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# Twist proteins as oxidative and hypoxic stress regulators

Jakub Kolodziejcki

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# THÈSE

Pour obtenir le grade de  
**Docteur**

Délivré par **UNIVERSITÉ de MONTPELLIER**

Préparée au sein de l'école doctorale  
**Sciences Chimiques et Biologiques pour la Santé**

Et de l'unité de recherche **IGMM-CNRS UMR5535**

Spécialité : **Biologie Santé**

Présentée par **Jakub Kolodziejski**

**Twist proteins as oxidative and hypoxic  
stress regulators**

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**“Nature was not designed to make life easy for biologists.”**

Tudge, 2007

For family.



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---

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Indeed, these were amazing four years.

# ABSTRACT IN ENGLISH

---

**Title:** Twist proteins as oxidative and hypoxic stress regulators.

**Key words:** Twist, cellular stress, ROS, hypoxia, apoptosis, tumorigenesis.

Twist1 and Twist2 are related transcription factors that play major roles both during embryonic development and in several pathologies, including cancer. Twists' oncogenic potential arises from a combination of their multiple functions in development. Notably, both Twist induce epithelial-to mesenchymal transition, thus promoting tumour invasiveness and possibly conferring to cells self-renewal properties. Furthermore, through disruption of both Rb- and p53-driven pathways, Twist override two major oncogene-induced fail-safe programs, namely senescence and apoptosis, thereby promoting malignant conversion. Twist has also been reported to participate in acquisition of drug resistance and in promotion of neo-angiogenesis.

Current knowledge of pleiotropic activities of Twist prompted us to postulate that these factors may be major regulators of stress response. Cancer cells survive and grow within a continuously changing environment that creates multiple stresses to which they must adapt in order to survive and thrive. Such adaptations often give rise to the acquisition of an aggressive phenotype. Consistent with this hypothesis, we recently unveiled new activities of Twist proteins that are related to stress response.

We have shown that Twist regulates response to oxidative stress, a condition exacerbated in cancer by stimuli such as inflammation, increased cellular metabolism and changes in tumour oxygenation. Our work has contributed to the understanding of molecular mechanisms through which Twist diminishes cellular ROS and thus participates in the escape from apoptosis and senescence. In the first part of my thesis, I worked on the antioxidant activity of Twist and described its molecular mechanisms.

The second part of my work addressed the impact of Twist proteins on cellular response to hypoxia that is insufficient oxygen supply, frequently found in solid tumours. Cellular response to hypoxic stress relies on stabilization and activation of HIF1 $\alpha$ , a key transcriptional mediator of the hypoxic response, regulating numerous genes involved



in glucose metabolism, oxygen transport, angiogenesis, cell growth and apoptosis. HIF1 $\alpha$  is beneficial for cancer cells in response to short hypoxic episodes, however its sustained activation in case of prolonged hypoxia may push cancer cells towards apoptosis. In this context, we have shown that Twist protects cancer cells from hypoxia-induced apoptosis. We have discovered HIF1 $\alpha$  and Twist physically interact, suggesting a possible mechanistic basis for Twist's protective effect. These results led us to postulate that Twist plays a role in cellular response to hypoxia and thus participates in cancer cell adaptation and acquisition of aggressive phenotypes triggered by lack of oxygen.

Our results reinforce the notion that Twist factors are major cellular stress modulators that might be important for adaptation of cancer cells to changing conditions in the process of tumour progression.

# ABSTRACT IN FRENCH

---

**Titre:** Etude des facteurs oncogéniques Twist dans la régulation du stress oxydatif et hypoxique.

**Mots clés:** Twist, stress cellulaire, ROS, hypoxie, apoptose, tumorigenèse.

Les facteurs de transcription Twist1 et Twist2 (famille Twist) jouent un rôle majeur dans le développement embryonnaire et dans la progression tumorale. Leur potentiel oncogénique dérive directement de la combinaison de leurs nombreuses activités développementales. Les gènes Twist peuvent notamment, en induisant la transition épithélio-mésenchymateuse (EMT), promouvoir l'invasion des cellules cancéreuses et participer de ce fait aux processus métastatique. De plus, en bloquant l'activité des voies de signalisation Rb et p53, ils peuvent inhiber les deux principaux programmes de sauvegarde cellulaire que sont l'apoptose et la senescence. Enfin, ils sont également impliqués dans la résistance des cellules cancéreuses aux agents chimio-thérapeutiques.

En plus de ces nombreuses activités, nos données préliminaires nous ont amené à considérer un rôle de Twist dans la réponse au stress. Les cellules cancéreuses doivent croître dans un environnement en perpétuel changement qui génère de nombreux types de stress. Seules les cellules capables de s'adapter, peuvent survivre et acquérir de nouvelles capacités les rendant plus agressives. La résistance au stress fait donc parti intégrante de la progression tumorale. Nos travaux révèlent que Twist en induisant une résistance au stress, plus particulièrement métabolique, est un acteur essentiel de l'acquisition d'un phénotype agressif des cellules cancéreuses.

Dans une première étude, nous avons montré que Twist module le stress oxydatif, une condition très fréquemment retrouvée dans les tumeurs. Ainsi, nos résultats indiquent que l'expression de Twist provoque une réduction du taux d'espèces réactives de l'oxygène (ROS) intracellulaire. Cette activité a pour conséquence directe d'induire une résistance accrue à l'apoptose déclenchée par divers traitements. Nous avons par la suite caractérisé cette activité et mis en évidence un programme génétique contrôlé par Twist impliquant divers facteurs possédant des propriétés anti-oxydantes.

Dans un second temps, nous nous sommes intéressés à un autre type de stress métabolique, l'hypoxie. L'hypoxie définie par un taux insuffisant d'oxygène, est retrouvée dans la plupart des tumeurs solides du fait de l'absence ou de l'anomalie de la vascularisation. L'hypoxie mène à la stabilisation d'un facteur de transcription, HIF1 $\alpha$ . Cette protéine est essentielle à l'adaptation hypoxique et contrôle l'expression de nombreux gènes impliqués dans le métabolisme du glucose, le transport de l'oxygène, l'angiogenèse ou l'apoptose. Dans les premiers temps d'hypoxie, l'effet d'adaptation induit par HIF1 $\alpha$  est bénéfique pour les cellules. Cependant, si l'absence d'oxygène se prolonge, HIF1 $\alpha$ , peut pousser les cellules vers la mort. Nos travaux démontrent que Twist est capable de rendre les cellules résistantes à une hypoxie prolongée. De plus, cette activité de protection contre le stress hypoxique agit via un effet paracrine. Enfin, nos données suggèrent que cet effet est médié par une interaction directe entre les protéines Twist et HIF1 $\alpha$ . Au final, cette étude indique que l'expression de Twist dans les cellules cancéreuses, en conférant une résistance accrue à l'environnement hypoxique, joue un rôle essentiel dans l'adaptation au stress et à l'acquisition de nouveaux phénotypes agressifs.

En résumé, l'objectif principal de ma thèse était de mettre en évidence de nouvelles propriétés cellulaires des oncogènes de la famille Twist. Nos résultats démontrent que Twist par ses capacités à contrôler le stress métabolique, permet à la cellule cancéreuse de mieux s'adapter et donc survivre dans un environnement en constante évolution. Nos travaux renforcent donc la notion de l'importance de ces facteurs dans la progression tumorale.

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# ABBREVIATIONS

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**Akt** - RAC-alpha serine/threonine-protein kinase

**BCL-2** - B cell lymphoma 2 protein family

**BH1-4** - BCL-2 homology domain 1-4

**bHLH** - basic helix-loop-helix

**BMI1** - B lymphoma Mo-MLV insertion region 1 homolog - polycomb complex protein

**CAF** - cancer associated fibroblast

**CDK** - cyclin-dependent kinase

**CSC** - cancer stem cells

**DDR** - DNA damage response

**DNA** - deoxyribonucleic acid

**ECM** - extra-cellular matrix

**EMT** - epithelial-mesenchymal transition

**ERK** - extracellular signal-regulated kinase

**FGF(R)** - fibroblast growth factor (receptor)

**FIH1** - factor inhibiting HIF 1

**GPx** - glutathione peroxidases

**GSH** - glutathione

**GST** - glutathione s-transferase

**H<sub>2</sub>O<sub>2</sub>** - hydrogen peroxide

**HAF** - hypoxia-associated factor

**HCC** - hepatocellular carcinoma

**HIF1/2 $\alpha$**  - hypoxia-inducible factor 1/2-alpha

**HLH** - helix-loop-helix

**HRE** - hypoxia responsive element

**Id** - inhibition of DNA binding

**IFN $\gamma$**  - interferon gamma

**JNK** - c-Jun N-terminal kinase

**KO** - knock-out

**MAPK** - mitogen-activated protein kinases

**MDM2/4** - mouse double minute 2/4 homolog

**MEF2** - myocyte enhancer factor 2

**MET** - mesenchymal-epithelial transition

**miRNA** - micro RNA

**MMP** - matrix metalloproteinase

**NADH** - reduced form of nicotinamide adenine dinucleotide

**NF- $\kappa$ B** - nuclear factor kappa-light-chain-enhancer of activated B cells

**NOX** - NADPH oxidase

**O<sub>2</sub><sup>-•</sup>** - superoxide anion radical

**OIS** - oncogene-induced senescence

**PGC1 $\alpha$**  - peroxisome proliferator-activated receptor gamma coactivator 1-alpha

**PHD** - prolyl hydroxylase

**PI3K** - phosphatidylinositol-3 kinase

**pRB** - gene product of retinoblastoma

**pRB** - gene product of retinoblastoma

**pVHL** - von Hippel–Lindau tumor suppressor  
**Rac1** - Ras-related C3 botulinum toxin substrate 1  
**RNA** - ribonucleic acid  
**ROS** - reactive oxygen species  
**Runx1-3** - Runt-related transcription factor 1-3  
**SC** - stem cells  
**SCS** - Saethre–Chotzen syndrome

**Sirt1-6** - sirtuin 1-6  
**SOD** - superoxide dismutase  
**Src** - steroid receptor coactivator  
**TAM** - tumour associated macrophages  
**TGF $\beta$**  - tumour growth factor beta  
**TME** - tumour microenvironment  
**TNF $\alpha$**  - tumour necrosis factor alpha  
**VEGF** - vascular endothelial growth factor

## INTRODUCTION

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# CHAPTER I. Mechanisms of tumorigenesis

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Over the past decades, cancer researchers have gradually started to recognize that tumours can be considered as organs whose complexity can approach that of healthy tissues. Cancer is a disease characterized by uncontrolled growth and spread of mutated malignant cells. If the growth and spreading of tumour is not prevented, it ultimately results in death of the whole organism. Appearance and progression of cancer is due to accumulation of inherited and somatically acquired mutations. These can arise spontaneously (the "bad luck" cause of cancer [1]) or be a result of environmental stress (pathogens, tobacco, radiation, chemicals). Despite many years of intensive research, we are still far from understanding cancer biology and the complexity of this disease. According to the International Agency for Research on Cancer, in 2012 cancer incidence rose up to an estimated 14 millions of new cases per year and mortality reached an estimated 8 millions per year worldwide. Furthermore, cancer incidence is predicted to increase by 70% (up to 22 millions) in next two decades, as cancer occurrence correlates with human longevity.

Before healthy cells evolve into a neoplastic state, they must acquire several distinctive phenotypes, named hallmarks of cancer by Hanahan and Weinberg [2]. Acquisition of these traits is usually a long multi-step process and is called tumorigenesis. This process and its main mechanisms will be summarized in following paragraphs. The first part of this chapter will describe the genesis of cancer traits and will present a few historical concepts of tumorigenesis. In the second part I will present a redefined and modern vision of tumorigenesis, which will be illustrated by novel concepts such as importance of cancer stem cells or the impact of evolution on cancer.

## **1 Genesis of cancer traits**

### **1.1 Characteristics of cancer cells**

What is a cancer or what is a tumour? These two terms are not interchangeable despite the fact that many publications use them as synonyms. Cancer could be defined as a collection of related malignant diseases of solid and liquid tissues, which when

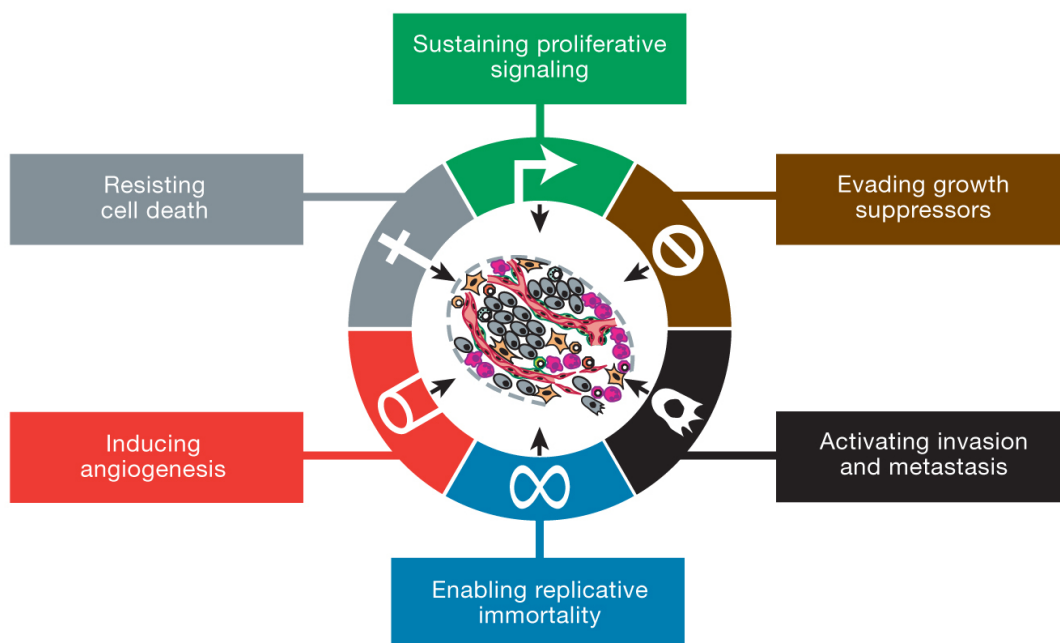


untreated inevitably result in death. Moreover, malignancy of cancer could be characterized by uncontrolled growth of cells accompanied by spread of these cells to surrounding tissues as well as to distant parts of the body. Physical manifestations of solid cancers are malignant tumours. Tumour is defined as an abnormal mass of cells that usually does not contain cysts or liquid areas. Interestingly, some tumours are benign and in contrast to malignant tumours lack an ability to invade neighbouring tissues or metastasize, which makes them easier to cure. To simplify, for the needs of this thesis each tumour will be considered malignant, as the main scope of this work is a malignant cancer disease. In 2000 Hanahan and Weinberg published their “opus magnum”, which defined malignant metastatic tumours by six distinctive and complementary features called hallmarks of cancer [3] (Figure 1):

- Sustaining proliferative signalling – in contrast to normal tissues, cancer cells must continue to proliferate in order for the tumour to grow. This can happen through several mechanisms, such as autocrine proliferative stimulation, reciprocal stimulation of cancer and stroma cells [4, 5] or receptors overexpression/affinity-mutation that increase cell growth in limited ligand environment (reviewed in [2]).
- Evading growth suppressors – invalidation of tumour suppressor genes (such as *RB* ([6], reviewed in [7]) and *TP53* ([8], reviewed in [9]) are the most frequent events. Loss of sensitivity to signalling by growth - controlling molecules, such as tumour growth factor beta ( $TGF\beta$ ) are also common in tumours.
- Resisting cell death – apoptosis serves as a natural barrier for cancer development. During transformation, apoptosis is induced in response to various cellular stresses (e.g. DNA damage); escaping elimination by programmed cell death is a sine qua non condition for tumour growth.
- Enabling replicative immortality – number of cell divisions is restricted for most of the normal cell lineages through a process called senescence. This control is implemented by the process of telomere erosion; in the absence of telomerase, shortening of telomeres at each cell division leads in fine to telomeric structures that can no longer be correctly replicated. Cancer cells acquire unlimited

replicative potential through reactivation of expression of the telomerase (reviewed in [2, 10–12]).

- Inducing angiogenesis – similarly to healthy tissues, tumours require the support of blood vessels in order to sustain nutrient and oxygen supply and to evacuate wastes such as carbon dioxide. Tumour vascularization promotes cancer growth and progression (reviewed in [13]).
- Activating invasion and metastasis – cancer cells disseminate to distant organs through lymphatic and vascular vessels. Successful metastasis is achieved when colonization is coupled with adaptation to the new microenvironment (reviewed in [14–18]).

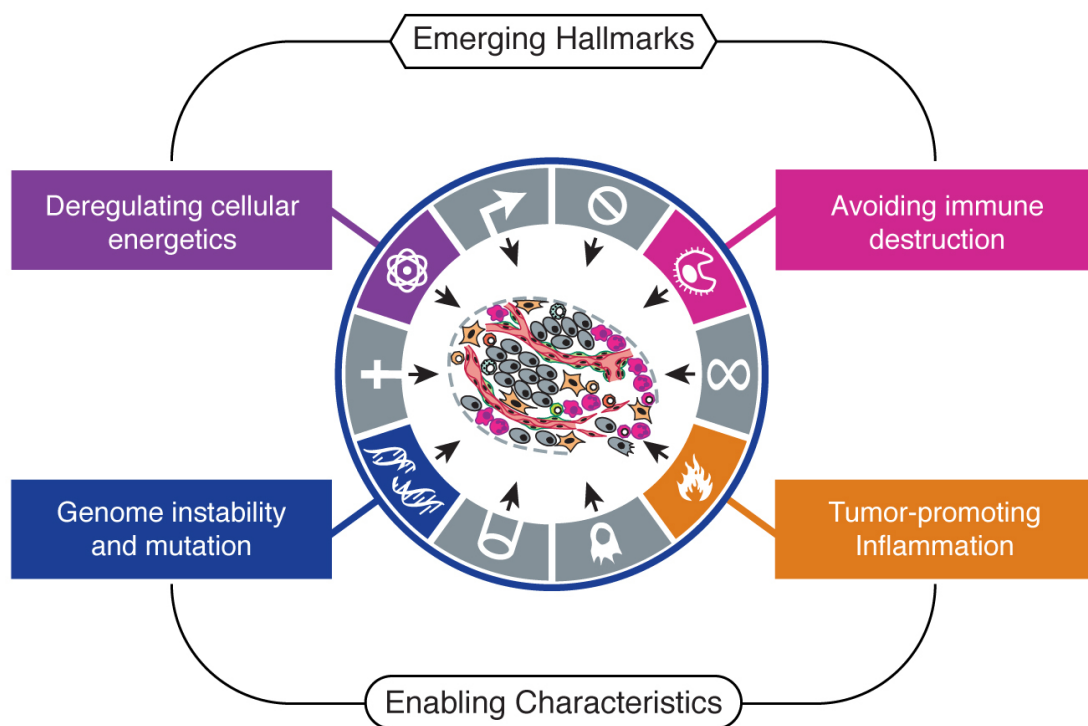


**Figure 1. Hallmarks of cancer originally proposed by Weinberg and Hanahan in 2000 [3].**

The last 10 years of intensive research greatly broadened our knowledge of tumorigenesis. These advances prompted Weinberg and Hanahan to revise and enlarge their original work, giving rise in 2011 to an updated version of their original article [2]. In addition to the originally described and now well-accepted hallmarks, the authors proposed to add new features. Two of them were called “enabling characteristics” and

their acquisition was proposed as a prerequisite for the establishment of the previously described hallmarks (Figure 2):

- Genome instability and mutations – alterations in the genome such as random mutations, chromosome rearrangements or DNA methylation promote acquisition of multiple hallmarks by cancer cells (reviewed in [19–21]).
- Tumour-promoting inflammation – inflammatory environment can foster cancer development (reviewed in [22, 23]) through supplementation of bioactive molecules, such as growth or survival factors (reviewed in [22, 24–26]). Moreover, inflammation is associated with production of reactive oxygen species (reviewed in [25]), which are mutagenic and thus accelerate the genetic evolution of cancer.



**Figure 2. Emerging hallmarks and enabling characteristics [2].**

Finally, two additional attributes called “emerging hallmarks”, believed to facilitate tumour development and progression were proposed (Figure 2):

- Deregulating cellular energetics – cancer cells undergo the glycolytic switch, which is characterized by a preference for glycolysis over oxidative phosphorylation, even in the presence of oxygen [27]. This Warburg-like metabolism allows cancer cells to supply glycolytic intermediates into various biosynthetic pathways, with the consequence of further supporting rapid cell growth [28, 29].
- Evading immune destruction – cancer cells express new epitopes and are thus immunogenic. They can thus be recognised and eradicated by the immune cells. In order to evade destruction, cancer cells develop several escape mechanisms, such as: secretion of immunosuppressive factors like TGFβ [30, 31], recruitment of immunosuppressive cells (T-regs) [32, 33] or by exerting selective pressure called immunoediting [34, 35].

## **1.2 Genes associated with tumorigenesis**

Genome instability and mutations were classified by Hanahan and Weinberg as one the enabling characteristics of cancer. These processes lead to accumulation of genetic changes, which in turn promote neoplastic cell escape from homeostatic regulation. Nowadays, large sets of genes are associated with oncogenesis and they can be arranged into two classes – oncogenes and tumour suppressor genes. Activation or inactivation of these genes can lead to acquisition of certain advantages such as apoptosis resistance or sustained proliferation.

### **1.2.1 Oncogenes**

An oncogene is defined as a gene whose increased expression acts in a dominant fashion to participate in the process of transformation of a normal to cancer cell. It is often a mutated or deregulated counterpart of a proto-oncogene, which is expressed in normal cells [36, 37]. Conversion from a proto-oncogene to an oncogene can be triggered through several mechanisms:

- Gain-of-function mutations – lead to hyperactivity;

- Mutations or epigenetic changes of proto-oncogene promoter – lead to increased transcription;
- Gene amplification or chromosomal translocation – lead to appearance of additional copies of the gene and results in its augmented expression;
- Chromosomal translocation coupled with proto-oncogene gene fusion – creates new gene with oncogenic potential.

According to Uniprot database, 299 proto-oncogenes have been discovered in humans so far. Often, proto-oncogenes encode proteins or recently discovered onco-miRs that function to stimulate cell division, inhibit cell differentiation or halt cell death, such as Ras, c-Myc, HER2/neu, hTERT, BCL-2 or mir-17-92 cluster [38]. As an examples I will briefly describe three proto-oncogenes affecting different signalling pathways: Ras – signal transduction, Myc – transcription and BCL-2 – protection against cell death.

#### **1.2.1.1 Ras family**

Ras family proteins belong to the superfamily of small GTPases [39–41]. They mostly act as signal transducers [42]. Activation of Ras proteins follows extracellular mitogenic signals, like EGF [43], which in turn promotes GTP binding mediated by guanine nucleotide exchange factors [44]. Upon activation, Ras proteins drive cellular proliferation and survival through various pathways such as Raf-MAPK cascade [45, 46] and PI3K/Akt pathway [47–49].

In humans, Ras family consists of 3 highly homologous members: *H-RAS*, *K-RAS*, *N-RAS*. Moreover, Ras proteins are not only the first identified oncogenes, but also so far the most frequently mutated ones in cancer. Sequencing of Ras genes revealed that the majority of tumour-isolated genes possessed a substitution G12D or G12V [50–53]. Such mutated Ras proteins are unable to form functional complexes between Ras and GTPase-activating proteins [54]. As such the biological consequence of these substitutions is a constant activation of Ras-dependent downstream effector pathways and thus uncontrolled proliferation.

### **1.2.1.2 Myc family**

The *myc* proto-oncogenes (comprising *c-myc*, *N-myc*, and *L-myc*) belong to basic helix–loop–helix zipper (bHLH-Zip) transcription factor family. Myc dimerizes with partner proteins, such as Max [55, 56] and the complex acts as an activator [57] or a repressor [58, 59] of transcription. Myc proteins are involved in various genetic programs like: growth [60], proliferation [61], death [62], energy metabolism [63–65], differentiation [66], self-renewal [67] and pluripotency [68]. These highly diverse activities result from the fact that the canonical Myc E-box consensus sequence (5'-CACGTG-3') [69] is among the most frequently occurring DNA-binding motifs in the human genome [70]. Moreover, this E-box can be bound by different transcription factors such as ChREBP, SREBP, HIF1 $\alpha$ , NRF1, USF, TFE3, Clock, and Bmal (reviewed in [71]). These facts led to consider Myc proteins as general transcription regulators with ability to regulate up to 15% of all mammalian genes ([72], reviewed in [73]). Additionally, Myc proteins were identified as possible mediators of gene regulation through microRNAs signalling (miRNA) [74, 75].

Spectacularly, constitutive Myc activation is observed in most, if not all tumours (reviewed in [76]). It occurs mostly through gene amplification [77, 78]. This mechanism was proven for all Myc proteins and has been found in various cancers [77, 79, 80]. In the case of Burkitt lymphoma Myc activation occurs through translocation into the immunoglobulin heavy chain locus (80% of translocations) [78, 81]. Interestingly, Myc oncogenes are associated with a phenomenon called oncogene addiction [82–84], which is characterized by tumoral dependence on the continuous activity of specific oncogenes for maintenance of their malignant phenotype. Although transcription factors are notoriously poor candidates for therapeutic targets, the importance of Myc in carcinogenesis prompted efforts in devising methods for its inactivation [85].

### **1.2.1.3 Bcl-2 family**

Bcl-2 (B-cell lymphoma 2) was identified as a bona fide oncogene in follicular lymphomas [86–88]. It is the first oncogene identified that acted not by inducing proliferation, but rather by inhibiting cell death [89]. Alterations in Bcl-2, or other anti-apoptotic members of the family, expression allow cancer cells to increase its apoptotic

threshold resistance. This, in consequence promotes survival under conditions that would normally induce cell death, such as deregulated oncogene expression, cytotoxic stresses or death signalling related to anti-cancer therapies and tumour microenvironment (reviewed in [90, 91]). Moreover, Bcl-2 expression was found to be necessary for tumour maintenance [92]. In follicular lymphoma, oncogenic activation occurs mainly through translocation of the Bcl-2 gene from chromosome 18 into chromosome 14, putting the gene under control of the immunoglobulin heavy-chain enhancer [88]. However, activation was also found to happen through gene amplification [93], miRNA deregulation [94] and DNA hypomethylation [95] in some malignancies.

### **1.2.2 Tumour suppressor genes**

Tumour suppressors are protective genes that limit the growth of tumours. Their loss of function mutations found in tumours inhibit or abrogate their defensive abilities. This category of genes is often involved in the maintenance of tissue homeostasis and their inactivation represents a second distinct genetic alteration observed during tumour development. In contrast to oncogenes, the proteins encoded by most tumour suppressor genes inhibit cell proliferation or survival. Tumour suppressors have been found also as important players in genome maintenance, especially in DNA repair mechanisms, which reduce the risk of detrimental mutations. According to Uniprot database, 701 genes with anti-tumoral properties have been identified up to date. Interestingly, this number is twice as high as the number of known oncogenes, suggesting a strong evolutionary pressure for cancer-protective mechanisms in a human organism.

Inactivation of this first-line defence system favours cancer development and progression. Inactivation of these genes occurs through genetic (deletions, loss of function mutations) or epigenetic modifications (promoter hypermethylation). Usually, these alterations need to be present in both gene alleles for its full inactivation. This mechanism called “two-hit hypothesis” was observed for the first time by Knudson in the retinoblastoma (*RB*) tumour suppressor gene. [96]. Notably, there are also exceptions to this rule, for example in *TP53*, encodes p53, mutation of one allele can give

rise to dominant-negative protein what prevents the activity of the wild type p53 protein. Nevertheless, in most human cancers mutant p53 bearing cells undergo loss of heterozygosity, eliminating the wild type allele.

#### **1.2.2.1 p53 – guardian of the genome**

The transcription factor p53 was discovered almost simultaneously by different teams in 1979 [97–99] and was initially considered as an oncogene ([100], reviewed in [101]). Glorious days of p53 as tumour suppressor begun in 1988, when tumour-derived *TP53* gene was sequenced and compared to wild type isoform [102, 103]. These results demonstrated that p53 was not a tumour promoting gene and show that the original studies have been carried out with cells expressing a dominant-negative mutant form of p53 protein ([104, 105] reviewed in [101]).

*TP53* gene is the most commonly mutated gene in human cancers [106] and alterations have been found throughout the gene [107]. Importantly, frame-shift or nonsense mutations could result in the loss of p53 protein expression. However, in most of the cases *TP53* gene is subjected to missense mutations resulting in substitutions of single amino acids in the p53 protein. Remarkably, more than 80% of these mutations in human tumours are localized within the DNA-binding region, leading to the loss or reduction of wild-type p53 transcriptional activity [108]. In addition, p53 normally acts as a tetramer; hence mutated proteins also function as dominant negative inhibitors over any remaining wild-type p53 [109].

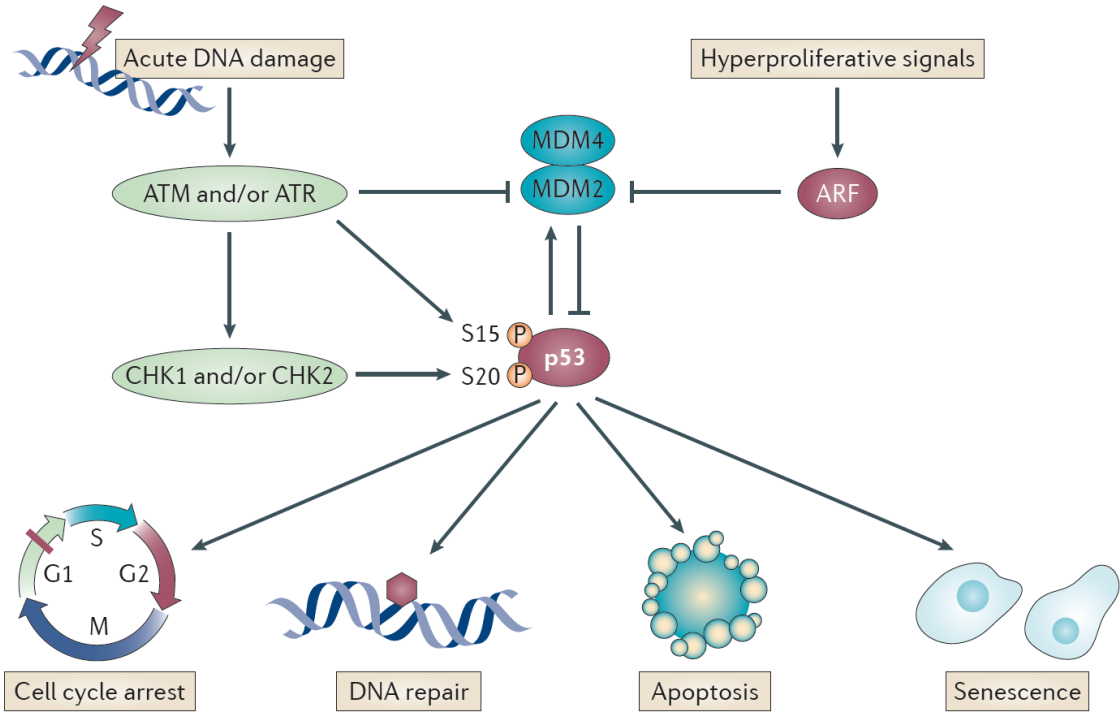
Under physiological conditions, p53 activity is maintained at low levels, through a continuous degradation mediated by MDM2 or MDM4, both E3-ubiquitin ligases [110–112]. Of note, even though p53 is usually activated by stabilisation of the protein, the basal expression of p53, is able to mediate various physiological functions such as: anti-apoptotic activity [113], fertility [114], cell metabolism [115] and mitochondrial respiration [116], autophagy [117], cell adhesion [118], stem cell maintenance [119] and development [120] (reviewed in [121]).

Exposition of cells to diverse stresses such as DNA damage, hyperproliferative signals, hypoxia, oxidative stress or nutrient starvation [122, 123] results in p53 stabilisation



and its concomitant activation. Displacing its negative regulators, MDM2 and MDM4, from the complex stabilizes and activates p53. Activated p53 regulates various transcriptional programs controlling cell cycle arrest, apoptosis or senescence (reviewed in [9]) (Figure 3). Additionally, recent studies showed that p53, acting both through transcriptional and non-transcriptional mechanisms, can also control other regulatory processes with onco-suppressive potential. Notably, p53 may act by down-regulating reactive oxygen species (ROS) accumulation, counteracting oncogenic metabolic reprogramming, activating autophagy, promoting communication within the tumour microenvironment, inhibiting both stem cell self-renewal and reprogramming of differentiated cells into stem cells, or controlling invasion and metastasis (reviewed in [9]).

Another important attribute of p53 is its ability to acquire gain-of-function mutations [124], which can lead to more aggressive and metastatic phenotype [125–128].



**Figure 3. Classical view on p53 activation and its most essential anti-tumoral properties [9].**

### **1.2.2.2 Tumour suppressor RB**

Almost 30 years ago retinoblastoma (*RB*, gene product is called pRB) gene was recognized as the first tumour suppressor gene [129]. In mammalian cells, pRB belongs to a family of three proteins that also include p107 and p130. Structurally and functionally both are related to pRB and belong to the same cellular pathway, but display distinct functions from pRB in specific contexts [6, 130].

The anti-oncogenic activity of pRB is based mainly on its ability to mediate cell cycle inhibition through the repression of the G1/S phase transition via two distinct mechanisms [131]. The first one is driven by the binding of pRB to the transcription factor E2F, consequently inhibiting DNA synthesis and leading to cell cycle arrest [132, 133]. This interaction is negatively regulated by pRB phosphorylation mediated by various cyclin-dependent kinases (CDKs) [134, 135]. In the second mechanism, pRB directly binds to Skp2 thus allowing APC-Cdh1 complex to ubiquitinate Skp2. Degradation of Skp2 promotes p27Kip (CDK inhibitor) accumulation, which contributes to cell cycle arrest [133, 136]. In addition to the cell cycle arrest, pRB possesses also other tumour-defensive abilities, such as senescence induction [137]. Moreover, pRB inactivation stimulates chromatin instability in E2F-dependent (e.g. via Mad2) or -independent manner (e.g. cohesin and condensin II) [138–140]. Finally, pRB was shown to regulate cell death and differentiation [133, 141–143].

Mutation of *RB* gene occurs prevalently in retinoblastomas, osteosarcomas and small-cell lung carcinomas [142]. However, functional inactivation caused by different events was found in the majority of cancers [142]. Indeed, frequent loss-of-function mutations affecting p16 or gain-of-function mutations affecting cyclin D, have the most prevalent impact on pRB inactivation in many tumour types ([144] reviewed in [142]).

### **1.2.2.3 Supervisors of genome integrity and stability**

During their lifespan, cells are constantly challenged by extrinsic and intrinsic stresses that may negatively influence the integrity of their genomes. To survive in such hostile conditions and transmit intact genetic information, cells have developed a series of mechanisms called DNA damage response (DDR), a highly organized and coordinated

system to withstand genotoxic stress. This response consists first of the ability of the organism to sense and signal problems in its DNA. Secondly, DDR leads to cell-cycle arrest and simultaneous activation of the appropriate DNA repair mechanisms. If the damage is not repairable DDR activates mechanisms leading to elimination of the injured cells [145]. Efficient DDR is essential for the maintenance of genome stability and constitutes therefore a major block of the passage to neoplasia.

DNA-damage response is complex and varies for different types of DNA lesions. The most common lesions and the cellular responses to them are listed below:

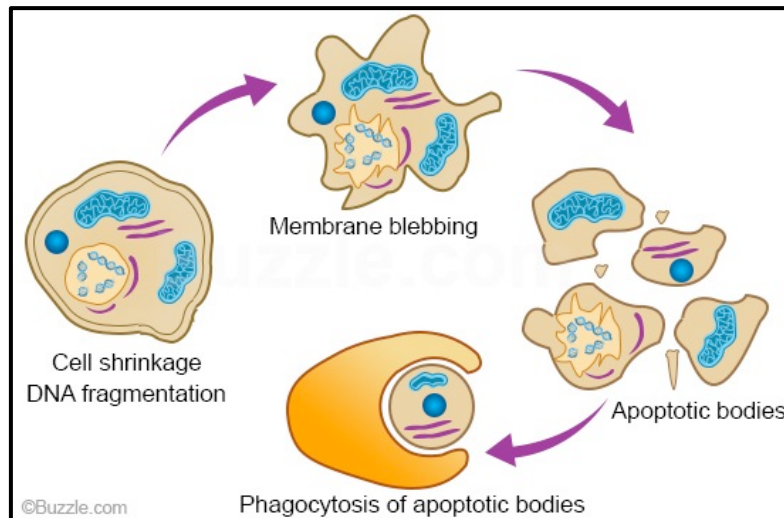
- Single-strand breaks (SSB) occur during replication or under environmental stresses. Repair is mediated by base excision repair (BER) mechanism with the involvement of genes such as: bifunctional DNA glycosylases, APE1 endonuclease, DNA polymerases and associated factors, flap endonuclease FEN1, ligase I or III (reviewed in [146]).
- Double-strand breaks (DSB), which are highly cytotoxic, and can arise from unrepaired SSB or an environmental stress, such as for example ionizing radiation. Major sensors and activators of repair response in the case of DSB are ATM, ATR and DNA-PK proteins (reviewed in [147, 148]). Repair response is composed of two major pathways:
  - Nonhomologous end-joining (NHEJ), which is the simplest way of repairing DSB without the need for a homologous template [149]. This mechanism relies on the XRCC4-XLF4-Ligase IV complex [150]. However, NHEJ response is highly mutagenic potential leading to frameshift mutations, deletions or chromosomal rearrangements [148].
  - Homologous recombination (HR), requires a homologous template, thus it is generally restricted to S and G2 phases. HR could also be used to restart stalled replication forks and to repair inter-strand DNA crosslinks [151]. The main factors involved in this pathway are RAD51 and related proteins, BRCA1 and 2 as well as replication protein A (RPA) [152].
- Mismatch repair removes miss-paired nucleotides due to replication errors, but also repairs DNA adducts such as those produced by platinum-based

chemotherapeutic agents [153]. The main factors involved in this mechanism are DNA mismatch repair proteins – MSH2, 3 and 6.

Additionally, DDR has been described also as initial event in cell transformation [154]. During cancer development both activation and inactivation of the DNA damage responses are observed. Mutational or epigenetic inactivation of DDR components promotes tumour development [155], but can be also used to enhance the efficiency of chemo- or radiotherapy. On the other hand, it has been reported that some cancers like glioblastomas [156] display a heightened DDR that results in increased resistance to chemo- and radiotherapy (reviewed in [147, 148]).

### **1.3 Apoptosis**

Apoptosis is a cellular suicide program that evolved as an efficient way to eliminate unnecessary or unhealthy cells from the body. It is a fundamental process associated with normal development and tissue homeostasis of all multicellular organisms [157]. Apoptosis is associated with several morphological changes such as cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, chromatin condensation and DNA fragmentation [158] (Figure 4). Apoptotic cells retain their membrane integrity during the whole process, thus preventing a non-specific inflammatory reaction that accompanies spillage of cellular contents during necrotic cell death. Indeed, elimination of dead cells occurs through phagocytosis, hence minimizing the impact of the process on neighbouring cells [159]. Interestingly, recent reports indicate that despite the general principle of apoptosis being a non-inflammatory cell death, specific apoptotic inducers, notably some common chemotherapeutic drugs, do in fact give rise to a pro-inflammatory form of apoptosis [160]. Interestingly, it would appear that such inflammatory response increases the therapeutic activity of the compounds used.



**Figure 4. Morphological changes occurring during apoptotic cell death.**

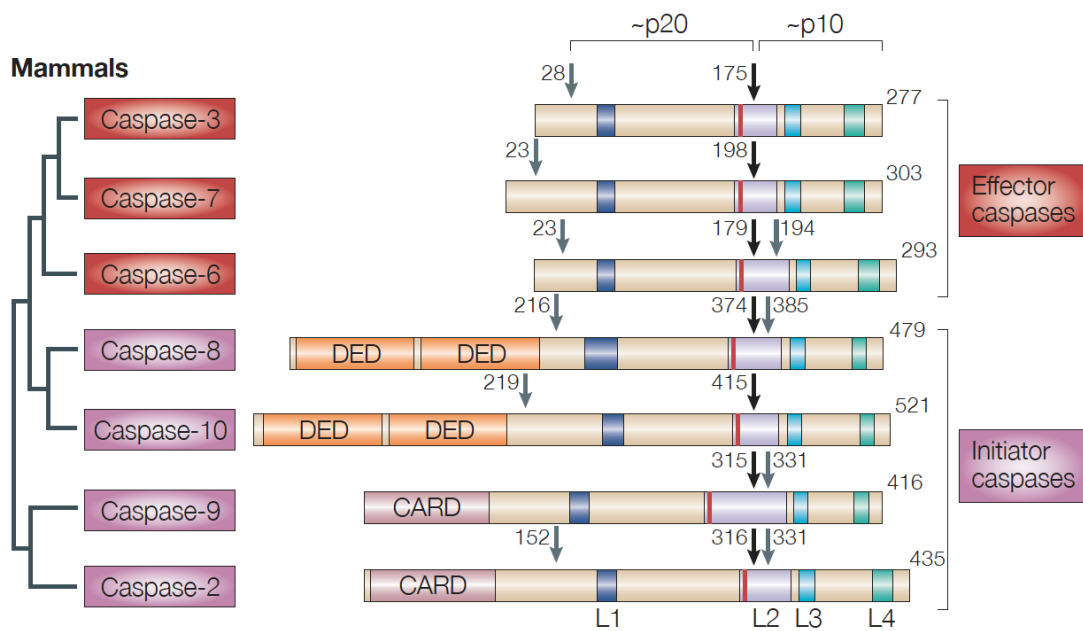
Apoptosis is crucial during the organism development as a sculptor of future organs. It helps removing unnecessary structures [161] or adjusting cell populations [162]. During adulthood, apoptosis is mainly involved in controlling tissue homeostasis and removal of unwanted cells e.g. autoreactive lymphocytes, infected cells, and potential cancer cell [163].

Despite its tight regulation, deregulation of apoptosis can happen, an event associated with various diseases. An excess of apoptosis favours neurodegeneration, immunodeficiency or infertility [164]. On the other hand, inefficient apoptosis may promote e.g. autoimmune disorders, where self-reactive B and T lymphocytes evade the quality control point, which is the negative selection. Finally, escape from apoptosis is one of the hallmarks of cancer. Mutated or defective apoptosis pathway allows cancer cells harbouring damaged DNA to survive and eventually proliferate, driving tumour progression and resistance to therapy [165].

### **1.3.1 Apoptotic cascade – caspases**

At the molecular level apoptosis is governed by caspases, a family of cysteine aspartate proteases. Under homeostatic conditions, caspases are ubiquitously expressed as inactive precursor enzymes (zymogenes), with limited or undetectable protease activity [159, 166]. Their activation is mediated by cleavage of the precursors, giving rise to a

small and a large subunit, which dimerize to produce the active enzyme [167]. Caspases involved in apoptosis are divided into two subfamilies called initiator and effector caspases (Figure 5). Following an apoptotic signal, initiator proteases (caspases 8, 10, 9 and 2) are recruited to activation platforms and activated by self-cleavage, producing enzymes that can in turn cleave other caspases and effector molecules. Activated effector caspases (3, 6 and 7) then cleave numerous protein substrates within the cell, thus executing the apoptotic program [167].



**Figure 5. Apoptotic caspases in mammals [168].**

**Legend:** caspase recruitment domains (CARDs), death effector domains (DEDs).

Upon activation, caspases' proteolytic activity affects major cellular components. For example cleavage of cytoskeleton (actin microfilaments, microtubules and intermediate filaments) and nuclear lamins promotes morphological changes characteristic of the apoptotic cell death [159]. Moreover, caspases mediate not only the shutdown of intracellular metabolic machinery but also stop transcription and translation [169].

### 1.3.1 Apoptotic cascade – extrinsic pathway

Extrinsic pathway of apoptosis is initiated on the cell surface by an interaction between ligands and their receptors, so called “death receptors” e.g. Fas/CD95, TNFR (tumour necrosis factor receptor) or TRAIL. Death receptors belong to the superfamily of TNF receptors and contain a protein/protein interaction motif called death domain (DD). Activation of these receptors promotes the recruitment of adaptor proteins such as FADD (Fas-Associated protein with Death Domain) that launches the formation of caspase 8 activating platforms at the cytosolic side of the plasma membrane (Figure 6).

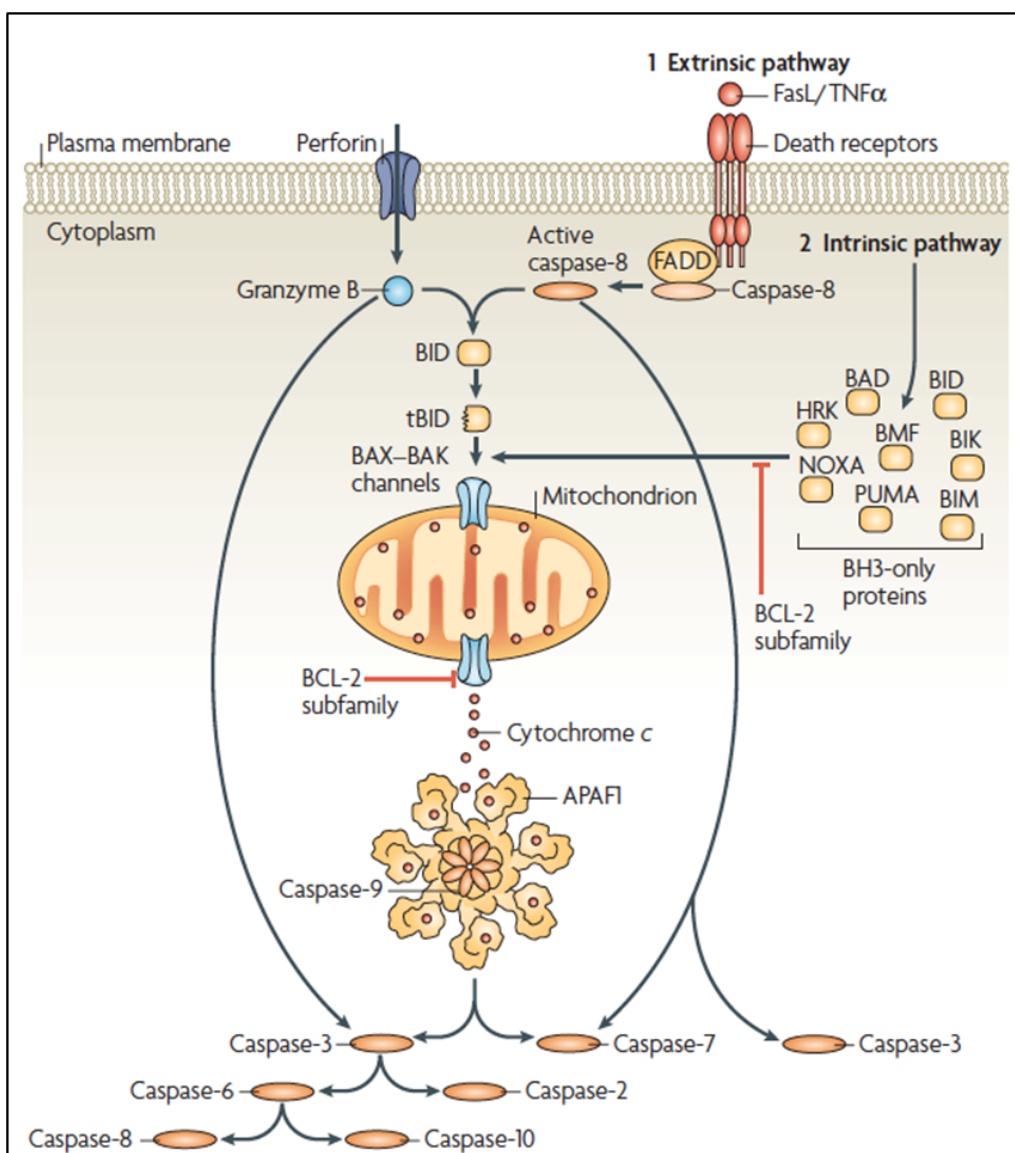


Figure 6. Caspase activation pathways [159].

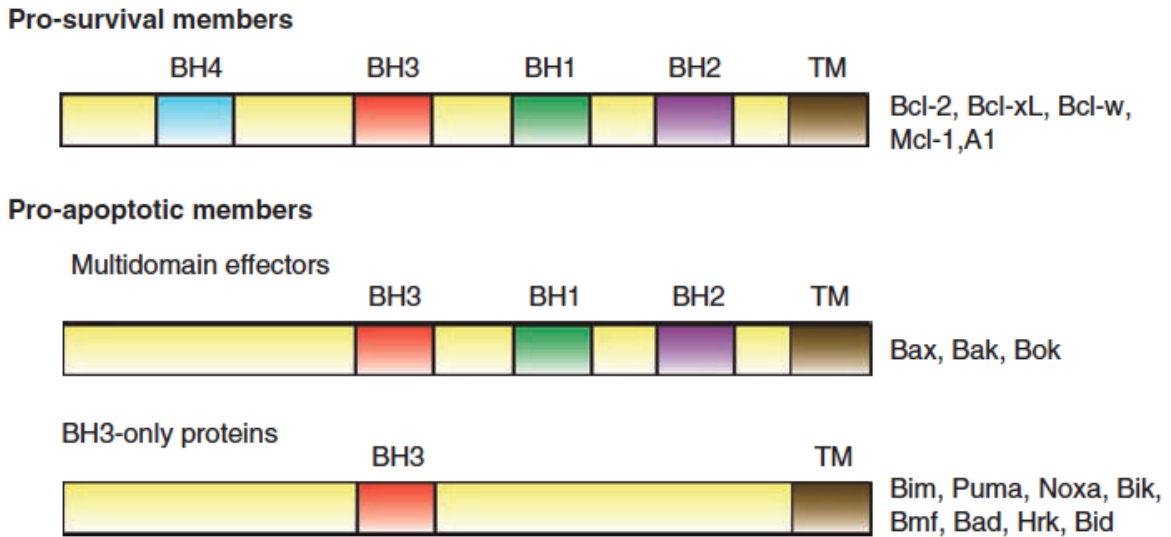
Formation of DISC complex (Death-Inducing Signalling Complex) induces caspase 8 activation through cleavage and dimerization [170]. Following caspase 8 activation, the apoptotic cascade is activated i.e. downstream cleavage of effector caspases 3 and 7. Moreover, the extrinsic pathway is linked to the intrinsic apoptotic signalling through caspase 8-mediated cleavage of a BH3-only protein BID.

### **1.3.2 Apoptotic cascade – intrinsic pathway**

This is the major apoptotic pathway that is activated by a plethora of stimuli, both in development, in homeostasis and in disease. The key element of the intrinsic cascade is the mitochondrion (Figure 6). Permeabilization of the mitochondrial outer membrane (MOMP) is the crucial step that promotes release of pro-apoptotic molecules such as cytochrome *c* from the intermembrane space into the cytoplasm. Cytosolic cytochrome *c* is a co-factor necessary for the formation of an apoptosome, a cytosolic platform on which the initiator caspase 9 is activated.

The mitochondrial permeability transition is controlled by proteins of the BCL-2 family. These proteins are characterized by the presence of at least one BH (BCL-2 homology) domain [86]. BCL-2 protein family consists of 3 subfamilies, defined by their structure and function (Figure 7) [171]. The first group consists of multi-domain anti-apoptotic BCL-2 proteins such as BCL-2, BCL-XL or MCL-1, which are characterized by 4 BH domains. The second group consists of BH multi-domain pro-apoptotic proteins (Bax, Bak, Bok), which are responsible for the induction of cytochrome *c* release. The last subfamily consists of single BH domain pro-apoptotic proteins (BIK, HRK, BIM, BAD, BID, PUMA, NOXA, BMF), characterized by low structural homology, except for the BH3 domain. BH3-only proteins serve as sensors of apoptotic signals and by communication with both multi-domain subfamilies regulate the balance between life and death [86].





**Figure 7. BCL-2 protein family structure and function [172].**

**Legend:** BCL-2 homology domain (BH1-4) transmembrane domain (TM).

### 1.3.3 Apoptosis, cancer and evolution

Apoptosis is believed to be one of the key defence mechanisms against tumour initiation and progression. However, recent studies added more complexity to this well-established paradigm. Over the last years, numerous data strengthened the notion that tumours are composed of a mixture of different clones, which evolve under constantly changing environments and are thus subject to changing selective pressures. These results in a heterogeneous cell populations with clones endowed with different abilities, such as resistance to apoptosis or proliferation potential. Under continuous selective pressure, apoptosis could be a driver of clonal expansion by generating vacant niches (Figure 8). In turn, these niches might become repopulated by cells with a more aggressive phenotype and that have evaded pro-apoptotic signalling and thus hold certain competitive advantages. According to this model, somewhat paradoxically, apoptosis might be considered as a driver of tumour evolution and thus a hallmark of aggressiveness. This phenomenon may be especially relevant during early steps of tumorigenesis (reviewed in [173]) Additionally, apoptotic cells can promote cell division of neighbouring cells through release of prostaglandin E2 [174]. While little data are yet available to validate this model [175–178], it brings an alternative view of the role of apoptosis during cancer progression.

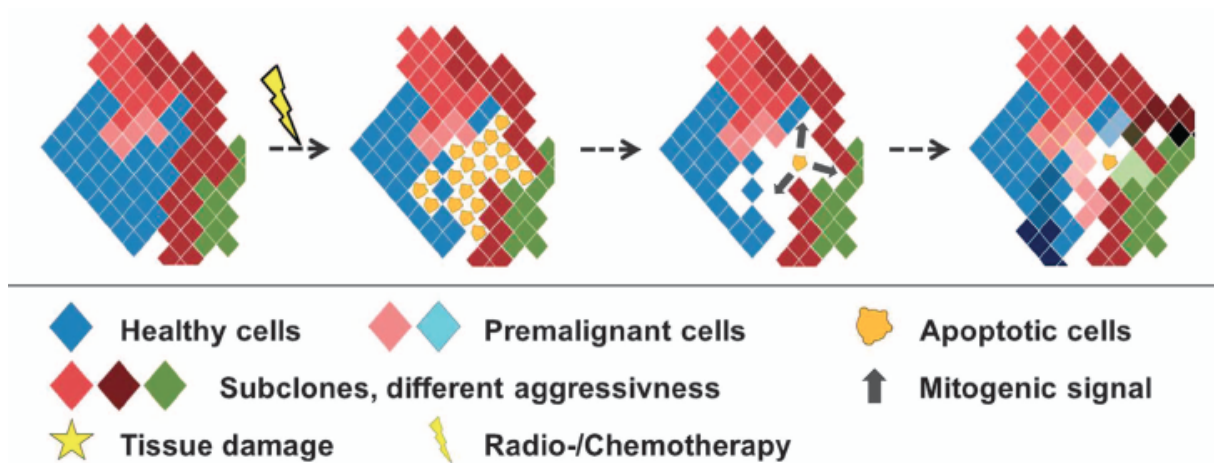


Figure 8. The model representing how apoptosis induced by chemo- radiotherapy drives malignant progression [173].

## 1.4 Senescence

Senescence was discovered more than 50 years ago by Leonard Hayflick and Paul Moorhead as a physiological process linked to cellular proliferative [179]. They showed that normal human fibroblasts in cell culture undergo cell cycle arrest after certain number of divisions. Following this fundamental discovery, it was later demonstrated that in the absence of telomerase activity, replicative senescence is induced by the shortening of telomeres at each division cycle, leading to their complete erosion after extensive proliferation ([179–181], reviewed in [182]).

Further studies not only significantly broadened our knowledge of senescence but also identified senescence as a key cellular process. Indeed, senescence is considered to act as one of the main mechanisms counteracting tumorigenesis and together with apoptosis, comprise the main defence mechanism of our organism. Senescence is induced by plethora of different stimuli and leads to permanent cell cycle arrest. There are two main molecular pathways of senescence. First, developmentally programmed senescence, mainly active during embryogenesis, especially involved in tissue remodelling [183]. Secondly, damage-induced senescence, which is activated under various circumstances such as DNA damage, telomere erosion, oxidative stress or an oncogenic event (Figure 9) [183].

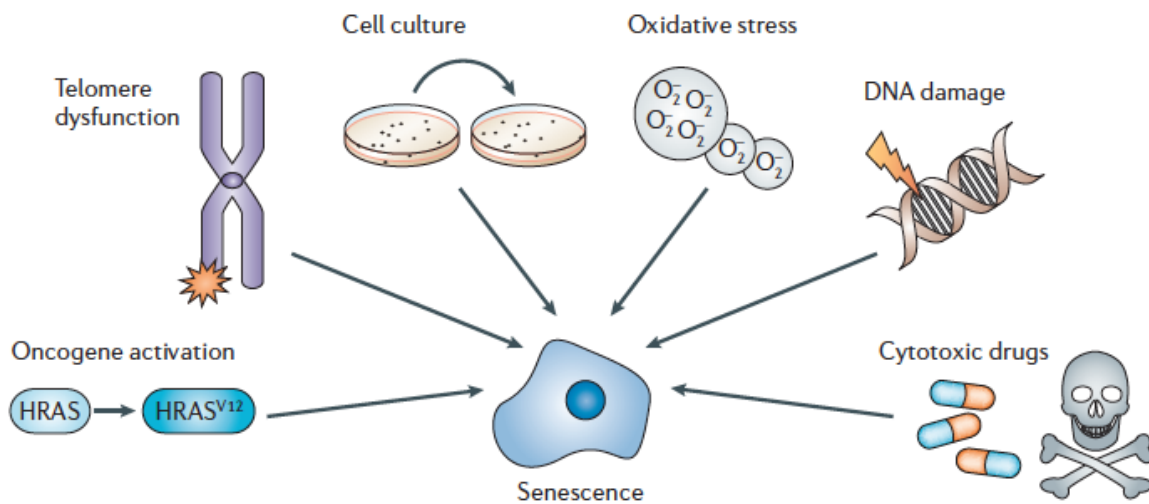


Figure 9. Stimuli promoting damage-induced senescence [184].

#### 1.4.1 Characteristics of a senescent cell

Senescence is accompanied by some distinct morphological changes, *in vitro*, cells become large, flat, vacuolized and occasionally multinucleated [183]. However, *in vivo*, senescent cells retain their normal morphology because of constraints imposed by the tissue architecture. Cell cycle-arrest for senescent cells occurs during G1 and G2 phases or in certain cases of oncogene-induced senescence in S-phase [185]. Notably, senescent cells are resistant to apoptosis, which under specific circumstances may promote cancer progression [186]. Senescence is accompanied by alteration in gene expression, such as upregulation of cyclin-dependent kinase inhibitors (p21 and p16) or downregulation of genes involved in cell cycle progression like cFOS, cyclin A, cyclin B and PCNA (reviewed in [185]). Another aspect of senescence is linked to foci of heterochromatin also known as senescence-associated heterochromatic foci (SAHF) [187]. Noteworthy, SAHF are preferentially formed during oncogene-induced senescence, but not during replicative senescence or upon ageing [188]. Finally, senescence is associated with several biomarkers such as senescence-associated  $\beta$ -galactosidase (SA  $\beta$ -GAL) [189]. Appearance of  $\beta$ -galactosidase expression serves as the most reliable sign of this process and probably reflects the increased occurrence of autophagy in senescent cells [190]. Among other senescence markers, while less specific, it is worth to mention a

downregulation of proliferative markers, such as Ki67 or lack of 5-bromodeoxyuridine (BrdU) incorporation into DNA.

#### **1.4.1.1 SASP**

Senescence-associated secretory phenotype (SASP) is a complex pro-inflammatory response of senescent cells. SASP is mediated by transcription involving factors such as nuclear factor- $\kappa$ B (NF- $\kappa$ B). Its “secretome” consist of pro-inflammatory cytokines (IL-6 and IL-8), chemokines, macrophage inflammatory proteins (MIPs), growth factors (TGF) and proteases [191, 192]. Secretion of these factors cause inflammation and may be essential for clearance of senescent cells by phagocytosis [193]. Furthermore, SASP can induce neighbouring cells to undergo TGF $\beta$ -mediated senescence mostly through ROS mediated DNA damage [194–196]. Therefore, SASP can be described as a powerful autocrine and paracrine trigger that creates an inflammatory microenvironment, which may contribute to the elimination of senescent cells (reviewed in [183]). However recent discoveries demonstrated that under certain conditions (such as obesity [197]), SASP could also act as a promoter of tumour progression (reviewed in [198]), in agreement with a strong tumour-promoting role of inflammation [199].

#### **1.4.2 Mechanisms of senescence**

Various signals may induce cellular senescence as a consequence of activation of different signalling pathways, such as for example the p53 pathway. Upon activation, signal transduction leads to stimulation of the main effectors of senescence, the CDK inhibitors p16 (also known as INK4A; encoded by CDKN2A), p15 (also known as INK4B; encoded by CDKN2B), p21 (also known as WAF1; encoded by CDKN1A) and p27 (encoded by CDKN1B) [183]. Consequently, inhibition of CDK–cyclin complexes promotes hypo-phosphorylation and thus activation of pRB and results in cell cycle arrest [200]. The following paragraphs briefly describe the main mechanisms involved in damage-induced senescence (Figure 10).

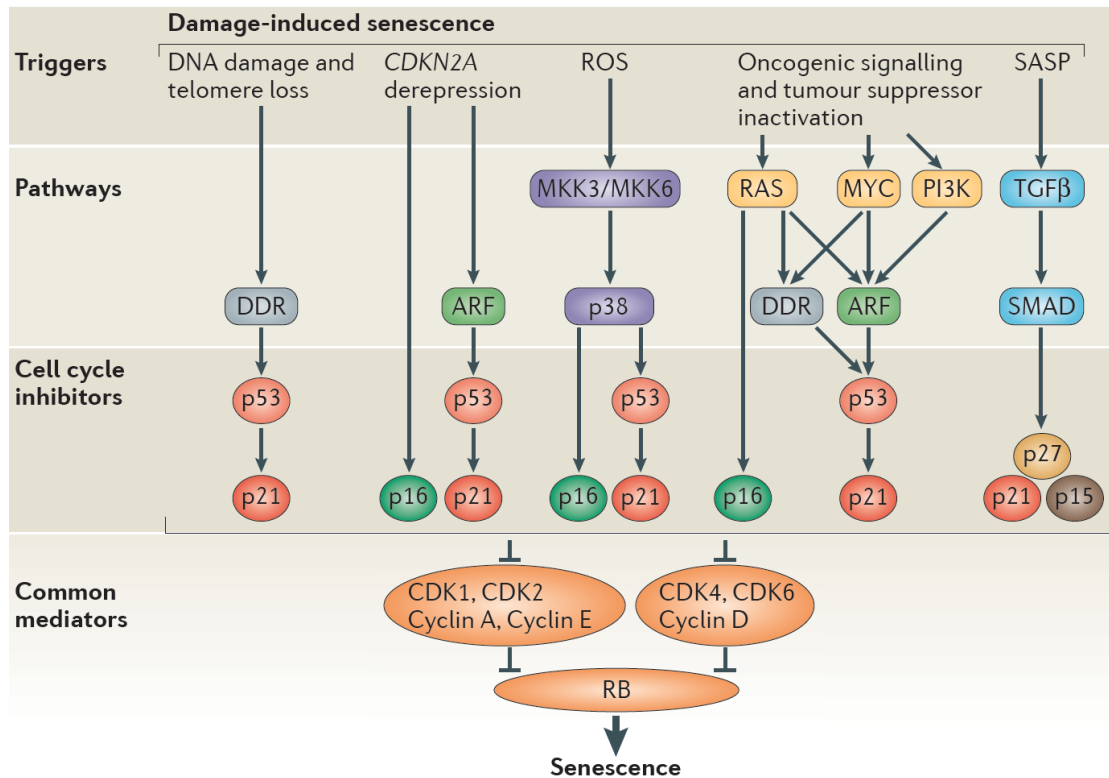


Figure 10. Molecular pathways of senescence (adapted from [183]).

#### 1.4.2.1 Telomere shortening and DNA-damage response

Telomeres serve as molecular clocks and at the same time as logbooks of replicative history of primary cells [180]. Notably, each consecutive cell division shortens telomeres, leading to telomere erosion and in consequence to “replicative senescence” [185]. Loss of telomeres is sensed by cells as DNA damage and therefore triggers a classical DDR, similar to the one induced by radiation or chemotherapy. DDR is mediated through DNA damage kinases, which phosphorylate and activate several cell cycle regulator proteins, including p53. As a consequence, activated p53 upregulates expression of p21 and therefore inhibits CDK-cyclin complexes [183] (Figure 10).

#### 1.4.2.2 Oncogene-induced senescence (OIS)

Senescence is one of the fail-safe programs carried out in response to oncogene activation. First example of this phenomenon was reported in human fibroblast transduced with a constitutively active Ras, H-Ras V12 [201]. Oncogenic Ras triggers senescence through the activation of p16-RB pathway (reviewed in [202]). More than 50 oncogenes have been described to date as capable of promoting OIS [203]. Furthermore,

loss of tumour suppressors can also trigger senescence, as exemplified by the loss of PTEN [204], NF1 [205] or von Hippel-Lindau disease tumour suppressor (VHL) [206].

Notably, a general feature of OIS is the derepression of the CDKN2A locus (also known as INK4A or ARF), which encodes two tumour suppressors - p16 and ARF [207, 208]. Indeed, p16 is an inhibitor of CDK4 and CDK6 while ARF regulates p53 stability through inactivation of the E3 ubiquitin protein ligase MDM2. In addition, this type of senescence can be induced after activation of oncogene mediated DDR [209, 210] or ROS generation [211]. While the different mechanisms leading to OIS are complementary, it is not clear which plays the predominant role in tumour suppression (Figure 10). In fact, it would appear that in mice ARF-p53 pathway is the main activator of OIS [212], whereas in human this role is played by the DDR-p53 pathway [155]. Similar observations were made regarding the p16 status; in mice p16 is a modest inducer of senescence, whereas in humans it is prominent [213]. These discrepancies contribute to the realisation that mouse models are often not a faithful representation of human cancers.

## **2 Growing complexity of tumorigenesis**

### **2.1 Clonal evolution in cancer**

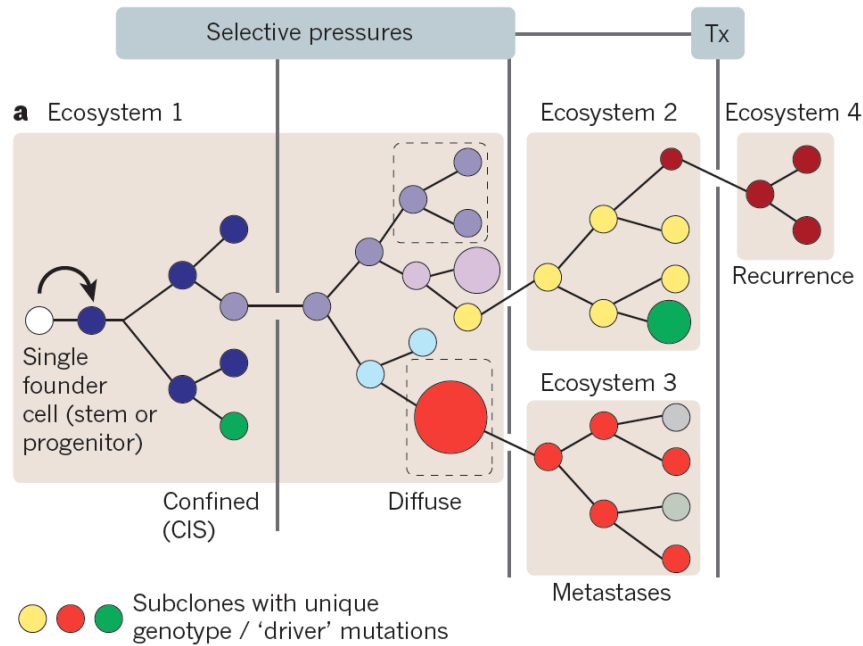
Cancer is a major cause of death throughout the world and despite exceptional effort and much money spent, progress in elimination of advanced cancer disease is slow [214]. Nevertheless, our understanding of cancer biology and its genetics significantly increased over past years. This led to the emergence of new concepts that could explain the complexity of cancer and thus the difficulty to find efficient cures. Among the new concepts, we will discuss in the following chapter the integration of evolution in tumour progression.

#### **2.1.1 General concept**

The concept of cancer as an evolutionary disease is not new and was presented for the first time by Peter Nowell in 1976 in his seminal article [215]. He described cancer as an evolutionary process that is driven by stepwise somatic-cell mutations with sequential, subclonal selection. Recent achievements in cancer biology validated and confirmed a

link between processes of tumorigenesis and Darwinian evolution (reviewed in [216, 217]).

Tumour microenvironment controls tumour progression through several aspects such as limitation in resources, environmental tumour architecture or other factors limiting the size of the tumour. In simple words, doubling time of a tumour is usually between 60 to 200 days, however doubling time of a cancer cell usually takes only a few days [218]. This suggests that under normal conditions either the majority of cancer cells die before their next division or cancer expansion is hampered by its microenvironment [219]. Thus, we can extrapolate that natural selection in tumours is based on similar principles as the selection of organisms – meaning the competition for space and resources [220]. On top of that, tumours are not only shaped by natural selection, but they also may be influenced in an artificial way. Therapies such as radiation or chemotherapy constitute a significant source of selection pressure, which changes the dynamic balance between cancer clones. In the most probable scenario, cancer therapies result in massive death of cancer cells, but also drive selective pressure for the proliferation of clones resistant to treatment. Finally, another important aspect of tumour shaping process introduced by therapies is their genotoxicity. Indeed, surviving cells exposed to such stress are likely to mutate and in consequence acquire new oncogenic properties. This, in a wider perspective fuels tumour regeneration with improved fitness and malignant potential [220] as summarized on Figure 11.



**Figure 11. Architecture of cancer evolution under selective pressure [220].**

Selective pressure: nutrients, oxygen and growth factors. **Legend:** Tx – therapy, CIS – carcinoma *in situ*.

### 2.1.2 Intratumour heterogeneity and driver mutations

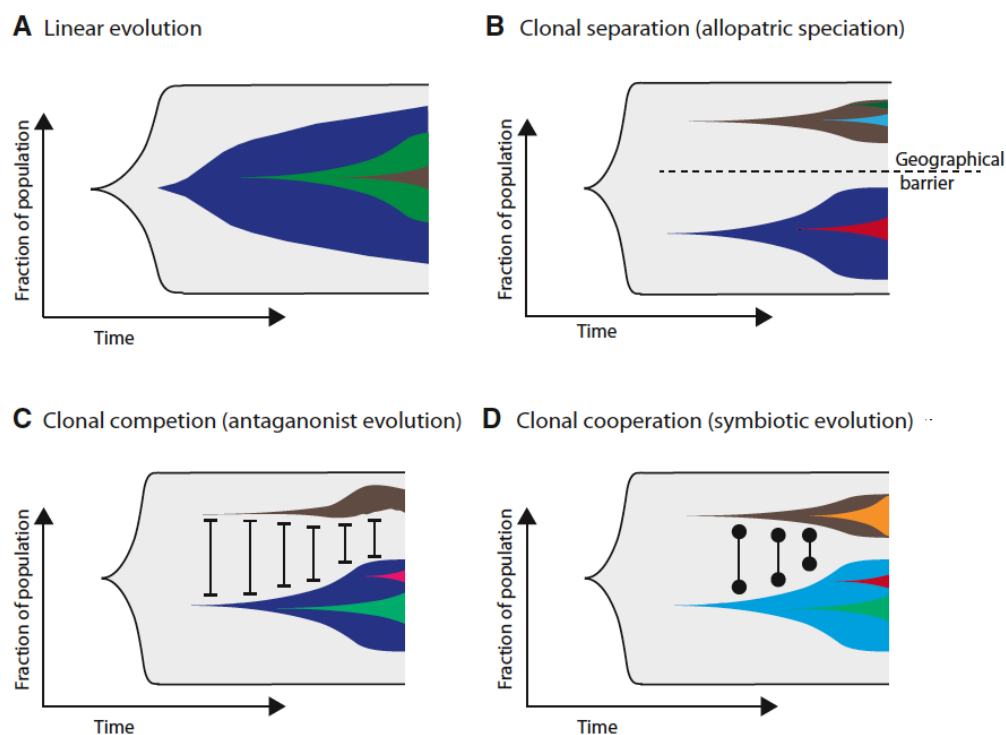
Each tumour is different and this is not only true when we compare the same cancer types in different patients. Strikingly, those differences are not only obvious between primary and secondary tumours in the same patient [221] but also between different regions of the primary tumour as it was observed in glioblastoma, lung, kidney or breast tumours [222–226].

Tumour diversity can be observed by differences in expression of protein biomarkers [227], or at multiple genetic [228], and epigenetic [229] levels. The main driver of genetic diversity, at least at the intracellular level, is genomic instability, which consists of several processes such as mismatch repair, telomere maintenance, DNA replication or chromosomal segregation [230]. As a consequence of poor fidelity of these processes, cancer clones evolution is driven by interaction of selectively advantageous ‘driver’ lesions, selectively neutral ‘passenger’ lesions and deleterious lesions [220, 231]. Currently, cancer is considered as dynamic disease, which remains under continuous



evolution and in consequence results in constant repopulation of its niche as new clones take over and replace ancestral clones.

Clone replacement could occur through different models that are summarized in the Figure 12. During linear evolution new clone can outcompete its predecessor, however it is not always the case, leading to distinct types of heterogeneity. In the case of branched tumour evolution, various subclones evolve in parallel, impacting on extensive subclonal diversity. Moreover, this model of evolution could be also coupled with either clonal competition, where distinct subclones compete for growth advantages or with cooperation that leads to a symbiotic relationship (reviewed in [232]).



**Figure 12. Models of tumour evolution [232].**

(A) Example of linear evolution where clones sequentially accumulate mutations over time. Additionally, linear evolution may result in heterogeneity when a subclone has failed to outcompete its predecessors. (B) Tumours subclones may evolve into distinct population because of geographical barrier within the tumour. (C) Example of clonal competition between subclones, where distinct subclones compete for growth advantages (equivalent to an antagonistic relationship). (D) Conversely, subclonal populations may cooperate, resulting in a symbiotic relationship [232].

In conclusion, genomic instability coupled with environmental contexts may give rise to a genetically distinct subclonal populations with distinct phenotypic advantages [215, 233].

### **2.1.3 Consequences and implication for therapy**

Intratour genetic heterogeneity results in diversity that affects clinically relevant parameters such as gene expression signatures that further influence prognosis and response to therapeutic agents [234, 235]. For example in, renal clear cell carcinoma analysis revealed that primary tumour contains different clones reflecting both good and poor prognosis [226]. Moreover, recent reports suggested that efforts to forecast outcome require the identification of distinct subclones at date of diagnosis [236]. This could be illustrated by recent study concerning myeloma, where a clone barely detectable at the stage of diagnosis was dominant at the end of disease [237].

Intra-tumour heterogeneity poses a challenge to targeted therapies and raises important questions regarding future drug development strategies. Of note, most programs of drug development do not consider clonal diversity or driver alteration frequencies in tumours, but simply target the driver events such as mutations in PTEN, PIK3CA, or mTOR. However, recently launched DARWIN clinical trial should give us some answers (<http://clinicaltrials.gov/show/NCT02183883>) [232]. It aims to assess whether targeting a clonally dominant driver event results in improved progression free survival outcomes relative to targeting the same driver event when it is present subclonally. In addition, these studies will monitor the subclonal dynamics throughout therapy and during the acquisition of drug resistance. Importantly, there is already some evidence that in case of drug-resistant disease, continuation of therapy could accelerate tumour progression through giving a selective advantage to the resistant subclone [238]. Meanwhile, two other possibilities have been raised. First one to be considered is immunotherapy, which does not depend on the clonality of a single molecular target and thus might overcome some of the challenges of intratumour heterogeneity. Second approach called adaptive or metronomic therapy has been only used in terminally ill patients, but might prove to be useful as first line therapy [239]. This therapy bases on the statement that instead of aggressive treatment, which may

lead to the emergence of resistant clones, lower doses of drug should be used. According to this hypothesis, such strategy should maintain drug sensitive clones and in consequence prevent competitive release of drug resistant, untreatable subclones [232]. Preliminary research on mice revealed that adaptive growth-related dose of drug in comparison to standard high dose treatment offered better survival than standard therapy [240].

## **2.1 Cancer stem cells (CSC)**

For a long time it has been recognized that tumours are composed by various cell populations at different stages of differentiation [241]. Extensive studies led to the suggestion that the undifferentiated tumour cells may be considered as tumour stem cells. The concept of cancer stem cell derives from the well-described and better understood phenomenon of normal stem cells, such as tissue specific stem cells (SCs), which are indispensable for tissues repair mechanism and tissue homeostasis. SCs are found in many different organs such as haematopoietic lineages, digestive tract, muscle, brain and skin. SCs have unique features and are characterized by a long-term self-renewal capacity and the capability to differentiate to many cell types ensuring the specific functions of a given tissue [242].

### **2.1.1 CSC – evidences**

CSCs were discovered by the group of John Dick in 1994 [243]. While studying human acute myeloid leukemia (AML), they isolated a population of cells characterized by expression of CD34+/CD38- markers, which usually were used to mark progenitor and pluripotent stem cells in the bone marrow. Moreover, xenotransplantation of CD34+/CD38- leukemic cells into NOD/SCID mice resulted in leukemia, that reproduces many features of human AML [243]. Since this pioneering work, CSCs have been identified as a component of many solid tumours such as breast cancer [244], brain tumours [245], colorectal cancer [246], skin squamous cell carcinoma (SCC) [247], head and neck cancer [248], lung cancer [249], pancreatic cancer [250], prostate cancer [251] or ovarian cancer [252] or liver cancer [253]. Notably, the variety of tumours in which CSCs were found suggests that these cells are a common feature of most cancers.

However, some reports indicate that it could not be the case at all stages of disease [254].

### 2.1.2 CSC - methods of identification

Tumour consists of cells characterized by different clonogenic potential with a specific infinite potential for CSC. In order to detect and test their tumour initiating capabilities different assays have been developed. For example, sphere-forming assay is used *in vitro*. In most of the cases cells that form spheres are more prone to give rise to secondary tumours in *in vivo* assays. *In vivo*, the gold standard of CSC identification is a serial xenotransplantation of limited dilutions of tumour cells into immunosuppressed mice. This method was used for the identification of the first CSCs and for later studies that allowed the isolation of CSCs in many human cancers (reviewed in [255]). However, the transplantation assay is not perfect, as it does not faithfully reproduce either a tumour microenvironment or a realistic immune environment [256].

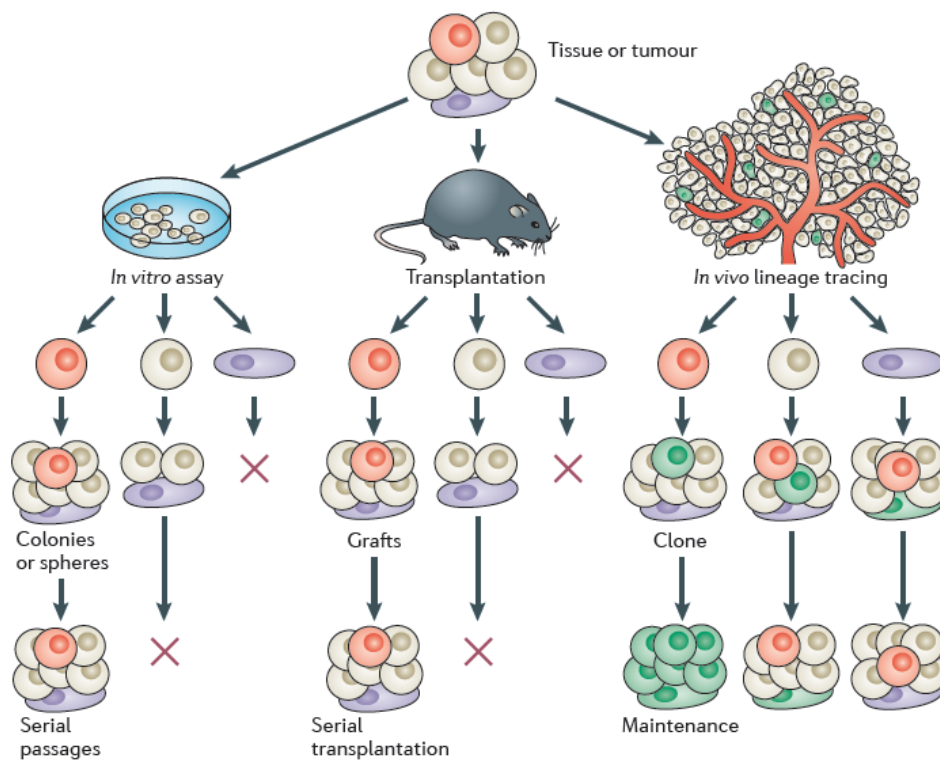
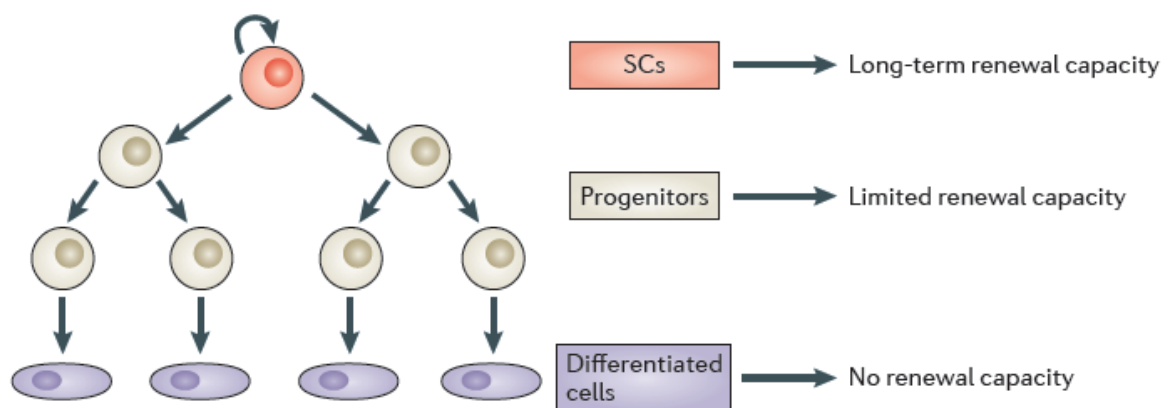


Figure 13. Functional assays for CSC identification [255].

More recently, an improved method has been developed: the genetic lineage-tracing approach that is becoming the gold-standard assay to assess the fate and long-term renewal potential of epithelial SCs in their natural environment [257]. Recently, several studies used this method to assess the fate of individual tumour cells [258] (Figure 13).

### 2.1.3 CSC - characteristics

According to their definition and as mentioned above, cancer stem cells possess the same functions as normal SC like clonal long-term repopulation and self-renewal capacity (Figure 14) [259, 260].



**Figure 14. Most common type of cellular hierarchy observed in tumour cells [255].**

Importantly, origin CSC remains unclear and it is still an area of ongoing research, however several hypotheses exist. More precisely, CSC can originate from mutated developing stem or progenitor cells [261] as well as adult stem or progenitor cells [262, 263]. Finally, the third possibility is that CSC may originate from any cells, which have acquired stem cell like characteristics through activation of stemness pathways [264]. Recently a lot of studies tried to established functional similarity between CSC and normal SC. These recent findings indicate that CSC self-renewal capabilities are regulated trough the same molecular pathways as normal stem cells such as Wnt [265], Notch [266], and Hedgehog (HH) signalling [267]. Over the time, significant efforts were deployed on identification of specific markers of CSC such as CD133 [268], CD24-/CD44+ [244] and ALDH [269]. However, no consensus or general markers have been identified so far. This is probably because of the phenotypic diversity of CSC and

the fact that markers expressed by tumorigenic cells differ among patients [256]. Finally, CSCs were also found to be resistant to chemo- or radiotherapy and therefore are considered to be potentially responsible for tumour relapse after therapy. Indeed, following studies found out that CSCs are enriched in xenografts following chemotherapy of colorectal cancer [270] or radiotherapy of breast cancer [271].

#### **2.1.4 CSC – challenges and problems**

Several issues exist regarding CSC and their biology. Until now, it was generally assumed that these cells present the greatest tumour propagating potential and thus are responsible for sustaining tumour growth *in vivo*. However, it is still unclear if cells that present the highest clonogenic potential in transplantation assays and promote tumour growth in lineage-tracing experiments are the same [255]. Cancer stem-cell markers need to be further tested in numerous patients to improve their reliability. Moreover, up to now, there is no evidence that combining these potential cancer stem-cell markers would improve the isolation of a pure cancer stem-cell population. It is a hurdle that needs to be overcome to really characterize the biology of these cells. Studies of genetic heterogeneity should be combined with studies of tumorigenic potential in order to develop a more realistic view of the potential that these cells represent in understanding the tumours they are derived from [256].

## **2.2 Tumour microenvironment**

A solid tumour is now viewed as a complex ecosystem that includes not only cancer cells but also numerous different cell types required for the development of the tumour. This essential component is called the tumour microenvironment (TME). The role of the TME is now recognised as a major part of cancer biology and is the subject of intense studies. TME is composed of immune, inflammatory and mesenchymal cells, vascular and lymphatic vessels and extra-cellular matrix (ECM) (Figure 15) that through coordinated actions will greatly impact on the behaviour of the cancer cells. As an example, chronic inflammation triggered by immune cells coupled with stimuli secreted by others members of TME promotes tumour progression [272]. Additionally, hypoxia and metabolic stress (see chapter 3) are a common feature of the TME and lead to an adaptive response that triggers cancer malignancy [273].

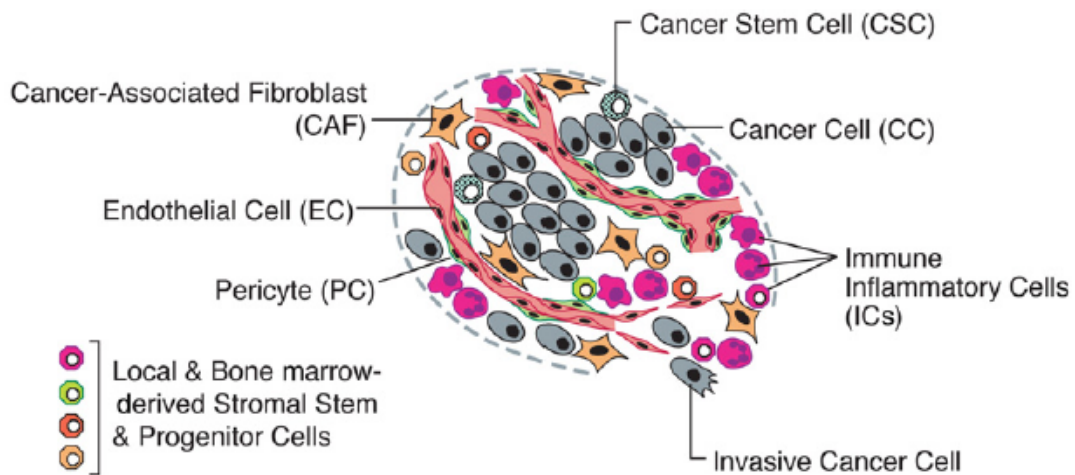


Figure 15. Cellular composition of TME [2].

### 2.2.1 Cellular composition of TME

Non-malignant cells of TME display tumour-promoting functions that are activated by their interaction with tumours cells [272]. They usually act through secretory processes that include cytokines, chemokines, growth factors and matrix remodelling enzymes. This secretome creates a complex and dynamic communication network between TME and cancer [274]. As noted above, TME consist of various cell types such as:

- Endothelial cells, which are the main component of vascular network. TME is usually characterized by an enrichment of angiogenic factors such as vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), that promote and orchestrate neovascularization [275]. However, angiogenesis triggered by the tumour results in formation of abnormal vessels that are usually characterized by leakiness [276] and drive uneven oxygenation as well as nutrient and drug distribution in the TME.
- Cancer associated fibroblasts (CAF) often resemble myofibroblasts found in maturing scars, chronic inflammations and fibrosis [277]. One of the most important function of CAFs in the TME is their ability to secrete mitogens such as EGF family members, hepatocyte growth factor (HGF), fibroblast growth factor

and insulin-like growth factor 1 (IGF1) [278–281]. Moreover CAFs can release transforming growth factor beta, which induces an epithelial–mesenchymal transition in malignant cells and contributes to the formation of an immune-suppressive microenvironment [282]. Finally CAFs are central in the formation of ECM (see below).

- Tumour associated macrophages (TAM) are abundant in most of human and mice tumours. Notably, their presence has been considered as pro-tumorigenic [22] and associated with poorer prognosis [283]. Their phenotype and response to environment is shaped by interaction with TME. Hypoxic or necrotic conditions act as attractants for TAM and lead to release of hypoxia-induced chemo-attractants, such as VEGF or endothelins [284] and in consequence to angiogenesis [285].
- Immune contexture describes all components of immune system, which can be found within tumour microenvironment. Precise knowledge of immune landscape may provide useful information for patients' survival. Main actors within immune contexture are macrophages, dendritic cells, natural killer cells and various subsets of T cells such as T helper cells, cytotoxic T cells or regulatory T cells (Treg) [286].

### **2.2.2 ECM and TME**

Extra-cellular matrix (ECM) is characterized as a multi-compound scaffold for cellular components of TME, but also possesses other important function such as modulation of cancer cell spreading. Tumours are considered to be stiffer than healthy tissue, because of excessive production of collagen by CAFs [287]. On the other hand, CAF and TAM release matrix metalloproteases (MMPs), which are responsible for ECM remodelling. Additionally, excessive remodelling through MMP or other proteases promotes tumour dissemination and metastasis [2, 288, 289].

### **2.2.3 TME and cellular stress**

Another important feature of tumour microenvironment is that it is a source of cellular stress. Tumours typically suffer from nutrient and oxygen deprivation caused by inefficient blood supply. Such hypoxic environment gives rise to a strong selective



pressure on tumour cells, leading to the acquisition of aggressive traits and thus is an essential component of tumour progression (see chapter 3). Severe oxidative stress (see chapter 3) caused by ROS release from TAM or CAF is also often found in the TEM. It consequently activates different cancer related transcription factors such as NF- $\kappa$ B, AP-1, p53, PPARc,  $\beta$ -catenin/Wnt, and NF-E2-related factor (NRF2) [290, 291]. Additionally, persistent oxidative stress up-regulates an adaptive antioxidant response that helps cells to survive under unfavourable environmental conditions, such as therapy or hypoxia [290].

# CHAPTER II. Twist oncogenes

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## 1 Twist proteins – discovery

Twist was discovered in *Drosophila* in 1987. Initially called *twist* or *twi*, it was shown to be a zygotic developmental gene involved in early mesoderm patterning [292]. The name of the protein is related to the twisted phenotype observed upon twist deletion in *Drosophila* embryos [293].

Twist genes are characterized by a high degree of homology in a broad range of species. Twist orthologs were identified in different organisms such as: jellyfish [294], *C. elegans* [295, 296], leech [297], lancelet [298], zebrafish [299] and *Xenopus* [300]. In vertebrates, there are two Twist genes, Twist1 and Twist2. The mouse ortholog of Twist was cloned and sequenced in 1991 [301]. Twist2 was identified 1995 from a yeast-two-hybrid screen using E12 as a bait and initially called Dermo1 for its restricted expression pattern in the dermis of mouse embryos [302]. In humans both Twist genes were identified in 1997 [303, 304]. According to the NCBI Gene database, 84 and 138 orthologs have been identified so far for Twist1 and Twist2, respectively. Because both factors share a high degree of sequence identity and functional redundancy the generic term, Twist, will be used in situations that consider both Twist proteins.

### 1.1 Molecular structure of Twist genes

Human Twist1 gene consists of two exons localized on 7q21.2 [303, 305]. The mRNA sequence contains an open reading frame encoding 202 amino-acid residues positioned in the first exon. The second exon contains an untranslated region with two potential polyadenylation sites. The Twist2 gene is found on 2q37 and similarly to Twist1 is composed of two exons divided by a large intronic sequence. Interestingly, Twist2 gene codes two transcript variants that differ in the 3' UTR region. However, both variants encode the same protein (NCBI gene).

## 1.2 Protein structure of Twist genes

### 1.2.1 bHLH family characterization

Twist proteins are members of the basic helix-loop-helix (bHLH) family of transcription factors. The bHLH proteins form a large superfamily of transcription factors that are present in a wide range of organisms from yeast to humans. They are key factors in critical developmental processes, including sex determination, neurogenesis, myogenesis, hematopoiesis and gut development [306]. The characteristic bHLH domain has approximately 60 amino acids and contains a DNA-binding region (basic region) followed by two  $\alpha$ -helices separated by a variable loop region (HLH). This domain promotes dimerization, which can lead to formation of homodimeric or heterodimeric structures between different family members (reviewed in [307, 308]) (Figure 16).



**Figure 16. Representative structure of bHLH heterodimer from Protein Data Bank.**

Heterodimer consists of E47 (orange) and NeuroD1 (grey) transcription factors (PDB code: 2QL2). Proteins are shown as a secondary-structure cartoon and DNA double helix is shown in sticks representation. Analysis was made in Pymol application.

The dimers interact with DNA through E-box sequences, which are elements mediating cell-type-specific gene transcription. They share a consensus hexanucleotide sequence: CANNTG. These sequences are found in a large set of gene promoters and enhancer elements (reviewed in [308]). The large number of combinations of dimeric structures

composed of bHLH proteins as well as the heterogeneity in E-box sequences translates into the vast range of processes in which these factors are involved [309].

#### **1.2.1.1 Classification of bHLH proteins**

The large number of helix-loop-helix (HLH) proteins described in the literature, led to their classification into 7 groups. Their discrimination was based on their tissue distribution, dimerization capabilities and DNA-binding specificities ([310], reviewed in [308]). The classification was described by Murre's group, who was also the first to demonstrate the presence of bHLH motifs in E12 and E47 proteins [306]:

- Class I proteins, also known as the E proteins, are expressed in many tissues. They can create homo- and heterodimers. Their DNA-binding specificity is limited to canonical E-box sequences. Examples: E12, E47, HEB.
- Class II proteins show tissue-restricted pattern of expression. They preferentially form heterodimers with E proteins and bind both canonical and non-canonical E-box sites. Examples: Twist, MyoD, NeuroD.
- Class III proteins, such as Myc family, possess leucine-zipper (LZ) adjacent to HLH motif.
- Class IV proteins include Mad, Max and Mxi proteins, which can dimerize with Myc proteins.
- Class V proteins, like Id or Emc, do not possess the basic region and hence do not bind DNA. They play a role of antagonists to proteins from classes I and II.
- Class VI proteins have a proline amino acid in their basic region, like Hairy protein in *Drosophila*.
- Class VII proteins have a PAS motif located at carboxy terminal site of bHLH region, which allows them to dimerize with other PAS, non-PAS proteins and small molecules like dioxin. Key members of class VII are hypoxia inducible factors or Arnt protein [311].

More recently Atchley and Fitch classified bHLH proteins into four major groups (A - D) using evolutionary relationships through a phylogenetic tree and differences regarding E-box binding sequences and amino acid sequences [292]. The sequencing of new genomes led to the identification of new bHLH families and classification was extended to two

more groups (E and F) by Ledent and co-workers [307] (reviewed in [311]). This recent classification is presented and compared with Murre's classification in the Table 1 below:

Classification of bHLH proteins by sequence			
Phylogenetic group	Description	Classification according to Murre <i>et al.</i>	Examples of proteins (family names)
A	Bind to CAGCTG or CACCTG	I, II	MyoD, Twist, Net
B	Bind to CACGTG or CATGTTG	III, IV	Mad, Max, Myc
C	Bind to ACGTG or GCGTG. Contain a PAS domain	VII	Arnt, HIF, Clock
D	Lack a basic domain and hence do not bind DNA but form protein-protein dimers that function as antagonists of group A proteins	V	ID
E	Bind preferentially to N-box sequences CACGCG or CACGAG. Contain an orange domain and a WRPW peptide	VI	Hairy
F	Contain an additional COE domain, involved in Coe dimerization and DNA binding	-	Coe (Col/Olf-1/EBF)

**Table 1. Comparison of bHLH proteins classifications.**

Newer classification based on sequence comparison, E-box binding, comparison of residues in certain parts of the protein and presence or absence of additional domains. Older classification was based on tissue distributions, DNA-binding specificities and dimerization, it was describing a smaller set of proteins.

### 1.2.2 Characteristic of protein structure of Twist genes

Both Twist proteins belong to the class II of bHLH factors, which means that they can create not only homodimers, but also heterodimers with class I E proteins. According to its amino-acid sequence, human Twist1 is around 21kDa and shares almost 96% sequence homology with the mouse isoform [301]. Twist's bHLH region is highly conserved among different species and its importance is highlighted by the fact that even a point mutation causes a Saethre–Chotzen syndrome (SCS) (discussed later) [304]. Similarly to others family members, Twist's bHLH region is responsible for DNA

sequence recognition and binding. Of note, single point mutation such as S144 or K145 in the bHLH can completely abolish its functions [312]. Another conserved region of Twist called Twist box, is located at the C-terminal part of the proteins and is necessary for protein folding and activity [313]. Furthermore, a transactivation domain has been characterized within the Twist box and genetic mutations within this region are associated with SCS of human patients [314]. Functionally, this region is involved in protein interaction (discussed later). Twist is a nuclear factor and in line with this its sequence contains two nuclear localization signal (NLS) domains, 37RKRR40 and 73KRGKK77. Interestingly, in the case of NLS mutation, Twist may still be translocated to nucleus, however this scenario is true only in the case of heterodimers as NLS is supplied by its non-mutated partner [315].

Overall sequence identity between Twist1 and Twist2 is around 66%, but it is increased up to 95% in the bHLH region and to 100% in the Twist box region [316] (Figure 17). The N-terminal region is much less conserved between both proteins, Twist2 lacks this glycine-rich domain, resulting in a significant difference in size: Twist1 is bigger than Twist2 by having 202 amino acids versus 160 amino acids respectively. The absence of glycine-rich domain results in lack of some interaction partners when compared to Twist1. This fact can explain some differences in functions and a lack of complete redundancy between Twist proteins ([302], reviewed in [316]).

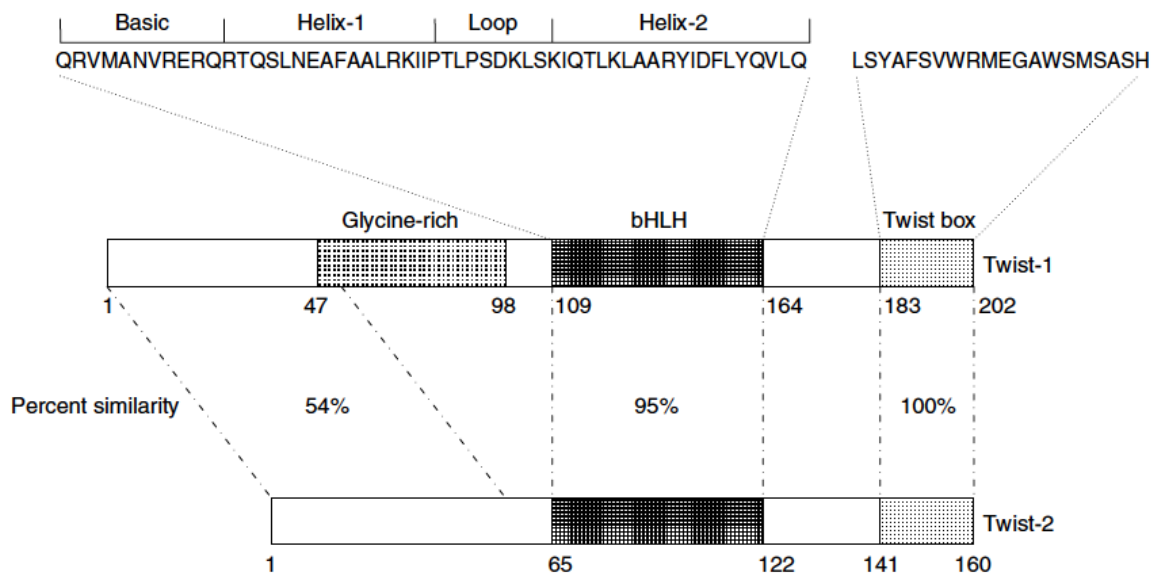
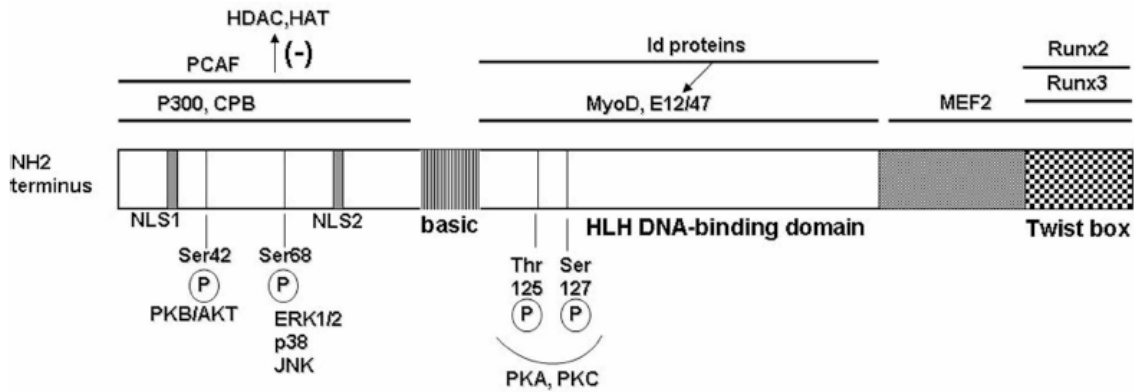


Figure 17. Comparison of human Twist1 and Twist2 protein structure [317].

### 1.2.3 Post-translational modifications of Twist

Similarly to other proteins, Twist is regulated through different post-translational modifications. Phosphorylation can dictate not only the stability of protein, but also its activity or predisposition for binding to different partners (Figure 18). Human Twist1 can be phosphorylated at different sites such as Ser18, Ser20, Ser42, Ser68, Thr125 and Ser127 [318]. Thr125 and Ser127 are located at the first  $\alpha$ -helix of the HLH domain and their phosphorylation status is controlled by protein kinases A and C, and protein phosphatase 2A [319]. Moreover, these modifications are conserved from fly Twist throughout the Twist subfamily of proteins (including Hand1 and Hand2) [320]. Their impairment is associated with SCS, it influences the choice of dimerization partners and as such deregulates limb development [320]. One of the best characterized post-translational modifications of Twist1 is its phosphorylation on Ser68 by mitogen-activated protein kinases (MAPK) such as p38, JNK (c-Jun N-terminal kinase) and ERK (extracellular signal-regulated kinase-1/2) [321]. This phosphorylation stabilizes Twist through inhibition of ubiquitylation-mediated degradation. In addition, phosphorylation mediated by PKB/Akt at Ser42 [322], similarly to previously mentioned modifications, promotes progression of breast cancer [318]. Recently, it has been shown that casein kinase 2 mediated phosphorylation on Ser18 and 20 occurs in response to interleukin 6 stimulation and promotes Twist stability in head and neck squamous cell carcinoma [323]. These examples demonstrate that phosphorylation generally stabilizes Twist proteins.

Even more recently, the group of Binhua P Zhou showed that Twist could be also acetylated by Tip60 on residues K73 and K76. Functionally, this acetylation induces the formation of a Twist-BRD4 complex and in consequence leads to the activation of WNT5A promoter, which promotes progression of basal-like breast cancer [324]. Lately, Twist was also found to be methylated at position R34 by protein arginine methyl transferase 1 (PRMT1). This modification has an impact on nuclear transport and E-cadherin gene repression during lung cancer progression [325].



**Figure 18. Molecular structure of human Twist protein [326].**

Additionally, on this model structure phosphorylation sites and possible interacting factors have been marked.

#### 1.2.4 Regulation of gene transcription by Twist

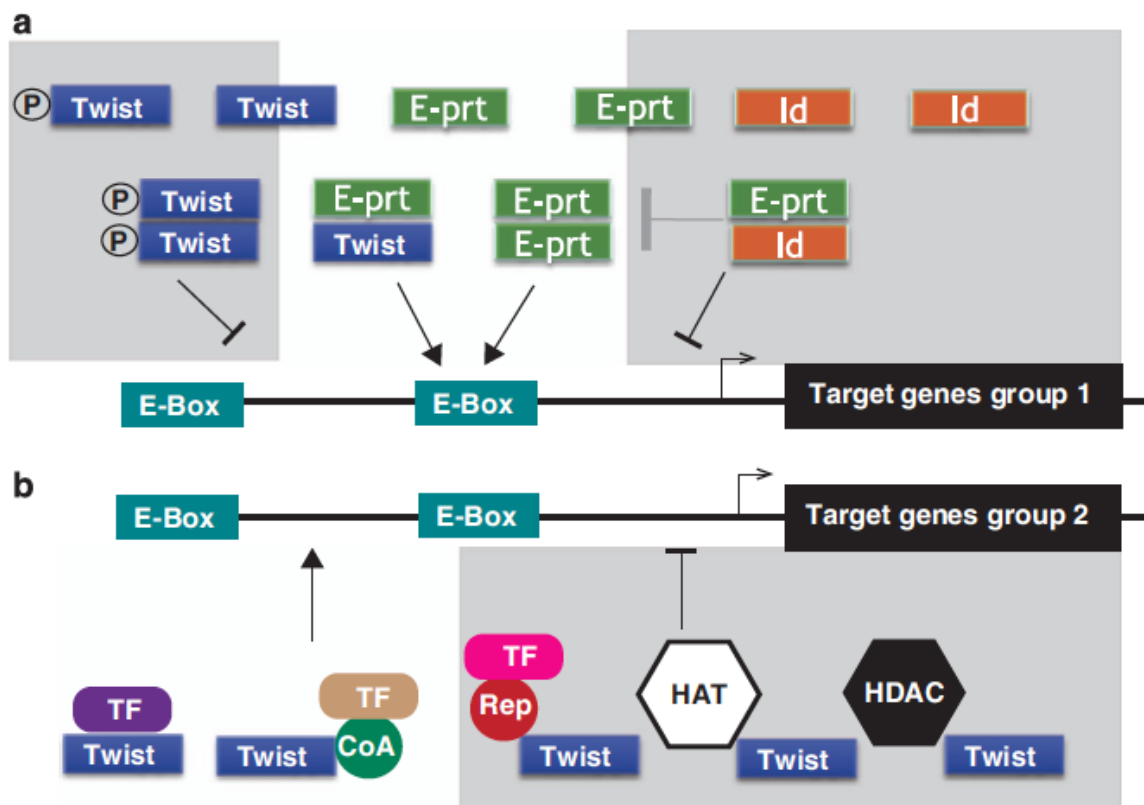
Twist functionality could be modulated by different factors such as dimer choice or other interacting partners. First of all, dimerization is mainly regulated through availability of possible partners as well as their phosphorylation or acetylation status [327, 328]. Class B of bHLH is known to form not only homodimers but also heterodimers with class A such as E12 or E47. Nonetheless, formation of heterodimers between class B has also been reported and could be exemplified by Twist and Hand1. Moreover, availability of E proteins, apart from their expression levels, could also be regulated by another class of bHLH factors - Id (inhibition of DNA binding) proteins such as Id1, Id2, Id3 and Id4. The Id proteins, which lack the DNA-binding domain, have a high affinity for E12 and compete against class B bHLH proteins for its binding. Therefore, Id proteins actively reduce the amount of available dimerization partners by binding and sequestering bHLH factors, without themselves being able to bind DNA. In conclusion, it is the relative stoichiometry of each E protein, Id repressors and Twist1–2 that will determine the cell fate via the control of certain expression profiles [327, 329].

The N-terminal part of Twist protein can interact with p300, cAMP-response element binding protein (CREB), CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF). This interaction results in inhibition of acetyltransferase activities of p300 and PCAF [330]. This mechanism is an example of Twist mediated transcriptional repression, since histone acetylation is usually coupled with transcriptional activation.



Finally, the Twist C-terminal part that contains both the Twist box and a transactivation domain, is known to modulate gene expression through binding to other transcription factors. Indeed, Twist may modulate activity of factors such as MEF2, Runx2, or Sox9 through direct binding [331–333] (Figure 18). Moreover, Twist-box is involved in one the best known Twist function – inhibition of p53. Recently, the group of Roberta Maestro demonstrated that binding between C-terminal ends of p53 and Twist1 destabilize p53 and leads to attenuation of its response [334].

Finally, Twist was shown to inhibit transcriptional activity of other proteins such as NF-κB or PGC1α. However, interactions between these proteins were described without pointing towards specific domains [335, 336]. All known modes of transcriptional activity of Twist are presented in (Figure 19).



**Figure 19. Transcription regulation mediated by Twist proteins [337].**

(A) bHLH partner competition. (B) Mechanism of activation and repression of transcription. More details in text. **Legend:** TF – transcription factor, CoA – co-activator, Rep – repressor, HAT – histone acetyltransferase, HDAC – histone deacetylase.

## **2 Physiological functions of Twist**

### **2.1 The role of Twist during development**

*Drosophila* twist is crucial for many processes during embryonic and tissue development. For example, it is required during dorsal-ventral patterning [338], where it is regulated through a nuclear gradient of Dorsal protein that restrains the expression of twist to the ventral mesoderm [339, 340]. *Drosophila* twist mutants are also impaired in the gastrulation process, during which twist was shown to be needed for the morphogenetic movement ([341], reviewed in master thesis [342]). More detailed developmental study will be presented on mammalian examples, as they are the main scope of this thesis. Short summary and comparison of Twist developmental functions between fly and mouse is presented below in Table 2.

#### **2.1.1 Twist1 expression pattern during mouse development**

During mouse embryogenesis Twist transcripts can be detected at embryonic day 7.5 (E7.5) in the extra embryonic mesoderm and it is strongly expressed in the allantois [343]. Interestingly, Twist1 protein is not detected until almost E8.5 despite its earlier mRNA expression patterns, the timing that coincides with the abnormalities observed in *Twist1* null mice [344, 345]. In summary, Twist1 expression in mouse embryo occurs first along a dorso-ventral gradient pattern until the headfold stage, and then moves along the rostro-caudal axis of the embryos as mesoderm cell layer- and neural crest cell-derived tissues develop.

Twist1 plays critical roles in diverse developmental processes such as cranial tube morphogenesis, mesoderm formation, myogenesis, cardiogenesis and neurogenesis [344, 346–349]. To better understand the role of Twist during development, Twist knockout was generated [344]. Unfortunately, Twist1 null embryos die at E11.5, displaying exencephaly, pharyngeal arch and somitic defects, as well as defects in both cranial and cardiac neural crest cell populations [344, 350, 351]. Moreover, Twist1-KO mice exhibited abnormalities in mesenchymal cell types and were characterized by the presence of significant apoptotic cell populations, especially in the developing

sclerotome [344]. Increased cell death during development could reflect Twist1 anti-apoptotic properties as a modulator of p53 or NF- $\kappa$ B pathways [335, 352].

Gene	Expression pattern	Functions
<i>Drosophila melanogaster</i>		
twist	presumptive mesoderm mesodermal segments thoracic flight muscle progenitors	Required for proper gastrulation. Twist homodimers specify mesoderm. Twist-Daughterless heterodimers antagonize mesodermal differentiation, properly patterning the mesoderm. Persistent expression in adult myoblasts maintains an undifferentiated state.
<i>Mus musculus</i>		
Twist1	extraembryonic mesoderm alantois head mesenchyme & cranial sutures pharyngeal arches cephalic and thoracic neural crest lateral mesoderm distal limb bud mesenchyme	Follows mesodermal induction. Required for proper cranial mesenchyme and neural crest migration and distribution, directly affecting neural tube patterning and cushioning. Patterns the limb and cranial suture where it functions in a dimer dependent manner, regulated by dosage and phosphorylation.
Twist2	sclerotome, myotome, dermatome branchial arches limb bud mesenchyme osteoblasts dermis	Essential for chondrogenesis, preventing differentiation of preosteoblasts. Dermal expression is required for proper skin formation and wound healing. Downstream of NF- $\kappa$ B directly repressing the cytokine p65 promoter. Helps in fatty acid synthesis.

**Table 2. Twist-family bHLH protein expression and function (based on [353])**

In addition, Twist displays other functions such as repressor of muscle differentiation, which occurs through two separate mechanisms. In the first one, Twist may sequester E-proteins, thus preventing the formation of functional myogenic complexes with the skeletal muscle specific bHLH factor, MyoD. Therefore, it would functionally block both *cis*- and *trans*- MyoD elements. Secondly, Twist inhibits myocyte enhancer factor 2 (MEF2) through a physical interaction [331, 346, 347].

Moreover, Twist plays an essential role during osteogenesis. Twist expression was found in osteoprogenitors of the mid sutural mesenchyme of the developing cranial

sutures [350], where it inhibited osteoblastic differentiation [327, 354] (discussed later). The importance of this function during development is demonstrated by Saethre-Chotzen syndrome caused by Twist1 haploinsufficiency, which is characterized by craniosynostosis (a developmental defect caused by a premature closure of the cranial sutures), as well as limb development abnormalities [304, 305, 313, 355] (discussed later).

### **2.1.2 Twist2 expression pattern during mouse development**

Spatial expression pattern of Twist2 generally overlaps with expression of Twist1 during mouse embryogenesis [301, 302, 356]. First detection of Twist2 occurs at E10.5 in mesenchymal cell populations in the sclerotome, myotome, dermatome, branchial arches, and limb bud [302]. Later, the expression becomes restricted to mesodermally derived cartilage and dermal tissues by E15.5 and finally it focalizes in dermal tissues near the end of embryogenesis (E17.5) [302].

Twist2, similarly to its homologue, is involved in myogenic and osteoblast maturation [302, 335, 356]. In contrast to Twist1 deficient animals, Twist2 knockout mice survive embryogenesis, however they suffer from developmental abnormalities such as deficient wound healing or hair regeneration [335, 357]. Additionally, these mice suffer from severe postnatal atrophy of multiple tissues, apoptosis, and ultimately die soon after birth due to cachexia. The cachectic phenotype is most probably mediated by increased pro-inflammatory cytokine gene expression [335]. Interestingly, Twist1-2 heterozygous mice can recapitulate this phenotype, suggesting strongly a partial redundancy between Twist proteins. Nonetheless, it is worth mentioning that Twist2-null mice developed in 129/C57 mixed genetic background survive to adulthood with milder phenotype, highlighting the fact that a number of factors influence the phenotype observed in these different genetic backgrounds [335] (discussed later).

Furthermore, Twist2, like Twist1 is also involved in muscle differentiation through repression of MyoD and MEF2 activities [357]. Finally, Twist2 is also able to maintain osteoblast progenitors in an undifferentiated state and as a consequence prevents osteogenesis [356, 357].

### 2.1.3 Mesenchymal stem cells and Twist

Mesenchymal stem cells can differentiate to multiple cell lineages such as chondrocytes, osteoblasts or adipocytes. As mentioned earlier, Twist proteins play a pivotal role in mesenchymal stem cells fate. The following Figure 20 describes the involvement of Twist1 in mesenchymal stem cell differentiation.

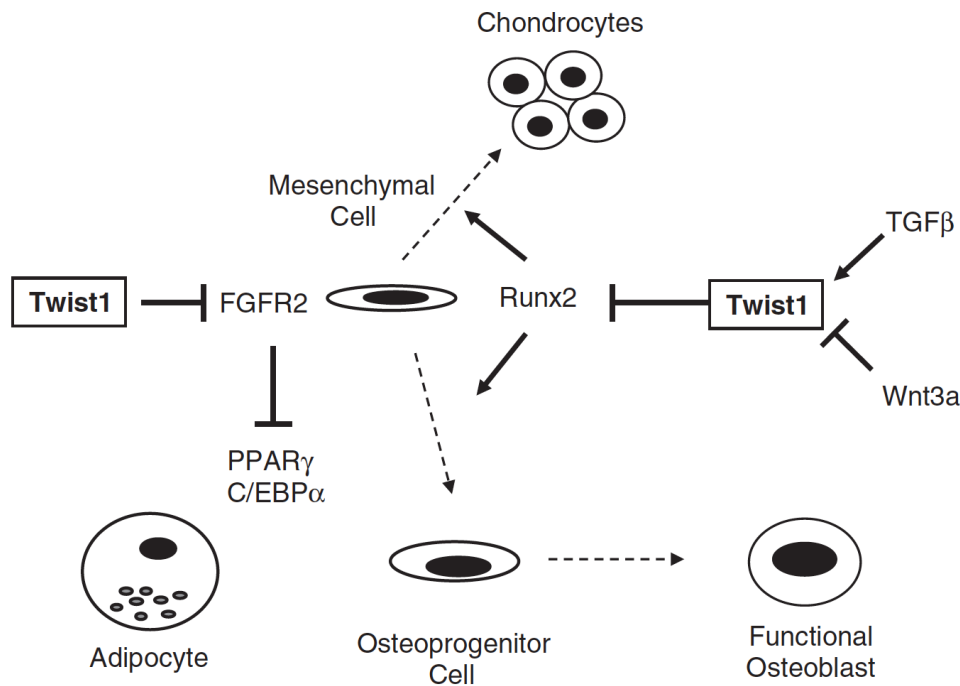


Figure 20. Role of Twist in mesenchymal cell lineage fate [358].

Chondrocyte, adipocyte and osteoblast differentiation is regulated through Twist-dependant mechanism involving genes such as Runx2 or FGFR2 (more details in text).

#### 2.1.3.1 Role of Twist1 in chondrogenic differentiation

Several studies indicated that Twist is involved in differentiation of mesenchymal cells into cartilage. *In vitro* studies demonstrated that overexpression of Twist inhibits differentiation [359], and its silencing leads to induction of chondrocyte phenotype both *in vitro* and *in vivo* [360]. Chondrocyte differentiation correlates with Twist inhibition and concomitant up-regulation of Runx2 [359]. Expression of these two proteins is most probably regulated by TGF $\beta$  and Wnt signalling pathways [359, 360]. Notably, Twist by inhibition of Runx2 expression downregulates its target - FGF18, which is crucial for

chondrocyte proliferation and maturation [361, 362]. Moreover, a recent report indicated that Notch pathway mediated inhibition of chondrogenesis may results in direct upregulation of Twist1 [363]. Overall, Twist1 negatively regulates chondrocyte differentiation toward terminal maturation by both direct and indirect mechanisms.

#### **2.1.3.2 Role of Twist in adipocyte differentiation**

Recently, several studies revealed that Twist is important for mesenchymal stem cells differentiation into adipocytes. Group of Marie P.J. showed that silencing of Twist increases expression of FGF receptor 2 (FGFR2) [358], which negatively controls adipocyte differentiation [364]. This suggests that Twist expression may promote adipogenic differentiation of mesenchymal stem cells in part via FGFR2. However, the proposed mechanism seems to be indirect and more work needs to be done to propose a more complete view of this process. Additionally, Twist may be further involved in the regulation of adipogenesis, as Twist1 is a PPAR $\delta$ -inducible regulator of PGC1 $\alpha$  (see below). This suggests a physiological link between Twist1 and maintenance of energy homeostasis in mice [336]. Thus, several mechanisms could be involved in the regulation of adipocyte biology by Twist1.

#### **2.1.3.3 Role of Twist in osteoblast differentiation**

Twist1 was found to control osteoblast development from osteoblast commitment throughout proliferation, differentiation and finally cell death [350, 365]. Observation of osteoblastogenesis process both *in vitro* and *in vivo* revealed that Twist expression decreases proportionally to the advancement of differentiation process [332, 366, 367]. The involvement of Twist in differentiation and maintenance of osteoblastic cells in an osteoprogenitor-like state [368, 369] was later validated by *in vitro* and *in vivo* studies [332, 370–372].

Twist regulates osteogenesis through several distinct mechanisms. First of all, Twist is implicated in the control of osteogenesis mediated by FGF signalling. Twist1 and FGFR2 mRNA expression overlap, suggesting that Twist1 may control the effect of FGFR2 signalling on the cell proliferation–differentiation balance [350, 373]. The link between Twist1 and FGFR2 is interesting firstly because Twist1 seems to inhibit FGFR2 expression in the mouse cranial suture [327, 350]. On the other hand, FGFR2 expression

is lowered in Twist haploinsufficient human osteoblasts and this expression could be restored by Twist overexpression [374]. This apparent discrepancy is due to different stoichiometric balance between Twist1 and its E-protein partners. Indeed, when Id1 levels are higher than those of Twist1, FGFR2 expression is induced. On the contrary, if the levels of Twist1 are higher than those of Id1, FGFR2 expression is inhibited, a situation that results in the terminal differentiation of osteoblasts [327].

Second, Twist1 has an effect on Runx2 - a master regulator of osteogenic program [332, 375]. It has been demonstrated that Twist1 modulates Runx2 function not only through physical interaction, but also through the control of its transcription [332, 374, 376, 377]. However, it is believed that Twist maintains mesenchymal cells in an undifferentiated state by negatively regulating Runx2 [332, 350]. Finally, both Twist proteins physically interact with Runx2 and block its binding to DNA at the osteocalcin promoter. The same study demonstrated also that Runx2 transactivation is inhibited through Twist box [332]. However, these two pathways do not exclude other possibilities of Twist action in osteogenesis as Twist1 was found to modulate periostin expression and BMP signalling that are potent activators of osteoblast differentiation *in vitro* [369, 371]. Twist2 is also known to regulate osteoblast differentiation, however its involvement occurs temporally after Twist1 [356]. Importantly, Twist2 mutations are not associated with SCS-like phenotypes, hence Twist2 appears to have no role in cranial bone development, which is consistent with its lack of expression in developing skull [302].

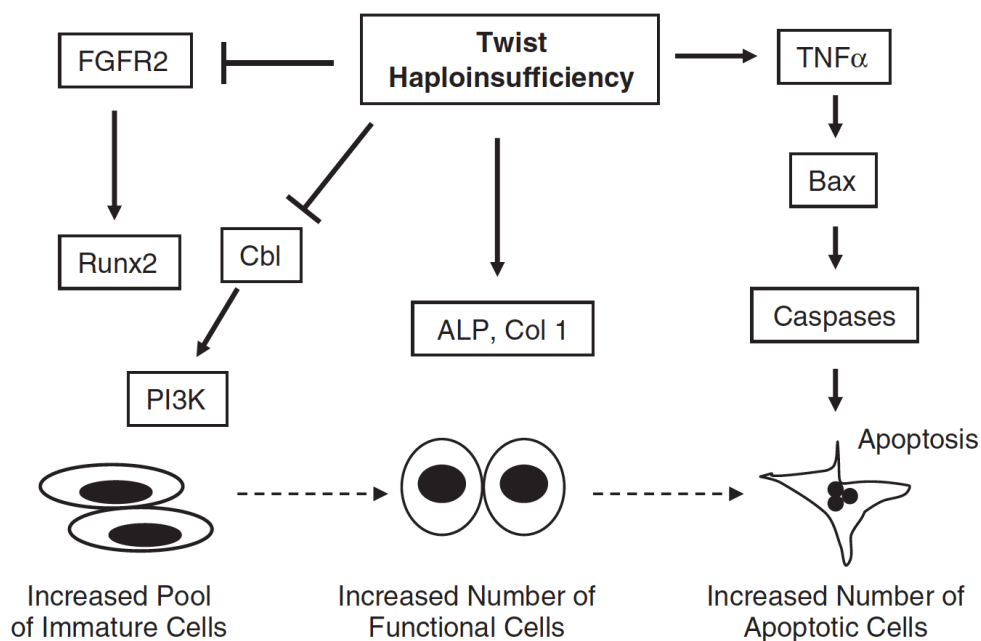
#### **2.1.4 Twist proteins and developmental diseases**

Twist1 knockout is lethal at embryonic stage, while Twist1 haploinsufficiency causes Saethre–Chotzen syndrome (SCS). As stated previously, Twist2 knockout mice complete embryogenesis, but die soon after birth due to cachexia. Nonetheless, similarly to bigger brother Twist1, point mutations in Twist2 also cause a pathology, which in this case is a Setleis syndrome called. Of note, the phenotype of Setleis syndrome is milder and different from SCS. Although human Twist1 and Twist2 encode bHLH transcription factors with a high degree of sequence identity, different phenotypical traits upon invalidation suggest some non-redundant functions in skin and bone development and

highlight the importance of studying Twist1 and Twist2 as separate entities [378]. Following section will described Twist proteins involvement in developmental diseases.

#### 2.1.4.1 *Twist1 and Saethre–Chotzen syndrome.*

Saethre-Chotzen syndrome also called acrocephalosyndactyly III (ACS III) is an autosomal dominant craniosynostosis characterized by premature fusion of the coronal sutures leading to skull deformations, distal limb abnormalities, dysmorphic facial features of variable severity and ptosis (falling of the eyelid) [304, 305, 355]. SCS has an estimated incidence of 1:25,000 to 1:50,000 live births [379]. Noteworthy, similar phenotype, called Muenka syndrome, results from mutations in the *FGFR3* gene [380]. SCS is associated with haploinsufficiency of human Twist1, which is caused by multiple mutations resulting in a stop codon or a missense mutation in its bHLH domain [313, 381]. Most of these mutations result in protein degradation, loss of dimerization with E proteins and reduced binding to the DNA canonical sequences in the promoter of target genes [312, 382]. From a molecular point of view, SCS results from an activation or repression of distinct molecular mechanisms, resulting in expansion of osteogenic cells producing collagen and in premature cranial suture fusion (Figure 21).



**Figure 21. Molecular mechanism of Twist1 haploinsufficiency in the Saethre–Chotzen syndrome [358].**



Basically, haploinsufficiency of Twist1 decreases its ability to compete with ID proteins for dimerization with E12 protein. This was reported to result in a higher proportion of Twist homodimers versus heterodimers within the cranial sutures [354]. Consequently, a reduced FGFR2 gene expression is observed, resulting in the alteration of Runx2 gene expression, which in turn reduces even further FGFR2 gene expression. Importantly, this molecular switch may be rescued by ectopic Twist1 expression, thus establishing a direct link between Twist1 and FGFR2 in SCS [374]. In addition, Twist1 may regulate FGFR2 gene expression via interaction with E12 and the histone acetyltransferase p300/CBP [330]. The second mechanism involves the regulation of Cbl, an E3 ubiquitin ligase that targets tyrosine kinase receptors and other signalling proteins, resulting in their ubiquitylation and downregulation [383]. Twist mutant cells are characterized by a downregulation of Cbl that leads to phosphatidylinositol-3 kinase (PI3K) accumulation and increases PI3K signalling, leading to osteoblast replication, which may contribute to the premature cranial suture ossification in SCS [384]. Additionally to alterations in proliferation or function of osteoblast cells, SCS patients may suffer also from increased apoptosis in cranial sutures [377]. That could be caused by limited Twist activity as it is known to display anti-apoptotic activities [352]. More detailed study demonstrated that Twist haploinsufficiency induces osteoblast apoptosis by mechanisms involving tumour necrosis factor alpha (TNF $\alpha$ ) and/or increased Bax levels [91, 377]. However, this mechanism may be a secondary effect and its activation could occur to compensate the expansion of osteogenic cells in this syndrome (reviewed in [358]).

#### 2.1.4.1.1 Twist1 haploinsufficiency in mice and SCS syndrome

Development of Twist1 heterozygous mice and their phenotype showed considerable similarity with human Saethre–Chotzen syndrome. Interestingly, Twist1 heterozygous mouse skeletal phenotype resembles those of human SCS with synostosis of the coronal suture [304, 314, 385]. The common phenotype in SCS patients with Twist haploinsufficiency and Twist1 heterozygous mice indicates that Twist1 mutation is the causal mechanism of the disease [313, 386].

#### **2.1.4.2 *Twist2 and Setleis syndrome***

Recently it has been described that homozygous missense mutations in human *Twist2* gene cause the Setleis syndrome (MIM 227260) [378]. Setleis syndrome is an inherited developmental disorder classified as a Focal Facial Dermal Dysplasia type III (FFDD III) and is characterized by bilateral temporal marks and additional facial features, including, absent eyelashes on both lids or multiple rows on the upper lids, absent Meibomian glands, slanted eyebrows and chin clefting [378]. These mutations (p.Q65X and p.Q119X) affect *Twist2* protein stability and DNA binding, providing the first genetic basis for a FFDD syndrome. Recently two other *Twist2* gene abnormalities were reported to cause Setleis syndrome, both being frameshift mutations - p.S57AfsX45 and p.R31GfsX71 [387, 388]. Notably, *Twist2* knockout mice developed in the 129/C57 mixed genetic background has revealed a facial phenotype similar to the one found in Setleis syndrome patients and has been established as a relevant mouse model for the study of FFDD [378]. The exact function of *Twist2* in this disease is not yet established because of still lacking clear molecular mechanism. However, a report published in 2011 suggested that the relevant target of *Twist2* in FFDD might be periostin, a secreted homophilic cell adhesion protein involved in connective tissue development and maintenance [389, 390]. *Twist2* mutants lacking the C-terminal part cannot enter the nucleus and thus do not induce transcription of periostin [389]. Consequently, dysregulation of periostin by *Twist2* mutant may alter the composition of the extracellular matrix and contribute to the development of the Setleis syndrome [389].

## **2.2 *Twist* roles in adulthood**

At the end of embryogenesis *Twist* expression is absent from most cell types. However its expression remains active in restricted number tissues. The highest expression of *Twist1* and *Twist2* were found in adipose tissue, and to a lesser extent in other mesoderm derived tissues such as muscle, heart, endometrium or placenta ([303, 391], [proteinatlas.org](http://proteinatlas.org)). However this is still controversial as another report studying the role of *Twist1* in adulthood showed the lack of expression of *Twist* in heart, skeletal muscle, small intestine, lung, liver, or kidney and testis, but detected a certain level of *Twist* in stromal cells surrounding the mammary gland ducts [392]. *Twist* expression was also

found in several cell types such as bone marrow-derived mesenchymal stromal/stem cells [372], hematopoietic stem cells (Twist1) [393], myeloid lineage (Twist2) [394] or fibroblasts of different origin [303]. Similarly to human, expression of Twist was found in the mouse white and brown fat tissue, and to a lower extent in muscle, heart or large intestine [336].

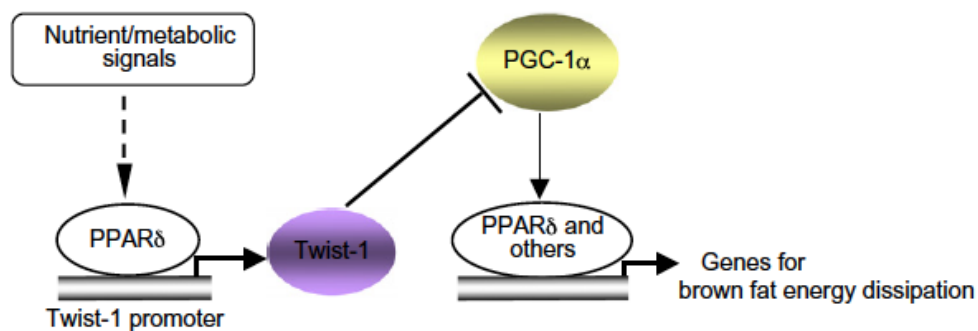
Twist proteins during adulthood are responsible for several functions such as maintenance of several stem cell types [372], adaptive thermogenesis [336] and inflammatory response [335], which will be described in the following paragraphs. Interestingly, although Twist1 knockout is an embryonic lethal, the conditional Twist1 knockout induced in 13 day-old mice unexpectedly showed only minor effects on general health, only resulting in impairment of hair follicle growth [392]. That is in striking contrast to Twist2 knockout and heterozygous Twist1/Twist2 mice, which showed severe defects in energy homeostasis and a strong increase in cytokine levels [335].

### **2.2.1 Role of Twist in the adipose tissue**

Twist proteins were found to be expressed both in white and brown fat tissue [336, 391, 395]. Interestingly, expression of Twist1 is several times higher in the subcutaneous (SAT) vs. visceral (VAT) human adipose tissue, suggesting that Twist1 plays potentially important roles in adipocyte development and fat distribution [395]. Moreover, Twist1 expression is correlated with body mass index (BMI), it is decreased in obesity and increases following surgical or caloric restriction weight loss [395] underlining the importance of Twist in cellular energetics. Additionally, Twist2 knockout and Twist1/Twist2 heterozygous mice are born with no SAT and severely reduced VAT [372]. Finally, it has been demonstrated that Twist2 acts as a physical inhibitor of the transcription factor ADD1/SREBP1c involved in adipocyte differentiation as well as regulation of the LDL receptor gene, fatty acid and sterol biosynthesis genes [389].

In brown adipocytes, Twist1 was found to orchestrate PGC1 $\alpha$  transcriptional activity [336]. Twist1 physically interacts with PGC1 $\alpha$  at the promoter site, giving rise to the recruitment of HDAC5 deacetylase. Finally, it inhibits the transcription of a battery of energy metabolism genes. In fact, the same study also revealed that PPARs induce Twist

expression upon activation through metabolic or nutrient signals. Therefore it appears that Twist1 serves as a negative feedback regulatory loop to finely tune PGC1 $\alpha$ /PPAR $\delta$  controlled brown fat metabolism in response to nutrient status and to thus participate in controlling the energy metabolism [336] (Figure 22). Since a decrease of energy expenditure was proposed to contribute to white fat accumulation and obesity this study suggests that Twist1 may be a positive indirect contributor to fat accumulation leading to obesity.



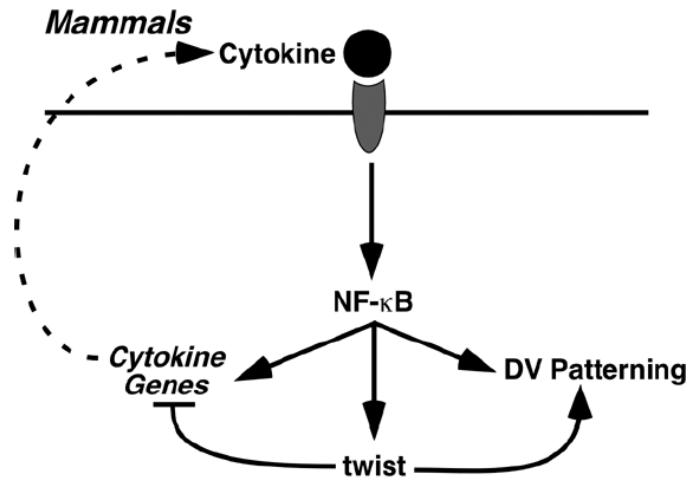
**Figure 22. Mechanism of Twist involvement in brown fat metabolism [336]** (more details in text).

To complete this paragraph, it is important to mention a recent article regarding the role of Twist1 in human white fat tissue and regulation of fatty acids. In this *in vitro* study, silencing of Twist1 led to a decrease of PGC1 $\alpha$  and CPT1, resulting in reduced fatty acid oxidation [391]. This result suggests that Twist1 is a promoter of fatty acid oxidation in white adipocytes. This work underlines an apparent discrepancy between results obtained in the human and in the mouse. Future studies should clarify these differences.

### 2.2.2 Twist, immune system and inflammation

The most significant evidence of Twist involvement in the regulation of the inflammatory response came from a study published in 2003. The group of Eric Olson showed that perinatal death of Twist2 KO and Twist1-2 heterozygous mice is due to high levels of pro-inflammatory cytokines [335]. Indeed, the phenotype of these mice is characterized by cachexia, defined as a severe wasting of body tissue, loss of appetite and anaemia. This may indicate a redundancy between Twist1 and Twist2 and suggests the existence of a critical threshold of expression of these genes [335]. Notably, Twist proteins were demonstrated to regulate expression of pro-inflammatory cytokines.

More precisely, they are able to physically bind p65/RelA, a subunit of NF- $\kappa$ B and inhibit NF- $\kappa$ B mediated transcription, suggesting a possible repression mechanism [335]. Moreover, both Twist proteins were able to bind E-boxes on promoters of pro-inflammatory cytokines such as TNF $\alpha$  or IL-1 $\beta$  [335]. The authors proposed a mechanism of negative feedback loop between NF- $\kappa$ B and Twist proteins where Twist expression could be triggered by TNF $\alpha$  in a p65-dependent manner [335] (Figure 23).



**Figure 23. Mechanism depicting Twist as a negative regulator of cytokine expression [335].**

**Legend:** DV patterning –dorsal-ventral patterning. More details in text.

Additionally, a more recent publication demonstrated an ability of Twist2 to downregulate pro-inflammatory cytokines such as IL-12 or IFN $\gamma$  while promoting the expression of anti-inflammatory cytokines e.g. IL-10 [394]. Twist1 and Twist2 were also described as key regulators of development of immune cells. Indeed, the expression of Twist1 is largely observed in CD34+ hematopoietic stem cells [303, 393, 396] whereas Twist2 is mostly expressed in the myeloid lineage [394]. More precisely, Twist2 was described as a major regulator of myeloid lineage development by inhibiting the proliferation and differentiation of macrophage progenitors through direct interaction and inhibition of Runx1 and C/EBP $\alpha$  transcription factors [394]. Moreover, in mature murine macrophages, IFN-induced Twist1 expression was shown to downregulate TNF $\alpha$  production [397].

Additionally, Twist1 expression in T cells considerably lowered their contribution to inflammation and tissue destruction in mice. Twist was shown to exhibit an important role in regulating the function and differentiation of immune cells, particularly T helper 1 (Th1) cells. Expression of Twist1 mediated by IL-12 via STAT4 and T cell receptor (TCR) signalling in TH1 cells acts as an endogenous regulator limiting the proinflammatory potential of TH1 cells, in the continuous presence of antigen with repeated activation of NFAT and NF- $\kappa$ B [398]. Additionally, Pham et al. further defined the role of Twist1 in Th1 cell-mediated inflammation. They showed that Twist1 modulates inflammation, as measured by inhibition of IFN $\gamma$  production, by decreasing the expression of T-bet, STAT4 and Runx3 and their binding to the IFN $\gamma$  locus [399]. Interestingly, Twist2 role in T lymphocytes and immature thymocytes seems to focus mainly around the prevention of galectin-1-induced apoptosis, a process normally occurring after NF- $\kappa$ B activation during the negative selection in the thymus [400, 401]. All these studies strongly support the critical role of Twist1 inflammatory response and its importance in pathological conditions associated with deregulation.

### **3 Twist proteins as oncogenes and their roles during tumorigenesis**

In addition to Twist's essential role during embryogenesis and in adulthood, Twist proteins were found to be expressed and associated with many types of aggressive tumours. Notably, Twist up-regulation was detected in different malignancies such as: breast cancer [402], hepatocellular carcinoma [403, 404], prostate cancer [405, 406], ovarian cancer [407] gastric cancer [408, 409], oesophageal squamous cell carcinoma [406, 410], bladder cancer [411, 412], pancreatic cancer [413] or neuroblastoma [414]. Interestingly, Twist expression is not only restricted to carcinomas but also occurs in haematological malignancies such as: myeloid malignancies [393, 396] or T-cell lymphomas [415]. Additionally, Twist expression is often associated with poorer prognosis and increased invasiveness of tumours (reviewed in [416]). Thus, Twist role in tumour development and progression has become an intense area of research. The following paragraphs will describe in detail why Twist factors are now being considered as major oncogenes.

### 3.1 Twist reactivation during oncogenesis

As already discussed, Twist proteins play crucial roles during development; however in most healthy adult tissues, they are usually silenced and only re-expressed during tumour development.

Twist reactivation can be triggered by a variety of stimuli. However, it seems to mostly depend on the deregulation of signalling pathways active during embryonic development. Several important examples will be mentioned below. First of all, the Notch1/STAT3 signalling axis, well known for its involvement in cancer [417] was demonstrated to induce Twist and thus promote cancer progression in breast cancer [417], hepatocellular carcinoma (HCC) [418] and in human gastric cancer [419]. NF- $\kappa$ B promoted phosphorylation increases Twist expression in breast and ovarian cancers [420, 421]. The nuclear protein HMGA2 (High Mobility Group A2) induces Twist expression by binding to A:T rich sequences of its promoter and thus promotes epithelial to mesenchymal transition (EMT) [422]. Additionally, similar mechanisms of Twist induction were observed by DLX4 (distal-less homeobox protein 4) [423], by Src1 (steroid receptor coactivator-1) [424] or N-Myc and c-Myc [425].

Interestingly, several models of Twist reactivation during oncogenesis are associated with hypoxia. An initial connection was made upon identification of a hypoxia responsive elements (HRE) on the Twist promoter. It was later demonstrated that HIF1 $\alpha$  upregulates Twist expression. Further studies demonstrated that HIF1 $\alpha$  cooperates with Twist to promote metastasis in response to intra-tumoral hypoxia [426]. Another report suggested that HIF2 $\alpha$  might be also responsible for Twist expression and promote cancer metastasis as a response to hypoxia [427]. Of course, aforementioned examples did not cover all the cases of Twist induction during cancer but they additionally prove that various pleiotropic signals may induce Twist expression (Figure 24). More detailed lists could be found in the following reviews [416, 428].

Of note, Twist activity during oncogenesis could be also negatively regulated by several factors such as frizzled-related proteins (sFRP1 and sFRP2) [429], bone morphogenetic protein 7 (BMP7) [430], Claudins 4 or 5 [431, 432].

Finally, post-translational modifications mentioned before, such as phosphorylation at Ser42 or Ser68 mediated by PKB/Akt1 or MAPK respectively, may stabilize Twist proteins and promote their oncogenic activities [321, 322]. Additionally, Twist mRNA translation, and thus its protein level, can be regulated by YB-1, a DNA/RNA-binding protein that promotes the translation of cap-independent mRNA transcripts [433].

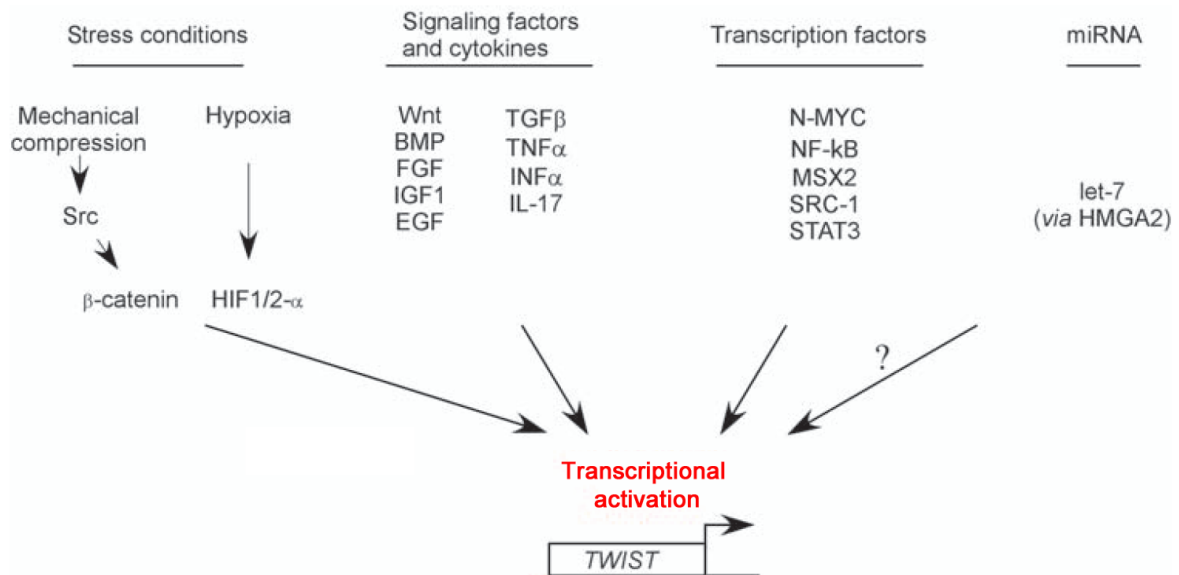


Figure 24. Regulation of Twist expression (adapted from [416]).

## 3.2 Inhibition of tumour-suppressive programs by Twist

### 3.2.1 Twist mediated cell death protection and cancer

One of the hallmarks of cancer is evasion of cell death. Interestingly, one of the Twist oncogenic properties is its ability to override apoptosis, a natural barrier against pre-malignant lesions.

First of all, Twist anti-apoptotic potential is linked to p53 signalling. Indeed, Myc oncoproteins are known to act both as growth, proliferation and apoptosis promoters. Remarkably, c-Myc overexpression followed by growth factors deprivation in rat fibroblast induces p53/ARF-dependant apoptosis [62, 434]. The first demonstration of Twist oncogenic potential was described in the same cellular context. The study published by the group of G. Hannon in 1999 [352] showed that expression of Twist1 or



Twist2 protects rat fibroblast from c-Myc induced apoptosis by repressing the ARF tumour suppressor. This suggested that Twist may affect p53 activity indirectly through a modulation of the ARF/MDM2/p53 pathway (Figure 25) [352]. In 2004 the group of Alain Puisieux reported an oncogenic cooperation between N-Myc and Twist1 in neuroblastoma. As in the fibroblasts expressing c-Myc, in this cancer model N-Myc induces both cell proliferation and cell death. Co-expression of Twist inhibits the N-myc induced apoptotic response. Consequently, this cooperation allows cells to override this cellular failsafe program, thus favouring tumour progression [414]. Importantly, inhibition of the p53 signalling pathway seems to occur at different levels. Besides its ability to downregulate p14/ARF, Twist can inhibit p53 activity through a direct interaction. Initial pull-down assays suggested that Twist binds p53 and in so doing inhibits its ability to bind DNA [435]. A more detailed study revealed that Twist interacts with p53 via its C-terminal region, called the Twist-box. This interaction hinders key posttranslational modifications of p53 and facilitates its MDM2-mediated degradation [334] (Figure 25). Finally, Twist may also inhibit p53 transcriptional activity in another indirect manner, through the inhibition of p300-mediated acetylation [330].

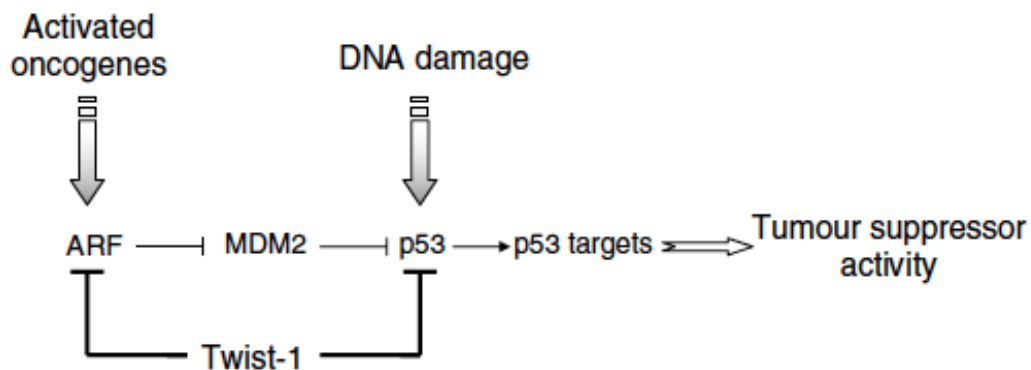
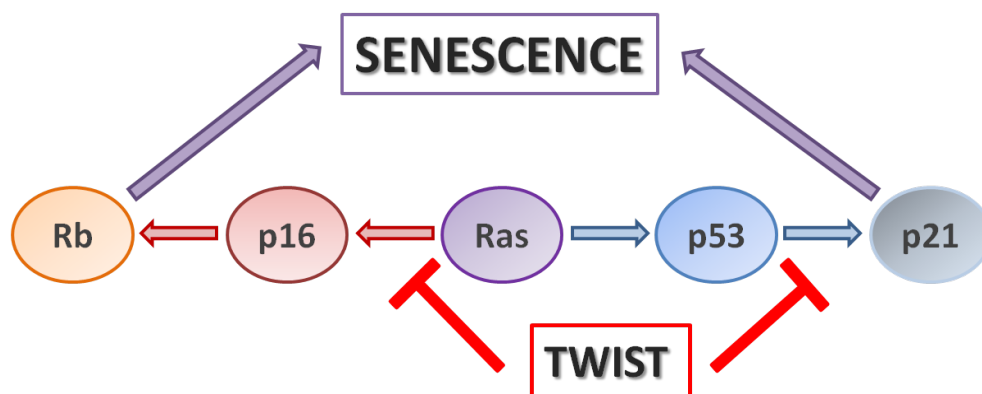


Figure 25. Mechanisms of Twist mediated p53 inhibition [317].

### 3.2.2 Twist as an inhibitor of oncogene induced senescence

Apoptosis is not the only fail-safe program in which Twist is involved. Indeed, years of research accumulated evidence establishing a role of Twist as an inhibitor of senescence. The initial connection was proposed in 2007 by the group of X. Wang, when they showed that Twist expression in non-malignant human epithelial cells protected

them from DNA damage induced senescence [436]. Indeed, treatment of these cells with genotoxic drugs, such as hydrogen peroxide or cisplatin, induces senescence due to accumulation of DNA damage. However, senescence could be suppressed through Twist overexpression. It was proposed that the senescence escape could be due to the inhibition of p14/ARF-mediated (mouse homolog - p19/ARF) MDM2/p53 and Chk1/2 DNA damage response pathways [436]. The involvement of Twist in inhibition of oncogene-induced senescence was confirmed by Stephane Ansieau and collaborators in 2008 [437]. Specifically, they showed that spontaneous mammary tumours in MMTV-ErbB2/Neu transgenic mice displayed increased levels of Twist2. Silencing of Twist by shRNA triggered cellular senescence characterized by flattened cell shape, G1 growth arrest and SA  $\beta$ -gal activity. Interestingly, these results were in agreement with the fact that aberrant activation of ErbB2 in mammary epithelial cells triggers a premature senescence response [438]. These observations were extended to human melanoma and breast cancer cell lines, where silencing of Twist1 and/or Twist2 resulted in induction of senescence. Finally, the authors performed oncogenic cooperation assays to confirm the ability of Twist1 and Twist2 to overcome oncogene-induced senescence. They found that while expression of ErbB2 in primary mammary epithelial cells or of activated form of H-Ras - H-RasV12 in primary mouse or human fibroblasts resulted in oncogene induced senescence, this phenotype was abolished by co-expression of Twist1 or Twist2. Furthermore, this study also provided a mechanistic explanation of Twist action. Senescence escape was mediated through inhibition of two pathways involved in the cell cycle control - p53 and RB (Figure 26). More precisely, Twist proteins repress transcription of two cyclin-dependent kinase inhibitors - p21<sup>CIP/WAF1</sup> and p16<sup>INK4A</sup> [437].



**Figure 26. Twist1 as a potent inhibitor of p53 and Ras pathway.**

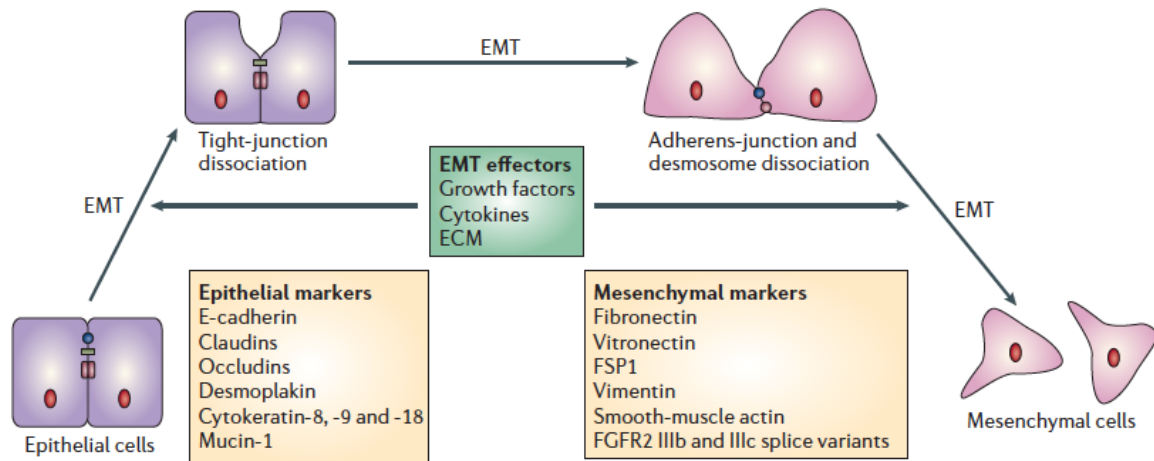
Additionally, this work established a link between EMT and failsafe programs, originally described as independent [439]. Cooperation assays in epithelial cells showed that both Twist proteins abrogate ErbB2- or RasV12-induced senescence and concomitantly cooperate with these mitogenic oncoproteins to promote EMT [437].

Later, this discovery was further validated in several *in vivo* models. A study in a pancreatic cancer model demonstrated that inflammatory stress can provoke a p16<sup>INK4A</sup>-dependent senescence of pancreatic ductal cells. It also showed that this response is abrogated by oncogenic K-Ras through the upregulation of Twist, which leads to a suppression of p16<sup>INK4A</sup> expression [440]. Two additional studies confirmed that Twist1 cooperates with K-RasG12D for acceleration of lung tumorigenesis by inhibition of cellular senescence programs and, as a consequence, progression from benign adenomas to adenocarcinomas [441, 442]. However, another study carried out on human non-small cell lung cancer (NSCLC) suggested that senescence occurs independently of either p53 or p16 mutational status. Furthermore, it does not require the previously defined mediators of senescence, namely p21 and p27 [442]. Finally, a recent study suggested that Twist expression in cancer-associated fibroblasts might inhibit senescence, which may have an impact on tumour-supporting features of the cancer microenvironment [443]. Taken together, these data suggest that Twist induces malignant conversion by inhibiting different failsafe programs.

### **3.3 Twist as a regulator of epithelial to mesenchymal transition**

The epithelial-mesenchymal transition (EMT) plays a crucial role in the formation of the body plan and in differentiation of multiple tissues and organs. EMT also contributes to tissue repair, but it can also cause organ fibrosis and promote carcinoma progression. EMT endows cells with migratory and invasive properties as well as resistance to programmed cell death; moreover it has been described to induce stem cell properties. Thus, EMT is fundamental for both embryonic development and for carcinogenesis. It promotes major phenotypic changes that include loss of cell-cell adhesion, loss of cell polarity and the acquisition of migratory and invasive properties and possibly acquisition of a cancer stem cell phenotype. EMT is characterized by dramatic

morphological changes associated with the down-regulation of epithelial markers, such as E-cadherin, and the expression of mesenchymal markers, such as vimentin, fibronectin or N-cadherin (reviewed in [444]). Following these changes, initially immobile and polarized epithelial cells undergo morphological changes and acquire motility properties. A mechanism of EMT process is summarized in Figure 27 below.



**Figure 27. Mechanism of EMT (adapted from [445]).**

Twist as an embryonic factor is involved in mesoderm formation and was considered among other transcription activators such as Snail and Slug as a promoter of the EMT process. However, the first report describing its involvement in EMT followed by acquisition of metastatic properties during tumorigenesis was only published in 2004 by group of Robert Weinberg [402]. In this study researchers used an established a model of metastasis using 4 murine mammary cancers cell lines of common origin [446, 447]. These cell lines differ in their metastatic progression capabilities. Yang *et al.* showed that expression of Twist is essential for metastasis to the lung of a tumour originating from the mammary gland. Moreover, Twist expression also favoured cancer by increasing the number of circulating cancer cell in the blood flow. Finally, Twist was identified as an EMT inducer, since its expression was sufficient to suppress expression of E-cadherin and to up-regulate mesenchymal markers such as fibronectin or vimentin in HMEC cells and in consequence promote migration [402]. Over the following years numerous reports confirmed Twist's involvement in acquisition of metastatic and invasive properties with the concomitant down-regulation of the E-cadherin expression

[405, 426, 448, 449]. Moreover, our observations suggest also that the 4 cell lines that have been isolated seem to have diverged from the original clones. As a consequence, these cells used by different labs, while maintaining the different invasive phenotypes that were originally described, differ both in their epithelial versus mesenchymal phenotype and in the pattern of Twist expression. This further suggests that the metastatic phenotype in this model is controlled in a rather more complex manner than originally described by Yang *et al.* (unpublished observations).

Interestingly, Jing Yang and Robert A. Weinberg suggested that Twist repression of E-cadherin is indirect and the mechanism of its downregulation is more complicated than that triggered by other EMT inducers, such as Snail or Zeb, which can repress its expression by a direct binding to its promoter [450]. A new light on Twist-mediated EMT, as well as the stem cell phenotype, was shed in 2010 [451], when an interaction of Twist with Bmi1, a member of polycomb-repressive complex 1 (PRC1) maintaining chromatin silencing, was described. Bmi1 is involved in the self-renewal of cells through repression of the INK4A-ARF locus [452] and its overexpression is frequently observed in tumour-initiating cells [248, 453]. This study demonstrated that Twist1 is able to induce BMI1 expression during tumorigenesis. Moreover, Twist and BMI1 interact with each other and mediate promoter repression of E-cadherin in a PRC-dependent fashion [451]. Finally, cooperation of Twist and BMI1 through induction of EMT and repression of the p16<sup>INK4a</sup> locus promotes tumour-initiating phenotype in cancer cells. Interestingly, another study carried out by group of Jing Yang suggested that Twist mediated E-cadherin repression occurs through the induction of Snail2 expression [454]. These researchers claim that Snail2 silencing attenuates the expression of mesenchymal markers and suppresses E-cadherin repression. Overall, all those studies strongly suggest the involvement of Twist in EMT process itself and the associated increase in cell plasticity and metastasis potential. Until now, a large number of studies confirmed the role of Twist in different types of carcinomas. However, Twist-mediated EMT mechanism is still not clear, as different studies tend to propose different models of Twist mediated E-cadherin repression. In addition, new EMT markers governed by Twist are currently being discovered. For example, Twist in cooperation with AP2 up-regulates the mesenchymal marker integrin  $\alpha 5$ , which is associated with EMT and tumour invasiveness [455]. Finally, a recent study demonstrated that increased matrix

stiffness in the tumour microenvironment directly activates EMT, tumour invasion, and metastasis through Twist1. Mechanistically, increasing matrix stiffness induces integrin-dependent phosphorylation events and releases Twist1 from its cytoplasmic anchor G3BP2, which allows it to translocate into the nucleus and drive transcription [456].

### 3.3.1 Twist, EMT and metastasis

The current state of knowledge allows a relative consensus in the research community accepting that classical EMT-associated features are strongly involved in the three major steps of malignant cancer progression: invasion, dissemination and metastasis [457, 458]. Initially, the increased invasive phenotype controlled by Twist was mainly connected to its ability to induce EMT. However, it is now clear that, the metastatic traits triggered by Twist are not solely mediated through the EMT pathway. Indeed, Twist was described as a transcriptional repressor of TIMP1 an inhibitor of matrix metalloproteinases (MMPs). Thus Twist promotes the activity of MMPs and leads to tumour microenvironment remodelling, favouring invasion [459]. Moreover, Twist was described as an indirect transcriptional regulator of MMP2 [408] and direct regulator of MMP1 [460]. Importantly, in breast cancer Twist was associated with invadopodia formation, a specialized membrane protrusions associated with cell motility and extracellular matrix degradation [461]. Notably, PDGFR $\alpha$  was identified as a direct transcriptional target of Twist, which in turn activates Src, to promote invadopodia formation. Consequently, Twist promotion of invadopodia-mediated matrix degradation increases cancer metastatic ability in breast cancer [461]. Furthermore, Twist mediated invasion can also occur through regulation of miRNA expression. The group of Robert Weinberg demonstrated Twist1 involvement in miR-10b signalling pathway [462]. Combination of the *in vitro* and *in vivo* studies revealed that Twist directly promotes miR-10b expression and in consequence inhibits the synthesis of the homeobox D10 (HOXD10) protein [462], known for its abilities to impair migration and invasion [463]. Downregulation of HOXD10 leads to the expression of RhoC, an important player of the metastasis process [462, 464, 465]. Overall, this study suggested an EMT independent Twist1-induced migration and invasion at least in breast cancer [462]. Another example describes Twist as a mediator of mesenchymal-mode movement through Rac1 (Ras-related C3 botulinum toxin substrate 1) activation [466]. Twist in cooperation with

BMI1 represses the expression of the *let-7i* miRNA. In turn, repression of *let-7i* was found to drive activation of Rac1 protein, which belongs to Rho family of GTPases and was found to regulate cell-cell adhesion and motility [467]. This suggest that activation of Rac1 by Twist enables a mesenchymal-mode movement in three-dimensional environments that promotes tumour invasiveness in *in vivo* studies and in head and neck cancer patients [466].

Metastasis is responsible for more than 90% of cancer-associated mortality. The mechanisms of metastasis are complex, but several crucial steps can be distinguished. First of all, a primary tumour must invade adjacent areas, then disseminate through blood vessels and finally seed at distant sites, eventually giving rise to macrometastases. Importantly, it was recently suggested that de-differentiation through EMT-inducers strongly enhances cancer cell motility and dissemination capabilities [444]. Moreover, gene expression patterns in human cancers indicated that de-differentiated cancer cells combine EMT with a stem-cell like phenotype, leading to the concept of “migrating cancer stem cells” as the basis of metastasis [468] (discussed later). However, metastases of the most common human cancers (well- to moderately-differentiated carcinomas) often show a re-differentiation through a mesenchymal-epithelial (re-)transition (MET). Consequently, transient dedifferentiation (EMT) and re-differentiation (MET) processes were proposed to be the driving force of metastasis [469]. Additionally, invasive and de-differentiated cells were shown to be quiescent, while cells in metastatic sites were found to become re-differentiated and proliferative. Overall, these observations suggest that EMT must be reversed in order to allow growth and colonization [469]. Recently, an elegant study about the role of Twist in metastasis in squamous cell carcinoma supported the role of an EMT in dissemination and the need of a MET for efficient metastasis [470] (Figure 28).

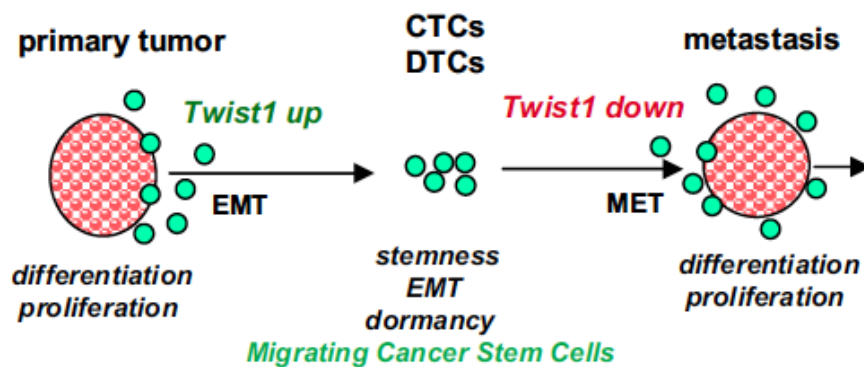


Figure 28. EMT, MET and during tumour progression [471].

The mouse model used in this study employs an inducible Twist1 under the control of doxycycline responsive promoter. After tumour induction, Twist activation was triggered through oral application of doxycycline and resulted in Twist expression in all cancer cells, irrespective of their localization (primary tumour, circulating or disseminated tumour cells, or metastasis), therefore modelling ‘an ‘irreversible’ Twist1/EMT activation. In contrast, topical application of doxycycline only induced Twist1 in the primary skin tumours, and allowed a transient Twist1 expression in disseminated tumour cells (“reversible” Twist1/EMT activation). Notably, both models were characterized by increased number of circulating cancer cells followed by higher number of extravasated cancer cells in the lung, as compared to control mice. However, the number of metastases was higher in mice with transient induction of Twist. Moreover, silencing of Twist in metastasis was associated with proliferation and reversion of EMT-associated growth inhibition. Overall, this study demonstrated the importance of EMT mediated dissemination of tumour cells, but also of the subsequent MET for colonization and macrometastasis formation [470].

### 3.3.2 Conclusions

Overall, Twist involvement in EMT induction is well demonstrated, as Twist expression was found to favour EMT in different carcinomas. However, Twist regulation of E-cadherin is not well understood and may vary depending on the cellular model and may be dependent on available oncogenes for cooperation. Moreover, Twist mediates tumour dissemination, promoting invasion and metastasis via EMT but also via other



pathways. Interestingly, a recent study suggests that Twist expression and/or activity may need to be silenced in order to increase the appearance of macrometastases via the MET process.

Finally, Twist induced EMT was linked to an acquisition of cancer stem cell like properties by tumour cells and this issue will be discussed in the next paragraphs. The following Figure 29 summarizes the function of Twist in the EMT process.

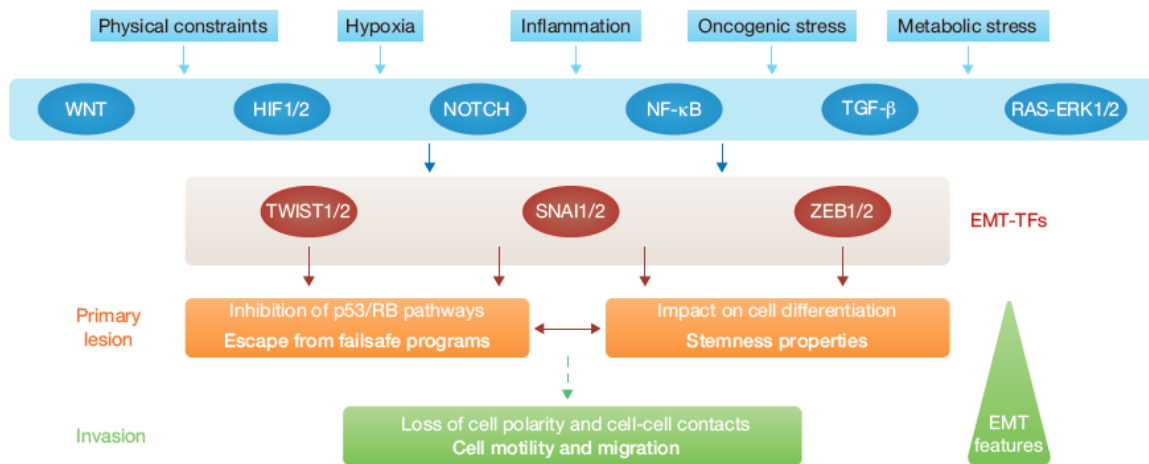


Figure 29. Overview of EMT pathways [472].

### 3.4 Twist and cancer stem cells

As mentioned before, Twist expression was recently associated with the acquisition of the cancer stem cell phenotype. Interestingly, this biological function of Twist may be dependent on its ability to induce EMT. In 2008 two reports came out suggesting that EMT endows normal and transformed mammary epithelial cells with stem-like properties, including self-renewal capabilities [473, 474]. Interestingly, both research groups found that Twist proteins were expressed in a population of breast cancer stem cells characterized by CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. Furthermore, ectopic expression of Twist in immortalized, differentiated mammary epithelial cells led to an enrichment of CD44<sup>+</sup>/CD24<sup>-</sup> cells [473]. Overall, these reports suggested that Twist expression in the course of the EMT concomitantly promoted the acquisition of stem cells properties, since transformed human mammary epithelial cells that underwent EMT formed mammospheres, soft agar colonies, and were more efficient in initiating *in vivo*

tumorigenesis. Moreover, co-expression of Twist and activated Ras in the mammary gland results in generation of breast tumours with characteristic features of human claudin-low breast cancers, a subtype of breast cancers that have stemness features [475]. A mechanistic explanation of Twist mediated stemness acquisition during cancer progression is linked to the interaction between BMI1 and Twist. Moreover, Twist overexpression activates  $\beta$ -catenin and Akt pathways, resulting in higher expression of a CSC marker - CD44 in cancer cells [476]. On the other hand, recent studies showed that the relationship between EMT and stemness is more complex than it was previously thought. As mentioned before, Rac1-mediated induction of the motile stem-like cancer cell phenotype already casted doubt on this relationship [466]. The EMT activator PRRX1 cooperates with Twist1 in induction of all EMT features relevant to cancer cell dissemination. Surprisingly, PRRX1 was shown to also suppress stemness properties during the EMT phase and had to be downregulated, both for their reactivation and for the metastatic colonization following intravasation into distant organs [477, 478]. These findings identify a distinct mechanism of metastatic colonization that uncouples stemness from EMT and growth arrest. Recently, an elegant *in vivo* study demonstrated that Twist expression is required for tumour initiation and progression in a dose dependent manner. Remarkably, the authors demonstrated that Twist1 confers cancer stem cell properties during tumour initiation independently of its role in EMT during tumour invasion. More precisely, Twist1-depleted cells from squamous cell carcinoma (SCC) or papilloma showed drastically reduced abilities to form secondary tumours. Additionally, papilloma cells with Twist1 deletion exhibit unaffected expression of EMT markers, thus uncoupling EMT from stemness in these models [479]. Mechanistically, it was found that Twist directly or indirectly regulates the expression of Nrp1, a VEGF co-receptor that promotes tumour stemness and proliferation in skin tumours [480, 481], but also of Phgdh, an enzyme overexpressed in tumour cells and essential for cancer cell proliferation [482, 483]. Interestingly, Twist mediated cell proliferation and tumour initiation occurs in a p53-independent manner [479]. Overall, despite an initial link between EMT and stemness properties, recent studies suggested that Twist induction of EMT and stemness traits can be separated. Moreover, recent findings highlighted the need for functional studies to identify precise mechanisms of Twist

activities. Furthermore, questions also should be asked to address the differences found between long-term versus transient Twist expression.

### **3.5 Twist and hypoxia**

Hypoxia and mechanisms involved in the response to hypoxic stress will be discussed in more detail in the next chapter. Because of that, precise mechanisms of hypoxic stress and their regulation will not be discussed in this part except for the direct interplay between Twist and HIF proteins.

HIF genes activation is a critical response to micro-environmental conditions during tumour progression. It regulates the expression of multiple genes participating in tumour growth, angiogenesis, EMT and metastasis. Interestingly, concomitant expression of HIF1 $\alpha$  and Twist during mouse development has been shown to play a role in the development of specific neural populations [484]. Furthermore, HIF1 $\alpha$  has been described as an inducer of Twist expression during tumour associated hypoxic stress [426]. This occurs through a direct binding of HIF1 $\alpha$  to HRE (hypoxia responsive element) on Twist promoter. The fact that Twist expression under hypoxic conditions promotes EMT and metastasis provides an insight into cancer biology, suggesting that in response to intra-tumoral hypoxia, Twist through its pro-invasive activity promotes cancer cells migration to a less hostile environment [426]. Another report demonstrated that Twist expression could also be modulated by HIF2 $\alpha$  [427]. Regulation of Twist expression by HIF2 $\alpha$  suggests a temporal continuity of Twist expression as HIF1 $\alpha$  activity during hypoxia decrease overtime and HIF2 $\alpha$  conversely increases [485].

### **3.6 Twist and angiogenesis**

Angiogenesis defines the process of creation of new vascular vessels. Its involvement in cancer progression has been largely studied as its inhibition can provide a reasonable way to shut down nutrition support and is considered to be a potential mechanism of cancer eradication. In breast cancer, Twist was shown to increase vascular volume and permeability [417, 449]. Twist overexpression has also been shown to promote lymph angiogenesis probably through the induction of VEGF-C and VEGFR-3 [486, 487]. In addition, in hepatocellular carcinoma and breast cancer, Twist overexpression was

correlated with induced angiogenesis and metastasis [488, 489]. Recently, Twist was presented as a factor able to induce vasculogenic mimicry [490, 491], which is an alternative blood supply mechanism in malignant tumours. Finally, Twist mediated angiogenesis can occur through macrophages recruitment, as Twist was shown to induce macrophage chemo-attractant CCL2 [492, 493].

### **3.7 Twist and chemoresistance**

Chemoresistance is a major limitation for a successful treatment of all types of cancers. Therefore, identification of molecular mechanisms associated with chemoresistance is a critical step toward preventing recurrent disease, the clinical outcome associated with the resistance of cancer cells to chemotherapy [494]. A number of emerging evidences suggest that Twist1 plays an important role in chemoresistance of different cancers. First of all, Twist was found to promote resistance to taxol, which is effective against a broad range of carcinomas, such as nasopharyngeal, bladder, ovarian and prostate cancer. For example, in the nasopharyngeal carcinoma cell line HNE1-T3, high expression of Twist was associated with acquisition of chemoresistance to this drug [495] through transcriptional activation of Akt2 [496]. In cisplatin and doxorubicin resistant cells, Twist mediated chemoresistance occurred through an up-regulation of its downstream gene YB-1 [497]. Another recent report suggests that Twist mediates cisplatin resistance via regulation of miR-186, at least in ovarian cancer [498]. Resistance to cisplatin could be also mediated by Twist via the AMPK-activated mTOR pathway in NSCLC cells [499]. In the case of anthracycline chemoresistance observed in bladder cancer, Twist was shown to activate the P-glycoprotein expression, known for its chemoresistant properties [500]. Interestingly, Twist mediated drug resistance has been associated also with increased expression of several ATP binding cassette (ABC) transporters. Indeed, Twist can stimulate the expression of ABC transporters through direct binding to their promoters [501]. Finally, Twist is also involved in NF- $\kappa$ B-mediated chemoresistance, where it can, as a downstream target of NF- $\kappa$ B protein, mediate chemoresistance through suppression of inhibitory phosphorylation of BCL-2 [502].

### **3.8 Summary of Twist oncogenic activities**

The examples presented in this chapter of Twist proteins involvement during the various processes of tumorigenesis highlight them as important players of cancer development. Twist activities are exhibited at different stages of tumour progression. Inactivation of tumour suppressive programs occurs early on, as their inhibition promotes cells survival. The most studied aspect of Twist oncogenic activities is the control of the EMT process. The exclusive group of EMT inducers, to which Twist belongs, makes them the key players of the most destructive stage of tumour progression – metastasis. Interestingly, recent reports suggest that Twist expression is needed for only a part of the metastatic process, namely for tumour dissemination, and must be silenced later to allow macrometastases to form. Another intensively studied aspect of Twist oncogenic properties is Twist mediated stemness. Twist was described as a factor promoting tumour-initiating phenotype and despite the initial connection to EMT it is now becoming clear that CSC properties and EMT are induced through independent mechanisms. Importantly, Twist could also mediate escape of cells from the hypoxic environment as HIF1 $\alpha$  can induce its expression. Furthermore, Twist abilities to promote angiogenesis could also favour cancer progression. Finally, Twist expression is often associated with poor survival prognosis, as it is associated with chemoresistance to several drugs. In conclusion, Twist proteins, beside their important roles during embryogenesis and adulthood, are currently recognized as major oncogenes with a broad spectrum of oncogenic abilities.

## CHAPTER III. Cellular stress

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Stress can be broadly defined as an impact of internal or external stimuli (physical, chemical or biological) that perturbs cellular homeostasis and triggers processes leading to its restoration of (reviewed in [503]). Homeostasis is a state of equilibrium between the net rate growth and the net rate of cell death [504]. This fine balance can be easily disturbed by exposition to stressful environmental stimuli and an appropriate cellular response should lead to a return to a homeostatic equilibrium. This cellular reaction depends not only on the type of stress but also on its severity. Thus, cells possess an intrinsic potential to "judge" the risk and respond adequately. More precisely, if the stressful impact does not exceed a certain threshold, cells should be able to handle it by activation of appropriate protective mechanisms that will result in cell survival. On the other hand, if the stressful agent is too strong or exposition to it is too long cells may fail to activate or to maintain protective responses but rather may activate signalling cascades that eventually fuel cell death pathways ([505, 506], reviewed in [507]). Unsuccessful response to stress thus leads to activation of various cell death mechanisms, such as apoptosis, necrosis, pyroptosis, or autophagic cell death.

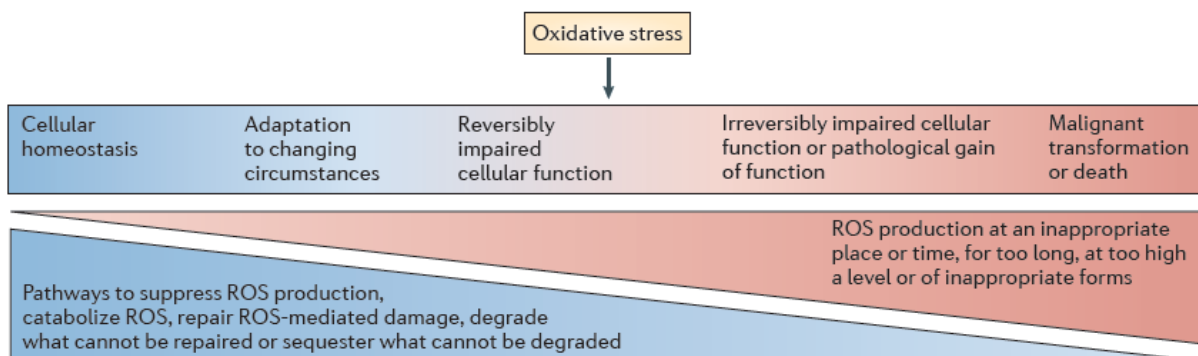
All cells in unicellular or multicellular organisms are exposed to different stress stimuli. Cancer cells survive and grow in a continuously changing environment and must adapt to multiple stresses. For example, most solid tumours are poorly vascularized, which puts cancer cells in hostile conditions, such as low oxygen supply, nutrient deprivation, and pH changes. These conditions lead to adaptive responses to counteract this harsh environment and in the long run result in selection of a population of cancer cells capable of surviving and adapting to the continuous challenge from the ever-evolving microenvironment. Such adaptations can give rise to the acquisition of an aggressive phenotype, characterized by increased invasiveness and general resistance to stresses, including those caused by chemo- and radiotherapy (reviewed in [507]).

Cells during their lifetime usually experience different types of stresses such as heat shock or oxidative stress. Importantly exposition to any of them activates a distinct type of action generally called cellular stress response. These are complex and versatile signalling processes, however some of the patterns are common between different

species e.g. antioxidant response and heat shock protein response, which occur in lower organisms as well as mammals [507]. For the needs of this thesis, I will focus only on two types of stress and their appropriate responses – oxidative and hypoxic stress that are key topics of my research.

## 1 Reactive oxygen species and response to oxidative stress

Reactive oxygen species (ROS) are molecules characterized by the presence of one or more unpaired electrons, giving rise to their high reactivity. History of free radicals began in 1954, when it was suggested that partially reduced forms of oxygen are toxic [508]. From that time on, our knowledge about ROS has been very much extended and clarified. Cellular effects of ROS are not all deleterious, indeed, they are beneficial in maintaining homeostasis, but deleterious as a cause of oxidative stress [509]. Of note, ROS are constantly produced by all living organisms. In mammals their main source is the mitochondria. As their overproduction is harmful for living cells, their level is controlled by a class of proteins, called ROS scavengers. As already mentioned, ROS play important roles during physiological processes, such as immune or mitogenic responses [509]. On the other hand, excessive ROS production causes oxidative stress and thus leads to various intracellular damages, such as oxidation of cellular lipids, proteins and DNA [510]. Adaptations to oxidative stress coupled with its genotoxic effect have an impact on cancer progression and usually favour an aggressive phenotype. General outlook of oxidative stress and its effects is presented in Figure 30.



**Figure 30. ROS production and catabolism as well as cellular adaptation impact the broad range of ROS signalling [511].**

In this chapter we will provide a brief description of the most important features of ROS biology, mechanisms of oxidative stress and their effects under physiological and pathological conditions with an emphasis on their role during tumorigenesis.

### **1.1 Reactive oxygen species – definition and classification**

Reactive oxygen species belong to the free radicals family, which defines them as molecules or molecular fragments containing at least one unpaired [512]. Such configuration makes them highly reactive with other molecular species. In living organisms, ROS and reactive nitrogen species are the most important classes of free radicals.

A broad range of reactive oxygen molecules are classified as ROS (Figure 31). Physiologically, the most important family member is the superoxide anion ( $O_2^{\bullet-}$ ). This radical is generated through the addition of an electron to the molecular oxygen ( $O_2$ , also called dioxygen). Superoxide anion is produced through different metabolic processes and is considered as a “primary” ROS, which could later through different reactions give rise to “secondary” ROS [513]. Additionally, production of superoxide occurs almost entirely within mitochondria [514].

Another important player in ROS biology is the hydroxyl radical,  $\bullet OH$ , which is a neutral form of the hydroxide ion. Combination of its high reactive potential with a very short *in vivo* half-life (around  $10^{-9}$  s) restricts the possible damage to the places close to its formation [515]. Production of hydroxyl radicals is largely linked to the redox states of iron and copper.

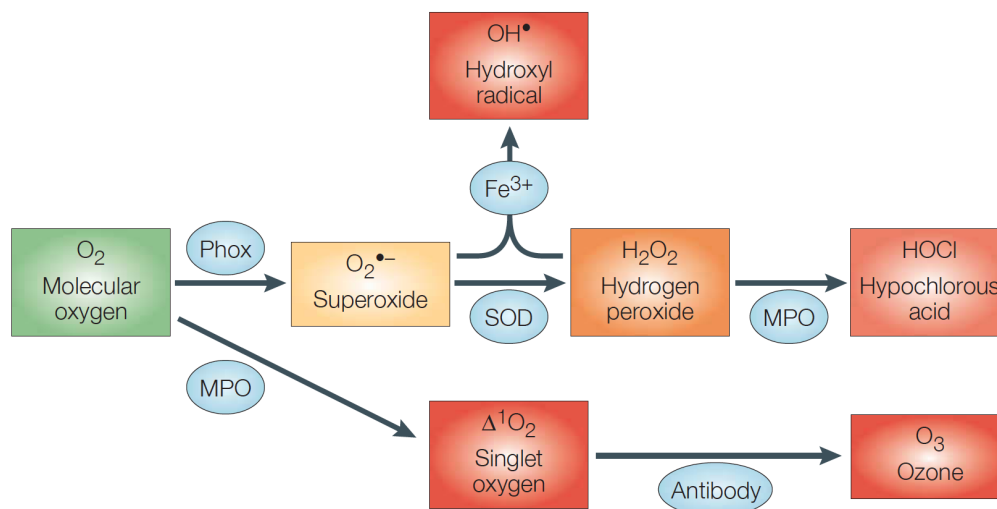
Peroxisomes are one of the major places of oxygen consumption. Their activity is connected to production of hydrogen peroxide ( $H_2O_2$ ), which is not a free radical per se, but is chemically more active than molecular oxygen and because of that, is included in the ROS category. Of note, over-production of  $H_2O_2$  can be easily extinguished by catalase [516].

Additional class of oxygen free radicals that are created in living organisms are peroxy radicals ( $ROO\bullet$ , where R correspond to a carbonic residue). The simplest form of



peroxyradical is  $\text{HOO}^\bullet$ , which is the protonated form of superoxide ( $\text{O}_2^{\bullet-}$ ) and is called either hydroperoxyl radical or perhydroxyl radical [517]. Physiologically, this type of radical is involved in fatty acid peroxidation [518].

Of note, the few examples of oxygen species presented above do not exhaust all known radicals but represent the most studied and biologically important ones. Additional non-described members of the ROS family are particles such as alkoxy radical ( $\text{RO}^\bullet$ ), hypochlorous acid ( $\text{HClO}$ ), singlet oxygen ( $^1\text{O}_2$ ) or ozone ( $\text{O}_3$ ). As mentioned before, the group of free radicals consists not only of oxygen particles but also of particles based on other active atoms such as nitrogen (nitric oxide, peroxyxynitrite), carbon or sulphur [519–521].



**Figure 31. Different types of reactive oxygen species [522].**

Superoxide anion, which is generated by mitochondria or NOX enzymes family, can be converted by the superoxide dismutase (SOD) to  $\text{H}_2\text{O}_2$ . In the presence of iron, hydrogen peroxide or superoxide radical can be transformed into a hydroxyl radical. In inflamed areas, hypochlorous acid is formed from  $\text{H}_2\text{O}_2$  and chloride by a phagocyte enzyme - myeloperoxidase (MPO). The same enzyme is involved in the production of singlet oxygen, which is formed from an oxygen particle in inflamed areas. Finally, ozone molecules can be converted to singlet oxygen by an antibody molecule ([523], reviewed in [522]).

## 1.2 Biology of ROS

As mentioned above, ROS play important roles during physiological processes occurring in all aerobic organisms. However, because of their possible deleterious effects, their production is strictly regulated. The regulation is mainly exercised by a group of enzymes that tightly control ROS production. It would appear that all living organisms also possess a second line of defence against overproduction of ROS that consists in a specific class of enzymes or compounds that scavenge free radicals. The following paragraphs will first describe the sources of ROS production and then the defence mechanisms against the excess of ROS.

### 1.2.1 Endogenous sources of ROS production

Different cellular organelles participate to endogenous ROS production (Figure 32). The main production of oxygen free radicals occurs in the mitochondria. Additionally, ROS generation also takes place in other organelles, such as endoplasmic reticulum (ER, particularly during ER stress) or peroxisomes (during metabolism of long-chain fatty acids). Finally, various cytoplasmic enzymes with oxidase or oxygenase activity can promote ROS generation during their physiological activity. The following sections will briefly describe the most important sources of ROS production.

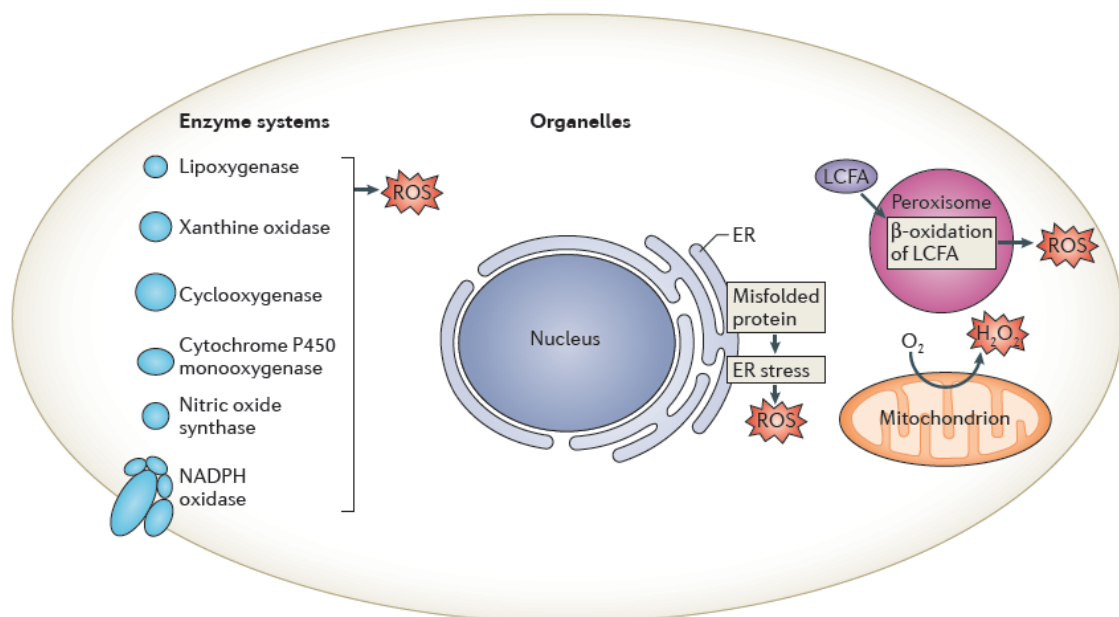
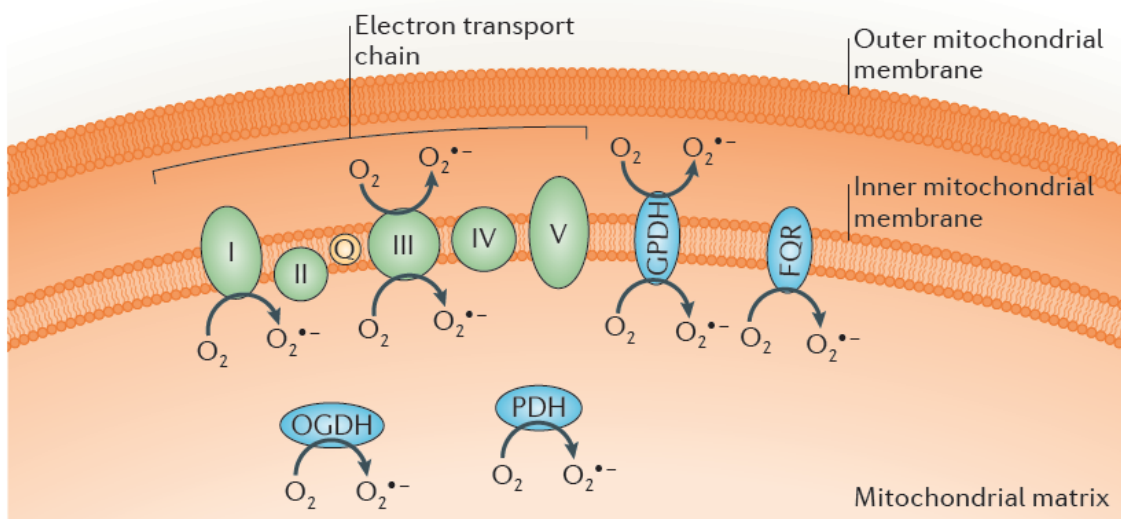


Figure 32. Intracellular sources of reactive oxygen species [524].

### 1.2.1.1 Mitochondria

Mitochondria are the main energy supplier in different cell types. ATP production through oxidative phosphorylation is strictly connected with changes in oxidation states; mitochondria are massive ROS producers. Basically, this process consists of electrons flow through the respiratory chain that finally results in reduction of oxygen particles to water and generation of a proton transmembrane potential. During this process, molecular oxygen can also undergo a one-electron reduction and generate a superoxide anion [525]. Experiments on isolated mitochondria demonstrated that the main source of oxidative particles are complex I and III of the electron transport chain (Figure 33) [526].



**Figure 33. Intracellular ROS generation inside the mitochondria [524].**

Several actors of the electron transport chain participate in the superoxide anion ( $O_2^{\bullet -}$ ) production, such as complex I and complex III (see text for details). Beside these main contributors of ROS production, its generation also occurs through other mitochondrial enzymes such as OGDH (2-oxoglutarate dehydrogenase) and PDH (pyruvate dehydrogenase), and the mitochondrial membrane forms of GPDH (glycerol 3-phosphate dehydrogenase; also known as GPDM) as well as the FQR (electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial) system or coenzyme Q10 [524–526].

Our knowledge about the *in vivo* regulation of mitochondrial ROS production is still incomplete, however various factors such as the mitochondrial membrane potential are believed to influence ROS generation [525, 527]. In general, an increase of the gradient

across the inner mitochondrial membrane fuels the production, whereas a weaker gradient diminishes ROS production [527].

#### 1.2.1.1.1 Complex I

Complex I (also called NADH dehydrogenase or NADH ubiquinone oxidoreductase) is an entry point to the electron transport chain from NADH. Mechanistically, transfer of an electron from NADH to flavin mononucleotide (FMN) promotes its transport through a chain of seven FeS (iron-sulphur) clusters to the coenzyme Q10 (ubiquinone). The involvement of Complex I in superoxide anion production depends on two mechanisms [528, 529]. The first one occurs when NADH/NAD<sup>+</sup> ratio is high, resulting in a reduction of FMN site. The second takes place during a reverse electron transport, when electron transfer to the coenzyme Q10 pool is coupled with a high membrane potential ([530, 531], reviewed in [525]).

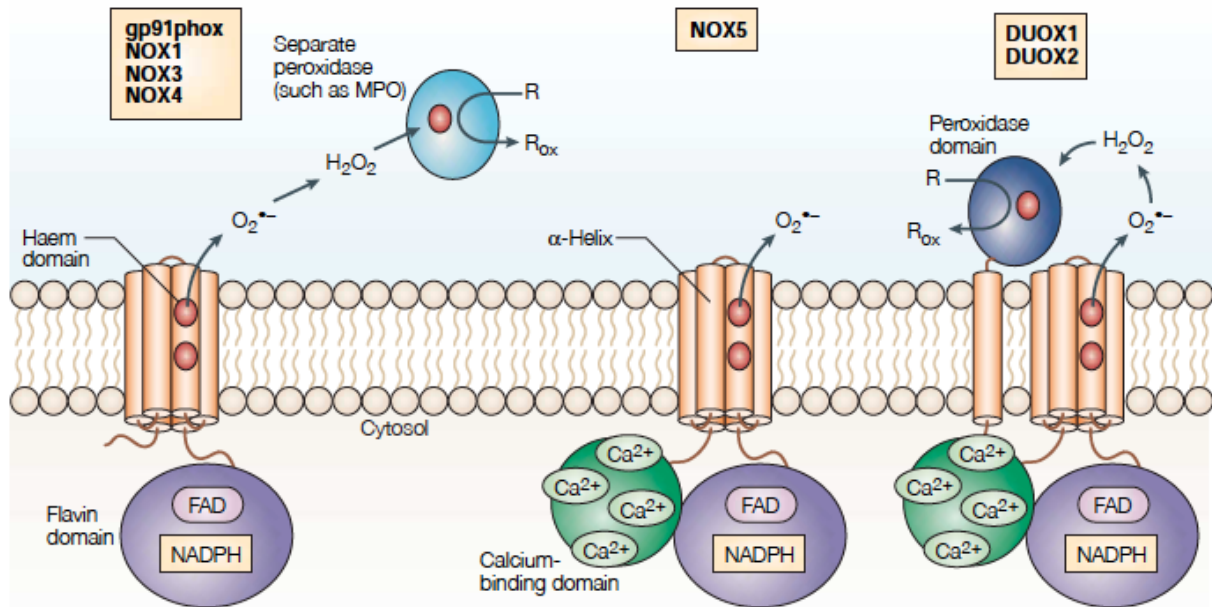
#### 1.2.1.1.2 Complex III

Complex III, also called cytochrome *bc1*, is responsible for electron transport from the reduced form of coenzyme Q10 to cytochrome *c*. Complex III possesses a high ability to produce superoxide anions under inhibition of Qi site by antimycin [532, 533]. However, under physiological conditions, production of O<sub>2</sub><sup>•-</sup> is negligible compared to the maximum potential production of complex I ([534], reviewed in [525]).

#### 1.2.1.2 NADPH oxidases

Intracellular enzymes called collectively NOX – NADPH oxidases are important players in ROS production. Activation of these enzymes occurs in neutrophils during inflammatory responses, when high level of ROS production is essential for an efficient host defence against infection. More precisely, these cells internalize microorganisms through phagocytosis and then neutralize them through secretion of a mixture of ROS and secreted enzymes into preformed phagosomes [535]. In this case, ROS production occurs through a flavin- and heme-containing protein complex that transfers electrons from cytosolic NADPH to molecular oxygen that results in production of superoxide anion. The catalytic subunit of NOX enzymes is called gp91phox (also named NOX2), which along with its regulators such as p22phox, p47phox or small GPTase RAC, forms

an active complex. It is now known that there are seven human NOX homologues (NOX1–5, dual oxidase 1 (DUOX1) and DUOX2) that produce ROS to sustain a range of host defence and signalling functions [536] (Figure 34).



**Figure 34. Structural representation of NOX and DUOX family [522].**

Structural study allowed researchers to classify NOX family into 3 groups. The first group consists of 4 members (NOX1-4). The second group is only composed of NOX5, whose catalytic core is the same as in the previous group, however is additionally supplied with Ca-binding domains. The last group consists of duo oxidases 1-2 (DUOX1-2), which are built in the same way as NOX5, but contain an additional domain that is homologous to peroxidases such as MPO (reviewed in [522]).

### 1.2.1.3 Other enzymes

Mitochondria and NOX are the best characterized intracellular sources of ROS, however ROS production is not restricted to these sources. Importantly, a wide variety of enzymes have the ability to produce ROS, such as xanthine oxidase, nitric oxide synthase, cyclooxygenases, cytochrome P450 enzymes and lipoxygenases (reviewed in [524]). Of note, the general pattern of ROS production varies from one cell type to another. Additionally, metal ions, such as iron or copper, also have an impact on cellular levels of ROS. For example during a Fenton reaction, reduction of ionic iron (Fe<sup>2+</sup>) is

coupled with generation of a more reactive hydroxyl radical from the hydrogen peroxide (reviewed in [524]).

### **1.2.2 Exogenous sources of ROS**

Plethora of exogenous sources can promote ROS formation. One of the most common is radiation; gamma and X radiation, as examples of ionizing radiation, cause augmentation of free radicals. Low wavelength ultraviolet light also produces free radicals through a process of photolysis. In addition, urban air contains a mixture of oxidizing gases (ozone and nitric oxides) and particles (polycyclic aromatic hydrocarbons) arising from a variety of sources, such as power plants, motor vehicles, wildfires and waste incinerators. Evidence suggests that chronic exposure to polluted air provokes irreversible damage to cellular macromolecules via ROS production and their accumulation in cells and tissues [537]. Moreover, consumption of popular and legal drugs such as tobacco or alcohol are known to increase ROS concentration [538, 539]. Of note, some chemotherapeutics, such as cisplatin, increase intracellular ROS levels, which participate in the therapeutic effect (reviewed in [540]). Finally, ROS production can be triggered by exposure to a variety of stresses such as hypoxic, metabolic or oncogene-activated stress.

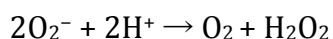
### **1.2.3 Defence mechanisms against ROS**

Homeostasis ensures a balance between production of ROS during cellular metabolism and antioxidant cellular activities. Of note, exposure to an excess of free radicals from external sources triggers cells to activate their defence mechanism [532]. These mechanisms are complex with several pathways having been described so far. First of all, a tight regulation of enzymes involved in ROS production both in the mitochondria and in the cytoplasm is a crucial first-line defence mechanism. A second line of defence relies on antioxidative agents, which react with free radicals and disable them. This class consists of a variety of enzymes such as catalases or superoxide dismutases and of non-enzymatic scavengers, such as ascorbic acid (vitamin C) or glutathione (GSH). Finally, repair mechanisms are considered as the last line of cellular ROS defence mechanism. The following paragraph will be focused on the enzymatic and non-enzymatic antioxidants.

### **1.2.3.1 Enzymatic antioxidant defence**

#### 1.2.3.1.1 Superoxide dismutase (SOD)

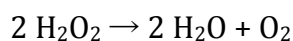
Superoxide dismutases play an important role in scavenging one of the most deleterious of ROS – the superoxide radical. The first enzyme of the SOD family was discovered in 1969 by Fridovich [541]. Up till now, several SODs have been identified among various species and are characterized by a particular metal composition in their reactive cores. In mammals three distinct metalloproteinases were discovered. The first one, called CuZn-SOD (SOD1), is the most common and is mainly localized in the cytoplasm [542]. The second, Mn-SOD (SOD2) is expressed only in the mitochondria [543] and the third, EX-SOD (SOD3, tetramers CuZn-SOD) is specific of the extracellular compartment [544]. The functional SOD are dimers (SOD1) or tetramers (SOD2, 3) and their activity is closely connected to the metal cofactors in their catalytic cores: SOD1, 3 - copper and zinc, SOD2 – manganese. Functionally, the main role of SOD is to promote a reaction of dismutation of the superoxide radical into molecular oxygen and hydrogen peroxide:



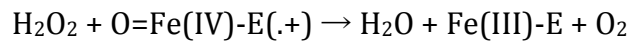
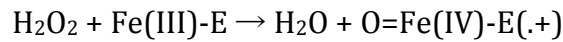
SODs are among the most important components of defence against oxidative stress, as shown by the observation that SOD2-KO mice die several days after birth due to massive oxidative stress [545]. Mice lacking SOD1 are viable, but have a short lifespan and are prone to numerous pathologies, such as hepatocellular carcinoma [546]. In contrast, mice with invalidated SOD3 do not show any obvious defects and exhibit a normal lifespan, but are more prone to oxidative injuries [547].

#### 1.2.3.1.2 Catalase

Catalase was discovered more than 100 years ago as an enzyme involved in hydrogen peroxide decomposition [548, 549]. Catalase acts as a tetramer and is commonly found in various animals and plants. Catalase is one of the most abundant peroxisomal proteins within mammalian cells. Mechanistically, catalase is a homotetrameric heme-containing enzyme that removes  $\text{H}_2\text{O}_2$  through a dismutation reaction:



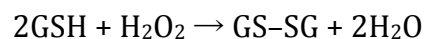
It is one of the fastest cycling enzymes with the capacity of 200 000 rounds per second. Even though this enzyme was discovered early on, its specific mechanism is still a little puzzling, but it is believed to occur through a temporary reaction of iron ion with oxygen [549]:



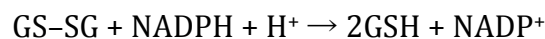
Finally, the exact physiological function of catalase is most probably to prevent the accumulation of  $\text{H}_2\text{O}_2$ , which in turn may fuel hydroxyl radical formation through the Fenton reaction (reviewed in [549, 550]).

#### 1.2.3.1.3 Glutathione peroxidase (GPx)

Glutathione peroxidases are a family of enzymes involved in hydrogen peroxide decomposition. However, in contrast to catalase, they can also reduce lipid hydroperoxides to their corresponding alcohols. Their normal functions are exerted as tetramers, which contain the unique amino acid selenocysteine within their active sites. In comparison to catalase, GPx uses low-molecular-weight thiols, such as glutathione, to fuel their biological activity. Overall reaction of hydrogen peroxide can be summarized as:



As mentioned above, the activity of GPxs depends on the availability of the reduced form of glutathione. Oxidized GSH consists of two molecules of GSH bound together through a sulphur-bridge – GS-SG. Glutathione disulphide is recycled back to glutathione by glutathione reductases, using NADPH as a cofactor:



Until today, 8 different isoforms of GPx were identified in human (reviewed in [551]). The main isoform, GPx1, is found in nearly all mammalian tissues. Despite its abundance and its important function, KO of GPx1 in mice shows no profound phenotype. The



animals have a normal lifespan and do not develop any severe pathology, suggesting that this enzyme is not critical [552, 553].

#### 1.2.3.1.4 Other enzymes

An additional function of antioxidant enzymes is to detoxify secondary metabolites of free radicals, such as aldehydes, epoxides and hydroperoxides. For example, the glutathione *S*-transferases (GSTs) family enzymes catalyse conjugation of the reduced form of glutathione to substrates such as peroxidised lipids, leading to their detoxification. GSTs family contains 3 separate classes (cytosolic, mitochondrial and membrane-associated microsomal) and they are highly expressed in human [554].

The human antioxidant response comprises other enzymes, not discussed here, highlighting its high degree of complexity. For example, the metabolism of H<sub>2</sub>O<sub>2</sub>, while predominantly controlled by catalase and GPx, can also be processed by other enzyme families such as thioredoxin reductases (TRX) or thioredoxin peroxidases (PRX).

#### **1.2.3.2 Non-enzymatic antioxidant defence**

Additionally to the enzymatic response, cells also carry a non-enzymatic barrier based on low molecular weight antioxidants. These small molecules are either water- or lipid-soluble and can be supplemented with diet (such as vitamins or flavonoids) or synthesized by cells (such as GSH). Below I will present only the main examples of these molecules.

##### 1.2.3.2.1 Glutathione (GSH)

Glutathione is constitutively synthesized within each cell and is abundant in the cytosol (majority), the mitochondria and the nucleus. A GSH molecule consists of 3 amino-acids (L-cysteine, L-glutamic acid, and glycine), where the sulfhydryl group (SH) of the cysteine serves as a proton donor and is responsible for its antioxidant activity (reviewed in [555]). Oxidized glutathione (GS-SG) can be recycled to its active form by glutathione reductases. Its abundance, the ease of its recycling combined with its small size makes GSH a major soluble antioxidant within tissues [556]. Additionally, a too high concentration of GS-SG may have damaging effect on functionality of many enzymes and the ratio between GSH and GS-SG is a good indicator of intracellular oxidative

stress [557]. GSH performs several antioxidant functions during cellular life. Notably, GSH is an important cofactor of several detoxifying enzymes such as glutathione peroxidases (GPx) or GSTs. Another function is connected to its ability to directly scavenge hydroxyl radicals and singlet oxygen. Finally, glutathione is able to regenerate several important antioxidant molecules to their active form, such as vitamin A and E ([556], reviewed in [558]).

#### 1.2.3.2.2 Ascorbic acid

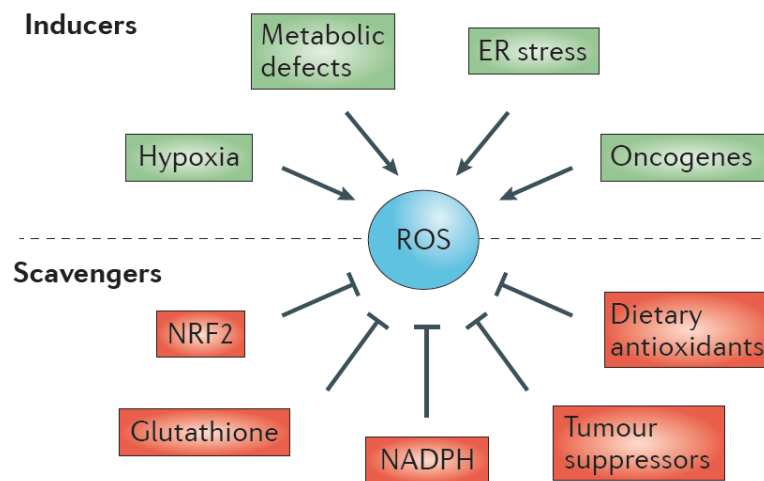
Ascorbic acid (vitamin C) is a water-soluble dietary supplement that can act as a cofactor and an antioxidant [559]. This small molecule is characterized by an ability to scavenge oxygen species such as hydroxyl radicals, and has the capacity to regenerate vitamin E back to its active form (reviewed in [560]). Mechanistically, ascorbic acid exerts its function as an electron donor, resulting in a relatively unreactive dehydroascorbic acid. However, although considered as an antioxidant compound, under certain metabolic situations, vitamin C can display some pro-oxidative properties in the presence of metal ions (such as  $\text{Fe}^{2+}$ ) because of its potential as an electron donor (reviewed in [561]).

#### 1.2.3.2.3 $\alpha$ -tocopherol

Vitamin E refers to a group of small molecules built from tocopherols and tocotrienols. Among these,  $\alpha$ -tocopherol is the most biologically active form of vitamin E [562]. In contrast to vitamin C,  $\alpha$ -tocopherol is a lipid-soluble antioxidant with an important role in the protection of hydrophobic sites in the cell membrane [563]. Functionally, vitamin E acts as a scavenger of peroxy radical – a by-product of lipid peroxidation chain reaction (reviewed in [564]). As mentioned before, the biological activity of  $\alpha$ -tocopherol can be restored back to its reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol [562]. Of note, it was demonstrated that supplementation with  $\alpha$ -tocopherol during cancer progression inhibits free radical formation and triggers apoptosis of cancer cells [565].

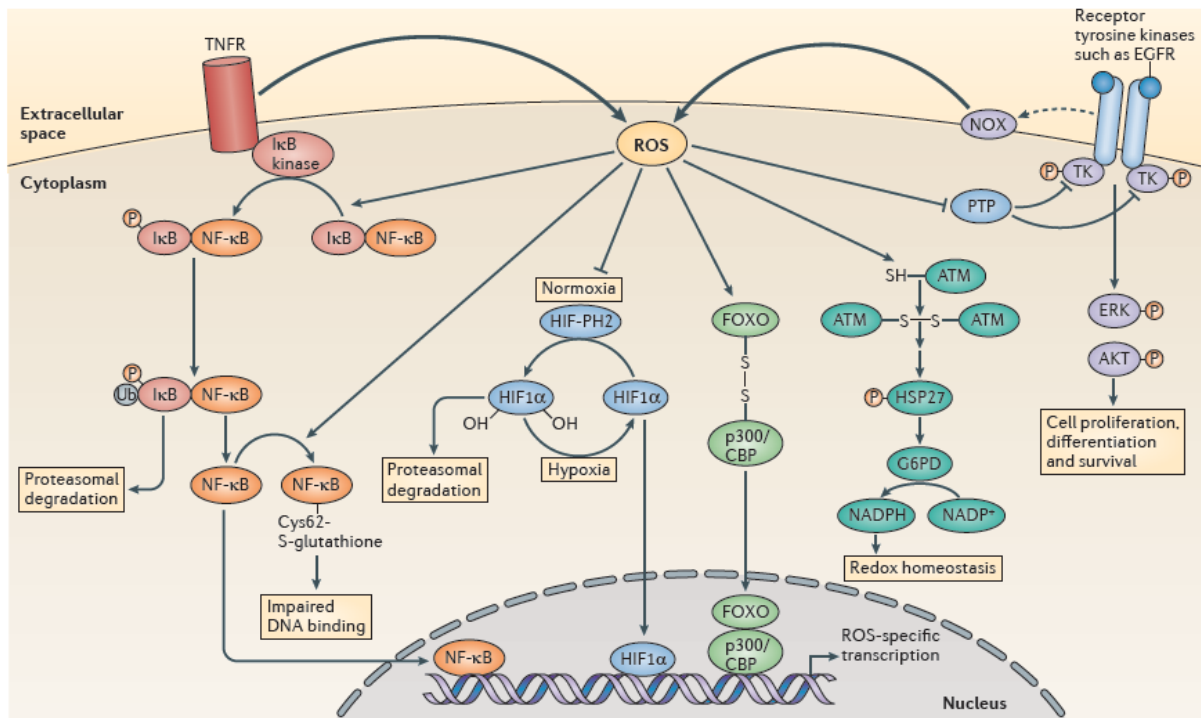
### 1.3 Physiological functions of ROS

As previously stated, free radicals are important players in regulating cellular homeostasis, however their presence inside the cell must be tightly controlled and kept at low levels. The steady state of ROS results from a balance between their rate of production and the rate of detoxification. This particular balance is usually called a “redox state” and is kept within narrow range under homeostatic conditions. The redox state is often measured as the ratios of GS-SG /GSH,  $\text{NAD}^+/\text{NADH}$  or  $\text{NADP}^+/\text{NADPH}$  (reviewed in [558]). Importantly, under pathological conditions, these ratios can be altered in either direction and these oscillations have a major impact on the fate of affected cells [566] (Figure 35).



**Figure 35. The cellular redox status is an outcome of a balance between ROS inducers and ROS scavengers [567].**

Fluctuations of the redox state through redox reactions are detected by the cell through signal transduction pathways, called redox signalling. In order to activate redox signalling a physiological redox balance has to be disturbed, either by an increase in ROS formation or a decrease in the activity of antioxidant systems. This temporary imbalance results in signal transduction and activation of response mechanisms. Notably, several targets were identified as mediators of redox signalling such as the transcription factors AP-1, NF- $\kappa$ B, the Src family kinases and JNK and p38 MAPK (reviewed in [558]). The ability of ROS to transduce signals through different effector proteins allows them to regulate various cellular processes (Figure 36).



**Figure 36. Examples of transcriptional regulation by ROS acting at the plasma membrane or in the cytosol [511].**

For example, it has been shown that cells generate a small oxidative burst of ROS upon stimulation with factors such as: IL-1 $\beta$ , TNF $\alpha$ , TGF $\beta$ -1, GM-CSF or FGF2 (reviewed in [558]) (Figure 36). Thus, activation of several signal transduction pathways is accompanied by ROS signalling, suggesting their importance as physiological messengers of different processes, including proliferation, immunity, autophagy or differentiation. In the following section I will describe the role of ROS as regulators of two crucial processes – innate immunity and differentiation of stem cells.

### 1.3.1 ROS and innate immunity

An oxidative burst in the context of inflammation is characterized by a massive production of reactive oxygen species, which play a key role in the defence against environmental pathogens. Neutrophils and macrophages are recruited sites of infection by chemotactic factors such as IL-8, C5a, LTB<sub>4</sub>, and formyl peptides released either by mitochondria or by bacteria. In inflammatory areas, activated immune cells trigger the production of NOX2-dependant H<sub>2</sub>O<sub>2</sub> [511, 568]. Increasing ROS concentration guides migration of immune cells towards pathogens. Moreover, high ROS levels promote

pathogens' destruction and retention of the immune cells in the inflammatory site (reviewed in [511]). One of the mechanisms of ROS production occurs through stimulation of cell-surface Toll-like receptor 4 (TLR4) by lipopolysaccharides (LPS), mediating a response through TRAF6 and ECSIT signalling [569]. Of note, various types of non-phagocytic cells, such as fibroblasts, vascular smooth muscle cells and endothelial cells, are known to produce ROS by NADPH oxidase [570, 571]. Production of such free radicals occurs mainly intracellularly upon stimulation by growth factors and cytokines such as TNF $\alpha$ , IL-1, PDGF or angiotensin II (reviewed in [558]).

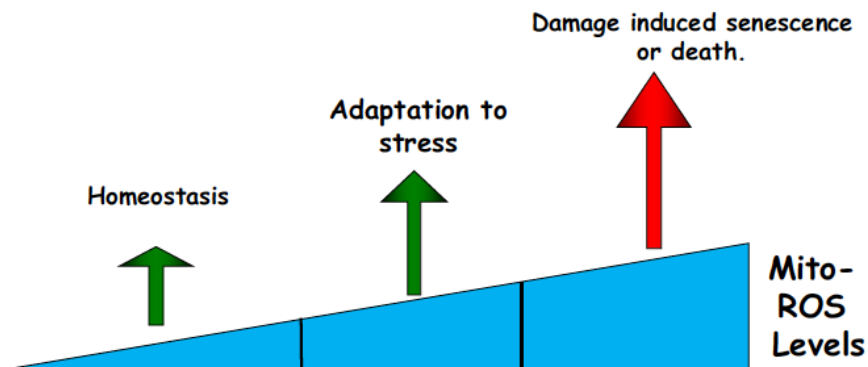
### **1.3.2 ROS and differentiation**

Embryonic stem cells give rise to all organs and tissues, while adult stem cells serve to replenish tissue-specific cell populations throughout the life of the organism. In stem cells, mitochondrial activity is low, but it increases during the differentiations process. For example, bone marrow-derived human mesenchymal stem cells are characterized by increased mitochondrial content, driven by PGC1 $\alpha$  during differentiation [572]. Additionally, an increase in mitochondrial mass and oxygen consumption was observed during differentiation of embryonic stem cells [573]. Indeed, several studies reported that stem cells with impaired ROS generation systems (such as complex I and III) or with an overexpression of ROS scavenging machinery (such as GPx) displayed a delayed differentiation [574, 575]. Moreover, some studies suggested that differentiation of stem cells rely on NOX4-mediated H<sub>2</sub>O<sub>2</sub> generation [576].

## **1.4 Oxidative stress**

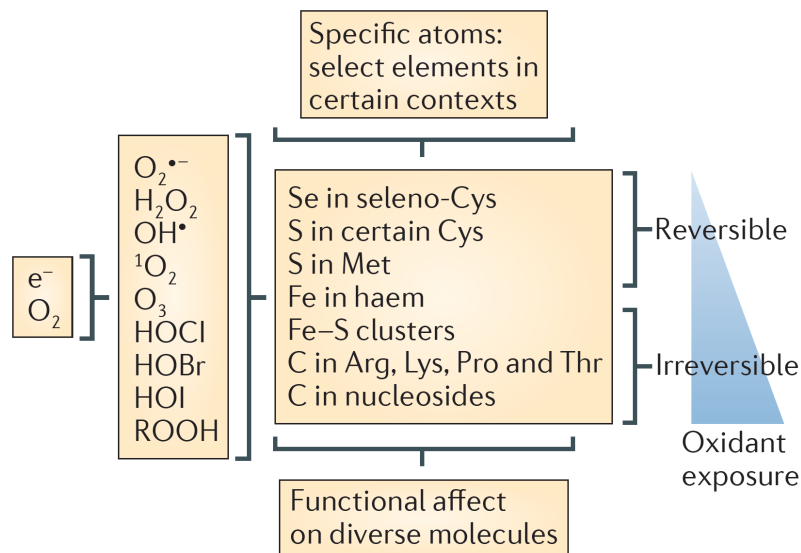
Homeostasis is maintained by a tight balance between production and scavenging of ROS. Any disturbance of this fragile equilibrium can lead to oxidative stress. Oxidative stress can occur under several circumstances, such as excess in ROS production, which cannot be counterbalanced by endogenous scavenging mechanisms. Similarly, oxidative stress builds up when impaired antioxidative mechanism cannot detoxify the normal rate of ROS production. Disequilibrium of the redox state has an important impact on many cellular processes. Initially, cells respond by counteracting oxidative stress through activation of antioxidant mechanism, resulting in adaptation. However, when

ROS production exceeds defence potential of cells and damages suffered by cells are too high, cells activate mechanisms leading to senescence or cell death (Figure 37).



**Figure 37. Oxidative stress and its impact on cellular fate [527].**

Remarkably, when oxidative stress is not transient, but becomes chronic, it may result in promotion of certain pathologies such as cancer, cardiovascular or neurodegenerative diseases (reviewed in [577]). Mechanistically, ROS overproduction causes damage to the most basic components of cells such as DNA, proteins or lipids. As a result of such damage, these biomolecules lose their functions, increasing the rate of mutagenesis, which increases the risk of malignant conversion. Importantly, ROS exhibit some form of “atomic specificity” during their reaction with cellular macromolecules. This “atomic specificity” is also responsible for the potential of its reversibility [578]. The main target of reversible reactions is sulphur, one of the least abundant atoms in macromolecules. However, if the oxidative stress overcomes a certain threshold, ROS can interact irreversibly with carbon or iron atoms (reviewed in [511]) (Figure 38).



**Figure 38. ROS and their atomic specificity [511].**

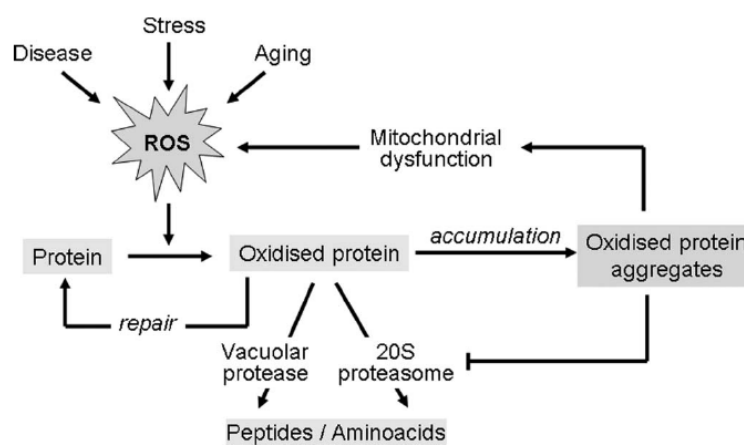
#### 1.4.1 Oxidative target: DNA

Oxidative stress induces different types of DNA damage. First of all, oxidative stress can induce both single and double strand breaks. Moreover, ROS oxidize purines and pyrimidines, turning them into an abasic components of DNA. Oxidation of DNA can create a wide variety of changes in bases, but two of the most common are thymine glycol (TG) and 8-oxodG, which often serve as reliable markers of oxidative DNA damage [579]. Importantly, oxidative lesions are usually not lethal for cells, but are considered as highly mutagenic ([580], reviewed [581]). While DNA repair mechanisms are able to detect and repair most of these lesions, some remain unrepaired or repaired with low fidelity. Thus, the final outcome is determined by the balance between the repair efficiency and the severity of oxidative stress.

#### 1.4.2 Oxidative target: proteins

During oxidative stress, proteins can be either directly oxidized by ROS or via secondary attack of products resulting from lipid peroxidation. Protein oxidation can lead to conformational changes associated with the decrease in their activity when targeted amino acid residues are at or in a close proximity to active sites. Protein oxidation can affect different atoms, for example oxidation of sulphur in methionine and cysteine. This

alteration can be reversed by the cellular machinery. On the other hand, irreversible damage to proteins (such as carbonylation) results in proteins degradation by the proteasome system. Interestingly, the high content of carbonylated proteins has been suggested to be a major determinant of ageing, providing a molecular link between oxidative stress and ageing [582, 583]. Moreover, extensively oxidized protein cross-links lead to formation of aggregates, which consequently impair the proteasome and mitochondrial functions, thereby increasing ROS production (reviewed in [584]) (Figure 39). Obviously, protein oxidation can also impair crucial DNA repair enzymes, which in turn increases the frequency of DNA aberrations and thus promotes mutations.



**Figure 39. Protein oxidation and its consequences (more details in text) [584].**

### 1.4.3 Oxidative target: lipids

Cell membranes are very sensitive to ROS-induced damage. The majority of polyunsaturated fatty acids in membranes contain a methylene group between two double bonds that makes the fatty acids particularly sensitive to oxidation [516]. This results in formation of reactive aldehydes, including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), known to be highly reactive with proteins and DNA [509]. This suggests strongly that lipid peroxides can decompose to a range of highly mutagenic carbonyl products ([585], reviewed in [586]). Indeed, ROS induced lipid peroxidation of cell membranes has been implicated in malignant transformation [587]. Levels of these lipid peroxides can serve as markers of cellular damage. Increased levels



of lipid peroxidation products such as lipid hydroperoxides, 4-HNE and MDA have been reported in oral cancer and pre-cancer patients [588, 589].

#### **1.4.4 Oxidative stress and pathology**

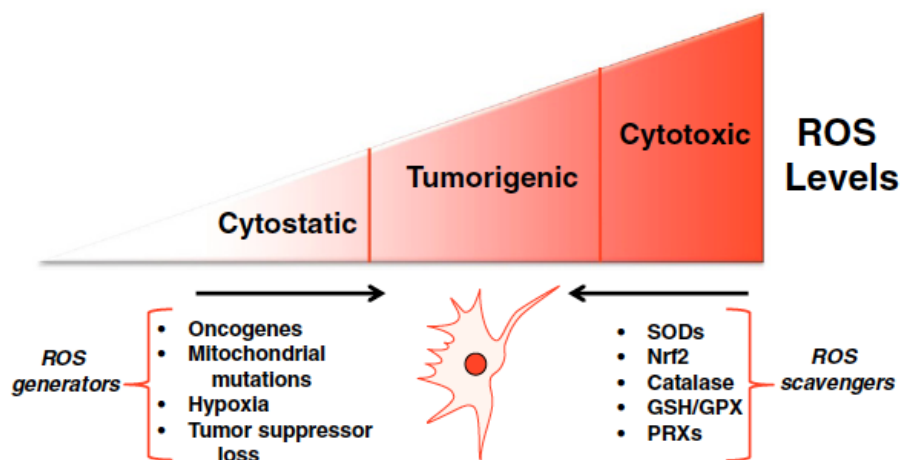
Oxidative stress has been implicated in various pathological conditions including cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion and ageing [590–593]. Among diseases caused by the oxidative stress we can distinguish two groups. The first one is connected with mitochondrial oxidative stress elicited through glutathione redox balance and damaged glucose tolerance, examples of which can be cancer and diabetes mellitus. The second group is linked to inflammatory oxidative stress, characterized by enhanced activity of NADPH and xanthine oxidases, which can cause atherosclerosis, chronic inflammation or ischemia (reviewed in [558]). In addition, oxidative stress is associated with yet another inevitable pathology – ageing [594]. According to this theory, free radicals, as by-products of oxidative metabolism, cause cumulative cellular damage (DNA, proteins, lipids) resulting in overall loss of organismal fitness over time. Finally, oxidative stress during cancer progression can serve as a powerful ally. Indeed, ROS mutagenic potential enhances cancer cells potential to acquire driver mutations. In addition, it can promote tumour adaptation to the tumour microenvironment, resulting in the acquisition of more aggressive phenotypes. ROS involvement in tumorigenesis will be discussed in the next paragraph.

#### **1.5 ROS and cancer**

The first link between cancer and ROS was presented in 1981 when researchers showed that insulin up-regulates  $H_2O_2$  levels and thus increases tumour cell proliferation [595]. However, more than 30 years later, the exact role of ROS in tumorigenesis remains controversial, as many researches demonstrated either pro-oncogenic or anti-oncogenic properties of ROS (reviewed in [596]). Cancer cells are characterized by a high demand for ATP in order to sustain their rapid growth. Consequently, elevated metabolism is coupled with increased ROS generation, the effects of which seem to be dose dependent.

Notably, low and moderate amounts of ROS may contribute to tumour formation. At these concentrations, ROS can work two ways: on the one hand, ROS can promote

tumour cell growth and survival through activation of signalling pathways involving various kinases such as MAPK, ERK and JNK [597, 598] (Figure 40).



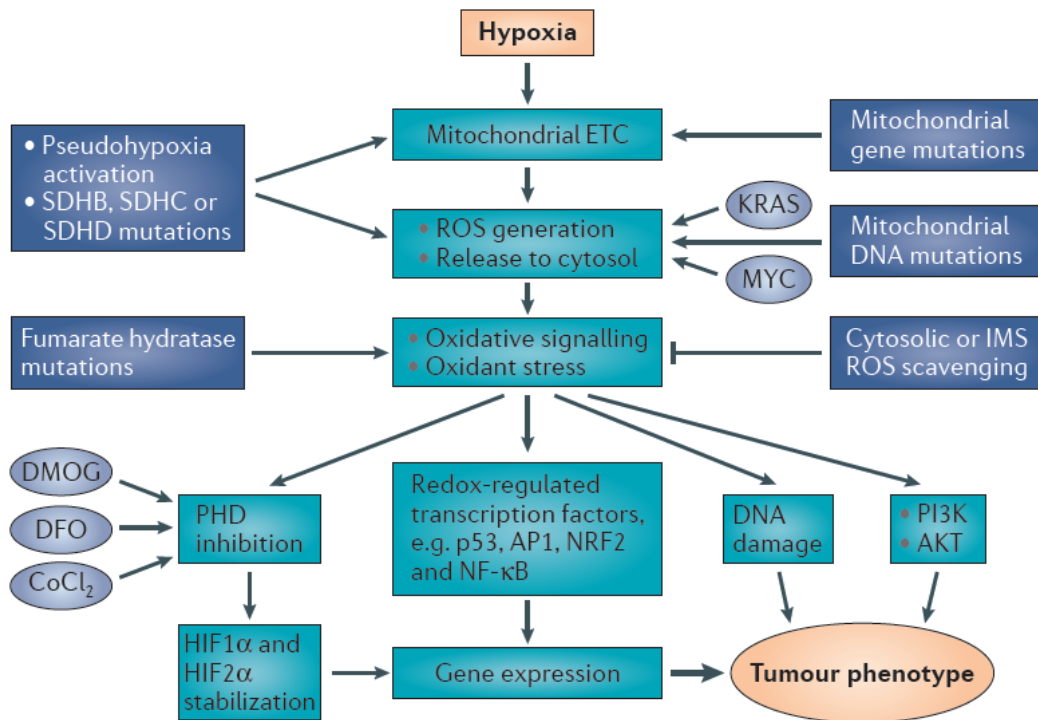
**Figure 40. Balance between ROS generation and ROS scavengers allows cancer cells to remain in the tumorigenic range of ROS levels [599].**

On the other hand, ROS may contribute to DNA damage and therefore generate mutations. As explained previously, ROS can induce several different changes in DNA sequence including modification of bases, production of base free sites, deletions, frame shifts, strand breaks, DNA–protein cross-links and chromosomal rearrangements [516]. The increase in DNA-damage occurrence leads to an extensive mutagenesis and thus may result in acquisition of genetic advantages in strongly selective environments.

A too high concentration of ROS results in major cellular damage and consequently leads to senescence or cell death (Figure 40). Of note, cancer cells respond to this challenge by activating or enhancing existing antioxidant mechanisms. This in consequence gives cancer cells advantages such as survival or opportunity for adaptation in hostile environments [271]. Moreover, this cancerous ability to reduce oxidative stress could explain some failures in cancer therapy. Recent studies demonstrated that certain therapies fail because cancer stem cells maintain low ROS levels through increased expression of ROS-scavenging systems [271, 600]. Interestingly, during cancer cell migration to distant organs, elevated ROS levels were observed during cell detachment [601]. The next paragraphs will describe the main roles of ROS during cancer progression.

### 1.5.1 ROS and hypoxia

As discussed above, increased aerobic metabolism as well as environmental stresses can induce intracellular ROS production. Rather counter-intuitively, ROS production can also be stimulated by hypoxic conditions. Hypoxia is a common feature of many solid tumours that reach a critical size that no longer allows efficient oxygen and nutrient supply to all cells. At this point, tumours overcome this restriction to their growth by triggering the process of neo-angiogenesis. However, in a vast majority of cases tumour vasculature is leaky and the blood supply is insufficient to prevent hypoxia in poorly vascularised areas of the tumour (reviewed in [602]). While it would appear that under limited oxygen supply, normal pathways of ROS generation from the mitochondria decrease, they may increase again when cell become oxygenated [603, 604]. In parallel, certain mechanisms of ROS production seem to actually increase during hypoxia, such as for example the release of oxygen species from complex III into the mitochondrial inter membrane space ([605, 606], reviewed in [607]). Importantly, elevation of ROS under hypoxia stabilizes the master regulator of hypoxia – the transcription factor HIF1 $\alpha$  [608] (more details in 2<sup>nd</sup> part of this chapter). Recent studies demonstrated that activation of prolyl hydroxylases (PHDs), which control HIF1 $\alpha$  stability, can occur through ROS-mediated mechanisms [605, 609, 610]. Precise molecular involved have not yet been elucidated, but they are likely to involve oxidation of Fe<sup>2+</sup> within PHDs. Importantly, the phenomenon of hypoxia-induced ROS signalling has been described both in primary and in malignant cells [604, 611]. Interestingly, this mode of HIF activation can also occur in normoxia, which in this case can be considered as "pseudohypoxia" [610]. Taken together, these data suggest that hypoxia and pseudohypoxia can behave as activators of mitochondrial ROS formation and signalling towards tumour progression (reviewed in [607]). A schematic outline of these phenomena is presented in Figure 41.



**Figure 41. Model of tumour progression driven by hypoxia or pseudohypoxia-mediated ROS formation [607].**

Hypoxic or pseudohypoxic conditions trigger mitochondrial ROS formation. Increased ROS generation followed by their cytosolic release activates signalling pathways, including HIF stabilization and redox signalling. **Legend:** AP1, activating protein 1; IMS, intermembrane space; NRF2, nuclear respiratory factor 2.

### 1.5.2 ROS and fail-safe programs

Excess of oxidative stress eventually results in cell death and cancer cells escape their demise by increasing their antioxidant response. Notably, they tend to increase the expression of the transcription factor NRF2 (nuclear factor erythroid 2-related factor 2), considered as a master inducer of antioxidant genes [567]. However, if production of ROS overcomes the antioxidant response, cancer cells will undergo senescence or apoptosis.

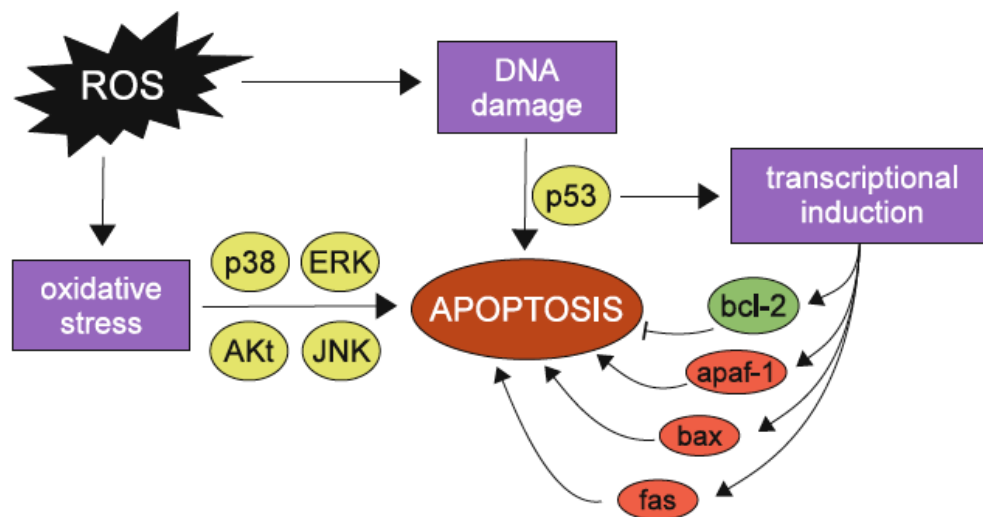
#### 1.5.2.1 Senescence

As previously stated, senescence is a mechanism related to aging of the organism, which inhibits old, likely damaged cells, from continuous division and therefore protects an organism from spreading of mutations (more details in Chapter 1). Interestingly, ROS

accumulation was correlated with induction of senescence [612]. The link between replicative senescence and oxidative stress was further strengthened when researchers found that exogenous treatment of primary cells with sub-lethal concentrations of hydrogen peroxide triggered rapid senescence entry [613]. In the same report, it was shown that treatment with hydrogen peroxide induces the tumour suppressor protein p53 and stimulates p53-dependent cell cycle regulator p21/waf-1, leading to G1 arrest [613]. Additionally, a number of experiments demonstrated that ROS-induced senescence could be rescued through treatment with an antioxidant N-acetylcysteine (NAC) or by lowering the level of ambient oxygen [614].

### 1.5.2.2 Apoptosis

While sub-lethal doses of ROS provoke senescence, if oxidative stress is high and gives rise to severe cellular damage, cells undergo programmed cell death [615]. ROS mediated apoptosis induction occurs through activation of several pathways, such as p53-mediated response to DNA damage [616] or signalling through p38/JNK (Figure 42).



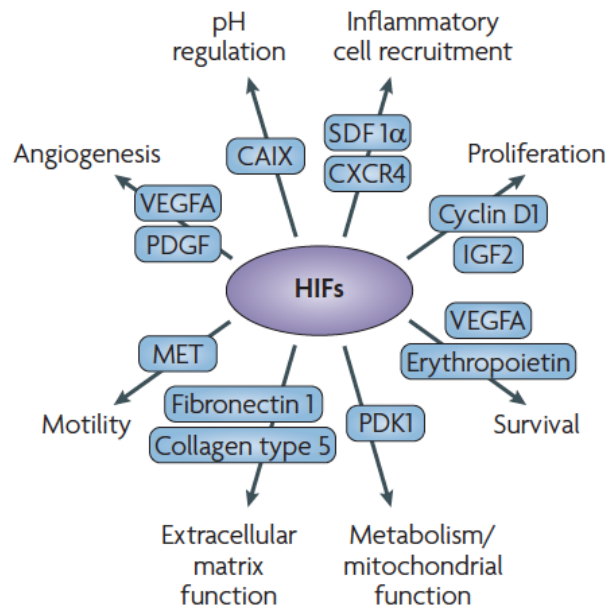
**Figure 42. ROS mediated activation of apoptosis [617].**

ROS simultaneously provoke a response to oxidative stress and to DNA damage. In case of DNA damage, the p53 tumour suppressor gene triggers apoptosis through down-regulation of Bcl-2 expression and up-regulation of BAX, FAS, and Apaf-1 (apoptosis-activating factor 1) expression (reviewed in [617]).

Interestingly, ROS are also involved in oncogene-induced apoptosis. Notably, c-Myc activation is associated with an intracellular increase of ROS, that promotes genomic instability [618, 619]. Moreover, c-Myc overexpression in immortalized fibroblasts coupled with serum deprivation provokes oxidative stress followed by cell death [620]. This study demonstrated that ROS accumulation occurs through c-Myc mediated NF- $\kappa$ B inactivation [620]. Additionally, c-Myc mediated oxidative stress and apoptosis could also occur through down-regulation of peroxiredoxin III [621].

## 2 Hypoxic stress and HIF genes

Oxygen is an essential nutrient that serves as a key substrate in cellular metabolism and bioenergetics. Hypoxia, defined as insufficient oxygen supply occurs in different physiological and pathological conditions during the life of organisms. Hypoxia activates specific transcriptional mechanisms to cope with this stress. This response is mainly controlled by a family of transcriptional regulators called hypoxia-inducible factors (HIFs). HIFs regulate numerous adaptive processes such as metabolism, angiogenesis, proliferation and motility (Figure 43).



**Figure 43. Processes and genes activated by HIF genes during hypoxic stress [622].**

In order to keep this powerful transcriptional program under control, tight regulatory mechanisms such as oxygen sensing through prolyl hydroxylase domain-containing enzymes (PHDs) operate in cells. The genes involved in these mechanisms are mutated in many human cancers as their deregulation leads to HIF genes activation, thus promoting cancer progression.

The following paragraphs will cover the description of oxygen sensing, hypoxia condition and stress, molecular defence mechanisms against hypoxic stress and their function in physiological and pathological conditions.

## 2.1 Physiological oxygen concentration and definition of hypoxia

Molecular oxygen ( $O_2$ ) is indispensable for aerobic metabolism to maintain intracellular bioenergetics and to serve as an electron acceptor in many organic and inorganic reactions. Oxygen is the second component of ambient air with tension reaching 21%, however physiological tissue oxygen concentration is significantly lower. For most of mammalian tissues the oxygen concentration varies between 2% to 9%  $O_2$  [623] (Figure 44). Such differences in oxygen supply result from the dramatic decrease in blood oxygen content as it travels from the lungs throughout the body.

Organ	Normal $pO_2$ (mmHg)	% $O_2$
Trachea	150	19.7
Alveoli	110	14.5
Arterial blood	100	13.2
Pulmonary arterial blood	40	5.3
Brain	35	4.4
Intestinal tissue	58	7.6
Liver	41	5.4
Kidney	72	9.5
Muscle	29	3.8
Bone marrow	49	6.4

**Figure 44. Physiological oxygen tensions in a selection of normal human tissues [485].**

Increased or decreased oxygen availability results in hyperoxia or hypoxia, respectively. Hyperoxia appears physiologically from excessive angiogenesis and clinically is characterized through delivery of inappropriately high oxygen concentrations [624].

Hypoxia exists as a physiological stimulus for embryogenesis, wound healing and maintenance of pluripotent stem cells [625–627]. However, hypoxia that involves oxygen tensions below the normal physiological range can restrict the function of organs, tissues, or cells (Figure 45).

Condition	pO <sub>2</sub> (mmHg)	% O <sub>2</sub>
Normoxia (ambient)	159	21%
Physiological hypoxia	15–68	2–9%
Mild hypoxia	8–38	1–5%
Hypoxia	<8	<1%
Anoxia	<0.08	<0.1%

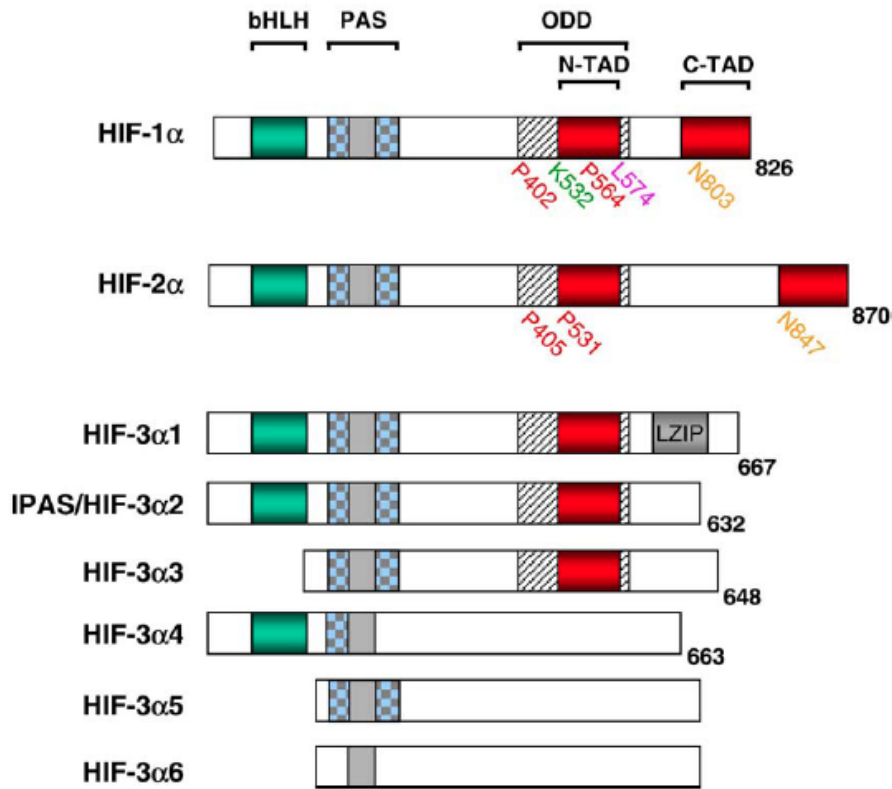
**Figure 45. Frequently used definitions describing low oxygen [485].**

Pathological hypoxia can result from a reduction in oxygen supply either due to high altitude or localized ischemia caused by the disruption of blood flow in a given area. Additionally, most solid tumours contain hypoxic regions because of severe structural abnormalities in their vascularisation [628]. The cellular response to hypoxic conditions is orchestrated by transcription factors, mainly HIFs, which control the expression of a plethora of genes promoting angiogenesis, anaerobic metabolism and resistance to apoptosis.

## 2.2 Hypoxia inducible factor genes

Hypoxia inducible factors are the main regulators of hypoxic stress and they are present in all metazoans [629]. Mammals possess three isoforms of HIF $\alpha$ , of which HIF1 $\alpha$  and HIF2 $\alpha$  (also known as EPAS1) are the most structurally similar and best characterized [630, 631]. The last one, HIF3 $\alpha$  (or IPAS), exists as multiple splice variants [632]. Some of these isoforms may act as inhibitors of HIF1 $\alpha$  and HIF2 $\alpha$  activity in a dominant-negative manner ([633, 634], reviewed in [635]) (Figure 46). Each subunit contains a bHLH-PAS domain [636], which is responsible for HIF heterodimerization and DNA binding [637].





**Figure 46. Schematic representation of HIF $\alpha$  subunits [638].**

Functional domains are indicated by compartments: bHLH – basic helix–loop–helix domain, PAS – Per/ARNT/Sim domains, ODD – oxygen-dependent degradation domain, N-TAD – N-terminal activation domain, C-TAD – C-terminal activation domain and LZIP – leucine zipper.

HIF1 $\alpha$  is expressed ubiquitously, whereas the expression of HIF2 $\alpha$  and HIF3 $\alpha$  is restricted to certain tissues, including vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells, and cells of the myeloid lineage (reviewed in [622]). All HIF $\alpha$  subunits heterodimerize with the HIF1 $\beta$  subunit, or ARNT, through their bHLH and PAS domains [639] (Figure 47) in order to control the expression of various target genes.



**Figure 47. Representation of HIF1 $\beta$  gene [485].**

HIF heterodimers bind to specific DNA sequences called hypoxia response elements (HREs). These sequences are similar to enhancer box (E-box) motifs and are characterized by the consensus sequence G/ACGTG [630, 640]. Several studies using genome-wide chromatin immunoprecipitation showed a strong correlation between HRE occupancy and hypoxic gene induction for HIF1 $\alpha$ -regulated genes, while the correlation for both HIF2 $\alpha$ -induced genes and HIF-repressed genes was considerably weaker [641, 642]. Despite the fact that HIF1 $\alpha$  and HIF2 $\alpha$  share 48% amino-acid identity as well as some common functions, they are considered nowadays as unique factors. Analyses of mice invalidated for either HIF1 $\alpha$  and HIF2 $\alpha$  revealed their embryonic lethality, however the precise phenotype of each knock-out was distinct [643, 644]. More precisely, HIF1 $\alpha$  KO mice exhibit severe defects in blood vessel system and die at E11 [643]. On the other hand mice invalidated for HIF2 $\alpha$  mostly die at E13.5 and suffer from bradycardia, abnormal lung maturation and blood vessel defects [644, 645]. Further studies demonstrated that HIF1 $\alpha$  preferentially induces expression of genes of the glycolytic pathway [646, 647], while HIF2 $\alpha$  is rather involved in the regulation of genes important for tumour growth, cell cycle progression and maintenance of stem cell pluripotency, like the proto-oncogene c-Myc [648] and the stem cell factor OCT-3/4 [649].

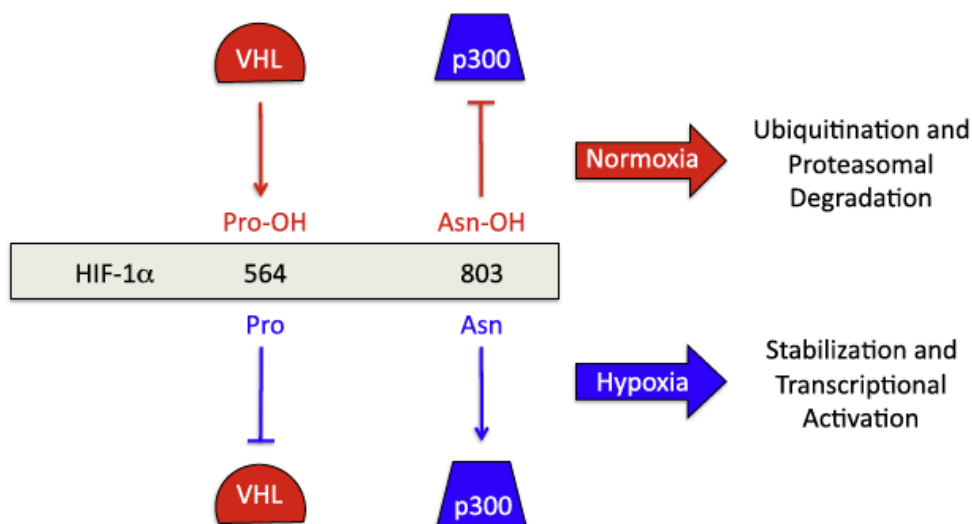
### **2.3 Regulation of HIF proteins**

HIF1 $\alpha$  and HIF1 $\beta$  mRNAs are constitutively and ubiquitously expressed, regardless of the oxygen tension. The regulation of HIF1 $\alpha$  and HIF2 $\alpha$  occurs mostly at the level of protein stability, whereas HIF1 $\beta$ /ARNT is constitutively expressed and stable [650–652]. In order to tackle their powerful transcriptional potential, cells carry complex regulation mechanisms of HIF $\alpha$  genes. The best studied regulatory mechanism of HIF $\alpha$  factors is based on oxygen availability. However, HIF proteins can also be regulated by other modifications such as phosphorylation or acetylation, which are independent of the available oxygen supply.

#### **2.3.1 HIF $\alpha$ Regulation by O<sub>2</sub> Availability**

In a well-oxygenated environment HIF $\alpha$  proteins are unstable with a half-life lower than 5 minutes. Stability of HIF $\alpha$  proteins is mediated through their hydroxylation by PHDs.

HIF $\alpha$  transcription factors contain two conserved proline residues (P402/P564 and P405/P531 for human HIF1 $\alpha$  and HIF2 $\alpha$ , respectively), located within the oxygen-dependent degradation domain (ODD). Hydroxylation of HIF $\alpha$  factors creates a binding site for the von Hippel-Lindau (pVHL) tumour suppressor protein, which is a component of an ubiquitin ligase complex [653–655]. A specific substrate recognition module of an E3 ubiquitin ligase complex (composed of elongin C, elongin B, cullin2, and ring-box 1) is formed upon pVHL binding. This event drives the recognition of HIF $\alpha$ 's as substrates for polyubiquitylation and proteasomal degradation [656]. The PHD proteins belong to the Fe(II) and 2-oxoglutarate-dependent oxygenase superfamily, whose activity is dependent on oxygen. Under hypoxic conditions, lack of hydroxylation allows HIF1 $\alpha$  and HIF2 $\alpha$  to dimerize with HIF1 $\beta$ , translocate to the nucleus and fulfil their transcriptional function [656] (Figure 48). Interestingly, oxygen-based regulation of HIFs is not limited to PHD but also affects another oxygen dependent mechanism, based on HIF hydroxylation by the asparaginyl hydroxylase, a factor inhibiting HIF (FIH1) [657]. Similarly to PHD, FIH1 is a member of the Fe(II) and 2-oxoglutarate-dependent dioxygenase family [658]. Under physiological conditions, FIH1 hydroxylates a conserved asparaginyl residue within the HIF1 $\alpha$  and HIF2 $\alpha$  CTADs domains, creating a steric barrier that prevents the recruitment of co-activators, such as p300 and CBP ([657], reviewed in [659]) (Figure 48).



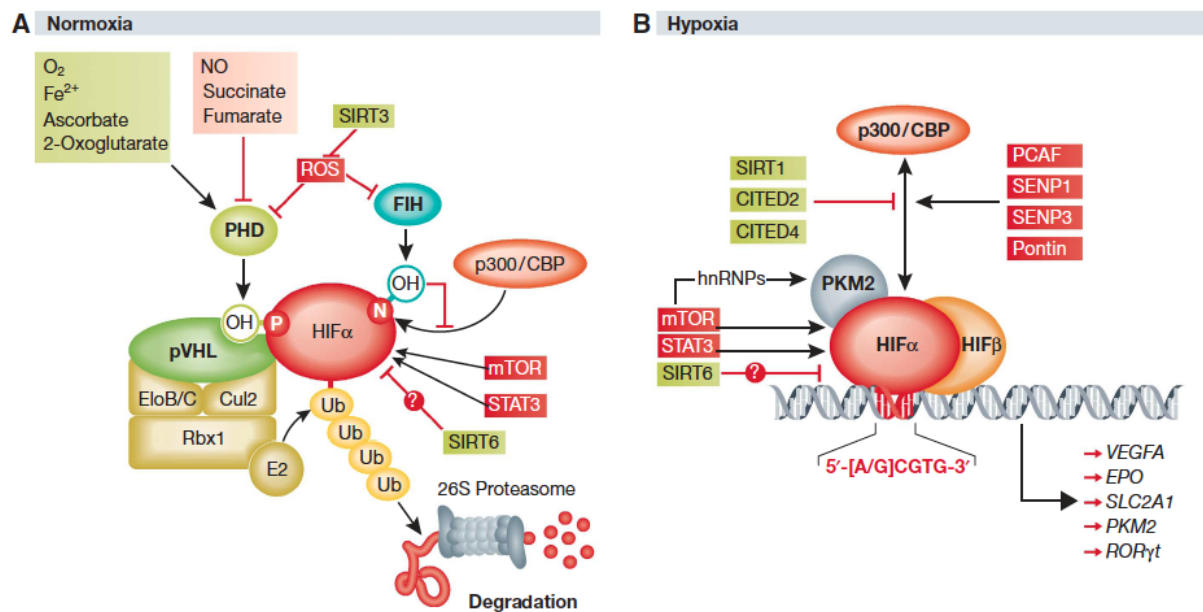
**Figure 48. Oxygen sensed regulation of HIF1 $\alpha$  stability and activity [660].**

Additionally, FIH1 remains active at lower oxygen concentrations than the PHDs and so might suppress the activity of HIF $\alpha$  proteins that escape destruction in moderate hypoxia [661, 662]. Interestingly, researchers showed that HIF2 $\alpha$  is more resistant to FIH1-mediated inactivation compared to HIF1 $\alpha$ , which could contribute to explaining differences in their transcriptional activities [663, 664].

### **2.3.2 Additional mechanisms of HIFs regulation**

Hydroxylation is the most prevalent and the best known mechanism of HIF1 $\alpha$  regulation, however several alternative HIF regulatory pathways have been described. The most prominent ones will be described in this part. It has been shown that the heat shock protein 90 (Hsp90) stabilized both HIFs through binding to their PAS motifs. In contrast, receptor of activated protein kinase C (RACK1) competes with Hsp90 for binding to HIF1 $\alpha$  and promotes HIF1 $\alpha$  degradation [665]. Interestingly, another HSP protein – HSP70 was shown to bind to the C-terminal part of HIF1 $\alpha$  and to cooperate with ubiquitin ligase CHIP to degrade it under prolonged hypoxia, but also under high glucose concentration [666, 667]. HIF proteins can also be regulated by phosphorylation. Indeed, HIF1 $\alpha$  activity has been is modulated by MAPK [668], casein kinase 1 (CK1) [669] and ataxia telangiectasia mutated (ATM) [670]. The activity of HIF $\alpha$  proteins is also modulated by their acetylation, which is mediated by multiple sirtuins. Sirtuins are a stress-responsive family of NAD<sup>+</sup>-dependent deacetylases that influence gene transcription, metabolism, DNA repair, and organismal life span [671]. Recent reports showed that Sirt1 interacts with both HIF1 $\alpha$  and HIF2 $\alpha$ , thus mediating their deacetylation at lysine residues. Interestingly, the effects of this modification are opposite for HIF1 $\alpha$  versus HIF2 $\alpha$ . For the latter, binding of Sirt1 creates a complex, which enhances HIF2 $\alpha$ 's transcriptional activity on the erythropoietin gene promoter [672]. In contrast, Sirt1 mediated deacetylation represses HIF1 $\alpha$  transcriptional activity. During hypoxic stress, HIF1 $\alpha$  acetylation increases because of Sirt1 NAD-mediated inactivation, thus promoting the expression of HIF1 $\alpha$  target genes [672]. Additionally, the mitochondrial deacetylase Sirt3 indirectly regulates HIF $\alpha$  stabilization. Indeed, it acts by suppressing the formation of mitochondrial (ROS) [673], thus destabilizing HIF1 $\alpha$  (reviewed in previous paragraphs). Moreover, it was observed that Sirt3-deficient cells show HIF1 $\alpha$ -dependent increase in glucose transport, glycolysis and

proliferation [673, 674]. Consistently, Sirt6 deficiency, affecting an enzyme identified as a HIF1 $\alpha$  repressor, increases HIF1 $\alpha$ -dependent glucose uptake and glycolytic activity at the expense of mitochondrial respiration [675]. Finally, an increasing number of reports demonstrate that HIF $\alpha$  proteins are subjected to numerous other post-translational modifications, including SUMOylation, S-nitrosylation, and neddylation, which mainly increase the stability of HIF $\alpha$  factors ([676–678], reviewed in [679]). All mechanisms of HIF $\alpha$  regulation are summarized in Figure 49.



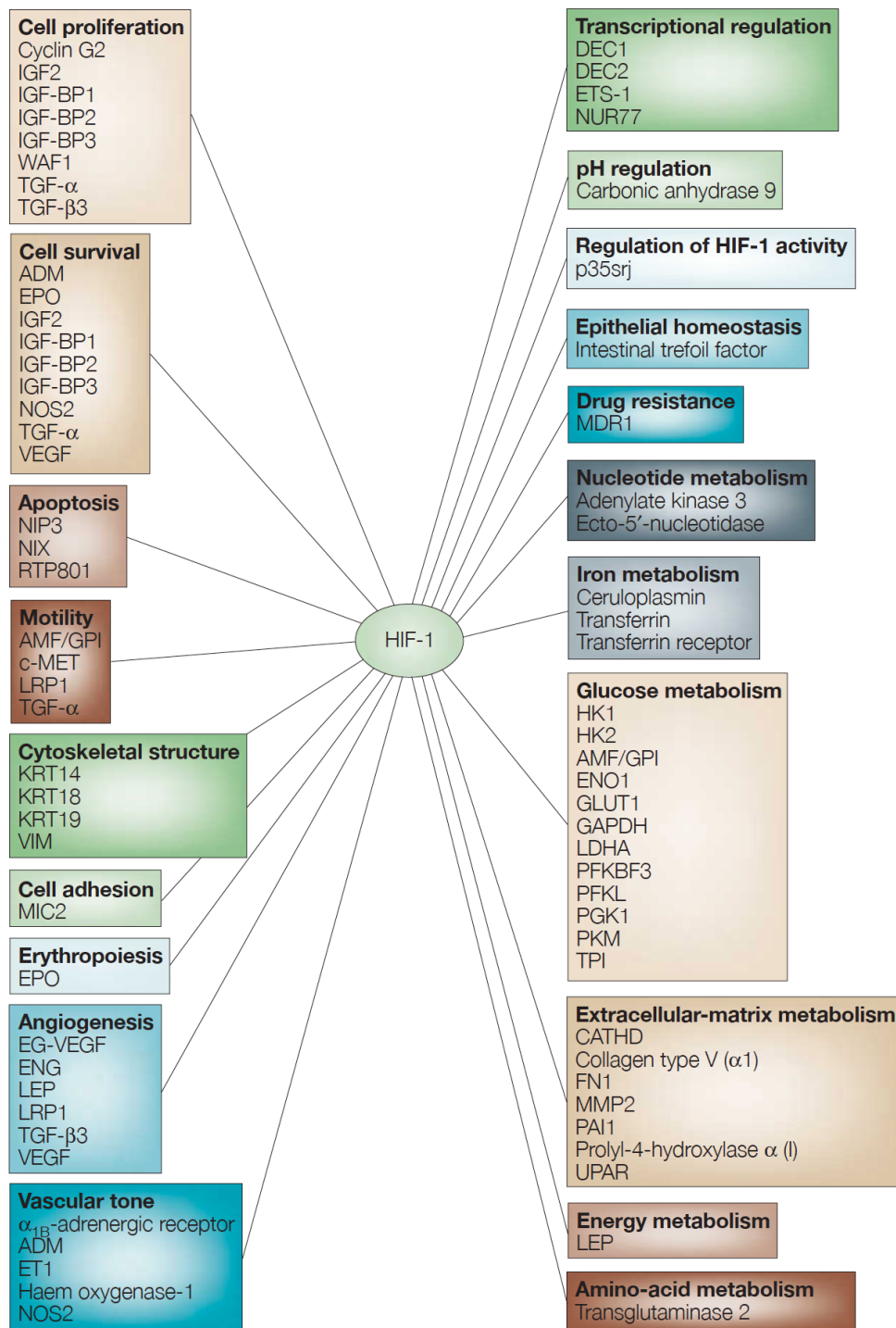
**Figure 49. Expanded model of HIF $\alpha$  proteins regulation [680].**

(A) Under physiological conditions HIF $\alpha$  is hydroxylated by PHD, which promotes its pVHL-mediated ubiquitylation and proteasomal degradation. Interestingly, PHD activity responds to various stimuli, including ROS and NO. The second step of regulation consists of FIH-mediated hydroxylation of asparagine residues, which in turn inhibits binding of HIF $\alpha$  co-activator p300/CBP. Additionally, the translation of HIF $\alpha$  mRNA is modulated through the mTOR pathway [681]. Moreover, the transcriptional activity of HIF $\alpha$  is controlled by factors such as STAT3 [682] or Sirt6 (described in the text). (B) During hypoxia, hydroxylation mediated both by PHD and FIH is inhibited, leading to stabilisation of HIF $\alpha$  and its dimerization with HIF1 $\beta$ . The active heterodimer can bind p300/CBP and PKM2 co-activators, promoting transcription of target genes [683]. In addition, HIF $\alpha$  activity may be further modulated by different factors, such as Sirt1, SUMOylation (SEN1,3), chromatin remodelling factor Pontin [684], p300/CBP-associated factor (PCAF) [685] or CBP/p300 interacting transactivator with ED-rich tail (CITED)2 and CITED4 [686, 687]. **Legend** (not mentioned in the text): NO – nitric oxide, mTOR – mammalian target of rapamycin, CBP – Creb-binding protein, STAT3 – signal transducer and activator of transcription 3, ub – ubiquitin, Elob/C – elongins B and C, Cul2 – cullin 2, Rbx 1 – RING-box protein 1, SEN1/3 – sentrin-

specific protease 1/3, PKM2 – pyruvate kinase isoform M2, hnRNPs – heterogeneous nuclear ribonucleoproteins.

## **2.4 Physiological functions of HIF $\alpha$ proteins**

A plethora of gene targets have been identified for both HIF1 $\alpha$  and HIF2 $\alpha$ . Indeed, hypoxic stress is a severe condition, which forces cells to activate or reprogram many essential mechanisms as summarized in Figure 50. The following paragraphs will focus mainly on the different activities of HIF1 $\alpha$ , as this factor is more relevant to the research topics presented in this thesis. However, some general information will be given regarding the consequences of activation of HIF1 $\alpha$  and HIF2 $\alpha$  that despite their similarities modulate some distinct cellular processes.

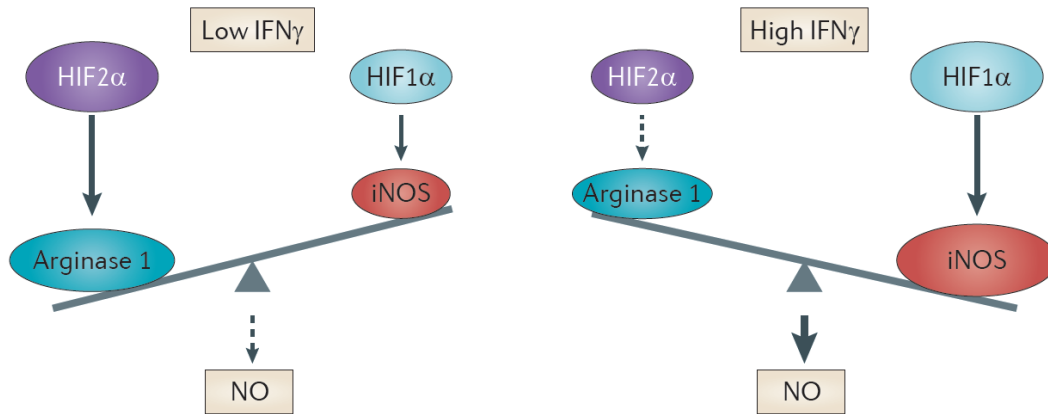


**Figure 50. Complexity and diversity of HIF1 $\alpha$  transcriptional activity [688].**

#### 2.4.1 Outcomes of HIF1 $\alpha$ versus HIF2 $\alpha$ activation

Initially only HIF1 $\alpha$  had been described as a master regulator of hypoxic response [636], since HIF2 $\alpha$  was discovered as an endothelium-specific HIF $\alpha$  isoform (EPAS-1), and as a

consequence was initially believed to have a more specialized function than HIF1 $\alpha$  [631]. However, further studies showed that both HIF $\alpha$  proteins are involved in gene regulation under hypoxic conditions in many cell types [689]. However, their specific roles are not identical and there are even examples of antagonistic outcomes of HIF1 $\alpha$  and HIF2 $\alpha$  activation [690] (Figure 51).



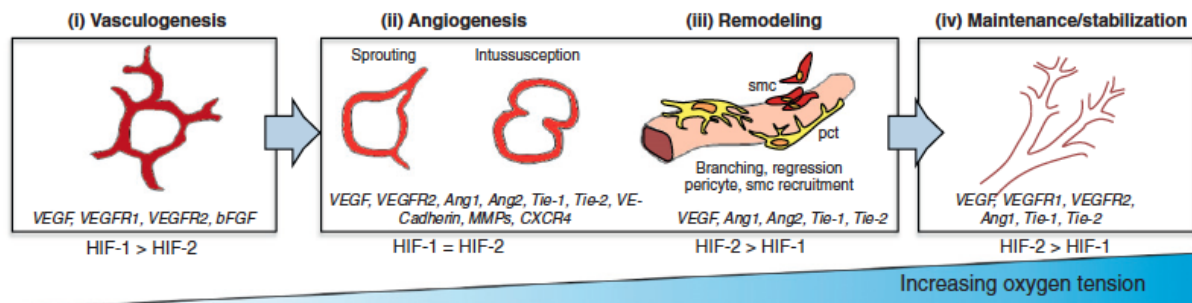
**Figure 51. HIF1 $\alpha$  and HIF2 $\alpha$  exhibit antagonistic function in respect to NO production [679].**

In conditions of low amount of IFN $\gamma$ , HIF2 $\alpha$  activity is increased, leading to an upregulation of arginase1 expression and therefore a decreased production of nitric oxide (NO). In contrast, when IFN $\gamma$  concentration is high, HIF2 $\alpha$  activity is diminished while HIF1 $\alpha$  promotes the production of NO acting on the inducible nitric oxide synthase (iNOS) [691]. These physiologically antagonistic functions allow the HIF $\alpha$  subunits to co-ordinately regulate the production of NO in a cytokine-induced and transcription-dependent fashion (reviewed in [679]).

In general, apart from some common genes such as vascular endothelial growth factor A and glucose transporter 1 (Glut1), HIF1 $\alpha$  preferentially activates genes involved in glycolysis and in apoptosis. In contrast, HIF2 $\alpha$  tends to control the expression of genes involved in stem cell phenotype and in invasion (reviewed in [679]). Of note, if one of the HIF $\alpha$  factors is missing, the other takes over the control of some of the classical targets of the missing homologue, suggesting a partial functional redundancy [692, 693]. In addition to distinct transcriptional profiles, the HIF1 $\alpha$  and HIF2 $\alpha$  exhibit different temporal activation pattern in hypoxic conditions. Affinity of PHDs and FIH1 for HIF2 $\alpha$  is lower than for HIF1 $\alpha$ , as a result HIF2 $\alpha$  is stabilised and activated at higher oxygen tensions than HIF1 $\alpha$  [662]. Moreover, differences in gene activation have been linked to the sequence diversities identified in N-terminal transactivation domains of HIF1 $\alpha$



versus HIF2 $\alpha$  [694]. The balance between HIF1 $\alpha$  and HIF2 $\alpha$  activities during hypoxic stress linked to diversities in their target genes enables the coordinate regulation of the complex physiological hypoxia-dependent processes, such as formation of the vascular (Figure 52) and skeletal systems (reviewed in [485]).



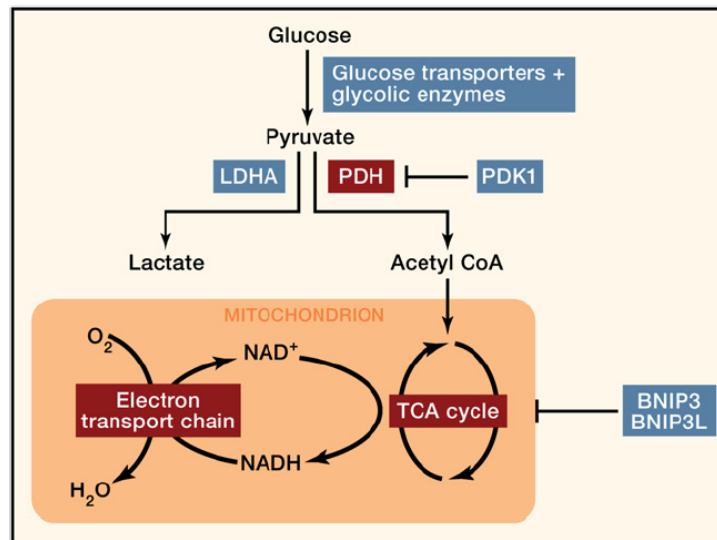
**Figure 52. Transition between HIF1 $\alpha$  and HIF2 $\alpha$  transcriptional programs during vessel formation is based on changes in oxygen tension [485].**

Vasculogenesis is mainly driven by HIF1 $\alpha$ , as it occurs under acute hypoxia. Increased oxygen concentration allows a complementary input of HIF1 $\alpha$  and HIF2 during, while vascular remodelling and stabilization phases occur in more oxygenated conditions and are mainly driven by HIF2 $\alpha$  (reviewed in [485]).

#### 2.4.2 Glucose and energy metabolism

The first challenge faced by cells under hypoxia is to survive. To do so they adapt to their new environment, notably through dramatic metabolic changes. The main source of energy in all aerobic organisms is oxidative phosphorylation, where molecular oxygen serves as electron acceptor. In hypoxia, cells switch their cellular metabolism to non-oxidative form of ATP production, that is, anaerobic glycolysis (reviewed in [648]). As the main regulator of this adaptive response, HIF1 $\alpha$  enhances glucose metabolism and decreases oxidative phosphorylation. First of all, HIF1 $\alpha$  promotes higher glucose uptake through SLC2A1 and SLC2A3 (glucose transporters Glut1 and Glut3 respectively) [695]. Then the response is shifted towards upregulation of glycolysis and gives rise to activation of genes such as hexokinases (HK), phosphofruktokinase (PFK) or 6-phosphofrukt-2-kinase/fructose-2,6-bisphosphatase (PFKFB) [695–697]. Pyruvate represents the main metabolite of glycolysis, which normally fuels mitochondrial metabolism. During hypoxia, HIF1 $\alpha$  inhibits the flux of pyruvate into acetyl-CoA through

pyruvate dehydrogenase kinase 1 (PDK1) [698, 699]. This process diverts metabolism from mitochondria and in consequence shuts down oxygen consumption (reviewed in [700, 701]). Accumulated pyruvate is converted by lactate dehydrogenase A (LDHa) into lactate, which in turn is transported out of the cell through monocarboxylate transporter 4 (MCT4). Both genes are upregulated by HIF1 $\alpha$  [695, 702] (Figure 53).



**Figure 53. Regulation of glucose metabolism by HIF1 $\alpha$  [703].**

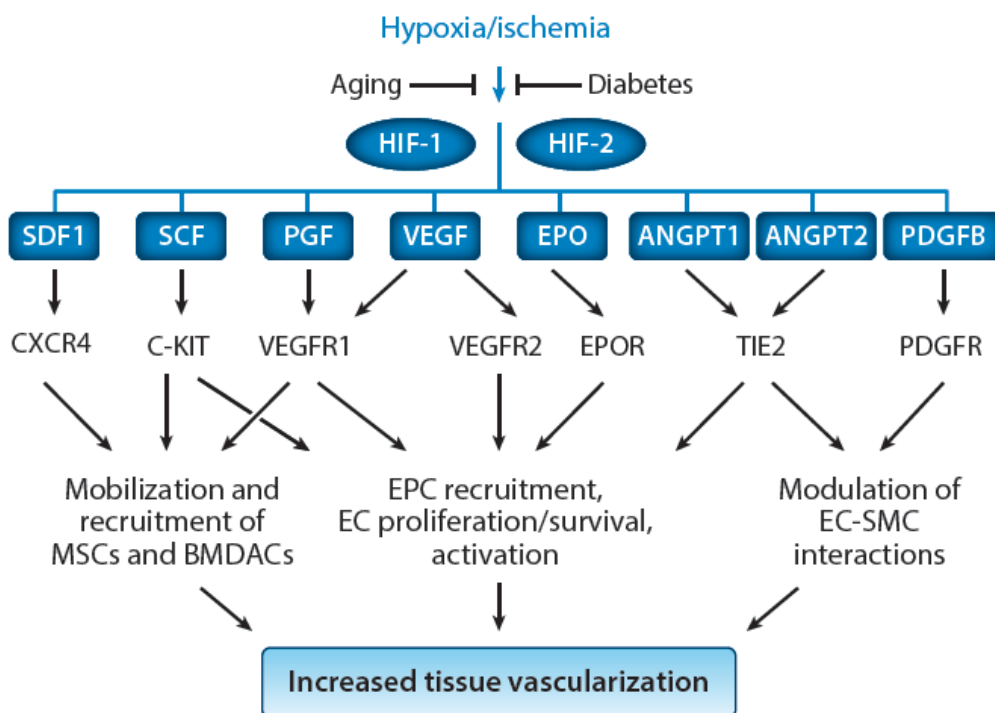
HIF1 $\alpha$  activates the expression of glycolytic genes, which counteracts deficits in nutrient supplies, as encountered in hypoxia. Induced PDK1, inactivates pyruvate dehydrogenase (PDH), the mitochondrial enzyme that converts pyruvate to acetyl CoA for entry into the tricarboxylic acid (TCA/citric acid/Krebs) cycle. Conversion of pyruvate into lactate is controlled by LDH, lactate is then exported into the extracellular milieu by specific transporters. HIF1 $\alpha$  also triggers mitochondrial-selective autophagy through induction of BNIP3 and BNIP3L proteins. Finally, downregulation of mitochondria biogenesis reduces energy yield, but prevents excess ROS production that occurs due to inefficient electron transport under hypoxic conditions (reviewed in [703]).

Remarkably, lactate produced by hypoxic cancer cells can be taken up by non-hypoxic cells and used as a respiratory substrate [704]. HIF1 $\alpha$  possesses also a modulatory function in the respiratory chain. More precisely, HIF1 $\alpha$ , by orchestrating a subunit switch (from COX4I1 to COX4I2) in cytochrome *c* oxidase, increases the efficiency of electron transfer to O<sub>2</sub>. This is important because it allows cells' respiration in environments with limited oxygen supply without excessively increasing ROS levels [705]. Adaptation to hypoxic stress can be additionally strengthened by HIF-dependant

upregulation of BH3 domain protein BNIP3, which triggers selective mitochondrial autophagy and therefore protects cells from mitochondrial ROS under hypoxia [706].

### 2.4.3 HIF and vascular response to hypoxia

HIF $\alpha$  proteins are major actors of embryonic vascular development [707, 708]. During adulthood, pro-angiogenic properties of HIF $\alpha$  subunits still play an important role, as they are involved in both physiological and pathological processes, such as wound healing and response to limb ischemia or myocardial infarction [709–712]. Ischemic conditions are characterized by deficits in blood supply and in consequence lead to nutrient, including oxygen, deprivation. The essential HIF1 $\alpha$  function during ischemia-related hypoxia is an activation of angiogenic factors, such as VEGF, stromal cell-derived factor 1 (SDF1), angiopoietin 2, placental growth factor (PGF) or platelet-derived growth factor B (PDGFB) [709, 713–716] (Figure 54).



**Figure 54. Hypoxia inducible factors as master regulators of ischemia-induced vascularization [717].**

Ischemia induced hypoxia activates HIF genes leading to expression of various angiogenic cytokines and growth factors. These bind to their cognate receptors to mediate their biological effects that promote tissue vascularization. **Legend:** BMDAC – bone marrow-derived angiogenic cell, EC – endothelial cell, EPC

- endothelial progenitor cell, EPOR – EPO receptor, MSC – mesenchymal stem cell, PDGFR – PDGF receptor, SMC – smooth muscle cell, VEGFR – VEGF receptor.

Finally, to underline the importance of HIF1 $\alpha$  involvement in angiogenic processes, we should mention that HIF1 $\alpha$ -KO mouse embryos fail in proper development of vasculature [695, 718]. Apart from angiogenesis, both HIF proteins are involved in erythropoiesis throughout the regulation of erythropoietin gene, which encodes a crucial hormone responsible for survival and differentiation of erythroid progenitor cells [630, 716, 719, 720].

## **2.5 Hypoxia inducible factors and cancer**

HIF factors are often upregulated in cancer and their activation correlates with poor prognosis in a large set of solid tumours, including pancreatic, cervical, colorectal and ovarian cancer (reviewed in [679]). Interestingly, expression of HIF1 $\alpha$  in neuroblastoma and renal cell carcinoma is rather a favourable prognostic factor [679, 721, 722]. In the following part of this thesis the mechanisms of HIF induction during oncogenesis and its consequences will be discussed.

### **2.5.1 Mechanism of HIF factors activation during cancer progression**

Numerous molecular mechanisms, involving the responses of tumour microenvironment and the impact of genetic alterations, contribute to the increased HIF activity in human cancers.

#### **2.5.1.1 Hypoxia and environmental factors**

Cancer cells are characterized by rapid, uncontrolled growth, which clearly requires sufficient nutrient and oxygen supply. When a tumour grows beyond a critical size, its growth is thwarted unless its own blood vessel network is established. Importantly, newly generated tumoral blood vessels are functionally impaired, due to the lack of precise control mechanism of vascular system such as inadequate coverage with pericytes [723]. Furthermore, because of these abnormalities in vascular vessels, distant areas are still predisposed to hypoxia and metabolic stress. That can eventually lead to cell necrosis in these areas. Importantly, when hypoxia within the tumour

microenvironment is coupled with HIF1 $\alpha$  mediated adaptation, cancer cells gain potential driving capabilities towards malignant progression and therapy resistance, which ultimately limits the patient's chance of survival [724, 725]. The functional relevance of hypoxia to malignant progression was nicely demonstrated by Amato Giaccia's group, providing experimental evidence for a crucial role of hypoxia in the generation of tumour cell subpopulations with diminished apoptotic sensitivity due to inactivation of p53 [726]. Of note, tumour-associated hypoxia is not the only mechanism responsible for HIF induction during tumorigenesis. Among others, some of the most important mechanisms implicate ROS and RNS. As mentioned before, oxidative stress promotes HIF factor stabilization through different mechanisms, e.g. an alteration in mitochondrial metabolism (reviewed in [727]). In addition, in the case of nitric stress, upregulation of nitric oxide synthase and its production have been shown to increase HIF1 $\alpha$  stability in human oral squamous cell carcinoma [728].

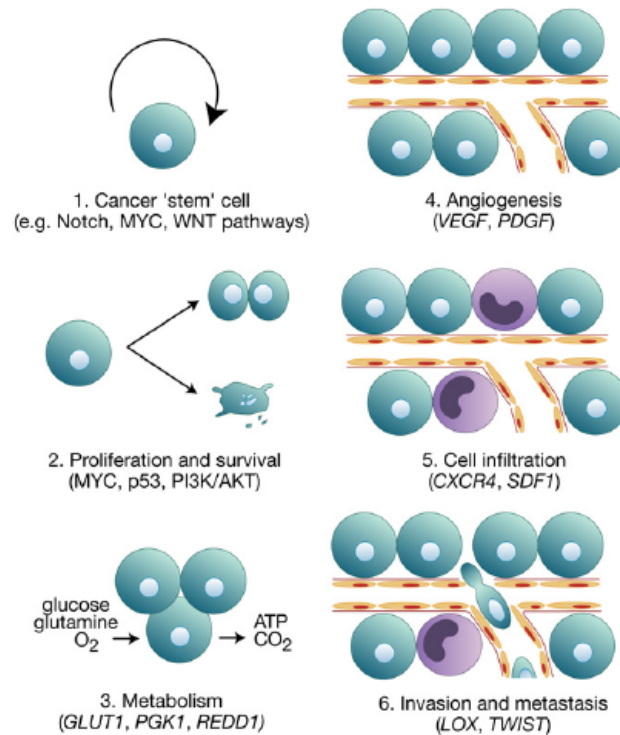
#### **2.5.1.2 Genetic alterations**

Various genetic alterations, including both oncogene gain of function and tumour-suppressor gene loss of function, can result in HIF $\alpha$  stabilization independently of oxygen concentration. For example, loss of the tumour suppressor pVHL increases the stability of HIF $\alpha$  subunits within normoxic environment (reviewed in [729]). Another classical mechanism of HIF $\alpha$  induction appears to operate via deregulation of the mammalian target of rapamycin (mTOR), which can also be activated by gain of function mutation in tyrosine kinases (e.g. epidermal growth factor receptor, HER2/neu, Ras, and/or the downstream PI3K/Akt pathway ([730, 731], reviewed in [727]). mTOR is a serine–threonine protein kinase that phosphorylates ribosomal protein S6 kinase and eIF4E binding protein 1, which in turn increase the rate of translation of HIF1 $\alpha$  mRNA into protein [731]. In addition, HIF factors may be stabilized by deregulation of mitochondrial metabolism. Loss of function mutations in genes encoding enzymes of mitochondrial tricarboxylic acid cycle cycle, such as succinate dehydrogenase (SDH) or fumarate hydratase (FH), lead to increased levels of the metabolic substrate of the enzyme – succinate and fumarate, respectively. Increased level of this TCA intermediates inhibit PHD2 by competing with  $\alpha$ -ketoglutarate for binding to its catalytic centre,

thereby reducing hydroxylation, ubiquitylation, and proteasomal degradation of HIF1 $\alpha$  [732–735].

### 2.5.2 HIF mediated tumour progression

The following paragraphs will characterize the most classical tumorigenic activities of HIF proteins, summarized in Figure 55.



**Figure 55. Impact of HIF activation on multiple aspects of cancer development [736].**

#### 2.5.2.1 Metabolic reprogramming

Hypoxic stress, often observed in solid tumours, drives HIF1 $\alpha$ -mediated metabolic reprogramming. Importantly, this includes increased glucose uptake, decreased oxygen consumption, increased flux from glucose to lactate and decreased flux to acetyl coenzyme A. These metabolic changes may also occur in normoxic conditions, following constitutive HIF $\alpha$  activation, for example due to VHL loss of function [64, 737, 738]. Another hallmark of metabolic reprogramming of cancer cells is the ratio of intracellular and extracellular pH. More precisely, cancer cells can increase the intracellular pH and decrease the extracellular pH, a situation that favours cancer progression (reviewed in

[739]). These changes in acid–base balance are mediated by HIF1 $\alpha$  through transactivation of the carbonic anhydrase 9 (CAIX), monocarboxylate (lactate) transporter 4 (MCT4) and sodium–hydrogen exchanger 1, (NHE1) genes, all of which are responsible for the maintenance of ion balance (reviewed in [740, 741]).

#### **2.5.2.2 Invasion and metastasis**

As discussed earlier epithelial-to-mesenchymal transition is a key feature of invasive cells and can be characterized by the loss of epithelial cell-cell contact together with acquisition of mesenchymal features and motility. Hypoxia and HIF $\alpha$  subunits influence the expression of many EMT regulators, thus promoting metastasis. For example, HIF can mediate activation of two master regulators of EMT and metastasis processes: Snail and Twist. In the case of Twist, HIF is able to directly induce its expression [426] and for Snail, HIF1 $\alpha$  promotes its nuclear localization [742]. Apart from activation of EMT inducers, HIF is also involved in extracellular matrix remodelling, for example through induction of lysyl oxidase (LOX) [743–746]. Moreover, the process of invasion into a tumour blood vessel (intravasation) is facilitated by increased vessel permeability that is triggered by VEGF [747] and angiopoietin 2 [748]. For example, cancer cell metastasis into lungs is augmented through expression of L1 cell adhesion molecule and angiopoietin-like 4, which decrease endothelial cell–cell interactions and thereby facilitate invasion through the vessel wall and into the lung parenchyma [749]. These data highlight the manner in which the hypoxic tumour microenvironment promotes metastasis via the activation of multiple HIF-responsive genes at all stages of cancer spread, including invasion, intravasation, and distant extravasation.

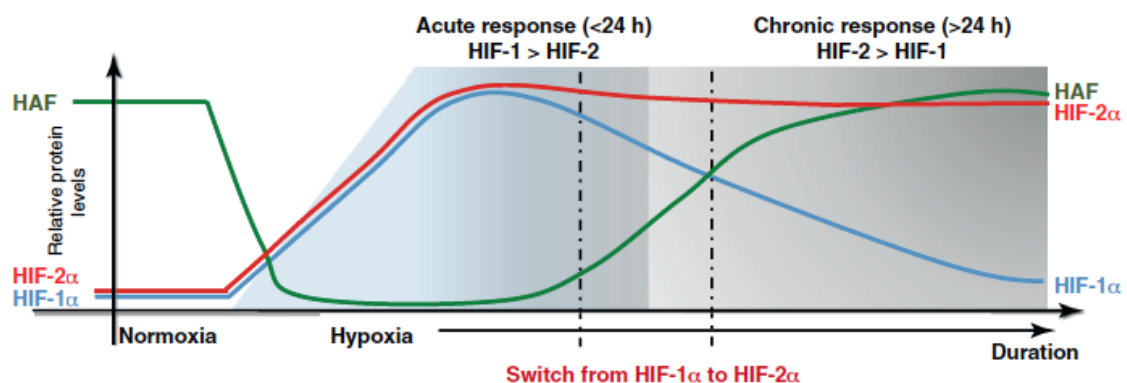
#### **2.5.2.3 Cancer stem cells**

Another important feature of cancer cell response to hypoxia is the dedifferentiation of cancer cells toward a stem and progenitor cell phenotype [750, 751]. Next, hypoxia and HIFs may contribute to the maintenance of putative cancer “stem” cells. HIF1 $\alpha$  and HIF2 $\alpha$  both promote the hypoxia-induced undifferentiated phenotype by activating the Notch pathway and inducing the transcription of other stem-cell-specific factors [752]. HIF2 $\alpha$  depletion in CD133+ (GBM stem cell marker) glioblastoma (GBM) cells reduces these cells' tumorigenic and angiogenic potential *in vitro* and *in vivo* [753]. On the other

hand, HIF2 $\alpha$  seems to be required for their survival [753]. Interestingly, the requirement of HIF1 $\alpha$  for the maintenance of the undifferentiated phenotype in GBM stem cells is apparently limited to hypoxic conditions [754]. However, further studies are needed to clarify the involvement of HIF1 $\alpha$  and HIF2 $\alpha$  in normal and malignant stem cell survival.

### 2.5.3 HIF switch and differential effect of HIF $\alpha$ proteins

Differences of HIF $\alpha$  factors regulation were already mentioned. Recent studies confirmed previous observations by describing HIF switches, and more precisely the mechanisms that alter HIF $\alpha$  isoform dependency (reviewed in [485]). On the one hand, Sirt1 increases HIF2 $\alpha$  activity in hypoxia by deacetylating the protein [755, 756]. On the other hand, Sirt1 also mediates transcriptional inactivation of HIF1 $\alpha$  [755, 756]. Another part of this interesting regulation depends on the hypoxia-associated factor (HAF). Initially, this factor was described as a HIF1 $\alpha$  specific E3 ligase, which catalyses its ubiquitination and leads to its proteasomal degradation [757]. The same group later reported that in contrast to its effect on HIF1 $\alpha$ , HAF increased the transcriptional activity of HIF2 $\alpha$  independently of its E3 ligase activity [758]. Additionally, it is known that HIF1 $\alpha$  predominantly controls cellular reaction to an acute hypoxia, whereas HIF2 $\alpha$  drives the response to chronic oxygen shortage [759]. This observation is in accordance with the fact that HAF levels decrease during acute hypoxia, but increase in prolonged hypoxia [757, 758] (Figure 56).

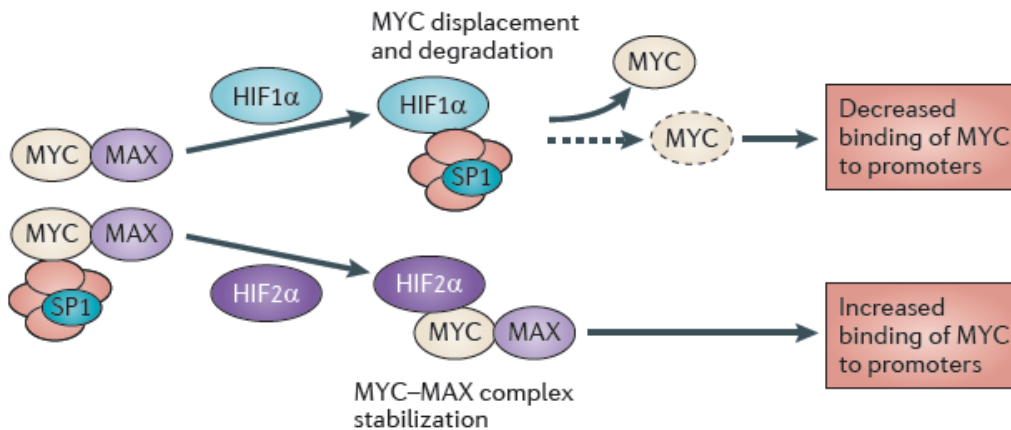


**Figure 56.** HAF-dependent switch between HIF1 $\alpha$  and HIF2 $\alpha$  during acute and chronic hypoxia (more detailed description in previous paragraph) [485].



### 2.5.3.1 HIF factors and Myc oncogene

Myc proteins are overexpressed in more than 40% of human cancers and are potent promoters of cell proliferation and growth (reviewed in [485]). How do such strong oncogenes behave under prolonged hypoxia, which is with associated growth inhibition? These opposite predicted molecular outcomes raised the question of possible interactions between those two prominent factors (Figure 57).



**Figure 57. Distinct actions of HIF1α and HIF2α on Myc complex formation and promoter occupancy [679].**

Indeed, it turns out that HIF1α activation during hypoxia produces cell cycle arrest by inhibiting of Myc [760]. Several molecular mechanisms can account for this effect. First, HIF1α binds to SP1 and can displace Myc from promoters of multiple target genes [761]. Second, HIF1α can disrupt Myc-Max dimerization, inhibiting its transcriptional activity [648]. Finally, HIF1α promotes Myc degradation under hypoxic conditions [64, 762]. Interestingly, transformed cells expressing HIF2α showed an enhancement in MYC activity [648]. In contrast to HIF1α, HIF2α promotes cell cycle progression in hypoxic cells through transcriptional effects on both MYC-activated (such as CCND2 and E2F1) and MYC-repressed (such as CDKN1A and CDKN1B) target genes. Just like HIF1α, HIF2α can interact with MAX and SP1 ([648], reviewed in [679]), but the resulting outcomes of these interactions are very different. In fact, the overall picture is even more complex. Indeed, in as yet unexplained manner, HIF1α can also cooperate with MYC in the control of expression of a limited set of specific target genes, namely, the glycolytic enzymes and VEGFA [763]. Finally, high levels of N-Myc have been reported to override the

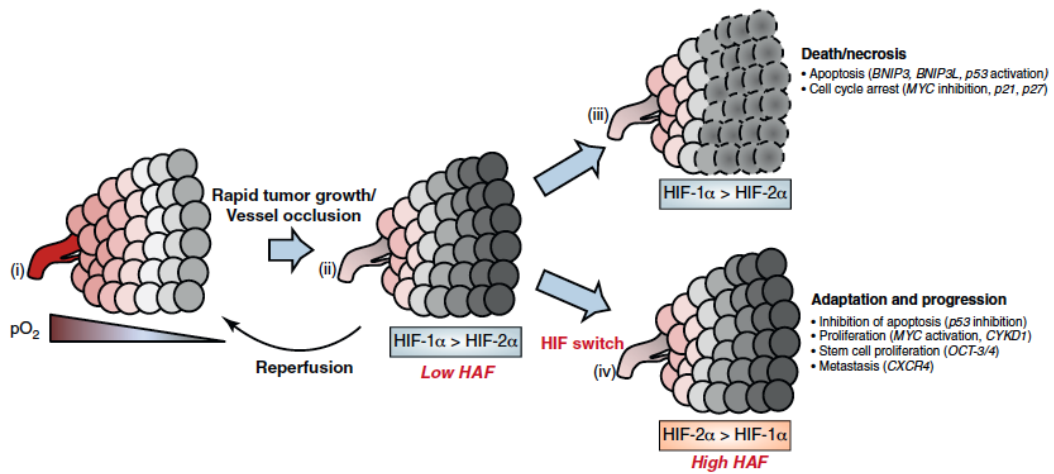
HIF1 $\alpha$ -mediated inhibition of cell cycle progression [764]. This observation may explain the anti-tumorigenic potential of HIF1 $\alpha$  in renal cell carcinoma, as tumours with lower Myc levels are more susceptible to HIF1 $\alpha$ -mediated growth inhibition.

### ***2.5.3.2 HIF $\alpha$ proteins and p53 tumour suppressor***

Similarly to other stressful condition, hypoxic stress leads to p53 stabilization and activation [765]. HIF1 $\alpha$  and HIF2 $\alpha$  have opposing effects on the p53 pathway. Numerous studies have shown that p53 accumulation occurs within hypoxic regions of solid tumours and correlates with apoptosis, although this might happen only when accompanied by acidosis and nutrient deprivation [766]. Indeed, HIF1 $\alpha$  can stabilize p53 protein either through direct interaction [767, 768] or by interaction with MDM2, leading to inhibition of MDM2-dependent ubiquitylation and degradation of p53 [769]. Conversely, HIF2 $\alpha$  seems to inhibit p53 indirectly by multiple mechanisms. First, HIF2 $\alpha$  was shown to inhibit p53 phosphorylation and stabilization [770] and second, HIF2 $\alpha$  can indirectly regulate the MDM2 pathway [771]. More precisely, HIF2 $\alpha$ -induced growth factors, such as TGF $\alpha$ , can stimulate a growth factor receptor–Akt–MDM2 pathway that promotes p53 inhibition.

### ***2.5.3.3 Conclusions***

The examples discussed in this section highlight differential effects of HIF1 $\alpha$  and HIF2 $\alpha$  on tumour development. HIF1 $\alpha$ -mediated growth inhibition is part of the cellular response to insufficient nutrient supply and thus deficient energy production under hypoxic conditions in solid tumours. HIF1 $\alpha$  favours metabolic reprogramming, which provides an important adaptation mechanism for cancer cell survival and progression. In contrast, HIF2 $\alpha$  promotes cancer cell proliferation through cooperation with Myc or inhibition of p53. This suggests that both HIF factors have distinct roles during cancer progression. Moreover, HIF switch (controlled by the level of HAF) can induce sequential cooperation between HIF $\alpha$  factors, which in consequence will allow tumour adaptation to hostile environment followed by tumour progression and expansion (Figure 58).



**Figure 58. Effect of the “HIF switch” on the process of tumorigenesis [485].**

Acute hypoxia causes stabilization of both HIF $\alpha$  proteins, however the main transcriptional response is orchestrated by HIF1 $\alpha$ . If a low oxygen supply persists, the level of hypoxia-associated factor (HAF) will increase, favouring HIF2 $\alpha$  stabilization and thus the HIF switch. The HIF switch allows sequential tumour adaptation, proliferation and progression. In an unfavourable genetic background, HIF1 $\alpha$  may remain dominant over HIF2 $\alpha$ . In that case, despite the initial adaptation to stress, HIF1 $\alpha$  response suppresses cancer development through cell cycle arrest and apoptosis (reviewed in [485]).

## OBJECTIVES

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Despite impressive progress in understanding molecular and cellular mechanisms of oncogenesis, most cancers are still incurable. Each cancer is a unique entity composed of tumour and stromal cells that interact with a continuously changing environment. This creates selective pressures that push cancer cells towards constant adaptation and evolution. Cellular stress is a cornerstone of these processes and its impact on tumour progression merits to be investigated with particular attention.

The role of Twist transcription factors in tumorigenesis was initially believed to consist of their classical role as members of the EMT-inducing transcription factor family. A little later, a closer look at Twist proteins and their oncogenic activities revealed their role in protecting cancer cells from two major barriers of tumorigenesis, namely apoptosis and senescence. This discovery placed Twist among factors involved in cellular adaptation to stress during tumorigenesis. It also provided the general framework of my PhD thesis, whose main objective was to unveil new cellular activities of Twist and understand their role in tumorigenesis.

Previous work in the laboratory led to a discovery of an unexpected activity of Twist proteins, namely its role as a regulator of oxidative stress. While ROS, as by-products of oxidative phosphorylation, are generated in all aerobic cells, cancer cells suffer from exacerbated oxidative stress caused by multiple stimuli: inflammation, increased cellular metabolism, chemo- or radiotherapy. Elevated levels of ROS are harmful both for healthy and for transformed cells. Indeed, excessive production of these highly reactive molecules leads to oxidative damage of DNA, proteins and lipids and is mutagenic or lethal at higher doses. Initial data obtained in the laboratory showed that Twist diminishes cellular ROS levels and thus participates in the escape from apoptosis, a major cellular response to severe damage. In the first part of my thesis work, I continued this project, initiated by a previous PhD student, by uncovering the molecular mechanisms responsible for the antioxidant activity of Twist.

The specific objectives for this part of the study were:

- *To evaluate the consequences of the antioxidant activity of Twist oncoproteins.* For that I have mainly investigated the link between Twist's antioxidant properties and its control of cell death.

- *To identify target genes responsible for Twist antioxidant and anti-apoptotic activities.* In order to do so, a microarray analysis was performed that allowed the identification of sets of anti- and pro-oxidant genes regulated by Twist. The microarray data was confirmed by RTqPCR, selected candidates were studied in functional assays.

This study provided new insights into Twist biology and in particular shed light on its novel biological activity in oncogenesis (article 1).

In the second part of my thesis work, I concentrated on a protective role of Twist in an additional stressful setting, namely its role in cellular stress caused by a limited oxygen supply (hypoxia). Hypoxia is a common feature of solid tumours, where cells suffer from lack, or insufficient, blood flow, limiting the amount of oxygen needed to sustain efficient oxidative phosphorylation. Hypoxia leads to stabilization and activation of HIF1 $\alpha$ , which is a key transcriptional mediator of the hypoxic response. HIF1 $\alpha$  regulates expression of numerous genes involved in glucose metabolism, oxygen transport, angiogenesis, cell growth, invasion and apoptosis. These pleiotropic regulatory functions make HIF1 $\alpha$  a very potent oncogene. Indeed, elevated levels of HIF1 $\alpha$  in tumours correlate with poor patient's prognosis. Interestingly, Twist was reported to be a transcriptional target of HIF1 $\alpha$  in some cell types. The second objective of my thesis was to define and characterize the possible interplay of Twist and HIF1 $\alpha$  in the context of tumorigenesis. The specific objectives for this part of my PhD project were:

- *To establish if Twist is involved in adaptation of cancer cells to hypoxic stress.* In order to do so, I either abrogated or on the contrary, increased, Twist expression in hepatic cancer cells and studied their response to hypoxia.
- *To characterize mechanisms by which Twist protects cells from hypoxic stress.* First, I have tested the physical interactions between Twist and HIF1 $\alpha$  proteins. Having demonstrated that these two transcriptional regulators are part of the same molecular complex, I then investigated the genetic expression profiles of cells expressing each protein separately or both together.

Our data shed a new light on the mechanistic bases of cellular adaptation to hypoxia and highlight the importance of Twist as a major regulator of cellular stress response in the course of tumorigenesis (manuscript of the article 2).





## RESULTS

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# ARTICLE I

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## **Modulation of Oxidative Stress by Twist Oncoproteins**

Nicolas Floc'h\*, Jakub Kolodziejski\*, Leila Akkari, Yannick Simonin, Stephane Ansieau, Alain Puisieux, Urszula Hibner, Patrice Lassus

\* - These authors contributed equally to this work.

### **1 Introduction**

Twist proteins are potent regulators of cell fate, crucial during embryonic development and often reactivated in various cancers. However, the current state of knowledge does not provide us with a full mechanistic explanation for diverse pleiotropic roles that Twist play in various situations. Thus, our goal was to investigate whether Twist proteins possess other, yet undiscovered molecular activities important for their physiological and pathophysiological roles. Initially we focused our research on reactive oxygen species as their actions overlap with known Twist properties. For example, ROS are able to trigger the induction of the same cellular failsafe programs [620, 772] that are overridden by Twist proteins. This might mean that Twist proteins may somehow impact the ROS regulatory properties. Furthermore, Twist anti-apoptotic properties were discovered through a genetic screen aimed at identifying genes that counteracted c-Myc induced apoptosis [352]. Interestingly, several subsequent reports showed that generation of ROS accompanied c-Myc overexpression and was linked to the associated cell death phenotype [772]. Therefore, we further hypothesized that Twist proteins might be involved in the regulation of ROS levels. This was later confirmed by a number of preliminary experiments.

When I arrived in the laboratory, this study was already well advanced, thanks to the work of a PhD student Nicolas Floc'h. At that point, the importance of Twist in the

regulation of ROS levels was confirmed. Moreover, Nicolas showed that Myc-induced apoptosis occurs via an initial burst of ROS. This process could be modulated by Twist's genes expression. To gain further insight on this research project, we aimed at unveiling the molecular mechanism responsible for Twist antioxidative properties. Initial experiments showed that none of the classical detoxifying enzymes (Catalase, MnSOD, Cu/ZnSOD, Gpx1) that we tested, were significantly modulated in presence of Twist, at least at the mRNA level. In order to investigate alternative mechanisms, a series of experiments was designed:

1. Firstly, we intended to uncover genes linked to Twist mediated antioxidative properties. In order to identify these genes, a micro-array approach comparing cells with up-regulation of Twist1 to control cells, was used.
2. Next, the expression mRNA levels of preselected genes (known to be regulators of ROS), upon Twist overexpression and silencing were verified. Additionally, their Redox regulatory potential in our model system was determined.
3. Finally, we already demonstrated that Twist antioxidative properties protect cells from c-Myc-induced apoptosis. Therefore, modulation of the validated target genes was tested for their ability to mimic Twist anti-apoptotic properties.

Obtaining these data was indispensable in order to propose and further validate a molecular mechanism governing this newly identified, antioxidative activity of Twist.

# Modulation of Oxidative Stress by Twist Oncoproteins

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## Abstract

Expression of developmental genes Twist1 and Twist2 is reactivated in many human tumors. Among their oncogenic activities, induction of epithelial to mesenchymal transition is believed to increase cell motility and invasiveness and may be related to acquisition of cancer stem cell phenotype. In addition, Twist proteins promote malignant conversion by overriding two oncogene-induced failsafe programs: senescence and apoptosis. Reactive oxygen species (ROS) are also important mediators of apoptosis, senescence and motility and are tightly linked to disease, notably to cancer. We report here that Twist factors and ROS are functionally linked. In wild type cells both Twist1 and Twist2 exhibit antioxidant properties. We show that Twist-driven modulation of oncogene-induced apoptosis is linked to its effects on oxidative stress. Finally, we identify several targets that mediate Twist antioxidant activity. These findings unveil a new function of Twist factors that could be important in explaining their pleiotropic role during carcinogenesis.

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## Introduction

The two Twist genes (Twist1 and Twist2), related in sequence and function, belong to the super-family of basic Helix-Loop-Helix (bHLH) transcription factors. They are essential for embryonic development as they are involved in mesoderm patterning and the differentiation of multiple cell lineages, including muscle, cartilage and osteogenic cells [1]. Their embryonic functions appear to be related to their ability to induce cell migration by promoting the epithelial to mesenchymal transition (EMT) [2], to protect cells from apoptosis [3] and to control inflammation [4]. Twist expression was reported in several types of precursors and stem cells [5,6], where it was suggested to control the maintenance of their pluripotency. In contrast, Twist proteins are not detectable in most differentiated tissues [7], but their expression is reactivated in many human cancers, including carcinomas (breast, liver, ovarian, prostate), sarcomas, melanomas, gliomas and neuroblastomas [8]. Their oncogenic potential is thought to arise from a combination of multiple properties. First, by promoting the epithelial to mesenchymal transition (EMT), Twist proteins favour invasiveness and may confer to cells self-renewal properties [9,10,11,12]. Second, by disrupting both Rb- and p53-dependent pathways, they override the two major oncogene-induced cellular failsafe programs, which are senescence and apoptosis [13,14,15], thereby promoting malignant conversion. Third, several reports describe a

role for Twist1 in the acquisition of resistance to therapeutic drugs [16,17], linking Twist to tumor recurrence.

Reactive oxygen species (ROS), which include superoxide anions, H<sub>2</sub>O<sub>2</sub>, organic peroxides, and hydroxyl radicals, are by-products of oxidative phosphorylation and as such are constantly generated in all aerobic cells. ROS are eliminated by antioxidant enzymes: scavenger enzymes, superoxide dismutases (SOD), convert superoxide anions to H<sub>2</sub>O<sub>2</sub>, which is subsequently detoxified by catalase, glutathione peroxidase or thioredoxin peroxidase systems [18]. ROS, although essential for processes such as immune response, angiogenesis and stem cell renewal [19], can also be harmful for cells. An uncontrolled production of these molecules provokes damage, including DNA lesion, protein oxidation, and lipid peroxidation [19]. Cellular response to major unrepaired damage includes apoptosis or senescence. Because Twist and ROS overproduction both impact on activities related to the regulation of several common cellular processes [13,15], we explored the possibility that Twist is involved in the control of ROS and, more generally, in cellular response to oxidative stress.

We report here that Twist lowers the level of intracellular ROS, displaying an antioxidant activity in several cell types. Twist-driven inhibition of oxidative stress participates in the protection of cells against c-Myc induced apoptosis. Analysis of molecular mechanisms involved in this new function reveals the existence of an oxidative genetic program mediated by several new targets. Our

findings unveil a novel property of Twist proteins that could have important implications for their role during tumor progression.

## Results

### Twist proteins display antioxidant properties

To investigate the effect of Twist proteins on ROS production, we first generated foreskin-derived human diploid fibroblasts (HDF) ectopically expressing Twist1 or Twist2. The cells displayed no noticeable morphological changes and grew at a similar rate to both parental and control cells (data not shown). As assessed by labeling cells with DiHydroEthidium (DHE) (Figure 1A–C) or with DiChlorodihydroFluorescein DiAcetate (DCFDA) (Figure S1A–B), two ROS fluorescent indicators, ectopic expression of either Twist1 or Twist2 was associated with a significant reduction of intracellular ROS. Expression of Twist was as efficient in inhibiting ROS as was the treatment of cells with 10 mM N-acetyl-cysteine (NAC), a widely used antioxidant compound (Figure S1C–D). The antioxidant property of both Twist proteins was next confirmed using additional cellular models, including primary Mouse Embryonic Fibroblasts (MEFs) (Figure 1D–F), immortalized Rat Embryonic Fibroblasts (REF52) (Figure 1G–I) and immortalized human mammary epithelial cells (HMEC-hTert) (Figure S1E). These results indicate that the antioxidant activity of Twist is neither cell type- nor species-dependent.

We reasoned that since ectopic expression of Twist diminished ROS levels, inhibition of endogenous Twist expression should give rise to the opposite effect. Since HDFs mainly express Twist2 (data not shown), we explored the consequences of its depletion in these cells. This was achieved through the use of either siRNA or shRNA that recognized unrelated sequences of Twist2 mRNA. Both gave rise to inhibition of Twist2 expression and, in accord with our hypothesis, both led to a significant increase of endogenous ROS (Figure 2). Of note, this increase was reverted by NAC (Figure S2). Overall, our results strongly suggest that Twist proteins exhibit antioxidant properties in several cellular models.

### Twist, ROS and c-Myc-induced apoptosis

As ROS drive induction of the same cellular failsafe programs [20,21] that are overridden by Twist proteins, we assessed whether the newly identified antioxidant property of Twist had an impact on its oncogenic potential. In light of the role of ROS in c-Myc induced cell death and transformation [21,22,23], we focused our attention on Twist's anti-apoptotic activity, which was originally discovered through a genetic screen aimed at identifying genes that counteracted c-Myc induced apoptosis [14]. Interestingly, several subsequent reports showed that generation of ROS accompanied c-Myc overexpression and was linked to the cell death phenotype, as well as to DNA damage, genetic instability and cellular transformation [21,22,23].

To investigate whether the anti-apoptotic function of Twist is functionally related to its antioxidant properties, we ectopically expressed a tamoxifen inducible c-Myc-ER fusion construct in rat embryonic fibroblasts (REF52), the cell type that was used to unveil the apoptotic activity of c-Myc [24]. We first confirmed that c-Myc activation sensitized REF52 cells to apoptosis upon growth factor withdrawal (Figure 3A). Interestingly, these conditions are associated with a significant increase in endogenous ROS production (Figure 3B–C) provoked mainly by the removal of serum (Figure S3A). To evaluate whether ROS production is instrumental in c-Myc induced cell death in this model, cells were cultured in the presence of Tiron, an efficient antioxidant compound [25]. Of note, unlike NAC, Tiron did not display

any cytotoxic effects in REF52. As shown in Figure 3D–F, reduction of ROS by this antioxidant compound was associated with improved cell survival, confirming the reported role played by ROS in c-Myc-induced apoptosis [26]. As expected, we found that ROS induction was necessary but not sufficient to induce a strong apoptotic response in this model (Figure S3B).

As previously described, ectopic expression of Twist proteins protected cells from c-Myc-induced apoptosis [14] upon growth factor withdrawal (Figure 3G). Strikingly, the increased survival was associated with a significant reduction in ROS (Figure 3H–I, Figure S3A), suggesting that the antioxidant property of Twist is indeed related to its oncogenic potential. Comparable results were obtained in experiments performed using mouse embryonic fibroblasts (MEFs) (Figure 4).

We conclude that Twist-mediated inhibition of apoptosis is accompanied by the repression of either the production or the accumulation of reactive oxygen species.

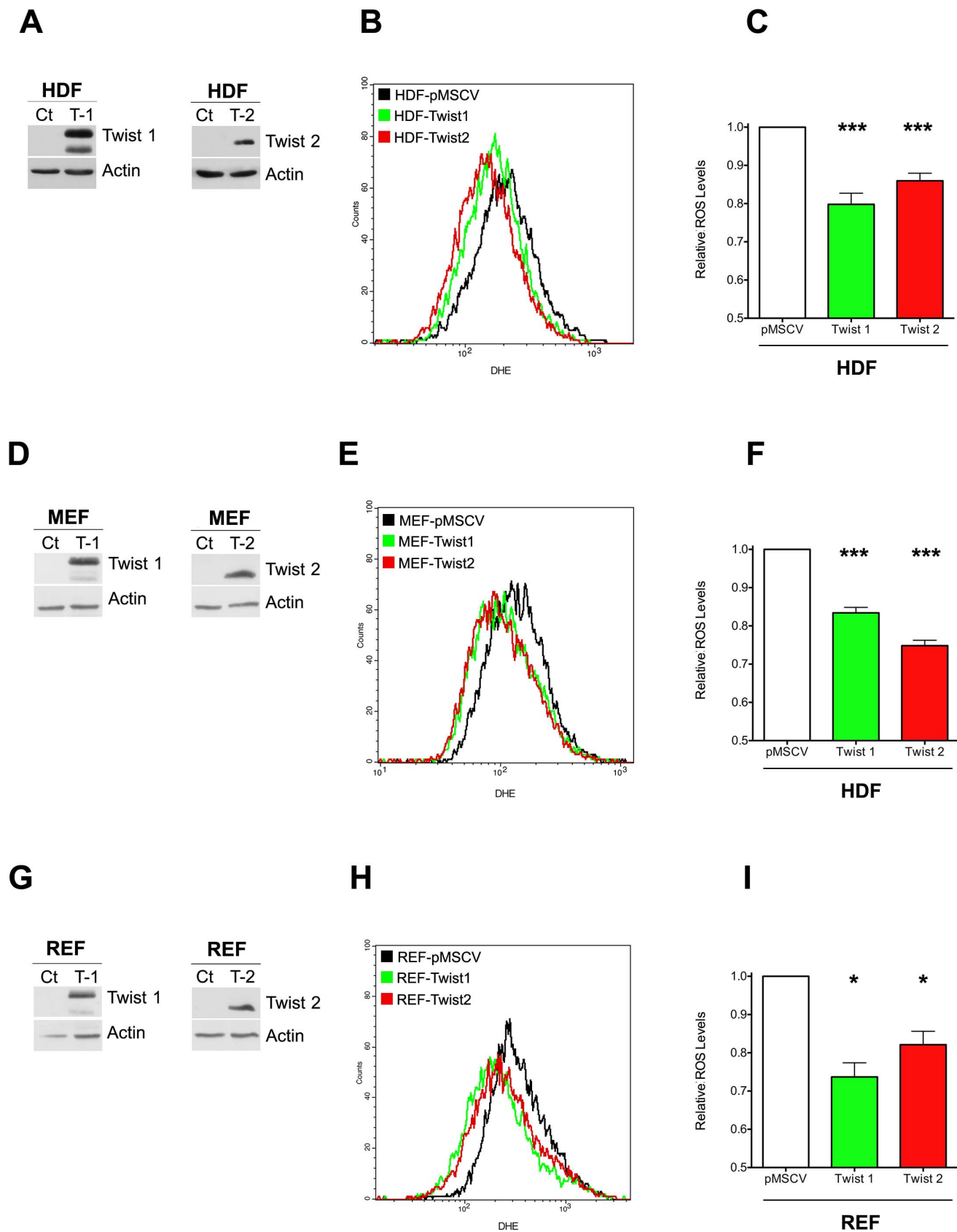
### Molecular mechanisms regulating the antioxidant activity of Twist

We next investigated the molecular mechanisms involved in the antioxidant activity of Twist. Because Twist proteins are transcription factors, we first tested whether they modulate the expression of the main cellular detoxifying enzymes. We quantified mRNA levels of Catalase, MnSOD, Cu/ZnSOD and Gpx1 by RT-qPCR in MEF cells transduced with Twist1, Twist2 or a control empty retroviral vector. As shown in Figure S4, none of the genes tested showed a significant differential expression in the presence of Twist. Similar results were obtained in HDF and REF52 (data not shown).

In search of Twist target genes involved in this new activity, we next performed a microarray analysis comparing MEF cells expressing Twist1 with MEF transduced with an empty vector. 188 genes were found to be up-regulated and 246 repressed by more than 50% following Twist1 expression. The results confirmed that none of the main antioxidant enzymes were deregulated (data not shown). However, the analysis identified several genes, previously described as being involved in the control of oxidative stress, whose expression is regulated by Twist1 (Figure 5A). More precisely, we found four up-regulated genes: Cdo1, an enzyme required for the synthesis of taurine, a well known antioxidant compound [27], Mgst3, a microsomal glutathion S transferase carrying a peroxidase activity [28], ApoD, a protein involved in the inhibition of lipid peroxidation [29] and Dhcr24, described recently as a ROS scavenger [30]. Two genes were down-regulated: Aox1, an enzyme that produces ROS by oxidizing aldehydes [31] and Osgin1, a mitochondrial oxidative stress induced gene that was recently shown to increase intracellular ROS levels when overexpressed [32].

Because both Twist factors display an antioxidant activity, we next investigated whether the genes we identified were also modulated by Twist2. We generated MEF-empty vector, Twist1 and 2 lines and mRNA level of each target was quantified by RT-qPCR. As shown in Figure 5B, similarly to Twist1, 2 regulates the expression of the oxidative stress factors revealed by the microarray analysis. This result suggests that both Twist proteins modulate intracellular ROS level in a similar manner by controlling a common set of factors.

Interestingly, none of the genes we identified was highly deregulated, suggesting that none by itself would account for Twist's antioxidant properties. We reasoned that an alternative mechanism of Twist's action might be a more global modulation of a genetic program of oxidative stress regulators. A similar situation has been reported for Nrf2, a master gene of intracellular



**Figure 1. Twist proteins lower intracellular ROS levels.** (A–C) HDF, (D–F) MEF and (G–I) REF52 cells were transduced with retroviruses encoding either Twist1 (T–1) or Twist2 (T–2) tagged with a Myc epitope. The empty vector (pMSCV) was used as a control. (A, D and G) Expression of the transgenes was verified by western-blotting (CT:pMSCV; T-1:Twist1, T-2:Twist2). (B, E and H) ROS levels were analyzed by flow cytometry after DHE

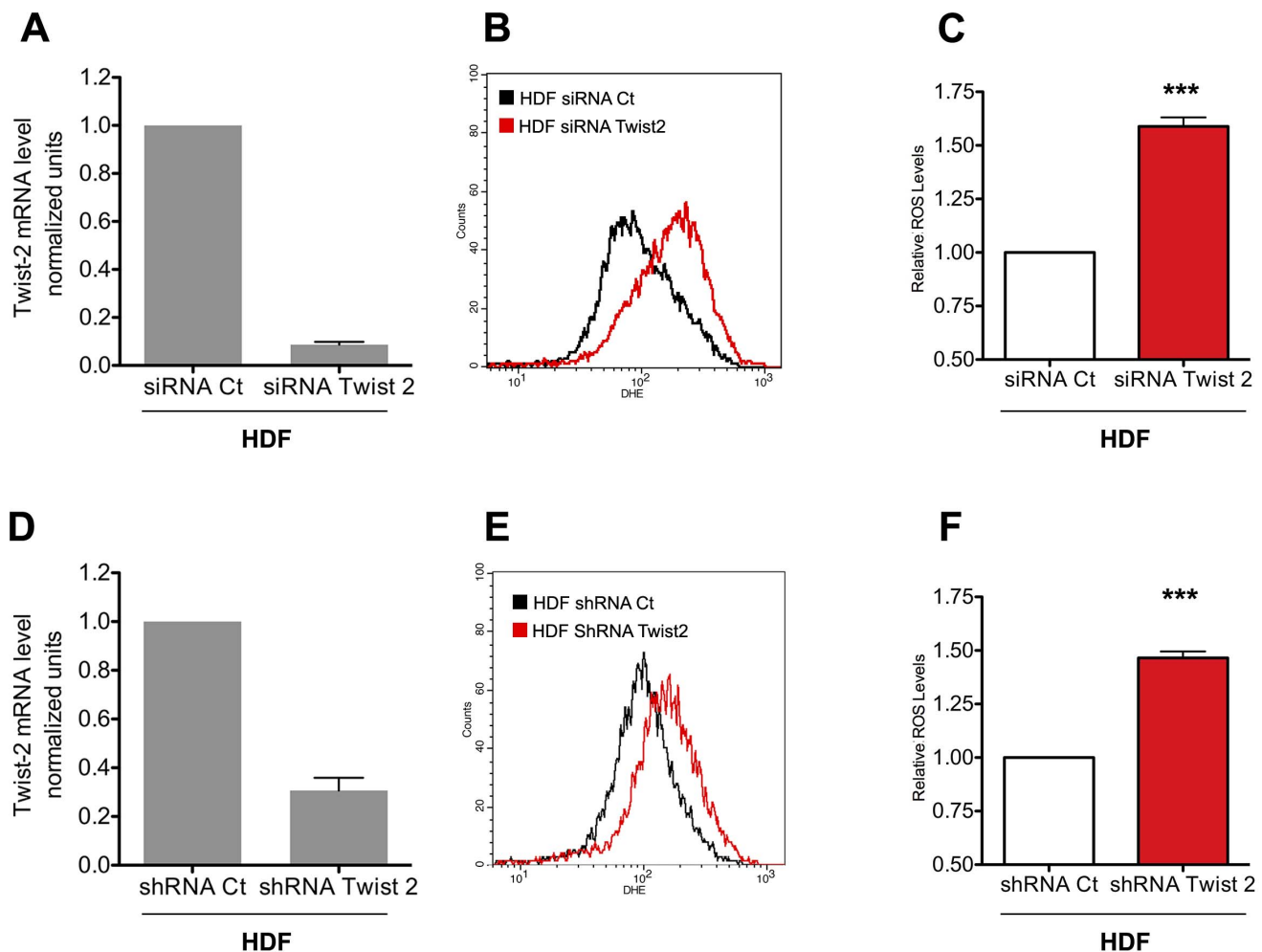


labeling. (C, F, and I) Quantitative analysis of ROS levels in HDF (C), MEF (F) and REF52 (I) cells with data representing the mean values  $\pm$  SEM of the fluorescence geometric means (HDF:  $n = 10$ ; MEF:  $n = 8$ ; REF:  $n = 3$ , \*: compared to control). doi:10.1371/journal.pone.0072490.g001

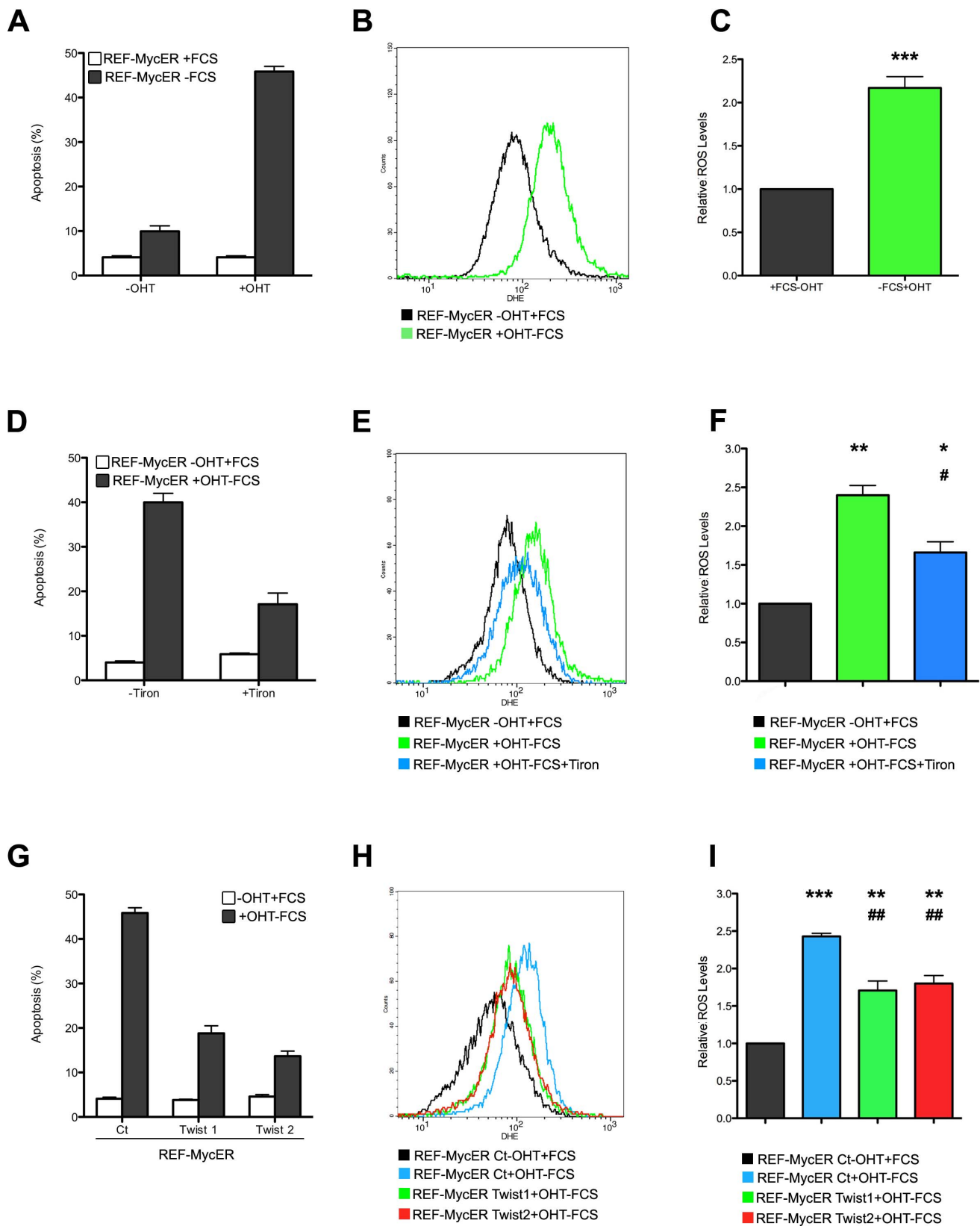
ROS level control [33]. Upon induction of oxidative stress, this transcription factor is activated and triggers expression of numerous genes encoding enzymes involved in ROS detoxification [33]. It is the joint action of these proteins that efficiently removes excess of ROS from the cell. If such was indeed the mode of action of Twist, one would expect that modulation of individual target gene expression would only partially mimic Twist antioxidant activity. To test this hypothesis we overexpressed *Mgst3*, which is one of the genes up-regulated by Twist. In parallel, we silenced *Osgin1*, for which Twist is a repressor. Twist1 overexpressing MEF cells were used as a positive control. Ectopic expression and silencing were verified by western-blot and RT-qPCR, respectively (Figure 6A–C). Intracellular ROS levels were decreased similarly by up-regulation of *Mgst3* or down-regulation of *Osgin1* (Figure 6D). As expected, their antioxidant activity was

lower than that displayed by Twist1 (Figure 6E), suggesting that each gene participates but does not fully substitute for Twist in decreasing ROS levels. Next, we tested the impact of the modulation of the same genes on c-Myc-induced apoptosis. As shown in Figure 6F, perturbation of the expression of *Mgst3* and *Osgin1* partially protects cells from cell death. Once again, this effect was not as marked as that produced by Twist1 (Figure 6G). We conclude that both Twist target genes are indeed involved in, but are not sufficient for, the Twist anti-apoptotic activity. Thus, our data indicate that Twist antioxidant and anti-apoptotic activities are mediated by a joint action of several of its downstream effectors.

In addition to their anti-apoptotic activity, Twist proteins have been very well described in terms of inducing EMT and enhancing cell motility [7]. We thus asked if the antioxidant and pro-



**Figure 2. Twist2 inhibition increases oxidative stress.** (A) HDF cells were transfected with a siRNA control or a siRNA directed against Twist2 and the silencing efficiency was measured by RT-qPCR normalized to GAPDH. (B) The level of ROS in control and Twist2-inhibited HDF cells was monitored by labeling with DHE and a representative FACS histogram is shown. (C) ROS levels are presented as mean geometric fluorescence intensities ( $\pm$  SEM) in cells transfected with a siRNA against Twist2 relative to a control siRNA (\*:  $n = 6$ , relative to control). (D) HDF were infected with a retroviral vector (pRetro-SUPER-puromycin) containing a shRNA sequence directed against Twist2 or an irrelevant sequence as a control, selected and analyzed 3 to 5 days post-selection. Knockdown efficiency was measured by RT-qPCR relative to GAPDH. (E) DHE fluorescence was analyzed as in panel B. (F) Quantitative analysis of ROS was determined as in C (\*:  $n = 6$ , relative to control). doi:10.1371/journal.pone.0072490.g002

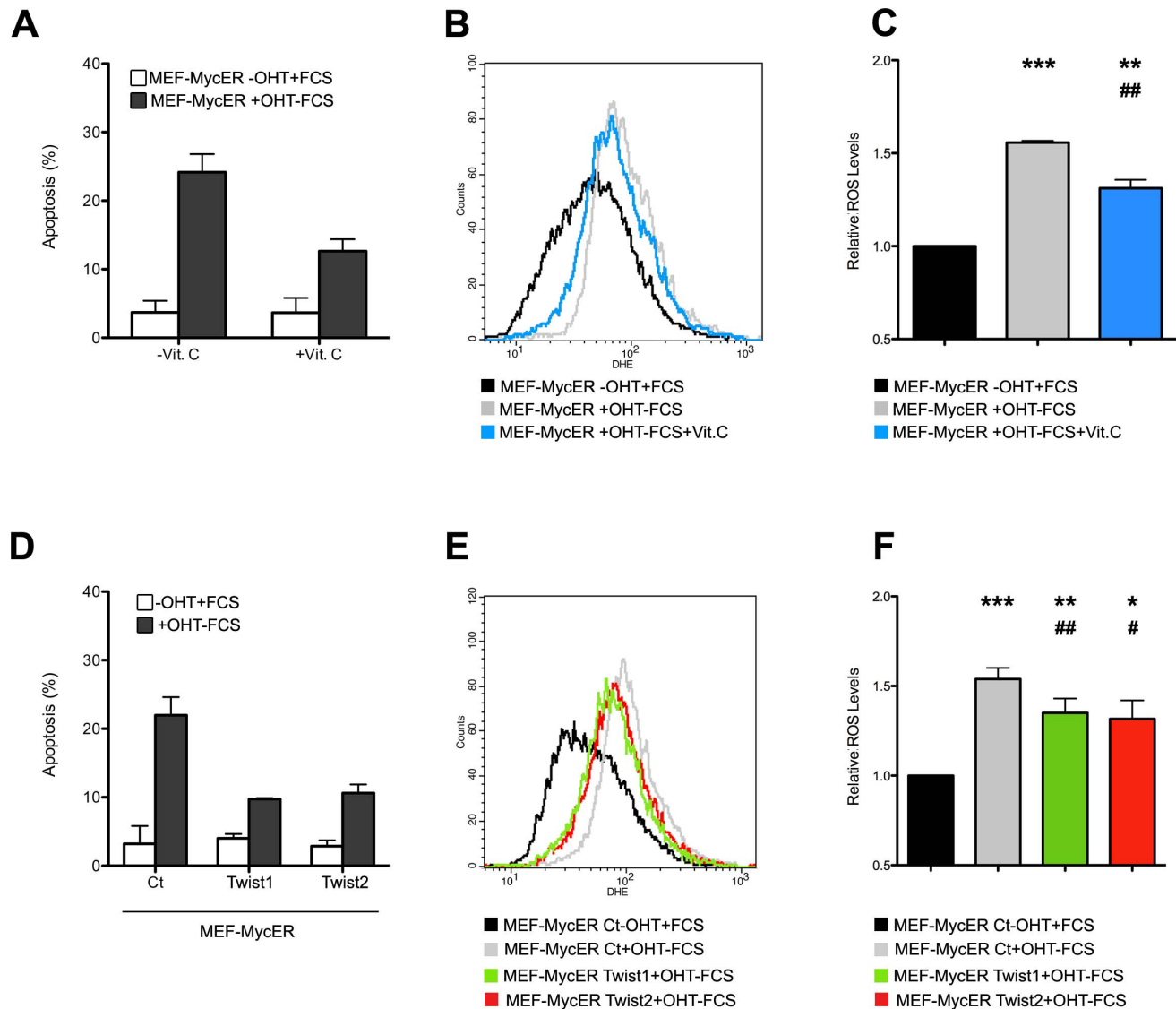


**Figure 3. Twist inhibits c-Myc-induced ROS accumulation and apoptosis.** (A) REF52 cells expressing a MycER construct were cultured for 24 hours in 0.1% serum (dark bars) in the presence or absence of 4-hydroxytamoxifen (OHT), as indicated, to induce c-Myc activity. Control cells were cultured in 10% serum (white bars). Cells were collected, labelled with Cy3-Annexin-V and apoptosis was quantified by flow cytometry. Error bars represent mean  $\pm$  SEM of three independent experiments. (B) REF52-MycER cells were treated for 8 hours as in A and DHE fluorescence was measured as in Figure 1B. (C) Represents the quantitative analysis of B (\*: n = 7, compared to control). (D) REF52-MycER cells were cultured in the

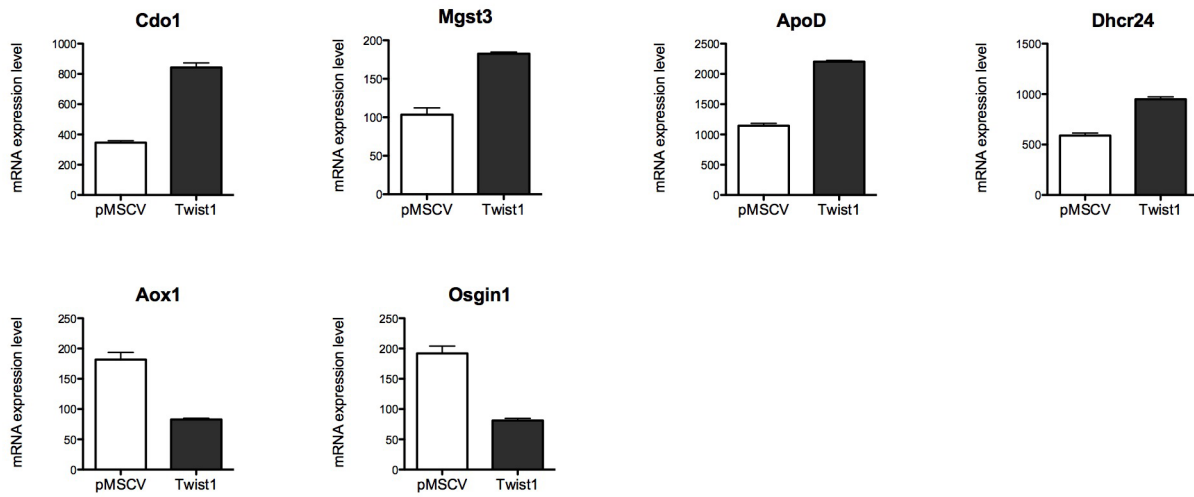
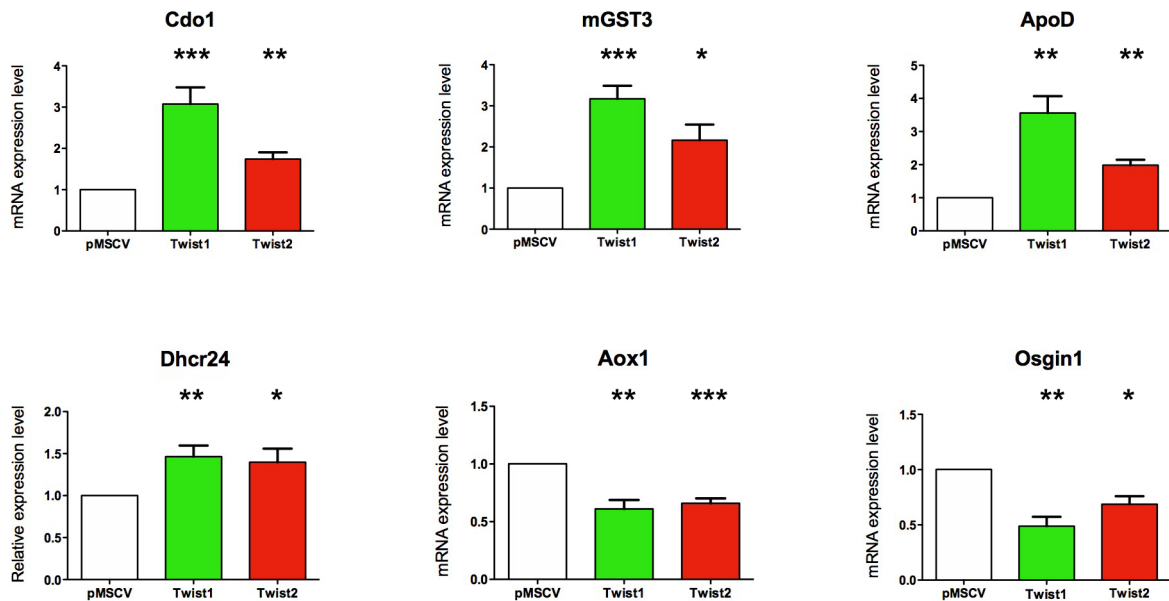
presence of 10% FBS and absence of OHT (white bars) or in 0.1% serum with OHT (dark bars) in the presence or absence of 2 mM Tiron, as indicated. Apoptosis was quantified as in A. (E) Cells were cultured as in D and ROS levels were monitored as in B. (F) Quantification of E (\*: n = 6, compared to black bar. #: n = 6, compared to green bar). (G) Quantification of apoptosis in REF52-MycER cells expressing Twist1 or Twist2. Black bars: cells are grown under pro-apoptotic conditions (active MycER, 0.1% FBS), white bars: control cultures. Cell death was quantified by Cy3-Annexin-V staining as in A. (H) Cells expressing Twist1 or Twist2 were treated as in G and ROS levels were determined as in D. (I), quantification of ROS levels from H (\*: n = 6, compared to black bar. #: n = 6, compared to blue bar). doi:10.1371/journal.pone.0072490.g003

migratory properties of Twist might be linked. We performed a wound-healing assay with cells expressing either Twist1 or Mgst3 or transduced with an empty vector as a control (Figure S5A and B). Whereas Twist expression accelerated wound closure, as

expected, Mgst3 had no effect on cell migration in this assay. It would thus appear that lowering the intracellular ROS level has no significant influence on cell migration in this experimental model, suggesting that these two activities of Twist are uncoupled.



**Figure 4. Twist antioxidant activity is associated with modulation of apoptosis in primary mouse embryonic fibroblasts.** (A) MEF cells expressing the MycER construct were cultured in the presence of 0.5% or 10% FBS, with or without OHT, and in the presence or absence of Vitamin C, used as an antioxidant. Apoptosis was quantified by Annexin-V staining and is presented as means  $\pm$  SEM of three independent experiments. (B) MEF-MycER cells were treated as in A and ROS was measured by DHE staining by flow cytometry. (C) Quantification of B (\*: n = 5, compared to black bar. #: n = 5, compared to grey bar). (D) MEF-MycER cells were infected with either Twist1, Twist2 or pMSCV vectors and selected with puromycin. Cells were then cultured for 24 hr. in 10% FBS in the absence of OHT (white bars) or in 0.5% FBS supplemented with OHT (dark bars). Apoptosis was determined as in A. (E) ROS levels in all cell lines was monitored as in B. (F) Quantitative analysis of E (\*: n = 6, compared to black bar. #: n = 6, compared to grey bar). doi:10.1371/journal.pone.0072490.g004

**A****B**

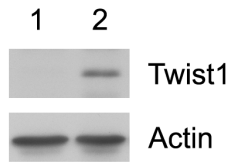
**Figure 5. Molecular mechanisms regulating Twist antioxidant activity.** (A) Microarray results for genes modulated by Twist1 and involved in the regulation of oxidative stress. Data show expression mRNA level of MEF-empty vector and MEF-Twist1. (B) mRNA levels for Cdo1, Mgst3 ApoD, Dhcr24, Aox1 and Osgin1 were assessed in MEF cells infected with empty pMSCV vector, pMSCV-Twist1 or pMSCV-Twist2. RNA levels were monitored by RT-qPCR, normalized with Hprt and adjusted relative to levels in pMSCV-transduced control cells. doi:10.1371/journal.pone.0072490.g005

## Discussion

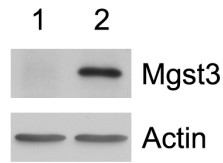
Twist transcription factors regulate several distinct cellular functions, [7]. Our study uncovered a novel activity carried by both Twist1 and Twist2, namely modulation of intracellular levels of reactive oxygen species (ROS). This finding might have important implications for understanding consequences of Twist activation in physiological and in physiopathological settings.

We concentrated our analysis on the impact of ROS on Twist's control of apoptosis. Twist displays anti-apoptotic activities in many cellular contexts and in response to multiple stimuli [7] and whether they are all governed by the regulation of ROS remains to be tested. For example, several reports have suggested that Twist expression in cancer cells is correlated with therapeutic drug resistance [16,17] and it was shown that ROS are induced by therapeutic drugs and might participate in their cytotoxic action

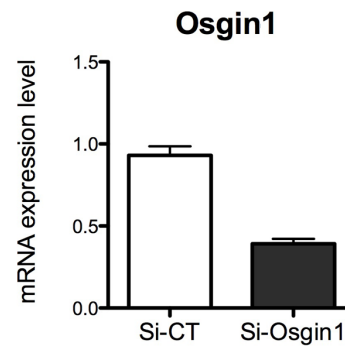
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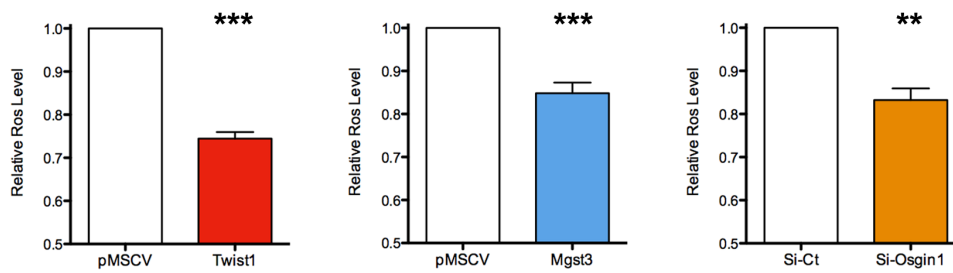
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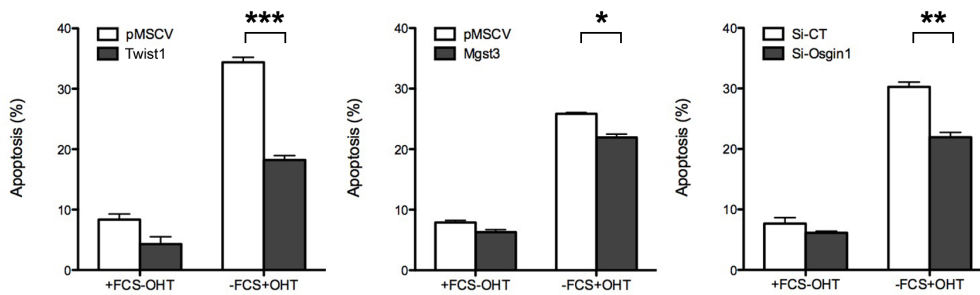
**C**



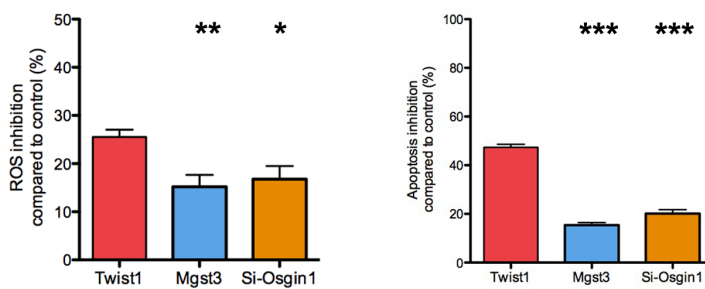
**D**



**E**



**F**



**Figure 6. Role of Twist target genes in the control of ROS levels and apoptosis.** (A, B) MEF cells were transduced with retroviruses encoding either Twist1 tagged with a Myc epitope (A), or with Mgst3 tagged with an HA epitope (B). Empty vector was used as a control. Expression of the transgenes was verified by western-blotting (1: Empty vector; 2: Transgene). (C) MEF cells were transfected with a siRNA control or a siRNA directed against Osgin1 and the silencing efficiency was measured by RT-qPCR normalized to HPRT. (D) Quantitative analysis of ROS levels of cell lines from A, B and C (\*: n = 6, compared to control). (E) MEF-MycER cells were infected with either control (pMSCV), Twist1, or Mgst3 or were transfected with corresponding siRNA as in C (n = 3). Apoptosis was induced and determined as in Figure 4. (F) Alternative presentation of data from D and E, allowing better visualization of partial effects on ROS (\*: n = 6, compared to red bar) and apoptosis (\*: n = 3, compared to red bar) exercised by Mgst3 and Osgin-1. These graphs show the percentage of inhibition compared to control (pMSCV of si-CT). doi:10.1371/journal.pone.0072490.g006

[34]. It is thus possible that the effect of Twist on drug resistance results, at least in part, from its antioxidant activity.

We demonstrated that the inhibition by Twist of c-Myc oncogene - induced apoptosis is correlated with its ability to lower ROS levels. This observation is especially interesting in the context of reports showing that mice invalidated for Twist1 display an increased apoptotic index during development [3]. Moreover, MEFs invalidated for Twist2 show an increased sensitivity to stimuli such as TNF-alpha [4], a cytokine that induces and requires ROS to exert its apoptotic activity. Thus, the antioxidant effect could participate in Twist regulation of cell death during development.

Modulation of ROS by Twist may also be important in adult tissues. It is well established that stem cells, in particular hematopoietic stem cells (HSC), display low levels of ROS [34]. This is essential for their maintenance and renewal, whereas an increase of ROS is required for differentiation [34]. The pattern of Twist expression is coherent with its role in regulation of this process. Specifically, Twist factors have been shown to control the maintenance of mesenchymal stem cells [5] and Twist2 inhibits differentiation of the myeloid lineage [6]. It will be of great interest to evaluate whether the antioxidant activity of Twist is conserved, and relevant, in these cellular settings.

Twist plays a major role in mediating metastasis [12]. In our experimental model Twist pro-migratory activity does not appear to be linked to its effect on the intracellular ROS levels. However, a recent study showed that Snail, another epithelial to mesenchymal transition inducing factor, triggers EMT and gives rise to a cancer stem cell phenotype by inhibiting ROS [35]. It is thus possible that Twist antioxidant activity, while not involved in the migration of mesenchymal cells, might act upstream during the transition process. These results also suggest that the control of intracellular ROS levels might be a common mechanism employed by different EMT-inducing factors. It will be of interest to test this hypothesis, notably in the context of the well-documented role of ROS in physiology and pathology and particularly in the process of cancer initiation and progression.

## Materials and Methods

### Ethics Statement

Experiments were performed according to national regulations and this study was specifically approved (ID approval N° D34-172-16) by the regional ethics committee of Languedoc-Roussillon (Comité Régional d'Éthique sur l'Expérimentation Animale-Languedoc-Roussillon), France.

### Constructs

N-terminal Myc-tagged Twist1 and Twist2 and HA-tagged Mgst3 murine cDNAs were cloned in pMSCV-puromycin retroviral vectors (Clontech). pBabe-MycER was a kind gift from Dr A. Gandarillas. ShRNA sequences against Twist2 were cloned into the previously described pRetro-SUPER vector [13]. An irrelevant sequence, with no significant hits when blasted against

the NCBI nucleotide sequence NR database (5'-GTACGTAC-GATCACAGTCA-3') was used as an shRNA control.

### Cell culture

Human diploid fibroblasts (HDF) were a kind gift from Dr J. Piette [36]. Mouse embryonic fibroblasts (MEF), derived from C57BL/6 mice, were prepared from 13.5 DPC embryos. All cell lines including 293T cells [37] were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 ng/mL streptomycin, and 100 U/mL penicillin at 37 °C with 5% CO<sub>2</sub>. HMEC were grown as recommended by the American Type Culture Collection (ATCC).

When appropriate, cells were treated with 1 μM 4-hydroxytamoxifen (Sigma Aldrich H7904), 2 mM Tiron (Sigma Aldrich 33724), 10 mM N-acetyl-L-cysteine (Sigma Aldrich A9165) or 20 μM Vitamin C (Ascorbic acid Sigma Aldrich A4403).

### Retroviral infection

$1.5 \times 10^6$  293T cells were transfected twice at 24 hours intervals, with 3 μg of retroviral vector using jetPEI™ (Polyplus). Forty-eight hours after the last transfection, supernatants were collected, filtered (0.45 μm, Millipore), supplemented with 8 μg/ml of polybrene and added to cells to be transduced for 16 hr. M.O.I was always  $\leq 1$  as we noted that high viral titers induced a significant amount of non-specific oxidative stress. Infected cells were selected after 48 hr. with the appropriate antibiotics.

### Short Interfering RNA (siRNA) Transfection

Cells were seeded in 6-well plates 1 day before transfection. Cells were then transfected with 100 nM siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. siRNAs were from Thermo Scientific except for siRNA against Osgin1, which was from IDT. Sequences are: siRNA Control: GUACGUACGAUCACAGUCA, siRNA TWIST2: siGENOME SMARTpool M-012862 siRNA Osgin1: MMC.RNAL.N027950.12.1.

### Immunoblot Analysis

Cells were lysed in boiling Laemmli buffer supplemented with protease inhibitors, then sonicated and complemented with DTT. Protein concentration was determined by BCA (Thermo Scientific) assay. Fifteen to twenty micrograms of total protein was loaded onto SDS-PAGE gels and transferred onto nitrocellulose membranes. The membrane was blocked with TBST (1 × TBS with 0.1% Tween 20) + 5% milk at room temperature for 1 hr. and incubated with primary antibody and then with horseradish peroxidase (HRP)-coupled secondary antibody (Amersham). Activity was visualized by electrochemiluminescence. Antibodies used in this study are anti-Myc (9E10), anti-Actin (Sigma Aldrich) and anti-HA High Affinity (Roche).

### Reverse Transcription and Real-Time PCR

Total mRNA was isolated using a RNeasy mini kit (Qiagen). Reverse transcription was performed with random hexamers and

M-MLV Reverse Transcriptase (Invitrogen). Real-time PCR was performed in triplicates with LC FastStart DNA Master SYBR Green I on a LightCycler rapid thermal cycler system (Roche Diagnostics), according to the manufacturer's instructions. House-keeping genes GAPDH or HPRT were used for normalization. Primers sequences are listed in supplementary table (Table S1).

### ROS detection

Cells were incubated with 30 mM DHE (Invitrogen D11347) for 25 min or with 5  $\mu$ M of CM-H<sub>2</sub>DCFDA (Invitrogen C6827) for 30 min, at 37°C in PBS. The cells were collected and fluorescence was quantified on a FACSCalibur flow cytometer using CellQuestPro software (BD Biosciences).

### Apoptosis analysis.

Cells were plated at low density (10<sup>5</sup> cells/well) in 6 well plates 24 hours before induction of apoptosis. c-Myc-induced apoptosis was triggered by treatment with 1  $\mu$ M 4-hydroxytamoxifen in 0.1% FBS. Apoptosis was monitored 24 hr. post-induction. To determine the percentage of apoptotic cells with externalized phosphatidylserine (PS), adherent and floating cells were collected and labeled with the Annexin V-Cy3 Apoptosis Detection Kit (Abcam) according to the manufacturer's instructions. Labeled cells were then analyzed on a FACSCalibur flow cytometer using CellQuestPro software (BD Biosciences).

### Microarray

mRNA purified from three different preparations of MEF-Empty vector and MEF-Twist1 were used for analysis. Microarray processing and subsequent analysis were performed by The Genomic Research Unit of the Luxembourg CRP (<http://www.crp-sante.lu/Competence-centers/Luxembourg-Biomedical-Research-Resources/Genomics-Research-Unit>). The full dataset analysis will be published in a future study (manuscript in preparation).

### Statistical analysis

Experiments were repeated at least three times. Data are presented as mean  $\pm$  SEM. An Independent Student's t test was performed to analyze the assay results; a two-tailed Student's t test was used to compare the intergroup differences. Significance was accepted for values where  $P \leq 0.05$  (\*/#),  $P \leq 0.01$  (\*\*/##),  $P \leq 0.0005$  (\*\*\*/###).

## Supporting Information

**Figure S1 The antioxidant property of Twist is confirmed by DCFDA staining and is maintained in an epithelial cell line.** (A) ROS levels in the HDF cells used in Figures 1B and 1C were analyzed by flow cytometry following DCFDA staining and representative histograms are shown. (B) Quantitative analysis of ROS levels in HDF expressing either Twist1 or Twist2 relative to control HDF cells (\*: n = 11, compared to white bar). (C) ROS measurements in HDF cells in the absence or presence of 5 mM NAC. (D) Quantitative analysis of ROS levels in cells incubated with NAC (\*: n = 4, compared to

white bar). (E) ROS level analysis in HMEC-hTert cells expressing Twist1 or Twist2. (TIF)

**Figure S2 Antioxidant treatment protects cells from oxidative stress induced by Twist2 depletion.** HDF cells expressing a shRNA directed against Twist2 were cultured with 10 mM NAC for 24 hours. ROS levels were analyzed by DHE staining. (TIF)

**Figure S3 ROS increase is mainly induced by serum starvation.** (A-B) REF52-MycER expressing or not Twist1 were cultured in 10% FBS or 0.1% FBS in the presence or absence of 4-hydroxytamoxifen (OHT). (A) ROS levels were measured after 8 hours and quantified as in figure 3B (\*: n = 4, Twist1 compared to pMSCV). (B) Apoptosis was determined as in Figure 3C (presented as mean  $\pm$  SEM of three independent experiments). (TIF)

**Figure S4 Molecular mechanisms regulating Twist antioxidant activity.** mRNA levels for MnSOD, Cu/znSOD, catalase and Gpx1 were assessed in MEF cells infected with empty pMSCV vector, pMSCV-Twist1 or pMSCV-Twist2. RNA levels were monitored by RT-qPCR, normalized with Hprt and adjusted relative to levels in pMSCV-transduced control cells (n = 4, ns: non significant). (TIF)

**Figure S5 Twist antioxidant activity is not linked to enhanced migration.**  $1 \times 10^6$  MEF-pMSCV, MEF-Twist1 and MEF-Mg3 were seeded on a six well plate and 24 hours later a wound was made with a pipet tip. Wound width was monitored at time 0 and 16 hours. Distance of the wound was measured using ImageJ software. (A) Representative pictures of the three cell lines at 0 and 16 hours. (B) Quantification of wound width (presented as mean  $\pm$  SEM of three independent experiments. \*: compared to control at 18 hr.). (TIF)

### Table S1 Primer sequences.

(DOC)

### Text S1

(DOC)

## Acknowledgments

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## Author Contributions

Conceived and designed the experiments: NF JK UH PL. Performed the experiments: NF LA JK PL. Analyzed the data: NF JK UH PL. Contributed reagents/materials/analysis tools: YS SA AP. Wrote the paper: PL.

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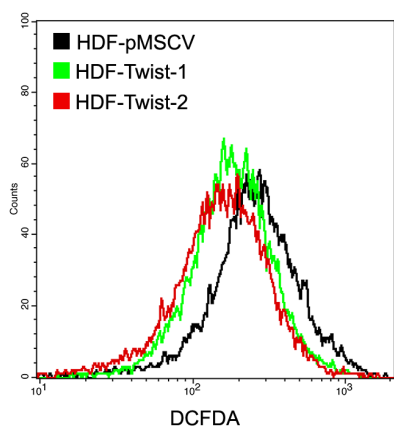
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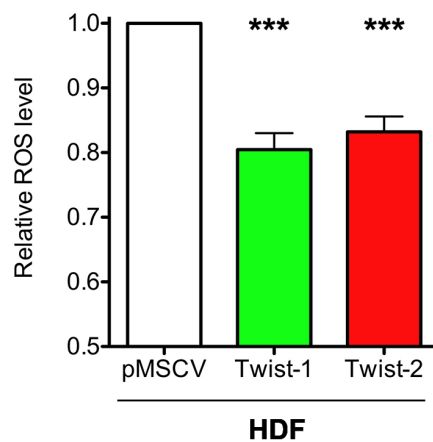


# Figure S1

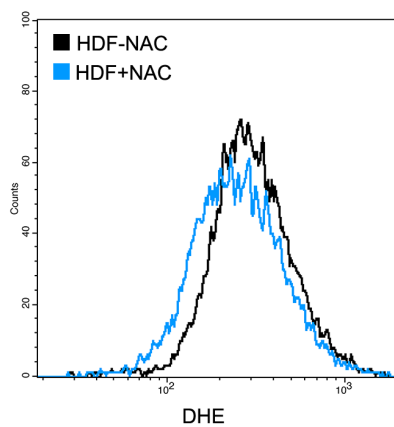
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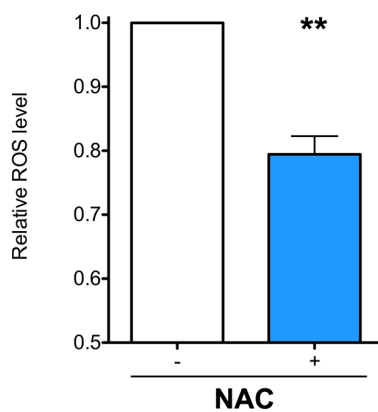
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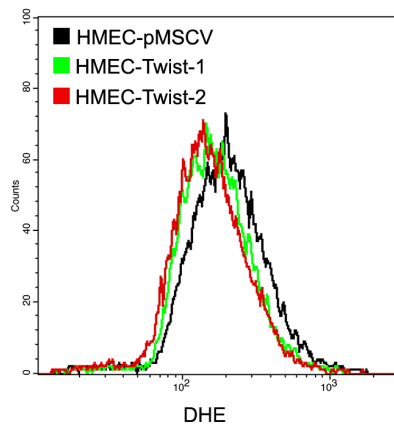
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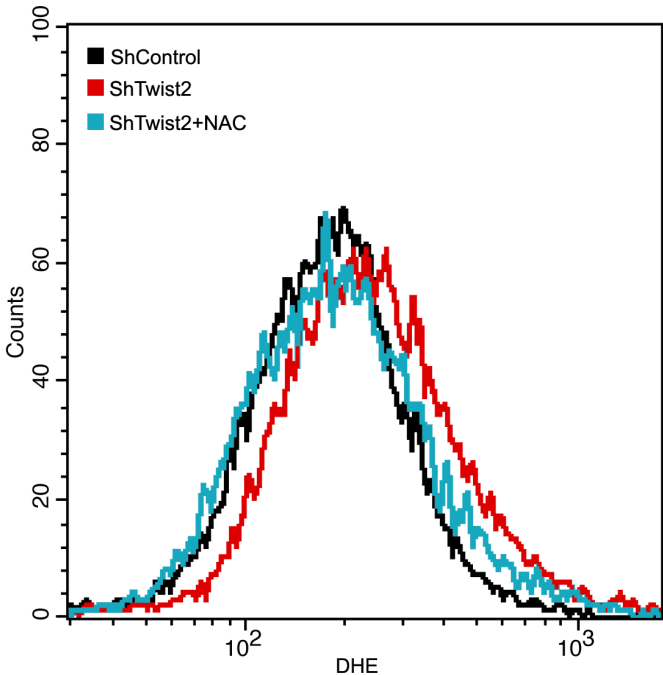
## D



## E

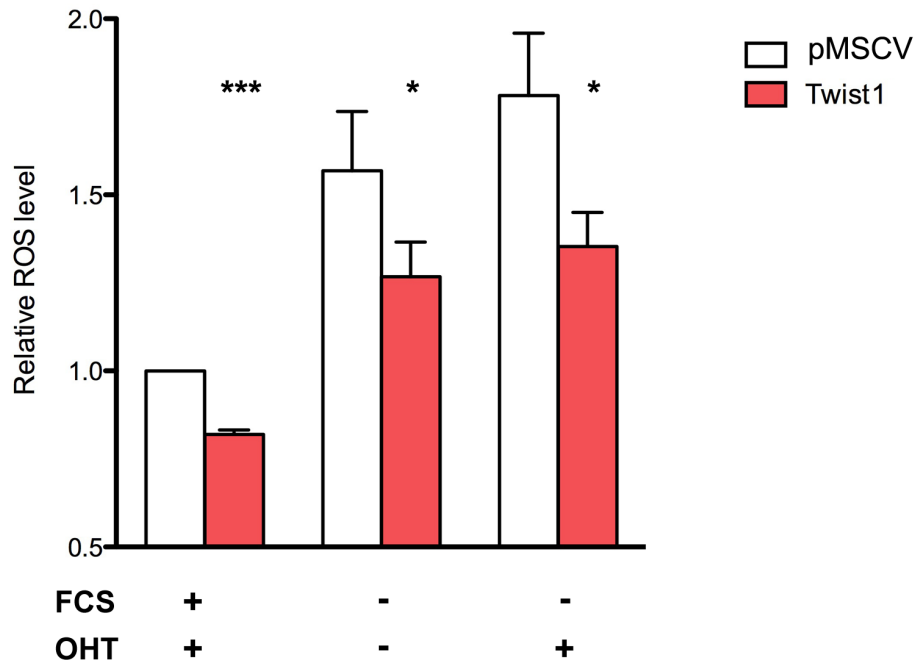


**Figure S2**

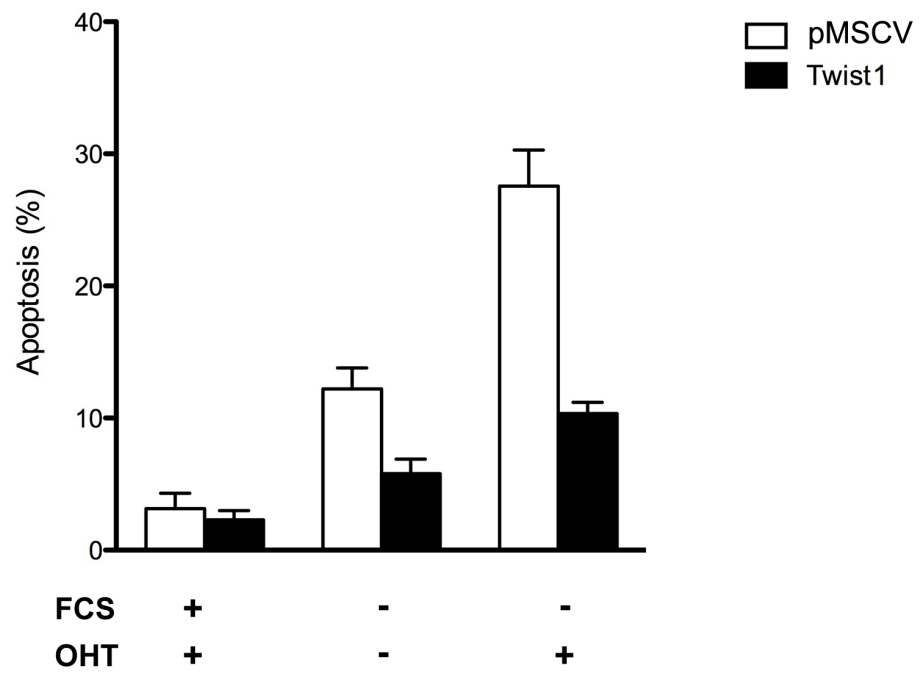


**Figure S3**

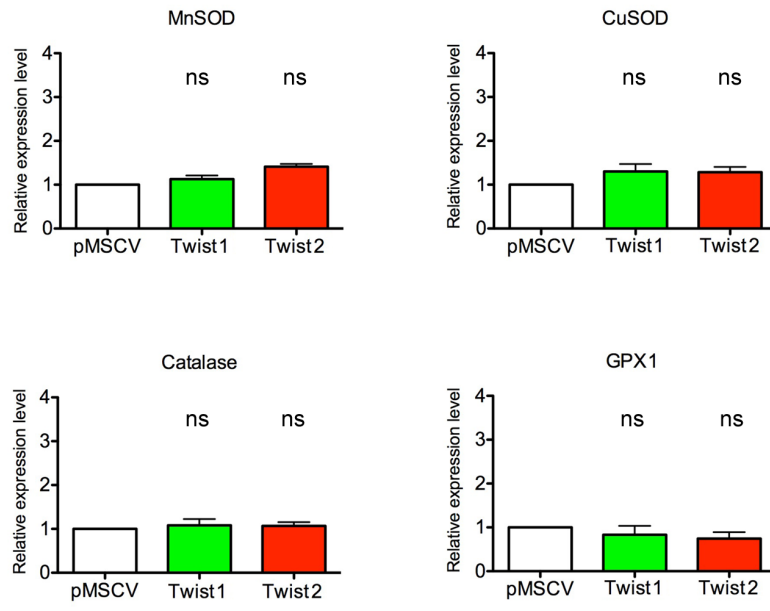
**A**



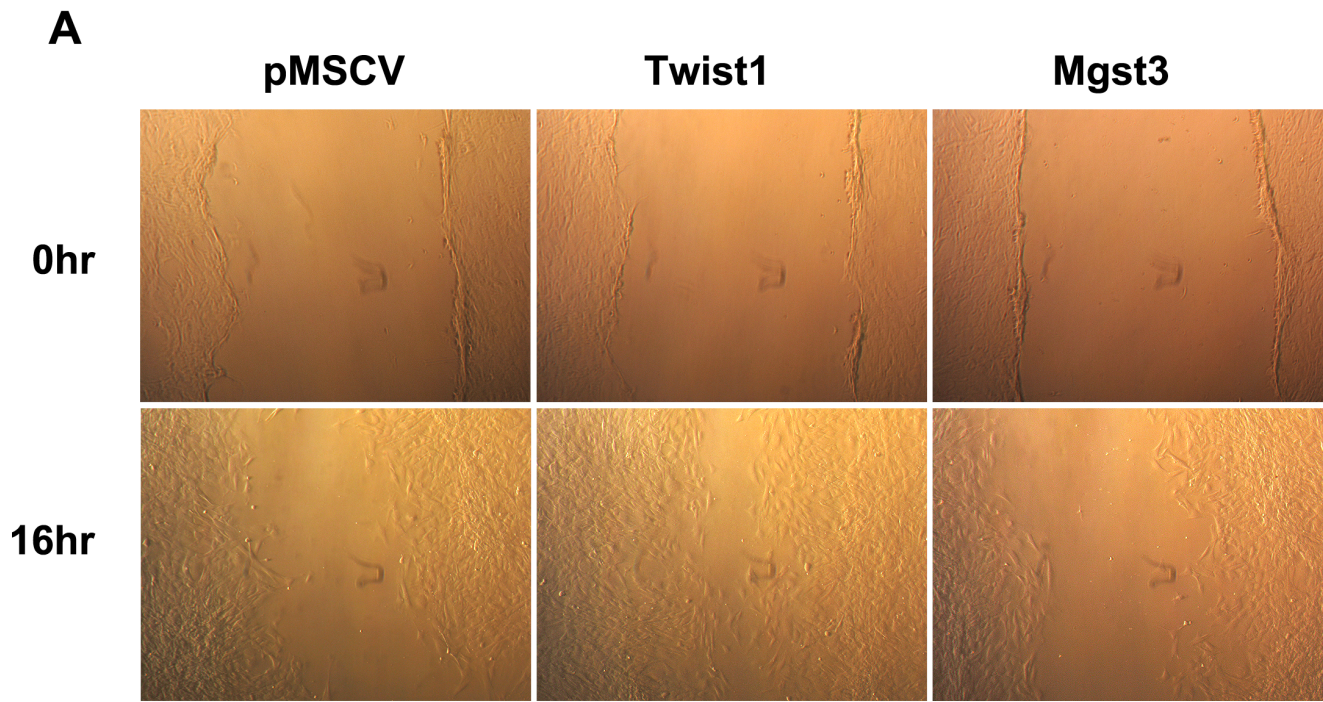
**B**



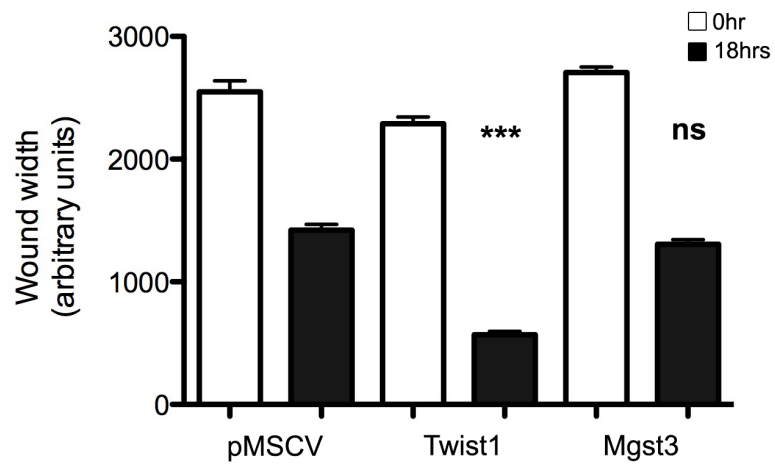
# Figure S4



**Figure S5**



**B**



**Table S1**

<b>Genes</b>	<b>Sequence forward</b>	<b>Sequence reverse</b>
<b>Twist2 (human &amp; mouse)</b>	GCAAGAAGTCGAGCGAAGAT	GCTCTGCAGCTCCTCGAA
<b>MnSOD (mouse)</b>	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
<b>Cu/ZnSOD (mouse)</b>	AACCAGTTGTGTTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG
<b>Catalase (mouse)</b>	AGCGACCAGATGAAGCAGTG	TCCGCTCTCTGTCAAAGTGTG
<b>Gpx1 (mouse)</b>	AGTCCACCGTGTATGCCTTCT	GAGACGCGACATTCTCAATGA
<b>Cdo1 (mouse)</b>	GTGGATCAAGGAAATGGGA	CTTGATCATCTCGTTGGA
<b>Mgst3 (mouse)</b>	ATGGCTGTCCTCTCTAAGGAG	CTCTACCTTGACTTCTTGCGG
<b>ApoD (mouse)</b>	GAACGGAAACATCGAAGTGCT	CTGGCTCTGAGACGTTGCTC
<b>Dhcr24 (mouse)</b>	TGCGAGTCGGAAAGTACAAG	GCCAATGGAGTTCAGCAAAG
<b>Aox1 (mouse)</b>	AGTGATGATCTCGCGGTACAA	CTGGTGTTGCCTAAGCCTTCT
<b>Osgin1 (mouse)</b>	CGGTGACATCGCCCACTAC	GCTCGGACTTAGCCCACTC
<b>Hprt (mouse)</b>	GCAGTACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAAGCT
<b>GAPDH (human)</b>	ACACCCACTCCTCCACCTTT	TCCACCACCCTGTTGCTGTA

## Supporting Text

**Supplementary Figure 1: The antioxidant property of Twist is confirmed by DCFDA staining and is maintained in an epithelial cell line.** (A) ROS levels in the HDF cells used in Figures 1B and 1C were analyzed by flow cytometry following DCFDA staining and representative histograms are shown. (B) Quantitative analysis of ROS levels in HDF expressing either Twist1 or Twist2 relative to control HDF cells (\*: n=11, compared to white bar). (C) ROS measurements in HDF cells in the absence or presence of 5mM NAC. (D) Quantitative analysis of ROS levels in cells incubated with NAC (\*: n=4, compared to white bar). (E) ROS level analysis in HMEC-hTert cells expressing Twist1 or Twist2.

**Supplementary Figure 2: Antioxidant treatment protects cells from oxidative stress induced by Twist2 depletion.** HDF cells expressing a shRNA directed against Twist2 were cultured with 10mM NAC for 24 hours. ROS levels were analyzed by DHE staining.

**Supplementary Figure 3: ROS increase is mainly induced by serum starvation.** (A-B) REF52-MycER expressing or not Twist1 were cultured in 10% FBS or 0.1% FBS in the presence or absence of 4-hydroxytamoxifen (OHT). (A) ROS levels were measured after 8 hours and quantified as in figure 3B (\*: n=4, Twist1 compared to pMSCV). (B) Apoptosis was determined as in Figure 3C (presented as mean  $\pm$  SEM of three independent experiments).

**Supplementary Figure 4: Molecular mechanisms regulating Twist antioxidant activity.** mRNA levels for MnSOD, Cu/znSOD, catalase and Gpx1 were assessed in MEF cells infected with empty pMSCV vector, pMSCV-Twist1 or pMSCV-Twist2. RNA levels were monitored by RT-qPCR, normalized with Hprt and adjusted relative to levels in pMSCV-transduced control cells (n=4, ns: non significant).

**Supplementary Figure 5: Twist antioxidant activity is not linked to enhanced migration.**  $1 \times 10^6$  MEF-pMSCV, MEF-Twist1 and MEF-Mgst3 were seeded on a six well plate and 24 hours later a wound was made with a pipet tip. Wound width was

monitored at time 0 and 16 hours. Distance of the wound was measured using ImageJ software. (A) Representative pictures of the three cell lines at 0 and 16 hours. (B) Quantification of wound width (presented as mean  $\pm$  SEM of three independent experiments. \*: compared to control at 18 hr.)





## 2 Summary

Our study demonstrated a novel activity exhibited by both Twist1 and Twist2, namely regulation of intracellular ROS levels. Additionally, Twist mediated antioxidative properties display an ability to protect cells from ROS-induced cell death.

For this article I performed the necessary experiments required for revision of the manuscript. Notably, I showed that Twist antioxidative properties are exhibited via the regulation of a genetic program of oxidative stress regulators (Figure 5A). Further analysis of our microarray data identified several genes, previously described as involved in the control of oxidative stress, whose expression is regulated by Twist1. More precisely, we found four up-regulated genes: Cdo1, Mgst3, ApoD and Dhcr24 as well as two down-regulated genes: Aox1, Osgin1. Thereafter, I validated selected groups of genes by measurement of their mRNA levels in cells upon overexpression of Twist1 compared to cells expressing a control plasmid (Figure 5B). Furthermore, using the same technique I also demonstrated that both Twist proteins modulate intracellular ROS level by impacting a common set of factors (Figure 5B). Next, in order to determine if the validated genes mimic Twist properties, we performed the same type of analysis on two selected genes - Mgst3 and Osgin1. Indeed, modulation of their expression translated into changes in intracellular ROS levels (Figure 6D). However, in both cases these results only partially reproduce the effects observed for Twist proteins suggesting that they are only partially responsible for Twist antioxidative properties (Figure 6F) Furthermore, I tested the impact of modulation of those two selected genes on c-Myc-induced apoptosis. The results showed that, similarly to changes observed for ROS levels, both genes protected cells from cell death (Figure 6E) although their effect was not as strong as expression of Twist1 (Figure 6F). Finally, I tested whether Twist antioxidative and pro-migratory activities might be linked. Wound-healing assay indicated that consistently with our expectations Twist expression accelerates wound closure. However, up-regulation of one of Twist target - Mgst3, had no effect on cell migration at least in this assay (Supplementary Figure 5).

Overall, this study shed new light on Twist proteins and their ability to protect cells against oxidative stress. Furthermore, discovery of Twist's ability to regulate

intracellular ROS levels provides a new important insight into its biology. Indeed, this study demonstrated that Twist anti-apoptotic properties might be at least partially explained by its antioxidative activities. Finally, the establishment of a link between Twist and cellular stress provides an important implication for the adaptation process, which is considered as a crucial event during cancer initiation and progression.

## **Twist mediates adaptation to hypoxia through non-cell autonomous effects.**

Jakub Kolodziejcki, Barbara Mojsa, Urszula Hibner, Patrice Lassus

### **1 Introduction**

In our previous article we have demonstrated for the first time, a link between metabolic cellular stress and Twist proteins. Of note, induction of metabolic stress is not limited to ROS accumulation, but might be caused by diverse stimuli such as oxygen concentration or nutrient availability. While going further with the idea of adaptation to cellular stress, our attention was drawn to HIF1 $\alpha$ , master regulator of hypoxia response. Indeed, HIF1 $\alpha$  was found to up-regulate Twist expression under hypoxia [426]. Interestingly HIF1 $\alpha$  plays a dual role in the determination of cell fate following hypoxic stress. Notably, short-term hypoxia induces a beneficial, protective HIF1 $\alpha$  response. However during prolonged oxygen deprivation, HIF1 $\alpha$  activity might rather induce cell death [773, 774]. This induction of cell death during prolonged hypoxia provides a potential crossroad for Twist and HIF1 $\alpha$ , where Twist could act as a protective factor. Interestingly, beside the fact of Twist up-regulation during hypoxia, there is no data concerning a possible interaction between those two transcription factors. A phenomenon of protein up-regulation coupled with following transcriptional cooperation was already observed for Twist proteins, notably in relation to NF- $\kappa$ B and PGC1 $\alpha$  [335, 336]. As such, a possible hypothesis might be a specific negative-feedback loop, in which HIF1 $\alpha$  up-regulated Twist protects cells from HIF1 $\alpha$ - induced cell death under hypoxia. Consequently, if proven right this hypothesis might strengthen Twist role as cellular stress modulator. This could have important implications for tumour development as metabolic stress, is a common condition found in solid tumours [775].

Moreover, increased chances for successful adaptation during cancer development may provide a significant advantage towards cancer malignancy [485, 776].

Hypoxic stress drew our attention also due to another reason, precisely changes in medium acidity. In comparison to normal cells, cancer cells produce increased amount of H<sup>+</sup> (including lactic and carbonic acids) [29, 777]. This extensive medium acidification promotes cellular stress, that results in alteration of intracellular pH regulating machinery and might in consequence drastically affect cell signalling and metabolic functions of all living cells (reviewed in [778]). Therefore, increased protons production coupled with defective vascularization and poor extracellular clearance, creates a hypoxic and acidic niche that could rapidly become lethal to cells (reviewed in [778]). On top of that, several reports demonstrated an involvement of HIF1 $\alpha$  in pH regulation through up-regulation of various pH modulators such as CAIX or MTC4 [702, 779]. These facts made our preliminary observations particularly interesting. Indeed, overexpression of Twist factors in different cell types resulted in lower medium acidification when compared to control cells. Thus we hypothesized that the combined effects of Twist and HIF1 $\alpha$  on medium acidification might increase overall survival rate of cancer cells under hypoxic stress and increase its adaptive properties.

Adaptation and resistance to hypoxia and pH acidification are crucial for cancer development. We based our research on hepatocellular carcinoma, a type of cancer in well adapted to our working hypothesis and in which both Twist and hypoxia play a major role: First of all, this type of solid cancer is often characterized by vast hypoxic areas [780]. Secondly, HIF1 $\alpha$ -mediated response to hypoxia plays an important role in HCC development as it may trigger metastasis or angiogenesis process [781]. Additionally, hepatocytes exposed to hypoxia undergo apoptosis what provides a reliable model for *in vitro* studies. Finally, Twist expression in HCC is associated with poorer patient prognosis [403].

# **Twist mediates adaptation to hypoxia through non-cell autonomous effects**

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JK, BM, UH and PL analysed the data

PL wrote the paper

\*PL and UH contributed equally to this work

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## Introduction

Twist1 and Twist2 are bHLH transcription factors that are essential in embryogenesis and are no longer detectable in most adult tissues (Ansieau et al., 2010). Similarly to many other developmental regulators, their expression is reactivated in a large number of human cancers, including hepatocellular carcinoma (HCC), glioblastoma, sarcomas, neuroblastoma and melanoma (Puisieux et al., 2006). Multiple activities of Twist proteins contribute to their oncogenic potential. First of all, they promote epithelial to mesenchymal transition (EMT) and, as a consequence, enhance invasiveness and may induce a cancer stem cell phenotype (Puisieux et al., 2014). They have also been shown to inhibit both apoptosis and senescence by interfering with p53 and Rb pathways (Puisieux et al., 2014) or by modulating oxidative stress (Floc'h et al., 2013). Finally, several studies reported a role of Twist in resistance to chemotherapeutic drugs (Ansieau et al., 2010) and in promotion of angiogenesis (Ansieau et al., 2010), linking these factors to tumour aggressiveness and recurrence. While all cells of higher eukaryotes need oxygen for survival, there is a broad range of oxygen pressures that are physiological for different tissues (Aragonés et al., 2009). Hypoxia, defined as insufficient oxygen concentration, is encountered in pathologies, such as ischemia and is found in virtually all solid tumours (Semenza, 2012). Initially, it arises because oxygen diffusion is limited in avascular primary tumours, later, the tumour vasculature, induced in part as a response to the initial hypoxia, is abnormal and fails to fully rectify the oxygen deficit (De Bock et al., 2011). In order to survive and thrive in this hostile environment, cancer cells adapt and undergo profound phenotypic changes, such as for example a metabolic reprogramming. This adaptive process is driven by a family of transcription factors named HIFs (Hypoxia Inducible Factors) (Keith et al., 2012). A decrease of oxygen concentration leads to stabilization and activation of HIF1 $\alpha$  and HIF2 $\alpha$ . They form a complex with the constitutive HIF1 $\beta$  subunit, which modulates the expression of a multitude of genes involved in glucose metabolism, pH homeostasis, angiogenesis, autophagy and apoptosis, to name a few (Semenza, 2012).

It is well established that the hypoxic environment is an important feature of tumour progression. Indeed, numerous studies have shown that adaptation and resistance of

cancer cells to this particularly harsh environment leads to acquisition of traits specific of aggressiveness. These include immunosuppression, increased invasiveness through EMT, resistance to therapy, genomic instability and dedifferentiation toward a tumour initiating cell phenotype (Jain, 2014). Hypoxia could thus constitute a trigger for generating and selecting aggressive cancer cells, which would help to explain the relative failure of anti-angiogenic therapies (Jain, 2014). It is thus very important to understand mechanisms involved in resistance to hypoxic stress.

Several recent reports described a link between HIF $\alpha$  and Twist factors (Barriga et al., 2013; Gort et al., 2008; Yang et al., 2008). It was shown that Twist1 is a transcriptional target of HIF1 $\alpha$  and HIF2 $\alpha$ , both during development (Barriga et al., 2013) and in some cancer cells (Yang et al., 2008). It was further reported that invasiveness induced by HIF in response to hypoxia is mediated by Twist (Barriga et al., 2013; Gort et al., 2008; Yang et al., 2008). Because we and others have discovered novel activities of Twist that participate in cellular resistance to stress (Ansieau et al., 2008; Floc'h et al., 2013), we hypothesized that this oncogene could also be involved in protection against the hypoxic stress.

We report here that both Twist1 and Twist2 inhibit hepatocyte cell death triggered by prolonged hypoxia. Furthermore, we show that this new activity is exercised in non-cell autonomous manner. Indeed, we report that cells expressing Twist protect non-expressors grown in co-culture through preventing, extracellular medium acidification. Finally, we demonstrate that, at the molecular level, this new property of Twist involves the modulation of HIF1 $\alpha$  activity and suggest that this occurs through a direct interaction. Our results establish for the first time a link between hypoxic stress and the oncoproteins Twist. They also suggest that Twist might play a major role in the selection of cancer cells with increased aggressiveness during the process of tumour progression.



## **Results**

### **Twist factors protect hepatic cells from hypoxia-mediated apoptosis**

Twist genes have been reported to be over-expressed in hepatocellular carcinoma (Lee et al., 2006; Yang et al., 2009). Moreover, hypoxia is detected in some of the main steps, such as fibrosis and cirrhosis, leading to liver cancer (Nath and Szabo, 2012; Wilson et al., 2014; Wu et al., 2007), making hepatic cells a pertinent model to study hypoxic stress. We first confirmed published reports of toxicity of prolonged hypoxia for different hepatocyte-like cell types. We thus assayed apoptosis of primary progenitors (BMELs), immortalized (AML12) and transformed cells (HepG2) cultured for several days in low oxygen (1%). As shown in Figure 1A, the three lines died after 2 to 3 days, demonstrating their sensitivity to hypoxic stress. We next addressed the effect of Twist proteins on hypoxia-induced apoptosis. To this end, we generated cells lines (HepG2, AML12 and BMELs) stably expressing either Twist1 or Twist2 and placed them in hypoxic conditions. We found that both factors protect all cell types tested here from cell death induced by oxygen deprivation (Figure 1B). These experiments demonstrated that Twist proteins increase resistance of hepatocytes to hypoxic stress.

### **The mechanism of Twist induced resistance to hypoxic stress is non-cell autonomous**

Two pathways have been described to trigger hypoxia-mediated apoptosis: HIF1 $\alpha$  controlled modulation of expression of several members of the Bcl2 family and activation of the p53 pathway (Greijer and van der Wall, 2004). To gain insight into the mechanisms involved in the protective effect of Twist, we assayed the mRNA expression levels of different Bcl2 family members and of several p53 target genes under hypoxic conditions in the presence or absence of Twist. Somewhat unexpectedly, we found no variation in any of the mRNAs tested (data not shown), suggesting that in our experimental models Twist-induced resistance to hypoxic

stress is mediated by an alternative mechanism. As many apoptotic pathways are controlled by extracellular signals, we next investigated whether the culture medium from Twist expressing cells had an impact on wild type cells. To this end, we collected media conditioned by control HepG2, as well as Twist1- and Twist2-expressing HepG2 lines cultured for 24 hours in hypoxia. These media were then added to wild type HepG2, grown under the same conditions and apoptosis was analysed 48 hours later. As shown in Figure 2A, cells incubated with medium conditioned by Twist1 or Twist2 lines were significantly more resistant to cell death. These data indicate that the anti-apoptotic activity of Twist is mediated by an extracellular signal. To confirm this result, we used transwells to co-culture wild type and Twist-expressing cells, thus physically separating the two cell lines, but allowing them to share the same medium. Analysis of cell death after 3 days under hypoxic condition, showed that HepG2 cultured either with Twist1 or Twist2 lines are less prone to apoptosis mediated by oxygen starvation (Figure 2B). A similar co-culture experiment, in which wild type cells were stained beforehand with a fluorescent dye and then co-cultured with Twist expressing cells confirmed this result (Supplementary Figure 1). We conclude that Twist-induced resistance to hypoxic stress relies on an external signal. Consistently, this effect acts in trans, as the cells that do not express Twist are protected almost as efficiently as the cells that do.

### **Control of extracellular pH by Twist accounts for its anti-apoptotic activity**

We reasoned that the extracellular signal that protects cells from hypoxia-induced apoptosis could be a growth factor, a cytokine, a nutrient or a metabolite. In the course of the experiments, we noticed a difference in the colour of the media conditioned by Twist-expressing and by control cells, especially when cultured under hypoxic conditions. Hypoxia is known to induce metabolic reprogramming by switching glucose metabolism to glycolysis (Semenza, 2013). As a consequence, metabolites such as lactic or carbonic acid are released from cells, leading to acidification of the extracellular environment, which is visible by the yellow colour of the standard culture medium. Our observation of the Twist cells' medium turning less yellow in comparison to wild type cells, suggested a role of Twist in the modulation of

the pH balance. To confirm this interpretation, we measured the pH of media from cultures of control HepG2, as well as Twist1 and Twist2 expressing HepG2 grown in hypoxic conditions (Figure 3A). Whereas, as expected, the pH decreased in all cell cultures, both Twist factors slowed down medium acidification. Interestingly, we measured extracellular concentration of lactate, one of the main acidic by-products of glycolysis, and found no difference as a function of Twist expression (Supplementary Figure 2). This suggests that Twist factors regulate pH homeostasis through a different, as yet unidentified, pathway.

Cancer cells can cope with a low pH environment to a certain extent (Casey et al., 2010; Parks et al., 2013). However, below a certain threshold that may differ from one cell type to another, all cells, including cancer cells, undergo apoptosis (Parks et al., 2013). To test if acidification is responsible for triggering cell death of HepG2 exposed to hypoxia, we buffered the culture medium with 5mM of sodium bicarbonate. Measuring the pH after 2 days of oxygen starvation confirmed that it was higher when NaHCO<sub>3</sub> was present (Figure 3B). We next quantified apoptosis of cells grown in the buffered medium and found that sodium bicarbonate has a strong protective effect against hypoxia-induced apoptosis (Figure 3C).

Overall, these data suggest that Twist induces resistance of hepatocytes to hypoxic stress by modulating the extracellular pH. This mode of action is consistent with the non-cell autonomous protection afforded by Twist to cells under hypoxic stress.

### **HIF1 $\alpha$ depletion mimics the protective activity of Twist against hypoxic stress**

HIF factors, especially HIF1 $\alpha$ , are master regulators of cellular response to hypoxia. Among other activities, HIF1 $\alpha$  has been linked to both apoptosis modulation (Keith et al., 2012) and the control of extra- and intracellular pH (Parks et al., 2013). We hypothesized that Twist-mediated protection against hypoxia-induced apoptosis could be linked to HIF1 $\alpha$ . To test this idea, we generated cell lines of HepG2 expressing either an shRNA directed against HIF1 $\alpha$  or an shRNA control, directed against GFP. We cultured both cell lines in hypoxia and quantified their apoptotic

response. Although HIF1 $\alpha$  stabilization is part of a general cell protective mechanism in hypoxia (Keith et al., 2012), in some cell types its increased activity is associated with cell death (Greijer and van der Wall, 2004). Consistently, inhibiting HIF1 $\alpha$  expression in HepG2 cells increased the resistance to hypoxia induced cell death (Figure 4A). Next, we investigated whether the protective effect of HIF1 $\alpha$  depletion relied on changes of the extracellular environment. HepG2-shControl and HepG2-shHIF1 $\alpha$  cells were cultured for 24 hours in low oxygen and their respective media were used to replace the medium of the wild type HepG2, grown in the same setup and kept in culture for an additional 48 hours. Similarly to medium conditioned by Twist expressing cells, medium from cells depleted of HIF1 $\alpha$  protected wild type cells from the hypoxic stress (Figure 4B). Again in parallel to the results obtained with Twist expressors, we found that the extracellular pH of HepG2-shHIF1 $\alpha$  cell culture was less acidic compared to their control counterparts (Figure 4C).

Thus, HIF1 $\alpha$  inhibition recapitulates the hypoxia-protective phenotype of cells over-expressing Twist1 or Twist2. Indeed, in both cases cells are more resistant to hypoxic stress; this is a trans-acting effect, which apparently involves modulation of pH homeostasis. We reasoned that a simple explanation of these results, would be that Twist might inhibit some activities of HIF1 $\alpha$ . Because both proteins are transcription factors belonging to the bHLH family, we hypothesized that Twist could modulate HIF1 $\alpha$  function through a direct protein-protein interaction.

### **Twist1 and Twist2 form a complex with HIF1 $\alpha$**

To test whether Twist and HIF1 $\alpha$  proteins can form a complex, we first transiently transfected 293T cells with Twist1 or Twist2 and with HIF1 $\alpha$  cDNAs tagged, respectively, with a FLAG or a Hemagglutinin (HA) epitope. We cultured the cells in hypoxia and verified transgenes expression by western blot (Figure 5A). Immunoprecipitates obtained with either an anti-HA or with an anti-FLAG antibody were analysed by western blots. As shown in Figure 5B, HIF1 $\alpha$  co-precipitates with both Twist1 and with Twist2. The reverse immunoprecipitation experiment, using an anti-FLAG antibody directed against Twist1 or Twist2 transgenes, confirmed this

result (Figure 5C). To our knowledge, this is the first demonstration that Twist and HIF1 $\alpha$  physically interact.

However, a co-immunoprecipitation observed with transiently over-expressed proteins does not necessarily reflect a physiologically significant interaction. We thus sought to confirm these results with the endogenous proteins. Unfortunately, despite several attempts, we were unable to find a reliable antibody that could efficiently immunoprecipitate the endogenous HIF1 $\alpha$ . We therefore used an alternative method to detect endogenous protein-protein interactions, namely *in situ* PLA (proximity ligation assay) (Weibrecht et al., 2010). This experimental approach requires antibodies validated for immunofluorescence that additionally come from different species. This constraint did not allow us to use the PLA to confirm Twist2 interaction with HIF1 $\alpha$ . We thus concentrated on Twist1/HIF1 $\alpha$  complex. First we verified the specificity of each antibody by immunostaining. As shown in Figure 6A, the signal for Twist1 was detected specifically in HepG2-Twist1 cells and not in HepG2 empty vector control cell line (HepG2-pMSCV). Similarly, HIF1 $\alpha$  was detected only in HepG2 incubated in hypoxic condition (Figure 6B). The PLA experiment was conducted using as negative controls the HepG2-pMSCV cells incubated in 1% oxygen and the HepG2-Twist1 cells incubated in 21% O<sub>2</sub>. The experimental condition was the HepG2-Twist1 cells incubated in hypoxia (1% O<sub>2</sub>). An interaction between two proteins is visualized by a fluorescent dot. No, or very few, dots were present in both the control samples (Figure 6C), while numerous dots could be seen in HepG2-Twist1 cells cultivated in low oxygen. These data provide a strong confirmation of a physical interaction between Twist1 and HIF1 $\alpha$ .

Overall, our results indicate that Twist factors modulate HIF1 $\alpha$  activity and suggest that this effect is achieved through a direct protein-protein interaction.

## **Conclusions**

Many of the processes controlled by Twist1 and Twist2 qualify these transcription factors as powerful oncogenes. They are best known for mediating increased

invasiveness and allowing the bypass of two major cellular fail-safe genetic programs, namely apoptosis and senescence (Puisieux et al., 2014). Moreover, Twist proteins are involved in the cellular response to mechanical and to oncogenic stress (Ansieau et al., 2008; Desprat et al., 2008) and we have recently shown that Twist's oncogenic activities extend to modulation of oxidative stress (Floc'h et al., 2013). With the present study, we further emphasize the role of the two Twist proteins in dealing with metabolic cellular stress. Why are these transcription factors, whose primary activity appears to be initiation of migration, so tightly associated with cellular response to stress? A possible explanation might come from their role in embryonic development, notably in the process of neural crest delamination and subsequent migration. Neural crest cells are formed at the border of the neural plate and move away from the neuroepithelium into different regions of the embryo, where they differentiate into many cell types, such as the cartilage and bone cells, neurons and glial cells of the peripheral nervous system, and smooth muscle cells (Theveneau and Mayor, 2011). In the process of migrating away from their specific niche towards many different new locations, the cells encounter, and must adapt to, strong environmental pressure that induce potent cellular stress. It follows that adaptation to environmental stress is an essential element of embryogenesis that must accompany Twist genes expression, which is central in the migratory process. Importantly, it was recently reported that HIF1 $\alpha$  is required for activation of Twist in neural crest cells migration during *Xenopus* and zebrafish embryonic development (Barriga et al., 2013).

Hypoxia, which constitutes one of the environmental metabolic stresses to which both embryonic and cancer cells need to adapt in order to survive, is a potent driver of tumour progression (Jain, 2014). Indeed, resistance to this particular metabolic environment generates and selects cancer cells with increased aggressive traits as a consequence of increased genomic instability. Our results suggest that Twist oncogenes could be players in the acquisition of this phenotype. By extending the capacity of resistance of cells to oxygen deprivation, Twist could increase their chance to acquire mutations required for the tumour to progress toward a more malignant state.

It is now well accepted that despite originating from a single cell, most tumours are heterogeneous, since they are composed of multiple cancer clones (Marusyk et al., 2012). While it has been shown that Twist can be induced by HIF $\alpha$  factors (Yang et al., 2008), it is reasonable to assume that some heterogeneity in Twist expression occurs also in the hypoxic compartment. This is interesting in light of our results of the non-cell autonomous mode of action of Twist in the inhibition of hypoxia-induced apoptosis. This phenomenon could be an example of cooperative behaviour of cancer cell clones, which is expected to be beneficial for the tumour growth and progression, since it allows to share the evolutionary cost of adaptation, thus increasing the overall fitness (Tabassum and Polyak, 2015). However, the overall effect on tumour resistance to hypoxia is expected to be significant only if the Twist expressors are present above a certain threshold. Indeed the efficiency of the non cell-autonomous effect is expected to be negligible if the proportion of Twist expressors is low. They should still have a survival advantage over non-expressors, due for example to the cell-autonomous anti-apoptotic activities of Twist. Over time, the population of cells expressing Twist will grow and at some point will increase the capacity of all cells to resist the oxygen shortage. If this interpretation is correct, even a small initial number of Twist expressing cells could have a significant influence on the dynamics of tumour growth and the acquisition of malignant traits.

We found that Twist proteins modulate pH homeostasis. Our data indicate that inhibition of acidification is causally linked to Twist-mediated resistance to hypoxic stress. In apparent discrepancy to our interpretation, it has been shown that acidification of the extracellular milieu under hypoxia facilitates cancer cells invasion by inducing the breakdown of extracellular matrix and activating several proteins involved in migration (Parks et al., 2013). Indeed, because Twist proteins are also pro-invasive factors, our discovery might appear paradoxical in this context. However, it is clearly established that a strong pH imbalance is incompatible with cell survival (Parks et al., 2013). We can therefore postulate that the first consequence of Twist expression in hypoxia is to preserve cell integrity by keeping the pH above a certain critical threshold and only then facilitating cells migration away from this environment. Indeed, our results show that whereas Twist expressing cells display a higher extracellular pH in hypoxia, it is nevertheless more acidic than the pH of normoxic

cultures. In consequence, this more moderate fall of the pH would be expected to allow cell survival, but still favour invasive behaviour.

HIF1 $\alpha$  inhibition and Twist over-expression display very similar phenotypes in regard to apoptosis and to pH regulation. Strikingly, we show that both Twist1 and Twist2 bind to HIF1 $\alpha$  protein, suggesting that Twist could inhibit HIF1 $\alpha$  transcriptional activity through a physical interaction. In an attempt to test this hypothesis we quantified the expression of several classical HIF1 $\alpha$  target genes (Keith et al., 2012), namely Glut1, VEGF-A, NHE1 and CAIX (the two last known to be involved in pH homeostasis). However, we could detect no effect of Twist on their upregulation in hypoxia (data not shown). These negative results do not eliminate the possibility that Twist regulates a specific transcriptional sub-program of HIF1 $\alpha$  pleiotropic activities. The test of this hypothesis by global transcriptional analysis is underway.

There is an interesting parallel between the link between Twist and HIF1 $\alpha$  and the impact of Twist on another transcription factor, namely p65 NF- $\kappa$ B (Šošić et al., 2003). Indeed, similarly to HIF1 $\alpha$  under hypoxia, NF- $\kappa$ B, upon activation by pro-inflammatory cytokines, induces the expression of Twist. Twist in turn binds to p65 and inhibits its transcriptional activity, but only in relation to a specific subset of NF- $\kappa$ B target genes, specifically those involved in the control of apoptosis. Our results suggest that such a feedback loop might operate in a similar manner with regard to Twist interaction with HIF1 $\alpha$ . In these two physiological situations, Twist appears to function as a molecular brake that protects cells from over-reacting to stress by decreasing potentially deleterious signals that might endanger cellular survival, unless carefully uncontrolled.

In summary, our work unveils a novel oncogenic activity of Twist factors that is likely to participate significantly in their ability to trigger cancer cells malignant conversion.



## Materials and Methods

### Constructs

Twist1 and Twist2 murine cDNAs were cloned into pMSCV-puromycin retroviral vectors (Clontech) and p3X-FLAG-CMV10 plasmid (Sigma). N-terminal HA-tagged HIF1 $\alpha$  murine cDNA was cloned into pMSCV-puromycin. ShRNA sequences against GFP (5'-TACAACAGCCACAACGTCTAT-3') and HIF1 $\alpha$  (5'-CCCATTCTCATCCGTCAAAT-3') were cloned into pSIREN-RetroQ vector (Clontech).

### Cell culture

HepG2 and 293T cell lines were grown in Dulbecco's modified Eagle medium (Life Technologies) containing 4.5g/l glucose supplemented with 10% fetal bovine serum (Sigma), 100 ng/mL streptomycin, and 100 U/mL penicillin. AML12 cells were cultured in DMEM/F12 medium (Life Technologies) supplemented with 10% FBS (Sigma), 5  $\mu$ g/ml transferrin (Sigma), 5  $\mu$ g/ml selenium (Sigma), 10  $\mu$ g/ml insulin (Sigma) and antibiotics. Bipotential mouse embryonic liver (BMEL) cells, derived from c57/B6xC3H E14 embryonic mouse livers (), were cultured on collagen (Corning) coated dishes in RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum (Biowest), 50 ng/ml epidermal growth factor (PeproTech), 30 ng/ml insulin-like growth factor II (PeproTech), 10  $\mu$ g/ml insulin (Sigma) and antibiotics. All cell lines were grown in humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. Oxygen deprivation was carried out 24h after culture initiation in an incubator with 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub> for indicated time. When appropriate, cells were treated with 5 mM sodium bicarbonate (Sigma).

### Co-culture experiments

For medium replacement experiments, cells were plated on 6-well plates with 1.5x10<sup>6</sup> cells, after 48h of incubation in hypoxia, conditioned media were transferred onto control cells for further incubation.

For the transwell co-culture assay, chambers were assembled using 0.4  $\mu$ m pore transwell-inserts (BD Falcon) as upper chambers and 24-well plates as lower

chambers.  $10^5$  (WT cells) cells were placed in the upper chamber and  $2 \times 10^5$  cells (pMSCV or Twist cells) in the lower chamber. At the end of incubation times, cells in the upper chamber were collected for further analysis.

For stained co-culture experiments cells (either WT, Twist1 or Twist2 lines) were plated on 6-well plates in 1:1 ratio with WT HepG2 pre-stained with CellTracker Green CMFDA Dye according to manufacturer protocol.

### **Apoptosis analysis**

BMEL and AML12 cell were plated at  $4 \times 10^5$  cells/well and  $1.5 \times 10^6$  HepG2 cells per well in 6-well plates was used for apoptosis measurement experiments. Cells were transferred to hypoxic conditions 24h after plating. Apoptosis was monitored for 48h for AML12 and 72h for BMEL and HepG2. To determine percentage of apoptotic cells, adherent and floating cells were collected and labelled with the NucView488 Caspase 3 Substrate (Biotum) according to the manufacturer's protocol. Labelled cells were then analysed on a FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences). Apoptosis for prestained co-culture experiments was determined after labelling with the Annexin V-Cy3 Apoptosis Detection Kit (Abcam) according to the manufacturer's instructions. Stained cells were then analysed on a FACS Fortessa flow cytometer using Diva software (BD Biosciences).

### **pH measurements and lactate assay**

For these measurements AML12 and HepG2 cells were plated as described in apoptosis section. After the indicated times, medium was collected and pH was measured using a standard calibrated pH meter at 37 °C. The concentration of lactate in these supernatants was also measured by colorimetric assays using a L-lactate assay kit (Sigma-Aldrich) according to the manufacture's instructions.

### **Retroviral infection and transient transfection**

For retroviral infection,  $1.5 \times 10^6$  293T cells were transfected twice at 24 hours intervals, with 3  $\mu\text{g}$  of retroviral vector using jetPEI™ (Polyplus). Forty-eight hours after the last transfection, supernatant was collected, filtered (0.45  $\mu\text{m}$ , Millipore), supplemented with 4  $\mu\text{g}/\text{ml}$  of polybrene and added to cells to be transduced for 24h

M.O.I was always  $\leq 1$  as we noted that high viral titers significantly stress cells. Infected cells were selected after 48h with the appropriate antibiotics.

For transient transfections  $1.5 \times 10^6$  293T cells were transfected for 24h with 2.5  $\mu\text{g}$  of the indicated vectors using jetPEI (Polyplus) and used in subsequent western blot and co-immunoprecipitation experiments.

### **Co-immunoprecipitation.**

Following transfection with the indicated plasmids for 24h, 293T cells were incubated for 8h with 20  $\mu\text{M}$  MG-132 (Sigma) under hypoxic conditions as indicated in figure legends. They were then homogenized in lysis buffer A (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM NaF, 5 mM sodium pyrophosphate, 25 mM  $\beta$ -glycerophosphate, 5 mM EDTA, 20  $\mu\text{M}$  MG-132 and protease inhibitor cocktail) containing 1% of NP-40. For anti-HA and anti-FLAG immunoprecipitations, cell lysates (200  $\mu\text{l}$ ) were diluted with 300  $\mu\text{l}$  dilution buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM NaF, 5 mM sodium pyrophosphate, 25 mM  $\beta$ -glycerophosphate, 0.5 mM EDTA, 20  $\mu\text{M}$  MG-132 and protease inhibitor cocktail) and cell debris was removed by centrifugation at 9000 g for 1 min at 4 °C. Resulting supernatants were rotated for 2 h at 4 °C with 20  $\mu\text{l}$  anti-HA coupled agarose beads (Sigma) or 12  $\mu\text{l}$  of anti-FLAG coupled agarose beads (Sigma). Beads were recovered by centrifugation and washed five times for five minutes with lysis buffer A containing 0.5 M NaCl. Protein complexes bound to the beads were eluted by addition of 3x Laemmli sample buffer and incubation at 95 °C for 5 min in the case of HA immunoprecipitation. For anti-FLAG immunoprecipitation, material bound to beads was eluted with anti-FLAG peptide according to manufacturer's protocol in order to reduce unspecific IgG light-chain signal. Precipitated proteins were analysed by immunoblotting. Protein lysates were loaded onto SDS-PAGE gels and transferred onto nitrocellulose membranes. The membrane was blocked with TBST (1x TBS with 0.1% Tween 20) + 5% milk at room temperature for 1h and incubated with primary antibody and then with horseradish peroxidase (HRP)-coupled secondary antibody (Jackson). Activity was visualized by electrochemiluminescence. Antibodies used in this study are anti-HA High Affinity (Roche) and anti-FLAG M2 (Sigma).

### ***In situ* PLA**

Transduced HepG2 cells were seeded onto glass coverslips. Following the indicated treatment, cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 min, at room temperature. The interaction between overexpressed Twist1 proteins and endogenous HIF1 $\alpha$  was detected using the Duolink In Situ Kit (Sigma), according to the manufacturer's instructions. Briefly, cells were successively incubated with blocking solution for 30 min at 37 °C, with primary antibodies against Twist1 (1:200) (Abcam) and HIF1 $\alpha$  (1:200) (Cayman) overnight at 4 °C and with secondary antibodies conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS) for 1 h at 37 °C. Cells were then incubated with two connector oligonucleotides together with DNA ligase for 30 min at 37 °C. If the two secondary antibodies are in close proximity, this step allows the connector oligonucleotides to hybridize to the PLA probes and form a circular DNA strand after ligation. Incubation, for 100 min at 37 °C, with DNA polymerase consequently leads to rolling circle amplification, the products of which are detected using fluorescently labelled complementary oligonucleotides. Cells were washed with Duolink In Situ Wash Buffers following each of these steps. In the last wash, 1 mg/ml DAPI was added to the cells for 5 min at room temperature to stain the nuclei. Coverslips were mounted onto glass slides with Mowiol (polyvinyl alcohol 4-88; Sigma) and analysed by confocal fluorescence microscopy.

### **Immunofluorescence**

For immunofluorescence, HepG2 cells were seeded onto glass coverslips. Following the indicated treatment, cells were fixed and permeabilized as described in PLA section. Overexpressed Twist1 and endogenous HIF1 $\alpha$  were detected using anti-Twist1 (1:200) and anti-HIF1 $\alpha$  (1:200) antibodies, respectively and nuclei were stained with DAPI. Coverslips were analysed by fluorescent microscopy. Image acquisition and analysis were performed at the Montpellier RIO imaging facility.

### **Statistical analysis**

All experiments were repeated at least three times. Data are presented as mean  $\pm$  SEM. An Independent Student's t test was performed to analyse the assay results; a

two-tailed Student's t test was used to compare the intergroup differences. Significance was accepted for values, where  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*),  $P \leq 0.0005$  (\*\*\*)

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## Figure legends

**Figure 1. A:** BMEL, AML12 and HepG2 were seeded and incubated the next day either in hypoxia or normoxia. Cells were harvested after 48 (AML12) or 72 hours (HepG2, BMEL) and stained with NucView 488 for apoptosis analysis. Cell death was quantified by flow cytometry. **B:** The three cell lines were transduced with the following retroviral constructs: pMSCV empty, pMSCV-Twist1 or pMSCV-Twist2. After puromycin selection, cells were cultured in hypoxia for 2 or 3 days as in (A). Apoptosis was measured as above.

**Figure 2. A:** HepG2-pMSCV, HepG2-Twist1 and HepG2-Twist2 lines were cultured in 1% oxygen for 1 day in parallel with wild type HepG2. Medium from wild type cells was then removed and replaced by medium from the aforementioned lines. Cells were left 48 hours before being harvested, and quantified for apoptosis as in figure 1. **B:** Cells were seeded in Transwell chambers with wild type cells in the upper one and HepG2-pMSCV, HepG2-Twist1 or HepG2-Twist2 in the lower compartment. Cells were then placed in 1% oxygen for 72 hours. Apoptotic ratio from the upper chamber culture was determined as above.

**Figure 3. A:** HepG2 empty vector, HepG2 expressing Twist1 or Twist2 cells were placed in hypoxia and medium was collected at the indicated times to measure extracellular pH with a calibrated pH meter. **B:** Wild type HepG2 were incubated in 1% oxygen in the presence or absence of 5 mM sodium bicarbonate. pH was quantified 48 hours later as in (A). **C:** Wild type HepG2 were grown in hypoxic conditions with or without NaHCO<sub>3</sub> for 72 hours and apoptosis was determined as in Figure 1.

**Figure 4. A:** HepG2 were transduced with retroviral vector expressing either an shRNA control directed against GFP or targeting HIF1 $\alpha$ . After selection, the two cell lines were incubated in hypoxic conditions for 3 days. Cells were then collected and cell death was quantified as in Figure 1. **B:** Wild type HepG2, HepG2-shGFP and HepG2-shHIF1 $\alpha$  cells were grown for 24 hours in hypoxia. Medium from the parental line was replaced by medium from the shRNAs expressing cells. Apoptosis was determined as in (A), 48 hours later. **C:** The lines used in (A) and (B) were cultured in 1% O<sub>2</sub> for 24 hours. Medium was then collected to measure extracellular pH as in Figure 3.

**Figure 5. A:** 293 T cells were transfected with vectors encoding HIF1 $\alpha$  tagged with HA, Twist1 or Twist2 tagged with FLAG epitope. Cells were incubated in hypoxia and lysed in immunoprecipitation buffer. An aliquot of each transfection condition was used to verify the expression of the constructs by western-blot. HIF1 $\alpha$  expression was visualized with an antibody anti-HA while both Twist protein levels were detected with an anti-FLAG antibody. **B:** HIF1 $\alpha$  was immunoprecipitated with agarose beads linked to an HA antibody. After elution, complexes were revealed by western-blot. **C:** Twist factors were immunoprecipitated with FLAG antibody crosslinked to beads and visualized as in (B).

**Figure 6. A:** Wild type HepG2 cells were seeded on coverslip and incubated for 24 hours either at 21% or 1% oxygen concentration. An immunofluorescence was performed to detect HIF1 $\alpha$  protein. **B:** HepG2-pMSCV control or HepG2 expressing Twist1 cells were cultured on coverslip and Twist1 expression was revealed by fluorescent immuno-staining. As in (A) nuclei were visualized with Dapi. **C:** cell lines used in (B) were grown on glass support and cultivated either in normoxia or hypoxia for 1 day. Cells were then fixed to perform the *in situ* proximity ligation assay with HIF1 $\alpha$  and Twist1 antibodies. Red dots represent a positive interaction. Nuclei were counterstained with Dapi.

**Supplementary Figure 1.** Parental HepG2 were pre-stained with CellTracker Green CMFDA Dye and seeded together at a 50:50 ratio with either HepG2-pMSCV,

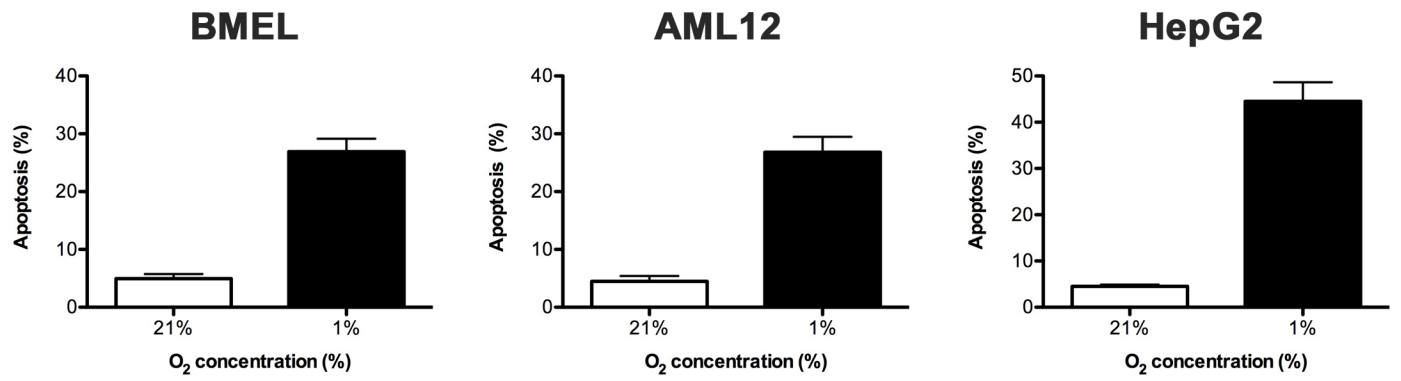


HepG2-Twist1, HepG2-Twist2 lines. Cells were then grown in hypoxia for three days. Apoptosis was quantified on green stained cells by annexin V-Cy3 labelling.

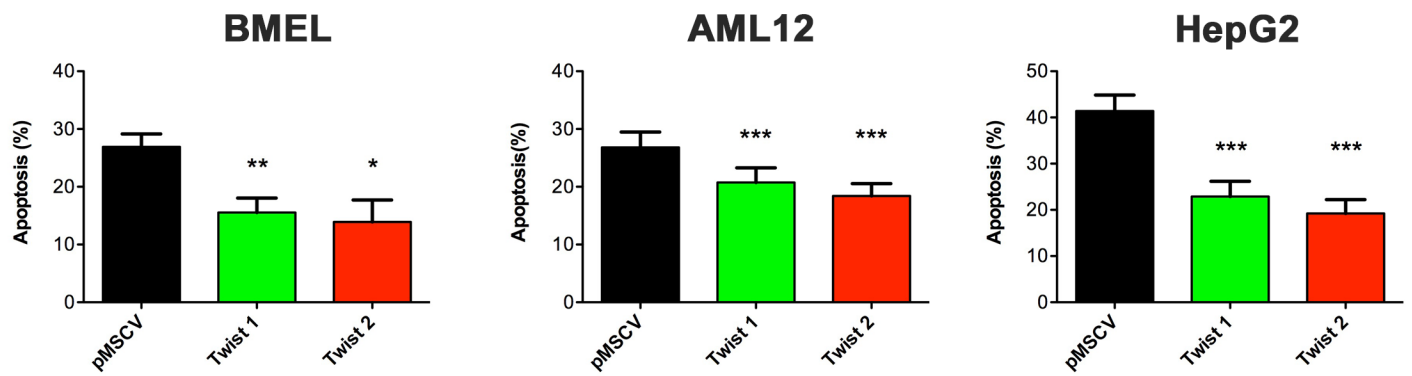
**Supplementary Figure 2. A:** Wild Type HepG2 were incubated either at 21% or 1% oxygen for the indicated time. Released lactate concentration was measured from the corresponding culture medium. **B:** Extracellular lactate from the pMSCV, Twist1 and Twist2 HepG2 lines was quantified at 24 and 48 hours of hypoxia incubation as in (A).

# Figure 1

**A**

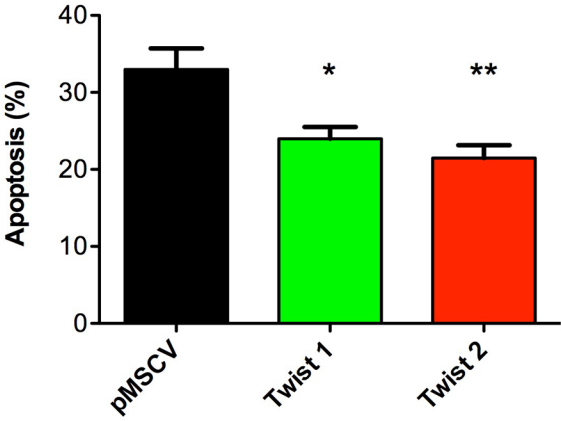


**B**

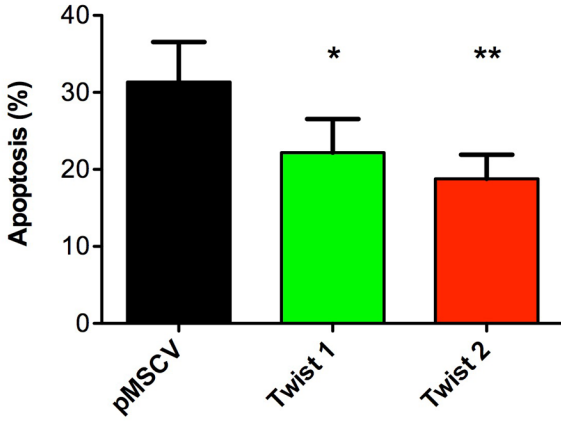


# Figure 2

**A**

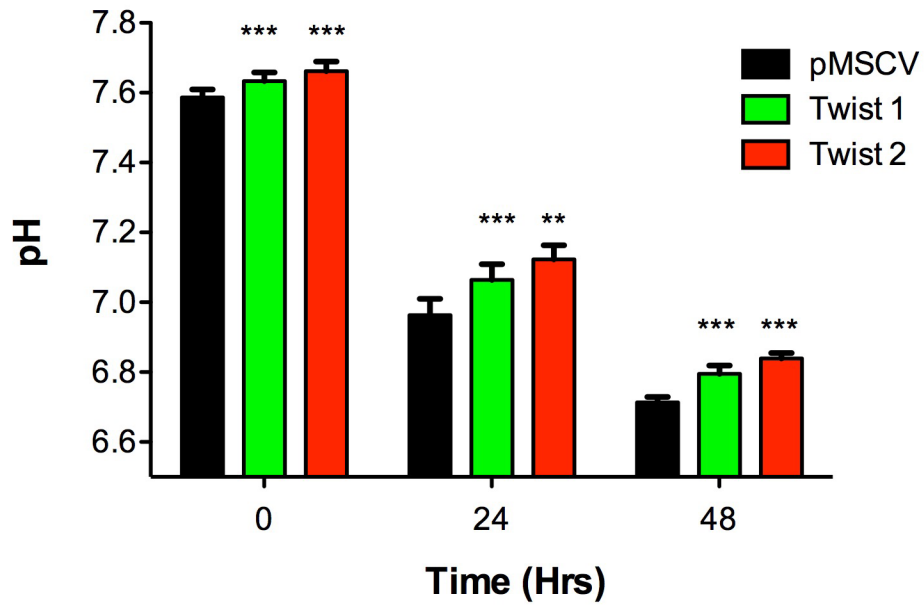


**B**

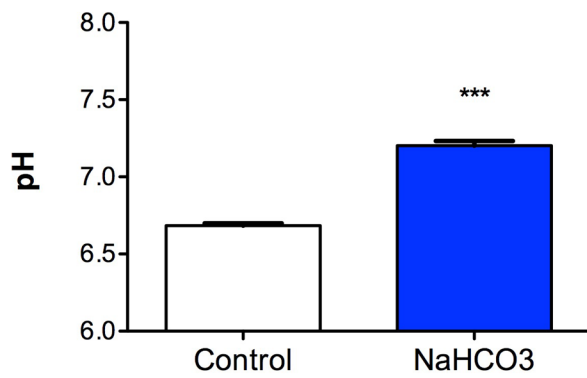


# Figure 3

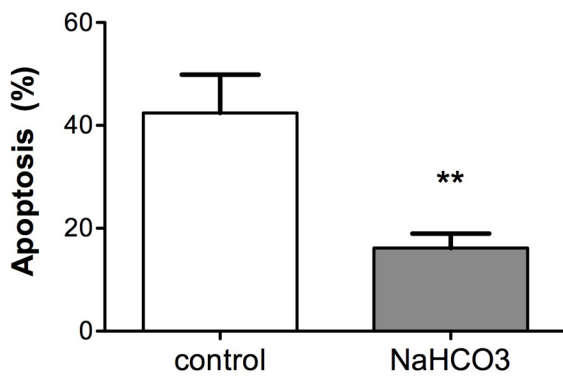
**A**



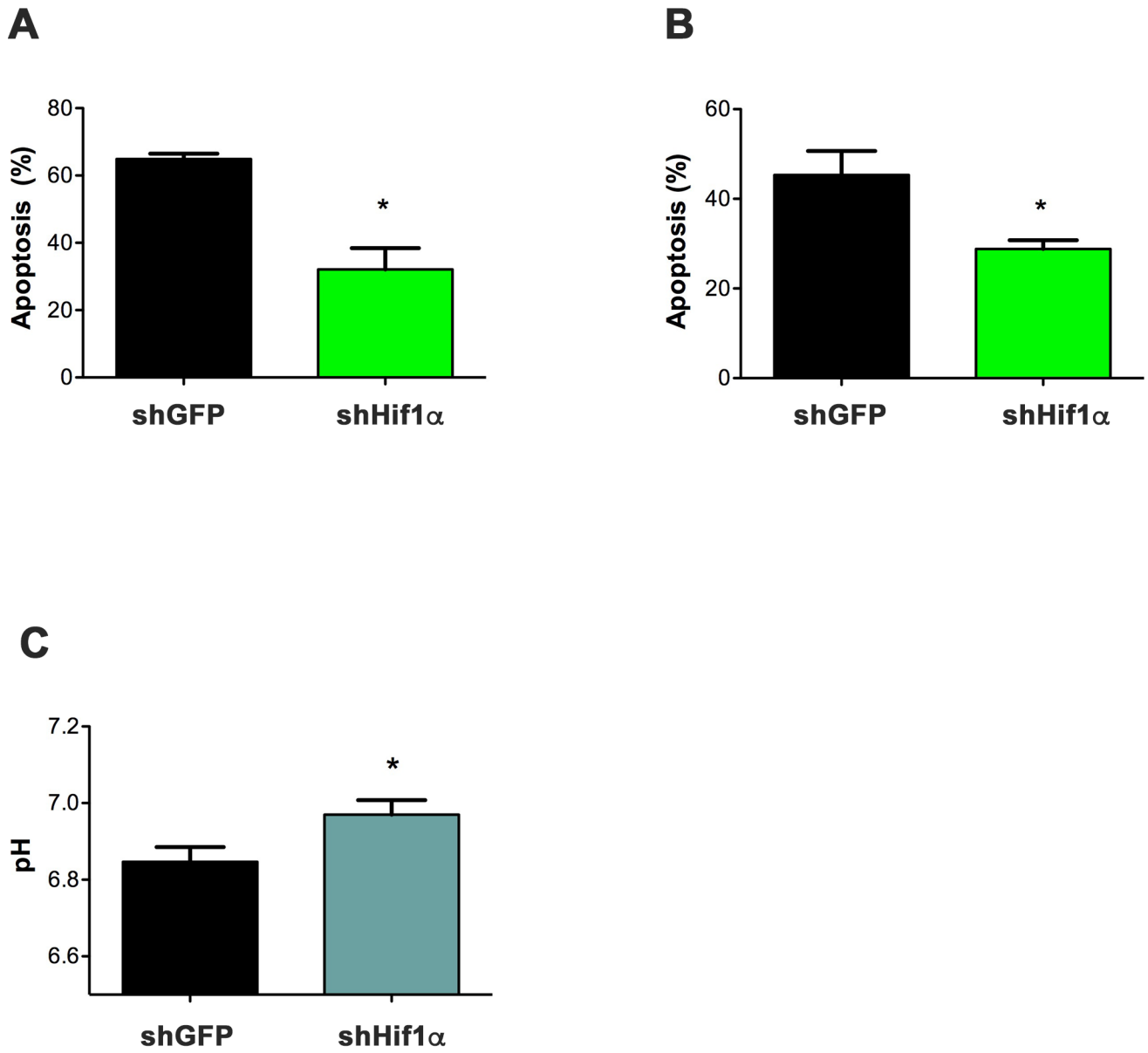
**B**



**C**



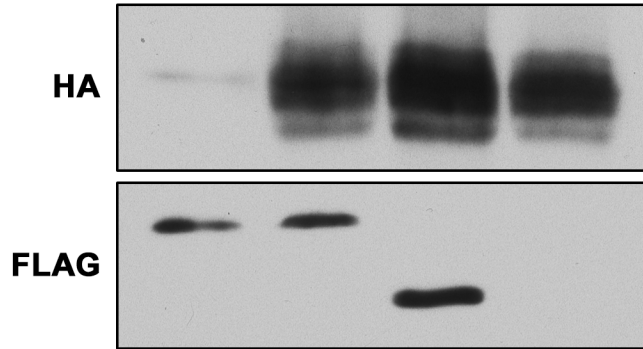
# Figure 4



# Figure 5

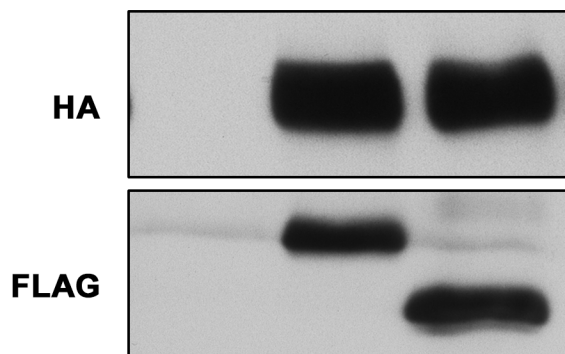
## A

HIF1 $\alpha$ -HA	-	+	+	+
Twist2-FLAG	-	-	+	-
Twist1-FLAG	+	+	-	-



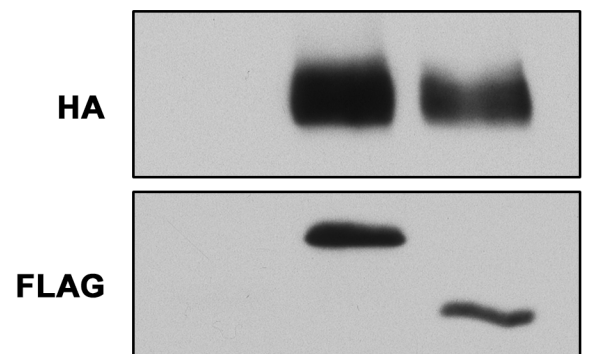
## B

HIF1 $\alpha$ -HA	-	+	+
Twist2-FLAG	-	-	+
Twist1-FLAG	+	+	-



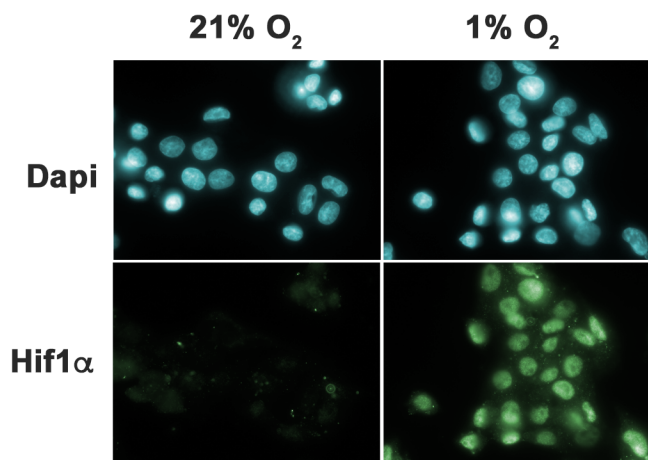
## C

HIF1 $\alpha$ -HA	+	+	+
Twist2-FLAG	-	-	+
Twist1-FLAG	-	+	-

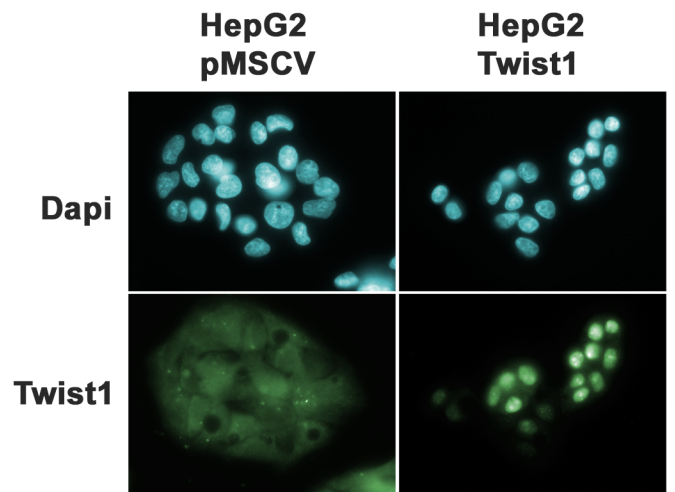


# Figure 6

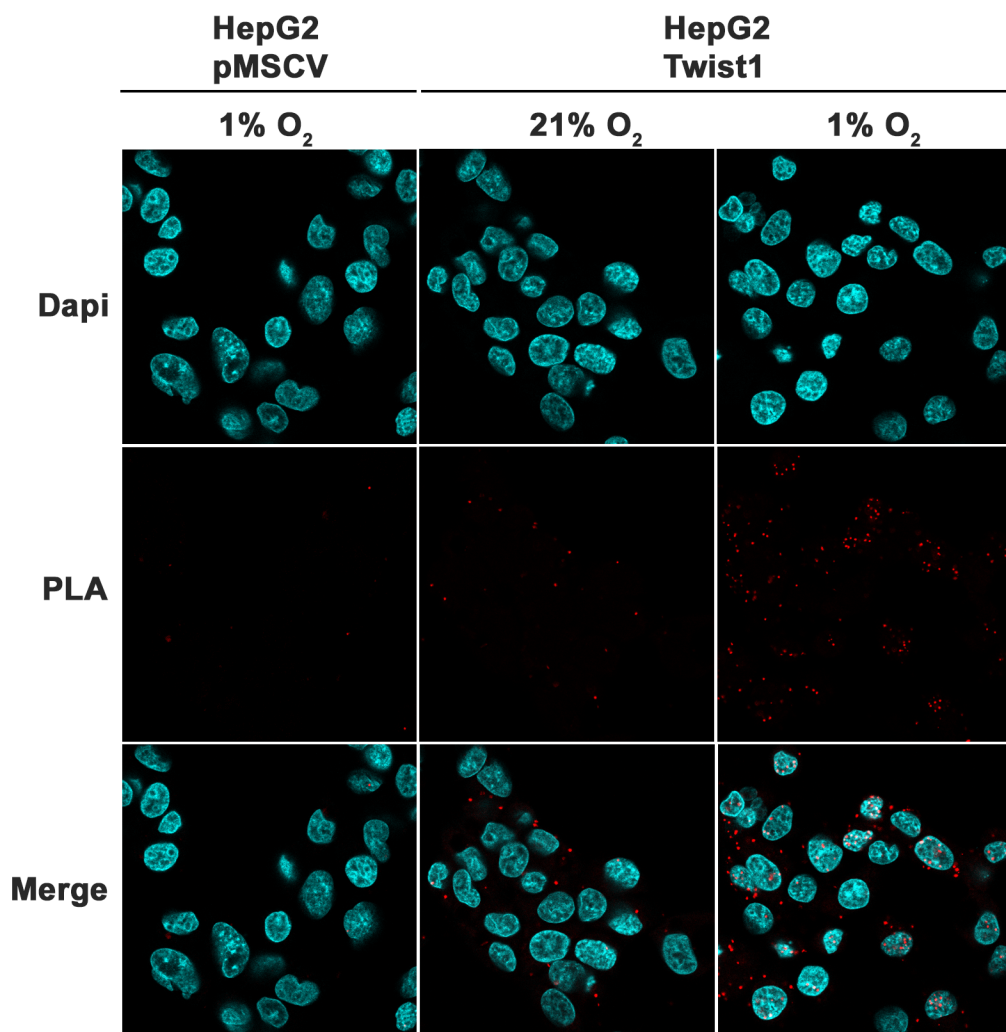
**A**



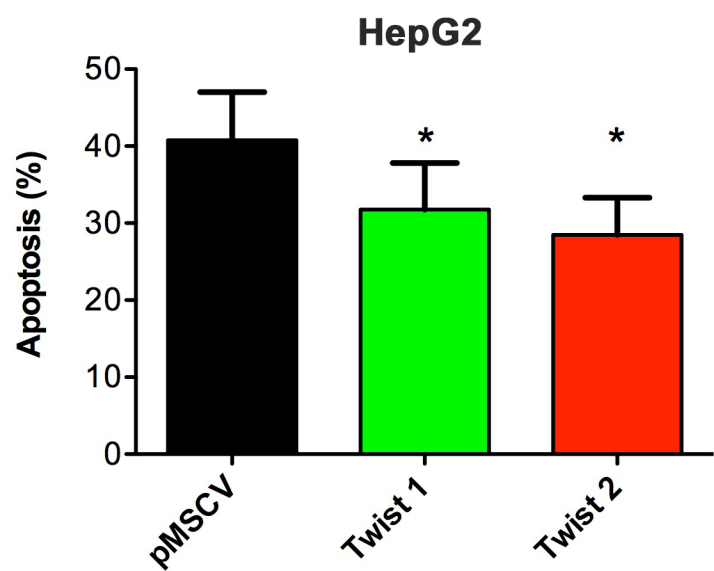
**B**



**C**



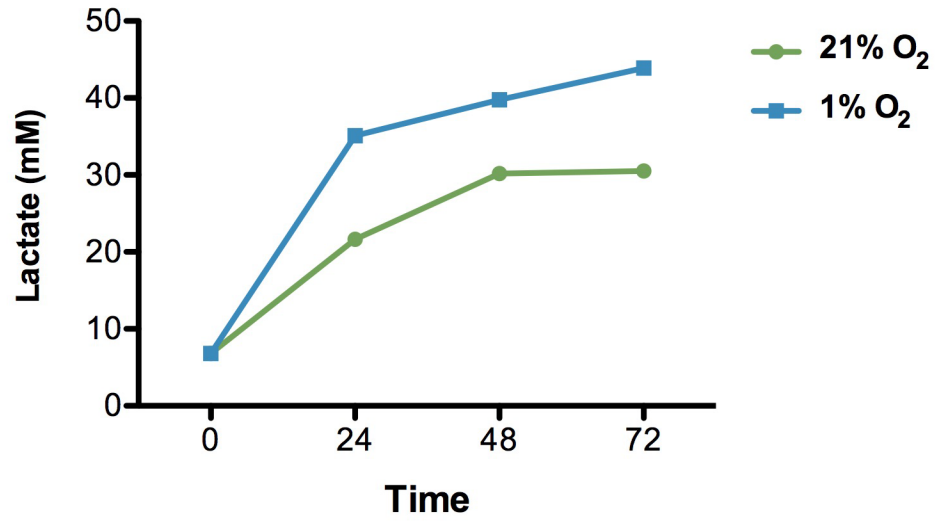
# Supplementary Figure 1



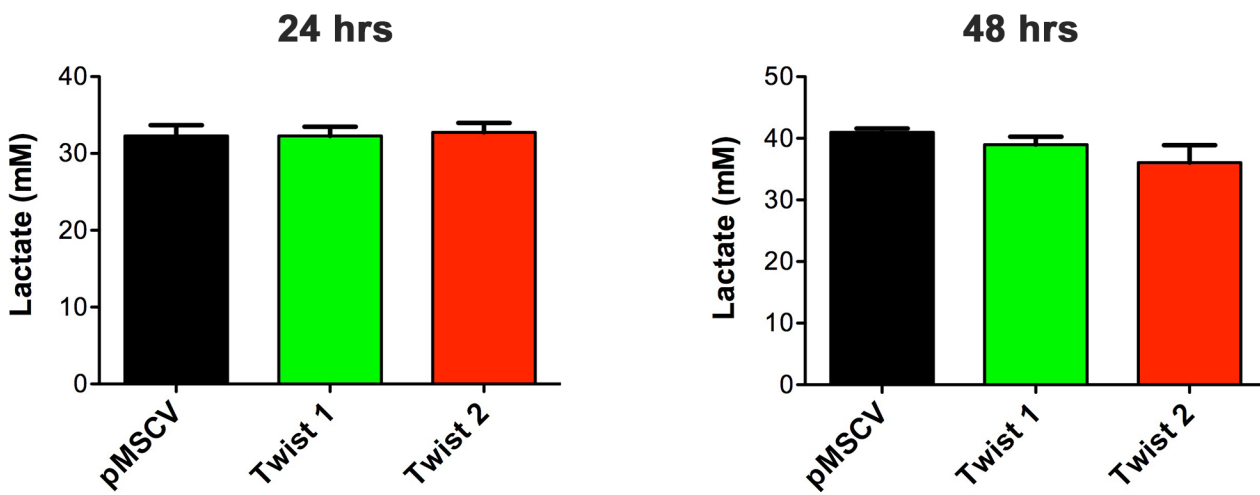


# Supplementary Figure 2

**A**



**B**



## 2 Summary

In this study, we have shown that both Twist proteins protect cells from hypoxia-induced apoptosis. Furthermore, Twist protective abilities were observed for both primary, immortalized and transformed hepatic cells. Measurements of medium acidosis in normoxic and hypoxic conditions confirmed our initial observations that Twist overexpression reduces medium acidity. Those results suggest that Twist might exert its protective abilities by regulating extracellular pH as acidosis under hypoxic condition can lead cancer cell apoptosis. In order to confirm this hypothesis, we used a widely used buffering compound - sodium bicarbonate, which increases pH when added to culture medium. Our data showed indicate that Twist protective effect is mediated through pH regulation as sodium bicarbonate treatment protected cells from hypoxia induced cell death. In addition, we have found that the effect of Twist up-regulation might be transferred to wild type cells resulting in their increased adaptation under hypoxic conditions. Indeed, various cooperation experiments such as medium swapping, co-culture or insert co-culture experiments demonstrated that Twist protective effect can be transferred onto neighbouring cells demonstrating a non-cell autonomous effect. In order to investigate a possible molecular mechanism, we focused on hypoxia master regulator - HIF1 $\alpha$ . Importantly, cell lines invalidated for HIF1 $\alpha$  were characterized by higher pH balance and lower sensitivity to hypoxia mediated cell death. Furthermore, swapping medium experiments performed on cells depleted for HIF1 $\alpha$  resulted in higher protection against apoptosis when compared to control cells. The similar results obtain from Twist over-expression and HIF1 $\alpha$  depletion suggested that Twist properties might be mediated through inhibition of HIF1 $\alpha$ . Following this hypothesis, we then demonstrated an interaction between Twist factors and HIF1 $\alpha$ . Interestingly, we showed, using by both classical co-IP and *in situ* proximity ligand assay (PLA) that both proteins interact with each other independently of oxygen concentration. Furthermore, PLA experiments additionally demonstrated that the interaction mostly takes place in the nucleus. Overall, our results suggest that Twist may act via regulation of cellular metabolism, as it is widely demonstrated that hypoxia reprograms glucose metabolism toward glycolysis and thus an increased release of lactate in the extracellular compartment. Unfortunately, our lactate measurements demonstrated that Twist does

not alter lactate production during hypoxia. Consistent results were obtained for mRNA analysis of HIF1 $\alpha$  target genes involved in pH regulation – CAIX and MCT4 (data not shown). This suggests that the complete mechanism of Twist action might be more complex. It is possible, that Twist and HIF1 $\alpha$  together regulate exclusive sets of genes (as was already observed in case of ROS regulation). However detailed mechanism has to be determined and additional study such as micro-array analysis or protein studies of HIF1 $\alpha$  -Twist1. These experiments are currently underway.

As such, we described a novel property of Twist proteins and further confirmed their role as regulator of cellular stress response. We also provided a demonstration of Twist factors ability to cooperate with neighbouring WT cells in order to increase their adaptive potential against hypoxic stress. We also shed new light on the possible mechanism regulating the protective effect of Twist factors, underlining the role of pH regulation and interaction with HIF1 $\alpha$ . Finally, the discovery of novel Twist properties further increases the notion of its oncogenic potential, as adaptation to cellular stress is considered to be a crucial step for tumour development.

## DISCUSSION

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Carcinogenesis is a complex, multistep, multipath process often described as “somatic evolution.” On one hand conventional models of cancer progression is based on the genetic and epigenetic changes observed in malignant and premalignant tumours. On the other hand cancer development might be portrayed as selection forces that are governing growth and evolution of cancer within adaptive landscapes. These selection forces are determined by environment-specific proliferative barriers that establish the optimal set of phenotypic properties indispensable for tumour growth and invasion. Thus, the phenotypic properties acquired by cancer cells can be viewed as successful adaptations to these micro-environmental selection forces. Of note, only weak to moderate stress might be advantageous for cancer cells as extensive stress will rather provoke cell death and thus failure in tumour development. Importantly, it is widely accepted that challenging environment promotes evolution of aggressive and invasive phenotypes through the sequential acquisition of adaptive properties [558, 782].

During my PhD, we have demonstrated that, Twist proteins protected cells from oxidative- and hypoxia-induced cell death. Our data suggest that Twist proteins could be considered as cellular stress regulators. Notably, we demonstrated that Twist proteins down-regulate intracellular ROS levels and therefore protect cells from c-Myc induced apoptosis. This data are consistent with the fact that c-Myc overexpression is accompanied by ROS up-regulation that is clearly linked to this cell death phenotype [618, 620]. The precise molecular mechanism responsible for Twist protective properties is exhibited through alteration of a genetic program consisting of anti- (Cdo1, Mgst3, ApoD, Dhcr24) and pro-oxidant (Aox1, Osgin1) genes. This mechanism was validated using examples of Mgst3 and Osgin1. Modulation of the selected genes showed that both of them decrease intracellular ROS levels and protect cells from c-Myc-induced cell death. However, these effects only partially mimic the impact of Twist proteins, which suggests that Twist protective abilities are determined by a joint action of several genes. Furthermore, wound-healing assay revealed that Twist antioxidative properties are not related to its pro-migratory properties. This study unveils for the first time a connection between Twist proteins and metabolic stress. This mechanism can explain how Twist factors might exhibit protective and adaptive properties toward oxidative stress and thus broadens their oncogenic potential.

Pursuing the idea of Twist as modulator of cellular stress, we have found that these factors also supply adaptive properties against hypoxic stress. Indeed, we have demonstrated that Twist proteins protect cells from hypoxia-induced cell death. Interestingly, Twist protective effect depends on its ability to decrease medium acidification, as we have shown that pH increase through sodium bicarbonate treatment also protects cells from hypoxia-induced cell death. Indeed, increased acid production due to hypoxia-mediated glycolytic-switch results in micro-environmental acidosis. This change induces further adaptation through somatic evolution in order to select phenotypes resistant to acid-induced toxicity (reviewed in [783]). Additionally, Twist seems to co-operate with neighbouring wild type cells to protect them from hypoxic cell death phenotype. Interestingly, this additionally favours Twist adaptive abilities toward cellular stress and indicates new pro-oncogenic properties of Twist proteins during tumour development. Further efforts focused on the molecular mechanisms indicated that Twist properties can act through HIF1 $\alpha$ . Indeed, HIF1 $\alpha$  invalidation decreases medium acidification and protects cells from death. In contrast to the initial adaptive response, during prolonged hypoxia HIF1 $\alpha$  may rather activate pro-apoptotic traits through induction of pro-apoptotic genes such as BNIP3, BNIP3L [773, 784] or p53 activation ([769], reviewed in [774]). Moreover, experiments in which medium from HIF1 $\alpha$  depleted cells was swapped to WT cells confirmed that pH phenotype may confer Twist anti-apoptotic properties. Finally we demonstrated that Twist proteins interact with HIF1 $\alpha$ , which provides further insights into the possible Twist protective mechanism. Overall, these data characterize a new Twist activity as a protector against hypoxic stress. Furthermore, Twist adaptive properties strengthen the previously proposed role of Twist as modulator of cellular stress and additionally provide further insight into Twist oncogenic activities.

Twist proteins are considered to be potent and powerful oncogenes acting through regulation of fail-safe programs, metastasis or cancer stem cells. In light of our current discoveries we may add an additional activity of Twist proteins, namely as a regulator of cellular stress, which creates a new niche within its oncogenic activities. Additionally, this modulatory characteristic may be linked to previously described oncogenic properties such as chemoresistance.

Therefore, Twist would play a crucial regulatory role in stressful situations, which are commonly occurring during cancer development. Indeed, manageable and moderate oxidative stress positively contributes to oncogenesis and malignant progression of cancer cell. In such conditions, ROS drive DNA damage leading to genome instability, activate signalling pathways that trigger mitogenic and pro-survival inputs, promote cell motility and finally shape tumour microenvironment by inducing inflammation and angiogenesis (reviewed in [785, 786], discussed in introduction). However, extensive and in consequence unmanageable levels of oxidative stress often become factors limiting cancer cells survival. In this case, novel properties of Twist proteins may provide adaptive mechanisms shielding cells from extensive ROS accumulation. Importantly, ROS have been suggested to induce cell death either by stabilizing p53 or by influencing the effectors acting downstream of p53 [787, 788]. With this in mind our results become even more interesting and are consistent with other studies on Twist mediated apoptosis regulation. Notably, we showed that Twist overexpression protects cells from c-Myc induced apoptosis and this effect is mediated through ROS down-regulation. Indeed, activation of c-Myc is classically associated with ROS up-regulation as it prevents NF- $\kappa$ B-dependant up-regulation of MnSOD that in normal cells opposes death by serum starvation [618, 620]. According to those findings Twist proteins might provide two-phase mechanism responsible for the protection against cell death. On one hand, it has been demonstrated that Twist proteins block apoptosis through direct interaction with p53 [334] and on the other hand Twist may protect cells from death by ROS down-regulation, which may later act up- and down-stream of p53. To close this regulatory loop, it is important to mention that both c-Myc and ROS were previously described to activate p53 and therefore induce apoptosis [335, 434]. In agreement with our results on c-Myc induced apoptosis, other groups have shown that mouse embryonic fibroblast invalidated for Twist2 display an increased sensitivity towards other pro-apoptotic stimuli such as TNF $\alpha$  [335]. Of note, this cytokine induces and furthermore requires ROS to exert its pro-apoptotic activity. This thus suggests that the antioxidant effect could participate in Twist regulation of cell death induced by various stimuli during development [789]. One could be tempted to speculate that Twist factors may regulate other cell death processes, in which ROS levels are elevated e.g. necrosis [790].



Apoptosis is not the only fail-safe program affected during tumorigenesis that is regulated by both ROS and Twist. Indeed, for example, H-Ras induced senescence, is associated with elevated oxidative stress [614]. Again similarly to controlling apoptosis, Twist was also described to prevent Ras induced senescence through down-regulation of p16<sup>INK4A</sup> and p21<sup>CIP</sup> [437]. Thus, this suggests that Twist may act in two complementary ways to prevent senescence induction.

Nevertheless, it is important to mention that Twist adaptive properties against oxidative stress may impact also on other Twist oncogenic activities. First of all, Twist expression is associated with chemoresistance against several chemotherapeutic drugs such as paclitaxel, cisplatin or doxorubicin. Interestingly, anti-cancer drugs are often associated with elevated ROS. Indeed, each of the drugs sensitive to Twist levels, promote oxidative stress, which may contribute to their cytotoxicity ([791–794], reviewed in [795]). This is certainly not a simple coincidence, but rather a crucial fact that prompts a question whether Twist antioxidative properties may contribute to Twist mediated chemoresistance. Above all, a demonstration of such relation would significantly strengthen the role of Twist during adaptation towards oxidative stress.

It is also important to mention the involvement of Twist proteins in biology of normal and cancer stem cells (discussed in introduction). It has been demonstrated that physiological stem cells contain lower levels of ROS than their more mature progeny and that these differences appear to be critical for maintaining stem cell function [796, 797]. Interestingly, it also appears to be true for subsets of CSCs in some human and murine breast tumours, which were described to contain lower ROS levels than corresponding non-tumorigenic cells (NTCs) [271]. Notably, lower ROS levels, particularly in CSCs, are associated with increased expression of free radical scavenging systems [271]. Additionally, Twist proteins are involved in maintenance of immature phenotype of mesenchymal stem cells and as such their expression promotes acquisition of stem-cell phenotypes by cancer cells during tumour progression. Those findings raise a question if the antioxidative properties of Twist proteins as well as Twist mediated up-regulation of enzymatic machinery might impact ROS biology in physiological and pathological stem cells and thus regulating their differentiation.

As mentioned before, Twist factors are known for their pro-metastatic properties. Indeed, those factors mediate tumour dissemination by promoting invasion and metastasis via EMT, but also via other pathways (discussed in introduction). Indeed, disseminating cancer cells require adequate ROS defence as they are exposed to high levels of ROS during tumour progression and secondary site colonization [798]. Furthermore, a group of Sean Morrison demonstrated that oxidative stress increases in metastasizing cells when compared to established subcutaneous tumours and limits distant metastasis in melanoma cell [799]. This finding additionally raises a question if Twist antioxidative properties may contribute to metastasis progression by increasing adaptation of disseminating cancer cells towards oxidative stress. However, our results suggest that Twist mediated lowering of the intracellular ROS level has no significant influence on cell migration, at least *in vitro*.

Presented examples indicate that Twist-mediated adaptive response against oxidative stress might not be limited only to protection from ROS-induced apoptosis. In my opinion, Twist due to its role of a potent oxidative-stress modulator might be involved in regulation of other aspects of cancer cell biology that provide them with a precise advantage over healthy cells thus promoting tumour progression on different levels. As such, Twist mediated protection from ROS can be seen as an additional or rather complementary activity supporting its already described oncogenic properties.

The second part of my thesis provides further evidence of Twist involvement in regulation of cellular stress. We proposed that Twist by protecting cells against hypoxia-induced apoptosis trigger adaptive response towards hypoxic stress. On one hand hypoxia can be seen as a hostile environment, which is a characteristic feature of most solid tumours that can clearly induce cell death [800, 801]. However on the other hand, development of an intra-tumoral hypoxia has been shown to be a critical step in tumorigenesis. Indeed, low tumour  $pO_2$  correlates with poor patient survival and advanced metastasis [802]. This may suggest that adaptation to hypoxia triggered by Twist proteins promotes cancer development, probably in cooperation with others, previously described Twist properties. Hypoxia is mainly controlled by the activity of HIF1 $\alpha$ . This master-regulator of hypoxic response affects many key aspects of tumour initiation, progression, invasion and metastasis. In spite of that, HIF1 $\alpha$  may also exert an

anti-tumoral activity. Notably, during prolonged hypoxia, HIF1 $\alpha$  promotes apoptosis through the induction of BNIP3 and BNIP3L expression or p53 stabilization ([773], reviewed in [774]). In view of these considerations, Twist mediated anti-apoptotic activity may provide additional time for cells to adapt to stressful environment. As such, it would create an opportunity for cooperation between Twist and HIF1 $\alpha$  what may lead to selection and thus evolution, a process indispensable for tumour progression. In addition, the article of M.H. Yang demonstrated that HIF1 $\alpha$  might trigger expression of Twist1 and thus promote EMT and consequently a metastatic phenotype [426]. Furthermore, Twist expression can be also modulated through HIF2 $\alpha$  [427]. This provides a temporal continuity of Twist expression, as HIF1 $\alpha$  activity decreases during hypoxia while the one of HIF2 $\alpha$  increases [485]. These findings suggest that Twist mediated adaptation might continues to occur during cancer progression, which may result in promotion of cancer cells migration to a less hostile environment.

Increased acid production resulting from up-regulation of glycolysis leads to micro-environmental acidosis, which in turn requires further adaptation to phenotypes resistant to acid-induced toxicity, mainly through somatic evolution. Furthermore, cell populations that emerge from acidosis have a powerful growth advantage. Indeed, they alter their environment through increased glycolysis in a way that is toxic to other phenotypes, but harmless to themselves. In addition, the environmental acidosis facilitates invasion through destruction of adjacent normal cell populations, degradation of the extracellular matrix and promotion of angiogenesis (reviewed in [783]). Noteworthy, prolonged exposure of normal cells to an acidic microenvironment typically induces necrosis or apoptosis by affecting p53- and caspase-3-dependent mechanisms [803, 804]. Interestingly, the mechanism of Twist action under hypoxia could rely on up-regulation of extracellular pH, therefore it seems plausible that the Twist adaptive phenotype observed under hypoxia could also apply to acidosis.

Of note, hypoxic stress and acidosis are closely linked as HIF1 $\alpha$ -mediated metabolic reprogramming directly promotes acidosis. Therefore, it is probable that when both stresses occur simultaneously, Twist would co-operate with HIF1 $\alpha$  to regulate extracellular pH and thus protect cells from both stresses. However, it should be determined whether this effect can still be observed during normoxic acidosis, when the

activity of HIF1 $\alpha$  is low. Interestingly, in our hands Twist is also able to regulate pH in normoxia, strongly suggesting that it might be generally protecting cells from the consequences of acidosis.

Strikingly, our results demonstrate that Twist-mediated protection can be transferred to neighbouring cells, possibly through local modulation of extracellular pH. Indeed, we have shown that Twist expressing cells protect WT cells in three different co-culture experiments – medium swapping, co-culture and insert co-culture. Protection seems to occur through regulation of medium acidification. This is consistent with co-culture experiments, in which 1:1 mix of cells demonstrated 50% smaller protection when compared to control Twist cells. Interestingly, during co-culture experiments the percentage of death was similar in Twist expressing and WT cells, further implying that Twist protective properties depend mostly on its ability to modulate pH rather than on acting as a direct blocker of apoptosis through interaction with p53. Those experiments support the idea that Twist proteins protect neighbour cells through the modification of their microenvironment, which clearly has important implications e.g. in cancer therapy.

However, these results additionally raise another crucial question: are Twist cells able to co-operate with other cancer clones in order to gain and provide a selective advantage. In most cases individual sub-clones within a malignant microenvironment are depicted as self-interested competitors [215, 220]. Although, a number of reports propose that the phenomena of inter-clonal cooperation may be highly beneficial for a developing tumour. Indeed, it has been shown to drive tumorigenesis not only in fruit fly models [805, 806], but also on various stages of tumorigenesis in mammalian models, especially during tumour maintenance and progression [807, 808]. In the context of Twist proteins, the article published by the group of L.M. Ellis deserves a particular attention. Notably, they have shown that colorectal cancer cells resistant to Oxaliplatin could cooperate with chemo-naïve cells towards tumour progression [807]. More precisely, they demonstrated that conditioned media from OxR cells, when placed on chemo-naïve cells, led to their increased survival both in the presence or absence of Oxaliplatin. Secondly, subcutaneous injections of 1:1 ratio of resistant and parental chemo-naïve cells into mice resulted in the development of larger tumours when compared to independent injections of either of the pure populations. In addition,

investigators observed that resistant cells grew slower than the parental cells. Moreover, the larger size of the mixed *in vivo* tumours suggests that the cell lines were non-cell autonomously interacting to increase tumour growth. Thus, these studies demonstrate that inter-clonal cooperation is highly beneficial, if not necessary for tumour maintenance and progression. In light of these findings our results become even more interesting, as one could consider that Twist mediated regulation of pH may have a cooperative effect on neighbouring cells, which in turn may promote tumour progression and acquisitions of new properties by winning clones.

In summary, all the present examples of Twist involvement during both oxidative and hypoxic stress provide evidence for its protective function in response to numerous cellular stresses. Twist protective properties might be not only limited to protection against stress-induced apoptosis, but also complement other Twist oncogenic activities. Additionally, provided data strongly suggest that Twist protective activities might be extended towards neighbouring WT cells thus promoting their adaptation. Furthermore, in light of recent discoveries, Twist protective properties at least against hypoxic stress and acidosis might result in co-operation with other cancer subpopulations.

However, one question remains open, are Twist activities limited to Twist genes family or may it rather be a universal property of a bigger group of similar genes. Notably, Twist proteins belong to a family of EMT inducers comprising Snail, Slug or Zeb. Interestingly, a recently published report indicated that Snail-mediated FBP1 down-regulation results in metabolic reprogramming and concomitant down-regulation of ROS production through suppression of the activity of the mitochondrial complex I [809]. That in consequence, leads to an increase in CSC-like properties and enhancement of tumorigenicity. As such it suggest that other EMT inducers might be also involved in the regulation of oxidative stress. However, to fully establish a general connection between EMT inducers and cellular stress response, further studies are indispensable, especially focusing on other EMT factors such as Slug or Zeb.

Following part of the discussion will switch our attention to the molecular mechanism of these new properties of Twist proteins. Surprisingly, the mechanism of action of Twist does not base on changes in mRNA levels of any of the main cellular detoxifying

enzymes such as catalase, MnSOD, Cu/ZnSOD or GPx1. Instead, Twist rather relies on the modulation of a different genetic program of oxidative stress regulators. Indeed, we have demonstrated that Twist proteins down-regulate two pro-oxidative genes, namely Aox1 and Osgin1, and up-regulate four antioxidative genes: Cdo1, Mgst3, Dhcr24 and ApoD. Additionally, our results indicated that Twist do not possess a single target gene as all effectors were modified moderately. It was further confirmed through a number of experiments, which showed that direct modulation of individual genes only partially recapitulated Twist effect both on ROS levels and apoptosis. Our findings are contrary to what was observed for Snail mediated regulation, as Snail's activity is based on the repression of one gene – FBP1. However, a similar regulatory network has been also reported for NRF2, a master regulator of intracellular ROS levels [810].

Of course as our mechanism was only validated on two target genes, Mgst3 and Osgin1, we cannot conclude if all discovered genes equally contribute to Twist phenotype. Additionally, as this study was focused on the measurements of mRNA levels, we cannot undeniably conclude if all presented genes are direct transcriptional targets of Twist. Thus additional experiments, such as CHIP or promoter analysis, should be performed in order to further clarify this issue. Furthermore, our experiments were restricted to a particular cellular model and thus identification of six genes is probably not exhaustive. Taking into account the limitations of the system used, we anticipate that Twist antioxidative program may vary from one cellular model to another.

Following the course of my thesis, one topic was clearly reappearing at its different stages. Noteworthy, our results evidently point towards an interesting interplay between p53 status and Twist activities. Indeed, it has been shown that Twist inhibits p53 transcriptional activity through a direct interaction that results in inhibition of p53-dependent apoptosis. Furthermore, several reports suggested the involvement of p53 in ROS biology. For example, overexpression of p53 was shown to up-regulate pro-oxidant enzymes – NQO1 and POX (quinone oxidoreductase and proline oxidase) that leads to oxidative stress and consequently to apoptosis [787, 811]. In contrast, physiological levels of p53 have a subtle, but vital function in containing ROS at non-toxic levels through transactivation of antioxidant genes [812, 813]. Therefore, it would be very interesting if Twist mediated inactivation of p53 might impact intracellular ROS levels

even though the Twist target genes that we identified are not known to be modulated by p53.

An additional question arises regarding PGC1 $\alpha$  as it has been demonstrated that Twist can inhibit its transcriptional activity through direct interaction. Indeed, PGC1 $\alpha$  is an antioxidative factor, which modulates several ROS-detoxifying enzymes such as GPx1 or SOD2 [814]. In this context it would be very interesting to study whether Twist could impact on ROS levels by modulating PGC1 $\alpha$ . Overall, I believe that our study unveiled a new mechanism of Twist activity against oxidative stress, through the joint action of several antioxidant enzymes.

In contrast to the first article, the mechanisms proposed in the manuscript of the second article is yet less complete. Our data strongly suggest that in the case of hypoxia-induced cell death, Twist exerts its protective activity at least partially by modulating medium acidification. Importantly, that notion is supported by the fact that using sodium bicarbonate significantly protected cells against apoptosis by increasing the pH of culture medium. However, we cannot exclude that Twist may exert other pro-survival effects, which could be independent of its role as a pH modulator. They could all nonetheless converge and finally result in efficient protection against hypoxia-induced cell death. Furthermore, taking into account the co-culture and medium swap experiments lead us to believe that Twist activity may rely also on the expression of extracellular protective factors (e.g. IGF1 [815, 816]). Summarising, all these data rather favour the idea that Twist's anti-apoptotic activity in hypoxic condition is mediated by an impact of external factors than by internal regulatory mechanisms such as we observed in case of oxidative stress. Furthermore, experiment performed on cells with invalidated HIF1 $\alpha$  suggested that Twist mediated mechanism might occur through interaction with HIF1 $\alpha$  activity. Indeed, down-regulation of HIF1 $\alpha$  not only protected cells from hypoxia-induced apoptosis, but also significantly increased the medium pH. More importantly, similarly to Twist conditioned medium, supernatants from these cells also efficiently protected wild type cells from hypoxia-induced apoptosis.

Therefore, the most likely mechanism that we are currently taking under consideration is based on the interaction between HIF1 $\alpha$  and Twist proteins, shown by both PLA and

co-IP experiments. Interestingly, this interaction occurs both during normoxia and hypoxia, suggesting that Twist can bind HIF1 $\alpha$  independently from its hydroxylation. Although, this information is interesting per se, it also raises a series of currently unanswered questions. First of all, this interaction is not yet completely characterized, as we are not sure whether it is direct. Secondly, as HIF1 $\alpha$  is a highly modified protein, characterizing the sites, which are required for this interaction, would be important for learning how modulate their respective activities are modulated. Additionally, experiments such as double-IP could provide useful information about complex composition what would further help us to determine the biological outcome of this interaction. Finally, basing on PLA experiments we know that this interaction occurs mainly in the nucleus. However in order to determine if those two proteins can form an active common complex on chromatin, series of CHIP on CHIP experiments would be indispensable.

Moreover, the results obtained with HIF1 $\alpha$  invalidation suggest that Twist proteins may act via down-regulation of HIF1 $\alpha$  transcriptional activity to protect cells from hypoxia-induced apoptosis. Of note, we have verified that Twist expression does not alter HIF1 $\alpha$  mRNA and proteins level under hypoxia. Surprisingly, we discovered that Twist is unable to regulate many of well-known targets of HIF1 $\alpha$ , such as Glut1, LDH, PDK1, VEGF (data not shown), which are crucial during HIF1 $\alpha$ -mediated adaptation. Consistently, a further study of HIF1 $\alpha$  targets mRNA levels revealed that Twist does not affect the levels of BNIP3 or BNIP3L – classical HIF1 $\alpha$  pro-apoptotic targets. As we have shown that Twist phenotype is linked to medium acidification, we focused on HIF1 $\alpha$  targets involved in pH regulation – CAIX and MCT4. However, Twist factors do not regulate these genes at least at the transcriptional level (data not shown). Similar results were obtained for other pH regulators; whose expression is modulated independently of HIF1 $\alpha$ , such as NHE1, AE1 or NBC (reviewed in [778]). These data support our current hypothesis, that Twist action on HIF1 $\alpha$  is rather directed towards a specific gene or group of genes. We suspect that Twist may impact only those genes, promoters of which contain combined binding sequences for both HIF1 $\alpha$  and Twist. It is however also possible, that Twist regulates the extracellular pH by affecting the activity of membrane localized channels and transporters.



As mentioned before, all experiments were performed on p53 wild type cells. It is important, as it is known that during prolonged hypoxia or very low oxygen concentration HIF1 $\alpha$  stabilizes p53 (reviewed in [774]). However, our results suggested that classical p53 target genes such as Bax, Noxa or Puma are not regulated by Twist in our conditions (data not shown). Therefore we can conclude that Twist described properties are independent from p53 status.

To sum up, in this PhD thesis I described new Twist activities, that underline its role as a potent modulator of cellular metabolic stress. Notably, we showed that Twist proteins protect cells from oxidative and hypoxic stress and probably also acidosis. Remarkably, we also provided a mechanical explanation for the impact of Twist during oxidative stress. Indeed, we showed that Twist through regulation of genetic programme of antioxidant enzymes protects cells from ROS-induced apoptosis. In the case of hypoxic stress, we showed that Twist protection is likely to be mediated through the regulation of medium acidification. Additionally, we linked Twist phenotype with HIF1 $\alpha$ , a master regulator of hypoxic response. Remarkably, we demonstrated for the first time that Twist proteins interact with HIF1 $\alpha$ . Additionally, we hypothesised that Twist proteins through down-regulation of selective group of HIF1 $\alpha$  target genes protect cells from hypoxia-induced apoptosis. As such our results shed light on novel activities of Twist proteins, granting them a place among other regulators of cellular stress. However, further studies, especially on the exact molecular mechanisms controlling Twist-mediated response towards hypoxia, are still required to fully address the question of Twist oncogenic potential.

Up to now Twist was known to play crucial role in chemoresistance, escape from apoptosis and senescence as well as metastasis and CSC. Our results not only describe new activities of Twist per se, but more importantly provide a clear link between all these processes. Indeed, considering Twist as major regulators of stress and thus factors crucial for cell adaptation greatly strengthens its role in tumour development.

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## ANNEXE I. Résumé de la thèse

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## **Etude des facteurs oncogéniques Twist dans la régulation du stress oxydatif et hypoxique.**

Dans cette thèse, j'ai décrit mes travaux se concentrant sur de nouvelles activités de protéines oncogéniques Twist. La connaissance actuelle sur des activités pléiotropes de Twist nous a incité à postuler que ces facteurs peuvent être les principaux régulateurs de la réponse au stress. Les cellules cancéreuses survivent et croissent dans un environnement en constante évolution qui crée de multiples contraintes auxquelles ils doivent s'adapter pour survivre et s'efforcer. Ces adaptations donnent souvent lieu à l'acquisition d'un phénotype agressif. Conformément à cette hypothèse, nous avons récemment dévoilé de nouvelles activités de protéines Twist qui sont liés à la réponse au stress. Nous avons montré que Twist régule réponse au stress oxydatif et hypoxiques causé par la surproduction d'espèces réactives de l'oxygène et la privation d'oxygène respectivement.

### **INTRODUCTION**

#### **1. Mécanismes de la tumorigenèse**

Au cours des dernières décennies, les chercheurs de cancer ont peu à peu commencé à reconnaître que les tumeurs peuvent être considérées comme des organes dont la complexité peut rapprocher de celui des tissus sains. Le cancer est une maladie caractérisée par une prolifération incontrôlé et propagation des cellules malignes mutées. Si la croissance et la propagation de la tumeur n'a pas été empêchée, elle aboutit en définitive à la mort de l'organisme entier. Avant que cellules saines évoluent dans un état néoplasique, ils doivent acquérir plusieurs phénotypes distinctifs, qui sont nommés de cancer par Hanahan et Weinberg. L'acquisition de ces traits est souvent un processus long en plusieurs étapes et il est appelé la tumorigenèse.

L'instabilité du génome et ces mutations conduisent à l'accumulation de modifications génétiques, qui à son tour favorisent l'évasion des cellules néoplasiques de régulation homéostatique. Actuellement, de grands ensembles de gènes sont associés à l'oncogénèse et ils peuvent être arrangés en deux classes - oncogènes et gènes suppresseurs de tumeurs. L'activation ou l'inactivation de ces gènes peuvent conduire à

l'acquisition de certains avantages tels que la résistance à l'apoptose ou de la prolifération soutenue.

Un oncogène (par exemple, Ras, Myc ou Bcl-2) est défini comme un gène dont l'expression accrue agit de façon dominante à participer au processus de transformation de la normale à une cellule cancéreuse. Il est souvent un homologue muté ou dérégulé d'un proto-oncogène, qui est exprimée dans les cellules normales. La conversion d'un proto-oncogène à un oncogène peut être déclenchée par plusieurs mécanismes, comme des mutations gain de fonction.

D'autre part, des suppresseurs de tumeurs (par exemple p53 ou RB) sont des gènes de protection qui limitent la croissance des tumeurs. Leur "perte de fonction" mutations trouvées dans les tumeurs inhibent ou abrogent leurs capacités défensives. Cette catégorie de gènes est souvent impliqué dans le maintien de l'homéostasie tissulaire et leur inactivation représente une deuxième modification génétique distincte observée au cours du développement de la tumeur. Contrairement aux oncogènes, les protéines codées par la plupart des gènes suppresseurs de tumeurs inhibent la prolifération cellulaire ou la survie. Acquisition des événements du pilote de mutation permet aux cellules cancéreuses de surmonter deux programmes importantes et critiques pour la sécurité intrinsèque - l'apoptose et la sénescence. L'apoptose est un programme de suicide cellulaire qui a évolué comme un moyen efficace pour éliminer les cellules inutiles ou malsaines du corps. Il est un processus fondamental associé avec le développement normal et l'homéostasie tissulaire de tous les organismes multicellulaires. Au niveau moléculaire l'apoptose est dirigé par les caspases, une famille de protéases cystéine aspartate. L'un des régulateurs essentiels de l'apoptose sont protéines de la famille BCL-2, qui peuvent être fonctionnellement divisé en trois sous-familles. Notamment, les protéines famille Bcl-2 multi-domaines peuvent être anti-apoptotique (par exemple Bcl-2, Mcl-1) ou pro-apoptotique (par exemple Bax, Bak). La dernière sous-famille se compose de protéines pro-apoptotiques uniquement BH3 comme Bid, Bad, PUMA.

La sénescence est considéré d'agir comme l'un des principaux mécanismes de la tumorigenèse et avec l'apoptose ils comprennent le principal mécanisme de défense de

notre organisme. La sénescence est induite par pléthore de différents stimuli et conduit à l'arrêt permanente du cycle cellulaire. Différents signaux peuvent induire la sénescence cellulaire à la suite de l'activation de voies de signalisation différentes, telles que la voie p53. Lors de l'activation, la transduction du signal conduit à la stimulation des effecteurs principaux de la sénescence, les inhibiteurs de CDK et conduit à un arrêt du cycle cellulaire au cours de phases G1 et G2. Néanmoins, notre compréhension de la biologie du cancer et sa génétique considérablement augmenté au cours des dernières années. Cela a conduit à l'émergence de nouveaux concepts qui pourraient expliquer la complexité du cancer et donc la difficulté de trouver des remèdes efficaces. Aujourd'hui, le cancer est décrit comme un processus évolutif qui est entraînée par étapes des mutations des cellules somatiques avec la sélection subclonal séquentielle. En outre, les réalisations récentes dans la biologie du cancer validées et confirmées un lien entre les processus de la tumorigenèse et de l'évolution darwinienne. Chaque tumeur est différente et cela est vrai non seulement lorsque nous comparons les mêmes types de cancer chez des patients différents. Diversité de la tumeur peut être observée par des différences dans l'expression de biomarqueurs protéiques, ou à des niveaux multiples génétique et épigénétiques. Le principal moteur de la diversité génétique, au moins au niveau intracellulaire, est l'instabilité génomique. L'hétérogénéité génétique Intratumorale résulte de diversité qui affecte les paramètres cliniquement pertinents tels que les signatures d'expression de gènes qui influencent en outre le pronostic et la réponse aux agents thérapeutiques.

Une tumeur solide est maintenant considérée comme un écosystème complexe qui comprend non seulement les cellules cancéreuses, mais aussi de nombreux types différents de cellules nécessaires pour le développement de la tumeur. Ce composant essentiel est appelé le microenvironnement tumoral (ang. *tumor microenvironment* - TME). Le rôle de la TME est désormais reconnu comme une partie importante de la biologie du cancer et fait l'objet d'études intenses. En outre, microenvironnement tumoral contrôle la progression tumorale par plusieurs aspects tels que la limitation des ressources, de l'architecture de l'environnement de la tumeur ou d'autres facteurs qui limitent la taille de la tumeur.

Enfin, pendant une longue période, il a été reconnu que les tumeurs sont composées par diverses populations de cellules à différents stades de différenciation. Des études approfondies ont conduit à la suggestion que les cellules tumorales indifférenciées peuvent être considérées comme des cellules souches de la tumeur (*ang. cancer stem cells - CSCs*). De nos jours, les CSCs ont été identifiés en tant que composant d'un grand nombre de tumeurs solides telles que le cancer du sein, les tumeurs du cerveau ou le cancer colorectal. Selon leur définition CSC possède les mêmes fonctions que SC normal comme clonale repeuplement à long terme et la capacité d'auto-renouvellement. En outre, les cellules souches cancéreuses se sont également révélées être résistantes à la chimiothérapie ou à la radiothérapie et par conséquent sont considérées comme étant potentiellement responsable de la rechute de la tumeur après traitement. Cependant, il existe plusieurs questions concernant les CSCs et leur biologie comme la détermination de cellules souches spécifiques en basant sur leurs marqueurs.

## **2. Oncogènes Twist**

Twist a été découvert en 1987 chez la *Drosophila* comme un gène du développement zygotique impliqué au début de la structuration du mésoderme. Chez les mammifères existe deux orthologues de la famille Twist - Twist1 et Twist2 - et les deux facteurs partagent un degré élevé d'identité de séquence et la redondance fonctionnelle. Protéines Twist sont des membres de la famille hélice-boucle-hélice basique (*ang. basic helix-loop-helix - bHLH*) de facteurs de transcription. Les protéines bHLH forment une grande superfamille de facteurs de transcription, qui sont présents dans une large gamme d'organismes de la levure à l'homme, impliqués dans de nombreux processus critiques tels que la détermination, la neurogenèse, ou la myogenèse. Le domaine bHLH caractéristique a environ 60 acides aminés et contient une région d'ADN-Binding (région de base) suivi de deux hélices séparées par une région de boucle variable (HLH). Ce domaine favorise la dimérisation, ce qui peut conduire à la formation de structures homodimères ou hétérodimères entre différents membres de la famille et l'apparition de leurs formes actives de la transcription.

Les deux protéines Twist appartiennent à la classe II de facteurs bHLH, ce qui signifie qu'ils peuvent créer non seulement des homodimères, mais aussi hétérodimères avec

des protéines de classe I E. Il faut noter que la fonctionnalité de Twist pourrait être modulée par différents facteurs tels que le choix d'un dimère ou d'autres partenaires d'interaction. Tout d'abord, la dimérisation est principalement régulée par la disponibilité des partenaires possibles ainsi que leur état de phosphorylation ou acétylation. Twist1 joue un rôle critique dans les processus de développement divers, tels que la morphogenèse de la tube crânienne, la formation de mésoderme, myogenèse, cardiogenèse et la neurogenèse. En outre, Twist affiche d'autres fonctions comme répresseur de la différenciation musculaire, ou comme un gène essentiel au cours de l'ostéogenèse.

Twist2, de façon similaire à son homologue, est impliqué dans la maturation myogénique et de ostéoblastes. Cependant, contrairement aux animaux déficients de Twist1 (E11.5), les souris knock-out de Twist2 survivent embryogénèse, mais ils souffrent d'anomalies du développement telles que la cicatrisation ou la régénération cheveux déficientes. A la fin de l'embryogenèse l'expression de Twist est absente de la plupart des types de cellules. Cependant, son expression reste active dans un nombre restreint de tissus. L'expression la plus élevée de Twist1 et Twist2 a été trouvée dans le tissu adipeux, et dans une moindre mesure dans d'autres tissus dérivés du mésoderme comme les muscles, le cœur, de l'endomètre ou du placenta. Au cours de l'âge adulte les protéines Twist sont responsables de plusieurs fonctions comme l'entretien de plusieurs types de cellules souches, la thermogenèse adaptative et la réponse inflammatoire.

En plus de son rôle essentiel de Twist au cours de l'embryogenèse et à l'âge adulte, les protéines Twist ont été trouvées être exprimées et associées à de nombreux types de tumeurs agressives. Notamment, la sur-régulation de Twist a été détectée dans différentes tumeurs malignes telles que: le cancer du sein ou de carcinome hépatocellulaire. En outre, l'expression de Twist est souvent associée à un mauvais pronostic et à une augmentation de l'invasivité des tumeurs. Ainsi, le rôle de Twist dans le développement et la progression tumorale est devenu un domaine de recherche intense.

Une des caractéristiques du cancer est l'évasion de la mort cellulaire. Fait intéressant, l'une des propriétés oncogéniques de Twist est sa capacité à déroger l'apoptose médiée par p53, une barrière naturelle contre les lésions pré-malignes. L'apoptose est pas le

seul programme de fail-safe dans lequel Twist est impliqué. En effet, des années de recherche ont accumulé des preuves établissant un rôle de Twist en tant qu'inhibiteur de la sénescence. En plus de l'inactivation des programmes de suppresseurs de tumeurs protéines Twist présentent aussi d'autres propriétés oncogéniques. L'aspect le plus étudié des activités oncogènes de Twist est le contrôle du processus de EMT. Le groupe exclusif d'inducteurs EMT, à laquelle appartient Twist, il en fait les principaux acteurs de la scène la plus destructrice de la progression tumorale - métastase. Fait intéressant, des rapports récents suggèrent que l'expression de Twist est seulement nécessaire pour une partie du processus métastatique, notamment pour la diffusion de la tumeur, et ensuite elle doit être réduite au silence pour permettre formation de macrométastases. Un autre aspect des propriétés oncogéniques Twist intensivement étudié est "souchitude" médiée par Twist. Il a été décrit comme un facteur favorisant phénotype tumoral-initiant et en dépit de la connexion initiale à EMT il est désormais clair que les propriétés du CSCs et EMT sont induits par des mécanismes indépendants. Surtout, Twist pourrait médiate évaison des cellules de l'environnement hypoxique parce que HIF1 $\alpha$  induit son expression. En outre, capacités à promouvoir l'angiogenèse de Twist pourraient également favoriser la progression du cancer. Enfin, l'expression de Twist est souvent associée à un mauvais pronostic de survie car elle est associée à la chimiorésistance à plusieurs médicaments. En conclusion, les protéines Twist, à côté de leurs rôles importants au cours de l'embryogenèse et l'âge adulte, sont actuellement reconnues comme des oncogènes avec un large éventail de capacités oncogènes.

### **3. Le stress cellulaire**

Le stress peut être généralement définie comme un impact des stimuli internes ou externes (physique, chimique ou biologique) qui perturbe l'homéostasie cellulaire et déclenche des processus menant à sa restauration. L'homéostasie est un état d'équilibre entre le taux net de la croissance et le taux net de la mort cellulaire. Ce subtil équilibre peut être facilement perturbé par l'exposition à des stimuli environnementaux stressants et une réponse cellulaire appropriée devrait conduire à un retour à un équilibre homéostatique. Cette réaction cellulaire dépend non seulement du type de stress, mais également de sa gravité. Plus précisément, si l'impact stressant ne dépasse pas un certain seuil, les cellules doivent être en mesure de le gérer par l'activation des

mécanismes de protection appropriés qui se traduiront dans la survie cellulaire. D'autre part, si l'agent stressant est trop fort ou l'exposition est trop longue, les cellules peuvent échouer à activer ou à maintenir des réponses protectrices mais plutôt d'activer les cascades de signalisation qui finalement attisent les voies de la mort cellulaire. Pour les besoins de cette thèse, je vais me concentrer uniquement sur les deux types de stress et leurs réponses appropriées - le stress oxydatif et hypoxique qui sont des sujets clés de ma recherche.

L'homéostasie est maintenue par un équilibre précaire entre la production et l'élimination des ROS. Toute perturbation de cet équilibre fragile peut conduire au stress oxydatif. Le stress oxydatif peut se produire dans plusieurs circonstances, tels que l'excès de production de ROS, qui ne peut être compensée par des mécanismes endogènes d'élimination. De même, le stress oxydatif s'accumule lorsque le mécanisme antioxydant affaibli ne peut pas détoxifier le taux normal de la production de ROS. Un déséquilibre de l'état redox a un impact important sur de nombreux processus cellulaires. Initialement, les cellules répondent en contrecarrant le stress oxydatif grâce à l'activation d'un mécanisme antioxydant, résultant dans l'adaptation. Toutefois, lorsque la production de ROS dépasse le potentiel défensif des cellules et que les dommages subis par les cellules sont trop élevés, les cellules activent les mécanismes conduisant à la sénescence ou la mort cellulaire. Remarquablement, lorsque le stress oxydatif est passager, mais devient chronique, il peut en résulter la promotion de certaines pathologies telles que le cancer, les maladies cardiovasculaires ou les maladies neurodégénératives. Mécaniquement, la surproduction de ROS provoque des dommages aux composants les plus élémentaires de la cellule telles que l'ADN, des protéines ou des lipides. En conséquence d'un tel dommage, ces biomolécules perdent leurs fonctions, ce qui augmente le taux de mutagenèse et le risque de transformation maligne.

L'oxygène est un nutriment essentiel qui sert de substrat clé dans le métabolisme cellulaire et la bioénergétique. L'hypoxie, définie comme l'apport d'oxygène insuffisant, se produit dans des conditions physiologiques et pathologiques au cours de la vie des organismes. L'hypoxie active les mécanismes de transcription spécifiques pour faire face à ce stress. Cette réponse est principalement contrôlée par une famille de régulateurs de transcription appelée facteur induit par l'hypoxie (ang. hypoxia inducible factors - HIFs).



HIFs régulent de nombreux processus d'adaptation telles que le métabolisme, l'angiogenèse, la prolifération et la motilité. Afin de garder ce puissant programme de transcription sous contrôle, les mécanismes réglementaires strictes telles que la détection de l'oxygène par enzymes contenant de domaine prolyl hydroxylase (ang prolyl hydroxylase domain-containing enzymes - PHDs) opèrent dans les cellules. Les gènes impliqués dans ces mécanismes sont mutés dans de nombreux cancers humains parce que leur dérégulation conduit à l'activation de HIF, favorisant ainsi la progression du cancer. Inhibition de la croissance médiée par HIF1 $\alpha$  fait partie de la réponse cellulaire à l'apport de nutriments insuffisant et ainsi de la production d'énergie déficiente dans des conditions hypoxiques dans les tumeurs solides. HIF1 $\alpha$  favorise la reprogrammation métabolique, ce qui fournit un mécanisme d'adaptation important pour la survie de cellules cancéreuses et sa progression. En revanche, HIF2 $\alpha$  favorise la prolifération de cellules cancéreuses par la coopération avec Myc ou l'inhibition de la p53. Cela donne à penser que les deux facteurs de HIF ont des rôles distincts au cours de la progression du cancer. En outre, changement de niveau de HIF commutateur (contrôlée par le niveau de HAF) peut induire une coopération séquentielle entre les facteurs HIF $\alpha$ , qui permettront à la suite adaptation de la tumeur à l'environnement hostile suivie par la progression tumorale et l'expansion.

## **OBJECTIFS**

Le rôle des facteurs de transcription Twist dans la tumorigénèse a été initialement lié à leur rôle classique comme des facteurs de transcription induisant EMT. Après, protéines Twist et leurs activités oncogènes révélés leur rôle dans la protection des cellules cancéreuses de deux obstacles majeurs de la tumorigénèse, notamment l'apoptose et la sénescence. Cette découverte a placé Twist parmi les facteurs impliqués dans l'adaptation cellulaire au stress pendant la tumorigénèse. Il a également fourni le cadre général de ma thèse de doctorat, dont le principal objectif était de dévoiler de nouvelles activités cellulaires de Twist et de comprendre leur rôle pendant la tumorigénèse.

Dans ce manuscrit, je décris les travaux que j'ai conduits au cours de ma thèse sur de nouveaux rôles des facteurs oncogéniques Twist. Dans la première partie de mon travail

de thèse, je continuais le projet initié par un doctorant précédente, afin de comprendre les mécanismes moléculaires responsables de l'activité antioxydante de Twist. Cette étude a fourni de nouvelles connaissances sur la biologie de facteur Twist et en particulier sur sa nouvelle activité biologique dans l'oncogenèse.

Dans la deuxième partie de mon travail, je me suis concentré sur un rôle protecteur de Twist pendant un autre type de stress, notamment le stress cellulaire induite par un apport limité d'oxygène (hypoxie). Ainsi, le deuxième objectif de ma thèse était de définir et de caractériser l'interaction possible de Twist et HIF1 $\alpha$  dans le contexte de la tumorigenèse. Nos données apportent un éclairage nouveau sur les bases mécanistes d'adaptation cellulaire à l'hypoxie et soulignent l'importance de Twist comme un régulateur majeur de la réponse au stress cellulaire au cours de la tumorigenèse (manuscrit de l'article 2).

## **RESULTATS**

### Article I. Modulation of Oxidative Stress by Twist Oncoproteins.

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\* - These authors contributed equally to this work.

Pendant ma thèse, nous avons montré que, TWIST1 et Twist2 contrôlent les niveaux de ROS intracellulaires. En outre, nos données indiquent que les propriétés antioxydantes médiée par Twist sont cruciales pour protéger les cellules de la mort cellulaire induite par ROS. Pour cet article, je effectué les expériences nécessaires pour la révision du manuscrit. Notamment, j'ai montré que les propriétés antioxydantes de Twist sont mediees via la régulation d'un programme génétique controlent les niveaux de régulateurs de stress oxydatif (Figure 5A).

Une analyse complète de nos données des biopuces a identifié plusieurs gènes, décrits précédemment comme impliqué dans le contrôle du stress oxydatif, dont l'expression est régulée par TWIST1. Plus précisément, nous avons trouvé quatre gènes induites: Cdo1, Mgst3, APOD et DHCR24 ainsi que deux gènes diminués: aox1, Osgin1. Par la suite,

je validé des changements dans l'expression de ces gènes par la mesure de leurs niveaux d'ARNm dans les cellules lors de la surexpression de TWIST1 par rapport à des cellules exprimant un plasmide vide (figure 5B). En outre, en utilisant la même technique je également démontré que les deux protéines Twist modulent le niveau de ROS en impactant un ensemble commun de facteurs (figure 5B). Ensuite, afin de déterminer si les gènes validés reproduire les propriétés de Twist, nous avons réalisé le même type d'analyse sur deux gènes sélectionnés - Mgst3 et Osgin1. En effet, la modulation de leur expression traduit en changements dans les niveaux de ROS intracellulaires (figure 6D). Cependant, dans les deux cas, ces résultats ne font que reproduire partiellement les effets observés pour les protéines Twist suggérant qu'ils ne sont que partiellement responsables de son propriétés antioxydantes (Figure 6F) Par ailleurs, j'ai testé l'impact de la modulation de ces deux gènes sélectionnés sur l'apoptose induite par c-Myc. Nos résultats ont montré que, de manière similaire aux changements observés pour des niveaux de ROS, les deux gènes que on a teste protéger les cellules de la mort cellulaire (figure 6E), bien que leur effet est moins forte que celui observer pour l'expression de TWIST1 (figure 6F). Enfin, j'ai testé si les activités antioxydantes et pro-migrateurs de Twist pourraient être liés. Des expériences de cicatrisation ont montré que, conformément à nos hypothèses, l'expression des protéines TWIST favorisent la fermeture des plaies. Cependant, l'augmentation du niveau de un de cibles de Twist - Mgst3, n'a eu aucun effet sur la migration de cellules, au moins dans cet expérience (figure 5 complémentaire).

Dans l'ensemble, cette étude apportent un éclairage nouveau sur les protéines Twist et leur capacité à protéger les cellules contre le stress oxydatif. En effet, cette étude a démontré que des propriétés anti-apoptotique de Twist peuvent être partiellement expliqués au moins par ses activités antioxydantes. Enfin, l'établissement d'un lien entre le stress cellulaire et Twist peut être important pour le processus d'adaptation, qui est considéré comme un événement crucial lors de l'initiation et la progression du cancer.

Manuscript of article II. Twist mediates adaptation to hypoxia through non-cell autonomous effects.

Jakub Kolodziejcki, Barbara Mojsa, Urszula Hibner, Patrice Lassus

Dans cette étude, nous avons montré que les protéines de la famille TWIST sont capables de protéger les cellules de l'apoptose induite par l'hypoxie. De plus, les capacités protectrices des TWIST ont été observées dans des cellules hépatiques primaires, immortalisées et transformées. Des mesures de pH dans des conditions normales et hypoxiques semblent confirmer nos observations précédentes suggérant que la surexpression de TWIST réduit l'acidité du milieu. Ces résultats semblent indiquer que twist pourrait exercer ces fonctions protectrices en régulant le pH extracellulaire de la même manière que l'acidose qui peut entraîner l'apoptose des cellules cancéreuses en hypoxie. Afin de confirmer cette hypothèse, nous avons utilisé du bicarbonate de sodium, un tampon qui entraîne une augmentation du pH lorsqu'il est ajouté au milieu de culture. Nos résultats nous ont indiqué que l'effet protecteur de TWIST est médié par la régulation du pH tout comme le bicarbonate de sodium qui protégé les cellules de la mort induite par l'hypoxie. De plus, nous avons montré que l'effet de la surexpression de TWIST pourrait être transposé aux cellules normales afin de favoriser leur adaptation en condition hypoxique. Plus précisément, différentes expériences de coopérations cellulaire (comme l'échange de milieu ou la co-culture) ont permis de démontrer que l'effet protecteur de TWIST peut-être transmis aux cellules voisines, indiquant ainsi un effet indépendant du contexte cellulaire.

Afin d'identifier un possible mécanisme moléculaire, nous nous sommes intéressé à un facteur majeur de régulation de l'hypoxie: HIF1 $\alpha$ . De façon intéressante, il a été montré que des lignées cellulaires invalidées pour HIF1 $\alpha$  sont caractérisées par un fort équilibre du pH et une faible sensibilité à la mort induite par l'hypoxie. De plus, les expériences d'échange de milieu ont montré que comparé aux cellules contrôles, les cellules KO HIF1 $\alpha$  sont protégées de façon accrue contre l'apoptose. Comme des résultats similaires sont obtenus lors de la déplétion de HIF1 $\alpha$  et la surexpression de TWIST, cela suggère que les effets de TWIST pourraient être médiés par l'inhibition de HIF1 $\alpha$ . En suivant cette hypothèse, nous avons ensuite démontré que les facteurs TWIST interagissent avec

HIF1 $\alpha$ . De façon notable, nous avons montré, à la fois par co-IP classique et par « *in situ* proximity ligand assay » (PLA), que ces interactions se produisent indépendamment de la concentration en oxygène. Aussi, les expériences de PLA nous ont également permis de démontrer que les interactions ont surtout lieu au niveau du noyau.

Globalement, nos résultats suggèrent que TWIST agit via la régulation du métabolisme cellulaire. En effet, il a été plusieurs fois montré que l'hypoxie entraîne une reprogrammation de métabolisme du glucose vers la glycolyse, induisant ainsi une augmentation du relargage extracellulaire de lactate. Malheureusement, nos mesures du taux de lactate ont indiqué que TWIST n'altère pas la production de lactate pendant l'hypoxie. Des analyses de mRNA réalisées sur des gènes cible de HIF1  $\alpha$  impliqués dans la régulation du pH - CAIX et MCT4- ont néanmoins permis d'obtenir des résultats cohérents (résultats non montrés). Cela suggère que le mécanisme d'action de TWIST pourrait être encore plus complexe. Il est en effet possible que TWIST et HIF1 $\alpha$  régulent ensemble un set particulier de gène (comme cela a déjà été observé dans le cas de la régulation des ROS).

Cependant, le mécanisme détaillé doit encore être déterminé et des études complémentaires, comme des analyses micro-array ou protéiques, sont actuellement en cours. Ainsi, nous avons décrit une nouvelle propriété de protéines TWIST et confirmé leurs rôles de régulateurs du stress cellulaire. Nous avons également démontré la capacité des protéines TWIST à coopérer avec les cellules saines voisines afin d'accroître leur potentiel d'adaptation contre le stress hypoxique. Nous avons également mis en lumière un mécanisme possible de régulation de l'effet protecteur des Twist impliquant à la fois la régulation du pH et l'interaction avec HIF1 $\alpha$ . Enfin, la découverte de nouveaux effets des Twist augmente son potentiel oncogénique, l'adaptation au stress cellulaire étant considéré comme une étape cruciale pour le développement de la tumeur.

## **RESUME**

Mon travail a permis de montrer des nouvelles activités de Twist, qui soulignent son rôle comme un puissant modulateur de stress métabolique au niveau cellulaire. Notamment, nous avons montré que les protéines Twist protègent les cellules contre le stress oxydatif et hypoxique et probablement aussi acidose. Remarquablement, nous

avons également fourni une explication mécanique pour l'effet de Twist au cours de stress oxydatif. En effet, nous avons montré que Twist par la réglementation du programme génétique des enzymes antioxydantes protège les cellules de l'apoptose induite par ROS. Dans le cas de stress hypoxique, nous avons démontré que la protection de Twist est susceptible d'être médiée par la réglementation de l'acidification de milieu. En outre, nous avons relié les effets de Twist avec HIF1 $\alpha$ , un régulateur majeur de réponse hypoxique. Remarquablement, nous avons démontré pour la première fois que les protéines Twist interagissent avec HIF1 $\alpha$ . En outre, nous émettons l'hypothèse que les protéines Twist régulent sélectivement un ensemble de gènes cibles de HIF1 $\alpha$  afin de protéger les cellules contre l'apoptose induite par l'hypoxie. Par conséquent nos résultats soulignant des nouvelles activités des protéines Twist, ont leur accordée une place parmi les autres facteurs de réglementation de stress cellulaire. Cependant, d'autres études, notamment sur les mécanismes moléculaires contrôlent la réponse du Twist vers l'hypoxie, sont encore nécessaires pour répondre à la question de Twist potentiel oncogène.