Ligorio M, Zheng Y, *et al*. HER2 expression identifies dynamic functional states within circulating breast cancer cells. *Nature* (2016) 537:102–6. doi: 10.1038/nature19328

292. Guo D, Ye J, Dai J, Li L, Chen F, Ma D, *et al*. Notch-1 regulates Akt signaling pathway and the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 in T-ALL cell lines. *Leuk Res*. (2009) 33:678–85. doi: 10.1016/j.leukres.2008.10.026

293. Ramakrishnan G, Davaakhuu G, Chung WC, Zhu H, Rana A, Filipovic A, *et al*. AKT and 14-3-3 regulate Notch4 nuclear localization. *Sci Rep*. (2015) 5:8782. doi: 10.1038/srep08782

294. Eliasz S, Liang S, Chen Y, De Marco MA, Machek O, Skucha S, *et al*. Notch-1 stimulates survival of lung adenocarcinoma cells during hypoxia by activating the IGF-1R pathway. *Oncogene* (2010) 29:2488–98. doi: 10.1038/onc.2010.7

295. Nefedova Y, Sullivan DM, Bolick SC, Dalton WS, Gabrilovich DI. Inhibition of Notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy. *Blood*. (2008) 111:2220–9. doi: 10.1182/blood-2007-07-102632

296. Yao J, Qian C. Inhibition of Notch3 enhances sensitivity to gemcitabine in pancreatic cancer through an inactivation of PI3K/Akt-dependent pathway. *Med Oncol.* (2010) 27:1017–22. doi: 10.1007/s12032-009-9326-5

297. Meng RD, Shelton CC, Li YM, Qin LX, Notterman D, Paty PB, *et al*. gamma-Secretase inhibitors abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells resulting in enhanced chemosensitivity. *Cancer Res*. (2009) 69:573–82. doi: 10.1158/0008-5472.CAN-08-2088

298. McDonald ER, 3rd, de Weck A, Schlabach MR, Billy E, Mavrakis KJ, Hoffman GR, *et al*. Project DRIVE: a compendium of cancer dependencies and synthetic lethal relationships uncovered by large-scale, deep RNAi screening. *Cell* (2017) 170:577–92 e10. doi: 10.1016/j.cell.2017.07.005

299. Yeo SK, Guan JL. Breast cancer: multiple subtypes within a tumor? *Trends Cancer* (2017) 3:753–60. doi: 10.1016/j.trecan.2017.09.001

300. Gomez-Miragaya J, Palafox M, Pare L, Yoldi G, Ferrer I, Vila S, *et al*. Resistance to Taxanes in Triple-Negative Breast Cancer Associates with the Dynamics of a CD49f+ Tumor-Initiating Population. *Stem Cell Reports* (2017) 8:1392–407. doi: 10.1016/j.stemcr.2017.03.026

301. Dall GV, Vieusseux JL, Korach KS, Arao Y, Hewitt SC, Hamilton KJ, *et al*. SCA-1 Labels a Subset of Estrogen-Responsive Bipotential Repopulating Cells within the CD24+ CD49fhi Mammary Stem Cell-Enriched Compartment. *Stem Cell Reports* (2017) 8:417–31. doi: 10.1016/j.stemcr.2016.12.022

302. Lu H, Clauser KR, Tam WL, Fröse J, Ye X, Eaton EN, *et al*. A breast cancer stem cell niche supported by juxtacrine signaling from monocytes and macrophages. *Nature Cell Biol*. (2014) 16:1105–17. doi: 10.1038/ncb3041
303. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, *et al*. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* (2008) 133:704–15. doi: 10.1016/j.cell.2008.03.027
304. Farnie G, Clarke RB. Mammary stem cells and breast cancer–role of Notch signalling. *Stem Cell Rev*. (2007) 3:169–75. doi: 10.1007/s12015-007-0023-5
305. Bhola NE, Jansen VM, Koch JP, Li H, Formisano L, Williams JA, *et al*. Treatment of Triple-Negative Breast Cancer with TORC1/2 Inhibitors Sustains a Drug-Resistant and Notch-Dependent Cancer Stem Cell Population. *Cancer Res.* (2016) 76:440–52. doi: 10.1158/0008-5472.CAN-15-1640-T
306. Azzam DJ, Zhao D, Sun J, Minn AJ, Ranganathan P, Drews-Elger K, *et al*. Triple negative breast cancer initiating cell subsets differ in functional and molecular characteristics and in gamma-secretase inhibitor drug responses. *EMBO Mol Med.* (2013) 5:1502–22. doi: 10.1002/emmm.201302558
307. D'Angelo RC, Ouzounova M, Davis A, Choi D, Tchuenkam SM, Kim G, *et al*. Notch reporter activity in breast cancer cell lines identifies a subset of cells with stem cell activity. *Mol Cancer Ther.* (2015) 14:779–87. doi: 10.1158/1535-7163.MCT-14-0228
308. Zhou W, Wang G, Guo S. Regulation of angiogenesis via Notch signaling in breast cancer and cancer stem cells. *Biochim Biophys Acta* (2013) 1836:304–20. doi: 10.1016/j.bbcan.2013.10.003
309. Perez-Tenorio G, Alkhori L, Olsson B, Waltersson MA, Nordenskjold B, Rutqvist LE, *et al*. PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer. *Clin Cancer Res.* (2007) 13:3577–84. doi: 10.1158/1078-0432.CCR-06-1609
310. Whelan TJ, Pignol JP, Levine MN, Julian JA, MacKenzie R, Parpia S, *et al*. Long-term results of hypofractionated radiation therapy for breast cancer. *N Engl J Med.* (2010) 362:513–20. doi: 10.1056/NEJMoa0906260
311. Yarnold J, Bentzen SM, Coles C, Haviland J. Hypofractionated whole-breast radiotherapy for women with early breast cancer: myths and realities. *Int J Radiat Oncol Biol Phys.* (2011) 79:1–9. doi: 10.1016/j.ijrobp.2010.08.035
312. Smith BD, Bentzen SM, Correa CR, Hahn CA, Hardenbergh PH, Ibbott GS, *et al*. Fractionation for whole breast irradiation: an American Society for Radiation Oncology (ASTRO) evidence-based guideline. *Int J Radiat Oncol Biol Phys.* (2011) 81:59–68. doi: 10.1016/j.ijrobp.2010.04.042
313. Zhang P, He D, Chen Z, Pan Q, Du F, Zang X, *et al*. Chemotherapy enhances tumor vascularization via Notch signaling-mediated formation of tumor-derived endothelium in breast cancer. *Biochem Pharmacol.* (2016) 118:18–30. doi: 10.1016/j.bcp.2016.08.008
314. Stender JD, Nwachukwu JC, Kastrati I, Kim Y, Strid T, Yakir M, *et al*. Structural and molecular mechanisms of cytokine-mediated endocrine resistance in human breast cancer cells. *Mol Cell* (2017) 65:1122–35 e5. doi: 10.1016/j.molcel.2017.02.008
315. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, *et al*. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat Genet*. (2013) 45:1446–51. doi: 10.1038/ng.2823

316. Ma CX, Reinert T, Chmielewska I, Ellis MJ. Mechanisms of aromatase inhibitor resistance. *Nat Rev Cancer* (2015) 15:261–75. doi: 10.1038/nrc3920
317. Hart CD, Migliaccio I, Malorni L, Guarducci C, Biganzoli L, Di Leo A. Challenges in the management of advanced, ER-positive, HER2-negative breast cancer. *Nat Rev Clin Oncol*. (2015) 12:541–52. doi: 10.1038/nrclinonc.2015.99
318. Rodon J, Dienstmann R, Serra V, Tabernero J. Development of PI3K inhibitors: lessons learned from early clinical trials. *Nat Rev Clin Oncol.* (2013) 10:143–53. doi: 10.1038/nrclinonc.2013.10
319. O'Leary B, Finn RS, Turner NC. Treating cancer with selective CDK4/6 inhibitors. *Nat Rev Clin Oncol.* (2016) 13:417–30. doi: 10.1038/nrclinonc.2016.26
320. Sidaway P. Breast cancer: brain metastasis detectable in CTCs. *Nat Rev Clin Oncol.* (2017) 14:588. doi: 10.1038/nrclinonc.2017.138
321. Goel S, Wang Q, Watt AC, Tolaney SM, Dillon DA, Li W, *et al*. Overcoming Therapeutic Resistance in HER2-Positive Breast Cancers with CDK4/6 Inhibitors. *Cancer Cell* (2016) 29:255–69. doi: 10.1016/j.ccell.2016.02.006
322. Malumbres M. CDK4/6 Inhibitors resTORe therapeutic sensitivity in HER(2)(+) breast cancer. *Cancer Cell* (2016) 29:243–4. doi: 10.1016/j.ccell.2016.02.016
323. Aberle MR, Burkhart RA, Tiriac H, Olde Damink SWM, Dejong CHC, Tuveson DA, *et al*. Patient-derived organoid models help define personalized management of gastrointestinal cancer. *Br J Surg*. (2018) 105:e48–60. doi: 10.1002/bjs.10726
324. Sachs N, de Ligt J, Kopper O, Gogola E, Bounova G, Weeber F, *et al*. A Living biobank of breast cancer organoids captures disease heterogeneity. *Cell* (2018) 172:373–86 e10. doi: 10.1016/j.cell.2017.11.010
325. Krop I, Demuth T, Guthrie T, Wen PY, Mason WP, Chinnaiyan P, *et al*. Phase I pharmacologic and pharmacodynamic study of the gamma secretase (Notch) inhibitor MK-0752 in adult patients with advanced solid tumors. *J Clin Oncol*. (2012) 30:2307–13. doi: 10.1200/JCO.2011.39.1540

CHAPTER 3

Hypoxia induced Notch activity in a Notch overexpression lung cancer model

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In preparation

CHAPTER 4

A lineage-tracing tool to map the fate of hypoxic tumour cells

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Abstract

Intratumoural hypoxia is a common characteristic of malignant treatment-resistant cancers. However, hypoxia-modification strategies for the clinic remain elusive. To date, little is known on the behaviour of individual hypoxic tumour cells in their microenvironment. To explore this issue in a spatial and temporally controlled manner, we developed a genetically encoded sensor by fusing the O_2-labile hypoxia-inducible factor 1α (HIF-1α) protein to eGFP and a tamoxifen-regulated Cre recombinase. Under normoxic conditions, HIF-1α is degraded but, under hypoxia, the HIF-1α-GFP-Cre-ER^{T2} fusion protein is stabilised and in the presence of tamoxifen activates a tdTomato reporter gene that is constitutively expressed in hypoxic progeny. We visualise the random distribution of hypoxic tumour cells from hypoxic or necrotic regions and vascularised areas using immunofluorescence and intravital microscopy. Once tdTomato expression is induced, it is stable for at least 4 weeks. Using this system, we could show *in vivo* that the post-hypoxic cells were more proliferative than non-labelled cells. Our results demonstrate that single-cell lineage tracing of hypoxic tumour cells can allow visualisation of their behaviour in living tumours using intravital microscopy. This tool should prove valuable for the study of dissemination and treatment response of post-hypoxic tumour cells *in vivo* at single-cell resolution.

Introduction

Many solid tumours contain areas of hypoxia, which is the result of O_2 demand (rapid proliferation) exceeding O_2 supply (aberrant vasculature) (Thomlinson and Gray, 1955; Brahimi-Horn, Chiche and Pouysségur, 2007). Due to limits on O_2 diffusion from the blood vessels, tumours experience chronic hypoxia and necrosis in regions distant from the vasculature. Acute or cycling hypoxia also occurs in tumours due to temporary occlusion of blood vessels obstructing perfusion leading to areas of hypoxia, which become re-oxygenated when the obstruction is relieved (Dewhirst, 2009; Salem *et al.*, 2018). Tumour hypoxia is strongly associated with a worse outcome in many different cancers irrespective of treatment (Vaupel and Mayer, 2007) and tumour hypoxia is a direct factor in resistance to some chemotherapy and radiation therapy. Direct O_2 measurements in clinical studies using oxygen needle electrodes indicate hypoxia is strongly associated with local regional control in head and neck squamous cell carcinoma (Nordsmark *et al.*, 2005), prostate (Milosevic *et al.*, 2012) and cervix (Fyles *et al.*, 1998) cancer patients treated with radiotherapy. Hypoxia imaging using PET tracers such as ^{18}F-Hx4,^{18}F-MISO, and ^{18}F-FAZA have demonstrated strong associations of tracer uptake with outcome (Lehtiö *et al.*, 2004; Dubois *et al.*, 2011; Fleming *et al.*, 2015).

The main adaptive response to hypoxia is the stabilisation of the oxygen regulated hypoxia inducible factor alpha (HIF-α) proteins HIF-1α HIF-2α and HIF-3α. HIF-α proteins are predominantly regulated post-translationally through oxygen dependent prolyl and asparagine hydroxylases, which hydroxylate specific proline and asparagine residues on the HIF-α oxygen dependent degradation (ODD) domain. Hydroxylation of asparagine inhibits the recruitment of the transcriptional regulator p300. Prolyl hydroxylation promotes interaction with the von Hippel-Lindau (pVHL) protein, which recruits an E3 ubiquitin ligase, targeting HIF-α for proteasomal degradation (Ivan *et al.*, 2001). Under hypoxic conditions, the activity of prolyl and asparagine hydroxylases is attenuated leading to accumulation of HIF-α proteins. HIF-α then translocates to the nucleus where it binds the constitutively expressed HIF-1β protein and the co-activator p300. The HIF transcriptional complex is known to trans-activate over 1,500 target genes through binding to hypoxia response elements (HREs) located in the target genes or flanking sequences (Prabhakar and Semenza, 2015). HIF target genes play a role in a broad range of pathways including those involved in angiogenesis (VEGF), metabolism (GLUT-1), cell proliferation (TGF-α), cell adhesion (MIC2), pH regulation (CAIX) and cell survival (TGF-α) among others (Jubb, Buffa and Harris, 2010; Wilson and Hay, 2011; Muz *et al.*, 2015; LaGory and Giaccia, 2016). It has long been known that the concentration of oxygen within the tumour correlates with the efficacy of radiotherapy (Gray *et al.*, 1953). HIF-α can be stabilised in relatively mild hypoxia however much more severe hypoxia plays a larger role in radiotherapy resistance due to the decreased effectiveness of radiotherapy at these very low oxygen conditions. Even so, hypoxia and higher levels of HIF are associated with,

and their presence also correlates with more aggressive tumours, therapy resistance, immunosuppression, metastasis, and poor prognosis (LaGory and Giaccia, 2016). Elevated HIF-1α and HIF-2α levels have also been shown to be associated with poor prognosis in a number of cancers (Giatromanolaki *et al.*, 2001; Ioannou *et al.*, 2009; Zheng *et al.*, 2013; Ren *et al.*, 2016; Roig *et al.*, 2018).

Because of its strong correlation with adverse patient outcome, hypoxic modification in tumours has been an area of intense basic and translational research and drug development. A systematic review of 10,108 patients across 86 trials that were designed to modify tumour hypoxia in patients that received primary radiotherapy alone showed that overall modification of tumour hypoxia significantly improved the effect of radiotherapy but had no effect on metastasis (Overgaard, 2007).

Accelerated radiotherapy with carbogen and nicotinamide (ARCON) which increases tumour oxygenation to improve radiotherapy treatment has shown limited success in a Phase III clinical trial. ARCON with improved 5 year regional control specifically in patients with hypoxic tumours, however, no improvement in disease free or overall survival was found (Janssens *et al.*, 2012).

The hypoxia activated pro-drug Evofosfamide was studied in a Phase III clinical trial. Evofosfamide improved progression free survival as well as higher objective response rate, however the trial failed as the primary endpoint (overall survival time) was not significantly improved (Van Cutsem *et al.*, 2016). Unfortunately, while a few clinical trials have been successful, many hypoxia modification or targeting trials have failed because of underpowered studies and the lack of hypoxia biomarkers to stratify responders among others (Spiegelberg *et al.*, 2019).

Although many of the molecular mechanisms of how cells respond to hypoxia are known, how the hypoxic cells may contribute to poor prognosis is still poorly understood. It is known that hypoxic cells are more resistant to treatment and more likely to disseminate and develop into metastases (Harada *et al.*, 2012; Muz *et al.*, 2015; Godet *et al.*, 2019). However, a direct demonstration of the cell autonomous phenotypes of hypoxic tumour cells within the primary tumour and their interplay with the tumour microenvironment remains understudied.

Using the ODD domain of the HIF-1α protein as an oxygen sensor fused to a tamoxifen inducible $CreER^{T2}$ recombinase, Harada and colleagues elegantly used lineage tracing of hypoxic cells and their progeny in colon carcinoma xenografts (Harada *et al.*, 2012). They showed that hypoxic cells were able to survive irradiation and formed a large proportion of the recurrent tumour after 25 Gy irradiation. High HIF-1 activity was also found in cells that experience radiation-induced re-oxygenation. HIF-1 positive cells after irradiation induced re-oxygenation also translocated towards blood vessels and this translocation was suppressed by HIF inhibitors. Godet *et al*. recently showed through an alternative hypoxia lineage tracing system, that post-hypoxic tumour cells in mice maintain a ROS-resistant phenotype. This provides a survival advantage in the blood stream therefore

promoting their ability to form distant metastases (Godet *et al.*, 2019). One limitation of the aforementioned systems is the relatively long time in continuous hypoxia needed before labelling of cells was achieved, limiting the systems predominantly to areas of sustained chronic hypoxia. These studies also did not visualise individual hypoxic tumour cells within the tumour microenvironment.

In this present study we developed an alternative approach to lineage trace the fate of hypoxic tumour cells that directly reports HIF-1α stabilization rather than the hypoxia transcriptional response. The continuous expression of the system we created allows identification of cells experiencing acute as well as chronic hypoxia and is achieved through a genetically encoded hypoxia sensor composed of a GFP-tagged HIF-ODD-GFP-CreER^{T2} fusion protein herein known as MARCer. Once HIF-1α is stabilised the addition of tamoxifen leads to the Cre-mediated activation of a ubiquitously expressed tdTomato, labelling hypoxic cells and their progeny. These fluorescent markers enabled intravital imaging using window chambers (Kedrin *et al.*, 2008), tracing the fate of hypoxic tumour cells at the single cell level within the primary tumour.

Results

Hypoxia induces eGFP and tdTomato expression in HIF-MARCer reporter cells

To establish a HIF-cell-tracing method (HIF-MARCer) amenable for *in vivo* hypoxia imaging, H1299 non-small cell lung carcinoma cells were transduced with HIF-1α-eGFP-CreER^{T2} complementary DNA (cDNA) (MARCer fusion protein) expression vector and with a loxP-flanked STOP tdTomato cassette (H1299-MR cells; Fig. 1A). Thus, under hypoxia, the tamoxifen-regulated HIF-Cre fusion protein will excise the STOP cassette leading to tdTomato expression, which will persist under normoxia. To test this system, H1299-MARCer cells were exposed to hypoxia (0.2% O_2) or deferoxamine mesylate (DFO; a hypoxia mimetic) *in vitro*, resulting in induction of eGFP and HIF-1α protein expression, which was degraded within minutes after re-exposure to normoxia and corresponded with the levels of the endogenous HIF-1α protein (Fig. 1B,C).

H1299-MR cells were also cultured under hypoxia in the presence of 4-hydroxytamoxifen (4-OHT) (Fig. 1D; Fig. S1B), and eGFP and tdTomato expression were measured by flow cytometry (Fig. 1C-F). Hypoxia (0.1% O_2) induced the expression of eGFP from 6 h of treatment onwards (Fig. S1A,C,E). MARCer stabilisation is visualised through eGFP expression after 24 h exposure to hypoxia (0.2%, Fig. 1C) and cytoplasmic distribution of eGFP after treatment with DFO is visualised in Fig. S1D. tdTomato expression was not visible immediately after exposure to hypoxia and was therefore assessed after re-oxygenation for up to 24 h, and until then tdTomato expression kept increasing, whereas eGFP rapidly decreased upon re-oxygenation (Fig. 1D,F; Fig. S1B). In the absence of 4-OHT, tdTomato

expression was not induced (Fig. S1C,F). The HIF-1 target gene *VEGF* was induced by 0.2% hypoxia and 4-OHT only slightly further induced these levels (Fig. S1E).

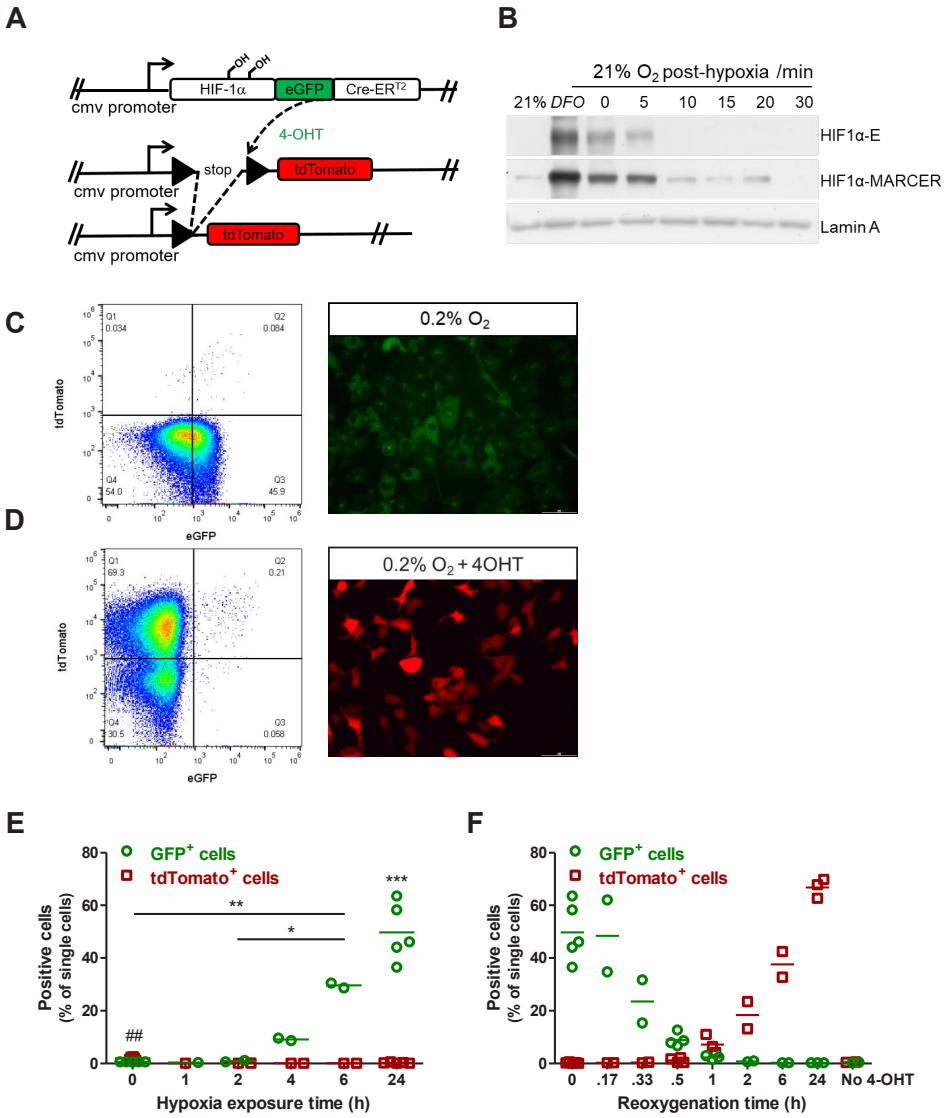

Figure1: H1299-MARCer reporter (H1299-MR) cells were created.

(A) Constructs used for transduction of H1299 cells. (B) Western blot analysis of HIF-1α-MARCer and endogenous HIF-1α (HIF-1α-E) after exposure of H1299-MARCer cells to hypoxia (0.2% O_2) *in vitro* and re-oxygenation. Lamin A was used as a loading control and the HIF-stabilising agent DFO was used as a positive control. (C,D) Fluorescence-activated cell sorting (FACS) plots of eGFP and tdTomato expression after exposure to hypoxia (0.1% O_2) for 24 h (C, left) and 24 h of hypoxia followed by 24 h of re-oxygenation (D, left). Representative images of MARCer stabilisation via eGFP

taken after 24 h exposure to hypoxia (0.2% O_2) (C, right) and after a further 24 h of re-oxygenation of tdTomato expression (D, right) are also shown. Scale bars: 200 µm. (E) Flow cytometric analysis of eGFP and tdTomato expression after exposure to increasing times of hypoxia (0.1% O_2) in the presence of 4-hydroxytamoxifen (4-OHT). Time point '0' is showing cells cultured under normoxia in the presence of 4-OHT for 24 h. Dots represent independent experiments carried out in duplicate and coloured bars indicate averages. $^{\#\#}P<0.01$ indicates a difference in tdTomato expression between no hypoxia and 2 h, and $^{\#\#\#}P<0.001$ shows the difference in tdTomato expression for 0 versus 4, 6, and 24 h. $^{*}P<0.05$ and $^{**}P<0.01$ show a difference for eGFP expression as indicated, and $^{***}P<0.001$ indicates a significantly higher eGFP expression after 24 h compared to 0, 2 and 4 h, as calculated by one-way ANOVA followed by Bonferroni's multiple comparison. (F) Flow cytometric analysis of eGFP and tdTomato expression after exposure to 24 h hypoxia (0.1% O_2) followed by increasing times of re-oxygenation. It should be noted that time point '0' is showing the same data presented in E as 24 h. Dots represent independent experiments carried out in duplicate and coloured bars indicate averages.

Once tdTomato expression was induced, it was stably expressed in H1299-MR cells under normoxic conditions in the absence of 4-OHT for up to at least 4 weeks (Fig. S1F). When tdTomato$^+$ cells were re-exposed to hypoxia, tdTomato expression remained stable (Fig. S1G); however, the fluorescence intensity gradually and significantly declined over time (Fig. S1H).

We conclude that the HIF MARCer allele reliably reports on endogenous hypoxia and HIF activity, and only slightly increases the HIF transcriptional response when 4-OHT is present. By stably inducing tdTomato expression upon administration of tamoxifen we created a reliable tracer of cells exposed to hypoxia with little background fluorescence.

A single administration of tamoxifen induces tdTomato expression in H1299-MR xenografts

H1299-MR cells were injected subcutaneously into the flank of female Balb/c nude mice to grow as xenografts. Once tumour size reached ~100 mm^3, tamoxifen was administered by oral gavage and eGFP and tdTomato expression were assessed by flow cytometry 2 days later (Fig. 2A). eGFP expression could not be detected as this is rapidly degraded after exposure to oxygen (Fig. 1B,F) during sample processing. tdTomato was induced by both 5 mg and 10 mg tamoxifen, and 10 mg was used in further experiments as expression appeared more robust (Fig. 2A). tdTomato expression was followed over time and significantly induced from 5 days after tamoxifen administration onwards and expression did not significantly increase beyond 5 days (Fig. 2B).

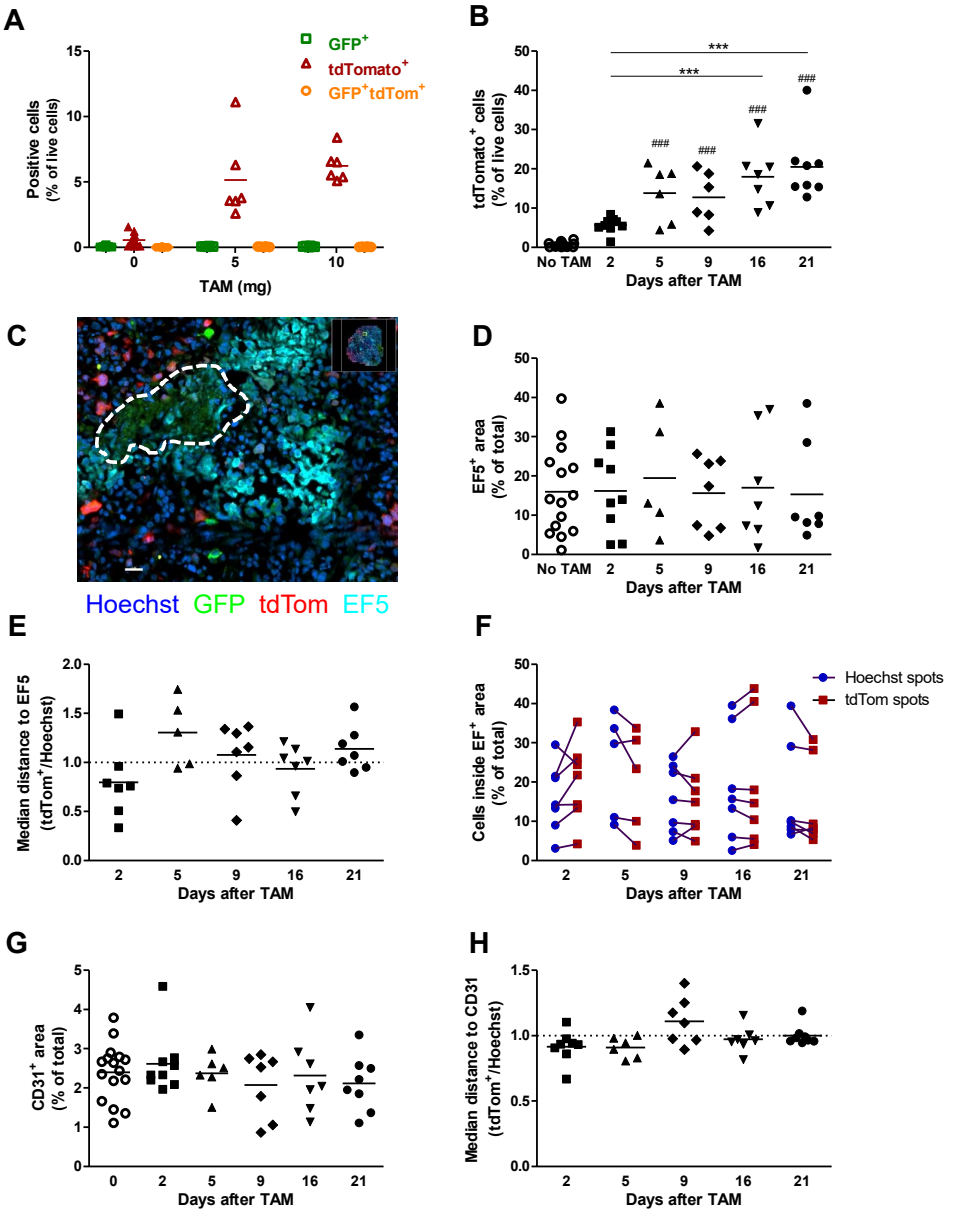

Figure 2: eGFP and tdTomato expression and quantification of immunofluorescent staining of H1299-MR xenografts.

(A) One single administration of tamoxifen by oral gavage induced tdTomato expression in H1299-MR xenografts as measured by flow cytometry 2 days after administration. Dots represent individual mice and bars indicate averages. (B) From 5 days after administration of 10 mg tamoxifen, tdTomato expression was significantly induced. ###P<0.001 compared to no tamoxifen, ***P<0.001 as determined by one-way ANOVA and Bonferroni's multiple comparison. (C) Micrograph of EF5 staining showing a necrotic area (dashed line) surrounded by close and more distant EF5+ staining.

Scale bar: 30 µm. (D) EF5 quantification showing hypoxic area as a percentage of total tumour area. (E) Distance of tdTomato$^+$ cells to EF5$^+$ areas normalised to all cells (Hoechst; Fig. S2C). (F) Cells inside hypoxic areas as a percentage of all cells or tdTomato$^+$ cells. (G) CD31 staining showing vessel density as a percentage of total tumour area. (H) Distance of tdTomato$^+$ to CD31$^+$ areas normalised to all cells (Hoechst; Fig. S2D). For staining (D-H), one to five sections per tumour, separated by ~1 mm, were analysed and the average was indicated by the dots, whereas the bars indicate the average of all mice in the group. TAM, tamoxifen.

tdTomato expression does not significantly correlate with severe hypoxia

To assess whether expression of tdTomato correlated with the extent of hypoxia and vascularisation, we stained frozen tumour sections for EF5 (an exogenous hypoxia marker) (Fig. 2C; Fig. S2A) and CD31 (PECAM1) (Fig. S2B). The EF5$^+$ area did not change over time (Fig. 2D) and did not significantly correlate with tumour size as assessed by a Pearson correlation test on all time points combined (Fig. S3A). EF5$^+$ areas were located both in proximity to and more distant from necrotic areas (Fig. 2C; Fig. S2A). The distance of tdTomato$^+$ cells to EF5$^+$ regions was not different from the general population, nor did it change over time (Fig. 2E; Fig. S2C). tdTomato$^+$ cells were also equally likely to be inside an EF5$^+$ area as the total cell population (Fig. 2F). From these results we conclude that post-hypoxic cells are not more likely to reside in hypoxic areas than other tumour cells. Also, the number of tdTomato$^+$ cells did not correlate with the EF5$^+$ area for any of the assessed time points (Fig. S3B). Finally, eGFP$^+$ cells were occasionally visible (Fig. 2C; Fig. S2A,B). Quantification of eGFP expression appeared impossible due to high autofluorescence of necrotic areas (Fig. 2C, dashed line), and staining for GFP protein did not clearly improve eGFP$^+$ cell detectability (not shown).

Tumour sections were stained for CD31 (Fig. S2B) and the percentage of the tumour area covered by vessels was determined. The percentage of vessel area did not change over time (Fig. 2G), nor did the closest distance of each of the tdTomato$^+$ cells or of all cells to the nearest vessel (Fig. 2H; Fig S2D). Surprisingly, the total areas of EF5 and CD31 positivity did not significantly correlate (Pearson correlation test on all time points combined, Fig. S3C), as we would expect a larger EF5$^+$ area to correlate with a lower vessel density and therefore the CD31 area. This could be due to areas of increased oxygen demand, limited perfusion or vessel leakiness, in addition to cycling hypoxia.

RFP staining and tdTomato fluorescence show a similar expression pattern after administration of tamoxifen

Tumour sections were stained using antibodies against RFP by immunofluorescence in order to detect the tdTomato protein (Fig. S4). More RFP$^+$ cells were detected by immunofluorescence than by imaging intrinsic tdTomato, indicating that not all tdTomato$^+$ cells are detected by direct fluorescence (Fig. 3A). However, tdTomato fluorescence and RFP immunofluorescence showed a strong correlation (Fig. 3B) and the same expression pattern after tamoxifen (Fig. 3A), a trend that was also similar to

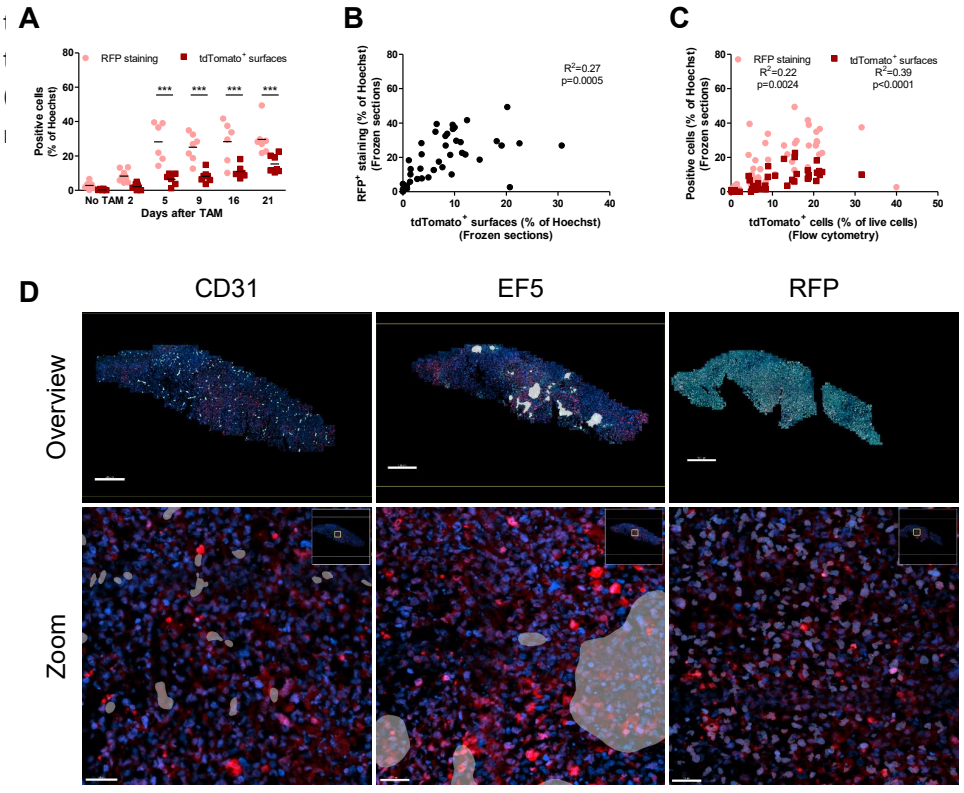

Figure 3: Immunofluorescent staining of H1299-MR xenografts.

(A) Quantification of RFP staining showing that more tdTomato$^+$ cells can be detected after staining than when only intrinsic tdTomato was imaged by epifluorescence microscopy. Dots represent the average of one to five sections per tumour, separated by ~1 mm. ***P<0.001 as determined by two-way ANOVA followed by Bonferroni's multiple comparison. (B) RFP staining and intrinsic tdTomato on frozen sections significantly correlate. (C) RFP staining and intrinsic tdTomato significantly correlate with tdTomato cells measured by flow cytometry. (D) Micrographs of consecutive sections showing that CD31 staining (left) and RFP staining (right) do not show a clear correlation with EF5 staining (middle). Scale bars: 500 µm (top row), 50 µm (bottom row). TAM, tamoxifen.

Post-hypoxic H1299-MR cells proliferate faster than non-hypoxic tumour cells

Next, we assessed the fate of the tdTomato$^+$ post-hypoxic tumour cells. 5-Ethynyl-2'-deoxyuridine (EdU) was administered to mice 3 h before sacrifice, and proliferation in xenografts was measured by flow cytometric analysis of EdU incorporation. At all measured time points, tdTomato$^+$ cells proliferated faster than tdTomato$^-$ cells, comprising both non-hypoxic tumour cells and host cells within the tumour microenvironment (Fig. 4A). This was confirmed by immunofluorescent staining of EdU on 4% paraformaldehyde (PFA)-fixed frozen sections (Fig. 4B; Fig. S5A). Fig. S5B shows that EdU background staining

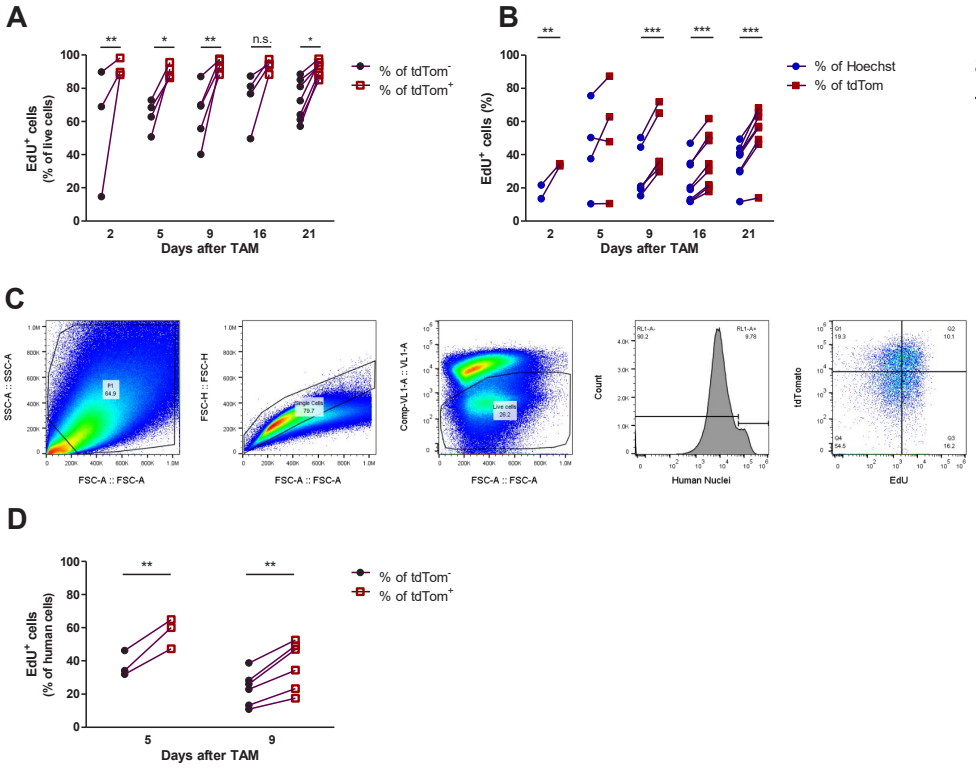

Figure 4: Post-hypoxic H1299-MR cells proliferate faster than non-hypoxic tumour cells.

(A,B) EdU assay showing that tdTomato$^+$ cells proliferated faster than the tdTomato$^-$ population as measured by flow cytometry (A) and immunofluorescence (B). (C) Gating strategy of EdU incorporation in tdTomato$^-$ and tdTomato$^+$ human cells (RL1-A$^+$). (D) EdU proliferation assay showing that tdTomato$^+$ human cells proliferated faster than the tdTomato$^-$ human cells. Dots represent individual mice and paired observations were connected with a line. n.s., non-significant; **P*<0.05, ***P*<0.01, ****P*<0.001 as determined by two-way ANOVA followed by Bonferroni's multiple comparison. TAM, tamoxifen.

Increased proliferation of post-hypoxic H1299-MR tumour cells is a non-cell-autonomous feature

To address whether the increased proliferation was a cell-autonomous acquired and stable feature of post-hypoxic tumour cells, H1299-MR xenografts were excised at 5-17 days after administration of tamoxifen and single-cell suspensions were cultured *ex vivo*. Antibiotic selection enriched for tumour cells and the percentage of tdTomato$^+$ cells in culture increased initially, after which it stabilised (Fig. 5A). EdU incorporation showed that tdTomato$^+$ and tdTomato$^-$ cells proliferated at the same rate at all passages (Fig. 5B). H1299-MR cells were also incubated at 21% and 0.2% O_2 with 200 nm 4-OHT for 24 h, mixed in a 1:1 ratio and grown in Dulbecco's modified Eagle medium (DMEM) containing

1% foetal bovine serum (FBS). Approximately 20-30% of the cells were tdTomato$^+$ and expression did not change as measured by flow cytometry after 3 and 15 days, indicating that proliferation of tdTomato$^+$ and tdTomato$^-$ cells was similar *in vitro* (Fig. 5C). These results show that tumour cells previously exposed to hypoxia *in vivo* do not proliferate faster *ex vivo*, and that the observed increased proliferation of post-hypoxic tumour cells is influenced by factors in the tumour microenvironment.

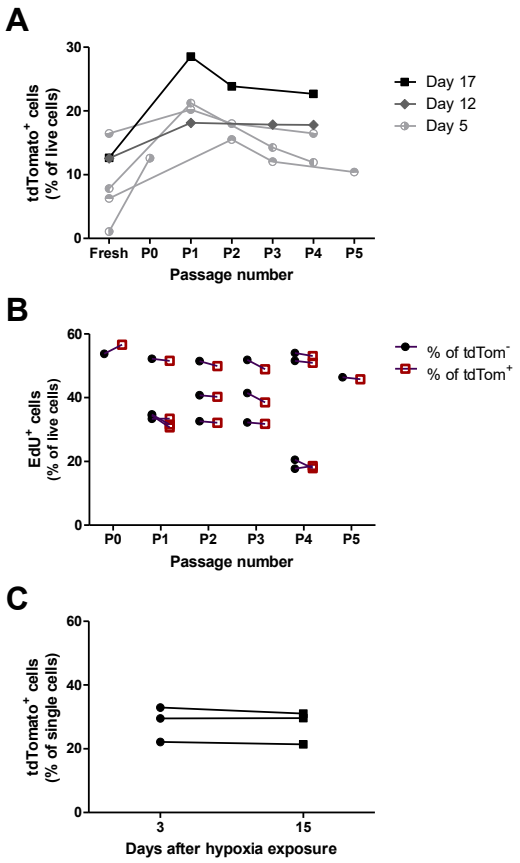

Figure 5: Post-hypoxic tumour cells and non-hypoxic cells proliferate at similar rates *ex vivo* and *in vitro*.

(A,B) Cells isolated from H1299-MR xenografts were cultured *ex vivo* under geneticin and blasticidin selection. Individual tumours were depicted and connected with a line. (A) tdTomato expression initially increased and stabilised after several cultured passages as measured by flow cytometry. (B) In *ex vivo* culture, tdTomato$^+$ and tdTomato$^-$ cells proliferate similarly, as shown by EdU incorporation measured by flow cytometry. (C) H1299-MR cells were incubated at 21% and 0.2% O_2 with 200 nm 4-OHT for 24 h before being mixed in a 1:1 ratio and grown in DMEM containing 1% FBS. tdTomato expression was then analysed and proved similar after 3 and 15 days.

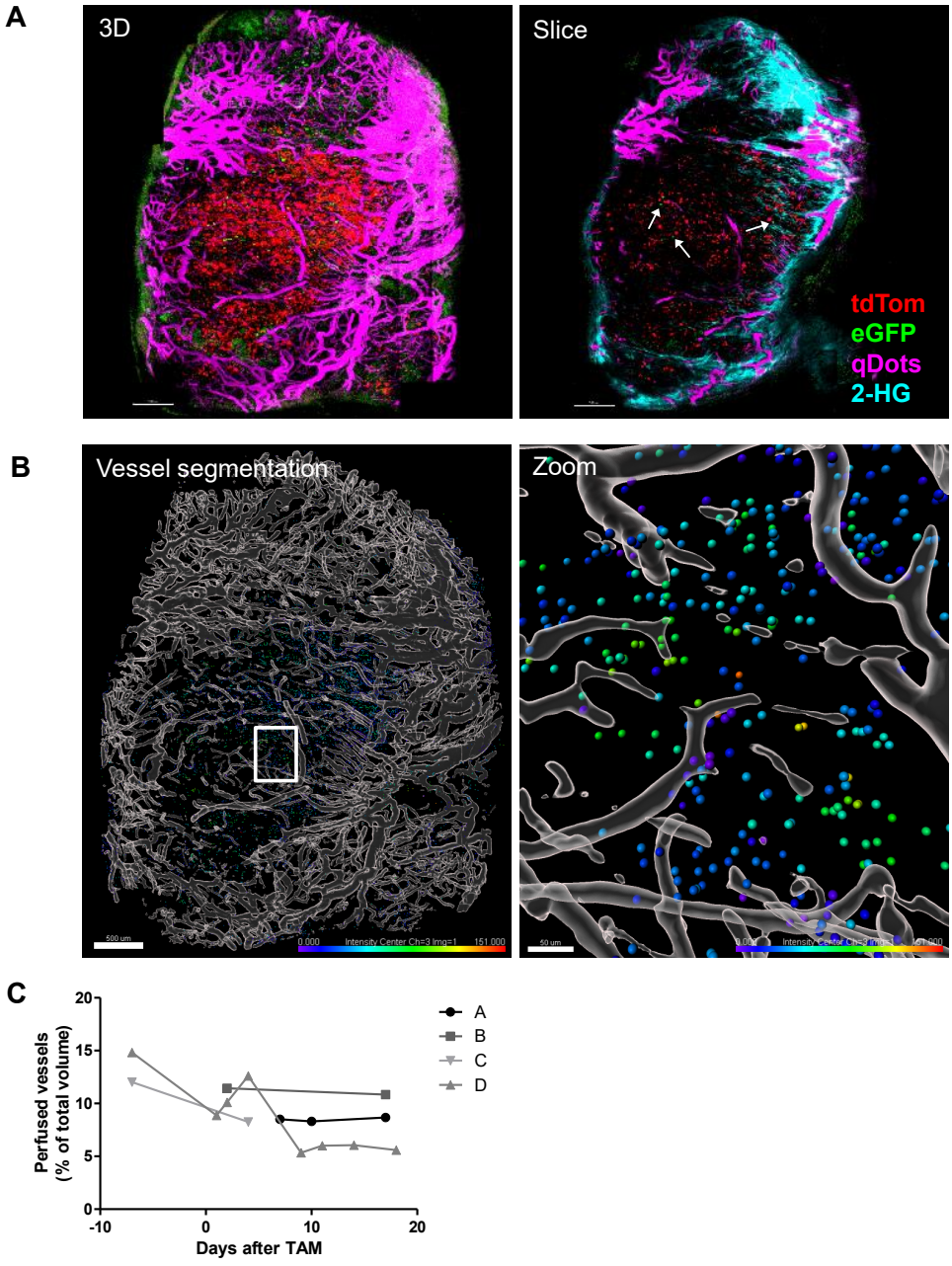

Figure 6: H1299-MR xenografts visualised using intravital microscopy.

(A) 3D maximum-intensity projection (left) and one slice of the same tumour (right) imaged 4 days after administration of tamoxifen. Perfused vessels are shown in purple, eGFP^{+} cells in green, tdTomato^{+} cells in red and collagen in cyan (second harmonic generation microscopy, right panel only). Channel arithmetics was applied using MATLAB to subtract GFP bleed through into the

tdTomato channel and tdTomato bleed through into the Qtracker 705 channel. Scale bars: 500 µm (B) Vessel segmentation (grey) and tdTomato$^+$ cells shown in the colour spectrum, indicating the distance to perfused vessels (0 µm in purple to 151 µm in red). Scale bars: 500 µm (left) and 50 µm (right). (C) Perfused vessel volume as a percentage of total tumour volume in four mice followed over time. TAM, tamoxifen.

Intravital imaging visualises hypoxic cell tracing at the single-cell level in xenografts

Next, we used intravital microscopy to identify hypoxia lineage tracing *in vivo* in xenograft tumours. H1299-MR xenografts were covered by an imaging window to allow intravital imaging by multiphoton microscopy and tumours were followed for up to 18 days after administration of tamoxifen (Fig. S6). FITC-Dextran or qTracker 705 were injected intravenously and tumours were imaged for vessel perfusion, eGFP and tdTomato, and by second harmonic generation microscopy (Fig. 6A; Fig. S6A-D). Fig. 6A shows an example of a tumour imaged 4 days after administration of tamoxifen and other time points are shown in Fig. S6D. tdTomato was not observed before administration of tamoxifen (Fig. S6B-D). Occasionally eGFP$^+$ cells were observed (Fig. 6A, arrows). These results demonstrate that we were able to track post-hypoxic tumour cells in a spatiotemporal manner using intravital microscopy.

To assess tumour perfusion, we injected qTracker 705 and segmented and reconstructed the vessels with Imaris (Fig. 6B). The colour spectrum indicates the distance of tdTomato$^+$ cells to perfused vessels. The median distance of tdTomato$^+$ spots in this tumour was 27.4 µm, with a distribution of 0-151 µm. These results indicate that tdTomato cells were located in proximity to, as well as further away from, the vessels (Fig. 6B, right); however, this was not normally distributed. Cells were more likely to be close to a vessel, with 50% of the cells being within 27.4 µm of a vessel in the displayed tumour and an average of 35.5 µm for all tumours (not shown). Purple cells (Fig. 6B, right) with a distance of 0 µm from a vessel appeared to be touching the vessel, rather than circulating inside it. Total vessel perfusion was calculated from the segmented vessels and remained constant over time in two of the four mice (Fig. 6C, curve A and B, corresponding to mice represented in Fig. S6A,B), whereas vessel volume decreased in mouse C and D (Fig. S6C,D). Overall, tumours seemed well perfused, which is in line with total vessel area as shown by CD31 staining (Fig. 2G).

Discussion

Here, we describe a novel system to lineage trace hypoxic cells. We show that the system is robust *in vitro* with hypoxia-regulated eGFP expression and constitutive tdTomato labelling upon addition of tamoxifen in the progeny of these hypoxic tumour cells. We also see that the MARCer reporter system does not interfere with endogenous

HIF-1α protein expression and the transcription of the HIF target gene *VEGF*, and this expression is only mildly affected by tamoxifen and only during the short period of tamoxifen administration. *In vivo*, tdTomato expression was induced; however, the amount of expression or the distribution did not correlate with EF5 positivity at any of the investigated time points. Intravital imaging showed that the tumours were well perfused, which is supported by the regular distribution of vessels throughout the tumour and the lack of correlation between vessels and $EF5^+$ areas, as shown by immunofluorescence, suggesting also alternative mechanisms of HIF stabilisation.

With the H1299-MR model, *in vitro* exposure to hypoxia induced eGFP expression. However, eGFP was barely detectable *in vivo* with our microscopy systems However, given the clear presence of hypoxic areas as shown by EF5 staining, lack of HIF-1α – and thus eGFP – seems unlikely. This is likely to be due to the requirement of O_2 for GFP folding and fluorescence (Heim *et al*., 1994; Kumagai *et al*., 2013), making it a suboptimal fluorescent marker to track current hypoxia. On the other hand, Godet *et al*. were able to visualise GFP under 0.5% O_2 (Godet *et al*. 2019). Also, processing of the samples exposes them to atmospheric oxygen, possibly introducing enough oxygen for GFP maturation *ex vivo*. Despite these limitations we were able to detect eGFP in H1299-MR xenografts using immunofluorescence and intravital microscopy.

Whether limited eGFP expression in our model is related to lack of expression or lack of visibility requires further investigation. An alternative approach could be to replace eGFP with fluorescent proteins not requiring oxygen such as UnaG (Kumagai *et al*., 2013). Possibly, eGFP expression is truly low in the currently studied tissues, which could be due to the length of time the cells are exposed to cycling hypoxia, not allowing enough time for sufficient MARCer accumulation before re-oxygenation and MARCer degradation. This is supported by our current finding that the amount of EF5 expressed in the tissue does not correlate with the number of $tdTomato^+$ cells found in the tissue. An alternative explanation is that H1299 NSCLC cells express high levels of ROS, a potent activator of HIF-1α (Jung *et al*., 2008; Lee *et al*., 2010). Activation of HIF via ROS could partially explain the lack of overlap and correlation between EF5, HIF-eGFP and tdTomato labelling.

Surprisingly, neither the fraction of $tdTomato^+$ cells and extent of EF5, nor the distance of those cells to EF5 correlated significantly, indicating that post-hypoxic tumour cells are randomly distributed with regard to the current hypoxic status of the tumour. This may be due to the dynamic nature of hypoxia in tumours and the time of assessment, i.e. the time after which the labelled cells were exposed to hypoxia, at least 48 h after tamoxifen administration. Because the MARCer construct is constitutively expressed, short episodes of hypoxia could induce labelling, but this might not be seen in proximity to EF5 due to re-oxygenation between the time tamoxifen was given and injection of EF5. Other research has also shown that hypoxic cells move out of hypoxic $EF5^+$ or $pimonidazole^+$ areas (Harada *et al*., 2012; Erapaneedi *et al*., 2016; Conway *et al*., 2018; Godet *et al*., 2019), followed by random distribution. The transient nature of the hypoxic state may explain that the

induction of tdTomato expression does not correlate with the level of acute hypoxia 2, 5, 9, 16 or 21 days later. One way to further investigate this would be through the use of a second marker of hypoxia, given together with tamoxifen. This would allow visualisation of the hypoxia dynamics and see the change in hypoxia between labelling and endpoint. Taken together, EF5 reports on chronic severe hypoxia and anoxia, whereas HIF labelling captures also transient tumour hypoxia at moderate O_2 levels. It also seems evident that many cells that have previously been exposed to hypoxia have redistributed to much less hypoxic regions.

In preliminary results with this system, it was apparent that a greater proportion of post-hypoxic cells tdTomato$^+$ cells were undergoing proliferation compared to the non-hypoxic cells. In a range of common cancer types, HIF-1α was positively associated with the proliferation marker Ki67 (Mki67) (Zhong *et al*., 1999), and several studies report that HIF overexpression in cells can promote cell proliferation (Medici and Olsen, 2012).

While many cell types including cancer cells proliferate more slowly under hypoxia, here we are tracking the uptake of EdU by cells previously exposed to hypoxia. Whether this enhanced proliferation proves to be generalisable remains to be investigated, but might help explain why hypoxia may trigger more aggressive tumours. (Hubbi and Semenza, 2015; Al Tameemi *et al.*, 2019). Blouw and co-workers found a microenvironment-dependent effect of HIF-1α knockdown on tumour progression which might be relevant here. Koshiji et al. (Koshiji *et al.*, 2004) and Hubbi et al. (Hubbi *et al.*, 2013) showed HIF-1α stabilisation induced cell cycle arrest, whereas in our model, HIF-1α was most likely degraded at the time we measured proliferation. It remains to be investigated what mechanisms are responsible for the long-term effect of hypoxia on proliferation in post-hypoxic tumour cells and whether this is dependent on HIF-1α. The H1299-MR model proves to be a promising tool to study this and other long-term effects of hypoxia including its role in metabolic plasticity and metastasis. Moreover, our finding that post-hypoxic tumour cells proliferated faster *in vivo* but not *in vitro* or *ex vivo* demonstrate that this acquired feature is un-stable and non-cell autonomous. It will be of interest to identify what factors in the tumour microenvironment contribute to this.

Other approaches exploiting fluorescent markers driven by hypoxia-responsive elements and oxygen-dependent degradation domains have been described (Erapaneedi *et al.*, 2016; Wang *et al.*, 2016). Using intravital microscopy, Wang et al. visualised migration of individual normoxic and hypoxic MDA-MB-231 cells in a xenograft model. Similar to our findings, they also reported the presence of hypoxic cells both in proximity and distant from blood vessels. However, they did not quantify whether the distance was different from normoxic cells and different to our current study, Wang et al. studied cells currently experiencing hypoxia whereas we mainly focused on post-hypoxic cells (Wang *et al.*, 2016). Tracing of recently re-oxygenated cells was also performed by Erapaneedi et al. (Erapaneedi *et al.*, 2016), however their system using the fluorescent marker mOrange is dependent on PEST-sequence-dependent decay, making it less robust and more

challenging to visualise by intravital microscopy, whereas we show stable expression of tdTomato for at least 4 weeks in post-hypoxic cells. Thus, the HIF-MARCer system is a useful addition to the armamentarium to visualize hypoxic, HIF-expressing cells. We show here that H1299-MR cells are a valuable tool to study the long-term fate of hypoxic cells, for example by using intravital microscopy.

In conclusion, our results demonstrate that single-cell lineage tracing of post-hypoxic tumour cells using the H1299-MARCer system allows visualisation of their behaviour in living tumours using intravital microscopy. We provide a valuable tool to study the dissemination and treatment response of post-hypoxic tumour cells *in vivo* and *ex vivo* at a single-cell resolution. Using this system, we provide evidence that post-hypoxic tumour cells may have a proliferative advantage over non-hypoxic tumour cells and that this is influenced by the tumour microenvironment.

Materials and Methods

Generating the Lenti MARCer system

First, an eGFP CreER^{T2} fusion single primer PCR was performed on plasmid pL451-Dll1(GFP-ires-CreER^{T2}), a kind gift from Johan van Es (van Es et al., 2012), with primer 5'-GGCATGGACGAGCTGTACAAGTCCAATTTACTGACCGTACAC-3' and on pEGFP-C1 (Clontech) with primer 5'-GGCATGGACGAGCTGTACAAG-3'. After the reaction, these template plasmids were digested with DpnI, before mixing 1 µl of each reaction for a fresh PCR with primer pair 5'-GTGAGCAAGGGCGAGGA-3' and 5'-CCAGACATGATAAGATACATTGATGAG-3' to amplify the fusion product with proofreading Phusion DNA Polymerase (FynnZyme). This GFP-CreER^{T2} PCR fragment was gel purified, and T overhangs were added with normal Taq polymerase before sub cloning it in a pCR-XL-TOPO® vector (Invitrogen), generating pCR-GFP-CreER^{T2} vector. Afterwards, this vector was used as a template to generate in a similar approach the fusion with HIF-1α. A HIF-1α '1.5 kb guide DNA fragment was amplified from BAC clone RP11: 618G20 (GenBank: AL137129.4), using primers 5'-TGGATCCGAGCTCGGTACCATAGATCTGAACATTATAACTTGATAAATGAGG-3' and 5'-AGCTCCTCGCCCTTGCTCACCTGGAATACTGTAACTGTGC-3'. The product, a fusion of HIF-1α in exon 12 with 20 nucleotides of the cDNA GFP, was cloned by use of the NEBuilder® HiFi DNA assembly cloning kit in the pCR-GFP-CreER^{T2} plasmid, generating pCR-HIF-LHA-GFP-CreER^{T2}. On this plasmid, a single primer PCR with primer 5'-GCAAGCCCTGAAAGCGCAAG-3' and the cDNA human HIF-1α in a p3XFLAG-CMV™-10 expression vector (Sigma-Aldrich, St Louis, MO, USA) (Gort et al., 2008) was used as a template with a complementary single primer PCR with primer 5'-CTTGCGCTTTCAGGGCTTGC-3'. After the reaction, the template plasmids were digested with DpnI, before mixing 1 µl of each reaction for a fresh PCR with primer pair 5'-GATATCGGTACCAGTCGACTC-3' and 5'-GTGGTACCCGTCATCAAGCTGTGGCAGGGA-3', amplifying the MARCer cDNA (Fig. 1A,

top), which was subcloned in a pCR®-Blunt II-TOPO® vector, generating pCR-BluntII-MARCer. Flanked by BstXI sites, the MARCer cDNA was, after digestion, retrieved by gel purification to replace luciferase in the BstXI-digested pLenti CMV V5-LUC Blast (w567-1), Addgene plasmid #21474, deposited by Eric Campeau (Campeau et al., 2009), generating pLenti-CMV-MARCer-Blast. This plasmid expresses amino acids 1-603 of the human HIF-1α protein fused to eGFP-CreER^{T2}.

The Ai65(RCFL-tdT) targeting vector, Addgene plasmid #61577, deposited by Hongkui Zeng (Li et al., 2015), was digested with BstBI-AscI, and a 6267 bp fragment was isolated and cloned into an empty BstBI-AscI-digested pLVX-puro vector with an introduced unique AscI site to fuse proteins to FLAG and HA tags at the carboxy terminus (Groot et al., 2014). Next, the FLAG-HA-PGK-Puro-WPRE fragment was removed from this vector with an AscI-KpnI digest followed by blunting of the DNA ends with Mung Bean and ligated back. Finally, the FRT-STOP-FRT cassette was removed with a digest with XhoI and the vector was back ligated to generate the pLV(cmv)-NEO-SFS-tdTomato Cre reporter plasmid. The full integrity of all constructs was confirmed by DNA sequencing.

Generation of the H1299-MR cell line

Viral particles were produced using viral vectors and packaging plasmids in 293FT cells as previously published (Groot et al., 2014). H1299 cells on which we performed STR analysis were first transduced with MARCer viruses and cells were selected with 10 µg/ml blasticidin in the 10% FBS RPMI culture medium, supplemented with penicillin/streptomycin. Transduced cells were single-cell seeded to form clones on 15 cm dishes. Next, clones were harvested by use of glass clonal cylinders (Sigma-Aldrich) with Baysilone-Paste (GE Bayer Silicones) and expanded. Clones were split and transiently transfected with the reporter plasmid described above and screened for their switching capacity with the addition of 100 nM DFO and 100 nM 4-OHT (H7904-5MG, Sigma-Aldrich). We identified clone number 12 to show most tdTomato expression after treatment. Next, clone 12 was subsequently transduced with SFS-tdTomato viruses and clones were selected after this second transduction and were selected with 10 µg/ml blasticidin and 1000 µg/ml G418 in the 10% FCS RPMI culture medium, supplemented with penicillin/streptomycin. The polyclonal cell population was exposed to 1% of oxygen for 24 h in a Russkinn INVIVO 1000 hypoxic chamber. Next, the recovered cells were passed in culture at normoxic conditions for 2 days. To these cells, 100 nM 4-OHT was added to the medium for 24 h. To select for cells without leakage, cells were single-cell seeded and colourless clones were picked as described above. Clones were expanded and split into 250,000 cells per six-well plate and exposed to 200 nM 4-OHT under hypoxia and normoxia for 24 h, before being trypsinised and expanded for 3 days in culture flasks. We identified H1299 clone 12.3 (H1299-MR) as a robust hypoxia reporter cell line that we characterised and used in our further experiments.

Cell culture and hypoxia

H1299-MR cells were cultured in 10% FBS (Gibco) RPMI culture medium (Sigma-Aldrich) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich), 10 µg/ml blasticidin (InvivoGen) and 1000 µg/ml G418 (Sigma-Aldrich). Cells were regularly tested for mycoplasma and incubated in a humidified incubator or hypoxia chamber Ruskinn InVivo 300 (Fig. 1C, left, D, left, E,F; Fig. S1A,B,G,H) or InVivo 1000 (Fig. 1B,C, right, D, right, Fig. 5C; Fig. S1C-F) with or without 4-OHT. Images during cell culture were taken with a Nikon eclipse Ts2 microscope.

Western blotting

Cells were lysed in RIPA buffer, containing 50 mM Tris·HCl, 0.5% DOC, 0.1% SDS, 1 mM EGTA, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 150 mM NaCl and 1 mM PMSF, and protease inhibitors (ROCHE pill Complete Inhibitor). Protein concentrations were determined with the Bradford Protein Assay (Bio-Rad). Proteins (30 g) were separated by 6% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat dry milk (Marvel) and subsequently incubated (overnight, 4°C) with primary antibodies anti-HIF-1α (1:1000; #610959, BD Biosciences) and anti-lamin A (1:1000; #L1293, Sigma-Aldrich), and then horseradish peroxidase-linked secondary antibodies (horse anti-mouse and horse anti-rabbit; 1:2500; Cell Signaling Technology). ECL Prime Western Blotting Detection Reagent (GE Healthcare) was used for visualisation.

RNA isolation and quantitative PCR

mRNA was extracted using the Nucleospin RNA II kit (Bioke), and cDNA conversion was performed using an iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. Quantitative PCR was performed on a CFX96 (Bio-Rad). The expression of VEGF (F: 5'-GACTCCGGCGGAAGCAT-3'; R: 5'-TCCGGGCTCGGTGATTTA-3') was detected with SYBR Green I (Eurogentec). Gene expression was normalised to *Rpl13a* (F: 5'-CCGGGTTGGCTGGAAGTACC-3'; R: 5'-CTTCTCGGCCTGTTTCCGTAG-3') mRNA expression.

Mice

All animal studies were performed according to the Animals Scientific Procedure Act 1986 (UK) and approved by local ethical review. Female Balb/c nude mice were obtained from Envigo and kept in individually ventilated cages with unlimited supply of food and water and 12 h light-dark cycles, and mice were weighed twice a week. H1299-MR cells were harvested, dissolved in a single-cell suspension with Matrigel (Corning Life Sciences, 1:1) and 10^6 cells were injected subcutaneously into the flank of 6- to 10-week-old mice. Once tumours reached ~100 mm^3, tamoxifen (TAM, Sigma-Aldrich) was dissolved in vegetable oil containing 5% ethanol and administered through oral gavage. At the end of the experiment, mice were injected intraperitoneally with EF5 [2-(2-nitro-1/-/-imidazol-l-yl)-*N*-(2,2,3,3~-pentafluoropropyl)acetamide], a nitroaromatic compound stabilised in

the absence of oxygen (Lord et al., 1993), a kind gift from Prof. Cameron Koch (University of Pennsylvania, Philadelphia, PA, USA) and EdU (Santa Cruz Biotechnology) 3 h before sacrifice. Tumours were harvested, rinsed in PBS, cut in half and processed for flow cytometry or immunofluorescence.

Flow cytometry

Tumours were collected, halved, kept in Hanks' balanced salt solution (HBSS; Gibco) and chopped into small pieces using a scalpel. Tumours were then digested in HBSS (Gibco) containing Collagenase Type 2 (Worthington Biochemical Corporation) and DNAse I (Thermo Fisher Scientific) for 40 min at 37°C in a shaking incubator. Cells were filtered through a 50 µm strainer (Sysmex Partec) and rinsed with FACS buffer (PBS containing 5% FBS) and centrifuged for 5 min at 300 *g*, 4°C and rinsed again. Cells were incubated for 15 min with LIVE/DEAD Fixable Violet Dead Cell Stain (Thermo Fisher Scientific), and a maximum of 2×10^6 cells was used for EdU staining using the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay according to manufacturer's instructions (Thermo Fisher Scientific). The remaining cells were fixed using intracellular fixation buffer (1:1 in PBS, Thermo Fisher Scientific) for ~10 min at room temperature (RT). Cells were stored in IC fixation buffer at 4°C until analysis on the Attune NxT Flow Cytometer (Thermo Fisher Scientific), when they were centrifuged for 5 min at 400 *g* and dissolved in FACS buffer. For compensation purposes, H1299-MR cells were cultured *in vitro* and subjected to DFO treatment (eGFP, channel BL-1), TAM+ hypoxia and re-oxygenation (tdTomato, channel YL-1), 5 min at 65°C (LIVE/DEAD, channel VL-1, 1:1 with untreated cells), EdU (Click-iT assay, channel BL-1) or left untreated (unstained control and human nuclei, channel RL-1). When stained for human nuclei (1:100; MAB1281, Merck Millipore; Fig. 4C,D), this was performed for 30 min at RT in the dark after EdU staining (Click-iT Assay) and blocking. AF647 donkey anti-mouse IgG (1:500; Thermo Fisher Scientific) was used as a secondary antibody.

Cells cultured *in vitro* that were used for flow cytometry were rinsed and scraped in PBS, and added to IC fixation buffer (1:1 in PBS) inside the hypoxia chamber. They were then taken out of the chamber and incubated, together with cells not exposed to hypoxia, for ~30 min at RT. Cells were stored in IC fixation buffer at 4°C until analysed, they were centrifuged for 5 min at 400 *g* and dissolved in FACS buffer. An example of the gating strategy was shown in Fig. 1C,D and analyses were performed in FlowJo (BD).

Ex vivo cell culture

When cells from xenografts were cultured *ex vivo*, tumours were harvested and put in RPMI complete medium without selection antibiotics. They were then digested as described above. From this point, cells were kept under sterile conditions, filtered through a 30 µm strainer (Sysmex Partec) and rinsed with PBS containing 2% BSA and 5 mM EDTA (both Sigma-Aldrich). After centrifugation for 5 min, 300 ***g*** at 4°C, the pellet was resuspended and incubated for 3 min in red cell lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM

EDTA). Cells were rinsed twice with PBS/BSA/EDTA and dissolved in PBS. Half of the cells was taken into culture with RPMI complete medium and 10 µg/ml blasticidin and 1000 µg/ml G418 and the other half was stained for LIVE/DEAD, fixed and analysed by flow cytometry. At several culture passages, cells were harvested with Trypsin/EDTA (Sigma-Aldrich), filtered through a 30 µm strainer and stained for LIVE/DEAD and EdU as described above, and analysed by flow cytometry.

Immunofluorescence and microscopy

Tumour halves were rinsed in PBS and fixed for 3-4 h in 4% PFA at 4°C. They were transferred to 30% sucrose in PBS solution and kept overnight in the fridge and consecutively snap frozen in OCT embedding medium (Thermo Fisher Scientific) and stored at −20°C until cutting. Cryosections (10 µm) were cut using a Bright Cryostat or a Leica CM1950, dried overnight at 37°C and stored at −80°C until staining.

Sections were allowed to dry at room temperature for at least 30 min, rinsed in PBS and permeabilised with 0.5% Triton X-100 in PBS. For staining of CD31, 5% BSA (Sigma-Aldrich) and 5% donkey serum (Sigma-Aldrich) were added to the permeabilisation solution and this was also used for blocking for 1 h at RT. Blocking for RFP staining was done with 10% normal goat serum (Sigma-Aldrich) in PBS for 1 h at RT and with TNB blocking buffer (PerkinElmer) for 30 min for EF5 staining. Staining was performed overnight at 4°C with Cy5-conjugated EF5 antibody or Cy5-EF5 antibody containing competitor (both purchased from University of Pennsylvania and diluted 1:1 in PBS), rabbit anti-RFP (600-401-379, Rockland) or rabbit IgG (BD Biosciences) at 1:500 in PBS, and goat anti-CD31 (AF3628, R&D Systems) or goat IgG (R&D Systems) at 1 µg/ml. Sections stained for EF5 were washed with PBS/Tween 20 0.3% twice for 45 min. Sections stained for RFP and CD31 staining were washed 3× in PBS and incubated for 30 min at RT with secondary antibodies AF633 goat anti-rabbit and AF647 donkey anti-goat, respectively, at 1:500 dilution. The Click-iT EdU Cell Proliferation Kit for Imaging (Thermo Fisher Scientific) was used according to the manufacturer's instructions, and a solution containing only the Alexa Fluor picolyl azide in PBS/BSA 3% was used as a negative control. All sections were counterstained with Hoechst 33342 (Thermo Fisher Scientific) and mounted in Prolong Diamond Antifade Mountant (Thermo Fisher Scientific). Entire sections were imaged using a Nikon-NiE epifluorescence microscope with a 20× objective. Images were stitched with NIS Elements (Nikon) and processed with Imaris software (Bitplane). To get an overview of the entire tumour, one to five sections per tumour, separated by ~1 mm, were analysed and the average was displayed.

Abdominal imaging window and intravital microscopy

Under inhalation anaesthesia with isoflurane, an imaging window was placed onto the abdominal wall of a mouse as previously described (Ritsma et al., 2013) with the following adjustments. A titanium window was used and a coverslip was glued on top with BBRAUN

Histoacryl (Akin Global Medical). Vetergesic was injected subcutaneously as analgesic. An incision was made in the skin, after which 5×10^5 H1299-MR cells in 30% Matrigel in 5 µL were injected into the thin fat layer above the abdominal muscle. The window was stitched to the muscle layer using 5-0 silk and then secured to the skin with 5-0 prolene sutures (Ethicon). After ~6 weeks, when tumours became visible by eye, 10 mg tamoxifen in 100 µl 5% ethanol/oil mixture was administered by oral gavage. At every imaging session, windows were carefully cleaned with an insulin syringe using 0.9% NaCl and all liquid and air between the coverslip and the tumour was removed. FITC-Dextran (MW 500, Sigma-Aldrich) or Qtracker 705 (Thermo Fisher Scientific), diluted 1:10 in sterile saline, was administered via the cannulated tail vein by a bolus injection of 2×12.5 µl, followed by a rate of 70 µl/h using an automated pump (Harvard Instruments). Tumours were imaged using a ZEISS LSM 880 microscope with a Mai-Tai laser (Newport Spectra-Physics, 940 nm excitation) using a ZEISS 20×/1.0 NA water objective covered with ultrasound gel. For detection of Qtracker 705, a 670 nm shortpass filter was used, whereas bandpass filters of 457-487 nm were used for collagen (second harmonic generation microscopy, 2-HG), of 488-512 nm for eGFP/FITC and of 562.5-587.5 nm for detection of tdTomato. Tile scans were taken up to 350 µm deep with a voxel size of 0.83×0.83 in *x-y* and 5 µm in *z*, and stitched using ZEN Black Software (Carl ZEISS AG). Quantitative analyses were performed using Imaris.

Image analyses

All analyses were performed using Imaris software. First, the total tumour was outlined, background such as folds were excluded as much as possible and signal outside the tumour area was removed. For EF5 staining, hypoxic areas directly underneath the skin were excluded from the analysis. For stained sections, masks were created for Hoechst, tdTomato, and staining using the 'surfaces' or 'spots' functions and thresholds adjusted for each imaging session. All analyses were checked by eye and when the mask did not visually represent the positively stained area, the data were excluded from the analysis. A distance map from $CD31^+$ and $EF5^+$ areas was created using MATLAB and the median distance of $tdTomato^+$ spots and $Hoechst^+$ spots to $CD31^+$ and $EF5^+$ surfaces was calculated. For analyses of EdU, $Hoechst^+$ cell surfaces were masked. These were filtered for an EdU^+ threshold, either or not preceded by the $tdTomato^+$ threshold and expressed as a percentage of total. RFP^+ cells were also filtered by an intensity threshold on $Hoechst^+$ surfaces.

For intravital images, channel arithmetic was applied using MATLAB to subtract GFP bleed through into the tdTomato channel and tdTomato bleed through into the Qtracker 705 channel. A surfaces mask was created on the Qtracker 705 signal and the perfused-vessel volume was expressed as a percentage of total tumour volume. A distance map from vessels was created using MATLAB and the distance of $tdTomato^+$ spots to vessels was represented as a colour spectrum (0-151 µm).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software. This was also used to create the figures that are showing the mean and individual measurements carried out in duplicate unless stated otherwise. Paired observations are displayed with a connecting line. The statistical tests performed are indicated for each figure and $P<0.05$ was considered significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.A.F.V., B.M., R.J.M., M.A.V.; Methodology: J.A.F.V., J.I., B.M., J.K., L.M.O.B., A.J.G.; Validation: J.A.F.V., J.I.; Formal analysis: J.A.F.V., J.I.; Investigation: J.A.F.V., J.I., B.M., J.K., L.M.O.B., A.J.G.; Resources: A.J.G.; Writing - original draft: J.A.F.V., J.I., A.J.G., M.A.V.; Writing - review & editing: J.A.F.V., J.I., B.M., J.K., A.J.G., R.J.M., M.A.V.; Visualization: J.A.F.V., J.I., A.J.G.; Supervision: R.J.M., M.A.V.; Project administration: M.A.V.; Funding acquisition: B.M., R.J.M., M.A.V.

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References

1. Al Tameemi, W., Dale, T. P., Al-Jumaily, R. M. K., & Forsyth, N. R. (2019). Hypoxia-Modified Cancer Cell Metabolism. *Frontiers in Cell and Developmental Biology*, *7*(January), 1–15. https://doi.org/10.3389/fcell.2019.00004
2. Blouw, B., Song, H., Tihan, T., Bosze, J., Ferrara, N., Gerber, H.-P., Johnson, R. S. and Bergers, G. (2003). The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 4, 133-146. doi:10.1016/s1535-6108(03)00194-6
3. Brahimi-Horn, M. C., Chiche, J., & Pouysségur, J. (2007). Hypoxia and cancer. *Journal of Molecular Medicine*, *85*(12), 1301–1307. https://doi.org/10.1007/s00109-007-0281-3
4. Campeau, E., Ruhl, V. E., Rodier, F., Smith, C. L., Rahmberg, B. L., Fuss, J. O., … Kaufman, P. D. (2009). A versatile viral system for expression and depletion of proteins in mammalian cells. *PLoS ONE*, *4*(8). https://doi.org/10.1371/journal.pone.0006529
5. Conway, J. R. W., Warren, S. C., Herrmann, D., Murphy, K. J., Cazet, A. S., Vennin, C., … Timpson, P. (2018). Intravital Imaging to Monitor Therapeutic Response in Moving Hypoxic Regions Resistant to PI3K Pathway Targeting in Pancreatic Cancer. *Cell Reports*, *23*(11), 3312–3326. https://doi.org/10.1016/j.celrep.2018.05.038
6. Dewhirst, M. W. (2009). Relationships between cycling hypoxia, HIF-1, angiogenesis and oxidative stress. *Radiation Research*, *172*(6), 653–665. https://doi.org/10.1667/RR1926.1
7. Dubois, L. J., Lieuwes, N. G., Janssen, M. H. M., Peeters, W. J. M., Windhorst, A. D., Walsh, J. C., … Lambin, P. (2011). Preclinical evaluation and validation of [18F]HX4,a promising hypoxia marker for imaging. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(35), 14620–14625. https://doi.org/10.1073/pnas.1102526108
8. Erapaneedi, R., Belousov, V. V, Schäfers, M., & Kiefer, F. (2016). A novel family of fluorescent hypoxia sensors reveal strong heterogeneity in tumor hypoxia at the cellular level. *The EMBO Journal*, *35*(1), 102–113. https://doi.org/10.15252/embj.201592775
9. Fleming, I. N., Manavaki, R., Blower, P. J., West, C., Williams, K. J., Harris, A. L., … Gilbert, F. J. (2015). Imaging tumour hypoxia with positron emission tomography. *British Journal of Cancer*, *112*(2), 238–250. https://doi.org/10.1038/bjc.2014.610
10. Fyles, A. W., Milosevic, M., Wong, R., Kavanagh, M. C., Pintilie, M., Sun, A., … Hill, R. P. (1998). Oxygenation predicts radiation response and survival in patients with cervix cancer. *Radiotherapy and Oncology*, *48*(2), 149–156. https://doi.org/10.1016/S0167-8140(98)00044-9
11. Giatromanolaki, A., Koukourakis, M. I., Sivridis, E., Turley, H., Talks, K., Pezzella, F., … Harris, A. L. (2001). Relation of hypoxia inducible factor 1α and 2α in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. *British Journal of Cancer*, *85*(6), 881–890. https://doi.org/10.1054/bjoc.2001.2018
12. Godet, I., Shin, Y. J., Ju, J. A., Ye, I. C., Wang, G., & Gilkes, D. M. (2019). Fate-mapping post-hypoxic tumor cells reveals a ROS-resistant phenotype that promotes metastasis. *Nature Communications*, *10*(1), 1–18. https://doi.org/10.1038/s41467-019-12412-1

13. Gort, E. H., Van Haaften, G., Verlaan, I., Groot, A. J., Plasterk, R. H. A., Shvarts, A., … Vooijs, M. (2008). The TWIST1 oncogene is a direct target of hypoxia-inducible factor-2α. *Oncogene*, *27*(11), 1501–1510. https://doi.org/10.1038/sj.onc.1210795
14. Gray, L. H., Conger, A. D., Ebert, M., Hornsey, S., & Scott, O. C. (1953). The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *The British Journal of Radiology*, *26*(312), 638–648. https://doi.org/10.1259/0007-1285-26-312-638
15. Groot, A. J., Habets, R., Yahyanejad, S., Hodin, C. M., Reiss, K., Saftig, P., … Vooijs, M. (2014). Regulated Proteolysis of NOTCH2 and NOTCH3 Receptors by ADAM10 and Presenilins. *Molecular and Cellular Biology*, *34*(15), 2822–2832. https://doi.org/10.1128/mcb.00206-14
16. Harada, H., Inoue, M., Itasaka, S., Hirota, K., Morinibu, A., Shinomiya, K., … Hiraoka, M. (2012). Cancer cells that survive radiation therapy acquire HIF-1 activity and translocate towards tumour blood vessels. *Nature Communications*, *3*, 710–783. https://doi.org/10.1038/ncomms1786
17. Heim, R., Prashert, D. C., Tsien, R. Y., & Kandel, E. R. (1994). Wavelength mutations and posttranslational autoxidation of green fluorescent protein (Aequorea victoria/blue fluorescent protein/Escherichia coli/imidazolidinone). *Biochemistry*, *91*(December), 12501–12504.
18. Hubbi, M. E., Kshitiz, Gilkes, D. M., Rey, S., Wong, C. C., Luo, W., … Semenza, G. L. (2013). A nontranscriptional role for HIF-1α as a direct inhibitor of DNA replication. *Science Signaling*, *6*(262), 1–22. https://doi.org/10.1126/scisignal.2003417
19. Hubbi, M. E., & Semenza, G. L. (2015). Regulation of cell proliferation by hypoxia-inducible factors. *American Journal of Physiology - Cell Physiology*, *309*(12), C775–C782. https://doi.org/10.1152/ajpcell.00279.2015
20. Ioannou, M., Papamichali, R., Kouvaras, E., Mylonis, I., Vageli, D., Kerenidou, T., … Koukoulis, G. K. (2009). Hypoxia inducible factor-1alpha and vascular endothelial growth factor in biopsies of small cell lung carcinoma. *Lung*, *187*(5), 321–329. https://doi.org/10.1007/s00408-009-9169-z
21. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., … Kaelin, J. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: Implications for O2 sensing. *Science*, *292*(5516), 464–468. https://doi.org/10.1126/science.1059817
22. Janssens, G. O., Rademakers, S. E., Terhaard, C. H., Doornaert, P. A., Bijl, H. P., Van Ende, P. Den, … Kaanders, J. H. (2012). Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: Results of a phase III randomized trial. *Journal of Clinical Oncology*, *30*(15), 1777–1783. https://doi.org/10.1200/JCO.2011.35.9315
23. Jubb, A. M., Buffa, F. M., & Harris, A. L. (2010). Assessment of tumour hypoxia for prediction of response to therapy and cancer prognosis. *Journal of Cellular and Molecular Medicine*, *14*(1–2), 18–29. https://doi.org/10.1111/j.1582-4934.2009.00944.x
24. Jung, S. N., Yang, W. K., Kim, J., Kim, H. S., Kim, E. J., Yun, H., … Ha, J. (2008). Reactive oxygen species stabilize hypoxia-inducible factor-1 alpha protein and stimulate transcriptional activity via AMP-activated protein kinase in DU145 human prostate cancer cells. *Carcinogenesis*, *29*(4), 713–721. https://doi.org/10.1093/carcin/bgn032

25. Kedrin, D., Gligorijevic, B., Wyckoff, J., Verkhusha, V. V., Condeelis, J., Segall, J. E., & van Rheenen, J. (2008). Intravital imaging of metastatic behavior through a mammary imaging window. *Nature Methods*, *5*(12), 1019–1021. https://doi.org/10.1038/nmeth.1269
26. Koshiji, M., Kageyama, Y., Pete, E. A., Horikawa, I., Barrett, J. C., & Huang, L. E. (2004). HIF-1α induces cell cycle arrest by functionally counteracting Myc. *EMBO Journal*, *23*(9), 1949–1956. https://doi.org/10.1038/sj.emboj.7600196
27. Kumagai, A., Ando, R., Miyatake, H., Greimel, P., Kobayashi, T., Hirabayashi, Y., … Miyawaki, A. (2013). A bilirubin-inducible fluorescent protein from eel muscle. *Cell*, *153*(7), 1602–1611. https://doi.org/10.1016/j.cell.2013.05.038
28. LaGory, E. L., & Giaccia, A. J. (2016). The ever-expanding role of HIF in tumour and stromal biology. *Nature Cell Biology*, *18*(4), 356–365. https://doi.org/10.1038/ncb3330
29. Lee, S. J., Hwang, A. B., & Kenyon, C. (2010). Inhibition of respiration extends C. elegans life span via reactive oxygen species that increase HIF-1 activity. *Current Biology*, *20*(23), 2131–2136. https://doi.org/10.1016/j.cub.2010.10.057
30. Lehtiö, K., Eskola, O., Viljanen, T., Oikonen, V., Grönroos, T., Sillanmäki, L., Minn, H. (2004). Imaging perfusion and hypoxia with PET to predict radiotherapy response in head-and-neck cancer. *International Journal of Radiation Oncology Biology Physics*, *59*(4), 971–982. https://doi.org/10.1016/j.ijrobp.2003.12.014
31. Li, L., Bourg, A. Van Der, Niino, Y., Egolf, L., Gu, H., Mills, M., … Zeng, H. (2015). Transgenic mice for intersectional targeting of neural sensors and effectors with high specificity and performance. *Neuron*, *85*(5), 942–958. https://doi.org/10.1016/j.neuron.2015.02.022.Transgenic
32. Medici, D., & Olsen, B. R. (2012). Rapamycin inhibits proliferation of hemangioma endothelial cells by reducing HIF-1-dependent expression of VEGF. *PLoS ONE*, *7*(8). https://doi.org/10.1371/journal.pone.0042913
33. Milosevic, M., Warde, P., Meńard, C., Chung, P., Toi, A., Ishkanian, A., … Bristow, R. (2012). Tumor hypoxia predicts biochemical failure following radiotherapy for clinically localized prostate cancer. *Clinical Cancer Research*, *18*(7), 2108–2114. https://doi.org/10.1158/1078-0432.CCR-11-2711
34. Muz, B., de la Puente, P., Azab, F., & Azab, A. K. (2015). The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia*, 83. https://doi.org/10.2147/hp.s93413
35. Nordsmark, M., Bentzen, S. M., Rudat, V., Brizel, D., Lartigau, E., Stadler, P., … Overgaard, J. (2005). Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. *Radiotherapy and Oncology*, *77*(1), 18–24. https://doi.org/10.1016/j.radonc.2005.06.038
36. Overgaard, J. (2007). Hypoxic radiosensitization: Adored and ignored. *Journal of Clinical Oncology*, *25*(26), 4066–4074. https://doi.org/10.1200/JCO.2007.12.7878
37. Prabhakar, N. R., & Semenza, G. L. (2015). Oxygen Sensing and Homeostasis. *Physiology*, *30*(5), 340–348. https://doi.org/10.1152/physiol.00022.2015
38. Ren, H. Y., Zhang, Y. H., Li, H. Y., Xie, T., Sun, L. L., Zhu, T., … Ye, Z. M. (2016). Prognostic role of hypoxia-inducible factor-1 alpha expression in osteosarcoma: A meta-analysis. *OncoTargets and Therapy*, *9*, 1477–1487. https://doi.org/10.2147/OTT.S95490

39. Ritsma, L., Steller, E. J. A., Ellenbroek, S. I. J., Kranenburg, O., Borel Rinkes, I. H. M., & Van Rheenen, J. (2013). Surgical implantation of an abdominal imaging window for intravital microscopy. *Nature Protocols*, *8*(3), 583–594. https://doi.org/10.1038/nprot.2013.026
40. Roig, E. M., Yaromina, A., Houben, R., Groot, A. J., Dubois, L., & Vooijs, M. (2018). Prognostic role of hypoxia-inducible factor-2α tumor cell expression in cancer patients: A meta-analysis. *Frontiers in Oncology*, *8*(JUN). https://doi.org/10.3389/fonc.2018.00224
41. Salem, A., Asselin, M. C., Reymen, B., Jackson, A., Lambin, P., West, C. M. L., … Faivre-Finn, C. (2018). Targeting hypoxia to improve non–small cell lung cancer outcome. *Journal of the National Cancer Institute*, *110*(1), 14–30. https://doi.org/10.1093/jnci/djx160
42. Spiegelberg, L., Houben, R., Niemans, R., de Ruysscher, D., Yaromina, A., Theys, J., … Dubois, L. J. (2019). Hypoxia-activated prodrugs and (lack of) clinical progress: The need for hypoxia-based biomarker patient selection in phase III clinical trials. *Clinical and Translational Radiation Oncology*, *15*, 62–69. https://doi.org/10.1016/j.ctro.2019.01.005
43. Thomlinson, R. H., & Gray, L. H. (1955). The Histological Structure of Some Human Lung Cancers and the Possible Implications for Radiotherapy. *British Journal of Cancer*, *9*, 539–549. https://doi.org/10.1038/bjc.1955.55
44. Van Cutsem, E., Lenz, H.-J., Furuse, J., Tabernero, J., Heinemann, V., Ioka, T., … Bendell, J. C. (2016). MAESTRO: A randomized, double-blind phase III study of evofosfamide (Evo) in combination with gemcitabine (Gem) in previously untreated patients (pts) with metastatic or locally advanced unresectable pancreatic ductal adenocarcinoma (PDAC). *Journal of Clinical Oncology*, *34*(15_suppl), 4007. https://doi.org/10.1200/JCO.2016.34.15_suppl.4007
45. van Es, J. H., Sato, T., van de Wetering, M., Luybimova, A., Gregorieff, A., Zeinstra, L., … Clevers, H. (2012). Dll1 marks early secretory progenitors in gut crypts that can revert to stem cells upon tissue damage. *Nature Cell Biology*, *14*(10), 1099–1104. https://doi.org/10.1038/ncb2581.Dll1
46. Vaupel, P., & Mayer, A. (2007). Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev*, *26*(2), 225–239. https://doi.org/10.1007/s10555-007-9055-1
47. Wang, Y., Wang, H., Li, J., Entenberg, D., Xue, A., Wang, W., & Condeelis, J. (2016). Direct visualization of the phenotype of hypoxic tumor cells at single cell resolution in vivo using a new hypoxia probe. *IntraVital*, *5*(2), e1187803. https://doi.org/10.1080/21659087.2016.1187803
48. Wilson, W. R., & Hay, M. P. (2011). Targeting hypoxia in cancer therapy. *Nat Rev Cancer*, *11*(6), 393–410. https://doi.org/10.1038/nrc3064
49. Zheng, S.-S., Chen, X.-H., Yin, X., & Zhang, B.-H. (2013). Prognostic significance of HIF-1α expression in hepatocellular carcinoma: a meta-analysis. *PloS One*, *8*(6), e65753. https://doi.org/10.1371/journal.pone.0065753
50. Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., … Simons, J. W. (1999). Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. *Cancer Research*, *59*(22), 5830–5835.

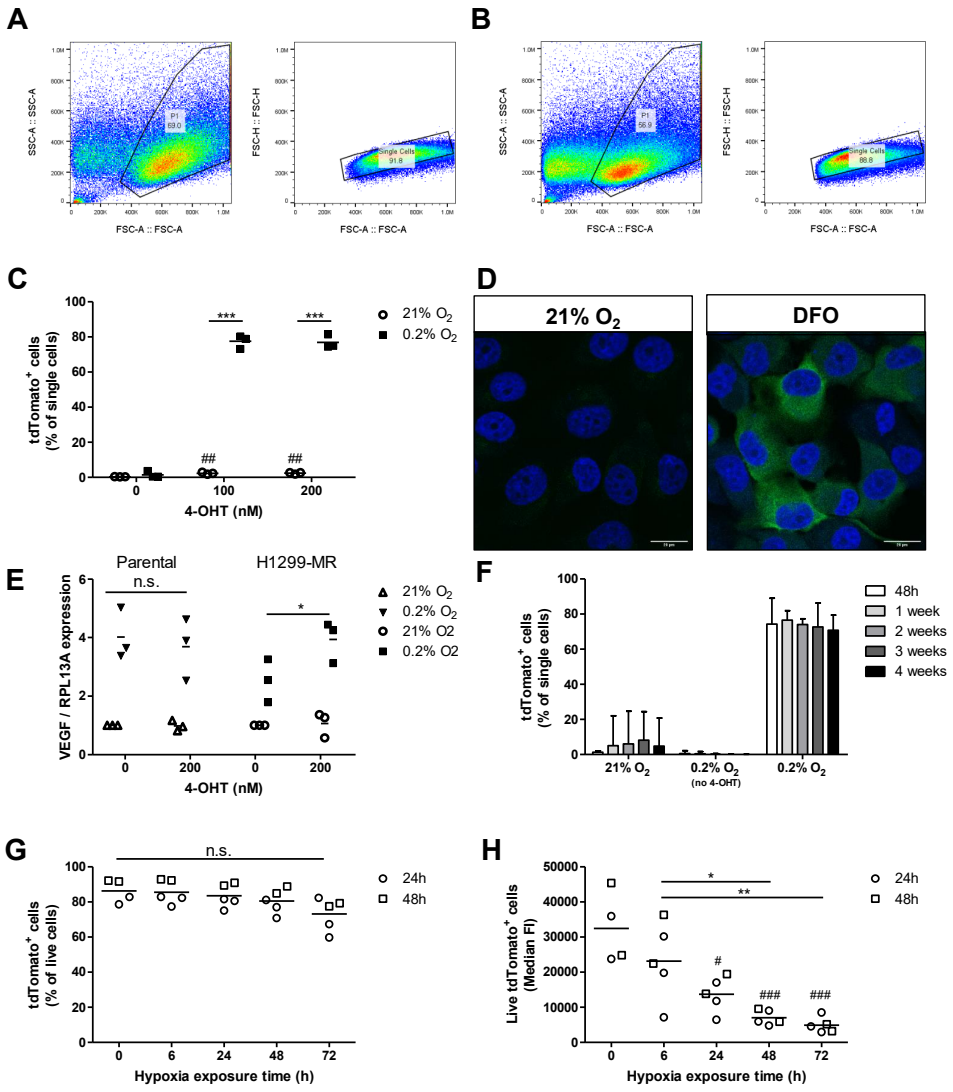


Figure S1: H1299-MR characteristics.

Gating strategy of eGFP and tdTomato expression after exposure to hypoxia (0.1% O2) for 24h (A) and 24h of hypoxia followed by 24h of re-oxygenation (B) (C) Flow cytometric analysis of tdTomato expression 72 hours after 0.2% O2 and different concentrations of 4-OHT. *** p<0.001 as indicated using 2-way ANOVA followed by Bonferroni post-test. ## p<0.01, 4-OHT versus without 4-OHT under normoxia using 1-way ANOVA followed by Bonferroni's multiple comparison. (D) Distribution of eGFP after 24h treatment of H1299-MR with 100µM DFO. Scale bar: 20µm. (E) VEGF gene expression normalised for the housekeeping gene RPL13A after exposure to tamoxifen in the parental H1299 cell line and H1299-MR Dots represent independent experiments carried out in triplicate and bars indicate averages. (F-H) H1299-MR cells stably expressing tdTomato were re-exposed to hypoxia. (F) Stable expression of tdTomato in normoxia once it was induced by hypoxia (0.2% O2) and 4-OHT Bars represent mean expression of 3 independent experiments and error bars indicate 95% c.i. (G)

tdTomato was induced with 4-OHT and 24h (circles) or 48h (squares) of 0.1% O2 as indicated. Re-exposure to hypoxia for up to 72 hours did not significantly affect the percentage of tdTomato+ cells. Dots represent independent experiments carried out in duplicate and bars indicate averages (H) Re-exposure to hypoxia gradually reduced the tdTomato median fluorescence intensity. Dots represent independent experiments carried out in duplicate and bars indicate averages #p<0.05, ###p<0.001 compared to no hypoxia re-exposure and *p<0.05, **p<0.01 as indicated and calculated by One-way ANOVA followed by Bonferroni's multiple comparison.

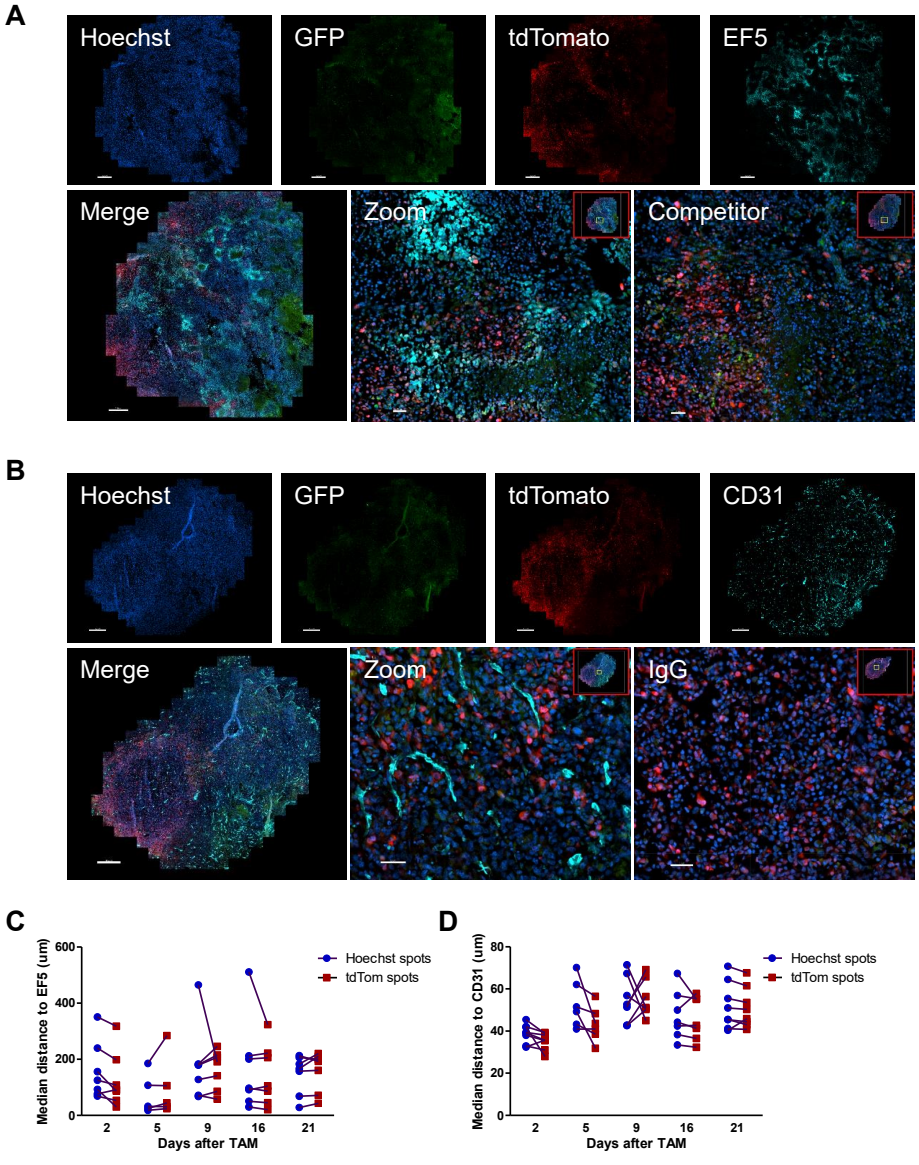


Figure S2: Immunofluorescent staining of H1299-MR xenografts at different time points after administration of tamoxifen.

(A) Micrographs of EF5 staining. An EF5-Cy5 antibody mixture containing a competitive inhibitor of the antibody was used as a negative control (competitor). (B) CD31 and IgG control staining (C) Distance of tdTomato-positive cells and all cells to EF5-positive areas. (D) Distance of tdTomato-positive cells and all cells to CD31-positive areas. Scale bars overview: 500 µm, scale bars zoom and controls: 50 µm.

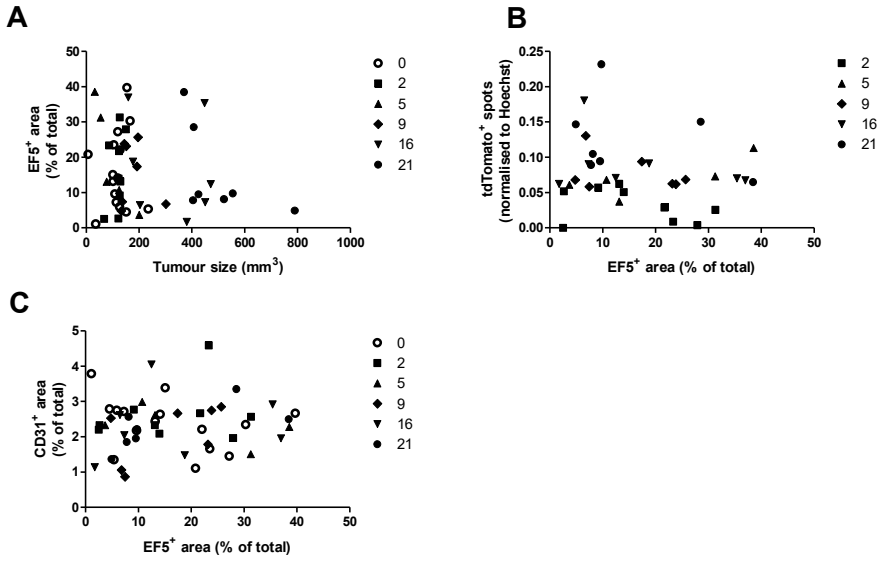


Figure S3: Quantification of immunofluorescent staining of H1299-MR xenografts.

(A) Correlation between EF5 and tumours size (not significant). (B) Correlation between EF5 and tdTomato (not significant). (C) Correlation between EF5 and CD31 (not significant).

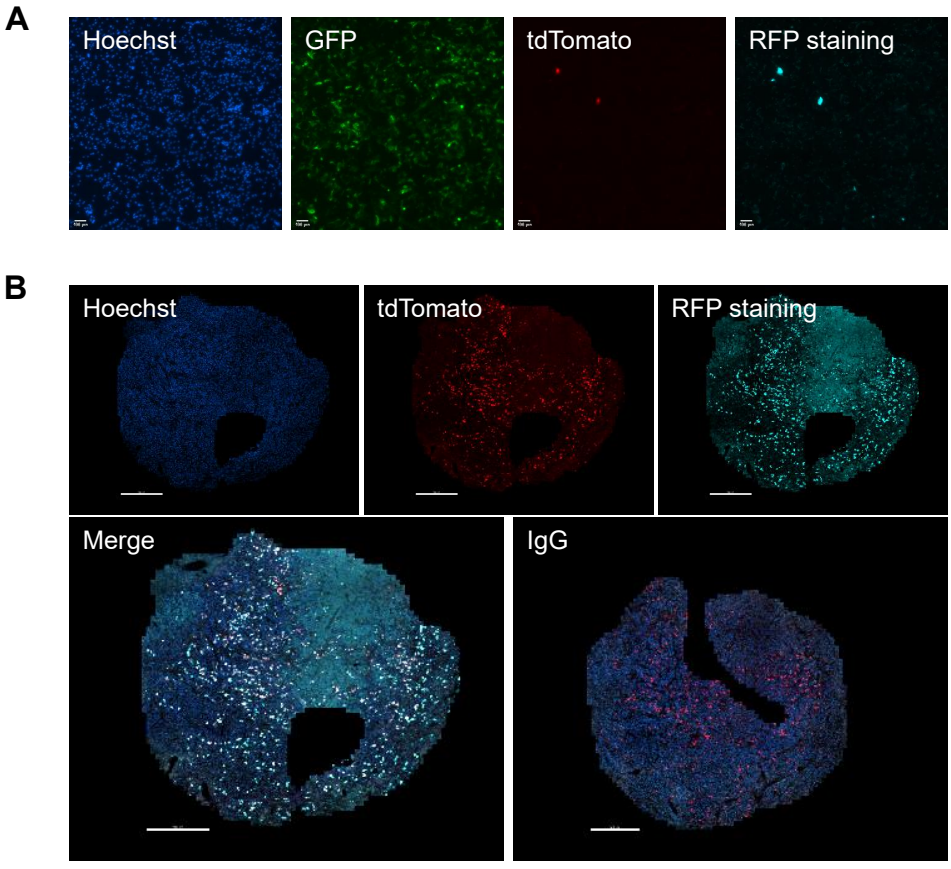


Figure S4: Immunofluorescent RFP staining.

(A) Staining of GFP-positive cells, cultured *in vitro* with 100 µM DFO, confirming the antibody against RFP does not recognise GFP. (B) Micrographs of RFP staining and IgG control on xenograft sections. Scale bars A: 100 µm, B: 500 µm.

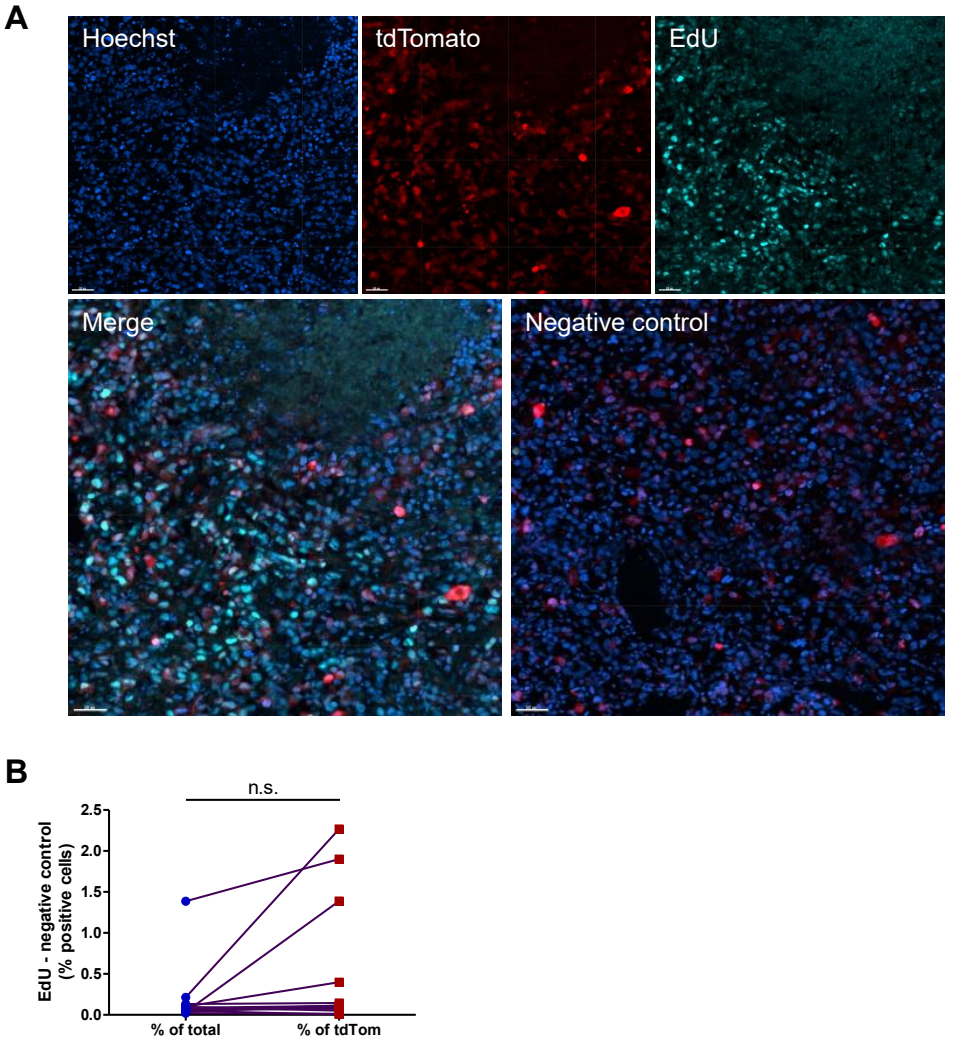
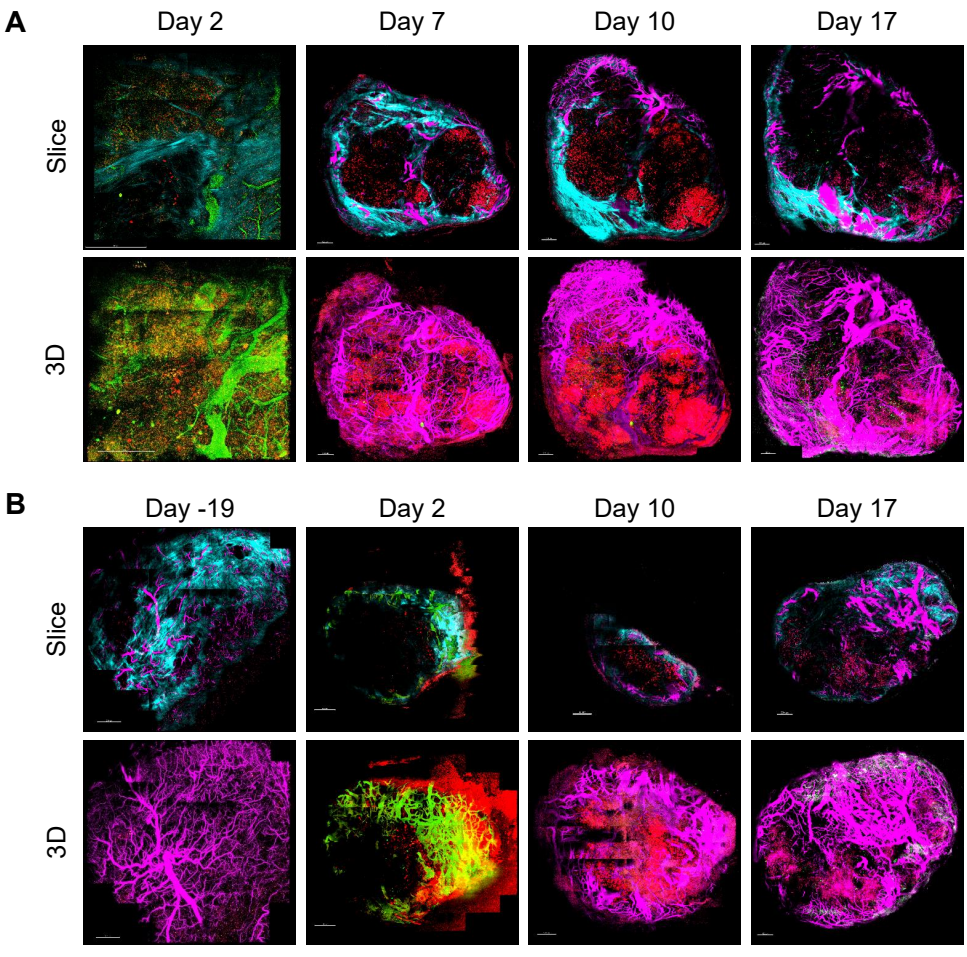


Figure S5: EdU staining.

(A) Micrographs of xenograft sections stained for EdU and a negative control with only the AF-dye. Scale bars 50 µm. (B) Background staining as percentage positive cells of total (Hoechst) or tdTomato (n=13).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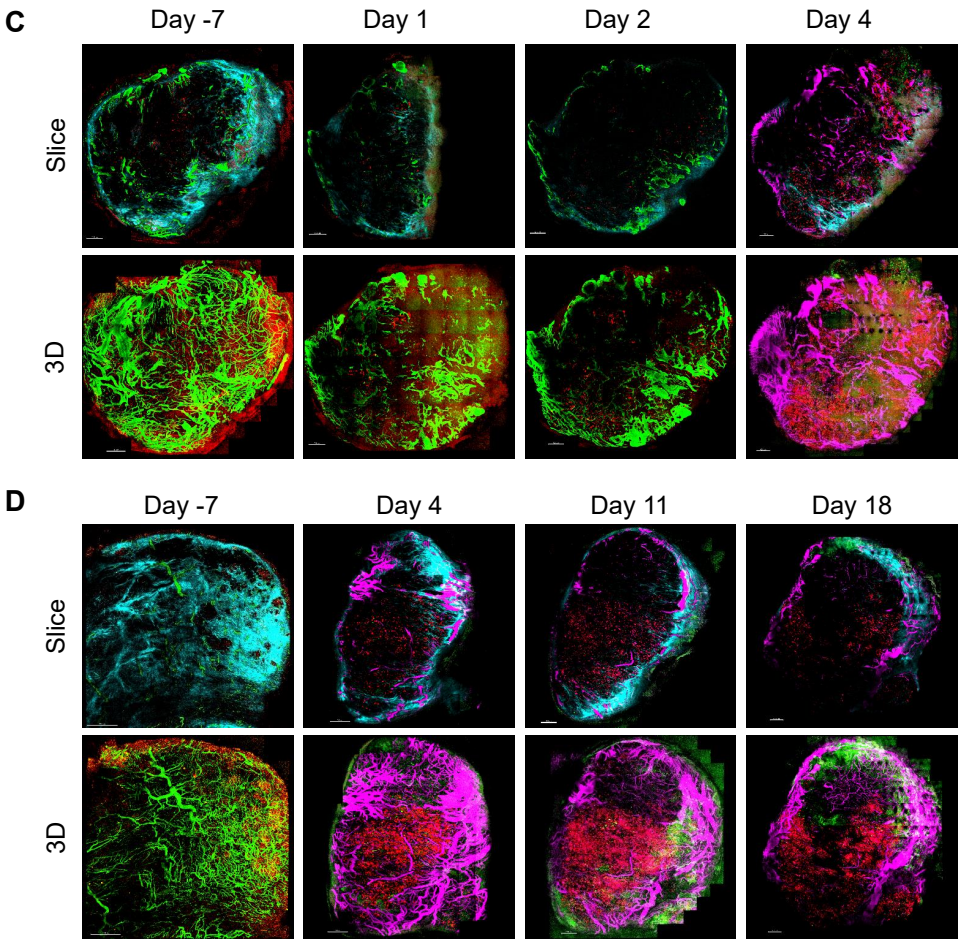

Figure S6: H1299-MR xenografts in 4 mice were followed by intravital microscopy.

(A-D) Perfused vessels are shown in green (days -7, 1, and 2) or purple, tdTomato-positive cells in red, and collagen in cyan (second harmonic generation microscopy, top panels only). Days indicate the time after tamoxifen administration and scale bars are 500 µm. Channel arithmetics was applied using MATLAB to subtract GFP bleed through into the tdTomato channel and tdTomato bleed through into the Qtracker 705 channel (A-D).

CHAPTER 5

Hypoxia lineage tracing and genetic ablation to study the role of hypoxic cells in radiotherapy resistance

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In preparation

CHAPTER 6

Generation of a novel mouse model that enables lineage tracing of the hypoxia inducible factor response

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In preparation

CHAPTER 7

7

General Discussion

Jonathan Ient

Hypoxia

Tumour hypoxia and high levels of HIF have long been known to be negative prognostic and predictive factors in cancer patient outcome, due to its contributions to therapy resistance, invasiveness, metastasis, altered metabolism, angiogenesis, vasculogenesis, cell survival and genomic instability. Intratumoral hypoxia is a general feature of many solid tumours and one of the strongest independent prognostic and predictive factors for patient outcome. In **chapters 4-6** we develop a new system for lineage tracing hypoxic and post-hypoxic cells. In **chapter 4** we used the MARCer (HIF-CreER^{T2}) protein in combination with a tdTomato reporter in a H1299 non-small cell lung cancer model. We demonstrate that this system can be used to lineage trace hypoxic and post-hypoxic cells *in vivo* and live microscopy can be achieved with the use of intravital microscopy. We find a proliferative advantage *in vivo* for labelled post-hypoxic cells than unlabelled cells. In **chapter 5** we use the MARCer system in combination with a floxed 'dsred' DTR-eGFP reporter which allows us to ablate labelled cells with diphtheria toxin. We generated the metastatic 4T1 mammary carcinoma cell line with our system. We characterise this model *in vitro* and *in vivo* and test its ability to report on hypoxia and the efficiency of ablation of labelled cells with diphtheria toxin. We find that the model can report on hypoxia when tamoxifen is added and (post) hypoxic labelled cells can be efficiently ablated. The model in this chapter will be used in future experiments to investigate the effects of radiotherapy on hypoxic cells and the benefits that hypoxia targeting therapies may have on radiotherapy efficacy. In **chapter 6** we generate a transgenic mouse which has the MARCer gene inserted into one allele of the HIF-1A locus. We characterise the embryonic stem cell clone that was used to create these mice. We find that the MARCer fusion protein is stabilised under hypoxic conditions and when 4-hydroxy tamoxifen is added is able to label hypoxic cells when combined with a reporter. The lineage tracing system developed in these chapters should provide a useful tool to study hypoxic cells and their behaviour as well as therapies designed to target the hypoxic fraction in a tumour.

Due to the fact that hypoxia has a negative prognostic and predictive outcomes for patients, this has led to intense research into strategies that target this population of cells in tumours. A broad range of strategies have been developed from small molecule inhibitors to oxygen supplementation and have been around for the past 3 decades. In 1912 Swartz first observed the enhancing effects of oxygen on irradiation. He noted that the skin reaction caused by radium was reduced when it was pressed into the arm, which he attributed to reduced blood flow and therefore less oxygen. 40 years later Gray et al. did the first formal experiments to investigate the efficacy of radiotherapy at different oxygen concentrations on cancer cells (Gray et al., 1953). They observed that tumour cells are three times more sensitive to X-rays when well oxygenated compared to when they are anoxic.

Early clinical studies measured the oxygen concentrations in tumours using the Eppendorf pO_2 histograph which utilised needle oxygen electrodes. These studies showed that tumour progression and response to radiotherapy was dependent on the oxygenation of tumours in breast cancer (Okunieff et al., 1993), squamous cell carcinoma metastases (Gatenby et al., 1988), cervical cancer (Hockel et al., 1996; Höckel et al., 1993) and head and neck cancer (M Nordsmark, 1996). Collectively, these studies show that the more hypoxic tumours lead to worse patient outcome. These and other studies also showed that tumour oxygenation was linked to tumour progression. Since then it has been found that patients whose primary tumour is poorly oxygenated have increased risk of metastasis and poorer prognosis (Bos et al., 2003; P Vaupel et al., 2007; Peter Vaupel et al., 2004). Many steps in the metastatic cascade have been linked to hypoxia including epithelial to mesenchymal transition, local tissue invasion, intravasation into the blood and survival in the bloodstream and extravasation into distant organs and formation of a secondary tumour (Godet et al., 2019; Rankin et al., 2016).

As well as radiotherapy resistance, hypoxia also contributes to the reduced efficacy of chemotherapeutics for several different reasons. First, like oxygen, the chemotherapeutics must diffuse a large distance which is further hampered by the composition of the extracellular matrix leading to a reduced concentration in less perfused areas (Minchinton & Tannock, 2006). Furthermore, hypoxia is known to upregulate drug efflux proteins further reducing the intracellular concentration of chemotherapy drugs in a number of cancers (Ding et al., 2010; Kruh & Belinsky, 2003; L. Liu et al., 2008). Reduced proliferation and downregulation of apoptotic proteins in hypoxic cells also reduces the efficacy of chemotherapy because the rapid proliferation of tumour cells is often required for DNA damaging chemotherapy drugs to work efficiently. Additionally, the increased acidity of the tumour microenvironment reduced the capacity of small molecules to pass through the cellular membrane, and can also lead to localisation in unfavourable cellular compartments and the altered pH can also directly lead to reduced activity of pH dependant drugs (Stubbs et al., 2000).

While early cancer research focused on chronic hypoxia, Brown et al. showed evidence of a second type; Acute hypoxia (Brown, 1979). This is hypoxia that was postulated to be caused by intermittent opening and closing of tumour blood vessels leading to intermittent periods of hypoxia and re-oxygenation. Further research since then has shown the negative prognostic effects that acute/cycling hypoxia can have. Re-oxygenation leads to the production of reactive oxygen species which as well as producing DNA damage, also lead to stabilisation of HIF-1α, and thus the HIF-1 transcriptional complex, which can protect the cells from cytotoxic therapies. Cycling hypoxia can also alter the function and activity of HIF1 which can influence tumour development and progression, response to therapy and promote tumour cell survival (Dewhirst, 2009). Acute hypoxia can last from a few minutes to a few hours making the distinction between acute and chronic hypoxia virtually impossible to distinguish. The combination of chronic and acute hypoxia leads to

a very dynamic tumour oxygenation profile, which is constantly changing (Peter Vaupel et al., 2004).

While the HIF family of proteins is the most well-studied oxygen regulated protein, general protein expression itself is also downregulated in hypoxia via a different mechanism. Hypoxia leads to the accumulation of misfolded proteins, due to the oxygen-dependent nature of disulphide formation post-translation (Koritzinsky et al., 2013). This activates the unfolded protein response (UPR) which results in autophosphorylation of protein kinase RNA-like endoplasmic reticulum kinase (PERK). PERK in turn phosphorylates the translation initiation factor eIF2α kinase (Koumenis et al., 2002). This leads to transient attenuation of protein translation, increased expression of chaperones to aid folding and increased degradation and autophagy. This reduces ER stress to increase cell survival, however, if ER stress persists apoptosis is induced. This reduction in protein synthesis is essential to reduce energy use, with the ATP demand for protein synthesis reducing about 10 fold compared to normoxic cells (Hochachka et al., 1996). Cancer cells also use the UPR as a mechanism to survive oncogenic and environmental stress and even though they experience extended ER stress, cancer cells are able to bypass the apoptotic signalling and avoid apoptosis. This allows cancer cells to exploit the UPR to promote cancer progression. The UPR has also been shown to contribute to the development of chemoresistance via various downstream mechanisms (Daishi Chen et al., 2017; Feng et al., 2011; Jiang et al., 2009; Salaroglio et al., 2017).

Targeting Hypoxia

Hypoxia modification

Hypoxia modification has been the focus of therapeutic investigation since the 60s. It aims to re-sensitise areas of tumour hypoxia to irradiation through different strategies from using hyperbaric oxygen to mitochondrial inhibitors (Fig. 1). Clinical trials utilising hyperbaric chambers with radiotherapy found significant improvements in local tumour control in carcinoma of the head and neck and the uterine cervix (Dische, 1978). However due to the complexities of this strategy and the schedules of hyperfractionation used as well as the sensitisation and toxicity of normal tissue this technique was not generally accepted. However, it did show that hypoxia modification can lead to improved patient outcome.

Oxygen mimetics are compounds that like oxygen, are able to 'fix' radiation-induced DNA damage leading to cell death. Unlike oxygen however, the compounds are not consumed by cells during respiration and therefore have increased diffusion into areas of anoxia and hypoxia. Nitroimidazoles are the most well studied and showed promise in pre-clinical studies. However, in clinical trials, misonidazole caused severe central and peripheral neuropathy which reduced the tolerable dose making it ineffective (Rosenberg

& Knox, 2006). Hence a second generation of these drugs were developed with better pharmacokinetics and less toxicity. Of these drugs only nimorazole has shown a benefit for patients in clinical trials (Overgaard et al., 1998). Improved locoregional control was found in a phase III clinical trial in 414 patients with pharynx and supraglottic carcinoma in patients who were treated with nimorazole in combination with radiotherapy as opposed to radiotherapy alone. Another phase III clinical trial in head and neck squamous cell carcinoma is currently ongoing (NCT01950689)

Another strategy to increase tumour oxygenation that has been tested is the combination of nicotinamide (a vasodilator) in combination with breathing carbogen (95% oxygen 5% carbon dioxide). This both improved tumour perfusion which has the benefit of decreasing levels of acute hypoxia (Chaplin et al., 1990) while also increasing blood oxygenation, reducing the levels of chronic hypoxia. This technique was used in the clinical trials, ARCON (Accelerated Radiation, CarbOgen, and Nicotinamide) (Janssens et al., 2012) and BCON (Bladder, CarbOgen, and Nicotinamide) (Peter J. Hoskin et al., 2010). ARCON showed limited success in a Phase III clinical trial with improved 5-year regional control specifically in patients with hypoxic laryngeal carcinoma tumours, however, no improvement in disease-free or overall survival was found. The lack of improvement in overall survival is likely due to the high local and regional control rates in both treatment arms and the ability to perform salvage surgery in case of recurrence. The results of the BCON trial showed improvements in overall survival, risk of death and local relapse in bladder cancer. These trials show the benefit of combining oxygen modification strategies to improve tumour oxygenation to further radio sensitise the tumour.

Another vasodilator that has been tested is nitroglycerin. A Phase II clinical trial in NSCLC used nitroglycerin with chemotherapy and cisplatin showed improved overall response, time to disease progression and survival time. This was thought to be due to a decreased hypoxic fraction however levels of hypoxia were not measured (Yasuda et al., 2006). A second phase II trial in NCLS combining nitroglycerin with chemo/ radiotherapy was stopped early when no reduction in tumour hypoxia or increased overall survival was seen (Reymen et al., 2020). The lack of overall survival was in part due to the heterogeneous treatment modalities and small sample size, but this cannot explain the lack of decreased hypoxia.

Giving patients erythropoietin (EPO) to stimulate red blood cell production, reducing anaemia and increasing blood and tumour oxygenation has also made it to clinical trials. However no significant benefits of EPO have been found, and in a number of trials, it led to poorer patient outcomes (Lazzari & Silvano, 2020).

Another method for reducing hypoxia is to reduce the oxygen consumption of tumour cells. Metformin, which has the off-target effect of inhibiting mitochondrial complex I, has been used to decrease oxygen consumption in tumours and has been shown in pre-clinical studies to sensitise tumours to radiation. Metformin was also tested in patients with prostate cancer in combination with radiotherapy and significantly decreased early

biochemical relapse rates (Zannella et al., 2013). It is also currently in phase II clinical trial in cervix cancer in combination with cisplatin and radiation (NCT02394652). Whether the concentration needed to sufficiently inhibit O_2 consumption can be reached without serious side effects is uncertain. Metformin was first used in the treatment of diabetes and a meta-analysis found that metformin was associated with a 14% reduction of cancer incidence and a 40% reduction in cancer mortality (Wu et al., 2015) likely through the activation of AMP-activated protein kinase (Zhou et al., 2001) and reducing the expression of cyclin D1 (Sahra et al., 2008).

Other drugs that were initially used to treat other diseases have also found a new lease of life as ways to combat cancer. This brings with it the advantage that they are often already approved for use and more is known about their toxicity profiles and mechanisms of action reducing the cost of reaching the clinic. Atovaquone, originally an anti-malarial drug has been found to reduce tumour hypoxia by inhibiting mitochondrial complex III. *in vivo* studies showed a significant reduction in tumour hypoxia and a significant growth delay when combined with radiotherapy (Ashton et al., 2016). Atovaquone is currently in clinical trials (NCT02628080). Papaverine, originally used as smooth muscle relaxant was found to have the off-target effect of inhibiting mitochondrial complex I. Papaverine both increases perfusion by acting as a vasodilator as well as reducing oxygen consumption alleviating hypoxia. *In vivo* studies showed that it was able to sensitise tumours to irradiation (Benej et al., 2018). Phase I clinical trials in combination with stereotactic radiation therapy are currently recruiting for patients with non-small cell lung cancer or lung metastases (NCT03824327).

A systematic review of 10,108 patients across 86 trials designed to modify tumour hypoxia in patients receiving primary radiotherapy alone showed that modification of tumour hypoxia significantly improved the effect of radiotherapy, but had no effect on metastasis (Overgaard, 2007). Another meta-analysis looking specifically in squamous cell carcinoma of the head and neck found similar results using data from 4805 patients in 32 randomised clinical trials (Overgaard, 2011). Overall, hypoxia modification in combination with radiotherapy resulted in significantly improved therapeutic benefit in loco-regional control, disease-specific survival and to a lesser extent overall survival while no benefit was seen to distant metastasis. Despite the benefits, no hypoxia modification strategies are currently in routine global use.

HAPs

Another strategy is to eliminate hypoxic tumour cells is the use of hypoxia-activated pro-drugs (HAPs) (Fig. 1). These drugs are inert until they are activated through enzymatic reduction under hypoxic conditions, most commonly via two, one-electron reduction steps. The first one-electron reduction happens readily, but in the presence of oxygen is rapidly converted back to the pro-drug. When oxygen is not present the second reduction is able to occur, which causes the pro-drug to fragment, releasing the active moiety. The

majority of HAPs work through inducing DNA damage leading to apoptosis especially when combined with chemo/radiotherapy.

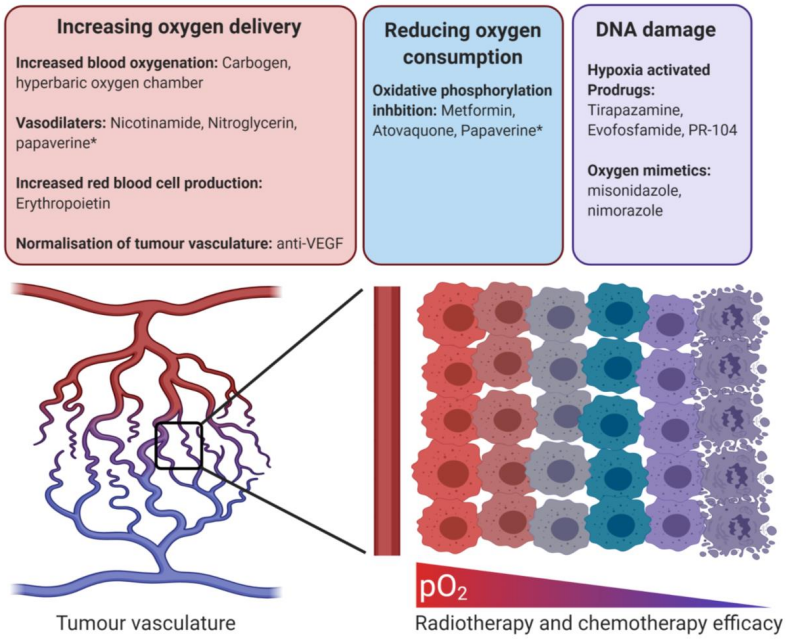

Figure 1:

Tumour microenvironment and hypoxia. The high rate of tumour growth coupled with aberrant tumour vasculature leads to areas distant from vessels becoming hypoxic. This reduces the efficacy of radiotherapy and chemotherapy. Hypoxia modification strategies aim to increase tumour oxygenation, alleviating hypoxia to increase the sensitivity to radio and chemotherapy. Hypoxia targeting strategies aim to produce or increase DNA damage in the hypoxic regions to produce cell killing or increase the efficacy of radio and chemotherapy. Created with BioRender.com.

The first HAP to be clinically tested was Tirapazamine (TPZ) (Doherty et al., 1994). TPZ has shown promise in a number of clinical trials, however only one Phase III trial has had a positive outcome; the addition of TPZ to cisplatin was shown to be superior to cisplatin alone (Reddy & Williamson, 2009). The only other completed trial, however, was negative, possibly due to the large deviations in the radiotherapy dose delivered. A further sub-study of the data from a phase II clinical study showed that TPZ was able to dramatically lower locoregional failure if the patients were selected based on tumour hypoxia (Rischin et al., 2006). Studies have shown that the there is a large range of hypoxia levels within tumours and so stratification of patients based on hypoxia biomarkers is essential, however more studies are needed to standardise these biomarkers and optimise the hypoxia threshold (Mortensen et al., 2010; Rasey et al., 1996; Spiegelberg et al., 2019).

Unfortunately, the hypoxic status was also left out of the design of another HAP in clinical trials; TH-302 (Evofosfamide). Both clinical trials conducted failed, with the MAESTRO trial (NCT01746979) only just failing to reach significance ($p = 0.0588$). Differences between murine and human drug tolerance, tumour size and levels of hypoxia also play a factor in the clinical failure of HAPs. Because areas of hypoxia are not well perfused, the HAPs must diffuse large distances to reach their intended target and lowering the dose can significantly affect the local concentration of the drug in the tumour. TPZ has been shown to not diffuse to the most hypoxic regions and this is likely one factor that led to the failure in clinical trials (Abou-Alfa et al., 2011). Analogues of TPZ with far better tissue penetration properties have been created but not reached clinical trials yet (K. O. Hicks et al., 2010).

Another factor that must be considered in the design of HAPs is the expression and tissue specificity of the reductase or reductases that are able to activate the HAP. PR-104 was another HAP that showed promising pre-clinical data and had the advantage over TPZ that its stable cytotoxic metabolite had a bystander effect and could diffuse to more oxygenated areas of the tumour enhancing its cytotoxic properties (Kevin O. Hicks et al., 2007). However, upon initiation of clinical trials, it was found in multiple trials that the drug was poorly tolerated in patients leading to the termination of the trials (Abou-Alfa et al., 2011; McKeage et al., 2012). Further investigation showed that as well as being activated in hypoxia, aldo-keto reductase 1C3 (AKR1C3) was able to reduce PR-104 into its active form in the presence of oxygen. AKR1C3 was also shown to have high expression in several cancers but it was also expressed in some normal tissues and this was likely the reason for the failure of the dose-escalation studies (Guise et al., 2010). This highlights the difficulty of designing HAPs that are tumour specific as many cell types express reductases and they are often not hypoxia-induced.

The failure of these drugs highlights the complex properties that HAPs need to possess, for them to be successful. The optimal HAP should have good tissue penetration, strong oxygen inhibition, be selective for reductases highly expressed in tumours only and ideally have a moderate bystander effect so that it can affect a greater proportion of the tumour without increasing normal tissue toxicity. To create and test new candidates requires the use of *in vitro* assays and *in vivo* models that can model these properties. Selection of an optimal HAP has been shown to benefit from the use of multi-parameter modelling to optimise the balance between drug diffusion and consumption so that the drug can penetrate far enough for optimal hypoxic cell killing (Kevin O. Hicks et al., 2003, 2006, 2007). Key to their success will also be the use of hypoxia biomarkers that can guide the decision to use them or not based on the hypoxia status of individual tumours. This is a necessity to avoid the same mistakes that hampered the clinical testing of TPZ and TH-302.

Hypoxia Biomarkers

Part of the reason for the lack of patient stratification was the lack of reliable hypoxia biomarkers. Tissue based biomarkers such as pimonidazole have been used which is reduced and deposited in areas of hypoxia. This has been used in trials such as ARCON and hypoxia levels measure by pimonidazole correlated with ARCON benefit. Pimonidazole has also been used to find endogenous markers such as CAIX and GLUT-1 which correlated with pimonidazole deposition (Airley et al., 2003; P. J. Hoskin et al., 2003).

Hypoxia signatures have also been developed which look at the expression of a cluster of genes and these have proven to be highly prognostic (Buffa et al., 2010; Winter et al., 2007). They have also been used to successfully predict the response to agents such as nimorazole that specifically sensitise hypoxic cells to radiation (Toustrup, Sørensen, Alsner, et al., 2012; Toustrup, Sørensen, Lassen, et al., 2012). The major issue with these biomarkers is the requirement of a biopsy, which is not always possible and repeat measurements are not always possible. Markers found in blood serum such as Osteopontin have also been used in clinical trials and allow easier samples acquisition (Overgaard et al., 2005). However, both of these methods can not give information on the heterogeneity of hypoxia across the tumour which is important when testing hypoxia targeting therapies. Several studies have used a median oxygen measurement of $HP_{2.5}$ (2.5mmHg) or the tumour fraction $HP_{2.5}$>19% as a cut off for hypoxia as these fractions have been linked to poor treatment outcome (Graves et al., 2011; Marianne Nordsmark et al., 2005), however, no threshold has been commonly accepted.

As such non-invasive techniques to measure tumour hypoxia may be a better method in many situations as it allows repeated measurements and the evaluation of hypoxia across the entire tumour. At the moment, PET-based methods are the most well developed but bioluminescence and photoacoustic imaging methods are being developed (Carolina, 2017; O'Connor et al., 2019). To date, no hypoxia imaging techniques are used routinely in the clinic but they have been utilised within clinical trials. ^{18}F-fluoromisondazle (^{18}F-FMISO) is the most extensively studied hypoxia tracer and is based on nitroimidazole reduction in hypoxic cells resulting in deposition of the tracer. ^{18}F-FMISO accumulation has been found to correlate with hypoxic regions in gliomas, head and neck, breast, lung and renal tumours, however, ^{18}F-FMISO did not work in sarcomas, rectal and pancreatic tumours due to variable ^{18}F-FMISO retention, normoxic accumulation or lack of any detectable retention (Fleming et al., 2015). Despite the utility of ^{18}F-FMISO it has not been generally accepted due to slow pharmacokinetics and moderate contrast impeding diagnostics.

Other tracers are being developed with improved pharmacokinetics that will be more clinically applicable. These include ^{18}F-HX4, ^{18}F-FETNIM and ^{18}F-FAZA which generally have improved clearance from normoxic tissues improving the hypoxia-normoxia contrast and tumour blood ratio. They have been tested in a selection of tumours and the results have been promising with improved clinical characteristics as compared to ^{18}F-FMISO. ^{18}F-HX4 was tested in head and neck squamous cell carcinoma and allowed monitoring of

therapy-induced decreases in hypoxia during treatment (Zegers et al., 2014). CAIX, VEGF and osteopontin blood biomarkers were also sampled, and interestingly both CAIX and VEGF remain unchanged while only a weak correlation between hypoxia and osteopontin was observed. This raises questions about the utility of these blood biomarkers for the detection of treatment-associated decreases in hypoxia.

[^{18}F]FDG PET is the most frequently used clinical imaging technique for the detection and staging of cancer. It is dependent on the rate of glycolysis and the upregulation of glucose transporters. Because glycolysis is upregulated in hypoxia and subvolumes of tumours displaying high glucose metabolism were shown to often be responsible for local recurrences (Aerts et al., 2009; Due et al., 2014). A clinical study compared [^{18}F]FDG PET and ^{18}F-HX4 uptake in head and neck cancer to see whether [^{18}F]FDG PET could be used to image tumour hypoxia (Zegers et al., 2015). While there was some correlation on a general tumour level between [^{18}F]FDG and ^{18}F-HX4, the hypoxic volume as measured by ^{18}F-HX4 was smaller than the high metabolic tumour volume. This shows that [^{18}F]FDG cannot be used as hypoxia marker, but may be of use in concert with a dedicated hypoxia imaging modality to guide and predict treatment success (Thorwarth et al., 2006).

Hypoxia biomarkers will be an essential tool for the selection of patients that receive hypoxia targeting therapies. The use of PET tracers has already been shown to be predictive of treatment response in several cancers and further refinement will only broaden their applicability and use in the clinic. Further to this, more biological knowledge is needed to improve clinical success. A greater understanding of hypoxia and the phenotypes it elicits are crucial to the development of treatments and biomarkers alike. Research to identify the optimal window of opportunity for treatment will also be important in increasing the efficacy of hypoxia targeting therapies.

Models for tracing hypoxic cells

Models for tracing hypoxic cells *in vivo* will be of use in the testing of hypoxia targeting or modifying therapies by facilitating the analysis of hypoxic cell killing or the hypoxic fraction *in vivo*. Recently several methods for tracing hypoxic cells have been developed including in this thesis each with different mechanisms and characteristics(Erapaneedi et al., 2016; Godet et al., 2019; Harada et al., 2012; Wang et al., 2016). Both Wang and Erapaneedi used the hypoxia-responsive HRE promoter to drive expression of the reporter fluorescent proteins, allowing them to visualise hypoxic tumour cells and recently re-oxygenated tumour cells. Erapaneedi et al., used the properties of UnaG and the oxygen requiring mOrange to distinguish between hypoxic cells and recently re-oxygenated cells. These are excellent models for studying hypoxic cells while they are still in or have recently left the hypoxic niche. However, once cells migrate into more well-perfused areas, expression of these proteins stops, making it very difficult to track these cells further and study them after re-oxygenation.

Similarly, Harada et al. and Godet et al. also used the HRE promoter, but they used it to drive expression of an oxygen regulated Cre-ODD similar to our construct. This was coupled with a floxed 'stop' luciferase reporter (Harada et al., 2012) or floxed 'DsRed' GFP reporter (Godet et al., 2019). This allows the tracing of cells long after they have left the hypoxic niche and enables the study of their behaviour in this setting. One drawback to this approach was the relatively low efficiency of cell tagging upon short hypoxia exposure. This limits the system to predominantly research into chronic hypoxia as acute hypoxia does not allow enough time for expression before re-oxygenation and degradation occur.

In our system, we make use of constitutive promoters (cmv, EF1α and the endogenous HIF1A promoter), which reduces the lag time between the onset of hypoxia and sufficient Cre-ODD expression to efficiently label cells. This should allow for more efficient labelling of cells in acute as well as chronic hypoxia although further analysis needs to be done to confirm this. Each of these systems has its own strengths and weaknesses and a combination of approaches may be needed to fully explore the remaining questions about the effects of hypoxia on tumours as well as in other physiological and pathological contexts.

Optimisation of these strategies will also be important to maximise the utility of these systems. Within this thesis, we have been refining our labelling strategy and combining it with reporters that can be used to study different questions about hypoxic cells. As talked about in previous chapters the use of UnaG as a fluorescent protein is preferable due to it not requiring oxygen to fluoresce (Erapaneedi et al., 2016; Kumagai et al., 2013). The only co-factor that UnaG requires is bilirubin, which is a metabolite of haem, found after degradation of red blood cells and is therefore readily available in serum. Erapaneedi et al. were able to show *in* vivo that enough bilirubin was present in areas of hypoxia for UnaG to fluoresce, confirming its utility *in* vivo.

Therefore we have further developed a floxed 'stop' UnaG-HA reporter. We have tested this reporter in the H1299 cell line in combination with the MARCer construct in **chapter 5** (i.e without GFP). Testing of this model in spheroids showed robust labelling of cells within the hypoxic core of spheroids (Fig 2), and fluorescence was able to visualised without the need for antibody staining although the addition of the HA tag allows antibody staining in cases where fixation quenches intrinsic fluorescence. The addition of diphtheria toxin receptor in chapter 5 was added to facilitate investigation into the effect that killing hypoxic cells has on the tumour and the efficacy of combining therapies that target hypoxia with radio and chemotherapy.

In **chapter 6** we developed a new transgenic mouse that can label hypoxic cells in tumours that can better mimic the patient due to the spontaneity of tumour development. This mouse has been successfully crossed with a PyMT mouse on an FVB background that also contained an R26STOP Tomato reporter. This will aid us in studying the behaviour of hypoxic cells within the tumour and can help to understand the effects of therapies on the hypoxic population.

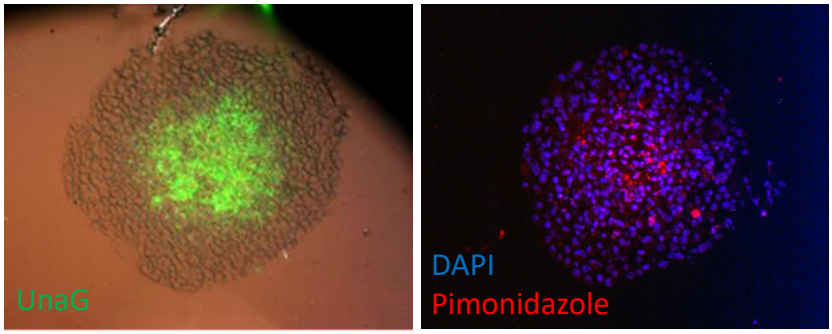

Figure 2:

H1299 MARCer + UnaG reporter spheroid, treated with 200nm 4-OHT. Unfixed (Left) or DAPI and pimonidazole stained (right). Image shows UnaG labelling of hypoxic cells in the hypoxic core of the spheroid.

Hypoxia / HIF activation also occurs naturally during embryonic development with oxygen concentrations varying from 1-5% in the uterus. Also, in adult tissues, physiological hypoxia is common (e.g. bone-marrow, kidney) and also occurs in response to wound healing and repair, infection and inflammation. For example, HIF is required for the correct development of the foetus, and in mice it has been shown that knockout of Hif-1α, Hif-2α or Hif-1β leads to embryonic lethality (Dahl et al., 2005) Adult tissue stem cells often reside deeper into the tissue architecture in hypoxic niches. HIF is known to maintain stemness by activating stem cell transcription factors (i.e Oct4) and protects against DNA damage from ROS produced by oxidative phosphorylation (Covello et al., 2006). Lineage tracing would aid in the detection of these cells that reside in hypoxic areas such as embryonic, hematopoietic, mesenchymal, kidney and neural stem cells (Mohyeldin et al., 2010). The Notch stem cell pathway and hypoxia are intimately connected in cell fate decisions, morphogenesis, vascularisation, proliferation and differentiation in development but also in cancer initiation, progression and treatment resistance (Dunwoodie, 2009; Siebel & Lendahl, 2017)

Notch and hypoxia

Notch's role in cancer has been well studied, acting as both as a driver and as a tumour suppressor in cancer with crosstalk with many other signalling pathways. In **chapter 2** we reviewed the current knowledge on its effect in breast cancer. The evidence overwhelmingly shows its correlation with a more aggressive disease and worse outcome. Furthermore, Notch plays an important role in the response to radiotherapy, chemotherapy, hormonal therapies and targeted therapies. Numerous studies have shown the ability of Notch inhibition to re-sensitise treatment-resistant cancers (Domingo-Domenech et al., 2012; McAuliffe et al., 2012; Meng et al., 2009; Nefedova et al., 2008; Yao & Qian, 2010).

Both hypoxia and Notch affect the DNA damage response and apoptosis which can affect the efficacy of radiotherapy on top of the reduced efficacy of radiotherapy in severe hypoxia. Hypoxia represses DNA repair in chronic hypoxia, however, it also activates ATM and ATR in the absence of detectable DNA damage however this activation is transient and is repressed following exposure to chronic hypoxia (Isabel M. Pires et al., 2010). ATM has also been shown to phosphorylate and stabilise HIF-1α in mild hypoxic (0.2-1% O_2) conditions (Cam et al., 2010). Furthermore, ATR has been shown to affect HIF-1α, with ATR inhibition leading to delayed HIF-1α stabilisation and induction of HIF-1 target genes (I M Pires et al., 2012). Notch signalling also plays a role in DNA repair where Notch binds to ATM inactivating its kinase activity. This has been shown to contribute to the survival of Notch driven human leukaemia and notch inhibition in the presence of DNA damage increased radiation sensitivity (Vermezovic et al., 2015). The same study also observed that activated Notch1 was inversely correlated with pATM in human primary breast cancer by immunohistochemistry and expression microarray datasets. Both pathways also affect the tumour suppressor TP53. A number of mechanisms have been postulated for HIFs interaction with P53 including direct interaction (An et al., 1998), indirect regulation through the ubiquitin ligase Mdm2 (Delin Chen et al., 2003) , competition for the coactivator p300 (Schmid et al., 2004) and regulation by HIF target genes such as nucleophosmin, which directly interacts with p53 regulating its activation by inhibiting p53 phosphorylation at serine 15 (J. Li et al., 2004). On top of this Notch also works in synergy with hypoxia to maintain the undifferentiated cell state, which is important for stem cell maintenance (Gustafsson et al., 2005; Sahlgren et al., 2008). Notch signalling has also been found to mediate hypoxia-induced EMT. Notch regulated expression of Snail-1 and LOX both of which are critical for EMT (Sahlgren et al., 2008).

Hypoxia and Notch also play roles in angiogenesis. Notch signalling is crucial for the correct formation of the (neo) vasculature and both over and underexpression of Notch leads to the production of aberrant vasculature. Studies have shown that the Notch ligand Delta-like ligand 4 (DLL4) is induced by the HIF target gene VEGF and is highly expressed in tumour vasculature (Mailhos et al., 2001). DLL4 acts as a negative feedback loop, restraining vascular sprouting and branching. Much research has gone into targeting angiogenesis with the best validated being blockade of VEGF signalling. Targeting VEGF has been shown to be able to normalise tumour vasculature, and reduce vessel density (R. K. Jain, 2005). This normalisation increases oxygen and drug delivery, and has been shown in clinical trials to prolong survival in several cancers (Coleman et al., 2017; Hurwitz et al., 2004; Johnson et al., 2004).

However not all cancers are sensitive to this blockade and tumours may develop resistance, so other targets are needed (Rakesh K. Jain et al., 2006). DLL4 has been shown *in vivo* to be a mechanism of anti-angiogenic therapy resistance in tumours, and therefore may be a target to overcome this resistance (J. L. Li et al., 2011). Targeting DLL4, although leading to increased tumour vascularity, has been seen to decrease tumour growth even

in tumours that are resistant to VEGF targeting (Mailhos et al., 2001; Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). This is presumably because the vasculature is non-functional, and accordingly an increase in hypoxia was seen in DLL4 targeted tumours. The combination of VEGF and DLL4 targeting has been shown to be greater than either treatment alone (Ridgway et al., 2006). Combining DLL4 or Notch inhibition with radiation has been shown *in vivo* to significantly delay tumour growth compared to irradiation alone and lead to extensive necrosis (S. K. Liu et al., 2011). In a H460 NSCLC model with either high or blocked Notch activity, Notch activity did not affect proliferation or intrinsic radiosensitivity, however *in vivo* high Notch activity lead to faster tumour growth greater radioresistance and tumours were also more hypoxic (Theys et al., 2013). DLL4 has been shown to be present in exosomes from endothelial cells (Sheldon et al., 2010) and from high DLL4 expressing tumour cells, which can lead to tubule formation *in vitro* giving an explanation for this observation.

In **chapter 3** we extend this work to investigate the interaction between hypoxia and Notch signalling on angiogenesis. We incubate H460 ΔE NSCLC overexpressing an activated form of Notch1 cells in normoxia, hypoxia, anoxia, or hypoxia with DBZ for 24h before collecting this medium and putting it on receiver cells. We find increased Notch activity in receiver cells when cultured with conditioned medium from hypoxia and anoxia compared with normoxia.

Due to the synergy between hypoxia and Notch, which increase tumour progression, aggression, levels of hypoxia and make tumours more therapy-resistant, we have been developing and testing a series of hypoxia-activated gamma-secretase inhibitors (GSIs). Unlike most HAPs which work through the creation of DNA damage to induce apoptosis, the HAP GSI will be activated in areas of hypoxia releasing the active GSI, inhibiting Notch activity. This will have a similar effect to targeting DLL4, due to DLL4s role as a Notch ligand. We postulate that reducing Notch activity should have the effect of creating aberrant vasculature, slowing tumour growth, and improving the efficacy of other treatments and re-sensitising treatment-resistant tumours as well as other anti-tumour effects as discussed in **chapter 2**. Combining this with anti-angiogenic therapies could lead to even greater tumour control and overcome resistance to anti-angiogenic therapies. The GSI also has the ability to diffuse to nearby cells in a bystander effect to inhibit notch in a greater proportion of the tumour. This has the advantage over normal GSIs of allowing higher doses and longer treatment schedules to be used by reducing normal tissue toxicity which has historically limited the applicability of GSIs in the clinic. Normal tissues are also able to tolerate 50% Notch inhibition which is another advantage over other HAPs which often kill normal cells better than tumour cells which are often resistant.

References

1. Abou-Alfa, G. K., Chan, S. L., Lin, C.-C., Chiorean, E. G., Holcombe, R. F., Mulcahy, M. F., Carter, W. D., Patel, K., Wilson, W. R., Melink, T. J., Gutheil, J. C., & Tsao, C.-J. (2011). PR-104 plus sorafenib in patients with advanced hepatocellular carcinoma. *Cancer Chemotherapy and Pharmacology*, *68*(2), 539–545. https://doi.org/10.1007/s00280-011-1671-3
2. Aerts, H. J. W. L., van Baardwijk, A. A. W., Petit, S. F., Offermann, C., Loon, J. van, Houben, R., Dingemans, A. M. C., Wanders, R., Boersma, L., Borger, J., Bootsma, G., Geraedts, W., Pitz, C., Simons, J., Wouters, B. G., Oellers, M., Lambin, P., Bosmans, G., Dekker, A. L. A. J., & Ruysscher, D. De. (2009). Identification of residual metabolic-active areas within individual NSCLC tumours using a pre-radiotherapy 18Fluorodeoxyglucose-PET-CT scan. *Radiotherapy and Oncology*, *91*(3), 386–392. https://doi.org/10.1016/j.radonc.2009.03.006
3. Airley, R. E., Loncaster, J., Raleigh, J. A., Harris, A. L., Davidson, S. E., Hunter, R. D., West, C. M. L., & Stratford, I. J. (2003). Glut-1 and CAIX as intrinsic markers of hypoxia in carcinoma of the cervix: Relationship to pimonidazole binding. *International Journal of Cancer*, *104*(1), 85–91. https://doi.org/10.1002/ijc.10904
4. An, W. G., Kanekal, M., & Blagosklonny, M. V. (1998). *Stabilization of wild-type p53 by hypoxia-inducible factor 1* . *392*(March), 7416–7419.
5. Ashton, T. M., Fokas, E., Kunz-Schughart, L. A., Folkes, L. K., Anbalagan, S., Huether, M., Kelly, C. J., Pirovano, G., Buffa, F. M., Hammond, E. M., Stratford, M., Muschel, R. J., Higgins, G. S., & McKenna, W. G. (2016). The anti-malarial atovaquone increases radiosensitivity by alleviating tumour hypoxia. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms12308
6. Benej, M., Hong, X., Vibhute, S., Scott, S., Wu, J., Graves, E., Le, Q. T., Koong, A. C., Giaccia, A. J., Yu, B., Chen, C. S., Papandreou, I., & Denko, N. C. (2018). Papaverine and its derivatives radiosensitize solid tumors by inhibiting mitochondrial metabolism. *Proceedings of the National Academy of Sciences*, *115*(49), E11561–E11561. https://doi.org/10.1073/pnas.1818732115
7. Bos, R., Van der Groep, P., Greijer, A. E., Shvarts, A., Meijer, S., Pinedo, H. M., Semenza, G. L., Van Diest, P. J., & Van der Wall, E. (2003). Levels of hypoxia-inducible factor-1?? independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer*, *97*(6), 1573–1581. https://doi.org/10.1002/cncr.11246
8. Brown, J. M. (1979). Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. *British Journal of Radiology*, *52*(620), 650–656. https://doi.org/10.1259/0007-1285-52-620-650
9. Buffa, F. M., Harris, A. L., West, C. M., & Miller, C. J. (2010). Large meta-analysis of multiple cancers reveals a common, compact and highly prognostic hypoxia metagene. *British Journal of Cancer*, *102*(2), 428–435. https://doi.org/10.1038/sj.bjc.6605450
10. Cam, H., Easton, J. B., High, A., & Houghton, P. J. (2010). mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1α. *Molecular Cell*, *40*(4), 509–520. https://doi.org/10.1016/j.molcel.2010.10.030

11. Carolina, N. (2017). *Photoacoustic Imaging for the Detection of Hypoxia*. *46*(5), 527–530. https://doi.org/10.1097/SHK.0000000000000644.PHOTOACOUSTIC
12. Chaplin, D. J., Horsman, M. R., & Trotter, M. J. (1990). Effect of nicotinamide on the microregional heterogeneity of oxygen delivery within a murine tumor. *Journal of the National Cancer Institute*, *82*(8), 672–676. https://doi.org/10.1093/jnci/82.8.672
13. Chen, Daishi, Rauh, M., Buchfelder, M., Eyupoglu, I. Y., & Savaskan, N. (2017). The oxido-metabolic driver ATF4 enhances temozolamide chemo-resistance in human gliomas. *Oncotarget*, *8*(31), 51164–51176. https://doi.org/10.18632/oncotarget.17737
14. Chen, Delin, Li, M., Luo, J., & Gu, W. (2003). Direct interactions between HIF-1 alpha and Mdm2 modulate p53 function. *The Journal of Biological Chemistry*, *278*(16), 13595–13598. https://doi.org/10.1074/jbc.C200694200
15. Coleman, R. L., Brady, M. F., Herzog, T. J., Sabbatini, P., Armstrong, D. K., Walker, J. L., Kim, B.-G., Fujiwara, K., Tewari, K. S., O'Malley, D. M., Davidson, S. A., Rubin, S. C., DiSilvestro, P., Basen-Engquist, K., Huang, H., Chan, J. K., Spirtos, N. M., Ashfaq, R., & Mannel, R. S. (2017). Bevacizumab and paclitaxel–carboplatin chemotherapy and secondary cytoreduction in recurrent, platinum-sensitive ovarian cancer (NRG Oncology/Gynecologic Oncology Group study GOG-0213): a multicentre, open-label, randomised, phase 3 trial. *The Lancet Oncology*, *18*(6), 779–791. https://doi.org/10.1016/S1470-2045(17)30279-6
16. Covello, K. L., Kehler, J., Yu, H., Gordan, J. D., Arsham, A. M., Hu, C. J., Labosky, P. A., Simon, M. C., & Keith, B. (2006). HIF-2α regulates Oct-4: Effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes and Development*, *20*(5), 557–570. https://doi.org/10.1101/gad.1399906
17. Dahl, K. D. C., Fryer, B. H., Mack, F. a, Compernolle, V., Maltepe, E., Adelman, D. M., Carmeliet, P., & Simon, M. C. (2005). Hypoxia-Inducible Factors 1 and 2 Regulate Trophoblast Differentiation. *Mol Cell Biol*, *25*(23), 10479–10491. https://doi.org/10.1128/MCB.25.23.10479–10491.2005
18. Dewhirst, M. W. (2009). Relationships between cycling hypoxia, HIF-1, angiogenesis and oxidative stress. *Radiation Research*, *172*(6), 653–665. https://doi.org/10.1667/RR1926.1
19. Ding, Z., Yang, L., Xie, X., Xie, F., Pan, F., Li, J., He, J., & Liang, H. (2010). Expression and significance of hypoxia-inducible factor-1 alpha and MDR1/P-glycoprotein in human colon carcinoma tissue and cells. *Journal of Cancer Research and Clinical Oncology*, *136*(11), 1697–1707. https://doi.org/10.1007/s00432-010-0828-5
20. Dische, S. (1978). Hyperbaric oxygen: The Medical Research Council trials and their clinical significance. *British Journal of Radiology*, *51*(611), 888–894. https://doi.org/10.1259/0007-1285-51-611-888
21. Doherty, N., Hancock, S. L., Kaye, S., Norman Coleman, C., Shulman, L., Marquez, C., Mariscal, C., Rampling, R., Senan, S., & Roemeling, R. V. (1994). Muscle cramping in phase i clinical trials of tirapazamine (SR 4233) with and without radiation. *International Journal of Radiation Oncology, Biology, Physics*, *29*(2), 379–382. https://doi.org/10.1016/0360-3016(94)90293-3

22. Domingo-Domenech, J., Vidal, S. J., Rodriguez-Bravo, V., Castillo-Martin, M., Quinn, S. A., Rodriguez-Barrueco, R., Bonal, D. M., Charytonowicz, E., Gladoun, N., de la Iglesia-Vicente, J., Petrylak, D. P., Benson, M. C., Silva, J. M., & Cordon-Cardo, C. (2012). Suppression of acquired docetaxel resistance in prostate cancer through depletion of notch- and hedgehog-dependent tumor-initiating cells. *Cancer Cell*, *22*(3), 373–388. https://doi.org/10.1016/j.ccr.2012.07.016
23. Due, A. K., Vogelius, I. R., Aznar, M. C., Bentzen, S. M., Berthelsen, A. K., Korreman, S. S., Loft, A., Kristensen, C. A., & Specht, L. (2014). Recurrences after intensity modulated radiotherapy for head and neck squamous cell carcinoma more likely to originate from regions with high baseline [18F]-FDG uptake. *Radiotherapy and Oncology*, *111*(3), 360–365. https://doi.org/10.1016/j.radonc.2014.06.001
24. Dunwoodie, S. L. (2009). The Role of Hypoxia in Development of the Mammalian Embryo. *Developmental Cell*, *17*(6), 755–773. https://doi.org/10.1016/j.devcel.2009.11.008
25. Erapaneedi, R., Belousov, V. V, Schäfers, M., & Kiefer, F. (2016). A novel family of fluorescent hypoxia sensors reveal strong heterogeneity in tumor hypoxia at the cellular level. *The EMBO Journal*, *35*(1), 102–113. https://doi.org/10.15252/embj.201592775
26. Feng, R., Zhai, W. L., Yang, H. Y., Jin, H., & Zhang, Q. X. (2011). Induction of ER stress protects gastric cancer cells against apoptosis induced by cisplatin and doxorubicin through activation of p38 MAPK. *Biochemical and Biophysical Research Communications*, *406*(2), 299–304. https://doi.org/10.1016/j.bbrc.2011.02.036
27. Fleming, I. N., Manavaki, R., Blower, P. J., West, C., Williams, K. J., Harris, A. L., Domarkas, J., Lord, S., Baldry, C., & Gilbert, F. J. (2015). Imaging tumour hypoxia with positron emission tomography. *British Journal of Cancer*, *112*(2), 238–250. https://doi.org/10.1038/bjc.2014.610
28. Gatenby, R. A., Kessler, H. B., Rosenblum, J. S., Coia, L. R., Moldofsky, P. J., Hartz, W. H., & Broder, G. J. (1988). Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. *International Journal of Radiation Oncology, Biology, Physics*, *14*(5), 831–838. https://doi.org/10.1016/0360-3016(88)90002-8
29. Godet, I., Shin, Y. J., Ju, J. A., Ye, I. C., Wang, G., & Gilkes, D. M. (2019). Fate-mapping post-hypoxic tumor cells reveals a ROS-resistant phenotype that promotes metastasis. *Nature Communications*, *10*(1), 1–18. https://doi.org/10.1038/s41467-019-12412-1
30. Graves, E. E., Vilalta, M., Cecic, I. K., Erler, J. T., Tran, P. T., Sayles, L., Sweet-cordero, A., Le, Q. T., & Amato, J. (2011). *Hypoxia in Models of Lung Cancer: Implications for Targeted Therapeutics*. *16*(19), 4843–4852. https://doi.org/10.1158/1078-0432.CCR-10-1206.Hypoxia
31. Gray, L. H., Conger, A. D., Ebert, M., Hornsey, S., & Scott, O. C. (1953). The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *The British Journal of Radiology*, *26*(312), 638–648. https://doi.org/10.1259/0007-1285-26-312-638
32. Guise, C. P., Abbattista, M. R., Singleton, R. S., Holford, S. D., Connolly, J., Dachs, G. U., Fox, S. B., Pollock, R., Harvey, J., Guilford, P., Doñate, F., Wilson, W. R., & Patterson, A. V. (2010). The bioreductive prodrug PR-104A is activated under aerobic conditions by human aldo-keto reductase 1C3. *Cancer Research*, *70*(4), 1573–1584. https://doi.org/10.1158/0008-5472.CAN-09-3237

33. Gustafsson, M. V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U., & Bondesson, M. (2005). Hypoxia requires Notch signaling to maintain the undifferentiated cell state. *Developmental Cell*, *9*(5), 617–628. https://doi.org/10.1016/j.devcel.2005.09.010
34. Harada, H., Inoue, M., Itasaka, S., Hirota, K., Morinibu, A., Shinomiya, K., Zeng, L., Ou, G., Zhu, Y., Yoshimura, M., McKenna, W. G., Muschel, R. J., & Hiraoka, M. (2012). Cancer cells that survive radiation therapy acquire HIF-1 activity and translocate towards tumour blood vessels. *Nature Communications*, *3*, 710–783. https://doi.org/10.1038/ncomms1786
35. Hicks, K. O., Siim, B. G., Jaiswal, J. K., Pruijn, F. B., Fraser, A. M., Patel, R., Hogg, A., Liyanage, H. D. S., Dorie, M. J., Brown, J. M., Denny, W. A., Hay, M. P., & Wilson, W. R. (2010). Pharmacokinetic/Pharmacodynamic Modeling Identifies SN30000 and SN29751 as Tirapazamine Analogues with Improved Tissue Penetration and Hypoxic Cell Killing in Tumors. *Clinical Cancer Research*, *16*(20), 4946–4957. https://doi.org/10.1158/1078-0432.CCR-10-1439
36. Hicks, Kevin O., Myint, H., Patterson, A. V., Pruijn, F. B., Siim, B. G., Patel, K., & Wilson, W. R. (2007). Oxygen Dependence and Extravascular Transport of Hypoxia-Activated Prodrugs: Comparison of the Dinitrobenzamide Mustard PR-104A and Tirapazamine. *International Journal of Radiation Oncology Biology Physics*, *69*(2), 560–571. https://doi.org/10.1016/j.ijrobp.2007.05.049
37. Hicks, Kevin O., Pruijn, F. B., Secomb, T. W., Hay, M. P., Hsu, R., Brown, J. M., Denny, W. A., Dewhirst, M. W., & Wilson, W. R. (2006). Use of three-dimensional tissue cultures to model extravascular transport and predict in vivo activity of hypoxia-targeted anticancer drugs. *Journal of the National Cancer Institute*, *98*(16), 1118–1128. https://doi.org/10.1093/jnci/djj306
38. Hicks, Kevin O., Pruijn, F. B., Sturman, J. R., Denny, W. A., & Wilson, W. R. (2003). Multicellular resistance to tirapazamine is due to restricted extravascular transport: A pharmacokinetic/pharmacodynamic study in HT29 multicellular layer cultures. *Cancer Research*, *63*(18), 5970–5977.
39. Hochachka, P. W., Buck, L. T., Doll, C. J., & Land, S. C. (1996). Unifying theory of hypoxia tolerance: Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(18), 9493–9498. https://doi.org/10.1073/pnas.93.18.9493
40. Höckel, M., Knoop, C., Schlenger, K., Vorndran, B., Baußnann, E., Mitze, M., Knapstein, P. G., & Vaupel, P. (1993). Intratumoral pO2 predicts survival in advanced cancer of the uterine cervix. *Radiotherapy and Oncology*, *26*(1), 45–50. https://doi.org/10.1016/0167-8140(93)90025-4
41. Hockel, M., Schlenger, K., Aral, B., Mitze, M., Schaffer, U., & Vaupel, P. (1996). Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Research*, *56*(19), 4509–4515. http://www.ncbi.nlm.nih.gov/pubmed/8813149
42. Hoskin, P. J., Sibtain, A., Daley, F. M., & Wilson, G. D. (2003). GLUT1 and CAIX as intrinsic markers of hypoxia in bladder cancer: Relationship with vascularity and proliferation as predictors of outcome of ARCON. *British Journal of Cancer*, *89*(7), 1290–1297. https://doi.org/10.1038/sj.bjc.6601260
43. Hoskin, Peter J., Rojas, A. M., Bentzen, S. M., & Saunders, M. I. (2010). Radiotherapy with concurrent carbogen and nicotinamide in bladder carcinoma. *Journal of Clinical Oncology*, *28*(33), 4912–4918. https://doi.org/10.1200/JCO.2010.28.4950

44. Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R., & Kabbinavar, F. (2004). Bevacizumab plus Irinotecan, Fluorouracil, and Leucovorin for Metastatic Colorectal Cancer. *New England Journal of Medicine*, *350*(23), 2335–2342. https://doi.org/10.1056/NEJMoa032691
45. Jain, R. K. (2005). Normalization of Tumor Vasculature: An Emerging Concept in Antiangiogenic Therapy. *Science*, *307*(5706), 58–62. https://doi.org/10.1126/science.1104819
46. Jain, Rakesh K., Duda, D. G., Clark, J. W., & Loeffler, J. S. (2006). Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nature Clinical Practice Oncology*, *3*(1), 24–40. https://doi.org/10.1038/ncponc0403
47. Janssens, G. O., Rademakers, S. E., Terhaard, C. H., Doornaert, P. A., Bijl, H. P., Van Ende, P. Den, Chin, A., Marres, H. A., De Bree, R., Van Der Kogel, A. J., Hoogsteen, I. J., Bussink, J., Span, P. N., & Kaanders, J. H. (2012). Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: Results of a phase III randomized trial. *Journal of Clinical Oncology*, *30*(15), 1777–1783. https://doi.org/10.1200/JCO.2011.35.9315
48. Jiang, C. C., Yang, F., Thorne, R. F., Zhu, B. K., Hersey, P., & Zhang, X. D. (2009). Human melanoma cells under endoplasmic reticulum stress acquire resistance to microtubule-targeting drugs through XBP-1-mediated activation of akt. *Neoplasia*, *11*(5), 436–447. https://doi.org/10.1593/neo.09208
49. Johnson, D. H., Fehrenbacher, L., Novotny, W. F., Herbst, R. S., Nemunaitis, J. J., Jablons, D. M., Langer, C. J., DeVore, R. F., Gaudreault, J., Damico, L. A., Holmgren, E., & Kabbinavar, F. (2004). Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *Journal of Clinical Oncology*, *22*(11), 2184–2191. https://doi.org/10.1200/JCO.2004.11.022
50. Koritzinsky, M., Levitin, F., Van Den Beucken, T., Rumantir, R. A., Harding, N. J., Chu, K. C., Boutros, P. C., Braakman, I., & Wouters, B. G. (2013). Two phases of disulfide bond formation have differing requirements for oxygen. *Journal of Cell Biology*, *203*(4), 615–627. https://doi.org/10.1083/jcb.201307185
51. Koumenis, C., Naczki, C., Koritzinsky, M., Rastani, S., Diehl, A., Sonenberg, N., Koromilas, A., & Wouters, B. G. (2002). Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2alpha. *Molecular and Cellular Biology*, *22*(21), 7405–7416. https://doi.org/10.1128/mcb.22.21.7405-7416.2002
52. Kruh, G. D., & Belinsky, M. G. (2003). The MRP family of drug efflux pumps. *Oncogene*, *22*(47 REV. ISS. 6), 7537–7552. https://doi.org/10.1038/sj.onc.1206953
53. Kumagai, A., Ando, R., Miyatake, H., Greimel, P., Kobayashi, T., Hirabayashi, Y., Shimogori, T., & Miyawaki, A. (2013). A bilirubin-inducible fluorescent protein from eel muscle. *Cell*, *153*(7), 1602–1611. https://doi.org/10.1016/j.cell.2013.05.038
54. Lazzari, G., & Silvano, G. (2020). From anemia to erythropoietin resistance in head and neck squamous cell carcinoma treatment: A carousel driven by hypoxia. *OncoTargets and Therapy*, *13*, 841–851. https://doi.org/10.2147/OTT.S242263

55. Li, J. L., Sainson, R. C. A., Oon, C. E., Turley, H., Leek, R., Sheldon, H., Bridges, E., Shi, W., Snell, C., Bowden, E. T., Wu, H., Chowdhury, P. S., Russell, A. J., Montgomery, C. P., Poulsom, R., & Harris, A. L. (2011). DLL4-Notch signaling mediates tumor resistance to anti-VEGF therapy in vivo. *Cancer Research*, *71*(18), 6073–6083. https://doi.org/10.1158/0008-5472.CAN-11-1704
56. Li, J., Zhang, X., Sejas, D. P., Bagby, G. C., & Pang, Q. (2004). Hypoxia-induced nucleophosmin protects cell death through inhibition of p53. *Journal of Biological Chemistry*, *279*(40), 41275–41279. https://doi.org/10.1074/jbc.C400297200
57. Liu, L., Ning, X., Sun, L., Zhang, H., Shi, Y., Guo, C., Han, S., Liu, J., Sun, S., Han, Z., Wu, K., & Fan, D. (2008). Hypoxia-inducible factor-1α contributes to hypoxia-induced chemoresistance in gastric cancer. *Cancer Science*, *99*(1), 121–128. https://doi.org/10.1111/j.1349-7006.2007.00643.x
58. Liu, S. K., Bham, S. A. S., Fokas, E., Beech, J., Im, J., Cho, S., Harris, A. L., & Muschel, R. J. (2011). Delta-like ligand 4-Notch blockade and tumor radiation response. *Journal of the National Cancer Institute*, *103*(23), 1778–1798. https://doi.org/10.1093/jnci/djr419
59. Mailhos, C., Lewis, J., Ish-Horowicz, D., Modlich, U., Harris, A., & Bicknell, R. (2001). Delta4, an endothelial specific Notch ligand expressed at sites of physiological and tumor angiogenesis. *Differentiation*, *69*(2–3), 135–144. https://doi.org/10.1046/j.1432-0436.2001.690207.x
60. McAuliffe, S. M., Morgan, S. L., Wyant, G. A., Tran, L. T., Muto, K. W., Chen, Y. S., Chin, K. T., Partridge, J. C., Poole, B. B., Cheng, K. H., Daggett, J., Cullen, K., Kantoff, E., Hasselbatt, K., Berkowitz, J., Muto, M. G., Berkowitz, R. S., Aster, J. C., Matulonis, U. A., & Dinulescu, D. M. (2012). Targeting Notch, a key pathway for ovarian cancer stem cells, sensitizes tumors to platinum therapy. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(43). https://doi.org/10.1073/pnas.1206400109
61. McKeage, M. J., Jameson, M. B., Ramanathan, R. K., Rajendran, J., Gu, Y., Wilson, W. R., Melink, T. J., & Tchekmedyian, N. S. (2012). PR-104 a bioreductive pre-prodrug combined with gemcitabine or docetaxel in a phase Ib study of patients with advanced solid tumours. *BMC Cancer*, *12*. https://doi.org/10.1186/1471-2407-12-496
62. Meng, R. D., Shelton, C. C., Li, Y.-M., Qin, L.-X., Notterman, D., Paty, P. B., & Schwartz, G. K. (2009). gamma-Secretase inhibitors abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells resulting in enhanced chemosensitivity. *Cancer Research*, *69*(2), 573–582. https://doi.org/10.1158/0008-5472.CAN-08-2088
63. Minchinton, A. I., & Tannock, I. F. (2006). Drug penetration in solid tumours. *Nature Reviews Cancer*, *6*(8), 583–592. https://doi.org/10.1038/nrc1893
64. Mohyeldin, A., Garzón-Muvdi, T., & Quiñones-Hinojosa, A. (2010). Oxygen in stem cell biology: A critical component of the stem cell niche. *Cell Stem Cell*, *7*(2), 150–161. https://doi.org/10.1016/j.stem.2010.07.007
65. Mortensen, L. S., Buus, S., Nordsmark, M., Bentzen, L., Munk, O. L., Keiding, S., & Overgaard, J. (2010). Identifying hypoxia in human tumors: A correlation study between 18F-FMISO PET and the Eppendorf oxygen-sensitive electrode. *Acta Oncologica*, *49*(7), 934–940. https://doi.org/10.3109/0284186X.2010.516274

66. Nefedova, Y., Sullivan, D. M., Bolick, S. C., Dalton, W. S., & Gabrilovich, D. I. (2008). Inhibition of notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy. *Blood*, *111*(4), 2220–2229. https://doi.org/10.1182/blood-2007-07-102632
67. Noguera-Troise, I., Daly, C., Papadopoulos, N. J., Coetzee, S., Boland, P., Gale, N. W., Chieh Lin, H., Yancopoulos, G. D., & Thurston, G. (2006). Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature*, *444*(7122), 1032–1037. https://doi.org/10.1038/nature05355
68. Nordsmark, M. (1996). Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. *Radiotherapy and Oncology*, *41*(9), 31–39. https://doi.org/10.1016/s0167-8140(96)01811-7
69. Nordsmark, Marianne, Bentzen, S. M., Rudat, V., Brizel, D., Lartigau, E., Stadler, P., Becker, A., Adam, M., Molls, M., Dunst, J., Terris, D. J., & Overgaard, J. (2005). Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. *Radiotherapy and Oncology*, *77*(1), 18–24. https://doi.org/10.1016/j.radonc.2005.06.038
70. O'Connor, J. P. B., Robinson, S. P., & Waterton, J. C. (2019). Imaging tumour hypoxia with oxygen-enhanced MRI and BOLD MRI. *British Journal of Radiology*, *92*(1096). https://doi.org/10.1259/bjr.20180642
71. Okunieff, P., Hoeckel, M., Dunphy, E. P., Schlenger, K., Knoop, C., & Vaupel, P. (1993). Oxygen tension distributions are sufficient to explain the local response of human breast tumors treated with radiation alone. *International Journal of Radiation Oncology*Biology*Physics*, *26*(4), 631–636. https://doi.org/10.1016/0360-3016(93)90280-9
72. Overgaard, J. (2007). Hypoxic radiosensitization: Adored and ignored. *Journal of Clinical Oncology*, *25*(26), 4066–4074. https://doi.org/10.1200/JCO.2007.12.7878
73. Overgaard, J. (2011). Hypoxic modification of radiotherapy in squamous cell carcinoma of the head and neck - A systematic review and meta-analysis. *Radiotherapy and Oncology*, *100*(1), 22–32. https://doi.org/10.1016/j.radonc.2011.03.004
74. Overgaard, J., Eriksen, J. G., Nordsmark, M., Alsner, J., & Horsman, M. R. (2005). Plasma osteopontin, hypoxia, and response to the hypoxia sensitiser nimorazole in radiotherapy of head and neck cancer: Results from the DAHANCA 5 randomised double-blind placebo-controlled trial. *Lancet Oncology*, *6*(10), 757–764. https://doi.org/10.1016/S1470-2045(05)70292-8
75. Overgaard, J., Hansen, H. S., Overgaard, M., Bastholt, L., Berthelsen, A., Specht, L., Lindeløv, B., & Jørgensen, K. (1998). A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5-85. *Radiotherapy and Oncology*, *46*(2), 135–146. https://doi.org/10.1016/S0167-8140(97)00220-X
76. Pires, I M, Olcina, M. M., Anbalagan, S., Pollard, J. R., Reaper, P. M., Charlton, P. a, McKenna, W. G., & Hammond, E. M. (2012). Targeting radiation-resistant hypoxic tumour cells through ATR inhibition. *British Journal of Cancer*, *107*(2), 291–299. https://doi.org/10.1038/bjc.2012.265

77. Pires, Isabel M., Bencokova, Z., McGurk, C., & Hammond, E. M. (2010). Exposure to acute hypoxia induces a transient DNA damage response which includes Chk1 and TLK1. *Cell Cycle*, *9*(13), 2502–2507. https://doi.org/10.4161/cc.9.13.12059
78. Rankin, E. B., Nam, J. M., & Giaccia, A. J. (2016). Hypoxia: Signaling the Metastatic Cascade. *Trends in Cancer*, *2*(6), 295–304. https://doi.org/10.1016/j.trecan.2016.05.006
79. Rasey, J. S., Koh, W. J., Evans, M. L., Peterson, L. M., Lewellen, T. K., Graham, M. M., & Krohn, K. A. (1996). Quantifying regional hypoxia in human tumors with positron emission tomography of [18F]fluoromisonidazole: A pretherapy study of 37 patients. *International Journal of Radiation Oncology Biology Physics*, *36*(2), 417–428. https://doi.org/10.1016/S0360-3016(96)00325-2
80. Reddy, S. B., & Williamson, S. K. (2009). Tirapazamine: A novel agent targeting hypoxic tumor cells. *Expert Opinion on Investigational Drugs*, *18*(1), 77–87. https://doi.org/10.1517/13543780802567250
81. Reymen, B. J. T., van Gisbergen, M. W., Even, A. J. G., Zegers, C. M. L., Das, M., Vegt, E., Wildberger, J. E., Mottaghy, F. M., Yaromina, A., Dubois, L. J., van Elmpt, W., De Ruysscher, D., & Lambin, P. (2020). Nitroglycerin as a radiosensitizer in non-small cell lung cancer: Results of a prospective imaging-based phase II trial. *Clinical and Translational Radiation Oncology*, *21*, 49–55. https://doi.org/10.1016/j.ctro.2019.12.002
82. Ridgway, J., Zhang, G., Wu, Y., Stawicki, S., Liang, W. C., Chanthery, Y., Kowalski, J., Watts, R. J., Callahan, C., Kasman, I., Singh, M., Chien, M., Tan, C., Hongo, J. A. S., De Sauvage, F., Plowman, G., & Yan, M. (2006). Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature*, *444*(7122), 1083–1087. https://doi.org/10.1038/nature05313
83. Rischin, D., Hicks, R. J., Fisher, R., Binns, D., Corry, J., Porceddu, S., & Peters, L. J. (2006). Prognostic significance of [18F]-misonidazole positron emission tomography-detected tumor hypoxia in patients with advanced head and neck cancer randomly assigned to chemoradiation with or without tirapazamine: A substudy of Trans-Tasman Radiation Oncolog. *Journal of Clinical Oncology*, *24*(13), 2098–2104. https://doi.org/10.1200/JCO.2005.05.2878
84. Rosenberg, A., & Knox, S. (2006). Radiation sensitization with redox modulators: A promising approach. *International Journal of Radiation Oncology Biology Physics*, *64*(2), 343–354. https://doi.org/10.1016/j.ijrobp.2005.10.013
85. Sahlgren, C., Gustafsson, M. V., Jin, S., Poellinger, L., & Lendahl, U. (2008). Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(17), 6392–6397. https://doi.org/10.1073/pnas.0802047105
86. Sahra, I. Ben, Laurent, K., Loubat, A., Giorgetti-Peraldi, S., Colosetti, P., Auberger, P., Tanti, J. F., Le Marchand-Brustel, Y., & Bost, F. (2008). The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene*, *27*(25), 3576–3586. https://doi.org/10.1038/sj.onc.1211024
87. Salaroglio, I. C., Panada, E., Moiso, E., Buondonno, I., Provero, P., Rubinstein, M., Kopecka, J., & Riganti, C. (2017). PERK induces resistance to cell death elicited by endoplasmic reticulum stress and chemotherapy. *Molecular Cancer*, *16*(1), 1–13. https://doi.org/10.1186/s12943-017-0657-0

88. Scehnet, J. S., Jiang, W., Ram Kumar, S., Krasnoperov, V., Trindade, A., Benedito, R., Djokovic, D., Borges, C., Ley, E. J., Duarte, A., & Gill, P. S. (2007). Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. *Blood*, *109*(11), 4753–4760. https://doi.org/10.1182/blood-2006-12-063933
89. Schmid, T., Zhou, J., Köhl, R., & Brüne, B. (2004). p300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (HIF-1). *Biochemical Journal*, *380*(1), 289–295. https://doi.org/10.1042/BJ20031299
90. Sheldon, H., Heikamp, E., Turley, H., Dragovic, R., Thomas, P., Oon, C. E., Leek, R., Edelmann, M., Kessler, B., Sainson, R. C. A., Sargent, I., Li, J., & Harris, A. L. (2010). *New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes*. *116*(13), 2385–2394. https://doi.org/10.1182/blood-2009-08-239228.The
91. Siebel, C., & Lendahl, U. (2017). Notch signaling in development, tissue homeostasis, and disease. *Physiological Reviews*, *97*(4), 1235–1294. https://doi.org/10.1152/physrev.00005.2017
92. Spiegelberg, L., Houben, R., Niemans, R., de Ruysscher, D., Yaromina, A., Theys, J., Guise, C. P., Smaill, J. B., Patterson, A. V., Lambin, P., & Dubois, L. J. (2019). Hypoxia-activated prodrugs and (lack of) clinical progress: The need for hypoxia-based biomarker patient selection in phase III clinical trials. *Clinical and Translational Radiation Oncology*, *15*, 62–69. https://doi.org/10.1016/j.ctro.2019.01.005
93. Stubbs, M., McSheehy, P. M. J., Griffiths, J. R., & Bashford, C. L. (2000). Causes and consequences of tumour acidity and implications for treatment. In *Molecular Medicine Today*. https://doi.org/10.1016/S1357-4310(99)01615-9
94. Theys, J., Yahyanejad, S., Habets, R., Span, P., & Dubois, L. (2013). *High NOTCH activity induces radiation resistance in non small cell lung cancer*. *108*(3), 440–445. https://doi.org/10.1016/j.radonc.2013.06.020.High
95. Thorwarth, D., Eschmann, S. M., Holzner, F., Paulsen, F., & Alber, M. (2006). Combined uptake of [18F]FDG and [18F]FMISO correlates with radiation therapy outcome in head-and-neck cancer patients. *Radiotherapy and Oncology*, *80*(2), 151–156. https://doi.org/10.1016/j.radonc.2006.07.033
96. Toustrup, K., Sørensen, B. S., Alsner, J., & Overgaard, J. (2012). Hypoxia Gene Expression Signatures as Prognostic and Predictive Markers in Head and Neck Radiotherapy. *Seminars in Radiation Oncology*, *22*(2), 119–127. https://doi.org/10.1016/j.semradonc.2011.12.006
97. Toustrup, K., Sørensen, B. S., Lassen, P., Wiuf, C., Alsner, J., & Overgaard, J. (2012). Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck. *Radiotherapy and Oncology*, *102*(1), 122–129. https://doi.org/10.1016/j.radonc.2011.09.010
98. Vaupel, P, Hockel, M., & Mayer, A. (2007). Detection and characterization of tumor hypoxia using pO2 histography. *Antioxid Redox Signal*, *9*(8), 1221–1235. https://doi.org/10.1089/ars.2007.1628
99. Vaupel, Peter, Mayer, A., & Höckel, M. (2004). Tumor hypoxia and malignant progression. *Methods in Enzymology*, *381*(2), 335–354. https://doi.org/10.1016/S0076-6879(04)81023-1

100. Vermezovic, J., Adamowicz, M., Santarpia, L., Rustighi, A., Forcato, M., Lucano, C., Massimiliano, L., Costanzo, V., Bicciato, S., & Sal, G. Del. (2015). Notch is a direct negative regulator of the DNA-damage response. *Nature Publishing Group*, *22*(5). https://doi.org/10.1038/nsmb.3013
101. Wang, Y., Wang, H., Li, J., Entenberg, D., Xue, A., Wang, W., & Condeelis, J. (2016). Direct visualization of the phenotype of hypoxic tumor cells at single cell resolution in vivo using a new hypoxia probe. *IntraVital*, *5*(2), e1187803. https://doi.org/10.1080/21659087.2016.1187803
102. Winter, S. C., Buffa, F. M., Silva, P., Miller, C., Valentine, H. R., Turley, H., Shah, K. A., Cox, G. J., Corbridge, R. J., Homer, J. J., Musgrove, B., Slevin, N., Sloan, P., Price, P., West, C. M. L., & Harris, A. L. (2007). Relation of a hypoxia metagene derived from head and neck cancer to prognosis of multiple cancers. *Cancer Research*, *67*(7), 3441–3449. https://doi.org/10.1158/0008-5472.CAN-06-3322
103. Wu, L., Zhu, J., Prokop, L. J., & Hassan Murad, M. (2015). Pharmacologic Therapy of Diabetes and Overall Cancer Risk and Mortality: A Meta-Analysis of 265 Studies. *Scientific Reports*, *5*(December 2014), 1–10. https://doi.org/10.1038/srep10147
104. Yao, J., & Qian, C. (2010). Inhibition of Notch3 enhances sensitivity to gemcitabine in pancreatic cancer through an inactivation of PI3K/Akt-dependent pathway. *Medical Oncology*, *27*(3), 1017–1022. https://doi.org/10.1007/s12032-009-9326-5
105. Yasuda, H., Yamaya, M., Nakayama, K., Sasaki, T., Ebihara, S., Kanda, A., Asada, M., Inoue, D., Suzuki, T., Okazaki, T., Takahashi, H., Yoshida, M., Kaneta, T., Ishizawa, K., Yamanda, S., Tomita, N., Yamasaki, M., Kikuchi, A., Kubo, H., & Sasaki, H. (2006). Randomized phase II trial comparing nitroglycerin plus vinorelbine and cisplatin with vinorelbine and cisplatin alone in previously untreated stage IIIB/IV non-small-cell lung cancer. *Journal of Clinical Oncology*, *24*(4), 688–694. https://doi.org/10.1200/JCO.2005.04.0436
106. Zannella, V. E., Pra, A. D., Muaddi, H., McKee, T. D., Stapleton, S., Sykes, J., Glicksman, R., Chaib, S., Zamiara, P., Milosevic, M., Wouters, B. G., Bristow, R. G., & Koritzinsky, M. (2013). Reprogramming metabolism with metformin improves tumor oxygenation and radiotherapy response. *Clinical Cancer Research*, *19*(24), 6741–6750. https://doi.org/10.1158/1078-0432.CCR-13-1787
107. Zegers, C. M. L., Van Elmpt, W., Hoebers, F. J. P., Troost, E. G. C., Öllers, M. C., Mottaghy, F. M., & Lambin, P. (2015). Imaging of tumour hypoxia and metabolism in patients with head and neck squamous cell carcinoma. *Acta Oncologica*, *54*(9), 1378–1384. https://doi.org/10.3109/0284186X.2015.1062913
108. Zegers, C. M. L., Van Elmpt, W., Reymen, B., Even, A. J. G., Troost, E. G. C., Öllers, M. C., Hoebers, F. J. P., Houben, R., Eriksson, J., Windhorst, A. D., Mottaghy, F. M., De Ruysscher, D., & Lambin, P. (2014). In vivo quantification of hypoxic and metabolic status of NSCLC tumors using [18f]hx4 and [18f] fdg-pet/ct imaging. *Clinical Cancer Research*, *20*(24), 6389–6397. https://doi.org/10.1158/1078-0432.CCR-14-1524
109. Zhou, G., Goodyear, L. J., Moller, D. E., Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J., & Moller, D. E. (2001). Role of AMP-activated protein kinase in mechanism of metformin action Find the latest version : Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of Clinical Investigation*, *108*(8), 1167–1174. https://doi.org/10.1172/JCI200113505.Introduction

Summary

Cancer is the second leading cause of death globally according to the World Health Organisation. While survival rates are increasing there is still a high unmet need in many cancers for new treatment modalities and personalised treatment to further increase survival. New methods to overcome treatment-resistant tumours are vitally important in instances where the tumour becomes resistant to a particular therapy.

In **chapter 2** we review the current knowledge on the role of Notch in breast cancer. Notch is a cell-cell communication system that, depending on the context, can act as both an oncogene and a tumour suppressor. In breast cancer, there is overwhelming evidence that Notch plays a role in both development and progression with high Notch activity being associated with a more aggressive disease and poor patient outcome. While mutational changes in Notch are limited, expression, activity and cross-talk with other oncogenic pathways are found in many breast cancers. Furthermore, Notch has been shown to play an important role in the response to radiotherapy, chemotherapy, hormonal and targeted therapies. Critically, there is strong evidence that treatment-resistant breast cancers can be re-sensitised through inhibition of Notch, providing a rationale for combining Notch inhibition with current therapies.

Hypoxia is a common feature found in solid tumours arising from an imbalance between oxygen consumption and delivery in the tumour. The high proliferation and metabolic activity of tumours coupled with the inefficient tumour vasculature leads to areas of hypoxia within the tumour. Hypoxia is associated with worse outcomes in many different cancers regardless of treatment type. Notch and hypoxia have also been shown to influence several of the same pathways including parts of the metastatic cascade and neoangiogenesis. The Notch target gene DLL4 and the hypoxia-inducible factor (HIF) target gene VEGF are critical for the formation and maturation of new blood vessels. Previous research in our lab has shown a larger hypoxic fraction in Notch overexpressing tumours suggesting reduced functional vasculature or increased survival and adaptation of hypoxic cancers cells with increased Notch activity.

In **chapter 3** we extend this work to investigate how hypoxia influences Notch activity in this model and how this can affect Notch activity in distant cells. We use conditioned medium from these cells exposed to different oxygen conditions to see whether Notch signalling could be induced in reporter cells independent of cell contact. We find that hypoxic cells are able to upregulate Notch activity in recipient cells. Importantly this upregulation in activity can be abrogated through the use of Notch inhibitors. This points to a possible application of Notch inhibitors to reduce the hypoxic fraction of tumours, however, whether this holds true *in vivo* is unknown. Future research into the effect of Notch inhibitors on the hypoxic fraction of Notch expressing tumour cells and their microenvironment is therefore needed.

In **chapter 4** we develop a novel strategy to label hypoxic cells in a temporally controlled manner. We created an 'oxygen sensing' HIF1α-GFP-CreER^{T2} fusion protein which also incorporates temporal control through the ER^{T2} domain which translocates the protein

to the nucleus when 4-hydroxytamoxifen (4OHT) is present. We coupled this with a fluorescent reporter and transduced them into a H1299 non-small cell lung cancer model. We characterise this model both *in vitro* and *in vivo* showing permanent labelling of hypoxic cells only when 4OHT is added. We show that hypoxic cells can be visualised at single-cell resolution *in vivo* via intravital microscopy, and *ex vivo* via immunohistochemistry. Using this model we find a proliferative advantage for post-hypoxic cells *in* vivo when compared with unlabelled cells. This implies that hypoxia permanently alters the cellular phenotype and behaviour, but what causes this increased proliferation is still unknown. Future research should be aimed at finding the (epi)genetic and proteomic changes that cause this phenotype.

In **chapter 5** we refine this system further with the substitution of the CMV promoter for EF1α in the oxygen sensing construct to facilitate its use in cell lines where CMV silencing occurs. We coupled this with a reporter construct that also contains the Diphtheria toxin receptor protein allowing labelled cells to be selectively ablated upon administration of Diphtheria toxin. We characterised this system *in vitro* using the 4T1 murine metastatic breast cancer model, showing oxygen and 4OHT dependent labelling of cells which can then be selectively ablated with Diphtheria toxin. Next, we optimised the 4T1 cell line *in vivo* in an immunocompetent orthotopic model. We determined the hypoxic fraction at different tumour volumes to determine the timing and dosing of tamoxifen. Next, we optimised the dose of diphtheria toxin needed to kill all labelled cells within days after tamoxifen administration in tumours and determined the growth response of 4T1 tumours to single and fractionated radiotherapy. We show that hypoxic cells can be labelled through the administration of tamoxifen and can then be subsequently ablated with diphtheria toxin. Our hypothesis is that Diphtheria toxin will function as the 'perfect' drug or hypoxic cytotoxin and will sensitize tumours to RT treatment. Ongoing studies are investigating the benefits of hypoxic cell depletion with Diphtheria toxin and tumour control after radiotherapy.

In **chapter 6** we describe the generation of a novel knock-in mouse strain which can be used to lineage trace hypoxic cells. At present, there are no mouse strains that can identify hypoxic cells in vivo at the single-cell level. Such systems would be invaluable in our further understanding of the role of hypoxia in normal tissue development, homeostasis, tissue regeneration and in pathological processes such as cancer inflammation, tissue ischemia including cardiovascular and neurodegenerative diseases. To enable lineage tracing of HIF-1 activated cells throughout development and in adult mouse tissues we fused the c-terminal oxygen dependent degradation domain of HIF1a HIF-1α (aa 1-603)- to a eGFP-CreER^{T2} into theHIF1 locus in C57Bl/6 embryonic stem cells. We show that the ES cells that were used to generate the mice are able to report on hypoxia when a reporter was introduced. We obtained germline transmission of the HIF1-Cre-ER allele and obtained hemizygous mice that are viable and fertile. The generated mouse strain should provide a valuable tool to study hypoxic cell behaviour *in vivo* and in the assessment of hypoxia modification and targeting strategies.

Valorisation Addendum

Societal relevance:

Cancer is one of the leading causes of death worldwide and rising as a consequence of our ageing population. Cancer also has the highest direct and indirect costs of any disease. While developments have been made improving the survival rate of less malignant breast cancer patients, there is still a need to find new treatment strategies to improve survival further especially in patients with metastatic disease. In this thesis, we start by reviewing the current evidence for the role of Notch in breast cancer. Notch is cell to cell communication system that plays a critical role in development, determining cell fate and the generation of new blood vessels. Notch has been shown to act as both an oncogene and a tumour suppressor depending on the cancer type and context. Notch plays a role in normal breast development, but there is also overwhelming evidence that it also plays a role in the development and progression of breast cancer. This is not always due to mutations, with Notch signalling found to be active and cross-talking with many other oncogenic signalling pathways. For example there is evidence that both ER and EGFR signalling (both targets in breast cancer) negatively regulate NOTCH signalling. When inhibited (tamoxifen or anti-EGFR/ HER2) they activate Notch providing a survival signal. The co-targeting of EGFR and ER with Notch would sensitise the tumour to these treatments.

Scientific relevance:

Hypoxia (low oxygen) is a common feature of tumours due to their high metabolic activity, proliferation, and suboptimal vasculature. Hypoxia is also linked to metastasis which is the ultimate cause of treatment failure in the majority of cancers. While there are sometimes efficient treatments for early disease, the metastatic burden often leads to eventual treatment failure. Hypoxia is also known to reduce the efficacy of radiotherapy due to the oxygen effect enhancing the DNA damage created by radiotherapy. This means that more DNA damage and ultimately cell death is produced in well-oxygenated regions than in hypoxic regions. The hypoxic fraction of tumours has also been shown to be more resistant to chemotherapeutics through multiple mechanisms. Hypoxia also affects many of the same pathways as Notch signalling. Both play major roles in the maintenance of (cancer) stem cells and affect tumour progression and response to therapy. Both Notch and hypoxia play a critical role in angiogenesis, and in cancer this can lead to the formation of aberrant vasculature further increasing the hypoxic fraction of tumours. Overall hypoxia is a negative predictive factor and is associated with poor patient outcome. Due to this, there have been several different strategies aimed at relieving tumour hypoxia to increase the efficacy of radiotherapy and chemotherapy. In addition to this, hypoxia has also been used to target drugs to the tumour to reduce side effects which often limit the dose of chemotherapeutics that can be used, reducing their efficacy. Despite many promising candidates no hypoxia modification or targeting strategies have reached the clinic. Part of the reason for these failures is a lack of knowledge and understanding into how the hypoxic population of cells behave and how they contribute to therapy resistance and

tumour recurrence as a population and at the single-cell level especially in the complex *in vivo* microenvironment.

In this thesis, we describe the generation and development of a genetic tool to permanently label hypoxic cells in a temporally controlled manner. Using this tool we have shown that labelled cells are able to be visualised both in living tumours using intravital microscopy and once the tumour has been removed at single-cell resolution. We find that post-hypoxic cells were more proliferative than unlabelled cells raising questions as to how and what effect this may have on the tumour and patient outcome. The hypoxic tracer method provides a useful quantitative tool with high specificity and sensitivity to study hypoxic and post-hypoxic cell behaviour *in vivo* at the single-cell level. More information on the effect of hypoxia will lead to new and improved strategies for targeting these cells, which can eventually translate to the clinic. This tool will facilitate study into the dynamics of hypoxic and post-hypoxic cells within the primary tumour and their contribution to metastases. The effects of treatments such as radio and chemotherapy on this population of cells and how they respond can also be investigated.

We further developed the tool to allow the selective killing of labelled cells allowing investigation into the role of hypoxic cells in therapy resistance. This system will be able to show what effects targeting the hypoxic population will have, and what benefits this has in combination with established treatments. This tool will also be useful in understanding the efficacy of hypoxia modification and targeting strategies. It will facilitate visualisation of the hypoxic population with and without these strategies and what effect they have on this population of cells. It will help in the optimisation of these strategies and how and when they are applied. The timing of these strategies in relation to other treatments such as radiotherapy can be investigated to elucidate the most effective schedule.

While cell lines are an indispensable tool in the study of cancer they often do not allow the use of immunocompetent mice or effectively replicate the clinical situation even in orthotopic models. To overcome this limitation we created a knockin transgenic mouse strain with this tool to facilitate the study of hypoxia in spontaneous tumours when crossed with mice lines such as MMTV-PYMT; a transgenic mouse model which spontaneously develop ductal mammary carcinomas. Spontaneous tumours better imitate the clinical situation due to undergoing initiation steps such as immunoediting as well as being influenced by organ-specific microenvironmental factors such as hormones and tissue architecture. This model can therefore give data that is more relevant to the clinical situation. In addition to applications in cancer research, this tool will be of use in other areas where hypoxia is a factor. Other problems such as tissue ischemia (heart failure, stroke) lead to hypoxia, where this tool can be of use to study the affected cells and tissues. On top of this certain aspects of development are dependent on hypoxia and hypoxia-inducible factors, with human embryos developing in a predominantly hypoxic environment.

Together, the work in this thesis provides a rationale for the use of Notch inhibitors in the treatment of breast cancers and provides the scientific community with a valuable new tool.

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The time I have spent in Maastricht doing my PhD has been life changing. 5 years ago I was working as a research assistant looking for PhD opportunities. At the time it had not really crossed my mind to do it in another country. One day Ester Hammond showed me the availability of a PhD position in Maastricht with Marc. I was both excited about the project and a bit uncertain about moving to another country. However, after thinking I realised it was a great opportunity and life experience and it turned out that way. I applied for the position and after a skype interview was brought over for another interview, to meet the lab and to see the city. This day was both intense with a full day of interviews with everyone, a presentation and journal club but also exciting with meeting a lot of new people from many different cultures. The city was also beautiful and reminded me a lot of old cities in the UK and a similar culture which helped to ease the reservations I had. After being offered the position I was elated and started to organise the move. I arrived in august and quickly settled in with the help of my new colleagues who made the transition so easy with their help. The people I have met in and outside of the lab have made this an amazing experience and without their help I would have never made it to the end and come out the other side a better researcher and person.

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Curriculum Vitae

Jonathan Ient was born on March 4th 1992 in Ascot, in the United Kingdom. In June 2013, he graduated with a 2.1 in Biochemistry from University College London. In 2014 he obtained a Masters of Research degree in Biosciences: Biochemistry receiving a distinction working on a project titled "Design, expression and NMR spectroscopy of a ZIP14 N-terminal construct". Following this he worked as a research assistant for 1.5 years in the laboratory of Ester Hammond specialising in hypoxia and DNA repair at Oxford university. He worked on "Preclinical testing of an ATR inhibitor demonstrates improved response to standard therapies for esophageal cancer" among other projects. In September 2016 he started his PhD under the supervision of Marc Vooijs and Arjan Groot in the Department of Radiotherapy, Faculty of Health, Medicine and Life Sciences (FHML), Maastricht university. During his PhD he attended the 3rd Course on Breast Cancer, Institute Curie (Paris) and received a travel award for his poster presentation. He also presented his work at the ICCR conference 2019, in Manchester. His work focused on the development of a novel technique to lineage trace and ablate hypoxic cells. He also worked on the role of Notch in breast cancer and its interaction with hypoxia in facilitating resistance to radiotherapy and formation of neovasculature. These results are described in this thesis.

List of publications

Vermeer, J. A. F., **Ient, J**., Markelc, B., Kaeppler, J., Barbeau, L. M. O., Groot, A. J., Muschel, R. J., & Vooijs, M. A. (2020). A lineage-tracing tool to map the fate of hypoxic tumour cells. *Disease Models & Mechanisms*, dmm.044768. https://doi.org/10.1242/dmm.044768

Giuranno, L., **Ient, J**., De Ruysscher, D., & Vooijs, M. A. (2019). Radiation-Induced Lung Injury (RILI). *Frontiers in Oncology*, *9*(September), 1–16. https://doi.org/10.3389/fonc.2019.00877

Moreno Roig, E., Groot, A., Yaromina, A., Hendrickx, T., Barbeau, L., Giuranno, L., Dams, G., **Ient, J**., Olivo Pimentel, V., van Gisbergen, M., Dubois, L., & Vooijs, M. (2019). HIF-1α and HIF-2α Differently Regulate the Radiation Sensitivity of NSCLC Cells. *Cells*, *8*(1), 45. https://doi.org/10.3390/cells8010045

Mollen, E. W. J., **Ient, J**., Tjan-Heijnen, V. C. G., Boersma, L. J., Miele, L., Smidt, M. L., & Vooijs, M. A. G. G. (2018). Moving breast cancer therapy up a notch. *Frontiers in Oncology*, *8*(November), 1–25. https://doi.org/10.3389/fonc.2018.00518

Fidler, D. R., Murphy, S. E., Courtis, K., Antonoudiou, P., El-Tohamy, R., **Ient, J**., & Levine, T. P. (2016). Using HHsearch to tackle proteins of unknown function: A pilot study with PH domains. *Traffic*, *17*(11), 1214–1226. https://doi.org/10.1111/tra.12432

Leszczynska, K. B., Dobrynin, G., Leslie, R. E., **Ient, J**., Boumelha, A. J., Senra, J. M., Hawkins, M. A., Maughan, T., Mukherjee, S., & Hammond, E. M. (2016). Preclinical testing of an Atr inhibitor demonstrates improved response to standard therapies for esophageal cancer. *Radiotherapy and Oncology*, *121*(2). https://doi.org/10.1016/j.radonc.2016.10.023

Leszczynska, K. B., Göttgens, E.-L., Biasoli, D., Olcina, M. M., **Ient, J**., Anbalagan, S., Bernhardt, S., Giaccia, A. J., & Hammond, E. M. (2016). Mechanisms and consequences of ATMIN repression in hypoxic conditions: roles for p53 and HIF-1. *Scientific Reports*, *6*(1), 21698. https://doi.org/10.1038/srep21698

Ramachandran, S., **Ient, J**., Göttgen, E.-L., Krieg, A. J. A., Hammond, E. M. E., Göttgens, E.-L., Krieg, A. J. A., Hammond, E. M. E., Göttgen, E.-L., Krieg, A. J. A., & Hammond, E. M. E. (2015). Epigenetic Therapy for Solid Tumors: Highlighting the Impact of Tumor Hypoxia. *Genes*, *6*(4), 935–956. https://doi.org/10.3390/genes6040935