

THE UNIVERSITY OF HULL

Investigating the Role of WSB-1 in Breast Cancer

Being a Thesis Submitted for the Degree of Doctor of
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by

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*"Books and pens are our most powerful weapons.
One child, one teacher, one book and one pen can change the world."*

Malala Yousafzai

"We are going to die, and that makes us the lucky ones. Most people are never going to die because they are never going to be born. The potential people who could have been here in my place but who will in fact never see the light of day outnumber the sand grains of Sahara. Certainly those unborn ghosts include greater poets than Keats, scientists greater than Newton. We know this because the set of possible people allowed by our DNA so massively exceeds the set of actual people. In the teeth of those stupefying odds it is you and I, in our ordinariness, that are here. We privileged few, who won the lottery of birth against all odds, how dare we whine at our inevitable return to that prior state from which the vast majority have never stirred?"

Professor Richard Dawkins

Abstract

Breast cancer is the second most represented cancer type worldwide and will affect 1 in 8 women in the UK. Overall patient survival can reach 90% when the disease is diagnosed early but rapidly drops as the tumour progresses and metastasises. The steps required for tumour formation are known but numerous factors are involved and the specific mechanisms controlling this phenomenon are still poorly understood. WSB-1 is a hypoxia-responsive E3 ubiquitin ligase which was found to be upregulated in metastatic tissues, compared to normal or non invasive tissues. In addition, studies demonstrated that WSB-1 was involved in pancreatic cancer, neuroblastoma, and osteosarcoma progression. However, its role in breast cancer has not been particularly studied.

The objective of this thesis is to study the role of WSB-1 in breast cancer. Initially, level of *WSB1* in patients' tumour cDNA samples was evaluated according to known clinical and biological variables. Impact of high *WSB1* levels on patients' distant metastasis-free survival, relapse-free survival and overall survival was also investigated. Then, the effect of WSB-1 knockdown on protein levels of epithelial to mesenchymal transition markers, as well as several matrix metalloproteinases (MMP) transcript and protein levels, and activity was examined. Consequence of these modifications on cell motility (migration, invasion) was studied, using single cells, cellular monolayers and spheroids. Finally, affinity purification followed by mass spectrometry was used to identify novel WSB-1 partners.

Overall, in MDA-MB-231 cells, WSB-1 appeared to drive metastasis formation by upregulating MMPs expression and activity, promoting EMT and inducing invasiveness. WSB-1 had a more conflicting effect in MCF7 cells. In fact, WSB-1 effect appeared to be depending on the ER- and ER+ status.

Together, these results validated WSB-1 as an important player in breast cancer development, particularly in metastasis formation.

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List of abbreviations

ADAM: a disintegrin and metalloproteinase
ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs
AHR: arylhydrocarbon receptor
ALDOA: aldolase A, fructose-bisphosphate
ANOVA: analysis of variance
APC/C: cullin-related anaphase-promoting complex/cyclosome complex
AP-MS: affinity purification followed by mass spectrometry
APS: ammonium persulfate
ARNT: arylhydrocarbon receptor nuclear translocator
ATM: ataxia telangiectasia mutated
ATR: ATM- and RAD3-related
B2M: β -2-microglobulin
bHLH: basic helix-loop-helix
bp: base pair
BRCA1: breast cancer 1, early onset
BRCA2: breast cancer 2, early onset
BSA: bovine serum albumin
°C: degree Celsius
CA9: carbonic anhydrase IX
CAD: C-terminal transactivation domain
CBP: CREB-binding protein
cDNA: complementary DNA
CHIP: C terminus of HSC70-interacting protein
CO₂: carbon dioxide
CRAPome: contaminant repository for affinity purification-ome
DAPI: 4', 6-diamidino-2-phenylindole
DEPC: diethylpyrocarbonate
DFS: disease-free survival
DIO2: 2-iodothyronine deiodinase
DMEM: Dulbecco's Modified Eagle Medium
DMFS: distant metastasis-free survival
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid

DNA-PK: DNA-dependent protein kinase
dNTP: deoxynucleotide triphosphate
ECM: extracellular matrix
ECS: Elongin B, C/Cul2,5/SOCS
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
EMT: epithelial to mesenchymal transition
EPO: erythropoietin
ER: oestrogen receptor
ERK: extracellular regulated kinase
FBS: Fetal Bovine Serum
FDA: food and drug administration
FIH: factor inhibiting HIF
g: gram
 $\times g$: gravity acceleration
G-CSF-R: granulocyte colony-stimulating factor receptor
GLUT1: glucose transporter 1
h: hour
HAF: hypoxia-associated factor
HCl: hydrochloric acid
HECT: homologous with E6-associated protein C-terminus
HER2: human epidermal growth factor receptor
HIF: hypoxia inducible factor
HIPK2: homeodomain-interacting protein kinase 2
HK2: hexokinase 2
HR: hazard ratio
HRE: hypoxia responsive element
HSP: heat shock protein
IGF: insulin-like growth factor
IL-21R: interleukin-21 receptor
IP: immunoprecipitation
IPAS: inhibitory PAS domain protein
kDa: kilodalton
L: litre
LC-MS: liquid chromatography-mass spectrometry

LOX: lysyl oxydase
M: molar (mol.l^{-1})
MALDI-MS: matrix assisted laser desorption/ionisation-mass spectrometry
MAPK: mitogen-activated protein kinase
min: minute
mm: millimetre
MMP: matrix metalloproteinases
mRNA: messenger ribonucleic acid
MT-MMP: transmembrane metalloproteinase
NaCl: sodium chloride
NAD: N-terminal transactivation domain
NMD: nonsense-mediated decay
NP-40: nonyl phenoxypolyethoxylethanol
NTC: no template control
O₂: oxygen
ODDD: oxygen-dependent degradation domain
OS: overall survival
PAS: PER-ARNT-Sim
PBS: phosphate-buffered saline
PEI: polyethylenimine 25kDa linear
pH: potential of hydrogen
PHD: prolylhydroxylase
PIKK: phosphatidylinositol 3-kinase-related kinases
PKM2: pyruvate kinase, muscle
PR: progesterone receptor
PTHrP: parathyroid hormone-related peptide
PVDF: polyvinylidene difluoride
pVHL: von Hippel-Lindau protein
qPCR: quantitative polymerase chain reaction
RB1: retinoblastoma 1
RhoGDI2: Rho GDP-dissociation inhibitor 2
RING: really interesting new gene
ROS: reactive oxygen species
rpm: rotations per minute
RPMI: Roswell Park Memorial Institute medium

RUNX2: runt related transcription factor 2
SCF: Skp1/Cul1/F-box protein
SD: standard deviation
SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis
sec: second
SEM: standard error mean
Shh: sonic hedgehog
shRNA: short hairpin RNA
siNT: non-targeting siRNA
siRNA: small interfering RNA
siWSB-1: siRNA against WSB-1
SOCS: suppressor of cytokine signalling
SPARC: secreted protein acidic cystein-rich
STIP1: stress-induced phosphoprotein 1
STUB1: STIP1 homology and U-Box containing protein 1
TAD: transactivation domain
TBS: Tris-buffered saline
TCEB1: transcription elongation factor B, polypeptide 1/elongin C
TCEB2: transcription elongation factor B, polypeptide 2/elongin B
TEA: Tris/acetic acid/EDTA solution
TGF: transforming growth factor
TIMP: tissue inhibitor of metalloproteinases
TNBC: triple negative breast cancer
TP53: tumour protein p53
U: unit
UBC: ubiquitin C
UTB: urea/Tris HCl buffer
V: volt
v: volume
VEGF: vascular endothelial growth factor
WSB: WD-40 repeat-containing SOCS box protein
ZEB: zinc finger E-box binding homeobox
ZO-1: zona occludens 1

Chapter 1

Introduction

1. General introduction

1.1. Cancer

1.1.1. Breast cancer

Cancer is one of the major causes of premature death in the world. In 2012 in England, 42% of deaths for people aged 75 years old or younger was due to cancer (Department of Health, 2014). Despite concerning mainly females, breast cancer is the most common cancer type in the UK and represents 30% of cancers cases in women (source: Cancer Research UK). It is one of the most hypoxic cancer types and metastasises principally to the lungs, bones, and liver (Leong et al., 2006; McKeown, 2014). The causes for breast cancer are still largely unknown, although mutations of specific genes, age, as well as family history and lifestyle can help predict the apparition of the disease. BRCA1 (breast cancer 1, early onset) and BRCA2 (breast cancer 2, early onset) are two genes whose link with breast cancer occurrence have been uncovered in the mid-1990s (Miki et al., 1994; Wooster et al., 1995). In fact, many more genetic variants have been identified that predispose to breast cancer and specific mutations on these genes can increase the risk of developing the disease (Couch et al., 2014). Classification of breast cancers in subtypes is a widely used way to tailor the therapeutic approach to the patient (Onitilo et al., 2009; Prat et al., 2015). Subtypes are determined by the expression status of three receptors: ER (oestrogen receptor), PR (progesterone receptor), and HER2 (human epidermal growth factor receptor) (Figure 1.1). ER-positive (ER+) and PR-positive (PR+) tumours represent 70% of all breast tumours. Monitoring the expression of these receptors is important to determine the tumour's specific metabolism, and predict the response to certain chemotherapeutic drugs (Heldring et al., 2007). For example, ER+ and PR+ tumours rely on the expression of oestrogen and progesterone to grow and proliferate. Therefore, use of endocrine therapy whereby patients are treated systemically with steroid hormones oestrogen or progesterone analogues is highly efficient (Puhalla et al., 2012).

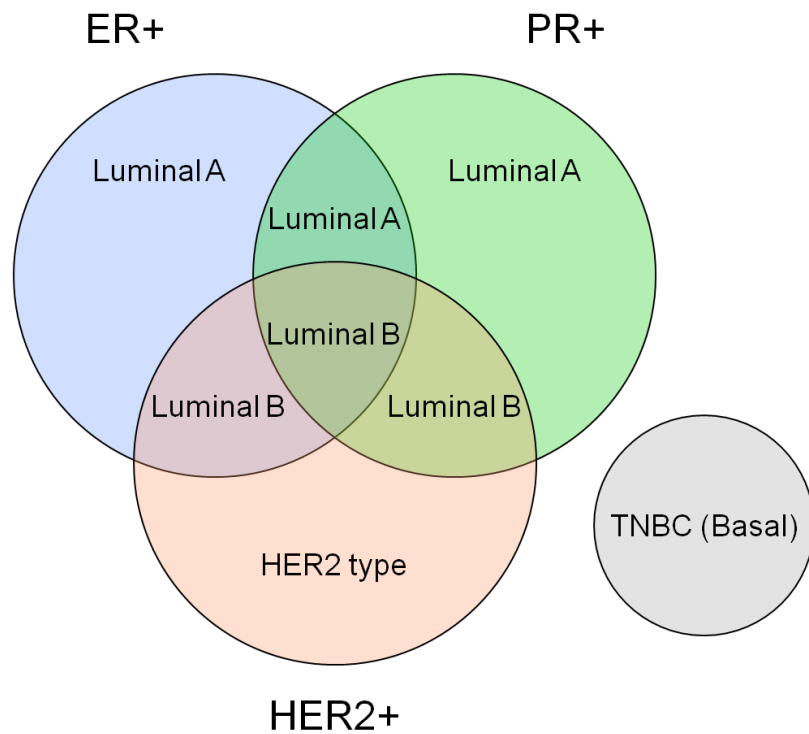


Figure 1.1: Venn diagram representing the main breast cancer subtypes according to receptors expression status

ER: oestrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor; TNBC: triple negative breast cancer.

Hormonal receptor status is also critical in appreciating survival rates (Haque et al., 2012). Triple negative breast cancer (TNBC) usually present the worst survival rates compared to any other subtype (Onitilo et al., 2009). However, the group of Bae and colleagues demonstrated in a large clinical study that the rarer tumours negative for HER2 (HER2-) and expressing only one steroid hormone receptor displayed similarly low survival rates and increased aggressiveness as tumours expressing neither of these steroid receptors (Bae et al., 2015). Similarly, patients displaying metastatic expression of ER and PR demonstrated a better survival rate than those not expressing either ER or PR, whereas triple negative patients (ER/PR/HER2 negative) represented the group with the lowest overall survival (Lower et al., 2005; Onitilo et al., 2009).

In addition, tumours are graded from stage I to stage IV according to their advancement. The size of the tumour, the number of lymph nodes where cancer cells are found, if any, as well as the presence of metastases determine the stage of the breast cancer (Table 1.1). Diagnosed early, 5 years overall survival can reach 90% but these figures dramatically decrease as the disease progresses to stage III and over (Polyak and Metzger Filho, 2012).

1.1.2. Cancer biology: the hallmarks of cancer

Cancer is a multifactorial disease for which the exact mechanisms involved are diverse and still poorly understood. However, several essential characteristics for cancer development have been identified over time and reviewed by Hanahan and Weinberg (2000, 2011). They identified the following hallmarks, key contributors to cancer progression:

Sustained proliferative signalling: Whereas normal cells rely on growth factors released in the milieu by other specialised cells, cancer cells are able to overcome this limitation through several means. By increasing the expression of growth factor receptors on their surface, cancer cells become hypersensitive to these signals (Velu et al., 1987; Gullick, 1991). In fact, some mutations act directly on the downstream signalling through ligand-independent

Table 1.1: Summary of the number stages of breast cancer

Stage	Characteristics			5 year survival
	Tumour	Lymph nodes affected	Met.	
Stage I				99%
A	< 2cm	None	No	
B	< 2cm	A few in nearby lymph nodes	No	
Stage II				90%
A	< 2cm	1-3 in arm pit or near breastbone	No	
	<i>or</i> Between 2cm and 5cm	None	No	
B	Between 2cm and 5cm	A few in nearby lymph nodes	No	
	<i>or</i> Between 2cm and 5cm	1-3 in arm pit or near breastbone	No	
	<i>or</i> > 5cm	None	No	
Stage III				60%
A	Any size	4-9 in arm pit or near breastbone	No	
	<i>or</i> > 5cm	A few in nearby lymph nodes	No	
	<i>or</i> > 5cm	1-3 in arm pit or near breastbone	No	
B	Spread to skin or chest wall	Up to 9	No	
C	Spread to skin and chest wall	> 10 in armpit, or above and below collar bone, or in armpit and breastbone	No	
Stage IV	Any size	Some or none	Yes	15%

receptors and constitutive activation of downstream effectors (Witsch et al., 2010). Alternatively, some cancer cells are able to send signals inducing growth factor expression to normal cells (Gleave et al., 1993). Combined, these elements provide high proliferative skills to cancer cells, and allow this fast and sustained growth often observed in tumours.

Energy metabolism reprogramming: Increased cell population equals increased energetic requirements. The most common energetic pathway in aerobic conditions is OX-PHOS (oxidative phosphorylation). In anaerobic conditions, however, oxygen resources are scarce and glycolysis using glucose as an energetic source and forming pyruvate and lactate as

by-products is preferred (Warburg, 1956). Cancer cells are able to use anaerobic metabolism, even when in an aerobic environment. This ensures they are freed from O₂ availability but also increases their need for glucose (Denko, 2008). As a result, glucose transporters such as GLUT1 (glucose transporter 1, coded by *SLC2A1*) as well as many key glycolysis enzymes are overexpressed in cancer cells (Mathupala et al., 2001). This ability to thrive regardless of high glucose or oxygen availability grants cancer cells with a greater flexibility and therefore, increase their chance of survival.

Cell death resistance: In normal condition, cell proliferation and cell death is a tightly monitored balance regulated by a complex interplay of pro- and anti-apoptotic factors. Cancer cells are able to disrupt this balance, and proliferate at an unusual pace. Loss of *TP53* (tumour protein p53, coding for p53), a tumour suppressor gene usually regulating pro-apoptotic signals, is a frequent mutation found in tumours. In parallel, changes in the expression of apoptosis and survival regulators, such as Bcl-2 and Bax are often observed in tumour tissues (Paul-Samojedny et al., 2005; Adams and Cory, 2007; Singh et al., 2015). Combined, these alterations to the cell death regulation mechanism allow cancer cells to live longer and override normal cellular response to abnormal circumstances.

Evasion from growth suppressors: Growth arrest factors are expressed by tumour suppressor genes. Their function is to induce cell cycle arrest or even drive the cell to apoptosis in response to abnormal proliferation signals. These tumour suppressor genes, which notably include *TP53* and *RB1* (retinoblastoma 1, coding for pRb), are often mutated in cancer cells. As a result, cancer cells are able to overcome usual growth limitations such as contact inhibition, and evade apoptosis induction. In addition, it has been found that the TGF (transforming growth factor)- β signalling, which usually plays an anti-proliferative role, can also promote tumour growth and tumour progression in advanced tumours (Wendt et al., 2012; Principe et al., 2014). This "TGF- β paradox" is the result of mutations disrupting the signalling pathway; indeed, increased levels of TGF- β signalling inhibitors have been observed

in human cancers (Syed, 2016). Other studies demonstrated that TGF- β signalling could actually promote epithelial to mesenchymal transition (EMT), which will be discussed in more details later on (Bhowmick et al., 2001).

Replicative immortality: As mentioned earlier, balance between cell proliferation and cell death is a finely tuned mechanism. Most cells are only capable of undergoing a limited number of cell division due to the shortening of their telomeres after each cycle (Hayflick, 1965; Harley et al., 1990). Once this number is reached, cells either attain senescence, an irreversible non-proliferative state, or enter apoptosis. Cancer cells that were able to overcome senescence and escape apoptosis will essentially become immortal. Modification in the telomerase activity of cancer cells also contributes to unlimited cell division (Akincilar et al., 2016; Kulić et al., 2016).

Invasion and metastasis activation: Most cells usually rely on cell-cell interactions and adhesion mechanisms to survive. Unable to adhere to a surface and form junctions between one-another, normal cells would die. Unsurprisingly, cancer cells are able to overcome this requirement (Wirtz et al., 2011). Loss of expression of adhesion molecules and cytostatic factors in cancer cells is usually accompanied by increased expression of molecules associated to migration and invasiveness, as well as morphological changes (Christofori, 2003). Along the road of transformation, cells gradually lose their apico-basal polarity and present a more mesenchymal phenotype. Cancer cells are therefore not only able to live without attachment to matrix or other cells but are also capable of degrading such matrix and disseminate to neighbouring tissues (Nisticò et al., 2012). Some cells will eventually metastasise and form tumours away from the initial site. This forms the so-called metastatic cascade, which will be explored in a later section.

Immune system evasion: More recently, some studies highlighted that immunosuppressed patients tended to develop more tumours. It transpired that most of these tumours were in fact due to viral infections whereas 80% of tumours in the general population have non-viral

origins. It was however observed that immunosuppressed mice developed more tumours following exposure to carcinogenic compounds than their wild type counterparts (Teng et al., 2008; Hanahan and Weinberg, 2011). The hypothesis drawn from these observations is that some cancer cells are more immunogenic than others and, while the most immunogenic cells are detected by the immune system and destroyed, the more discreet ones manage to escape the immune surveillance and grow undetected. The role of the immune system against cancer development was further proven when organ transplant in immunosuppressed patients resulted in the development of melanoma from the donor after the surgery (Strauss and Thomas, 2010).

Angiogenesis induction: Except during wound-healing or female menstrual cycle, adult vasculature is quiescent. Tumours, however, manage to successfully reactivate angiogenesis to maintain their growth (Carmeliet, 2005). Nevertheless, tumoural neovasculature is chaotic and loosely organised. This leads to the formation of poorly oxygenated areas, which are known as hypoxic (Brown and Giaccia, 1998; Brown and Wilson, 2004).

1.1.3. The metastatic cascade

Besides the changes in metabolism and function exposed above, malignant cells also present major phenotypic changes throughout cancer progression. During the epithelial to mesenchymal transition, cells lose their specific epithelial characteristics, such as apico-basal polarity and intercellular junctions, in favour of a more flexible mesenchymal phenotype. EMT is characterised by modifications in the expression of molecules linked to cellular junction and adhesion (*e.g.* ZO-1, E-cadherin), as well as invasion and migration (*e.g.* N-cadherin) (Yang and Weinberg, 2008). This phenotypic change is accompanied by secretion of proteins able to degrade the extracellular matrix (ECM) such as matrix metalloproteinases (MMPs), as well as a reorganisation of the collagen network composing the ECM (Nisticò et al., 2012; Bonnans et al., 2014). The EMT process itself is controlled by some well-known transcription factors which are also at play during normal processes such as wound healing and embryonic

morphogenesis (Hanahan and Weinberg, 2011; Lamouille et al., 2014). The Snail family of transcription factors comprises SNAI1 (Snail) and SNAI2 (Slug). Snail is responsible for the inhibition of several epithelial markers such as E-cadherin and other proteins involved in junction formation (occludin, desmoplakin, claudins) (Cano et al., 2000). On the contrary, Snail activates the expression of several matrix metalloproteinases MMPs as well as Twist, ZEB1 and ZEB2, three important transcription factors also involved in the EMT regulation (Aigner et al., 2007; Peinado et al., 2007; Lamouille et al., 2014). As a matter of fact, Twist and ZEB1 have also been shown to repress E-cadherin expression (Vesuna et al., 2008; Mazda et al., 2011). In addition, other studies demonstrated that the ZEB family of transcription factors repressed the expression of ZO-1, a component of tight junctions necessary to epithelial cells, and induced the expression of various MMPs (Lamouille et al., 2014).

This newly acquired mesenchymal phenotype provides cells with increased flexibility: they are able to survive without cellular adhesion, invade through the extracellular matrix and move to the nearby blood vessels (Figure 1.2). Very little circulating tumour cells actually survive the shear forces and attacks from the immune cells present in the blood stream (Cristofanilli, 2006). As a result, as little as 0.01% of circulating tumour cells are able to exit the blood circuit, colonise another part of the organism and establish in a new tissue (Wirtz et al., 2011). The existence of circulating tumour cells was first described by Thomas Ashworth in 1869 but due to their very limited number, use of these cells for diagnosis or any other purpose was hampered by technical limitations until recently (Plaks et al., 2013). Since the past decade, more and more approaches have been developed that take advantage of these circulating tumour cells, specifically to monitor tumours from a distance and establish patient-derived xenografts in mice (Morton and Houghton, 2007). This technique is particularly interesting to guide therapeutic decision as the reaction of the xenograft to a specific drug can give indication on the tumour response to that molecule (Garraalda et al., 2014). Patient-derived xenografts still require a significant amount of optimisation before

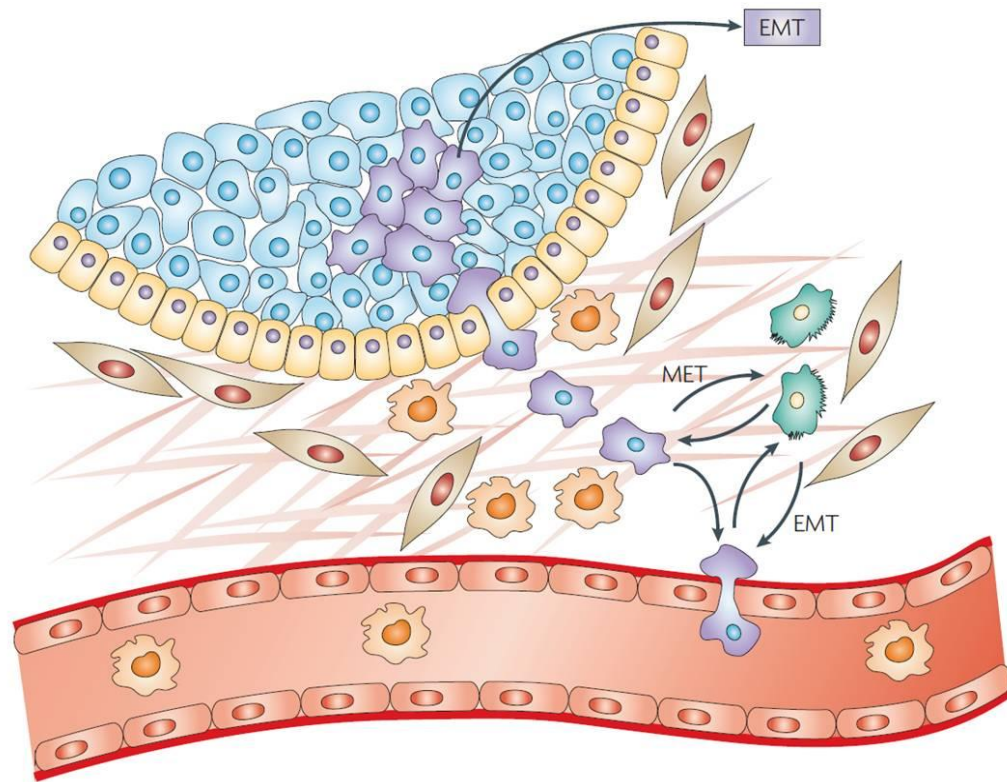


Figure 1.2: Illustration of the phenotypic changes allowing cancer cells to migrate from the tumour site to the blood stream

Cancer cells first undergo EMT, increasing their motility and potential to survive as single cells. Cells then migrate through the matrix and reach a blood vessel. Crossing the epithelium, cells then enter the blood stream and are able to reach distant tissues. EMT: epithelial to mesenchymal transition; MET: mesenchymal to epithelial transition. From Peinado et al. (2007).

being routinely used at the clinical level, but already represent a valuable tool for cancer research (Aparicio et al., 2015). Overall, circulating tumour cells are of great interest as they allow genotypic profiling of a patient's tumour without resorting to biopsy, which is an invasive procedure and complicated to carry out for tumours difficult of access. Furthermore, the quantity of circulating tumour cells was found to be linked to the advancement of the disease and could be used as an indicator of prognosis (Cristofanilli et al., 2004; Cristofanilli, 2006). Others claim that circulating tumour cells may also help determine the success of chemotherapy (Chaffer and Weinberg, 2011). Once in the blood stream, circulating tumour cells are able to disseminate through the organism and reach distant tissues. This process is illustrated in Figure 1.3. Depending on the venous drainage of the tissue of origin, circulating tumour cells are directed to specific organs in the body (Martin et al., 2000). For this reason, given types of cancer are likely to metastasise to the same organs, with a predominance for lungs and liver, as well as bone and brain (Martin et al., 2000; Leong et al., 2006).

1.2. Hypoxia

The atmospheric oxygen level is approximately of 20%. However, usual oxygen tension in the organism varies from one organ to the other, rarely exceeding 10% O₂ (Ivanovic, 2009; Hancock et al., 2015). Oxygen accessibility relies on the proximity from blood vessels. In fact, Thomlinson and Gray measured that cells distant of more than 160µm from a blood vessel will not have access to oxygen (Thomlinson and Gray, 1955). The irregular and leaky tumour vasculature leads to poor perfusion of the tumour mass (Figure 1.4) (Brown and Giaccia, 1998; Brown and Wilson, 2004). As a result, hypoxic regions with low access to oxygen are commonly observed (Zhong et al., 1999; Höckel and Vaupel, 2001; Vaupel and Mayer, 2007; McKeown, 2014). In these areas, oxygen level drops below 2% and can reach near anoxic levels (<0.02% O₂) (Strese et al., 2013). Interestingly, tumour hypoxia is often associated with more aggressive tumour types, increased metastasis formation and worse overall survival rates (Höckel et al., 1999; Höckel and Vaupel, 2001; Semenza, 2010, 2012).

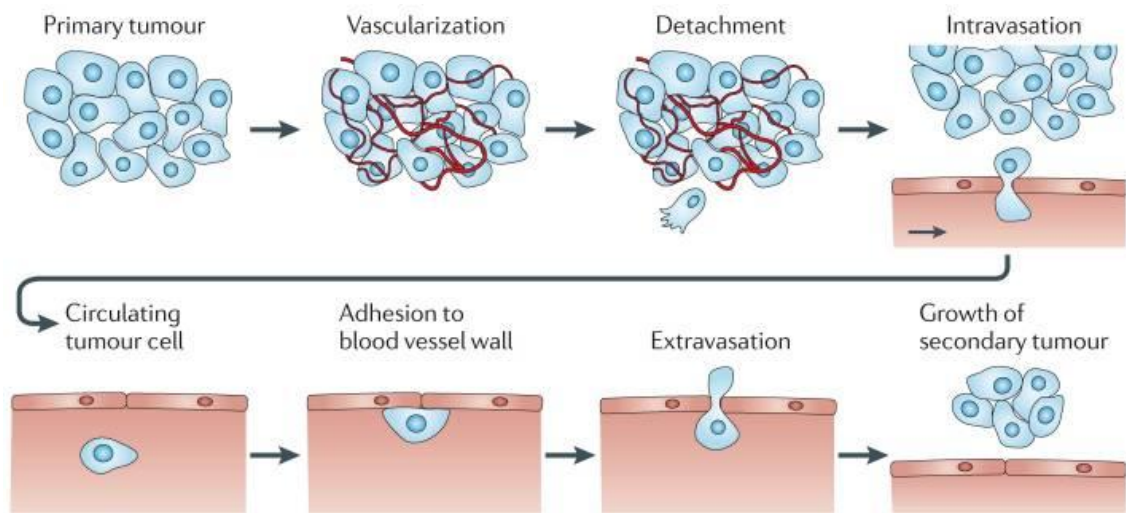


Figure 1.3: Diagram of the metastatic process

Mutant cells proliferate within a tissue to form a tumour. Vasculature develops in the tumour mass and soon, some tumour cells leave that original tumour to enter the blood stream (intravasation) and reach distant tissues in the body. The tumour cell exits the blood vessel (extravasation) and initiates the formation of a secondary tumour. From Wirtz et al. (2011).

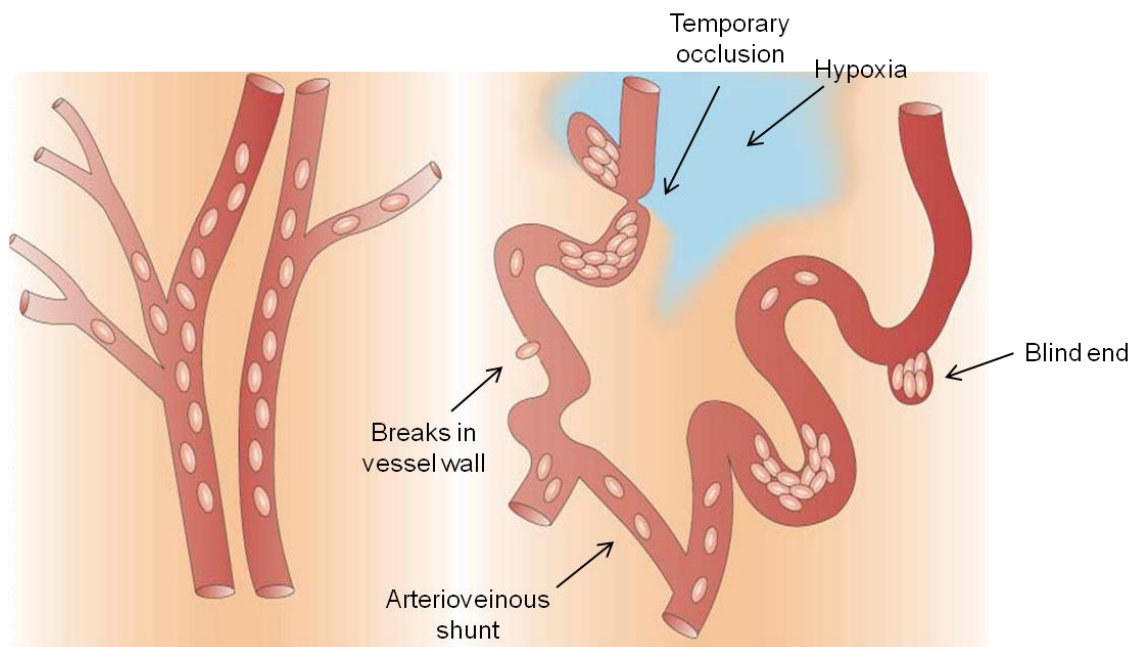


Figure 1.4: Vascular network of normal tissue (left) and tumour tissue (right)

Blue area represents the hypoxic zone. Adapted from Brown & Wilson (2004).

Several studies explained this phenomenon suggesting that tumour hypoxia hampers the efficacy of radio- and chemotherapies (Harrison et al., 2002; Williams et al., 2005; Cosse and Michiels, 2008; Dewhirst et al., 2008; Harada, 2011). The irregular vascularisation of tumours prevents a proper diffusion of chemotherapeutic agents which then fail to reach their target via the bloodstream (Brown and Wilson, 2004). Radiotherapy creates damage to the DNA in tumour cells by the reaction of ionising radiations with oxygen. Therefore, its effects are a lot weaker in hypoxia and cells need to be exposed to higher doses of radiation to get killed (Moeller et al., 2005; Williams et al., 2005; Rockwell et al., 2009; McKeown, 2014). Due to the energy metabolism reprogramming underwent by cancer cells, an acidification of the microenvironment has been observed in hypoxia (Wojtkowiak et al., 2011; Santi et al., 2013). This change in pH can affect the efficacy of certain chemotherapeutic drugs by altering the molecule conformation. Besides the mechanical effects of a lack of oxygen on the efficacy of therapy, hypoxia also modifies particular aspects of the cancer cell metabolism that are targeted by chemotherapeutic drugs. Indeed, a decrease in the proliferation of tumour cells has been described in hypoxia (Harris, 2002; Cosse and Michiels, 2008). Decrease in the apoptotic potential has also been observed, which renders cells more resistant to chemo- and radiotherapy (Erler et al., 2004; Finger and Giaccia, 2010). Cancer cells also tend to increase the expression of certain membrane transporters, which facilitate the export of drugs outside of the cells (Nooter et al., 1995; Burger et al., 2003; Fletcher et al., 2010). Finally, the genomic instability and deregulation of the DNA repair mechanisms often found in cancer cells make them more likely to develop drug resistance (Reynolds et al., 1996; Yuan and Glazer, 1998; Bristow and Hill, 2008; Pires et al., 2010; Bouwman and Jonkers, 2012).

The hypoxia inducible factor (HIF) is the main transcription factor mediating the hypoxic response. Semenza and colleagues stated that as many as 40% of human tumours present abnormally high levels of HIF-1 α (Semenza, 2007).

1.2.1. HIF

HIF is a heterodimeric oxygen-sensitive transcription factor. It is constituted of an α and a β subunit. The HIF- α subunit is approximately 120kDa and its activity is dependent on oxygen level. On the other hand, the HIF- β subunit, also known as ARNT (arylhydrocarbon receptor nuclear translocator), is approximately 90kDa and is constitutively expressed in the cytoplasm (Wang et al., 1995). It is inactivated in normoxia and mediates specific cell signalling response in hypoxia (Wang and Semenza, 1993a).

1.2.1.1. HIF- α

There are actually three different HIF- α proteins named HIF-1 α , HIF-2 α and HIF-3 α . HIF-1 α was the first hypoxia-inducible transcription factor described by the group of Greg Semenza (1993a, 1993b). The roles of **HIF-1 α** and **HIF-2 α** have first been investigated in the context of embryonic development. HIF null mice models were generated and both *hif-1 α ^{-/-}* and *hif-2 α ^{-/-}* mutations led to embryonic lethality and numerous developmental defects, including abnormal blood vessel formation (Iyer et al., 1998; Peng et al., 2000). However, embryos demonstrated different phenotypes subsequent to each knockdown, indicating that HIF-1 α and HIF-2 α played different roles during mice embryonic development, and by extension, during the hypoxic response (Huang and Bunn, 2003; Loboda et al., 2010). Interestingly, HIF-1 α and HIF-2 α have similar structures, sharing 48% of their amino acid sequence (Figure 1.5) (Tian et al., 1997). Both HIF-1 α and HIF-2 α possess a basic helix-loop-helix (bHLH) domain in N-terminal that constitutes HIF- α DNA-binding domain, a PAS (PER-ARNT-Sim) domain recognised by HIF- β thus allowing formation of HIF- α /HIF- β heterodimers, and a transactivation domain (TAD) in C-terminal that can be separated in N- and C-terminal transactivation domains (NAD and CAD, respectively). All these domains are conserved between HIF-1 α and HIF-2 α . Their bHLH domain even shares 85% of the amino acid sequence. HIF-1 α and HIF-2 α mostly differ in their TAD, which is responsible for the interaction between HIF and its target genes and co-activators. This is likely to explain the

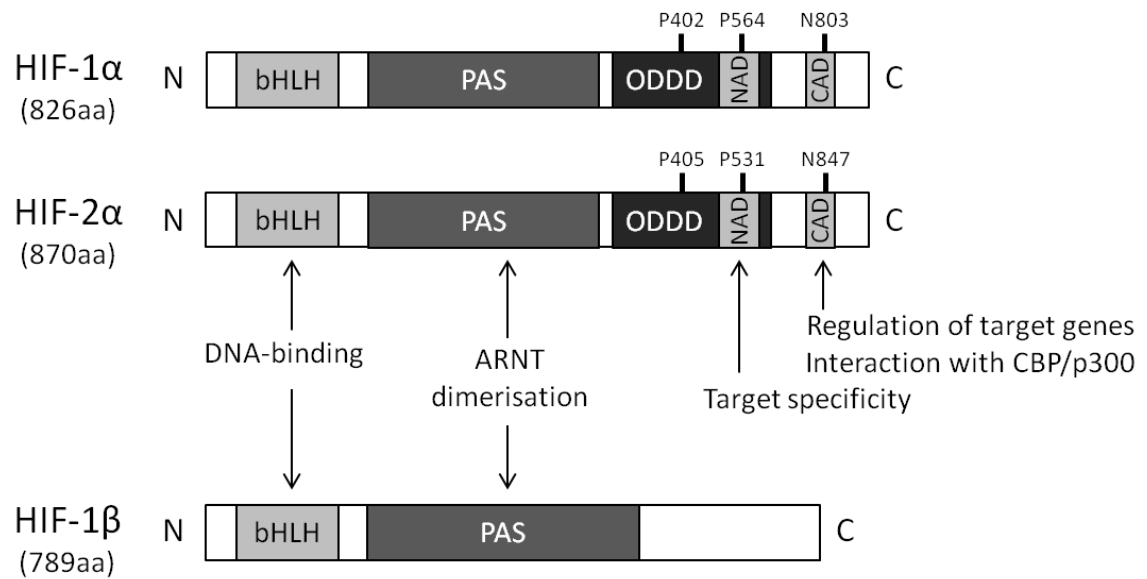


Figure 1.5: Diagram of HIF-1α, HIF-2α and HIF-1β structures

HIF-1α and HIF-2α have very similar structures and share 48% of their overall amino acid sequences. The α and β subunits dimerise via the PAS domain. bHLH: basic helix-loop-helix, PAS: PER-ARNT-SIM, ODDD: oxygen-dependent degradation domain, NAD: N-terminal transactivation domain, CAD: C-terminal transactivation domain.

differential roles of each subunit (Keith et al., 2012). Nonetheless, some key amino acid residues required for HIF- α post-translational regulation are conserved between HIF-1 α and HIF-2 α , explaining why the mechanisms regulating their activity are similar for both subunits (Hu et al., 2003; Loboda et al., 2010). Despite having analogue structures and comparable mechanisms of regulation, HIF-1 α and HIF-2 α observe distinct regulation patterns (Ratcliffe, 2007). For example, Wiesener and colleagues were unable to detect HIF-1 α in lungs and highlighted a longer expression of HIF-2 α than HIF-1 α in kidney and liver samples following hypoxia exposure (Wiesener et al., 2003). In addition, some studies demonstrated that HIF-1 α tends to be part of an early

response to severe hypoxia whereas HIF-2 α can be induced in more moderate oxygen tensions and its upregulation tends to last longer (Löfstedt et al., 2007; Carreau et al., 2011). In fact, 5% O₂ is enough to upregulate HIF-2 α (Holmquist-Mengelbier et al., 2006). As a result, considering that most tissues are subject to levels of oxygen between 3 and 10%, it is likely that HIF-2 α plays a role in the transcription of several genes in tissues that are in physiological normoxia. Similarly, HIF-1 α is induced from 6% O₂, although maximal response is observed at 0.5% O₂ and 1.5% - 2% O₂ is sufficient to elicit half of the maximal response (Jiang et al., 1996; Bertout et al., 2008).

Despite having similar structures, HIF-1 α and HIF-2 α do not have completely redundant roles in the cell (Hu et al., 2006; Imamura et al., 2009). As demonstrated by the results obtained with knocked-out mouse embryos mentioned earlier, the presence of either HIF-1 α or HIF-2 α is not sufficient to compensate for the loss of the other isoform. Moreover, the differences existing between the sequences of HIF-1 α and HIF-2 α transactivation domains suggest that each protein is able to interact with specific targets (Hu et al., 2007). In 2003, Hu and colleagues studied the differential roles of HIF-1 α and HIF-2 α and showed that only HIF-1 α was implicated in the hypoxia-induced activation of the glycolytic pathway (Hu et al., 2003). Using a cell line model expressing only HIF-2 α and not HIF-1 α , they were unable to activate

the glycolytic pathway in hypoxia. However, restoring HIF-1 α expression in these cells allowed the induction of glycolytic genes in response to hypoxia. Hu and colleagues suggested that the difference in HIF-1 α and HIF-2 α targets relied on the existence of another *cis*-acting element in the promoter sequence of target genes recognised only by HIF-2 α , yet to be identified.

In parallel, Wang and colleagues transfected HEK293T cells with mutated versions of either HIF-1 α or HIF-2 α and analysed the genes that were upregulated by HIF-1 α and/or HIF-2 α overexpression as well as by hypoxia (Wang et al., 2005). They demonstrated that, despite a majority of genes being upregulated by both HIF-1 α and HIF-2 α , some genes were specifically upregulated by either HIF-1 α or HIF-2 α . In addition, a study led on six cell lines showed that HIF-1 α and HIF-2 α expression levels not only displayed differential regulation when confronted to decreasing levels of oxygen, but also that this differential regulation pattern was cell-specific (Bracken et al., 2006). The level of oxygen required to induce HIF-1 α is higher in the liver and kidney than in the brain, but the hypoxic event must last longer in the latter, suggesting that hypoxic response is less easily triggered in the brain than in other organs. Holmquist-Mengelbier and colleagues demonstrated that, in neuroblastoma, the timing of HIF-1 α and HIF-2 α expression is more important than what is targeted by each isoforms when it comes to comparative analysis of their roles (Holmquist-Mengelbier et al., 2006). HIF-1 α appeared to be induced during acute hypoxia and soon after the start of hypoxia exposure. The half-life of the protein has been estimated to 5min (Wang et al., 1995; Huang et al., 1996). Additionally, increase of HIF-1 α level in tumours has previously been shown to be associated with poor prognosis (Semenza, 2003).

The role of **HIF-3 α** is more obscure. As it has been discovered later, fewer studies are available. The *HIF3A* gene produces at least 7 splice variants, some of which acting as negative regulators of HIF-1 α and HIF-2 α (Maynard et al., 2003, 2007). Specifically Makino and colleagues showed in mice that IPAS (inhibitory PAS domain protein), which prevents

heterodimerisation and activation of HIF by binding to the HIF- α subunit, is actually a splice variant of *HIF3A* (Makino et al., 2002). Moreover, IPAS mRNA expression was hypoxia-induced and paralleled a decrease in HIF-3 α mRNA expression level in heart and lungs of the animals. Later, HIF-3 α 4 was identified as the human ortholog of IPAS (Maynard et al., 2005). More recently, an extensive study was led on HIF-3 α splice variants in response to hypoxia in human and showed that all the HIF-3 α splice variants were able to interact with HIF-1 β , HIF-1 α and HIF-2 α (Jang et al., 2005; Maynard et al., 2005). Interaction of any HIF-3 α splice variant with another HIF- α subunit inhibited nuclear translocation in response to hypoxia. Similarly, HIF-3 α competed with the other HIF- α subunits for the binding of HIF-1 β and hence, negatively regulating cellular response to hypoxia. However, experiments involving siRNA knockdown and overexpression of specific HIF-3 α splice variants suggested that the role of HIF-3 α is more complex than a sole inhibitory activity of hypoxia-mediated HIF regulation (Heikkilä et al., 2011; Tanaka et al., 2009).

1.2.1.2. HIF- β

The ability of **HIF-1 β** , also known as ARNT (arylhydrocarbon receptor nuclear translocator), to form complexes was first described with arylhydrocarbon receptor (AHR) but it was soon discovered that HIF-1 β was also binding to HIF- α in hypoxia, resulting in the transcription of hypoxia-dependent proteins (Wood et al., 1996). It is now known that both HIF-1 α and HIF-2 α interact with HIF-1 β via the PAS domain (Figure 1.5), forming active heterodimers, which translocate to the nucleus and allow the transcription of hypoxia-responsive genes (Ke and Costa, 2006).

A novel bHLH/PAS domain protein (**ARNT2**) was later described, which showed important similarity to ARNT (Hirose et al., 1996). ARNT and ARNT2 were next proven to possess shared functions in mouse (Keith et al., 2001). One study focused on the differential expression of HIF-1 β and ARNT2 in developing kidney (Freeburg and Abrahamson, 2004). It was reported that ARNT2 formed heterodimers with HIF-1 α and HIF-2 α . However, as the level of ARNT2

decreased with age, and was not significantly overexpressed in the most hypoxic regions of the developing kidney, it was concluded that that ARNT2 was likely to play only a minor role in hypoxia-regulated gene expression. Interestingly, a recent report indicated a role for ARNT2 as a modulator of HIF-1 signalling in the human breast cancer cell line MCF7 (Qin et al., 2011).

A third member of the ARNT family, **ARNT3**, has also been described but its role in hypoxia and HIF signalling has not been thoroughly investigated (Takahata et al., 1998).

1.2.2. Oxygen-dependent regulation of HIF

In normoxia (20% O₂), the HIF- α subunit is hydroxylated on the proline residues located in the oxygen-dependent degradation domain (ODDD) by prolylhydroxylases (PHDs) (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001; Chan et al., 2005). It is subsequently ubiquitinated by von Hippel-Lindau protein (pVHL) and targeted for degradation by the proteasome (Maxwell et al., 1999). When the oxygen level falls below 5%, however, the PHDs, whose enzymatic activity has a strict requirement for oxygen, are no longer functional, leading to a decrease in HIF hydroxylation, which in turn stops its targeting to the proteasome (Schofield and Ratcliffe, 2004). Unhydroxylated HIF-1 α and HIF-2 α are therefore able to associate with the constitutively expressed HIF-1 β subunit. The α/β dimer then translocates to the nucleus and, combined with the coactivator CBP/p300, modulates the transcriptional levels of HIF target genes presenting an HRE (hypoxia responsive element) in their promoter (Figure 1.6) (Kallio et al., 1998; Depping et al., 2008). In fact, an early study by Chilov and colleagues suggested that HIF-1 α possessed the nuclear localisation signals allowing nuclear addressing of the HIF complex (Chilov et al., 1999). They found that HIF-1 α was actually able to translocate to the nucleus independently of HIF- β binding, although not with the same efficiency. In this study, it was also suggested that the α/β dimerisation occur in the nuclear compartment rather than the cytoplasm.

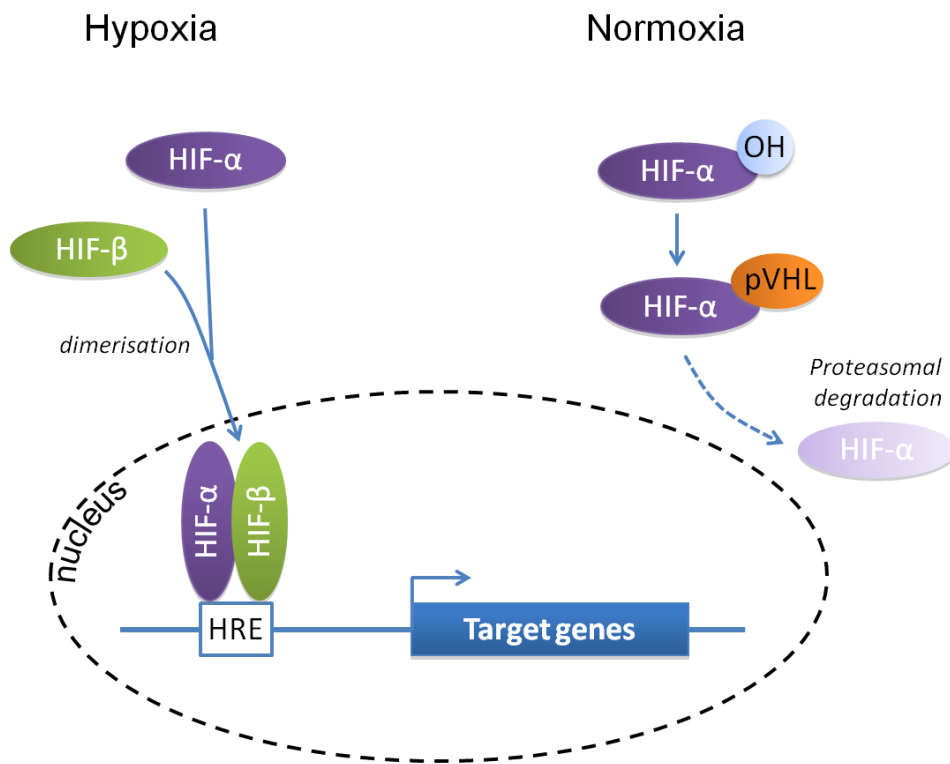


Figure 1.6: Diagram of the oxygen-dependent regulation of HIF

The HIF- β subunit is constitutively expressed in the cytoplasm whereas the hydroxylated HIF- α subunit is ubiquitinated and degraded in normoxia. In hypoxia however, the HIF- α subunit forms a dimer with HIF- β , which translocates to the nucleus and binds to hypoxia-responsive elements (HRE) located in the promoting sequence of the target genes.

In addition to proline hydroxylation, HIF activity is regulated by phosphorylation of specific residues in its transactivation domain (TAD). In hypoxia, HIF-1 α is phosphorylated by p42/44 MAPKs (mitogen-activated protein kinases) also known as ERK1/2 (extracellular regulated kinase) (Richard et al., 1999; Minet et al., 2000). The members of the phosphatidylinositol 3-kinase-related kinases (PIKKs) superfamily DNA-PK (DNA-dependent protein kinase) and ATM (ataxia telangiectasia mutated)-ATR (ATM- and RAD3-related) have also been shown to phosphorylate HIF-1 α (Cam et al., 2010; Bouquet et al., 2011). HIF-1 α phosphorylation appeared to increase the stability of the protein and as a result, increase HIF-1 transcriptional activity (Cam et al., 2010). However, it did not modify its DNA-binding activity. How HIF-1 α phosphorylation affects its transcriptional activity is still poorly understood but has been thought to be related to a higher affinity of HIF-1 β for phosphorylated HIF-1 α (Richard et al., 1999).

The histone acetyltransferase CBP/p300 is a coactivator of HIF transcriptional activity and interacts directly with HIF- α (Kwok et al., 1994; Gu et al., 2001). The interaction between HIF- α and CBP/p300 occurs in the CAD region of the transactivation domain of HIF-1 α and HIF-2 α (Eckner et al., 1994). The asparagine residue 803/847 (for HIF-1 α and HIF-2 α , respectively) is the critical amino acid controlling this interaction (Figure 1.5). In normoxia, this amino acid is hydroxylated whereas in hypoxia, the unhydroxylated asparagine residue allows interaction between the HIF- α subunit and the coactivator CBP/p300 (Lando et al., 2002a; Lisy and Peet, 2008).

pVHL is responsible for the polyubiquitination of HIF- α subunits in normoxia, targeting them for proteasomal degradation. In fact, loss of pVHL is an event commonly observed in renal cell carcinoma (Linehan et al., 2004). As a result, an increased level of HIF can be observed in cells upon pVHL loss along with a response similar to that observed in hypoxia, cohering with an accentuation of the pathology (Maynard and Ohh, 2004). In fact, mice models knocked out for pVHL are commonly used to constitutively activate the HIF signalling pathway *in vivo* and

study the hypoxic response (Fu et al., 2011).

1.2.3. Other HIF regulatory mechanisms

An increasing number of studies have shown that alternative mechanisms, independent of pVHL- and/or oxygen, can control HIF degradation. These include:

The hypoxia-associated factor (**HAF**), also known as SART1₈₀₀, was described by Koh and colleagues as an E3 ubiquitin-ligase allowing HIF-1 α degradation in an oxygen-independent manner (Koh et al., 2008). It was first characterised as a nuclear protein expressed only in proliferating cells and taking part in the spliceosome machinery (Makarova et al., 2001). HAF directly interacts with, and ubiquitinates HIF-1 α but does not target HIF-2 α . The E3 ubiquitin-ligase activity of HAF is independent of pVHL and unlike the latter, does not require specific oxygen level to trigger HIF-1 α proteasomal degradation. Although HAF expression has been described in all the tumour tissues and normal cell lines analysed, HAF does not appear to be expressed in normal, non-proliferative tissues (Shichijo et al., 1998).

Factor inhibiting HIF (**FIH**) is an asparaginylhydroxylase localised in the cytoplasm negatively regulating HIF-1 α in an oxygen-dependent manner (Lando et al., 2002b; Lisy and Peet, 2008). By hydroxylating the asparagine 803 residue in HIF-1 α C-terminal transactivation domain (CAD), FIH prevents the association of coactivators with the HIF- α/β heterodimer subsequently impairing the transcriptional activity of HIF (Mahon et al., 2001; Lando et al., 2002a). FIH acts as a homodimer (Dann et al., 2002) and interacts with pVHL and HIF-1 α to form a ternary complex (Mahon et al., 2001). Although FIH and PHDs both regulate HIF in an oxygen-dependent manner, they do not have the same oxygen sensitivity. Therefore, FIH and PHD would not be inactivated at the same time following a decrease in oxygen level, allowing a fine tuning of oxygen-dependent HIF regulation (Bracken et al., 2006; Lisy and Peet, 2008). Studies have been led regarding the implication of HIF-1 α inhibition by FIH in the context of cancer progression. Whereas FIH could be considered a tool to counterbalance HIF-1 α upregulation in renal cancer, Hyseni and colleagues observed high FIH expression

levels in invasive breast cancer that did not match the higher HIF-1 α expression also observed (Hyseni et al., 2011; Khan et al., 2011).

SHARP1 is a basic helix-loop-helix (bHLH)-containing transcription factor implicated in the invasiveness of triple negative breast cancer. A recent study by Montagner and colleagues presented SHARP1 as a novel HIF-1 α and HIF-2 α inhibitor (Montagner et al., 2012). SHARP1 was shown to induce HIF-1 α proteasomal degradation in an oxygen- and ubiquitination-independent manner by increasing the association between HIF-1 α and the 20S subunit of the proteasome. Interestingly, SHARP1 was found to be associated to HIF-1 α in several TNBC cell lines (Piccolo et al., 2013). Through its inhibitory activity of HIF-1 α , SHARP1 was identified as an anti-metastatic factor, particularly in breast and oral cancers. Another study demonstrated that SHARP1 negatively regulated angiogenesis in endometrial cancer by inhibiting VEGF (vascular endothelial growth factor) mRNA expression (Liao et al., 2014).

The **sirtuin family** is a group of seven related histone deacetylases (Vassilopoulos et al., 2011). Many members of this family have been recently described as inhibitors of HIF-1 α or HIF-2 α : SIRT1 has been shown to acetylate lysine residues in HIF-2 α TAD, preventing the binding between HIF-2 and CBP/p300 (Chen et al., 2012). SIRT3 is a mitochondrial protein that has been demonstrated to inhibit the ROS-dependent enzyme responsible for proline hydroxylation required for HIF-1 α activation (Bell et al., 2011). SIRT6 have been shown to hinder HIF-1 transcriptional activity. It is still unclear whether SIRT6 competes with HIF-1 to bind the promoter or directly interacts with HIF-1 α , thus preventing its binding to the promoter (Zhong et al., 2010). Finally, SIRT7 have been shown to inhibit HIF-1 and HIF-2 activity through a yet unclear mechanism, which seems to be independent of PHD and not relying on proteasomal degradation (Hubbi et al., 2013).

1.2.4. HIF-1 targets

HIF-transcriptional targets are characterized by the presence of hypoxia-responsive elements (HREs) in their promoter sequence, to which the HIF- α /HIF- β heterodimer binds (Wang and

Semenza, 1993b). The consensus sequence of HRE is RCGTG (where R represents A or G) but a larger motif have been recognised, analysing 108 core HRE sequences (Figure 1.7) (Semenza et al., 1996; Wenger et al., 2005).

Numerous HIF targets have been identified which are implicated in a wide range of cellular processes (Ke and Costa, 2006). HIF signalling plays a part in **angiogenesis**. In fact, the main regulator of vasculature, VEGF, is a direct target of HIF-1 α (Forsythe et al., 1996). **Erythropoiesis** is another important mechanism regulated by the HIF-signalling. EPO (erythropoietin) was one of the first HIF targets identified and controls the formation of red blood cells (Wang and Semenza, 1993c). These two mechanisms are a direct response to hypoxia. Indeed, prolonged lack of oxygen can be fatal for cells and extension of the vascular network, as well as increase of oxygen-carrying cells (red blood cells/erythrocytes) aim at correcting the situation. Nevertheless, other mechanisms are also regulated by the HIF signalling. In particular, the **glucose metabolism** pathway is an important target of the HIF signalling of which GLUT1 (glucose transporter 1, coded by *SLC2A1*) is a major component (Chen et al., 2001). Other key players of the glucose metabolism such as CA9 (carbonic anhydrase IX), HK2 (hexokinase 2), PKM2 (pyruvate kinase, muscle), ALDOA (aldolase A, fructose-bisphosphate) are also known as HIF targets (Semenza et al., 1994; Wykoff et al., 2000; Mathupala et al., 2001). Hypoxia also acts on **cell proliferation** and **apoptosis** by regulating the expression of several growth factors (TGF- α , IGF-2 (insulin-like growth factor 2)) and pro-apoptotic proteins such as BNip3 (Bcl-2/adenovirus E1B 19-kDa interacting protein 3) (Carmeliet et al., 1998; Bruick, 2000; Goda et al., 2003; Ke and Costa, 2006). HIF activity has also been demonstrated to upregulate the expression of the **pro-EMT** transcription factors Snail, Twist and ZEB1 (Yang et al., 2008; Zhang et al., 2013a, 2015). Contributing to cancer cell **invasiveness** as well, studies described that the expression of MMP2, MMP9 and MMP14 was mediated in part by the HIF signalling (Ben-Yosef et al., 2002; Muñoz-Nájjar et al., 2006; Choi et al., 2011). LOX (lysyl oxydase), which plays an important

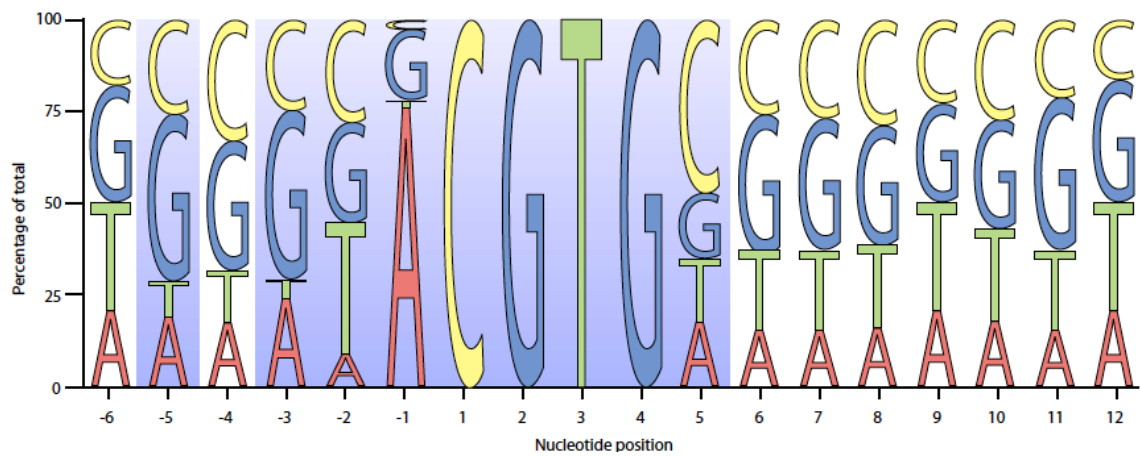


Figure 1.7: HRE consensus sequence diagram

Diagram representing the likely amino acid for each position of the HRE sequence. The size of the letter is proportional to its probability. Nucleotides in blue are the most conserved position of the motif. From Wenger et al. (2005).

role in metastasis formation was also identified as a HIF-1 target (Erler et al., 2006). Recently, the E3 ligase WSB-1 (WD-40 repeat-containing SOCS box protein) has been revealed as a target of the HIF signalling and is thought to modulate therapy response and metastatic spread in hypoxic conditions in some cancer types (Tong et al., 2013; Shichrur et al., 2014; Cao et al., 2015; Kim et al., 2015).

1.3. WSB-1

Archange and colleagues initially showed an upregulation of WSB-1 in response to hypoxia (Archange et al., 2008). In addition, the analysis of the WSB-1 promoter sequence demonstrated the presence of putative HREs upstream of its transcription starting site (Benita et al., 2009). In fact, a recent study demonstrated that one of these putative HREs located between 334 and 339 nucleotides upstream of the transcription start site was indeed mediating hypoxia-regulated transcription of WSB-1 via the binding of HIF-1 (Tong et al., 2013). In a study published last year, Cao and colleagues confirmed that, in hypoxia, HIF-1 α was binding WSB-1 on the -339bp (Cao et al., 2015). In addition, they suggested the existence of a positive feedback loop between WSB-1 and HIF-1 α . In parallel, a study led by Kim also suggested a positive feedback loop between WSB-1 and HIF-1 α and specified this was mediated by WSB-1 degradation of pVHL (Kim et al., 2015).

To date, this work on HRE characterisation was only conducted in hepatocarcinoma and osteosarcoma cell lines and the implication of HIF-2 in hypoxia-mediated WSB-1 expression has never been investigated.

WSB-1 is part of an E3 ubiquitin ligase that belongs to the WSB (WD-40 repeat-containing SOCS box protein) family (Hilton et al., 1998). It was first discovered in chicken, named cSWIP-1 and described as a target of Shh (Sonic hedgehog) during embryonic development (Vasiliauskas et al., 1999; Dentice et al., 2005). Its sequence is highly conserved between mouse, chicken and human, where it has been studied. WSB-1 possesses two characteristic domains giving the protein its name: WD-40 repeats and a SOCS (suppressor of cytokine

signalling) box motif in its C-terminus region (Dentice et al., 2005) (Figure 1.8).

The **WD-40 repeats** are a succession of tryptophan and aspartic acid residues within 40 amino acid-long sequences. These motifs recognise phosphorylated serine and threonine residues and allow the formation of reversible protein complexes by acting as a scaffolding protein (Smith et al., 1999; Kile et al., 2002; Smith, 2008). WD-40 repeats were initially observed in the sequence of the β subunit of G proteins but have since been highlighted in numerous proteins where they appear to interact with one another. Interestingly, most proteins sporting WD-40 repeats possess a regulatory role but no enzymatic activity (Neer et al., 1994).

The **SOCS box** domain was first identified as an important player in the signal transduction of cytokines. An initial study by Hilton and colleagues identified twenty proteins with SOCS box domains organised in five families (Hilton et al., 1998). Later, over forty proteins containing a SOCS box have been described and sorted into nine groups (Kile et al., 2002). Notably, a decreased half-life of the SOCS box-containing protein SOCS-1 has been reported when the SOCS box was deleted, highlighting the importance of this activity domain for the protein stability (Kamura et al., 1998). In addition, such domain is frequently observed in E3 ubiquitin ligases associated with elongin B/C complexes (Kamura et al., 1998).

1.3.1. Ubiquitin ligase activity

Quality control of the proteins present in a cell, as well as prompt degradation of misfolded or damaged proteins, is of high importance for correct cellular function and is mostly controlled by the ubiquitin proteasome pathway (Goldberg, 2003). For this reason, the ubiquitin proteasome pathway has been a point of interest for the understanding of cancer development mechanisms for a long time. In fact, infection by the human papillomavirus was shown to be responsible for altering the normal ubiquitin proteasome pathway, thus resulting in tumour development (Boyer et al., 1996; Spataro et al., 1998). As a result, an array of drugs

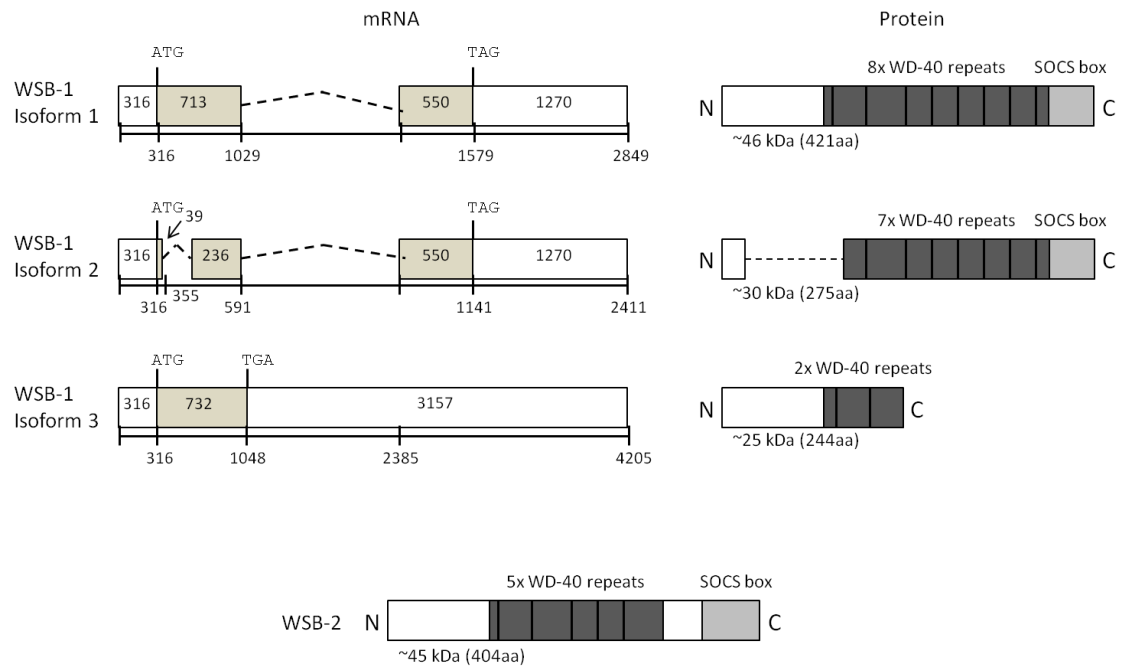


Figure 1.8: Diagram of mRNA and protein structures of WSB-1 isoforms

Transcripts of isoforms 1 and 2 share the same stop codon and produce the 2 longest isoforms. The isoform 3 uses a premature stop codon, producing a shorter protein lacking 6 WD-40 repeats and the SOCS box. The WD-40 repeats are protein-protein interaction domains and the SOCS box is responsible for the enzymatic activity.

have been designed that act on the ubiquitin proteasome pathway (Shen et al., 2013; Liu et al., 2015). Notably, Bortezomib is the first proteasome inhibitor that has been validated by the American FDA (food and drug administration) for cancer treatment (Kane et al., 2003; Johnson, 2015). The effect of this drug to prevent the progression of breast cancer has been proven in ER+ and TNBC tumours (Petrocca et al., 2013; Thaler et al., 2015). In addition, Bortezomib also showed positive effect against bone metastasis in mice (Jones et al., 2010).

Figure 1.9 illustrates the ubiquitin proteasome pathway. The ubiquitin molecule is first activated by the E1 ubiquitin activating enzyme in an ATP-dependent manner. The E2 ubiquitin conjugating enzyme then takes the ubiquitin in charge and recruits the E3 ubiquitin ligase, as well as the substrate and other scaffolding proteins in some cases. The E3 ubiquitin ligase is responsible to attach the ubiquitin molecules on the substrate protein. The poly-ubiquitinated substrate protein is subsequently degraded by the 26S proteasome. E3 ubiquitin ligases present a greater variability than E1 or E2 enzymes and determine the substrate specificity (Liu et al., 2015). E3 ligases have been organised in categories, according to their interactions while forming the complex with the substrate protein: **HECT** (homologous with E6-associated protein C-terminus) form a simple complex containing only the E2 and E3 enzymes and the substrate protein, whereas the **U-box** and **RING** (really interesting new gene) E3 ligases require the presence of scaffolding proteins as part of the ubiquitination complex. More specifically, RING E3 ligases are classified into single-molecular RING containing E3 ligases or multi-subunit E3 ligase complexes which usually comprise cullins such as ECS (Elongin B, C/Cul2, 5/SOCS), SCF (Skp1/Cul1/F-box protein) or APC/C (cullin-related anaphase-promoting complex/cyclosome complex) (Okumura et al., 2012).

Interestingly, pVHL, which is the protein responsible for hypoxia-dependent HIF- α degradation, is known to be part of the ECS subfamily of RING type E3 ubiquitin ligases (Kile et al., 2002). More specifically, pVHL, elongin B/C and a SOCS-containing E3 ligase form together a VCB complex which is believed to be involved with HIF- α downregulation in

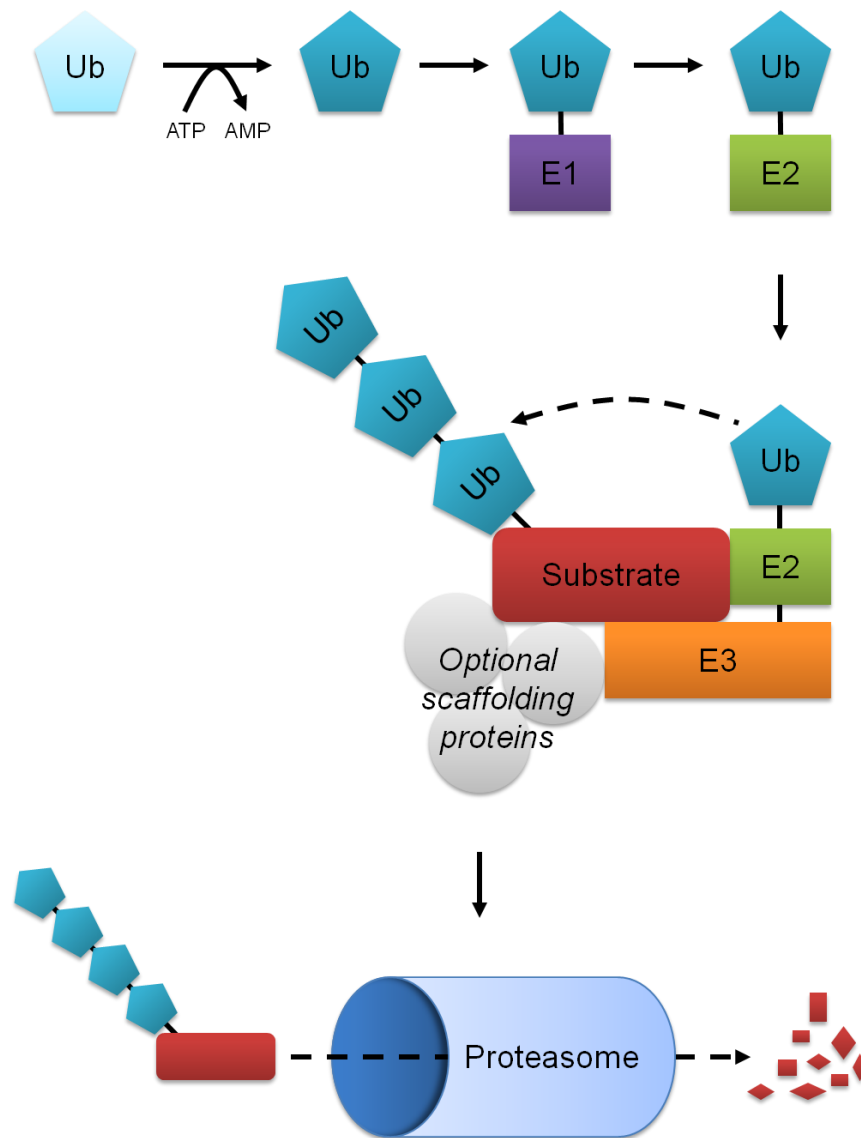


Figure 1.9: Ubiquitination process leading to proteasomal degradation of proteins

The ubiquitin molecule is activated in an ATP-dependent manner and associates to an E1 ubiquitin activating enzyme. The E1 enzyme is replaced by an E2 ubiquitin conjugating enzyme and then binds to the E3 ubiquitin ligase, the substrate protein and eventual scaffolding proteins to link the ubiquitin on the substrate protein. The substrate protein is then directed to the proteasome and degraded. Ub: ubiquitin.

normoxia (Ardley and Robinson, 2005). In fact, WSB-1 was also reported to interact with elongin B/C along with Cul5 and Rbx1 and was later defined as a member of a new family of E3 ligases that harbour both WD-40 repeats and a SOCS box (Dentice et al., 2005; Choi et al., 2008).

1.3.2. Known WSB-1 targets

The E3 ligase activity of WSB-1 has notably been described on **HIPK2** (homeodomain-interacting protein kinase 2), a nuclear serine/threonine protein kinase (Choi et al., 2008). Studies have shown an increase in HIPK2 degradation subsequent to HIF-1 α activation (Moehlenbrink et al., 2010; Nardinocchi et al., 2011). Tong and colleagues showed that this hypoxia-induced HIPK2 degradation was mediated by a hypoxia-induced upregulation of WSB-1, further confirming that HIPK2 is a target of WSB-1 ubiquitin-ligase activity (Tong et al., 2013). HIPK2 activates p53 and induces apoptosis, as well as phosphorylation and activation of p300, a HIF cofactor (Aikawa et al., 2006). In this specific context, WSB-1 activity could therefore be considered as anti-apoptotic based on its role on HIPK2 (Choi et al., 2008).

DIO2 (2-iodothyronine deiodinase), an enzyme which transforms inactive thyronine (T4) into active triiodothyronine (T3), is another target of WSB-1 ubiquitin ligase activity, as demonstrated by siRNA knockdown (Dentice et al., 2005). This study demonstrated that WSB-1 activity on DIO2 contributed to the secretion of PTHrP (parathyroid hormone-related peptide) in the growth plates of chicken embryos and stimulated skeletogenesis.

Recently, a study demonstrated that WSB-1 directly binds **RhoGDI2** (Rho GDP-dissociation inhibitor 2) which is then degraded by the proteasome (Cao et al., 2015). RhoGDI2 is a protein inhibiting the Rho pathway, regulating cell motility, migration and metastasis. In fact, Cao and colleagues revealed that the regulation of RhoGDI2 by WSB-1 played a role in the metastatic spread of osteosarcoma. Incidentally, RhoGDI2 has been noted as an invasion and metastasis suppressor gene in human cancer and is considered a possible therapeutic target (Gildea et al., 2002).

Another recent study suggested WSB-1 was able to degrade **pVHL** (Kim et al., 2015). As a result, HIF would be upregulated and promote cell motility and invasion during cancer metastasis. This hypothesis was further supported by the increased WSB-1 levels Kim and colleagues observed in metastatic tissues compared to non-metastatic tissues in several cancer types.

Besides its E3 ubiquitin ligase activity, WSB-1 has also been shown to interact with several other proteins, resulting in various outcomes. For example, Nara and colleagues described the interaction of WSB-1 with **IL-21R** (interleukin-21 receptor) (Nara et al., 2011). In HEK293T cells, WSB-1 was shown to induce the glycosylation of IL-21R, thus promoting its maturation. Coincidentally, IL-21 has an anti-tumour activity that is currently being investigated to be used for cancer therapy (Davis et al., 2015). IL-21 also plays a role in the development of immunopathologies (Søndergaard and Skak, 2009). WSB-1 has also been found to interact with several other proteins as part of the RING E3 ubiquitin ligase complex. In particular with **Cul5**, **TCEB1** (transcription elongation factor B, polypeptide 1/Elongin C) and **TCEB2** (transcription elongation factor B, polypeptide 2/Elongin B), which have already been mentioned in section 1.3.1, and with **UBC** (ubiquitin C) (Kamura et al., 2001; Mahrour et al., 2008; Lievens et al., 2009; Emanuele et al., 2011).

1.3.3. WSB-1 isoforms

WSB-1 is coded by the *WSB1* gene, located on chromosome 17. *WSB1* has actually been reported to produce three distinct isoforms resulting from the alternative splicing of the initial transcript (Archange et al., 2008). WSB-1 isoform 1 consists of the full length protein. It is 421 amino acids long and contains eight WD-40 repeats as well as complete N-terminal domain and SOCS box in C-terminus. WSB-1 isoform 2 is 275 amino acids long and misses parts of the N-terminus sequence, including one WD-40 repeat. WSB-1 isoform 3 has the longest mRNA sequence but codes for the smallest protein. It is 244 amino acids long and misses six WD-40 repeats and the SOCS box in C-terminus, challenging its catalytic ability. The

transcript sequences and protein structures of each isoform are presented in Figure 1.8.

Because most of the WD-40 repeats and the SOCS box present in the other two isoforms are missing in WSB-1 isoform 3, this isoform is likely to present a decreased ability for protein-protein interaction and no enzymatic activity. In addition, a previous study suggested that proteins deprived of their SOCS box presented a reduced half-life (Kile et al., 2002). For this reason, WSB-1 isoform 3 could also have a decreased life span and could be a candidate for nonsense-mediated mRNA decay (NMD). Its sequence has in fact been permanently deleted from the NCBI database for this reason. NMD is a mechanism whereby mRNA harbouring premature stop codons are sequestered and degraded to avoid accumulation of ineffective or even antagonistic proteins in the cell (Behm-Ansmant et al., 2007; Isken and Maquat, 2007). NMD is inhibited in hypoxia (Gardner, 2008). Therefore, NMD-mediated WSB-1 isoform 3 degradation would be suspended in hypoxia, potentially leading to an increase in WSB-1 isoform 3 levels in such circumstances. Indeed, Archange and colleagues observed a shift in the expression of WSB-1 isoforms by alternative splicing of the mRNA from the isoform 1 and 2 towards the isoform 3 in response to various cellular stresses in pancreatic cancer cells, including hypoxia (Archange et al., 2008). Similarly, Shichrur and colleagues reported an increase of WSB-1 isoform 3 levels in neuroblastoma (Shichrur et al., 2014). However, both studies highlighted opposite effect of WSB-1 isoform 3 on cellular growth. Whereas WSB-1 promoted growth by increasing cell proliferation and not by altering cell death in pancreatic cancer cells, knockdown of WSB-1 in neuroblastoma cells appeared to increase apoptotic cell death.

1.3.4. WSB-2

WSB-2 is another member of the WSB family. As for WSB-1, it is expressed in a broad range of mouse and human tissues (Hilton et al., 1998; Nara et al., 2010). As a matter of fact, WSB-1 and WSB-2 share 65% of their protein sequence (Sarraj et al., 2007). WSB-2 is smaller than WSB-1 isoform 1 and possesses only five WD-40 repeats (Figure 1.8).

The function of WSB-2 is not clear but it is believed to also be an E3 ubiquitin ligase (Kile et al., 2002). In fact, Nara and colleagues described an interaction of WSB-2 with IL-21R and, contrary to what was reported for WSB-1, WSB-2 was shown to downregulate the expression of IL-21R (Nara et al., 2010). Another study showed that WSB-2 interacts with G-CSF-R (granulocyte colony-stimulating factor receptor) in a murine myeloid cell line model, acting on the proliferation-differentiation balance (Erkeland et al., 2007). However, despite the similarity between WSB-1 and WSB-2 sequences and common binding partners, other interactions are specific. For example, WSB-2 has no effect on DIO2 (Dentice et al., 2005).

1.3.5. Relevance of WSB-1 in cancer

Previous studies found a connection between WSB-1 and cancer, and investigated the implication of WSB-1 in the spreading of certain tumours. In an attempt to validate a procedure to find cancer gene-expression signatures, Rhodes and Chinnaiyan highlighted WSB-1 as one of several overexpressed protein in salivary carcinoma (Rhodes and Chinnaiyan, 2005). Archange and colleagues uncovered a conflicting role for WSB-1 whereby WSB-1 isoforms 1 and 2 stimulated the proliferation of pancreatic cancer cells and WSB-1 isoform 3 inhibited pancreatic cancer cells growth by increasing cell proliferation rather than decreasing cell death (Archange et al., 2008). In fact, WSB-1 isoform 3 could have a protective effect against apoptosis in pancreatic cancer cells. Similarly, Tong and colleagues suggested that WSB-1 was involved in chemoresistance in hepatocellular cancer cells as WSB-1 knockdown resulted in an increased sensitivity to the apoptosis-inducing drug etoposide in hypoxia (Tong et al., 2013). In neuroblastoma, where gain of parts of or the entire chromosome 17 is frequent, increased WSB-1 copy number, and therefore increased WSB-1 expression, was a sign of a good prognosis for the patients (Chen et al., 2006). A more recent study refined these results by focusing more closely on WSB-1 isoform 3 (Shichrur et al., 2014). They observed higher levels of WSB-1 isoform 3 than WSB-1 isoforms 1 and 2 in human neuroblastoma cell lines, xenograft models and primary neuroblastoma samples. The

downregulation of WSB-1 isoform 3 resulted in an increase in apoptosis and a decrease in resistance to chemotherapy, which concurs with Tong findings and the positive outcome for neuroblastoma patients with high WSB-1 levels. On the contrary, the study by Cao and colleagues on osteosarcoma indicated that higher WSB-1 levels were observed in tumours with metastasis compared to tumours without (Cao et al., 2015). In fact, they argue that WSB-1 levels could be used as a prognostic marker for metastatic-free survival. In osteosarcoma cells, WSB-1 favoured tumour progression by activating the Rho proteins, which induce cell motility, migration and metastasis. Another study reported a potential association between WSB-1 and susceptibility to chronic myeloid leukemia whereby a mutation on *WSB1* locus would indicate an increased risk factor for developing leukemia (Kim et al., 2011a). In a recent study, Kim and colleagues compiled the level of WSB-1 in several cancer types and found that, in most cases, WSB-1 level was elevated in metastatic tissues (Kim et al., 2015). They also observed that high WSB-1 level was associated with poor survival rates for triple negative breast cancer patients.

WSB-1 appears to play an important role on tumour progression, metastasis and resistance to chemotherapy. Its specific effect, however, depends on the cancer type and precise WSB-1 mechanisms of action remains elusive.

1.4. Objectives and aims of this thesis

Breast cancer is a disease that will directly concern 1 in 8 women during their lifetime in the UK. Worldwide, breast cancer was the second most common cancer in 2012, behind lung cancer, and represented 25% of the total number of new cases diagnosed in women (Ferlay et al., 2015). For this reason, it is important to develop tools allowing early detection, to optimise the therapeutic approach for each individual patient, and to find effective drugs preventing further spreading of the disease. Some markers have already been identified, which allow the categorisation of breast cancers and help choosing the ideal therapy. Other proteins are used to inform on the invasiveness of a tumour and the probability of survival for the patient.

Finally, novel drugs are designed that target mechanisms involved in metastasis or resistance. Although survival rates for patients with stage I and II tumours are above 90%, these odds decrease massively for later stages patients (Table 1.1). Therefore, it is important to identify novel targets to prevent further spreading of the disease, particularly for stage III patients who do not present metastases yet and avoid their progression to stage IV. Consequently, this thesis aims at evaluating the role of WSB-1, a protein shown to be upregulated in several cancer types and associated with metastatic progression in some cases, in breast cancer.

1.4.1. Specific objectives

The specific objectives of this thesis are as follows:

- **Chapter 3:** Investigating the significance of WSB-1 expression in breast cancer samples and defining the effects of hypoxia on WSB-1 in breast cancer *in vitro*,
- **Chapter 4:** Studying the role of WSB-1 in the motility and invasiveness of breast cancer cells,
- **Chapter 5:** Identifying novel proteins interacting with WSB-1.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Cell culture

2.1.1. Cell lines

Several human cell lines were utilised throughout this study: HEK293T (embryonic kidney), MCF7 (pleural effusion of metastatic adenocarcinoma), MDA-MB-231 (pleural effusion of metastatic adenocarcinoma), BT474 (breast tissue of primary ductal adenocarcinoma), DU4475 (mammary gland of primary tumour), HMEpC (healthy mammary gland), MDA-MB-468 (pleural effusion of metastatic adenocarcinoma), and T47D (pleural effusion of metastatic ductal carcinoma). Table 2.1 summarises the characteristics of each cell line. Cell lines were originally purchased from ATCC or ECACC.

Cell culture media were regularly tested to ensure cell cultures were mycoplasma-free.

2.1.2. Subculture

DU4475 cells were grown in RPMI 1640 (Roswell Park Memorial Institute medium; Lonza, UK) supplemented with 10% FBS (Fetal Bovine Serum; Gibco, Life Technologies, UK), 1% Glutamax (Lonza) and 1% Penicillin-Streptomycin (Lonza). HMEpC cells were grown in Human Mammary Epithelial Cell Growth medium (Cell Applications, CA, USA). All the other cell lines were grown in 4.5g/L glucose DMEM (Dulbecco's Modified Eagle Medium; PAA, GE Healthcare Life Science, UK) supplemented with 1% Sodium Pyruvate (PAA, GE Healthcare Life Science) and 10% heat inactivated FBS.

When reaching 70-85% confluency, cells were passaged to keep the cell population in an exponential growth state. The cell monolayers were washed twice with 1X PBS (Phosphate-Buffered Saline; PAA, GE Healthcare Life Science) and incubated 5min at 37°C in 1X trypsin (Lonza). Detached cells were then resuspended in fresh medium and a fraction of the volume was transferred into fresh flasks. Suspension cells DU4475 were diluted 1:3 in fresh RPMI

every 2 to 3 days. Cells were incubated at 37°C with 5% CO₂ and a humid atmosphere.

2.1.3. Viable cells counting

In order to seed a constant number, cells were counted before experimental setup. A small volume of the cell suspension was diluted 1:2 in a 0.4% trypan blue solution (Gibco, ThermoFisher Scientific, UK) and viable cells were counted in 4 quadrants of a Neubauer haemocytometer (Marienfeld Superior, Germany). The number of cells per millilitre of cell suspension was obtained by multiplying the average number of cell per quadrant by the dilution factor and by 10,000.

2.1.4. Freezing down and thawing cells

To ensure constant stock of cells, new cells were frozen after a few passages. Once reaching 70-85% confluency, the cellular monolayer was trypsinised as per paragraph 2.1.2 and the cells were resuspended in media and centrifuged. The cell pellet was resuspended in a volume of 1X PBS, centrifuged again and the pellet was resuspended in a small volume of freezing media containing 10% DMSO (dimethyl sulfoxide) in FBS (Gibco, Life Technologies). The cell and freezing media suspension was aliquoted into cryovials (Nunc, Thermo Scientific, UK), which were placed in a freezing container (Nalgene, Thermo Scientific) in a -80°C freezer to allow gradual cooling of the cells. After 24 to 48h, cells were transferred into liquid nitrogen for long-term storage. When required, a cryovial was quickly brought to room temperature and the cells were transferred into prewarmed media, centrifuged, and the supernatant discarded to remove any traces of DMSO. The cell pellet was resuspended in fresh media and transferred into a T25 flask until they reached 70-85% confluency. Cells were then transferred into a T75 flask and grown normally.

Table 2.1: Summary of the characteristics of the cell lines used in this thesis

	HEK293T	HMEpC	BT474	DU4475	MCF7	MDA-MB-231	MDA-MB-468	T47D
Disease	Not Applicable (N/A)	N/A	Ductal adeno-carcinoma	Unknown	Adeno-carcinoma	Adeno-carcinoma	Adeno-carcinoma	Ductal carcinoma
Development	N/A	N/A	Primary	Primary	Metastatic	Metastatic	Metastatic	Metastatic
Origin	Embryonic kidney	Mammary gland	Breast/duct	Mammary gland	Pleural effusion	Pleural effusion	Pleural effusion	Pleural effusion
Culture type	Adherent	Adherent	Adherent	Suspension	Adherent	Adherent	Adherent	Adherent
Culture media	Dulbecco's Modified Eagle Medium (DMEM)	Human Mammary Epithelial Cell Growth Medium	DMEM	Roswell Park Memorial Institute medium (RPMI)	DMEM	DMEM	DMEM	DMEM
ER	N/A	N/A	+	-	+	-	-	+
PR	N/A	N/A	+	-	+	-	-	+
HER2	N/A	N/A	+	-	-	-	-	-
Classification	N/A	N/A	Luminal B	Basal	Luminal A	Basal	Basal	Luminal A
Ethnicity	Unknown	Unknown	Caucasian	Caucasian	Caucasian	Caucasian	Black	Unknown
Age (years)	Foetus	Unknown	60	70	69	51	51	54

2.1.5. Spheroids formation

25,000 cells were seeded per wells of ultra low adherence round bottom 96-well plates (Costar, Sigma-Aldrich, UK), as previously reported (Vinci et al., 2012). Cells were left undisturbed for 4 days in an incubator at 37°C with 5% CO₂ and a humid atmosphere in order to allow spheroids to aggregate. On day 4, spheroids were transferred in a well coated with 0.1% gelatine diluted in sterile water and supplemented with DMEM containing 2% FBS. Alternatively, spheroids were embedded in Matrigel (BD Bioscience, UK).

Plates were left to set for 30min and pictures of the spheroids were then taken using an inverted optic microscope (AxioVert, Zeiss, UK). Pictures were taken every day for 4 days.

2.2. Exposure to hypoxic conditions

Hypoxic conditions were achieved using H35 Hypoxystation hypoxia chamber (Don Whitley Scientific, UK). Humidity (75%), CO₂ (5%) and temperature (37°C) were monitored and specific oxygen concentrations were defined prior to experiment start. Media was replenished shortly before cells being placed in the chamber. At the end of the experiment, hypoxic samples were lysed directly in the chamber to prevent reoxygenation.

In parallel, normoxic control samples were incubated in a conventional incubator kept at 37°C with 5% CO₂ and a humid atmosphere in atmospheric oxygen tension (~20% O₂).

2.3. Cell transfection

2.3.1. Transfection of silencing RNA

Transfection with silencing RNA (siRNA) was performed using DharmaFECT transfection reagent #1 (Dharmacon, Thermo Scientific), following manufacturer instructions with some modifications. Briefly, depending on the cell line, 2×10^5 to 3×10^5 cells were seeded in 35mm plastic dishes (Thermo Scientific). Cells were left to adhere for 24h before being transfected. siRNA (see Table 2.2 for details) stocks were prepared by resuspending the

Table 2.2: Details of the siRNA sequences used for knockdown experiments

Target	Manufacturer	Final concentration	Additional information
HIF-1α	Custom synthesis (Sigma-Aldrich, USA)	50nM	5' CUGAUGACCAGCAACUUGA(dT) 3' 5' UCAAGUUGCUGGUCAUCAG(dT) 3'
HIF-2α	Custom synthesis (Sigma-Aldrich)	50nM	5' CAGCAUCUUUGAUAGCAGU(dT) 3' 5' ACUGCUAUCAAAGAUGCUG(dT) 3'
HIF-1β	Custom synthesis (Sigma-Aldrich)	50nM	5' GGUCAGCAGUCUCCAUGA(dT) 3' 5' UCAUGGAAGACUGCUGACC(dT) 3'
Non-targeting	siGENOME (Dharmacon/Thermo Scientific)	25nM	Non-targeting siRNA #2
WSB-1	siGENOME SMARTpool (Dharmacon/Thermo Scientific)	25nM	Targets all WSB-1 isoforms 5' UAUGGGACCUGAAAGAUGA 3' 5' GGAAACAUGAUGAAAGUAU 3' 5' GAAGUGGUCAGAGAUUUAA 3' 5' GAAAACUCCUCCUUAACUU 3'

lyophilised oligos in sterile DEPC-treated (diethylpyrocarbonate) water to a concentration of 100 μ M. Non-targeting siRNA (siNT) and siRNA against WSB-1 (siWSB-1) were used at a final concentration of 25nM, and siRNAs against HIF subunits (siHIF-1 α , siHIF-2 α , siHIF-1 β) were used at a final concentration of 50nM. The siRNA stocks were first diluted to 2 μ M (or 4 μ M for HIF) with DEPC water. The transfection mix was composed of two solutions. A: diluted siRNA in serum-free media (1:1) and B: DharmaFECT transfection reagent #1 in serum-free media (1:70). Each solution was incubated 5min at room temperature prior mixing them together (1:8, A:B). The transfection solution was incubated between 20min and 1h at room temperature to allow the formation of siRNA:transfection reagent complexes before transferring into the well. Complete media equivalent to four times the volume of transfection media was added to each well and the transfection mix was added drop-wise on the cells. Cells were allowed to incubate 24h with the transfection mix before replacing the media with fresh DMEM. In the case of HIF knockdown, cells were transfected a second time 24h after the first transfection, following the same protocol.

2.3.2. Transfection of plasmid DNA

The pFLAG-CMV2-WSB1 plasmid (Figure 2.1), a kind gift from Prof. Hironobu Asao, Yamagata University, Japan was used for the transfection (Nara et al., 2011). Transfection was performed using PEI (polyethylenimine 25kDa linear; Polysciences, Germany) on 60% confluent cell monolayer seeded in 15cm dishes, as previously described (Pires et al., 2014).

A 20mg/mL stock solution of PEI was prepared in MilliQ water heated to 80°C. Once cooled, the pH of the solution was adjusted to 7.0 using 5M HCl. The solution was then filter sterilised and aliquots were stored and -20°C.

Transfection mix contained 10µg/mL of DNA (an equivalent volume of sterile DEPC water was used for the mock transfected dishes) and 50µg/mL of PEI diluted in serum-free media per 15cm dish. After gently mixing the components together, the solution was incubated 30min at room temperature. The transfection mix was then added drop-wise to the cells and left for 24h before changing the media.

2.4. Protein analysis

2.4.1. Cell lysis and protein extraction

Cells were seeded in plastic dishes (Nunc, Thermo Scientific) and incubated in the respective experimental conditions. At the appropriate time points, media was removed and the cellular monolayer was gently washed with 1X PBS at room temperature. The cells were then scrapped from the dish into 1X PBS. This cell suspension was centrifuged at 20,000×*g* and the supernatant removed. The pellet was resuspended in a volume of UTB lysis buffer (9M Urea; 75mM Tris-HCl pH 7.5; 0.15M β-mercaptoethanol) appropriate to the cell pellet size. The whole cell lysate samples were sonicated using a Bioruptor (Diagenode, Belgium) on high setting for 15min by 30sec intervals. Samples were then centrifuged at 20,000×*g* to pellet all sheared material and clarified supernatants were transferred in fresh microcentrifuge tubes and stored at -20°C.

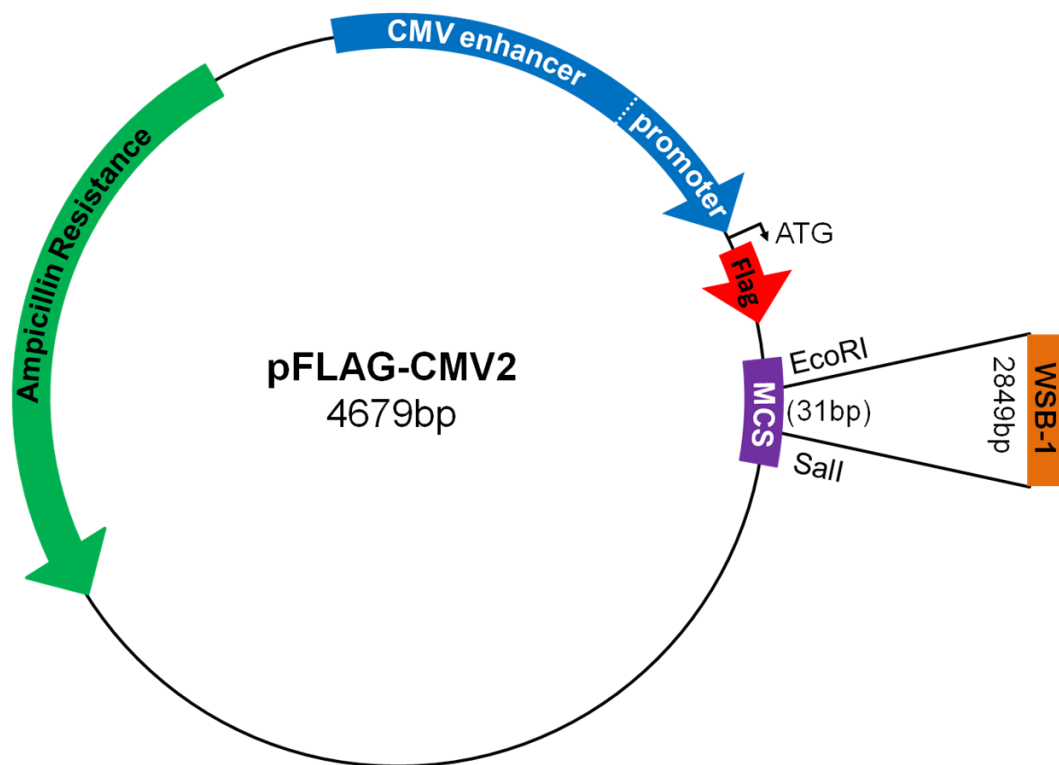


Figure 2.1: Map of the pFLAG-CMV2 plasmid

WSB1 sequence was inserted in the pFLAG-CMV2 plasmid between the Eco RI and Sal I restriction sites. The pFLAG-CMV2 plasmid is distributed by Sigma-Aldrich (UK) and the pFLAG-CMV2-WSB1 plasmid was a kind gift from Prof. Hironobu Asao (Yamagata University, Japan). MCS: multiple cloning site.

2.4.2. Protein quantification and sample preparation

Clarified whole cell lysates were quantified using Nanodrop Light (Thermo Scientific). Protein samples were diluted in UTB lysis buffer to a fixed concentration (*eg.* 2.5µg/µL) and a constant volume so that a consistent volume was loaded in each well. Sample buffer (3.3% SDS; 6M Urea; 17mM Tris-HCl pH 7.5; 0.01% bromophenol blue; 0.07M β-mercaptoethanol) was then added to each sample and the mixture was heated at 100°C for 5min in a heat block.

2.4.3. SDS-PAGE

Protein samples were analysed by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) followed by Western blotting. Protein samples were run through a concentrating stacking gel (1.5M Tris-HCl pH 6.8, 30% acrylamide/0.8% bis-acrylamide, 10% SDS, 10% TEMED and 10% APS in the appropriate volumes) and a 12% acrylamide separating gel (1.5M Tris-HCl pH 8.8, 30% acrylamide/0.8% bis-acrylamide, 10% SDS, 10% TEMED and 10% APS in the appropriate volumes) (see Table 2.3 for composition). Samples were prepared as described in section 2.4.2, so that 30-60µg of protein were loaded per well. A molecular weight ladder (Precision Plus Protein Western C Standard, BioRad, CA, USA) was loaded alongside the samples. Electrophoresis was performed in a tank filled with 1X running buffer (see Table 2.4) and ran at 100-120V for 1.5h.

Table 2.3: Composition of 12% acrylamide gels for SDS-PAGE (volumes given for casting two 1mm-thick minigels)

Reagent	Concentration gel	Separation gel
MilliQ water	6.1mL	5.2mL
1.5M Tris pH 8.8	-	3.75mL
0.5M Tris pH 6.8	2.5mL	-
Acrylamide	1.3mL	5.8mL
10% SDS	100µL	150µL
10% APS	100µL	75µL
TEMED	20µL	18µL

Table 2.4: Composition of running and blotting buffers (10X)

Reagent	Running buffer	Blotting buffer
Tris base	30.2g	30.2g
Glycine	144g	144g
SDS (pellets)	10g	-
MilliQ water	Top up to 1L	Top up to 1L

2.4.4. Western blotting

When the migration front reached 1cm from the bottom of the gel, electrophoresis was stopped and proteins were transferred onto a PVDF (polyvinylidene difluoride; GE Healthcare) membrane previously activated in methanol for 15sec. The transfer was performed for 1.5h at 100V in 1X blotting buffer containing 20% methanol (see Table 2.4).

The PVDF membrane was blocked for 1h in a milk solution (5% fat-free powdered milk in TBST (10% 10X TBS (Tris-Buffered Saline) (88g NaCl; 24g Tris base; pH 7.4 in a final volume of 1L MilliQ water) and 0.1% Tween 20 diluted in MilliQ water)) and washed thoroughly in TBST. The membrane was then incubated overnight at 4°C with the primary antibody diluted in a solution of 1% milk-TBST or a solution of BSA (bovine serum albumin), in accordance to the datasheet provided by the manufacturer (refer to Table 2.5 for information on primary antibodies used). The membrane was washed in TBST and incubated 1h at room temperature with the adequate HRP-conjugated polyclonal secondary antibody diluted to 1:2000 in 1% milk-TBST (Dako, Denmark). The membrane was incubated for 1min with Clarity Western ECL developing solution (BioRad) and imaged using the ChemiDoc XRS+ System and the Image Lab software (BioRad). Densitometry analyses of the blots were performed using the ImageJ software (NIH, USA).

2.4.5. Coomassie blue protein gel stain

Alternatively to Western blotting, acrylamide gels were incubated in a volume of InstantBlue Coomassie blue stain (Expedeon, UK) under agitation at room temperature for a minimum of

1h to stain the proteins in the gel. After extensive washes in MilliQ water, an image of the gel was taken using the ChemiDoc XRS+ System and the Image Lab software (BioRad).

Table 2.5: List of the antibodies commonly used for Western Blotting

Target	Manufacturer	Reference	Dilution	Origin	Clonality	Expected band size (kDa)
AUF1	Cell Signaling	12382	1:1000	R	Mono	37-48
β-Actin	Santa Cruz	sc-69879	1:10000	M	Mono	42
E-Cadherin	Cell Signaling	3195	1:1000	R	Mono	135
Flag	Sigma	F3165	1:2000	M	Mono	N/A
GAPDH	Ambion	AM4300	1:5000	M	Mono	36
GLUT1	Abcam	ab40084	1:500	M	Mono	55
GSK3β	Cell Signaling	9315	1:500	R	Mono	46
HIF-1α	BD Biosciences	610958	1:500	M	Mono	120
HIF-1β	Santa Cruz	sc-56620	1:500	M	Mono	95
HIF-2α	LSBio	LS-B517	1:500	R	Poly	120
HIPK2	Abcam	ab28507	1:1000	R	Poly	110
HSP90α/β	Santa Cruz	sc-1055	1:1000	G	Poly	90
MMP-1	R&D Systems	MAB901	1:1000	M	Mono	54
MMP-2	Cell Signaling	4022	1:500	R	Poly	72 (proform) 64 (cleaved)
MT1-MMP (MMP-14)	Abcam	ab38971	1:2000	R	Poly	62 (proform) 50 (cleaved)
p23	Santa Cruz	sc-101496	1:2000	M	Mono	23
Snail	Cell Signaling	3879	1:1000	R	Mono	29
STIP1	Abcam	ab126724	1:10000	R	Mono	63
Tid-1	Cell Signaling	4775	1:500	M	Mono	40 (Tid-1L) 37 (Tid-1s)
Vimentin	Cell Signaling	5741	1:1000	R	Mono	57
WSB-1	Genetex	GTX115792	1:1000	R	Poly	25 (isoform 3)
ZO-1	Cell Signaling	8193	1:1000	R	Mono	220

R: Rabbit, M: Mouse, G: Goat, Mono: monoclonal, Poly: polyclonal

2.5. LC-MS analysis

LC-MS (liquid chromatography mass spectrometry) was used to identify the peptides present within protein samples. The samples destined to LC-MS analysis were prepared following the method described in sections 2.3.2. Samples were run in 12% precast gels (BioRad) as per section 2.4.3 and the SDS-PAGE gel was stained using Coomassie blue, as described in section 2.4.5. Sections were then delineated in each lane in order to evenly distribute the protein quantity across the portions. Bands were then excised using a clean scalpel on a ceramic tile wiped with isopropanol, cut into pieces of approximately 1mm and transferred into low binding LoBind microcentrifuge tubes (Eppendorf, Sigma-Aldrich, UK). The pieces of gel were first destained by two washes in a solution of 25mM ammonium bicarbonate in 50% acetonitrile for 20min each wash. Supernatant was removed and pieces of gel washed in acetonitrile until turned white, for a maximum of 5min. The pieces were then dried in a SpeedVac (Thermo Scientific) for 20min on medium setting. Reduction of the samples was done by incubating the gel pieces in a solution of 10mM dithioerythritol in 100mM ammonium bicarbonate at 56°C for 1h. Once returned at room temperature, the samples were alkylated by incubating the gel pieces in a solution of 50mM iodoacetamide in 100mM ammonium bicarbonate for 30min in the dark. The samples were then washed by firstly incubating the gel pieces in 100mM ammonium bicarbonate for 15min, then in a solution of 25mM ammonium bicarbonate in 50% acetonitrile for 15min, thirdly in an acetonitrile solution for 5min, and finally dried in a SpeedVac for 20min on medium setting. The gel pieces were digested overnight at 37°C in a solution of trypsin 0.025µg/µL diluted in 25mM ammonium bicarbonate. Finally, peptides contained in the supernatant following trypsin digestion were retained and an additional extraction step was performed on residual gel pieces by incubating them twice in a solution of 50% acetonitrile for 15min. Supernatant was collected each time. Supernatants were combined and dried in a SpeedVac, and peptides were rehydrated in 10µL of 0.1% trifluoroacetic acid. Analysis of the peptides by LC-MS was

performed by the Proteomics laboratory in the University of York. Peptides were matched to protein sequences using the MASCOT database.

2.6. Immunoprecipitation

2.6.1. Cell lysis

Cells initially transfected with the pFLAG-CMV2-WSB1 plasmid (procedure described in section 2.3.2), were gently washed twice with 1X PBS after 24h of incubation and the cell monolayer was then collected in 1mL of 1X PBS using a cell scraper. Cell suspensions were spun and supernatant was removed. Cell pellets were resuspended in 500 μ L of lysis buffer (150mM NaCl; 20mM HEPES pH 7.5; 0.5mM EDTA; 0.5% NP-40; proteases and phosphatases inhibitors (cOmplete and PhosSTOP tablets, Roche, UK)). After rotating 20min at 30rpm at 4°C to allow complete cell lysis, cell lysates were clarified by centrifugation (20,000 $\times g$, 20min, 4°C) and supernatants were collected in fresh microcentrifuge tubes. At this stage, 40 μ L of the clarified lysates were collected for each sample in separate tubes and saved as input samples for later analysis.

2.6.2. Immunoprecipitation using Flag beads

50 μ L of Anti-Flag M2 Affinity Gel beads (Sigma-Aldrich, UK) were thoroughly washed three times by resuspending them in lysis buffer (described in section 2.6.1) and centrifugating at 2,000 $\times g$ for 2min at 4°C. Beads were then resuspended in clarified lysates, as per section 2.6.1, and incubated rotating at 30rpm at 4°C for 2h. Beads were spun (2,000 $\times g$, 2min, 4°C) to discard supernatant, and washed in lysis buffer. Finally, beads were resuspended in 1X Laemmli (2X Laemmli (65.8 mM Tris-HCl; pH 6.8; 2.1% SDS; 26.3% (w/v) glycerol; 0.01% bromophenol blue, BioRad) diluted in lysis buffer) and heated to 100°C for 5min. Input samples were also diluted in 2X Laemmli buffer and heated alongside. Samples were kept at -20°C until analysis, following protocol described in section 2.4.3.

2.6.3. Immunoprecipitation using protein A/G magnetic beads

Cell lysates obtained in section 2.6.1 were combined with 10µg of antibody and incubated 2h at room temperature, rotating. 25µL of Pierce Protein A/G Magnetic Beads (Thermo Scientific) were washed twice in wash buffer (TBS 1X; 0.05% Tween 20; 0.5M NaCl) and recovered using a magnet in order to collect beads and remove supernatant. Lysates combined with antibody were then incubated with the beads for 1h at room temperature, rotating. Beads were then collected using a magnet and the supernatant was discarded. Beads were washed three times by resuspending in 500µL of wash buffer, collection of beads with a magnet and removal of supernatant. A last wash using MilliQ water was performed and beads were finally resuspended in 50µL 1X Laemmli, heated to 100°C for 5min and stored at -20°C until analysis.

2.6.3.1. Elution step following immunoprecipitation with protein A/G magnetic beads

Alternatively, beads were resuspended in 100µL of low pH elution solution (0.1M Glycine pH 2) after a wash in 500µL of MilliQ water, and incubated for 10min at room temperature, rotating. Beads were then separated using a magnet and supernatant was collected and neutralised by adding 15µL of neutralising solution (1M Tris pH 7.5-9) per 100µL. Samples supplemented with an equal volume of Laemmli 2X were heated for 5min at 100°C and stored as described above.

2.7. Zymography

2.7.1. Conditioned media preparation

Just prior to the beginning of the incubation, the cell monolayer was washed in PBS and dishes were replenished with 2mL of serum-free media. Conditioned media was collected and spun 5min at 4°C, 2000rpm to discard debris and floating cells. Cleared conditioned media was concentrated down to approximately 50µL using Vivaspin 4 ultrafiltration spin column with molecular weight cut-off of 10kDa (Sartorius, Germany) by spinning 1.5h at 4°C, 4000rpm. Concentrated media was then collected in a fresh tube and frozen at -20°C.

Before running, samples were thawed on ice. 4X sample buffer (250mM Tris pH 6.8; 8% SDS; 40% Glycerol; 0.04% bromophenol blue) was added to an aliquot of concentrated conditioned media, as well as the control samples used. Control samples were: trypsin (diluted 1:20 in serum-free DMEM), 5% FBS DMEM (diluted 1:2 in sterile water), and serum-free DMEM only. All the samples were then heated at 100°C for 5min before use.

2.7.2. Gelatine SDS-PAGE

Concentrated conditioned media samples were run in a freshly casted 10% acrylamide, 1mg/mL gelatine gel at 120V for 1.5h. Composition of the gel is detailed in Table 2.6.

Table 2.6: Composition of 10% acrylamide gelatine gels for zymography (volumes given for casting two 1mm-thick minigels)

Reagent	Separation gel	Concentration gel
MilliQ water	-	6.1mL
2.65mg/mL gelatine	8.3mL	-
1.5M Tris pH 8.8	5.25mL	-
0.5M Tris pH 6.8	-	2.5mL
Acrylamide	7mL	1.3mL
10% SDS	165µL	100µL
50% glycerol	165µL	-
10% APS	100µL	100µL
TEMED	10µL	20µL

2.7.3. Renaturing and developing

Gelatine gel was briefly washed in MilliQ water and incubated 1h in 1X renaturing buffer (Novex, Life Technologies, Thermo Fisher Scientific), renewing the buffer 3 times. Gel was rinsed in MilliQ water and left in 1X developing buffer (Novex) at 37°C overnight. After a brief wash in MilliQ water, gel was stained 1h in Coomassie blue solution (45% methanol; 1% acetic acid; 0.125% Coomassie Brilliant Blue R-250 (ThermoFisher)). Destain was

achieved by incubating gel 15 to 30min in solution I (25% methanol; 10% acetic acid) and 1h in solution II (5% methanol; 7.5% acetic acid). Gel was stored in MilliQ water until imaging using the ChemiDoc XRS+ System and the Image Lab software (BioRad).

2.8. Transcript analysis

2.8.1. Total RNA extraction

At the appropriate time points, media was removed from cell monolayers and gently washed with 1X PBS at room temperature. Cells were lysed directly in the dish, using the lysis solution provided with the total RNA extraction kit (Aurum total RNA mini kit, BioRad), according to manufacturer's instructions. Briefly, lysates were collected in lysis buffer and thoroughly resuspended in equal amount of 70% ethanol. Cell lysates were transferred into a RNA binding column and washed several times in a succession of solutions and centrifugated. The membrane of the column was incubated 15min in 1X DNase I solution. Finally, the column was transferred into a fresh microcentrifuge tube and total RNA was eluted in a small volume of elution solution.

RNA samples were stored at -20°C until quantification using a Nanodrop Light (Thermo Scientific) and then placed at -80°C for long-term storage.

2.8.2. cDNA synthesis

mRNA from total RNA extracts were reverse transcribed into cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific), according to manufacturer's instructions. Briefly, a master mix containing 1mM dNTP, 10µM oligo d(T), 1U/µL RNase inhibitor, 10U/µL reverse transcriptase and 1X reaction buffer was prepared in DEPC water and distributed in small PCR tubes each containing 1µg of template RNA. A non-template control was also prepared in parallel, substituting the RNA template for the equivalent amount of DEPC water. Tubes were then incubated at 42°C for 1h and 70°C for 5min and kept at -20°C.

2.8.3. Quantitative PCR

Real time quantitative PCR (qPCR) analyses were performed using SYBR Green technology with QuantiFAST SYBR Green and QuantiTECT primer assays (Qiagen, NL) or in-house designed primers (synthesised by Sigma-Aldrich) in a StepOnePlus Real Time PCR machine (Applied Biosystems, Life Technologies). For a list of the primers used, see Table 2.7. In-house designed primers were used at a final concentration of 0.1 μ M.

The qPCR reaction was setup as follows: a master mix was prepared with SYBR Green (1:2, v:v) and primer assay (1:10, v:v). The cDNA samples were first diluted 1:10 in DEPC water and added into a 96-well plate to a concentration of 1:10 of the final volume (v:v). The final reaction volume in each well was 20 μ L, all samples were done in triplicates and each primer assay included NTC wells with cDNA substituted for water. The cycling protocol used is shown in Table 2.8.

2.8.4. Absolute quantitative PCR

A standard curve was prepared containing known number of WSB-1 copies. The pFLAG-CMV2 plasmid containing the *WSB1* gene (see section 2.3.2) was used as a template. 1 μ g of the plasmid was first linearised using the fast digest restriction enzyme Nde I (ThermoFisher Scientific, UK) as per manufacturer instructions. After a 5min digestion step at 37°C, the enzyme was heat inactivated 5min at 65°C. The digested product was then run in a 1% agarose gel (Fisher, Thermo Scientific) dissolved in 1X TAE (40mM Tris; 20mM acetic acid; 1mM EDTA). DNA detection was allowed by staining the agarose gel with GelRed (Biotium, USA). The gel part containing the band corresponding to the cut plasmid was excised from the gel and DNA was purified from the agarose using the GeneJET gel extraction kit (Thermo). Concentration of the recovered cut plasmid was measured using the Nanodrop and suitable dilutions were done in order to achieve tubes containing 300000, 30000, 3000, 300 and 30 copies of the plasmid. The standard samples were run alongside unknown samples on a qPCR plate using SYBR Green, as described in section 2.8.3 above.

Table 2.7: List of the primers used for SYBR Green qPCR

Target	Amplicon length	Sequences (5' → 3') or catalogue number	Supplier
<i>ARNT</i> (HIF-2 α)	134bp	F: TGTCATCCTGAAGACCA R: AAGGAGCTGGTTCTCATCCA	Sigma-Aldrich
<i>B2M</i>	98bp	QT00088935	Qiagen
<i>CA9</i>	160bp	F: GGAAGGCTCAGAGACTCA R: CTTAGCACTCAGCATCAC	Sigma-Aldrich
<i>EPAS1</i> (HIF-1 β)	84bp	F: GTGCTCCACGGCCTGTA R: TTGTCACACCTATGGCATATCACA	Sigma-Aldrich
<i>HIF1A</i>	88bp	F: GTACCCTAACTAGCCGAGGAAGAA R: GTGAATGTGGCCTGTGCAGT	Sigma-Aldrich
<i>HK2</i>	55bp	F: TGCCACCAGACTAAACTAGACG R: TGAATCCCTTGGTCCATGAGA	Sigma-Aldrich
<i>LOX</i>	174bp	F: GTTCCAAGCTGGCTACTC R: GGGTTGTCGTCAGAGTAC	Sigma-Aldrich
<i>MMP1</i>	103bp	QT00014581	Qiagen
<i>MMP7</i>	109bp	QT00001456	Qiagen
<i>MMP14</i>	144bp	F: CCCGAAGCCTGGCTACA R: GCATCAGCTTTGCCTGTTACT	Sigma-Aldrich
<i>SCL2A1</i> (GLUT1)	77bp	QT00068957	Qiagen
<i>SNAIL</i> (Snail)	69bp	F: GACCACTATGCCGCGCTCTT R: TCGCTGTAGTTAGGCTTCCGATT	Sigma-Aldrich
<i>VEGFA</i> (VEGF)	121bp	F: CTACCTCCACCATGCCAAGT R: CTCGATTGGATGGCAGTAGC	Sigma-Aldrich
<i>WSB1</i> (total)	107bp	QT00064127	Qiagen
<i>WSB1</i> (isoform 3)	120bp	QT01018248	Qiagen

Table 2.8: Cycling protocol used for SYBR Green qPCR

Initial denaturation	Denaturation	Annealing/Extension	Melt curve		
95°C	95°C	60°C	95°C	60°C	95°C
5min	10sec	3sec	15sec	1min	15sec
1x	-----Repeat 35x-----		+0.3°C per step		

2.9. Patient cDNA microarray analysis using TaqMan qPCR

TissueScan plates (panels I, II and IV) containing 2-5ng dried human breast first strand cDNA samples were purchased from OriGene Technologies, Inc. (USA). The qPCR analysis of these plates was performed using the TaqMan technology. A master mix containing SsoAdvanced Universal Probes Supermix (BioRad) (1:2, v:v), TaqMan probes for target and reference genes (see Table 2.9) (1:20 each, v:v) and DEPC water to total a final reaction volume of 30µL per well.

Table 2.9: TaqMan probe assays used for patient cDNA microarray analysis

Target	Supplier	Reference	Dye	Amplicon length
B2M	Applied Biosystem	4326319E	VIC	75bp
CA9	BioRad	qHsaCIP0031395	FAM	124bp
WSB1	BioRad	qHsaCEP0050519	FAM	74bp

A sufficient volume of master mix was pipetted in the first row of the plate (empty) and then distributed in the wells containing cDNA using a multichannel pipette. Once sealed, the plate was gently agitated using a vortex and left on ice for 15min, to allow the lyophilised cDNA to resuspend in the master mix. The plate was then placed in the StepOnePlus Real Time PCR machine and the cycling protocol used is described in Table 2.10.

Table 2.10: Cycling protocol used for patient cDNA microarray analysis

Initial denaturation	Denaturation	Annealing/Extension
95°C	95°C	60°C
30sec	10sec	60sec
1x	-----Repeat 40x-----	

2.10. RNA-Sequencing

mRNA samples obtained using the Aurum total RNA mini kit (BioRad) were quantified following the procedure described in section 2.8.1 and 4µg of each mRNA were transferred into fresh 1.5mL microcentrifuge tubes, kept on ice. One tenth of the volume of mRNA of 3M sodium acetate pH: 5.2 was added, as well as 3 times the volume of mRNA of absolute ethanol. Samples thus prepared were then packaged in dry ice and sent to LC Sciences (Houston, USA) for sequencing.

2.11. Wound-healing 2D migration assay

Cells were seeded in 35mm dishes and grown to near confluency (95-100%). Five to six parallel wounds were realised by scratching the cell monolayer with a 20µL pipette tip. Wells were thoroughly rinsed twice with PBS to get rid of all detached and loosely attached cells. Wells were replenished with fresh media containing 0.5% FBS. The low serum concentration is chosen to rule out any cell proliferation effects on the wound-healing assay. Images of the wounds were taken both immediately after scratching and at the end on the experiment, 16h later. The width of each wound was measured at the same position on 0h and 16h images. Wound closure was then calculated and the average wound closure for the control condition was normalised to 100%.

2.12. Transwell invasion assay

Invasion chambers containing growth factor reduced Matrigel (BD BioCoat GFR Matrigel Invasion Chamber, BD Bioscience, UK) were incubated in 500µL of serum-free media for 2h in CO₂ incubator in order to rehydrate the Matrigel. Wells of 24-well plates were filled with 750µL of complete media as described in section 2.1 and Matrigel inserts, as well as control inserts not containing Matrigel were carefully placed in the wells, avoiding bubbles. A cell suspension of 25000 cells in 0.1% BSA-containing DMEM per insert was prepared and placed on top of inserts. Plates were then incubated for 18h at the desired conditions. At the end of

the experiment, inserts were wiped from media and Matrigel with a cotton bud, rinsed in PBS and fixed in methanol for 15min. Inserts were then rinsed in PBS and Following a quick rinse in PBS, bottom of the inserts were cut using a sharp razor blade and mounted on glass slides with DAPI-stained mounting media (ProLong, Life Technologies). Slides were sealed and kept in the dark at 4°C until imaging.

2.12.1. Scoring of the Transwell invasion slides

Slides were imaged using an inverted microscope equipped with a DAPI filter (Axio Vert, Zeiss, UK). Using the 5X objective, pictures of three non-overlapping fields were taken for each condition. Cells were then counted using the cell counter script built in the ImageJ software.

2.13. Kaplan-Meier plots

Kaplan-Meier plots were obtained using the online tool KMplot (Györfy et al., 2010). Of the five Affymetrix probes specific for WSB-1 available, 201296_s_at, 210561_s_at recognised all the three WSB-1 isoforms, 201294_s_at, 201295_s_at matched WSB-1 isoform 3 only, and 213406_at could not be matched to any WSB-1 transcript sequences. Desired criteria regarding patient data were then selected and Kaplan-Meier plots drawn. Patients were split by the median to determine "high" and "low" expression.

2.14. *In silico* analysis of RNA-Sequencing and mass spectrometry data

2.14.1. RNA-Sequencing results

List of all the transcripts detected in the samples were presented in an Excel spreadsheet. Each transcript was associated with the fold change value between two given samples expressed as $\log_2(\text{fold change})$ and the *p*-value. Comparisons between every of the samples analysed were available. The protocol followed to obtain the samples was described in section 2.10.

2.14.2. Heat map generation

The software GeneE (Broad Institute, MIT, Cambridge, USA) was used to produce the heatmap. Transcript names along with $\log_2(\text{fold change})$ values were entered in the software database. Colour gradient was automatically allocated by the software according to the range of the values presented.

2.14.3. Pathway enrichment analysis

The software IPA (Qiagen) was used to perform pathway enrichment analysis. Information on the most represented pathways, biological processes and their up or downregulation. Gene or protein names, $\log_2(\text{fold change})$ or fold change, and p -values were uploaded to the software and data was then processed by IPA. Some items were not recognised by the IPA database and therefore removed from the list of "analysis-ready molecules".

2.14.4. Interactome analysis

Lists of relevant proteins were input on the online platform STRING (Szklarczyk et al., 2015). Interaction networks between these proteins were obtained based on experimental confirmation, co-occurrence in the literature and conservation of the interaction across species. Information on individual proteins and nature of the interaction were also available

2.14.5. Mass spectrometry results

Results of mass spectrometry were presented as an Excel spreadsheet containing the accession number of the molecule, its description and gene name, the value obtained for each of the six samples analysed, the average value for the samples in each condition (normoxia or hypoxia), the ratio normoxia/hypoxia, the fold change (ranges from -100 for proteins only found in hypoxia, to +100 for proteins found only in normoxia), and the t -test p -values. Mass spectrometry results were then analysed using IPA (section 2.14.3), STRING (section 2.14.4), and the CRAPome repository (section 2.14.6). Protocol followed to prepare the samples used was described in section 2.5.

2.14.6. Filtering of the mass spectrometry data

Identification of potential contaminant in mass spectrometry data was performed using the CRAPome (contaminant repository for affinity purification) repository (Mellacheruvu et al., 2013). Software returned amount of experiments in which the proteins yield by the LC-MS analysis were found in the 411 mass spectrometry analyses present in the database. An arbitrary threshold of 12% was set to filter potential contaminants from the dataset.

2.15. Statistical analysis

Every experiment was replicated at least three times unless otherwise stated. Inter- or intra-experimental repeats were pooled as described in following results chapters and any significance in observable differences was assessed with suitable statistical test: Student *t*-test to compare two groups, ANOVA to compare more than two groups with one-another. The standard deviations (SD) or standard error means (SEM) were calculated and reported on the graphs as error bars, as described in figure legends. If datasets were proven not to follow a Normal distribution, which was determined by Shapiro-Wilk test, non-parametric tests were used as follow: Mann-Whitney to compare two groups, and Kruskal-Wallis to compare more than two groups between one-another. The software Prism (GraphPad, USA) was used for all statistical analyses, unless stated otherwise.

Chapter 3

Hypoxic regulation of WSB-1 expression in breast cancer

3. Hypoxic regulation of WSB-1 expression in breast cancer

3.1. Introduction

WSB-1 was first described in the chicken by Vasiliauskas and colleagues (Vasiliauskas et al., 1999). Several studies have since been published on WSB-1 but only a handful focused on the role of WSB-1 in the context of cancer. In neuroblastoma, Chen and colleagues demonstrated that increased WSB-1 expression was associated with increased patient survival (Chen et al., 2006). Later, Shichrur and colleagues found that artificial downregulation of WSB-1 isoform 3 resulted in an increase of apoptosis and a decrease in chemoresistance, suggesting that high levels of WSB-1 could in fact have detrimental effect on patients' survival (Shichrur et al., 2014). Archange and colleagues also investigated the implication of WSB-1 isoforms in pancreatic cancer (Archange et al., 2008). Contrary to the results of Shichrur in neuroblastoma, Archange and colleagues found that WSB-1 isoform 3 inhibited pancreatic cancer cells growth whereas WSB-1 isoforms 1 and 2 stimulated cancer cells proliferation. Additionally, the shift in alternative splicing of WSB-1 in favour of the isoform 3 instead of isoforms 1 and 2 observed in condition of stress in pancreatic cancer cells resulted in increased resistance to apoptosis. In hepatocellular cancer, siRNA knockdown of WSB-1 resulted in an increased sensitivity to the apoptosis inducing drug etoposide in hypoxia, suggesting WSB-1 is involved in hypoxia-induced chemoresistance (Tong et al., 2013). In osteosarcoma, WSB-1 was associated with progression and metastasis (Cao et al., 2015). Based on the immunohistochemistry staining of human osteosarcoma samples, WSB-1 levels were always higher in tumours with metastases than those without. For this reason, Cao and colleagues suggested WSB-1 levels could be of prognostic value for metastatic-free survival. This observation was further confirmed by a wider study by Kim and colleagues, whereby higher WSB-1 levels were observed in metastatic tissues for most of the cancer types analysed (Kim et al., 2015). The results obtained by these studies on the implication of WSB-1 in cancer

progression are conflicting and the role of WSB-1 remains unclear. Moreover, no study has concentrated on the role of WSB-1 in breast cancer.

Breast cancer is considered to generate some of the most hypoxic tumours (Leong et al., 2006; McKeown, 2014). Interestingly, previous works showed that WSB-1 expression was upregulated in hypoxia, and could in fact be able to mediate the hypoxic response by direct feedback or through the degradation of the main regulator of HIF- α , pVHL (Benita et al., 2009; Tong et al., 2013; Cao et al., 2015; Kim et al., 2015).

This chapter will aim to answer the following questions:

- Is there a correlation between *WSB1* transcript level and breast tumour's subtype or stage?
- Can the tumour level of *WSB1* transcript be of interest to predict patient survival?
- Does basal *WSB1* transcript level correlates with a cell line's origin?
- How is WSB-1 expression affected by hypoxia in breast cancer cells *in vitro*?
- What is the relationship between WSB-1 and the HIF signalling pathway?
- How does WSB-1 knockdown affect the transcriptomic profile of breast cancer cells?

3.2. Experimental design

3.2.1. Analysis of WSB-1 transcript levels in patient cDNA samples

In order to investigate the existence of a correlation between WSB-1 expression and tumour progression, cDNA microarrays from breast tumour tissues of patients, covering a wide range of progression stages and characteristics, were analysed by qPCR, alongside healthy control tissue samples. This approach has been successfully used in the past by other research groups to investigate the implication of LEDGE/p75 (lens epithelium derived growth factor) or the FAM83 protein family in general cancer progression using an array of human cancers tissues (Basu et al., 2012; Cipriano et al., 2014). A similar approach was also used by van 't Veer and colleagues in order to determine a signature that would predict breast cancer prognosis

(van 't Veer et al., 2002). Transcript levels of *WSB1* and *CA9* (carbonic anhydrase IX) were monitored by real time relative qPCR analysis. Three commercially available 96-well microarray plates (OriGene) coated with patient cDNA samples corresponding to 144 human breast samples were analysed. The full panel consisted of sixteen normal tissue samples and 128 tumour tissue samples spanning all cancer development stages and presenting various profiles. The percentage of normal tissue, tumour tissue, hypercellular and hypo/acellular stroma as well as necrotic tissue within the tumour tissue sample was assessed through histopathology by OriGene. Information on the expression status of the oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) expression, determined by immunohistochemistry staining was also provided. The complete clinical information of the three TissueScan plates used (panel I, II and IV) is available in Table B1 (appendix disc) and a summary can be found in Table 3.1.

The TissueScan plates were analysed using TaqMan qPCR technology for it allows monitoring of the relative expression level of a target gene, as well as that of the reference gene in a single reaction. β -2 microglobulin (*B2M*) was chosen as a reference gene for its **reliability** (it has been previously established in the lab that *B2M* expression was not affected by exposure to hypoxia) and its **consistency** (*B2M* was the reference gene used throughout the work presented in this thesis). In addition, expression of *CA9*, a well-defined hypoxia-responsive gene, was chosen to assess the level of hypoxia in each patient cDNA sample.

3.2.2. Evaluation of the impact of WSB-1 expression level on patient survival

In 1958, Kaplan and Meier designed a specific process to plot nonparametric estimations from incomplete observations (Kaplan and Meier, 1958). These so-called KM (Kaplan-Meier) plots were of a particular interest in cancer research to illustrate data such as patient survival probabilities or response to a drug treatment (Goel et al., 2010; Rich et al., 2010). This type of incomplete data is frequent when analysing survival rates of patients across long periods of time, and KM plot enables statistical analysis of the entire population sampled as a whole,

Table 3.1: Summary of the clinical features of the human breast samples in the TissueScan arrays analysed in this study

Patients characteristics	Number of patients (n=128)
Age $\pm$ standard deviation (years)	56.3 $\pm$ 13.1
Tumour stage	
I	23
IIA	36
IIB	22
IIIA	23
IIIB	6
IIIC	13
IV	5
Tumour subtypes	
Luminal A	50
Luminal B	12
HER2 type	7
TNBC	25
Undefined	50
Hormone receptor expression	
ER+	57
ER-	45
PR+	55
PR-	40
HER2+	20
HER2-	70
ER: oestrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor, TNBC: triple negative breast cancer, +: positive, -: negative.	

instead of considering each patient individually. The effect of differential *WSB1* expression levels on patients' survival probabilities, including distant metastasis-free survival (DMFS), disease-free survival (DFS), and overall survival (OS) will be evaluated.

To evaluate the implication of *WSB-1* levels on survival, the KMplotter online tool was used (Györfy et al., 2010). This tool compiles gene expression data and survival information of 4,142 breast cancer patients from several curated databases with a mean follow-up of 69 months. The database allows the analysis of 22,277 genes using Affymetrix probes. Five Affymetrix probes were registered for *WSB-1*. The probes 201296_s_at and 210561_s_at were

recognising all three WSB-1 isoforms whereas the probes 201294_s_at and 201295_s_at were specific for isoform 3. The fifth probe 213406_at could not be matched to any of the WSB-1 sequences and was therefore discarded for further analyses.

3.2.3. Comparison of WSB-1 copy number in different breast cell lines

The basal level of *WSB1* transcript in various breast cancer cell lines was evaluated to complement the patient cDNA microarray analysis.

As the endogenous expression level of *B2M* varies in each cell line, relative quantitation methods, which rely on the comparison between transcript levels a gene of interest to those of *B2M*, would not be suitable for comparing the levels of *WSB1* transcript between various cell lines. Therefore, absolute qPCR was employed, which measures the intensity of the signal created by *WSB1* transcripts in each cell lines and deducts absolute *WSB1* transcript copy number using a standard curve of known copy numbers.

Cell pellets of the following breast cancer cell lines were harvested from exponentially growing populations from the following cell lines: BT474, DU4475, MCF7, MDA-MB-231, MDA-MB-468, and T47D. Total mRNAs were extracted in triplicates for each cell line and absolute qPCR was performed. In addition, samples from the primary non-cancer breast cell line HMEpC and the immortalised non-cancer embryonic kidney cell line HEK293T were also analysed to serve as benchmarks for non-cancer WSB-1 copy number. A summary of the characteristics of each cell line is available in Chapter 2, Table 2.1.

3.2.4. Monitoring of WSB-1 levels in response to hypoxia exposure

The effect of hypoxia exposure on WSB-1 transcript and protein levels was assessed *in vitro* using the two breast cancer cell lines MCF7 and MDA-MB-231 as they are widely used models for breast cancer experimental work (Lacroix and Leclercq, 2004). In addition, each cell line possesses specific characteristics, which cover different profiles of breast tumour: MCF7 cells are part of the luminal A subtype, whereas MDA-MB-231 belong the basal subtype also known

as triple negative (Holliday and Speirs, 2011). This is an important feature as outcome and therapeutic approaches differ with each subtype. Full information on each cell line is presented in Chapter 2, Table 2.1.

In order to mimic the oxygen level found in breast tumours, cells were exposed to various oxygen tensions using a hypoxia chamber (H35 Hypoxystation, Don Whitley, UK) with controllable parameters for various lengths of time. Western blotting was then used to assess alterations in WSB-1 protein levels.

3.2.5. Investigation of the relationship between WSB-1 and HIF signalling

As discussed in Chapter 1, HIF-1 α and HIF-2 α share a large portion of their targets but present also some specific effects. In order to determine whether hypoxia-induced WSB-1 upregulation was specifically regulated by one isoform in particular, HIF-1 α , HIF-2 α and HIF-1 β were selectively knocked down by siRNA in MCF7 cells. *WSB1* transcript level was then monitored by qPCR.

Any potential effect of WSB-1 knockdown on the level and functionality of HIF was also investigated. Firstly, the effect of WSB-1 knockdown by siRNA on HIF-1 α protein level was assessed using Western blotting. Secondly, the transcript levels of several well-characterised HIF targets, such as *VEGFA* and *SLC2A1* were also analysed by qPCR.

3.2.6. Analysis of the effect of WSB-1 knockdown and hypoxia exposure on MDA-MB-231 cells transcriptome by RNA sequencing

In a recent study, Kim and colleagues argued that WSB-1 could regulate HIF and affect its activity and the HIF-mediated transcription (Kim et al., 2015). Therefore, the effect of WSB-1 knockdown and hypoxia exposure on the whole transcriptome was analysed by RNA sequencing (RNA-Seq). This sequencing technology enables the identification of all the transcripts present in a total mRNA sample and compares the expression levels between two conditions, giving information on the modification in the expression of each transcript (Wang

et al., 2009b). Here, it will be used to establish which pathways are affected by WSB-1 knockdown in hypoxia and quantify the effect of such condition on specific biological functions.

For this analysis, total mRNA was extracted from MDA-MB-231 cells following 2% O₂ exposure for 24h and/or WSB-1 knockdown and analysed by RNA-Seq. RNA-Seq analysis of the sample was performed by LC Sciences (Houston, USA). The list of all mRNA differentially expressed between every samples was used to highlight specific genes of interest, whose expression level was significantly altered by WSB-1 knockdown.

To help with the analysis, several tools were used. The IPA software (Qiagen) allows the representation of key pathways and regulatory networks found in the dataset. In addition, heat maps were generated to better visualise the differences in protein expression between samples.

3.3. Results

3.3.1. Evaluation of *WSB1* expression in a cohort of breast cancer patients and its relationship with known clinical and biological variables

Raw data from the analysis of patient cDNA microarrays was obtained by relative qPCR using TaqMan probes for *WSB1* and *CA9*. and analysed according to the $\Delta\Delta C_T$ method, as described by Livak and Schmittgen, and expressed as fold change of the average of normal samples (Livak and Schmittgen, 2001). Before performing any further analyses, outliers were identified and eliminated from the normal (non-cancer) and tumour datasets. Values were considered out of range if not included in the following interval:

$$[average - (3 \times standard\ deviation) ; average + (3 \times standard\ deviation)]$$

One tumour sample was identified as outlier in the WSB-1 transcript dataset, and another one in the CA9 transcript dataset. Each point has been eliminated from both datasets.

Normal distribution of the data was verified performing a Shapiro-Wilk test (Shapiro and

Wilk, 1965). Since only the non-cancer subset of the samples analysed with the WSB-1 primers was following a Normal distribution ($p = 0.834$), further statistical analyses were done using non-parametric tests. Note that in the Shapiro-Wilk test, p -values > 0.05 are indicative of a Normal distribution of the dataset.

In Figure 3.1, fold change values of *WSB1* and *CA9* relative expressions were plotted against one another. Normal tissue samples are represented in green and tumour tissue samples in red. No visible groups stood out from the scattering of the samples on the graph and higher *CA9* expression did not always correspond to samples from higher grades tumours. However, it is important to note that all normal samples displayed an overall lower *CA9* expression than the cancer samples, suggesting a less active hypoxic signalling. *WSB1* expression was less variable than that of *CA9*, showing only a maximum value of 3.84-fold increase compared to the average *WSB1* expression of the normal samples, whereas *CA9* expression values were observed to be as high as 800-fold the expression of *CA9* in the normal samples average. Only eight tumour samples displayed a higher *WSB1* expression than the highest value recorded for the normal samples (Figure 3.1, right of the vertical dotted line). Interestingly, all eight samples could be qualified as higher-grade, as they were issued from tumour tissue samples from stage IIA and above (stage IIA: four samples, stage IIIA: one sample, stage IIIB: one sample, and stage IIIC: two samples).

WSB1 and *CA9* transcript levels were then analysed individually and datasets were divided into different subgroups. Initially, samples were divided into three categories: **normal** (non-cancer samples), **lower-grade** (stage I samples), and **higher-grade** (samples of stage IIA and above) (Figure 3.2). Although the average level of *WSB1* transcript in the higher-grade group ($\bar{x} = 0.902$) was higher than that of the lower-grade group ($\bar{x} = 0.669$), this difference was not significant ($p > 0.05$). However, *WSB1* level in the higher-grade group was significantly lower than in the normal tissue samples ($\bar{x} = 1.142$; $p < 0.05$). *CA9* transcript levels were significantly higher in the higher-grade group ($p < 0.001$; $\bar{x} = 57.342$) and the lower-grade

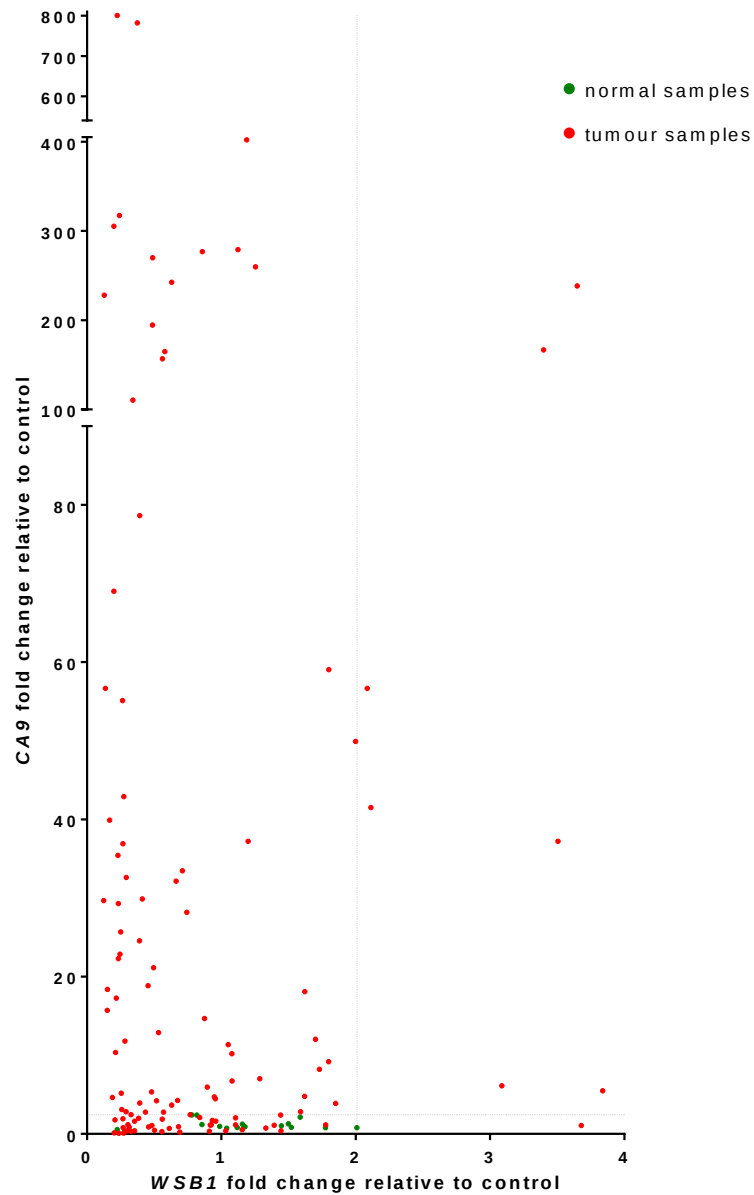


Figure 3.1: Relative *WSB1* expression versus relative *CA9* expression for normal and tumour breast tissue samples

cDNA from patient tumour samples coated on microarray plates were analysed by qPCR using TaqMan reagents. Relative expression of *WSB1* was plotted against relative expression of *CA9* for normal samples in green ($n=16$), and tumour samples in red ($n=121$). *CA9* expression could not be detected in 5 tumour samples, which are therefore not represented. *B2M* was used as the reference gene. Dotted lines represent the highest values for normal samples.

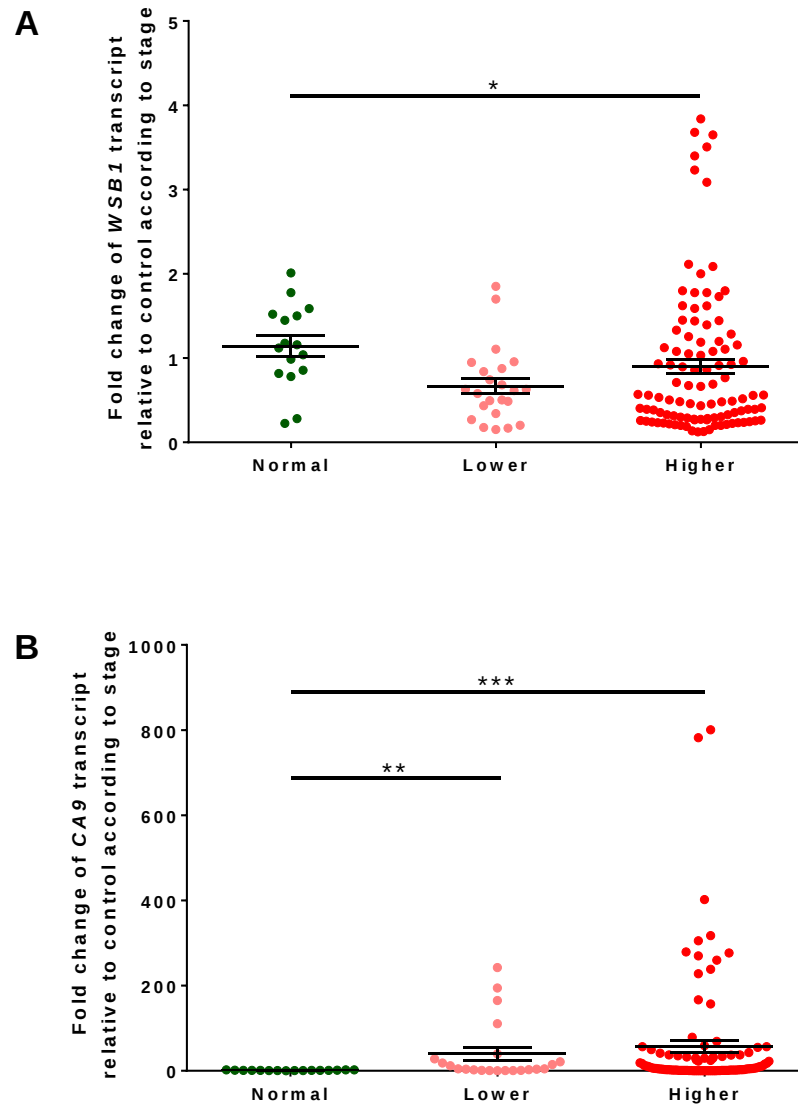


Figure 3.2: Relative level of *WSB1* and *CA9* transcripts in patient tissue samples according to the tumour grade

Transcript levels of *WSB1* (A) and *CA9* (B) of cDNA from patient tumour samples were analysed by qPCR using TaqMan reagents. Green: normal samples ($n=16$); pink: lower-grade samples (stage I) ($n=23$); red: higher-grade samples (stage IIA and above) ($n=102$). Statistical significance was determined by Kruskal-Wallis test for non-parametric data. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Error bars represent mean $\pm$ SEM.

group ($p < 0.01$; $\bar{x} = 39.65$) than in the normal tissue samples ($\bar{x} = 1.145$).

Breast cancer development and therapeutic strategy strongly depends on the expression of three receptors: oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Therefore, it was decided to verify whether *WSB1* expression could significantly vary with the expression of these receptors.

Firstly, the study focused on the oestrogen receptor. Figure 3.3 presents the relative transcript levels of *WSB1* and *CA9* in samples from normal tissue, from patients expressing the oestrogen receptor (**ER+**), and from patients not expressing it (**ER-**). *WSB1* transcript level was significantly lower in ER- samples compared to normal samples ($p < 0.01$) and to ER+ samples ($p < 0.05$). However, there was no statistical difference in *WSB1* transcript level between normal and ER+ samples.

Similarly, *CA9* transcript level was not altered in ER+ samples compared to normal samples. Additionally, ER- samples demonstrated a significantly greater *CA9* expression than both ER+ and normal samples ($p < 0.01$).

Secondly, any dependence of *WSB1* and *CA9* transcript levels to **PR expression** was investigated (Figure 3.4). *WSB1* transcript level was significantly down-regulated in PR- samples compared to normal samples ($p < 0.05$). Although a similar trend was observed for the PR+ samples, this difference was not significant. PR+ samples and PR- samples were expressing significantly more *CA9* than normal samples ($p < 0.001$ and $p < 0.05$, respectively). This could indicate that PR+ and PR- tumour samples were more hypoxic than the normal samples. The difference in *CA9* transcript level observable between PR+ and PR- samples, yet noticeable, was not statistically significant.

Finally, the expression of *WSB1* according to the **HER2 status** of the tumour samples was investigated. *WSB1* transcript levels were not significantly altered by HER2 expression, whereas the increased *CA9* transcript level in tumour samples indicates that HER2+ samples and HER2- samples were expressing significantly higher levels of *CA9* than normal samples

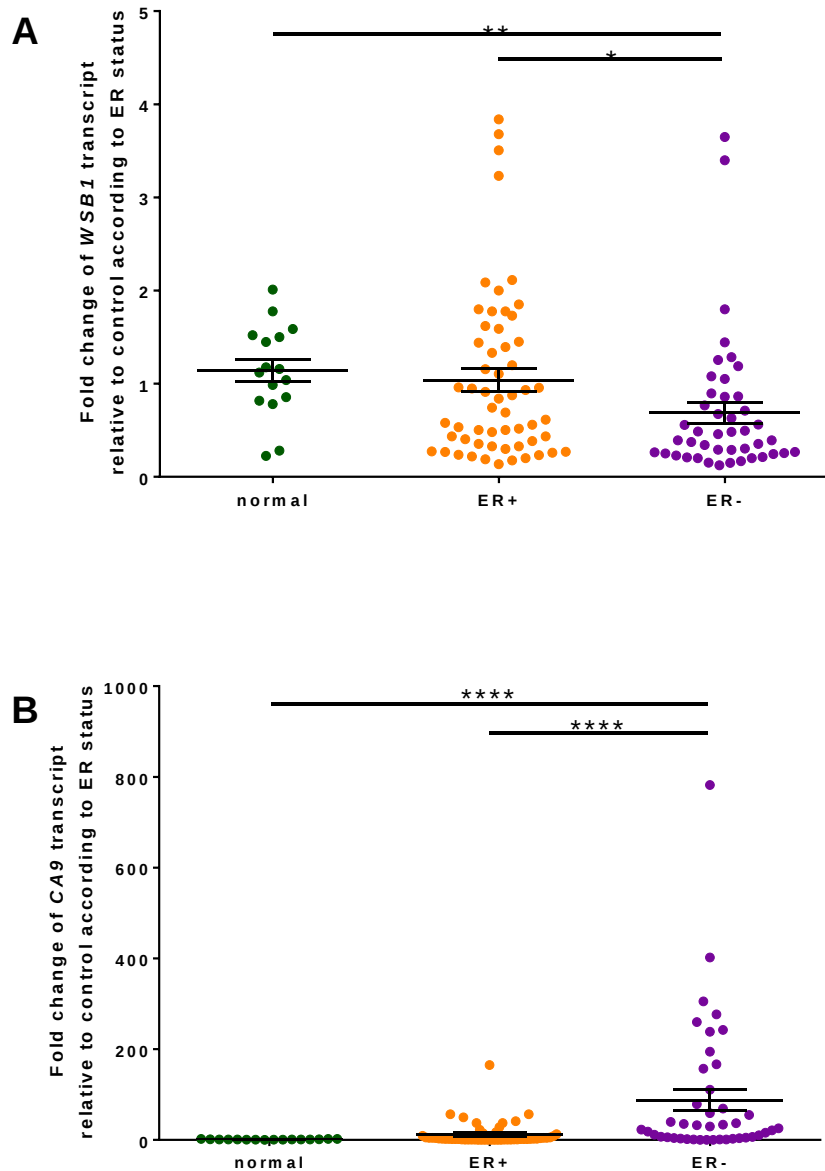


Figure 3.3: Relative level of *WSB1* and *CA9* transcripts in patient tissue samples according to oestrogen receptor (ER) expression

Transcript levels of *WSB1* (A) and *CA9* (B) of cDNA from patient tumour samples were analysed by qPCR using TaqMan reagents. Green: normal samples ($n=16$); orange: ER+ samples ($n=57$); purple: ER- samples ($n=44$). Error bars represent mean $\pm$ SEM. Statistical significance determined by Kruskal-Wallis test for non-parametric data. *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$.

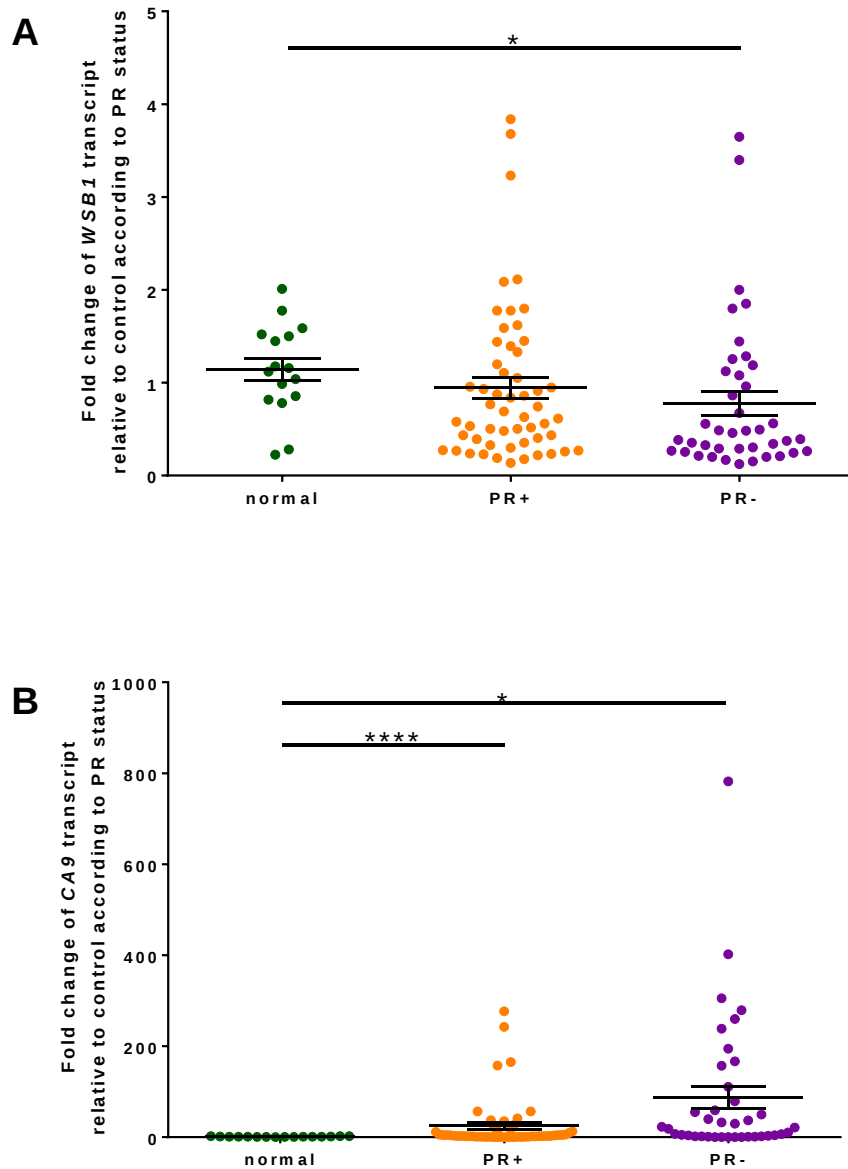


Figure 3.4: Relative level of *WSB1* and *CA9* transcripts in patient tissue samples according to progesterone receptor (PR) expression

Transcript levels of *WSB1* (A) and *CA9* (B) of cDNA from patient tumour samples were analysed by qPCR using TaqMan reagents. Green: normal samples ($n=16$); orange: PR+ samples ($n=55$); purple: PR- samples ($n=40$). Error bars represent mean $\pm$ SEM. Statistical significance determined by Kruskal-Wallis test for non-parametric data. *, $p < 0.05$; ****, $p < 0.0001$.

($p < 0.001$ and $p < 0.05$, respectively; Figure 3.5).

Breast tumours are conventionally organised in four main subtypes according to the expression status of ER, PR and HER2. This classification was discussed in Chapter 1 (Figure 1.1). Luminal A corresponds to HER2- tumours expressing ER, PR or both receptors. Conversely, luminal B corresponds to HER2+ tumours expressing ER, PR or both. Tumours expressing only HER2 are called HER2 type. Finally, tumours not expressing any of the receptors are called basal or triple negative (TNBC).

Relative *WSB1* and *CA9* transcript levels in the microarray samples were therefore analysed according to the **tumour subtype** (Figure 3.6). *WSB1* level varied for each of the subtypes but was consistently lower than that of the normal samples. TNBC and HER2 type displayed the lowest *WSB1* levels. *WSB1* expression levels in normal and TNBC samples were significantly different ($p < 0.05$). *CA9* transcript levels also varied between the subtypes, with *CA9* expression being significantly higher ($p < 0.0001$) in TNBC tumour samples than normal samples. Additionally, among the three subtypes luminal A, luminal B and HER2 type, luminal B tumour samples displayed the highest *WSB1* and *CA9* levels.

Finally, a Spearman correlation test for non-parametric datasets was used to evaluate the potential existence of a correlation between *WSB1* and *CA9* expression levels. This was performed for different subsets: normal (non-cancer) *versus* tumour datasets; lower (normal and stage I) *versus* higher (stage IIA and above) grades; according to ER, PR or HER2 status. None of these revealed a correlation between the expression levels of the two transcripts.

To summarise, this patient cDNA microarray analysis allowed the highlighting of a significant downregulation of *WSB1* transcript levels in ER-, PR- and TNBC tumours compared to normal samples. In addition, higher-grade tumours also demonstrated a lower *WSB1* transcript level than normal samples. However, there was no statistical correlation between *CA9* and *WSB1* transcript levels in any of the subgroups analysed.

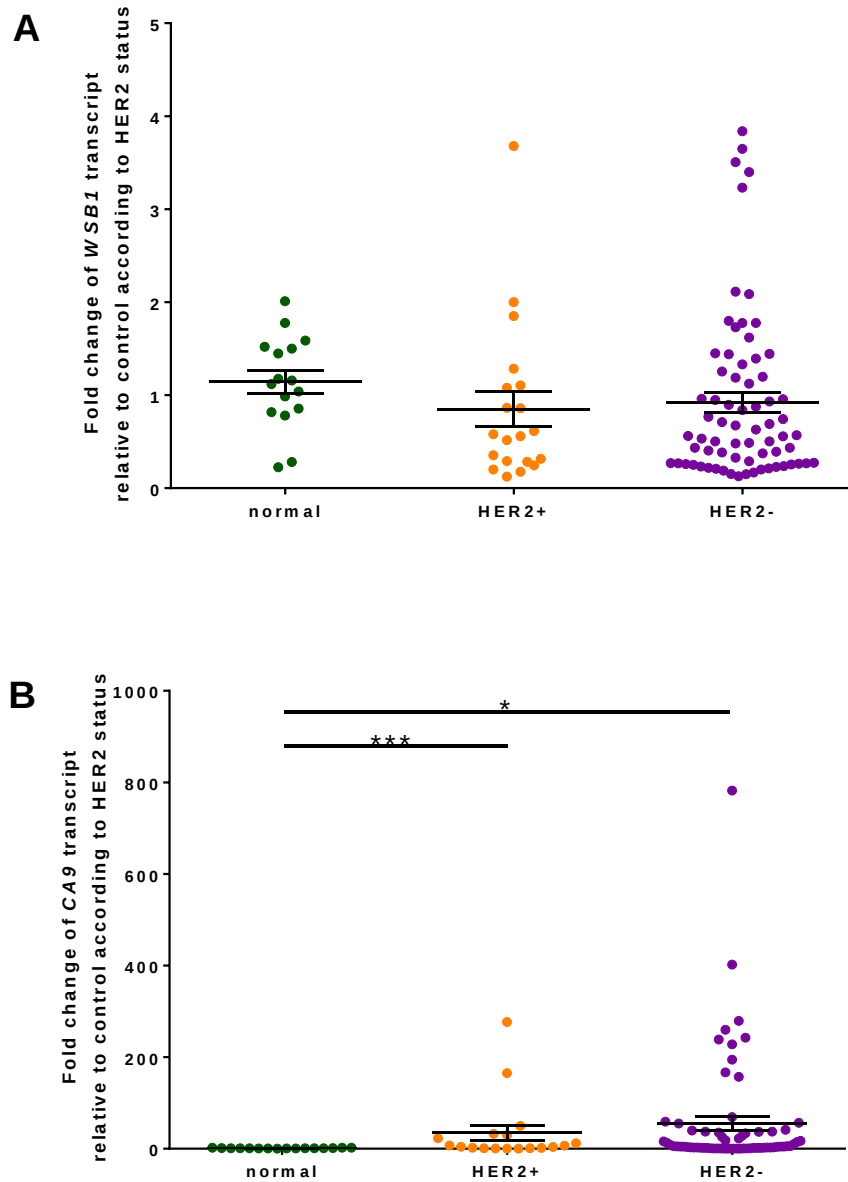


Figure 3.5: Relative level of *WSB1* and *CA9* transcripts in patient tissue samples according to human epidermal growth factor receptor (HER2) expression

Transcript levels of *WSB1* (A) and *CA9* (B) of cDNA from patient tumour samples were analysed by qPCR using TaqMan reagents. Green: normal samples ($n=16$); orange: HER2+ samples ($n=20$); purple: HER2- samples ($n=70$). Error bars represent mean $\pm$ SEM. Statistical significance determined by Kruskal-Wallis test for non-parametric data. *, $p<0.05$; ***, $p<0.001$.

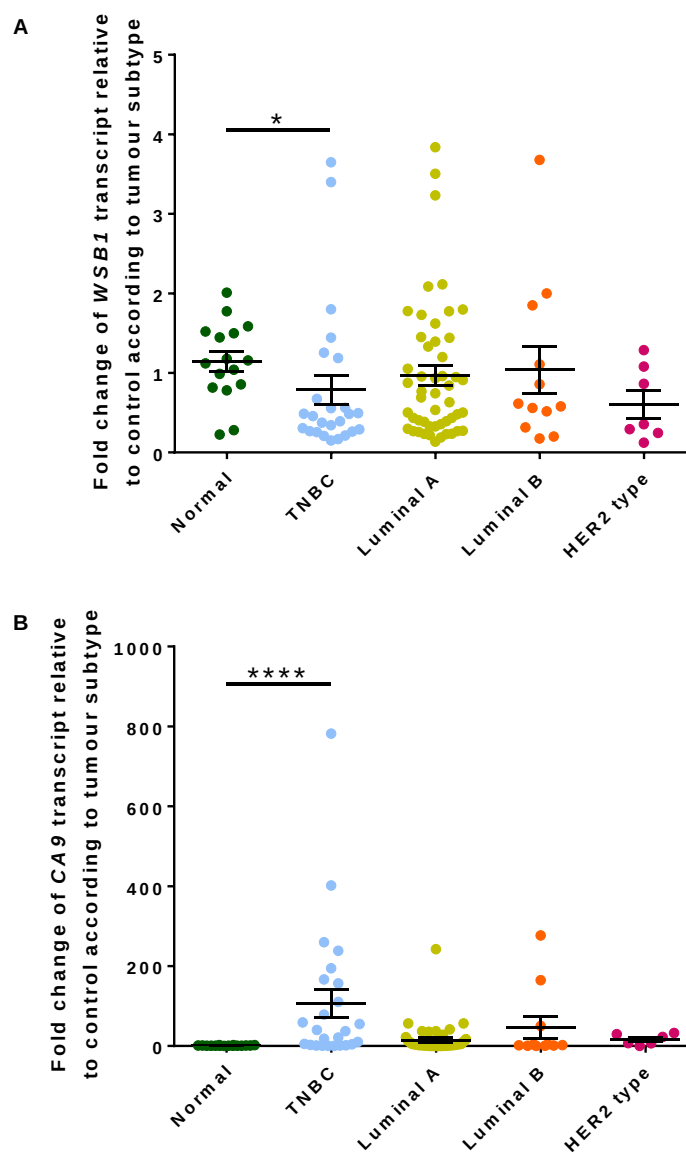


Figure 3.6: Relative level of *WSB1* and *CA9* transcripts in patient tissue samples according to the tumour subtype of the tissue of origin

Transcript levels of *WSB1* (A) and *CA9* (B) of cDNA from patient tumour samples were analysed by qPCR using TaqMan reagents. Green: normal samples ($n=16$); blue: TNBC samples ($n=25$); light green: luminal A ($n=50$); orange: luminal B samples ($n=12$); pink: HER2 type samples ($n=7$). Error bars represent mean $\pm$ SEM. Statistical significance determined by Kruskal-Wallis test for non-parametric data. *, $p < 0.05$; ****, $p < 0.0001$.

3.3.2. Effect of WSB-1 expression level on patient survival

Kaplan-Meier (KM) plots allow for the visualisation of the effect of the expression of a specific gene or factor on the survival or relapse risk of a patient (Kaplan and Meier, 1958). This representation also indicates the effect of high expression of the gene of interest on the patient's prognosis. When the hazard risk (HR) is greater than 1, it signifies that a high expression level of the target of interest is linked to a poor prognosis. Conversely, if $HR < 1$, a high expression level of the gene of interest is linked to a good prognosis (Spruance et al., 2004).

The effect and significance of the level of *WSB1* expression on distant metastasis-free survival (**DMFS**) in specific groups of patients are summarised in Table 3.2 to Table 3.4. The KM plot analyses were done using the KMplot tool (Györfy et al., 2010). This online platform indexes four Affymetrix probes for WSB-1. Two probes recognise all three WSB-1 isoforms and two others recognise only WSB-1 isoform 3. Table 3.2 displays the results obtained using the mean expression of the two Affymetrix probes matching all the three WSB-1 isoforms present in the database. Table 3.3 presents the results obtained using the mean expression of the two probes specific for WSB-1 isoform 3. Finally, Table 3.4 shows the results obtained using the mean expression of these four probes.

Independently of the combination of probes used, there is a significant difference in DMFS in patients harbouring ER- and PR- tumours (Table 3.2, Table 3.3, Table 3.4). Patients with HER2+ tumours also present a statistically significant difference in survival when considering the mean expression of the two probes specific for the three isoforms ($p = 0.041$; Table 3.2) or the two probes specific for the isoform 3 only ($p = 0.012$; Table 3.3). This is not the case when considering the four probes together ($p = 0.14$; Table 3.4). More specifically, patients from HER2 type tumours that express only HER2 and not ER and PR, also displayed a statistical difference in survival when using the two probes specific for the three WSB-1 isoforms ($p = 0.0061$; Table 3.2). This was not observed when using probes specific for the

Table 3.2: Summary of distant metastasis-free survival (DMFS) values from KM plots obtained using Affymetrix probes recognising all WSB-1 isoforms

	Luminal A	Luminal B	HER2 type	Basal/TNBC	ER+	ER-	PR+	PR-	HER2+	HER2-
p-value	0.3	0.96	0.0061	0.38	0.22	0.00095	0.8	0.00034	0.041	1
HR	1.17	0.99	2.54	0.79	0.8	2.4	1.16	4.24	5.68	1
n^{High}	459	181	55	109	289	85	61	47	33	41
n_{Low}	459	180	56	110	288	85	61	48	33	41

HR: hazard ratio; n^{High}: number of patients expressing high *WSB1*; n_{Low}: number of patients expressing low *WSB1*; green: high *WSB1* indicates good prognosis; red: high *WSB1* indicates poor prognosis; grey cells: significant *p*-value.

Table 3.3: Summary of DMFS values from KM plots obtained using Affymetrix probes specific for WSB-1 isoforms 3 only

	Luminal A	Luminal B	HER2 type	Basal/TNBC	ER+	ER-	PR+	PR-	HER2+	HER2-
p-value	0.69	0.83	0.12	0.52	0.6	0.00028	0.57	0.0041	0.012	0.49
HR	1.06	0.96	1.67	0.84	0.91	2.62	0.7	3.12	3.46	1.55
n^{High}	459	181	55	109	289	85	61	47	33	41
n_{Low}	459	180	56	110	288	85	61	48	33	41

HR: hazard ratio; n^{High}: number of patients expressing high *WSB1*; n_{Low}: number of patients expressing low *WSB1*; green: high *WSB1* indicates good prognosis; red: high *WSB1* indicates poor prognosis; grey cells: significant *p*-value.

Table 3.4: Summary of DMFS values from KM plots obtained using all Affymetrix probes matching WSB-1

	Luminal A	Luminal B	HER2 type	Basal/TNBC	ER+	ER-	PR+	PR-	HER2+	HER2-
<i>p</i>-value	0.38	0.98	0.052	0.5	0.73	0.0002	0.7	0.000039	0.14	1
HR	1.14	1.01	1.91	0.84	0.94	2.69	1.26	5.57	2.01	1
n^{High}	459	181	55	109	289	85	61	47	33	41
n_{Low}	459	180	56	110	288	85	61	48	33	41

HR: hazard ratio; n^{High}: number of patients expressing high *WSB1*; n_{Low}: number of patients expressing low *WSB1*; green: high *WSB1* indicates good prognosis; red: high *WSB1* indicates poor prognosis; grey cells: significant *p*-value.

Table 3.5: Summary of relapse-free survival (RFS) values from KM plots obtained using all Affymetrix probes matching WSB-1

	Luminal A	Luminal B	HER2 type	Basal/TNBC	ER+	ER-	PR+	PR-	HER2+	HER2-
<i>p</i>-value	0.0059	0.58	0.61	0.73	0.13	0.0013	0.53	0.16	0.000082	0.023
HR	0.78	0.94	1.12	0.96	0.87	1.5	1.12	1.25	2.99	0.74
n^{High}	882	501	104	290	900	335	263	241	84	378
n_{Low}	882	501	104	290	902	336	262	241	84	378

HR: hazard ratio; n^{High}: number of patients expressing high *WSB1*; n_{Low}: number of patients expressing low *WSB1*; green: high *WSB1* indicates good prognosis; red: high *WSB1* indicates poor prognosis; grey cells: significant *p*-value.

Table 3.6: Summary of overall survival (OS) values from KM plots obtained using all Affymetrix probes matching WSB-1

	Luminal A	Luminal B	HER2 type	Basal/ TNBC	ER+	ER-	PR+	PR-	HER2+	HER2-
<i>p</i>-value	0.55	0.74	0.11	0.88	0.29	0.35	n/a	n/a	0.99	0.52
HR	1.12	1.07	1.89	1.04	1.25	1.31	n/a	n/a	1	0.71
n^{High}	252	160	45	102	189	71	n/a	n/a	14	31
n^{Low}	252	160	44	102	188	71	n/a	n/a	14	31

HR: hazard ratio; n^{High}: number of patients expressing high *WSB1*; n^{Low}: number of patients expressing low *WSB1*; green: high *WSB1* indicates good prognosis; red: high *WSB1* indicates poor prognosis.

isoform 3 only ($p = 0.12$; Table 3.3). When analysing results for the four probes together, the difference in survival was not significant although very close to the threshold ($p = 0.052$; Table 3.4).

As mentioned previously, HR figures indicate the prognosis value of a high *WSB1* transcript level. To facilitate readings, values linked to a good prognosis ($HR < 1$) were written in green and values linked to poor prognosis ($HR > 1$) were written in red. To prevent incorrect interpretation, values comprised in the interval $0.95 < HR < 1.05$ were arbitrarily considered to be neutral and left in black.

In all the cases where a significant difference in survival between high and low *WSB1* expression level was seen (grey boxes), including HER2 type, high level of *WSB1* always corresponds to the worst survival outcome ($HR > 1$). Additionally, high *WSB1* expression levels were also seen to be linked to poor prognosis in patients with luminal A tumours. Conversely, high *WSB1* expression level was linked to good prognosis in TNBC and ER+ tumours. Finally, levels of WSB-1 transcript appeared to have no impact on survival in patients with luminal B tumours.

DMFS focuses on the probability of a patient to display distant metastases during the progression of the disease. This data can provide indication on the aggressiveness of a tumour, as more aggressive tumours are more likely to spread to distant organs or lymph nodes and form metastases. There are, however, other parameters available to analyse patient survival probabilities. Relapse-free survival (**RFS**) is presented in Table 3.5. This concentrates on the likelihood of a relapse and the original tumour to reappear post-treatment. The values obtained for RFS differ from those obtained for DMFS. Changes in *WSB1* level is associated with a significant difference in survival in luminal A patients, ER-, HER2+, and HER2- tumours ($p = 0.0059$; $p = 0.0013$; $p = 0.000082$; and $p = 0.023$, respectively). Interestingly, high *WSB1* expression level seems to be linked to poor RFS prognosis for HER2+ tumours ($HR > 1$), whereas it seems to be linked to a good RFS prognosis in HER2- tumours ($HR < 1$).

Patient presenting luminal A tumours expressing high level of *WSB1* also presented a better survival probability, as opposed to what was observed when considering DMFS. Outcome for patients presenting luminal B and TNBC tumours with high *WSB1* expression also differed when considering RFS rather than DMFS. High *WSB1* expression appeared to be linked to a good prognosis in luminal B tumours whereas it appeared to have no effect on survival of patients with TNBC tumours.

Finally, Table 3.6 displays overall survival (**OS**), which emphasises on general patient survival, without considering relapse or occurrence of metastasis. These values involved a smaller amount of patients, which rendered the analysis more difficult. As a result, none of the subgroup studied displayed significant differences in survival according to *WSB1* transcript level. In fact, PR+ and PR- tumours covered such a small population that no analysis could be performed. However, it appeared that high *WSB1* expression was mostly associated with poor prognosis, except for HER2- tumours where high *WSB1* expression actually linked to good prognosis.

The KM plots produced for ER+ and ER- tumours using the four Affymetrix probes are displayed in Figure 3.7, as an example. Black lines correspond to patients with low *WSB1* expression, and red lines correspond to patients with high *WSB1* expression. Whereas the two lines overlapped for the ER+ tumours, suggesting there was no difference in survival probability according to high and low *WSB1* expression, the red line rapidly dissociated from the black line for the ER- tumours, coherently with the highly significant *p*-value ($p = 0.0002$). The figures below the plots indicate the number of patients in each category.

As a conclusion, analysis of patient survival data indicated that high *WSB1* levels are associated with a poor prognosis for DMFS in patients presenting ER- or PR- tumours. Similarly, patients presenting ER- or HER2- tumours and high *WSB1* levels displayed a significantly worse RFS. However, high *WSB1* levels were associated with a good RFS prognosis in patients presenting luminal A or HER2- tumours.

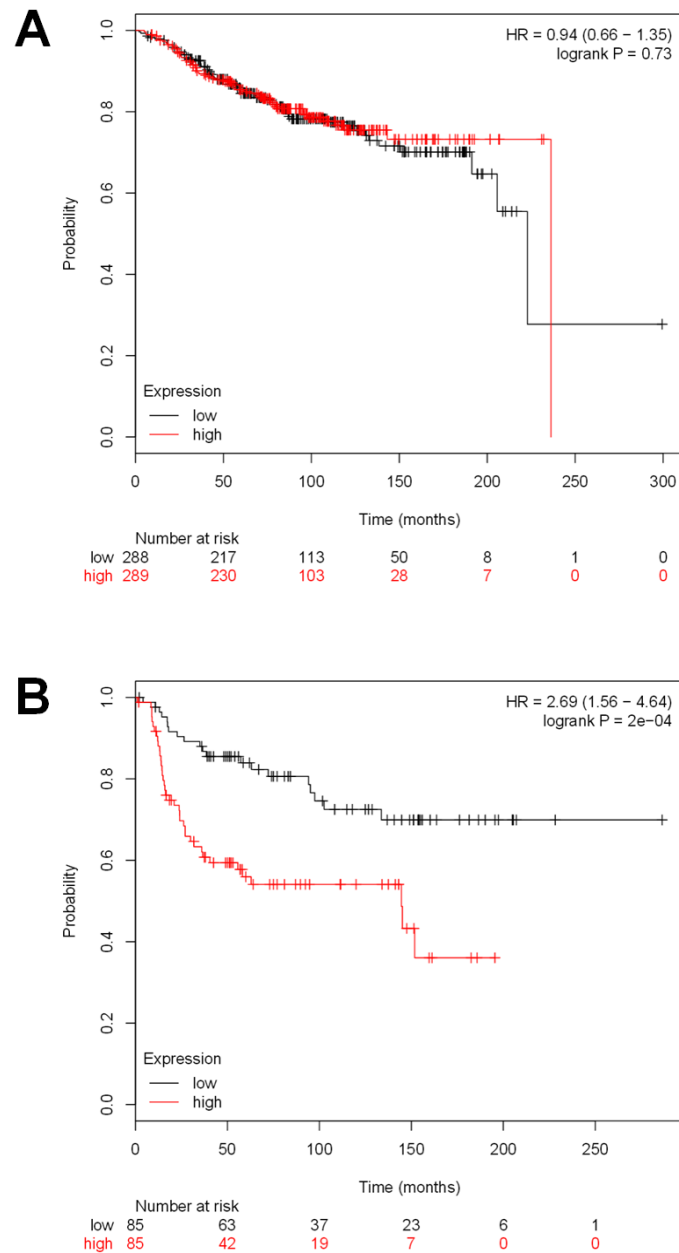


Figure 3.7: Impact of *WSB1* expression on distant metastasis-free survival (DMFS) in breast cancer patients

KM plots showing changes in DMFS probability according to level of *WSB1* expression in patients displaying ER+ tumours (A) and ER- tumours (B), using the four Affymetrix probes matching WSB-1 that are available in the KMplot platform. Red line: patients expressing high levels of *WSB1*; black line: patients expressing low levels of *WSB1*; HR: hazard ratio; logrank P: *p*-value;

3.3.3. Comparison of the basal *WSB1* expression level in a selection of breast cell lines

WSB1 transcript copy number was assessed by absolute qPCR in six breast cancer cell lines as well as the immortalised non-cancer embryonic kidney cell line HEK293T and the non-cancer primary breast epithelium cell line HMEpC. Details on the cell lines used can be found in Table 2.1. The selection was composed of two luminal A, one luminal B and three basal cell lines. Unfortunately, no HER2 subtype cell lines could be included in this panel.

Figure 3.8 presents the differences in *WSB1* transcript copy number across the eight cell lines analysed. The number of *WSB1* copies varied from nearly 13,000 copies (MDA-MB-468) to over 30,000 (MDA-MB-231). As evidenced by the difference in copy number in these two triple negative cell lines, there was no correlation between *WSB1* copy number and the characteristics of the breast cancer cell lines. However, with the exception of T47D and MDA-MB-231 cell lines, the breast cancer cell lines presented lower *WSB1* copy numbers than the average of the non-cancer cell lines HEK203T and HMEpC ($\bar{x} = 25357.68$).

From this experiment, it was clear that the basal transcription level of *WSB1* varied significantly between cell lines. However, there appeared to be no link between the basal *WSB1* expression in a cell line and its ER/PR/HER2 status.

3.3.4. Effect of hypoxia exposure on *WSB1* levels

Previous studies in different cancer types highlighted an upregulation of *WSB1* transcription following hypoxia exposure (Archange et al., 2008; Benita et al., 2009; Tong et al., 2013; Kim et al., 2015). However, the impact of hypoxia on *WSB1* expression in breast cancer cells *in vitro* is not known.

The existence of similar changes in breast cancer was investigated hereafter using two well-characterised breast cancer cell lines: MCF7 and MDA-MB-231. Changes in *WSB1* protein level, as evaluated by Western blot, are depicted in Figure 3.9. An increase of *WSB1* protein levels can be observed upon exposure of the cells to 2% O₂ (moderate hypoxia) for up to 30h. This hypoxia-driven upregulation was observed in both breast cancer cell lines. Figure 3.10

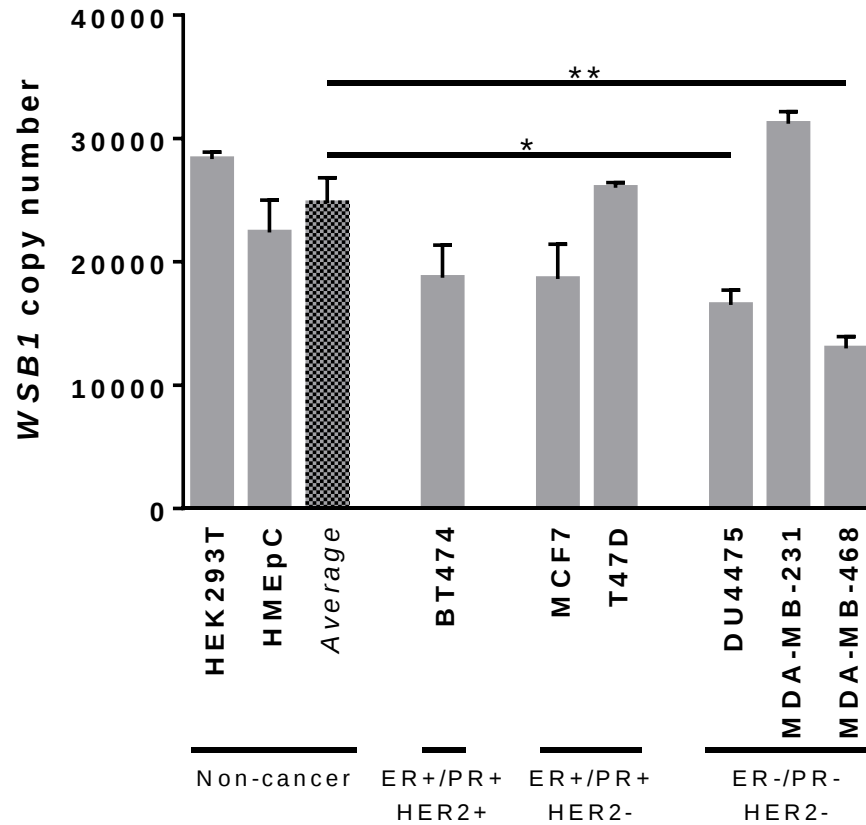


Figure 3.8: Comparison of *WSB1* transcript copy number across different cell lines

WSB1 copy number was measured by absolute qPCR in human embryonic kidney cell line HEK293T, non-cancer primary breast cell line HMEpC and a selection of breast cancer cell lines. Error bars represent mean $\pm$ SEM, statistical significance determined by 1-way ANOVA with Bonferroni correction between the non-cancer average (dark grey) and each cancer cell line, $n=3$. *, $p<0.05$; **, $p<0.01$.

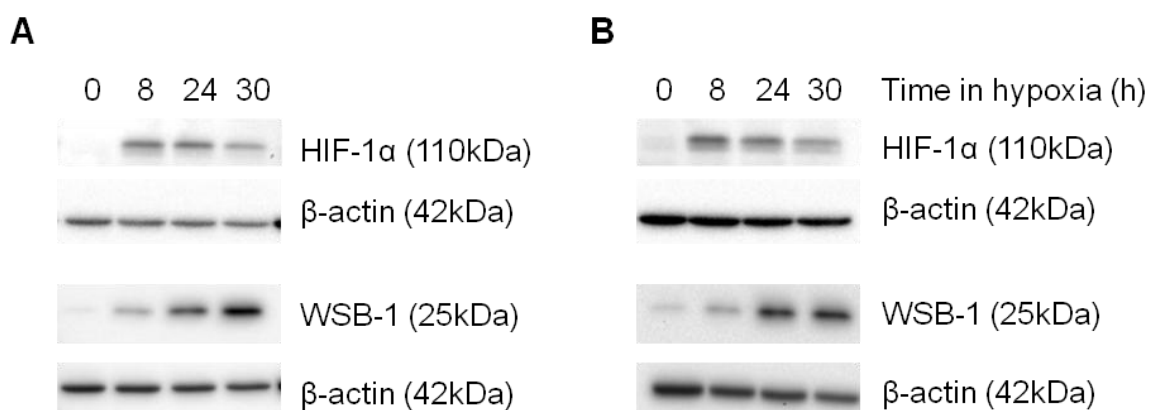


Figure 3.9: Effect of moderate hypoxia (2% O₂) on WSB-1 protein level

MCF7 (A) and MDA-MB-231 (B) cells were exposed to either 20% or 2% O₂ for a series of time points. Cells were lysed and protein expression in samples was analysed by Western blotting for HIF-1α, WSB-1 and β-actin. Western blots are representative of $n=3$ experiments.

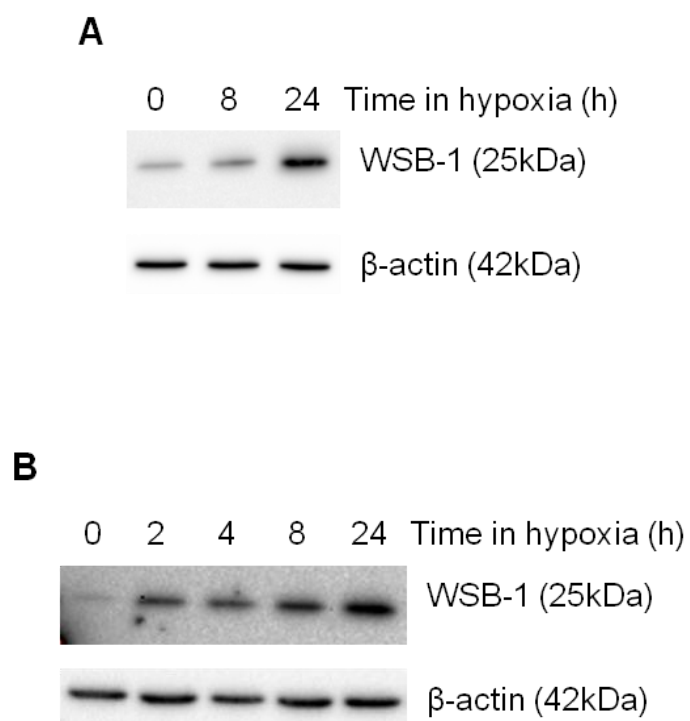


Figure 3.10: Effect of severe hypoxia (0.5% O₂) on WSB-1 protein level

MCF7 (A) and MDA-MB-231 (B) cells were exposed to either 20% or 0.5% O₂ for a series of time points. Cells were lysed and protein expression in samples was analysed by Western blotting for WSB-1 and β -actin. Western blots are representative of $n=3$ experiments.

presents a similar time course where cells were submitted to a more severe hypoxia level (0.5% O₂). As it is visible in these two figures, WSB-1 level demonstrated a time-dependent upregulation upon hypoxia exposure.

HIF-1 α levels were monitored in parallel in order to confirm the response of the cells to the hypoxia exposure. HIF-1 α protein level was visibly higher in the samples exposed to hypoxia (Figure 3.9).

In summary, moderate (2% O₂) and severe (0.5% O₂) hypoxia induced an upregulation of WSB-1 in breast cancer cell lines. This upregulation was visible at the protein and the transcript levels and 2h in hypoxia was sufficient to induce a noticeable change.

3.3.5. Regulation of WSB-1 by hypoxia-inducible factors

In order to evaluate if WSB-1 induction in breast cancer was dependent on HIF, the three HIF subunits HIF-1 α , HIF-2 α and HIF-1 β were selectively knocked down using siRNA in MCF7 cells, which were incubated at 2% O₂ for 24h. Cells were then lysed and total *WSB1* transcript level was measured by qPCR. In Figure 3.11, it can be observed that the hypoxia-induced upregulation of *WSB1* transcript, present in the siNT samples, was abolished when HIF-1 α and HIF-1 β were knocked down. In contrast, knockdown of HIF-2 α did not have an effect on *WSB1* transcript level. This indicated that hypoxia-mediated WSB-1 upregulation in breast cancer was exclusively mediated by HIF-1 α .

In order to analyse the role of WSB-1 on the HIF signalling and other cellular functions, siRNA transfection was used to knockdown *WSB1* transcription. Figure 3.12 presents the effect of this siRNA against WSB-1 (siWSB-1) on total *WSB1* (A, B) and *WSB1* isoform 3 (C, D) transcript levels compared to cells treated with non-targeting siRNA (siNT). This was performed in the two cell lines MCF7 (A, C) and MDA-MB-231 (B, D). Transfection with siWSB-1 significantly decreased the level of total *WSB1* transcript in hypoxia, although the effect on *WSB1* isoform 3 specifically was less important, particularly in MCF7 cells. This figure also showed that the hypoxia-induced *WSB1* upregulation was significant in MCF7 cells

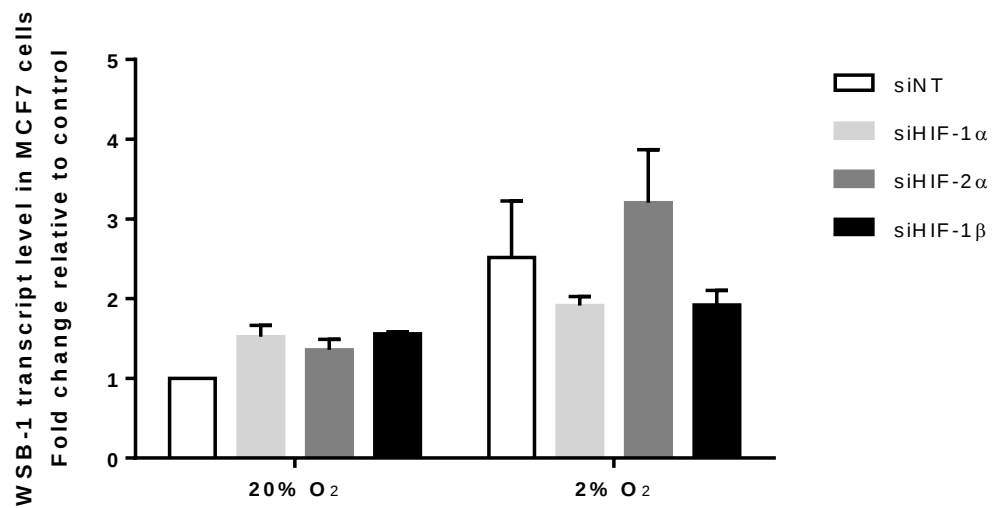


Figure 3.11: Effect of different HIF isoforms knockdown on *WSB1* transcript level in hypoxia in MCF7

MCF7 cells were treated with siRNA to selectively knock down HIF-1α (siHIF-1α), HIF-2α (siHIF-1α), HIF-1β (siHIF-1β) or a control non-targeting siRNA (siNT). Cells were then incubated at 20% O₂ or 2% O₂ for 24h, mRNA samples were prepared and effect of the treatment on total *WSB1* transcript level was assessed by qPCR. *B2M* was used as the housekeeping gene. Error bars represent mean $\pm$ SEM, statistical significance determined by 2-way ANOVA with Tukey correction for multiple comparisons, $n=3$.

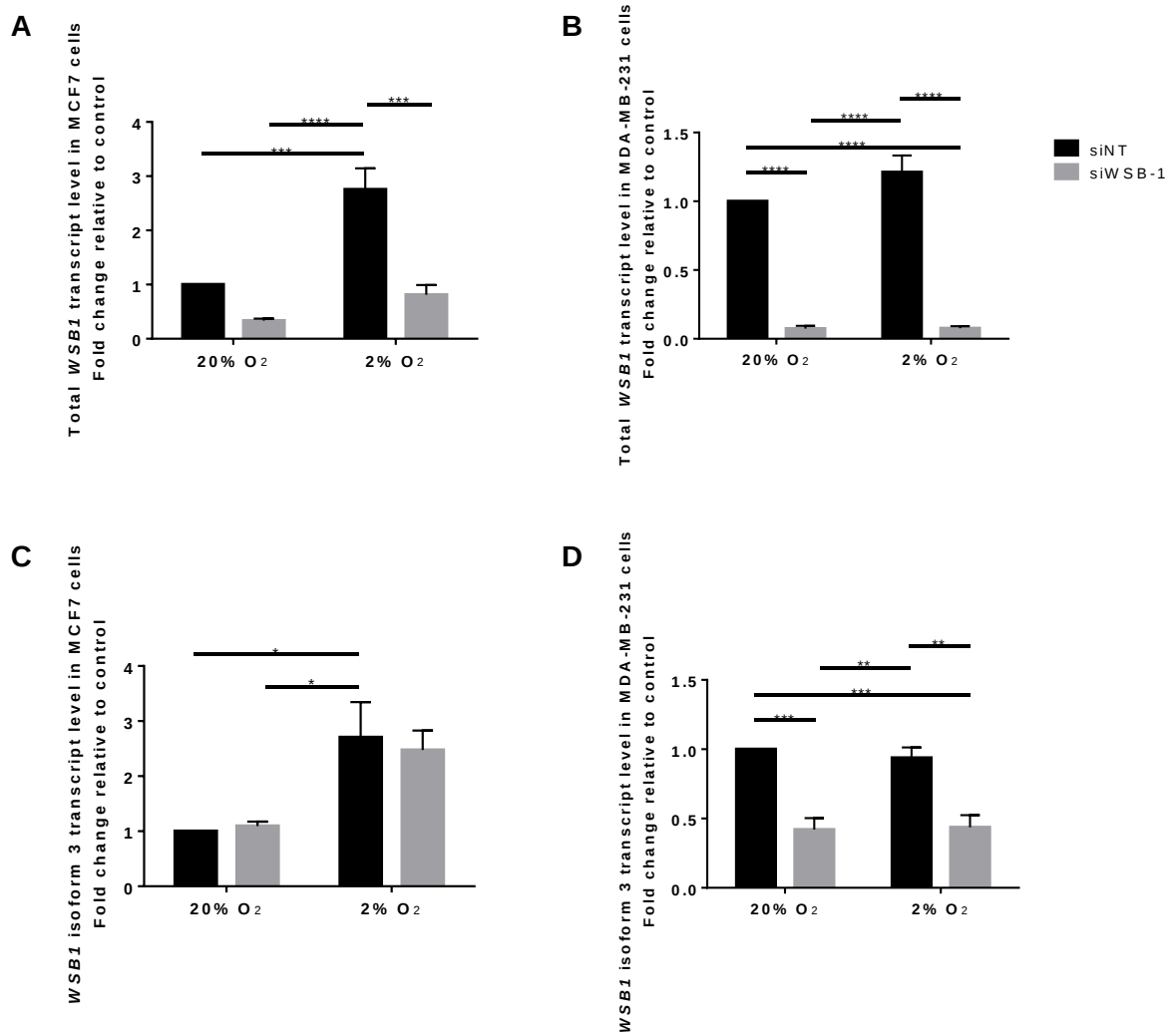


Figure 3.12: Effect of WSB-1 knockdown on *WSB1* transcript levels in hypoxia

MCF7 (A, C) and MDA-MB-231 (B, D) cells were treated with siRNA to knock down WSB-1 (siWSB-1) or a control non-targeting siRNA (siNT). Cells were then incubated at 20% O₂ or 2% O₂ for 24h, mRNA samples were prepared and effect of the treatment on total *WSB1* transcript (A, B) or *WSB1* isoform 3 transcript (C, D) was assessed by qPCR. *B2M* was used as the housekeeping gene. Error bars represent mean $\pm$ SEM, statistical significance determined by 2-way ANOVA with Tukey correction for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; $n = 4$.

but not in MDA-MB-231 cells.

To investigate the existence of a feedback between WSB-1 and HIF, the effect of siWSB-1 and moderate hypoxia on *HIF1A* transcript was assessed by relative qPCR in MCF7 (Figure 3.13A). Despite there being no statistically significant difference in transcript levels in response to WSB-1 knockdown, *HIF1A* transcript appeared to be slightly higher in siWSB-1 samples compared to their siNT counterparts. Then, WSB-1 or HIF-1 α were again knocked down in MCF7 to monitor the effects of such treatment on WSB-1 protein levels (Figure 3.13B). WSB-1 and HIF-1 α knockdowns effectively induced a decrease in WSB-1 and HIF-1 α protein levels. HIF-1 α knockdown also induced an important decrease of WSB-1 protein level in MCF7 cells, as expected from the results presented in Figure 3.11. Interestingly, no effect of WSB-1 knockdown was observed on HIF-1 α protein levels.

Finally, in order to evaluate any potential effect of WSB-1 on HIF function, qPCR analysis of the transcript levels of several known and well-characterised HIF targets in response to WSB-1 knockdown were evaluated by qPCR in MCF7 (Figure 3.14) and MDA-MB-231 (Figure 3.15): *VEGFA* (A), *SCL2A1* (B), *CA9* (C), *HK2* (D), and *LOX* (E). In this analysis, WSB-1 knockdown led to the modulation of the transcription of several genes, and this modulation was differential between MCF7 and MDA-MB-231 cells. In MCF7, *VEGFA* transcripts (Figure 3.14A) demonstrated the most important upregulation following WSB-1 knockdown and hypoxia of all the targets investigated. In fact, the effect of WSB-1 knockdown on the transcription *VEGFA* was noticeable even in normoxia. Similarly, *LOX* transcript levels (Figure 3.14E) were upregulated in response to WSB-1 knockdown in normoxia and in hypoxia. This specific result must, however, be considered with caution. *LOX* transcript levels were particularly low, affecting the reproducibility and reliability of the C_T values observed. *SCL2A1* (Figure 3.14B) and *HK2* (Figure 3.14D) transcript levels also increased in response to WSB-1 knockdown in hypoxia. Finally, *CA9* transcript level (Figure 3.14C) was not affected by WSB-1 knockdown in normoxia or in hypoxia and only increased in response to hypoxia.

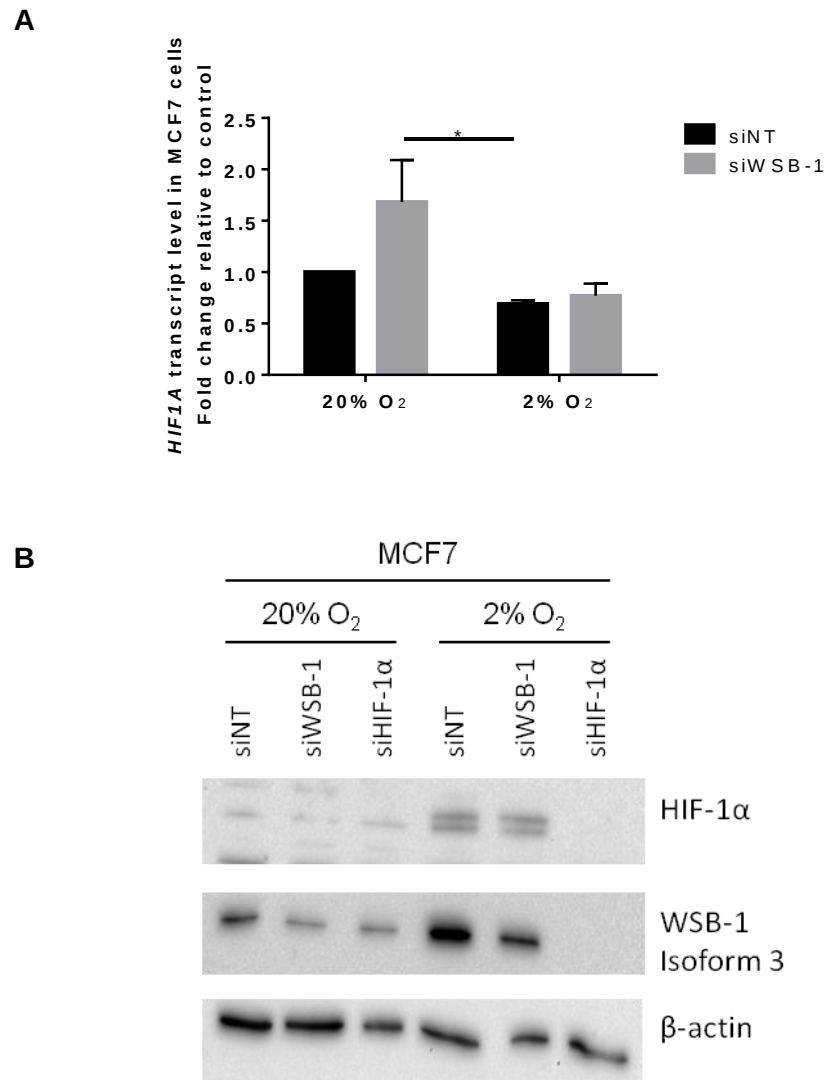


Figure 3.13: Effect of WSB-1 and HIF-1α knockdowns on WSB-1 levels

MCF7 cells were treated with siRNA against WSB-1 (siWSB-1) or HIF-1α (siHIF-1α), or a control non-targeting siRNA (siNT) and exposed to either 20% or 2% O₂ for 24h. *HIF1A* transcript level was assessed by qPCR from the prepared mRNA samples (A). *B2M* was used as the housekeeping gene. Error bars represent mean $\pm$ SEM, statistical significance determined by 2-way ANOVA with Tukey correction for multiple comparisons. *, $p < 0.05$; $n = 3$. Protein expression was analysed by Western blotting for WSB-1, HIF-1α and β-actin (B). Western blots were obtained using whole cell lysates collected after 24h exposure to 20% or 2% O₂. Images are representative of $n = 3$ experiments.

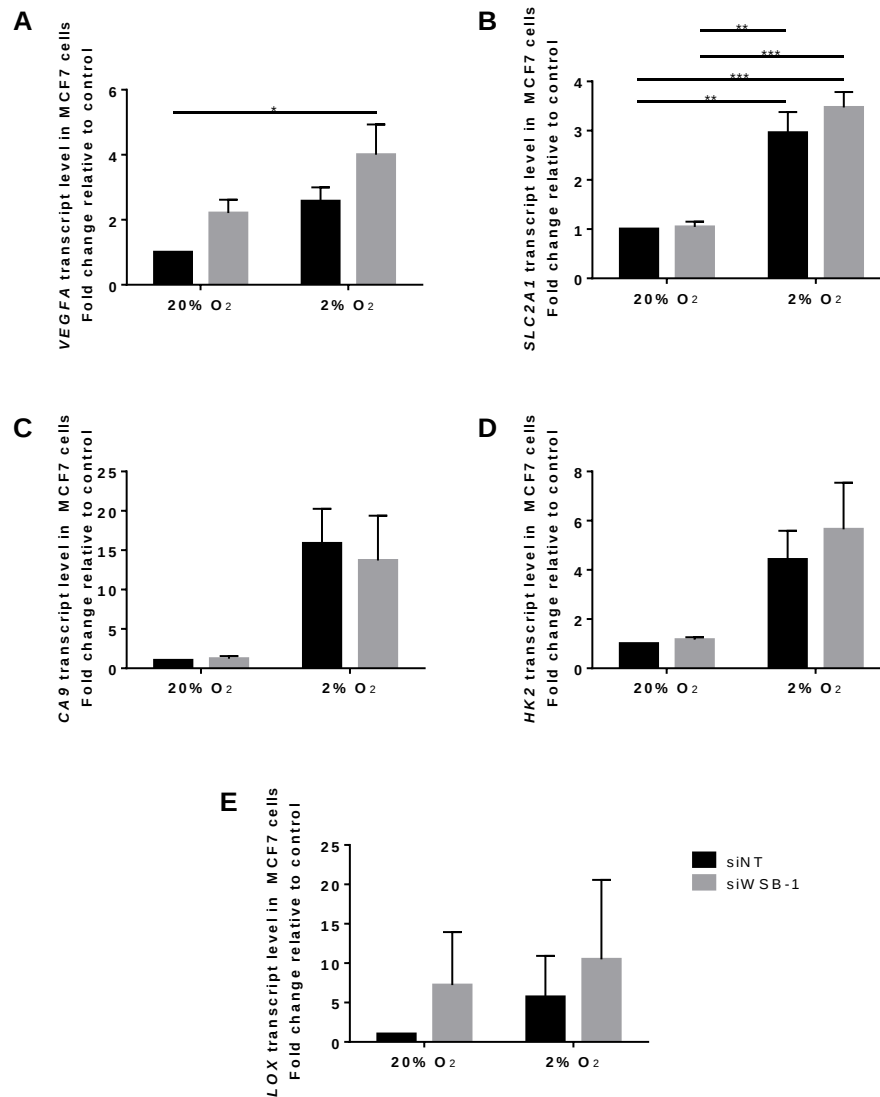


Figure 3.14: Analysis of the transcript levels of a selection of known HIF targets in response to WSB-1 knockdown by qPCR in MCF7 cells

MCF7 cells were treated with siRNA to knock down WSB-1 (siWSB-1) or a control non-targeting siRNA (siNT). Cells were then incubated at 20% O₂ or 2% O₂ for 24h, mRNA samples were prepared and effect of the treatment on transcript levels of several targets was assessed by qPCR. *B2M* was used as the housekeeping gene. Error bars represent mean $\pm$ SEM, statistical significance determined by 2-way ANOVA with Tukey correction for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 3$.

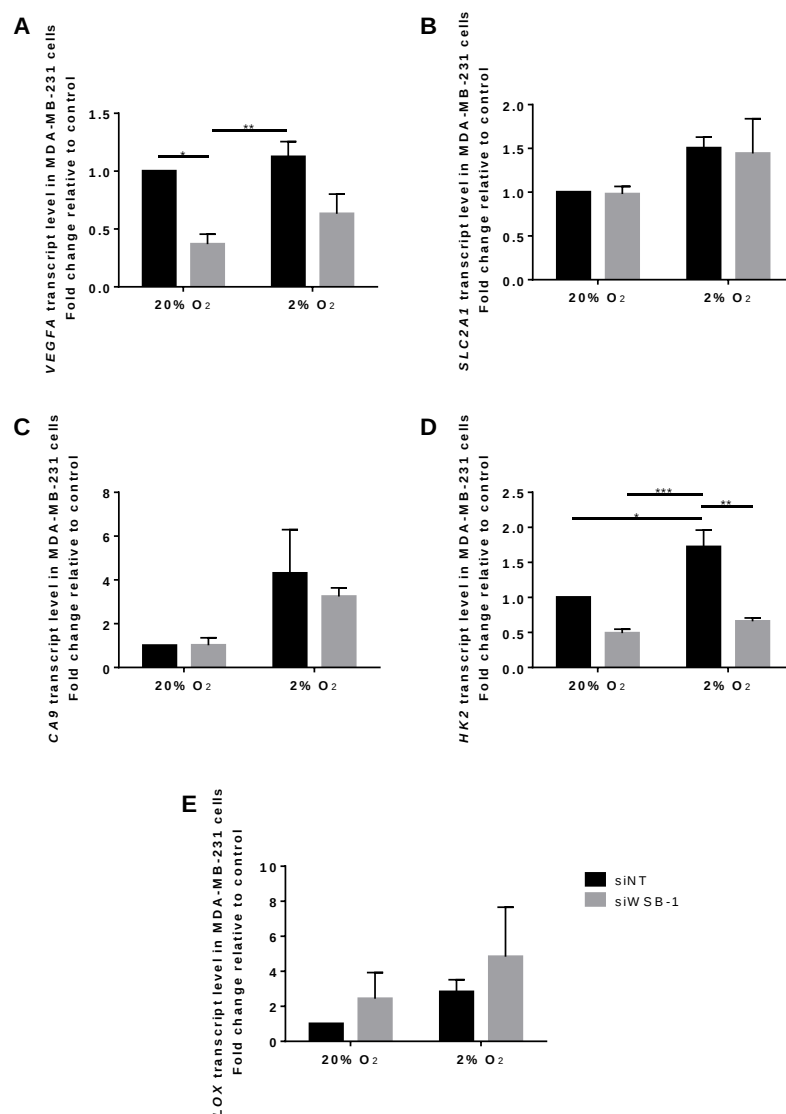


Figure 3.15: Analysis of the transcript levels of a selection of known HIF targets in response to WSB-1 knockdown by qPCR in MDA-MB-231 cells

MDA-MB-231 cells were treated with siRNA to knock down WSB-1 (siWSB-1) or a control non-targeting siRNA (siNT). Cells were then incubated at 20% O₂ or 2% O₂ for 24h, mRNA samples were prepared and effect of the treatment on transcript levels of several targets was assessed by qPCR. *B2M* was used as the housekeeping gene. Error bars represent mean $\pm$ SEM, statistical significance determined by 2-way ANOVA with Tukey correction for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 3$.

In MDA-MB-231 cells, WSB-1 knockdown induced a 25% decrease in *CA9* transcript (Figure 3.15C) in hypoxia. WSB-1 knockdown also induced a 44% decrease in *VEGFA* transcript (Figure 3.15A), significant in normoxic condition ($p < 0.05$), and in *HK2* transcript (Figure 3.15D), significant in hypoxia ($p < 0.01$). *HK2* transcript level was also significantly induced by 2 fold by hypoxia ($p < 0.05$). Although *SLC2A1* transcript level increased in response to hypoxia (Figure 3.15B), siWSB-1 did not appear to have any effect in transcript levels. Finally, WSB-1 knockdown induced an upregulation of *LOX* transcripts (Figure 3.15E) in MDA-MB-231 cells. However, as it was the case in MCF7 cells, the C_T values were low, corresponding to a low expression of the transcript, which impeded the accuracy of the readings.

Overall, this section demonstrated that the hypoxia-induced upregulation of WSB-1 transcript was mediated by HIF-1 α in MCF7 cells (Figure 3.11). In addition, knockdown of WSB-1 induced a decrease in *HIF1A* transcript but not protein levels (Figure 3.13) and a modification in the hypoxia-mediated upregulation of a selection of known HIF targets, suggesting a feedback between WSB-1 and the HIF signalling pathway in both breast cancer cell lines studied (Figure 3.14 and Figure 3.15).

3.3.6. Effect of WSB-1 knockdown on MDA-MB-231 cells transcriptome

Considering the results presented in the precedent section, WSB-1 knockdown appeared to modify the transcript level of several of the HIF targets investigated. Therefore, the effect of WSB-1 knockdown on the entire transcriptome was analysed by RNA Sequencing (RNA-Seq) of total mRNA samples extracted from MDA-MB-231 cells following WSB-1 knockdown and/or 24h exposure at 2% O₂ (Table B2 to Table B7, appendix).

The data generated was initially analysed in the following three dataset permutations: 20% O₂ siNT (A) vs 20% O₂ siWSB-1 (B); 2% O₂ siNT (C) vs 2% O₂ siWSB-1 (D); 20% O₂ siNT (A) vs 2% O₂ siWSB-1 (D). The first one presents the changes in the transcriptome following WSB-1 knockdown in normoxia. The second one focuses on the effect of WSB-1 knockdown in

moderate hypoxia, and the third one on the effect of WSB-1 knockdown and moderate hypoxia exposure combined. Genes that were regulated with more than 3 fold between siNT and siWSB-1 samples were represented in a heat map (Figure 3.16). Upregulated transcripts in siWSB-1 are presented in red and downregulated transcripts in siWSB-1 (including *WSB1*, as expected) are represented in blue.

Despite punctual differences, most transcripts presented a similar regulation pattern across the three datasets. Therefore, the next analyses will only be performed on the dataset comparing 2% O₂ siNT vs 2% O₂ siWSB-1, which takes into account the effect of WSB-1 knockdown in hypoxia.

The next figures present a summary of the RNA-Seq data analysed with the IPA software (Qiagen). All the transcripts demonstrating a change of at least 1.5 fold were included in this second analysis.

Table 3.7 presents the top ten transcripts that were the most regulated in response to WSB-1 knockdown in hypoxia. Values in the dataset were expressed as log₂(fold change) and the corresponding fold change values were given in the table.

OSBPL6, *RNF170* and *NDE1* were not present on the heat map (Figure 3.16) because the transcript was not expressed in all three datasets. Several upregulated transcripts were coding for proteins involved in cell cycle regulation (*CDK6*, *CDK2*) or cellular organisation (*LMNB1*, *CENPQ*). Some of the downregulated transcripts were coding for protein involved in cancer development (*SPARC*, *IL24*) or metabolism (*TM7SF2*). Finally, one transcript was directly associated with the ubiquitin proteasome pathway (*NEURL1B*).

The significant biological functions categories represented by the transcripts found in the dataset are listed in Figure 3.17. In agreement with the overview provided by the most regulated transcripts (Table 3.7), the most represented biological functions categories in this dataset implicated "cellular movement" (p -values between 1.24×10^{-4} and 4.76×10^{-3}), "cell cycle" and related processes, and "protein processing" which includes protein synthesis, post-

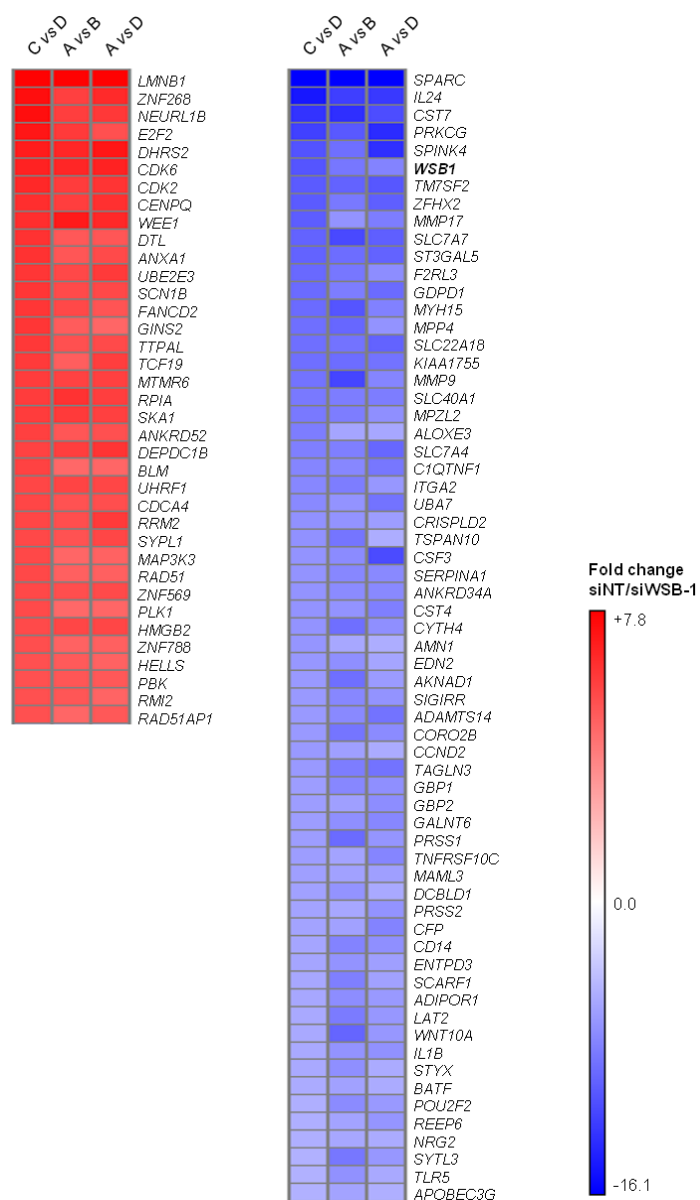


Figure 3.16: Heatmap for the most upregulated and downregulated genes following WSB-1 knockdown

MDA-MB-231 cells were treated with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). Cells were incubated at 20% O₂ or 2% O₂ for 24h, mRNA samples were prepared and analysed by RNA-Seq to identify all the transcripts present. Genes detected in the three conditions and which expression varied more than 3 fold are represented. A: 20% O₂ siNT, B: 20% O₂ siWSB-1, C: 2% O₂ siNT, D: 2% O₂ siWSB-1. Blue: downregulated genes in the siWSB-1 samples; red: upregulated genes in siWSB-1 samples. The intensity of the colour corresponds to the amplitude of the regulation.

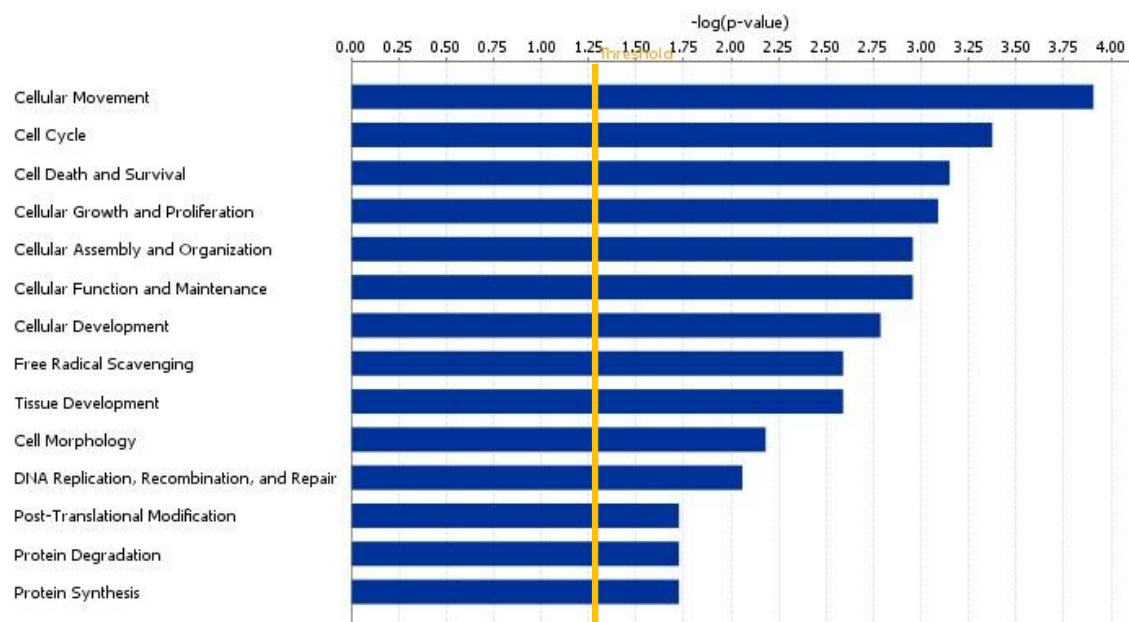


Figure 3.17: Biological function categories modified by WSB-1 knockdown in hypoxia
MDA-MB-231 cells were treated with siRNA against WSB-1 or a control non-targeting siRNA. Cells were then incubated at 2% O₂ for 24h, mRNA samples were prepared and analysed by RNA-Seq to identify all the transcripts present. The biological functions represented within the dataset, involving only genes which expression varied more than 1.5 fold between the 2% O₂ siNT and 2% O₂ siWSB-1 samples. Bar height represents the average p -value of all the biological functions comprised in each category, threshold represents (yellow line) a p -value of 0.05.

Table 3.7: Top upregulated and downregulated molecules in response to WSB-1 knockdown and hypoxia exposure

Gene name	Full name	Fold change	p-value
<u>Upregulated</u>			
<i>OSBPL6</i>	Oxysterol binding protein-like 6	27.96	5×10^{-5}
<i>RNF170</i>	Ring finger protein 170	9.66	5×10^{-5}
<i>LMNB1</i>	Lamin B1	5.93	5×10^{-5}
<i>NEURL1B</i>	Neuralized E3 ubiquitin protein ligase 1B	5.36	5×10^{-5}
<i>HIST1H1E</i>	H1 histone family, member 4	5.19	2.3×10^{-3}
<i>E2F2</i>	E2F transcription factor 2	5.00	5×10^{-5}
<i>DHRS2</i>	Dehydrogenase-reductase SDR family member 2	4.78	5×10^{-5}
<i>CDK6</i>	Cyclin-dependent kinase 6	4.54	5×10^{-5}
<i>CDK2</i>	Cyclin-dependent kinase 2	4.28	5×10^{-5}
<i>CENPQ</i>	Centromere protein Q	4.08	5×10^{-5}
<u>Downregulated</u>			
<i>NDE1</i>	NudE neurodevelopment protein 1	-21.22	5×10^{-5}
<i>SPARC</i>	Secreted protein acidic cysteine-rich/Osteonectin	-14.34	5×10^{-5}
<i>IL24</i>	Interleukin 24	-11.60	5×10^{-5}
<i>CST7</i>	Cystatin F/Leukocystatin	-9.03	5×10^{-5}
<i>PRKCG</i>	Protein kinase C, gamma	-8.02	1.15×10^{-3}
<i>SPINK4</i>	Serine peptidase inhibitor, kazal type 4	-6.82	5×10^{-5}
<i>WSB1</i>	WD repeat and SOCS box containing 1	-6.68	5×10^{-5}
<i>TM7SF2</i>	Transmembrane 7 superfamily member 2	-6.38	5×10^{-5}
<i>ZFH2</i>	Zinc finger homeobox 2	-6.35	5×10^{-5}
<i>MMP17</i>	Matrix metalloproteinase 17	-6.26	2×10^{-4}

translational modification, and protein degradation ($p = 1.88 \times 10^{-2}$). Interestingly, HIF-mediated signalling was not highlighted as being significantly affected.

The most significant biological functions for each category are detailed in Table 3.8. Each biological function was associated with a p -value and the number of transcripts from the dataset involved in this particular biological function. Some biological functions were common to several categories such as microtubules dynamics or proteolysis. The number of molecules from the dataset involved in each particular biological functions gives indication on which processes were the most represented in the dataset. From this, it appeared that "cellular movement", "cellular growth and proliferation", "cellular development", "cell death and survival", and "DNA replication, recombination and repair" were the biological functions the most significantly modified by WSB-1 knockdown in hypoxia, and were important for cell

Table 3.8: List of the most significant specific biological functions per category

Category	<i>p</i>-value	Number of molecules from dataset
<u>Biological function</u>		
<u>Cellular movement</u>		
Cell movement of tumour cell lines	1.24×10^{-4}	61
Cell movement of breast cancer cell lines	3.26×10^{-4}	58
Migration of tumour cell lines	7.26×10^{-4}	52
<u>Cell cycle</u>		
Senescence of breast cancer cell lines	4.22×10^{-4}	9
Cell cycle progression	1.43×10^{-3}	22
Arrest in G0/G1 phase transition of breast cancer cell lines	1.57×10^{-3}	4
<u>Cell death and survival</u>		
Cell death of breast cancer cell lines	7.07×10^{-4}	68
Apoptosis of breast cancer cell lines	1.69×10^{-3}	58
<u>Cellular growth and proliferation</u>		
Proliferation of cells	8.14×10^{-4}	89
Cell proliferation of breast cancer cell lines	1.64×10^{-3}	84
Outgrowth of breast cancer cell lines	1.56×10^{-2}	4
<u>Cellular assembly and organisation, Cellular function and maintenance</u>		
Microtubules dynamics	1.11×10^{-3}	13
Organisation of cytoskeleton	1.25×10^{-3}	14
Polymerisation of microtubules	2.57×10^{-3}	3
<u>Cellular development</u>		
Cell proliferation of breast cancer cell lines	1.64×10^{-3}	84
Outgrowth of breast cancer cell lines	1.56×10^{-2}	4
<u>Free radical scavenging</u>		
Production of superoxide	2.57×10^{-3}	3
Production of reactive oxygen species	8.72×10^{-3}	4
<u>Tissue development</u>		
Polymerisation of microtubules	2.57×10^{-3}	3
Formation of filaments	6.66×10^{-3}	5
<u>Cell morphology</u>		
Autophagy of breast cancer cell lines	6.57×10^{-3}	8
Formation of cellular protrusions	460×10^{-2}	7
<u>DNA replication, recombination, and repair</u>		
Proliferation of cells	8.14×10^{-4}	89
Cell proliferation of breast cancer cell lines	1.64×10^{-3}	84
Double-stranded DNA break repair	8.72×10^{-3}	4
<u>Post-translational modification, Protein degradation, Protein synthesis</u>		
Proteolysis	1.88×10^{-2}	2

proliferation. On the other hand, "free radical scavenging", "tissue development", "cell morphology", "post-translational modification", "protein degradation", and "protein synthesis" were less altered by WSB-1 knockdown in hypoxia exposure, despite being significantly represented in the dataset. Of note, these processes were also represented by a minimal number of molecules.

The main biological functions listed previously (Figure 3.17) are represented as a heat map in Figure 3.18. Biological functions activated in response to hypoxia and WSB-1 knockdown are in orange, whereas inhibited activity is represented in blue. Each box corresponds to specific pathways within each category and grey boxes correspond to pathways not represented in the dataset. In this figure, "cell cycle", and "cellular growth and proliferation" processes appeared to be mostly activated, whereas biological functions such as "cell morphology", "tissue development", "cellular assembly and organisation", or "cellular function and maintenance" appeared to be mainly inhibited. "Cellular movement", despite being the most significantly represented biological functions (p -values between 1.24×10^{-4} and 4.76×10^{-3} ; Table 3.8), did not appear to be clearly regulated. In fact, the pathways comprised in "cellular movement" were either weakly activated or inhibited.

Finally, the IPA analysis also highlighted the main canonical pathways represented in the dataset. As illustrated in Figure 3.19, three main pathways were represented: cancer (♦, 8/28 pathways), cell cycle (▲, 8/28 pathways), and DNA damage/repair mechanisms (●, 4/28 pathways). The majority of the molecules involved in these pathways were downregulated in response to WSB-1 knockdown in hypoxia (red bars) and at least about 20% of the molecules involved in each pathway were represented in the dataset studied.

To summarise, the RNA-Seq analysis of MDA-MB-231 cells revealed that WSB-1 knockdown and exposure to moderate hypoxia modified some key cellular pathways. Notably, mechanisms involved in cell cycle were generally activated by WSB-1 knockdown, whereas processes associated with cancer development were mostly downregulated. WSB-1

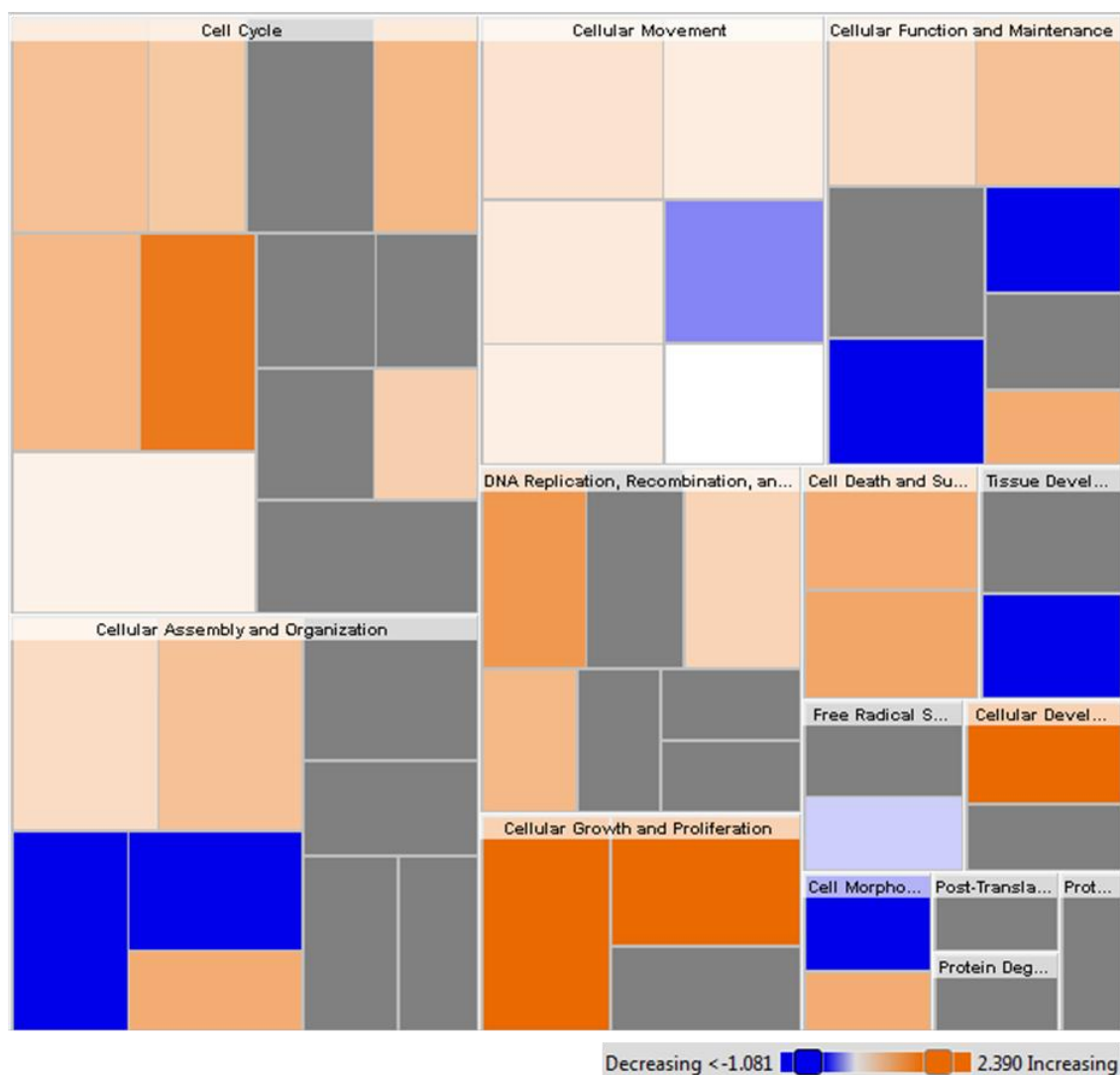


Figure 3.18: Main biological functions modified by WSB-1 knockdown and hypoxia exposure

MDA-MB-231 cells were treated with siRNA against WSB-1 or non-targeting. Cells were then incubated at 2% O_2 for 24h, mRNA samples were prepared and analysed by RNA-Seq to identify all the transcripts present. The biological functions represented within the dataset, involving only genes which expression varied more than 1.5 fold between the 2% O_2 siNT and 2% O_2 siWSB-1 samples. Boxes size was calculated using $-\log(p\text{-value})$ to be proportional to the p -value and square colour corresponds to the direction of the regulation, the more intense the colour, the more regulated the function. . Orange: positive z-score, activated process; blue: negative z-score, inhibited process.

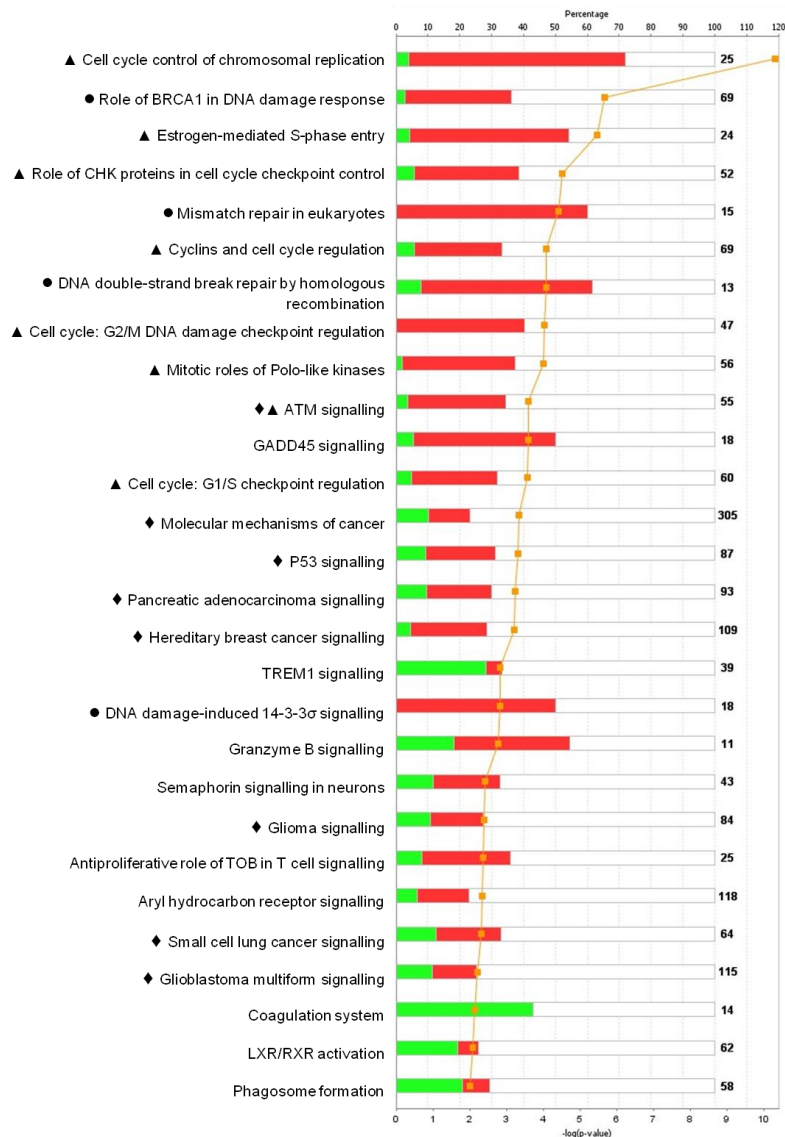


Figure 3.19: Canonical pathways modulated by WSB-1 knockdown in hypoxia

MDA-MB-231 cells treated with siRNA against WSB-1 or non-targeting were incubated at 2% O₂ for 24h and mRNA samples were prepared and analysed by RNA-Seq. Bar chart represents the statistically significant ($p \leq 0.01$) pathways present within the dataset, considering only genes which expression varied more than 1.5 fold between 2% O₂ siNT and 2% O₂ siWSB-1 samples. Bold figures: total number of genes involved in the pathway. Green bar: downregulated genes; red bar: upregulated genes; white bar: no overlap with dataset; yellow line: $-\log(p\text{-value})$. Signs indicate domain of the pathway: cancer (♦), cell cycle (▲), and DNA damage/repair mechanisms (●).

knockdown also tempered with cellular movement but the net effect was difficult to assess as some mechanisms were moderately activated and others moderately inhibited.

3.4. Discussion

In this chapter, the association of the expression level of *WSB1* with cancer progression and patient survival was investigated, along with the effect of hypoxia on *WSB1* protein and transcript levels. In addition, the relationship between *WSB1* and the HIF pathway was analysed. Finally, the effect of *WSB1* knockdown on the transcriptional response to hypoxia in breast cancer cells was examined.

Following is a brief summary of the results obtained, answering the questions stated at the beginning of this chapter:

- *Is there a correlation between WSB1 transcript level and breast tumour's subtype or stage?*

Analysis of cDNA from normal and breast cancer tissue samples by qPCR revealed the absence of correlation between *CA9* and *WSB1* expression in tumour or equivalent normal (non-cancer) tissues. Surprisingly, higher-grade tumours expressed significantly less *WSB1* than normal tissues. Absence of ER, PR, and HER2 expression appeared to be associated with smaller *WSB1* levels.

- *Can the tumour level of WSB1 transcript be of interest to predict patient survival?*

When significant, high general *WSB1* transcript level was associated with poor distant metastasis-free survival (DMFS). Effect of high general *WSB1* transcript level on relapse-free survival (RFS) was more conflicting.

- *Does basal WSB1 transcript level correlates with a cell line's origins?*

Basal *WSB1* transcript level varied in different cell lines and did not associate with a particular ER/PR/HER2 status, as revealed by absolute quantification of *WSB1* transcript level in a selection of breast cancer cell lines, compared to two non-cancer cell lines.

- *How is WSB-1 expression affected by hypoxia in breast cancer cells in vitro?*

HIF-1 α specifically mediates the hypoxia-induced WSB-1 upregulation observed in breast cancer cell lines.

- *What is the relationship between WSB-1 and the HIF signalling pathway?*

Analysis of a selection of mRNA by qPCR indicated that WSB-1 modulates transcript levels of some known HIF targets, possibly through a crosstalk with the HIF signalling pathway.

- *How does WSB-1 knockdown affect the transcriptomic profile of breast cancer cells?*

RNA-Seq data suggested that WSB-1 knockdown in hypoxia had an impact on several canonical pathways involved in cancer, cell cycle, cellular movement and DNA damage/repair mechanisms.

3.4.1. Correlation between WSB-1 level and breast tumour tissue characteristics

Initially, the expression level of WSB-1 in breast cancer patient samples was evaluated. Tissue cDNA microarrays combining sixteen normal breast and 128 breast tumour tissue samples were used. Relative levels of *WSB1* transcripts were also measured in each sample in parallel to that of *CA9*. Carbonic anhydrase IX is a known HIF target which is commonly used as a marker of hypoxia. *CA9* levels are also frequently upregulated in breast cancer tissues (Chia et al., 2001; Trastour et al., 2007; Tan et al., 2009). However, this information must be taken with caution as the HIF pathway can be altered in tumour cells (*e.g.* pVHL mutations in renal cancer, although loss of VHL has not been identified in breast cancer (Sourvinos et al., 2001; Seagroves et al., 2010)), leading to a constitutive stabilisation and activation of the HIF signalling without the need for the adequate oxygen tension (Wiesener et al., 2001). *CA9* transcript level can also increase due to the hypomethylation of its promoter region, leading to a HIF-independent *CA9* transcriptional regulation (Nakamura et al., 2011; Ivanova et al., 2015).

The analysis of the patient cDNA microarray samples revealed an inverse relationship between *WSB1* and *CA9* levels (Figure 3.1). Higher-grade tumour samples expressed

significantly less *WSB1* than normal tissue samples, but also expressed significantly more *CA9* (Figure 3.2). This inverse relationship was observed in every significant differences presented in the next analyses of the patient cDNA samples. The increase in *CA9* transcript level in higher-grade tumour samples compared to normal tissue samples is in agreement with the fact that hypoxia associates with poor prognosis, and more advanced tumours usually express higher levels of hypoxic markers (Höckel and Vaupel, 2001; Gruber et al., 2004; Buffa et al., 2010; Semenza, 2012). However, since WSB-1 was previously described as a hypoxia-inducible protein, it was not expected for *WSB1* expression not to parallel the expression of *CA9* (Benita et al., 2009; Tong et al., 2013). This could be due to various facts. Firstly, there is not a direct correlation between hypoxia and *CA9* expression. Secondly, WSB-1 expression can be upregulated in response to chemotherapeutic treatment and independently of hypoxia exposure, as shown by the study of Archange and colleagues (Archange et al., 2008). Thirdly, cancer proliferation is associated with genomic instability and duplication of parts or entire chromosomes is frequent (Pires et al., 2010). In fact, duplication of chromosome 17, where is located *WSB1*, is often observed in neuroblastoma (Chen et al., 2006). Finally, this could also be an experimental issue inherent to tissue cDNA microarrays. cDNA sequences were synthesised from tumour biopsies and coated onto PCR plates. As a result, cDNA samples may be comprised of tumour cells as well as cells from the neighbouring normal tissues. In addition, tumours are highly heterogeneous systems that include several other cell types (*i.e.* stromal cells, immune cells, cancer-associated fibroblasts), which can harbour very different transcriptional phenotypes (Russo et al., 2003; Hanahan and Weinberg, 2011). Unfortunately, such diversity is lost with this type of approach and any results obtained with this technique reflect the overall transcriptional status of a given sample, and can hide subtler differences that were present within the original tumour, such as the oxygen level variations (Russo et al., 2003).

The difference in the fold change amplitude of *WSB1* and *CA9* levels observed in the patient

microarray study suggests that *CA9* transcription could be more responsive to hypoxia than that of *WSB1*. This is in accordance with the results obtained by qPCR and presented in Figure 3.15, where hypoxia exposure induced a greater increase in *CA9* transcript than *WSB1*.

Importantly, ER- tumours presented a highly significant greater level of *CA9* than ER+ tumours and normal samples, indicating a more hypoxic environment. This is of particular interest, as increased tumoural hypoxia usually impairs treatment efficiency and is associated with lower survival (Semenza, 2003; Chi et al., 2006; Erler et al., 2006). Coincidentally, ER expression tends to be lost during cancer progression and ER- tumours are often the most advanced (Lower et al., 2005). This observation therefore reinforces the existence of a correlation between tumour hypoxia and cancer progression at least in breast cancer.

Interestingly, TNBC and HER2 type, the breast cancer subtypes presenting the worst survival rates, displayed the lowest *WSB1* levels (Haque et al., 2012; Polyak and Metzger Filho, 2012). This is in accordance with the work of Chen and colleagues who showed that *WSB1* overexpression correlated with increased survival in neuroblastoma (Chen et al., 2006). However, this is in contradiction with more recent studies in other tumour types, where it was demonstrated that increased *WSB-1* expression contributed to cancer progression (Archange et al., 2008; Silva et al., 2011; Tong et al., 2013; Shichrur et al., 2014; Cao et al., 2015; Kim et al., 2015).

In summary, the analysis of *WSB1* transcript level in tissues from breast cancer patients and non-tumour samples suggested the existence of an inverted relationship between *WSB-1* transcription and the potential aggressiveness of the tissue sample, which is in opposition to most of the studies of *WSB-1* implication published to this date and the results presented in this thesis.

3.4.2. Prognostic value of *WSB-1* for breast cancer patients

KM plots are a useful tool designed by Kaplan and Meier to allow the analysis of incomplete sets of data often obtained during drug trials or survival monitoring of cancer patients

(Kaplan and Meier, 1958; Rich et al., 2010). A similar approach was recently used by Kim and colleagues, while studying the role of *WSB-1* in tumour metastasis in several cancer types (Kim et al., 2015). This analysis complements the information gathered from the microarray analyses in the present work. Whereas microarray analyses compare *WSB1* expression levels between normal (non-cancer) samples and across different categories of tumour samples, KM plots focus on the effect of high *versus* low *WSB1* expression within each tumour subtype. Each approach enables the addressing of different questions. KM plots provide information on the survival probability and the prognostic value of *WSB1* expression level within a specific type of patient cohort. In contrast, the microarray data allow comparison between the different groups and whether *WSB1* expression level is higher in ER- or PR- subgroups. Therefore, the combination of KM plots data and the microarray results enable a deeper appreciation of the possible correlation between *WSB1* level and patients' survival probability. For instance, this microarray analysis showed that ER- and PR- negative tumours expressed significantly lower levels of *WSB1* than normal samples (Figure 3.3 and Figure 3.4). On the other hand, KM plots indicated that there is in fact a significant difference in survival probability in the ER- and PR- subgroups according to *WSB1* expression level (Table 3.4). The hazard risk (HR) figure superior to 1 in these cases suggests that a high level of *WSB1* is associated with a poor prognosis. Therefore, low *WSB1* levels, as revealed by the microarray analysis, would indicate a better prognosis for the patients. As a result, this analysis suggests that a high level of *WSB-1* in ER- and PR- tumours, which usually present a lower expression of *WSB-1* than normal tissues, would be an indicator of poor DMFS and RFS probability for this specific group of patients. This is in accordance with most of the relevant studies published to this date (Archange et al., 2008; Silva et al., 2011; Shichrur et al., 2014; Cao et al., 2015; Kim et al., 2015).

The KMplot database references 54,675 Affymetrix probes to assess the expression level of 22,277 genes. As mentioned in section 3.2.2, five probes were listed for *WSB1*. Two probes

(201296_s_at and 210561_s_at) recognised all three WSB-1 isoforms and two other (201294_s_at and 201295_s_at) were specific for WSB-1 isoform 3. Finally, the sequence of the fifth probe (213406_at) could not be matched anywhere on the WSB-1 sequences. This was surprising because the 213406_at probe, along with 201295_s_at, was used by Archange and colleagues to recognise the three isoforms and WSB-1 isoform 3, respectively (Archange et al., 2008).

BreastMark and PROGene are other available databases for KM plots (Madden et al., 2013; Goswami and Nakshatri, 2013, 2014). However, the tool designed by Györfy was found to be the most user-friendly, flexible, and open (Györfy et al., 2010). In fact, to attenuate the inherent bias to every study, Györfy's KM plot tool combines all the studies in the database together, totalling 4,142 breast cancer patients. However, depending on the type of study they were enrolled in, all will not give information for distant metastasis-free survival (DMFS), relapse-free survival (RFS) and overall survival (OS) at the same time. This is evidenced by the difference in *n* numbers between DMFS (Table 3.4), RFS (Table 3.5) and OS (Table 3.6). As a matter of fact, numbers were so low for OS that some analyses could not be performed due to insufficient data.

These results indicate that WSB-1 could be of relevance in breast cancer progression, as shown in other cancer types (Archange et al., 2008; Silva et al., 2011; Shichrur et al., 2014; Cao et al., 2015; Kim et al., 2015). As a result, further *in vitro* work was conducted.

3.4.3. Study of WSB-1 *in vitro* in breast cancer models

3.4.3.1. Absolute quantification of WSB1 in a selection of cell lines

Level of *WSB1* transcript was measured in six breast cancer cell lines, one primary non-cancer breast epithelium cell line (HMEpC) and another non-cancer embryonic kidney cell line (HEK293T). In accordance with the results obtained with the microarray analysis described previously, most breast cancer cell lines displayed a lower *WSB1* copy number than the

average of the two non-cancer cell lines and the HMEpC cell line on its own (Figure 3.8). It would have been interesting to study a greater number of cell lines displaying a wider array of characteristics. For example, no HER2 type cell lines (ER/PR-/HER2+) or cell lines expressing only either ER or PR were analysed. However, given that no statistical difference in WSB-1 level was observed in the microarray data (Figure 3.6), it is possible that WSB-1 copy number would not have significantly differed between all these cell lines.

3.4.3.2. Effect of hypoxia on WSB-1 level *in vitro*

Previous studies in pancreatic cancer, hepatocellular carcinoma and osteosarcoma also described an upregulation of WSB-1 expression in hypoxia (Archange et al., 2008; Benita et al., 2009; Tong et al., 2013; Cao et al., 2015). *In vitro* work led on MCF7 and MDA-MB-231 cells in the present thesis revealed that hypoxia exposure did induce an increase in WSB-1 protein level, as well as mRNA, validating WSB-1 as a valuable protein to study in the context of breast cancer.

A link between WSB-1 and the HIF pathway had already been suggested in previous studies. Benita and colleagues presented WSB-1 as a previously unknown putative HIF-1 target (Benita et al., 2009). More recently, Tong and colleagues identified HRE motifs in the promoter sequence of *WSB1* while studying hypoxia-induced chemoresistance in hepatocellular carcinoma cells (Tong et al., 2013). However, neither of these studies investigated the potential implication of HIF-2 α in WSB-1 regulation in hypoxia. Data presented in this thesis demonstrated that hypoxia-induced *WSB1* upregulation was exclusively mediated by HIF-1 α , and that HIF-2 α played no role in WSB-1 induction in hypoxia (Figure 3.11). Additionally, another study suggested the existence of a feedback loop between WSB-1 and HIF-1 α (Kim et al., 2015). Indeed, Kim and colleagues observed that WSB-1 overexpression in HEK293T cells induced an increase in HIF-1 α protein level, whereas WSB-1 knockdown resulted in a decrease of HIF-1 α protein levels, and explained this by the WSB-1 induced pVHL degradation. The fact that this phenomenon was not observed in breast cancer

cells used in the present study suggests that, in the breast cancer context, WSB-1 might not affect pVHL stability and therefore HIF stability. However, WSB-1 knockdown induced a minor increase in *HIF1A* levels in MCF7 (Figure 3.13) and MDA-MB-231 (Figure A1) which was not transferred to WSB-1 protein levels.

An important factor to take into consideration when studying hypoxia-mediated changes in WSB-1 expression levels, is that WSB-1 expression could also be induced by other stress factors such as starvation and confluency (Archange et al., 2008). To minimise such stresses, and avoid hypoxia-independent WSB-1 upregulation, cells were seeded at such a density that the monolayer would not be confluent by the end of the experiment, and the media was replenished in the dishes before the start of each experiment. In addition, the increase in *WSB1* transcript level following hypoxia exposure greatly varied between repeats. For this reason, the error bars, especially in the controls, tend to be rather large, sometimes masking trends that could have been visible when looking at every single repeats independently. This is notably illustrated by the error bars for the siNT and siHIF-2 α samples in hypoxia in Figure 3.11. The fold change for *WSB1* transcript in response to hypoxia varied for each repeat, but the absence of induction in the siHIF-1 α and siHIF-1 β samples was undeniable every time. This is denoted by the smaller error bars for these samples. Therefore, the fact that the hypoxia-induced WSB-1 upregulation is specifically mediated by HIF-1 α but not HIF-2 α can be ascertained with a certain degree of confidence, despite the results not showing any significance.

3.4.3.3. Transcriptome analysis following WSB-1 knockdown and hypoxia exposure in MDA-MB-231 cells

RNA-Seq is a technique that sequences and quantifies all the mRNA molecules present in a sample, providing a snapshot of a cell's transcriptome at a given time. It was used to obtain a list of all the transcripts modulated by WSB-1 knockdown and hypoxia exposure. This was done in MDA-MB-231 cells as this cell line displayed the most significant changes in the

transcript levels of the HIF target (Figure 3.15).

This analysis provided valuable outlook of the mechanisms in which WSB-1 could be involved. WSB-1 knockdown in MDA-MB-231 resulted in the modulation of processes involved in cellular movement, and an upregulation of cell cycle, cell death, and DNA repair mechanisms (Figure 3.18, Figure 3.19, Table 3.8). In addition, interesting molecules were highlighted in the list of the most up- and downregulated transcripts (Table 3.7). *SPARC* was downregulated over 14-fold in siWSB-1 samples compared to siNT samples. This transcript codes for osteonectin, an extracellular glycoprotein initially reported to be secreted by osteoblasts and described as an anti-adhesive protein (Lane and Sage, 1994; Murphy-Ullrich, 2001). It has already been studied in the context of breast cancer as it is believed to play a role in breast metastasis to the bone, particularly in MDA-MB-231 cells (Sloan and Anderson, 2002; Koblinski et al., 2005; Campo McKnight et al., 2006). In fact, MDA-MB-231 cells treated with exogenous osteonectin demonstrated increased invasiveness *via* the upregulation of MMP2 (matrix metalloproteinase 2) activity and the inhibition of the MMP inhibitor TIMP2 (tissue inhibitors of metalloproteinases) (Gilles et al., 1998). *SPARC* is therefore described as a promoter of cell invasiveness in breast and also in prostate cancer cell lines (Jacob et al., 1999). The fact that *SPARC* was downregulated in MDA-MB-231 where WSB-1 had been knocked down suggests that WSB-1 could in fact promote or regulate the expression of osteonectin, therefore contributing to the increased invasiveness associated with high *WSB1* levels described in the literature and, importantly, in this thesis.

LMNB1 is another molecule of interest, which was upregulated close to 6-fold in cells knocked down for WSB-1 in hypoxia. *LMNB1* codes for lamin B1, a component of the inner nuclear membrane which plays a role in DNA replication (Vergnes et al., 2004; Camps et al., 2015). A recent study suggested that clinical outcome of human breast cancer patients worsened with decreased *LMNB1* expression levels (Wazir et al., 2013). *LMNB1* was also reported as a potential circulating biomarker for colorectal cancer risk and early hepatocellular carcinoma

detection (Marshall et al., 2010; Wong and Luk, 2012). In our model, high WSB-1 would inhibit or repress *LMNB1* expression, which, according to Wazir and colleagues, could signify worse prognosis for the patient; corroborating again the studies presenting WSB-1 as a promoter of cancer progression, particularly in hepatocellular carcinoma by Tong and colleagues (Tong et al., 2013; Wazir et al., 2013).

Previous studies suggested that increased invasiveness was obtained at the expense of proliferation (Vega et al., 2004; Matus et al., 2015). This phenomenon has not only been described in early development but also during tumour progression in several cancer types, including melanoma, colorectal cancer, basal cell carcinoma and mammary tumours (Svensson et al., 2003; Wang et al., 2004; Hoek et al., 2008; Rubio, 2008; Patsos et al., 2010). This data is in agreement with the results presented in this chapter, whereby an activation of cellular growth and proliferation (Figure 3.18) and an upregulation of cell cycle proteins (Figure 3.19) are observed on the one hand, and a downregulation of MMPs (Figure 3.16) and a mild inhibition of cellular movement (Figure 3.18) are seen on the other hand.

To summarise, this chapter revealed a possible link between WSB-1 levels and breast cancer invasiveness. Indeed, WSB-1 knockdown resulted in a decrease of transcript levels of several MMPs and other genes associated with invasiveness (*SPARC*, *IL24*), and an activation of processes and pathways associated with cell movement. In addition, KM plot analyses indicated high WSB-1 levels were frequently associated with statistically significant poor prognosis for patients. These findings suggest that WSB-1 could in fact play a role in breast cancer invasiveness. Therefore, the following chapter will focus on this specific aspect and investigate the role of WSB-1 in cell motility and invasiveness in breast cancer.

Chapter 4

Role of WSB-1 in cell motility and invasiveness in breast cancer

4. Role of WSB-1 in cell motility and invasiveness in breast cancer

4.1. Introduction

Increased motility is one of the hallmarks of cancer described by Hanahan and Weinberg (2000, 2011). Previously published research described a correlation between WSB-1 levels and neuroblastoma, pancreatic cancer, and osteosarcoma progression (Chen et al., 2006; Archange et al., 2008; Cao et al., 2015). Importantly, analysis of breast cancer cells transcriptome following WSB-1 knockdown in hypoxia presented in the previous chapter highlighted "cellular movement and migration" as a pathway significantly regulated by WSB-1. Therefore, further investigation of the role of WSB-1 in breast cancer cell **migration** (ability to move) and **invasion** (capacity to degrade extra-cellular matrix and progress in a 3D environment) abilities is needed. Study of cell migration and invasion needs to take into account a wide range of factors, including acquisition of specific characteristics that allow increased cell motility, degradation of the surrounding environment (*i.e.* the extracellular matrix (ECM)), and expression of motility-specific factors. Importantly, *in vitro*, cell behaviour varies with condition such as the method of culture: whether cells are grown as a monolayer or in a 3D structure such as spheroids, or the type of matrix surrounding them, if any.

A key aspect of malignant transformation is the epithelial to mesenchymal transition (**EMT**) (Kalluri and Weinberg, 2009). This change in phenotype is characterised by a loss of cell polarity and other characteristics of epithelial cells, in favour of a more mesenchymal phenotype. Some key factors implicated in this transition are used as EMT markers and monitoring of their level of expression can give indication on the position of the cells in this transition process. E-cadherin is one of these markers (Yang and Weinberg, 2008). This glycoprotein plays a part in the forming of adherens junctions and is inhibited by the Snail family of transcription factors (*SNAI1* and *SNAI2*) (Cano et al., 2000). E-cadherin expression has been investigated in the context of breast cancer. Several studies focused on the

association between E-cadherin levels and ER status, as well as HER2 expression, although no clear conclusion were made (Parker et al., 2001; Ye et al., 2010; Zhou et al., 2015). Loss of E-cadherin is usually characteristic of a poor prognosis and increased metastatic potential in breast cancer (Berx and Van Roy, 2001). As a result, it would be interesting to validate whether changes in WSB-1 expression have an impact on the EMT profile of breast cancer cells *in vitro*.

Cells are surrounded by an extracellular matrix composed of a network of cross-linked fibrous proteins (Figure 4.1). Besides **collagens**, the ECM also comprises fibronectin, **laminin**, hyaluronic acid and proteoglycans (Theocharis et al., 2015). There are twenty-eight types of collagen: some widely expressed in most tissues, others more specialised (Table 4.1).

Several enzymes are responsible for the regulation of the ECM structure. The main group is the **matrix metalloproteinases** (MMPs). They were discovered in 1962 by Gross and Lapiere and are responsible for degrading the proteins of the ECM and disrupting its architecture (Gross and Lapiere, 1962). Twenty-three types of MMPs have been identified in humans, each with different substrates (Table 4.2). Some MMPs are specialised in collagens degradation (MMP1, 8, 13), whereas others have the ability to degrade gelatine (MMP2, 9) (Löffek et al., 2011). Most MMPs are secreted as zymogens (pro-MMPs) and activated extracellularly, some are activated within the cell and released in the extracellular space, and one category is membrane bound (MMP14-17, 24, 25) (Visse and Nagase, 2003; Itoh, 2015). Some MMPs can also activate other members of the family by cleaving the prodomain off the zymogen (Toth et al., 2003; Geurts et al., 2012). Interestingly, Köhrmann and colleagues demonstrated that MMP1, 2, 8-13, 15, 19, 23, 24, 27, and 28 were upregulated in breast cancer tissues compared to normal breast tissues, and, more recently, the group of Zhang showed that MMP2, 7, 9, and 14 transcripts were also significantly upregulated in breast cancer tissues compared to normal breast tissues (Köhrmann et al., 2009; Zhang et al., 2013b). Other molecules mediating ECM breakdown include the adamalysins, the meprins and the tissue inhibitors of

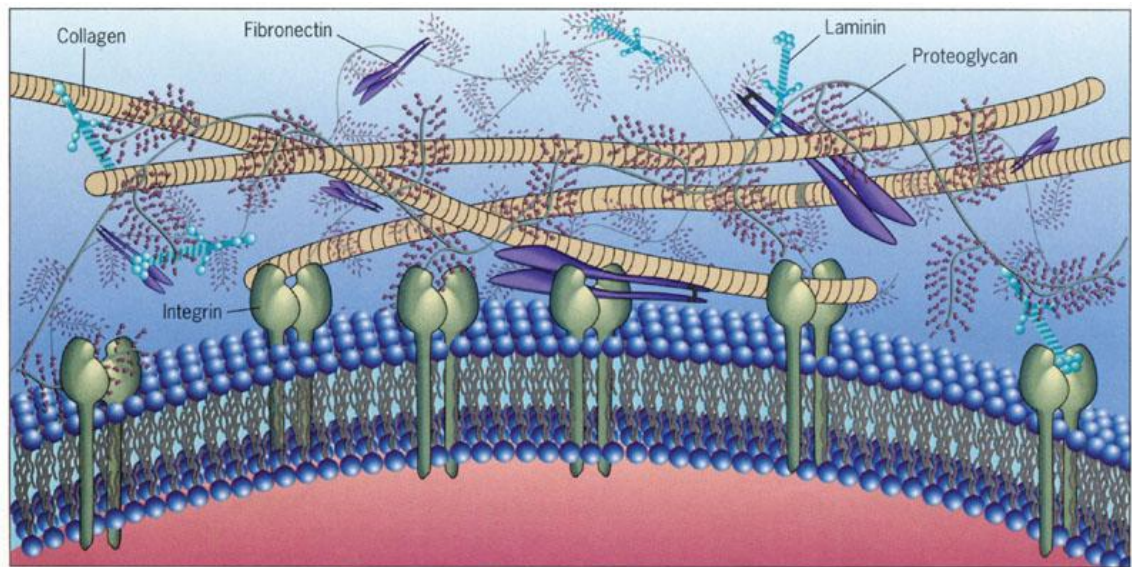


Figure 4.1: Diagram of the macromolecular organisation of the extracellular matrix

The extra-cellular matrix is a complex network composed of numerous fibrous proteins which interact with the intracellular milieu through the integrins. From Karp (2009).

Table 4.1: List of collagen types and main tissues of expression**Adapted from (Olsen et al., 2003; Shoulders and Raines, 2009; Ricard-Blum, 2011)**

Collagen type	Tissues
I	Most connective tissues: bones, skin, tendon, blood vessels, ...
II	Cartilage and vitreous of the eye
III	Blood vessels
IV	Basement membranes in all organs
V	Tendons, cornea, and interstitial tissues
VI	Liver, kidney, perichondrium
VII	Epidermal/dermal junction
VIII	Endothelial cells
IX	Cartilage
X	Hypertrophic and mineralizing cartilage
XI	Cartilage
XII	Tendons and fibril associated collagen
XIII	Epidermis, hair follicles, nail root cells
XIV	Most connective tissues: bones, skin, tendon, blood vessels, ...
XV	Many tissues, including liver, kidney
XVI	Skin, kidney
XVII	Hemidesmosomes, skin
XVIII	Liver, kidney
XIX	Eyes, brain, testes, basement membranes, embryonic tissues
XX	Cornea
XXI	Stomach, kidney
XXII	Tissue junctions
XXIII	Heart, retina
XXIV	Bone, cornea
XXV	Brain, heart, testis
XXVI	Testis, ovary
XXVII	Cartilage
XXVIII	Skin, sciatic nerve

Table 4.2: List of principal types of MMPs and their main substrates

Type MMP	Alternative name	Extracellular matrix substrate
<u>Collagenases</u>		
MMP1	Collagenase 1	Collagen I, II, III, VIII, X, gelatine, proteoglycans, tenascin, entactin, MMP2, MMP9, pro-MMP9
MMP8	Collagenase 2	Collagen I, II, II, V, VIII, X, aggrecan
MMP13	Collagenase 3	Collagen I, II, III, IC, IX, X, XIV, gelatine, tenascin, fibronectin, aggrecan, osteonectin, MMP9
<u>Gelatinases</u>		
MMP2	Gelatinase A	Collagen I, IV, V, VII, X, XIV, gelatine, elastin, fibronectin, laminin, aggrecan, versican, osteonectin, proteoglycans
MMP9	Gelatinase B	Collagen IV, V, VII, X, XIV, gelatine, elastin, aggrecan, versican, proteoglycans, osteonectin
<u>Stromelysins</u>		
MMP3	Stromelysin 1	Collagen II, IV, V, IX, gelatine, aggrecan, versican, tenascin, proteoglycan, fibronectin, laminin, osteonectin, pro-MMP9
MMP10	Stromelysin 2	Collagen III, IV, V, gelatine, casein, aggrecan, elastin, proteoglycans
MMP11	Stromelysin 3	Collagen IV, casein, laminin, fibronectin, gelatine, transferrin
<u>Membrane type</u>		
MMP14	MT1-MMP	Collagen I, II, III, casein, elastin, fibronectin, vitronectin, tenascin, proteoglycans, laminin, entactin, pro-MMP2
MMP15	MT2-MMP	Tenascin, fibronectin, laminin, pro-MMP2
MMP16	MT3-MMP	Collagen III, gelatine, casein, fibronectin, pro-MMP2
MMP17	MT4-MMP	Gelatine, fibrinogen, pro-MMP2
MMP24	MT5-MMP	Fibronectin, proteoglycans, gelatine, pro-MMP2
MMP25	MT6-MMP	Collagen IV, gelatine, fibronectin, pro-MMP2, pro-MMP9
<u>Others</u>		
MMP7	Matrilysin	Collagen IV, X, gelatine, aggrecan, proteoglycans, fibronectin, laminin, entactin, tenascin, casein, transferrin, integrin β 4, osteonectin, elastin
MMP12	Metalloelastase	Collagen IV, gelatine, elastin, casein, laminin, proteoglycans, fibronectin, vitronectin, entactin
MMP19	N/A	Collagen I, IV, gelatine, fibronectin, laminin
MMP20	Enamelysin	Amelogenin
MMP23A	MMP21	<i>Pseudogene: partially duplicated copy of MMP23B</i>
MMP23B	MMP22, MMP23	Gelatine
MMP26	Matrilysin 2	Collagen IV, fibrinogen, fibronectin, casein, pro-MMP9
MMP27	N/A	N.D.
MMP28	Epilysin	Casein
MMP: Matrix metalloproteinase; N/A: not applicable; N.D.: not determined.		

metalloproteinases (TIMPs). The adamalysins comprise the **ADAMs** (A disintegrin and metalloproteinase) family of transmembrane proteins and the secreted proteinases **ADAMTS** (A disintegrin and metalloproteinase with thrombospondin motifs). The **meprins** are coded by two genes resulting in a secreted α subunit and a transmembrane β subunit which can form homo- or heterodimers and are able to cleave collagen IV and fibronectin (Bonnans et al., 2014). Meprins also contribute to MMP3-mediated MMP9 activation (Geurts et al., 2012). Finally, there are four types of **TIMPs**, which are able to inhibit the catalytic activity of ADAMs, ADAMTS and MMPs, but not meprins (Bonnans et al., 2014).

Integrins form the link between the extracellular matrix and the intracellular signalling. There are twenty-four different heterodimeric integrin receptors resulting from a combination of eighteen α subunits and eight β subunits (Hynes, 2002). Integrins can be arranged in four groups depending on the type of elements they recognise (Figure 4.2). Certain integrins have been reported to play an important role in promoting or suppressing cancer progression. For example, integrin $\alpha V\beta 3$, which promotes cell migration and activates MMP2, appeared to be required for metastasis in breast cancer; whereas $\alpha 2\beta 1$ was reported to be downregulated in breast cancer patients (Zutter et al., 1995; Brooks et al., 1996; Felding-Habermann et al., 2001). Once bound to the correct ligand, integrins trigger specific pathways, such as survival, proliferation, and migration (Guo and Giancotti, 2004).

This chapter will aim to answer the following questions:

- What is the effect of WSB-1 on the expression of proteins associated with the ECM architecture in hypoxia?
- Does WSB-1 function affect the expression, activation or activity of matrix metalloproteinases?
- Does WSB-1 function affect the engagement of EMT?
- How is cell motility modulated by WSB-1?

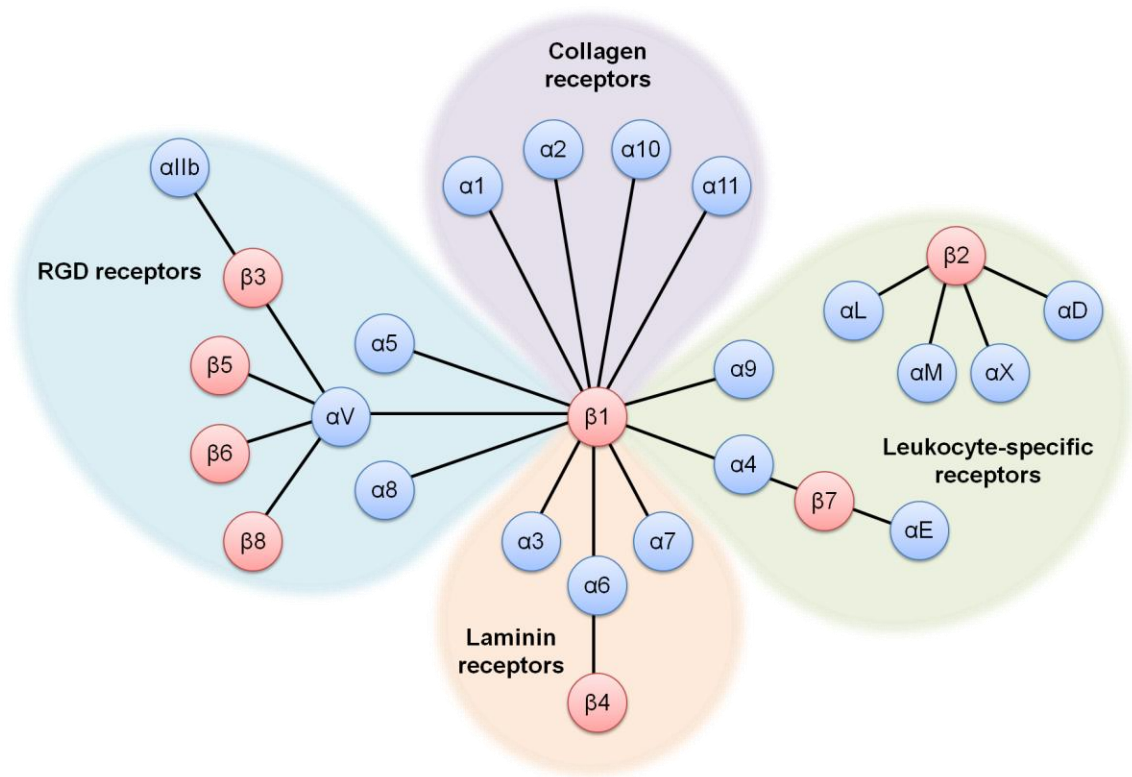


Figure 4.2: Map of interaction between the different integrin subunits

Integrins form α/β heterodimers in response to binding certain molecules (coloured areas). This dimerisation allows the transduction of a signal within the cell and activates specific pathways, depending on the dimer involved. RGD: Arg-Gly-Asp motif, notably present in vitronectin, fibronectin, thrombospondin.

4.2. Experimental design

4.2.1. Monitoring of MMP expression and activity

In order to further analyse the effect of WSB-1 knockdown on breast cancer cells invasiveness, the expression and activity of MMPs was monitored using qPCR, Western blot and gelatine zymography. MMPs such as MMP2 have the ability to degrade gelatine and their activity can be evaluated using gelatine zymograms (Toth et al., 2012). In brief, conditioned media from MDA-MB-231 cells treated with WSB-1 or non-targeting siRNA was concentrated and separated through a non-denaturing acrylamide-gelatine gel. Proteases present in the conditioned media were then able to digest the gelatine incorporated in the gel and visualised. To account for transmembrane MMPs (MT-MMPs) such as MMP14 (MT1-MMP), Western blot was performed using conditioned media as well as whole cell lysates.

Because MCF7 are not an invasive cell line, their endogenous MMP levels are low (Köhrmann et al., 2009). Therefore, these experiments were only done using MDA-MB-231 cells.

4.2.2. Expression of epithelial to mesenchymal transition markers

Epithelial to mesenchymal transition (EMT) is at the centre of cancer cell invasiveness (Yang and Weinberg, 2008). This transformation is driven by key transcription factors such as *SNAIL1*, which regulate the expression of proteins, notably involved in the formation of cell-cell junctions and ECM degradation (section 1.1.3; Hanahan and Weinberg, 2011; Lamouille et al., 2014). Some of these proteins are considered EMT markers and changes in their expression in response to WSB-1 knockdown and exposure to 2% O₂ for 24h was detected by Western blot.

4.2.3. Analysis of cell migratory ability on a 2-dimensional support

Two-dimensional cell migration was analysed using wound-healing assays. This technique allows the monitoring of cell migration over a plastic surface and benefits from an easy setup and a cost-effectiveness (Liang et al., 2007). Here, a monolayer of cells treated with WSB-1 or non-targeting siRNA was scratched using a sterile pipette tip. The widths of the wounds were

measured immediately after the scratch and again after 16h exposure to normoxic or hypoxic conditions. The wound closure was obtained by calculating the ratio between the width at the beginning and at the end of the experiment.

4.2.4. Analysis of single cell invasiveness through Matrigel

Single cell invasion was studied using Transwell assays. This *in vitro* method takes into consideration the existence of an extracellular matrix by seeding cells in an ECM-like matrix, such as Matrigel, as used in this study. Schematic of the experiment layout is visible in Figure 4.3.

4.2.5. Assessing changes in cellular motility using spheroids as 3D models

Spheroids are compact aggregates of cells held together in spherical shapes and usually presenting a necrotic core and a hypoxia gradient (Vinci et al., 2012). In this study, spheroids were formed by seeding MCF7 or MDA-MB-231 cells in ultra low adherence, round bottom, 96-well plates (Cheng et al., 2015). Spheroids were then either embedded in Matrigel or gelatine, and size and invasion or migration into the matrix, respectively, were monitored. A flowchart of the experimental process is presented in Figure 4.4.

4.3. Results

4.3.1. Effect of WSB-1 knockdown and hypoxia exposure on proteases expression, activation and activity

The RNA-Seq transcriptomic analysis presented in the previous chapter indicated "cellular movement" as an important biological function altered by WSB-1 knockdown in hypoxia (Figure 3.17). In this chapter, the effect of WSB-1 knockdown in hypoxia on the expression level of matrix metalloproteinases (MMP), integrins and collagens was analysed more closely. Table 4.3 presents the fold change and *p*-values of these transcripts, which presented a significant difference in expression between the siNT and siWSB-1 samples in hypoxia.

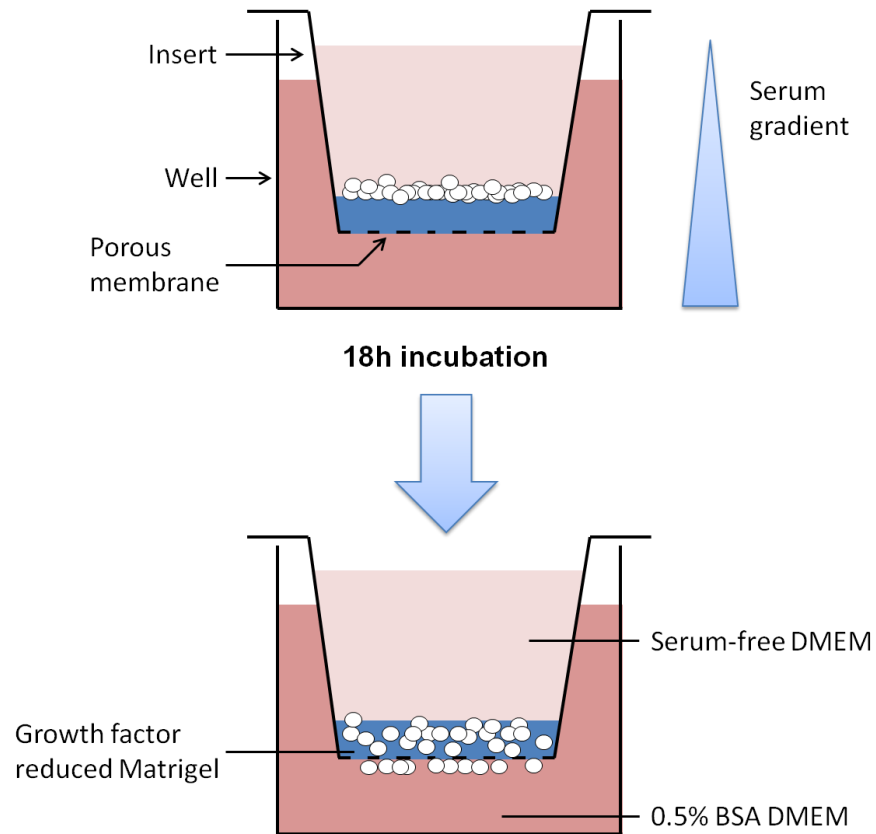


Figure 4.3: Diagram of the experimental layout for the invasion (Transwell) assays

Cells are seeded in the upper chamber (insert) in serum-free media. After 18h of incubation, some cells migrated through the Matrigel (blue) and crossed the porous membrane at the bottom of the inserts. Migration of the cells is triggered by a serum gradient between the upper chamber and the well.

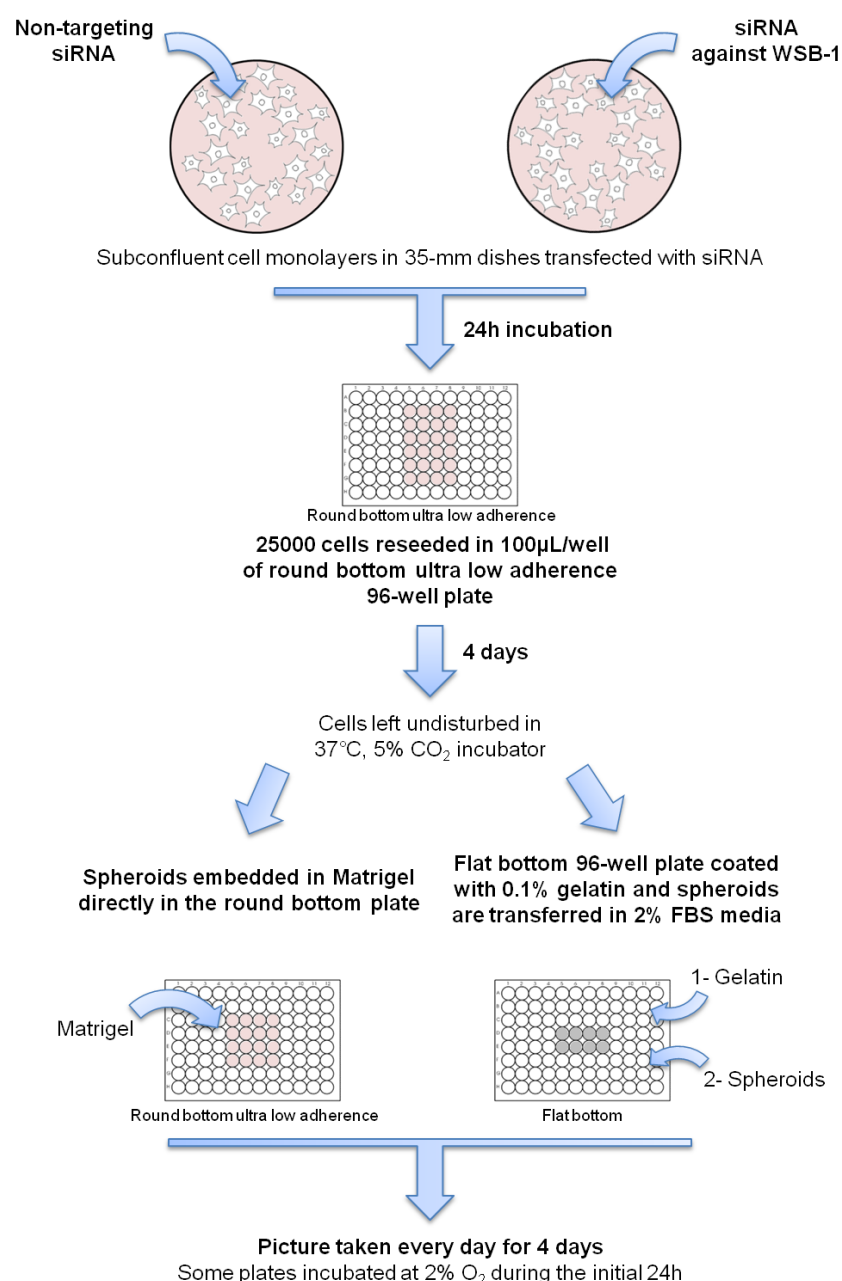


Figure 4.4: Flowchart summarising the setup of spheroid models

Cells are seeded in 35mm diameter dishes and are transfected with siRNA the next day. Cells are then trypsinised, counted and reseeded in 96-well plates (25000 cells in 100µL per well). Cells are left undisturbed for 4 days to allow spheroid formation. Spheroids are then either directly embedded in Matrigel in the plate or transferred on gelatine-coated wells. Each spheroid is photographed 0h, 24h, 48h and 72h after first contact with matrix.

Table 4.3: Fold change of collagen, metalloproteinase, laminin and integrin transcripts in response to WSB-1 knockdown in hypoxia in MDA-MB-231 cells

Gene Name	Log2(fold change)	Fold change	p-value
<u>Collagens</u>			
<i>COL4A5</i>	-1.16	-2.24	5.00E-05
<i>COL5A1</i>	-0.78	-1.71	5.00E-05
<i>COL17A1</i>	-0.71	-1.64	6.15E-03
<i>COL27A1</i>	-0.67	-1.59	4.25E-03
<u>Matrix metalloproteinases</u>			
<i>MMP1</i>	-1.05	-2.07	5.00E-05
<i>MMP9</i>	-2.37	-5.15	1.00E-04
<i>MMP11</i>	-0.94	-1.91	9.00E-04
<i>MMP14</i>	-0.79	-1.73	5.00E-05
<i>MMP17</i>	-2.65	-6.26	2.00E-04
<u>A disintegrin and metalloproteinases</u>			
<i>ADAM8</i>	-0.82	-1.77	5.00E-05
<u>A disintegrin and metalloproteinase with thrombospondin motifs</u>			
<i>ADAMTS1</i>	0.93	1.91	1.00E-04
<i>ADAMTS6</i>	-1.25	-2.38	5.00E-05
<i>ADAMTS9</i>	-1.86	-3.63	5.00E-05
<i>ADAMTS12</i>	-1.42	-2.67	5.00E-05
<i>ADAMTS14</i>	-1.90	-3.72	5.00E-05
<i>ADAMTS16</i>	-0.73	-1.66	9.00E-04
<u>Laminins</u>			
<i>LAMB1</i>	-0.57	-1.48	1.25E-03
<i>LAMC1</i>	0.83	1.77	5.00E-05
<i>LAMC2</i>	-0.77	-1.71	5.00E-05
<u>Integrins</u>			
<i>ITGA2</i>	-2.13	-4.37	5.00E-05
<i>ITGA6</i>	-0.87	-1.83	5.00E-05
<i>ITGAV</i>	-1.24	-2.37	5.00E-05
<i>ITGB1</i>	1.57	2.97	5.00E-05
<i>ITGB3</i>	1.07	2.10	5.00E-05
<i>ITGB8</i>	-0.65	-1.57	6.00E-03

Transcripts for **collagens** IV α 5, V α 1, XVII α 1, and XXVII α 1 were downregulated in absence of WSB-1 in hypoxia. The transcripts for the **MMPs** significantly represented, namely MMP1, 9, 11, 14 and 17, were also downregulated in response to WSB-1 knockdown. Another family of metalloproteinases, the A disintegrin and metalloproteinases (**ADAM**) and A disintegrin and metalloproteinases with thrombospondin motifs (**ADAMTS**) were also generally

downregulated in WSB-1 knocked down samples in comparison to the control samples. Transcripts coding for **laminins** (fibrous proteins constituent of the extracellular matrix) were significantly modified by WSB-1 knockdown in hypoxia: laminin β 1 (*LAMB1*) and γ 2 (*LAMC2*) were downregulated, whereas laminin γ 1 (*LAMC1*) was upregulated. Finally, **integrins** α 2, α 6, α V, and β 8 were downregulated, whereas integrins β 1 and β 3 were upregulated in response to WSB-1 knockdown. These integrins form dimers that interact with collagens, laminins and extra-cellular matrix molecules containing RGD motifs (Figure 4.2).

Since the transcript level of several MMPs appeared to be altered in the RNA-Seq analysis, the effects of WSB-1 knockdown on the transcript levels of MMP1, MMP7, and MMP14 were reanalysed by qPCR (Figure 4.5) in MDA-MB-231 cells. MMP1 and MMP14 transcript levels were clearly, although not significantly, decreased following WSB-1 knockdown. The absence of evident effect of siWSB-1 on MMP7 transcript levels can be due, in part, to a very low endogenous expression making any modifications in the signal hard to detect.

In summary, a decrease in transcript and mature protein levels of MMP1 and MMP14 was observed following WSB-1 knockdown. In addition, gelatine zymography revealed a decrease in MMP2 general level in response to WSB-1 knockdown in hypoxia.

Whole MDA-MB-231 cell lysates were also analysed by Western blot in order to monitor the protein level of the transmembrane protein MMP14, (Figure 4.6). Although WSB-1 knockdown did not appear to induce a change in pro-MMP14 levels, mature MMP14 showed an important decrease in the siWSB-1 samples compared to their siNT counterparts. In addition, it is important to note that MMP14 levels (both pro and mature forms) appeared to be upregulated by hypoxia exposure.

The conditioned media was also analysed by Western blot to evaluate the presence of MMPs in the cells. Proteins levels of MMP1 appeared to decrease with WSB-1 knockdown (Figure 4.7) whereas the antibody used failed to retrieve any signal for MMP2. Pro-MMP2 is known to be a target of and activated by MMP14. The gelatinase activity of MMP2 was monitored using

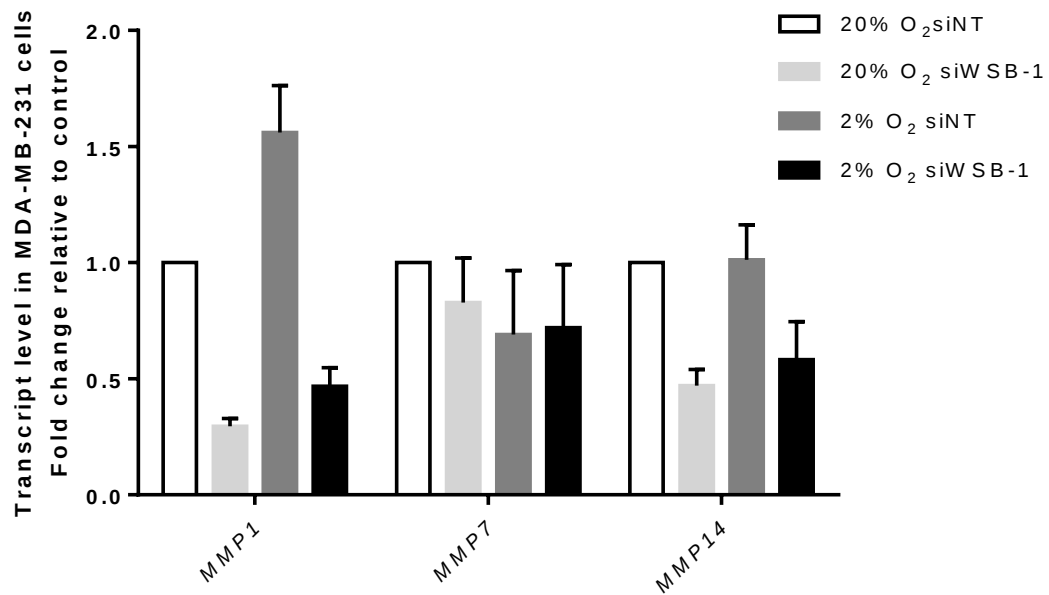


Figure 4.5: Effect of WSB-1 knockdown and moderate hypoxia on transcript levels of several matrix metalloproteinases

Cells were transfected with siRNA against WSB-1 and incubated 24h in normoxia or hypoxia (2% O₂). Transcript levels for *MMP1*, *MMP7* and *MMP14* were analysed by qPCR using SYBR Green. Housekeeping gene used was *B2M*. Error bars represent mean ± SEM. Statistical significance determined by 2-way ANOVA with Tukey correction for multiple comparisons. *MMP1*, *MMP7*: *n*=3; *MMP14*: *n*=4.

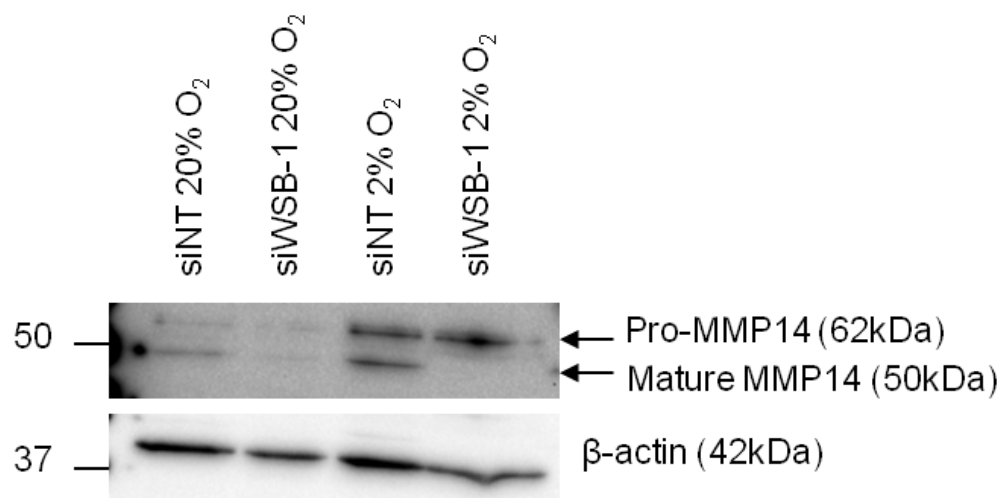


Figure 4.6: Effect of WSB-1 knockdown and hypoxia exposure on MMP14 protein levels
MDA-MB-231 cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT) and exposed to 20% O₂ or 2% O₂ for 24h in serum-free media. Whole cell lysates were prepared and 30 μ g of protein were analysed by Western blot for the expression of MMP14. This figure is representative of $n=5$ experiments.

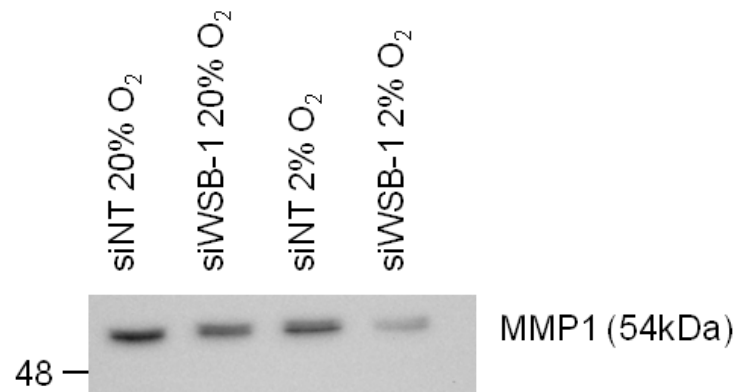


Figure 4.7: Effect of WSB-1 knockdown and hypoxia exposure on MMP1 protein levels
MDA-MB-231 cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT) and exposed to 20% O₂ or 2% O₂ for 24h in serum-free media. Conditioned media was collected and concentrated. Samples were analysed by Western blotting for the presence of MMP1. Blot is representative of $n=5$ experiments.

gelatine zymography. The zymogram shows the enzymatic activity of pro and mature MMP2 secreted by MDA-MB-231 cells grown in serum-free media (Figure 4.8). Due to the non-denaturing properties of the gel in which the conditioned media samples were run, zymogens (pro-form of the MMPs) were able to demonstrate a gelatinase activity. Therefore, the highest molecular weight band on the zymogram corresponds to the pro-MMP2 form, and the lower molecular weight band corresponds to the mature form of MMP2. The intensity of the highest molecular weight band decreased with WSB-1 knockdown, suggesting a decrease in MMP2 levels in response to a decrease in WSB-1. This was observed at both 20% and 2% O₂. A decrease in the intensity of the mature MMP2 band was also noticeable in response to hypoxia, whereas no effect of WSB-1 knockdown was seen at either 20% or 2% O₂. However, considering that the level of pro-MMP2 was lower in the siWSB-1 samples, this would mean that there was in fact more efficient activation of MMP2 in absence of WSB-1.

4.3.2. Modification in EMT markers expression in response to WSB-1 knockdown and hypoxia exposure

The effect of WSB-1 knockdown on ZO-1, E-cadherin and vimentin (known EMT markers) was evaluated in MCF7 and MDA-MB-231 using Western blotting (Figure 4.9). **ZO-1** belongs to a family of proteins associated with tight junctions. It is therefore an EMT marker indicative of an epithelial-like phenotype. ZO-1 expression in hypoxia was increased in siWSB-1 samples when compared to their siNT counterparts in both cell lines, although hypoxia alone markedly decreased ZO-1 levels. In addition, a similar upregulation pattern, albeit weaker, was seen in normoxia in MDA-MB-231. **E-cadherin** is a protein involved in cell-cell adhesion and used as a marker of epithelial phenotype. It is not expressed in MDA-MB-231 cells, but is present in MCF7 cells. In Figure 4.9, E-cadherin expression was seen to decrease upon WSB-1 knockdown for both exposures to 20% O₂ as well as 2% O₂. Conversely, **vimentin** is a protein of the cytoskeleton specific of mesenchymal-like cells, which is normally expressed by MDA-MB-231 but not by MCF7. Western blot analyses revealed a decrease in vimentin levels

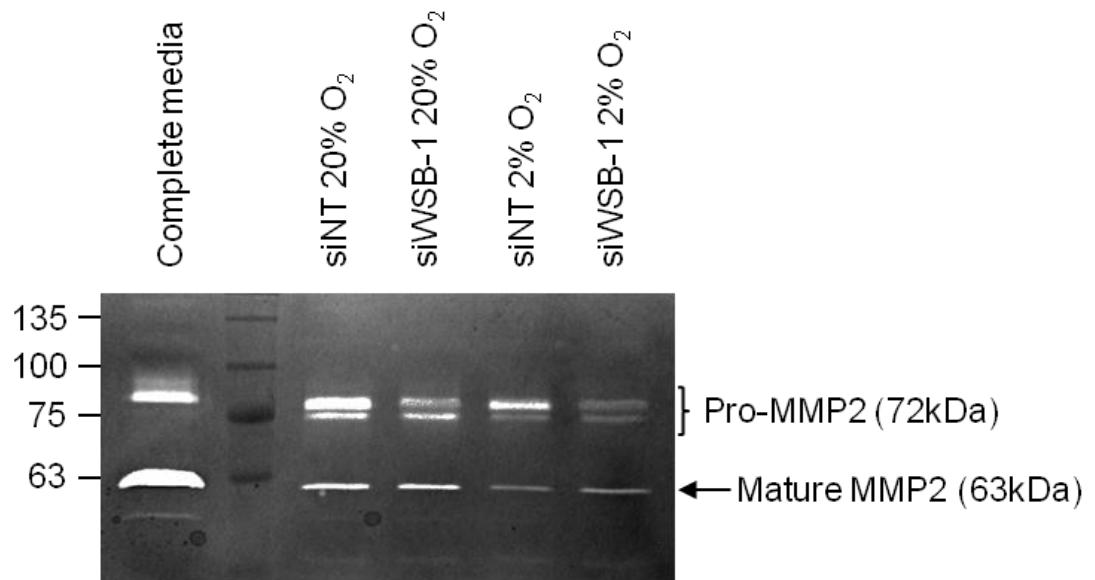


Figure 4.8: Effect of WSB-1 knockdown and hypoxia exposure on MMP2 expression, activation and activity

Following treatment with WSB-1 (siWSB-1) or non-targeting (siNT) siRNA, MDA-MB-231 cells were exposed to normoxia or hypoxia for 24h in serum-free media. Conditioned media was collected, concentrated was separated through a non-denaturing 10% acrylamide gel containing 1mg/mL gelatine. Complete media (DMEM supplemented with 10% FBS) was used as a control. Zymogram is representative of $n=5$ experiments.

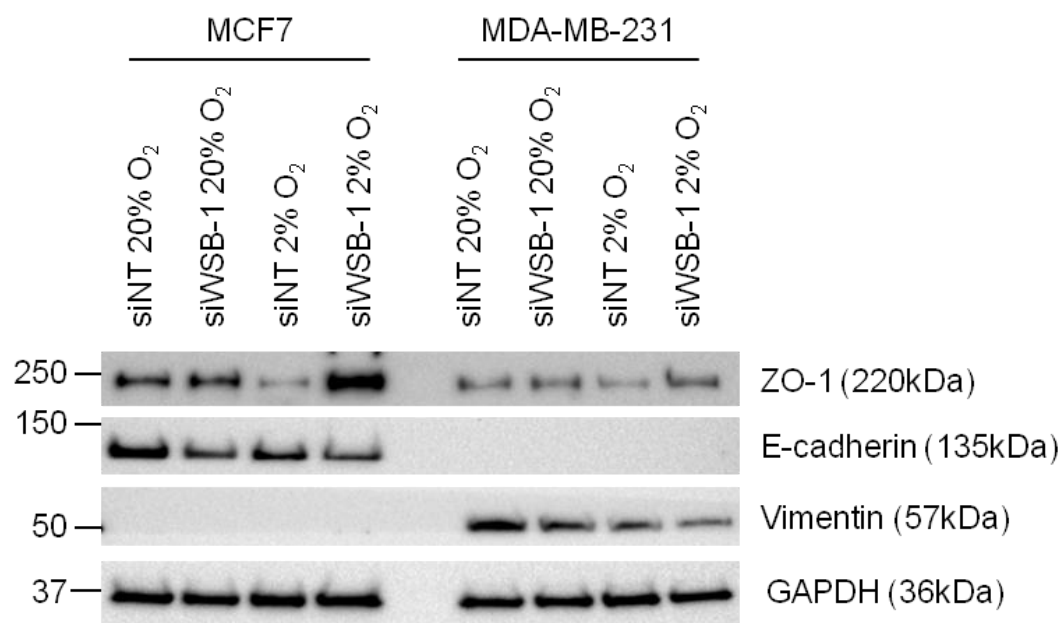


Figure 4.9: Expression of EMT markers in response to WSB-1 knockdown

MCF7 and MDA-MB-231 cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT) and exposed to 20% O₂ or 2% O₂ for 24h. Western blot of whole cell lysates (30µg protein/well) was then performed, probing for ZO-1, E-cadherin and vimentin. Figure representative of $n=4$ experiments.

In MDA-MB-231 cells treated with siWSB-1 compared to siNT.

In addition, the effect of WSB-1 knockdown on Snail transcript levels was analysed by qPCR in both cell lines (Figure 4.10). **Snail** is coded by *SNAI1* and is a transcription factor promoting epithelial to mesenchymal transition (EMT) by inhibiting the expression of epithelial-specific proteins such as E-cadherin. WSB-1 knockdown induced an upregulation of *SNAI1*, albeit non significant, in MDA-MB-231 cells (Figure 4.10A). However, no effect of WSB-1 knockdown on *SNAI1* was observed in MCF7 cells (Figure 4.10B).

In summary, WSB-1 knockdown appeared to induce alterations of EMT markers to different degrees in both cell lines studied.

4.3.3. Impact of WSB-1 knockdown on cell motility

4.3.3.1. Cellular migration

In order to assess the effect of WSB-1 knockdown on cellular migration in hypoxia, the method of wound healing assays was used (section 4.2.3). MCF7 and MDA-MB-231 cells were first transfected with siRNA against WSB-1 (siWSB-1) or non-targeting siRNA (siNT). The efficiency of the knockdown was confirmed by Western blotting (Figure 4.11A, C). Representative images of the cell monolayer immediately after scratching and 16h later are also presented (Figure 4.11B, D).

The effect of hypoxia exposure and WSB-1 knockdown on the migratory ability of MCF7 and MDA-MB-231 cells was then evaluated. Figure 4.12 depicts the evaluation of changes in cell motility when exposed to 2% O₂ in the presence or absence of WSB-1. Whereas no significant alterations could be seen for MDA-MB-231 cells, MCF7 migration ability significantly dropped when cells were both exposed to moderate hypoxia and knocked down for WSB-1 (Figure 4.12). Interestingly, hypoxia exposure or WSB-1 knockdown alone did not induce any alteration in cell migration for either cell line.

The same experiment was reproduced at 0.5% O₂ in an attempt to trigger a more important

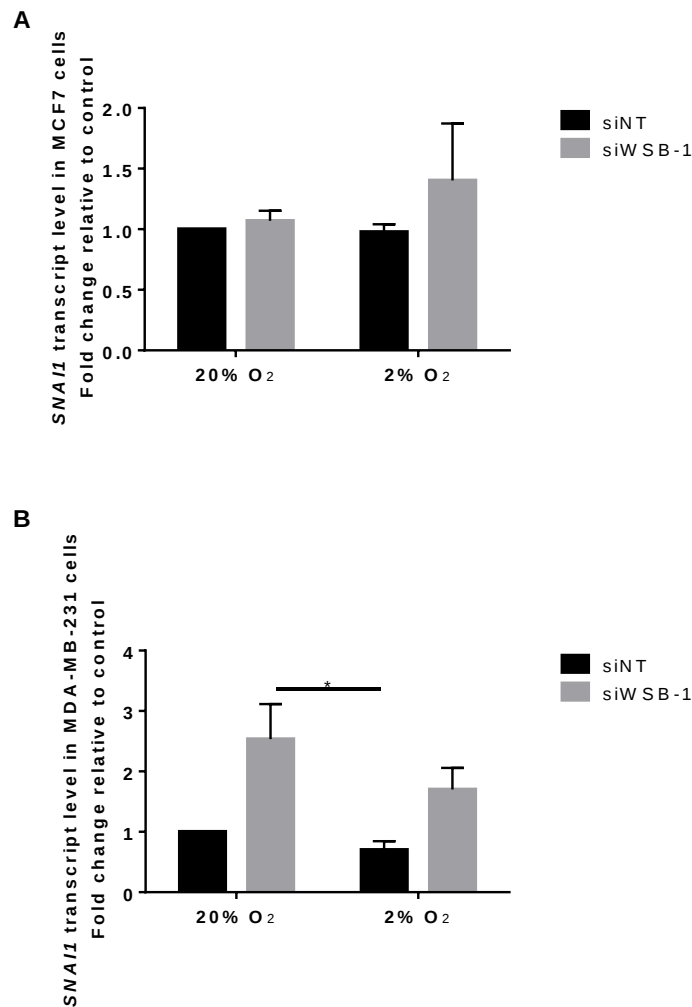


Figure 4.10: Effect of WSB-1 knockdown and moderate hypoxia on *SNAI1* transcript levels in MCF7 and MDA-MB-231

MCF7 (A) and MDA-MB-231 (B) cells were transfected with siRNA against WSB-1 (siWSB-1) of non-targeting (siNT) and incubated 24h in normoxia or hypoxia (2% O₂). Transcript levels for Snail (*SNAI1*) were analysed by qPCR using SYBR Green. Housekeeping gene used was *B2M*. Data represent the mean of $n=3$ experiments. Error bars represent mean $\pm$ SEM, statistical significance determined by 2-way ANOVA with Tukey correction for multiple comparisons.

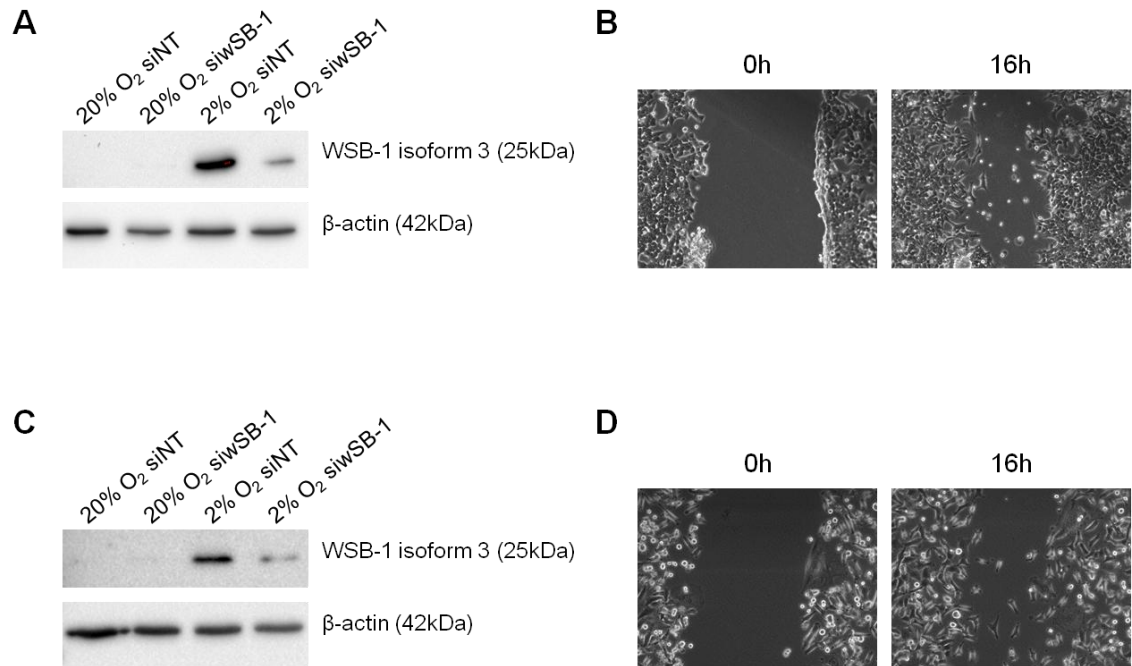


Figure 4.11: Efficiency of the WSB-1 knockdown and representative picture of wound-healing over 16h

Western blot used to demonstrate efficiency of the WSB-1 knockdown on MCF7 (A) and MDA-MB-231 (C) cells. MCF7 (A, B) and MDA-MB-231 (C, D) cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting siRNA (siNT) and incubated 24 hours in normoxia (20% O₂) or hypoxia (2% O₂). Pictures representative of wound closure from MCF7 (B) and MDA-MB-231 (D) cells treated with siRNA against WSB-1 immediately after scratching and 16h of incubation in normoxia later.

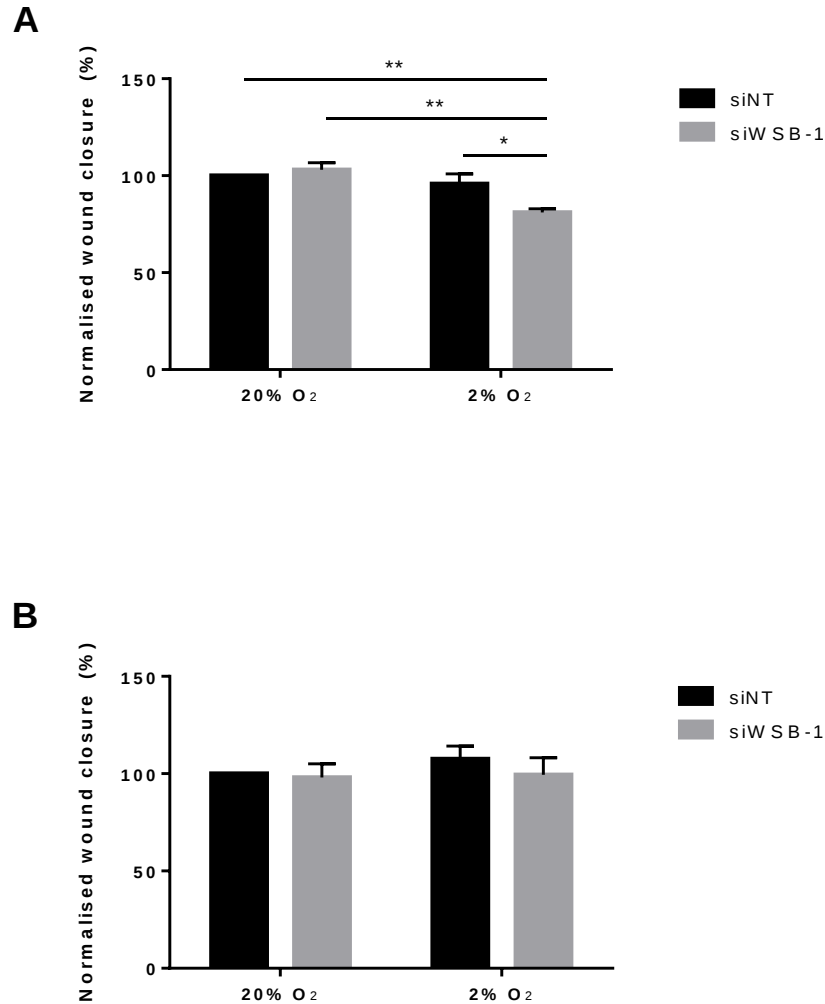


Figure 4.12: Effect of moderate hypoxia and WSB-1 knockdown on the migration ability of breast cancer cells

MCF7 (A) and MDA-MB-231 (B) cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). Cells were grown to confluency, and cell monolayers were scratched using a pipette tip. Cells were incubated in normoxia (20% O₂) or moderate hypoxia (2% O₂) for 16 hours. Wound images were taken immediately after scratching and post incubation. Data represents the mean of $n=4$ (MCF7) and $n=5$ (MDA-MB-231) experiments. Error bars represent mean $\pm$ SEM. *, $p<0.05$; **, $p<0.01$. Statistical significance was determined by 2-way ANOVA with Tukey correction for multiple comparisons.

response (Figure 4.13). Contrary to what was observed at 2% O₂, WSB-1 knockdown induced an increase in MDA-MB-231 migration, albeit not significantly. WSB-1 knockdown did not induce any change in MCF7 cells migration ability. However, surprisingly, exposure to hypoxia alone (siNT samples) resulted in a decrease in cell migration, which was not observed in any other instance.

4.3.3.2. Cellular invasion

Ability of cells to migrate through a matrix was studied using Transwell invasion assays coated with growth factor reduced Matrigel on which MCF7 or MDA-MB-231 cells were seeded (Figure 4.3). Control inserts without matrix were also used to normalise the data. To calculate the invasion indices presented, the number of cells that successfully crossed the Matrigel layer to reach the bottom of the insert was divided by the number of cells on the bottom on the control inserts. This ratio was then expressed as a fold change from the 20% O₂ siNT control condition to obtain the invasion index.

Invasion indices of MCF7 and MDA-MB-231 cells subjected to WSB-1 knockdown and/or 2% O₂ exposure are presented in Figure 4.14. For MCF7 cells, hypoxia exposure, WSB-1 knockdown, or combination of the two increased invasiveness, albeit not significantly. Interestingly, MDA-MB-231 cells invasiveness was negatively affected by WSB-1 knockdown and hypoxia exposure and combination of the treatments decreased the invasiveness further. It is important to note that none of these differences qualified for statistical significance, which will be discussed in section 4.4.3.

To investigate the effect of WSB-1 knockdown in association with a more severe hypoxia, the same invasion experiment was repeated at 0.5% O₂ (Figure 4.15). Treatment resulted in a very different pattern from the one observed previously at 2% O₂. However, similarities can be seen between these two datasets: combined hypoxia exposure and WSB-1 knockdown increased MCF7 invasiveness, whereas hypoxia exposure and WSB-1 knockdown induced a decrease in MDA-MB-231 invasiveness.

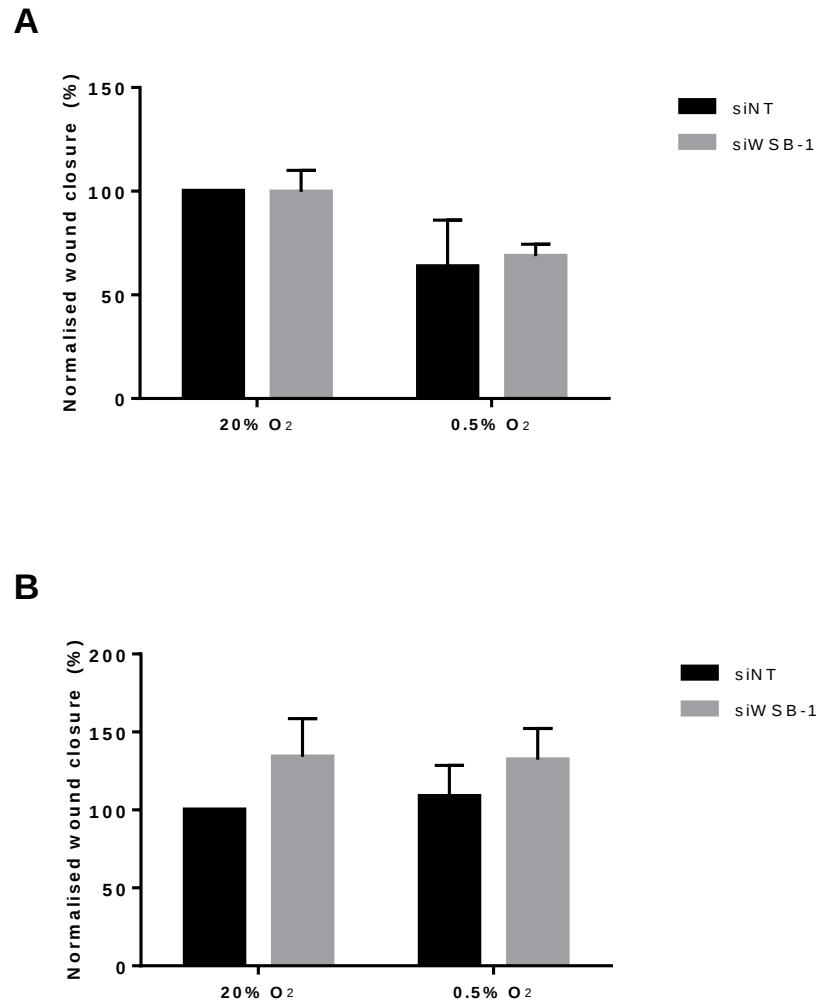


Figure 4.13: Effect of severe hypoxia and WSB-1 knockdown on the migration ability of breast cancer cells

MCF7 (A) and MDA-MB-231 (B) cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). Cells were grown to confluency, and cell monolayers were scratched using a pipette tip. Cells were incubated in normoxia (20% O₂) or moderate hypoxia (0.5% O₂) for 16 hours. Wound images were taken immediately after scratching and post incubation. Data represents the mean of $n=3$ (MCF7) and $n=4$ (MDA-MB-231) experiments. Statistical significance was determined by 2-way ANOVA with Tukey correction for multiple comparisons.

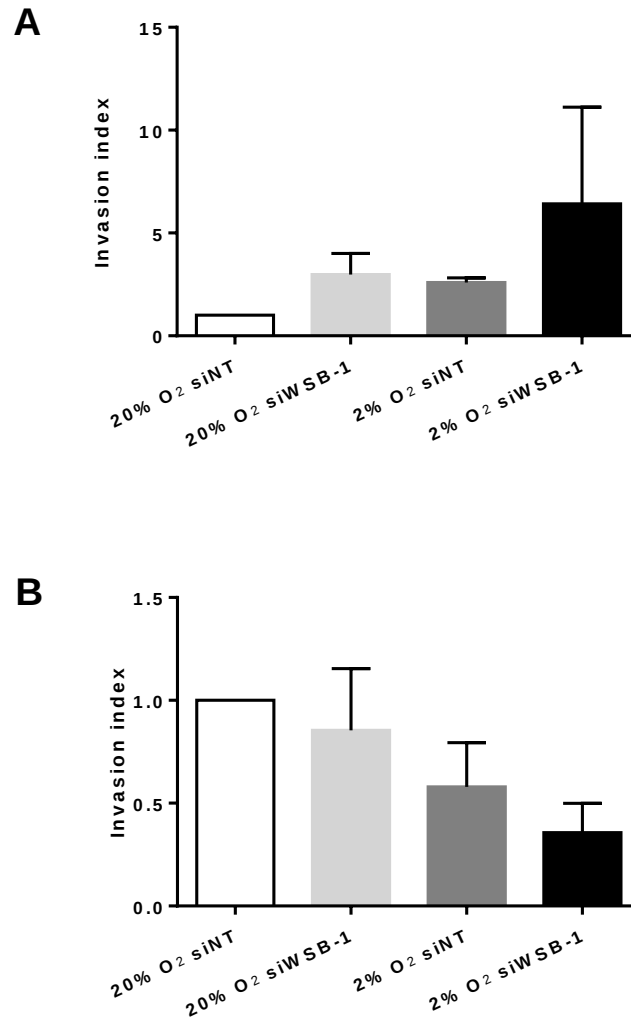


Figure 4.14: Invasion index of breast cancer cells at 2% O₂

MCF7 (A) and MDA-MB-231 (B) cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting siRNA (siNT). Cells were then seeded in inserts coated with Matrigel or control inserts (no Matrigel). Inserts were incubated in normoxia (20% O₂) or moderate hypoxia (2% O₂) for 18h. The number of cells in the underside of the chamber was then scored and used to determine the invasion index (see section X for details). Data represents the average of $n=4$ experiments. Error bars represent mean $\pm$ SEM. Statistical significance was determined by 2-way ANOVA with Tukey correction for multiple comparisons.

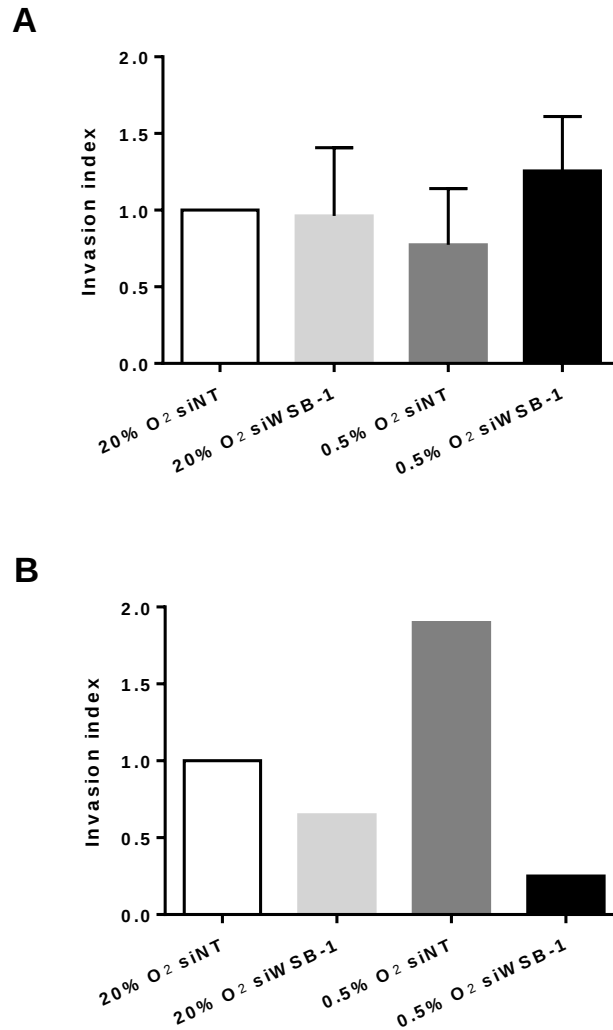


Figure 4.15: Invasion index of breast cancer cells at 0.5% O₂

MCF7 (A) and MDA-MB-231 (B) cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting siRNA (siNT). Cells were then seeded in inserts coated with Matrigel or control inserts (no Matrigel). Inserts were incubated in normoxia (20% O₂) or moderate hypoxia (0.5% O₂) for 18h. The number of cells in the underside of the chamber was then scored and used to determine the invasion index (see section X for details). Data represents the average of $n=3$ (MCF7) and $n=1$ (MDA-MB-231) experiments. Error bars represent mean $\pm$ SEM. Statistical significance was determined by 2-way ANOVA with Tukey correction for multiple comparisons.

In summary, these experiments revealed that MCF7 and MDA-MB-231 cells harbour two very different behaviours in response to WSB-1 knockdown and hypoxia exposure. Migration and invasion abilities of the cells were also differently affected by the treatments. Whereas WSB-1 knockdown induced a significant decrease in MCF7 cells migration in hypoxia, such treatment resulted in an increase in cellular invasiveness of MCF7 cells. Conversely, MDA-MB-231 cells migration increased in response to WSB-1 knockdown and hypoxia, whereas these conditions induced a decrease in invasiveness.

4.3.4. Impact of WSB-1 on invasiveness in 3D models

In order to further analyse the effect of WSB-1 knockdown on cell invasiveness and migration in a more relevant context, *in vitro* 3D tumour spheroid models were used. MCF7 cells aggregates were formed in ultra low adherence plates (section 2.1.5, Figure 4.4). Spheroids were then either embedded in the ECM-like matrix Matrigel to investigate the invasive potential, or deposited on gelatine to monitor the migratory pattern.

Two parameters were followed in these experiments (Figure 4.16). One was the **diameter** of the spheroids circled by a dashed line, the second was the **dispersion width** (or migratory/invasion front) represented by the arrows. This last parameter corresponds to the distance covered by the cells, which moved from the spheroid mass and through the surrounding matrix.

Representative examples of the pictures taken to monitor the growth of the spheroids are presented in Figure 4.17 and Figure 4.18. Evolution in the spheroid size over time, as well as the effect of WSB-1 knockdown and hypoxia exposure on spheroid aspect was observed. A noteworthy element is the absence of spread of MCF7 spheroids through Matrigel (Figure 4.17), compared to gelatine.

The growth kinetics of MCF7 spheroids embedded in Matrigel is presented in Figure 4.19. The line chart depicts the average of spheroid diameter over time for three independent repeats. No difference in spheroid growth could be seen in response to hypoxia exposure or WSB-1

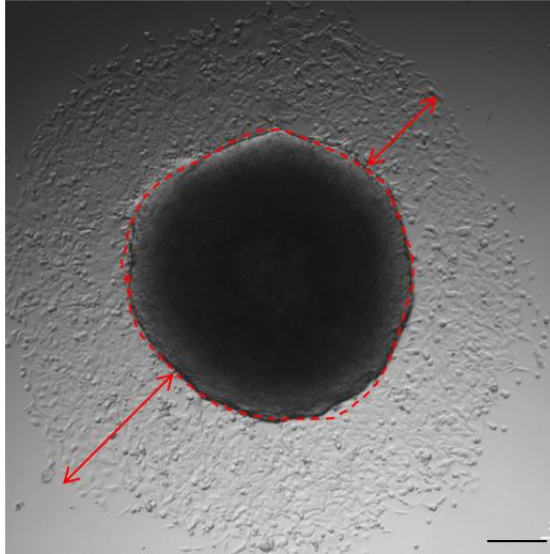


Figure 4.16: Parameters measured in the 3D invasion/migration assays

Microscopy image of MCF7 spheroids deposited on 0.1% gelatine. Dotted line represents the delineation of the spheroid itself, which diameter was measured; arrows exemplify dispersion width measured to assess invasiveness and migration of cells from the spheroid. Scale bar=200 μ m.

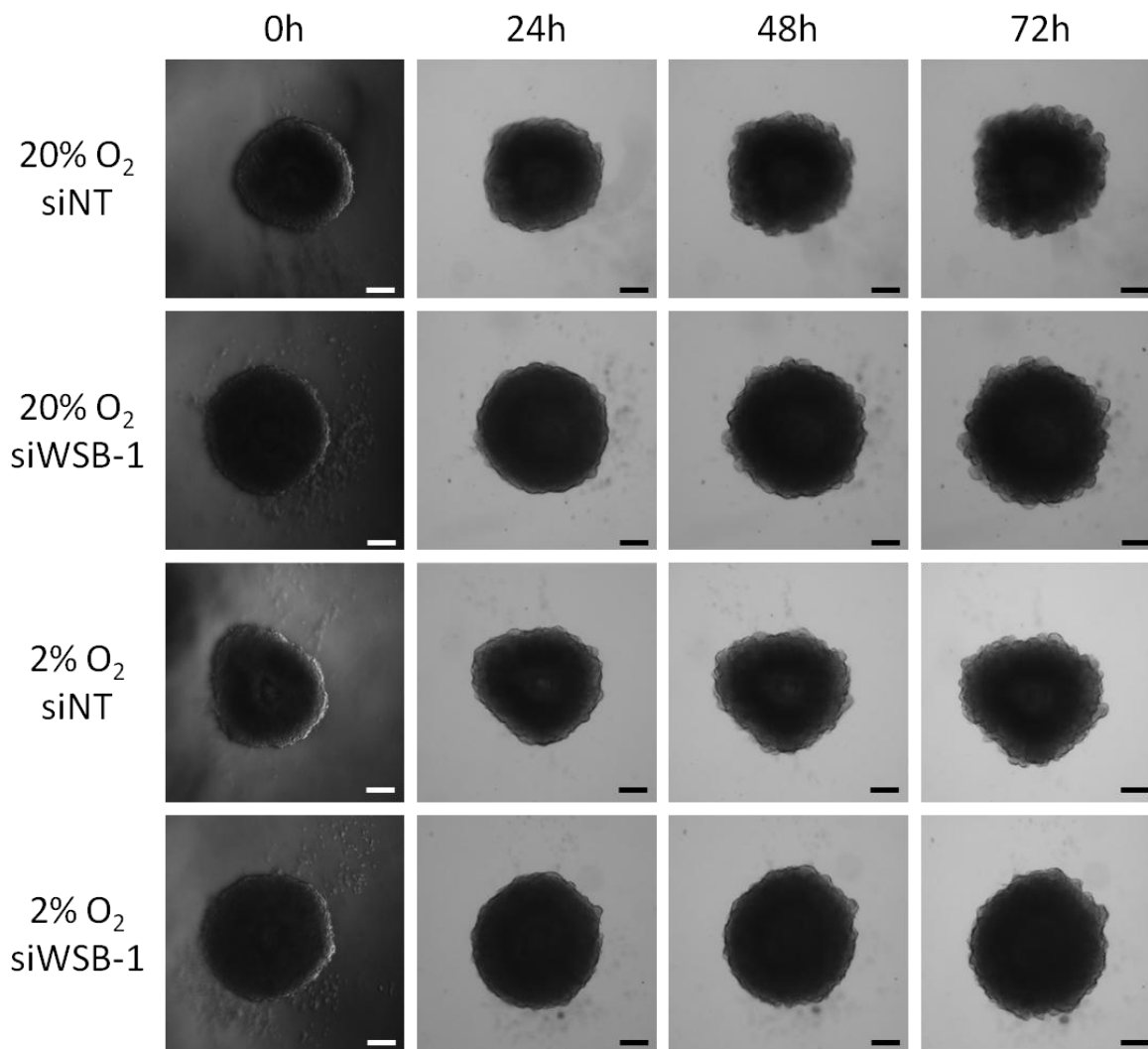


Figure 4.17: Representative images of MCF7 spheroids invasion assay

MCF7 cells were transfected with siRNA against WSB-1 (siWSB-1) or a non-targeting control (siNT). Spheroids were formed of 25,000 cells in ultra low adherence 96-well plates. Some spheroids were exposed to 2% O₂ for the first 24h of the experiment. Spheroids were imaged for 4 consecutive days. Scale bar=200μm.

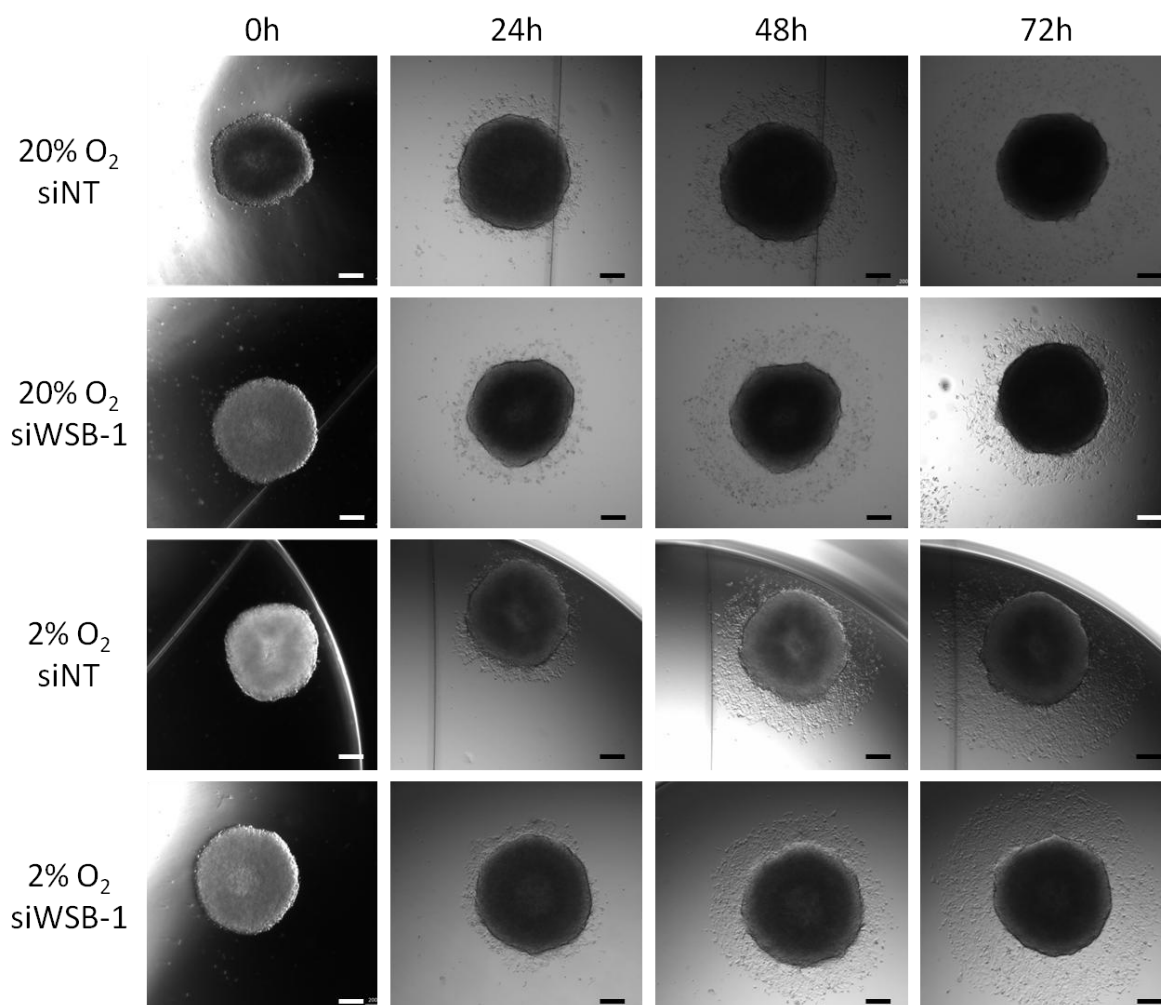


Figure 4.18: Representative images of MCF7 spheroids migration assay

MCF7 cells were transfected with siRNA against WSB-1 (siWSB-1) or a non-targeting control (siNT). Spheroids were formed of 25,000 cells in ultra low adherence 96-well plates. Some spheroids were exposed to 2% O₂ for the first 24h of the experiment. Spheroids were imaged for 4 consecutive days. Scale bar=200μm.

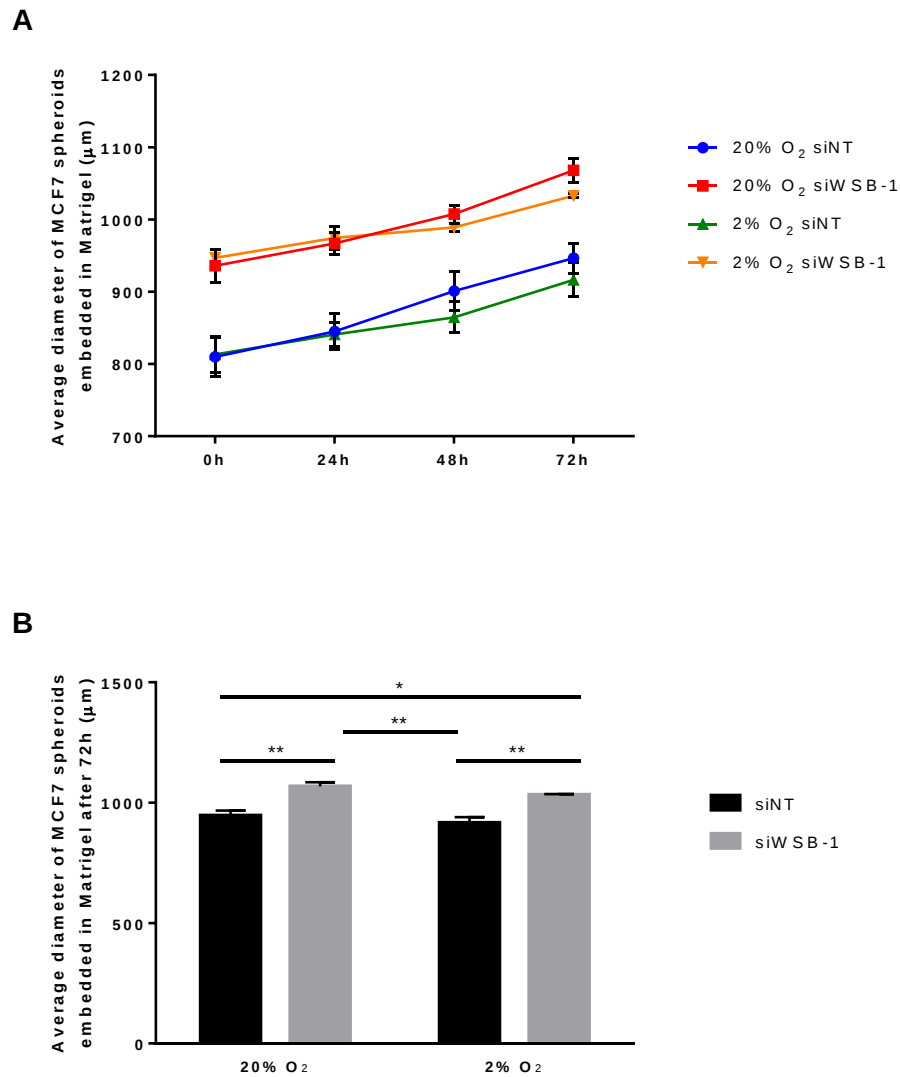


Figure 4.19: Growth kinetics of MCF7 spheroids embedded in Matrigel

Spheroids were generated using MCF7 cells transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). They were then embedded in Matrigel, exposed to normoxia (20% O₂) or hypoxia (2% O₂) for 24h and reoxygenated. Spheroids were imaged at 0, 24, 48 and 72h. A: Average of the spheroid diameter in μm. B: Average spheroid diameter 72h after embedding. Data represent the average of $n=3$ experiments, each with 4 intra-experimental replicates. Error bars represent mean $\pm$ SEM. Statistical significance determined by 1-way ANOVA with Tukey correction for multiple comparisons. *, $p<0.05$; **, $p<0.01$.

knockdown (Figure 4.19A). However, siNT spheroids were smaller than the siWSB-1 spheroids from 0h. The size of the spheroids after 72h of culture was significantly larger for siWSB-1 than siNT ($p < 0.01$), whereas hypoxia exposure had no effect on the diameter (Figure 4.19B). For this reason, spheroid size after 72h of culture was normalised to the initial size at 0h for each condition (Figure 4.20), which revealed no change in spheroid growth in response to treatment and therefore, no change in cell proliferation.

In parallel, other MCF7 spheroids were transferred on a layer of gelatine, in order to investigate cell migration. As observed for the invasion experiments, the siNT spheroids were smaller than the siWSB-1 ones at the beginning of the experiment (Figure 4.21A) but this difference was significant after 72h for those that had been exposed to hypoxia ($p < 0.05$) (Figure 4.21B). To account for this difference in size at the beginning of the experiment, the size of spheroid after 72h of culture was normalised to the initial size at 0h for each condition (Figure 4.22). This revealed once more that the growth of the spheroids was not affected by WSB-1 knockdown or hypoxia exposure, which indicated that cell proliferation was not modified by such treatment. The migration of cells from MCF7 spheroids onto the gelatine layer is presented in Figure 4.23. Initially, the dispersion front did not particularly differ in width between samples. However, cells from MCF7 siWSB-1 spheroids exposed to 20% O₂ appeared to disperse less than for the three other conditions (Figure 4.23A). After 72h, cells from MCF7 siWSB-1 spheroids incubated at 20% O₂ and 2% O₂ appeared to have spread less than their siNT counterparts, although this difference was not significant (Figure 4.23B).

To summarise, siWSB-1 spheroids were generally larger than their siNT counterparts at the beginning of the experiment, despite being generated in the same condition with the same number of cells. This difference in size between siNT and siWSB-1 was in fact significant after 72h for the spheroids generated from MCF7 cells. However, normalisation of the final spheroid size to the initial diameter revealed that there was no difference in growth among the different treatments, suggesting no changes in cellular proliferation during the course of

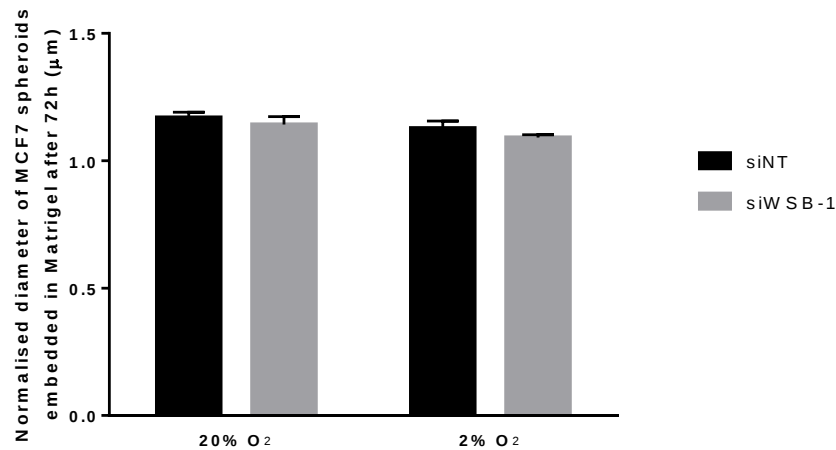


Figure 4.20: Relative size of MCF7 spheroids embedded in Matrigel

Spheroids were generated using MCF7 cells transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). They were then embedded in Matrigel, exposed to normoxia (20% O₂) or hypoxia (2% O₂) for 24h and reoxygenated. Spheroids were imaged at 0, 24, 48 and 72h. Spheroid diameter 72h after embedding normalised to the spheroid diameter at 0h. Data represent the average of $n=3$ experiments, each with 4 intra-experimental replicates. Error bars represent mean $\pm$ SEM. Statistical significance determined by 1-way ANOVA with Tukey correction for multiple comparisons.

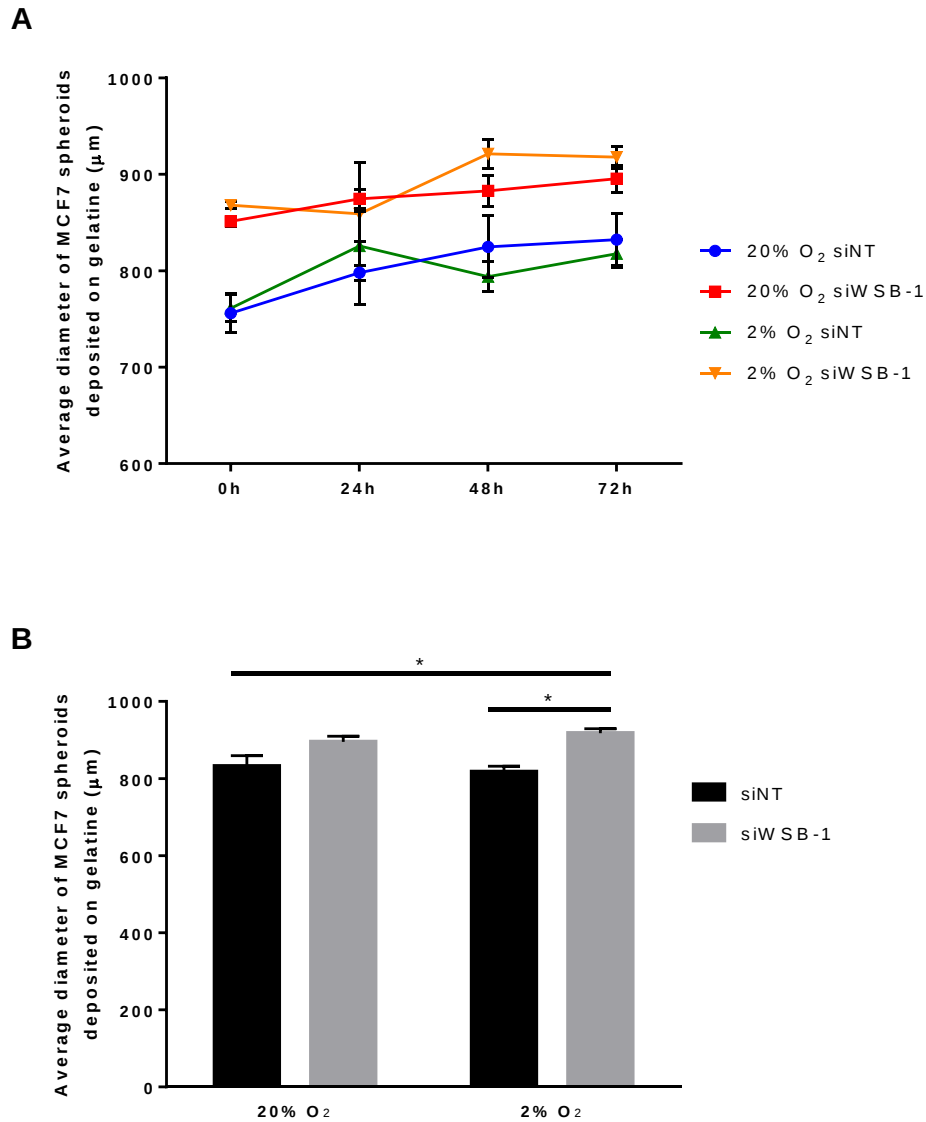


Figure 4.21: Growth kinetics of MCF7 spheroids embedded in gelatine

Spheroids were generated using MCF7 cells transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). They were then transferred on gelatine, exposed to normoxia (20% O₂) or hypoxia (2% O₂) for 24h and reoxygenated. Spheroids were imaged at 0, 24, 48 and 72h. A: Average of the spheroid diameter in µm. B: Average spheroid diameter 72h after transfer. Data represent the average of $n=3$ experiments, each with 4 intra-experimental replicates. Error bars represent mean $\pm$ SEM. Statistical significance determined by 1-way ANOVA with Tukey correction for multiple comparisons. *, $p<0.05$.

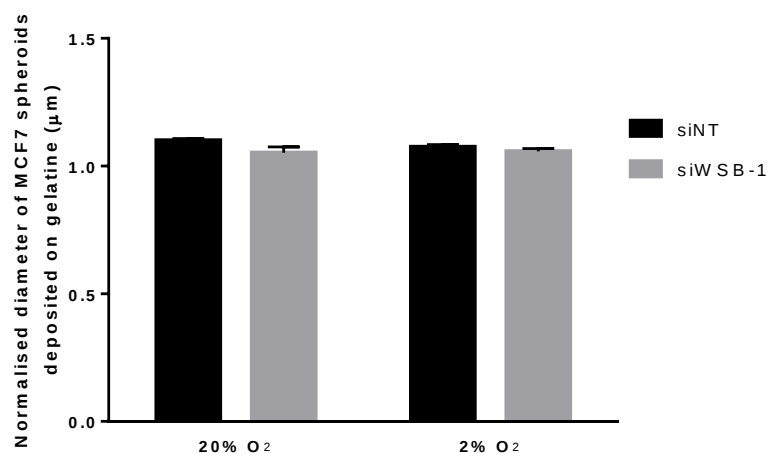


Figure 4.22: Relative size of MCF7 spheroids embedded in gelatine

Spheroids were generated using MCF7 cells transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). They were then transferred on gelatine, exposed to normoxia (20% O₂) or hypoxia (2% O₂) for 24h and reoxygenated. Spheroids were imaged at 0, 24, 48 and 72h. Spheroid diameter 72h after transfer normalised to the spheroid diameter at 0h. Data represent the average of $n=3$ experiments, each with 4 intra-experimental replicates. Error bars represent mean $\pm$ SEM. Statistical significance determined by 1-way ANOVA with Tukey correction for multiple comparisons.

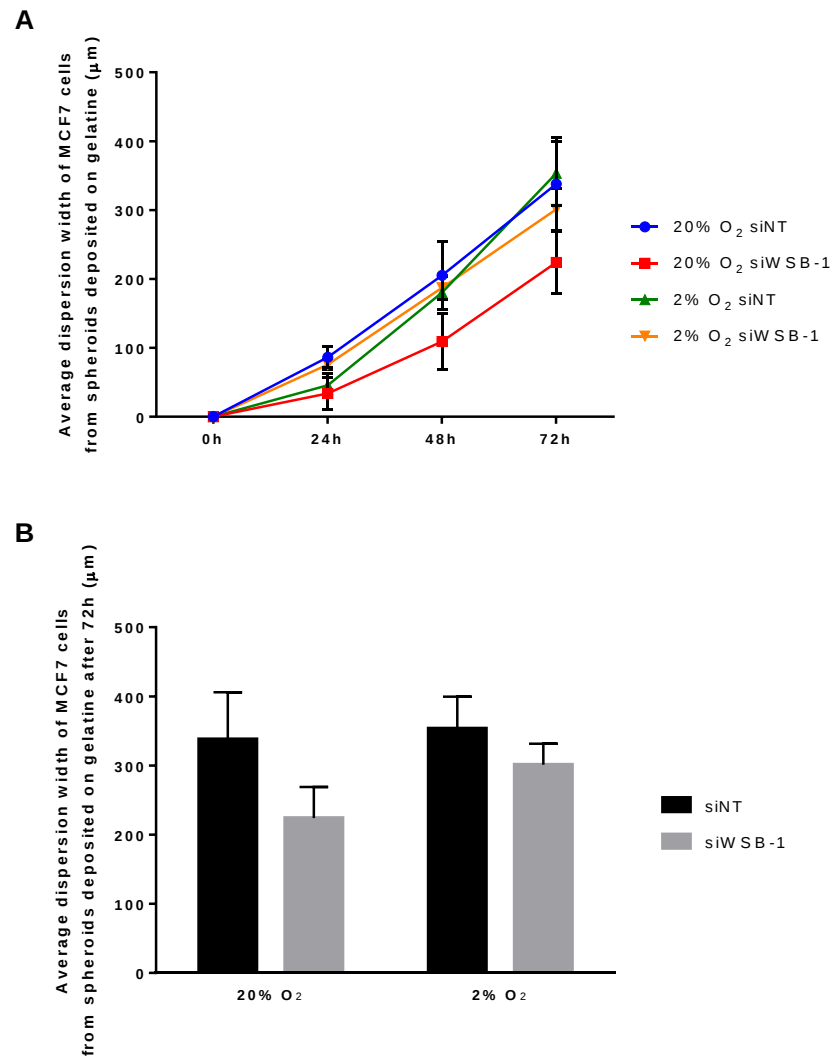


Figure 4.23: Spreading kinetics of MCF7 spheroids embedded in gelatine

Spheroids were generated using MCF7 cells transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). They were then transferred on gelatine, exposed to normoxia (20% O_2) or hypoxia (2% O_2) for 24h and reoxygenated. Spheroids were imaged at 0, 24, 48 and 72h. A: Average of the width of the invaded disc in μm . B: Average width of the invasive skirt 72h after transfer on gelatine. Data represent the average of $n=3$ experiments, each with 4 intra-experimental replicates. Error bars represent mean $\pm$ SEM. Statistical significance determined by 1-way ANOVA with Tukey correction for multiple comparisons.

the experiment. Interestingly, unlike the spheroids embedded in Matrigel, which did not invade to the ECM-like matrix at all in the migration assay, spheroids migrated onto the gelatine matrix. In this model, spheroids generated from siWSB1 cells appeared to spread less than the ones treated with siNT. As a result, siNT spheroids kept in normoxia were in fact those which spread the least.

4.4. Discussion

This chapter focused on investigating the role of WSB-1 in cell motility and invasiveness in breast cancer. To achieve this, further analysis of the RNA-Seq data presented in the previous chapter was undertaken, focusing on proteins known to be relevant to migration and invasion. Furthermore, qPCR, Western blot and zymography were used to investigate the activity of some of these factors. Finally, cell migration and invasion was evaluated using dedicated assays.

Following is a summary of the findings described in this chapter, answering the questions asked at the beginning:

- *What is the effect of WSB-1 on the expression of proteins associated with the ECM architecture in hypoxia?*

RNA-Seq analysis revealed alterations in the transcript levels of ECM components and proteins involved in its regulation in response to WSB-1 knockdown in hypoxia.

- *Does WSB-1 function affect the expression, activation or activity of matrix metalloproteinases?*

WSB-1 knockdown appeared to induce a decrease in the transcript and protein levels, as well as activity of the MMP studied in this chapter in MDA-MB-231 cells.

- *Does WSB-1 function affect the engagement of EMT?*

WSB-1 knockdown appeared to modify the protein levels of the three EMT markers analysed in MCF7 and MDA-MB-231 by Western blot: ZO-1, E-cadherin and vimentin. Additionally,

transcript levels of the pro-EMT transcription factor *SNAIL1* increased in response to siWSB-1 treatment in both cell lines.

- *How is cell motility modulated by WSB-1?*

Effect of WSB-1 knockdown on cell motility was investigated using different types of assays. Wound healing assays revealed WSB-1 knockdown in hypoxia could induce a decrease in 2D cell migration of MCF7 cells. Transwell invasion assays suggested MCF7 cells invasiveness decreased, whereas MDA-MB-231 cells invasiveness increased in response to WSB-1 knockdown. Finally, work with spheroids highlighted a decrease in migration of MCF7 cells on gelatine upon treatment with siRNA against WSB-1.

4.4.1. Extracellular matrix structure and remodelling

More specific analysis of the RNA-Seq data revealed changes in response to WSB-1 knockdown in hypoxia in the transcript levels of ECM components (collagens, laminins), ECM degrading enzymes (MMPs, ADAMs, ADAMTSs), as well as integrins. Only *ADAMTS1*, laminin $\gamma 1$, and integrins $\beta 1$ and $\beta 3$ transcripts were upregulated following WSB-1 knockdown (Table 4.3).

Integrins are an important ECM-related protein family that lies at the interface between the extracellular and intracellular spaces (Hynes, 2002). The role of the integrin signalling in tumour progression has been studied extensively (Guo and Giancotti, 2004). Integrins $\alpha 2\beta 1$ and $\alpha 6\beta 1$ have both been described as laminin receptors (Languino et al., 1989; Sroka et al., 2010). Furthermore, Maemura and colleagues reported that decreased laminin binding by integrin $\alpha 2\beta 1$ was associated with an increased malignancy and a progression in the EMT of breast carcinoma cells (Maemura et al., 1995). Considering that a downregulation of the transcripts coding for the $\alpha 2$ and $\alpha 6$ subunits was observed in response to WSB-1 knockdown, this could indicate a decrease in the signalling pathway normally triggered by the binding to ECM elements. In fact, some of these elements, the laminins, were also downregulated in response to WSB-1 knockdown in hypoxia.

The $\beta 3$ subunit has been associated with tumour progression in malignant melanoma (Albelda et al., 1990). Specifically, integrin $\alpha V\beta 3$ is known to activate MMP2 via MMP14, thus promoting the invasiveness of tumour cells (Brooks et al., 1996; Deryugina et al., 2001; Levental et al., 2009). Surprisingly, integrin $\beta 3$ transcript level was seen to increase with WSB-1 knockdown in the dataset presented in this thesis. The αV subunit, however, was downregulated, which would prevent the formation of $\alpha V\beta 3$ dimers.

MMPs are another family of ECM-related proteins responsible for matrix degradation discovered in 1962 (Gross and Lapiere, 1962). Their expression and activity has been frequently studied in oncology as increased MMP levels can indicate increased invasiveness of cancer cells and reveal an aggressive tumour (Haupt et al., 2006; Köhrmann et al., 2009; Kessenbrock et al., 2015). More specifically, the prognostic values of MMP1 and MMP14 expression were studied in breast cancer, where increased expression in patients was correlated with a poor prognosis (Boström et al., 2011; Yao et al., 2013). Kim and colleagues recently found that tumour metastasis presented a 1.55-fold and 1.84-fold increases of MMP2 and MMP9 levels, respectively, in patients with lung adenocarcinoma in never-smoker and presenting a high WSB-1 expression, compared to patients with low WSB-1 expression (Kim et al., 2015). The findings presented in this study support the results described in this chapter, whereby knockdown of WSB-1 resulted in a decrease in several MMP levels, including MMP9 which in fact demonstrated one of the most important decrease (Table 4.3). In light of these results, it was decided to analyse more closely the effects of WSB-1 knockdown on MMPs protein expression and activity.

WSB-1 knockdown was shown to decrease the transcript levels of MMP1 and MMP14 in MDA-MB-231 cells (Figure 4.5). The very high C_T values obtained with the MMP7 primers indicate that MMP7 transcript level is rather low in these cells, which is in accordance with what was previously described in the literature and prevents an accurate analysis of the results obtained (Sizemore and Keri, 2012).

In addition, protein levels of MMP1 and MMP14 were monitored by Western blot. Depending on the section of the peptide recognised by the antibody, it can be possible to detect both the pro- and mature forms. Interestingly, pro-MMP14 is only detectable in hypoxia (Figure 4.6). This is in accordance with previous studies which described a hypoxia-dependent MMP14 expression (Annabi et al., 2003; Noda et al., 2005). Interestingly, MMP14 is one of the mediators of MMP2 activation, which correlates with the decrease in MMP2 activation observed in Figure 4.8 (Kinoshita et al., 1996; Lehti et al., 1998). This MMP14-dependent MMP2 activation has in fact been described as a promoter of tumour progression and MMP14 activation resulted in increased tumour growth (Itoh et al., 2001; Albrechtsen et al., 2013). SPARC (secreted protein acidic cysteine-rich/osteonectin) has also been described to upregulate MMP14-mediated MMP2 activation (McClung et al., 2007). *SPARC* transcript was found to be importantly downregulated in MDA-MB-231 cells in response to WSB-1 knockdown (Table 3.7), which could also contribute to the decrease in MMP2 activation observed with the gelatine zymography in this chapter. Unfortunately, visualisation of MMP2 protein levels by Western blot could not be achieved in the present study as the antibody failed to return an exploitable signal. Interestingly, the cytoplasmic region of MMP14 was previously described to interfere with the HIF signalling pathway by binding FIH (factor inhibiting HIF-1) (Sakamoto et al., 2014). A decrease in MMP14 would therefore facilitate FIH binding to HIF-1 and inhibit HIF-1 activity.

Finally, investigation of MMP2 activity by gelatine zymography showed that WSB-1 knockdown resulted in a decrease in MMP2 activity, particularly in hypoxia (Figure 4.8). In addition, Western blot and qPCR analyses revealed that WSB-1 knockdown in hypoxia also induced a decrease in MMP1 and MMP14 expression (Figure 4.5 to Figure 4.7). Gelatine zymography is a commonly used technique that allows the visualisation of gelatinases pro- and mature forms. This is due to the fact that protein samples are denatured during the SDS-PAGE step of the procedure (see section 2.7 for details), exposing the gelatinolytic

domain of both the pro- and the mature forms, which remain active after the partial renaturation step (Toth et al., 2012). The main drawback of gelatine zymography is the impossibility to integrate an endogenous control. In addition, it is important to note that zymography only reveals the net activity of gelatinases found in the sample but does not take into consideration the activity of TIMPs (tissue inhibitors of metalloproteinases) or other endogenous inhibitors which might be present in the initial sample and would modulate the final MMP activity *in vivo* (Toth et al., 2012). Importantly, similarly to Western blot, the intensity of the signal can vary between repeats due to the many steps of the protocol, which can have different efficiencies each time. Therefore, gelatine zymography cannot be considered as a quantitative technique, *per se*.

4.4.2. Effect of WSB-1 knockdown on the epithelial to mesenchymal transition

EMT is an important aspect of cancer progression which has been vastly studied, particularly by Weinberg (Thiery, 2002; Yang and Weinberg, 2008; Kalluri and Weinberg, 2009; Polyak and Weinberg, 2009). In the present work (section 4.3.2), protein levels of three EMT markers were analysed: ZO-1 and E-cadherin, considered epithelial markers; and vimentin, considered a mesenchymal marker (Figure 4.9). WSB-1 knockdown was associated with an increase in ZO-1 protein level in MCF7 and MDA-MB-231, and a decrease in E-cadherin in MCF7 and in vimentin in MDA-MB-231. Whereas the results obtained in MDA-MB-231 are consistent with a shift from a mesenchymal to an epithelial profile in response to WSB-1 knockdown, suggesting WSB-1 upregulation could promote EMT, results obtained in MCF7 are contradictory. Sarrió and colleagues suggested that EMT in breast cancer was more characteristic of basal tumours (triple negative, TNBC), partly explaining the increased aggressiveness of this tumour subtype, which is further comforted by the fact that MDA-MB-231 cells are considered more advanced in the EMT process and therefore, more invasive (Lacroix and Leclercq, 2004; Sarrió et al., 2008). In addition, EMT must be seen as a transitional procedure, rather than an on/off switch. As a result, cancer cells, which are

already on the path of transformation, may present a combination of epithelial and mesenchymal markers at the same time.

Snail, coded by *SNAIL1*, is an important transcription factor involved in EMT as it inhibits E-cadherin expression and activates the expression of other pro-EMT transcription factors such as Twist and ZEB1/2 (Cano et al., 2000; Peinado et al., 2007). In breast cancer, Snail expression has been correlated with increased invasiveness. A study by Moody and colleagues demonstrated that increased *SNAIL1* level resulted in increased risk of tumour relapse and overall poorer survival rates in breast cancer patients (Moody et al., 2005). The increase in *SNAIL1* transcript observed in the present study in response to WSB-1 knockdown (Figure 4.10) is consistent with the decrease in E-cadherin protein level previously described in MCF7 cells (Figure 4.9), which has been presented as a hallmark of metastatic carcinoma (Cavallaro and Christofori, 2004; Lee et al., 2006). In fact, E-cadherin was described as an indicator of poor prognosis in breast cancer, and the group of Oesterreich proposed to decrease E-cadherin levels in ER+ patients using oestrogen treatment (Parker et al., 2001; Oesterreich et al., 2003). However, this increase in pro-EMT markers levels in MCF7 cells upon WSB-1 knockdown would indicate that WSB-1 upregulation is in fact acting against EMT progression, which is in contradiction with what was observed in MDA-MB-231 and in previous work (Archange et al., 2008; Silva et al., 2011; Shichrur et al., 2014; Cao et al., 2015; Kim et al., 2015). In MDA-MB-231 cells, *SNAIL1* increase is in contradiction with the changes in EMT markers expression observed by Western blot and challenges the impact of WSB-1 on EMT progression in this cell line as well. Despite numerous attempts, Snail levels could not be monitored by Western blotting as the antibody only yielded a very weak signal, even in lysates of positive control cell lines.

Interestingly, Lei and colleagues recently suggested that the hedgehog signalling regulated hypoxia-induced EMT (Lei et al., 2013). Previously, Vasiliauskas and colleagues revealed that, in the chicken, WSB-1 (then referred to as cSWiP) was under the control of Shh (Sonic

hedgehog) (Vasiliauskas et al., 1999). It could be interesting to verify this relationship further by investigating the role of the hedgehog signalling pathway in WSB-1 expression.

Overall, WSB-1 effect on EMT progression of MCF7 and MDA-MB-231 cells is unclear. Surprisingly, the pro-EMT impact expected from high WSB-1 levels in the triple negative cells MDA-MB-231, according to the results presented in this chapter, contradicts the fact that patients with triple negative breast cancer tumours displaying high *WSB1* levels presented better distant metastasis-free survival rates than those with low *WSB1* levels (section 3.4.2). Therefore, more markers need to be considered in order to achieve a more comprehensive analysis.

4.4.3. Changes in cellular motility and invasiveness

As suggested by several previous studies, changes in MMP levels result in modulation of invasiveness and aggressiveness (Kousidou et al., 2004; Muñoz-Nájjar et al., 2006; Köhrmann et al., 2009; Boström et al., 2011). Various techniques are available to measure these phenomena (Liang et al., 2007; Kramer et al., 2013). The effect of WSB-1 knockdown on cell motility was investigated using wound healing and Transwell invasion assays. In these assays, MCF7 and MDA-MB-231 cells displayed very different behaviours. Specifically, wound healing assays revealed that WSB-1 knockdown at 2% O₂ resulted in a significant decrease in MCF7 migration (Figure 4.12), but in a small non-significant increase in MDA-MB-231 at 0.5% O₂ (Figure 4.13).

A surprising and highly reproducible observation from the wound healing assay experiments was the absence of increase in cell migration in response to hypoxia, a phenomenon described numerous times in the literature (Daniel and Groves, 2002; Annabi et al., 2003; Sahlgren et al., 2008). Wound healing assays are cost-effective, easy to setup and interpret, but other methods have been developed to analyse cellular migration. The main difference resides in the technique used to clear an area within the cell monolayer (Riahi et al., 2012). Not physically scratching the cell monolayer guarantees the integrity of the coating on the tissue

culture dish and prevents injuries to the cells, which could trigger stress responses and modify their migration pattern.

It is also important to distinguish between individual and collective cell migration (Friedl and Gilmour, 2009). Wound healing assays can monitor collective cell migration as cells move together, without disrupting cell-cell contacts. On the contrary, the Transwell invasion assays used in this study, seeded with low cell numbers, reveals the motility of individual cells. To reach the bottom of the insert, cells need to move through the Matrigel layer by degrading the matrix; the more invasive cells reaching the bottom faster. In this study, it was observed that MCF7 cells invasiveness appeared to increase in response to WSB-1 knockdown in hypoxia, whereas it appeared to decrease in MDA-MB-231 (Figure 4.14, Figure 4.15). There are two ways to analyse invasion assay results. One, described in the manufacturer's guide, is to consider the invasion index, which normalises the number of cells visible on the bottom part of the Matrigel-coated inserts to the number of cells visible on the bottom part of the inserts which were not coated with Matrigel (Figure 4.14, Figure 4.15). Another method, used in several studies, is to only count the cells which crossed the Matrigel layer, and not normalise these values to the control inserts (Figure A2, Figure A3) (Jacob et al., 1999; Muñoz-Nájjar et al., 2006; Sahlgren et al., 2008; Nodale et al., 2012). This approach therefore differentiates between single cell migration (control inserts) and invasion (Matrigel inserts). Besides the difference between the invasion indices and the invasion considering solely the Matrigel inserts, separating the data reveals that WSB-1 knockdown in MCF7 resulted in a decrease of cell migration, whereas MDA-MB-231 demonstrated an increase in cell migration. Interestingly, this highlights changes in MCF7 and MDA-MB-231 cell migration in response to WSB-1 knockdown that were not visible using wound healing assays, particularly for MDA-MB-231 at 2% O₂ (Figure A2B) and MCF7 at 0.5% O₂ (Figure A3A). Importantly, due to the small number of cells seeded for the experiment, Transwell assays with control inserts informs on single cell migration, whereas wound healing focuses on collective cell migration.

These two mechanisms, although both resulting in cell movement, are mediated by different signalling pathways (Friedl and Gilmour, 2009; Hanahan and Weinberg, 2011).

Overall, the invasion assay results presented in this chapter lack significance and error bars are particularly large. This specific issue could be solved by repeating the experiment more times and adding further intra-experimental repeats, increasing the number of cells counted. Alternatively, similar assays are available, which measure invasiveness by staining the cells with cytological dyes and reading the optic density rather than by counting each individual cell under a microscope (Kramer et al., 2013).

Using 3D spheroids models allows a more comprehensive approach to the tumour system and has been increasingly used, particularly to test drug efficiency (Hirschhaeuser et al., 2010; Vinci et al., 2012; Benton et al., 2015). Embedding spheroids in Matrigel permits the evaluation of collective cell migration through a matrix, while the spheroids deposited on a layer of gelatine reports on collective migration. Unlike what could be expected following the Transwell invasion assay results, MCF7 spheroids did not invade through the Matrigel (Figure 4.19). However, similarly to what was observed with the wound healing assay (Figure 4.12), WSB-1 knockdown resulted in a decrease in cellular migration (Figure 4.23). In addition, MCF7 spheroids deposited on gelatine appeared to disaggregate and siNT cells tended to spread more than the siWSB-1 cells (Figure 4.23).

The sturdiness of a spheroid depends on the cell line and its ability to form tight junctions as well as the initial cell density. An attempt was made to generate spheroids using MDA-MB-231 cells. Unfortunately, MDA-MB-231 spheroids were too fragile to be handled and only the Matrigel embedding could be performed. As for the MCF7 spheroids, MDA-MB-231 cells were left for 4 days to aggregate and then embedded in Matrigel. The loose aspect of the MDA-MB-231 spheroids, particularly at 0h (Figure A4), prevented an accurate delineation of the outline. In addition, spheroids appeared to become more compact between 0h and 24h, making it difficult to estimate the stage at which cells spread away from the

spheroid mass (Figure A5A). After 72h of culture, the diameter of the spheroids was similar regardless of the knockdown or oxygen tension used (Figure A6). Nevertheless, MDA-MB-231 cells demonstrated important invasive capacity through Matrigel and formed an organised network. This is consistent with their more aggressive profile. In fact, the spreading was significantly more important when WSB-1 was knocked down ($p < 0.01$ in normoxia and $p < 0.05$ in hypoxia) (Figure A7), suggesting WSB-1 could prevent MDA-MB-231 cells invasion, which contradicts the effect observed of WSB-1 on EMT presented in section 4.4.2.

Spheroids from untransfected MDA-MB-231 cells had been successfully obtained by ours and by other groups, but never with transfected cells (Vinci et al., 2012). Therefore, it is possible that the process of siRNA transfection alters the cells' ability to develop the tight interactions required to form adequate spheroids. Generating stable knockdown models using shRNAs or even knockout WSB-1 entirely using the novel CRISPR/Cas9 approach would overcome this particular issue (Mali et al., 2013; Roy et al., 2015). In fact, cell lines knockdown for WSB-1 are presently being generated in the laboratory.

An alternative to spheroids are mammospheres (Shaw et al., 2012; Pires et al., 2014). Mammospheres take advantage of the ability of cancer cells to form spherical aggregates. Changes in the phenotype of these hollow structures indicate on the cells invasiveness.

To summarise, the role of WSB-1 in cell motility and invasiveness in breast cancer appears conflicting but real. Although WSB-1 knockdown results in a decrease of MMPs expression and activity in both cell lines, suggesting a pro-EMT role for WSB-1 and an increased ability for the cells to degrade the ECM, the effect of WSB-1 knockdown on EMT markers expression and motility differs for each cell lines. Given the effects of WSB-1 knockdown on the Snail/E-cadherin axis in MCF7, WSB-1 appears to prevent EMT progression. However, MCF7 cells migration and invasion tend to decrease upon siWSB-1 treatment, suggesting WSB-1 has the ability to increase MCF7 motility. On the contrary, effect of WSB-1 knockdown on EMT markers protein levels in MDA-MB-231 indicate WSB-1 promotes EMT progression.

Nevertheless, MDA-MB-231 cells demonstrated increased motility when treated with siWSB-1, making of WSB-1 an inhibitor of cell migration and invasion.

Chapter 5

Identification of novel WSB-1 binding partners

5. Identification of novel WSB-1 binding partners

5.1. Introduction

Little is known about WSB-1, and its E3 ubiquitin ligase activity has only been described on DIO2 and HIPK2 (Dentice et al., 2005; Choi et al., 2008). Importantly, WSB-1 is known as a RING (really interesting new gene) type E3 ubiquitin ligase and therefore, operates as the substrate recognition subunit, in a complex involving an E2 ubiquitin conjugating enzyme, Cullin 2/5, Elongin B/C, Rbx1 and the substrate protein (Chapter 1, Figure 1.9) (Ardley and Robinson, 2005; Dentice et al., 2005). In addition, WSB-1 has been described as interacting with IL-21 receptor, thus inducing its maturation (Nara et al., 2011). Furthermore, Cao and colleagues identified RhoGDI2 as a potential WSB-1 target using quantitative proteomic profiling (Cao et al., 2015). pVHL was also recently identified as a WSB-1 target (Kim et al., 2015). Many more proteins are suspected to interact with WSB-1 but only a few have been experimentally confirmed (Figure 5.1).

In order to better understand the role of WSB-1 in breast cancer progression and determine in which pathways this protein is involved, it is important to identify novel binding partners. To do so, affinity purification followed by mass spectrometry (AP-MS) is a common approach (Gingras et al., 2007; Nesvizhskii, 2012). AP-MS allows the isolation of a protein of interest along with any other interacting protein, and identification of these proteins by mass spectrometry. The amount of data obtained with such technique can be important and filtering of the numerous proteins identified is essential to isolate the key partners and pathways represented. Tools have been developed, which help analyse the interactome and identify contaminant proteins. STRING is an online database that lists protein interactions based on mining of the literature and generates interaction networks, which are useful to identify clusters of proteins interacting with one-another (Szklarczyk et al., 2015). The CRAPome (contaminant repository for affinity purification-ome) database indexes all the

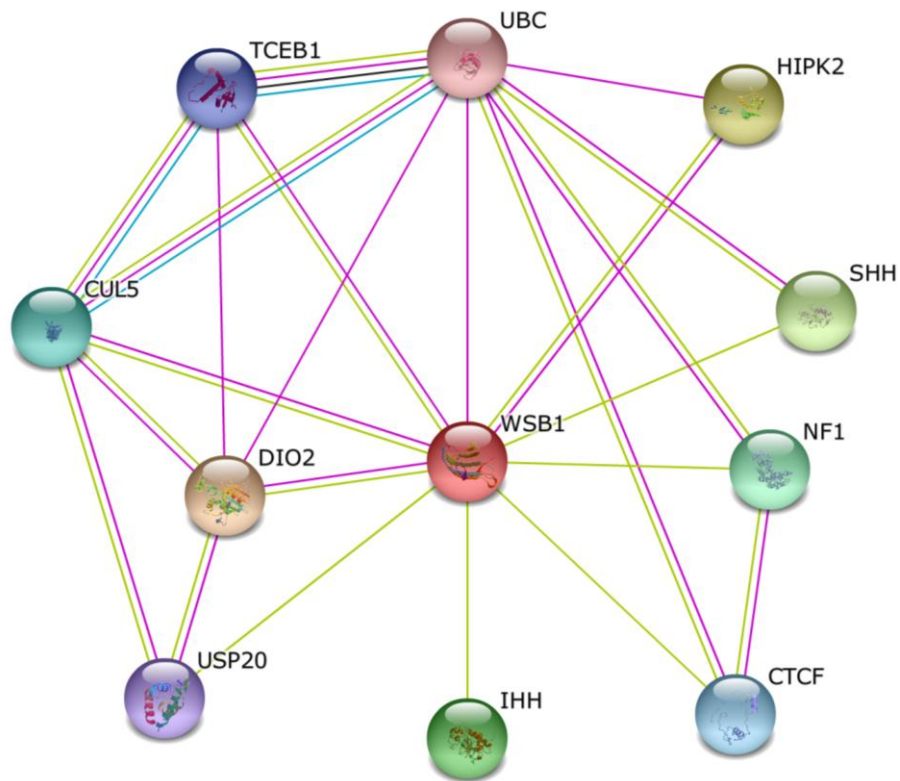


Figure 5.1: Network of proteins known and suspected to interact with WSB-1

Proteins that have been described to interact with WSB-1 or which have been mentioned in papers along with WSB-1 are reported in this network. The interactome was produced using the online STRING database (Szklarczyk et al., 2015). Colour of the connectors depends on the type of evidence for the interaction: yellow: text mining; pink: experiment; blue: databases; black: co-expression. CTCF: CCCTC-binding factor; CUL5: Cullin 5; DIO2: Deiodinase, iodothyronine, type II; HIPK2: Homeodomain interacting protein kinase 2; IHH: Indian hedgehog; NF1: Neurofibromin 1; SHH: Sonic hedgehog; TCEB1: Transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C); UBC: Ubiquitin C; USP20: ubiquitin specific peptidase 20.

proteins detected in 411 previous mass spectrometry studies, thus allowing the identification of probable non-specific binding (Mellacheruvu et al., 2013). IPA is a versatile software developed by Qiagen, which was already used in Chapter 3. It is able to highlight main biological processes in which the proteins from the interactome are involved, as well as other relevant information on interaction and roles.

WSB-1 is an E3 ubiquitin ligase which contributes to the ubiquitin proteasome pathway for proteasomal degradation of proteins. The 26S proteasome is a cylindrical multiprotein complex composed of a 20S subunit (hollow core in which marked proteins are degraded) and two 19S subunits (caps that regulate protein entry in the structure) at each extremity. Molecules blocking this ubiquitin proteasome system are available (Kisselev et al., 2012). In fact, some therapeutic strategies against cancer are based on proteasome inhibitors (Crawford et al., 2011). MG132 is one of these inhibitors and functions by inhibiting the activity of the 20S subunit (Guo and Peng, 2013).

To summarise, this chapter is aimed at answering the following questions:

- What proteins are likely to interact with WSB-1?
- What biological pathways are enriched in the list of putative WSB-1 binding partners?
- What is the effect of WSB-1 knockdown on the protein level of these putative WSB-1 partners?

5.2. Experimental design

5.2.1. Immunoprecipitation of WSB-1

Endogenous level of WSB-1 varies across cell types but is generally low and dependent on cellular stresses (Archange et al., 2008). In order to facilitate detection of WSB-1 and subsequent pull-down, it was decided to transfect cells with a plasmid coding for a Flag-tagged version of WSB-1 isoform 1 (Chapter 2, Figure 2.1). HEK293T cells were favoured as they display a good resistance to the transfection process, good transfection efficiency, and

a high proliferation rate (Durocher et al., 2002; Thomas and Smart, 2005). In order to maximise the hypoxic response in the cells and replicate the conditions used in a previous experiment (see section 5.2.2), an oxygen level of 0.5% for 24h was chosen for the hypoxia exposure.

Normoxic and hypoxic samples were lysed and Flag tagged WSB-1 was pulled down, along with any protein bound to it. Samples were run in an SDS-PAGE gel, which was stained with Coomassie blue to reveal the proteins or transferred on PVDF membranes to analyse particular proteins present by Western blot.

5.2.2. Liquid chromatography-mass spectrometry analysis

Liquid chromatography-mass spectrometry (LC-MS) is a powerful technique, which allows the detection and identification of tiny peptides within a large sample (Aebersold and Mann, 2003; Karpievitch et al., 2010). However, the most represented peptides will overshadow the less common ones. Therefore, it is important to separate the samples into more manageable portions, displaying bands of comparable intensity. The gel lanes for each sample were separated into eight pieces, as exemplified in Figure 5.2, where the proteomic workflow is illustrated. Each of the eight gel samples was then processed as a unique sample, dehydrated and digested until the LC-MS analysis. The latter was outsourced to the proteomics and analytical biochemistry facility at the University of York. The peptides isolated by LC-MS were matched to proteins using the MASCOT database.

LC-MS analysis of the samples yielded a large list of peptides eluted with the Flag-coated beads. This list of potential WSB-1 partners was refined with the help of the CRAPome database to remove possible contaminants. IPA and STRING software were used to highlight networks and relationships between proteins.

Similar work has previously been conducted in the lab, using MALDI-MS (matrix-assisted laser desorption/ionisation-mass spectrometry) instead of LC-MS. Less costly but also less sensitive, MALDI-MS is only capable of detecting a few hits per sample. The amount of

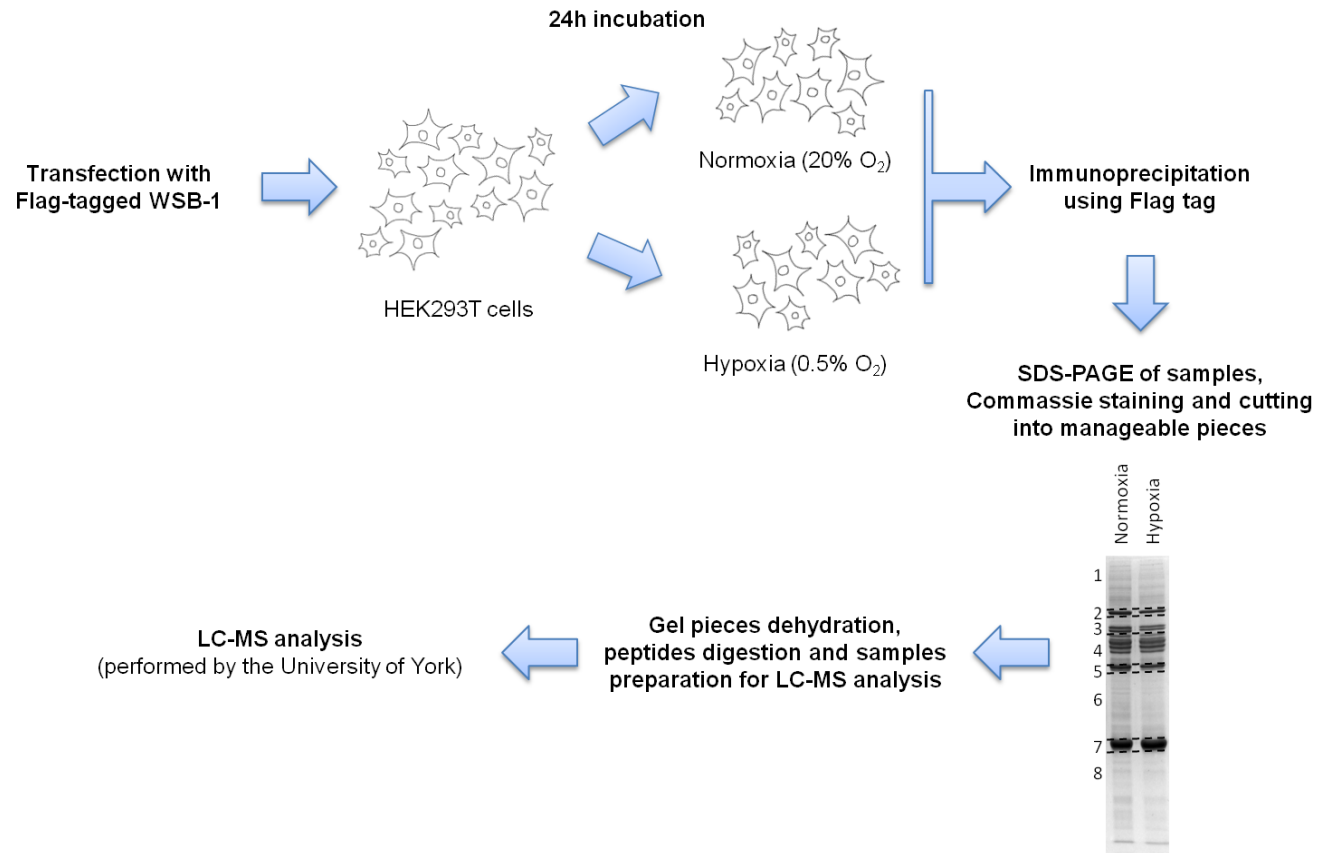


Figure 5.2: Flowchart summarising the steps leading to the LC-MS analysis

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1 and incubated at 20% or 0.5% O₂ for 24h. Flag-WSB-1 IP samples were run on an SDS-PAGE gel, stained with Coomassie blue and cut into pieces for LC-MS analysis. To ensure reliability of the results obtained, the immunoprecipitation was repeated three times and the three sets of samples were all analysed together by LC-MS at the University of York.

peptides matched to a given protein reinforces the reliability of the results. The proteins detected by the MALDI-MS analysis are listed in Table 5.1, along with the number of peptides matched for each hit.

5.2.3. Validation of the putative partners

From an initial list of 1066 hits, proteins for which a significant difference between normoxia and hypoxia samples was noted, as well as those relevant to breast cancer, were chosen for further investigation. Fresh WSB-1 Flag IP samples were produced, and the interaction of these putative targets with WSB-1 was validated by Western blot using antibodies specific for these proteins.

Treatment of cells with MG132, a proteasome inhibitor, was used to provoke the accumulation of potential WSB-1 targets in the cells and facilitate their detection.

The expression of the validated hits was analysed in WSB-1 Flag IP samples generated from breast cancer cells. Immunoprecipitation of the validated hits was also performed in HEK293T cells and the presence of WSB-1 in the immunoprecipitated samples was verified by Western blot. Finally, the effect of WSB-1 knockdown on the protein levels of these candidates in whole cell lysates was examined by Western blot.

5.3. Results

5.3.1. Selection of putative partners from LC-MS analysis

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1. Cells were incubated in normoxia or hypoxia (0.5% O₂) for 24h and WSB-1, along with any interacting proteins, was affinity purified. Immunoprecipitated proteins in each sample were subsequently analysed by LC-MS in order to identify all the peptides isolated, which are expected to directly or indirectly bind to WSB-1 in these condition. See Figure 5.3 for example of the Coomassie stained gels of whole cell lysates and IP samples. The similar intensity and band pattern between the three input samples indicates that similar amount of protein was

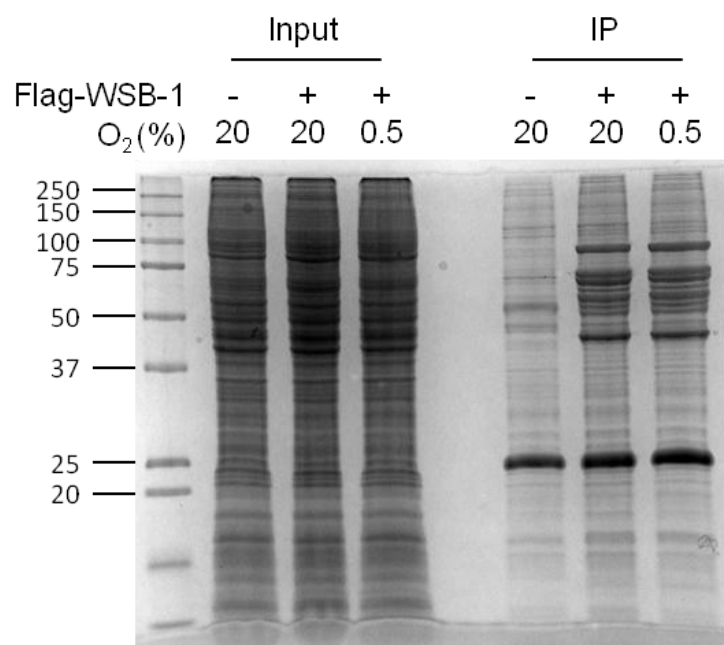


Figure 5.3: Immunoprecipitation of Flag-WSB-1 samples

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Whole cell lysates (input) or Flag-WSB-1 IP samples (IP) were run on an SDS-PAGE gel and stained with Coomassie blue. Image representative of $n=10$.

Table 5.1: List of proteins identified with MALDI-MS

Protein code	Full name	Peptides matched	
		20% O₂	2% O₂
CCT2	Chaperonin containing TCP1 subunit 2	1	1
CCT4	Chaperonin containing TCP1 subunit 4	1	1
CCT5	Chaperonin containing TCP1 subunit 5	-	1
HSP90AA1	Heat shock protein 90kDa alpha family class A member 1	8	6
HSP90AA2	Heat shock protein 90kDa alpha family class A member 2, pseudogene	1	1
HSP90AB1	Heat shock protein 90kDa alpha family class B member 1	7	5
HSPA1A	Heat shock protein family A (Hsp70) member 1A	4	6
HSPA8	Heat shock protein family A (Hsp70) member 8	3	4
KRT2	Keratin 2, type II	-	1
NUDC	NudC nuclear distribution protein	-	1
PTGES3	Prostaglandin E synthase 3/p23	3	-
RPS27A	Ribosomal protein S27a	1	-
TCEB1	Transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)	3	1
TCEB2	Transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B)	2	3
TUBA4A	Tubulin alpha 4a	1	-
TUBB2C	Tubulin beta 4B class IVb	1	-
WSB1	WD repeat and SOCS box containing 1	6	6

used for the immunoprecipitation. This is important given that no endogenous control can be incorporated in the IP protocol to confirm equal loading. In addition, Coomassie blue staining informed on the specificity of the pull down. Indeed, the mock IP sample presented less intense bands, which confirmed that the Flag beads did not non-specifically pull down every protein present in the samples.

A total of 1066 proteins were identified (Table B8, appendix). However, 606 of these hits were found only in one of the three replicates. Likely to be false positive, these orphaned molecules were discarded from the list leaving 460 potential WSB-1 partners. 369 hits were found in the normoxic sample, 386 hits were found in the hypoxic one, and 295 proteins were found in both conditions (Figure 5.4). Overall, the interaction with WSB-1 increased in hypoxia for 221 proteins, whereas for 239 proteins, the interaction decreased. This list of 460 hits was uploaded in the IPA software, where 423 analysis-ready molecules were detected.

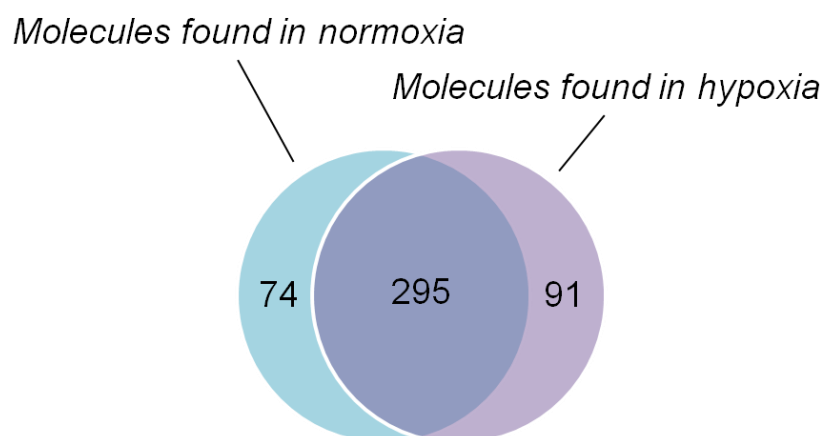


Figure 5.4: Venn diagram representation of the LC-MS hits for WSB-1 putative binding partners

460 molecules were identified in immunoprecipitated samples of HEK293T cells overexpressing a Flag-WSB-1 protein and incubated in normoxia or hypoxia (0.5% O₂) for 24h. 369 of these molecules were found in the normoxic samples, 386 were found in the hypoxic samples, and 295 were present in both sets of samples.

Interestingly, the IPA analysis revealed that "protein ubiquitination" ($p = 3.03 \times 10^{-36}$) was the second most represented canonical pathways in the dataset (Figure 5.5).

Affinity purification followed by mass spectrometry (AP-MS) analyses often produce important amounts of non-specific interactions. It is therefore important to discriminate specific from non-specific hits. To this end, the CRAPome (contaminant repository for affinity purification-ome) database was created (Mellacheruvu et al., 2013). The online CRAPome repository compiles 411 AP-MS analyses and the molecules identified in each of them. The number of analyses where a given protein is detected provides indication on its specificity; the more frequently the molecule is detected, the least likely it is to be specific. Consequently, the present list of 460 potential WSB-1 binding partners was re-examined for possible non-specific binding. Only 88 molecules out of 460 were found in less than 12% of the AP-MS studies indexed in the database. These 88 proteins were analysed again using IPA, yielding 80 analysis-ready molecules. The ten most represented pathways are listed in Table 5.2.

The "protein ubiquitination" pathway was found to be a highly significantly represented pathway again ($p = 8.91 \times 10^{-9}$), although the p -value was smaller than in the previous analysis ($p = 3.03 \times 10^{-36}$; Figure 5.5) and new pathways also appeared in the list of the ten most represented. Interestingly, significance of the HIF-1 α signalling pathways increased when removing the potentially non-specific hits: the p -value changed from 2.4×10^{-2} to 8.63×10^{-3} . On the other hand, pathways such as "eIF2 signalling", "regulation of eIF4 and p70S6K signalling", and "unfolded protein response" disappeared altogether from the pathways represented in the dataset, once likely contaminants were removed.

A network of interaction between these 88 proteins was obtained using the online STRING database and the result is shown in Figure 5.6. WSB-1 is highlighted in red and connects to several of the hits that were found in the analysis. In addition, many of the molecules are described to interact with each other, forming an articulated network. The interactions displayed by STRING are based on actual experiments as well as the frequency of these

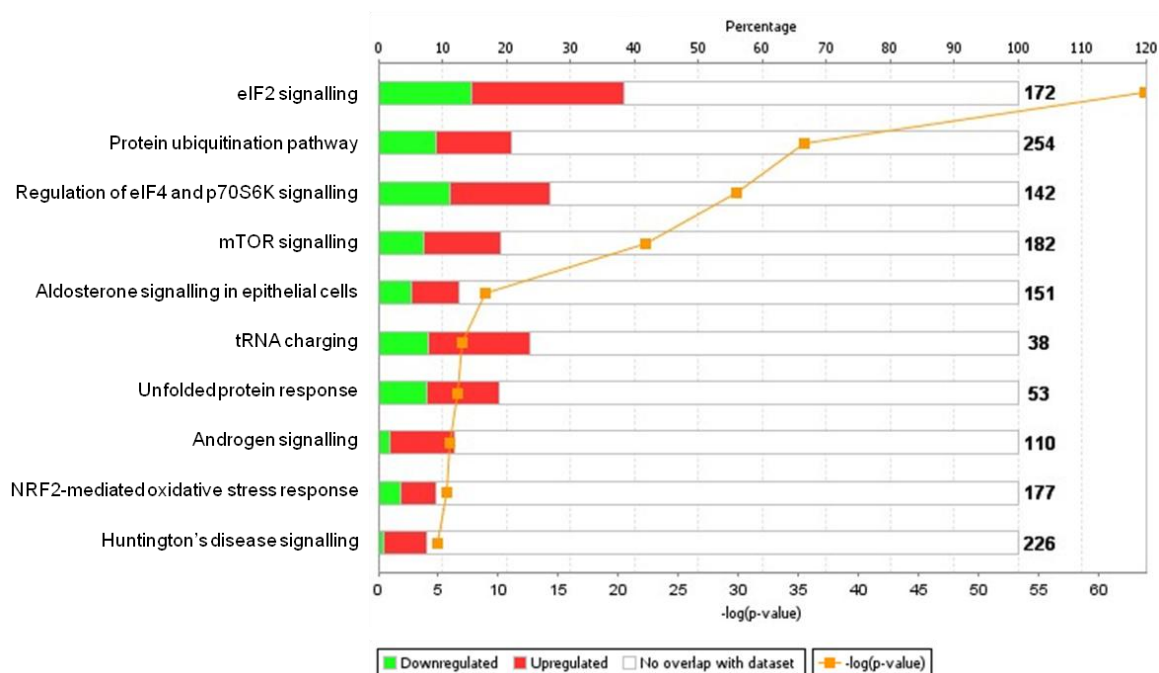


Figure 5.5: Ten most represented canonical pathways in the list of 460 proteins

Bar chart of the 10 most represented pathways within the dataset. Bold numbers corresponds to the number of genes involved in the pathway. Green bars correspond to downregulated genes within the dataset, red bars correspond to upregulated genes in the dataset and white bars correspond to genes not present in the dataset. Yellow line: $-\log(p\text{-value})$.

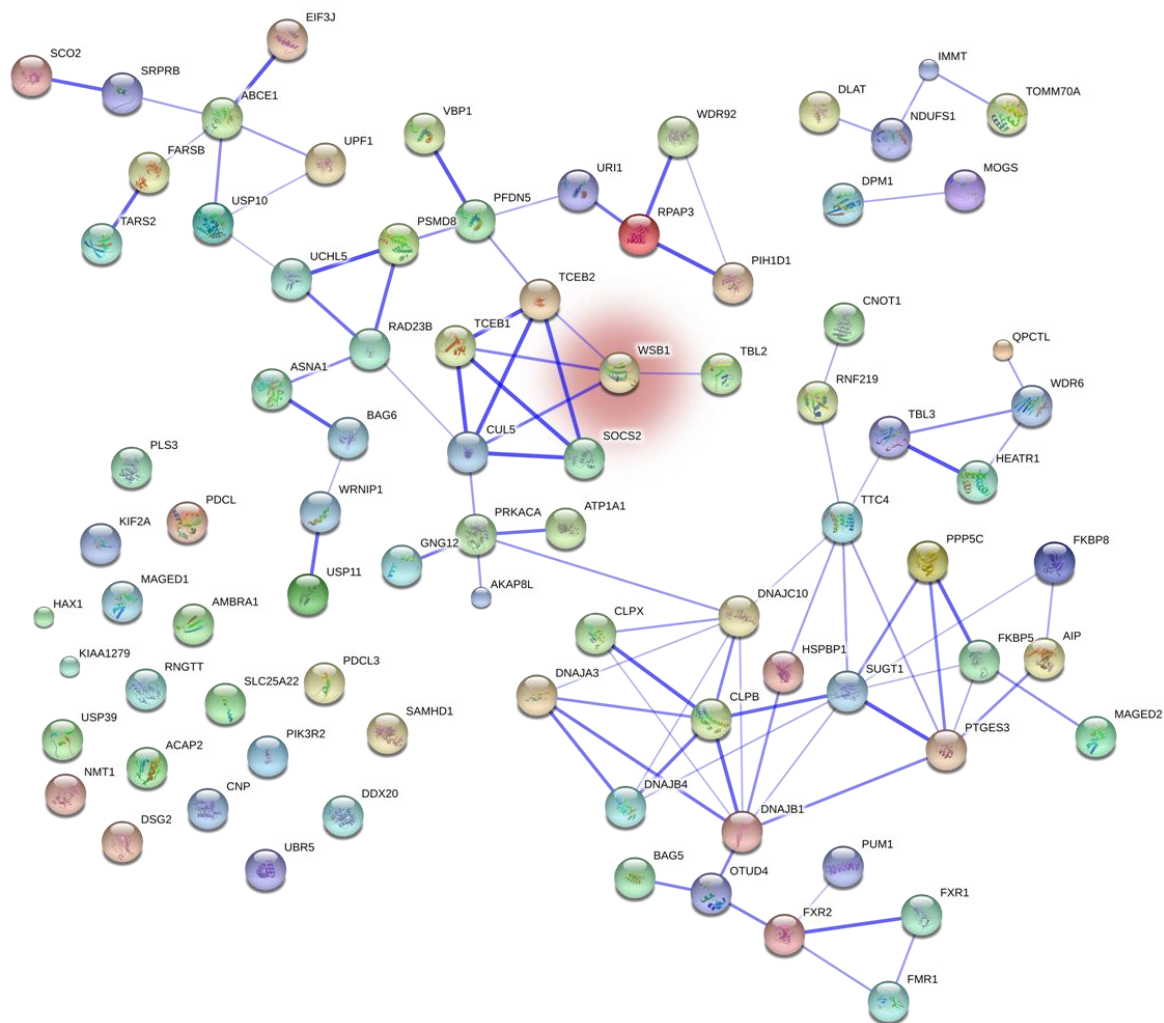


Figure 5.6: Interaction network of putative WSB-1 partners

Map of the interactions between the 88 molecules pulled down with Flag-WSB-1 and found in less than 12% of the studies indexed in the CRAPome database. The intensity of the blue connectors reflects the confidence of the relationship between the two proteins. WSB-1 is highlighted in red.

Table 5.2: Ten most represented canonical pathways in the list of 88 proteins

Canonical pathway	Molecules		<i>p</i> -value
	Downregulated	Upregulated	
Protein ubiquitination pathway	3/254	8/254	8.91×10^{-9}
NRF2-mediated oxidative stress response	2/177	4/177	1.01×10^{-4}
Renal cell carcinoma signalling	0/69	3/69	3.07×10^{-3}
Aldosterone signalling in epithelial cells	1/151	3/151	3.78×10^{-3}
IGF-1 signalling	2/97	1/97	7.94×10^{-3}
HIF-1 α signalling	0/100	3/100	8.63×10^{-3}
IL-9 signalling	1/34	1/34	8.97×10^{-3}
tRNA charging	1/38	1/38	1.11×10^{-2}
Androgen signalling	1/110	2/110	1.12×10^{-2}
P2Y purigenic receptor signalling pathway	1/118	2/118	1.35×10^{-2}

proteins being mentioned together in scientific articles. The more arguments are found supporting an interaction, the darker/thicker the blue connector. The LC-MS analysis was performed on three sets of samples. This allowed testing of the statistical significance of the difference in expression between normoxic and hypoxic conditions. This significance was tested with a *t*-test and only seven hits displayed a *p*-value < 0.05: HNRNPD/AUF1 (*p* = 0.0005), NDUFS1 (*p* = 0.0135), DNAJA3/Tid-1 (*p* = 0.0255), STIP1 (*p* = 0.031), PDIA6 (*p* = 0.0318), PSMD7 (*p* = 0.0348), and RPL12 (*p* = 0.0465). AUF1, Tid-1 and STIP1 were selected among the putative WSB-1 binding partners for further analysis. In addition, the MALDI-MS analysis realised previously permitted to highlight two other putative binding partners of WSB-1: HSP90 and PTGES3/p23 (Table 5.1). In fact, both HSP90 and p23 were detected in the LC-MS analysis but did not reach a significant *p*-value, which was in fact the case for most of the hits returned by the MALDI-MS approach. However, this *p*-value is based on the difference of expression between normoxia and hypoxia. Significance therefore informs on the effect of hypoxia on the quantity of protein pulled down, rather than its overall ability to bind WSB-1.

5.3.2. Validation of putative partners in HEK293T cells

AP-MS analyses provided five potential binding partners of WSB-1: AUF1, HSP90, p23, STIP1 and Tid-1. In addition, HIPK2 and GSK3 β were also investigated as part of the validation process of putative WSB-1 partners. HIPK2 is a known target of WSB-1 ubiquitin ligase activity and was intended as a control of the procedure (Choi et al., 2008). GSK3 β was highlighted as a potential WSB-1 partner in a yeast double hybrid experiment (Vinayagam et al., 2011). GSK3 β is a serine-threonine kinase involved in several cellular mechanisms including energy metabolism as it phosphorylates and inhibits glycogen synthase (Wang et al., 1994).

As illustrated by the interaction network between these seven selected molecules and WSB-1 (Figure 5.7), HSP90 appeared as a central protein, known to interact with four of the 6 other putative partners considered. GSK3 β is also shown to interact with two other proteins, but the possible link between GSK3 β and WSB-1, signalled in a yeast double hybrid experiment, is not represented.

Putative targets were first confirmed as positive hits by probing fresh WSB-1 Flag IP samples with the appropriate antibodies. In order to assess the quality of the pull-down, the membrane was probed with an antibody against the Flag tag. Equal intensity of the signal in the Flag-transfected samples confirmed equivalent transfection efficiency, whereas absence of signal in the mock samples highlighted specificity of the antibodies for the Flag tag (Figure 5.8).

These immunoprecipitated samples were then probed using antibodies against the proteins selected from the putative WSB-1 binding partners (Figure 5.9). The three repeats are shown as each yielded slightly different results and none could be selected as representative of the others. To clarify the results, a summary was made in Table 5.3. HSP90, STIP1, Tid-1 and p23 were successfully validated as they were consistently found in all the input samples and only in the transfected IP samples. On the other hand, AUF1, GSK3 β and HIPK2 could not be

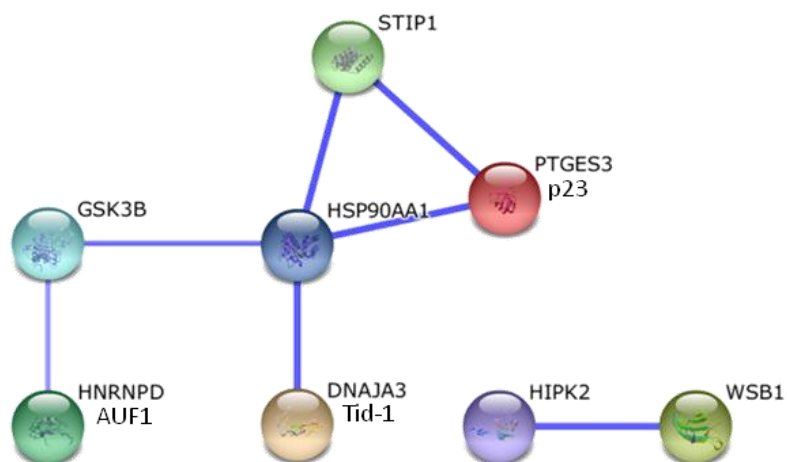


Figure 5.7: Interaction network between WSB-1 and putative partners

Map of the interactions between WSB-1 and the 8 molecules selected as putative WSB-1 partners. The intensity of the blue connectors reflects the confidence of the relationship between the two proteins.

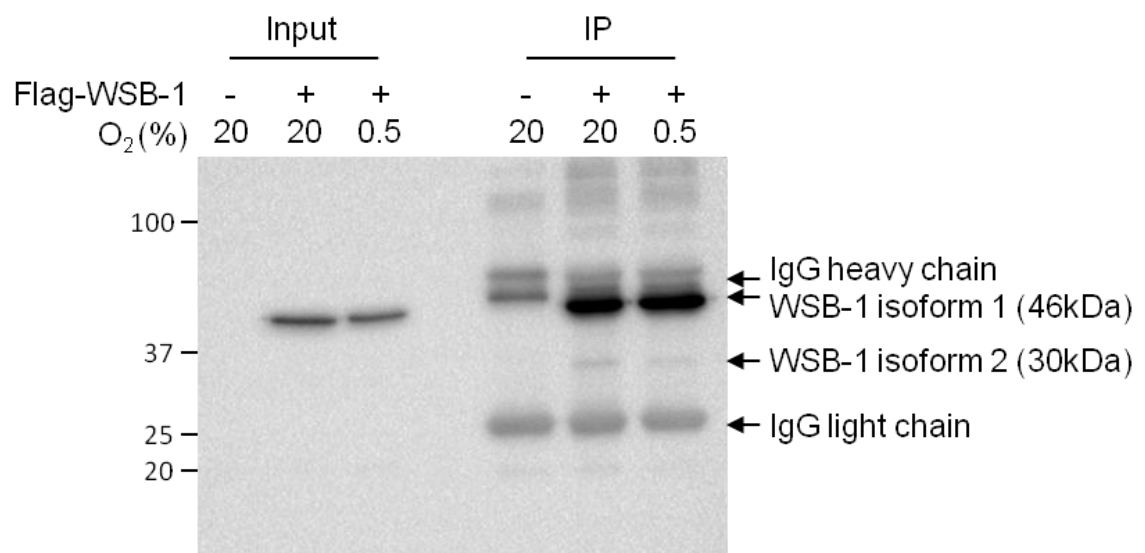


Figure 5.8: Immunoprecipitation efficacy of Flag-WSB-1 in transfected HEK293T samples

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Whole cell lysates (input) or Flag immunoprecipitation samples (IP) were run on an SDS-PAGE gel and resulting Western blot was probed with a Flag antibody. Image representative of $n=10$.

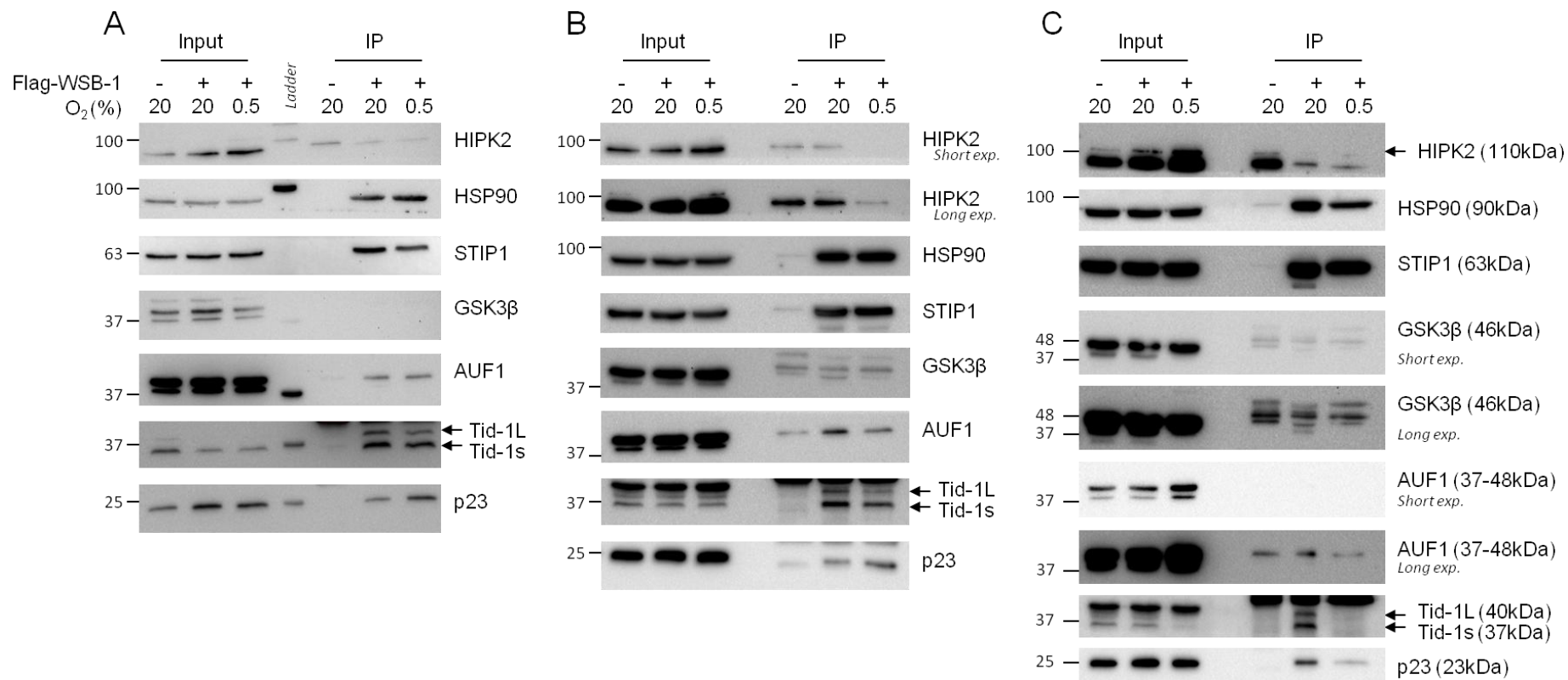


Figure 5.9: Western blot of the immunoprecipitated samples probed with antibodies against putative WSB-1 partners

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Whole cell lysates (input) or Flag immunoprecipitation samples (IP) were run on SDS-PAGE gel and the resulting Western blot was probed with antibodies against putative WSB-1 targets. Blots represent $n=3$ experiments (A, B, C).

Table 5.3: Summary of the validation process of the LC-MS hits in HEK293T cells

	<i>n</i> =1	<i>n</i> =2	<i>n</i> =3
HIPK2	✗	✗	✗
HSP90	✓	✓	✓
STIP1	✓	✓	✓
GSK3β	✗	✗	✗
AUF1	✓	~	✗
Tid-1	✓	✓	✓
p23	✓	~	✓

✓ : Found in input and absent in mock pull down; ✗ : Found in input and present in mock pull down or absent in all pull down; ~ : Found in input, faint band in mock pull down.

confirmed as possible WSB-1 targets by this process: AUF1 yielded conflicting results among repeats (Figure 5.9), making it impossible to conclude on its status; GSK3β and HIPK2 were consistently present in the mock-transfected control as well as in the Flag-transfected samples, or not found at all.

WSB-1 is an E3 ubiquitin ligase (Dentice et al., 2005). In addition, LC-MS analysis revealed that the protein ubiquitination pathway was highly represented in molecules pulled down with WSB-1 (Figure 5.5). Protein ubiquitination is a signal for proteasomal degradation and this particular mechanism can be blocked *in vitro* using MG132. Considering the role of WSB-1, any potential WSB-1 targets would therefore be signalled for proteasomal degradation. With this in mind, treating cells with MG132 would prevent the degradation of the putative WSB-1 targets and therefore facilitate their detection by Western blotting. The results of this experiment are represented in Figure 5.10. Coomassie blue staining (Figure 5.10A) suggested that MG132 had no clear effect on the quantity of protein present in the samples, as the samples treated with MG132 and those which were not displayed similar banding patterns. The same samples were probed with an antibody against Flag (Figure 5.10B). Most remarkably, this revealed an increase in the signal corresponding to WSB-1 isoform 3, whereas signals for WSB-1 isoform 1 (although saturated) and 2, appeared to be consistent

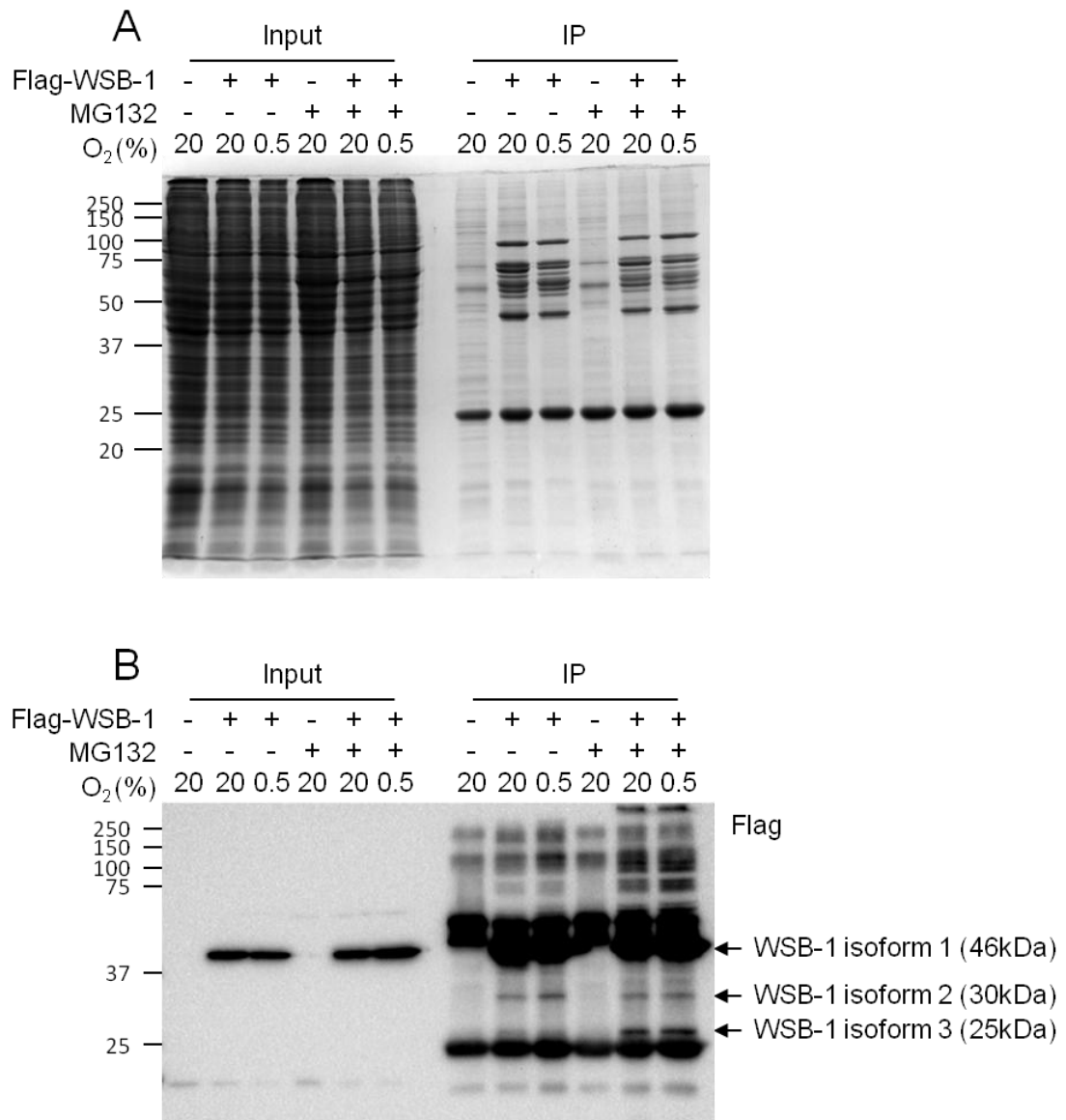


Figure 5.10: Effect of MG132 on the immunoprecipitation of Flag-WSB-1

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1 or mock transfected with water as a control and treated with MG132 (10 μ M) or equivalent volume of DMSO. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Whole cell lysates (input) or Flag immunoprecipitation samples (IP) were run on and SDS-PAGE gel and stained with Coomassie blue (A) or transferred by Western blotting and probed with a Flag antibody (B). *n*=1.

with and without MG132 treatment. Interestingly, higher molecular bands can also be detected in the MG132 samples only, which could potentially correspond to a post-translationally modified form of WSB-1 (such as ubiquitination). Next, the samples were probed with the antibodies against the putative WSB-1 targets being presently studied (Figure 5.11). Contrary to what was observed with WSB-1 isoform 3, the MG132 treatment appeared to have no effect on the quantity of protein pulled down for HSP90, HIPK2 and p23. Levels of AUF1 and Tid-1 actually appeared to decrease upon MG132 treatment. Finally, no signal could be detected for GSK3 β , regardless of the treatment. Overall, the results obtained with the MG132 treatment were similar to those obtained without MG132.

Considering that MG132 treatment did not alter the detection of the putative WSB-1 targets in immunoprecipitated samples, further work was carried out without MG132 treatment.

5.3.3. Validation of putative hits in breast cancer cell lines

To validate the interaction between the selected putative targets and WSB-1 in breast cancer, a similar experimental setup to that presented for HEK293T cells was applied to MCF7 and MDA-MB-231 cell lines. Figure 5.12 presents Coomassie blue stain (A) and Flag antibody probing (B) obtained with MCF7 cells. Although it appeared that the signal is generally stronger in the hypoxic samples compared to the normoxic samples, this experiment confirmed that WSB-1 could successfully be pulled down in MCF7 cells. Next, the MCF7 IP samples were probed with antibodies against the putative WSB-1 partners being investigated (Figure 5.13). Consistently with what was observed in Figure 5.12, signal intensities appeared weaker in the hypoxic samples compared to the normoxic samples. More importantly, with the exception of HIPK2, all the antibodies presented a band in the mock samples, suggesting a non-specific pull down of the proteins of interest by the Flag-coated beads.

In order to decrease the non-specific binding of the proteins to the Flag beads during the immunoprecipitation step, the protocol was amended. The washing steps were made more stringent by doubling the concentration of the detergent NP-40 (nonyl

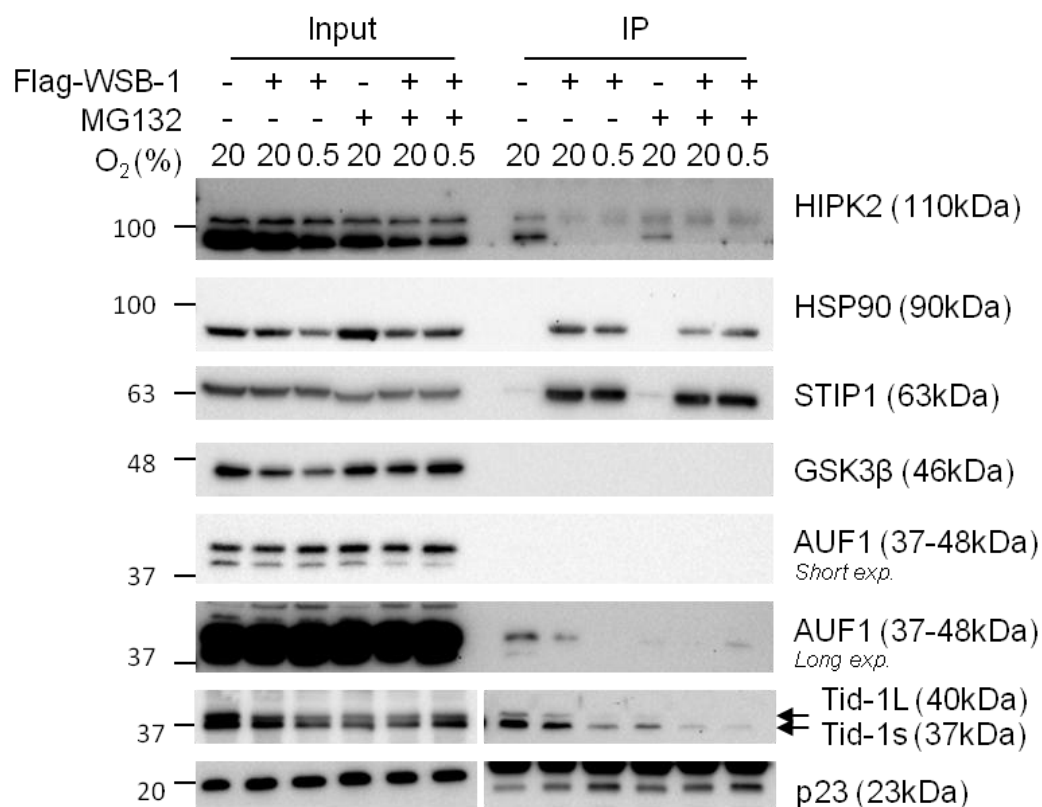


Figure 5.11: Effect of MG132 on binding of putative WSB-1 partners

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1 or mock transfected with water as a control and treated with MG132 (10μM) or equivalent volume of DMSO. Dishes were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Whole cell lysates (input) or Flag immunoprecipitation samples (IP) were run on and SDS-PAGE gel and the resulting Western blot was probed with antibodies against WSB-1 putative partners. Input and IP samples for Tid-1 and p23 probing were run on two separate gels. *n*=1.

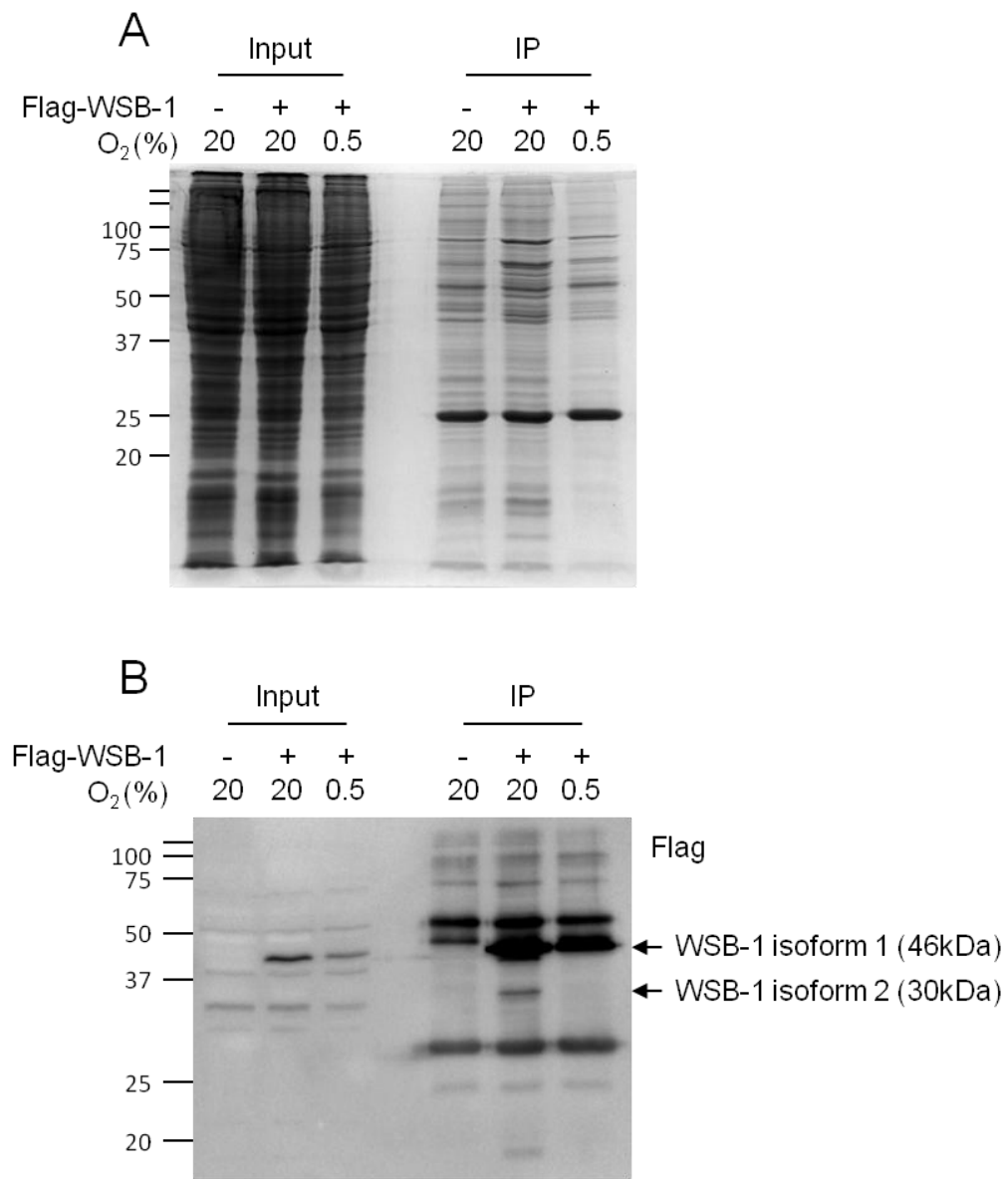


Figure 5.12: Validation of Flag-WSB-1 immunoprecipitation in MCF7 cells

SDS-PAGE of whole cell lysates (input) or Flag immunoprecipitation samples (IP) of MCF7 cells overexpressing Flag-WSB-1 or mock transfected with water as a control, stained with Coomassie blue (A) and Western blot of the same samples probed with an antibody against Flag (B). Images are representative of $n=2$. Results obtained following identical protocol used with HEK293T samples.

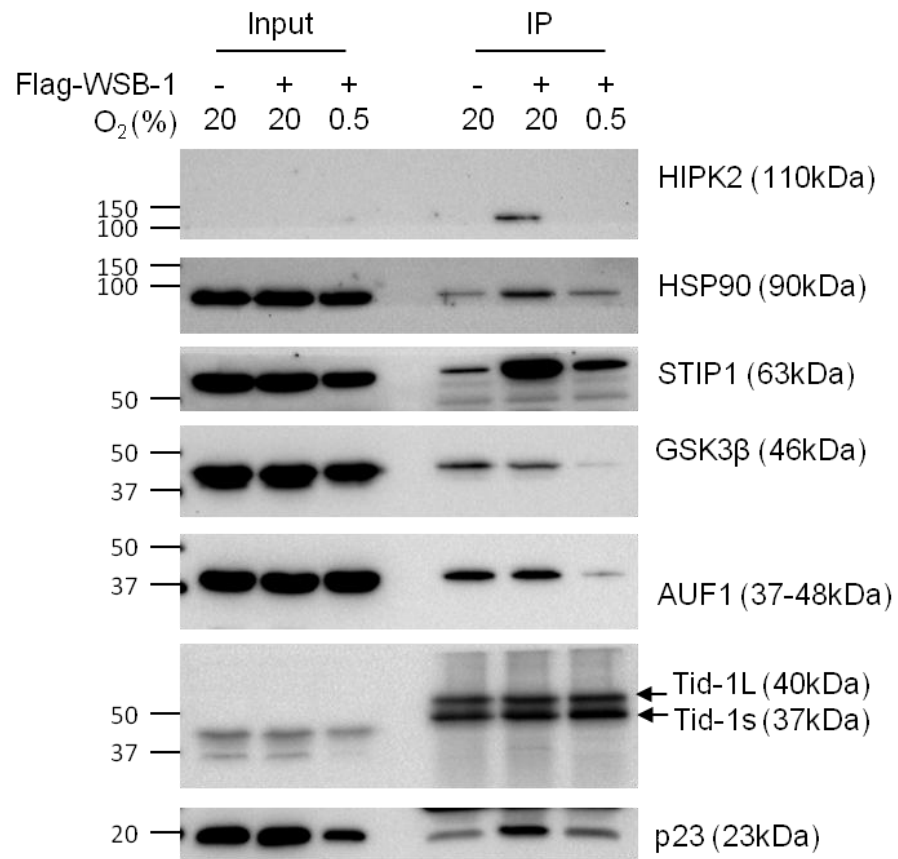


Figure 5.13: Validation of putative WSB-1 binding partners immunoprecipitation in MCF7 cells

MCF7 cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Whole cell lysates (input) or Flag immunoprecipitation samples (IP) were run on an SDS-PAGE gel and resulting Western blot was probed with antibodies against WSB-1 putative partners. Image representative of $n=2$.

phenoxyethoxyethanol) present in the original lysis buffer. All the other elements of the protocol were left unchanged.

As visible in Figure 5.14, the increase in the NP-40 content of the lysis buffer resulted in cleaner banding pattern. Less bands were visible in the Coomassie blue stain of the IP samples (A), as well as in the input samples probed with the Flag antibody (B). WSB-1 isoform 1 remained detectable in the IP samples, and WSB-1 isoform 2 also elicited a faint signal. As a result, the samples were then probed with the antibodies against the putative WSB-1 targets (Figure 5.15). The increased stringency was proven successful for HSP90 and STIP1, as the non-specific binding in the mock samples was cleared. In fact, the procedure also removed any signal for GSK3 β and AUF1. On the contrary, signals for Tid-1 and p23 remained as strong in the mock as in the transfected IP samples.

Table 5.4 below summarises the results obtained for MCF7 cells in the three repeats of the experiment. None of the hits could be validated with confidence but HSP90 and STIP1 appeared as strong candidates.

Table 5.4: Summary of the validation process of the LC-MS hits in MCF7 cells

	<i>n</i> =1	<i>n</i> =2	<i>n</i> =3
HIPK2	?	✗	✗
HSP90	✓	✗	✓
STIP1	~	~	✓
GSK3β	✗	✗	✗
AUF1	✗	✗	✗
Tid-1	~	✗	✗
p23	~	✗	✗
✓ : Found in input and absent in mock IP; ✗ : Found in input and present in mock IP or absent in all IP; ? : Absent in input; ~ : Found in input, faint band in mock IP.			

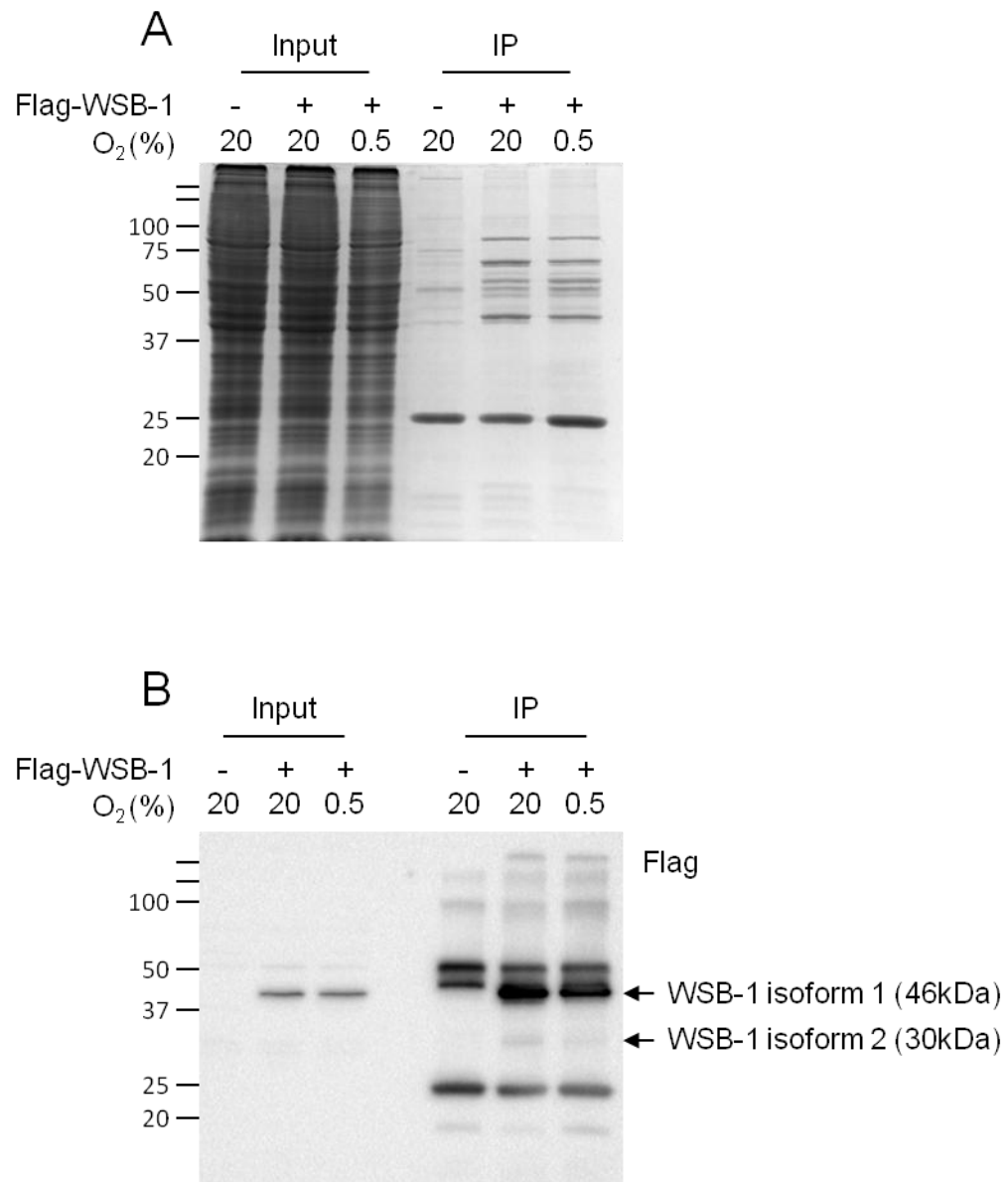


Figure 5.14: Validation of Flag-WSB-1 immunoprecipitation in MCF7 cells (stringent wash)

SDS-PAGE of whole cell lysates (input) or Flag-WSB-1 immunoprecipitation samples (IP) of MCF7 cells overexpressing Flag or mock transfected with water, stained with Coomassie blue (A) and Western blot of the same samples probed with an antibody against Flag (B). Immunoprecipitated samples washed in buffer containing two times more NP40 and followed by 2min rotation cycles at room temperature. $n=1$.

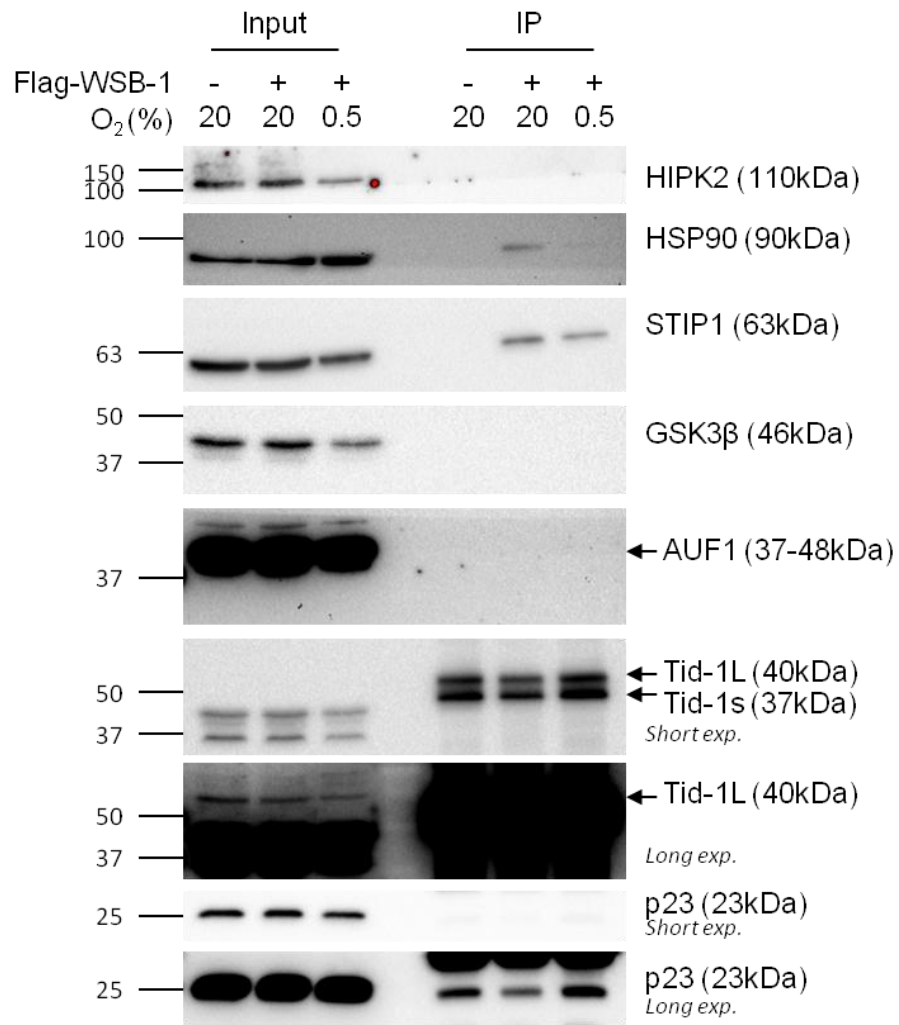


Figure 5.15: Validation of putative WSB-1 binding partners immunoprecipitation in MCF7 cells (stringent wash)

MCF7 cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Whole cell lysates (input) or Flag immunoprecipitation samples (IP) were run on an SDS-PAGE and resulting Western blot was probed with antibodies against WSB-1 putative partners. *n*=1.

The procedure described previously with MCF7 was repeated using MDA-MB-231 cells. Using the original lysis buffer, MDA-MB-231 cells transfected with the plasmid coding for the Flag tagged version of WSB-1 were immunoprecipitated using beads engineered to bind the Flag tag. The Coomassie blue stained SDS-PAGE gel as well as the Western blot of these samples probed with an antibody against Flag are presented in Figure 5.16. Figure 5.16B shows that WSB-1 isoform 1 was successfully pulled down in MDA-MB-231 cells. As a result, samples were then probed with antibodies against the putative WSB-1 targets (Figure 5.17). In this experiment, HIPK2 appeared to be undetectable, including in the input samples. STIP1 and AUF1, although presenting a clear signal in the input samples could not be detected in the IP samples. On the contrary, Tid-1 and p23, similarly to what was observed in MCF7 cells, were consistently pulled down, including in the mock samples. GSK3 β also presented a band in the mock IP samples. Interaction of these targets with WSB-1 could not be validated in MDA-MB-231 cells. However, the clear banding pattern of HSP90 suggested that it could be a binding partner of WSB-1.

With the intent of decreasing the non-specific binding observed in the mock samples for GSK3 β , Tid-1 and p23, the more stringent lysis buffer with increased detergent concentration described earlier was employed. Results are displayed in Figure 5.18 and Figure 5.19. More stringent washes did not alter WSB-1 pull down, as demonstrated by the strong signal corresponding to WSB-1 isoform 1 (Figure 5.18B). It did not disrupt Tid-1 and p23 non-specific binding either, as a signal in the mock IP samples was still observed. It did, however, decrease binding of STIP1, GSK3 β and AUF1 to the point where no signal could be detected in the IP samples for these three proteins. Surprisingly, HIPK2 was detected in the input this time but not in the IP samples. Finally, the banding pattern of HSP90, although absent in hypoxia, comforted its potential as a WSB-1 binding partner.

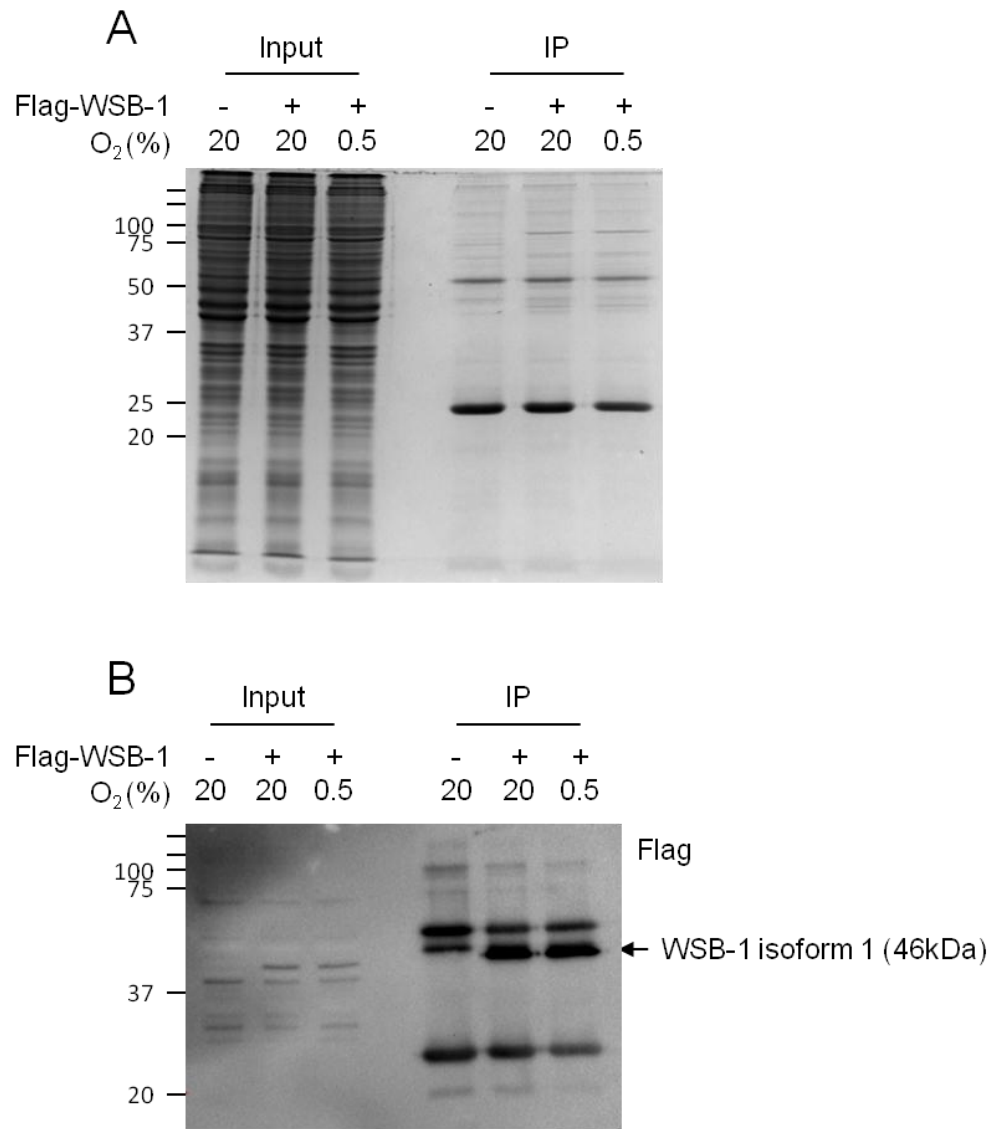


Figure 5.16: Validation of Flag-WSB-1 immunoprecipitation in MDA-MB-231 cells

SDS-PAGE of whole cell lysates (input) or Flag immunoprecipitation samples (IP) of MDA-MB-231 cells overexpressing Flag-WSB-1 or mock transfected with water as a control, stained with Coomassie blue (A) and Western blot of the same samples probed with an antibody against Flag (B). Images are representative of $n=2$. Results obtained following identical protocol used with HEK293T samples.

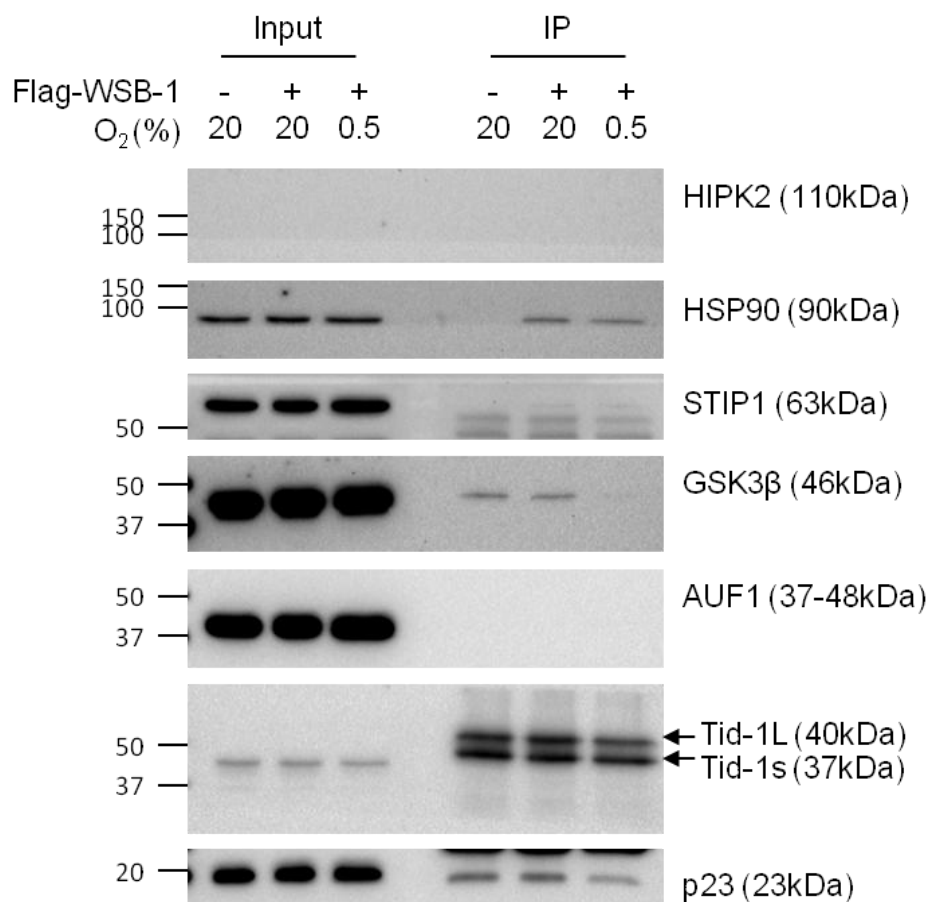


Figure 5.17: Validation of putative WSB-1 binding partners immunoprecipitation in MDA-MB-231 cells

MDA-MB-231 cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Whole cell lysates (input) or Flag immunoprecipitation samples (IP) were run on an SDS-PAGE gel and resulting Western blot was probed with antibodies against WSB-1 putative partners. Image representative of $n=2$.

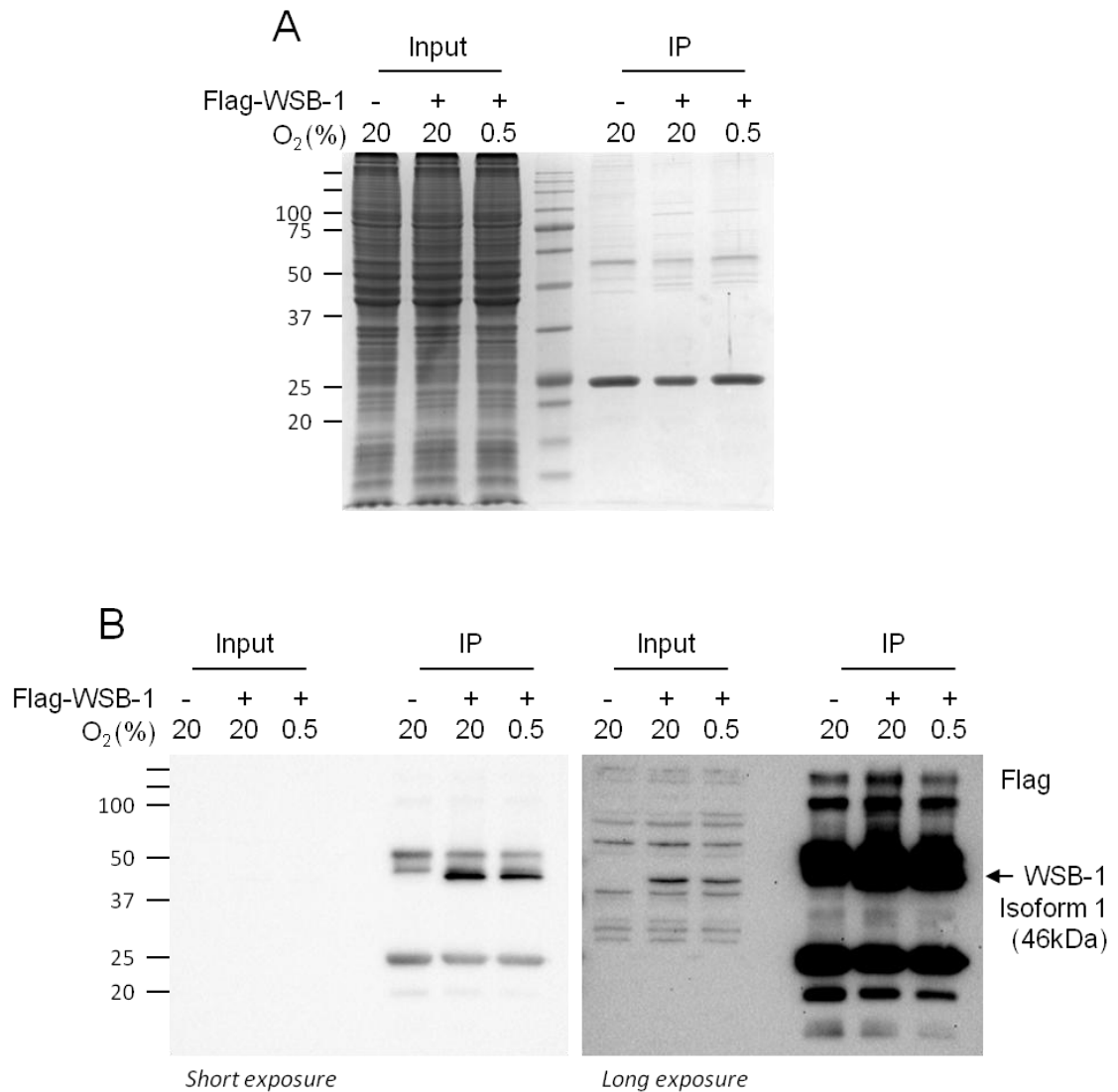


Figure 5.18: Validation of Flag-WSB-1 immunoprecipitation in MDA-MB-231 cells (stringent wash)

SDS-PAGE of whole cell lysates (input) or Flag immunoprecipitation samples (IP) of MCF7 cells overexpressing Flag-WSB-1 or mock transfected with water, stained with Coomassie blue (A) and Western blot of the same samples probed with an antibody against Flag (B). Immunoprecipitated samples washed in buffer containing two times more NP40 and followed by 2min rotation cycles at room temperature. $n=1$.

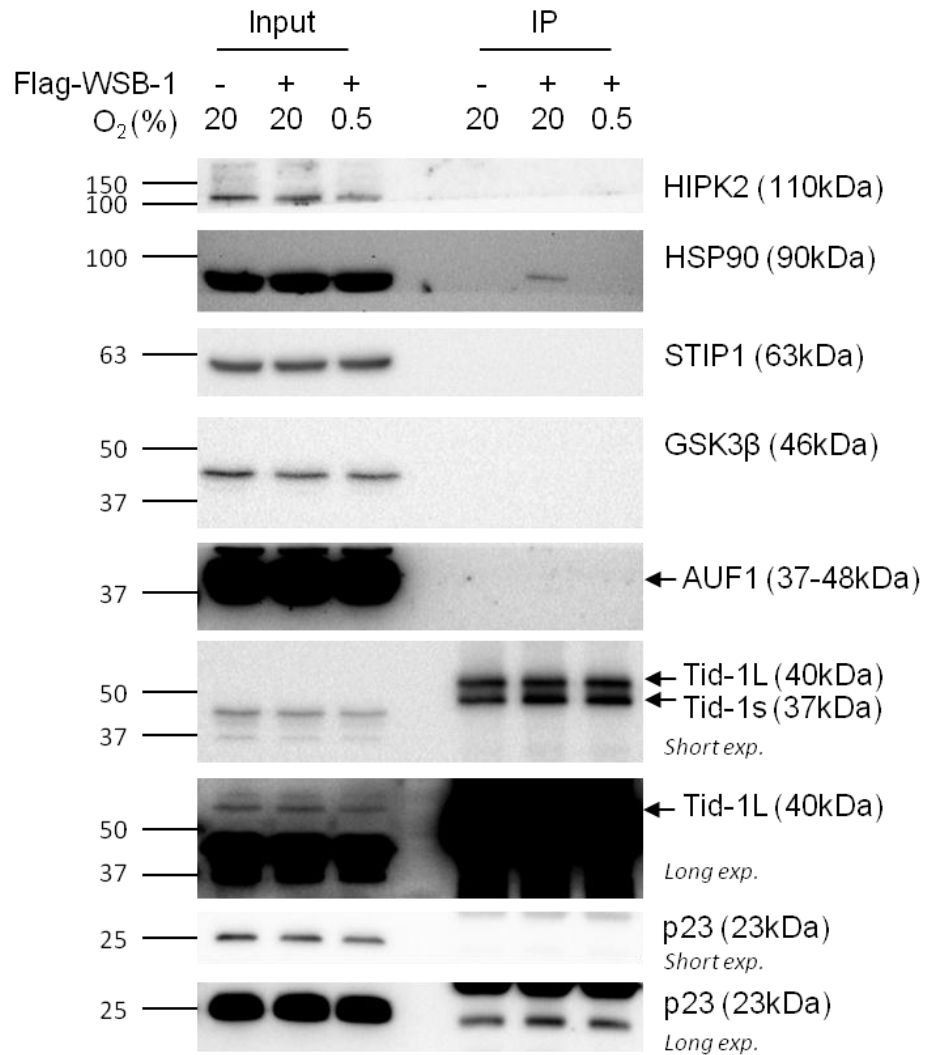


Figure 5.19: Validation of putative WSB-1 binding partners immunoprecipitation in MDA-MB-231 cells (stringent wash)

MDA-MB-231 cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Whole cell lysates (input) or Flag immunoprecipitation samples (IP) were run on an SDS-PAGE and resulting Western blot was probed with antibodies against WSB-1 putative partners. *n*=1.

To summarise, the results obtained in MDA-MB-231 are compiled in Table 5.5. Overall, none of the putative WSB-1 partners were validated in MDA-MB-231. HSP90, however, stood again as a strong possible candidate.

Table 5.5: Summary of the validation process of the LC-MS hits in MDA-MB-231 cells

	<i>n</i> =1	<i>n</i> =2	<i>n</i> =3
HIPK2	?	✗	✗
HSP90	✓	✗	✓
STIP1	✗	✗	✗
GSK3β	✗	~	✗
AUF1	✗	✗	✗
Tid-1	✗	✗	✗
p23	✗	✗	✗

✓ : Found in input and absent in mock IP; ✗ : Found in input and present in mock IP or absent in all IP; ? : Absent in input; ~ : Found in input, faint band in mock IP.

Taken together, results obtained in the three cell lines, as summarised in Table 5.6, revealed that, among the seven hits selected, HSP90 and STIP1 were likely to interact with WSB-1 in HEK293T cells as well as in breast cancer cell lines, and therefore presented as good candidates for WSB-1 partners.

Table 5.6: Summary of the validation process of the LC-MS hits for all three cell lines

	HEK293T	MCF7	MDA-MB-231
HIPK2	✗	✗	✗
HSP90	✓	✓	~
STIP1	✓	✓	✗ ^a
GSK3β	✗	✗	✗
AUF1	~	✗	✗
Tid-1	✓	✗ ^b	✗ ^b
p23	✓	✗ ^b	✗ ^b

✓ : Validated as interacting with WSB-1; ~ : Specificity uncertain; ✗ : Pull down not specific; ✗^a : No signal in IP samples; ✗^b : Target pulled down in mock samples.

5.3.4. Immunoprecipitation of HSP90 and STIP1 in HEK293T cells

In light of the results presented in the previous section, HSP90 and STIP1 were selected for further analyses to confirm their interaction with WSB-1. Lysates of HEK293T cells overexpressing Flag-tagged WSB-1 were incubated with magnetic beads coated with antibodies specific for HSP90 or STIP1. These reverse IPs allowed the pull down of proteins binding to this putative WSB-1 partners as well as any other proteins involved in the cluster.

Immunoprecipitation of HSP90 is shown in Figure 5.20. Coomassie blue staining of the SDS-PAGE gel revealed fewer proteins were pulled down in the mock than in the transfected IP samples (A). HSP90, however, was consistently pulled down in the three conditions, confirming the success of the procedure (B). The membrane was then probed with WSB-1 antibody, which revealed that WSB-1 was only pulled down in the transfected samples. WSB-1 signal appeared to decrease in hypoxia compared to normoxia, which could be explained by the lesser amount of protein in the hypoxic IP sample, or a decrease in HSP90/WSB-1 binding affinity in hypoxia. The same procedure was followed for STIP1 and the resulting IP is presented in Figure 5.21. Only faint bands were visible in the IP samples after staining of the SDS-PAGE gel with Coomassie blue (A). However, probing of the Western blot with STIP1 antibody revealed that the protein of interest was successfully pulled down (B). Probing of the same samples with WSB-1 antibody did not reveal any signal, signifying that WSB-1 was not pulled down alongside STIP1. This could be because STIP1 and WSB-1 actually do not interact, or because the binding is lost during the elution procedure. To eliminate this latter possibility, the experiment was reproduced without the elution step and the outcome is visible in Figure 5.22. STIP1 was still successfully pulled down but bands corresponding to WSB-1 were also visible in the IP samples, although they were difficult to identify with certainty.

In addition, the presence of bands in the mock IP sample suggested non-specific binding.

In conclusion, the present work allowed identifying HSP90 and STIP1 as strong candidates for WSB-1 binding from a list of over a thousand proteins pulled down along with a Flag-tagged

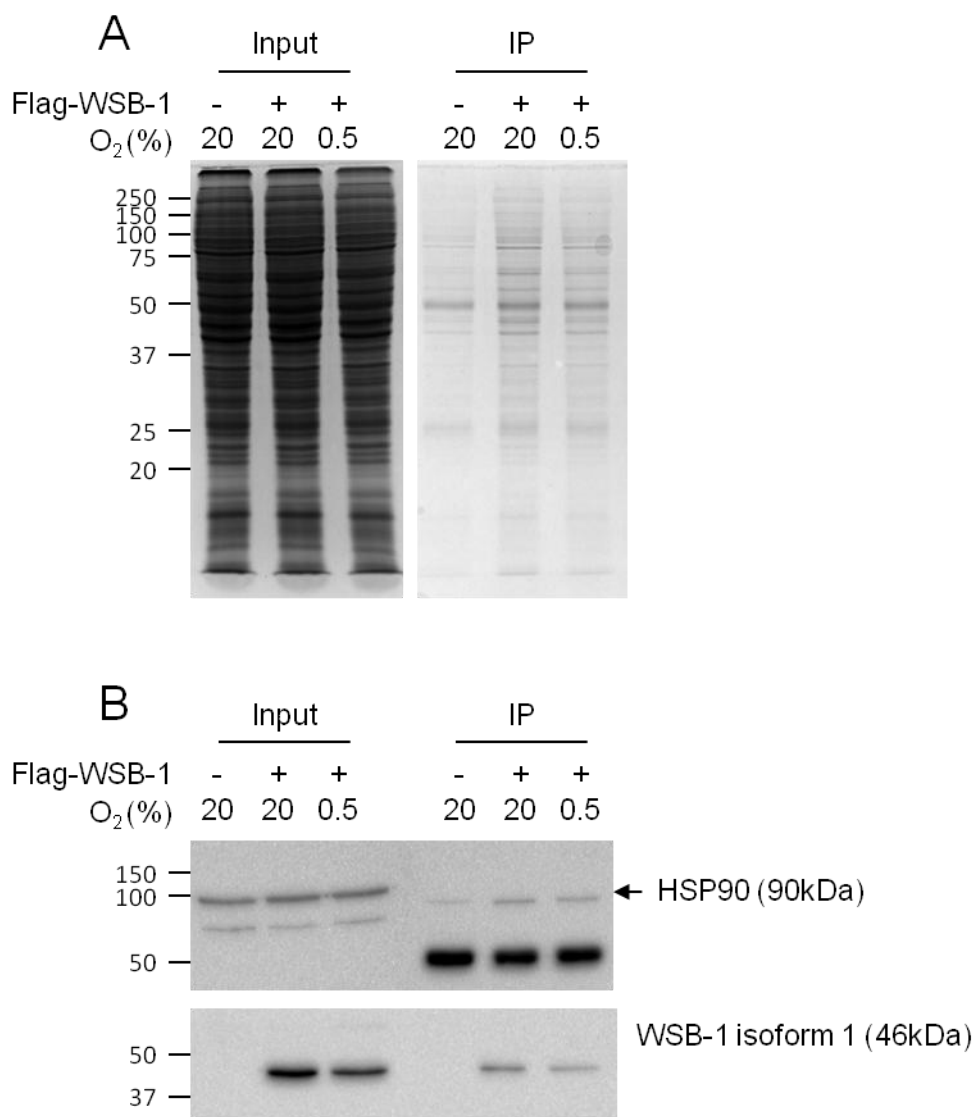


Figure 5.20: Immunoprecipitation of HSP90 from HEK293T samples overexpressing Flag-WSB-1

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Dishes were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Lysates were exposed to beads coated with HSP90 antibody. Whole cell lysates (input) or Flag immunoprecipitation samples (IP) were run on an SDS-PAGE gel, stained with Coomassie blue (A). Western blot was probed with HSP90 and WSB-1 antibodies (B). *n*=1.

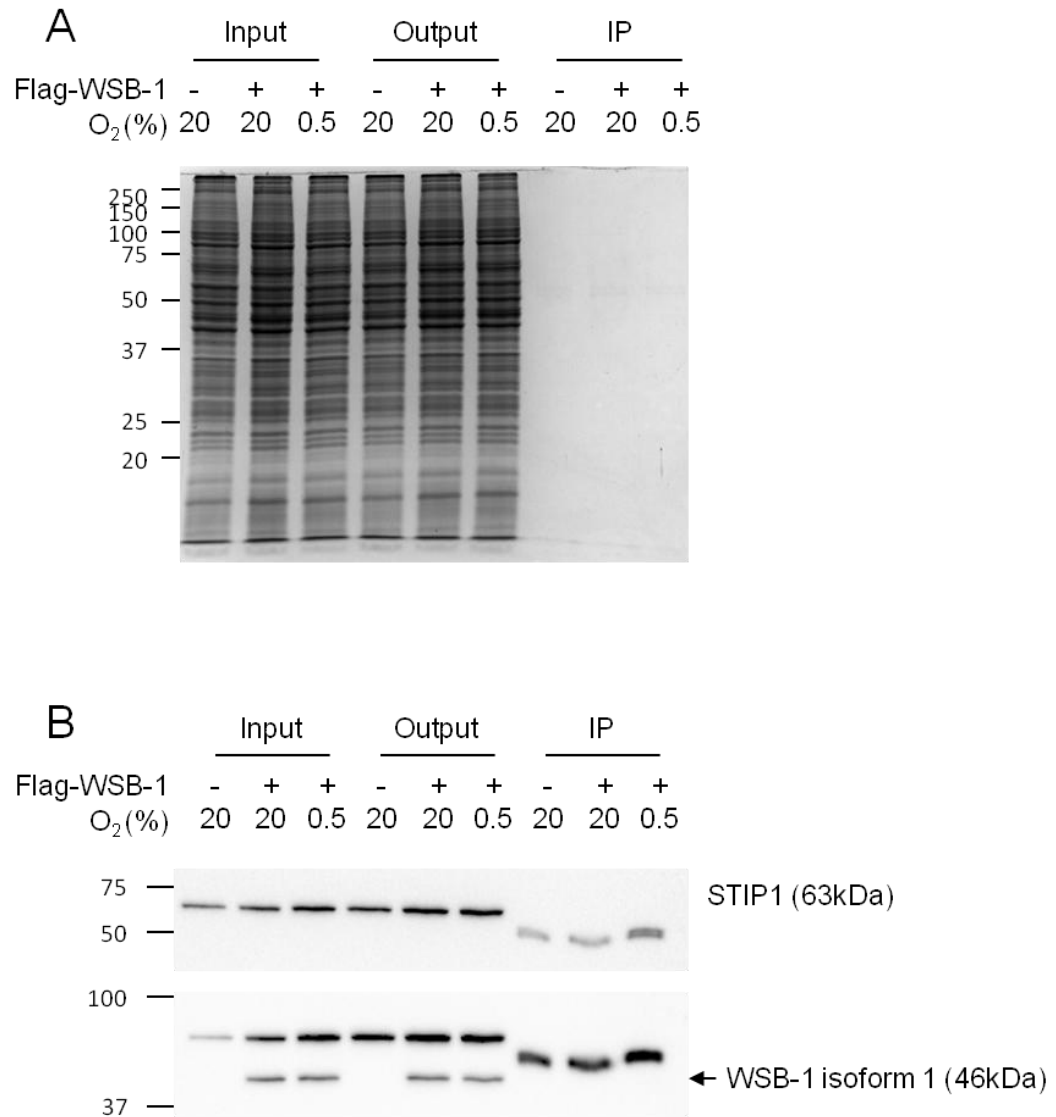


Figure 5.21: Immunoprecipitation of STIP1 from HEK293T samples overexpressing Flag-WSB-1

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Lysates were exposed to beads coated with STIP1 antibody. Resulting SDS-PAGE was stained with Coomassie blue (A) and Western blot was probed with STIP1 and WSB-1 antibodies (B). IP: immunoprecipitated samples; *n*=1.

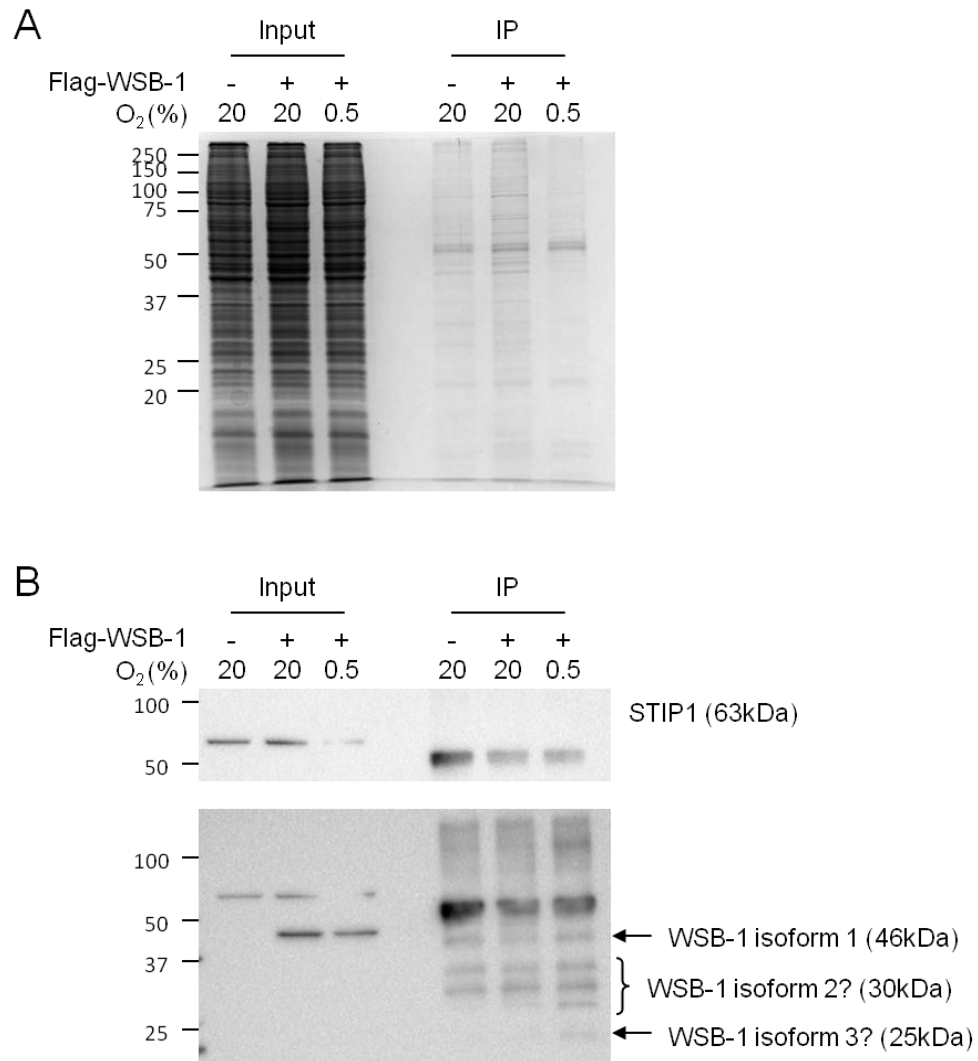


Figure 5.22: Immunoprecipitation without elution of STIP1 from HEK293T samples overexpressing Flag-WSB-1

HEK293T cells were transfected with a plasmid coding for a Flag tagged version of WSB-1, or mock transfected with water as a control. Cells were then exposed to normoxia (20% O₂) or hypoxia (0.5% O₂) for 24h. Lysates were exposed to beads coated with STIP1 antibody and no elution was performed at the end of the experiment. Whole cell lysates (input) or Flag-immunoprecipitation samples (IP) were run on an SDS-PAGE gel stained with Coomassie blue (A). Western blot was probed with STIP1 and WSB-1 antibodies (B). *n*=1.

version of WSB-1. The binding between HSP90 or STIP1 and WSB-1 was further validated by pulling down HSP90 or STIP1 from HEK293T cells overexpressing WSB-1 and verifying the presence of WSB-1. Present results suggested that HSP90 did interact with WSB-1, but the settings used failed to retrieve WSB-1 in proteins pulled down with STIP1, questioning its potential as WSB-1 partner.

5.3.5. Effect of WSB-1 knockdown on the expression of putative WSB-1 partners

In order to provide a better understanding of the interaction between WSB-1 and its putative partners previously identified, WSB-1 was knocked down in MCF7 and MDA-MB-231 cells and levels of the seven protein of interest were analysed by Western blot (Figure 5.23). STIP1 levels appeared not to be dependent on WSB-1 expression. Similarly, HSP90 levels, although they appeared to increase in response to hypoxia, did not change with WSB-1 knockdown in MDA-MB-231 cells. On the contrary, GSK3 β and Tid-1s levels consistently decreased in both cell lines in response to WSB-1 knockdown. HIPK2 and AUF1 levels appeared to increase in response to WSB-1 knockdown in MCF7 cells but decreased in MDA-MB-231. Conversely, WSB-1 knockdown resulted in a decrease of p23 level in MCF7 but an increase in MDA-MB-231.

Overall, most of the putative WSB-1 partners initially selected in the beginning of this chapter appeared to be affected by WSB-1 knockdown. Additionally, WSB-1 knockdown differentially influenced HIPK2, HSP90, AUF1 and p23 levels in MCF7 and MDA-MB-231 cell lines.

5.4. Discussion

The objective of this chapter was to investigate the WSB-1 interactome and identify novel WSB-1 interacting partners. To do so, samples of HEK293T cells overexpressing a Flag tagged version of WSB-1 were analysed by AP-MS. The list of proteins obtained was refined using the CRAPome database, to remove known potential contaminants from Flag AP-MS procedures. The refined list of hits was uploaded in IPA in order to highlight main pathways represented

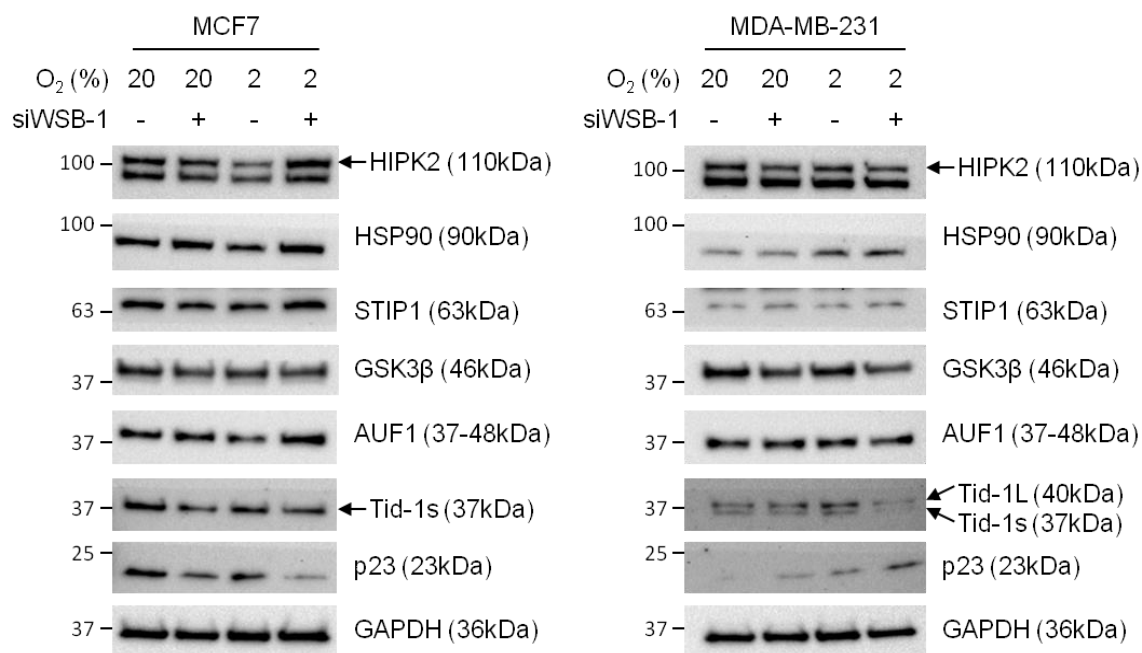


Figure 5.23: Effect of WSB-1 knockdown and hypoxia exposure to putative WSB-1 binding partners

MCF7 and MDA-MB-231 cells transfected with siRNA against WSB-1 (siWSB-1) or treated with non targeting siRNA (siNT) were exposed to hypoxia (24h at 2% O₂) or normoxia. The protein levels of putative WSB-1 partners were analysed by Western blot. GAPDH was used as housekeeping control. Images representative of $n=4$.

by the proteins pulled down with WSB-1. Subsequently, seven promising hits were chosen to be analysed more closely. Effectiveness of the pull down was confirmed in MCF7 and MDA-MB-231 cell lines and reverse IPs were also executed to further verify the interaction between WSB-1 and these possible partners. Finally, effect of WSB-1 knockdown on the protein level of these seven favoured proteins was monitored by Western blot.

To summarise, this chapter provided the following answers to the questions asked initially:

- *What proteins are likely to interact with WSB-1?*

AP-MS analyses provided an extensive list of proteins likely to interact directly or indirectly with WSB-1. Following careful filtration of the multiple hits, HSP90, STIP1, GSK3 β , AUF1, Tid-1 and p23 were retained as possible WSB-1 partners.

- *What biological pathways are enriched in the list of putative WSB-1 binding partners?*

"Protein ubiquitination pathway" and "HIF-1 α signalling" were among the most represented pathways within the WSB-1 interactome.

- *What is the effect of WSB-1 knockdown on the protein level of these putative WSB-1 partners?*

WSB-1 knockdown in MCF7 and MDA-MB-231 cells revealed that levels of HIPK2, GSK3 β , AUF1, Tid-1 and p23 varied with WSB-1 expression, and differed between the two cell lines. Interestingly, HSP90 in MDA-MB-231 and STIP1 in both cell lines appeared unaffected by WSB-1 knockdown, although they responded to hypoxia.

5.4.1. Mass spectroscopy analyses

A preliminary MALDI-MS analysis was previously undertaken in this laboratory. This study allowed highlighting of some proteins that appeared to be pulled down with WSB-1. HSPs (heat shock proteins) were highly represented in the results (Table 5.1). In particular, sixteen peptides in normoxia and twelve peptides in hypoxia matched to sequences of proteins in the HSP90 family. This could be explained by the fact that HSP90 is a very abundant protein, which represents 2 to 3% of the total proteins in a normal cell and up to 7% in melanoma cells

(Sahu et al., 2012).

Abundance of certain proteins can lead to artefacts and detection of false positives. Other false positives can arise from external contaminant such as keratin. For this reason, it is important to ensure the samples, gel and reagents are handled carefully in a clean environment ahead of the MS analysis. Afterwards, refining of the dataset obtained is possible using tools like the CRAPome database, which will filter the most common contaminants.

Because of the small scope of MALDI-MS, LC-MS was used in order to detect a larger range of proteins within WSB-1 interactome (Aebersold and Mann, 2003; Karpievitch et al., 2010). With the exception of RPS27A, HSP90AA2, TUBA4A, TUBB2C (which were recognised only by one peptide), all the other proteins detected by MALDI-MS were also present in the LC-MS hits list. These proteins can be either regulators of WSB-1 activity, targets of WSB-1, or binding partners. All proteins will not bind to each other with the same affinity. For example, interaction between regulator and regulated protein are transient and difficult to capture with conventional immunoprecipitation. As a result, some of these proteins might be under-represented in the hit list obtained. To increase the elution of unstable binding partners, samples can be incubated with cross-linking agents which will stabilise these otherwise weak interactions (Rappsilber, 2011). Similarly, ubiquitinated targets of WSB-1 activity are quickly degraded by the proteasome. The use of proteasome inhibiting drugs, such as MG132, could prevent proteasomal degradation of the target proteins and increase their half-life, facilitating their detection.

5.4.1.1. Effect of MG132

In the experiments presented in this chapter (Figure 5.10 and Figure 5.11), MG132 treatment did not appear to have an effect on the quantity of protein pulled down with WSB-1. Surprisingly, levels of HIPK2, a known WSB-1 target, actually decreased upon MG132 treatment (Choi et al., 2008). On the other hand, an increase in WSB-1 isoform 3 was observed in response to MG132 treatment. This corroborates the results obtained in the lab in a

preliminary study whereby treatment of HEK293T cells with MG132 resulted in an increase of WSB-1 isoform 3 protein levels (Bilbe and Pires, unpublished). Ectopically expressed full length WSB-1 isoform 1 was also observed to be ubiquitinated in an oxygen-independent manner. This particular aspect has previously been discussed in the literature where putative ubiquitination sites in WSB-1 sequence were described (Kim et al., 2011b; Wagner et al., 2011).

5.4.2. Analysis of the validity of putative WSB-1 partners

Following the AP-MS analyses using MALDI- and LC-MS, seven proteins were selected for further investigation. **HIPK2** was chosen as it is a well-known WSB-1 target and has been shown to be deregulated in cancer (Choi et al., 2008; Puca et al., 2010). This protein kinase is involved in the regulation of numerous biological processes, including cell proliferation, survival and differentiation (Saul and Schmitz, 2013). In fact, Nodale and colleagues demonstrated that HIPK2 overexpression induced downregulation of vimentin in MCF7 and MDA-MB-231 cells (Nodale et al., 2012). Vimentin being a mesenchymal marker, WSB-1 could contribute to the EMT progression by degrading HIPK2. In addition, HIPK2 has been shown to inhibit HIF-1 α , which could generate a positive feedback loop for WSB-1 (Nardinocchi et al., 2009).

DNAJA3/**Tid-1** was only found in hypoxic samples, with a significant *p*-value (*p* = 0.0255). This mitochondrial co-chaperone exists as two splice variants: Tid-1L (long) and Tid-1s (short) (Syken et al., 1999). It is believed to be implicated in cell survival, proliferation and cellular response to stress (Lo et al., 2004). In fact, Tid56, Tid-1 homolog in *D. melanogaster*, was identified as a tumour suppressor gene (Schilling et al., 1998). Antitumour activity of human Tid-1 was later described by Kim and colleagues (Kim et al., 2004). They proved that Tid-1 interacts with HER2, inhibiting its ability to induce proliferation of breast cancer cells *in vitro*. Therefore, increased level of Tid-1 would inhibit the effect of HER2 overexpression observed in 30% of human breast cancer patients and associated with poor prognosis.

Furthermore, Jan and colleagues revealed that high Tid-1 levels correlated with high levels of CHIP/STUB1 (STIP1 homology and U-Box containing protein 1), an ubiquitin ligase, in addition to low levels of HER2 in breast cancer tissues, prompting the authors to suggest that Tid-1 and CHIP could have a proteolytic action on HER2 (Jan et al., 2011). If Tid-1 was a target of WSB-1 E3 ubiquitin ligase activity, increased levels of WSB-1, as those observed in metastatic breast cancer, would prevent the inhibition of HER2 by Tid-1 and degradation of HER2 by CHIP/STUB1, further enhancing cell proliferation. On the contrary, WSB-1 knockdown appeared to induce a decrease in Tid-1 protein levels (Figure 5.23), suggesting that WSB-1 could have a positive effect on Tid-1 expression.

AUF1/hnRNP D (heterogeneous nuclear ribonucleoprotein D) was only detected in normoxic samples and was in fact the most significantly regulated protein in the LC-MS dataset ($p = 0.0005$). This protein binds to AU-rich elements in the 3'UTR region of mRNA and regulates their expression by increasing or shortening their half lives (Gouble et al., 2002). Recently, Xin and colleagues showed that AUF1 was ubiquitinated by pVHL in a hypoxia-dependent manner and degraded by the proteasome (Xin et al., 2012). A study in thyroid carcinoma found that AUF1 induced uncontrolled growth and proliferation of cancer cells by disturbing the stability of mRNAs coding for cyclin-dependent kinase inhibitors (Trojanowicz et al., 2009). This study even suggests that AUF1 could be a possible novel marker for thyroid carcinoma. In breast cancer cell lines, AUF1 was also demonstrated to have an impact on invasiveness and metastatic behaviour of MDA-MB-231 cells (Sommer et al., 2005). However, AUF1 exclusively bind to AU-rich elements in mRNA promoter sequences and no such elements were found in WSB-1 messenger sequence. AUF1 actually appeared several times in the LC-MS list. The "protein existence" parameter for each occurrence displayed "evidence at transcript level (e.g. the existence of cDNA)", which indicates that these sequences can be incomplete or fragments of the protein mentioned. With this in mind, the impossibility to pull down AUF1 (Table 5.6) could be explained by the fact that AUF1 simply do not interact with

WSB-1. In fact, the CRAPome tool indicates that AUF1 was found in 216 of 411 (52.6%) AP-MS analyses, making it a possible contaminant.

GSK3 β was not one of the LC-MS hits but was actually highlighted as a potential WSB-1 partner by yeast two hybrids experiments (Vinayagam et al., 2011). This kinase was first described as a mediator of glycogen metabolism and was later associated with tumourigenesis and cancer progression (Luo, 2009). Farago and colleagues suggested that GSK3 β downregulation promotes mammary tumour development by inducing the accumulation of β -catenin, which in turn results in the overexpression of cyclin D1, responsible for cell cycle progression, growth and tumour progression (Diehl, 2002; Farago et al., 2005). In addition, GSK3 β was shown to inhibit AUF1 activity (Tolnay et al., 2002). Finally, GSK3 β is known to phosphorylate and activate Snail, which in turn represses the transcription of E-cadherin (Lamouille et al., 2014). Thus, GSK3 β is considered to promote EMT progression. The present study found no proof of interaction between WSB-1 and GSK3 β , which would act against EMT progression and the general observation that WSB-1 contributes to the metastatic behaviour of cancer cells.

Another of GSK3 β substrate is **HSP90**, a chaperone of the heat shock protein family (Muller et al., 2013). Phosphorylation of HSP90 C-terminus determines with which co-chaperone it will interact. Among these are CHIP, STIP1 and p23 (Sims et al., 2011). As illustrated by Figure 5.7, HSP90 is a central protein, interacting with many of the other putative partners selected, which allowed the successful pull down of WSB-1 in HEK293T cells (Figure 5.20). In addition, HSP90 is known to interact with HIF-1 α (Gradin et al., 1996). A study in renal carcinoma cell lines revealed that treatment with geldamycin, an antibiotic known to prevent HSP90 interaction with other proteins, resulted in a disruption of the HSP90/HIF-1 α association and induced an oxygen- and pVHL-independent HIF-1 α degradation (Isaacs et al., 2002). A secreted form of HSP90 that demonstrated a pro-invasive role has been revealed (Wang et al., 2009a). This extracellular form has also been described to activate MMP2 and promote

invasiveness (Sims et al., 2011). Protein levels of HSP90 in the condition media of MDA-MB-231 cells upon WSB-1 knockdown was investigated by Western blot (Figure A8) but provided conflicted results and no conclusion could be drawn concerning the effect of WSB-1 on extracellular levels of HSP90.

p23 is the smallest molecule of the HSP90 machinery which was originally discovered as part of a complex with HSP90 and PR (Johnson et al., 1994). p23 interaction with HSP90 is very brief ($t_{1/2} = 2\text{min}$) and brings HSP90 in a highly active state. HSP90-p23 binding was found to increase in tumour cells and advanced-stage tumours (Oxelmark et al., 2006). In addition, this study also demonstrated that *in vitro* overexpression of p23 in MCF7 cells resulted in an increase in adhesion and invasion in fibronectin. In fact, another study found that overexpression of p23 doubled the transcriptional activity of ER in MCF7 cells and p23 actually appeared to be the limiting factor for ER signalling (Knoblauch and Garabedian, 1999). In a study using MCF7 cells overexpressing p23, it was shown that p23 was able to induce resistance to the chemotherapeutic drugs etoposide and doxorubicin by upregulating the expression of the ABCC3 transporter (Simpson et al., 2010). MCF7 cells overexpressing p23 also demonstrated increased invasiveness, although no change in oestrogen-dependent proliferation was observed. In addition, high levels of p23 in patient's tumours correlated with worse disease free survival. Overall, p23 was found to stimulate the hormone binding capacity of ER and PR (Felts and Toft, 2003). Although the levels of p23 detected in normoxia and hypoxia in HEK293T IP samples was not significantly different, WSB-1 knockdown appeared to differentially alter p23 levels in MCF7 (ER/PR+) and MDA-MB-231 (ER/PR-) cells. Particularly, in MCF7, WSB-1 knockdown resulted in a decrease in p23 levels, suggesting that WSB-1 overexpression could in fact upregulate p23 and therefore increase drug resistance and invasion.

Finally, **STIP1** (stress-induced phosphoprotein)/HOP (Hsp70-Hsp90 organizing protein) was significantly downregulated in hypoxia compared to normoxia in the LC-MS dataset

($p = 0.031$). STIP1 links HSP90 and HSP70 together and homologues of this co-chaperone were found in all living organisms (Odunuga et al., 2004). It was identified as a biomarker for ovarian cancer prognostic and STIP1 upregulation was described in colon carcinoma cells and hepatocellular carcinoma (Wang et al., 2010). Cho and colleagues demonstrated that high levels of STIP1 corresponded to higher grade ovarian tumours and associated with a worse overall survival (Cho et al., 2014). In addition, knockdown of STIP1 appeared to decrease invasion, migration and proliferation of epithelial ovarian cancer cells. These results are corroborated by Chao and his group who observed an increase in proliferation and migration following overexpression of recombinant STIP1 in ovarian cancer cells, and a study by Walsh and colleagues who demonstrated that STIP1 knockdown induced a decrease in the invasiveness of pancreatic cancer cell lines (Walsh et al., 2011; Chao et al., 2013). In that study, Walsh also showed that MMP2 coimmunoprecipitated with STIP1 in pancreatic cancer cells conditioned media. Finally, STIP1 knockdown resulted in a decrease in expression of HER2 and other client proteins. STIP1 binding to WSB-1 appeared to decrease in hypoxia and the interaction between the two proteins was confirmed in MCF7 as well as by reverse IP in HEK293T. However, WSB-1 knockdown did not modify STIP1 protein level in MCF7 nor MDA-MB-231, suggesting WSB-1 do not interact with STIP1 to induce its ubiquitination and proteasomal degradation, but possibly as an element of the HSP90 complex.

5.4.2.1. Immunoprecipitation

HIPK2 is a known target of WSB-1 ubiquitin ligase activity and their interaction has been discussed on several occasion in the literature (Choi et al., 2008; Tong et al., 2013). Hence, it is surprising that the present experimental design failed to retrieve this interaction. However, it is important to note that HIPK2 was not part of the initial LC-MS list of the hits. In addition, the interaction between WSB-1 and HIPK2 was never described in breast cancer cell lines, but in bone osteosarcoma (U2OS), colon carcinoma (RKO) and hepatocarcinoma (Hep2G). It is therefore a possibility that the interaction between WSB-1 and HIPK2 does not happen in a

breast cancer context.

GSK3 β is another of the putative partners that was disqualified during the validation process. Indeed, although the protein was present in the initial protein samples (input), no signal could be detected in the IP samples. Given that GSK3 β was identified as a possible WSB-1 partner *via* yeast two hybrid experiments and was not found in the LC-MS hit list, this result is not entirely surprising. GSK3 β however appears to interact with a few of the putative WSB-1 partners selected, including HSP90 (see section 5.4.2).

p23 takes part in the HSP90/HSP70 complex. Although it did not demonstrate a significant *p*-value in the LC-MS dataset (*p* = 0.5503) and was detected to similar levels in normoxic and hypoxic samples (fold change = -1.031), p23 appeared to bind specifically to WSB-1 in HEK293T cells (Figure 5.3, Table 5.3). The CRAPome database revealed that p23 was pulled down only in 47 of the 411 experiments (11.4%) in the database. When used in MCF7 and MDA-MB-231 cells, p23 consistently displayed a strong signal in the mock samples, rendering any conclusion regarding its interaction with WSB-1 difficult. However, given the results obtained with HEK293T cells, it is possible that p23 takes part in a protein cluster interacting with WSB-1.

Finally, AUF1 was also discarded following the immunoprecipitation experiments. Despite presenting a satisfactory signal in some of the replicates with HEK293T cells (Figure 5.9A, B; Table 5.3), overall results for AUF1 did not allow to confirm its interaction with WSB-1. The protein appeared to sometimes bind non-specifically to the beads (see band in mock IP sample in Figure 5.9B and Figure 5.13) or was not detected at all (Figure 5.9C, Figure 5.15, Figure 5.17, Figure 5.19). Interestingly, treating the cells with MG132 did not facilitate AUF1 pull down (Figure 5.11). In fact, following more detailed analysis of the LC-MS hits list, AUF1 was present in three distinct occurrences, once found only in normoxic samples and the other times found only in the hypoxic ones. This, combined with the fact that AUF1 preferred binding substrate is mRNA, as developed in section 5.4.2, concur to challenge the possibility of

an interaction between WSB-1 and AUF1. Furthermore, The CRAPome score of 216/411 (52.6%) suggests AUF1 is often pulled down during AP-MS experiments and could simply be a contaminant.

Several times, bands were detected in the mock IP samples. This results from proteins sticking to the beads during the immunoprecipitation, regardless of WSB-1 presence, or in the case of the reverse IPs, a cross reactivity between the beads and the antibody. To overcome this, control samples containing beads only, which were not incubated with protein lysate, could be run alongside the other samples. This would help determine the origin of the problem. Use of a more stringent washing buffer (Figure 5.15 and Figure 5.19), or addition of an elution step (Figure 5.22) can help decreasing the elution of non-specific proteins as well.

5.4.2.2. *siRNA*

The effect of WSB-1 knockdown on the levels of these putative WSB-1 partners was investigated in order to possibly identify WSB-1 targets (Figure 5.23). Indeed, protein level of a WSB-1 target is expected to decrease in response to WSB-1 activity. Consequently, WSB-1 target levels should all but decrease following WSB-1 knockdown. Therefore, the increase in HSP90 levels following WSB-1 knockdown observed in MCF7 cells suggest that HSP90 could actually be a target of WSB-1 E3 ubiquitin ligase activity.

Response of HIPK2 to WSB-1 knockdown was surprising. WSB-1 expression increases in response to hypoxia; however, HIPK2 levels were seen unchanged in MDA-MB-231 cells. Furthermore, WSB-1 knockdown appeared to coincide with a decrease in HIPK2 levels. Importantly, WSB-1 is not the only protein regulating HIPK2 levels and the changes observed in the present experiment could be the results of other HIPK2 regulators such as MDM2 or Siah1, another E3 ubiquitin ligase (Rinaldo et al., 2007; Kim et al., 2009).

5.4.3. Summarising diagram

To conclude, Figure 5.24 represents the interaction network between WSB-1, the seven

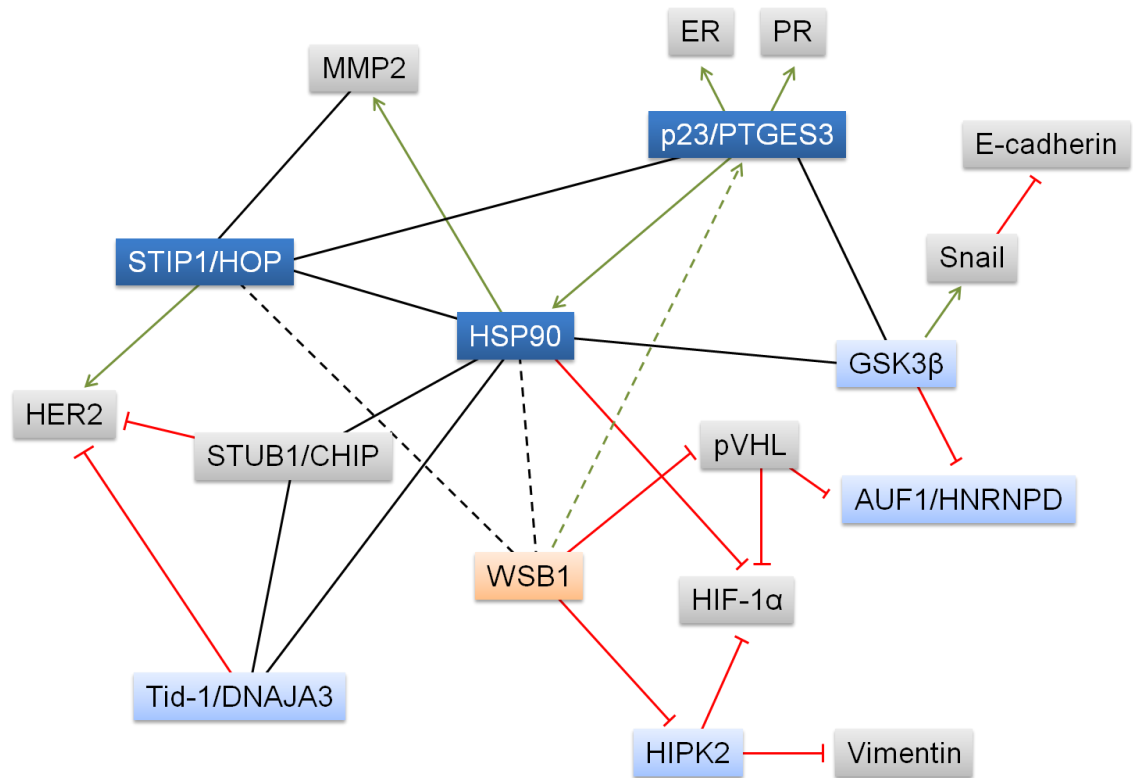


Figure 5.24: Diagram representing the interaction network between WSB-1, the putative partners studied and a selection of other proteins

Interactions are based on findings from the literature; every interaction might not be represented and the network might not be exhaustive. Blue boxes: putative partners investigated in this chapter; dark blue boxes: validated partners; grey boxes: other proteins. Green arrows: activation; red arrows: inhibition; black lines: unspecified interaction; dotted lines: interactions presented in this chapter.

putative partners studied here and other proteins that are associated with these proteins in the literature. Potential novel interactions were revealed in this chapter between HSP90, STIP1, p23 and WSB-1. These are symbolised by dotted lines in Figure 5.24. Importantly, HSP90, STIP1 and p23 are all part of the HSP90 machinery, which is involved in numerous cellular activities, including cellular transport and secretory pathways. McClellan and colleagues demonstrated that HSP90 is essential for cell cycle progression in situation of environmental stress (McClellan et al., 2007). HSP90 is also known to stimulate migration and invasion and is a known target for cancer therapy (Eustace et al., 2004; Snigireva et al., 2014). According to the results presented in this chapter, it is unclear how WSB-1 interaction with HSP90, STIP1 and p23 would affect the overall activity of the HSP90 complex. Nevertheless, hypoxia appeared to increase the binding of WSB-1 with proteins involved in the "protein ubiquitination pathway" and "HIF-1 α signalling".

Chapter 6

General discussion

6. General discussion

6.1. Summary of work presented in previous chapters

Breast cancer is the second most represented cancer type worldwide and will affect 1 in 8 women in the UK (Department of Health, 2014; Ferlay et al., 2015). Therefore, it is important to improve diagnosis and care and further understand the mechanisms driving this disease. In order to optimise the therapeutic approach, tumours were arranged in subtypes, according to their expression of ER, PR and HER2 (Figure 1.1) (Haque et al., 2012). However, studies have shown that this information is not sufficient to accurately predict the tumour response to given drugs (van 't Veer et al., 2002; Prat et al., 2015). In fact, more comprehensive gene expression profiles have been recognised to have an increasing importance (Weigelt et al., 2005). Such approaches allow the identification of a signature of markers whose levels inform on the tumour aggressiveness, the expected response to a given therapeutic drug or the estimated survival rate (Zajchowski et al., 2001; Bertucci et al., 2002; van de Vijver et al., 2002; Chang et al., 2003).

Studies with a similar approach revealed that WSB-1, an otherwise poorly studied protein, was often present in higher levels in metastatic tumour tissues, compared to healthy or less invasive tumour tissues (Rhodes and Chinnaiyan, 2005; Silva et al., 2011). WSB-1 involvement in tumour progression was later confirmed in more specific studies (Archange et al., 2008; Silva et al., 2011; Shichrur et al., 2014; Cao et al., 2015; Kim et al., 2015). In particular, Kim and colleagues very recently showed that high levels of WSB-1 correlated with lower metastasis-free survival for breast cancer patients, corroborating the work presented in this thesis (Kim et al., 2015).

The objective of this thesis was to provide a better understanding of the role WSB-1 might play in breast cancer. To this end, each of the previous chapters focused on a specific aspect of WSB-1 biology. In **Chapter 3**, the significance of WSB-1 expression in breast cancer samples

and the effects of hypoxia on WSB-1 in breast cancer *in vitro* were investigated. In **Chapter 4**, the role of WSB-1 in the motility and invasiveness of breast cancer cells was studied. Finally, in **Chapter 5**, novel proteins interacting with WSB-1 were identified.

6.2. Expression of WSB-1 in breast cancer

Breast cancer patient cDNA microarrays were analysed as a mean to assess the levels of *WSB1* in breast cancer tissue samples compared to healthy tissues (Chapter 3). Surprisingly, the highest *WSB1* levels were observed in the normal tissue samples. Nevertheless, higher-grade tumour tissue samples displayed higher *WSB1* levels than the lower-grade samples. In general, tissues samples not expressing ER, PR, and/or HER2 presented lower *WSB1* levels than positive tumour tissue samples. In particular, HER2 types and TNBC, which express only HER2 or no receptor at all, respectively, had the lowest *WSB1* levels of all subtypes. Caveats associated with patient cDNA microarrays have already been discussed in Chapter 3 (section 3.4.1). Importantly, these results are in contradiction with several studies published, as well as most of the observations presented in this thesis following experiments in breast cancer cell lines on the one hand, and analysis of patients' survival according to *WSB1* levels using KM plots on the other (Archange et al., 2008; Silva et al., 2011; Shichrur et al., 2014; Cao et al., 2015; Kim et al., 2015).

In fact, increased *WSB1* levels result in poorer distant metastasis-free (DMFS), relapse-free (RFS) and overall survival (OS) for the patients presenting HER2 type, HER2+ or ER- tumours. On the contrary, higher *WSB1* levels were associated with good DMFS, RFS in patients with ER+ tumours, and good RFS, OS in patient with HER2- tumours. In summary, high *WSB1* levels appear to be associated with good DMFS and RFS rates for patients presenting ER+/HER2- tumours, whereas patient with tumours presenting the opposite phenotype (ER-/HER2+) generally demonstrated poorer DMFS and RFS survival. Importantly, when considering OS, high *WSB1* levels in HER2- tumours is associated with good prognosis for the patients, but with poor prognosis in ER+ tumours, suggesting that ER negativity is linked to metastatic

proneness, an aspect that has already been discussed in the literature (Lower et al., 2005; Putti et al., 2005; Bae et al., 2015).

6.2.1. Potential differential impact of the different WSB-1 isoforms

The *WSB1* gene can generate three isoforms by alternative splicing (Archange et al., 2008). As the studies by Archange and Shichrur suggested, WSB-1 isoform 3 might not only be a candidate for nonsense-mediated mRNA decay (NMD) but in fact play an important role in pancreatic cancer and neuroblastoma development (Archange et al., 2008; Shichrur et al., 2014). Importantly, NMD notably targets proteins which could disrupt normal cellular processes (Kervestin and Jacobson, 2012). The fact that WSB-1 isoform 3 is a target of NMD could indicate that this isoform has a potential for increased cancer progression.

Owing to its structure lacking most of the WD-40 repeats and the SOCS box typically found in the other isoforms, WSB-1 isoform 3 is unlikely to possess an enzymatic activity and probably present a decreased ability to form protein-protein interactions (Figure 1.8). In fact, it is possible that WSB-1 isoform 3 acts as a dominant negative in hypoxia, competing with WSB-1 isoforms 1 and 2 for protein binding but unable to then act as an ubiquitin ligase. As a result, an increase in WSB-1 could in fact signify and increase in WSB-1 isoform 3, which would lead to a decrease in mechanisms normally associated with WSB-1 activity. The difference in dynamics of transcript levels in response to hypoxia between all WSB-1 isoforms combined (total WSB-1) and WSB-1 isoform 3 only is demonstrated in Figure A9, whereby maximum upregulation was reached after 4h of hypoxia exposure for total WSB-1 and after 8h for WSB-1 isoform 3.

To test whether WSB-1 isoform 3 acts as a dominant negative form of the other WSB-1 isoforms, it could be possible to overexpress this isoform specifically, or silence WSB-1 isoforms 1 and 2, and monitor the levels of known WSB-1 targets. Another approach would be to express a mutated version of WSB-1 with a deleted SOCS box, as it was attempted on other SOCS box-containing proteins, and observe protein levels of known WSB-1 targets (Zhang et

al., 2001; Kile et al., 2002).

Given the similarity in the sequences of WSB-1 isoforms, it is difficult to design tools that are specific for each isoform and detecting WSB-1 proteins by Western blot was limited by the efficacy of the antibodies. Four different antibodies were tested for WSB-1 detection and only one provided an exploitable signal. Interestingly, this antibody preferentially detected WSB-1 isoform 3 (25kDa) and only seldom detected WSB-1 isoform 1 (46kDa) and 2 (30kDa).

Like the primers used by Archange and colleagues for their study, the primers used for SYBR Green qPCR in this thesis were designed in such a way that they can detect either the three isoforms or WSB-1 isoform 3 specifically (Archange et al., 2008). Unfortunately, other primers had to be used for the analysis of the patient cDNA microarray, which did not allow for distinguishing between the different WSB-1 isoforms, which could explain the unexpected results observed.

6.3. Role of WSB-1 in breast cancer metastatic potential

The RNA-Seq analysis of MDA-MB-231 transcriptome revealed that WSB-1 knockdown impacted numerous biological processes in both normoxia and hypoxia (Chapter 3). In particular, WSB-1 appeared to positively act on "cell morphology", "tissue development", "cellular assembly and organisation", or "cellular function and maintenance". "Cell cycle", and "cellular growth and proliferation", on the other hand, seemed to be negatively regulated by WSB-1. This switch between invasiveness and proliferation is frequent during early development but has also been observed in mammary tumours (Wang et al., 2004). This means that increased metastatic potential for cancer cells is gained at the expense of cellular proliferation (Vega et al., 2004; Matus et al., 2015). These results suggested that WSB-1 would promote breast cancer cells aggressiveness and could repress cellular proliferation notably by altering cell cycle progression. In fact, tumour progression and metastasis is an extensive, complex process that can be broken down into several steps (Figure 6.1). Hypoxia plays a critical role in this mechanism but other proteins are also important. Highlighted in Figure 6.1

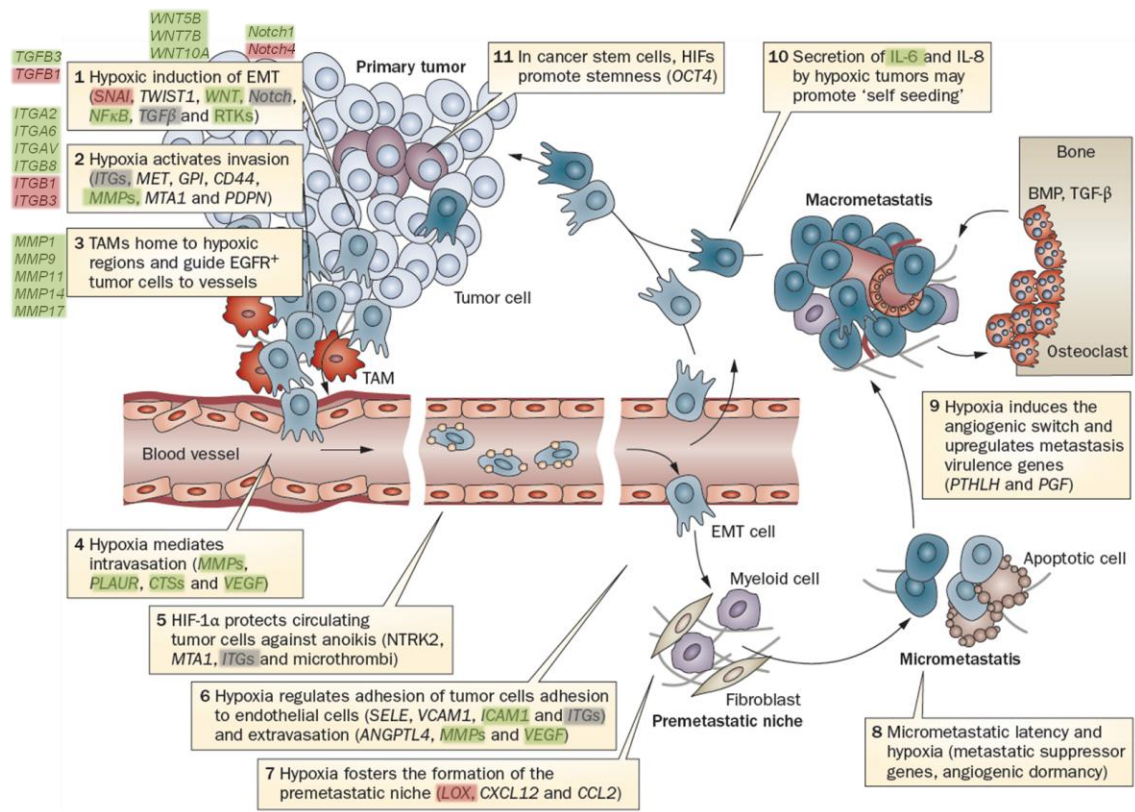


Figure 6.1: Schematic representation of the metastatic cascade

Representation of the different steps involved in the metastatic cascade, the role of hypoxia in each step and main proteins involved. Colours indicate what we described in this thesis (RNA-Seq, qPCR or/and ELISA (for VEGF)). Green: protein downregulated following WSB-1 knockdown, red: protein upregulated following WSB-1 knockdown. Grey: effect of WSB-1 knockdown unclear. TAM: tumour associated macrophages. From De Bock (2011).

are the proteins that were found up- or downregulated by WSB-1 knockdown in the RNA-Seq data, with the exception of *SNAI1*, *LOX* and *VEGFA*, which were not detected in the MDA-MB-231 transcriptome but analysed by qPCR. From this, it appears that, in MDA-MB-231 cells, WSB-1 activity primarily promotes genes involved in EMT induction (*WNT*, *NFKB*), invasion activation (*MMPs*, *ITGs*), intravasation (*MMPs*, *CTSS*, *PLAUR*, *VEGF*), and extravasation (*MMPs*, *VEGF*). WSB-1 also upregulates genes responsible for tumour cell adhesion to endothelial cells (*ICAM1*, *ITGs*), which is important for the survival of circulating tumour cells. From this, it becomes evident that the role of WSB-1 in breast cancer progression centres on the initial cellular changes of EMT and increasing the cellular ability to invade and migrate through the blood vessel.

6.3.1. Induction of EMT

Changes in the levels of certain EMT markers were observed in MCF7 and MDA-MB-231 cells following WSB-1 knockdown.

The EMT results were somewhat conflicting as effect on ZO-1 and vimentin indicated WSB-1 has a pro-EMT role whereas effect in *SNAI1* and E-cadherin suggested WSB-1 would in fact act against EMT progression. Taking into account the four EMT markers analysed, WSB-1 appeared to play opposite roles in MCF7 (ER+ cell line) where WSB-1 appear to prevent EMT, and in MDA-MB-231 (ER- cell line) where WSB-1 appear to promote EMT. Interestingly, this correlates with the observations from the patient survival analyses, which suggested that high *WSB1* levels in ER+ tumours was associated with a good prognosis (DMFS, RFS), whereas high *WSB1* levels in ER- tumours was associated with a poor prognosis (DMFS, RFS, OS). This reinforces the possibility that WSB-1 plays a different role in ER+ and ER- contexts.

6.3.2. Invasion and motility

The effect of WSB-1 on cell motility was investigated using dedicated assays (Chapter 4). Once more, WSB-1 knockdown affected differentially the behaviour of the two cell lines. MCF7

motility (migration and invasion) generally decreased following treatment with siWSB-1, revealing a pro-motility role for WSB-1 in the ER+ cell line. On the contrary, MDA-MB-231 motility tended to increase in response to WSB-1 knockdown, suggesting WSB-1 has in fact an anti-motility action in this ER- cell line. In addition, studies are underway in the lab to evaluate these effects *in vivo*. The preliminary data gathered so far appear to indicate that MDA-MB-231 cells stably expressing an shRNA against WSB-1 have a decreased metastatic potential compared to the control non-targeting shRNA MDA-MB-231 stable cells, which is in agreement with the literature and corroborates what was observed with MDA-MB-231 cells *in vitro*. However, these conclusions are preliminary as the work is still ongoing and these results are based only on macroscopic observations. H&E (hematoxylin and eosin) staining and analysis of the exact burden of microscopic metastatic foci is underway for lung samples from mice injected (tail vein) with the shRNA MDA-MB-231 stable cells.

Another important aspect for tumour metastasis is the expression of MMPs, which contribute to ECM degradation and help cells migrating and invading more easily to the blood vessels, as well as favour angiogenesis (Figure 6.1). In addition to the decrease in *MMP* transcript levels observed in the RNA-Seq data, levels of MMP1, MMP7 and MMP14, and activity MMP2 were specifically estimated in MDA-MB-231 cells. Because MCF7 cells are known to express little MMPs, these were only monitored in MDA-MB-231, where WSB-1 knockdown consistently resulted in a decrease of MMP1 and MMP14 levels, and MMP2 activity, suggesting WSB-1 in fact promotes MMPs expression. Notably, MMP1, MMP14 and MMP17 count MMP2 as one of their substrate. Therefore, based in the RNA-Seq data, the expected increase in MMP1, 9, 11, 14, and 17 levels in response to increased WSB-1 protein levels could result in a further upregulation of MMP2.

6.3.3. VEGF

Intravasation and extravasation are two critical steps in the metastatic process, whereby the cancer cells break through the endothelial cells delineating blood vessels to enter the blood

circulation and exit it, respectively. This allows circulating tumour cells to propagate in the organism and initiate tumour formation away from the initial site (Figure 1.3). VEGF is also important during metastasis development as formation of neovasculature and connection to the existing network is important to ensure blood supply of the novel cellular mass (Carmeliet, 2005).

As mentioned above, MMPs are key players in the metastatic cascade but VEGF has a central role in cancer cell spreading through the body. As seen in Chapter 3, *VEGFA* is upregulated in response to WSB-1 knockdown in MCF7 and downregulated in MDA-MB-231 cells. The upregulation in MCF7 cells suggests once more that WSB-1 has an anti-metastatic activity in ER+ cells, supporting what has been observed with the EMT markers, and the patient survival analyses. On the other hand, WSB-1 appears to have the opposite effect in ER- cells and promotes metastasis formation. *VEGFA* was not heavily modified by WSB-1 knockdown in the MDA-MB-231 transcriptome analysis. However, qPCR analysis of MDA-MB-231 mRNA showed that WSB-1 knockdown, in fact, induced a decrease in *VEGFA* transcript. In addition, an ELISA (enzyme-linked immunosorbent assay) detection of secreted VEGF in concentrated media from MDA-MB-231 cells performed in the lab revealed that WSB-1 knockdown decreased VEGF levels in the milieu (data not shown). Furthermore, branch forming assays are underway using HUVEC (human umbilical vein endothelial cells) exposed to conditioned media from MDA-MB-231 cells treated with siNT or siWSB-1. The preliminary results of this experiment suggest that WSB-1 acts on the length of vessels rather than the initial formation of the branching (data not shown).

To conclude, WSB-1 appears to have an important role in cancer cells spreading through the organism and metastasis growth.

6.3.4. SPARC and bone metastasis

SPARC/Osteonectin is an interesting molecule that was found to be highly downregulated in response to WSB-1 knockdown by the RNA-Seq analysis. SPARC plays an important role in

breast metastasis to the bone (Sloan and Anderson, 2002; Koblinski et al., 2005; Campo McKnight et al., 2006). In fact, SPARC upregulation has been associated with worse prognosis for invasive ductal carcinoma patients (Hsiao et al., 2010). In addition, *SPARC* was described as a promoter of cell invasiveness in breast and prostate cancer cell lines (Jacob et al., 1999; Maroni et al., 2015). Bellahcène and Castronovo detected that the expression of SPARC in breast cancer tumours contributes to the formation of microcalcification, often observed in such cancer (Bellahcène and Castronovo, 1995). They claim that this could be one of the reasons why bone is a preferred metastasis location for breast cancer.

Interestingly, transcriptional upregulation of SPARC in MCF7 cells resulted in increased cell motility and invasion (Briggs et al., 2002). Similarly, SPARC upregulation in MDA-MB-231 cells was shown to induce an increase in MMP2 activity and an inhibition of TIMP2 (Gilles et al., 1998). This particular aspect could explain how SPARC contributes to metastasis in breast cancer.

Another important element regarding SPARC is the effect of ER on its expression. Indeed, in an earlier study, Graham and colleagues showed an ER-mediated suppression of *SPARC* gene expression (Graham et al., 1997). It is therefore possible that the increased invasiveness and overall worse prognosis of ER- tumours is, in part, due to the elimination of the inhibitory effect of ER on SPARC. Similarly, the difference in behaviour that has been observed in this thesis between MCF7 and MDA-MB-231 cells could be associated with a differential expression of SPARC, due to their opposite ER statuses. To test this hypothesis, it would be interesting to evaluate the endogenous levels of SPARC in these two cell lines and assess the effect of WSB-1 knockdown on SPARC level in MCF7. Figure 6.2 presents a diagram summarising the involvement of WSB-1 within the metastatic cascade in each cell line.

6.3.5. RUNX2

The oPOSSUM platform is an online tool capable of detecting over-represented transcription factors binding sites within a large set of genes (Ho Sui et al., 2005). Using this platform, it was

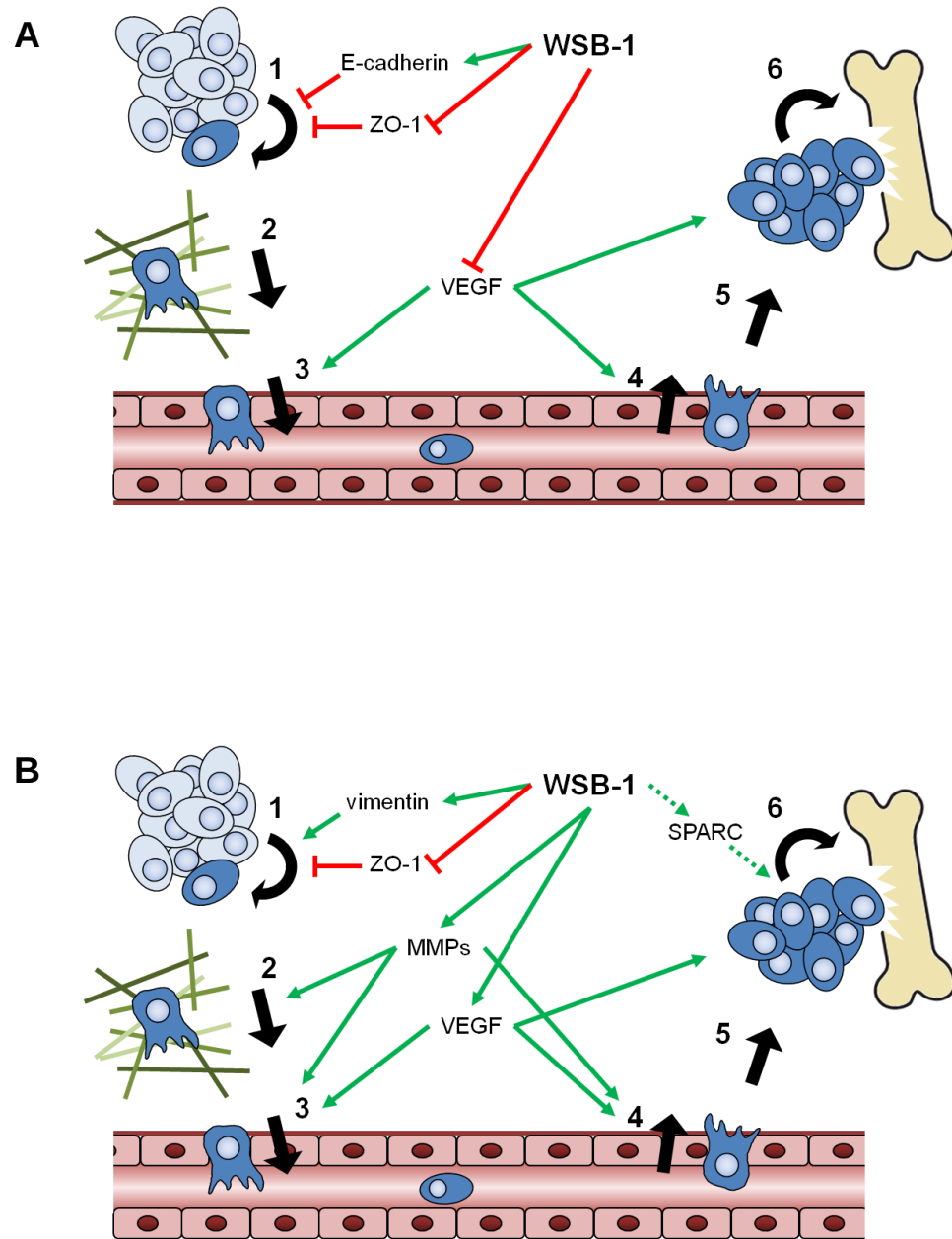


Figure 6.2: Summary diagram of WSB-1 effect on the metastatic cascade

Representation of the impact of WSB-1 in the metastatic cascade in MCF7 (A) and MDA-MB-231 (B) cells. 1: EMT, 2: invasion, 3: intravasation, 4: extravasation, 5: metastasis growth, 6: colonisation of the bone.

possible to highlight several transcription factors which were regulating the expression of several of the transcripts detected in the RNA-Seq analysis. In particular, RUNX2 (Runt related transcription factor 2) appeared as a particularly interesting candidate (data not shown). RUNX2 is the main transcription factor controlling osteogenesis but it is also involved in mammary gland development (Ferrari et al., 2013; Wysokinski et al., 2015). An important role for RUNX2 in carcinogenesis has been described, particularly in breast cancer (Wysokinski et al., 2015). In fact, overexpression of RUNX2 in MCF7 resulted in the induction of EMT, which corroborates the results of Briggs and colleagues upon SPARC upregulation described above (Briggs et al., 2002; Chinge et al., 2012).

Several of the transcripts that were downregulated in the RNA-Seq data in response to WSB-1 knockdown are among the genes regulated by RUNX2. Specifically, VEGF and SPARC have been particularly studied in the context of breast cancer (Zelzer et al., 2001; Kaye et al., 2007; Ferrari et al., 2013). MMP1, 2, 9 and 13 are also known targets of RUNX2, further explaining the role of RUNX2 in breast cancer metastasis (Gilles et al., 1998; Pratap et al., 2005; McClung et al., 2007; Wysokinski et al., 2015).

Observing the levels of this transcription factor in human breast cancer tissue microarray, the highest RUNX2 levels were found in TNBC samples (McDonald et al., 2014). In fact, ER signalling was shown to inhibit RUNX2 and it is therefore logical to observe higher RUNX2 levels in an ER- context (Khalid et al., 2008; Blyth et al., 2010).

Interestingly, RUNX2 has been demonstrated to be stimulated by PTHrP (parathyroid hormone-related protein) (Schroeder et al., 2005; Wysokinski et al., 2015). Dentice and colleagues previously revealed that PTHrP secretion was modulated by WSB-1 in the developing growth plate (Dentice et al., 2005). In the developing tibial growth plate, PTHrP defines the limit between cartilage and bone, preventing chondrocytes (cartilage cells) in the epiphysis (extremities of the bone) to undergo hypertrophic differentiation and form bone (Dentice et al., 2005). This is a very interesting aspect which will be explored in future work.

6.4. Identification of novel WSB-1 binding partners

Chapter 5 focused on the identification of novel WSB-1 binding partners. This is an important aspect of WSB-1 biology as it will contribute to better understand its role and implication within the cell. The work presented here highlighted three proteins involved in the heat shock protein system: HSP90, p23 and STIP1.

It is possible that the interaction between WSB-1 and the HSP complex contributes to the ubiquitination of HSP client proteins (Theodoraki and Caplan, 2012). Indeed, HSPs serve as quality control of the proteins present in the cell. If the protein is damaged or unable to fold properly, HSPs will target them for proteasomal degradation with the help of specific ubiquitin ligases such as STUB1 and cullin 5 (Ehrlich et al., 2009; Theodoraki and Caplan, 2012). Specifically, Samant and colleagues demonstrated that Cullin-RING E3 ligases such as cullin 5 play an important role in HSP90-client degradation, even in situations where HSP90 has been inhibited (Samant et al., 2014). Another role for HSPs is to mediate the proper folding of proteins. For example, in the case of DBC2, interaction with HSP90 is essential as it keeps DBC2 in such a conformation that the BTB (bric à brac, tramtrack, and broad complex) domain cannot interact with the RING E3 ligase cullin 3, and therefore, prevents the proteasomal degradation of DBC2 (Manjarrez et al., 2014). In a similar manner, maturation of steroid hormone receptors for mineralocorticoids, glucocorticoids and androgens strongly relies on binding with HSP70 and HSP90 (Echeverria and Picard, 2010). In fact, interaction with HSP90 keeps the steroid hormone receptors in a conformation allowing hormone binding (Pratt and Toft, 2003). Further work will be required to understand the implications of WSB-1 interaction with the HSP90 chaperones machinery and how this relates to WSB-1 role in breast cancer cell progression. For example, the use of HSP90 inhibitors such as geldanamycin and 17-AAG (17-N-allylamino-17-demethoxygeldanamycin), which are also clinically relevant, could help identify the role played by HSP90 in this system (Porter et al., 2010).

HSP90, p23 and STIP1 were selected based on their differential binding to WSB-1 in normoxia and hypoxia. However, the LC-MS analysis also detected a vast amount of proteins binding to WSB-1 independently of the oxygen level (Table B8). These proteins are of importance as they could still mediate important signalling pathways within the cells, which would be altered by a change in WSB-1 levels. Particularly, if the theory presenting WSB-1 isoform 3 as a dominant negative is verified, hypoxia would result in the blocking of the normal signalling observed in normoxia by increased WSB-1 isoform 3 levels, competing with WSB-1 isoform 1 for the binding of these proteins.

Among the proteins whose detection was not significantly different in normoxia and hypoxia are found elongin B (TCEB2), elongin C (TCEB1) and cullin 5 (CUL5), components of the ubiquitin ligase complex known to interact with WSB-1 (Dentice et al., 2005; Choi et al., 2008). Four other E3 ubiquitin ligases were also detected: TRIM21, UBR5, STUB1 and HUWE1. It is possible that these proteins are detected in the present analysis because they are also interacting with the HSP90 machinery, as described above. The absence of pVHL in the LC-MS data suggests that the degradation of pVHL by WSB-1 as described by Kim and colleagues in their study on HEK293T cells is in fact cell-specific and not occurring in breast cancer (Kim et al., 2015). Interestingly, STUB1 (STIP1 homology and U-Box containing protein 1)/CHIP (C terminus of HSC70-interacting protein) has been described to mediate the proteasomal degradation of HIF-1 α but, also, of RUNX2 (Li et al., 2008; Luo et al., 2010). STUB1, like STIP1, is capable of binding to HSP70 and HSP90 and the level of phosphorylation of the HSPs determines which of STIP1 and STUB1 will bind to them (Muller et al., 2013). Whereas binding of STUB1 with HSP90 induced protein degradation, binding of STIP1 induced protein folding.

6.5. Proposal for the WSB-1 role in breast cancer metastatic cascade

In summary, these elements combined suggest that WSB-1, although interfering in numerous steps of the metastatic cascade (Figure 6.1), including the initial EMT, is in fact the most

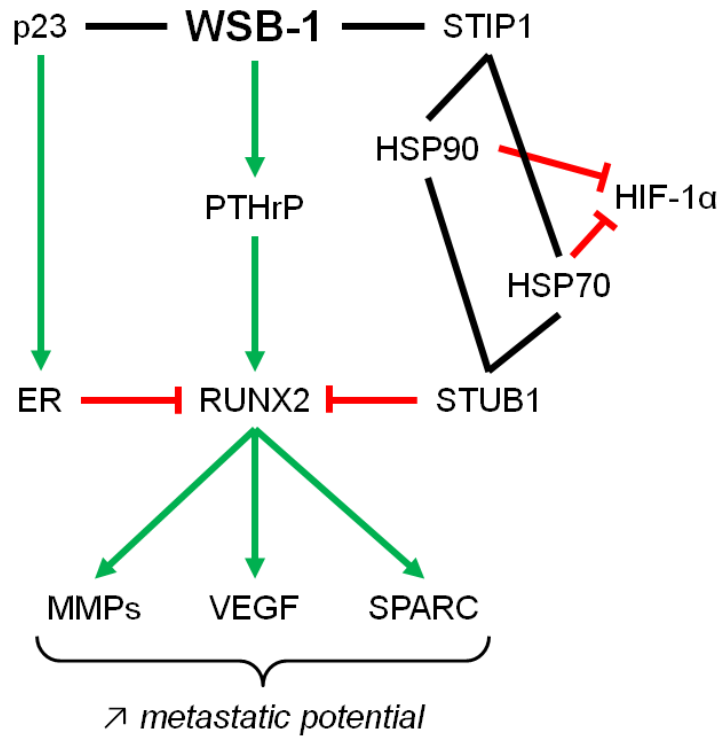


Figure 6.3: Suggestion of pathway resulting in WSB-1-mediated regulation of the metastatic potential in breast cancer

WSB-1 stimulates the secretion of PTHrP which in turn activates RUNX2. RUNX2 is a transcription factor responsible, in part, for the transcription of VEGF, SPARC and several MMPs. ER (oestrogen receptor) is a known RUNX2 inhibitor. Interaction of STIP1 or STUB1 with HSP70/90 is dependent on the HSPs phosphorylation pattern. STUB1 is able to promote the degradation of RUNX2 and HIF-1 α . Green arrows: activation/induction; red arrows: inactivation/inhibition; black lines: interaction.

important during intravasation/extravasation and establishment of metastasis, particularly to the bone. This project also revealed the importance of the ER+/ER- status in the role of WSB-1 (Figure 6.2). In this context, a pathway such as the one presented in Figure 6.3 could be imagined regarding WSB-1-mediated regulation of the metastatic potential in breast cancer.

6.6. Future directions

Based on the results presented in this thesis, several aspects could be investigated further.

These directions include:

- Generating *in vitro* tools that will allow to better investigating the role of WSB-1 is necessary. MDA-MB-231 cells stably knocked down for WSB-1 have already been generated in the lab using shRNA. Being able to overexpress WSB-1 isoform 3 specifically would be of a great help to study its implication within the WSB-1 biology. Attempts to clone a plasmid coding for WSB-1 isoform 3 have been unsuccessful so far.
- Further characterisation of the relationship between WSB-1 and HSP90, p23 and STIP1 is necessary. Treating cells with HSP90 inhibitors could help determine whether this interaction results in WSB-1 degradation. Selective knockdown of HSP90, p23 or STIP1 is also a possible approach.
- Identification of other transcription factors, besides RUNX2, that could be controlled by WSB-1. This can be done initially *in silico* (oPOSSUM, binding sites in promoter) using the MDA-MB-231 transcriptome and confirmed *in vitro* using reporter assays and ChIP (chromatin immunoprecipitation).
- Results regarding the effect of WSB-1 knockdown on the expression of EMT markers were conflicting. Monitoring the localisation of these markers by immunofluorescence would determine whether the effects on protein levels are likely to be transferred onto the cellular phenotype.

- Most of the analyses in this thesis were performed in MDA-MB-231 cells. Given the importance ER+/ER- status appears to have, it is important to verify how WSB-1 interferes in the metastatic cascade in ER+ cells, particularly in regards to the WSB-1/RUNX2/SPARC axis. Confirming the MDA-MB-231 cells results in other ER-/TNBC cell lines should be done.
- Further *in vivo* work would allow assessing of the importance of WSB-1 in tumour formation ability of breast cancer cells, using the previously mentioned stable cell lines.
- Finally, the RNA-Seq data suggested WSB-1 could play a role in cellular proliferation and cell cycle progression. These could be studied using clonogenic assays and flow cytometry, respectively, as well as 2D and 3D assays on cell proliferation.

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Appendix

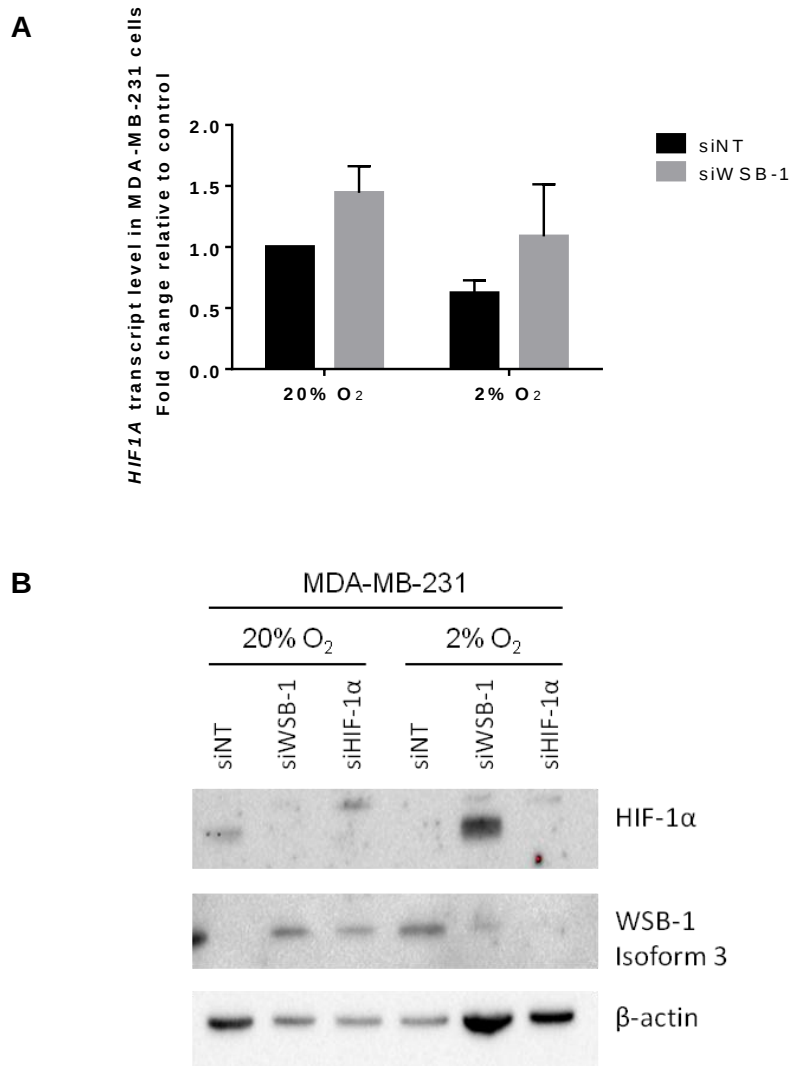


Figure A1: Effect of WSB-1 and HIF-1α knockdowns on WSB-1 levels

MDA-MB-231 cells were treated with siRNA against WSB-1 (siWSB-1) or HIF-1α (siHIF-1α), or a control non-targeting siRNA (siNT) and exposed to either 20% or 2% O₂ for 24h. *HIF1A* transcript level was assessed by qPCR from the prepared mRNA samples (A). *B2M* was used as the housekeeping gene. Error bars represent mean $\pm$ SEM, $n=3$. Statistical significance determined by 2-way ANOVA with Tukey correction for multiple comparisons. Protein expression was analysed by Western blotting for WSB-1, HIF-1α and β-actin (B). Western blots were obtained using whole cell lysates collected after 24h exposure to 20% or 2% O₂. Images are representative of $n=3$ experiments.

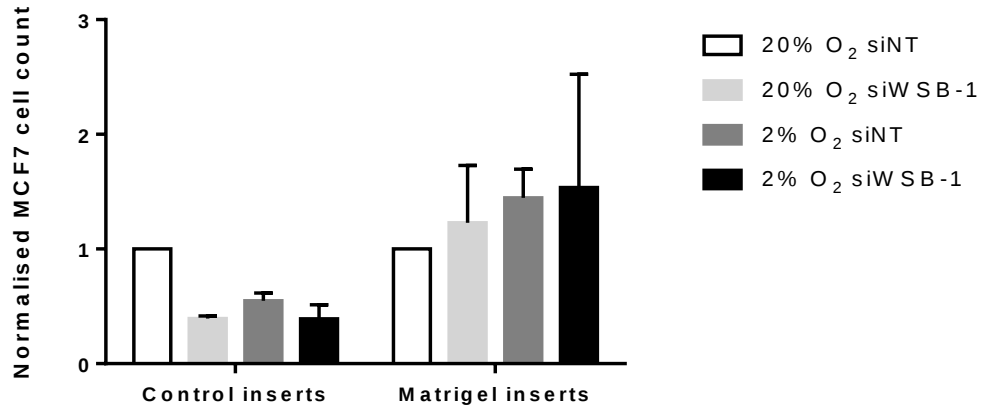
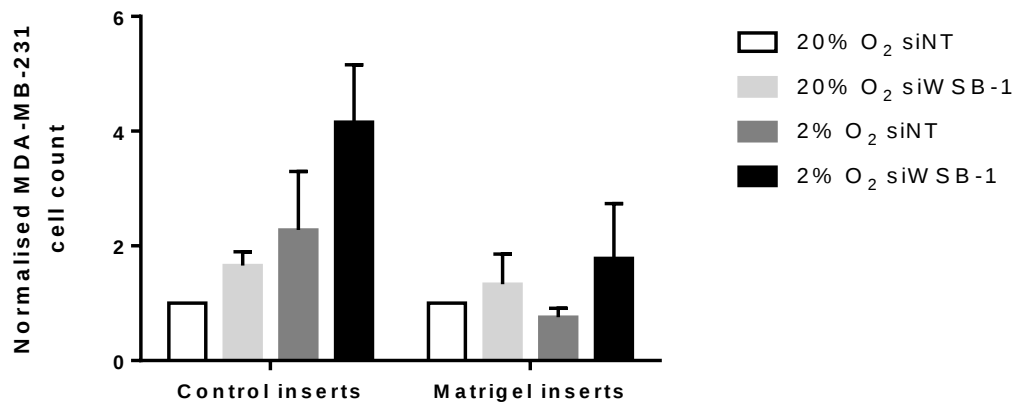
A**B**

Figure A2: Effect of WSB-1 knockdown on motility of breast cancer cells at 2% O₂

MCF7 (A) or MDA-MB-231 (B) cells treated with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT) were seeded in inserts coated with Matrigel or not (control inserts). Cells were then incubated in normoxia (20% O₂) or moderate hypoxia (2% O₂) for 18h. Cells crossing through the bottom were counted and values were normalised to 20% O₂ siNT control. Data represents the average $n=4$ experiments. Error bars represent mean $\pm$ SEM.

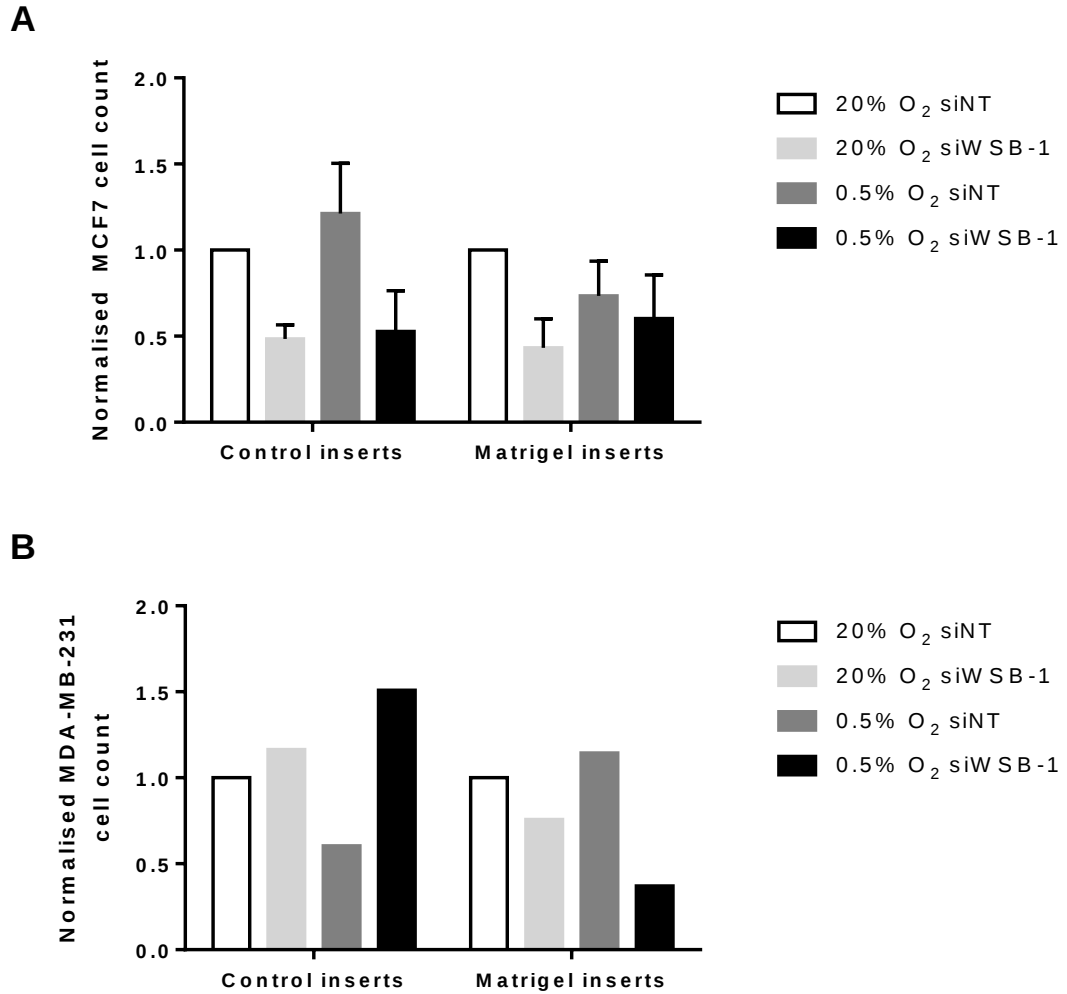


Figure A3: Effect of WSB-1 knockdown on motility of breast cancer cells at 0.5% O₂
MCF7 (A) or MDA-MB-231 (B) cells treated with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT) were seeded in inserts coated with Matrigel or not (control inserts). Cells were then incubated in normoxia (20% O₂) or moderate hypoxia (0.5% O₂) for 18h. Cells crossing through the bottom were counted and values were normalised to 20% O₂ siNT control. Data represents the average of $n=3$ (MCF7) and $n=1$ (MDA-MB-231) experiments. Error bars represent mean $\pm$ SEM.

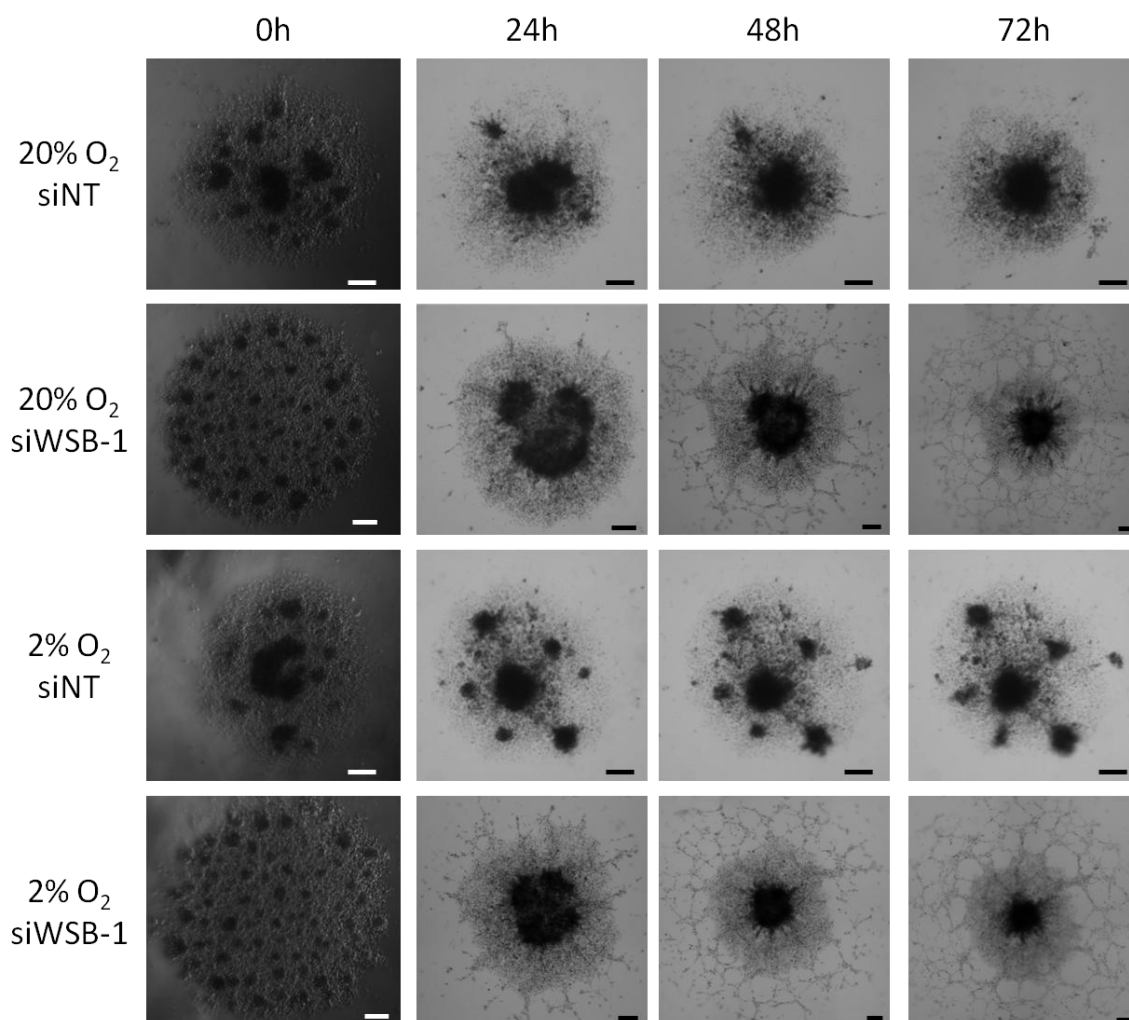


Figure A4: Representative images of MDA-MB-231 spheroids invasion assays

MDA-MB-231 cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting control (siNT). Spheroids were formed of 25,000 cells in ultra low adherence 96-well plates. Spheroids were imaged for 4 consecutive days. Scale bar=200μm.

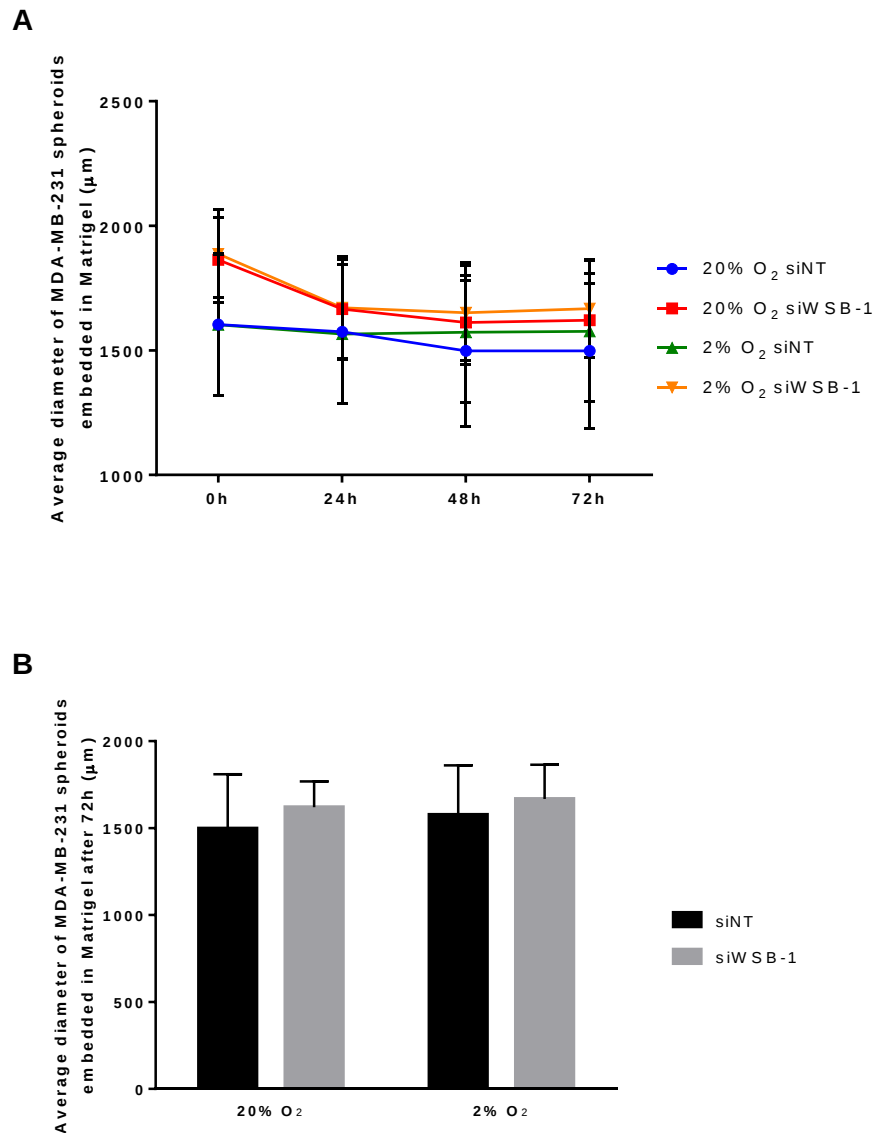


Figure A5: Growth kinetics of MDA-MB-231 spheroids embedded in Matrigel

Spheroids were generated using MDA-MB-231 cells transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). They were then embedded in Matrigel, exposed to normoxia (20% O_2) or hypoxia (2% O_2) for 24h and reoxygenated. Spheroids were imaged at 0, 24, 48 and 72h. A: Average of the spheroid diameter in μm . B: Average spheroid diameter 72h after embedding. Data represent the average of $n=3$ experiments, each with 4 intra-experimental replicates. Error bars represent mean $\pm$ SEM. Statistical significance determined by 1-way ANOVA with Tukey correction for multiple comparisons.

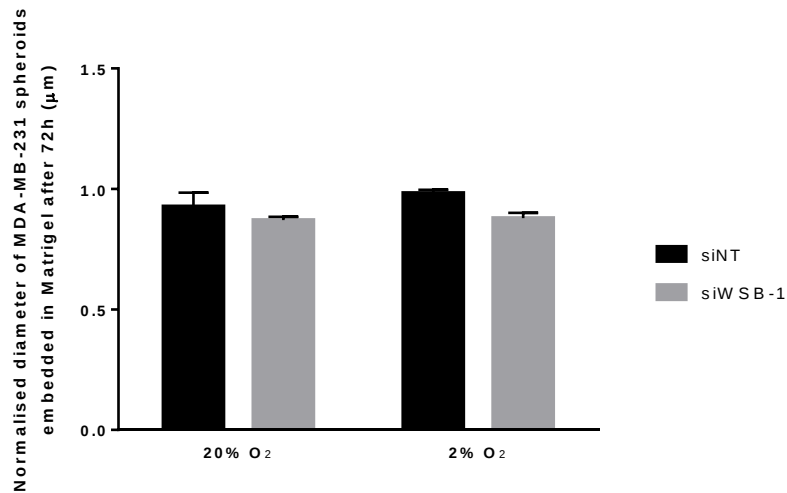


Figure A6: Relative size of MDA-MB-231 spheroids embedded in Matrigel

Spheroids were generated using MDA-MB-231 cells transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). They were then embedded in Matrigel, exposed to normoxia (20% O₂) or hypoxia (2% O₂) for 24h and reoxygenated. Spheroids were imaged at 0, 24, 48 and 72h. Spheroid diameter 72h after embedding normalised to the spheroid diameter at 0h. Data represent the average of $n=3$ experiments, each with 4 intra-experimental replicates. Error bars represent mean $\pm$ SEM. Statistical significance determined by 1-way ANOVA with Tukey correction for multiple comparisons.

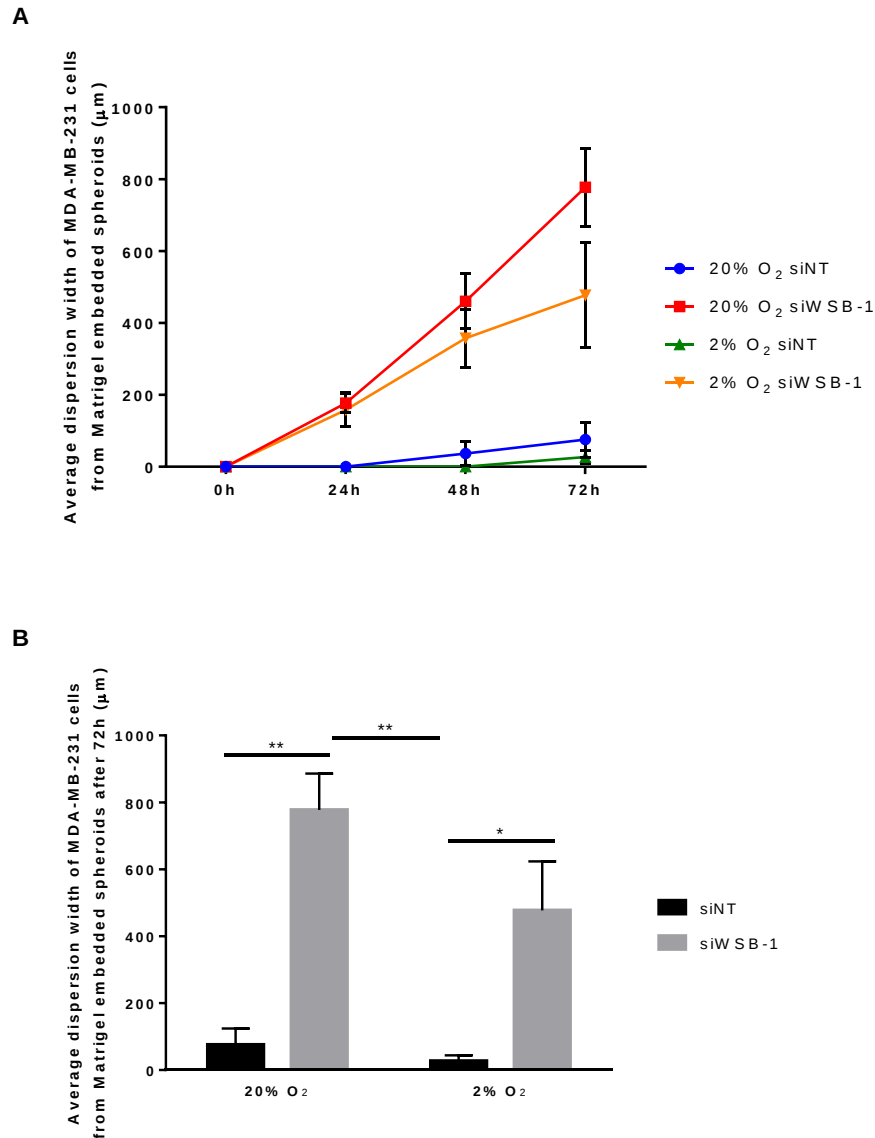


Figure A7: Spreading kinetics of MDA-MB-231 spheroids embedded in Matrigel

Spheroids were generated using MDA-MB-231 cells transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT). They were then embedded in Matrigel, exposed to normoxia (20% O_2) or hypoxia (2% O_2) for 24h and reoxygenated. Spheroids were imaged at 0, 24, 48 and 72h. A: Average of the width of the invasivedisc in μm . B: Average width of the invasive skirt 72h after embedding. Data represent the average of $n=3$ experiments, each with 4 intra-experimental replicates. Error bars represent mean $\pm$ SEM. Statistical significance determined by 1-way ANOVA with Tukey correction for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$.

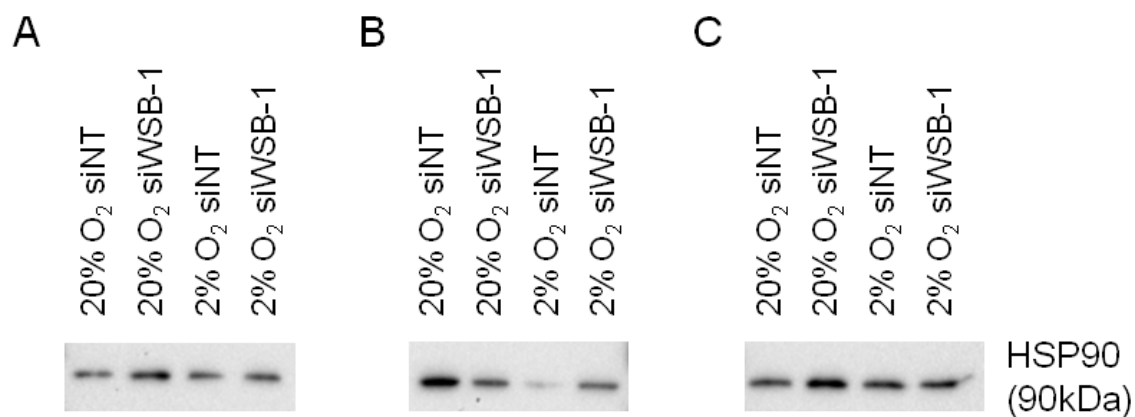


Figure A8: Effect of WSB-1 knockdown and hypoxia exposure on extracellular HSP90 protein levels

MDA-MB-231 cells were transfected with siRNA against WSB-1 (siWSB-1) or non-targeting (siNT) and exposed to 20% O₂ or 2% O₂ for 24h in serum-free media. Conditioned media was collected and concentrated. Samples were analysed by Western blotting for the presence of HSP90. Blot represent *n*=3 experiments.

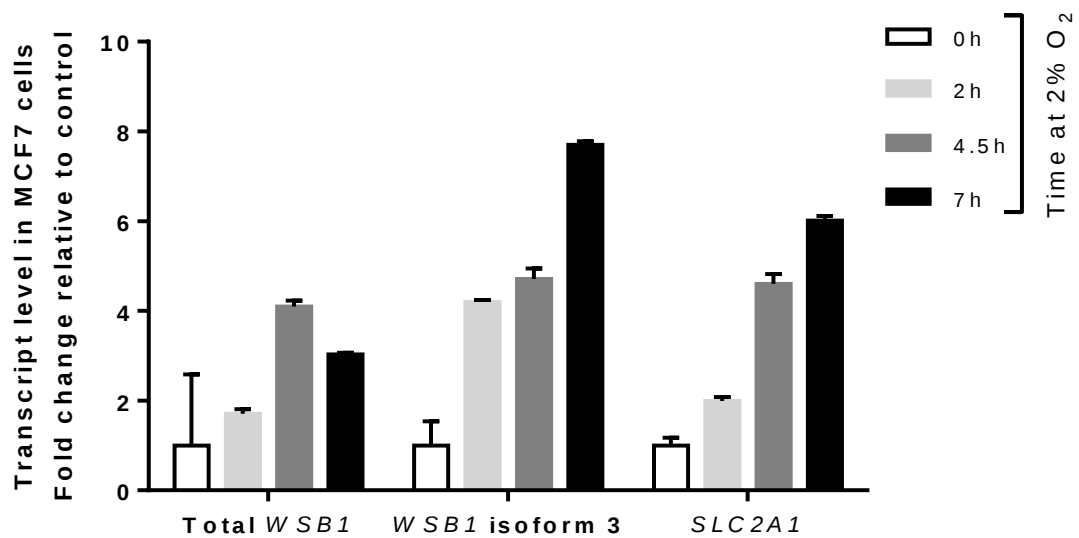


Figure A9: Effect of moderate hypoxia (2% O₂) on *WSB1* transcript level

MCF7 cells were exposed to 2% O₂ different lengths of time. Transcript level of total *WSB1*, *WSB1* isoform 3 and GLUT1 (*SLC2A1*) were analysed by relative qPCR. *B2M* was used as the housekeeping gene. Error bars represent average $\pm$ SEM for intra-experimental triplicate, $n=1$.