FRIZZLED RECEPTOR 6 AND RISK OF METASTATIC RECURRENCE IN EARLY TRIPLE NEGATIVE BREAST CANCER

A thesis submitted for the degree of Doctor of Philosophy

by

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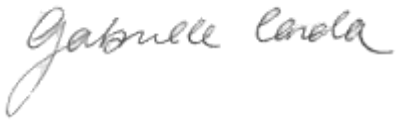
Declaration

I, Gabriele Corda, confirm that the work presented herein is my own. Work carried out by collaborators that was included in this thesis has been acknowledged.

March 2015

Signature



Abstract

WNT lipoglycoproteins (WNTs) modulate a plethora of cellular functions through the activation of the family of frizzled receptors (FZDs). Deregulation in components of the WNT signalling pathways is often observed in human cancers and associated with uncontrolled proliferation and metastasis. Frizzled receptor 6 (Fzd6), one of the ten human FZDs, is frequently overexpressed in cancer, but its role in tumorigenesis is still unclear.

In this study we investigated the role Fzd6 in breast cancer. We found that expression of Fzd6 predicts distant relapse in patients with localised breast cancers, particularly in those bearing the triple negative subtype. Using a loss of function approach, we demonstrated that Fzd6 is important to regulate motility and invasion of breast cancer cells *in vitro* and *in vivo.* Indeed, Fzd6 regulates the tropism of breast cancer cells the bone, liver and heart of mice. Mechanistically, we found that Fzd6 signalling activates the small GTPase Rho and is important in the organisation of the fibronectin matrix. Both Rho and fibronectin have been previously implicated in the development of metastasis in different systems.

All together, these results demonstrate that Fzd6 is an important driver of metastatic spread and a predictive marker of metastatic relapse in breast cancer patients. Fzd6 could therefore be used as a biomarker and target in metastatic breast cancer.

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Abbreviations

3D (Tridimensional)

A (Adenine)

AI (Aromatase Inhibitors)

AKT (V-akt murine thymoma viral oncogene homolog)

ALDH1A1 (Aldehyde Dehydrogenase 1 family, member A1)

ANOVA (Analysis of variance)

APC (Adenomatous Polyposis Coli)

APC (Allophycocyanin, a fluorochrome)

APS (Ammonium Persulphate)

BMP (Bone Morphogenic Protein)

BRCA1 (Breast cancer 1, early onset)

BRCA2 (Breast cancer 2, early onset)

BSA (Bovine Serum Albumin)

C (Cytosine)

C.I. (Confidence Interval)

$CaCl_2$ (Calcium Chloride)

CAM (Cell Adhesion Molecules)

CAMK2 (Calcium/calmodulin-dependent protein kinase II)

CDH1 (Epithelia-cadherin1)

c-DNA (Copy Deoxyribonucleic Acid)

CK1 (Casein Kinase 1)

CK14 (Cytokeratin 14)

CK17 (Cytokeratin 17)

CK5 (Cytokeratin 5)

CNV (Copy number variation)

COSMIC (Catalogue of Somatic Mutations in Cancer)

CRD (Cysteine Rich Domain)

DAG (Diacylglycerol)

DAPI (4′,6-diamidino-2-phenylindole)

DEP domain (Dishevelled, Egl-10 and Pleckstrin domain)

DFS (Disease Free Survival)

DKK (Dickkopf)

DMEM (Dulbecco's Modified Eagle's Medium)

DMSO (Dimethyl sulfoxide)

DNA (Deoxyribonucleic acid)

DRFS (Distant Relapse Free Survival)

ECL (Enhanced Chemiluminescence Substrate)

ECM (Extracellular Matrix)

EDTA (Ethylenediaminetetraacetic acid)

EGA (European Genome-Phenom Archive)

EGF (Epidermal Growth Factor)

EGFR (Epithelial Growth Factor Receptor)

ELISA (Enzyme-linked immunosorbent assay)

EMT (Epithelial Mesenchymal Transition)

ER (Oestrogen Receptor)

FACS (Fluorescence Activated Cell Sorting)

FBS (Foetal Bovine Serum)

FITC (Fluorescein isothiocyanate)

FOX (Forkhead Box Proteins)

FOXM1 (Forkhead box M1)

FRAP (Fluorescence Recovery After Photobleaching)

FZD (Frizzled)

g (Grams)

G (Guanosine)

GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)

GATA3 (GATA binding protein 3)

GDP (Guanosine diphosphate)

GEO (Gene Expression Omnibus)

GFP (Green Fluorescent Protein)

GFR (Growth Factor Reduced)

GSK3 (Glycogen Synthase Kinase 3)

GST (Glutathione S-transferase

GTP (Guanosine-5'-triphosphate)

HCl (Hydrochloric acid)

HER2 (Human Epidermal Growth Factor Receptor 2)

HIF-1(Hypoxia-inducible factor 1)

HR (Hazard Ratio)

HRP (Horseradish Peroxidase)

IF (Immunofluorescence)

Ig (Immunoglobulin)

Int-1 (Integration-1)

IP3 (inositol-1,4,5-trisphosphonate)

JNK (c-Jun N-Terminal Kinase)

KDa (Kilo Dalton)

Ki-67 (Marker of proliferation Ki-67)

KO (Knock Out)

L (Litres)

LB (Luria Broth)

LEF1, LEF/TCF (Lymphoid Enhancer-binding Factor 1)

LRFS (Local Relapse Free Survival)

LRP (Low-density lipoprotein receptor related protein)

M (Molar)

mA (milliAmpere)

MAPK (Mitogen Activated Phospho Kinase)

mg (Milligrams)

$MgCl_2$ (Magnesium Chloride)

mL (Millilitres)

MLC (Myosin Light Chain)

MLL3 (Mixed-lineage leukaemia 3)

mm (Millimetres)

mM (Millimolar)

MMP (Metalloproteinase)

MMTV (Mouse Mammary Tumour Virus)

MoAb (Monoclonal Antibody)

MRI (Magnetic Resonance Imaging)

mRNA (Messenger Ribonucleic Acid)

MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

MYC or c-MYC (V-myc avian myelocytomatosis viral oncogene homolog)

NaCl (Sodium Chloride)

NaN3 (Sodium Azide)

NF-AT (Nuclear Factor of Activated T-cells)

ng (nanograms)

NH_4Cl (Ammonium Chloride)

NLK (Nemo Like Kinase)

nm (Nanometres)

nM (Nanomolar)

NSG (NOD Scid Gamma)

OD (Optical Density)

PBS (Phosphate Saline Buffer)

PCP (Planar Cell Polarity)

PCR (Polymerase Chain Reaction)

PD (Passage doublings)

PDGF (Platelet-derived growth factor)

PDZ domain (Post synaptic density protein (PSD95), Drosophila disc large tumour suppressor (Dlg1), and zonula occludens-1 protein (zo-1) domain)

PFA (Paraformaldehyde)

PI (Propidium Iodide)

PI3K (Phosphatidylinositol 3-kinase)

PI3KA (Phosphatidylinositol-4,5-bisphosphate 3-kinase-catalytic alpha subunit)

PIP2 (Phosphatidylinositol-4,5-bisphosphate)

PIP3 (Phosphatidyl-inositol-3,4,5-triphosphate)

Pk (Prickle)

PKC (Phosphokinase C)

PLC (Phospholipase C)

PMS (Phenazine methosulfate)

PR (Progesterone Receptor)

PTEN (Phosphatase and Tensin homolog protein)

PTHrp (Parathyroid hormone-related Protein)

Q-RT-PCR (Quantitative Real Time Polymerase Chain Reaction)

Rb (Retinoblastoma Protein)

RIPA (Radio Immuno Precipitation Assay Buffer)

RNA (Ribonucleic acid)

ROCK (Rho Associated Kinase)

Rpm (Revolutions per minute)

RPMI medium (Roswell Park Memorial Institute medium)

SCF (Skp, Cullin, F-box containing complex β)

SCR (Scrambled)

SDS (Sodium Dodecyl Sulphate)

SDS-PAGE (Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis).

SE (Standard Error)

SEM (Standard error from the Mean)

SERD (Selective Oestrogen-receptor Downregulators)

SERM (Selective Oestrogen Receptor Modulators)

sFRP (Secreted frizzled related protein)

shRNA (Short Hairpin Ribonucleic Acid)

siRNA (Small Interfering Ribonucleic Acid)

T (Thymine)

TAK1 (Mitogen-activated protein kinase kinase kinase 7)

TBS (Tris Buffered Saline)

TBS-t (Tris Buffered Saline-Tween 20)

TCGA (The Cancer Genome ATLAS)

TEMED (Tetramethylethylenediamine)

TGF (Transforming Growth Factor)

TMA (Tissue Micro Arrays)

TNBC (Triple Negative Breast Cancer)

TNM (Tumour Nodes Metastasis staging system)

TP53 (Tumour protein 53)

Tris (Hydroxymethyl-aminomethane)

UV (Ultraviolet)

V (Volts)

VANGL (Van Gogh-like Protein)

VEGF (Vascular Endothelial Growth Factor)

WIF (WNT Inhibitor Factor)

WNT (Wingless-related integration site protein)

WT (Wild Type)

XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)

βTRCP (Beta-Transducin Repeat Containing Protein)

µg (Micrograms)

µL (Microliters)

µm (Micrometres)

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CHAPTER I

Introduction

1.1 Breast cancer

According to Cancer Research UK (http://publications.cancerresearchuk.org/downloads/Product/CS_KF_BREAST.pdf), breast cancer is the most common malignancy in the United Kingdom and the second most common cause of death from cancer in women after lung cancer. In 2012, about 11600 women died of breast cancer. The mortality rate per 100000 population was 24 % for females and 0.2 % for males. In women, the five-year net survival estimated between 2010 and 2011 was 86.6%, while the ten-year net survival was 78.4%. In Europe, an average of 464,000 new cases of breast cancer was diagnosed in 2012. Breast cancer affects also men but with a much lower rate (1 in 100000). Worldwide, less than 1% of all the breast cancer patients are male (Sasco, Lowenfels and Pasker-De Jong, 1993).

Women from western and developed countries are more likely to develop the disease (Ferlay *et al.*, 2010). Life style factors might explain why women of developed countries have an increased risk to develop breast cancer. These include having less children, giving birth at an older age and avoiding breastfeeding, all factors that have been reported to increase the risk of breast cancer (Porter, 2008; Parkin and Fernández, 2006; Hortobagyi *et al.*, 2005).

Breast cancer survival rates strongly depend on the disease stage at the time of diagnosis, but the majority of patients are alive after 5 years from the diagnosis. When breast cancer is diagnosed at early stages the outcome it is usually positive, but survival rates are low in metastatic breast cancer (Baade, Youlden and Chambers, 2011; Soerjomataram *et al.*, 2008). For this reason population screening and mammography are essential tools for the reduction of breast cancer mortality (Elmore *et al.*, 2005; Smith, 2003).

1.1.1 Breast cancer symptoms and diagnosis

According to the Merk Manual of Diagnosis and Therapy (Beers, 2006), the most common first symptom of breast cancer is a painless lump which is usually recognized by the patient. In the early stages the lump might be movable underneath the skin. In later stages, the lump adheres to the chest wall or to the skin over it. In very advanced stages, swollen bumps or festering sores may occur. Sometimes the skin over the lump can look dimpled, with a pattern similar to the orange skin, and lumps can become painful. A subtype of breast cancer called inflammatory breast cancer is characterized by a warm, red and swollen breast, which could occur with or without skin ridges or inhomogeneities. There might be nipple inversion or nipple discharge. Often in this kind of breast cancer there are no lumps (Beers, 2006). When symptoms of breast cancer are detected, the patient is referred to designated breast clinics where the diagnosis is made by “triple assessment”. This consists of a combination of mammography and ultrasound imaging. If a suspicious mass is found, core biopsy and/or fine needle aspiration cytology is performed (National Institute for Health and Care Excellence guidelines, https://www.nice.org.uk/). Magnetic resonance imaging (MRI) of the breast might also be used for further investigations in patients with confirmed breast cancer (Willett, Michell and Lee, 2010). In invasive breast cancer the axillary lymph nodes are usually the first sites of metastatic spread (Giuliano *et al.*, 1994). Therefore, ultrasound imaging of the axilla is also carried out in all patients when a malignancy is suspected. If lymph nodes are found to have an altered morphology on ultrasound, a biopsy is also taken. Following the triple assessment, a definitive diagnosis is made in most patients (Willett, Michell and Lee, 2010).

1.1.2 Breast cancer staging and grading

In United Kingdom, breast cancer is diagnosed following the TNM staging system (http://www.cancerresearchuk.org/about-cancer/type/breast-cancer/treatment/tnm-breast-cancer-staging#fit). TNM stands for Tumour, Node, Metastasis, and takes into account the size of the tumour (T), the presence of cancer cells in the lymph node (N) and the presence of metastasis (M). An overview of different breast cancer stages is summarized in table 1.1.

TNM is the most widely used system to classify the anatomic spread of a neoplasia (Sobin and Fleming, 1997). This staging system was developed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC). The TNM classification system is a tool for clinicians to describe the severity of cancer using standardized criteria (https://cancerstaging.org/references-tools/Pages/What-is-Cancer-Staging.aspx). To complete the diagnosis of breast cancer, biopsies are analysed by pathologists to assess tumour grade and tumour subtype. The grade of a tumour is evaluated depending on the appearance of cancer cells under a microscope, using the “Nottingham grading system”. Tissue morphology, mitotic index and cells nuclear shape are evaluated according to standardized criteria to give a final score between 1 and 3 (source: NHS, http://www.cancerscreening.nhs.uk/breastscreen/publications/nhsbsp58-poster.pdf). Grade 1 or low-grade is attributed when cancer cells look similar to normal cells (i.e. are well differentiated) and usually grow slowly. In this case cancer cells are less likely to spread. Grade 2 or moderate-grade is attributed when cancer cells look more abnormal and grow slightly faster than grade 1 cells. Grade 3 or high-grade is attributed when the cancer cells look very different from normal cells (are poorly differentiated) and may grow quicker than grade 1 or 2 cells (source: http://www.macmillan.org.uk/Cancerinformation/Cancertypes/Breast/SymptomsdiagnosSy/Stagingandgrading.aspx).

Biopsies are also analysed to assess the receptor status, i.e. if cancer cells express the oestrogen receptor (ER), progesterone receptor (PR) and Human epidermal growth factor receptor 2 (HER2). This is required to predict the response to certain therapies: Breast cancers that are oestrogen receptor positive are more likely to respond to hormone therapies, and HER2 positive

tumours can be treated with monoclonal antibodies (Trastuzumab) (source: http://www.cancerresearchuk.org/about-cancer/type/breast-cancer/diagnosis/further-tests-for-breast-cancer#hormone).

Table 1.1: TMN staging of breast cancer (Source: Cancer Research UK)

T STAGES	
Stage	**Description**
TX	The tumour size cannot be assessed
T1	The tumour is 2 cm across or less. T1 tumours are further classified in: T1mi – the tumour is 0.1cm across or less T1a – the tumour is more than 0.1 cm but not more than 0.5 cm T1b – the tumour is more than 0.5 cm but not more than 1 cm T1c – the tumour is more than 1 cm but not more than 2 cm
T2	The tumour is more than 2 centimetres, but no more than 5 centimetres across
T3	The tumour is bigger than 5 centimetres across
T4	T4 tumours are further classified in: T4a – The tumour has spread into the chest wall T4b – The tumour has spread into the skin and the breast may be swollen T4c – The tumour has spread to both the skin and the chest wall T4d – Inflammatory carcinoma – this is a cancer in which the overlying skin is red, swollen and painful to the touch
N STAGES	
Stage	**Description**
NX	The lymph nodes cannot be assessed (for example, if they were previously removed)
N0	No cancer cells found in any nearby nodes
ITCs	(Isolated tumour cells) When small clusters of cancer cells, less than 0.2 mm across, or a single tumour cell, or a cluster of fewer than 200 cells are found in one area of a lymph node. Lymph nodes containing only isolated tumour cells are not counted as positive lymph nodes
N1	Cancer cells are in the lymph nodes in the armpit but the nodes are not stuck to surrounding tissues
pN1mi	One or more lymph nodes contain micrometastases that are larger than 0.2mm or contain more than 200 cancer cells, but are less than 2mm
N2	N2 is divided into 2 groups: N2a – there are cancer cells in the lymph nodes in the armpit, which are stuck to each other and to other structures N2b – there are cancer cells in the lymph nodes behind the breast bone (the internal mammary nodes), which have either been seen on a scan or felt by the doctor. There is no evidence of cancer in lymph nodes in the armpit

Table 1.1 continued

N3	N3 is further divided in N3a – there are cancer cells in lymph nodes below the collarbone N3b – there are cancer cells in lymph nodes in the armpit and behind the breast bone N3c – there are cancer cells in lymph nodes above the collarbone
M STAGES	
M0	There is no sign of cancer spread to distant organs
cMo(i+)	Means there is no sign of the cancer on physical examination, scans or X-rays but cancer cells are present in blood, bone marrow, or lymph nodes far away from the breast cancer as detected by laboratory tests
M1	means the cancer has spread to another part of the body

1.1.3 Breast carcinogenesis

The most accredited theory to explain the onset of cancer is based on the clonal expansion of a population of cells carrying driver mutations that confer a selective growth advantage; certain mutations are observed with a high frequency in tumours, therefore are believed to be the initial events in the process of tumorigenesis (Stephens *et al.*, 2012). During the clonal expansion of cancer cells, more mutations, defined as “passenger mutations”, can be acquired, conferring a further growth advantage and/or a metastatic phenotype (Greaves and Maley, 2012; Bozic *et al.*, 2010). A plethora of somatic and germline mutations in oncogenes and tumour suppressor genes have been linked to the onset and development of breast cancer. According to the Catalogue of Somatic Mutations in Cancer (COSMIC) (Forbes *et al.*, 2008), the most common somatic mutations occurring in breast cancer are affecting, in order of decreasing frequency, the following genes: PIK3CA (encoding the phosphatidylinositol-4,5-bisphosphate 3-kinase-catalytic alpha subunit), TP53 (encoding the protein p53), CDH1 (encoding for E-Cadherin), MLL3 (Gene encoding the Histone-lysine N-methyltransferase), GATA3 (encoding for Trans-acting T-cell-specific transcription factor) and PTEN (encoding for the Phosphatase and Tensin homolog protein). These mutations are therefore likely drivers responsible for the aetiology of breast cancer (Stephens *et al.*, 2012).

PI3KCA encodes the p110α catalytic subunit of the class IA phosphatidylinositol 3-kinases (PI3Ks). PI3Ks catalyses the phosphorylation of a class of inositol-containing lipids called phosphatidylinositols (PIs), giving rise to several signalling molecules involved in the control of a broad range of cell functions (Vanhaesebroeck and Waterfield, 1999). Particularly, the phosphatidyl-inositol-3,4,5-triphosphate (PIP3) can activate the AKT serine/threonine kinase which regulates cell proliferation, survival, and motility. The tumour suppressor gene PTEN, also often mutated in breast cancer, inhibits the PI3K pathway by dephosphorylating PIP3 (Samuels *et al.*, 2005; Sun *et al.*, 1999; Li *et al.*, 1997). Deregulation of the PI3K/AKT/PTEN pathway is a common event in cancer and considered a frequent driver event in the development of breast tumours (Wood *et al.*, 2007; Samuels *et al.*, 2005).

The tumour suppressor gene p53 is commonly defined as the “guardian of the genome” (Lane, 1992). This definition originates from the propriety of p53 to control proteins involved in the cell cycle, DNA repair or apoptosis, in case of DNA damage; the regulation of these processes by p53 prevents the propagation of potentially toxic mutations during cell division and, therefore, defines p53 as an essential tumour suppressor gene (Sengupta and Harris, 2005; Kastan, Canman and Leonard, 1995; Lowe *et al.*, 1993). Not surprisingly, mutations of p53 are the most common in human cancers and very frequent in breast tumours (Pharoah, Day and Caldas, 1999; Levine, Momand and Finlay, 1991).

E-cadherin is part of the cadherins family, a group of transmembrane proteins involved in cell-cell junctions (Angst, Marcozzi and Magee, 2001). E-cadherin is often downregulated in breast cancer. This is associated with an unfavourable prognosis and aggressive phenotypes (Siitonen *et al.*, 1996; Pierceall *et al.*, 1995; Oka *et al.*, 1993). E-cadherin can suppress cell proliferation by dephosphorylating the retinoblastoma protein (Rb), reducing Cyclin D1 and elevating p27 levels (St Croix *et al.*, 1998). Moreover, E-cadherin can inhibit tumour growth by reducing the transcriptional activity of β-catenin and interacting with members of the Hippo pathway (Kim *et al.*, 2011; Stockinger *et al.*, 2001). Therefore, mutations of CDH1 can be driver events of breast cancer tumorigenesis by enhancing uncontrolled cell proliferation (Berx and Van Roy, 2001).

MML3 encodes the Histone 3-lysine-4 -methyltransferase and is commonly mutated in breast cancer, although is function in this tumour is still poorly characterized (Stephens *et al.*, 2012). Studies on mice suggest that MML3 interacts with p53 in the response to DNA damage, and since its removal results in urinary tumours, MML3 is a putative tumour suppressor gene (Wang *et al.*, 2011; Lee *et al.*, 2009).

GATA3 (Trans-acting T-cell-specific transcription factor 3) belongs to the family of GATA transcription factors and is important to regulate the differentiation of luminal cells in the mammary gland (Kouros-Mehr *et al.*, 2006). Array-based studies have linked high expression of GATA3 with lower tumour grade and reduced proliferation index in breast tumours. Moreover, lower expression of GATA3 was associated with poor outcome and lower survival in breast cancer patients (Yoon *et al.*, 2010; Mehra *et al.*, 2005; Usary *et al.*, 2004). Mechanistically, mutations in GATA3 might result in the failure to maintain a differentiated low proliferative phenotype of the luminal cells of the breast, promoting tumorigenesis through the epithelial mesenchymal transition (EMT) (Yan *et al.*, 2010; Usary *et al.*, 2004).

In addition to point mutations, gene copy number variations (CNV) are known to give a major contribution to the genetic landscape of tumours (Pollack *et al.*, 2002). Particularly, vast areas of the chromosome 1,8 and 17 are often subjected to extensive CNV in breast cancer (fig.1.1) (Forbes *et al.*, 2008; Fridlyand *et al.*, 2006; Pollack *et al.*, 2002; Kallioniemi *et al.*, 1994). For example, the copy number gain of HER2 was associated with a worse outcome and tumour relapse before the introduction of Trastuzumab in the therapy of HER2 positive tumours (Slamon *et al.*, 1987).

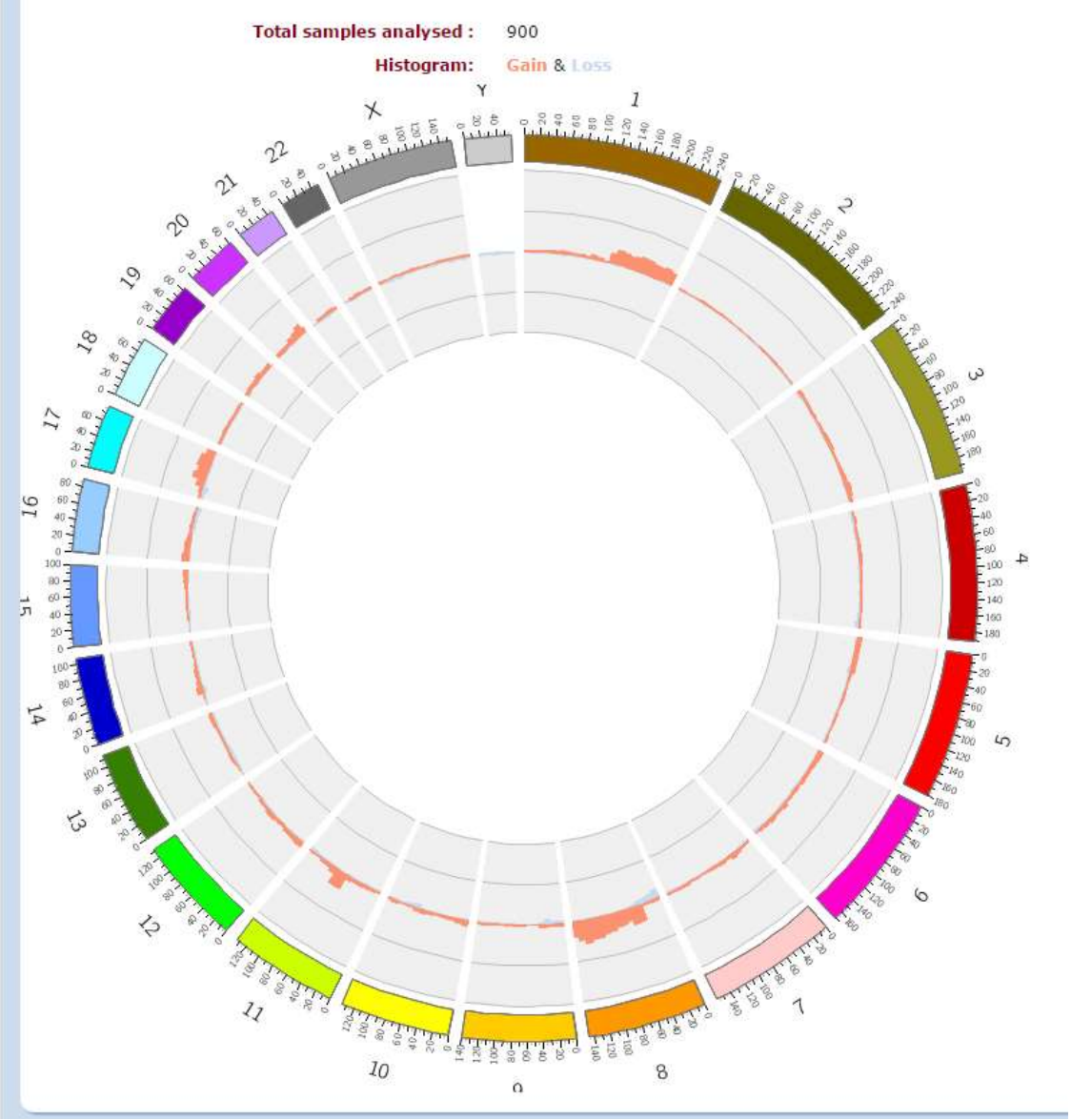

Figure 1.1: Circos image displaying CNV in breast cancer: the height of the bar is the total number of samples which had a CNV event in that region, with blue representing loss and pink representing gain (Source: COSMIC, (Forbes *et al.*, 2008).

Other genes commonly subjected to CNV in breast cancer are MYC and the fibroblast growth factor receptor 1 (FGFR1), both in the chromosome 8 (Stephens *et al.*, 2012). Amplification of FGFR1 has been associated with poor prognosis and resistance to hormone therapy in breast cancer (Turner *et al.*, 2010). MYC or (c-MYC) is a transcription factor which is thought to regulate about 15 % of all the human genes (Gearhart, Pashos and Prasad, 2007). MYC regulates cell proliferation and growth, and is often overexpressed in human cancers (Dang, 2012). In breast tumours, the amplification of c-MYC strongly

reduces the survival end increases the risk of tumour relapse (Sotiriou *et al.*, 2003; Deming *et al.*, 2000; Berns *et al.*, 1992).

Several other transcription factors are often found overexpressed in breast cancer and could participate to the early stages of the disease. The forkhead box proteins (Fox) are a class of transcription factors which are often deregulated in human cancers (Myatt and Lam, 2007). FOXM1, a member of the Fox family, is frequently overexpressed in numerous malignancies (Koo, Muir and Lam, 2012). In the COSMIC database, 15.17 % of the breast cancer samples overexpress FOXM1 (Forbes et al. 2014). FOXM1 was shown to regulate the expression of the oestrogen receptor α, which plays a major role in the proliferation of breast cancer cells (Millour and Lam, 2010). Indeed, RNA interference of FOXM1 inhibits cell growth and invasion of breast cancer cell lines (Ahmad *et al.*, 2010).

Another transcription factor which is commonly deregulated in breast cancer is the Hypoxia-inducible factor 1 (HIF-1), which controls the expression of genes involved in cancer metabolism and tumour vascularization, required for the survival and propagation of cancer cells (Semenza, 2003). HIF-1α, a functional subunit of HIF-1, is often overexpressed in breast tumours and its upregulation is associated with unfavourable prognosis in patients (Dales *et al.*, 2005; Schindl *et al.*, 2002; Zhong *et al.*, 1999).

Epigenetic modifications, i.e. DNA methylations and histones modifications could also contribute to the breast cancer landscape influencing the expression of oncogenes and tumour suppressor genes. For example, the hypermethytlation of tumour suppressor genes such as BRCA1 or MLH1 is a common events in breast cancer which strongly enhances cancer progression (Veeck et al. 2010).

1.1.4 Breast cancer aetiology

There are several factors that are known to increase the risk of breast cancer and these can be grouped in the following categories: genetic/familial, reproductive/hormonal, lifestyle related, and environmental (Salehi *et al.*, 2008). Genetic predisposition influences up to 10% of all breast cancers (McPherson, Steel and Dixon, 2000). Germline mutations have been involved in breast cancer, but two in particular, BRCA1 and BRCA2, have been largely studied and considered as the principal cause of familiar breast cancer (Ford *et al.*, 1998). The functions of BRCA1 and BRCA2 have not been fully elucidated yet, but it is clear that they are implicated in the processes of DNA repair and cell cycle control. Mutations in these genes can therefore facilitate the onset of driver mutations in breast cancer (Venkitaraman, 2002; Moynahan *et al.*, 1999; Patel *et al.*, 1998). Taken together, germline mutations of BRCA1 and BRCA2 encounter for 80–90% of all the familial breast cancer cases (Duncan, Reeves and Cooke, 1998). Germline mutations of the tumour suppressor gene p53 are less common (Li-Fraumeni syndrome) and can cause early onset of breast cancer (Malkin et al. 1990).

Age is one of the main risk factors. The risk of breast cancer doubles about every 10 years until the menopause, when the risk drops dramatically (McPherson, Steel and Dixon, 2000).

The altered homeostasis of ovarian hormones is another factor known to increase breast cancer incidence. In normal tissue, oestrogens and progesterone are finely regulated to modulate proliferation and differentiation of breast cells (Pike *et al.*, 1993). Reproductive factors that enhance oestrogens levels (such as early age at menarche, nulliparity and late onset of menopause) are known to enhance the risk of breast cancer. Conversely, reproductive factors associated with a reduced exposure to oestrogens, such as low number of ovulatory cycles, have been shown to be protective against breast cancer (Martin and Weber, 2000). Mechanistically, the increase in cell proliferation caused by oestrogens also increases the chances that genetic errors occur during cell replication; if the mutations occur in tumour suppressor genes or oncogenes, this could give rise to breast cancer (Preston-Martin *et al.*, 1990).

Progesterone levels might also influence breast cancer occurrence. Progesterone is crucial for normal breast tissue regulation, particularly lobular-alveolar development (Ismail *et al.*, 2003). Its role in breast cancer is still controversial, but it is probably associated with the different progesterone receptors isoforms subtypes expressed in the tumour tissue and is probably exercised indirectly through the regulation of oestrogens levels (Salehi *et al.*, 2008).

Lifestyle is another key factor in breast cancer incidence. Obesity has been shown to increase two fold the risk of developing the disease. Other habits such as alcohol consumption and smoking have been suggested as potential risk factors, although other investigators have reported a minimal or null increase of the risk (Romieu *et al.*, 2015; Ali *et al.*, 2014; Dossus *et al.*, 2014; McPherson, Steel and Dixon, 2000).

1.2 Breast cancer subtypes

Breast tumours can be categorized into different subtypes depending on the origin of the cells and molecular features. 90% of all breast cancers derive from epithelial cells of the milk ducts, therefore are called ductal carcinomas. Breast tumours originating from the milk producing glands (lobules) are called lobular carcinomas (figure 1.2). A third rare subtype, breast sarcoma, originates from the fatty or connective tissue (Beers 2006).

If breast cancer cells are confined where the lesion first occurred, the tumour is defined as “in situ” carcinoma. Ductal carcinomas in situ accounts for 20 to 30% of all breast cancers (Beers 2006). Nearly all women diagnosed at this early stage can be cured (Source: American Cancer Society website, http://www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-breast-cancer-types). Lobular carcinomas in situ accounts for 1 to 2 % of all breast cancers (Beers 2006).

Invasive ductal carcinomas are characterized by the tumour cells capability to pass through the walls of the ducts, invading the surrounding tissue. This type can generate metastases. It accounts for 65 to 85% of all breast cancers and it is the most common subtype (Beers 2006). Invasive lobular carcinomas are

more likely to occur in both breasts and they account for 10 to 15% of all breast cancers (Beers, 2006).

In about 20 % of the women diagnosed with early breast cancer, the tumour can spread to secondary organs and develop metastases. Currently, there is no cure for metastatic breast cancer and therapies are aimed to prolong as much as possible the survival of these patients (Stevanovic, Lee and Wilcken, 2006).

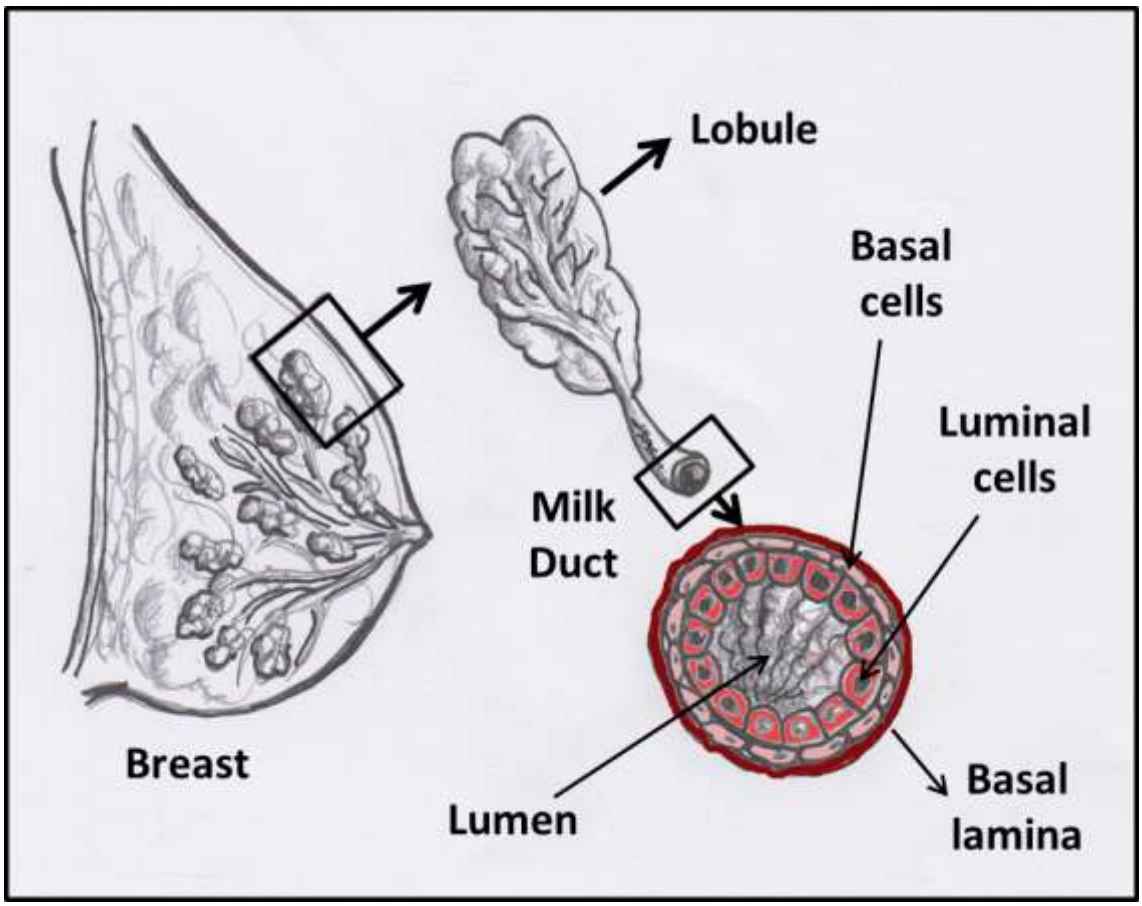

Figure 1.2: Schematic representation of the mammary gland: The mammary gland is constituted of milk secreting cells organized in lobules and ducts that convoy the milk towards the nipple. The milk duct is composed of two different cell types: the luminal cells which constitute the inner part of the duct and form the duct lumen, and the basal/myoepithelial cells which constitute a more external layer of cells. The basal lamina separates the duct from the surrounding stroma.

The most aggressive type is inflammatory breast cancer. This is characterized by skin erythema, peau d'orange and poor prognosis. Molecular profiling and histopathology might vary, but it always implicates invasion of tumour cells into the surrounding lymph nodes (Cariati et al., 2005).

A very rare form of breast cancer is the Paget's disease of the nipple. It is caused by the infiltration of carcinoma cells with glandular features in the epidermal layer of the nipple and areola. It accounts for 1% of all breast cancers (Lohsiriwat *et al.*, 2012; Beers, 2006).

Breast cancer biopsies are required to identify the molecular changes relevant for the establishment of appropriate therapeutic protocols. According to the St. Gallen International Expert Consensus, breast cancer cases can be categorized in 4 intrinsic subtypes: Luminal A, Luminal B, HER2 positive and Basal-like (Goldhirsch *et al.*, 2013). Recently, gene expression studies identified a claudin-low subtype, similar to the basal subtype but distinctive for the overexpression of 40 genes involved in the immune response. However, at present there are no standardized procedures for the identification of this subtype in the clinical practice (Eroles *et al.*, 2012).

1.2.1 Luminal breast cancer

Luminal breast tumour cells have features similar to the epithelial cells constituting the milk ducts lumen and express similar proteins. These tumours are ER positive and express the transcription factors downstream the oestrogens pathway (Rakha and Ellis, 2009; Sims *et al.*, 2007). Moreover, luminal tumours express cytokeratins 8 and 18 (Eroles *et al.*, 2012). Luminal breast cancer can be further categorized in two subgroups: Luminal A and Luminal B.

Luminal A. This subtype accounts for about 50-60% of all the breast cancers and is the most common subtype. Luminal A tumours usually have good prognosis, with lower probability of relapse and the highest rate of survival (Eroles et al., 2012). The Luminal A subtype can be identified by immunohistochemistry: these tumours are positive for the Oestrogen Receptor (ER) and Progesterone Receptor (PGR), negative for HER2, and < 14% of the cells are positive for the proliferative marker Ki-67 (although this cut point might vary between laboratories) (Goldhirsch *et al.*, 2013).

Luminal B. This subtype accounts for about 10-20 % of all breast cancers. It has a worse prognosis, a more aggressive phenotype and a higher histological

grade compared to the Luminal A subtype (Eroles *et al.*, 2012; Cheang *et al.*, 2009). This subtype can be further divided into HER2 positive or HER2 negative. The Luminal B HER2 positive tumours can have any grade of Ki-67 or PGR to be defined as luminal B, whereas Luminal B HER2 negative tumours must have in addition high levels of Ki-67 (≥ 20 % of the cells positive for Ki-67) or low or absent PGR (Goldhirsch *et al.*, 2013).

1.2.2 HER2 amplified breast cancer

ERBB2 gene encodes the protein HER2 which belongs to the family of the epidermal growth factor receptors. These receptors have intrinsic tyrosine kinase activity and mediate the response to extracellular cues by regulating complex cellular functions such as proliferation, survival and migration (Wieduwilt and Moasser, 2008). HER2 function seems to be exerted by the dimerization with other receptors of the same family, rather than direct activation by specific ligands (Olayioye, 2001). 15-20% of all breast cancers show overexpression of HER2. This is correlated with higher tumour size, increased invasiveness and high histological grade (Yarden and Sliwkowski, 2001). HER2 activation enhances the cell proliferation rate and reduces apoptosis by activating signalling pathways involving PI3K, AKT, PLC and PKC (Roy and Perez, 2009). In the COSMIC repository, about 12% of the breast cancer samples present a gene copy number gain of ERBB2 (Forbes et al. 2014). A direct correlation betweenERBB2 copy number gain and HER2 overexpression has been observed in breast cancer cell lines (Szollosi *et al.*, 1995).

HER2 overexpressing tumours had a poor prognosis before the introduction of a monoclonal antibody against HER2 (Trastuzumab), which drastically improved the survival rate of patients bearing this breast cancer subtype (Eroles *et al.*, 2012). Trastuzumab is the first successful antibody used in breast cancer targeted therapy (Roy and Perez, 2009). The mechanism of action is still not fully understood, but it has been proposed that the bond of Trastuzumab with the juxstamembrane portion of HER2 results in increased receptor endocytosis, hampers the receptor dimerization, and activates the immune system (Hudis, 2007). Moreover, downregulating the signalling cascades downstream HER2

(Such as PI3K and MAPK signalling), Trastuzumab can activate the cyclin dependent kinase inhibitor p27kip1, leading to cell cycle arrest and apoptosis (Nahta and Esteva, 2006).

1.2.3 Basal-like breast cancer

The basal-like group of breast tumours have a genetic expression profile that resembles the basal/myoepithelial cells of the mammary gland. These tumours are recognisable by the positive immunohistochemical staining of cytokeratins 5, 14 and 17 (CK5, CK14 and CK17), and the expression of other basal markers such as P-cadherin, caveolin1/2 and nestin (Sims *et al.*, 2007). This breast cancer subtype is the most aggressive and the one with the poorest prognosis (Liedtke *et al.*, 2008). Basal-like breast cancers account for 10-20 % of all breast carcinomas (Eroles *et al.*, 2012).

1.2.4 Triple negative breast cancer

Triple negative breast cancers (TNBC) are defined as breast tumours negative for ER, PGR and HER2 in immunohistochemical analysis. The vast majority of basal-like tumours (around 80%) are also triple negative (Goldhirsch *et al.*, 2013). Often the terms basal-like and triple negative are used as synonyms, although not all the basal-like tumours are also triple negative and vice versa (Eroles *et al.*, 2012; Rakha, Reis-Filho and Ellis, 2008). Indeed, 18 to 40 % of the basal tumours do not show a triple negative phenotype (Bertucci *et al.*, 2008). TNBC is characterized by a bigger tumour mass and a higher histological grade compared to the other breast cancer subtypes (Dent *et al.*, 2007). The incidence of TNBC is higher in black and in Hispanic women compared to other ethnic groups (Lara-Medina *et al.*, 2011; Stead *et al.*, 2009; Bauer *et al.*, 2007) and more common in young women (Dent *et al.*, 2007). An increasing waist-to-hip ratio and parity raises the risk of TNBC (Millikan *et al.*, 2008).

Several lines of evidence have linked the phenotypical characteristics of TNBC/basal-like breast cancers with the ones deriving from germline mutations

of the BRCA1 gene (Reis-Filho and Tutt, 2008). In fact, the vast majority of patients carrying a mutation of BRCA1 exhibit a triple negative/basal like phenotype (Haffty *et al.*, 2006; Foulkes *et al.*, 2003).

Triple negative breast cancer patients have a poorer outcome compared to patients bearing other subtypes (Dent *et al.*, 2007). Few therapeutic options for TNBC are available, since hormone and HER2 targeted therapies are not beneficial for these patients (Foulkes, Smith and Reis-Filho, 2010). Although the response to chemotherapy is usually good, the recurrence and disease progression are more common than in any other breast cancer subtype (Perou, 2011). Therefore, the development of novel therapeutic approaches is urgently needed.

Another feature of TNBC is that, contrarily to other subtypes, it is more likely to metastasize to organs such as the lungs and the brain, and less likely to metastasize to the bones (Foulkes, Smith and Reis-Filho, 2010; Dent *et al.*, 2009).

1.2.5 Claudin low breast cancer

In 2007 a new breast cancer subtype, the claudin low subtype, was identified by Herschkowitz and colleagues. This subtype is characterized by low expression of genes involved in cell adhesion such as Claudins 3, 4 and 7, Occludin and E-Cadherin. Claudin-low subtypes also have a low expression of luminal genes and high expression of lymphocyte markers (Herschkowitz *et al.*, 2007). Most tumours of this subtype are TNBC, although about 20% of them are positive for hormones receptors (Prat and Perou, 2011). Another feature of this group is the low expression of proliferation genes which are usually high in luminal B, basal like and HER2 enriched subtypes (Eroles et al., 2012; Prat and Perou, 2011)

Interestingly, this subtype expresses a class of genes which are associated with cancer stem cells, such as ALDH1A1. Some authors hypothesized that all of the previously described breast cancers subtypes derive from cancer stem cells, with claudin-low subtype being an early progenitor (Hennessy et al., 2009).

1.2.6 Normal like breast cancer

This group is a heterogeneous subset of tumours characterized by having similar features to the normal breast tissue. Common aspects of this class are the high expression of genes typical of adipose cells and other non-epithelial cells, and low expression of luminal genes. Clinical outcome for these tumours varies from good to very poor prognosis (Eroles et al., 2012; Rakha, Reis-Filho and Ellis, 2008).This class accounts for 5 to 10% of all breast tumours and can also be classified as TNBC, as they lack ER, PGR and HER2. Since normal like breast cancer cells are negative for CK5, they cannot be considered basal-like. This subtype is still poorly studied, and some authors hypothesize it derives from artefacts caused by sample contamination with normal tissue before microarray analysis (Eroles et al., 2012).

1.3 Breast cancer therapy

Therapy for breast cancer varies depending on the different molecular subtypes, but the first act is the surgical resection of the tumour, followed or not by radiotherapy (Beers, 2006). When the size of the tumour is large, patients receive chemotherapy before surgery (Neoadjuvant chemotherapy) to facilitate tumour excision, improve breast conservation and suppress occult systemic metastases (Smith *et al.*, 2002). Physical or chemical treatments can be used to eliminate cancer cells that have been left after surgery, referred to as adjuvant therapy. These treatments include chemotherapy, hormonal therapy, monoclonal antibodies, radiation therapy, or a combination of these (Davidson and Abeloff, 1994).

1.3.1 Hormone therapy

The hormone therapy includes two classes of drugs, anti-oestrogens and progestins. The anti-oestrogens strategy includes the selective oestrogen-

receptor modulators (SERM), selective oestrogen-receptor downregulators (SERD) and the aromatase inhibitors (AI). The therapeutic effect is due to the anti-proliferative action of antagonizing the ER pathway (Goodman, 1990). When other therapies have failed, progestins are used as second line defence in advanced breast cancer. Progestins reduce the levels of systemic oestrogens and the expression of ER (Lundgren *et al.*, 1990; Blumenschein, 1983).

One of the most widely used drugs in the treatment of breast cancer is Tamoxifen. It is used both in early and late stages of the disease, in women of all the ages and also as preventive treatment in women carrying BRCA1 or BRCA2 mutations. It belongs to the class of SERM and works antagonizing oestrogens in the mammary gland (Goodman, 1990).

Fulvestrant is a member of the SERD family and it is a pure antagonist of the oestrogen receptor. It is often used in women with advanced disease when the treatment with tamoxifen has failed (Goodman, 1990; Lundgren *et al.*, 1990; Blumenschein, 1983).

Anastrozole, letrozole and exemestane belong to the class of the aromatase inhibitors which act reducing the levels of circulating oestrogens. The enzyme aromatase converts the androstenedione in the oestrogens oestrone and estradiol. This conversion occurs predominantly in the ovaries of pre-menopausal women and peripherally in post-menopausal women. For this reason these drugs are often used in the treatment of breast tumours in post-menopausal women, where the side effects affecting the ovarian hormonal homeostasis are reduced (Goodman, 1990).

Megestrol acetate is one of the most used progestin in the therapy of breast cancer. Progestins are used in patients with metastatic relapsing breast cancers as second choice therapy where treatment with tamoxifen failed (Goodman, 1990).

1.3.2 Monoclonal antibodies

Trastuzumab and Pertuzumab are humanized monoclonal antibodies used alone or in combination for the treatment of HER2 positive breast cancers. HER2 activation generates downstream signal that increase the metastatic

potential and reduce apoptosis. HER2 overexpression is associated with resistance to cytotoxic and hormonal therapy (Baselga *et al.*, 2012; Vogel *et al.*, 2002). The mechanisms of action of the monoclonal antibodies in killing breast cancer cells are still unclear. Different theories have been proposed, such as direct toxicity mediated by the antibody itself, toxicity mediated by the complement, or induction of cell apoptosis (Beers, 2006; Goodman, 1990). Lapatinib is an inhibitor of both the Epidermal Growth factor receptor 1 (EGFR1) and HER2, and can be used in the treatment of HER2 positive metastatic breast cancer (Source: Cancer Research UK, http://www.cancerresearchuk.org/about-cancer/type/breast-cancer/treatment/which-treatment-for-breast-cancer#hormchem).

1.3.3 Chemotherapy

Chemotherapy includes a number of cytotoxic and cytostatic drugs that are used to eliminate fast dividing cells. It cannot be used as a single therapy in breast cancer, but always together with surgery, radiation therapy or other adjuvant therapies (Beers, 2006). Commonly used drugs in breast cancer are cyclophosphamide, doxorubicin, epirubicin, 5-fluorouracil, methotrexate, mitomycin, mitozantrone, docetaxel, gemcitabine and paclitaxel (source: Cancer Research UK).

1.4 WNT signalling pathway

The WNT (Wingless-related integration site protein) pathway consists of a group of cell signalling cascades activated by a class of lipoglycoproteins called WNTs (Wingless-related/integration site proteins). In humans, there are 19 WNTs (WNT1, WNT2, WNT2B, WNT3, WNT3a, WNT4, WNT5a, WNT5b, WNT6, WNT7a, WNT7b, WNT8a, WNT8b, WNT9a, WNT9b, WNT10a, WNT10b, WNT11 and WNT16). WNTs ligands mediate the signal transduction from the extracellular environment to the cell through the activation of 7-segments transmembrane receptors called frizzled (FZDs). In human, there are ten frizzled receptors (FZD_{1-10}) (Schulte, 2010).

The first WNT gene was discovered by Nusse et al. in 1982, during experiments where mice were infected with the mouse mammary tumour virus (MMTV). They found that the MMTV oncogenic properties derived from the capacity to activate a gene, following viral genome integration, that was first called int-1 (integration 1) (Nusse *et al.*, 1984). It was later discovered that the gene int-1 was the homologous of the gene Wingless in drosophila melanogaster (Sharma and Chopra, 1976). Due to the discovery of new Int-1 related genes and to avoid confusion in the nomenclature, the name Int-1 was later changed to WNT-1, (Wingless-related integration site protein 1).

WNT signalling is divided into two different branches, the canonical and the non canonical pathways. The WNT canonical pathway is characterised by the stabilization and translocation into the nucleus of the protein β-catenin. β-catenin has a dual function: together with other proteins constitutes the cellular adherens junctions (Hartsock and Nelson, 2008) and also promotes the transcription of WNT-associated genes in the nucleus (Daniels and Weis, 2005). The term non-canonical pathway usually refers to a group of signalling pathways activated by WNTs which do not lead to the cytoplasmic stabilization of soluble β-catenin (Pandur, Maurus and Kühl, 2002). Two of these pathways have been well characterised: the planar cell polarity (PCP) and the WNT-Calcium pathway (Strutt, 2003).

Although for simplicity the WNT signalling is often dichotomized in two branches, the canonical and the noncanonical pathways often overlap and cross interact to coordinate complex cellular responses. The two investigators

that first discovered the WNT pathway, Renée van Amerongen and Roel Nusse, recently proposed an integrated model where the total net balance of canonical and non canonical signals ultimately determines the response of the receiving cell (van Amerongen and Nusse, 2009).

The WNT pathway regulates several cellular functions during embryonal development and is also required for the homeostasis of adult tissues. WNT signalling has been shown to regulate multiple cellular functions, such as proliferation, stem cell maintenance, cell motility and polarity, cell differentiation and fate, in multiple physiological processes (van Amerongen and Nusse, 2009). Despite recent advances, many aspects of the WNT pathway are still poorly understood (Cadigan and Nusse, 1997). The existence of 19 ligands and 10 receptors makes the WNT pathway intrinsically complicated. Several aspects such as the specificity of ligand-receptor interactions and the signal specificity for each frizzled receptor are still poorly characterised (Wodarz and Nusse, 1998).

The WNT pathway is further complicated by the need of co-receptors like the low-density lipoprotein receptor related proteins 5 and 6 (LRP5/6). These co-receptors don't bind directly to WNT ligands but form a FZD/WNT/LRP5/6 ternary complex which is required for the activation of the WNT canonical signal (Zeng *et al.*, 2008; Mao *et al.*, 2001; Tamai *et al.*, 2000).

The WNT pathway can be inhibited by several secreted antagonists which act either binding the WNT ligands or the FZD/LRP5/6 receptor complex (Kawano and Kypta, 2003). Amongst these are some members of the Dickkopf family of proteins (Niehrs, 2006). Dkk_1 is the most characterised member of the family. It inhibits the canonical WNT pathway by inducing the endocytosis of the LRP5/6 co-receptor following the formation of a complex with LRP5/6 and the family of transmembrane proteins Kremen (Niida *et al.*, 2004; Huelsken and Behrens, 2002).

Another class of WNT pathway inhibitors act primarily by binding with WNT ligands and preventing their interaction with the frizzled receptors. This class includes the secreted frizzled related proteins (sFRPs), the WNT inhibitory factor 1 (WIF) and Cerberus.

1.4.1 Canonical WNT pathway

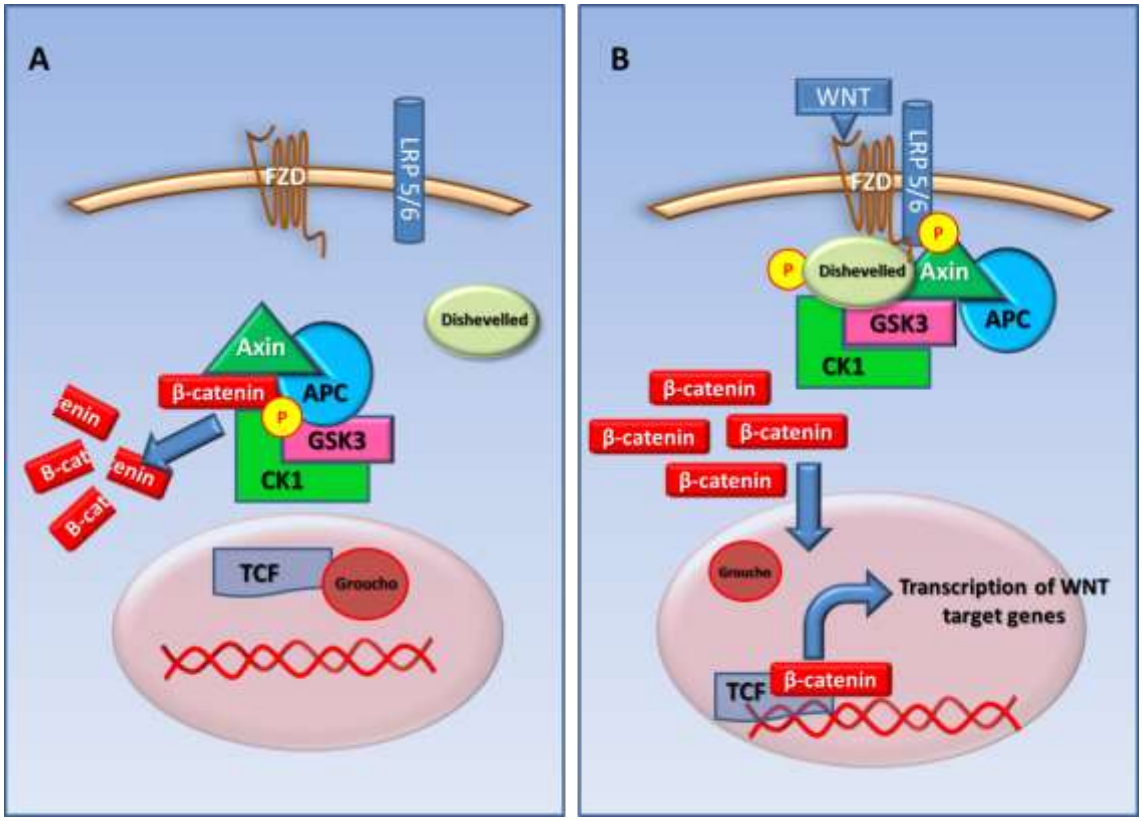

Figure 1.3: Schematic representation of the WNT canonical pathway: (A) In the absence of WNT ligand the levels of cytoplasmic soluble β-catenin are kept low by a heteromeric complex constituted by Axin, CK1, GSK3 and APC (refer to the main text for details). This complex phosphorylates β catenin which is then degraded through an ubiquitin/proteasome mechanism. **(B)** When the WNT ligand binds to FZD, dishevelled is recruited and phosphorylated; this induces the binding with the co-receptor LRP5/6 and the β-catenin destruction complex, leading to the increase of stabilized β-catenin in the cytoplasm. β-catenin can then translocate in the nucleus where it displaces the protein Groucho to bind to the transcription factor LEF/TCF. This induces the transcriptions of several WNT-associated genes (adapted from Clevers and Nusse, 2012).

In the WNT canonical pathway, in the absence of canonical WNT ligands, β-catenin cytoplasmic levels are kept low by a β-catenin destruction complex (fig.1.3 A). The destruction complex is constituted of Axin, Adenomatous Polyposis Coli (APC) and the two protein kinases CK1α/δ and GSK3α/β (Clevers and Nusse, 2012). Axin constitute a scaffold for the destruction complex binding with β-catenin, GSK3 and APC (Luo and Lin, 2004; Nakamura *et al.*, 1998). APC is a large protein that binds both β-catenin and Axin. Its

function is required for the activity of the destruction complex, although its specific molecular role is still unclear (Clevers and Nusse, 2012). The two kinases CK1 and GSK3 phosphorylate β-catenin in multiple residues (Liu *et al.*, 2002). Phosphorylated β-catenin is a target of the SCF-βTRCP ubiquitin ligase complex, which catalyses the addition of ubiquitin residues (Winston *et al.*, 1999). Ubiquitinated β-catenin is then degraded by the ubiquitin-proteasome complex (Aberle *et al.*, 1997).

In the presence of WNT ligands (fig. 1.3 B), the frizzled receptor changes conformation and associates with the co-receptor LRP5/5 (He *et al.*, 2004). LRP5/6 is then phosphorylated by GSK3 and CK1, leading to the association of Axin and the β-catenin destruction complex to the receptors heteromer (Zeng *et al.*, 2008). In these conditions the destruction complex is saturated by phosphorylated β-catenin and cannot induce further β-catenin phosphorylation, leading to the accumulation of stabilized β-catenin in the cytoplasm (Li *et al.*, 2012). The scaffold protein Dishevelled also binds to FZD and is phosphorylated upon WNT activation; although its specific function remains unclear, it is required for the phosphorylation of LRP5/6 (Zeng *et al.*, 2008; Bilic *et al.*, 2007; Wallingford and Habas, 2005; Wong *et al.*, 2003). Recent findings suggest that dishevelled might have a role in facilitating the interaction between LRP5/6 and Axin and in the formation of the FZD/LRP5/6 complex (Clevers and Nusse, 2012).

Stabilized β-catenin is then transported inside the nucleus where it interacts with the family of TCF/LEF transcription factors (Fagotto, Glück and Gumbiner, 1998; Behrens *et al.*, 1996; Molenaar *et al.*, 1996). β-catenin directly displaces the transcription inhibitor Groucho, which normally binds to TCF/LEF transcription factors; this in turns promotes the TCF/LEF mediated transcription of WNT target genes (Daniels and Weis, 2005).

The β-catenin-dependent activation of TCF/LEF1 leads to the transcription of several genes involved in a plethora of functions, including cell proliferation, tissue homeostasis, stem cell maintenance and cell differentiation (Clevers, 2006). The overexpression of WNT target genes such as c-myc and cyclin D1 were shown to be key factors in the development of colon cancer and other tumours (Anastas and Moon, 2012; Bejsovec, 2005; Tetsu and McCormick, 1999).

1.4.2 WNT Planar Cell Polarity

During development cells are organised in tissues through mechanisms that require cell polarization. The regulation of cell polarity is also essential during physiological processes requiring the directional migration of cells, for example during convergent extension movements (i.e. the narrowing and lengthening of a group of cells occurring during gastrulation) or wound healing (Caddy *et al.*, 2010; Roszko, Sawada and Solnica-Krezel, 2009). The acquisition of a specific position of a cell in a plane is referred to as planar cell polarity. This process has been extensively studied in drosophila and requires WNT signalling (Seifert and Mlodzik, 2007). A schematic overview of the PCP pathway is shown in figure 1.4.

Several PCP proteins have been identified in vertebrates through gene loss/gain experiments, but their specific molecular functions remain largely obscure (Simons and Mlodzik, 2008; van Amerongen and Berns, 2006). The principal core components of the PCP signalling cascade in vertebrates are FZD (FZD3 and FZD6 have been shown to mediate PCP signalling in mice (Stuebner *et al.*, 2010), Dishevelled (Dvl1, Dvl2), Vangl1/2, Celsr 1, Prickle (Pk1 and Pk2) and Diversin (or Ankrd6) (Seifert and Mlodzik, 2007; Wang and Nathans, 2007). Two WNT ligands, WNT5a and WNT11, have been implicated in the control of the PCP/WNT pathway, although it is likely that more WNT ligands can activate this pathway (Strutt, 2003).

Vangl2 is a membrane bound protein required for the stereociliary bundle orientation in the mouse cochlea and to regulate the convergent extension movements during neural tube closure in humans (Lei *et al.*, 2010; Montcouquiol *et al.*, 2003). Vangl 1/2 binds to Dishevelled and to Pk1/2 (Jenny *et al.*, 2005; Torban *et al.*, 2004).

Celsr1 is a 7 pass transmembrane protein required for the patterning of sensory hair cells and neural tube closure in mice (Curtin *et al.*, 2003). Recently, it was found that Celsr1 physically interacts with Vangl2 (Lei *et al.*, 2014).

Mutations of the Prickle 1/2 genes in humans, mice and zebrafish have been associated with seizures (Tao *et al.*, 2011). Studies in drosophila and zebrafish suggest that Pk1/2 are cytoplasmic proteins that physically interact with dishevelled, Strabismus (Vangl in humans) and Diego (Diversin or Ankrd6 in

humans) (Das *et al.*, 2004; Bastock, Strutt 2003; Tree *et al.*, 2002), and modulate PCP signalling by competing with Diego for the binding with Dishevelled. In drosophila the binding of Diego with Dishevelled facilitates the FZD/Dishevelled interaction, while the interaction between Pk and Dishevelled exerts the opposite effect (Jenny *et al.*, 2005).

Ankrd6 has been involved in the PCP-dependent orientation of the inner ear sensory organs (Jones *et al.*, 2014). The ankyrin repeat domain of Ankrd6 binds to the DEP domain of Dishevelled. This was shown to be required to activate the WNT/JNK pathway in HEK293 cells (Jones *et al.*, 2014). *In silico* studies predicted that the ankyrin repeats present in this protein are putative binding domains for Prickle1, Prickle2, Vangl1, and Vangl2 (Katoh, 2005). In drosophila the homologous of Ankrd6, Diego, is a cytoplasmic protein recruited by Dishevelled that facilitates FZD/PCP signal (Jenny *et al.*, 2005).

Although it is still unclear how the PCP core elements regulate cell polarity, it seems that their subcellular localization during PCP signalling is a key factor. In drosophila, before the onset of a FZD/PCP signal, the core components are localized around the apical-lateral cortex, while following a PCP stimulus, they distribute asymmetrically to form functional complexes (Seifert and Mlodzik, 2007). In other words, the precise localization of PCP core components in the cell is a prerequisite for PCP-dependent functions such as convergent extension movements and tissue polarity (Strutt, 2003).

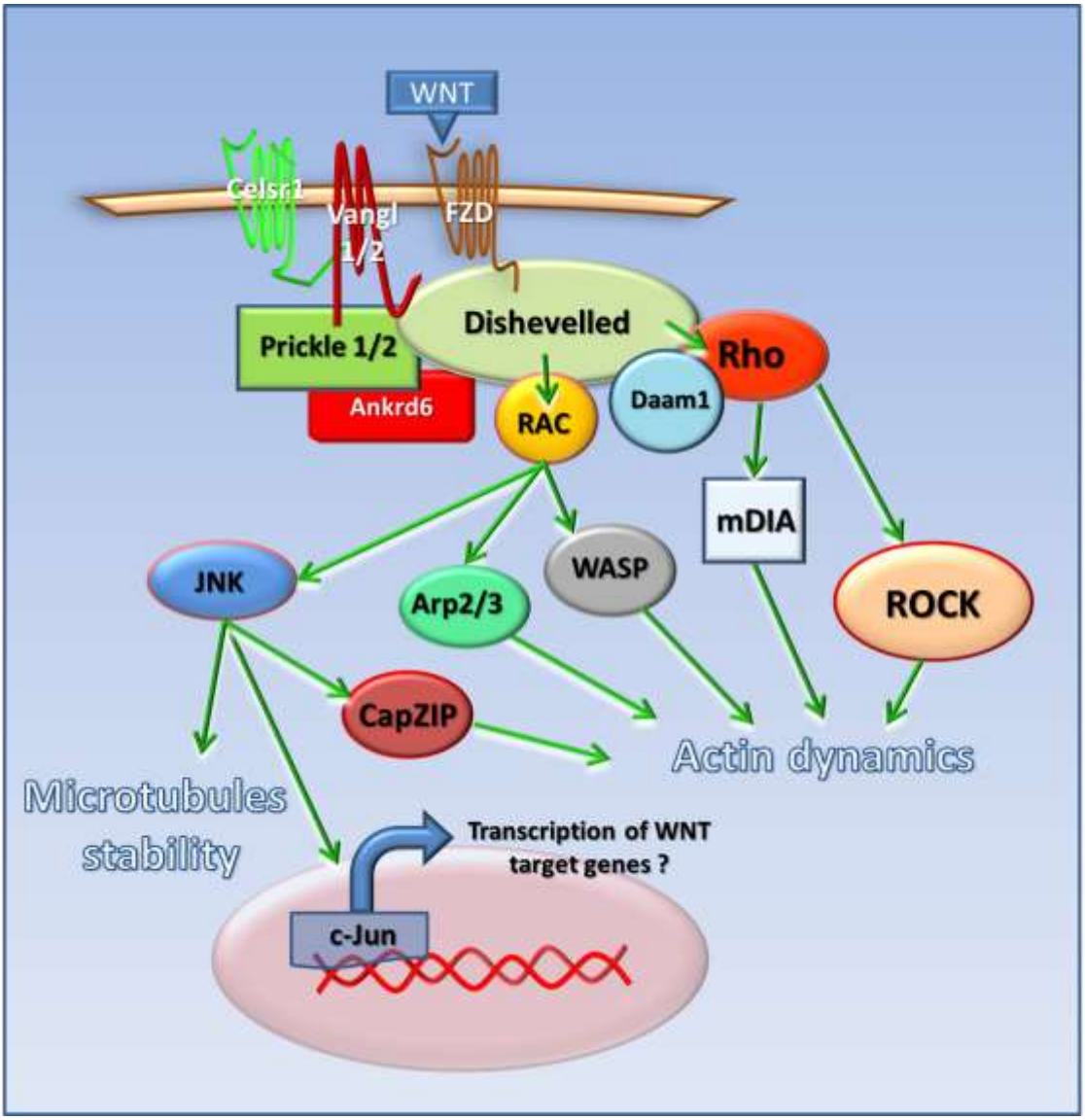

Figure 1.4: Schematic representation of the WNT/PCP pathway: The WNT/PCP pathway is activated by WNT ligands and requires the core components FZD, Vangl 1/2, Prickle 1/2, Ankrd6, Celsr1 and Dishevelled. Although their molecular functions are not fully understood, their presence is required for PCP functions such as the orientation of hairs and stereocilia in the ear and during gastrulation. The most characterised effectors of the WNT/PCP signalling are the small GTPAses Rho and Rac, which are involved in the regulation of actin dynamics (refer to the main text for more details).

Downstream the PCP core components are several effectors which mediate different cell behaviours; these include the Rho family of GTPases (Rho, Rac and Cdc42) and JNK (Roszko, Sawada and Solnica-Krezel, 2009).

The Rho family of GTPases belong to the family of Ras-related small GTP binding proteins, comprising RhoA, RhoB and RhoC, Rac1 and Rac2, Cdc42,

RhoG and TC10 (Boguski and McCormick, 1993). Rho GTPases are in an active state when bound to GTP and in an inactive state when bound to GDP (Symons, 1996). The WNT/FZD-dependent activation of Rho GTPases is mediated by Dishevelled (Schlessinger, Hall and Tolwinski, 2009). Dishevelled activates RAC1 by direct binding with the DEP domain (Habas, Dawid and He, 2003), whereas Rho interacts with Dishevelled through the protein Daam1, which forms a complex with Rho and Dishevelled (Habas, Kato and He, 2001). Rho and Rac collaborate together in the reorganization of the actin cytoskeleton during polarized cell shape changes and cell movement (Schlessinger, Hall and Tolwinski, 2009). Rac is involved in the formation of actin ruffles and focal adhesions at the leading edge of motile cells. These actin structures, called lamellipodia, constitute a molecular motor that push the cell forward during cell migration (Machesky and Hall, 1997; Nobes and Hall, 1995). This is achieved through the activation of the WAVE and Arp2/3 complexes (Jaffe and Hall, 2005). Rac can also induce genes transcription through the activation of the c-Jun N-terminal Kinase (JNK) pathway (Boutros *et al.*, 1998; Minden *et al.*, 1995). JNK signalling is required for the correct gastrulation in Xenopus, suggesting that this pathway is relevant in the morphogenic cell movements controlled by WNT/PCP (Yamanaka *et al.*, 2002). However, little is known about the molecular mechanisms by which JNK participates to PCP. A possible mechanism is through the phosphorylation of a protein that specifically interacts with the F-actin capping protein (CapZIP) that is involved in the remodelling of actin filaments (Eyers *et al.*, 2005). Moreover, JNK was shown to participate in the WNT/PCP by controlling microtubules stability (Ciani and Salinas, 2007). JNK can activate the transcription of target genes by phosphorylating c-Jun, which together with c-Fos forms the AP-1 transcription factor complex (Chiu *et al.*, 1988; Halazonetis *et al.*, 1988); through this pathway, JNK can activate genes involved in cell proliferation and survival (Leppa and Bohmann, 1999). It is likely that JNK could promote the transcription of genes which are relevant for PCP, although this aspect is still poorly investigated (Roszko, Sawada and Solnica-Krezel, 2009).

Rho can control different aspects of the actin cytoskeleton through the activation of the downstream effectors mDIA1 and the Rho associated kinase (ROCK) (Yamana *et al.*, 2006; Maekawa *et al.*, 1999). Rho has been implicated in the formation of actin stress fibres and focal adhesion, and regulates the

contractility of the actin cytoskeleton (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992).

Several experiments have demonstrated a requirement for WNT dependent Cdc42 activation during convergent extension movements in frog gastrulation, but it is not clear if this is activated by dishevelled or other Frizzled-dependent pathways (Schlessinger, Hall and Tolwinski, 2009).

1.4.3 WNT Calcium pathway

In some systems WNTs can increment calcium release through the activation of the WNT/Calcium pathway. This was demonstrated for the first time in the zebra fish model, where WNT5a and WNT11 were shown to increase the intracellular level of calcium (Westfall et al., 2003; Slusarski et al., 1997). The WNT/Calcium pathway is an important regulator of the embryonic development and also regulates physiological functions in adult tissues, such as slow muscle fibres formation and intracellular signalling in the retina (Kohn and Moon, 2005).

Calcium concentration is finely regulated in the cytoplasm, and temporary increments of Ca^{2+} function as important signal mediators in cells (Clapham, 1995). FZD receptors can activate heterotrimeric G-proteins, which in turn activate Phospholipase C (PLC) through the G-protein β/γ dimers (Slusarski et al., 1997) (fig. 1.5). PLC catalyses the formation of inositol-1,4,5-trisphosphonate (IP3) and diacylglycerol (DAG) from the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2); IP3 binds to intracellular receptors that lead to the release of Ca2+ from intracellular stores (Kuhl, 2004). Both Ca2+ and DAG activate the Protein Kinase C (PKC) (Sheldahl et al., 1999; Liu and Heckman, 1998).

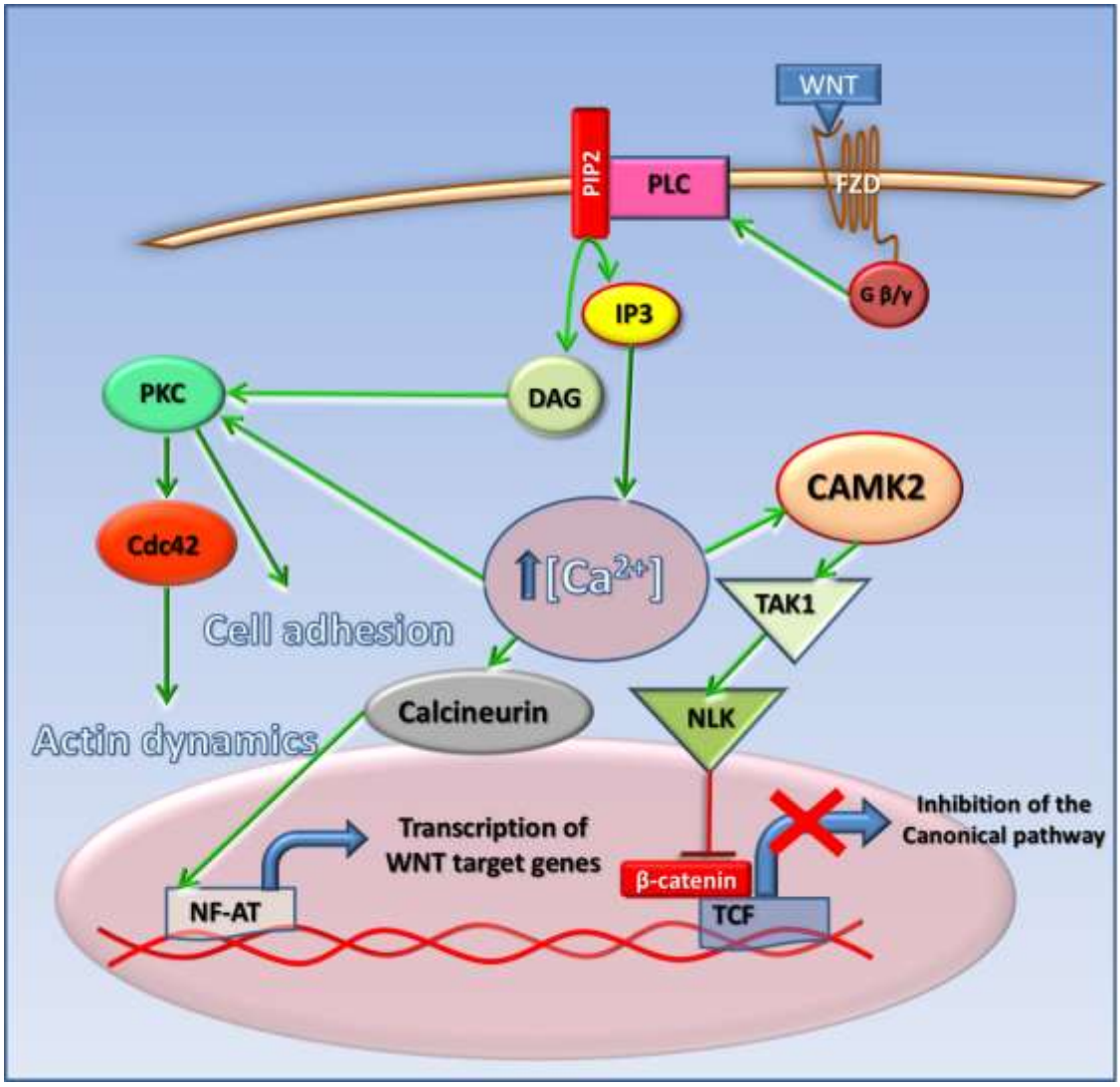

Figure 1.5: Schematic representation of the WNT/Calcium pathway: WNT ligands activate G-protein signals through the interaction with FZD. FZD receptors interact with the G protein subunits β/γ to activate PLC, which in turn catalyses the production of PIP3 and DAG from PIP2. The production of PIP3 leads to the release of intracellular Ca2+ and to the activation of the Ca2+ dependent proteins CAMK2 and Calcineurin. DAG and Ca2+ also activate PKC. PKC, CAMK2 and Calcineurin modulate the cellular response through the transcriptional control of target genes and cytoskeletal rearrangements.

Other proteins which are activated by the release of calcium are the calmodulin-dependent protein kinase II (CAMK2) and/or Calcineurin (Hogan et al., 2003; Kuhl et al., 2000).

WNT-dependent PKC activation was shown to regulate cell-cell adhesion, cell movement during gastrulation and tissue separation during embryogenesis (Kinoshita et al., 2003; Winklbauer et al., 2001; Pauken and Capco, 1999). PKC

can also control cytoskeletal dynamics through the activation of the small GTPase Cdc42 during gastrulation (Choi and Han, 2002).

CAMK2 is essential during embryonic development and is involved in the dorso-ventral axis formation in the Xenopus embryo (Kuhl et al., 2000). Moreover CAMK2 signal was shown to inhibit the WNT canonical pathway through the activation of the MAP kinases TAK1 and NLK, which block the transcriptional activation of TCF/LEF (Ishitani et al., 2003; Ishitani et al., 1999).

Calcineurin is a serine/threonine protein phosphatase which regulates the activity of several target proteins (Jain et al., 1993), including the Nuclear Factor of activated T-cells (NF-AT). When NF-AT is dephosphorylated by calcineurin, it enters the nucleus and regulates the expression of target genes. The WNT/Calcineurin/NF-AT signalling was shown to be required for the embryo dorsoventral axis formation in Xenopus, T-cells differentiation and activation, and cardiac valves development in mice (Saneyoshi et al., 2002; Serfling et al., 2000; Ranger et al., 1998).

1.4.4 WNT signalling in the development and maintenance of the mammary gland

Studies in mice have shown that the WNT pathway is essential for the normal development of the mammary gland. Canonical WNT signalling is required during the early stages of mammary gland development, and mice embryos stimulated with WNT3A or the WNT pathway activator lithium chloride (LiCl) show increased formation of mammary placodes. Conversely, the expression of the secreted WNT inhibitor Dickkopf 1 blocks mammary placode formation (Chu *et al.*, 2004). WNT10b and WNT6 display specific spatio-temporal expression patterns during mouse mammary gland development (Veltmaat *et al.*, 2004). The activation of canonical WNT signalling during mammary gland development was demonstrated in transgenic mice that express a TOPGAL Wnt reporter gene. This is based on a multimerized TCF binding site that drives the expression of LacZ, allowing a colour-base identification of the regions were TCF promoter is activated. These experiments showed that the β-catenin-dependent activation of the transcription factor TCF/LEF1 is required for the

formation of the mammary gland in mice (Chu *et al.*, 2004; van Genderen *et al.*, 1994).

The non canonical WNT pathway was also shown to be involved in mammary gland development. For example WNT5a, a non canonical ligand, is required for the correct extension and lateral branching of milk ducts in mice (Roarty and Serra, 2007).

The mammary gland undergoes numerous morphologic changes to adapt to the different stages of sexual development. These modifications require a tight control in cell proliferation, differentiation and migration. For example, during puberty, the rudimentary ductal tree develops in an elaborate epithelial network that constitutes the adult mammary gland; further modifications are required during pregnancy to adapt the gland for lactation. The plasticity of the mammary tissue implies the involvement of mammary stem cells (van Amerongen, Bowman and Nusse, 2012). WNT signalling is important for the maintenance of mammary stem cells both in the embryo and adult tissue (Zeng and Nusse, 2010). Van Amerongen and colleagues used a recombinant mice model were the expression Axin2 is traceable through a fluorescent reporter system. Axin2 is a well established target gene of the β-catenin pathway and also marks mammary stem cells. In these mice, WNT/β-catenin responsive stem cells localize in specific regions of the mammary epithelial network in a time dependent manner: Axin2 positive cells mark the prospective luminal cell in the embryo, while they become committed exclusively to the basal cell lineage in the 2 week-old pups (van Amerongen, Bowman and Nusse, 2012).

Ovarian hormones are essential to regulate the development of the mammary gland. Indeed, ovariectomy causes abnormalities in the development of the mammary buds and ducts during embryogenesis. Weber-Hall et al. observed that ovariectomy causes the reduction of WNT2, WNT4 and WNT5b mRNAs in the mammary gland, suggesting that ovarian hormones could control the mammary gland development through the expression of WNTs (Weber-Hall *et al.*, 1994). Indeed, the absence of PGR leads to the failure in the ductal side-branching of the mammary gland, but this can be rescued by the ectopic expression of WNT-1. Moreover, the same authors observed that progesterone controls the expression of WNT4 during pregnancy (Brisken *et al.*, 2000).

1.4.5 WNT Signalling in cancer

The first observation of the oncogenic effects of WNT signalling was made by Nusse and Varmus in 1982, when they found that MMTV infection induces mammary tumours in mice through the activation of WNT1 (Nusse et al., 1984). Since then, several other mutations in components of the WNT pathway have been observed during carcinogenesis (Polakis, 2000). Deregulation in β-catenin target genes such as cyclin D1 and c-MYC could lead to uncontrolled proliferation in several tissues and, therefore, give rise to tumour formation (Shtutman *et al.*, 1999; He *et al.*, 1998). The over activation of the β-catenin pathway is often originated by mutations in β-catenin itself, or in onco-supressor genes that control its degradation (Polakis, 2000).

Mutations affecting the amino-terminal region of the β-catenin gene hamper the interaction with APC and, therefore, prevent its degradation; this in turn leads to the constitutive activation of β-catenin and to oncogenic transformation (Polakis, 2000; Morin *et al.*, 1997; Rubinfeld *et al.*, 1997). β-catenin mutations occur with low frequency (Polakis, 2000), but mutations of the β-catenin destruction complex component APC are common in human cancers and considered the principal gatekeeper mutations in sporadic and familial colon tumours (Hussain and Harris, 1998; Morin *et al.*, 1997).

Axin1 is another protein of the destruction complex which is essential for the degradation of β-catenin (Clevers and Nusse, 2012). Mutations in Axin1 have been reported in hepatocarcinoma, particularly those lacking mutations of APC or β-catenin (Satoh *et al.*, 2000). Axin downregulation is also associated with poor prognosis in lung cancer (Xu *et al.*, 2007; Xu *et al.*, 2006).

Although the non canonical WNT pathway has been understudied in cancer, more scientists are now focusing the attention to the role of β-catenin independent signals in tumorigenesis (Wang, 2009). For example, WNT5a, generally considered a noncanonical ligand (although it was shown that it can stabilize β-catenin depending on the receptor context (Mikels and Nusse, 2006), promotes melanoma cells invasion and metastases through the activation of PKC (Weeraratna *et al.*, 2002). Moreover, WNT5a was shown to enhance motility and invasion of gastric cancer cells (Kurayoshi *et al.*, 2006). FZD10 has been implicated in the tumorigenesis of human synovial sarcomas through the

activation of the non canonical WNT/RAC1/JNK pathway (Fukukawa *et al.*, 2009). VANGL1, a member of the PCP core component (fig 1.4), has been implicated the polarity and migration of breast cancer cells (Anastas *et al.*, 2012).

Recently, a new role for PCP in the communication between stroma and cancer cells has emerged. Luga and Wrana showed that cancer-associated fibroblasts promote the mobilization of PCP proteins in cancer cells through the secretion of exosomes; this in turn promotes the migration and invasion of breast cancer cells (Luga and Wrana, 2013; Luga *et al.*, 2012). However, the activation of noncanonical WNT signalling has been associated with tumour-suppressive effects in leukaemia (Roman-Gomez *et al.*, 2007; Liang *et al.*, 2003). In other studies, non-canonical signalling mediated by WNT5a promoted leukaemia, probably by deregulation of stem cells maintenance (Sugimura and Li, 2010).

The WNT/Calcium pathway has also been linked to tumorigenesis, particularly in relation to cell motility and invasiveness (Le Floch *et al.*, 2005; Weeraratna *et al.*, 2002).

1.4.6 WNT signalling and cancer stem cells

Stem cells can produce differentiated cells and new undifferentiated stem cells by asymmetric cell division(Tuch, 2006). There are two subtypes of stem cells: embryonic stem cells, which are totipotent and can generate all tissues, and adult stem cells, which only differentiate into specific tissues (Bajada *et al.*, 2008). While embryonic stem cells are essential for embryogenesis, adult stem cells provide a source of new differentiated cells in tissues with high cells turnover and during tissue repair (Young and Black, 2004).

Many human cancers contain cells with stem like features, responsible for several aspects of cancer malignancy such as drug resistance, tumour recurrence, metastasis and tumour heterogeneity (Merlos-Suárez *et al.*, 2011; Campbell and Polyak, 2007; Li *et al.*, 2007; Jordan, Guzman and Noble, 2006; Dean, Fojo and Bates, 2005).

WNT signalling is involved in stem cells self-renewal and differentiation. Alterations in tightly regulated signals in cancer stem cells are implicated in the

malignant proliferation and metastasis of human tumours (Reya and Clevers, 2005). An emblematic example is the deregulation of β-catenin signalling in colon cancer, which leads to the activation of the same genetic programs characteristic of the stem/progenitor cells of the colon crypt (Vermeulen *et al.*, 2010; Reya and Clevers, 2005; Van De Wetering *et al.*, 2002). Similarly, the deregulation of WNT signalling is required for the maintenance of leukaemia stem cells (Wang *et al.*, 2010; Reya and Clevers, 2005).

The WNT pathway is thought to contribute to tumorigenesis by stimulating the transition between a differentiated epithelial state to an undifferentiated mesenchymal phenotype (epithelial-mesenchymal transition, EMT). Cells that undergo EMT present stem cells features and are considered the principal responsible for the onset of cancer metastasis (Singh and Settleman, 2010; Morel *et al.*, 2008; Neth *et al.*, 2007).

1.4.7 WNT signalling in breast cancer

As previously mentioned, the oncogenic effect of the WNT pathway in the mammary gland was firstly demonstrated by the seminal work of Nusse and Varmus that lead to the discover of WNT-1 as an oncogene in 1982 (Nusse *et al.*, 1984). Following studies have demonstrated that MMTV viral insertion can cause cancer through the activation of other WNTs, such as WNT3 and WNT10b (Howe and Brown, 2004; Smalley and Dale, 2001; Bowcock, 1999).

The role of canonical WNT signalling in human breast tumorigenesis has been confirmed with immunohistochemical studies in patients tissues: Two independent studies showed that about 60 % of the clinical samples analysed present nuclear/cytoplasmic localization of β-catenin corresponding to an activated status (Ryo *et al.*, 2001; Lin *et al.*, 2000). Moreover, in the study of Lin et al., it was shown that the activation of β-catenin positively correlated with increased Cyclin D1 expression, which is a poor prognostic factor in breast cancer (Lin *et al.*, 2000; McIntosh *et al.*, 1995). Collectively, these findings suggest a pivotal role of WNT canonical signalling in breast tumorigenesis. However, since mutations of β-catenin, APC or Axin are very rare in breast cancer, it is likely that the deregulation of the WNT canonical pathway is caused by other unknown factors (Howe and Brown, 2004).

Several studies have been carried out to understand abnormalities in the expression of WNT ligands, and many authors reported mRNA overexpression of several WNTs in breast cancer (table 1.2); however, there is still a lack of information regarding WNTs proteins expression and its correlation with breast tumorigenesis, mainly for the lack of high quality antibodies (Howe and Brown, 2004).

Table 1.2: Selected studies reporting alteration of WNT signalling proteins in breast cancer (Adapted from Howe and Brown, 2004)

Signalling component	Reported abnormality	% cases (Numbers)	Tumor type/sample
SFRP1	Loss or underexpression	80% (85/107)	Carcinomas
WIF1	Reduced imunostaining	60% (21/35)	Carcinomas
WNT2	Overexpression, RNA	45% (5/11)	Carcinomas
WNT2	Overexpression, RNA	22% (2/9)	Primary breast cancer
WNT5a	Overexpression, RNA	80%(4/5)	Carcinomas
WNT5a	Loss of immunostaining	36%(10/28)	Carcinomas
WNT7b	Overexpression, RNA	10% (2/20)	Carcinomas
WNT10b	Overexpression, RNA	6% (3/50)	Carcinomas
WNT13/2b	Overexpression, RNA	14%(2/14)	Carcinomas
WNT14	Overexpression, RNA	11%(1/9)	Primary breast cancer
APC	Truncation mutation	4% (1/24)	Cell lines
	Truncation mutation	0.5% (1/227)	Carcinomas
	Truncation mutations	6% (3/54)	Carcinomas
	Reduced immunostaining	41% (11/27)	Carcinomas
β-Catenin	Nuclear/cytoplasmic staining	60% (74/123)	Primary breast cancer
	Nuclear/cytoplasmic staining	63%(25/40)	Primary breast cancer
	Increased protein	13% (7/54)	Carcinomas

Deregulation of WNT signalling in breast cancer could be a consequence of the altered expression of secreted WNT inhibitors. Indeed, epigenetic downregulation of WNT antagonists such as SFRP1/2 and DKK1 were reported to be common in breast cancer (Suzuki *et al.*, 2008; Ugolini *et al.*, 2001). The tumour suppressor activity and the prognostic significance of SFRP1 have been described by several independent authors (Matsuda *et al.*, 2009; Shulewitz *et al.*, 2006; Klopocki *et al.*, 2004). Another secreted WNT inhibitor, Wif1, was shown to be hypermethylated and consequently downregulated in a large number of tumour samples and breast cancer cell lines (Ai *et al.*, 2006). These

findings were confirmed independently by other investigators (Veeck *et al.*, 2009; Wissmann *et al.*, 2003). Overall, these results suggest that epigenetic alterations of WNT antagonists, rather than mutations of β-catenin and APC, can explain the over-activation of canonical WNT signalling in breast cancer.

The role of β-catenin independent WNT pathways in breast cancer is still largely unknown. However, recent findings suggest that the noncanonical signalling could have a role in regulating the interaction of cancer cells with the tumour microenvironment and might be important to regulate the metastatic spread of invasive breast cancers (Alderton, 2013). The ground-breaking work from Luga et al. demonstrated that fibroblasts can induce the invasion, motility and protrusive activity of breast cancer cells through the activation of the PCP pathway (Luga and Wrana, 2013; Luga *et al.*, 2012).

1.5 Frizzled receptors

Frizzled receptors (FZDs) are seven-transmembrane-spanning proteins considered as a sub-class of the G protein-coupled receptor family. In humans there are ten FZDs. The name “frizzled” derives from the irregularly organized and curled hairs and bristles on thorax, wings, and feet of the frizzled mutants of Drosophila Melanogaster (Schulte, 2010). Human FZDs can be clustered in 4 groups depending on the protein homology: A first group includes FZD1, 2, 7 with 75% homology; a second group includes FZD5,8 with 70% of protein homology; a third group includes FZD4,9,10 with a 50% homology, and the last group consist of FZD3 and 6 with 50% of homology (Fredriksson and Schioth, 2005).

FZD receptors contain 7 transmembrane domains, an extracellular N-terminus and an intracellular C-terminus (fig. 1.6) (Foord *et al.*, 2005; Vinson, Conover and Adler, 1989). The extracellular region of all FZDs feature a conserved Cysteine Rich Domain (CRD) which is believed to be the binding site for WNTs (Xu and Nusse, 1998) and Soluble Frizzled-related proteins (Rattner *et al.*, 1997). The seven transmembrane regions are connected by 3 extracellular loops and 3 intracellular loops and a c-terminus which varies depending on the specific FZD (Schulte, 2010). The intracellular domain is required for the interaction with a plethora of proteins, which include, amongst others,

heterotrimeric G-proteins (Slusarski, Corces and Moon, 1997) and Dishevelled (Wong *et al.*, 2003).

All Frizzled receptors present a KTxxxW domain in the C terminus which is required for the binding with proteins containing the PDZ domain, such as Dishevelled (Wong *et al.*, 2003). A second PDZ motif is present in some, but not in all FZDs, at the terminal end of the C-terminus (Schulte, 2010).

FZDs bind to WNT ligands, but the specificity of the ligand/receptor interaction is largely obscure. Moreover, there is a certain degree of promiscuity for the binding to different WNT ligands (Hsieh *et al.*, 1999). Table 1.3 summarises the known FZDs/WNTs combinations.

Interestingly, studies in Xenopus embryos have shown that that Xfz3, the homologous of human FZD3, can dimerize to activate the canonical WNT signalling (Carron *et al.*, 2003). This study indicates that receptors dimerization could further increase the complexity of the WNT pathway.

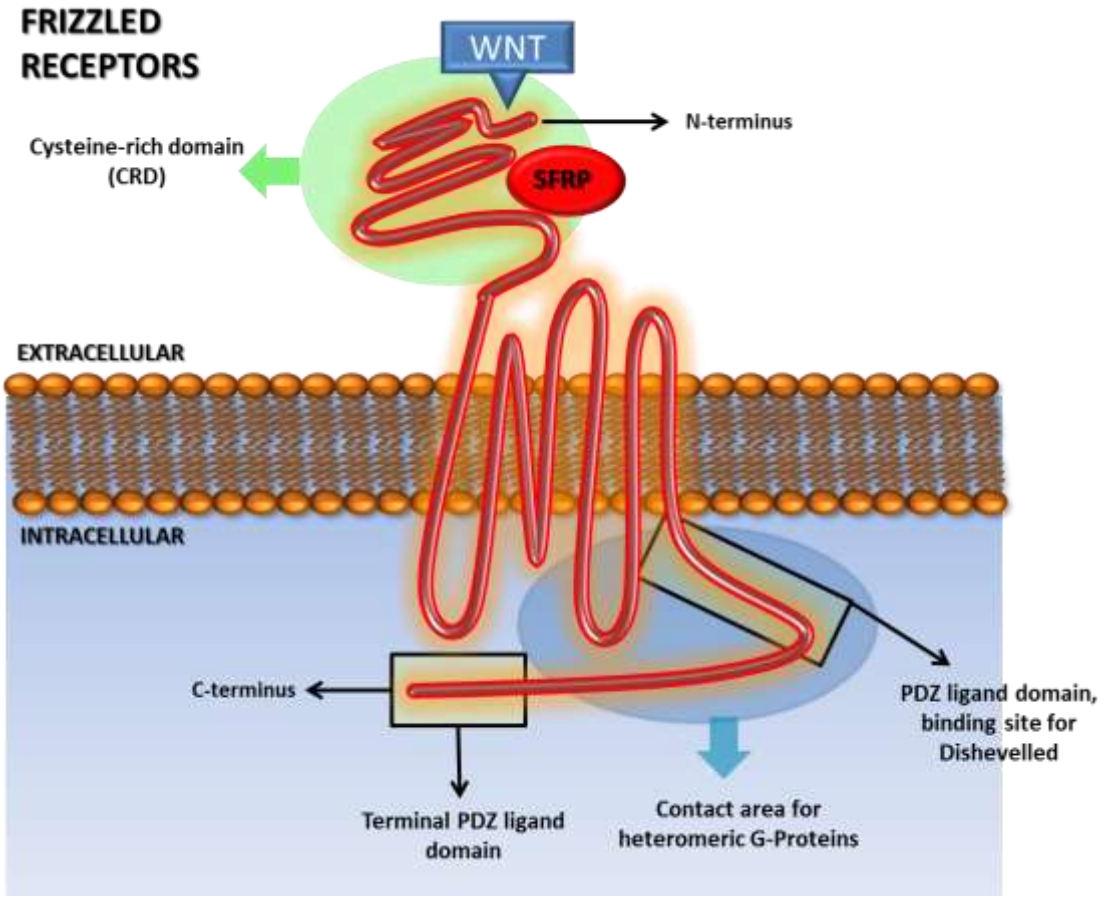

Figure 1.6: Schematic representation of the class of frizzled receptors: The frizzled receptors consist of 7 transmembrane domains, a N- terminus extracellular domain and a C-terminus intracellular domain. The extracellular CRD domain interacts with WNT ligands and secreted WNT inhibitors. The intracellular domain is a docking site for heteromeric G proteins and Dishevelled. FZD receptors also present 1 or 2 intracellular PDZ ligand domains for the interaction with PDZ proteins (adapted from Schulte, 2010).

Table 1.3: Reported interactions between FZD receptors and WNT ligands: "x" indicate binding interactions demonstrated with immunoprecipitation, while "o" indicate co-localization or other methods of receptor/ligand interaction identification (adapted from Dijksterhuis, Petersen and Schulte, 2014).

	WNTs																		
	1	**2**	**2b**	**3**	**3a**	**4**	**5a**	**5b**	**6**	**7a**	**7b**	**8a**	**8b**	**9a**	**9b**	**10 a**	**10 b**	**11**	**16**
FZD1	X	X		O	X		X	O		O	X								
FZD2		O		O	O		X		O	O	O		O						
FZD3		X	X		O		O				X								
FZD4																			
FZD5		O			X		X			X					O		O		
FZD6					X	X	X	O		O								O	
FZD7				X	X		O			O									
FZD8															O				
FZD9		X																	
FZD10											X								

1.5.1 Frizzled receptors in cancer

Several studies have shown that FZD receptors could promote tumorigenesis by controlling proliferation or cancer invasion. For example, FZD1 and 2 were found overexpressed in breast cancer compared to normal tissue (Milovanovic *et al.*, 2004). Moreover, FZD1 was shown to enhance drug resistance in breast cancer and neuroblastoma through the activation of a β-catenin dependent signal (Zhang *et al.*, 2012; Flahaut *et al.*, 2009).

FZD3 is overexpressed in chronic lymphocytic leukaemia and associated with increased transendothelial migration, and poorer prognosis in patients (Kaucka *et al.*, 2013; Lu *et al.*, 2004).

FZD4 was reported to be a mediator of EMT in prostate cancer with TMPRSS2-ERG fusions (Gupta *et al.*, 2010). Moreover a siRNA targeting FZD4 was shown to reduce the motility and migration of bladder cancer cells (Ueno *et al.*, 2012).

Ueno et al. reported that the overexpression of FZD7 in colon cancer cells leads to increased activation of the WNT canonical pathway and augmented invasion and proliferation of tumour cells (Ueno *et al.*, 2009; Ueno *et al.*, 2008). FZD7 overexpression and β-catenin stabilization were also suggested as the initiating events in hepatocellular carcinomas in mice models (Merle *et al.*, 2005). FZD7 was found overexpressed in TNBC compared to non TNBC tumours, and downregulation of FZD7 in TNBC cell lines resulted in a reduction of cell proliferation and invasion in vitro and in vivo (Yang *et al.*, 2011).

Wang et al. reported that FZD8 is overexpressed in lung cancer compared to matched normal tissue. Knock down of FZD8 resulted in reduced proliferation and colony number formation of lung cancer cell lines in vitro, and reduced tumour growth in mice xenografts (Wang *et al.*, 2012).

FZD9 was shown to have tumour suppressor activity in non-small cells lung cancer by activating JNK and supressing the anchorage-independent growth and proliferation of lung cancer cells (Winn *et al.*, 2005).

FZD10 mRNA was found upregulated in colorectal cancer compared to normal matched tissue by Terasaki et al, although the number of colon cancer cases analysed were only two (Terasaki *et al.*, 2002). FZD10 was also found overexpressed in synovial sarcomas, and the use of a monoclonal antibody

against FZD10 was effective in reducing cancer cells growth in vivo and in vitro (Fukukawa *et al.*, 2009).

1.6 Frizzled receptor 6

FZD6 receptor 6 (Fzd6) is the product of the FZD6 gene, located in chromosome 8 (8q22.3-q23.1). Fzd6 is a single peptide sequence of 706 aminoacids and like other FZDs, contains an extracellular CRD and seven transmembrane domains. However, in contrast to other FZDs, it does not contain a C-terminal PDZ domain-binding motif (McEntyre *et al.*, 2012; Tokuhara *et al.*, 1998).

Golan et al. reported that FZD6 does not transduce a canonical signal, but acts as a repressor of the FZD1-dependent activation of β-catenin in HEK293 cells. This is mediated by the activation of TAK1/NLK kinases (Golan *et al.*, 2004). These findings are in agreement with Sato et al., who showed that FZD6 does not mediate the activation of β-catenin (Sato *et al.*, 2010). The same group also reported that the CRD of FZD6 does not bind to WNT5a and WNT3a (Sato *et al.*, 2010). However, Fröjmark et al. reported that the stabilization of β-catenin, induced by WNT3a in primary fibroblasts was abolished in fibroblasts of patients affected by nail Dysplasia bearing FZD6 mutations (Fröjmark *et al.*, 2011). Moreover, a Fzd6 dependent stabilization of β-catenin after activation with WNT3a was observed in human mesenchymal stem cells (Kolben *et al.*, 2012). Consistent with a non-canonical role for Fzd6, Lyons et al showed that Fzd6 strongly binds WNT4 and does not transduce a β-catenin canonical pathway in kidney epithelial cells (Lyons *et al.*, 2004). Heinonen et al. suggested that WNT4 activates the PCP pathway through Fzd6 in murine hematopoietic precursor cells (Heinonen *et al.*, 2011). A recent study of Kilander et al., based on fluorescence recovery after photobleaching (FRAP), showed that WNT3A,WNT4, WNT-1, WNT5A, WNT9B, WNT10B and WNT16B all cause, to a different extent, the membrane shift of Fzd6 conjugated with GFP, suggesting a possible interaction of these WNTs with Fzd6 and a broad receptor/ligand promiscuity (Kilander, Dahlström and Schulte, 2014).

A role of FZD6 in PCP is further suggested by the phenotype observed in FZD6 -/- mice and patients affected by neural tube defects bearing germ line

mutations of FZD6 (De Marco *et al.*, 2012; Wang, Guo and Nathans, 2006; Guo, Hawkins and Nathans, 2004). FZD6 -/- and FZD3 -/- double mutants (but non mice with single knock down) present defects in neural tube closure and in the orientation of a subset of auditory and vestibular cells (Wang, Guo and Nathans, 2006). This suggests a certain level of redundancy between FZD6 and FZD3. FZD6 is also important for the hair patterning in mammals; FZD6 null mice are healthy and viable, but show a phenotype with disorganized orientation of the hair follicles (Wang, Chang and Nathans, 2010; Guo, Hawkins and Nathans, 2004). A role in platelet number regulation has been reported in FZD6 double knock down mice, where the number of platelets was increased in comparison to control mice (Steele *et al.*, 2009).

In humans, mutations of FZD6 have been associated with neural tube defects and a congenital nails disorder called Autosomal-Recessive Nail Dysplasia (De Marco *et al.*, 2012; Fröjmark *et al.*, 2011; Naz *et al.*, 2011).

1.6.1 Frizzled receptor 6 in cancer

Frizzled 6 is overexpressed in breast cancer, hepatocarcinoma, colon cancer, prostate cancer, leukaemia and squamous cell carcinoma (Ma *et al.*, 2009; Wu, Zierold and Ranheim, 2009; Bengochea *et al.*, 2008; Finak *et al.*, 2008; Smid *et al.*, 2008; Vincan and Barker, 2008; Haider *et al.*, 2006; Saramäki *et al.*, 2006). However, few studies have investigated the role of FZD6 in tumorigenesis. Wu et al. reported that ablation of FZD6 in chronic lymphocytic leukaemia B cells results in a delay in tumour progression and reduction of active β-catenin in mice (Wu, Zierold and Ranheim, 2009). Our group reported that FZD6 expression is associated with drug resistance and increased invasiveness in neuroblastoma, marking a cancer stem cell subpopulation (Cantilena *et al.*, 2011). Finally, a FZD6 polymorphism was associated with a greater risk of papillary thyroid cancer (Neta *et al.*, 2011).

1.7 Aims

On the basis of previous studies that suggested that FZD6 is implicated in tumorigenesis, we aimed to elucidate its role breast cancer. We wanted to determine whether FZD6 was overexpressed in breast cancer cells compared to normal breast tissue and if its expression is correlated with clinical features in patients. Next, using a loss of function approach, we wanted to assess the potential role of FZD6 in the proliferation and invasion of breast cancer cells and the signalling pathway downstream of the Fzd6 receptor.

CHAPTER II

Materials and methods

2.1 Reagents

All reagents, if not otherwise stated, were purchased from Fisher Scientific UK.

2.2 Oncomine data mining

In silico expression studies were made using the online software Oncomine (https://www.oncomine.org, powered by Life Technologies). Only datasets containing normal tissue compared to tumour tissue were considered for the analysis (fig. 2.1).

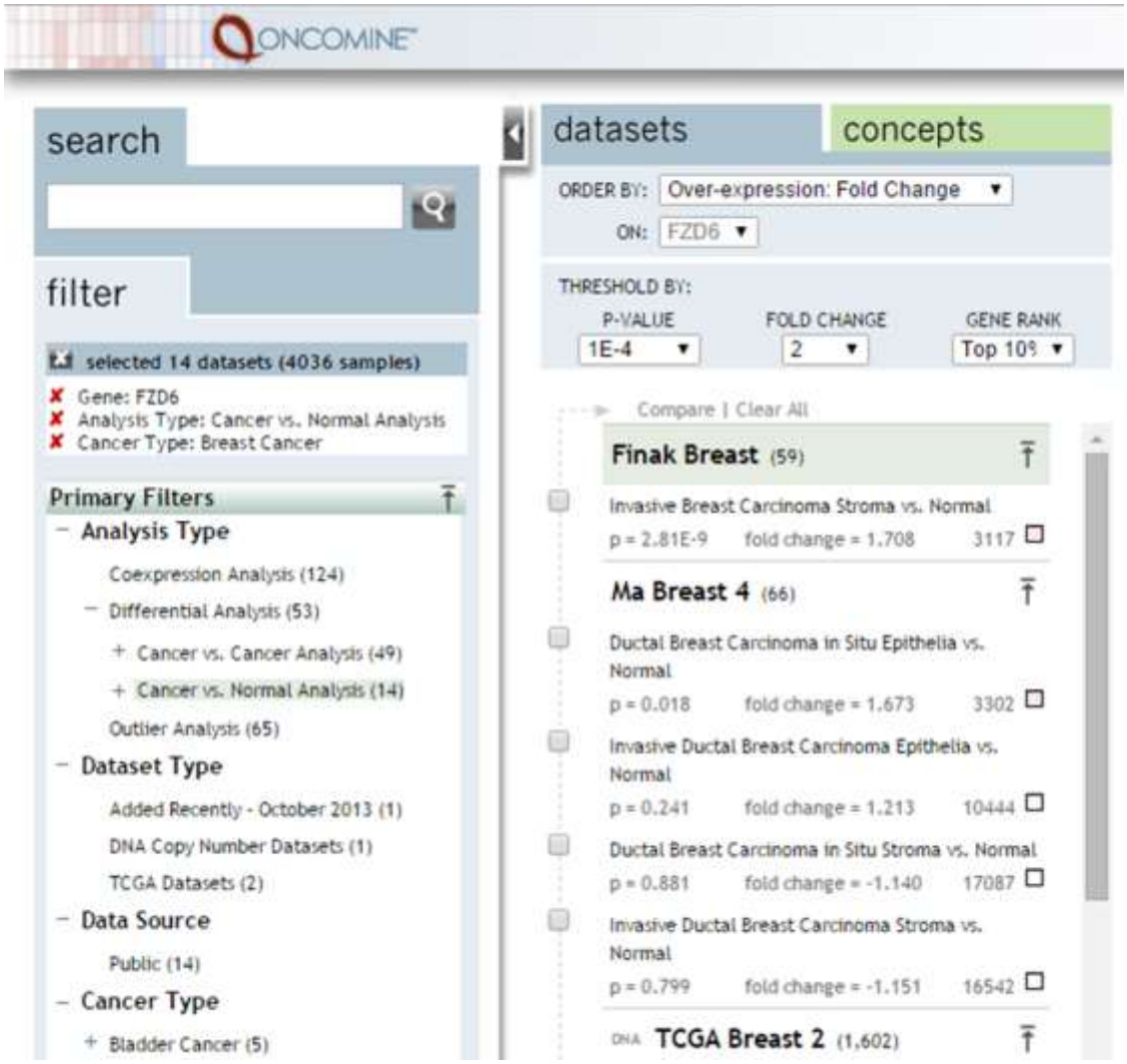

Figure 2.1: Search criteria in the Oncomine platform.

2.3 Kaplan Meier plotter survival studies

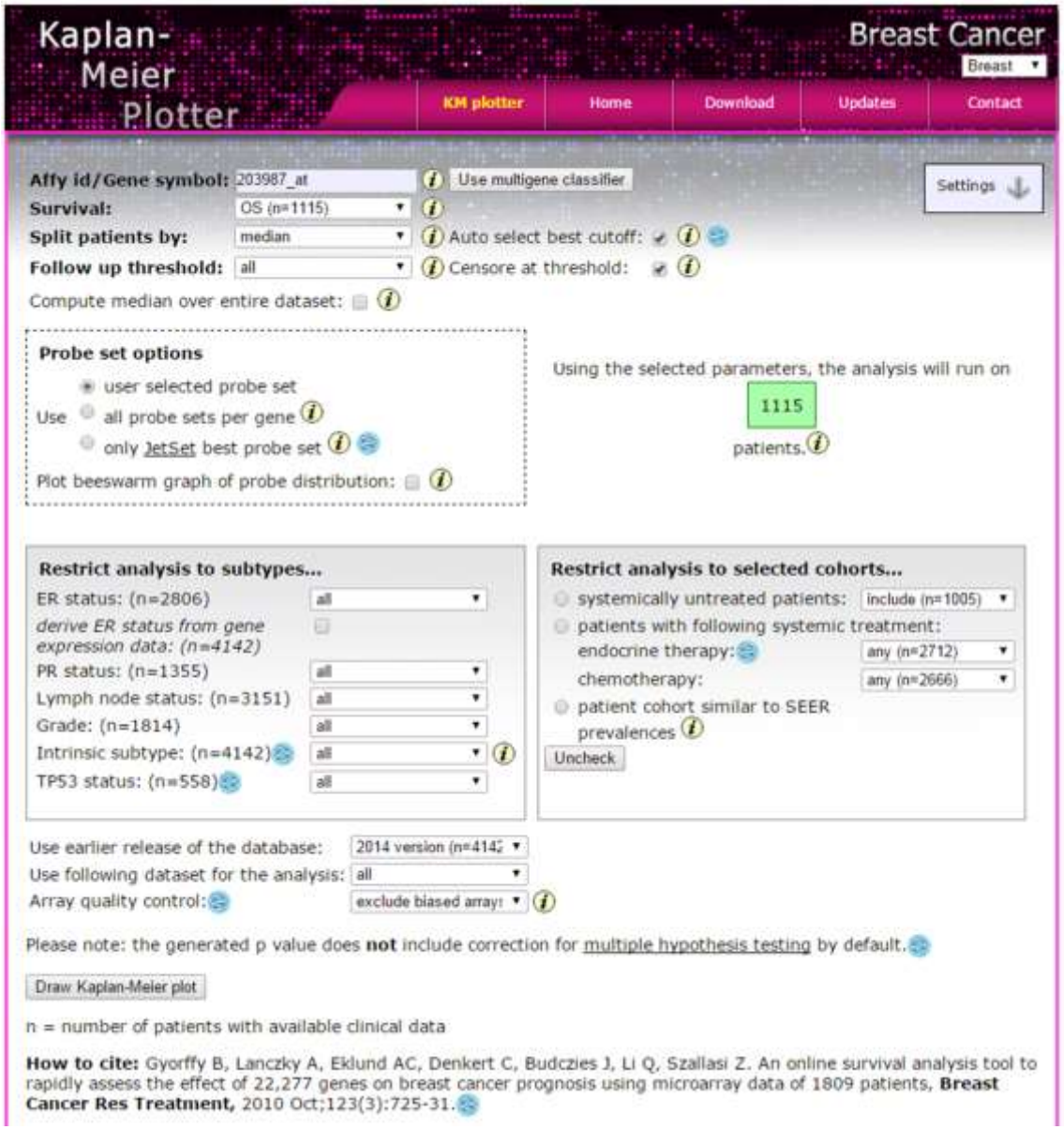

Figure 2.2: Search criteria in the Kaplan Meier plotter platform.

FZD6-associated survival studies were obtained using the online tool Kaplan Meier plotter (http://kmplot.com/analysis/) (Györffy *et al.*, 2010). The software uses microarrays expression data and the matched clinical information of breast cancer patients to generate survival curves. The expression data is provided by databases such as GEO (Gene Expression Omnibus), EGA (European Genome-Phenom Archive), and TCGA (The Cancer Genome Atlas).

Kaplan Meier curves correlate the survival of a cohort of patients for a certain amount of time with parameters such as the expression level of a certain gene or a given therapy. In this study three parameters were analysed in correlation with FZD6 expression:

- The overall survival of patients, expressed as the probability of survival from the time of diagnosis.
- The relapse free survival, intended as the probability of survival without symptoms after primary treatment.
- Distant relapse free survival, expressed as the probability of survival without the onset of metastasis after primary treatment.

Curves were generated selecting the best cut-off, i.e. all the percentiles between the lower and the upper quartiles were computed, and the best performing threshold was used as a cut off (figure 2.2).
Patients were then stratified in three different breast cancer subgroups: Luminal A, Luminal B and Basal, and further stratified in lymph node positive and lymph node negative using the appropriate drop-down lists (figure 2.2). All the other settings were maintained as default. The affimetrix probe 203987_at was used for FZD6.

2.4 COSMIC analysis

The Catalogue of somatic mutations in cancer (COSMIC) is a collection of published databases containing gene expression data and the record of somatic mutations of a large amount of tumours specimens. The repository is available through the web page http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/ and is managed by The Sanger Institute in Cambridge.

The cancer browser was used to filter results of breast cancer samples containing expression data for FZD receptors. Copy number variation and gene expression were computed and displayed as in figure 2.3.

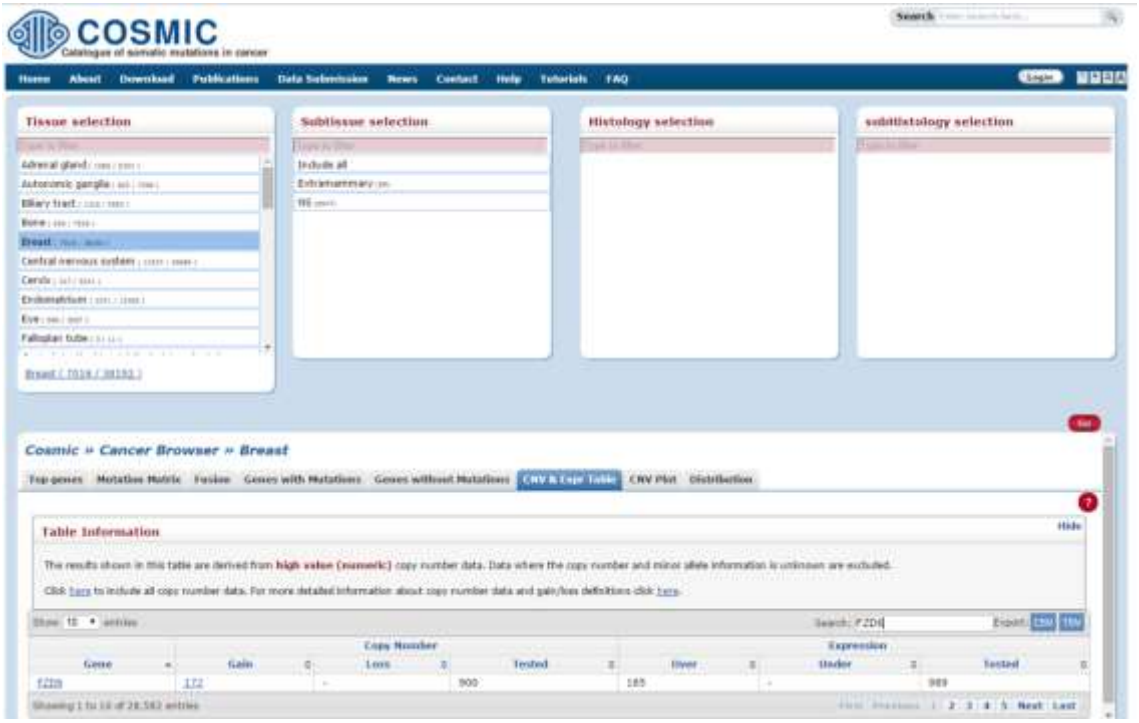

Figure 2.3: Analysis of the copy number variation and expression of FZD6 in breast cancer samples using the online platform COSMIC.

2.5 Cell lines

All the cell lines used in this study, except for the cell lines HMEC-184D, HMEC-184D-HT, HEK 293FT and L-cells were validated and certified by the accredited company DDC medical.

BT474: Derived from a luminal-like metastatic ductal breast carcinoma, isolated for the first time by Lasfargues and colleagues from the primary tumour (Lasfargues, Coutinho and Redfield, 1978). This cell line is positive for ER, PG and HER2 (Neve *et al.*, 2006). The cell line was a kind gift of Professor Robert Newbold, Brunel University.

MDA-MB-231: This cell line was isolated in 1976 from the metastatic pleural effusion of a breast adenocarcinoma (Cailleau *et al.*, 1976). It does not express the receptors for oestrogen and progesterone, and it is negative for HER2, thereby it is classified as triple negative cell line. This cell line is also basal-like. The cell line was a kind gift from Dr Pier Francesco Marra, King's College London.

MDA-MB-436: This cell line is derived from the pleural effusion of a triple negative ductal metastatic breast carcinoma (Neve *et al.*, 2006). It was isolated for the first time by Cailleau and colleagues in 1978 (Cailleau, Olivé and Cruciger, 1978). The cell line was a kind gift from Dr Pier Francesco Marra, King's College London.

HCC1143: Derived from an invasive ductal breast carcinoma, this cell line is a basal like, triple negative cell line that was isolated by Gazdar and colleagues in 1994 (Neve *et al.*, 2006; Gazdar *et al.*, 1998). The cell line was a kind gift from Dr Pier Francesco Marra, King's College London.

MCF7: This cell line derives from the pleural effusion of a metastatic ductal breast carcinoma. It presents luminal characteristics and expresses ER and PR, but it is negative for HER2. It was isolated for the first time in the 1973 by Soule and colleagues (Soule *et al.*, 1973). The cell line was a kind gift of Professor Robert Newbold, Brunel University.

SKBR3: This cell line was isolated from the pleural effusions originated from a breast adenocarcinoma in 1970 (ATCC website, http://www.lgcstandards-atcc.org/Products/All/HTB-30.aspx#history). The cell line is hormone receptors negative, but expresses HER2. SKBR3 cell line was a kind gift of Dr Gianluca Sala, G.D'Annunzio University, Italy.

BT20: This breast tumour line was established by E.Y. Lasfargues and L. Ozzello in 1958. It derives from the primary mass of an invasive ductal carcinoma and it is a basal triple negative cell line (Neve *et al.*, 2006; Lasfargues and Ozzello, 1958). The cell line was a kind gift of Professor Robert Newbold, Brunel University.

T47D: This breast cancer cell line was isolated by I. Keydar from a pleural effusion obtained from a female patient with an infiltrating ductal carcinoma of the breast. This cell line is luminal-like and positive for PR and ER (Neve *et al.*, 2006; Keydar *et al.*, 1979). The cell line was a kind gift of Dr Gianluca Sala, G.D'Annunzio University.

HMEC-184D: The 184D strain of human mammary epithelial cells was originally isolated by Professor Martha Stampfer and was obtained via reduction mammoplasty of a 21 years old disease-free individual. The primary HMEC-184D cell line has a finite lifespan of 20-40PD when grown in a stress-free

medium formulation of M87A supplemented with 0.5 ng/mL cholera toxic and 0.1nM oxytocin (Garbe *et al.*, 2009). The cell line was kindly provided by Dr Hemad Yasaei and Professor Robert Newbold, Brunel University.

HMECT-184D-HT: This cell line was a kind a gift from Dr Hemad Yasaei and Professor Robert Newbold, and derives from a small population of pre-stasis HMEC-184D transfected with the pCi neo-hTERT plasmid using Neon electroporation system. Subsequently, the cells were put in selection for 2-3 weeks in the presence of G418 Geneticin at a concentration of 150µg/mL. A small population of cells originating from a single transfected clone emerged and was named HMEC-184D-HT. When assayed for post-splice hTERT gene expression, it was shown to be 4-5 fold overexpressed compared to an empty vector counterpart. These cells proliferated for an extra 100 passages and are now deemed immortal.

HEK 293 FT: These cells are human embryonic kidney cells transformed with the SV40 large T antigen under the control of the human cytomegalovirus promoter. The SV40 antigen derives from the polyomavirus SV40 and it is capable to transform a vast number of cells through the perturbation of the Retinoblastoma and p53 proteins. The SV40 large T antigen also promotes the episomal replication of transfected plasmids containing the SV40 origin of replication (Fanning, 1992). In this study HEK 293 FT cells were used for the expression of viral packaging vectors and virus production. HEK 293 FT cells were purchased from Life Technologies.

Control, WNT3a and WNT5a L cells: These cells are mouse fibroblasts that have been stably transfected with empty vector or vectors expressing human WNT3a, or WNT5a (Willert *et al.*, 2003). Conditioned media produced by these cells can be therefore used in signalling experiments involving WNT3a and WNT5a. L-cells were a kind gift from Professor J.P. Medema.

2.6 Cell culture

MDA-MB-231, HCC1143, MDA-MD-436 cell lines were grown in RPMI medium (Gibco) supplemented with 1mM in sodium pyruvate (Gibco) and 10% foetal bovine serum (FBS) (Gibco). HEK 293 FT, MCF7, SKBR3, T47D and L cells

were grown in DMEM (Gibco) supplemented with 10% FBS. BT20 and BT474 were grown in a mixture of 50% DMEM and 50% F12 (Gibco), supplemented with 10% FBS. HMEC-184D and HMEC-184D-HT cells were grown in M87A medium (Lonza) supplemented with 0.25%FBS, 0.5 ng/ml cholera toxic (Sigma) and 0.1nM oxytocin (Bachem). WNT3a L-cells were maintained in constant selection with 125 µg/mL of Zeocin (Life Technologies) and WNT5a L-cells with 400µg/mL G418 (Life Technologies). All the cell lines were maintained in an incubator (Sanyo) at 37°C in a humidified atmosphere with 5% CO2 and regularly sub-cultured, using phosphate saline buffer (PBS) (Sigma) for washings and trypsin-EDTA (Sigma) to harvest cells. Cells were grown in flasks, dishes or multi-well plates (Nunc). Cells manipulations were carried out with graduated serological pipettes (Fisher Brand) in a class II sterile cabinet (Gelman Sciences). All the cells centrifugations were carried out in a Thermo Scientific Haraeus Biofuge Primo at 1200 rpm unless otherwise specified.

Cell number was estimated pipetting 10 µL in an improved Neubauer haemocytometer (Marienfeld) with a depth of 0.1 mm and by averaging the number of cells in 4 areas of 1 mm each.

For long term storage, cells were resuspended in 10% DMSO (Fisher), 40% FBS and 50% complete medium, and kept in 2 mL cryovials (Nalgene). To ensure a gradual freezing and to avoid the formation of intracellular ice crystals, the cryovials were kept overnight in a container containing isopropanol at – 80 °C and were then stored in liquid nitrogen. To recover cells from liquid nitrogen the vials were rapidly immersed in a water bath at 37°C until the cell suspension was thawed. Next, cells were resuspended in 20 mL of pre-warmed medium and pelleted at 1200 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in fresh medium and plated in flasks or dishes.

2.6.1 Preparation of WNT conditioned media

Supernatants from confluent 10 cm dishes containing mouse fibroblasts L cells expressing control plasmid, WNT3a, or WNT5a plasmids, were harvested and stored at + 4 °C. For signalling experiments, the conditioned media were used diluted 1:10.

2.7 Protein extracts preparation

Cells were counted and seeded in equal number for each experimental condition. When cells had to be exposed to an exogenous activator, a 24 hours starvation was performed beforehand. Cells were then washed with cold PBS and lysed by adding ice cold RIPA (Radio Immuno Precipitation Assay buffer, 150 mM sodium chloride (Fisher), 1.0% Igepal (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (Fisher), 50 mM Tris pH 8.0 (Fisher), 1nM sodium orthovanadate (New England Biolab), HALT protease inhibitor cocktail (Thermoscientific). Cells were scraped with a cell scraper, collected in Eppendorf tubes and kept on ice for 30 minutes. Every 5 minutes the lysates were vortexed. To separate the cell debris from soluble extracts, the lysates were centrifuged at 15.000 rpm at 4°C for 15 minutes in a Thermos Scientific Haraeus Fresco 21 table-top centrifuge. Next, supernatants were collected in fresh tubes and stored at – 80 ° C, or used for proteins analysis.

For the signalling experiment showed in figure 6.7, cell lysis was performed by adding 100µL of 2X Laemli buffer (8% SDS, 20% 2-mercaptoehtanol (Sigma), 40% glycerol (Fisher), 0.008% bromophenol blue (Fisher), 0.25 M Tris-HCl pH 6.8)(Laemmli, 1970) and scraping cells with a cell scraper on ice. Cell lysate were briefly sonicated and boiled at 100 °C for 5 minutes, then stored at - 20°C or used for acrylamide gel electrophoresis.

2.8 Estimation of total proteins concentration with the Bradford method

To estimate the concentration of total proteins in RIPA lysates, a microliter of the protein extract was resuspended with 200 µL of Bradford reagent (SIGMA). In parallel, 1 µL of different BSA standards (0, 2, 5, 6, 8 and 9 µg/µL) was resuspended in Bradford reagent with the same ratio to generate a standard curve. The samples were assayed after 5 minutes in a plate spectrophotometer

(Biorad) at the wavelength of 595 nm. The incognito concentration of the protein samples was extrapolated from the standard curve.

2.9 Western blot analysis

The lysates were mixed with 4x Laemmli Buffer and water to obtain a 4 fold dilution of the laemmli buffer. The samples were then vortexed and boiled at 100℃ for 5 minutes in a heat block, then stored at -20℃ or used for SDS-PAGE (Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis).

Polyacrylamide gels were made using gel casting cassettes from Biorad. The resolving gels were made according to the Table 2.1, varying the acrylamide percentage depending on the size of the proteins to be resolved. Stacking gels were casted according to the table 2.2 to obtain a concentration of acrylamide of 4% v/v.

Table 2.1: SDS-PAGE resolving gel composition for 10 mL

	Acrylamide final concentration % v/v			
Solution	8%	9%	10%	12%
Water	7.25 mL	6.75 mL	6.25 mL	5.25 mL
1,5 M Tris-HCl pH 8.8, 0,4% SDS	3.75 mL	3.75 mL	3.75 mL	3.75 mL
30% acrylamide/0.8% bisacrylamide (National Diagnostic)	4 mL	4.5 mL	5 mL	6 mL
10 % APS (Sigma)	0.1 mL	0.1 mL	0.1 mL	0.1 mL
TEMED (Fisher)	0.02 mL	0.02 mL	0.02 mL	0.02 mL

Table 2.2: SDS-PAGE stacking gel composition for 10 mL

Solution	Volume
Water	6 mL
0.5 M Tris-HCl pH 6.8, 0.4 % SDS	2.5 mL
30% acrylamide/0.8% bisacrylamide	1.3 mL
10 % APS	0.1 mL
TEMED	0.02 mL

Protein samples were loaded in equal amount into polyacrylamide gels and electrophoresed using a Biorad electrophoresis apparatus. A constant voltage of 100 V was kept until the protein bands were well resolved. To estimate the proteins molecular weight the samples were run in parallel with a protein ladder (Thermoscientific). The electrophoresis buffer composition was the following: 25 mM Tris base, 192mM glycine (Fisher), SDS 0.1% in distilled water (Green and Sambrook, 2012). Proteins were then transferred into nitrocellulose membranes (Amersham) using a Biorad apparatus and a transfer buffer with the following composition: 24 mM Tris base, 192 mM glycine, 20% SDS and 0.0375 % SDS in distilled water (Green and Sambrook, 2012). The transfer was performed on ice, applying a constant current of 350 mA. The membranes were then blocked in 5% non-fat dry milk (Marvel) dissolved in TBS-tween (TBS-t, 50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 0.1 % Tween 20 (Fisher).

The primary antibodies used and the corresponding experimental conditions are summarized in table 2.3. All the primary antibodies were incubated overnight, except for anti β-Actin, which was incubated for 1 hour at room temperature. To remove the excess of primary antibody and reduce the unspecific binding, the membranes were washed thrice with TBS-t for 10 minutes. The membranes were then incubated for 45 minutes with the appropriate secondary HRP-conjugated antibody. The secondary antibodies used in this study are listed in table 2.4. Membranes were then washed in TBS-t thrice for ten minutes and incubated for one minute in ECL (Enhanced chemiluminescence substrate, Pierce). The membranes were then placed in an autoradiography cassette and overlapped with light sensitive films (GE Healthcare) in the dark. The films were developed with a Kodak automatic developer.

If the nitrocellulose membrane had to be re-probed with a different primary antibody, the following stripping protocol was performed: membranes were incubated for 20 minutes in stripping buffer (0,6 M Tris-HCl pH 6.8, 0.7 % β-mercapto-ethanol and 2 %SDS in water) pre-warmed at 50 ° C, then washed 5 times for 4 minutes each with TBS-t and re-blocked in TBS-t 5 % milk.

Table 2.3: List of primary antibodies used in western blot analysis

Antibody	Company	Origin	Buffer	Dilution
FZD6 (D16E5)	Cell Signaling	Rabbit	PBS-t	1:1000
B-Actin (I-19)	Santa Cruz	Goat	TBS-t 5% milk	1:1000
Phospho-SAPK/JNK (Thr183/Tyr185)	Cell Signaling	Rabbit	TBS-t 5% BSA	1:1000
Active β-Catenin (8E7)	Millipore	Mouse	TBS 5% milk	1:1000
Phospho-AKT (Ser473)	Cell Signalling	Rabbit	PBS-t	1:1000
AKT	Cell Signaling	Rabbit	PBS-t	1:1000
Phospho-p44/42 MAPK (Erk1/2) Thr202/Tyr204	Cell Signaling	Rabbit	PBS-t	1:1000
p44/42 MAPK (Erk1/2)	Cell Signaling	Rabbit	PBS-t	1:2000
Phospho-PLCγ1 (Tyr783)	Cell Signaling	Rabbit	PBS-t	1:1000
PLCγ1	Cell Signaling	Rabbit	PBS-t	1:1000
E-Cadherin	Santa Cruz	Mouse	TBS-t 5% milk	1:500
Vimentin	Sigma Aldrich	Mouse	TBS-t 5% milk	1:1000

Table 2.4: List of HRP secondary antibodies used in western blot analysis

Antibody	Company	Origin	Buffer	Dilution
Anti-rabbit HRP	GE-healthcare	Goat	TBS-t 5% milk.	1:10000
Anti- Mouse HRP	GE-healthcare	Sheep	TBS-t 5% milk.	1:10000
Anti-Goat HRP	Santa Cruz	Donkey	TBS-t 5% milk.	1:10000

2.10 RNA extraction and purification

To extract the total RNA, cells were harvested and resuspended in complete medium, then pelleted and washed with PBS. The cell pellet was thoroughly resuspended in 1 mL of Trifast reagent (Peqlab) in 1.5 mL Eppendorf tubes and kept at room temperature for 5 minutes. Samples were then shaken vigorously

after the addition of 0.2 mL of chloroform (Fisher). Next, Samples were centrifuged at 12000 g for 5 minutes to allow a neat separation between the aqueous phase and the phenol phase. The aqueous phase containing the RNA was transferred in a fresh tube. The RNA was precipitated by adding 0.5 mL of isopropanol (Fisher) and the sample were vortexed. Following an incubation of ten minutes on ice, the samples were centrifuged at 12000 g for 10 minutes at 4 °C and the isopropanol was discarded. Next, the RNA pellet was washed twice with 75 % ethanol, let to air-dry and resuspended in water.

2.11 RNA retrotranscription and cDNA synthesis

Purified RNA was assayed with a Nanodrop spectrophotometer (Thermoscientific) to estimate the RNA concentration. To remove the contaminant DNA remaining from the previous purification steps, 1 µg of purified RNA was treated with DNAase I (Invitrogen) following the manufacturer instructions. The RNA was then retrotranscribed using the High Capacity RNA-cDNA Reverse Transcription Kit (Invitrogen). The retrotranscription reaction was carried out in a thermal cycler by incubating the samples for 1 hour at 37 °C and stopped by heating at 95 °C for 5 minutes. The c-DNA obtained was used for Q-RT-PCR (quantitative real time PCR) or stored for further use at – 20 °C.

2.12 Quantitative Real Time PCR (Q-RT-PCR)

The c-DNA deriving from the retrotranscription reaction was diluted 1:50 in RNAse/DNAse free water, and 5 µL of this solution were used for real time PCR. TAQman mastermix, FAM-conjugated probes (Applied Biosystems), and a 7900HT Fast Real-Time PCR System (Applied Biosystems) were used following the manufacturer instructions. Each sample was run in triplicate in a 96 well microtiter plate. The Q-RT-PCR was carried out by pre-incubating the samples for 2 minutes at 50 °C and at 95 °C for ten minutes. The samples were then subjected to 40 cycles of amplification, constituted of a first step of incubation at 95 °C for 15 seconds, followed by a second step of incubation at 60 °C for one minute. GAPDH was used as housekeeping reference gene.

The probes used in this study were FZD6 (Hs00171574_m1) and GAPDH (Hs99999905_m1). The expression data were analysed using the software SDS 2.3 from Applied Biosystems. Relative quantities were calculated using the comparative Ct method (ΔΔCt).

2.13 FZD6 indirect immunostaining and flow cytometry

Cells were harvested with trypsin EDTA, resuspended in complete medium and washed in PBS. The cell pellet was then resuspended in 100 µL of blocking solution (BSA 2% (Sigma) in PBS) and incubated on ice for 30 minutes. Cells were then pelleted and resuspended in 100µL of blocking solution containing 20 ng/µL of goat anti-FZD6 antibody (R&D systems). Following an incubation of 30 minutes on ice, cells were washed with 3 mL of blocking buffer and resuspended in 100µL of either fluorescein-conjugate donkey anti-goat secondary antibody (20ng/µL R&D systems) or anti-goat APC-conjugated antibody (20ng/µL, R&D) for 30 minutes on ice and in the dark. Cells were then washed with 3 mL of PBS, pelleted and resuspended in 100µL of blocking buffer. Samples were then analysed either with the Imagestream imaging cytometer or a standard flow cytometer. Mock stainings without the primary antibody were carried out in parallel to assess the unspecific binding of the secondary antibodies and used as a blank for flow cytometry. All the centrifugations were performed at 1200 rpm at 4 °C.

The samples were then assayed with a BD Facscalibur, an EPICS XL flow cytometer (Beckman Coulter), or an Imagestream X (Amnis). Cells resuspended in secondary antibody only, but not primary antibody, were used to set a gate for FZD6 negative cells. Test samples were run afterwards, and only the cells with fluorescence intensity above this gate were considered FZD6 positive. The Imagestream combines the functions of a flow cytometer with the ones of a fluorescence microscope. Cells running in a liquid flow are photographed, allowing the localization of the protein of interest (fig.3.9).

2.14 siRNA transfections

Cells were harvested and counted with a haemocytometer. 170000 cells/well were plated in a 6 well plate the day before transfection in 2 mL of complete medium. Transfections were carried out using 5 µL of Lipofectamine 2000 (Invitrogen) and 10 µL of a 10µM siRNA solution in water, following the transfection reagent manufacturer instructions. The medium was changed 24 hours after the transfection and cells were used for biological assays 48h after transfection. A pool of 4 different siRNA targeting FZD6 and a negative control siRNA were purchased from Qiagen. The pool was assessed firstly by real time PCR, and the two best performing siRNAs were used for the biological assays. The siRNAs target sequences used in this study were: (siRNA1 FZD6) CAGGGAGGTGGTTGTCATTCA; (siRNA2 FZD6) AAGAGAGATCCAATCAGTGAA.

2.15 Preparation of competent TOP10 F' Escherichia Coli (E.Coli)

TOP10F´ Chemically Competent E. coli were purchased from Life Technology. Bacteria were inoculated from the glycerol stock in 3mL of LB medium (Luria Broth, 1% NaCl, 1% tryptone (Fisher), 0.5 % yeast extract (Fisher) and incubated at 37 °C for two hours, at constant agitation. The culture was then centrifuged at 3000 rpm for 3 minutes and the supernatant was discarded. The bacteria pellet was resuspended in 100 µL of LB which were streaked in LB-agar plates (1.5 % Agar in LB). Following a 37 °C overnight incubation, one single colony was picked from the plate and inoculated in 50mL of LB medium. The culture was incubated overnight at 37 °C at constant agitation. 10 mL of the overnight culture were transferred into 200 mL of pre-warmed LB medium. This culture was incubated at 37 °C, at constant agitation, until the 600 nm optical density was between 0.4 and 0.6. The culture was then pelleted at 5000 rpm at 4 °C and the supernatant was discarded. The pellet was resuspended in 80mL of ice cold 0.1M $MgCl_2$ (Sigma) and incubated for 30 minute on ice. Bacteria were centrifuged at 5000g for 5 minutes at 4 °C, and the supernatant was

discarded. Next, the bacteria pellet was resuspended in 80 mL of ice cold $CaCl_2$ (Sigma) and incubated for 30 minutes on ice. Following another centrifugation, the supernatant was discarded and the pelleted was resuspended in a mixture of 8 mL ice cold 0.1 M $CaCl_2$ with 2.2 mL of glycerol (Fisher). Bacterial stocks were aliquoted in pre chilled 1.5 mL Eppendorf tubes and stored at -80 °C.

2.16 Lentiviral shRNA plasmids

The pGIPZ-shRNA lentiviral vector allows the stable expression of a human microRNA-30 which was modified to yield an efficient target silencing of the gene of interest (Silva *et al.*, 2005). This vector also contains a green fluorescent protein (GFP) expression cassette which allows the identification of cells transducing the viral construct. The map of the pGIPZ plasmid is shown in figure 2.4. This vector allows ampicillin selection in bacteria and puromycin selection in mammalian cells.

Bacterial cultures expressing three different pGIPZ-shRNA constructs used for FZD6 silencing were purchased from Life Technology. The antisense shRNA sequences were (Sh1 FZD6: ATCTGAATGACAACCACCT; Sh2 FZD6: TTAACTTTAGAATTGTGCT; Sh3 FZD6: TAACCTGCACATTTTCTGT). The negative control unspecific sequence (Scrambled) was ATCTCGCTTGGGCGAGAGTAAG.

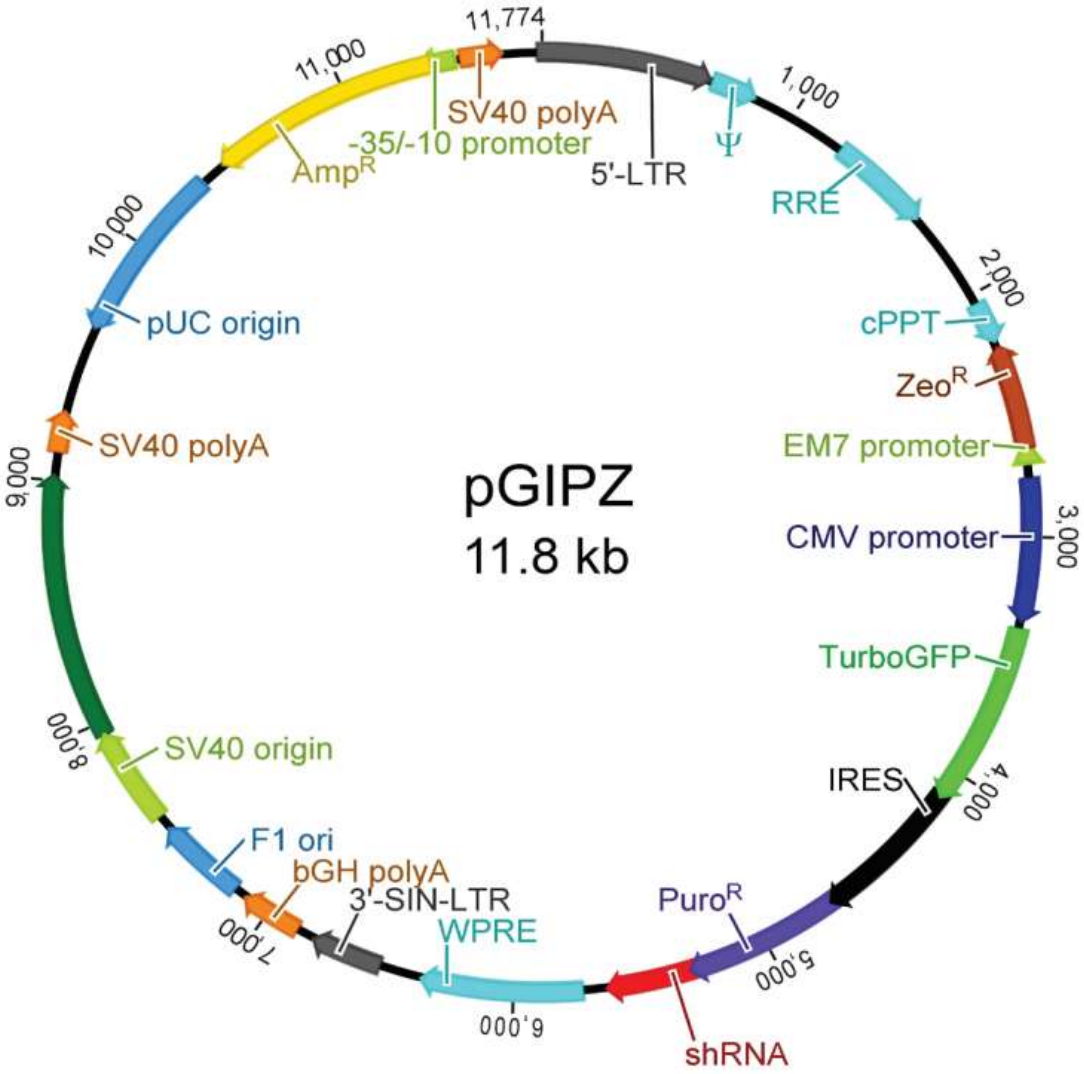

Figure 2.4: The pGIPZ-shRNA lentiviral vector map. (Extracted from the product manual available online at http://dharmacon.gelifesciences.com/uploadedFiles/Resources/GIPZ%20Lentiviral%20shRNA%20Technical%20Manual.pdf).

2.17 Expansion of bacterial cultures and plasmids purification

Bacteria expressing pGIPZ-shRNA constructs were inoculated from the glycerol stocks into 3mL of selective LB medium (100 µg/mL ampicillin in LB) and incubated at 37 °C for two hours, at constant agitation. The bacteria were then pelleted and resuspended in 100 µL of selective LB medium which were streaked onto LB agar plates containing 100 µg/mL of ampicillin. The plates were incubated at 37 °C overnight and a single colony was picked and

inoculated in 5 ml of LB selective medium. The culture was incubated overnight with vigorous shaking. 3 mL of this culture were then inoculated into 500 mL of selective LB medium and incubated at 37 °C overnight with constant agitation. Plasmid DNA was purified using a Genopure Plasmid Maxi Kit (Roche) according to the manufacturer instructions.

2.18 Transformation of competent TOP 10 F' Escherichia Coli with plasmid DNA

Plasmid propagation was carried out transforming TOP 10 F' competent E. Coli. Approximately 200ng of plasmid DNA were added to a 100µL suspension of competent bacteria which were then left on ice for 20 minutes. Next, bacteria were heat shocked at 42 ° C for 45 seconds, placed on ice for two minutes and resuspended in LB broth. 100 µL of bacterial suspension were then streaked into selective agar plates containing ampicillin. The plates were then incubated overnight at 37 °C, and a single colony was inoculated in selective LB broth. Bacterial colonies were expanded and plasmid purified as described in the paragraph 2.18.

2.19 Virus production in HEK 293 FT cells

$3x10^6$ HEK 293 FT cells were plated in 10 cm dishes in 7 mL of complete medium. 24 hours later, cells were co-transfected with 3.75 µg of pPAX2 and 1.75 µg of pMDG2 lentiviral packaging plasmids (a kind gift of Dr Owen Williams), and 5 µg of shRNA encoding vector, using lipofectamine 2000 and following the manufacturer instructions. The medium was replaced 24 hours after the transfection. Supernatants containing the viruses were harvested 48 hours after the transfection and filtered through a 0.45-mm filter unit (Sartorius). The supernatants were immediately used to infect MDA-MB-231 cells.

2.20 Lentiviral transduction in MDA-MB-231 cells

2.5×10^5 MDA-MB-231 cells were plated onto 6 well plates in 3 mL of complete medium. The following day media were replaced with 2.5 mL of supernatants containing the viral particles, in the presence of 8 µg/mL polybrene (Sigma). Media were replenished 24 hours after infection. 48 hours following the infection, cells were observed with a UV light microscope (NanoEntek) to assess the expression of GFP indicating a successful viral transduction (fig. 2.5). Next, culture media were replaced with complete media containing 1 µg/mL puromycin to select infected cells. 4 days after the infection, the puromycin concentration was increased to 2 µg/mL and kept at this concentration for the expansion and growth of cells. After several passages, approximately 100% of the cells resulted GFP positive (fig.2.5).

The FZD6 knock down efficiency in these cells was assessed by real time PCR, FACS and western blot analysis following one week of puromycin selection.

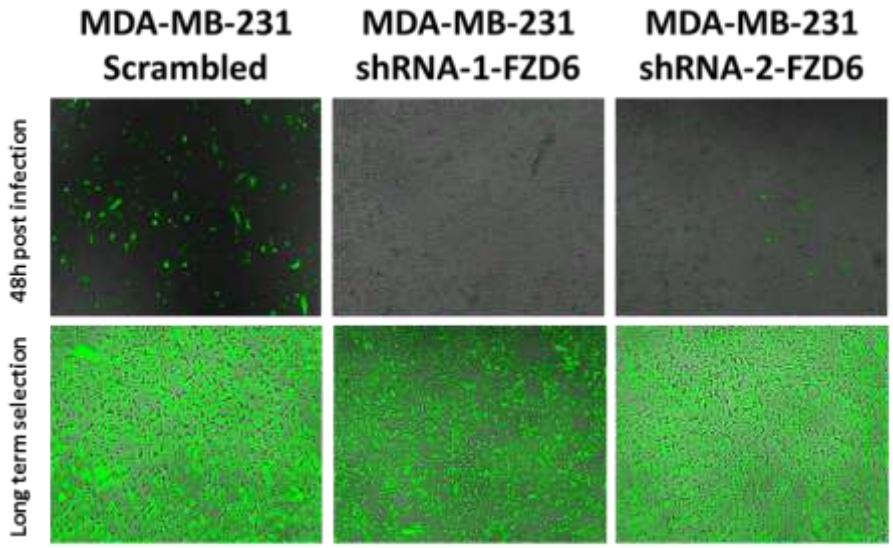

Figure 2.5: Viral transduction efficiency in MDA-MB-231 cells. Cells were observed at to assess the expression of the viral GFP cassette as indication of the infection efficiency. The photographs shown are overlays Bright field/GFP and were taken 48 hours post infection and after several passages in culture.

2.21 Proliferation assays

To assess the cell viability I used the MTS and XTT assays. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is a compound which in the presence of phenazine methosulfate (PMS) and cellular reducing enzymes, produces a red formazan product that has an absorbance maximum at 490-500 nm and a colour intensity that is proportional to the number of living cells (figure 2.6).

Similarly, The XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by the metabolic enzymes of living cells. Hence, these two formazan assays can be used to assess cell viability in vitro.

Cells transfected with siRNA or stably expressing shRNAs were counted and plated in complete medium at a seeding density of 5000 cells/100 µL into 96 well microtiter plates. 20 µL of CellTiter 96 AQueous One Solution (Promega) containing MTS and PMS were added to each well 24 hours or 48 hours later. Alternatively, 50µL of XTT reagent (1 µL of electron coupling reagent in 49 µL of XTT (Roche), were used. Cells were placed back in the incubator, and the absorbance at 490 nm was read 2 hours later using a plate spectrophotometer (Biorad).

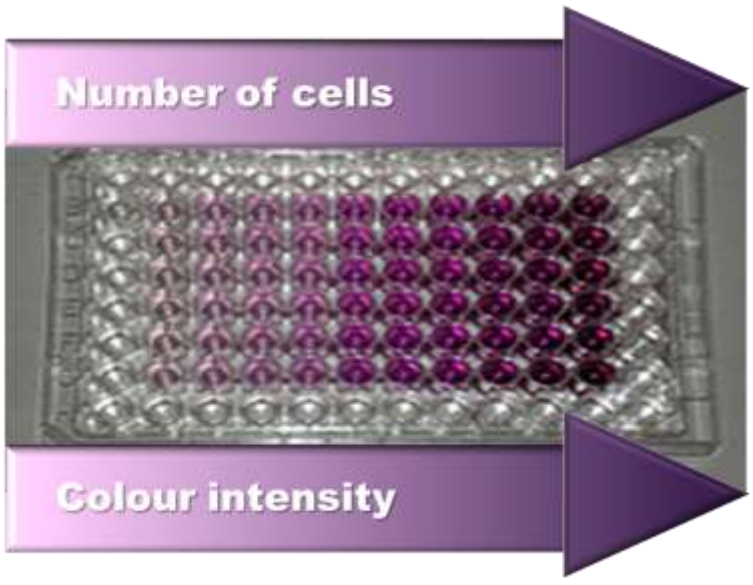

Figure 2.6: Schematic representation showing the principle of the formazan assays.

2.22 Cell cycle analysis

Propidium Iodide (PI) is a fluorescent intercalating agent that binds stoichiometrically to nucleic acids with a ratio of 1 molecule for 4-5 base pairs. When bound to DNA, PI fluorescence increases 20 to 30 fold. This feature is exploited to estimate the amount of DNA in cells. In proliferating cells the amount of DNA changes depending on the phases of the cell cycle: Cells in G_2 phase have two fold the amount of DNA of cells in G_0 and G_1. The amount of DNA during The S phase is in between the amount found during the G_0 and the G_1 phases. Thus, PI is commonly used in flow cytometry to quantify the percentage of cells in different phases of the cell cycle (Krishan, 1975). With this method it is also possible to identify apoptotic cells by their lower florescence emission caused by the loss of DNA fragments during apoptosis (Nicoletti *et al.*, 1991). Since PI also binds to RNA, a RNA digestion step must be carried out before cell cycle analysis.

120000 cells were seeded in 6 cm dishes and grown for 72 hours to reach about 80% confluency. Cells were then harvested together with the medium in order to retain floating apoptotic cells. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. The pellet was then washed in PBS and resuspended in 200 µL of PBS. Cells were fixed by adding 70 % ice cold ethanol in constant agitation and then were stored overnight at -20 °C. Next, fixed cells were centrifuged at 1500 rpm for five minutes and the ethanol discarded. The cell pellet was washed twice with PBS, resuspended in 100µL RNAse (0.1 µg/µL in PBS, Sigma) and incubated at room temperature for 5 minutes. Next, 2 µL of PI (50µg/mL in PBS) were added, and samples were incubated for 15 minutes at room temperature. Cells were analysed with an Imagestream X. All the cell cycle data was analysed using the software Ideas (Amnis).

2.23 Invasion assay

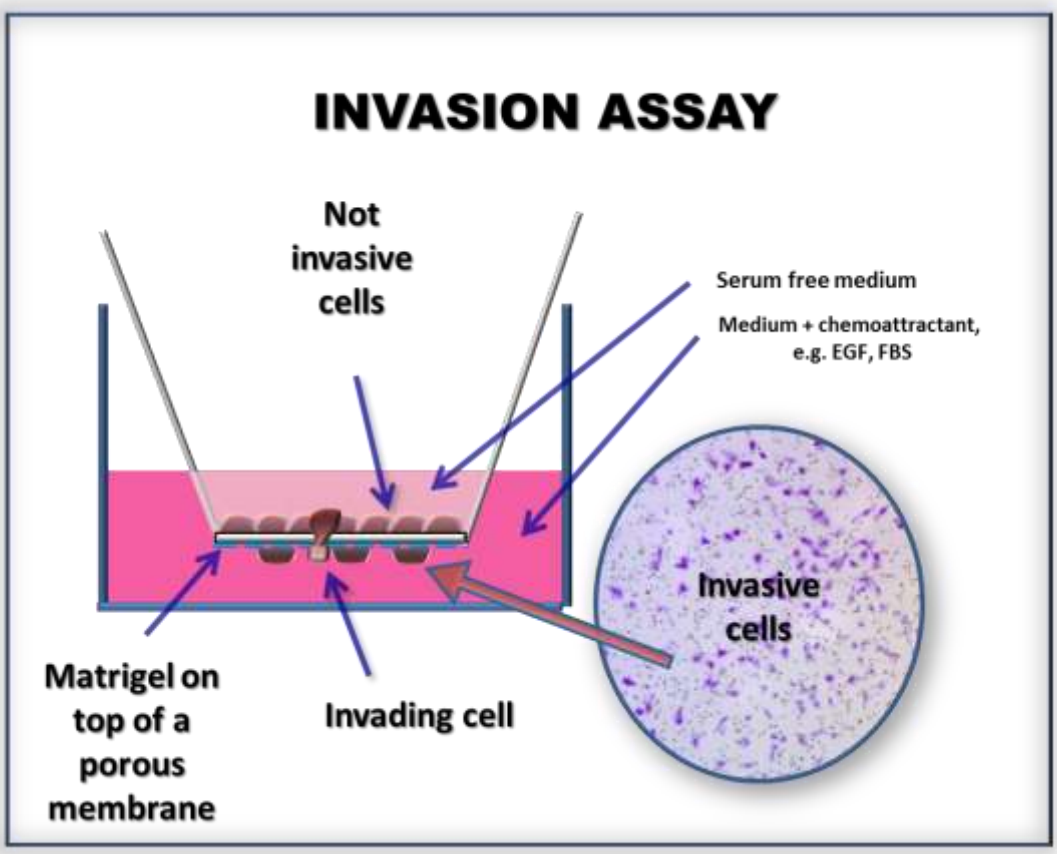

Figure 2.7: Schematic representation of the invasion assay

A rapid *in vitro* invasion assay to assess the metastatic potential of cancer cells was described for the first time in 1987 by Albini et al. (Albini *et al.*, 1987). A schematic representation of this assay is shown in figure 2.7. The assay uses Boyden chambers consisting of an upper compartment where cells are seeded, and a lower compartment which is replenished with medium containing a chemoattractant (e.g. EGF or FBS). In the bottom of the upper compartment lies a porous membrane that maintains a chemical gradient across the two compartments. The upside of the porous membrane is covered with Matrigel, a gelatinous protein mixture derived from mouse tumour cells. This matrix is rich in laminin, collagen IV and heparan sulphate proteoglycans, therefore is often used as a model of basal membrane and stromal tissue in a variety of biological assays (Hughes, Postovit and Lajoie, 2010). Malignant cells must invade basal

membranes to migrate towards distal organs (Liotta, 1984). In the invasion assay the basal membrane is mimicked by a layer of Matrigel. Metastatic cells are able to digest the matrigel and to migrate towards the underside of the filter attracted by a chemotactic gradient, whereas the cells that did not migrate remain in the upper side of the filter. The latter are removed with a cotton bud, and the membrane is stained with crystal violet. In this way, the cells that invaded the matrigel can be visualised and counted.

BioCoat Matrigel Invasion Chambers were purchased from BD biosciences. Chambers were rehydrated filling both compartments with 0.5 mL of serum free medium and placed in the incubator for 2 hours before each experiment.

Cells were harvested with trypsin/EDTA, washed in PBS, and resuspended in serum free medium. The hydration medium was removed from the chambers, and 0.750 mL of serum free medium plus a chemoattractant (FBS or EGF) were added in the lower compartment, while 20000 cells/well were plated in 0.5 mL of medium in the upper compartment. The invasion chambers were then incubated at 37 °C in a humidified incubator for 22 hours. The upper compartment was then displaced, and the cells from the upside of the filter were removed with a cotton bud. Next, the filters were fixed with methanol for two minutes and incubated in a solution of crystal violet (0.1% w/v,10% methanol in water) for 20 minutes. The excess of crystal violet was washed away by submerging the filters in distilled water three times. The filters were then let to air-dry and were removed from the chambers with a scalpel. Glass coverslips were used to mount the filters in glass slides with a drop of microscope oil. The filters were divided in 4 quadrants as shown in figure 2.8, and photographs of each quadrant were taken with an Axioskop 2 microscope (Zeiss) using a 5 X magnification. Cells in each quadrant were counted using the software ImageJ (NIH), and for each sample, an average of 3 filters (a total of 12 quadrants) was counted.

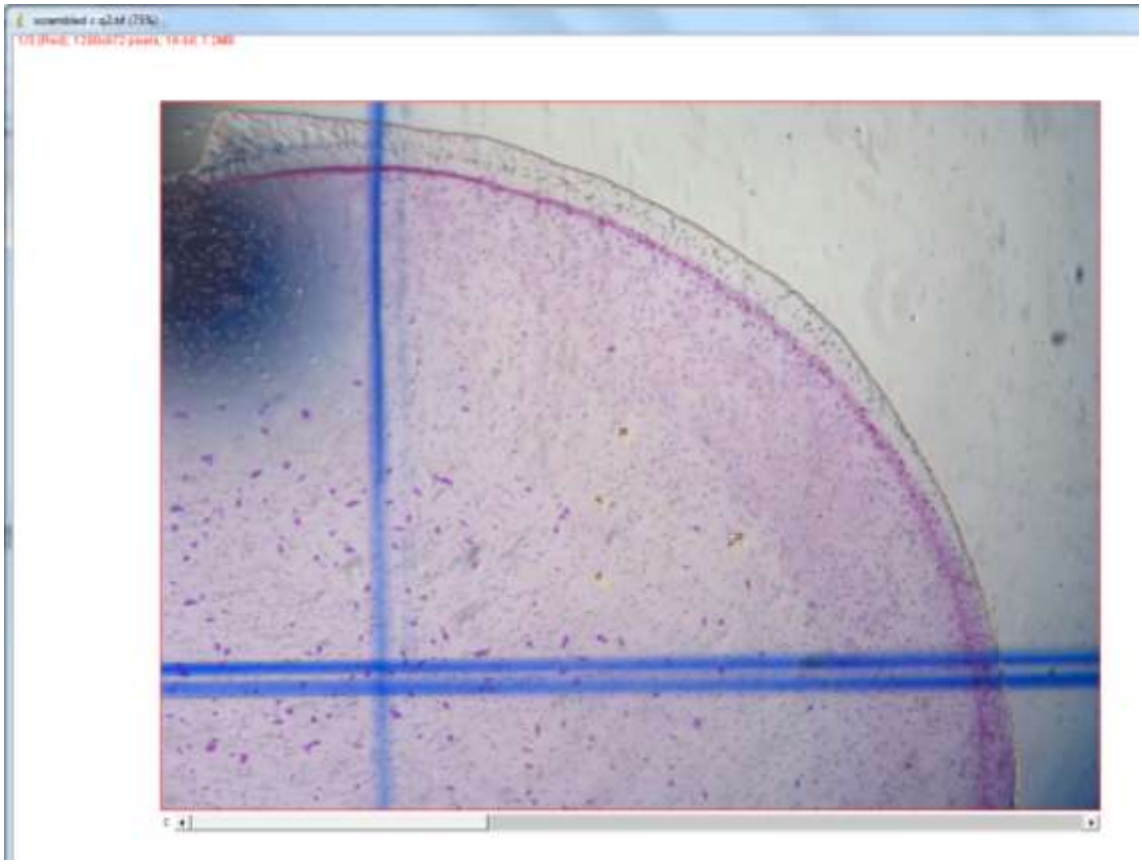

Figure 2.8: Representative photograph showing the porous filter of the invasion assay. The filters were fixed in methanol and incubated in a crystal violet solution. To facilitate the counting, the filters were divided with a fine tip marker in 4 quadrants, and pictures of each quadrant were taken. Cells were then counted using the multi-point tool in imageJ.

2.24 Wound healing assay

The wound healing assay is commonly used to study the directional migration of cells *in vitro*. When a confluent layer of adherent cells is scratched with a pipette tip, cells at the edge of the wound migrate towards the scratch, mimicking what happens during wound healing (Rodriguez, Wu and Guan, 2005). Cell migration is also a key step in the development of cancer metastasis, therefore, the wound healing assay can be used to assess migration rates of cancer cells *in vitro*.

Control cells and FZD6 depleted cells were analysed in parallel using bipartite chamber for live imaging (Fisher). 3.8 x 10^5 Cells were seeded into each compartment of the bipartite chamber and let to grow for 24 hours in 2 mL of complete medium. The medium was then replaced with 2 mL of Leibovitz's L-15

medium (Gibco), and the cells were placed back in the incubator for 4 hours. Cells were then wounded with a sterile pipette tip and placed in a live imaging microscope (Nikon Eclipse Ti). 4 different areas of each wound were photographed every 20 minutes for 24 hours. Cells trajectories were then tracked and their length measured over a period of 4 hours with the image analysis software NIS-Elements (Nikon). The speed of a minimum of 10 cells was scored in 4 areas of each wound analysed.

2.25 Threedimensional cultures data analysis

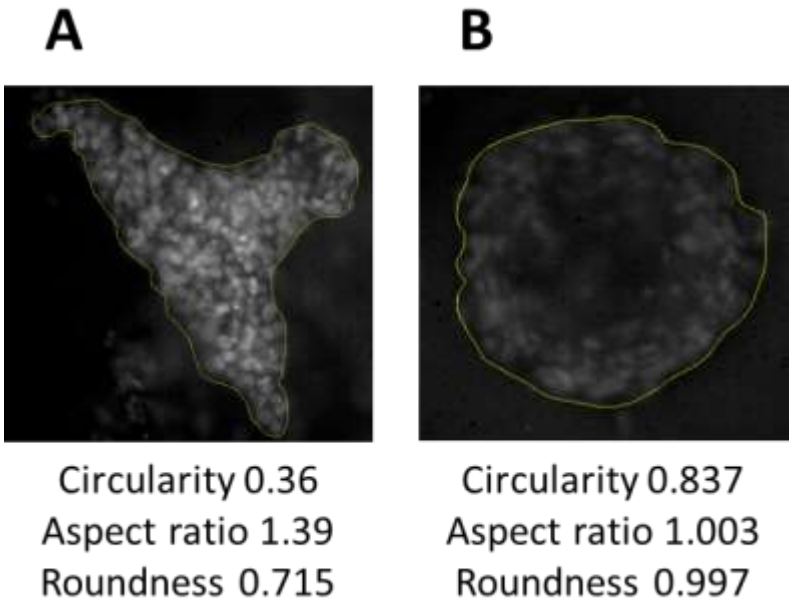

Figure 2.9: Representative photographs describing the assessment of the geometrical features of breast cancer acini growing in 3D cultures: The software ImageJ was used to draw a perimeter (indicated by a yellow line) around the image of the acini. The software computes the geometrical parameters of the shape delineated by this perimeter. **(A)** Representative photograph of a typical irregular acinus characterized by low circularity, aspect ratio >> 1 and low roundness (<<1). **(B)** An example of a regular acinus, characterized by circularity, aspect ratio and roundness close to 1.

Image analysis was performed by assessing, using the imageJ software, a minimum of 20 images of acini per well in a blinded fashion. The perimeter of each acinus was drawn using the free hand tool on the software imageJ as shown in figure 2.9. The software computes the geometrical features of the

acini delineated by the drown perimeter. Parameters analysed were roundness (defined as 4 × (Area of the acinus):(π × [Major axis of the acinus]^2)); circularity (defined as 4π × ([Area of the acinus]/[Perimeter of the acinus]^2), aspect ratio (defined as (Major Axis)/(Minor Axis) of the acinus). Value of 1.0 of circularity indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape. Data analysis was performed using the Prism Software (GraphPad Software, version 5.04, La Jolla, CA, USA). ANOVA followed by Bonferroni's post-hoc test was used to compare the samples.

2.26 Active Rho pull down assay

The Active Rho Pull-Down and Detection Kit (Pierce) was used to assess the levels of active Rho A, B and C in cultured breast cancer cells, following the manufacturer protocol. Cells were grown in T75 flasks until 60-70 % confluent, then were washed twice with PBS and starved overnight in serum free medium. Cells were then washed once with cold TBS-t and lysed in 300 µL of the lysis/washing buffer (25mM Tris•HCl, pH 7.2, 150mM NaCl, 5mM MgCl2, 1% NP-40, 5% glycerol, HALT protease inhibitor cocktail, 1nM sodium orthovanadate). 270 µL of total lysate were then pipetted into spin columns that were previously loaded with glutathione resin and 400 µg of GST-Rhotekin-RBD, which binds selectively to the active form of Rho binding GTP. The columns were then incubated at 4 ° C with gentle rocking for 1 hour. The resin was then washed three times with 400 µL of lysis/washing buffer. The complex GST-Rhotekin/Rho-GTP was then precipitated from the resin with 50 µL of 2 X Laemmli buffer and boiled at 100 °C for 5 minutes. 30µL of these preparations were electrophoresed into 12 % acrylamide gels and analysed by immunoblotting as described in the paragraph 2.10. To perform a loading control, 30 µL of the original total lysate which were excluded from the pull down were mixed with 10 µL of 4X Laemmli buffer, boiled for five minutes at 100 °C and resolved in a separate gel. Proteins were then transferred into nitrocellulose membranes which were blocked in 3 % BSA in TBS and incubated overnight with a rabbit anti-Rho antibody, diluted 1:1000 in TBS-t 3 % BSA. A goat anti-rabbit IgG (H+L) peroxidase-conjugated antibody and ECL were used to detect the Rho band. Densitometric analysis was carried out using the software ImageJ.

2.27 Fibronectin immunofluorescence

2.5×10^5 Cells were resuspended in 1 mL of complete medium and seeded onto 10 mm diameter coverslips in 24 wells plates. The cells were let to settle at 37 °C in a humidified incubator for 24 hours and then were starved overnight following two washes in PBS. Next, the coverslips were washed twice with PBS and the cells were fixed with PFA (4% in PBS) for ten minutes. The coverslips were then washed in PBS thrice and incubated with permeabilization buffer (0,1 % Triton X in PBS) for 10 minutes. Cells were then washed thrice with PBS and incubated in blocking solution (1% BSA in PBS) for 30 minutes. 100 µL of mouse anti-Fibronectin clone 10 antibody (BD biosciences), diluted 1:100 in blocking solution, were added on top of the coverslips for 1 hour at room temperature. The coverslips were then washed three times with PBS and incubated in 120 µL of horse anti-mouse texas red conjugated antibody, diluted 1:100 in blocking solution, for 1 hour at room temperature. After three washes of 5 minutes each with PBS, cells were incubated in DAPI (4', 6-diamidino-2'-phenylindole, dihydrochloride, Thermo Scientific) diluted 1:1000 in PBS for 10 minutes and washed three times with PBS. The coverslips were then mounted in microscope glass slides with a drop of FluorSave reagent (Calbiochem) and analysed with a Leica DM4000 microscope. For each cell line, pictures of ten random fields from 2 separate coverslips were taken using a 20 X magnification objective. Images were acquired under non-saturating conditions and using the same exposure settings for all the samples. The software Leica Application Suite was used to measure the intensity of fluorescence. The background fluorescence of an area of the coverslip without cells was subtracted to each measure.

2.28 Fibronectin competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA)

To assess the levels of soluble fibronectin released in the culture medium of breast cancer cells, the QuantiMatrix Human Fibronectin ELISA (Millipore) was used according to the manufacturer instructions.

2.5×10^5 Cells were resuspended in 1 mL of complete medium and seeded onto 24 wells plates in triplicate. The cells were let to settle at 37 °C in a humidified incubator for 24 hours. The culture medium was then discarded and the cells starved in serum free medium following two washes in PBS. The supernatants were harvested at 72 hours and the cells lysed directly in the plate by adding 30 µL of 2x Laemmli buffer. Supernatants and cell lysates were stored at – 20 ° C until use. 30µL of cells supernatant were then diluted with 30µL of diluent buffer (1% BSA in PBS + 0.01% Thimerosal) and 60µL of Rabbit anti-Human Fibronectin, and incubated at room temperature for 30 minutes. 100 µL of this solution were pipetted into pre-hydrated fibronectin coated strips and incubated at room temperature for 30 minutes. In parallel, fibronectin standards were analysed to build a standard curve. The strips were then washed four times with 200 µL of washing buffer (0.05% Tween-20 and 0.01% Thimerosal in water) and incubated with 100 µL of Goat anti-Rabbit IgG-HRP at room temperature for 30 minutes. Following 4 washes with 200 µL of wash buffer, 100 µL of TMB/E substrate were added to each well containing the strips. When the colour of the wells with the highest concentration became bright blue, the reaction was stopped by adding 100 µL of Stop Solution (0.5M HCl in water) to each well. The absorbance was immediately read at 450 nm with a plate spectrophotometer (Biorad), and the concentration of the samples was determined by extrapolation from the fibronectin standard curve.

To ensure that the assayed fibronectin was secreted by a similar amount of cells amongst the samples, the cells lysates were immunoblotted for β-actin following the procedure described in paragraph 2.10.

2.29 Statistical analysis

All the experiments were carried out in triplicate three times, unless otherwise stated. The unpaired two-tailed t-test was used to compare the differences amongst the experimental groups, unless otherwise specified. Differences were considered significant when the p values obtained were ≤ 0.05. NS indicates non significant differences ($P > 0.05$), * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$ and *** indicates $p \leq 0.001$.

RESULTS

CHAPTER III

Deregulation of FZD6 gene structure and expression in breast cancer

3.1 Introduction

To understand the role of FZD6 in breast cancer, we carried out a comparative analysis between normal and tumour samples in *in silico* databases, patients tissue arrays and breast cancer cell lines. The recent advances in sequencing technologies made available to public consultation a large amount of genomic data. Using specific tools, it is possible to compare gene expression of tumour specimens with the matched normal tissues. Moreover, it is possible to identify the frequency of mutations and to associate the expression of specific genes to the clinical condition of patients. In breast cancer, genomics studies allowed to identify different molecular signatures that can predict different outcomes and different responses to therapies (Bild *et al.*, 2005; Sorlie *et al.*, 2001). Databases are important tools to study oncogenes and oncosupressors, and also to help clinicians deciding the correct therapeutic strategies. The first step in this study was to use bioinformatic tools to investigate whether FZD6 expression and/or mutations were relevant in breast cancer. The *in silico* findings were integrated with immunohistochemical analysis on a large cohort of Italian breast cancer patients. The expression of FZD6 was also assessed in a panel of breast cancer cell lines, both at the mRNA and protein level.

3.2 *In silico* analysis of FZD6 expression in microarrays datasets

We used the online platform ONCOMINE (www.oncomine.org) to investigate whether FZD6 expression was altered in breast cancer compared to normal tissue. We found that FZD6 mRNA levels were higher in tumour samples compared to normal tissue (Fig. 3.1 A) and in the tumour stroma compared to the normal stroma (fig. 3.1 B). In the Ma database (Ma et al., 2009), we found that FZD6 expression in ductal breast carcinoma in situ was 1.673 fold higher than normal tissue (fig. 3.1 A). In the Finak dataset (Finak et al., 2008), FZD6 expression was 1.7 fold higher in invasive carcinomas stroma when compared to normal breast stroma (fig. 3.1 B). In the cancer genome atlas (TCGA), we found a 1.33 fold increase in the FZD6 gene copy number of ductal breast carcinomas compared to normal tissue or blood (fig.3.1 D).

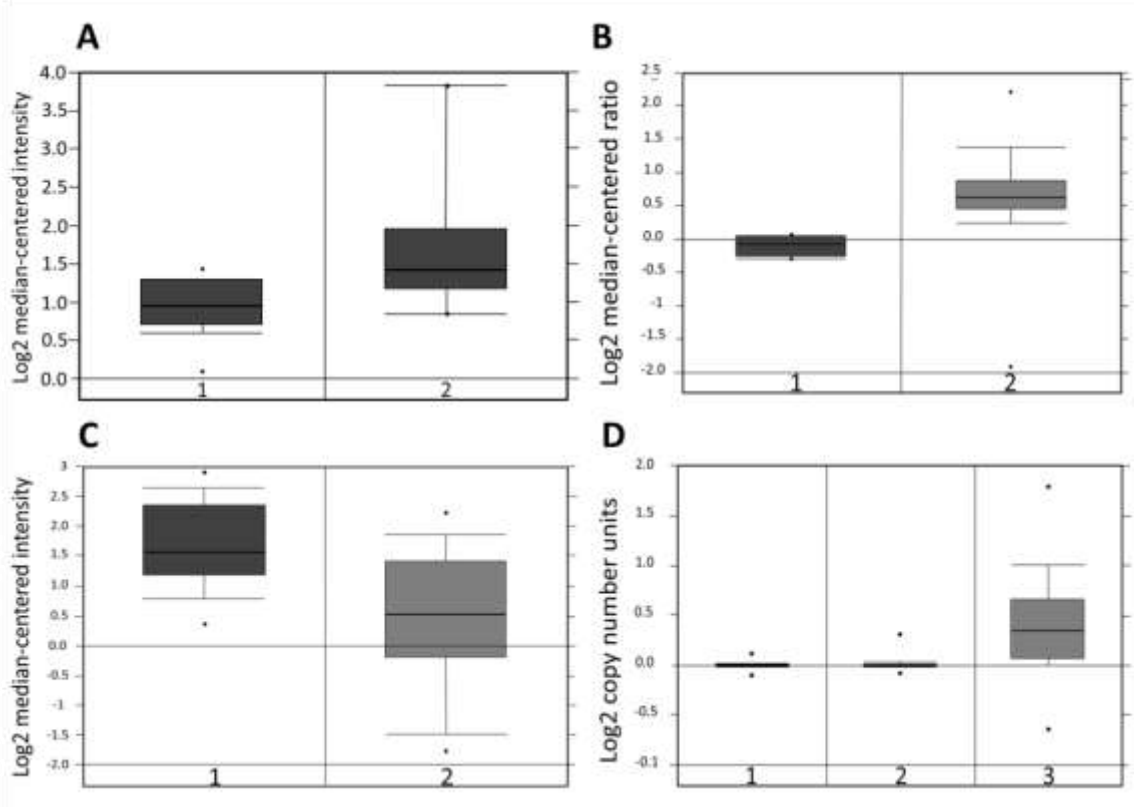

Figure 3.1: FZD6 expression in breast cancer datasets. The online platform ONCOMINE was used to assess FZD6 mRNA levels in normal breast and breast cancer from available microarrays datasets. **(A)** FZD6 Expression in the Ma dataset. 1, normal breast (n= 14); 2, ductal breast carcinoma in situ (n= 9). Fold change: 1.673, p= 0.018. **(B)** FZD6 Expression in the Finak dataset. 1, normal breast stroma (n= 6); 2, invasive breast carcinoma associated stroma (n=53). Fold change: 1.708; p= 2.81E-9. **(C)** FZD6 Expression in the Farmer dataset. 1, basal-like invasive breast carcinoma (n=16); 2, luminal-like invasive breast carcinoma (n=27) fold change: 2.303; p= 4.47E-5. **(D)** FZD6 copy number in TCGA dataset. 1, Blood (n=702); 2. Normal breast (n=111); 3. Invasive ductal breast carcinoma (n=639). Fold change 1.33; p=1.12E-101. • indicates extreme values.

These observations were consistent with the data extracted from the online platform COSMIC (Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/), where on 900 breast cancer samples analysed, 172 (19,11%) showed gene copy number gains for FZD6, and 185 out of 989 samples displayed FZD6 overexpression (18.71%) (table 3.1). Amongst all the FZD receptors, FZD6 was the most frequently amplified and overexpressed in breast cancer (Table 3.1).

To understand if FZD6 overexpression was linked to specific breast cancer subtypes, we performed a comparative analysis using the Farmer dataset

(Farmer *et al.*, 2005). We found that FZD6 expression was 2.3 fold higher in basal-like breast carcinoma compared to luminal-like carcinoma (fig. 3.1 C).

Table 3.1: Copy Number Variation (CNV) and overexpression of Frizzled (FZD) receptor 1 to 10 in primary breast cancer samples. Data was mined using the COSMIC repository (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/about).

FZD	Loss/gain (out of 900 samples)	Overexpression (out of 989 samples)
1	Loss 2 (0.22%) Gain 0	21 (2.12%)
2	Loss 3 (0.33) Gain 0	47 (4.75)
3	Loss 40 (4.44%) Gain 3 (0.33%)	59 (5.97%)
4	Loss 4 (0.44%) Gain 14 (1.56%)	31 (3.13)
5	Loss 1 (0.11) Gain 0	41 (4.15)
6	Loss 0 Gain 172 (19.11%)	185 (18.71%)
7	Loss 2 (0.22) Gain 0	40 (4.04%)
8	Loss 0 Gain 9 (1%)	12 (1.21%)
9	Loss 1 (0.11%) Gain 9 (1%)	27 (2.73%)
10	Loss 0 Gain 1 (0.11%)	40 (4.04%)

3.3 *In silico* survival studies

In order to understand the clinical significance of FZD6 overexpression in patients, we used the online tool Kaplan-Mayer Plotter (http://kmplot.com/analysis/) that allows the generation of Kaplan-Meier curves linking patients gene profiling to their outcome (Györffy *et al.*, 2010).

High expression of FZD6 was associated with a lower overall survival (HR=1.48, C.I. 95% 1.17-1.89, p=0.0013), lower relapse free survival (HR=1.42, C.I. 95% 1.26-1.6, $p=8.9x10^{-9}$) and lower distant relapse free survival (HR=1.48 C.I. 95% 1.2-1.82, p=0.00022) of the totality of breast cancer patients (fig.3.2).

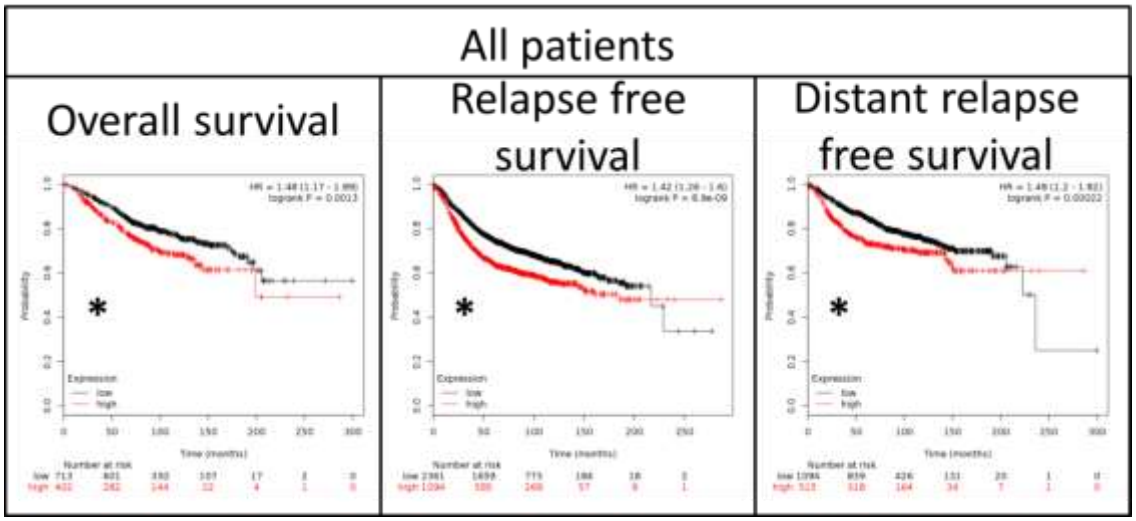

Figure 3.2: Kaplan Meier survival analysis of breast cancer patients with high or low expression of FZD6: The online tool Kaplan Meier plotter was used to predict overall survival, relapse free survival and distant relapse free survival of breast cancer patients with high (red) or low (black) FZD6 expression. ***** Indicates statistical significance. Hazard ratios, 95% confidence intervals and p values are indicated in the top-right corner of each plot.

We also compared the survival plots of patients with basal like, luminal A and luminal B breast cancer. HER2 positive patients were excluded due to the insufficient number of cases to carry out the analysis. Patients were further divided into those having or not having tumour spread in the lymph nodes at the time of diagnosis. We found that FZD6 expression status was not sufficient to predict overall survival when patients were divided into subgroups, except for basal like, lymph node positive patients, where high FZD6 expression was associated with lower overall survival (HR=3.36, C.I. 95% 0.94-11.97, p=0.048 (fig.3.3).

High expression of FZD6 was associated with a lower relapse free survival independently from the lymph node status when patients were not stratified into subgroups (fig. 3.4). When considering only the lymph node negative patients instead, a significant association was observed only for the luminal A subgroup. In the lymph node positive cohort high FZD6 was associated with a higher probability of tumour relapse in all the subgroups, particularly in those patients affected by basal breast cancer, where the hazard ratio was 1.9, C.I. 95% 1.09-3.31, p=0.022 (fig.3.4).

High FZD6 expression correlated with lower distant relapse free survival when all the lymph node negative subgroups were considered together (fig.3.5). A

significant association was also observed in basal like patients and luminal A patients with lymph node negative status, but not in the luminal B cohort (fig.3.5). FZD6 expression was not able to predict distant relapse free survival in those patients that were lymph nodes positive at the time of diagnosis, independently from the tumour subtype (fig.3.5). The risk of metastatic relapse was particularly high in basal like, lymph node negative patients with high FZD6 (HR=2.35 C.I.95% 1.16-4.75, p=0.014) (fig.3.5).

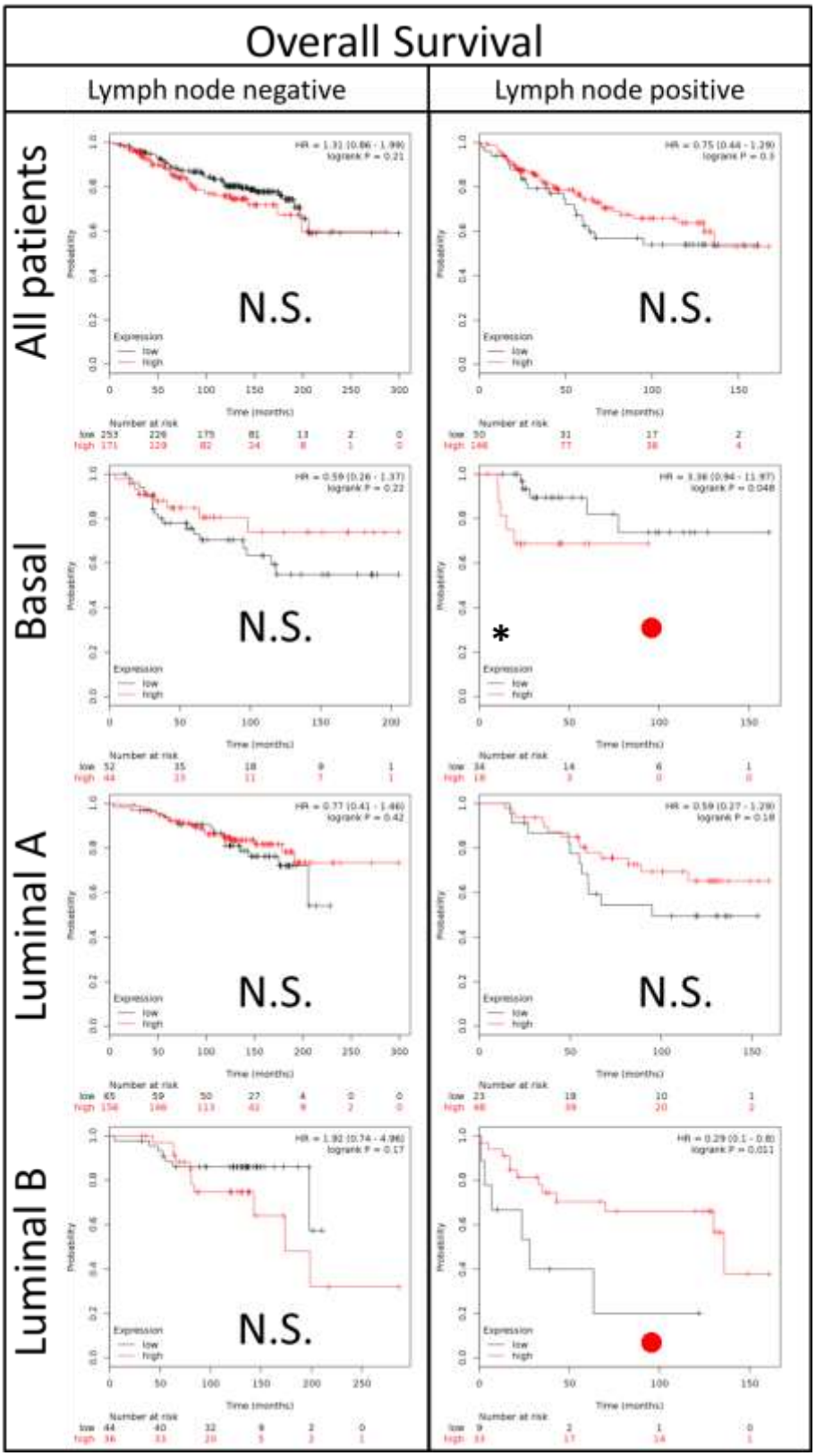

Figure 3.3: Kaplan-Meier survival analysis in different breast cancer subtypes: Plots were obtained as described in fig.3.2 and stratifying patients belonging to different breast cancer subgroups and with different lymph nodes status. Black lines indicate low FZD6, red lines high FZD6. Hazard ratios, 95% confidence intervals and p values are indicated in the top-right corner of each plot. ***** indicates statistical significance, N.S. indicates non significant differences and ● indicates that the number of patients available was low.

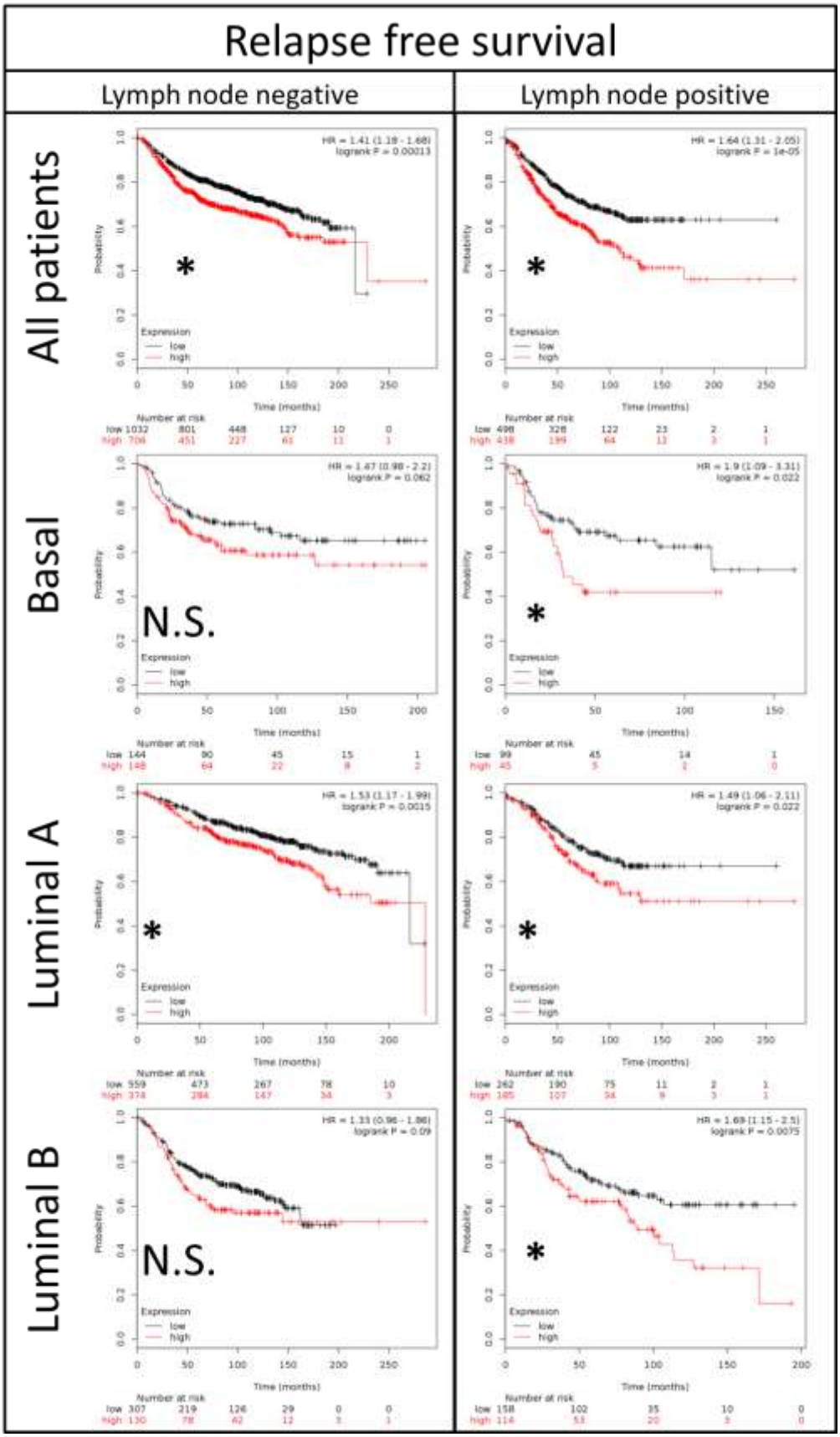

Figure 3.4: Kaplan-Meier relapse-free survival analysis: plots were obtained as described in figure 3.3. Black lines indicate low FZD6, red lines high FZD6. Hazard ratios, 95% confidence intervals and p values are indicated in the top-right corner of each plot. N.S. indicates non significant differences whereas ***** indicates statistical significance.

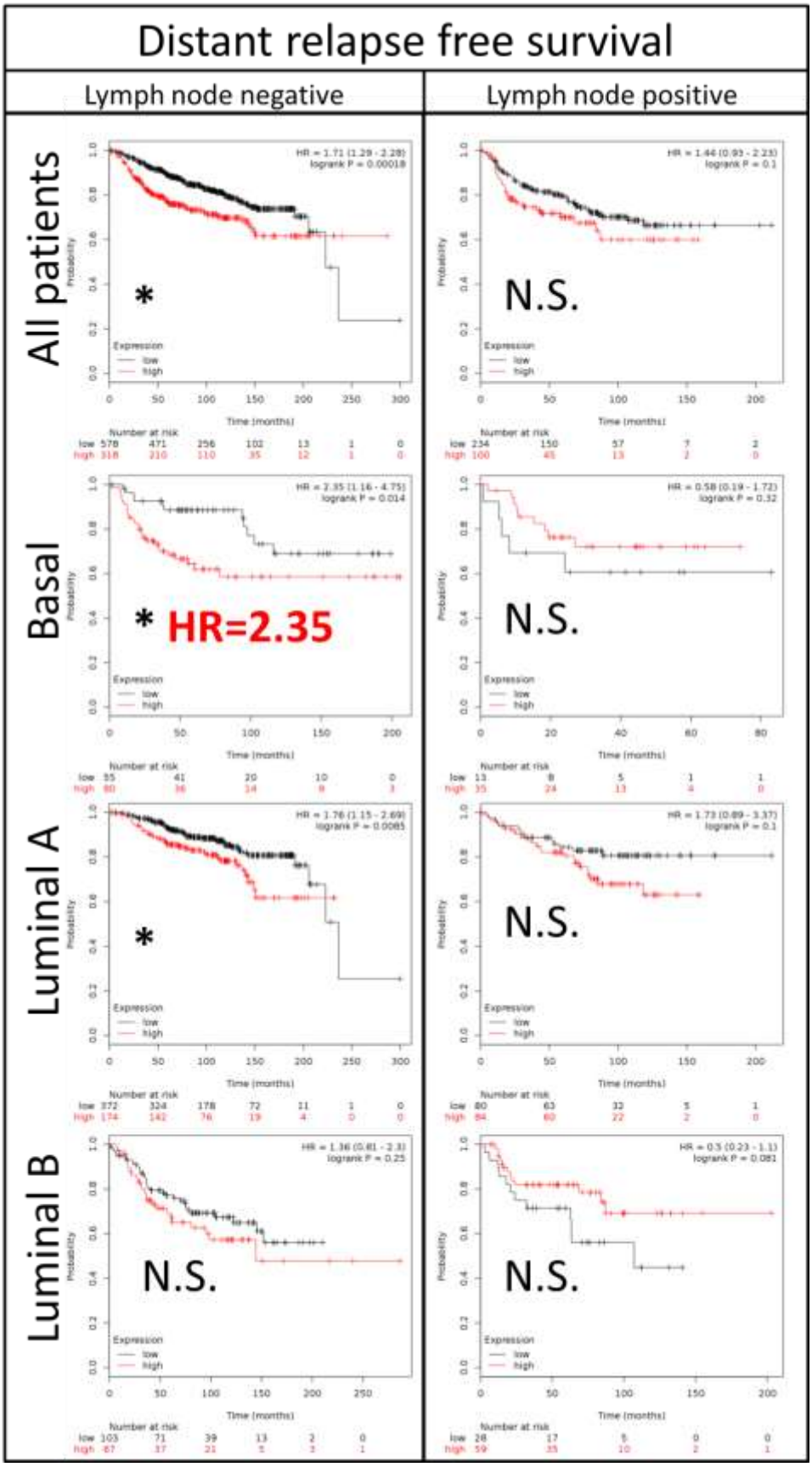

Figure 3.5: Kaplan-Meier distant relapse free survival analysis: plots were obtained as described in figure 3.3. Black lines indicate low FZD6, red lines high FZD6. Hazard ratios, 95% confidence intervals and p values are indicated in the top-right corner of each plot. N.S. indicates non significant differences whereas ***** indicates statistical significance.

3.4 Immunohistochemical analysis of FZD6 expression in an Italian cohort of breast cancer patients

The data presented in this section was produced by our collaborators Dr Rossano Lattanzio and Professor Mauro Piantelli from the Department of Experimental and Clinical Sciences, University G. D'Annunzio, Chieti, Italy, and Marcella Mottolese and Letizia Perracchio from the Regina Elena Cancer Institute, Rome, Italy.

FZD6 immunohistochemical staining was carried out on tissues arrays as described in material and methods. The study includes 352 primary infiltrating breast cancers from N0 and T1/T2 tumours from patients that were presenting primary unilateral breast carcinoma. Patient and tumour characteristics are summarized in table 3.2.

20.2% of the specimens showed membrane expression of FZD6, with or without concurring cytoplasmic staining (Figure 3.6). When considering the whole patient cohort, expression of Fzd6 was not significantly associated with clinical features (Table 3.3). However, Kaplan–Meier analysis indicated that expression of Fzd6 was significantly associated with lower distant relapse-free survival (DRFS) in the triple negative breast cancer patients subgroup (fig. 3.7). In multivariate analysis, membranous Fzd6 expression was an independent prognostic indicator for low disease free (DFS) and DRFS survival in triple negative cases (table 3.4). Tumour grade was the only independent factor influencing DFS and DRFS in the whole patient population (table 3.3).

Table 3.2: Patients and tumour Characteristics (n = 352)

Variable	Value (%)
Age at diagnosis (years)	
Median	60.5
<50	78 (22.1)
50-65	147 (41.8)
>65	127 (36.1)
Menopausal status	
Pre/perimenopausal	84 (23.9)
Postmenopausal	268 (76.1)
Molecular subtypes	
Luminal A-like	128 (36.4)
Luminal B-like (HER2 negative)	123 (34.9)
Luminal B-like (HER2 positive)	33 (9.4)
HER2 positive (non-luminal)	21 (6.0)
Triple negative (ductal)	47 (13.4)
Tumor size	
≤ 2 cm	228 (64.8)
> 2 cm	124 (35.2)
Tumour grade	
1	58 (16.5)
2-3	294 (83.5)
ER	
Negative	74 (21.0)
Positive	278 (79.0)
PGR	
Negative	161 (45.7)
Positive	191 (54.3)
Ki-67	
Low	237 (67.3)
High	115 (32.7)
HER2	
Negative	298 (84.7)
Positive	54 (15.3)
Fzd6	
Negative	281 (79.8)
Positive	71 (20.2)
Patient outcome	
Without recurrence	266 (75.6)
Local recurrence	32 (9.1)
Distant recurrence	54 (15.3)

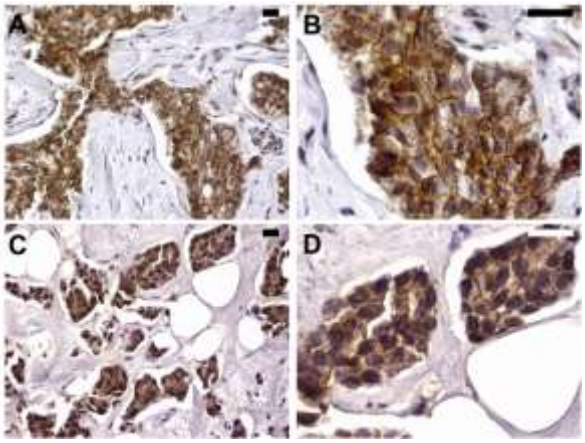

Figure 3.6: Immunohistochemical analysis of Fzd6 expression in breast cancer. (A, C) Images showing Fzd6 expression in two invasive triple negative human breast cancers (magnification 40X). **(B, D)** Enlargement of the images shown in panels A and C. Bars indicate 50µm.

Table 3.3: Multivariate analysis of Fzd6 expression in the totality of breast tumours

Variable	HR	95% CI	P
Disease-free survival			
Tumor size, cm (≤ 2 *vs* > 2)	1.0	0.7-1.6	0.840
Tumor grade (2-3 *vs* 1)	2.5	1.1-5.8	0.038*
ER (positive *vs* negative)	1.1	0.6-1.9	0.844
PGR (negative *vs* positive)	1.4	0.8-2.3	0.210
Ki-67 (high *vs* low)	1.5	0.9-2.4	0.096
HER2 (positive *vs* negative)	1.3	0.8-2.2	0.308
Fzd6 (negative *vs* positive)	1.3	0.7-2.2	0.417
Local Relapse-Free Survival			
Tumour size, cm (> 2 *vs* ≤ 2)	1.3	0.7-2.6	0.425
Tumor grade (2-3 *vs* 1)	1.5	0.5-4.5	0.445
ER (positive *vs* negative)	1.6	0.6-4.2	0.349
PGR (negative *vs* positive)	1.6	0.8-3.3	0.226
Ki-67 (high *vs* low)	1.2	0.6-2.5	0.658
HER2 (negative *vs* positive)	1.2	0.4-3.1	0.744
Fzd6 (negative *vs* positive)	1.1	0.5-2.5	0.833
Distant Relapse-Free Survival			
Tumour size, cm (≤ 2 *vs* > 2)	1.3	0.7-2.3	0.376
Tumor grade (2-3 *vs* 1)	4.8	1.1-20.2	0.032*
ER (positive *vs* negative)	1.1	0.5-2.2	0.875
PGR (negative *vs* positive)	1.4	0.7-2.6	0.291
Ki-67 (high *vs* low)	1.6	0.9-3.0	0.102
HER2 (positive *vs* negative)	1.5	0.8-2.8	0.244
Fzd6 (positive *vs* negative)	1.4	0.7-2.8	0.384

*Statistically significant

Table 3.4: Multivariate analysis of Fzd6 expression in triple negative tumours

Variable	HR	95% CI	P
Disease-free survival			
Tumor size, cm (> 2 *vs* ≤ 2)	4.1	0.7-21.5	0.098
Tumor grade (1 *vs* 2-3)	1.7	0.3-11.5	0.879
Fzd6 (positive *vs* negative)	5.7	1.5-22.9	0.011*
Local Relapse-Free Survival			
Tumour size, cm (>2 *vs* ≤2)	1.1	0.2-10.6	0.950
Tumor grade (1 *vs* 2-3)	1.0	0.1-10.4	0.941
Fzd6 (negative *vs* positive)	2.5	0.3-25.0	0.421
Distant Relapse-Free Survival			
Tumour size, cm (> 2 *vs* ≤ 2)	2.1	0.3-12.6	0.435
Tumor grade (1 *vs* 2-3)	1.0	0.1-15.2	0.992
Fzd6 (positive *vs* negative)	6.8	1.2-37.4	0.027*

*Statistically significant

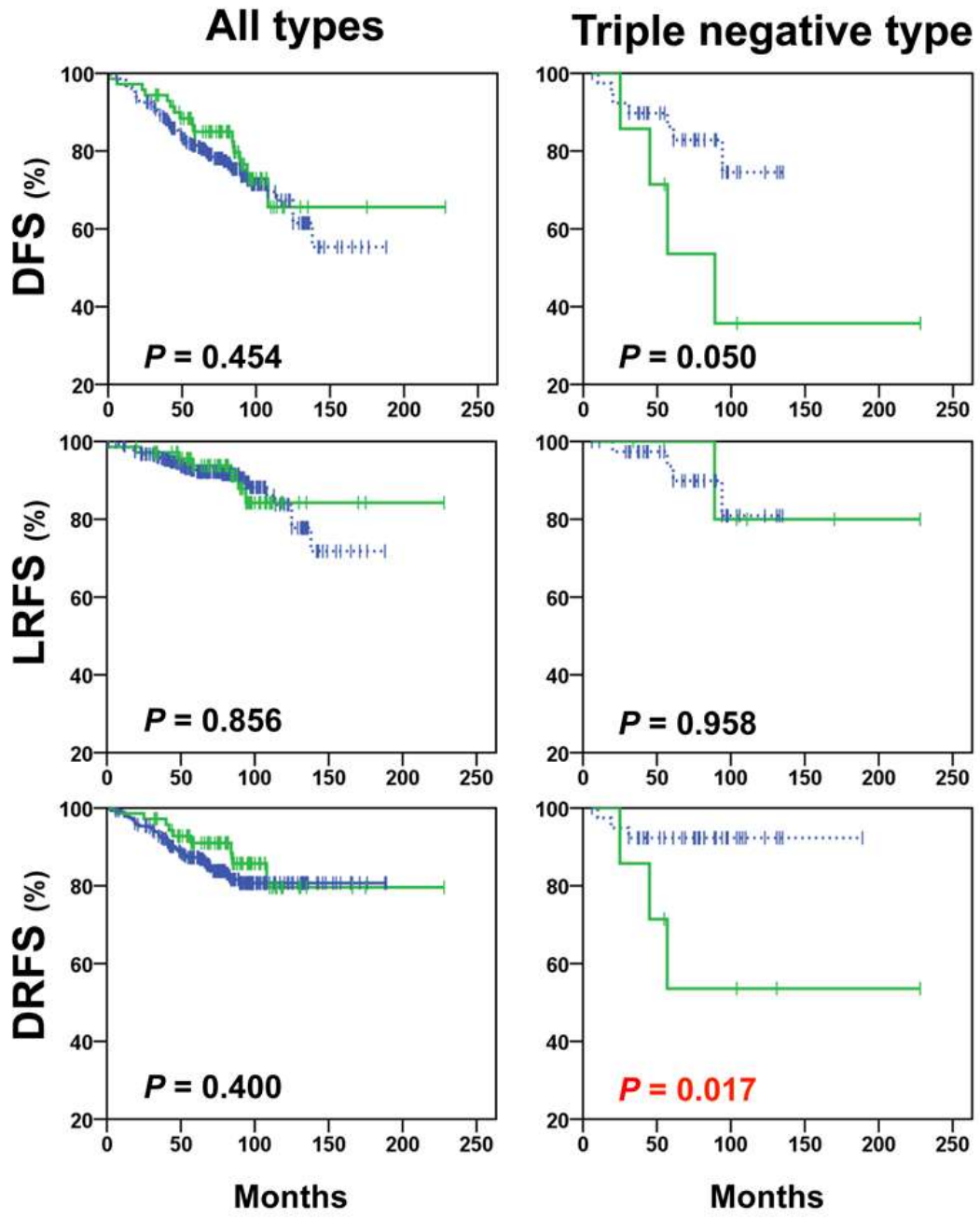

Figure 3.7: Survival curves. Kaplan–Meier estimates of disease-free survival (DFS), local relapse-free survival (LRFS) and distant relapse-free survival (DRFS) in breast cancer cases (*n* = 352) with high (green solid line) or low (blue dashed line) expression of Fzd6 assessed by immunohistochemistry. Statistical significance was assessed using the log-rank test.

3.5 Expression of FZD6 in breast cancer cell lines

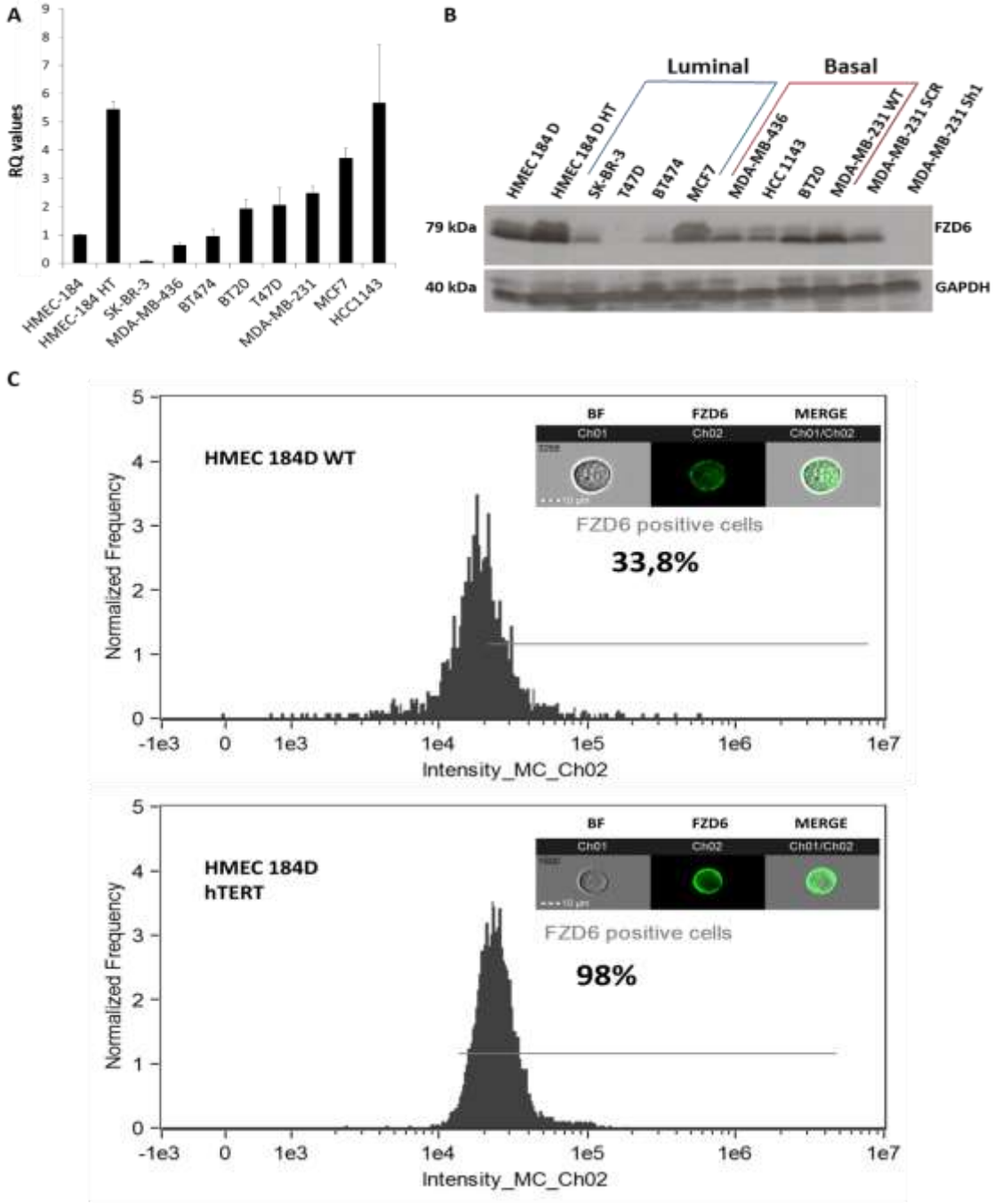

Figure 3.8: Expression of FZD6 in breast cancer cell lines: (A) Reverse transcription, Q-PCR analysis of FZD6 mRNA expression in a panel of breast cancer cell lines. **(B)** Western blot analysis showing expression of Fzd6 in different breast cancer cell lines. RNAi depleted MDA-MB-231 cells (MDA-MB-231-Sh1) were used as a negative control in comparison with the control cell line (MDA-MB-231-Scrambled) to unequivocally identify Fzd6-specific bands. **(C)** Imagestream analysis demonstrating increased percentages of human mammary epithelial cells (HMEC) expressing Fzd6 in the cell membrane after immortalization with the hTERT enzyme. Inset shows representative images of cells in bright field (BF) or stained with the Fzd6 antibody (green).

To complete our analysis we assessed FZD6 expression levels in a panel of breast cancer cell lines. Expression levels were compared to those of normal human mammary epithelial cells (HMEC-184D). HMEC-184D cells immortalized with H-TERT plasmid were also included in the analysis. FZD6 mRNA expression was assessed by RT-QPCR (fig.3.8 A), whereas protein levels were assessed by western blot analysis and Imagestream analysis (fig.3.8 B and C).

HMEC-184D-HT transformed cells showed a 5.45 fold increase in the mRNA levels and increased Fzd6 protein expression compared to normal HMEC-184D (Fig.3.8 A and B). Moreover, HMEC-184D-HT showed a 2.9 fold increase in the percentage of cells positive to membranous FZD6 expression compared to the normal conterparts (Fig. 3.8 C). Overall, the majority of breast cancer cell lines expressed higher levels of FZD6 mRNA compared to HMEC cells. However, the same difference was not evident at a total protein level (Fig. 3.8 B). Furthermore, basal like cells expressed more Fzd6 than non basal cell lines (fig. 3.8 B).

Imagestream analysis in a panel of breast cancer cell lines was carried out to assess membrane expression of the FZD6 receptor (fig.3.9). All the cell the cell lines, to different extent, showed membrane staining of FZD6, with the only exception of MDA-MB-436 cells.

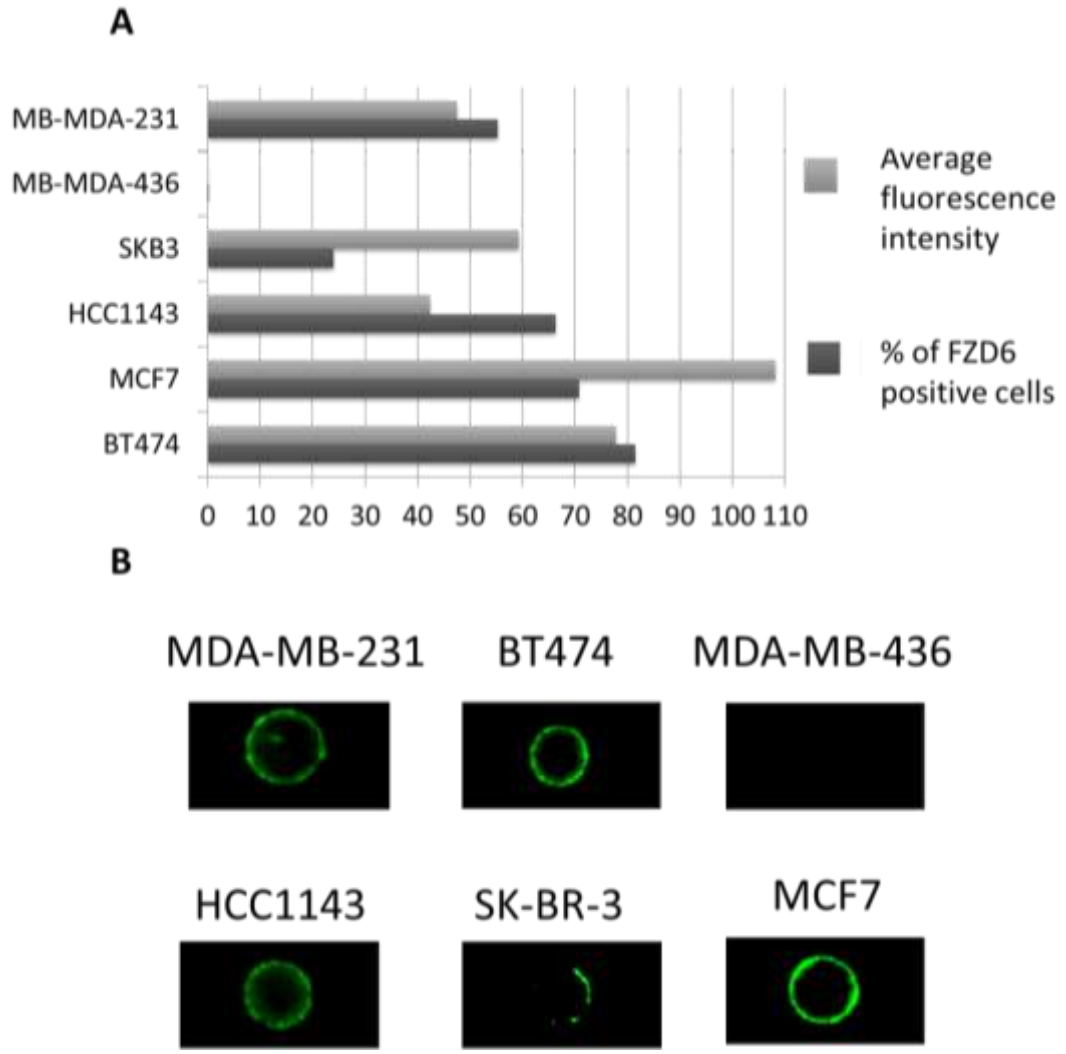

Figure 3.9: Expression of Fzd6 on the surface of breast cancer cell lines: (A) Cells were labelled with a Fzd6 antibody and subjected to flow cytometry-Imagestream analysis. The percentages of positive cells and the geometrical mean fluorescence intensities of the Fzd6 signal are indicated in the graph plot. **(B)** Representative photographs of cells from each of the cell lines taken during the Imagestream analysis and showing different degrees of Fzd6 staining on the cell surface.

3.6 Discussion

The results presented in this chapter demonstrate that the overexpression of FZD6 is a common event in breast cancer. It is likely that the up-regulation of FZD6 is a consequence of gene amplification, since a high percentage of the breast cancer cases in the COSMIC database (19.11%) showed a FZD6 copy number gain (table 3.1). A similar percentage of cases (18.71%) also showed

high FZD6 expression, suggesting that the two events could be causally linked (table 3.1). Given the importance of WNT signalling in cancer, altered expression of FZD6 has the potential to have an important role in breast tumorigenesis. Indeed, increased activation of the WNT pathway through overexpression of frizzled receptors has been reported in many tumours. Moreover, overexpression of FZD receptors has been shown to be critical in promoting tumour progression, metastasis and drug resistance in breast cancer (Zhang *et al.*, 2012; Yang *et al.*, 2011; Ueno *et al.*, 2008; Milovanovic *et al.*, 2004).

Our results indicate that FZD6 might have a specific role in breast cancer. This is suggested by the comparison between normal mammary epithelial cells (HMEC) and their immortal counterpart expressing hTERT (human Telomerase Reverse Transcriptase). Indeed, we observed a marked upregulation of FZD6 in HMEC in the presence of ectopic expression of hTERT, both at mRNA and protein levels (fig. 3.8). The forced expression of hTERT is widely used to immortalize primary cells that would otherwise rapidly undergo senescence in culture. This is achieved by the synthesis of new telomeres at the end of the chromosomes by hTERT. Telomeres are repeated nucleotide sequences that protect chromosome ends from degradation. Every time a somatic cell undergoes mitosis, telomeres are shortened, reducing the lifespan of the cell. This is thought to be a control mechanism that reduces the risk of propagation of cells with chromosome instability and mutations (Lee, Choi and Ouellette, 2004). The activation of telomerases is also a common event in cancer and is required by cancer cells to escape senescence and become immortal. Immortalization of primary cells with hTERT also promotes the acquisition of new mutations, as observed in tumorigenesis. For example, Noble et al. showed that ectopic expression of hTERT in human fibroblasts could result in deletions of p16 and mutations of p53 (Noble *et al.*, 2004). Mutations in relevant check point proteins can lead to genomic instability and the acquisition of mutations. We therefore hypothesise that genome instability in breast cancer cells might promote FZD6 rearrangements that results in its overexpression.

To understand the clinical implications of the overexpression of FZD6, we carried out survival studies based on gene expression, microarrays and protein expression in patients. Up-regulation of FZD6 reduces overall survival, relapse free survival and distant relapse free survival, suggesting that FZD6

upregulation could be a general risk factor in breast cancer. When patients were divided into different cohorts, i.e. in lymph node negative or positive and in breast cancer subtypes, FZD6 expression was not informative in terms of overall survival (fig. 3.3). Nevertheless, high FZD6 expression was associated with a lower relapse free survival, particularly in patients with positive lymph nodes (fig.3.4). This suggests that the upregulation of FZD6 could play a role in tumour recurrence. High expression of FZD6 was also associated with high risk of metastatic relapse in all the lymph node negative subgroups, but not in lymph node positive patients (fig.3.5). A possible explanation for this is that FZD6 could be important to trigger metastatic spread, but its expression might be irrelevant for the survival of patients with late stage disease, i.e. with lymph node positive for the presence of cancer cells.

The basal-like cohort was the one with the higher hazard ratio between high and low FZD6 expression for the metastatic relapse survival, suggesting a particular importance of FZD6 up-regulation in the metastatic progress for this subtype. These observations are consistent with the multivariate analysis carried out in a cohort of Italian patients. In these patients, high FZD6 expression was predictive of low relapse free survival and distant relapse free survival specifically in the triple negative breast cancer subgroup. We also observed higher expression of FZD6 in basal cell lines when compared to HMEC or luminal cell lines. Basal carcinomas are usually associated with a poorer prognosis and a more invasive phenotype when compared to luminal subtypes (Sorlie et al., 2001). We therefore hypothesize that FZD6 expression could be relevant in explaining the phenotypic differences between luminal and basal breast cancers. Although triple negative tumours initially show a good response to therapy, they tend to relapse at distant sites more frequently than other subtypes (Carey et al., 2010; Foulkes, Smith and Reis-Filho, 2010; Nguyen et al., 2008). Altogether, our findings suggest that surface expression of Fzd6, besides serving as a strong predictive marker of distant relapse, could be exploited as a target for therapy of triple negative cancers. The need to predict patients outcome in early breast cancer is of primary importance in order to select the appropriate therapeutic regimens. For example, in lymph node negative patients adjuvant systemic chemotherapy could be unnecessary and even harmful for low risks patients, whereas it could be crucial for high risk patients (Cianfrocca and Goldstein, 2004). FZD6 expression status could be

used to discern high risk lymph-node negative patients from the ones with low risk, helping clinicians to design adequate therapies.

Understanding the role of FZD6 in breast cancer could give new insights in the biological mechanisms underlying tumour recurrence and metastasis. This is of primary importance, since a vast number of tumours recur, even many years after primary treatment (Karrison, Ferguson and Meier, 1999). Different explanations have been proposed to explain tumour recurrence, but this process is still poorly understood. Recurrence is originated by cancer cells that remain in the patient's body after primary treatment and that cannot be detected with conventional diagnostic techniques. These cells undergo a period of quiescence, but eventually proliferate to reform the tumour (Aguirre-Ghiso, 2007). Many investigators argue that tumour repopulation is caused by cancer stem cells that survived surgery or adjuvant therapy (Merlos-Suárez *et al.*, 2011; Donnenberg and Donnenberg, 2005). Stem cells resist chemotherapy due to their high expression of membrane transporters that excrete cytotoxic drugs (Dean, Fojo and Bates, 2005). Cancer stem cells have also a pivotal role in the development of metastases. This is thought to be caused by the aberrant activation of pathways that are responsible of cell migration during organogenesis (Singh and Settleman, 2010; Karnoub *et al.*, 2007; Li *et al.*, 2006). In this contest, the role of the WNT pathway is well established (Reya and Clevers, 2005).The WNT pathway has been shown to be required for the activation of the epithelial to mesenchymal transition (EMT), essential for cancer cells to leave the primary tumour site and invade to distant organs (Neth *et al.*, 2007). Indeed, our group previously reported that FZD6 marks stem-like cells and confers drug resistance in neuroblastoma cells (Cantilena *et al.*, 2011). In breast cancer, the overexpression of FZD6 could provide a survival advantage to cancer stem cells to overcome primary treatment. Patient bearing high FZD6 would therefore have a higher risk for tumour recurrence and metastatic relapse.

CHAPTER IV

Depletion of FZD6 expression affects motility, invasion and three-dimensional growth, but not proliferation of breast cancer cell lines

4.1 Introduction

Several investigators have linked abnormalities in the WNT pathway with increased proliferation and invasion of breast cancer cells (Zhang *et al.*, 2012; Klemm *et al.*, 2011; Yang *et al.*, 2011; Matsuda *et al.*, 2009; Lindvall *et al.*, 2007). In the previous chapter I have shown that FZD6 overexpression is common in breast cancer and associated with low survival. To understand the role of the FZD6 receptor in breast cancer, we used RNA interference to downregulate its expression in cell lines. This approach is commonly used to understand the function of a gene by observing the phenotypic effects caused by its depletion (Hannon, 2002). Transient knock down was obtained using siRNAs, whereas stable knock down was achieved through the infection of cells with lentiviral vectors targeting FZD6. Non-specific RNA sequences were used as controls.

4.2 FZD6 knock down validation

Firstly, I transiently transfected two small interfering RNA (siRNA1 FZD6 and siRNA2 FZD6) targeting different regions of the FZD6 transcript. A nonspecific siRNA sequence was used as a negative control. RT-QPCR analysis demonstrated robust knock down in all the cell lines analysed (fig.4.1).

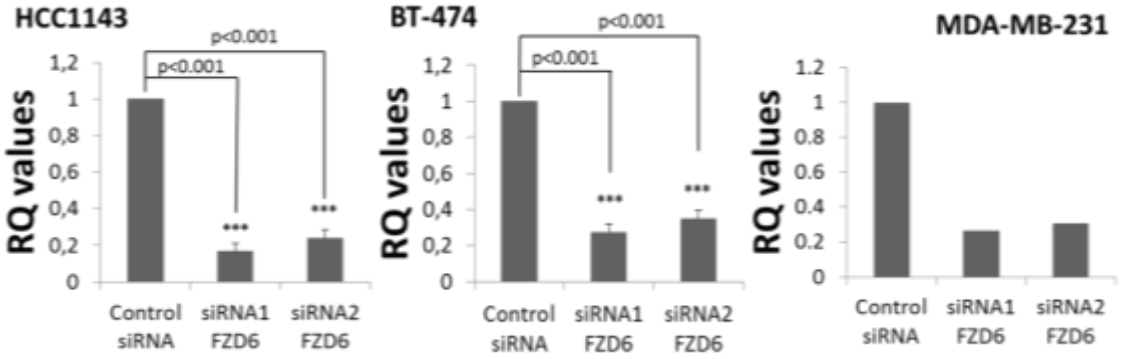

Figure 4.1: Quantification of FZD6 knockdown in breast cancer cells. Real-time Q-PCR showing knockdown of FZD6 mRNA expression levels in breast cancer cell lines transfected with the indicted siRNAs. FZD6 expression was normalized to GAPDH and expressed as relative quantities to the control siRNA. Error bars indicate standard deviation, asterisks indicate statistical significance (Student t-test, HCC1143, n=3; BT474, n=3; MDA-MB-231, n=1)

Knock down efficiency was also evaluated at protein level using flow cytometry and Imagestream analysis. Indirect immunostaining was performed on living cells using a FZD6 antibody and a FITC-conjugated secondary antibody. Cells were analysed with an EPICS XL flow cytometer (Beckman Coulter) or an Imagestream X Imaging flow cytometer (Amnis). FZD6 membrane staining was drastically reduced in the presence of both the siRNAs (Fig. 4.2 and 4.3) in the HCC1143 and MDA-MB-231 cell lines.

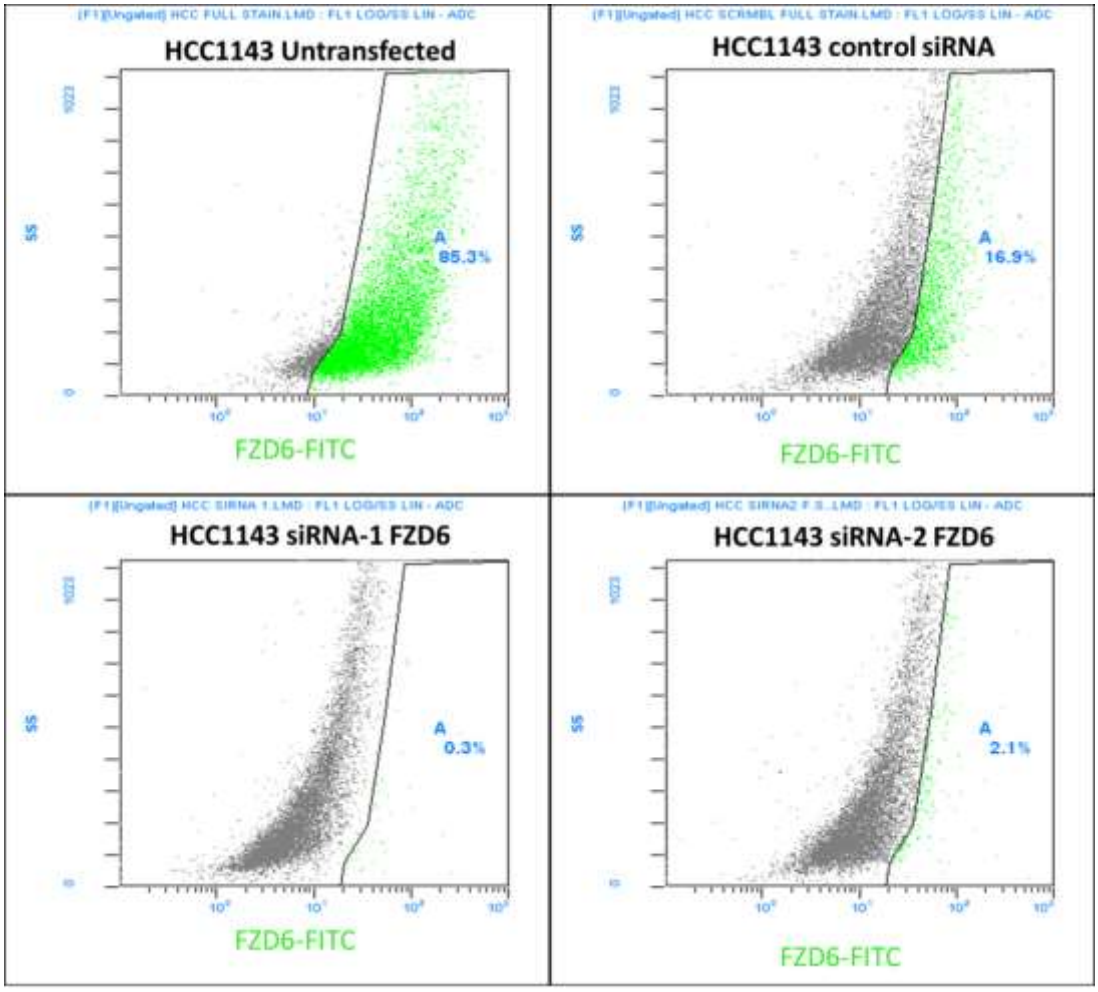

Figure 4.2: FACS analysis of the HCC1143 cell line transfected with siRNAs targeting FZD6. HCC1143 cells were transfected with the indicated siRNAs and subjected to FZD6 indirect immunostaining. Cells stained with secondary antibody only were used as a blank. FZD6-positive cells are shown in green.

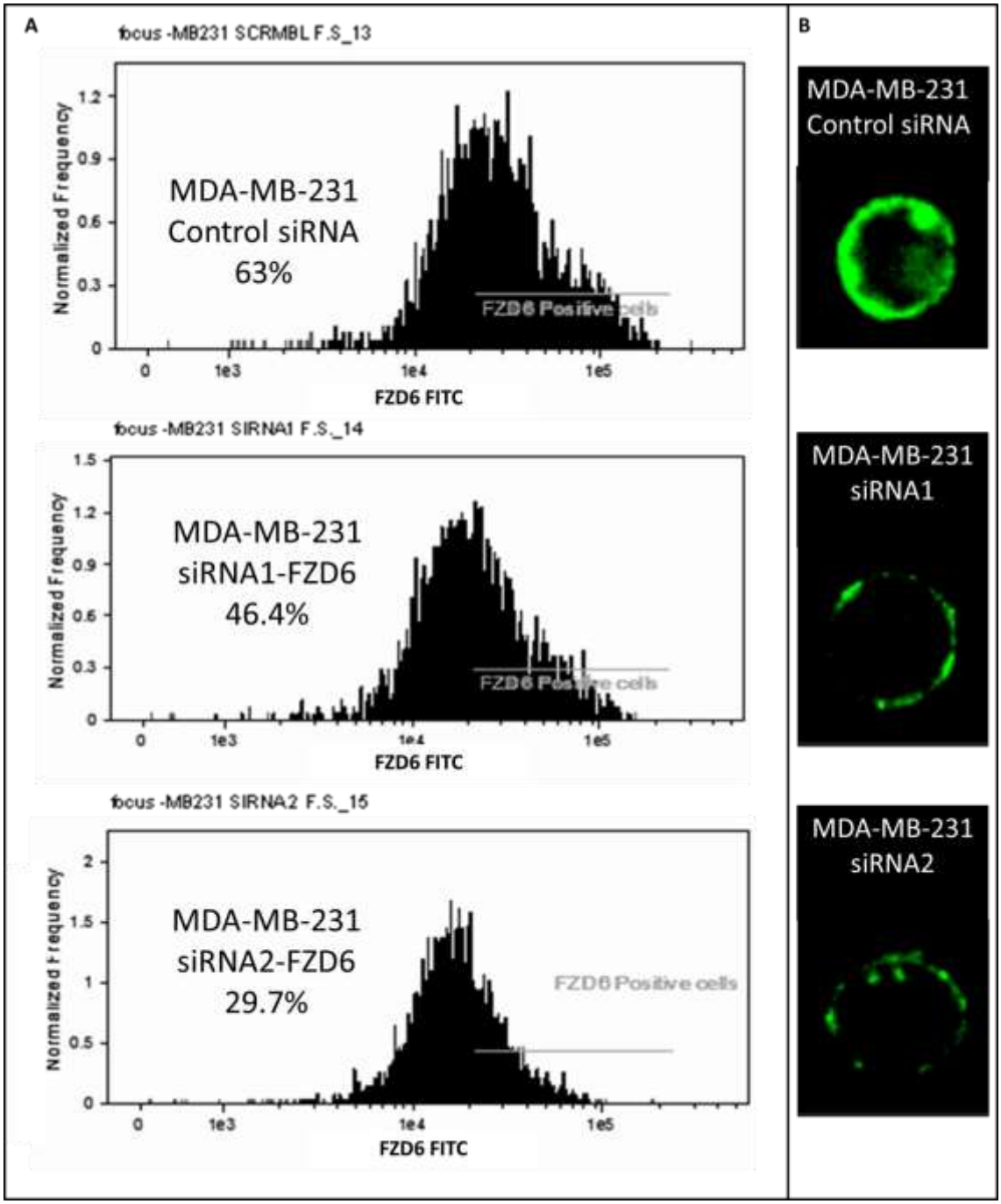

Figure 4.3: Knock down efficiency on MDA-MB-231 cells. (A) Imagestream analysis showing quantification of FZD6 expression in MDA-MB-231 cells after transfection with siRNAs-FZD6 or control siRNAs. Cells stained with secondary antibody only were used as a blank. **(B)** Imagestream analysis showing single cells with surface expression of FZD6 in the presence of the indicated siRNAs.

For a stable knock down the cell line MDA-MB-231 was infected with lentiviral vectors expressing shRNAs targeting FZD6 (Sh1 and Sh2 FZD6). A nonspecific shRNA (Scrambled) was used as a negative control. Infected cells were then selected with puromycin and analysed with RT-QPCR, western blot and FACS to assess knock down efficiency. Both sh1 and sh2 lentiviral constructs were

able to produce an almost complete abrogation of FZD6 protein expression (fig.4.4).

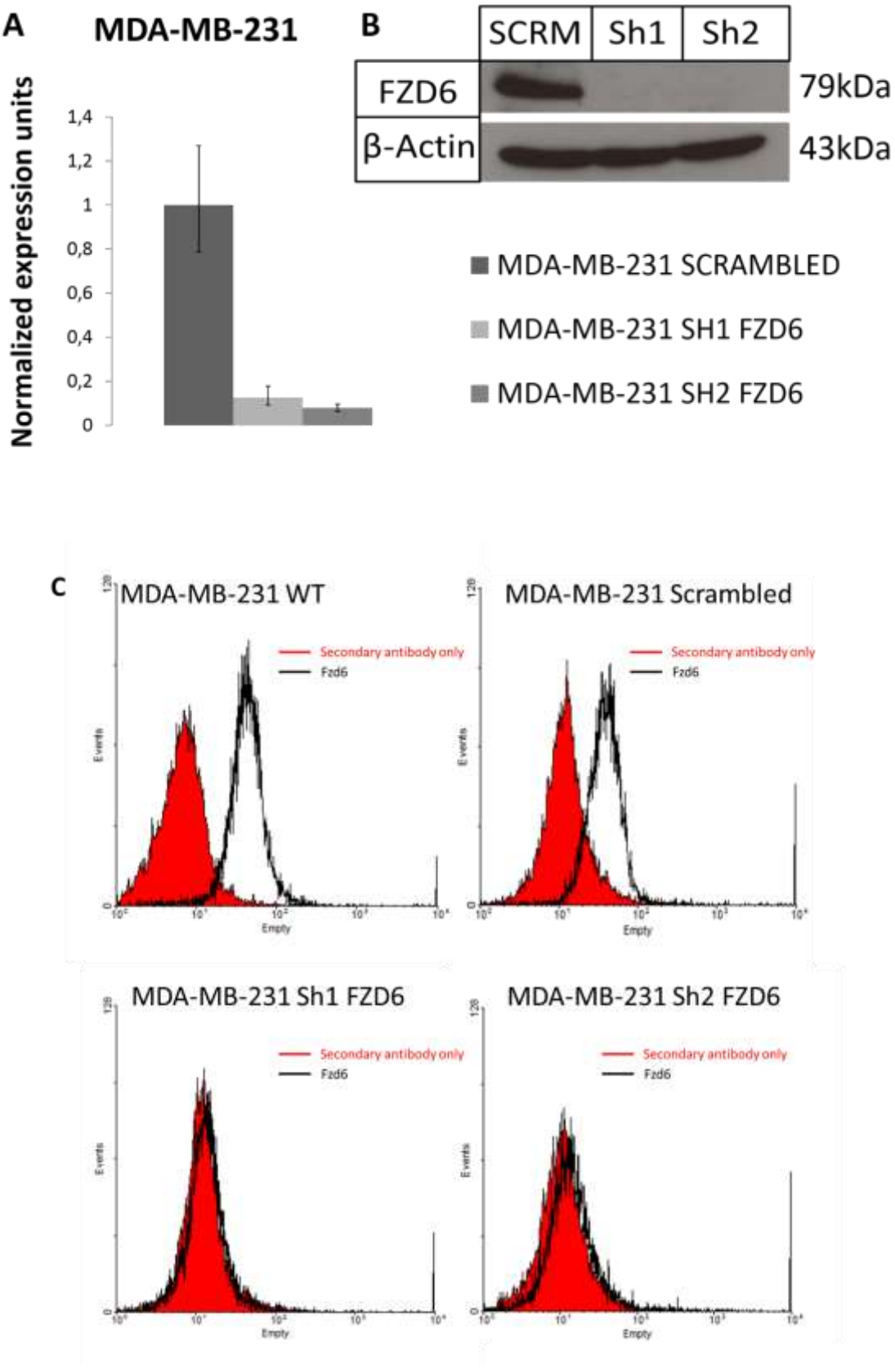

Figure 4.4: Knock down efficiency on MDA-MB-231 cells stably expressing control shRNA (Scrambled) or FZD6 shRNAs (Sh1 and Sh2 FZD6). (A) RT-QPCR analysis **(B)** Western blot analysis **(C)** FACS analysis of live cells showing membrane staining of FZD6. Wild type cells (WT) were also included in the analysis. Knock down assessment was carried out regularly throughout this research. Cells resuspended with secondary antibody, but not primary antibody, were used as a blank.

4.3 FZD6 knock down does not affect cellular proliferation

We used the MTS or XTT assays to assess cell metabolism/proliferation. These assays exploit the capacity of tetrazolium salts (MTS and XTT) to develop a red colour when reduced in a formazan product by living cells. The intensity of the colour is proportional to the cell metabolic activity and, indirectly, to cell number (Buttke, McCubrey and Owen, 1993). To test the effects of FZD6 knock down in cellular proliferation, the different breast cancer cell lines were transfected with FZD6 or control siRNAs and then plated in 96 well plates. 24 hours later, MTS was added to the cells and the absorbance read after 2 hours. Relative cell proliferation/metabolic activity of cells was expressed as optical density relative to controls. FZD6 knock down did not perturb metabolism/proliferation of breast cancer cells with the exception of the HCC1143 cell line (Fig. 4.5).

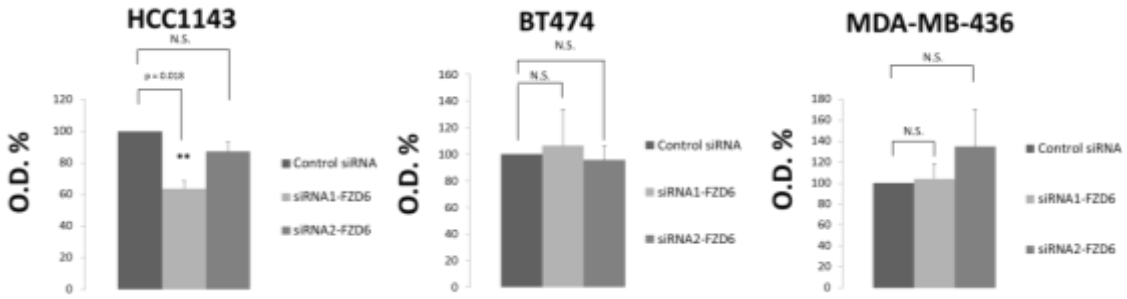

Figure 4.5: MTS assay of breast cancer cells after FZD6 knock down with siRNAs. Cells were transfected with control siRNA or siRNAs targeting FZD6 (siRNA 1 and siRNA 2). Metabolic activity is expressed as optical density (O.D.) units normalized to the control siRNA. Absorbance was read 24 hours after the cells were plated. Error bars indicate standard errors and asterisks indicate statistical significance (student t-test, n=3). N.S. indicates non significant differences.

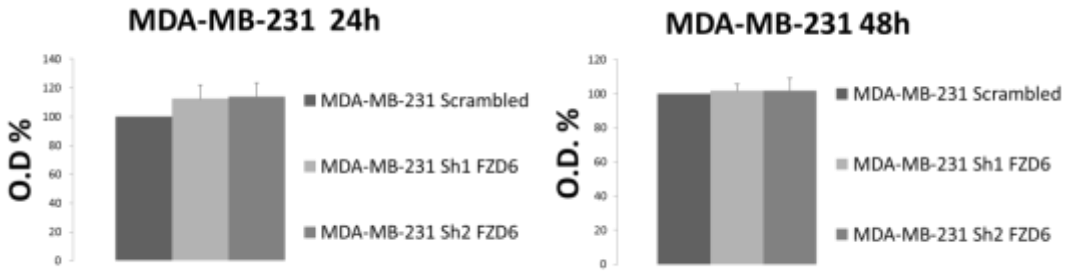

Figure 4.6: XTT assay of the MDA-MB-231 cell line expressing FZD6-shRNAs or control shRNA. Metabolic activity is expressed as optical density (O.D.) units normalized to the scrambled shRNA. Absorbance readings were made 24 hours and 48 hours after cells were plated. Three independent experiments were carried out in triplicate, error bars indicate standard errors. No significant differences were observed (student t-test, n=3).

Next, I assessed the proliferation of MDA-MB-231 cells stably expressing shRNAs targeting FZD6 (sh1-FZD6 and sh2-FZD6) or control shRNA (Scrambled). Cells were plated in 96 well plates, and the XTT assay was performed after 24 or 48 hours (fig.4.6). No significant differences were observed in cell proliferation in the presence of FZD6 downregulation.

Cell cycle analysis was also carried out on these cells (fig.4.7). The knock down of FZD6 did not alter significantly cell cycle profiles nor the Sub G1 DNA content of MDA-MB-231 cells.

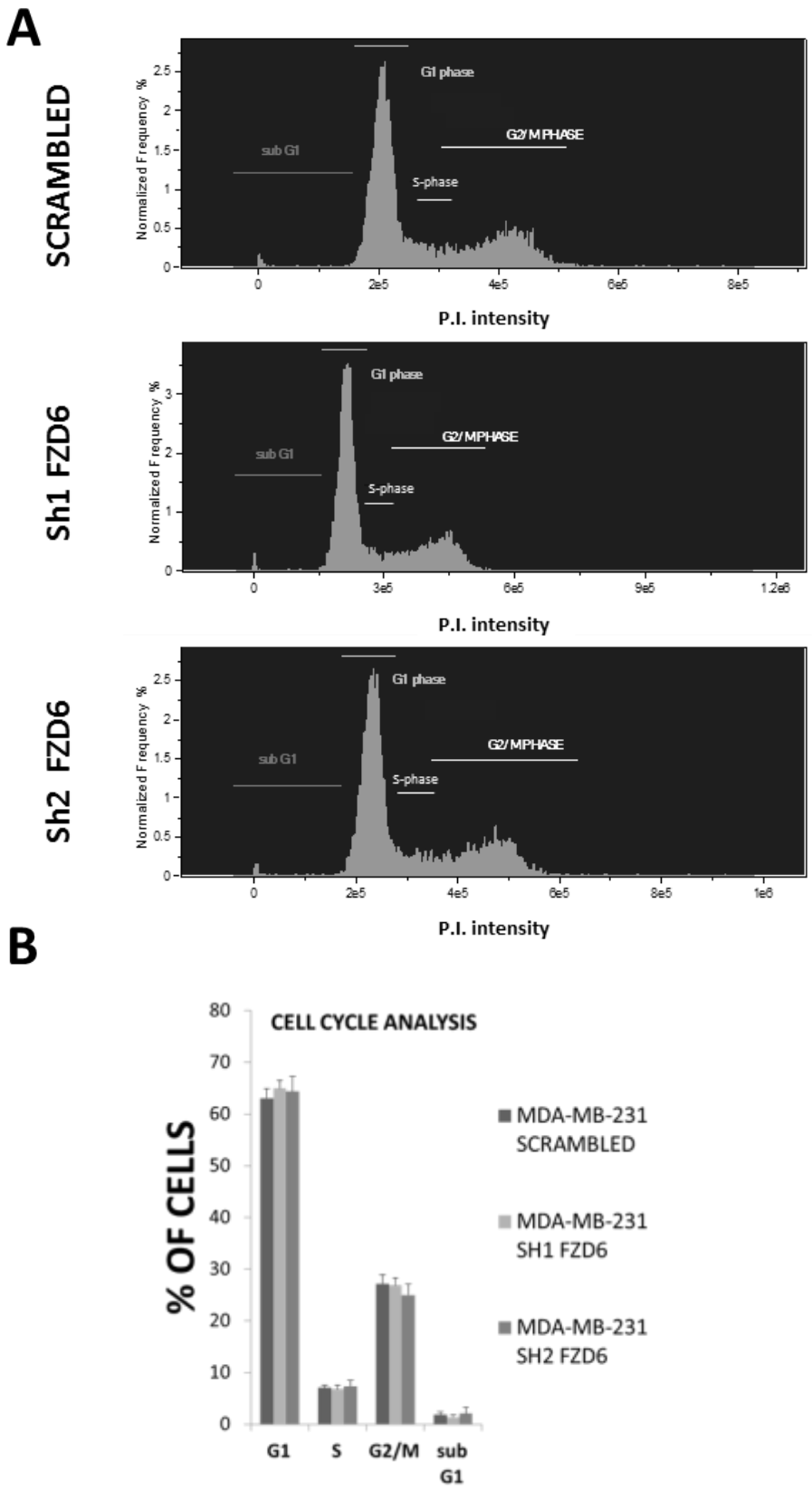

Figure 4.7: Cell cycle analysis on MDA-MB-231 cells in the presence of control shRNA or FZD6 shRNAs. (A) Representative experiment showing cell cycle profiles after propidium iodide staining and FACS analysis. **(B)** Plot showing the percentage of cells in different phases of the cell cycle. Error bars indicate standard error. The results shown represent the average of three independent experiments. No significance differences were observed (student t-test, n=3).

4.4 FZD6 knock down reduces the invasion and motility of breast cancer cells

To assess the invasion of breast cancer cells *in vitro*, I carried out invasion assays based on the capacity of cells to trespass a matrigel membrane. Cells were resuspended in serum free medium and seeded in the upper compartment of a Boyden chamber. The bottom of this compartment consists in a porous membrane covered in matrigel. The lower compartment of the Boyden chamber was replenished with medium containing a chemoattractant (FBS or EGF) to generate a chemotactic gradient.

In the presence of FZD6 siRNAs, the invasion capacity of breast cancer cells was drastically reduced (fig. 4.8). The cell line MDA-MB-436, that does not express membranous FZD6 (fig. 3.9), was used to rule out off-target effects of the siRNAs. Transfection of FZD6 siRNAs in MDA-MB-436 cells did not produce any effect in cell invasion, confirming siRNA specificity.

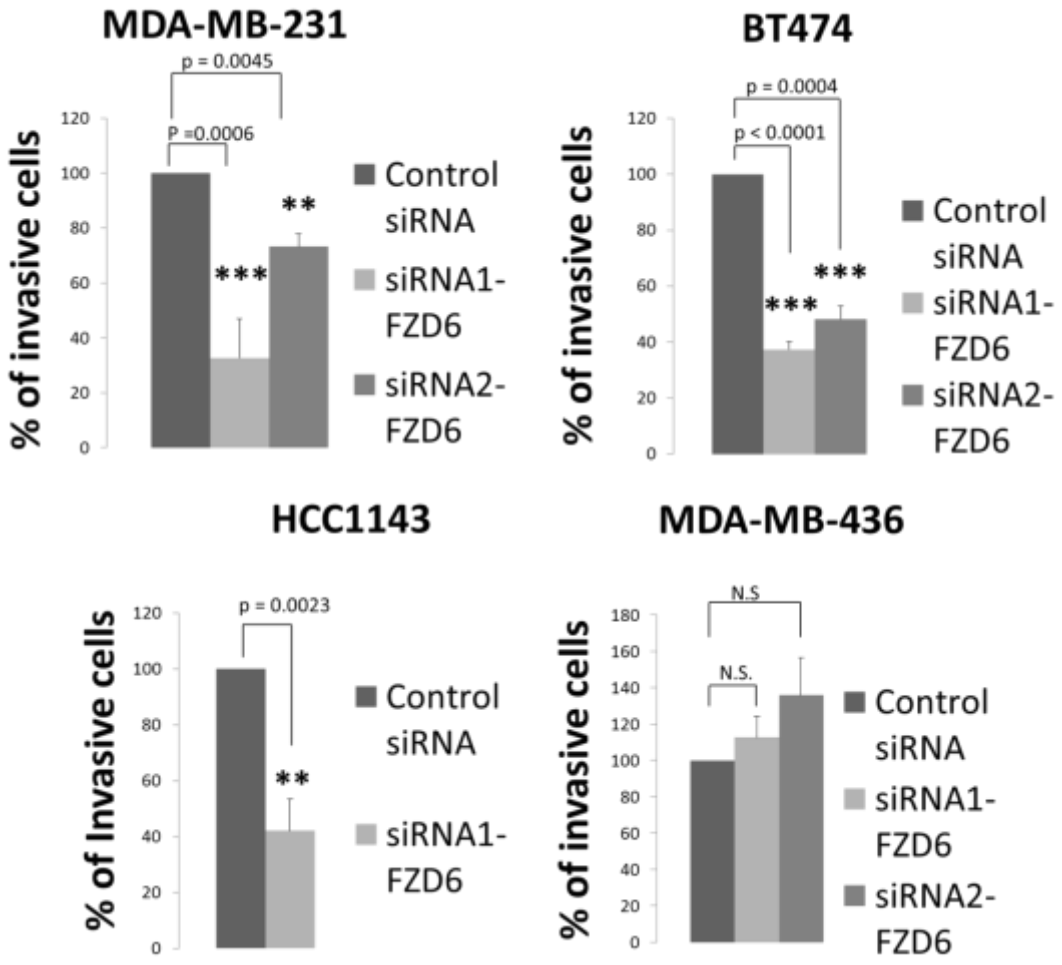

Figure 4.8: In vitro invasion assays of breast cancer cell lines transfected with control siRNA or siRNAs targeting FZD6. Invasive cells are expressed as percentage relative to the control siRNA. Medium containing 10% FBS was used as chemoattractant. Error bars indicate standard errors and asterisks indicate statistical significance (student's t-test, n=3). N.S. indicates non significant differences.

In agreement with the siRNA experiments, we also observed a marked reduction of the FBS-mediated invasion of MDA-MB-231 cells stably expressing FZD6 shRNAs (Fig.4.9). Similar results were observed when invasion was stimulated with EGF. Cells expressing control shRNA were 2.5 fold more invasive in the presence of EGF compared to non stimulated cells. Conversely, Sh1-FZD6 cells were not responsive to EGF stimulation (fig. 4.9).

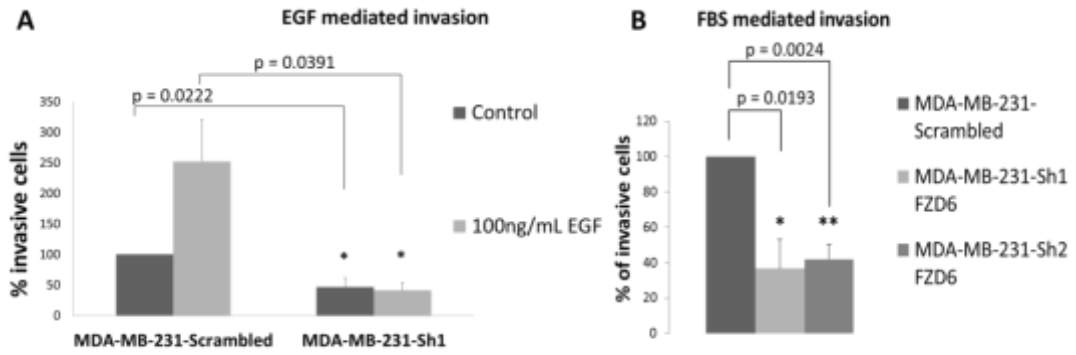

Figure 4.9: Invasion assays of the MDA-MB-231 cell line in the presence of control ShRNA or shRNAs targeting FZD6: (A) EGF mediated invasion. Cells resuspended in serum free media were seeded in a Boyden chamber where the bottom well was replenished with serum free medium (Control) or serum free medium with the addition of 100ng/mL EGF. **(B)** FBS mediated invasion. Cells were resuspended in serum free media and seeded in a Boyden chamber where the bottom well was replenished with medium containing 10% FBS. Invasion rates are expressed as percentage of the controls. Error bars indicate standard error. Asterisks indicate statistical significance (student t-test, n=3).

To assess whether FZD6 was important for cell motility, I carried out wound healing assays. This technique is widely used to assess directional motility of cells *in vitro (Rodriguez, Wu and Guan, 2005).* Confluent cells growing in monolayer are scratched with a pipette tip and placed in a live imaging system microscope. The scratch induces a migratory response that stimulates cells to migrate in a polarized fashion towards the wound. MDA-MB-231 cells expressing scrambled or sh2 FZD6 shRNAs were analysed simultaneously after the wound. Photographs from 4 different areas of the scratch were taken every 20 minutes over the range of 24 hours. Control cells were able to close the wound completely after 14h, whereas the wound was still visible in FZD6 depleted cells at 14 and 19 hours (Fig. 4.10 A). Cell motility was quantified tracking single cells trajectories and quantifying average speed using image analysis software. FZD6 knock down reduced cell speed to 16μm/h compared to the 22 μm/h of control cells (fig.4.10 B).

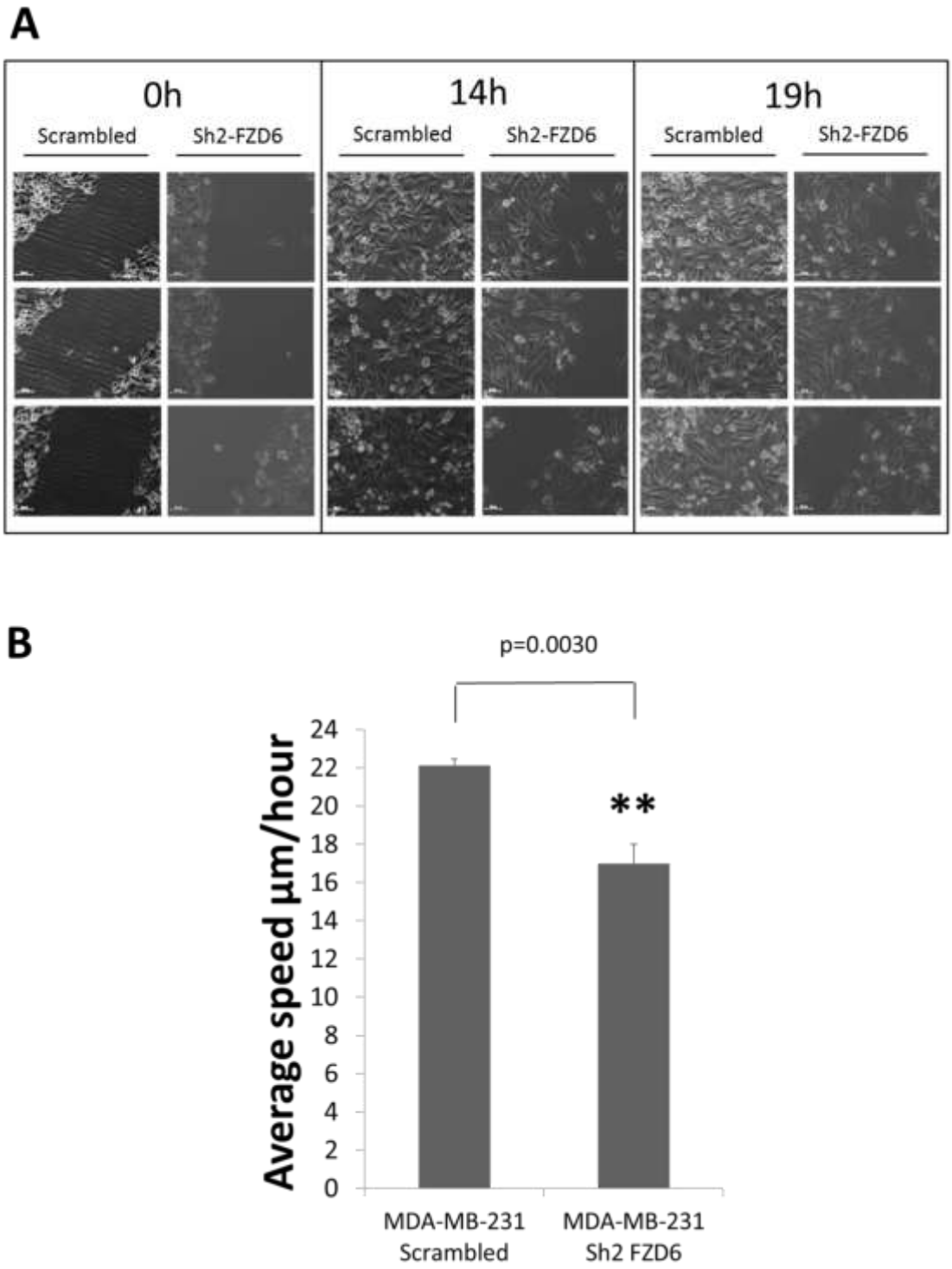

Figure 4.10: Wound healing assay. MDA-MB-231 cells infected with lentiviruses containing control (Scrambled) or Fzd6 shRNA (Sh2) were grown as a monolayer and then scratched with a pipette tip. Cells were placed in a live imaging system and filmed for 24 hours. **(A)** Examples of pictures taken at the indicated times showing the closure of the wound in control (scrambled) but not in FZD6 downregulated (Sh2 –FZD6) cells. **(B)** Quantification of locomotion of FZD6-expressing versus non-expressing cells. The average speed of 10 cells for each wound was calculated. 4 wounds per shRNA type were analysed. Error bars indicate standard errors and asterisks statistical significance (student's t-test, n=4).

4.5 FZD6 knock down changes the shape of breast cancer acini in a 3D culture system

The data described in this section was produced in collaboration with Sibylle Ermler and Elisabete Silva from the Institute for the Environment, Department of Life Sciences, Brunel University London. Sibylle Ermler and Elisabete Silva carried out the 3D cultures and the immunofluorescence staining, and I performed the image analysis on the acini photographs.

Many features of cells are not reproducible in a bidimensional culture, as they require the interaction with a tridimensional matrix (Lee *et al.*, 2007). The morphology of organoids growing in a tridimensional environment can be indicative of the malignancy of cells. For example, transformed mammary epithelial cells maintain the capacity to form organized acini in 3D culture, whereas breast cancer cells generate organoids in which the normal tissue organization and architecture are lost (Petersen *et al.*, 1992).

To study the role of FZD6 in the structural organization of breast cancer organoids, we cultured MDA-MB-231 cells expressing control or FZD6 shRNAs in a 3D matrigel matrix. Notably, in the absence of FZD6 the symmetrical shape of breast cancer acini, measured as aspect ratio, circularity and roundness, was significantly increased (fig. 4.11).

The acini were also stained for laminin (data not shown). All the samples did not express laminin. Acini were also stained for fibronectin, but the stain did not work well enough to make conclusions about expression levels, especially because only 2 dimensional pictures were available.

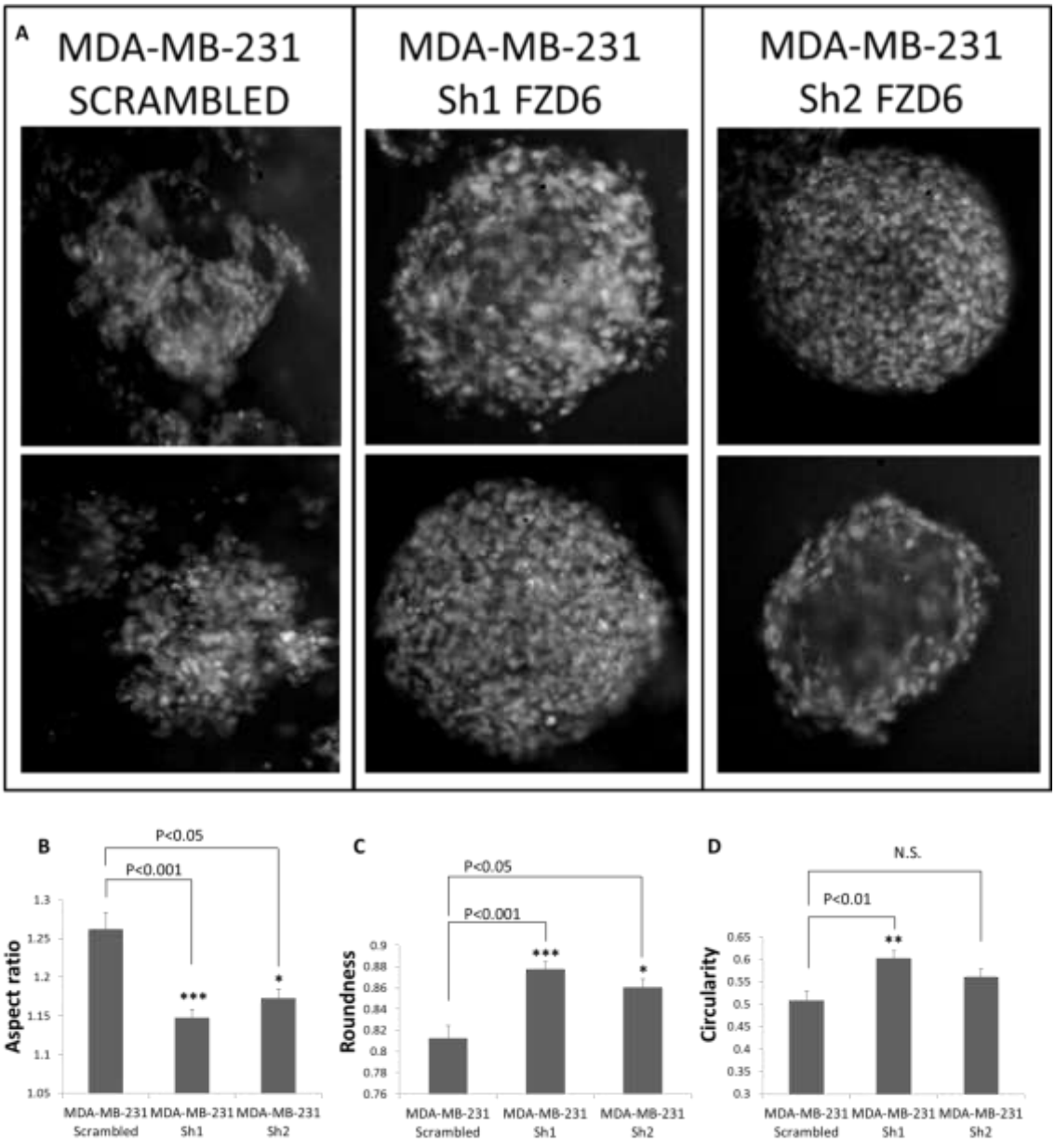

Figure 4.11: 3-D cultures of MDA-MB-231 cells. (A) Photographs of organoids formed by the different MDA-MB-231 cell lines expressing (scrambled) or non expressing (Sh1 and Sh2) FZD6 after 3 weeks in culture (DAPI staining). **(B-D)** Quantification of the aspect ratio, circularity and roundness, of the organoids described in A. The software imageJ was used to analyse the geometrical characteristics of the organoids. Error bars indicate standard errors and asterisks statistical significance (ANOVA followed by Bonferroni's post-hoc test on a minimum of 20 images of acini per well). The data shown is representative of two independent experiments.

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4.6 Discussion

The results described in this chapter demonstrate that FZD6 receptor is important for the motility and invasion of breast cancer cells (fig. 4.8, 4.9 and 4.10). On the other hand, FZD6 is not involved in cell proliferation (fig. 4.5, 4.6 and 4.7). The marginal reduction in proliferation of HCC1143 cells, in the presence of siRNA1 FZD6, is likely caused by off target effects, since the transfection of siRNA2 FZD6 did not perturb proliferation (fig. 4.5). Downregulation of FZD6 did not alter the cell cycle, nor induced apoptosis in MDA-MB-231 cells. Several investigators reported that the Wnt β-catenin-dependent pathway regulates cell proliferation. Conversely, the non-canonical pathway has been implicated mostly in cell migration (Reya and Clevers, 2005). Thus, our findings may suggest a non-canonical function for FZD6. Indeed, the knock down of FZD6 strongly supressed the invasion of all the breast cancer cell lines tested, with the exception of the MDA-MB-436 cell line which does not express membranous FZD6 (fig. 4.8 and 4.9). These results might help to explain why patients bearing tumours expressing high level of FZD6 have a higher chance to incur into metastatic relapse. Other frizzled receptors were previously linked to metastasis. For example, FZD7 and FZD5 play a role in the proliferation and invasion of colon, breast, and melanoma cancer cells, respectively (Yang *et al*., 2011; Ueno *et al*., 2008; Weeraratna *et al*., 2002). It is therefore likely that FZD6 regulates breast cancer metastasis in a similar fashion.

FZD6 might be important to coordinate the polarized motility of breast cancer cells, as suggested by the wound healing assay (fig.4.10). Cell movements are necessary for the coordination of many biological functions during morphogenesis, tissue healing and immune response (Ananthakrishnan and Ehrlicher, 2007). During morphogenesis, the WNT/planar cell polarity plays an important role in the regulation of embryonic cells polarized movements. This is a well conserved mechanism that was observed in the organogenesis of drosophila, caenorhabditis elegans and in vertebrates (Seifert and Mlodzik, 2007). Aberrant activation of these signals, normally active solely during morphogenesis, was also reported in cancer (Wang, 2009). Despite the canonical pathway has been an object of more studies, the PCP pathway has emerged as an important component of cancer development and progression

(Kaucka et al., 2013; Luga and Wrana, 2013). Recently, Luga and colleagues showed that FZD6 co-localise with PCP components in breast cancer cell protrusions during cell locomotion (Luga *et al.*, 2012). It is thus likely that the upregulation of FZD6 in breast cancer cells could lead to increased motility and invasion through the activation of the PCP pathway. A further confirmation of these assumptions is given by studies on the nervous system. FZD6 is required for correct convergent cell extension during neurulation and its abrogation or mutation results in neural tube and midbrain morphogenetic defects in mice (De Marco *et al.*, 2012; Stuebner *et al.*, 2010; Wang *et al.*, 2006). Thus, we could hypothesize that FZD6 mediates the polarized extension movement of breast cancer cells during the metastatic process activating similar signalling pathways.

The role of FZD6 in regulating invasion is further suggested by 3D culture experiments. Non-transformed breast epithelial cells, growing in a 3D extracellular matrix, form normal and structurally organized acini. Malignant cells instead, form irregular organoids that lack tissue polarity (Petersen *et al.*, 1992). The architecture of breast cells in a 3d matrix influences cellular behaviour. For example, the disruption of the three dimensional organization with the ectopic expression of a dominant RAC mutant (RAC is an important member of the PCP signalling), results in increased VEGF secretion and cell migration of breast cancer cells (Chen *et al.*, 2009). Highly malignant MDA-MB-231 cells form “stellate” structures with disorganized nuclei and elongated invasive processes (Kenny *et al.*, 2007). The depletion of FZD6 in these cells resulted in the formation of more regular acini, similar to the ones formed by non-malignant breast cells (fig.4.11). Furthermore, FZD6 depletion caused the reduction of elongated invasive protrusion branching from the acini’s central bulk. These results suggest that the overexpression of FZD6 in breast cancer cells could disrupt tissue architecture and promote invasion.

All together these findings underline the importance of FZD6 in promoting breast cancer cells invasion and motility and its role in the maintenance of tissue polarity.

CHAPTER V

Assessment of the role of FZD6 in the *in vivo* growth of breast cancer cells

5.1 Introduction

To understand the role of FZD6 in tumour growth and metastatic spread, we carried out orthotopic transplantations of MDA-MB-231 cells, with or without FZD6 expression, in immunodeficient mice. MDA-MB-231 cells are highly tumorigenic when injected into immunodeficient mice and are commonly used to model breast cancer metastasis *in vivo*. Mice used in the study belong to the strain NOD-SCID-γ (NSG). These animals bear mutations that result in the complete loss of immunity, allowing xenotransplantation of human cells. The first is a mutation of the PRKDC gene, commonly known as $Prkdc^{scid}$, a loss of function mutation that results in the disruption of adaptive immunity by drastically reducing the number of mature B and T cells (Greiner, Hesselton and Shultz, 1998). The second is the $Il2rg^{tm1Wjl}$ mutation, which affects the gene encoding the interleukin 2 receptor gamma chain, resulting in the arrest of the differentiation of natural killer cells (Willerford *et al.*, 1995).

The *in vivo* transplantation and the analysis of tumour tissue sections were carried out by our collaborators Dr Manuela Iezzi and Dr Alessia Lamolinara at the Aging Research Center, G. D'Annunzio University, Chieti, Italy.

5.2 FZD6 depletion does not affect the growth of MDA-MB-231 primary tumours

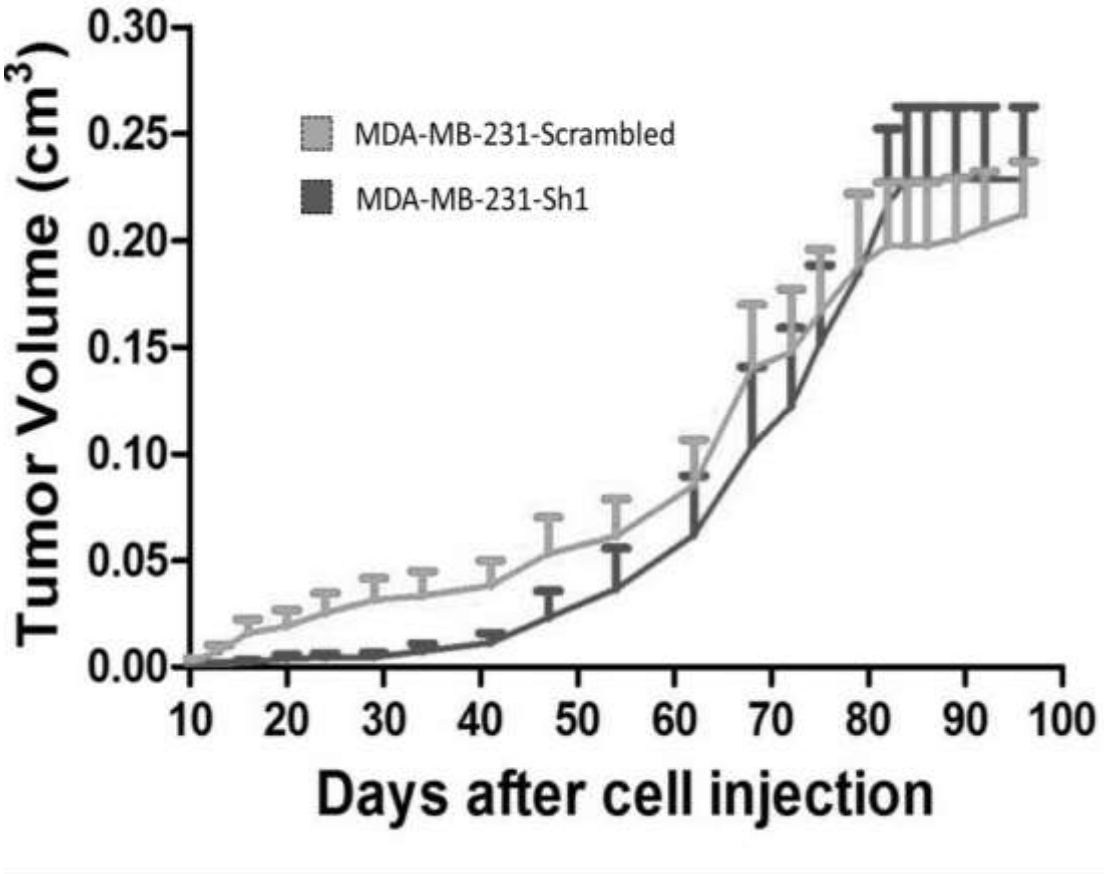

Figure 5.1: Orthotopic xenotransplantation of MB-MDA-231 cells with or without Fzd6 expression in NSG mice. MB-MDA-231 cells infected with a lentivirus containing a FZD6 (Sh1) or control shRNA (scrambled) were injected into the fat pad of immunocompromised NSG mice. Growth of the primary tumour was measured two times per week using callipers. Error bars indicate standard errors. 10 mice per group were used in this study. This experiment was carried out by our collaborators Dr Manuela Iezzi and Dr Alessia Lamolinara at the Aging Research Centre, G. D'Annunzio University, Chieti, Italy.

Mice were divided in two groups: 10 mice were injected with MDA-MB-231 cells infected with the control shRNA. A second cohort of 10 mice was injected with FZD6 depleted MDA-MB-231 cells. Despite the growth of FZD6 negative cells was slightly delayed in comparison to control cells, at the end of the experiment the size of the primary tumour masses was similar in the two groups (fig.5.2).

5.3 FZD6 depletion inhibits the invasion of breast cancer cells to the bone, liver and the heart of mice

Mice were sacrificed after 96 days from tumour cells injection or when tumour size reached the volume of 0.3 cm^3. The organs were harvested, fixed and embedded in paraffin. Histological sections were stained with haematoxylin /eosin and analysed by two pathologists independently.

The number of mice bearing metastases is plotted in figure 5.3. Tumour metastases were detected in lymph nodes, kidneys, pancreas, heart, bones, lungs and liver, but not in brain, stomach and intestine (figure 5.4 and 5.5).

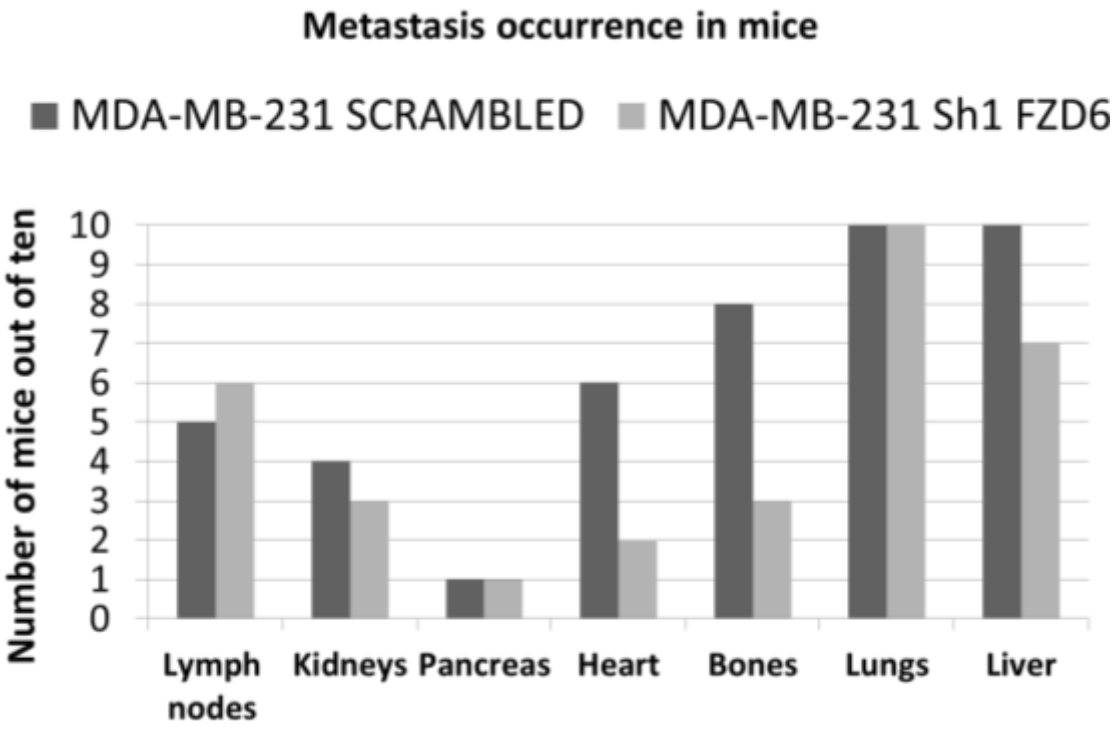

Figure 5.2: Metastatic occurrence in NSG mice injected with MDA-MB-231 cells, with or without FZD6 expression. The graph shows the number of mice with breast cancer metastasis in specific organs, as determined after histological analysis. This experiment was carried out by our collaborators Dr Manuela Iezzi and Dr Alessia Lamolinara at the Aging Research Centre, G. D'Annunzio University, Chieti, Italy.

Depletion of FZD6 only marginally changed the frequency of metastasis in the lymph nodes, kidneys, pancreas, lungs and liver compared to the FZD6 proficient counterparts. However, FZD6 negative cells were less prone to invade the heart (Scrambled group = 6/10 mice, Sh1 FZD6 group 2/10 mice) and bones (Scrambled group 8/10, sh1 group 3/10).

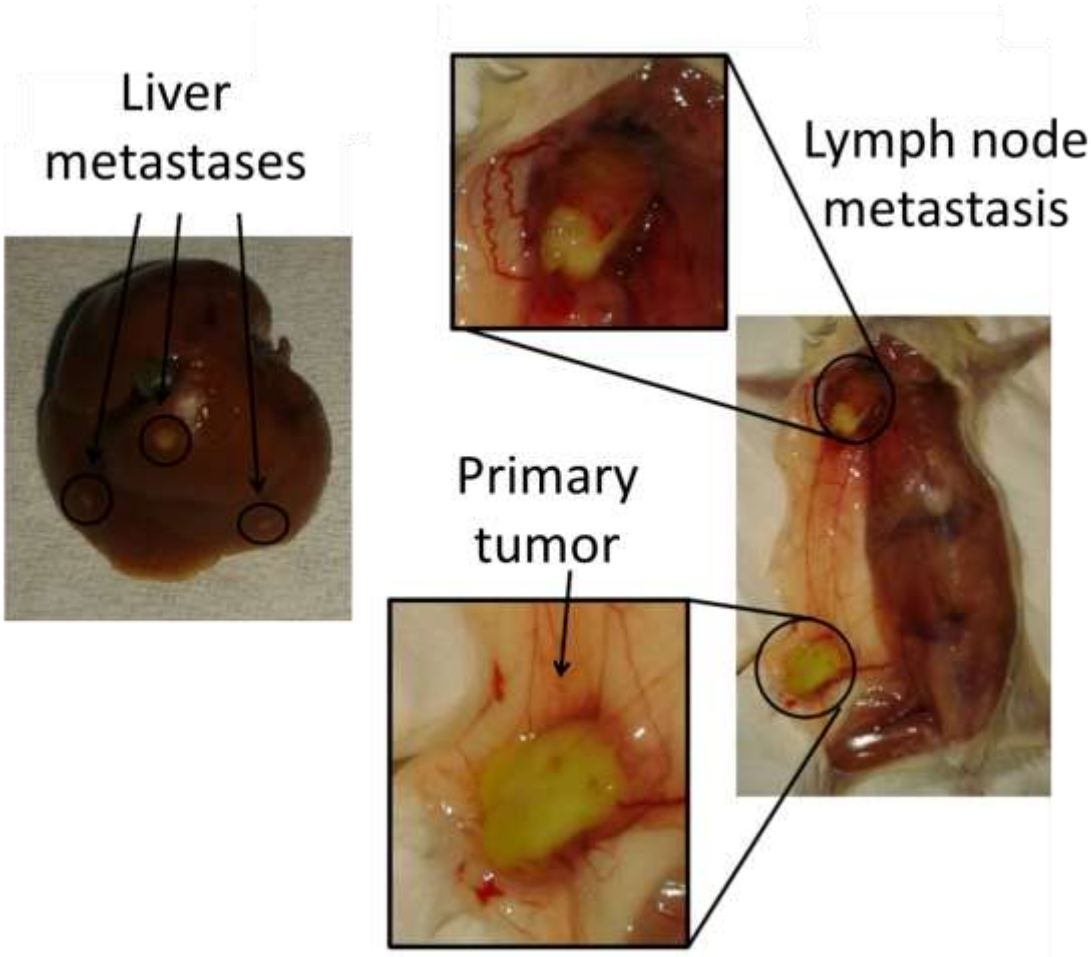

Figure 5.3: Representative photographs of MDA-MB-231 tumours in NSG mice: Breast cancer tumour cells are easily identifiable, since the lentiviral construct used to infect cells also express GFP.

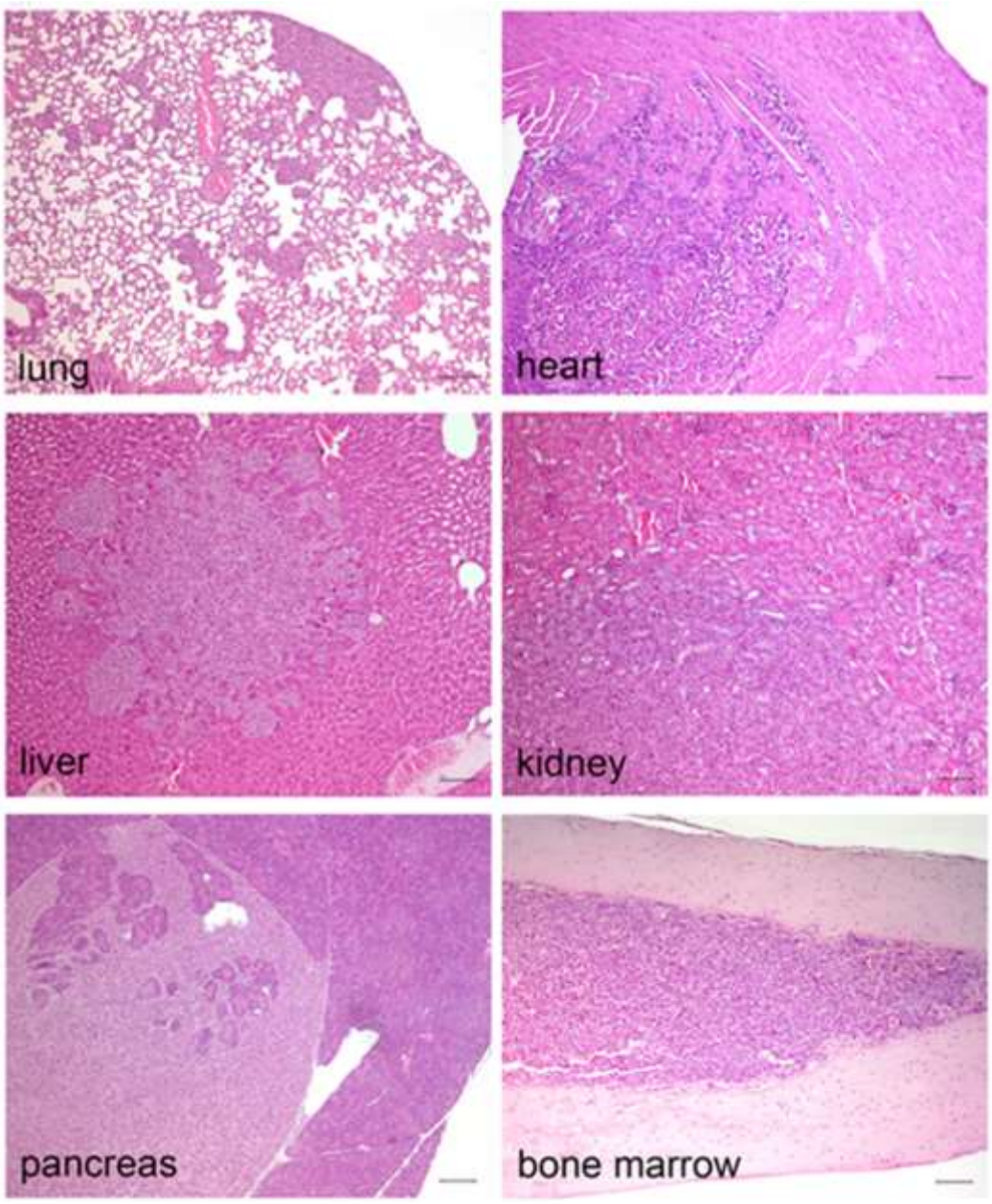

Figure 5.4: haematoxylin-eosins staining of representative organ sections showing metastatic dissemination of MDA-MB-231 cells. Tissue sections were prepared and stained by our collaborators Dr Manuela Iezzi and Dr Alessia Lamolinara at the Aging Research Centre, G. D'Annunzio University, Chieti, Italy.

To quantify microscopic metastases, lungs and livers were cut into thin sections. A semiquantitative evaluation based on the number and size of metastases was carried out (Table 5.1).

In the lungs, metastases were similar in number and shape in the two groups, whereas in the liver, metastases originated from FZD6 positive cells where bigger and more numerous compared with the FZD6 negative counterparts (metastatic score scrambled: 27, metastatic score sh1 FZD6: 13).

METASTASTIC SCORE										
LUNGS										
	MDA-MB-231 Scrambled					MDA-MB-231 Sh1				
METASTATIC SCORE	0	1	2	3	4	0	1	2	3	4
NUMBER OF MICE OUT OF 10	0	2	6	0	2	0	3	2	1	4
SCORE	0	2	12	0	8	0	3	4	3	16
TOTAL SCORE LUNGS	22					26				

METASTASTIC SCORE										
LIVER										
	MDA-MB-231 Scrambled					MDA-MB-231 Sh1				
METASTATIC SCORE	0	1	2	3	4	0	1	2	3	4
NUMBER OF MICE OUT OF 10	0	3	2	0	5	3	3	2	2	0
SCORE	0	3	4	0	20	0	3	4	6	0
TOTAL SCORE LIVER	27					13 *				

Table 5.1: Metastatic score in lungs and liver of NGS mice injected with MDA-MB-231 cells with or without FZD6. A semiquantitative evaluation was performed on organs sections, attributing to each sample a value from 0 to 4, based on the number and size of metastases; 0 was assigned to mice without metastases; 1 was attributed to organs with few small metastases, 4 to organs with numerous large metastases. Asterisk indicates statistical significance, p=0.0265, (student t-test, n=10). This experiment was carried out by our collaborators Dr Manuela Iezzi and Dr Alessia Lamolinara at the Aging Research Centre, G. D'Annunzio University, Chieti, Italy.

5.4 Discussion

The results described in this chapter are in agreement with the *in vitro* studies, further suggesting a role for FZD6 in breast cancer metastasis. FZD6 is largely dispensable for breast cancer cell proliferation *in vivo*, but is important to drive the metastatic spread of breast cancer cells to the bone, liver and heart of mice.

Cancer cells acquire a metastatic phenotype in a complex multistep process that involves modification of a number of signalling pathways. The first step in this process is the loss of the molecular bonds that keep cells together, such as cell adhesion molecules (CAM) and cadherins (Cavallaro and Christofori, 2004; Edelman and Crossin, 1991). In order to migrate, metastatic cells need to promote dynamic interaction with the extracellular matrix (ECM). In this process, the receptors for the integrins play a key role (Desgrosellier and Cheresh, 2010). Metastatic cells also require the secretion of proteases to open a way through the stroma (Koblinski, Ahram and Sloane, 2000) and reach blood or lymph vessels. The most common way of dissemination of cancer cells is through the blood stream. Metastatic cells are capable to traverse the blood vessels in a process called intravasation (Wyckoff *et al.*, 2000). An alternative transportation way is through the lymph vessels. The inverse process, the extravasation, allows metastatic cells to get from the blood stream to distant organs, where eventually, they can initiate new tumour colonies (Chambers, Groom and MacDonald, 2002). The capability of malignant cells to form metastases largely depends on the characteristics of the microenvironment that harbour these cells in the host organ (often referred as metastatic niche), according to the “seed and soil” model described for the first time by Paget (Paget, 1889). The metastatic niche could also be induced by the primary tumour through the secretion of growth factors, which in turn, lead to biochemical changes in distal organs. These changes could result in a receptive microenvironment defined as a “pre-metastatic niche”, which promotes the migration and spread of metastatic cells at these sites (Psaila and Lyden, 2009).

It is a widely held view that cancer stem cells are crucial in the malignant progression of tumours (Li *et al.*, 2006). Cancer stem cells are characterized by a mesenchymal, motile phenotype, therefore are likely candidates to promote cancer metastasis (Mani *et al.*, 2008). Although a lot of progresses have been

made to uncover the molecular basis of cancer metastasis, it is still unclear how certain tumours have privileged sites of metastatization. For example, breast and prostate cancer often metastasise in the bones. This selectivity cannot be explained merely by the anatomical disposition of the blood vessels draining the primary tumours, but implies the existence of biological processes that promote the specific engraftment to these organs (Nicolson, 1988). The WNT pathway has a pivotal role in controlling cancer stem cells maintenance and self-renewal, through both autocrine and paracrine signals (Reya and Clevers, 2005). It is thus possible that tissue specific WNT signals are required for harbouring cancer stem cells migrating from a primary tumour to a metastatic site. The tissue specific expression of WNT proteins could thus explain the selective organotropism of certain tumours.

The metastatic niche is maintained also through the recruitment of stromal cells such as macrophages and fibroblasts that intimately interact with cancer cells (Mantovani and Sica, 2010; Kalluri and Zeisberg, 2006; Pollard, 2004). Since these cells have a different morphology and secrete different growth factors depending on the tissue of origin (Baum and Duffy, 2011), it is likely that they also play a role in the organ-specific tropism of cancer cells. The WNT pathway is important in the communication between cancer cells and stromal cells. For instance, it has been shown that mammary murine fibroblasts secrete WNT-1 to promote breast tumorigenesis in mice (Jue *et al.*, 1992). Furthermore, human mammary fibroblasts release exosomes that stimulate breast cancer cells to produce WNT11 that in turn promotes cell motility through the PCP pathway (Luga *et al.*, 2012). Mammary fibroblasts also have a role in promoting a pre-metastatic niche: Melanchi and colleagues showed that mouse mammary cancer cells stimulate fibroblasts to produce periostin, a component of the extracellular matrix, in the lungs. Periostin in turn, recruits WNT signals that are essential for maintenance of cancer stem cells colonizing to the lungs. Block of Periostin prevents lungs metastasis in mice (Malanchi *et al.*, 2012). Macrophages might also be important in defining the organ colonization of metastatic cells by releasing tissue specific factors (Stout and Suttles, 2004). Macrophages are involved in all the limiting steps of cancer metastasis, including the generation of the metastatic niche. Their role in tumorigenesis is, at least in part, controlled by the secretion of WNT ligands. It is therefore possible that macrophages could be important in controlling the WNT signals

that are responsible for the homing of metastases in secondary site (Qian and Pollard, 2010; Oguma *et al.*, 2008; Pukrop *et al.*, 2006).

The selective organotropism of cancer metastasis is well documented for the bones, since this is the elected site of metastasis for two of the most common tumours, prostate and breast cancer (Bubendorf *et al.*, 2000; Coleman and Rubens, 1987). The Bone is mainly structured in a hard calcified matrix, therefore early events should occur to degrade this matrix (osteolysis) and make it receptive to harbour cancer cells. Furthermore, release of growth factors trapped within the bone matrix could be important survival signals for the colonization of metastatic cells (Yoneda and Hiraga, 2005). The WNT pathway is essential for bone development and bone remodelling from early to adult life (Macsai, Foster and Xian, 2008). Indeed, DKK1 or sFRP-1 knock-out mice, two endogenous WNT inhibitors, have severe bone development deficiency (Hall and Keller, 2006). The knock down of LRP5, which is a frizzled co-receptor, leads to low bone mass, deformities, and fractures. Not surprisingly, the WNT pathway also mediates molecular events that are important for the metastatic spread of certain cancers to the bones. For example, in mice models of prostate cancer metastasis, Dickkopf-1 (DKK-1), which antagonizes WNT activity, is secreted by prostate cancer cells in early stage to promote osteolysis and colonization in the bone. As metastases progress, DKK1 is downregulated and this results in abnormal production of bone tissue (osteoblastic metastasis) through the inhibition of the WNT pathway (Hall *et al.*, 2005). This would suggests that cross talk of cancer cells with the metastatic niche through the WNT pathway could be, at least in part, responsible for the organ selectivity of pancreatic cells. The WNT inhibitor DKK-1 could be also involved in the organotropism of multiple myeloma metastases, where its overexpression has been linked to the onset osteolytic lesions (Tian *et al.*, 2003). In breast cancer numerous secreted factors are known to enhance tropism of cancer cells to the bones, although the mechanisms are still largely unknown. It is a widely held view that osteoclasts and osteoblasts, that respectively digest and produce new bone tissue, play an important part in the onset of breast cancer bone metastases. Indeed, it has been reported that breast cancer cells can alter the balance between the activity of osteoblasts and osteoclast, leading to osteolytic and, less frequently, osteoblastic lesions (Yoneda and Hiraga, 2005). It has been proposed that breast cancer cells mimic in the bones what happens during lactation. Normal mammary cells release the parathyroid hormone-related

protein (PTHrP) that stimulates osteoclasts, resulting in bone resorption and calcium release. This is a physiological process aimed to the enrichment of calcium in the milk. Breast cancer cells use the same mechanism to digest the bone matrix and create docking sites to colonize these organs (Lu and Kang, 2007). PTHrP also regulates the epithelial and mesenchymal expression of LEF1 and β catenin during breast development, suggesting a cross talk with the WNT pathway (Foley *et al.*, 2001). Moreover, it has been shown that PTHrp and WNT pathway cross talk to regulate endochondral bone development (Guo *et al.*, 2009). As previously mentioned, osteolysis results in the release of numerous growth factors from the bone matrix. This in turn stimulates cancer cell growth and the release of new bone metastasis factors, creating a vicious loop. Amongst these factors are IGFs, TGFβ, PDGF and BMP, which all activate pathways that have been shown to interact with the WNT signalling (Reis *et al.*, 2012; Kamiya *et al.*, 2008; Nakashima, Katagiri and Tamura, 2005; Richard-Parpaillon *et al.*, 2002; Nishita *et al.*, 2000; Coleman and Rubens, 1987). It is thus likely that the WNT pathway plays an important role in the organotropism of breast cancer cells to the bone.

In our model, the ablation of FZD6 resulted in the reduced engraftment of breast cancer cells to the bone, the heart and the liver of immunodeficient mice. Although we demonstrated that FZD6 knock down leads to a reduction of motility and invasion *in vitro*, cells with the downregulation of FZD6 are still able to metastasise efficiently in lungs and other organs. Preliminary analysis on lung tissue sections suggests that Fzd6 expression could have been re-activated in metastases originating from cells infected with the FZD6 shRNA targeting retrovirus, probably due to the absence of selective pressure (data not shown). However, since the knock down of FZD6 did not lead to a general reduction of metastasis, but resulted in an organ specific reduction, the decreased invasion and motility alone are not sufficient to justify this effect. A possible explanation could be that the different WNT signalling landscapes present in different organs contribute to create a selective metastatic niche. Thus, it is possible that the knock down of FZD6 could reduce the interactions between cancer cells and their niche in specific organs, reducing the intensity of survival signals necessary for a successful colonization. FZD6 might be also relevant in mediating the interaction between cancer cells and macrophages and/or fibroblasts in a tissue dependent manner, since these stromal cells are able to secrete WNT ligands and to stimulate cancer cell to release WNTs.

However, further experiments are necessary to elucidate the role of FZD6 in the selective organotropism of breast cancer.

CHAPTER VI

Assessment of the signalling pathways regulated by FZD6 in breast cancer cells

6.1 Introduction

In the previous chapters I have demonstrated the involvement of FZD6 in the modulation of cell invasion and migration in breast cancer. To further these findings, we investigated which signalling pathways are activated by FZD6 using a loss of function approach.

Deregulation of EGF signalling is observed in the majority of solid cancers, contributing to tumorigenesis (Woodburn, 1999). One of the effectors of the EGF receptor (EGFR) is PLC-γ1, which mediates downstream signals that regulate cell motility and cancer progression (Chen *et al.*, 1994). EGFR also mediates the activation of AKT through the PI3K signalling cascade. The AKT pathway has a crucial role in survival, proliferation and motility of cancer cells (Vivanco and Sawyers, 2002; Kim *et al.*, 2001). Two other important effectors downstream EGFR are the Extracellular-signal Regulated kinases 1 and 2 (ERK 1 and 2) which belong to the family of the MAP kinases. They also have been implicated in processes like cell proliferation, differentiation and cell invasion in breast cancer (Jorissen *et al.*, 2003; Krueger *et al.*, 2001). Several researches have previously reported interactions between EGFR and WNT signalling (Hu and Li, 2010). For instance, Schroeder et al. found that EGFR and β-catenin form heterodimers in breast tumours growing in mice, demonstrating for the first time, a direct interaction between the two pathways (Schroeder *et al.*, 2002). Moreover, ectopic expression of WNT5a and WNT-1 activates ERK 1/2 and EGFR in mammary cells (Civenni, Holbro and Hynes, 2003). Therefore, we investigated whether FZD6 could have a role in mediating the activation of EGFR in breast cancer.

We also assessed the expression of markers of the Epithelial to Mesenchymal Transition (EMT). EMT is defined as the process by which epithelial cells

acquire a mesenchymal phenotype, i.e. become motile, are able to invade in the surrounding tissue, acquire resistance to apoptosis and are able to produce extracellular matrix components (Kalluri and Weinberg, 2009). The WNT pathway participates in the activation of the EMT program in cancer (Vincan and Barker, 2008), suggesting that frizzled receptors could be involved in this process. Vimentin belongs to the family of intermediate filaments and participates to the organization of the cytoskeleton. This protein is generally not expressed in epithelial cells, but it is present primarily in cells of mesenchymal origin. For this reason vimentin is also widely considered a marker of EMT in cancer. In breast cancer, vimentin expression is associated with an aggressive phenotype and poor prognosis and is relevant in the coordination of cell invasion (Vuoriluoto *et al.*, 2010). Loss of cell-cell adhesion is a fundamental step that confers to cells the capacity to migrate from the native epithelium and it is characteristic of cells that underwent EMT. E-Cadherin is one of the proteins that are essential to regulate cell-cell adhesion, and its loss is frequently observed in invasive breast carcinomas (Lombaerts *et al.*, 2006; Cano *et al.*, 2000). Fibronectin is a glycoprotein widely expressed in connective tissue, where it forms fibrillar structures which are essential components of the extracellular matrix. Fibronectin is implicated in a plethora of cell functions, such as cell adhesion, growth, migration, and differentiation (Pearlstein, Gold and Garcia-Pardo, 1980). In Breast cancer fibronectin is often found overexpressed and it enhances tumorigenesis through various mechanisms, including the activation of EMT (Park and Schwarzbauer, 2013).

In the following chapter I present a series of experiments aiming to elucidate the role of FZD6 in the regulation of signalling pathways and molecules described above.

6.2 FZD6 signalling is not involved in the regulation of AKT, PLC-γ and ERK 1/2

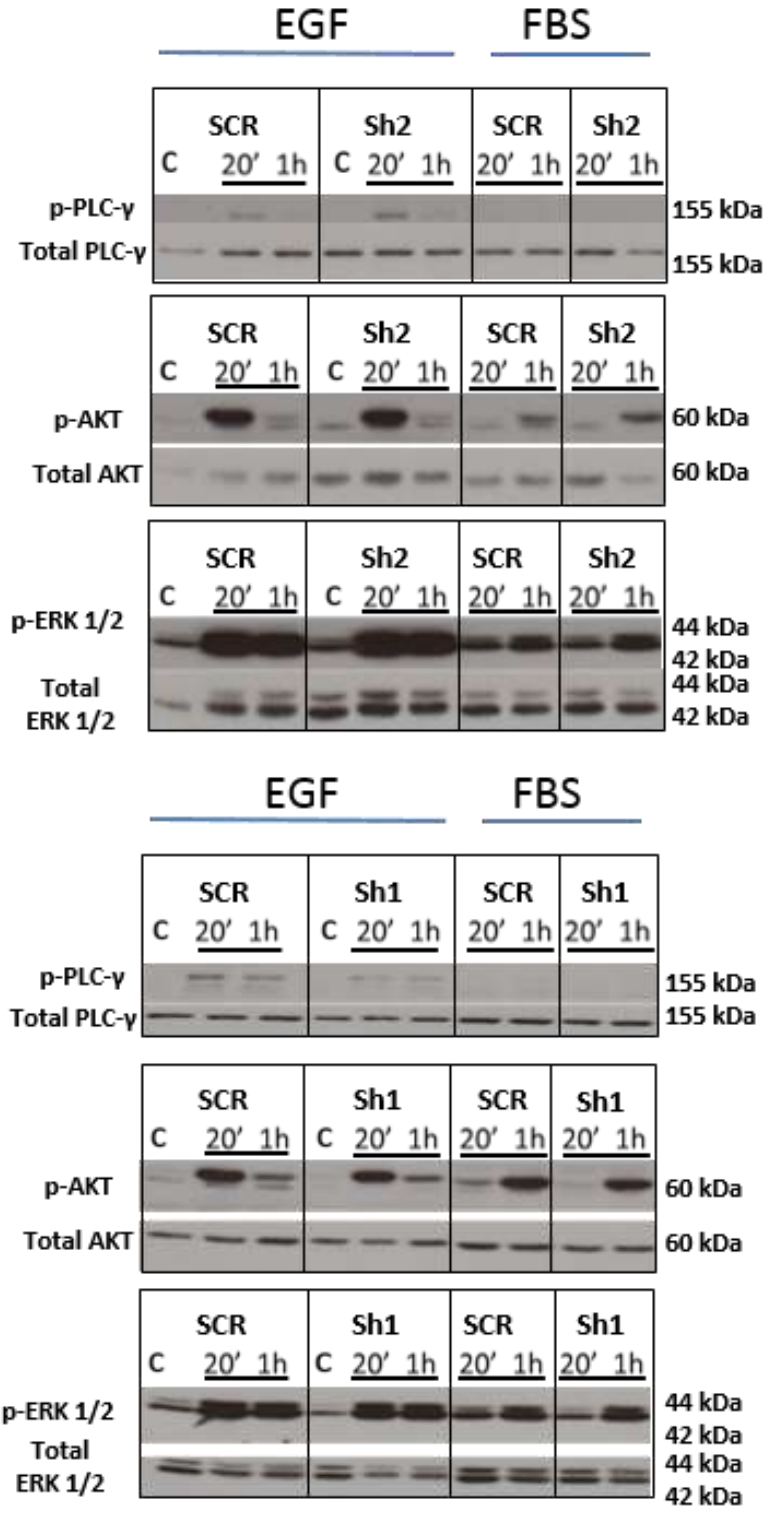

Figure 6.1: EGFR receptor pathway analysis on MDA-MB-231 cells expressing control shRNA (Scrambled) or FZD6 shRNAs (Sh1 and Sh2). Cells were starved overnight and then exposed for the indicated time to serum free medium containing 100ng/mL EGF or medium containing 10% FBS. Antibodies against the phosphorylated form of PLC-γ, p-AKT and p-ERK 1/2 were used to assess the EGFR pathway activation. C indicates untreated controls. Total PLC-γ1, total AKT and total ERK 1/2 were used as loading controls.

To assess whether FZD6 mediates a crosstalk between EGFR and WNT pathways, we assessed the activation of PLC-γ1, AKT and ERK1/2 in the presence of control shRNA (SCR) or FZD6 shRNA (sh1 and sh2) (fig.6.1).

In control cells, the activation of PLC-γ1 was detectable 20 minutes following the exposure to EGF, but became undetectable after 1 hour. Conversely, FBS did not activate PLC-γ1. We observed a mild reduction in the activation of PLC after the exposure with EGF in Sh1 cells compared to control cells (Figure 6.1, bottom panel). However, we did not observe the same effect in Sh2 cells when compared to the control shRNA (fig. 6.1, upper panel).

EGF induced a marked activation of AKT when administered to cells for 20 minutes and, to a lesser extent, for 1 hour (fig. 6.1). AKT was also activated in the presence of FBS but only after 1 hour of exposure. However, the downregulation of FZD6 did not change the activation pattern of AKT (fig.6.1).

EGF strongly activated ERk1/2 in control cells. FBS also induced the activation of ERK 1/2, particularly after one hour. However in cells infected with FZD6 shRNAs ERK 1/2 activation was unperturbed compared to cells infected with Scramble shRNA (fig.6.1).

6.3 FZD6 signalling does not regulate the expression of the EMT markers vimentin and E-cadherin

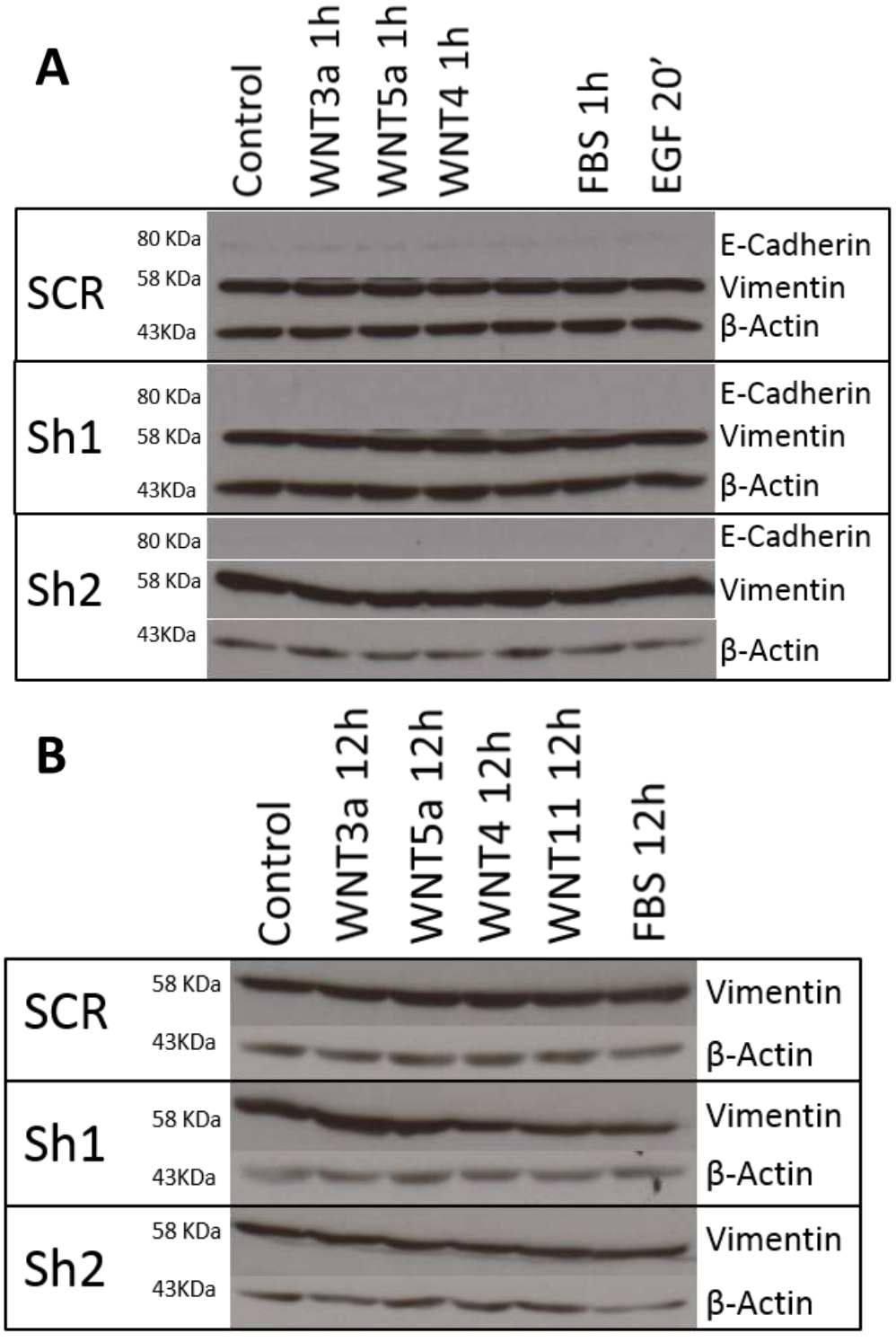

Figure 6.2: Western blot analysis of EMT markers in MDA-MB-231 breast cancer cells expressing control shRNA (SCR) or FZD6 shRNAs (Sh1 and Sh2). Cells were starved overnight and exposed to the indicated ligands for the indicated times **(A)** Expression of E-Cadherin and Vimentin after 1 hour exposure with the indicated WNT ligands or FBS, or after 20 minutes exposure to EGF. **(B)** Expression of Vimentin after 12h exposure to the indicated WNTs or to FBS. β-actin was used to control on samples loading.

To assess whether FZD6 is involved in the regulation of EMT, we analysed the expression of EMT markers in in MDA-MB-231 breast cancer cells. This cell line expresses the mesenchymal markers vimentin and fibronectin, but does not express E-Cadherin (Blick *et al.*, 2008). Moreover, we studied whether the administration of exogenous WNT ligands, FBS or EGF, was able to influence the expression of EMT markers. Cells were starved overnight and treated with WNT ligands, FBS or EGF for the indicated times (fig. 6.2), then subjected to western blot analysis.

The addition of WNTs ligands, FBS or EGF did not result in any significant changes in the expression of vimentin or E-cadherin, both in cells expressing FZD6 and in FZD6 depleted cells (figure 6.2 A and B).

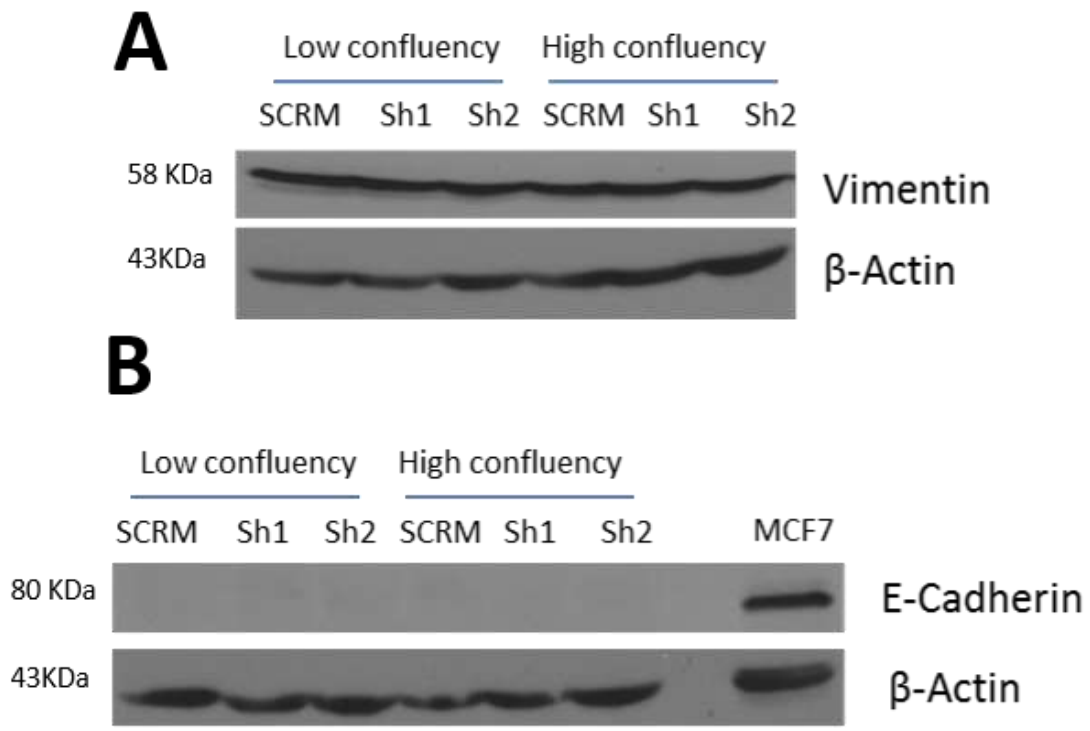

Figure 6.3: EMT markers expression in MDA-MB-231 cells expressing control shRNA (SCR) or FZD6 shRNAs (Sh1 and sh2) growing in presence of serum at different confluency levels. Cells were lysed at about 50% and 100% confluency and subjected to western blot analysis for the expression of vimentin **(A)** or E-Cadherin **(B)**. MCF7 were used as a positive control for E-Cadherin.

To assess whether the expression of these markers is dependent on the number of cells and/or on cell-cell interactions, the expression of vimentin and E-cadherin was analysed in cells growing in complete medium and lysed at low confluency (approximately 50%) or at high confluency (approximately 100 %) (fig. 6.3). We found that Vimentin and E-Cadherin did not change in different conditions.

6.4 FZD6 signalling regulates fibronectin matrix deposition, but not the secretion of soluble fibronectin

Fibronectin is a well established marker of EMT and enhances adhesion and motility of cancer cells (Park and Schwarzbauer, 2013; Friedl and Alexander, 2011). Therefore, we assessed fibronectin matrix deposition in MDA-MB-231 cells in the presence or absence of FZD6, using indirect immunofluorescence. The depletion of FZD6 with two shRNA (sh1 and sh2) resulted in a significant reduction of the fibronectin immunostaining in MDA-MB-231 cells (fig. 6.4).

In collaboration with Michele Sallese and Giorgia Fragassi at the Fondazione Mario Negri Sud, S.Maria Imbaro, Italy, we also analysed the structure of the fibronectin matrix assembly using confocal microscopy (fig.6.5). We found that MDA-MB-231cells expressing FZD6 have a more dispersed and homogenous distribution of fibronectin, whereas, in cells where FZD6 expression was abrogated, fibronectin was distributed more in perinuclear blobs and less dispersed in the cytoplasm (figure 6.5 A). Cells showing fibronectin aggregates were counted and plotted in figure 6.5 B. The knock down of FZD6 resulted in a 20% increment of in the formation of perinuclear fibronectin blobs.

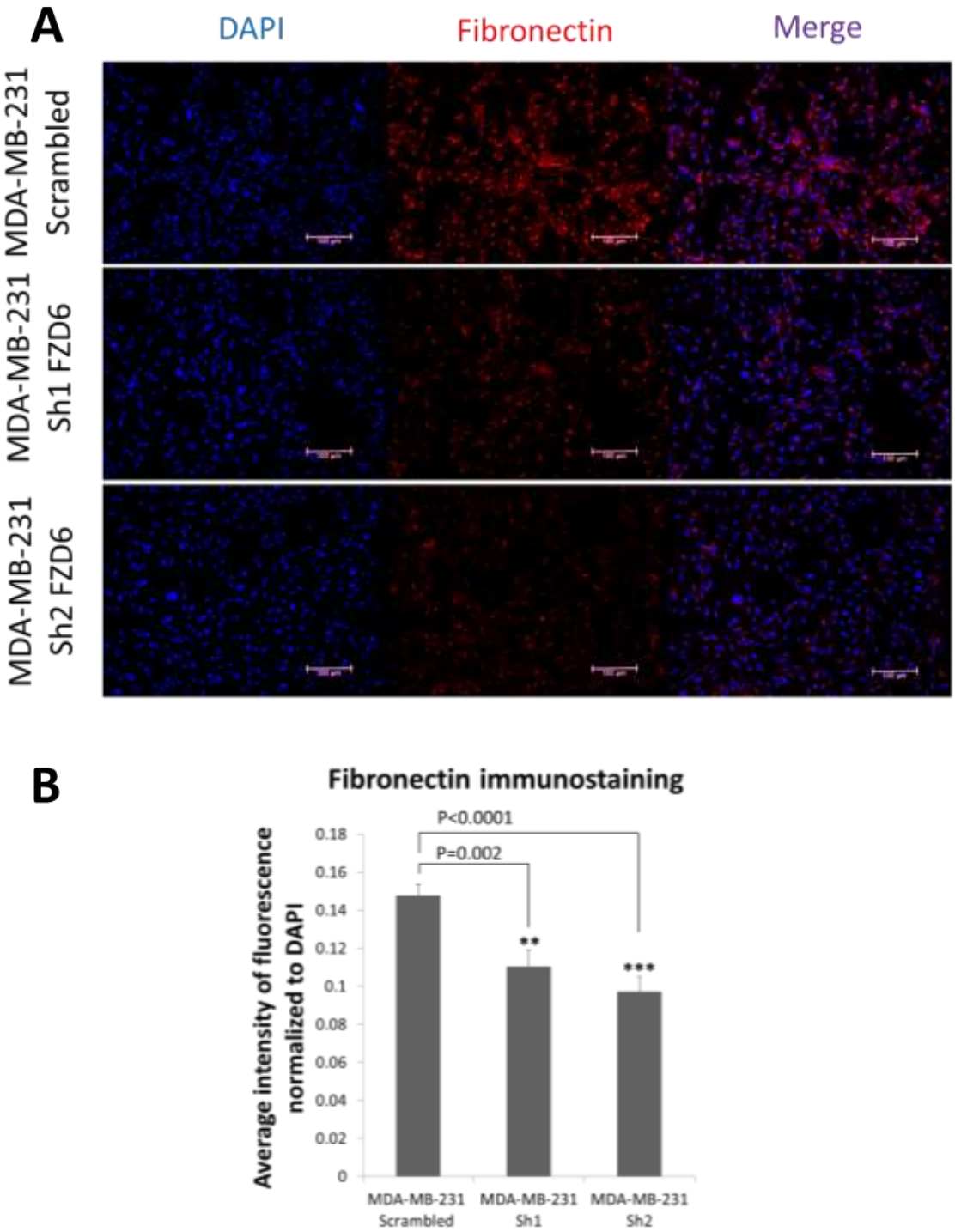

Figure 6.4: Immunofluorescence analysis of the fibronectin matrix in FZD6 depleted MDA-MB-231 cell lines. (A) Images showing expression of fibronectin (red colour) in the control (scrambled) or FZD6 depleted (Sh1, Sh2) cell lines. Nuclei were stained using DAPI (blue colour). **(B)** Quantification of the experiment shown in A. The fibronectin fluorescence relative to DAPI was quantified using imaging software in at least ten random fields per sample. Error bars indicate standard errors and the asterisk statistical significance (student's t-test, n=10). The data shown is representative of two independent experiments. Scale bars indicate a length of 100μm.

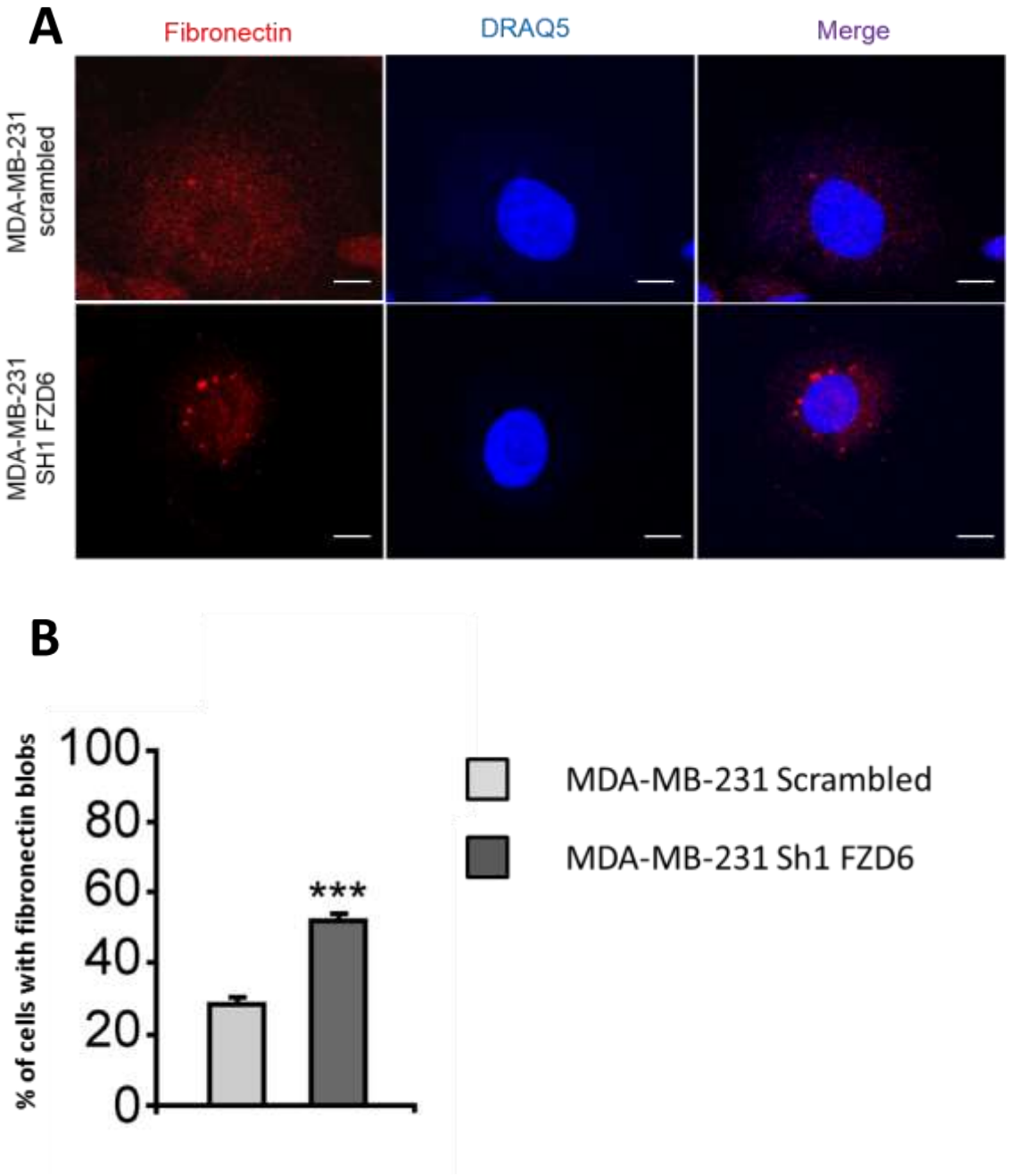

Figure 6.5: Deposition of fibronectin in dependence to FZD6 expression assessed by confocal microscopy. Two phenotypes were noted, dispersed in control cells, dots/blobs in the absence of FZD6 expression. **(A)** Representative photographs showing the organization of fibronectin in MDA-MB-231 cells expressing control shRNA or sh1 FZD6**. (B)** Percentage quantification of cells presenting blobs aggregates of fibronectin in the cells described in A. Data are expressed as % of total, as means ±SEM of three independent experiments, with at least 100 cells quantified per experiment. *** indicates $p<0.001$ compared to control cells (student's t-test). Scale bars indicate 10µm. This experiment was carried out by our collaborators Michele Sallese and Giorgia Fragassi at Fondazione Mario Negri Sud, S.Maria Imbaro, Italy.

Fibronectin exists also in a soluble form that cells release in the culture media (Proctor, 1987). We next assessed the expression of soluble fibronectin in cell culture supernatants using an ELISA assay. We found that the secretion of fibronectin was unchanged after the knockdown of FZD6 (fig. 6.6).

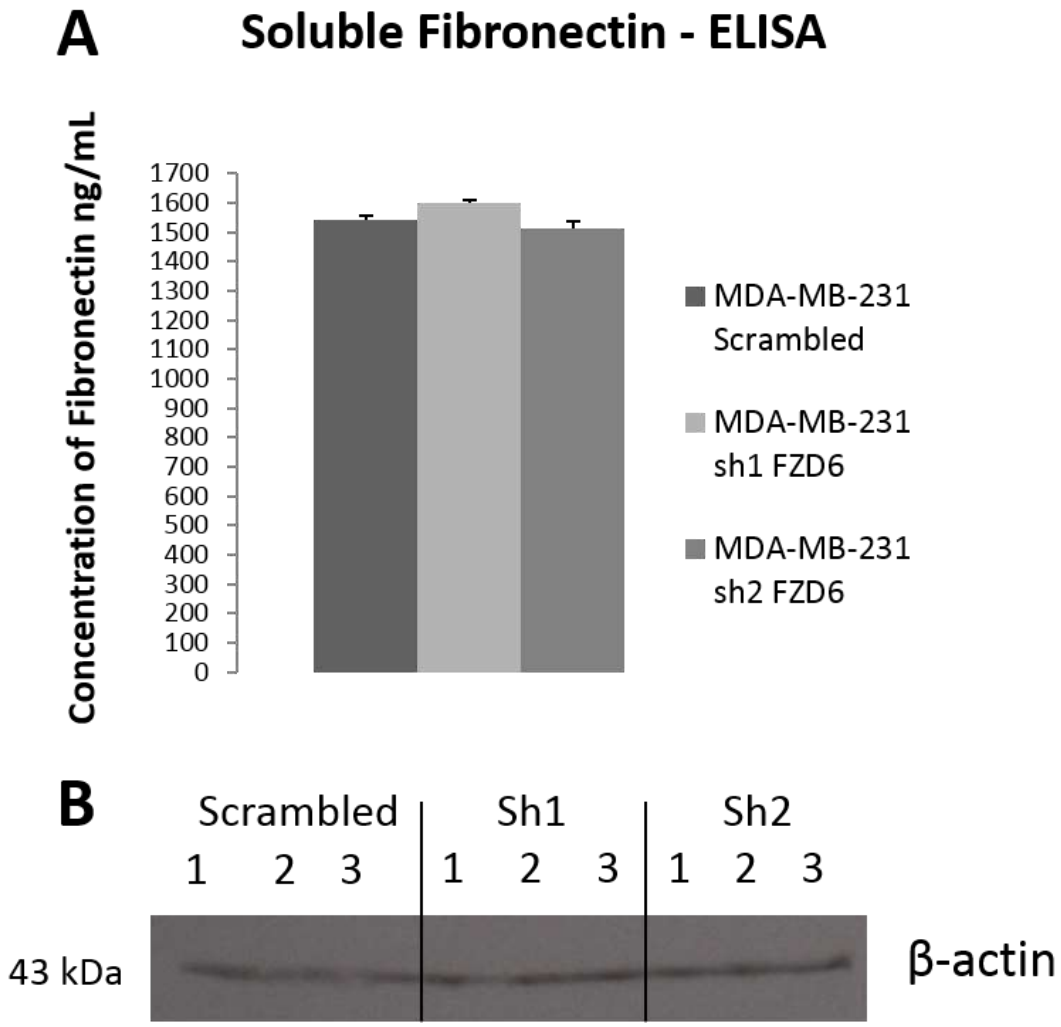

Figure 6.6: ELISA for the detection of soluble fibronectin secreted by MDA-MB-231 cells expressing control shRNA (Scrambled) or FZD6 shRNAs (sh1 and sh2). (A) Soluble fibronectin in the culture media was quantified through indirect ELISA 72 hours following serum starvation **(B)**. The cells used for the assay described in A were lysed in Laemmli buffer and subjected to western blot analysis to confirm a similar number of cells amongst the samples. Three wells per cell line were used and are indicated as 1 2 and 3. The data is representative of one experiment carried out in triplicate.

6.5 FZD6 mediates a non-canonical signal in breast cancer cells

To assess if FZD6 mediates a β-catenin dependent (canonical) signal or β-catenin independent (non canonical) signal, we performed western blot analysis of MDA-MB-231 expressing control or FZD6 shRNA using an antibody recognising active β-catenin or anti phospho c-Jun N-terminal kinase (p-JNK) following the activation with WNT ligands (Fig.6.7).

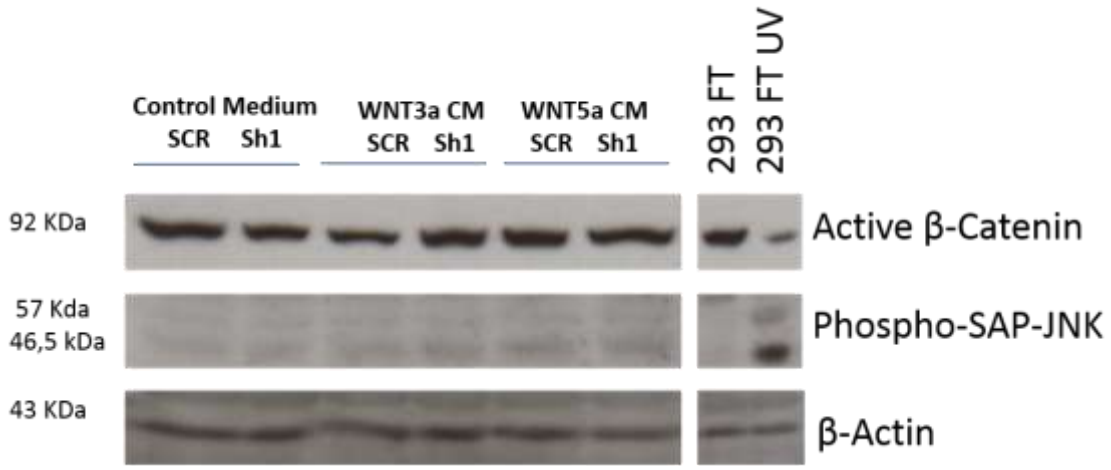

Figure 6.7: Assessment of the WNT canonical and WNT/JNK non-canonical pathways in MDA-MB-231 breast cancer cells in the presence (Scrambled) or absence (Sh1) of Fzd6 expression. Phospho-SAP/JNK or active β-catenin antibodies were used to monitor the non-canonical or canonical WNT pathways in the presence of the indicated WNT conditioned media. Cells were starved overnight and exposed to conditioned media produced by L-control cells or L-cells expressing WNT3a or WNT5a (diluted 1:10) for two hours and then subjected to western blot analysis. β-actin was used to control on proteins loading. Lysates of HEK 293 FT cells exposed to UV light were used as a p-JNK positive control. The blot shown is representative of at least three independent experiments.

FZD6 knock down did not influence the activation of β-catenin. With these experimental settings, we could not detect an activation of p-SAP/JNK.

We next explored the activation of another non-canonical effector, the small GTPase Rho (Fig.6.8). The recombinant protein Rhotekin conjugated with GST was used as a bait protein for its capacity to bind selectively the active form Rho-GTP, but not the inactive form Rho-GDP. A glutathione resin was used to isolate the complex Rhotekin-GST-Rho-GTP. A preliminary experiment was carried out on cells activated with different recombinant WNTs, but since no differences were observed in comparison with the controls (data not shown), cells were analysed only in basal conditions. A substantial decrease of active Rho was observed in MDA-MB-231 cells in the presence of two different shRNAs, sh1 (52% decrease) and sh2 (32% decrease).

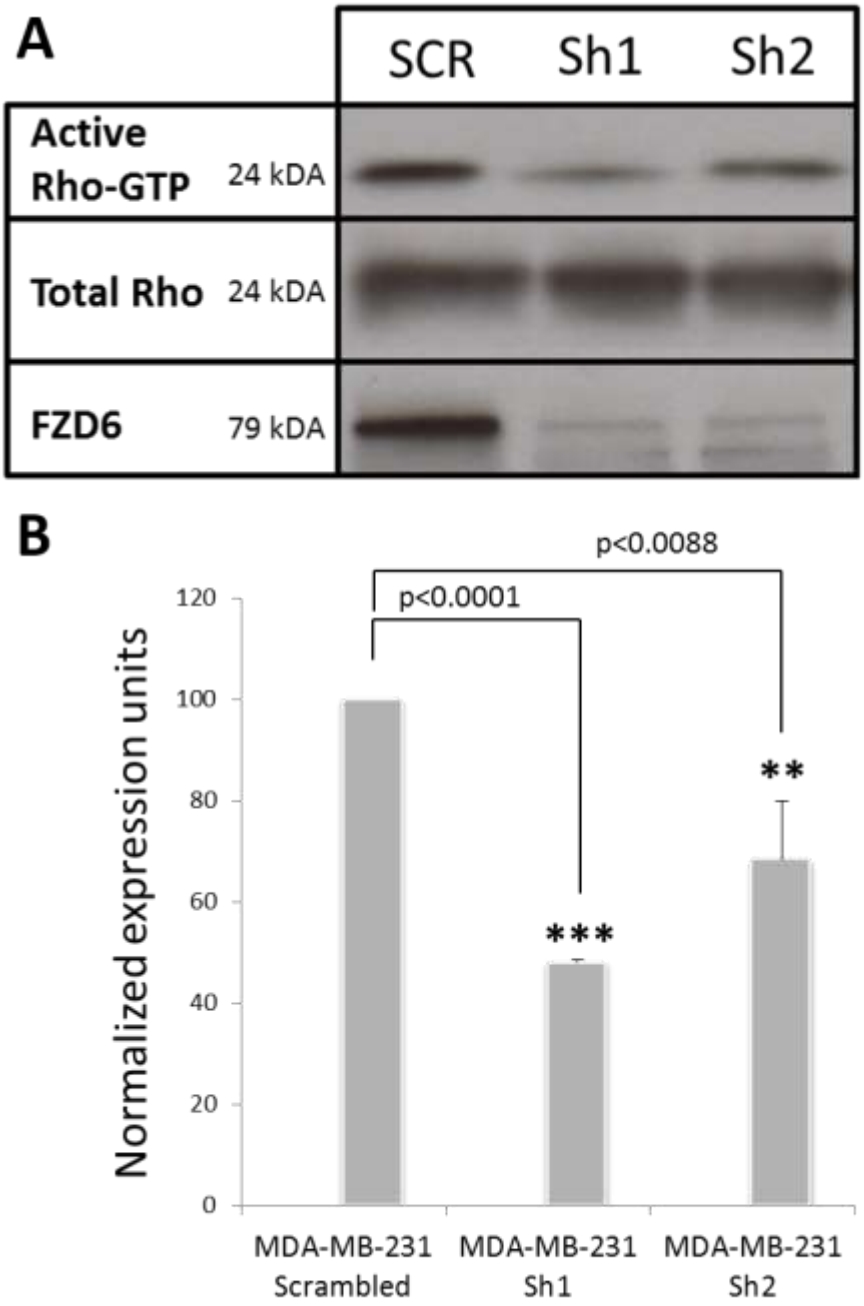

Figure 6.8: Pull down assay for the quantification of Rho-GTP in MDA-MB-231 cells expressing control shRNA (SCR) or shRNAs targeting FZD6 (Sh1 and Sh2). (A) Representative western blot analysis for the quantification of active Rho-GTP in MDA-MB-231 cells in the presence of the indicated shRNAs. A Pull down assay was carried out to isolate the active form of Rho bound to GTP. One tenth of the total lysate was excluded from the pull down procedure and subjected to a separate western blot analysis to confirm equal loading. Active Rho-GTP or total Rho were detected with an anti-Rho antibody and a HRP conjugated secondary antibody. A FZD6 antibody was used to demonstrate the knock down of FZD6. **(B)** Densitometric quantification of active Rho-GTP normalized to total Rho and expressed as percentage of the control (Scrambled). The data shown is the average of three independent experiments. Asterisks indicate statistical significance (student t-test).

6.6 Discussion

Activated β-catenin is usually not detectable in healthy mammary tissue, whereas it is in breast tumours (Howe and Brown, 2004). Our results indicate that constitutively active canonical WNT signalling is detectable in the MDA-MB-231 cell line, consistent with what found by other investigators (Bilir, Kucuk and Moreno, 2013; Benhaj, Akcali and Ozturk, 2006). The knock down of FZD6 did not result in a reduction of the levels of activated β-catenin, suggesting a non-canonical role of the receptor (fig.6.7).

In humans there are 10 different frizzled receptors and it is possible that each one mediates a specific signalling, although a certain level of redundancy might also occur. Furthermore, 19 different WNT ligands have been found in humans, but their affinity for specific FZD receptors and the intracellular signals are still poorly characterized. WNT ligands could have different effects depending on the biological contest; for example it was reported that WNT5a can activate both canonical and non canonical pathways, depending on the expression of specific frizzled receptors (Mikels and Nusse, 2006). The overexpression of WNT ligands could have oncogenic or oncosupressing effects, depending on the cancer type. Even within the same tumour, WNTs overexpression can have different effects (Anastas and Moon, 2012). It is therefore extremely complicated to define the role of WNT signalling outside a very specific context.

We found that the downregulation of FZD6 results in reduced activation of the small GTPase Rho (figure 6.8). Rho is a key regulator of the actin cytoskeleton, required for numerous biological processes during development and adult life. An important function of Rho is to regulate the cytoskeleton contractility that leads to cell movement (Hall, 1998). Moreover, Rho is activated by the WNT/FZD signalling to coordinate cell polarity required for the frog gastrulation (Habas, Kato and He, 2001). The first evidence of the involvement of Rho in tissue polarity derives from studies in drosophila mutants, which exhibit defects in the spatial orientation of omatidia in the eyes, defective organization of the hairs in the wings and present morphological defects of the embryos (Strutt, Weber and Mlodzik, 1997). Interestingly similar phenotypes are observed in frizzled and dishevelled mutants, suggesting that frizzled receptor and dishevelled could be upstream elements from Rho signal (Strutt, Weber and Mlodzik, 1997). FDZ6 KO mice present spatial asymmetry in the organization of

hair follicles and brain morphogenic abnormalities (De Marco et al., 2012; Stuebner et al., 2010; Wang et al., 2006a) which recall the disorganized orientation of drosophila omatidia and hair wings, and the embryonic defects observed in drosophila FZD and Rho mutants. These findings further suggest that FZD6 mediates a PCP signal through the activation of Rho.

In breast cancer, the over-activation of Rho in cells overexpressing FZD6 could also explain the increased motility and invasion. Rho and other member of the small GTPases family have a fundamental role in the cytoskeleton rearrangements that occur during cell locomotion (Nobes and Hall, 1999) . Rho regulates the actin-myosin interaction that generates the contractile force necessary for cell protrusion (Yee Jr, Melton and Tran, 2001) and the generation of focal adhesions with the ECM (Chrzanowska-Wodnicka and Burridge, 1996). Given the role of Rho GTPase in cell motility, it is reasonable to think of a role in cancer invasion and metastasis. Indeed, several studies linked the activation of Rho in all the stages of metastasis. For example, Itoh and colleagues demonstrated that the expression of an active dominant mutant of ROCK, a downstream effector of the Rho GTPase, confers a greater invasive activity to hepatoma cells in vivo, whereas the expression of a dominant negative ROCK mutant exerted the opposite effect (Itoh *et al.*, 1999). Studies on leukocytes transendothelial migration have shown that RhoA is required for monocytes tail retraction and efficient diapedesis, suggesting a possible role of Rho in cancer cells extravasation (Worthylake *et al.*, 2001). Rho is critical also during tumour angiogenesis. Indeed, conditional activation of ROCK in colon cancer cells results in more invasive and vascularized tumours, (Croft *et al.*, 2004; van Nieuw Amerongen *et al.*, 2003).

RhoA, RhoB and RhoC are often overexpressed in breast cancer (Jiang *et al.*, 2003; Fritz *et al.*, 2002; van Golen *et al.*, 2000). Experiments in MDA-MB-231 cell line, also used in our study, revealed that knock down of RhoA and RhoC reduces cell proliferation, invasion and angiogenesis in vitro and in vivo (Pillé *et al.*, 2005). Liu and colleagues showed that ROCK is overexpressed in metastatic breast cancer cell lines and tumours compared with the non metastatic matched tissues, and that the ectopic expression of ROCK induces the onset of bone and liver metastasis in mice injected with the otherwise not invasive MCF7 cell line (Liu *et al.*, 2009). Interestingly, in our experiments we show that the knock down of FZD6 reduces breast cancer metastasis in bones

and liver, the same sites of metastasis of MCF7 cells expressing conditional ROCK. This further suggests that FZD6-mediated activation of Rho has a role in the tropism of breast cells.

TGF-β-mediated loss of E-Cadherin and expression of N-Cadherin are abolished by the ectopic expression of a dominant negative form of RhoA in mammary epithelial cells, indicating a role of RhoA in the EMT and therefore in tumorigenesis (Bhowmick *et al.*, 2001). In addition, Rho could also have a pivotal role in breast cancer angiogenesis. Indeed, the overexpression of RhoC in HMEC was reported to increase the expression of VEGF and other angiogenetic factors (van Golen *et al.*, 2000). Inactivation of Rho signalling through the antagonism of FZD6 could therefore hinder several steps of the metastatic process.

The MDA-MB-231 cell line presents typical characteristics of EMT transition, i.e. expresses fibronectin and vimentin and does not express E-cadherin (Blick *et al.*, 2008; Olmeda *et al.*, 2007). Knock down of FZD6 did not change the expression of Vimentin nor restored the expression of E-cadherin in MDA-MB-231 cells, both in basal conditions and in cells stimulated with WNT3a, WNT4, WNT11, WNT5a, FBS or EGF, suggesting that FZD6 signalling does not interact with these proteins. However, we observed reduced deposition and an alteration of the distribution patterns of fibronectin in FZD6 depleted cells compared to the controls (fig. 6.4 and 6.5). Soluble fibronectin instead, was not regulated by FZD6 (fig. 6.6).

Fibronectin is a large glycoprotein that participates in numerous functions that require dynamic interactions of the cells with the extracellular environment, such as cell adhesion, growth, migration, and processes like wound healing and embryonic development. Fibronectin exists in humans in at least 27 different splicing variants that can be divided in two groups: soluble fibronectins and insoluble fibronectins. Fibronectins monomers contain 6 domains that allow the binding with other components of the extracellular matrix, such as fibrin, heparin sulphate, collagen, proteoglycans and other fibronectin molecules. Fibronectin also interacts with a class of membrane receptors called integrins. Cells secrete fibronectin in form of soluble dimers held together by disulphide bonds. With mechanisms that are not yet fully understood, cells can promote the formation of an insoluble network of fibrillar fibronectin, most probably through the

activation of the integrins receptors (Mosher, 2012; Plopper, 2012; Pankov and Yamada, 2002).

Increased expression of fibronectin has been observed in breast cancer and associated with poor prognosis (Helleman *et al.*, 2008; Ioachim *et al.*, 2002), suggesting a potential role of fibronectin in breast cancer tumorigenesis. Fibronectin could promote tumorigenesis in different ways. The primary mechanism could be by providing to malignant cells adhesion to the ECM to generate mechanical forces (Ruoslahti, 1984). Stimulation of cells with fibronectin has been shown to increase cell spreading and motility in a variety of systems (McCarthy, Hagen and Furcht, 1986; Schor, Schor and Bazill, 1981). In the MDA-MB-231 breast cancer cell line the interaction with fibronectin coated plates enhances cell spreading end lamellar protrusions, with recruitment of myosin IIA and IIB to the marginal lamellar zone, indicating the activation of a motile phenotype (Betapudi, Licate and Egelhoff, 2006). Fibronectin promotes cell motility both in its soluble form (chemotaxis) and when is bound to the ECM in insoluble form (haptotaxis) (Klominek, Robert and Sundqvist, 1993; Aznavoorian *et al.*, 1990). Although the culture of cells in a fibronectin substrate often results in enhanced motility, excessive adhesion to a fibronectin substrate might lead to the opposite effect. For example, the fibronectin matrix has been shown to inhibit dispersal and invasion of glioblastoma cells in vitro (Jia *et al.*, 2012; Sabari *et al.*, 2011). Cell motility is a complex multistep process in which adhesion and detachment with the ECM have to be dynamically modulated. Different classes of integrins participate to this process and their cell-specific differential expression could explain the variable interaction of cancer cells with fibronectin (Akiyama, Olden and Yamada, 1995). The role of fibronectin in cell invasion was demonstrated in vivo using synthetic peptides that inhibit the interaction between fibronectin and integrins. Melanoma and glioblastoma cells, exposed to these peptides, penetrate much less trough human amniotic basement membranes when compared to cells exposed to control peptides (Gehlsen *et al.*, 1988). Moreover, co-injection of B16-F10 murine melanoma cells with the pentapeptide containing the motive Gly-Arg-Gly-Asp-Ser, which competitively inhibits the binding of cells to fibronectin, prevents lung metastasis and greatly increases survival in mice (Humphries, Yamada and Olden, 1988). One possible mechanism through which fibronectin could promote cancer metastasis is by increasing the expression of matrix metalloproteinases which are required by cancer cells to invade through tissues. It has been shown that

exogenous addiction of fibronectin to cultures of breast cancer cells enhances the expression of metalloproteinases such as MMP-2 and MMP-9 through the activation of α5β1 integrin signalling (Das *et al.*, 2008). MMP-9 expression was also induced in the culture of human breast cancer cell line MDA-MB-231 growing on fibronectin-coated surface (Maity *et al.*, 2011). Consistently with these findings, Saad and colleagues demonstrated that the membrane-bound fibronectin on MDA-MB-231 cells is required to stimulate the release of MMP-2 by bone marrow fibroblasts (Saad *et al.*, 2002). The fibronectin matrix could also be important for the arrest of circulating cancer cells to the endothelium of secondary organs, a necessary step before extravasation. This was demonstrated with blood-borne rat breast cancer cells, which require a membrane coating of fibronectin to metastasize to the lungs and was independently observed by different investigators (Huang *et al.*, 2008; Felding-Habermann, 2003; Cheng *et al.*, 1998).

Another possible mechanisms through which fibronectin could enhance tumorigenesis is by activating the EMT. Exposure of the non transformed mammary epithelial cell line MCF10A to fibronectin induces an EMT phenotype characterized by the increased mRNA expression levels of vimentin, N-cadherin, Snail and MMP2. Exogenous fibronectin also upregulates its own expression in these cells and induces a migratory behaviour (Park and Schwarzbauer, 2013). When MCF10 cells are grown in a 3D culture system, addition of fibronectin results in the development of abnormal acini characterized by larger dimension and in a 50% reduction in the number of hollow acini when compared to the control cultures (Williams *et al.*, 2008). Vice versa, inhibition of fibronectin with antibodies in the malignant breast cancer cell line T4-2 prevents the formation of irregular grape-like acini and results in the formation of normal spherical acini similar to the ones formed by the non transformed cell line MCF10 (Sandal *et al.*, 2007). Interestingly, we observed a similar phenotype in MDA-MB-231 cells in which FZD6 was depleted (fig. 4.11), further suggesting a link between FZD6 and fibronectin matrix organization in breast cancer. Taken together, these findings suggest that fibronectin plays a pivotal role in breast cancer tumorigenesis through multiple mechanisms.

A question still to be addressed is how FZD6 could modulate the assembly of the fibronectin matrix surrounding breast cancer cells. The knock down of FZD6 did not perturb the amount of secreted fibronectin in breast cancer cells

supernatants (figure 6.6), whereas we observed a reduction of the insoluble fibronectin matrix formation and a perturbation of the distribution patterns. The perinuclear fibronectin blobs observed in FZD6 depleted cells might be deposits of soluble fibronectin that fail to be incorporated in the extracellular matrix. This suggests that FZD6 is implicated in the organization of the insoluble fibronectin matrix assembly but not in its expression and secretion. This would also be in agreement with a role of FZD6 in the PCP pathway. Indeed, the planar cell polarity pathway is required for the polarized surface assembly of the fibronectin matrix which is essential for the convergent extension movement during frog gastrulation. Perturbation of the expression of PCP components such as xfzd7 (analogue of the human FZD7 receptor in Xenopus) or WNT11 leads to disruption of the fibronectin matrix assembly and consequent defects in the frog morphogenesis (Williams *et al.*, 2008; Goto *et al.*, 2005). Interestingly, ectopic expression of a constitutively active form or Rho in the embryos expressing a dominant negative form of WNT11, rescues the fibronectin matrix assembly. This suggests that the WNT pathway controls the fibronectin matrix through the activation of Rho (Dzamba *et al.*, 2009). This is consistent with our observation in breast cancer, where the interference of the PCP pathway, through the knock down of FZD6, led to the reduction of Rho signalling and in a defective fibronectin matrix assembly. Fibronectin itself could regulate WNT non canonical signalling through the co-receptor Syndecan-4, indicating the existence of a positive feedback between fibronectin and the WNT pathway. Indeed, Syndecan-4 was shown to interact directly with xFzd7 and dishevelled to activate the PCP pathway in Xenopus Laevis, and the perturbation of Syndecan4 expression led to impairment of the convergent extension movement during Xenopus morphogenesis (Muñoz *et al.*, 2006).

The Fibronectin matrix assembly is a complex multistep process which is initiated by the interaction of soluble fibronectin with the α5β1 integrin receptor (Fogerty *et al.*, 1990). Initially, integrins receptors contribute to organize fibronectin in short fibrils on the cellular surface (Mao and Schwarzbauer, 2005). Consequentially, integrins interact with the actin cytoskeleton to promote myosin mediated contractility. This in turn causes the integrin receptor to stretch the fibronectin fibres, exposing new binding sites for the interaction with other fibronectin molecules, resulting in the development of an insoluble matrix (Plopper, 2012; Wu *et al.*, 1995; Hynes, 1990). In this process, Rho is necessary to couple the activation of the integrin receptor with the contraction of

the actin cytoskeleton (Huveneers and Danen, 2009). Indeed, interference of Rho activation prevents the fibronectin matrix assembly in mouse embryonic fibroblasts (Zhong *et al.*, 1998).

Given the role of Rho and fibronectin in cancer, we propose that overexpression of FZD6, through the activation of Rho, could lead to several process that enhance the malignant proprieties of breast cancer cells. The activation of Rho could directly increase cell motility, angiogenesis and promote the cytoskeletal retraction during intravasation/extravasation of breast cancer cells. Rho over activation could also lead to an increased production of fibronectin matrix, which in turn can promote haptotaxis, provide an adhesion substrate for breast cancer cells in the endothelium of host organs and induce the secretion of metalloproteinases. As previously mentioned, an analogy can be made between the directional migration of cells during development and the migratory process occurring during cancer metastasis. The non canonical PCP pathway and fibronectin matrix assembly are essential in both cases, and FZD6 could be a key regulatory element in these processes.

CHAPTER VII

FZD6 and fibronectin in the regulation of the actin cytoskeleton

7.1 Introduction

Cell migration can be considered as a cycle of molecular events (Ridley *et al.*, 2003). Initially, cells respond to migratory stimuli by extending polarized protrusions towards their direction. These protrusions consist of actin-rich regions organized in actin ruffles and meshes called lamellipodia, and of filamentous actin-rich structures protruding radially called filipodia (Etienne-Manneville, 2004). The leading edge protrusions engage interactions with ECM elements such as fibronectin, providing both anchorage and intracellular signals through the interaction with the membrane receptors integrins (Friedl *et al.*, 1997). Tension inside the cell is provided by the strain of actin fibres through focal adhesions, which are macromolecular complexes that mediate the chemo-mechanical interactions between the cell and the ECM (Pellegrin and Mellor, 2007; Wozniak *et al.*, 2004). The protrusive force is generated by the interaction between myosin II and actin fibres, resulting in the actomyosin contraction (Ridley *et al.*, 2003). Cell contraction has to be accompanied by the gradual loss of adhesion at the rear of the cell, allowing the cell trail to slide forward (Friedl and Alexander, 2011). Actin rearrangements are therefore essential throughout all the steps of cell migration. Since we observed drastic differences in the migratory behaviour of breast cancer cells following the depletion of FZD6, we investigated whether this effect was mediated by actin rearrangements.

7.2 Downregulation of FZD6 results in actin cytoskeleton rearrangements in MDA-MB-231 cells

To investigate whether FZD6 could influence the assembly of F-actin stress fibres, we analysed MDA-MB-231 cell expressing control vector or FZD6 shRNA after staining with fluorescent phalloidin. We observed a striking rearrangement of F-actin in the absence of FZD6: actin fibres were much thicker compared to the ones observed in control cells (figure 7.1). Cells presenting thick actin fibres where scored as shown in figure 7.1 B. The downregulation of FZD6 resulted in a ~40% increase in the number of cells with thick actin fibres.

Since we observed reduced fibronectin matrix assembly after the removal of FZD6 in MDA-MB-231 cells (figure 6.4), we wondered if exogenous addition of fibronectin could rescue the actin phenotype. We therefore analysed the structure of actin stress fibres after exposing FZD6 depleted cells to plasma fibronectin for 24 hours or 48 hours (figure 7.2). In the presence of exogenous fibronectin we observed a time dependent reduction of the number of cells presenting thick actin stress fibres. At 24 hours and at 48 hours the number of cells with thick actin fibres were respectively ~ 20% and ~ 40% less compared to control. After 48 hours exposure to exogenous fibronectin, FZD6 depleted cells presented an actin cytoskeleton indistinguishable from that observed in MDA-MB-231 control cells (fig.7.2).

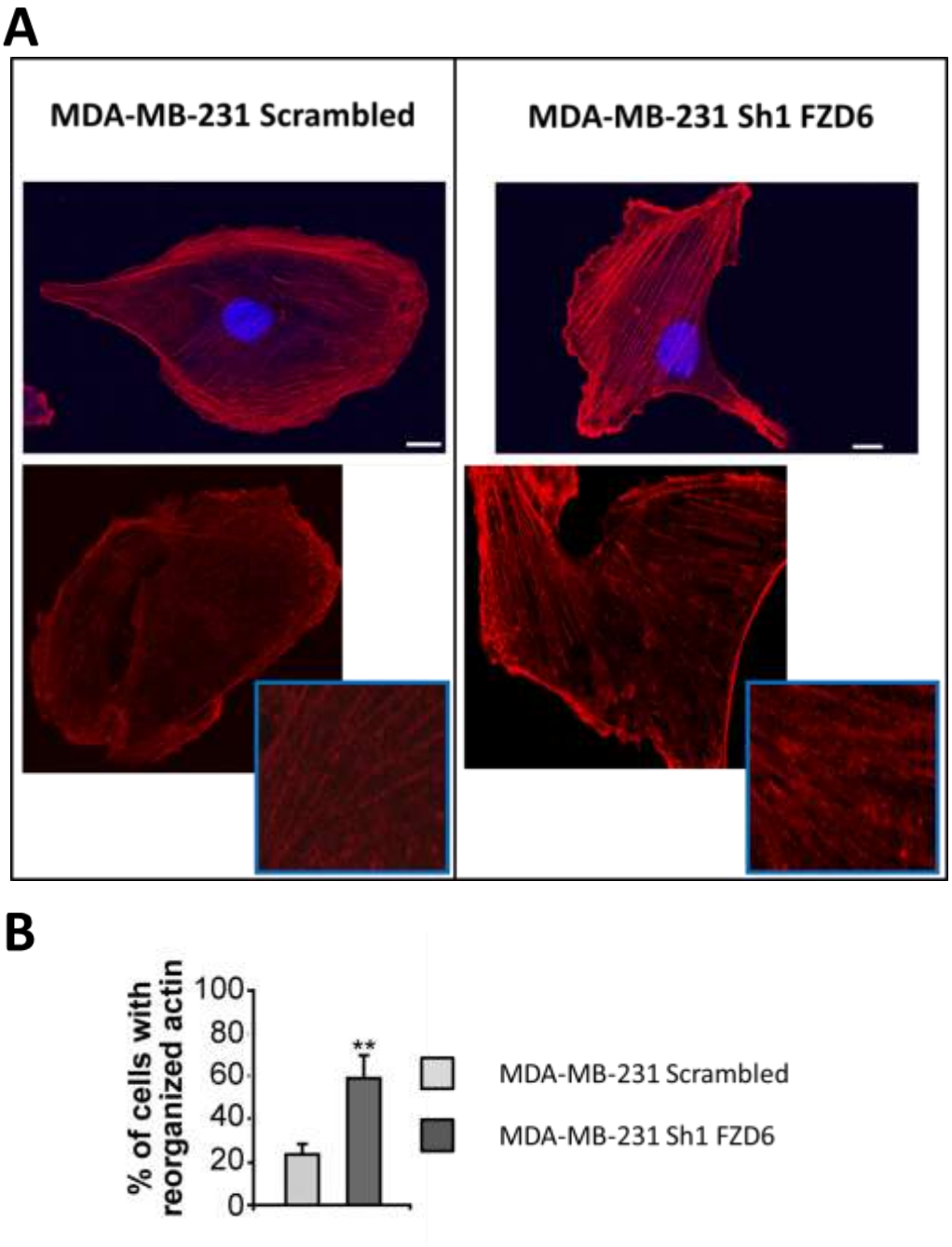

Figure 7.1: The actin cytoskeleton is rearranged in the absence of Fzd6. (A) Assembly of F-actin fibres in control (scrambled) or FZD6 depleted MDA-MB-231cells (sh1 FZD6) was detected by confocal microscopy using phalloidin staining. Two predominant phenotypes were observed: in the absence of FZD6 F-actin was organized in thick fibres, running throughout the cell. In contrast, in control cells F-actin fibres were thinner and concentrated in discrete areas in the proximity of the plasma membrane. Right bottom corners show in closer detail the actin patterns. Cells showing a “thick phenotype” were quantified and expressed as percentage of the total as means ±SEM of three independent experiments, with at least 100 cells quantified per experiment. ** indicates $p<0.001$ compared to control cells (student’s t-test). Scale bars indicate 10μm. This experiment was carried out by our collaborators Michele Sallese and Giorgia Fragassi at Fondazione Mario Negri Sud, S.Maria Imbaro, Italy.

7.3 Exogenous fibronectin rescues the actin phenotype and invasive potential of MDA-MB231 cells lacking FZD6

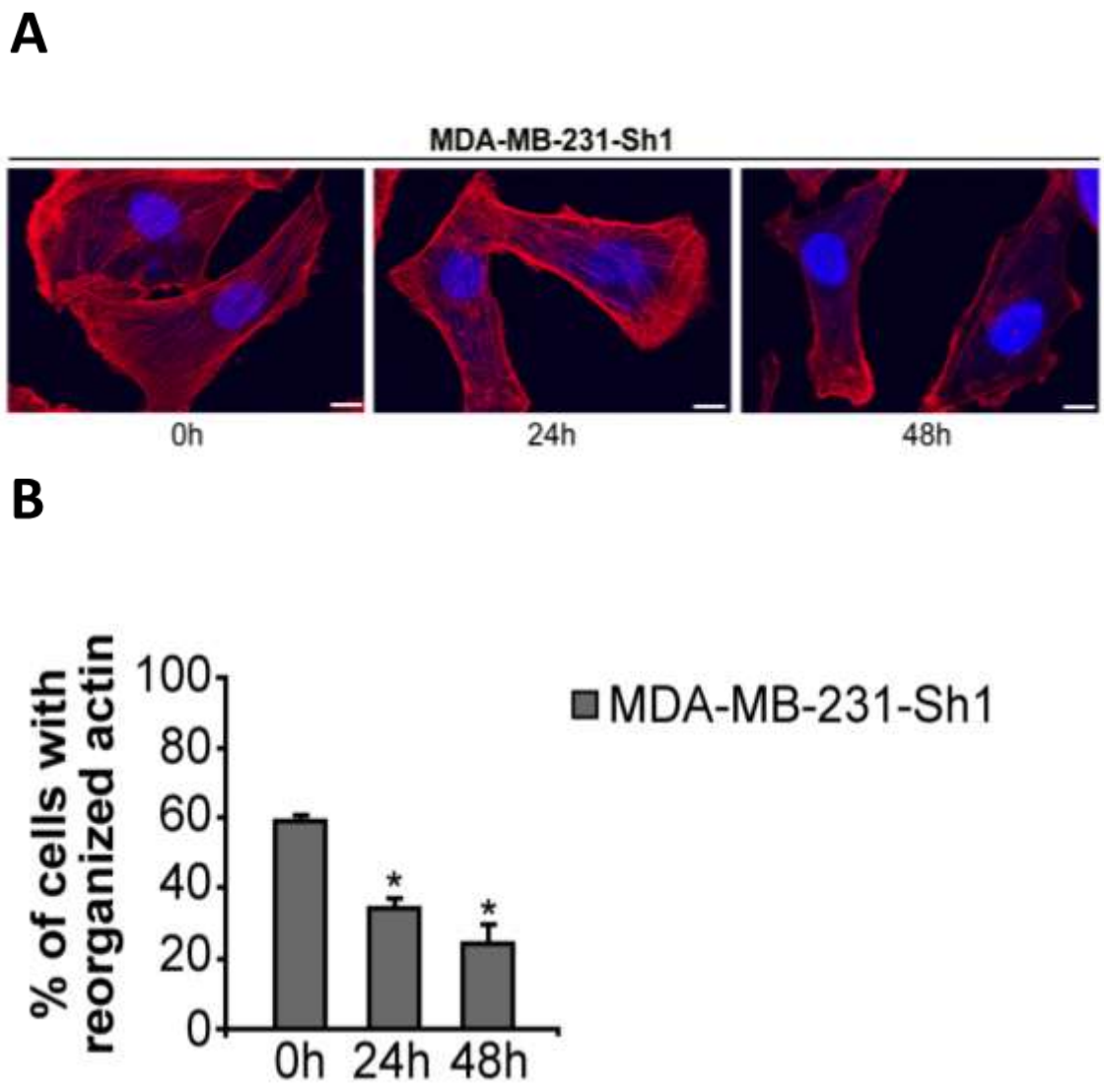

Figure 7.2: Exogenous addition of Fibronectin induces depolymerisation of actin stress fibres in FZD6 depleted cells. (A) Actin stress fibres were detected by confocal microscopy using phalloidin staining in MDA-MB-231 Sh1-FZD6 cells after the exposure for the indicated times to 20µg/mL fibronectin of human plasma origin. **(B)** Quantification of cells showing thick actin stress fibres, referred to the experiment described in A. Data is expressed as percentage of the total as means ±SEM of three independent experiments, with at least 100 cells quantified per experiment. * indicates $p<0.05$ compared to control cells (Student's t-test). Scale bars indicate 10µm. This experiment was carried out by our collaborators Michele Sallese and Giorgia Fragassi at Fondazione Mario Negri Sud, S.Maria Imbaro, Italy.

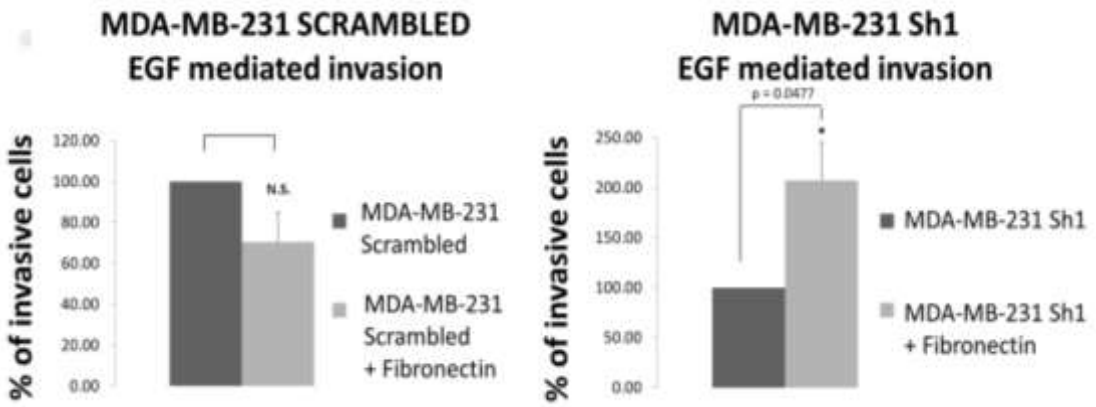

Figure 7.3: Invasion assay on MDA-MB-231 cells expressing control shRNA (scrambled) or FZD6 shRNA (sh1) in the presence or absence of exogenous fibronectin. Cells were resuspended in serum free medium alone or containing 20µg/mL of human plasma fibronectin and seeded in invasion chambers. The bottom compartments of the invasion chambers were replenished with Serum free medium containing 100ng/mL of EGF to provide a chemotactic gradient. The number of invasive cells is expressed as a percentage relative to control cells not exposed to fibronectin. Data is shown as means of three independent experiments. Error bars indicate standard error. * indicates $p<0.05$ (student t-test). N.S. indicates a non significant difference.

To assess whether the exposure to exogenous fibronectin had any effect on cell invasion, we carried out cell invasion assays using EGF as chemoattractant in serum free conditions. Briefly, cells expressing control shRNA or FZD6 sh1 RNA were resuspended in serum free medium alone or containing 20µg/mL of fibronectin and were seeded in invasion chambers. We found that exogenous fibronectin did not cause any significant perturbation of the invasion of cells expressing scrambled shRNA. Conversely, exogenous fibronectin partially restored the invasive phenotype of FZD6 depleted cells, resulting in a two fold increase of cell invasion when compared to cells that were not stimulated with fibronectin (figure 7.3).

7.4 Discussion

Actin reorganization is involved in the migration of cancer cells. In physiological conditions, actin is organized in helical filaments that can be modified with the addition or removal of subunits. The elongation, shortening, or thickening of these filaments, result in modifications of their mechanical proprieties. Cells modulate actin polymerization in a variety of conditions, including cell migration (Cooper, 1991). For example, actin polymerization is required at the leading edge of migrating cells to constitute lamellipodia and filipodia and to form contractile stress fibres throughout the cell. Like in a treadmill, actin monomers are disassembled from the rear of the fibre to become again available at the front for de novo polymerization, pushing the cell membrane forward (Pollard and Borisy, 2003). Moreover, the interaction of myosin II with actin filaments provides the contractility and the transmission of tension to the sites of adhesion (Ridley *et al.*, 2003). A plethora of proteins modulate actin dynamics during cells migration, through direct or indirect mechanisms. These proteins are required for the control of the spatial-temporal polymerisation/depolymerisation of actin filaments or contractility. The Rho family of GTPases are pivotal in various aspects of actin dynamics. In Particular, Rac has been shown to be involved in the actin polymerization at the leading edge lamellipodia, where Rho is required for the formation of stress fibres and focal adhesions (Nobes and Hall, 1995; Ridley and Hall, 1992). This was demonstrated through the Injection of constitutive active forms of Rho or ROCK that resulted in the formation of actin stress fibres in cell lines (Amano *et al.*, 1997; Ridley and Hall, 1992; Paterson *et al.*, 1990). As previously mentioned, WNT/PCP signalling regulates Rho activity and thereby actin dynamics (Kim and Davidson, 2011; Habas, Dawid and He, 2003; Habas, Kato and He, 2001). Our own experiments demonstrated that FZD6 is upstream of Rho activity. The depletion of FZD6 resulted in the reduced activation of Rho, however, this did not result in a reduction of actin stress fibres, but rather in the thickening and rearrangement of fibres. The antibody used recognises RhoA, RhoB and RhoC, therefore it was not possible to assess whether FZD6 activates specific isoforms. Interestingly, it was previously observed that the selective knock down of RhoA or RhoC results in different rearrangement of actin in MDA-MB-231 cells (Vega *et al.*, 2011), suggesting different roles and functions of the two isoforms. We could therefore hypothesise that FZD6 controls selectively the activation of one of the Rho

isoforms, and that the inhibition of one isoform, rather than a pan-inhibition of Rho, results in the thickening of actin stress fibres.

Two proteins downstream of Rho have been implicated in the reorganization of actin fibres: The Diaphanous related formin protein (mDIA) and ROCK (Narumiya, Tanji and Ishizaki, 2009). The work of Watanabe and colleagues has elegantly explained how the balance between these two Rho effectors controls the structure and thickness of actin stress fibres. They demonstrated that the relative expression of constitutively active forms of mDIA1 and ROCK results in different organization and thickness of actin stress fibres (Watanabe *et al.*, 1999). Moreover, there are several studies suggesting that mDIA1 and ROCK pathways crosstalk to modulate actin dynamics. For example, ROCK can inhibit the mDIA1 dependent activation of RAC to inhibit the polymerization of actin ruffles (Tsuji *et al.*, 2002). Unbalanced ratio between mDIA1 and ROCK could therefore be the cause of the striking modifications we observed in the actin cytoskeleton in cells lacking FZD6. It would be interesting to investigate whether FZD6 dependent activation of Rho leads to specific activation of one of these Rho effectors.

FZD6-dependent actin dynamics are likely to influence cell migration. Although this would require further investigations, we can think of a number of mechanisms by which actin rearrangements could impair cell invasion in FZD6 depleted cells. A first possibility is that, although actin stress fibres are thicker following the depletion of FZD6, they could be less contractile. Indeed, during cell migration, actin polymerization alone is not sufficient to create the mechanical force that leads the cell forward. Actin polymerization has to be accompanied by the interaction of actin with myosin to generate the tension force indispensable for cell migration. The phosphorylation of the regulatory light chain of myosin (MLC) is necessary for actomyosin contractility (Ridley *et al.*, 2003). Rho is essential to regulate actomyosin contractility, both directly and through the activation of ROCK. ROCK has a dual function: it can promote cell contraction by phosphorylating MLC or by inhibiting the MLC phosphatase. Rho-GTP can also bind directly to the MLC phosphatase preventing its function (Kimura *et al.*, 1996). We observed that depletion of FZD6 results in the inhibition of Rho signalling and reduced cell migration. This might lead to a reduction of stress fibres contraction caused by the inhibition or ROCK activity and the increased activation of MLC phosphatase.

Another explanation for the increased actin polymerisation and reduced migration in the absence of FZD6 could be an excessive adhesion of cells to the extracellular matrix. Although the adhesion of cells at the leading edge is required for migration, the disassembly of focal adhesion at the rear of the cell is equally important (Ridley *et al.*, 2003). It has been demonstrated that the actomyosin contractions are capable to physically break the bound between integrins and the actin cytoskeleton, leaving the integrins behind while the cell moves forward (Lauffenburger and Horwitz, 1996).This process is mediated by the contraction of actomyosin. Since the removal of FZD6 in breast cancer cells resulted in thicker actin stress fibres, we could speculate that the latter are strained between larger focal adhesions that engage stronger interaction with the ECM, hampering the detachment at the rear of the moving cell. It would be interesting to confirm this hypothesis assessing the phosphorylation of MLC or the structure of focal adhesions in FZD6 depleted cells.

Since we observed a marked reduction in fibronectin matrix deposition following the knock down of FZD6, we wondered whether exogenous delivery of fibronectin was able to rescue the invasion and the actin phenotype of FZD6 depleted cells. The addition of fibronectin to FZD6 depleted cells resulted in a twofold increase in cell invasion (figure 7.3). As previously discussed, the fibronectin matrix could promote cell invasion in several ways, such as promoting haptotactic migration, EMT, or increasing the expression of MPP2.

We wondered whether exogenous fibronectin could also modulate the rearrangements of the actin cytoskeleton in MDA-MB-231 cells. It was previously reported that the inhibition of fibronectin matrix assembly results in the formation of thick actin stress fibres in rat smooth muscle cells (Shi *et al.*, 2014). This is similar to what we observed in breast cancer cells lacking FZD6, where the reduction of the fibronectin matrix is accompanied by the formation of thick actin stress fibres. Altogether these results suggest that the fibronectin matrix could modulate the organization of the actin cytoskeleton. When FZD6 depleted cells are exposed to exogenous fibronectin, actin stress fibres become thinner and more similar to the ones observed in control cells (fig. 7.2 and 7.1); This highlights the presence of a signalling mechanism that couples the fibronectin matrix to the cytoskeleton. Several lines of evidence demonstrated a link between the extracellular matrix and the Rho family of GTPases, mainly through the communications via integrins and heparan sulphate proteoglycans

(Schwartz and Shattil, 2000; Ren, Kiosses and Schwartz, 1999). For instance, plating A549 human lung adenocarcinoma cells onto fibronectin coated dishes, activates Rho in a time dependent manner. Moreover, the interaction between α5β1 integrins and fibronectin stimulates RhoA activation in fibroblasts (Danen *et al.*, 2002; Gu *et al.*, 2001). Therefore, we could hypothesize that the addition of exogenous fibronectin to FZD6 depleted MDA-MB-231 cells could partially restore Rho signalling through integrin activation. This in turn could lead to a recovery of the actin dynamics and cell invasion. Thus, the Rho GTPase could be the link between WNT signalling, the extracellular matrix and the actin cytoskeleton. The FZD6-Actin-Fibronectin axis could therefore be pivotal in the regulation of breast cancer invasion and metastasis.

CHAPTER VIII

Conclusions

8.1 Overview of the findings and potential clinical implications

Metastasis is the main cause of death in cancer patients. Thus, it is of paramount importance to understand the molecular basis underlying this process, in order to develop new therapeutic strategies. Predicting the outcome of a tumour at an early stage is a major challenge, but it would be a major advance since would allow the design of patient-specific therapies. For this reason, a lot of effort has been put to find novel biomarkers that predicted the aggressiveness of cancer (Ludwig and Weinstein, 2005).

It is now established that many cancers contain a subpopulation of cells with characteristics of stem cells. These present the activation of pathways normally active during organogenesis, that in the context of cancer, confer a metastatic phenotype (Jordan, Guzman and Noble, 2006). The WNT pathway is essential to orchestrate different aspects of organogenesis, and signalling molecules belonging to this pathway are often altered in cancer. For this reason, elements of the WNT pathway could be used in cancer diagnosis and intervention (Barker and Clevers, 2006).

Overall, our findings suggest that:

- FZD6 is amplified and overexpressed in 19% of breast cancers

- FZD6 expression in TNBC is associated with an increased risk of relapse

- FZD6 expression is required for the metastatic growth of human breast cancer cells *in vitro* and *in vivo*

- FZD6 regulates non-canonical WNT signalling and assembly of the fibronectin matrix and actin cytoskeleton

WNT signalling is essential for the normal development of the mammary gland. Studies in transgenic mice which bare a β-catenin/TCF-responsive β-galactosidase reporter system revealed that the WNT pathway is active in early embryogenesis (Chu *et al.*, 2004; van Genderen *et al.*, 1994). WNT signalling is detectable in the epithelial cells of mammary placodes and developed mammary buds, but it is reduced during milk ducts outgrowth (Chu *et al.*, 2004). During mammary development, stem cells renewal and differentiation are strictly controlled; WNT signalling is pivotal in this regard (Zeng and Nusse, 2010). Zeng and colleagues demonstrated that breast stem cells can expand clonally remaining undifferentiated when exposed to the canonical ligand WNT3a (Zeng and Nusse, 2010). It is still unclear how non-canonical pathways participate in breast development, but there is some evidence suggesting that non canonical could be as important as the canonical signalling. For instance, WNT5a, a non canonical ligand, inhibits the extension and branching of mammary ducts during mammary development (Roarty and Serra, 2007). The PCP pathway is essential for the orientation and migration of cells during convergence extension movements required for vertebrates gastrulation (Roszko, Sawada and Solnica-Krezel, 2009) . It is likely that the polarization of epithelial cells during mammary gland development requires PCP signalling. It is tempting to speculate that the expression of FZD6 in breast cancer cells could activate a cell signalling normally active during breast development. Cells overexpressing FZD6 could therefore mimic the highly motile mesenchymal cells responsible for breast development, acquiring enhanced capacity to travers basal membranes and invade surrounding tissues, enhanced chemotaxis, and loss of cell polarity and cell-cell interactions.

Abrogation of FZD6 expression in the breast cancer cell line MDA-MB-231 resulted in the formation of more symmetrical and organized acini, similar to the ones formed by non transformed mammary cells (fig. 4.11). Moreover, our experiments in mice suggest that FZD6 is not involved in tumour growth, but mediates the organotropism of breast cancer cells, consistent with the hypothesis that FZD6 mediates PCP signalling.

Why does the knock down of FZD6 reduce the metastasis only in bones, liver and heart, but not in other organs? Different factors could influence the metastatic niche, such as the ECM, fibroblasts, and macrophages. Our results suggest that fibronectin matrix assembly could have a role in the organotropism

of FZD6 expressing cells. Indeed, fibronectin clustering was previously reported to be pivotal in directing the metastatic colonization of cancer cells: an elegant work by Kaplan and colleagues showed that conditioned media, deriving from distinct tumour types with peculiar patterns of metastatic spread, can modify the fibronectin matrix clustering redirecting the privileged metastatic site (Kaplan *et al.*, 2005). It is therefore possible that FZD6 could support increased fibronectin matrix assembly enhancing the adhesion of cancer cells to the endothelium of specific organs. The aberrant increase of the fibronectin matrix could also enhance the ectopic survival of breast cancer stem cells in secondary sites as the bones and the liver. Indeed, it was previously reported that binding to fibronectin could provide survival signals to stem-cells and fibroblasts (Tate *et al.*, 2002; Almeida *et al.*, 2000; Dao *et al.*, 1998).

The use of therapeutic antibodies targeting FZD6 might be a valuable tool to reduce or prevent bone and liver metastasis. Breast cancer metastases to the heart are very rare (Reynen, Kockeritz and Strasser, 2004). Conversely, bones are the most common site of distant metastasis in breast cancer patients. Pain, hypercalcaemia and pathological fractures are common symptoms that largely compromise the quality of life (Coleman and Rubens, 1987). Patients bearing breast cancer metastases in the liver have a very poor prognosis, with a median survival that is less than 6 months from diagnosis (Selzner *et al.*, 2000). Therefore, reduction of metastases in the bones and liver could greatly improve the survival and the quality of life of breast cancer patients.

What signalling molecules are activated downstream of FZD6? We found that FZD6 regulates the downstream activation of the small GTPase Rho affecting the actin cytoskeleton dynamics. These findings are consistent with a role of FZD6 in the PCP pathway. Other investigators have previously linked FZD6 to the PCP (De Marco *et al.*, 2012; Cantilena *et al.*, 2011; Stuebner *et al.*, 2010; Wang, Chang and Nathans, 2010; Wang, Guo and Nathans, 2006; Guo, Hawkins and Nathans, 2004). The PCP pathway controls the extensive movements required for the gastrulation and neurulation of vertebrates. These developmental processes are guided by coordinated modulation of cell migration and polarized matrix deposition. Rho and the actin cytoskeleton are required for the migration of germ cell layers during these processes (Roszko, Sawada and Solnica-Krezel, 2009; Goto *et al.*, 2005; Habas, Dawid and He, 2003; Habas, Kato and He, 2001). We propose that the aberrant activation of

FZD6 could facilitate breast cancer cell migration through the activation of the PCP pathway (fig.8.1).

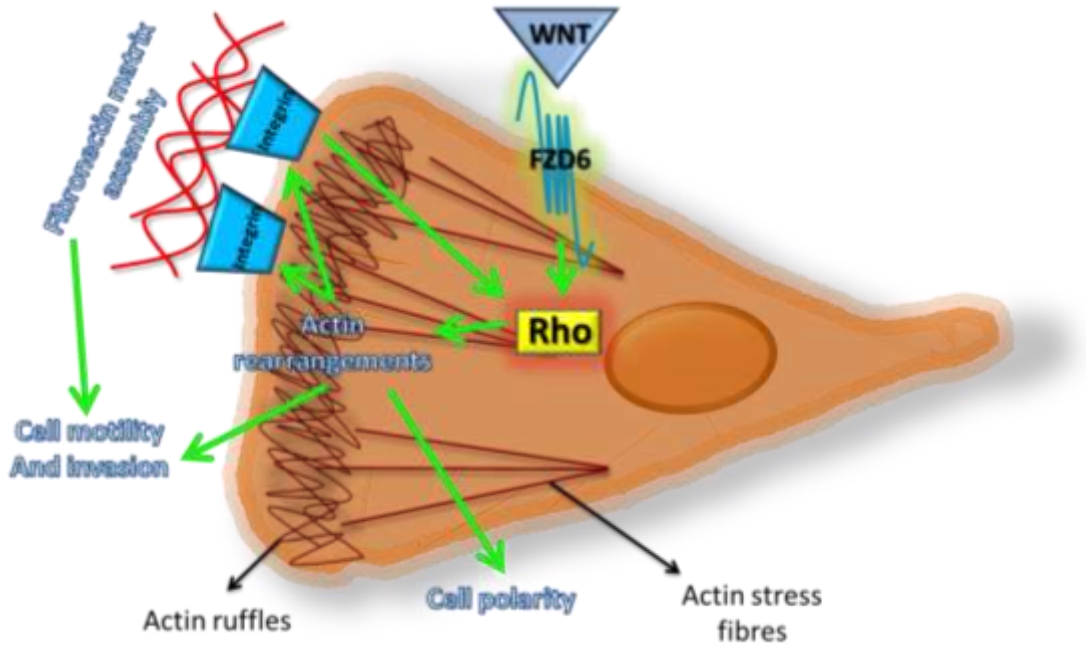

Figure 8.1: Schematic representation of the proposed molecular pathways activated by FZD6 in breast cancer. The binding of WNT ligands to Fzd6 leads to the activation of Rho. Rho in turns, promotes the rearrangement and contractility of actin stress fibres and other actin structures that regulate directional motility, cell polarity and invasion. The interaction between actin and integrins also regulates the deposition of fibronectin matrix. A positive feedback between matrix deposition and Rho activation might also exist through the activation of integrins. Fibronectin matrix assembly contributes synergistically with actin rearrangements to promote cell motility and invasion.

This could cause increased metastasis, higher risk of relapse and overall lower survival in patients, particularly those bearing the triple negative subtype.

The regulation of Rho by FZD6 could induce the activation of EMT, increased migration, angiogenesis and enhanced transendothelial migration by regulating actin dynamics (Croft *et al.*, 2004; van Nieuw Amerongen *et al.*, 2003; Bhowmick *et al.*, 2001; Worthylake *et al.*, 2001; Itoh *et al.*, 1999). Rho can also facilitate the polarized deposition of the fibronectin matrix assembly (Dzamba *et al.*, 2009; Zhong *et al.*, 1998). The fibronectin matrix surrounding cancer cells could contribute to the metastatic process in different ways, as reported by

several research groups (Park and Schwarzbauer, 2013; Das *et al.*, 2008; Huang *et al.*, 2008; Felding-Habermann, 2003; Ruoslahti, 1999; Friedl *et al.*, 1997; Klominek, Robert and Sundqvist, 1993; Aznavoorian *et al.*, 1990; Gehlsen *et al.*, 1988; Humphries, Yamada and Olden, 1988; Ruoslahti, 1984). For example, fibronectin provides an adhesion substrate that enhances cell adhesion to the endothelium of metastatic sites and promotes haptotactic motility (Huang et al., 2008; Klominek et al., 1993). Moreover, fibronectin could activate integrins and syndecan receptors to transduce intracellular signals that promote EMT and provide a positive feedback through the activation of Rho (Park and Schwarzbauer, 2014; Felding-Habermann, 2003). The coating of breast cancer cells with a fibronectin matrix stimulates fibroblasts to secrete metalloproteinases, which in turn enhance ECM degradation to promote invasion and colonization to metastatic sites (Maity et al., 2011; Saad et al., 2002). Inhibitors of the α5β1 receptor, also known as the fibronectin receptor (Schaffner, Ray and Dontenwill, 2013), are currently tested in multiple clinical trials (Lahlou and Muller, 2011). For example, ATN-161, an inhibitor peptide that mimic the fibronectin molecular structure, was shown to have beneficial effects in a phase 1 clinical trial carried out in patients with various solid tumours (Cianfrocca *et al.*, 2006). The same peptide was shown to be extremely efficient in inhibiting tumour growth and metastasis in mice injected with MDA-MB-231 breast cancer cells (Khalili *et al.*, 2006). These findings suggest that interfering with the fibronectin/integrin signalling could be a promising therapeutic strategy in breast cancer.

The direct targeting of Rho could also be exploited as a potential therapeutic approach, since Rho GTPases are involved in many aspects of carcinogenesis (Sahai and Marshall, 2002). Recently, rhosin, a novel inhibitor of the RhoA subfamily of GTPase, was shown to inhibit the proliferation and invasion of breast cancer cells. Interestingly, rhosin was also able to reduce the formation of mammospheres, suggesting a possible implication of Rho in breast cancer stem cells homeostasis (Shang *et al.*, 2012). Chen and collaborators tested a class of inhibitors of the enzymes farnesyltransferase and geranylgeranyltransferase, which are required for the farnesylation and geranylgeranylation of the C-terminal CAAX motif of the Rho family GTPases, respectively. These post-transcriptional modifications modulate the correct cellular localization and therefore the correct functions of Rho GTPases. The inhibitors caused the reduction of the 2D and 3D invasion of MDA-MB-231

breast cancer cells (Chen *et al.*, 2014). Inhibition of the Rho associated kinase ROCK was shown to arrest the invasion of breast cancer cells in vivo and in vitro (Liu *et al.*, 2009). An inhibitor of ROCK, K-115, was successfully tested in a phase II clinical trial for open-angle glaucoma, so its introduction in the clinic for breast cancer might be facilitated (Tanihara *et al.*, 2013). It would be interesting to assess whether this molecule or related compounds could find applications in breast cancer, particularly in those subtypes still lacking efficient therapies.

8.2 Future perspectives and limitations

Following this study, we need to extend our findings to validate FZD6 as a novel oncogene and candidate target for cancer therapy. A key step would be to test naked or toxin-conjugated antibodies in mice models of breast cancer. The lentiviral transduction of shRNA targeting FZD6 in MDA-MB-231 cells does not cause cell death; therefore, cells might still be capable to metastasise. Conversely, cytotoxic antibodies would kill FZD6 positive cells, and this might result in a stronger reduction of the metastatic spread in mice. The *in vivo* validation of our findings should be repeated using FZD6 knock-out cell lines to avoid re-expression of the protein and in vivo selection of FZD6 positive populations. This could be achieved by deleting the FZD6 locus in breast cancer cells trough the CRISPR-CAS9 technology, a powerful tool commonly used for genome editing.

Another limitation of our study is the limited number of triple negative breast cancer patients that were considered in the Kaplan Meier and multivariate analyses. In order to validate FZD6 as a marker of metastatic relapse in TNBC patients, we need to compare our data with other independent patients datasets.

Another question to be addressed is whether FZD6 is a marker of cancer stem cell in breast cancer. Our group previously reported that this is the case in neuroblastoma (Cantilena *et al.*, 2011). It is tempting to speculate that this might also happen in breast cancer. It is a widely held view that cancer stem cells might contribute to cancer metastasis, drug resistance and tumour recurrence (Visvader and Lindeman, 2008). If the expression of FZD6 is mainly restricted to

subpopulations of breast cancer stem cells, cytotoxic antibodies would be useful in deleting this aggressive cancer cell subtype.

Another aspect that was not addressed in this study is the contribution of FZD6 to angiogenesis, one of the hallmarks of cancer (Hanahan and Weinberg, 2011). Rho is involved in multiple aspects of this process (Bryan and d'Amore, 2007). Since FZD6 regulates Rho activity, it is reasonable to think it could be relevant in breast cancer angiogenesis.

Several aspects of FZD6 signalling still remain unclear, such as which signalling proteins link FZD6 to the actin cytoskeleton or the fibronectin matrix assembly. Moreover, it remains to be determined if FZD6 interacts specifically with one of the Rho isoforms, RhoA, RhoB and RhoC. In this thesis, I reported that the knock down of FZD6 results in the thickening of actin stress fibres and their structural rearrangement (fig. 7.1). However, it is not known what molecules downstream of Rho are implicated in this process. In this respect, it would be interesting to assess the activation of Rho effectors such as ROCK 1/2 and mDIA in the absence of FZD6 expression.

FZD6 could promote cell migration through Rho-mediated contraction of actin stress fibres. This could be verified by assessing the phosphorylation status of MLC in the absence or presence of FZD6 expression.

In this study FZD6 signalling has been investigated predominantly in one cell line, MDA-MB-231. We should confirm our signalling data using more breast cancer cell lines and using not only loss of function approaches but also overexpression experiments.

Finally, we have not investigated whether a specific WNT ligand mediates the pro-tumorigenic effects of FZD6 in breast cancer. Recent data gathered by FRAP membrane mobility analysis suggests that multiple ligands including WNT1, -2, 3A, -4, -5A, -7A, -9B and -10B modulate FZD6 surface mobility and are likely to activate or repress the receptor (Kilander, Dahlström and Schulte, 2014). This suggests the existence of a complex network of ligands and pathways linked to FZD6.

Appendix

Methods used by research collaborators

A.1 Patients characteristics and immunohistochemistry

The following methods were used by Rossano Lattanzio and Mauro Piantelli from G. D'Annunzio University, Chieti, Italy.

The study included 352 primary infiltrating breast cancers from N0 and T1/T2 tumours diagnosed between 1985 and 2003 at the Regina Elena National Cancer Institute, Rome, Italy, and presenting primary unilateral breast carcinomas. The study was reviewed and approved by the ethics committee of the ''Regina Elena'' National Cancer Institute and written informed consent was obtained from all patients. All patients received radiation therapy. One hundred and thirty-eight patients received hormonal therapy and 140 patients were treated with adjuvant chemotherapy (followed or not by hormonal therapy). Patients with HER2-positive tumours did not receive trastuzumab, as it was not used in breast tumour therapy in the study period. Follow-up data were obtained from institutional records or from referring physicians. The median follow-up was of 80 months (range 6-298 months). During follow-up, 32 patients (9.1%) developed a local recurrence and distant metastases were observed in 54 cases (15.3%).

Tissue microarrays (TMA) were constructed by extracting 2-mm diameter cores of histologically confirmed invasive breast carcinoma paraffin-embedded tissue. The cores were then embedded into gridded paraffin blocks, using a precision instrument (MTA, Beecher Instruments). After antigen retrieval (microwave treatment at 750 W for 10 min in 10 mM sodium citrate buffer, pH 6.0), five-micrometres sections were incubated overnight at 4°C with the anti-Fzd6 antibody (Novus Biological, Littleton, CO) at 1:100 dilution. The anti-rabbit EnVision kit (K4003, Dako, Glostrup, Denmark) was used for signal amplification. In control sections the specific primary antibody was replaced with

non-immune serum. The percentage of tumour cells with positive membrane staining for FZD6 ranged from 6 to 85%, with a mean ± SE of 23.8% ± 2.2. The following antibodies were used for the identification of tumour subtypes: anti-ER-α MoAb 6F11 (Novocastra, Leica Biosystems, Newcastle, UK), the anti-PGR MoAb 1A6 (Novocastra), the anti-Ki-67 MoAb MIB-1 (Dako) and the anti-HER2 (Herceptest, Dako). Immunohistochemical analysis was done by two pathologists (MP, RL) by consensus without knowledge of the clinicophatological information.

Ki-67 expression was dichotomized according to the St Gallen criteria: Ki-67 was considered high when the percentage of cells expressing the marker was ≥ 20% (Goldhirsch *et al.*, 2013). HER2 membranous staining was assessed with Herceptest (Dako) and classified as positive if the intensity was scored 3+ (30% of cells showing complete membrane staining), or, if scored 2+ (between 10% and 30% of positive cells), was considered positive in the presence of an amplification of the HER-2 gene as assessed by fluorescent in situ hybridization. Tumours were classified positive for PR or ER if ≥10% of cells were positive to the staining. Tumour size and tumour grade were dichotomized according to the Saint Gallen criteria for the definition of risk categories (T ≤ 2cm vs. T > 2 cm; grade 1 vs. grade 2-3). The relation between Fzd6 expression and the clinicophatological parameters were assessed by Pearson's $\chi 2$ or Fisher's exact test, as appropriate. Fzd6 expression was studied in different breast cancer molecular subtypes: Luminal A-like (n = 128), Luminal B-like, HER2 negative (n = 123), Luminal B-like, HER2 positive (n = 33), HER2 positive, non-luminal (n = 21) and Triple negative breast cancers (TNBC, n = 47). DFS was defined as the time from surgery to the first of the following events: tumour recurrence at local site or at distant sites. LRFS and DRFS were defined as the times from surgery to the occurrence of relapse at local and distant sites, respectively. Kaplan–Meier plots were used to illustrate the survival in specified cohorts and the log-rank test to test for equality of survival curves. The association of Fzd6 expression with outcome, adjusted for other prognostic factors, was tested by multivariate analysis (Cox's proportional hazards model). The following covariates were included in the multivariate DFS models: tumour size and grade, ER, PGR, Ki-67, HER2 and FZD6 status. Appropriateness of the proportional hazard assumption was assessed by plotting the log cumulative hazard functions over time and checking for

parallelism. SPSS Version 15.0 (SPSS, Chicago, IL) was used throughout and $P<0.05$ was considered statistically significant.

A.2 Threedimensional cultures

The methods described in this section were performed by Sibylle Ermler and Elisabete Silva from the Institute for the Environment, Department of Life Sciences, Brunel University London.

Wells in an eight-chamber slide (Millipore) were coated with 60 µl/well of growth factor reduced (GFR) matrigel and allowed to settle for 15 minutes. Cells were resuspended in complete medium containing 2% GFR matrigel (assay medium) and seeded in duplicate wells. Cells were incubated for 20 days and the medium replaced every 3–4 days with fresh assay medium. At the end of the incubation, the assay medium was discarded and the acini were fixed with 2% PFA (in PBS, pH 7.4) for 20 min at room temperature. Next, cells were permeabilized with 0.5% Triton-X in PBS for 10 min at 4° C and washed thrice with 100 mM glycine in PBS at room temperature. Acini were then blocked with immunofluorescence blocking buffer (IF buffer; 130 mM NaCl, 7.7 mM NaN3, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20) containing 10% of goat serum for 1.5 hours at room temperature. A secondary blocking step was performed incubating cells with Immunofluorescence blocking buffer containing 10% goat serum and 20 mg/ml of goat anti-mouse F(ab')2 fragment for 30 minutes. Primary antibodies were diluted 1:200 in the secondary blocking solution, incubated overnight at 4° C and rinsed thrice with IF buffer. The primary antibodies used were: Mouse anti-laminin V (ABcam; 1:200 in IF buffer) or anti-fibronectin (BD; 1:200 in IF buffer). Incubation with secondary antibody (Alexa Fluor 555 goat anti-mouse, Molecular Probes, Invitrogen) was in IF buffer containing 10% goat serum for 50 minutes (Dilution of 1:200), followed by three washes at room temperature. Cell nuclei were counterstained with a 300 nM solution of DAPI in PBS for 5 minutes and mounted with Vectashield HardSet mounting medium (Vector Laboratories, Peterborough, UK). Photographs of the acini were taken with an Olympus BX41 fluorescence microscope with IMSTAR Pathfinder™ software.

A.3 Orthotopic transplantations in immunodeficient mice

The *in vivo* transplantation and the analysis of tumour tissue sections were carried out by our collaborators Dr Manuela Iezzi and Dr Alessia Lamolinara at the Aging Research Centre, G. D'Annunzio University, Chieti, Italy.

NOD scid gamma (NSG) mice were purchased from the Jackson Laboratory and bred in the animal facility of the Aging Research Centre, G. D'Annunzio University, Chieti. Animal care and experimental procedures were approved by the Ethics Committee for Animal Experimentation of the institute, according to the Italian law. Eight weeks old female mice (10 mice per group) were injected unilaterally with 3.5×10^6 cells into the fourth mammary fat pad. Tumour growth was monitored biweekly using callipers up to 96 days or when tumours reached 0.3 cm3 of volume. Tumour volume was calculated as 0.5 X d12 X d2, where d1 and d2 are the smaller and larger diameters, respectively. Primary tumours and organs were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned and stained with haematoxylin and eosin. Slides were independently evaluated by two pathologists. To quantify microscopic metastases, lungs and livers were cut transversally into 2.0 mm thick parallel slabs starting from a random position, resulting in 5-8 slabs for lungs and 8-10 slabs for livers. A semiquantitative evaluation was performed attributing to each sample a value from 0 to 4, based on the number and size of metastases; 0 was attributed to organs with no detectable metastases; 4 to organs with numerous large metastases. Fisher's exact test was utilised to compare differences in metastatic spread.

A.4 Confocal microscopy

Confocal microscopy experiments were carried out by Michele Sallese and Giorgia Fragassi at Fondazione Mario Negri Sud, S.Maria Imbaro, Italy.

70000 cells were plated in 1 mL of complete medium in coverslips placed into 24 well plates. Cells were then washed with PBS and fixed with 4% PFA in PBS for 15 minutes. Cells were then washed 4 times with PBS and incubated in blocking solution (0.1 % saponin, 0.5 % BSA, 50 mM NH_4Cl pH 7.4, 0.02 % NaN_3) for 1 hour.

Cells were then incubated either with Phalloidin Alexafluor546-conjugated (Invitrogen) diluted 1:400 in blocking buffer for 30 minutes or with mouse anti-Fibronectin (BD) diluted 1:100 in blocking buffer for 2 hours. In the case of the Fibronectin staining, cells were then washed 4 times with PBS and stained with anti-mouse texas red (Invitrogen) diluted 1:400 in blocking buffer for 1 hour. The following steps were analogous for the phalloidin and fibronectin staining: Cells were washed in PBS and stained with DRAQ5 (Cell Signaling) diluted 1:1000 in PBS for 15 minutes. The coverslips were then washed 4 times with PBS and mounted with Mowiol (Sigma) into microscope glass slides.

Confocal images were acquired using a Zeiss LSM510 inverted confocal microscope system (Carl Zeiss, Gottingen, Germany). Cells were analysed using a 63x oil-immersion objective, maintaining the pinhole of the objective at 1 Airy unit. Images were acquired under non-saturating conditions and using the same settings for all samples.

Bibliography

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997) 'Beta-Catenin is a Target for the Ubiquitin-Proteasome Pathway', *The EMBO journal,* 16(13), pp. 3797-3804.

Aguirre-Ghiso, J.A. (2007) 'Models, mechanisms and clinical evidence for cancer dormancy', *Nature Reviews Cancer,* 7(11), pp. 834-846.

Ahmad, A., Wang, Z., Kong, D., Ali, S., Li, Y., Banerjee, S., Ali, R. and Sarkar, F.H. (2010) 'FoxM1 down-regulation leads to inhibition of proliferation, migration and invasion of breast cancer cells through the modulation of extra-cellular matrix degrading factors', Breast cancer research and treatment, 122(2), pp. 337-346.

Ahmad, O.B., Boschi-Pinto, C., Lopez, A.D., Murray, C.J., Lozano, R. and Inoue, M. (2001) *Age standardization of rates: a new WHO standard.* World Health Organization Geneva.

Ai, L., Tao, Q., Zhong, S., Fields, C.R., Kim, W.J., Lee, M.W., Cui, Y., Brown, K.D. and Robertson, K.D. (2006) 'Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer', *Carcinogenesis,* 27(7), pp. 1341-1348.

Akiyama, S.K., Olden, K. and Yamada, K.M. (1995) 'Fibronectin and integrins in invasion and metastasis', *Cancer and metastasis reviews,* 14(3), pp. 173-189.

Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M. and McEwan, R.N. (1987) 'A rapid in vitro assay for quantitating the invasive potential of tumor cells', *Cancer research,* 47(12), pp. 3239-3245.

Alderton, G.K. (2013) 'Metastasis: Polarizing metastasis', *Nature Reviews Cancer,* 13(2), pp. 75-75.

Ali, A.M., Schmidt, M.K., Bolla, M.K., Wang, Q., Gago-Dominguez, M., Castelao, J.E., Carracedo, A., Garzon, V.M., Bojesen, S.E., Nordestgaard, B.G., Flyger, H., Chang-Claude, J., Vrieling, A., Rudolph, A., Seibold, P., Nevanlinna, H., Muranen, T.A., Aaltonen, K., Blomqvist, C., Matsuo, K., Ito, H., Iwata, H., Horio, A., John, E.M., Sherman, M., Lissowska, J., Figueroa, J., Garcia-Closas, M., Anton-Culver, H., Shah, M., Hopper, J.L., Trichopoulou, A., Bueno-de-Mesquita, B., Krogh, V., Weiderpass, E., Andersson, A., Clavel-Chapelon, F., Dossus, L., Fagherazzi, G., Peeters, P.H., Olsen, A., Wishart, G.C., Easton, D.F., Borgquist, S., Overvad, K., Barricarte, A., Gonzalez, C.A., Sanchez, M.J., Amiano, P., Riboli, E., Key, T. and Pharoah, P.D. (2014) 'Alcohol consumption and survival after a breast cancer diagnosis: a literature-based meta-analysis and collaborative analysis of data for 29,239 cases', *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* 23(6), pp. 934-945.

Almeida, E.A., Ilic, D., Han, Q., Hauck, C.R., Jin, F., Kawakatsu, H., Schlaepfer, D.D. and Damsky, C.H. (2000) 'Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase', *The Journal of cell biology,* 149(3), pp. 741-754.

Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y. and Kaibuchi, K. (1997) 'Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase', *Science (New York, N.Y.),* 275(5304), pp. 1308-1311.

Ananthakrishnan, R. and Ehrlicher, A. (2007) 'The forces behind cell movement', *International journal of biological sciences,* 3(5), pp. 303-317.

Anastas, J.N. and Moon, R.T. (2012) 'WNT signalling pathways as therapeutic targets in cancer', *Nature Reviews Cancer,* 13(1), pp. 11-26.

Anastas, J., Biechele, T., Robitaille, M., Muster, J., Allison, K., Angers, S. and Moon, R. (2012) 'A protein complex of SCRIB, NOS1AP and VANGL1 regulates cell polarity and migration, and is associated with breast cancer progression', *Oncogene,* 31(32), pp. 3696-3708.

Angst, B.D., Marcozzi, C. and Magee, A.I. (2001) 'The cadherin superfamily: diversity in form and function', *Journal of cell science,* 114(Pt 4), pp. 629-641.

Aznavoorian, S., Stracke, M.L., Krutzsch, H., Schiffmann, E. and Liotta, L.A. (1990) 'Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells', *The Journal of cell biology,* 110(4), pp. 1427-1438.

Baade, P.D., Youlden, D.R. and Chambers, S.K. (2011) 'When do I know I am cured? Using conditional estimates to provide better information about cancer survival prospects', *Medical Journal of Australia,* 194(2), pp. 73.

Bajada, S., Mazakova, I., Richardson, J.B. and Ashammakhi, N. (2008) 'Updates on stem cells and their applications in regenerative medicine', *Journal of tissue engineering and regenerative medicine,* 2(4), pp. 169-183.

Barker, N. and Clevers, H. (2006) 'Mining the Wnt pathway for cancer therapeutics', *Nature Reviews Drug Discovery,* 5(12), pp. 997-1014.

Baselga, J., Cortés, J., Kim, S., Im, S., Hegg, R., Im, Y., Roman, L., Pedrini, J.L., Pienkowski, T. and Knott, A. (2012) 'Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer', *New England Journal of Medicine,* 366(2), pp. 109-119.

Bastock, R., Strutt, H. and Strutt, D. (2003) 'Strabismus is asymmetrically localised and binds to Prickle and Dishevelled during Drosophila planar polarity patterning', *Development (Cambridge, England),* 130(13), pp. 3007-3014.

Bauer, K.R., Brown, M., Cress, R.D., Parise, C.A. and Caggiano, V. (2007) 'Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype', *Cancer,* 109(9), pp. 1721-1728.

Baum, J. and Duffy, H.S. (2011) 'Fibroblasts and myofibroblasts: what are we talking about?', *Journal of cardiovascular pharmacology,* 57(4), pp. 376-379.

Beers, M. (2006) 'Merk Manual Diagnosis and Theraphy'

Behrens, J., von Kries, J.P., Kühl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996) 'Functional interaction of β-catenin with the transcription factor LEF-1', .

Bejsovec, A. (2005) 'Wnt pathway activation: new relations and locations', *Cell,* 120(1), pp. 11-14.

Bengochea, A., De Souza, M., Lefrancois, L., Le Roux, E., Galy, O., Chemin, I., Kim, M., Wands, J., Trepo, C. and Hainaut, P. (2008) 'Common dysregulation of Wnt/Frizzled receptor elements in human hepatocellular carcinoma', *British journal of cancer,* 99(1), pp. 143-150.

Benhaj, K., Akcali, K.C. and Ozturk, M. (2006) 'Redundant expression of canonical Wnt ligands in human breast cancer cell lines', *Oncology reports,* 15(3), pp. 701-708.

Berns, E.M., Klijn, J.G., van Putten, W.L., van Staveren, I.L., Portengen, H. and Foekens, J.A. (1992) 'c-myc amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer', *Cancer research,* 52(5), pp. 1107-1113.

Bertucci, F., Finetti, P., Cervera, N., Esterni, B., Hermitte, F., Viens, P. and Birnbaum, D. (2008) 'How basal are triple-negative breast cancers?', *International journal of Cancer,* 123(1), pp. 236-240.

Berx, G. and Van Roy, F. (2001) 'The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression', *Breast Cancer Research,* 3(5), pp. 289-293.

Betapudi, V., Licate, L.S. and Egelhoff, T.T. (2006) 'Distinct roles of nonmuscle myosin II isoforms in the regulation of MDA-MB-231 breast cancer cell spreading and migration', *Cancer research,* 66(9), pp. 4725-4733.

Bhowmick, N.A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C.A., Engel, M.E., Arteaga, C.L. and Moses, H.L. (2001) 'Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism', *Molecular biology of the cell,* 12(1), pp. 27-36.

Bild, A.H., Yao, G., Chang, J.T., Wang, Q., Potti, A., Chasse, D., Joshi, M., Harpole, D., Lancaster, J.M. and Berchuck, A. (2005) 'Oncogenic pathway signatures in human cancers as a guide to targeted therapies', *Nature,* 439(7074), pp. 353-357.

Bilic, J., Huang, Y.L., Davidson, G., Zimmermann, T., Cruciat, C.M., Bienz, M. and Niehrs, C. (2007) 'Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation', *Science (New York, N.Y.),* 316(5831), pp. 1619-1622.

Bilir, B., Kucuk, O. and Moreno, C.S. (2013) 'Wnt signaling blockage inhibits cell proliferation and migration, and induces apoptosis in triple-negative breast cancer cells', *Journal of translational medicine,* 11, pp. 280-5876-11-280.

Blick, T., Widodo, E., Hugo, H., Waltham, M., Lenburg, M., Neve, R. and Thompson, E. (2008) 'Epithelial mesenchymal transition traits in human breast cancer cell lines', *Clinical & experimental metastasis,* 25(6), pp. 629-642.

Blumenschein, G.R. (1983) 'The role of progestins in the treatment of breast cancer', *Seminars in oncology,* 10(4 Suppl 4), pp. 7-10.

Boguski, M.S. and McCormick, F. (1993) 'Proteins regulating Ras and its relatives', *Nature,* 366(6456), pp. 643-654.

Boutros, M., Paricio, N., Strutt, D.I. and Mlodzik, M. (1998) 'Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling', *Cell,* 94(1), pp. 109-118.

Bowcock, A.M. (1999) *Breast cancer: molecular genetics, pathogenesis, and therapeutics.* Humana Press.

Bozic, I., Antal, T., Ohtsuki, H., Carter, H., Kim, D., Chen, S., Karchin, R., Kinzler, K.W., Vogelstein, B. and Nowak, M.A. (2010) 'Accumulation of driver and passenger mutations during tumor progression', *Proceedings of the National Academy of Sciences of the United States of America,* 107(43), pp. 18545-18550.

Brisken, C., Heineman, A., Chavarria, T., Elenbaas, B., Tan, J., Dey, S.K., McMahon, J.A., McMahon, A.P. and Weinberg, R.A. (2000) 'Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling', *Genes & development,* 14(6), pp. 650-654.

Bryan, B. and d'Amore, P. (2007) 'What tangled webs they weave: Rho-GTPase control of angiogenesis', *Cellular and Molecular Life Sciences,* 64(16), pp. 2053-2065.

Bubendorf, L., Schöpfer, A., Wagner, U., Sauter, G., Moch, H., Willi, N., Gasser, T.C. and Mihatsch, M.J. (2000) 'Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients', *Human pathology,* 31(5), pp. 578-583.

Buttke, T.M., McCubrey, J.A. and Owen, T.C. (1993) 'Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines', *Journal of immunological methods,* 157(1), pp. 233-240.

Caddy, J., Wilanowski, T., Darido, C., Dworkin, S., Ting, S.B., Zhao, Q., Rank, G., Auden, A., Srivastava, S. and Papenfuss, T.A. (2010) 'Epidermal wound repair is regulated by the planar cell polarity signaling pathway', *Developmental cell,* 19(1), pp. 138-147.

Cadigan, K.M. and Nusse, R. (1997) 'Wnt signaling: a common theme in animal development', *Genes & development,* 11(24), pp. 3286-3305.

Cailleau, R., Cruciger, Q., Hokanson, K., Olive, M. and Blumenschein, G. (1976) 'Morphological, biochemical and chromosomal characterization of breast tumor lines from pleural effusions', *IN VITRO-JOURNAL OF THE TISSUE CULTURE ASSOCIATION.* SOC IN VITRO BIOLOGY 9315 LARGO DR WEST, STE 25, LARGO, MD 20774, 331-331.

Cailleau, R., Olivé, M. and Cruciger, Q.V. (1978) 'Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization', *In vitro,* 14(11), pp. 911-915.

Campbell, L.L. and Polyak, K. (2007) 'Breast tumor heterogeneity: cancer stem cells or clonal evolution?', *Cell Cycle,* 6(19), pp. 2332-2338.

Cano, A., Pérez-Moreno, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., del Barrio, M.G., Portillo, F. and Nieto, M.A. (2000) 'The transcription factor snail controls epithelial–mesenchymal transitions by repressing E-cadherin expression', *Nature cell biology,* 2(2), pp. 76-83.

Cantilena, S., Pastorino, F., Pezzolo, A., Chayka, O., Pistoia, V., Ponzoni, M. and Sala, A. (2011) 'Frizzled receptor 6 marks rare, highly tumourigenic stem-like cells in mouse and human neuroblastomas', *Oncotarget,* 2(12), pp. 976-983.

Carey, L., Winer, E., Viale, G., Cameron, D. and Gianni, L. (2010) 'Triple-negative breast cancer: disease entity or title of convenience?', Nature reviews Clinical oncology, 7(12), pp. 683-692.

Cariati, M., Bennett-Britton, T., Pinder, S. and Purushotham, A. (2005) '"Inflammatory" breast cancer', Surgical oncology, 14(3), pp. 133-143.

Carron, C., Pascal, A., Djiane, A., Boucaut, J.C., Shi, D.L. and Umbhauer, M. (2003) 'Frizzled receptor dimerization is sufficient to activate the Wnt/beta-catenin pathway', *Journal of cell science,* 116(Pt 12), pp. 2541-2550.

Cavallaro, U. and Christofori, G. (2004) 'Cell adhesion and signalling by cadherins and Ig-CAMs in cancer', *Nature Reviews Cancer,* 4(2), pp. 118-132.

Chambers, A.F., Groom, A.C. and MacDonald, I.C. (2002) 'Metastasis: dissemination and growth of cancer cells in metastatic sites', *Nature Reviews Cancer,* 2(8), pp. 563-572.

Cheang, M.C., Chia, S.K., Voduc, D., Gao, D., Leung, S., Snider, J., Watson, M., Davies, S., Bernard, P.S. and Parker, J.S. (2009) 'Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer', *Journal of the National Cancer Institute,* 101(10), pp. 736-750.

Chen, M., Knifley, T., Subramanian, T., Spielmann, H.P. and O'Connor, K.L. (2014) 'Use of Synthetic Isoprenoids to Target Protein Prenylation and Rho GTPases in Breast Cancer Invasion', *PloS one,* 9(2), pp. e89892.

Chen, A., Cuevas, I., Kenny, P.A., Miyake, H., Mace, K., Ghajar, C., Boudreau, A., Bissell, M.J. and Boudreau, N. (2009) 'Endothelial cell migration and vascular endothelial growth factor expression are the result of loss of breast tissue polarity', *Cancer research,* 69(16), pp. 6721-6729.

Chen, P., Xie, H., Sekar, M.C., Gupta, K. and Wells, A. (1994) 'Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but mitogen-activated protein kinase activity is not sufficient for induced cell movement', *The Journal of cell biology,* 127(3), pp. 847-857.

Cheng, H.C., Abdel-Ghany, M., Elble, R.C. and Pauli, B.U. (1998) 'Lung endothelial dipeptidyl peptidase IV promotes adhesion and metastasis of rat breast cancer cells via tumor cell surface-associated fibronectin', *The Journal of biological chemistry,* 273(37), pp. 24207-24215.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T. and Karin, M. (1988) 'The c-Fos protein interacts with c-JunAP-1 to stimulate transcription of AP-1 responsive genes', *Cell,* 54(4), pp. 541-552.

Choi, S. and Han, J. (2002) 'Xenopus Cdc42 regulates convergent extension movements during gastrulation through Wnt/Ca 2 signaling pathway', Developmental biology, 244(2), pp. 342-357.

Chrzanowska-Wodnicka, M. and Burridge, K. (1996) 'Rho-stimulated contractility drives the formation of stress fibers and focal adhesions', *The Journal of cell biology,* 133(6), pp. 1403-1415.

Chu, E.Y., Hens, J., Andl, T., Kairo, A., Yamaguchi, T.P., Brisken, C., Glick, A., Wysolmerski, J.J. and Millar, S.E. (2004) 'Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis', *Development (Cambridge, England),* 131(19), pp. 4819-4829.

Cianfrocca, M., Kimmel, K., Gallo, J., Cardoso, T., Brown, M., Hudes, G., Lewis, N., Weiner, L., Lam, G. and Brown, S. (2006) 'Phase 1 trial of the antiangiogenic peptide ATN-161 (Ac-PHSCN-NH2), a beta integrin antagonist, in patients with solid tumours', *British journal of cancer,* 94(11), pp. 1621-1626.

Cianfrocca, M. and Goldstein, L.J. (2004) 'Prognostic and predictive factors in early-stage breast cancer', *The oncologist,* 9(6), pp. 606-616.

Ciani, L. and Salinas, P.C. (2007) 'c-Jun N-terminal kinase (JNK) cooperates with Gsk3beta to regulate Dishevelled-mediated microtubule stability', *BMC cell biology,* 8, pp. 27.

Civenni, G., Holbro, T. and Hynes, N.E. (2003) 'Wnt1 and Wnt5a induce cyclin D1 expression through ErbB1 transactivation in HC11 mammary epithelial cells', *EMBO reports,* 4(2), pp. 166-171.

Clapham, D.E. (1995) 'Calcium signaling', Cell, 80(2), pp. 259-268.

Clevers, H. (2006) 'Wnt/β-catenin signaling in development and disease', *Cell,* 127(3), pp. 469-480.

Clevers, H. and Nusse, R. (2012) 'Wnt/β-catenin signaling and disease', *Cell,* 149(6), pp. 1192-1205.

Coleman, R.E. and Rubens, R.D. (1987) 'The clinical course of bone metastases from breast cancer', *British journal of cancer,* 55(1), pp. 61-66.

Cooper, J.A. (1991) 'The role of actin polymerization in cell motility', *Annual Review of Physiology,* 53(1), pp. 585-605.

Croft, D.R., Sahai, E., Mavria, G., Li, S., Tsai, J., Lee, W.M., Marshall, C.J. and Olson, M.F. (2004) 'Conditional ROCK activation in vivo induces tumor cell dissemination and angiogenesis', *Cancer research,* 64(24), pp. 8994-9001.

Curtin, J.A., Quint, E., Tsipouri, V., Arkell, R.M., Cattanach, B., Copp, A.J., Henderson, D.J., Spurr, N., Stanier, P. and Fisher, E.M. (2003) 'Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse', *Current Biology,* 13(13), pp. 1129-1133.

Dales, J., Garcia, S., Meunier-Carpentier, S., Andrac-Meyer, L., Haddad, O., Lavaut, M., Allasia, C., Bonnier, P. and Charpin, C. (2005) 'Overexpression of hypoxia-inducible factor HIF-1α

predicts early relapse in breast cancer: Retrospective study in a series of 745 patients', International journal of cancer, 116(5), pp. 734-739.

Danen, E.H., Sonneveld, P., Brakebusch, C., Fassler, R. and Sonnenberg, A. (2002) 'The fibronectin-binding integrins alpha5beta1 and alphavbeta3 differentially modulate RhoA-GTP loading, organization of cell matrix adhesions, and fibronectin fibrillogenesis', *The Journal of cell biology,* 159(6), pp. 1071-1086.

Dang, C.V. (2012) 'MYC on the path to cancer', *Cell,* 149(1), pp. 22-35.

Daniels, D.L. and Weis, W.I. (2005) 'β-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation', *Nature structural & molecular biology,* 12(4), pp. 364-371.

Dao, M.A., Hashino, K., Kato, I. and Nolta, J.A. (1998) 'Adhesion to fibronectin maintains regenerative capacity during ex vivo culture and transduction of human hematopoietic stem and progenitor cells', *Blood,* 92(12), pp. 4612-4621.

Das, S., Banerji, A., Frei, E. and Chatterjee, A. (2008) 'Rapid expression and activation of MMP-2 and MMP-9 upon exposure of human breast cancer cells (MCF-7) to fibronectin in serum free medium', *Life Sciences,* 82(9), pp. 467-476.

Das, G., Jenny, A., Klein, T.J., Eaton, S. and Mlodzik, M. (2004) 'Diego interacts with Prickle and Strabismus/Van Gogh to localize planar cell polarity complexes', *Development (Cambridge, England),* 131(18), pp. 4467-4476.

Davidson, N.E. and Abeloff, M.D. (1994) 'Adjuvant therapy of breast cancer', *World journal of surgery,* 18(1), pp. 112-116.

De Marco, P., Merello, E., Rossi, A., Piatelli, G., Cama, A., Kibar, Z. and Capra, V. (2012) 'FZD6 is a novel gene for human neural tube defects', *Human mutation,* 33(2), pp. 384-390.

Dean, M., Fojo, T. and Bates, S. (2005) 'Tumour stem cells and drug resistance', *Nature Reviews Cancer,* 5(4), pp. 275-284.

Deming, S.L., Nass, S.J., Dickson, R.B. and Trock, B.J. (2000) 'C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance', *British journal of cancer,* 83(12), pp. 1688-1695.

Dent, R., Hanna, W.M., Trudeau, M., Rawlinson, E., Sun, P. and Narod, S.A. (2009) 'Pattern of metastatic spread in triple-negative breast cancer', *Breast cancer research and treatment,* 115(2), pp. 423-428.

Dent, R., Trudeau, M., Pritchard, K.I., Hanna, W.M., Kahn, H.K., Sawka, C.A., Lickley, L.A., Rawlinson, E., Sun, P. and Narod, S.A. (2007) 'Triple-negative breast cancer: clinical features and patterns of recurrence', *Clinical cancer research : an official journal of the American Association for Cancer Research,* 13(15 Pt 1), pp. 4429-4434.

Desgrosellier, J.S. and Cheresh, D.A. (2010) 'Integrins in cancer: biological implications and therapeutic opportunities', *Nature Reviews Cancer,* 10(1), pp. 9-22.

Dijksterhuis, J., Petersen, J. and Schulte, G. (2014) 'WNT/Frizzled signalling: receptor–ligand selectivity with focus on FZD-G protein signalling and its physiological relevance: IUPHAR Review 3', *British journal of pharmacology,* 171(5), pp. 1195-1209.

Donnenberg, V.S. and Donnenberg, A.D. (2005) 'Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis', *The Journal of Clinical Pharmacology,* 45(8), pp. 872-877.

Dossus, L., Boutron-Ruault, M., Kaaks, R., Gram, I.T., Vilier, A., Fervers, B., Manjer, J., Tjonneland, A., Olsen, A. and Overvad, K. (2014) 'Active and passive cigarette smoking

and breast cancer risk: Results from the EPIC cohort', *International Journal of Cancer,* 134(8), pp. 1871-1888.

Duncan, J.A., Reeves, J.R. and Cooke, T.G. (1998) 'BRCA1 and BRCA2 proteins: roles in health and disease', *Molecular pathology : MP,* 51(5), pp. 237-247.

Dzamba, B.J., Jakab, K.R., Marsden, M., Schwartz, M.A. and DeSimone, D.W. (2009) 'Cadherin adhesion, tissue tension, and noncanonical Wnt signaling regulate fibronectin matrix organization', *Developmental cell,* 16(3), pp. 421-432.

Edelman, G.M. and Crossin, K.L. (1991) 'Cell adhesion molecules: implications for a molecular histology', *Annual Review of Biochemistry,* 60(1), pp. 155-190.

Elmore, J.G., Armstrong, K., Lehman, C.D. and Fletcher, S.W. (2005) 'Screening for breast cancer', *JAMA: the journal of the American Medical Association,* 293(10), pp. 1245-1256.

Eroles, P., Bosch, A., Alejandro Perez-Fidalgo, J. and Lluch, A. (2012) 'Molecular biology in breast cancer: intrinsic subtypes and signaling pathways', *Cancer treatment reviews,* 38(6), pp. 698-707.

Etienne-Manneville, S. (2004) 'Actin and microtubules in cell motility: which one is in control?', *Traffic,* 5(7), pp. 470-477.

Eyers, C.x., McNeill, H., Knebel, A., Morrice, N., Arthur, S.x.x., Cuenda, A. and Cohen, P. (2005) 'The phosphorylation of CapZ-interacting protein (CapZIP) by stress-activated protein kinases triggers its dissociation from CapZ', *Biochem.J,* 389, pp. 127-135.

Fagotto, F., Glück, U. and Gumbiner, B.M. (1998) 'Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of β-catenin', *Current Biology,* 8(4), pp. 181-190.

Fanning, E. (1992) 'Simian virus 40 large T antigen: the puzzle, the pieces, and the emerging picture', *Journal of virology,* 66(3), pp. 1289-1293.

Farmer, P., Bonnefoi, H., Becette, V., Tubiana-Hulin, M., Fumoleau, P., Larsimont, D., MacGrogan, G., Bergh, J., Cameron, D. and Goldstein, D. (2005) 'Identification of molecular apocrine breast tumours by microarray analysis', *Breast Cancer Research,* 7(Suppl 2), pp. P2. 11.

Faulstich, H., Zobeley, S., Rinnerthaler, G. and Small, J. (1988) 'Fluorescent phallotoxins as probes for filamentous actin', *Journal of Muscle Research & Cell Motility,* 9(5), pp. 370-383.

Felding-Habermann, B. (2003) 'Integrin adhesion receptors in tumor metastasis', *Clinical & experimental metastasis,* 20(3), pp. 203-213.

Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C. and Parkin, D.M. (2010) 'Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008', *International journal of cancer,* 127(12), pp. 2893-2917.

Finak, G., Bertos, N., Pepin, F., Sadekova, S., Souleimanova, M., Zhao, H., Chen, H., Omeroglu, G., Meterissian, S. and Omeroglu, A. (2008) 'Stromal gene expression predicts clinical outcome in breast cancer', *Nature medicine,* 14(5), pp. 518-527.

Flahaut, M., Meier, R., Coulon, A., Nardou, K., Niggli, F., Martinet, D., Beckmann, J., Joseph, J., Mühlethaler-Mottet, A. and Gross, N. (2009) 'The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/β-catenin pathway', *Oncogene,* 28(23), pp. 2245-2256.

Fogerty, F.J., Akiyama, S.K., Yamada, K.M. and Mosher, D.F. (1990) 'Inhibition of binding of fibronectin to matrix assembly sites by anti-integrin (alpha 5 beta 1) antibodies', *The Journal of cell biology,* 111(2), pp. 699-708.

Foley, J., Dann, P., Hong, J., Cosgrove, J., Dreyer, B., Rimm, D., Dunbar, M., Philbrick, W. and Wysolmerski, J. (2001) 'Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development', *Development (Cambridge, England),* 128(4), pp. 513-525.

Foord, S.M., Bonner, T.I., Neubig, R.R., Rosser, E.M., Pin, J.P., Davenport, A.P., Spedding, M. and Harmar, A.J. (2005) 'International Union of Pharmacology. XLVI. G protein-coupled receptor list', *Pharmacological reviews,* 57(2), pp. 279-288.

Forbes, S., Bhamra, G., Bamford, S., Dawson, E., Kok, C., Clements, J., Menzies, A., Teague, J., Futreal, P. and Stratton, M. (2008) 'The catalogue of somatic mutations in cancer (COSMIC)', *Current protocols in human genetics,* , pp. 10.11. 1-10.11. 26.

Ford, D., Easton, D., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D., Weber, B., Lenoir, G. and Chang-Claude, J. (1998) 'Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families', *The American Journal of Human Genetics,* 62(3), pp. 676-689.

Foulkes, W.D., Smith, I.E. and Reis-Filho, J.S. (2010) 'Triple-negative breast cancer', *New England journal of medicine,* 363(20), pp. 1938-1948.

Foulkes, W.D., Stefansson, I.M., Chappuis, P.O., Begin, L.R., Goffin, J.R., Wong, N., Trudel, M. and Akslen, L.A. (2003) 'Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer', *Journal of the National Cancer Institute,* 95(19), pp. 1482-1485.

Fredriksson, R. and Schioth, H.B. (2005) 'The repertoire of G-protein-coupled receptors in fully sequenced genomes', *Molecular pharmacology,* 67(5), pp. 1414-1425.

Fridlyand, J., Snijders, A.M., Ylstra, B., Li, H., Olshen, A., Segraves, R., Dairkee, S., Tokuyasu, T., Ljung, B.M., Jain, A.N., McLennan, J., Ziegler, J., Chin, K., Devries, S., Feiler, H., Gray, J.W., Waldman, F., Pinkel, D. and Albertson, D.G. (2006) 'Breast tumor copy number aberration phenotypes and genomic instability', *BMC cancer,* 6, pp. 96.

Friedl, P. and Alexander, S. (2011) 'Cancer invasion and the microenvironment: plasticity and reciprocity', *Cell,* 147(5), pp. 992-1009.

Friedl, P., Maaser, K., Klein, C.E., Niggemann, B., Krohne, G. and Zanker, K.S. (1997) 'Migration of highly aggressive MV3 melanoma cells in 3-dimensional collagen lattices results in local matrix reorganization and shedding of alpha2 and beta1 integrins and CD44', *Cancer research,* 57(10), pp. 2061-2070.

Fritz, G., Brachetti, C., Bahlmann, F., Schmidt, M. and Kaina, B. (2002) 'Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters', *British journal of cancer,* 87(6), pp. 635-644.

Fröjmark, A., Schuster, J., Sobol, M., Entesarian, M., Kilander, M.C., Gabrikova, D., Nawaz, S., Baig, S., Schulte, G., Klar, J. and Dahl, N. (2011) 'Mutations in Frizzled 6 Cause Isolated Autosomal-Recessive Nail Dysplasia', *The American Journal of Human Genetics,* 88(6), pp. 852-860.

Fukukawa, C., Nagayama, S., Tsunoda, T., Toguchida, J., Nakamura, Y. and Katagiri, T. (2009) 'Activation of the non-canonical Dvl–Rac1–JNK pathway by Frizzled homologue 10 in human synovial sarcoma', *Oncogene,* 28(8), pp. 1110-1120.

Garbe, J.C., Bhattacharya, S., Merchant, B., Bassett, E., Swisshelm, K., Feiler, H.S., Wyrobek, A.J. and Stampfer, M.R. (2009) 'Molecular distinctions between stasis and telomere attrition senescence barriers shown by long-term culture of normal human mammary epithelial cells', *Cancer research,* 69(19), pp. 7557-7568.

Gazdar, A.F., Kurvari, V., Virmani, A., Gollahon, L., Sakaguchi, M., Westerfield, M., Kodagoda, D., Stasny, V., Cunningham, H.T. and Wistuba, I.I. (1998) 'Characterization of paired tumor

and non-tumor cell lines established from patients with breast cancer', *International journal of cancer,* 78, pp. 766-774.

Gearhart, J., Pashos, E.E. and Prasad, M.K. (2007) 'Pluripotency redux—advances in stem-cell research', *New England Journal of Medicine,* 357(15), pp. 1469-1472.

Gehlsen, K.R., Argraves, W.S., Pierschbacher, M.D. and Ruoslahti, E. (1988) 'Inhibition of in vitro tumor cell invasion by Arg-Gly-Asp-containing synthetic peptides', *The Journal of cell biology,* 106(3), pp. 925-930.

Giuliano, A.E., Kirgan, D.M., Guenther, J.M. and Morton, D.L. (1994) 'Lymphatic mapping and sentinel lymphadenectomy for breast cancer', *Annals of Surgery,* 220(3), pp. 391-8; discussion 398-401.

Golan, T., Yaniv, A., Bafico, A., Liu, G. and Gazit, A. (2004) 'The human Frizzled 6 (HFz6) acts as a negative regulator of the canonical Wnt· β-catenin signaling cascade', *Journal of Biological Chemistry,* 279(15), pp. 14879-14888.

Goldhirsch, A., Winer, E.P., Coates, A.S., Gelber, R.D., Piccart-Gebhart, M., Thurlimann, B., Senn, H.J. and Panel members (2013) 'Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013', *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO,* 24(9), pp. 2206-2223.

Goodman, L.S. (1990) *Goodman and Gilman's the pharmacological basis of therapeutics.* Pergamon Press New York.

Goto, T., Davidson, L., Asashima, M. and Keller, R. (2005) 'Planar cell polarity genes regulate polarized extracellular matrix deposition during frog gastrulation', *Current biology,* 15(8), pp. 787-793.

Greaves, M. and Maley, C.C. (2012) 'Clonal evolution in cancer', *Nature,* 481(7381), pp. 306-313.

Green, M.R. and Sambrook, J. (2012) 'Molecular cloning: a laboratory manual', .

Greiner, D.L., Hesselton, R.A. and Shultz, L.D. (1998) 'SCID mouse models of human stem cell engraftment', *Stem cells,* 16(3), pp. 166-177.

Gu, J., Sumida, Y., Sanzen, N. and Sekiguchi, K. (2001) 'Laminin-10/11 and fibronectin differentially regulate integrin-dependent Rho and Rac activation via p130(Cas)-CrkII-DOCK180 pathway', *The Journal of biological chemistry,* 276(29), pp. 27090-27097.

Guo, N., Hawkins, C. and Nathans, J. (2004) 'Frizzled6 controls hair patterning in mice', *Proceedings of the National Academy of Sciences of the United States of America,* 101(25), pp. 9277-9281.

Guo, X., Mak, K.K., Taketo, M.M. and Yang, Y. (2009) 'The Wnt/β-catenin pathway interacts differentially with PTHrP signaling to control chondrocyte hypertrophy and final maturation', *PLoS One,* 4(6), pp. e6067.

Gupta, S., Iljin, K., Sara, H., Mpindi, J.P., Mirtti, T., Vainio, P., Rantala, J., Alanen, K., Nees, M. and Kallioniemi, O. (2010) 'FZD4 as a mediator of ERG oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells', *Cancer research,* 70(17), pp. 6735-6745.

Győrffy, B., Lanczky, A., Eklund, A.C., Denkert, C., Budczies, J., Li, Q. and Szallasi, Z. (2010) 'An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients', *Breast cancer research and treatment,* 123(3), pp. 725-731.

Habas, R., Kato, Y. and He, X. (2001) 'Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1', *Cell,* 107(7), pp. 843-854.

Habas, R., Dawid, I.B. and He, X. (2003) 'Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation', *Genes & development,* 17(2), pp. 295-309.

Haffty, B.G., Yang, Q., Reiss, M., Kearney, T., Higgins, S.A., Weidhaas, J., Harris, L., Hait, W. and Toppmeyer, D. (2006) 'Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer', *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 24(36), pp. 5652-5657.

Haider, A.S., Peters, S.B., Kaporis, H., Cardinale, I., Fei, J., Ott, J., Blumenberg, M., Bowcock, A.M., Krueger, J.G. and Carucci, J.A. (2006) 'Genomic analysis defines a cancer-specific gene expression signature for human squamous cell carcinoma and distinguishes malignant hyperproliferation from benign hyperplasia', *Journal of investigative dermatology,* 126(4), pp. 869-881.

Halazonetis, T.D., Georgopoulos, K., Greenberg, M.E. and Leder, P. (1988) 'c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities', *Cell,* 55(5), pp. 917-924.

Hall, C.L. and Keller, E.T. (2006) 'The role of Wnts in bone metastases', *Cancer and metastasis reviews,* 25(4), pp. 551-558.

Hall, A. (1998) 'Rho GTPases and the actin cytoskeleton', *Science (New York, N.Y.),* 279(5350), pp. 509-514.

Hall, C.L., Bafico, A., Dai, J., Aaronson, S.A. and Keller, E.T. (2005) 'Prostate cancer cells promote osteoblastic bone metastases through Wnts', *Cancer research,* 65(17), pp. 7554-7560.

Hanahan, D. and Weinberg, R.A. (2011) 'Hallmarks of cancer: the next generation', *Cell,* 144(5), pp. 646-674.

Hannon, G.J. (2002) 'RNA interference', *Nature,* 418(6894), pp. 244-251.

Hartsock, A. and Nelson, W.J. (2008) 'Adherens and tight junctions: structure, function and connections to the actin cytoskeleton', Biochimica et Biophysica Acta (BBA)-Biomembranes, 1778(3), pp. 660-669.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B. and Kinzler, K.W. (1998) 'Identification of c-MYC as a target of the APC pathway', *Science (New York, N.Y.),* 281(5382), pp. 1509-1512.

He, X., Semenov, M., Tamai, K. and Zeng, X. (2004) 'LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way', *Development (Cambridge, England),* 131(8), pp. 1663-1677.

Heinonen, K.M., Vanegas, J.R., Lew, D., Krosl, J. and Perreault, C. (2011) 'Wnt4 enhances murine hematopoietic progenitor cell expansion through a planar cell polarity-like pathway', *PloS one,* 6(4), pp. e19279.

Helleman, J., Jansen, M.P., Ruigrok-Ritstier, K., van Staveren, I.L., Look, M.P., Meijer-van Gelder, M.E., Sieuwerts, A.M., Klijn, J.G., Sleijfer, S., Foekens, J.A. and Berns, E.M. (2008) 'Association of an extracellular matrix gene cluster with breast cancer prognosis and endocrine therapy response', *Clinical cancer research : an official journal of the American Association for Cancer Research,* 14(17), pp. 5555-5564.

Hennessy, B.T., Gonzalez-Angulo, A., Stemke-Hale, K., Gilcrease, M.Z., Krishnamurthy, S., Lee, J., Fridlyand, J., Sahin, A., Agarwal, R. and Joy, C. (2009) 'Characterization of a

naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics', Cancer research, 69(10), pp. 4116-4124.

Herschkowitz, J.I., Simin, K., Weigman, V.J., Mikaelian, I., Usary, J., Hu, Z., Rasmussen, K.E., Jones, L.P., Assefnia, S., Chandrasekharan, S., Backlund, M.G., Yin, Y., Khramtsov, A.I., Bastein, R., Quackenbush, J., Glazer, R.I., Brown, P.H., Green, J.E., Kopelovich, L., Furth, P.A., Palazzo, J.P., Olopade, O.I., Bernard, P.S., Churchill, G.A., Van Dyke, T. and Perou, C.M. (2007) 'Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors', *Genome biology,* 8(5), pp. R76.

Hogan, P.G., Chen, L., Nardone, J. and Rao, A. (2003) 'Transcriptional regulation by calcium, calcineurin, and NFAT', Genes & development, 17(18), pp. 2205-2232.

Hortobagyi, G.N., de la Garza Salazar, Jaime, Pritchard, K., Amadori, D., Haidinger, R., Hudis, C.A., Khaled, H., Liu, M., Martin, M. and Namer, M. (2005) 'The global breast cancer burden: variations in epidemiology and survival', *Clinical breast cancer,* 6(5), pp. 391-401.

Howe, L.R. and Brown, A.M. (2004) 'Wnt signaling and breast cancer', *Cancer Biology and Therapy,* 3(1), pp. 36-41.

Hsieh, J.C., Rattner, A., Smallwood, P.M. and Nathans, J. (1999) 'Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein', *Proceedings of the National Academy of Sciences of the United States of America,* 96(7), pp. 3546-3551.

Hu, T. and Li, C. (2010) 'Convergence between Wnt-b-catenin and EGFR signaling in cancer', *Mol Cancer,* 9, pp. 236.

Huang, L., Cheng, H., Isom, R., Chen, C., Levine, R.A. and Pauli, B.U. (2008) 'Protein Kinase Cε Mediates Polymeric Fibronectin Assembly on the Surface of Blood-borne Rat Breast Cancer Cells to Promote Pulmonary Metastasis', *Journal of Biological Chemistry,* 283(12), pp. 7616-7627.

Hudis, C.A. (2007) 'Trastuzumab—mechanism of action and use in clinical practice', New England Journal of Medicine, 357(1), pp. 39-51.

Huelsken, J. and Behrens, J. (2002) 'The Wnt signalling pathway', *Journal of cell science,* 115(Pt 21), pp. 3977-3978.

Hughes, C.S., Postovit, L.M. and Lajoie, G.A. (2010) 'Matrigel: a complex protein mixture required for optimal growth of cell culture', *Proteomics,* 10(9), pp. 1886-1890.

Humphries, M.J., Yamada, K.M. and Olden, K. (1988) 'Investigation of the biological effects of anti-cell adhesive synthetic peptides that inhibit experimental metastasis of B16-F10 murine melanoma cells', *The Journal of clinical investigation,* 81(3), pp. 782-790.

Hussain, S.P. and Harris, C.C. (1998) 'Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes', *Cancer Res,* 58(18).

Huveneers, S. and Danen, E.H. (2009) 'Adhesion signaling - crosstalk between integrins, Src and Rho', *Journal of cell science,* 122(Pt 8), pp. 1059-1069.

Hynes, R.O. (1990) 'Fibronectins', .

Ioachim, E., Charchanti, A., Briasoulis, E., Karavasilis, V., Tsanou, H., Arvanitis, D., Agnantis, N. and Pavlidis, N. (2002) 'Immunohistochemical expression of extracellular matrix components tenascin, fibronectin, collagen type IV and laminin in breast cancer: their prognostic value and role in tumour invasion and progression', *European journal of cancer,* 38(18), pp. 2362-2370.

Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Meneghini, M., Barker, N., Waterman, M., Bowerman, B., Clevers, H. and Shibuya, H. (1999) 'The TAK1–NLK–MAPK-related

pathway antagonizes signalling between β-catenin and transcription factor TCF', Nature, 399(6738), pp. 798-802.

Ishitani, T., Kishida, S., Hyodo-Miura, J., Ueno, N., Yasuda, J., Waterman, M., Shibuya, H., Moon, R.T., Ninomiya-Tsuji, J. and Matsumoto, K. (2003) 'The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling', Molecular and cellular biology, 23(1), pp. 131-139.

Ismail, P.M., Amato, P., Soyal, S.M., DeMayo, F.J., Conneely, O.M., O'Malley, B.W. and Lydon, J.P. (2003) 'Progesterone involvement in breast development and tumorigenesis—as revealed by progesterone receptor "knockout" and "knockin" mouse models', *Steroids,* 68(10), pp. 779-787.

Itoh, K., Yoshioka, K., Akedo, H., Uehata, M., Ishizaki, T. and Narumiya, S. (1999) 'An essential part for Rho–associated kinase in the transcellular invasion of tumor cells', *Nature medicine,* 5(2), pp. 221-225.

Jain, J., McCafffrey, P.G., Miner, Z., Kerppola, T.K., Lambert, J.N., Verdine, G.L., Curran, T. and Rao, A. (1993) 'The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun', .

Jaffe, A.B. and Hall, A. (2005) 'Rho GTPases: biochemistry and biology', *Annu.Rev.Cell Dev.Biol.,* 21, pp. 247-269.

Jenny, A., Reynolds-Kenneally, J., Das, G., Burnett, M. and Mlodzik, M. (2005) 'Diego and Prickle regulate Frizzled planar cell polarity signalling by competing for Dishevelled binding', *Nature cell biology,* 7(7), pp. 691-697.

Jia, D., Entersz, I., Butler, C. and Foty, R.A. (2012) 'Fibronectin matrix-mediated cohesion suppresses invasion of prostate cancer cells', *BMC cancer,* 12, pp. 94-2407-12-94.

Jiang, W.G., Watkins, G., Lane, J., Cunnick, G.H., Douglas-Jones, A., Mokbel, K. and Mansel, R.E. (2003) 'Prognostic value of rho GTPases and rho guanine nucleotide dissociation inhibitors in human breast cancers', *Clinical cancer research : an official journal of the American Association for Cancer Research,* 9(17), pp. 6432-6440.

Jones, C., Qian, D., Kim, S.M., Li, S., Ren, D., Knapp, L., Sprinzak, D., Avraham, K.B., Matsuzaki, F. and Chi, F. (2014) 'Ankrd6 is a mammalian functional homolog of Drosophila planar cell polarity gene diego and regulates coordinated cellular orientation in the mouse inner ear', *Developmental biology,* 395(1), pp. 62-72.

Jordan, C.T., Guzman, M.L. and Noble, M. (2006) 'Cancer stem cells', *New England Journal of Medicine,* 355(12), pp. 1253-1261.

Jorissen, R.N., Walker, F., Pouliot, N., Garrett, T.P., Ward, C.W. and Burgess, A.W. (2003) 'Epidermal growth factor receptor: mechanisms of activation and signalling', *Experimental cell research,* 284(1), pp. 31-53.

Jue, S.F., Bradley, R.S., Rudnicki, J.A., Varmus, H.E. and Brown, A.M. (1992) 'The mouse Wnt-1 gene can act via a paracrine mechanism in transformation of mammary epithelial cells', *Molecular and cellular biology,* 12(1), pp. 321-328.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K. and Perrimon, N. (1996) 'The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing', *Genes & development,* 10(24), pp. 3116-3128.

Kallioniemi, A., Kallioniemi, O.P., Piper, J., Tanner, M., Stokke, T., Chen, L., Smith, H.S., Pinkel, D., Gray, J.W. and Waldman, F.M. (1994) 'Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization', *Proceedings of the National Academy of Sciences of the United States of America,* 91(6), pp. 2156-2160.

Kalluri, R. and Zeisberg, M. (2006) 'Fibroblasts in cancer', *Nature Reviews Cancer,* 6(5), pp. 392-401.

Kalluri, R. and Weinberg, R.A. (2009) 'The basics of epithelial-mesenchymal transition', *The Journal of clinical investigation,* 119(6), pp. 1420-1428.

Kamiya, N., Ye, L., Kobayashi, T., Mochida, Y., Yamauchi, M., Kronenberg, H.M., Feng, J.Q. and Mishina, Y. (2008) 'BMP signaling negatively regulates bone mass through sclerostin by inhibiting the canonical Wnt pathway', *Development (Cambridge, England),* 135(22), pp. 3801-3811.

Kaplan, R.N., Riba, R.D., Zacharoulis, S., Bramley, A.H., Vincent, L., Costa, C., MacDonald, D.D., Jin, D.K., Shido, K. and Kerns, S.A. (2005) 'VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche', *Nature,* 438(7069), pp. 820-827.

Karnoub, A.E., Dash, A.B., Vo, A.P., Sullivan, A., Brooks, M.W., Bell, G.W., Richardson, A.L., Polyak, K., Tubo, R. and Weinberg, R.A. (2007) 'Mesenchymal stem cells within tumour stroma promote breast cancer metastasis', *Nature,* 449(7162), pp. 557-563.

Karrison, T.G., Ferguson, D.J. and Meier, P. (1999) 'Dormancy of mammary carcinoma after mastectomy', *Journal of the National Cancer Institute,* 91(1), pp. 80-85.

Kastan, M.B., Canman, C.E. and Leonard, C.J. (1995) 'P53, cell cycle control and apoptosis: implications for cancer', *Cancer and metastasis reviews,* 14(1), pp. 3-15.

Katoh, M. and Katoh, M. (2005) 'Identification and characterization of rat Ankrd6 gene in silico.', *International journal of molecular medicine,* 15(2), pp. 359.

Kaucka, M., Plevova, K., Pavlova, S., Janovska, P., Mishra, A., Verner, J., Prochazkova, J., Krejci, P., Kotaskova, J., Ovesna, P., Tichy, B., Brychtova, Y., Doubek, M., Kozubik, A., Mayer, J., Pospisilova, S. and Bryja, V. (2013) 'The planar cell polarity pathway drives pathogenesis of chronic lymphocytic leukemia by the regulation of B-lymphocyte migration', *Cancer research,* 73(5), pp. 1491-1501.

Kawano, Y. and Kypta, R. (2003) 'Secreted antagonists of the Wnt signalling pathway', *Journal of cell science,* 116(Pt 13), pp. 2627-2634.

Kenny, P.A., Lee, G.Y., Myers, C.A., Neve, R.M., Semeiks, J.R., Spellman, P.T., Lorenz, K., Lee, E.H., Barcellos-Hoff, M.H. and Petersen, O.W. (2007) 'The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression', *Molecular oncology,* 1(1), pp. 84-96.

Keydar, I., Chen, L., Karby, S., Weiss, F., Delarea, J., Radu, M., Chaitcik, S. and Brenner, H. (1979) 'Establishment and characterization of a cell line of human breast carcinoma origin', *European Journal of Cancer (1965),* 15(5), pp. 659-670.

Khalili, P., Arakelian, A., Chen, G., Plunkett, M.L., Beck, I., Parry, G.C., Donate, F., Shaw, D.E., Mazar, A.P. and Rabbani, S.A. (2006) 'A non-RGD-based integrin binding peptide (ATN-161) blocks breast cancer growth and metastasis in vivo', *Molecular cancer therapeutics,* 5(9), pp. 2271-2280.

Kilander, M.B., Dahlström, J. and Schulte, G. (2014) 'Assessment of Frizzled 6 membrane mobility by FRAP supports G protein coupling and reveals WNT-Frizzled selectivity', *Cellular signalling,* .

Kim, D., Kim, S., Koh, H., Yoon, S.O., Chung, A.S., Cho, K.S. and Chung, J. (2001) 'Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 15(11), pp. 1953-1962.

Kim, H.Y. and Davidson, L.A. (2011) 'Punctuated actin contractions during convergent extension and their permissive regulation by the non-canonical Wnt-signaling pathway', *Journal of cell science,* 124(Pt 4), pp. 635-646.

Kim, N.G., Koh, E., Chen, X. and Gumbiner, B.M. (2011) 'E-cadherin mediates contact inhibition of proliferation through Hippo signaling-pathway components', *Proceedings of the National Academy of Sciences of the United States of America,* 108(29), pp. 11930-11935.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) 'Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase)', *Science (New York, N.Y.),* 273(5272), pp. 245-248.

Kinoshita, N., Iioka, H., Miyakoshi, A. and Ueno, N. (2003) 'PKC delta is essential for Dishevelled function in a noncanonical Wnt pathway that regulates Xenopus convergent extension movements', Genes & development, 17(13), pp. 1663-1676.

Kinoshita, N., Sasai, N., Misaki, K. and Yonemura, S. (2008) 'Apical accumulation of Rho in the neural plate is important for neural plate cell shape change and neural tube formation', Molecular biology of the cell, 19(5), pp. 2289-2299.

Klemm, F., Bleckmann, A., Siam, L., Chuang, H., Rietkötter, E., Behme, D., Schulz, M., Schaffrinski, M., Schindler, S. and Trümper, L. (2011) 'β-Catenin-independent WNT signaling in basal-like breast cancer and brain metastasis', *Carcinogenesis,* 32(3), pp. 434-442.

Klominek, J., Robert, K.H. and Sundqvist, K.G. (1993) 'Chemotaxis and haptotaxis of human malignant mesothelioma cells: effects of fibronectin, laminin, type IV collagen, and an autocrine motility factor-like substance', *Cancer research,* 53(18), pp. 4376-4382.

Klopocki, E., Kristiansen, G., Wild, P.J., Klaman, I., Castanos-Velez, E., Singer, G., Stöhr, R., Simon, R., Sauter, G. and Leibiger, H. (2004) 'Loss of SFRP1 is associated with breast cancer progression and poor prognosis in early stage tumors', *International journal of oncology,* 25(3), pp. 641-649.

Koblinski, J.E., Ahram, M. and Sloane, B.F. (2000) 'Unraveling the role of proteases in cancer', *Clinica Chimica Acta,* 291(2), pp. 113-135.

Kohn, A.D. and Moon, R.T. (2005) 'Wnt and calcium signaling: beta-catenin-independent pathways.', Cell calcium, 38(3-4), pp. 439.

Kolben, T., Peröbner, I., Fernsebner, K., Lechner, F., Geissler, C., Ruiz-Heinrich, L., Capovilla, S., Jochum, M. and Neth, P. (2012) 'Dissecting the impact of frizzled receptors in Wnt/β-catenin signaling of human mesenchymal stem cells', *Biological chemistry,* 393(12), pp. 1433-1447.

Koo, C., Muir, K.W. and Lam, E.W. (2012) 'FOXM1: From cancer initiation to progression and treatment', Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 1819(1), pp. 28-37.

Kouros-Mehr, H., Slorach, E.M., Sternlicht, M.D. and Werb, Z. (2006) 'GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland', *Cell,* 127(5), pp. 1041-1055.

Krishan, A. (1975) 'Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining', *The Journal of cell biology,* 66(1), pp. 188-193.

Krueger, J.S., Keshamouni, V.G., Atanaskova, N. and Reddy, K.B. (2001) 'Temporal and quantitative regulation of mitogen-activated protein kinase (MAPK) modulates cell motility and invasion', *Oncogene,* 20(31), pp. 4209-4218.

Kuhl, M. (2004) 'The WNT/calcium pathway: biochemical mediators, tools and future requirements', Front Biosci, 9(1), pp. 967-974.

Kuhl, M., Sheldahl, L.C., Malbon, C.C. and Moon, R.T. (2000) 'Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus', The Journal of biological chemistry, 275(17), pp. 12701-12711.

Kurayoshi, M., Oue, N., Yamamoto, H., Kishida, M., Inoue, A., Asahara, T., Yasui, W. and Kikuchi, A. (2006) 'Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion', *Cancer research,* 66(21), pp. 10439-10448.

Laemmli, U.K. (1970) 'Cleavage of structural proteins during the assembly of the head of bacteriophage T4', *Nature,* 227(5259), pp. 680-685.

Lahlou, H. and Muller, W.J. (2011) 'β1-integrins signaling and mammary tumor progression in transgenic mouse models: implications for human breast cancer', *Breast Cancer Res,* 13(6), pp. 229.

Lane, D.P. (1992) 'Cancer. p53, guardian of the genome', *Nature,* 358(6381), pp. 15-16.

Lara-Medina, F., Pérez-Sánchez, V., Saavedra-Pérez, D., Blake-Cerda, M., Arce, C., Motola-Kuba, D., Villarreal-Garza, C., González-Angulo, A.M., Bargalló, E. and Aguilar, J.L. (2011) 'Triple-negative breast cancer in Hispanic patients', *Cancer,* 117(16), pp. 3658-3669.

Lasfargues, E.Y. and Ozzello, L. (1958) 'Cultivation of Human Breast Carcinomas2', .

Lasfargues, E.Y., Coutinho, W.G. and Redfield, E.S. (1978) 'Isolation of two human tumor epithelial cell lines from solid breast carcinomas', *Journal of the National Cancer Institute,* 61(4), pp. 967-978.

Lauffenburger, D.A. and Horwitz, A.F. (1996) 'Cell migration: a physically integrated molecular process', *Cell,* 84(3), pp. 359-369.

Le Floch, N., Rivat, C., De Wever, O., Bruyneel, E., Mareel, M., Dale, T. and Gespach, C. (2005) 'The proinvasive activity of Wnt-2 is mediated through a noncanonical Wnt pathway coupled to GSK-3β and c-Jun/AP-1 signaling', *The FASEB journal,* 19(1), pp. 144-146.

Lee, G.Y., Kenny, P.A., Lee, E.H. and Bissell, M.J. (2007) 'Three-dimensional culture models of normal and malignant breast epithelial cells', *Nature methods,* 4(4), pp. 359-365.

Lee, K.M., Choi, K.H. and Ouellette, M.M. (2004) 'Use of exogenous hTERT to immortalize primary human cells', *Cytotechnology,* 45(1), pp. 33-38.

Lee, J., Kim, D.H., Lee, S., Yang, Q.H., Lee, D.K., Lee, S.K., Roeder, R.G. and Lee, J.W. (2009) 'A tumor suppressive coactivator complex of p53 containing ASC-2 and histone H3-lysine-4 methyltransferase MLL3 or its paralogue MLL4', *Proceedings of the National Academy of Sciences of the United States of America,* 106(21), pp. 8513-8518.

Lei, Y., Zhang, T., Li, H., Wu, B., Jin, L. and Wang, H. (2010) 'VANGL2 mutations in human cranial neural-tube defects', *New England Journal of Medicine,* 362(23), pp. 2232-2235.

Lei, Y., Zhu, H., Yang, W., Ross, M.E., Shaw, G.M. and Finnell, R.H. (2014) 'Identification of novel CELSR1 mutations in spina bifida', *PloS one,* 9(3), pp. e92207.

Leppa, S. and Bohmann, D. (1999) 'Diverse functions of JNK signaling and c-Jun in stress response and apoptosis', *Oncogene,* 18(45), pp. 6158-6162.

Levine, A.J., Momand, J. and Finlay, C.A. (1991) 'The p53 tumour suppressor gene', *Nature,* 351(6326), pp. 453-456.

Li, F., Tiede, B., Massagué, J. and Kang, Y. (2007) 'Beyond tumorigenesis: cancer stem cells in metastasis', *Cell research,* 17(1), pp. 3-14.

Li, F., Tiede, B., Massagué, J. and Kang, Y. (2006) 'Beyond tumorigenesis: cancer stem cells in metastasis', *Cell research,* 17(1), pp. 3-14.

Li, V.S., Ng, S.S., Boersema, P.J., Low, T.Y., Karthaus, W.R., Gerlach, J.P., Mohammed, S., Heck, A.J., Maurice, M.M. and Mahmoudi, T. (2012) 'Wnt signaling through inhibition of β-catenin degradation in an intact Axin1 complex', *Cell,* 149(6), pp. 1245-1256.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H. and Parsons, R. (1997) 'PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer', *Science (New York, N.Y.),* 275(5308), pp. 1943-1947.

Liang, H., Chen, Q., Coles, A.H., Anderson, S.J., Pihan, G., Bradley, A., Gerstein, R., Jurecic, R. and Jones, S.N. (2003) 'Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue', *Cancer cell,* 4(5), pp. 349-360.

Liedtke, C., Mazouni, C., Hess, K.R., Andre, F., Tordai, A., Mejia, J.A., Symmans, W.F., Gonzalez-Angulo, A.M., Hennessy, B., Green, M., Cristofanilli, M., Hortobagyi, G.N. and Pusztai, L. (2008) 'Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer', *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 26(8), pp. 1275-1281.

Lin, S.Y., Xia, W., Wang, J.C., Kwong, K.Y., Spohn, B., Wen, Y., Pestell, R.G. and Hung, M.C. (2000) 'Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression', *Proceedings of the National Academy of Sciences of the United States of America,* 97(8), pp. 4262-4266.

Lindvall, C., Bu, W., Williams, B.O. and Li, Y. (2007) 'Wnt signaling, stem cells, and the cellular origin of breast cancer', *Stem cell reviews,* 3(2), pp. 157-168.

Liotta, L.A. (1984) 'Tumor invasion and metastases: role of the basement membrane. Warner-Lambert Parke-Davis Award lecture', *The American journal of pathology,* 117(3), pp. 339-348.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G., Tan, Y., Zhang, Z., Lin, X. and He, X. (2002) 'Control of β-catenin phosphorylation/degradation by a dual-kinase mechanism', *Cell,* 108(6), pp. 837-847.

Liu, W. and Heckman, C. (1998) 'The sevenfold way of PKC regulation', Cellular signalling, 10(8), pp. 529-542.

Liu, S., Goldstein, R.H., Scepansky, E.M. and Rosenblatt, M. (2009) 'Inhibition of rho-associated kinase signaling prevents breast cancer metastasis to human bone', *Cancer research,* 69(22), pp. 8742-8751.

Lohsiriwat, V., Martella, S., Rietjens, M., Botteri, E., Rotmensz, N., Mastropasqua, M.G., Garusi, C., De Lorenzi, F., Manconi, A. and Sommario, M. (2012) 'Paget's disease as a local recurrence after nipple-sparing mastectomy: clinical presentation, treatment, outcome, and risk factor analysis', *Annals of surgical oncology,* 19(6), pp. 1850-1855.

Lombaerts, M., Van Wezel, T., Philippo, K., Dierssen, J., Zimmerman, R., Oosting, J., Van Eijk, R., Eilers, P., van De Water, B. and Cornelisse, C. (2006) 'E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines', *British journal of cancer,* 94(5), pp. 661-671.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A. and Jacks, T. (1993) 'p53 is required for radiation-induced apoptosis in mouse thymocytes', *Nature,* 362(6423), pp. 847-849.

Lu, X. and Kang, Y. (2007) 'Organotropism of breast cancer metastasis', *Journal of mammary gland biology and neoplasia,* 12(2-3), pp. 153-162.

Lu, D., Zhao, Y., Tawatao, R., Cottam, H.B., Sen, M., Leoni, L.M., Kipps, T.J., Corr, M. and Carson, D.A. (2004) 'Activation of the Wnt signaling pathway in chronic lymphocytic leukemia', *Proceedings of the National Academy of Sciences of the United States of America,* 101(9), pp. 3118-3123.

Ludwig, J.A. and Weinstein, J.N. (2005) 'Biomarkers in cancer staging, prognosis and treatment selection', *Nature Reviews Cancer,* 5(11), pp. 845-856.

Luga, V., Zhang, L., Viloria-Petit, A.M., Ogunjimi, A.A., Inanlou, M.R., Chiu, E., Buchanan, M., Hosein, A.N., Basik, M. and Wrana, J.L. (2012) 'Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration', *Cell,* 151(7), pp. 1542-1556.

Luga, V. and Wrana, J.L. (2013) 'Tumor-stroma interaction: Revealing fibroblast-secreted exosomes as potent regulators of Wnt-planar cell polarity signaling in cancer metastasis', *Cancer research,* 73(23), pp. 6843-6847.

Lundgren, S., Lønning, P., Utaaker, E., Aakvaag, A. and Kvinnsland, S. (1990) 'Influence of progestins on serum hormone levels in postmenopausal women with advanced breast cancer-I. General findings', *Journal of steroid biochemistry,* 36(1), pp. 99-104.

Luo, W. and Lin, S.C. (2004) 'Axin: a master scaffold for multiple signaling pathways', *Neuro-Signals,* 13(3), pp. 99-113.

Lyons, J.P., Mueller, U.W., Ji, H., Everett, C., Fang, X., Hsieh, J., Barth, A.M. and McCrea, P.D. (2004) 'Wnt-4 activates the canonical beta-catenin-mediated Wnt pathway and binds Frizzled-6 CRD: functional implications of Wnt/beta-catenin activity in kidney epithelial cells.', *Experimental cell research,* 298(2), pp. 369.

Ma, X., Dahiya, S., Richardson, E., Erlander, M. and Sgroi, D.C. (2009) 'Gene expression profiling of the tumor microenvironment during breast cancer progression', *Breast Cancer Res,* 11(1), pp. R7.

Machesky, L.M. and Hall, A. (1997) 'Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization', *The Journal of cell biology,* 138(4), pp. 913-926.

Macsai, C.E., Foster, B.K. and Xian, C.J. (2008) 'Roles of Wnt signalling in bone growth, remodelling, skeletal disorders and fracture repair', *Journal of cellular physiology,* 215(3), pp. 578-587.

Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K. and Narumiya, S. (1999) 'Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase', *Science (New York, N.Y.),* 285(5429), pp. 895-898.

Maity, G., Choudhury, P.R., Sen, T., Ganguly, K.K., Sil, H. and Chatterjee, A. (2011) 'Culture of human breast cancer cell line (MDA-MB-231) on fibronectin-coated surface induces pro-matrix metalloproteinase-9 expression and activity', *Tumor Biology,* 32(1), pp. 129-138.

Malanchi, I., Santamaria-Martínez, A., Susanto, E., Peng, H., Lehr, H., Delaloye, J. and Huelsken, J. (2012) 'Interactions between cancer stem cells and their niche govern metastatic colonization', *Nature,* 481(7379), pp. 85-89.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F.,Jr, Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z. and Tainsky, M.A. (1990) 'Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms', Science (New York, N.Y.), 250(4985), pp. 1233-1238.

Mani, S.A., Guo, W., Liao, M., Eaton, E.N., Ayyanan, A., Zhou, A.Y., Brooks, M., Reinhard, F., Zhang, C.C. and Shipitsin, M. (2008) 'The epithelial-mesenchymal transition generates cells with properties of stem cells', *Cell,* 133(4), pp. 704-715.

Mantovani, A. and Sica, A. (2010) 'Macrophages, innate immunity and cancer: balance, tolerance, and diversity', *Current opinion in immunology,* 22(2), pp. 231-237.

Mao, J., Wang, J., Liu, B., Pan, W., Farr, G.H., Flynn, C., Yuan, H., Takada, S., Kimelman, D. and Li, L. (2001) 'Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway', *Molecular cell,* 7(4), pp. 801-809.

Mao, Y. and Schwarzbauer, J.E. (2005) 'Fibronectin fibrillogenesis, a cell-mediated matrix assembly process', *Matrix biology,* 24(6), pp. 389-399.

Martin, A. and Weber, B.L. (2000) 'Genetic and hormonal risk factors in breast cancer', *Journal of the National Cancer Institute,* 92(14), pp. 1126-1135.

Matsuda, Y., Schlange, T., Oakeley, E.J., Boulay, A. and Hynes, N.E. (2009) 'WNT signaling enhances breast cancer cell motility and blockade of the WNT pathway by sFRP1 suppresses MDA-MB-231 xenograft growth', *Breast Cancer Res,* 11(3), pp. R32.

McCarthy, J.B., Hagen, S.T. and Furcht, L.T. (1986) 'Human fibronectin contains distinct adhesion- and motility-promoting domains for metastatic melanoma cells', *The Journal of cell biology,* 102(1), pp. 179-188.

McEntyre, J., Ostell, J., Pruitt, K., Brown, G., Tatusova, T. and Maglott, D. (2012) 'The Reference Sequence (RefSeq) Database', .

McIntosh, G.G., Anderson, J.J., Milton, I., Steward, M., Parr, A.H., Thomas, M.D., Henry, J.A., Angus, B., Lennard, T.W. and Horne, C.H. (1995) 'Determination of the prognostic value of cyclin D1 overexpression in breast cancer', *Oncogene,* 11(5), pp. 885-891.

McPherson, K., Steel, C. and Dixon, J. (2000) 'ABC of breast diseases: breast cancer—epidemiology, risk factors, and genetics', *BMJ: British Medical Journal,* 321(7261), pp. 624.

Mehra, R., Varambally, S., Ding, L., Shen, R., Sabel, M.S., Ghosh, D., Chinnaiyan, A.M. and Kleer, C.G. (2005) 'Identification of GATA3 as a breast cancer prognostic marker by global gene expression meta-analysis', *Cancer research,* 65(24), pp. 11259-11264.

Merle, P., Kim, M., Herrmann, M., Gupte, A., Lefrançois, L., Califano, S., Tre, C., Tanaka, S., Vitvitski, L. and de la Monte, S. (2005) 'Oncogenic role of the frizzled-7/β-catenin pathway in hepatocellular carcinoma', *Journal of hepatology,* 43(5), pp. 854-862.

Merlos-Suárez, A., Barriga, F.M., Jung, P., Iglesias, M., Céspedes, M.V., Rossell, D., Sevillano, M., Hernando-Momblona, X., da Silva-Diz, V. and Muñoz, P. (2011) 'The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse', *Cell stem cell,* 8(5), pp. 511-524.

Mikels, A.J. and Nusse, R. (2006) 'Purified Wnt5a protein activates or inhibits β-catenin–TCF signaling depending on receptor context', *PLoS biology,* 4(4), pp. e115.

Millikan, R.C., Newman, B., Tse, C., Moorman, P.G., Conway, K., Smith, L.V., Labbok, M.H., Geradts, J., Bensen, J.T. and Jackson, S. (2008) 'Epidemiology of basal-like breast cancer', *Breast cancer research and treatment,* 109(1), pp. 123-139.

Millour, J. and Lam, E. (2010) 'FOXM1 is a transcriptional target of ERα and has a critical role in breast cancer endocrine sensitivity and resistance', Breast Cancer Research, 12(Suppl 1), pp. P2.

Milovanovic, T., Planutis, K., Nguyen, A., Marsh, J.L., Lin, F., Hope, C. and Holcombe, R.F. (2004) 'Expression of Wnt genes and frizzled 1 and 2 receptors in normal breast epithelium and infiltrating breast carcinoma', *International journal of oncology,* 25(5), pp. 1337-1342.

Minden, A., Lin, A., Claret, F., Abo, A. and Karin, M. (1995) 'Selective activation of the JNK signaling cascadeand c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs', *Cell,* 81(7), pp. 1147-1157.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destrée, O. and Clevers, H. (1996) 'XTcf-3 transcription factor mediates β-catenin-induced axis formation in Xenopus embryos', *Cell,* 86(3), pp. 391-399.

Montcouquiol, M., Rachel, R.A., Lanford, P.J., Copeland, N.G., Jenkins, N.A. and Kelley, M.W. (2003) 'Identification of Vangl2 and Scrb1 as planar polarity genes in mammals', *Nature,* 423(6936), pp. 173-177.

Morel, A., Lièvre, M., Thomas, C., Hinkal, G., Ansieau, S. and Puisieux, A. (2008) 'Generation of breast cancer stem cells through epithelial-mesenchymal transition', *PloS one,* 3(8), pp. e2888.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K.W. (1997) 'Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC', *Science (New York, N.Y.),* 275(5307), pp. 1787-1790.

Mosher, D. (2012) *Fibronectin.* Elsevier.

Moynahan, M.E., Chiu, J.W., Koller, B.H. and Jasin, M. (1999) 'Brca1 controls homology-directed DNA repair', *Molecular cell,* 4(4), pp. 511-518.

Muñoz, R., Moreno, M., Oliva, C., Orbenes, C. and Larraín, J. (2006) 'Syndecan-4 regulates non-canonical Wnt signalling and is essential for convergent and extension movements in Xenopus embryos', *Nature cell biology,* 8(5), pp. 492-500.

Myatt, S.S. and Lam, E.W. (2007) 'The emerging roles of forkhead box (Fox) proteins in cancer', Nature Reviews Cancer, 7(11), pp. 847-859.

Nahta, R. and Esteva, F.J. (2006) 'Molecular mechanisms of trastuzumab resistance', Breast Cancer Res, 8(6), pp. 667-674.

Nakamura, T., Hamada, F., Ishidate, T., Anai, K., Kawahara, K., Toyoshima, K. and Akiyama, T. (1998) 'Axin, an inhibitor of the Wnt signalling pathway, interacts with β-catenin, GSK-3β and APC and reduces the β-catenin level', *Genes to Cells,* 3(6), pp. 395-403.

Nakashima, A., Katagiri, T. and Tamura, M. (2005) 'Cross-talk between Wnt and bone morphogenetic protein 2 (BMP-2) signaling in differentiation pathway of C2C12 myoblasts', *The Journal of biological chemistry,* 280(45), pp. 37660-37668.

Narumiya, S., Tanji, M. and Ishizaki, T. (2009) 'Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion', *Cancer and metastasis reviews,* 28(1-2), pp. 65-76.

Naz, G., Pasternack, S.M., Perrin, C., Mattheisen, M., Refke, M., Khan, S., Gul, A., Simons, M., Ahmad, W. and Betz, R.C. (2011) 'FZD6 encoding the Wnt receptor frizzled-6 is mutated in autosomal-recessive nail dysplasia', *British Journal of Dermatology,* , pp. no-no.

Neta, G., Brenner, A.V., Sturgis, E.M., Pfeiffer, R.M., Hutchinson, A.A., Aschebrook-Kilfoy, B., Yeager, M., Xu, L., Wheeler, W., Abend, M., Ron, E., Tucker, M.A., Chanock, S.J. and Sigurdson, A.J. (2011) 'Common genetic variants related to genomic integrity and risk of papillary thyroid cancer', *Carcinogenesis,* 32(8), pp. 1231-1237.

Neth, P., Ries, C., Karow, M., Egea, V., Ilmer, M. and Jochum, M. (2007) 'The Wnt signal transduction pathway in stem cells and cancer cells: influence on cellular invasion', *Stem cell reviews,* 3(1), pp. 18-29.

Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J. and Tong, F. (2006) 'A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes', *Cancer cell,* 10(6), pp. 515-527.

Nguyen, P.L., Taghian, A.G., Katz, M.S., Niemierko, A., Abi Raad, R.F., Boon, W.L., Bellon, J.R., Wong, J.S., Smith, B.L. and Harris, J.R. (2008) 'Breast cancer subtype approximated

by estrogen receptor, progesterone receptor, and HER-2 is associated with local and distant recurrence after breast-conserving therapy', Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 26(14), pp. 2373-2378.

Nicoletti, I., Migliorati, G., Pagliacci, M., Grignani, F. and Riccardi, C. (1991) 'A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry', *Journal of immunological methods,* 139(2), pp. 271-279.

Nicolson, G.L. (1988) 'Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites', *Cancer and metastasis reviews,* 7(2), pp. 143-188.

Niehrs, C. (2006) 'Function and biological roles of the Dickkopf family of Wnt modulators', *Oncogene,* 25(57), pp. 7469-7481.

Niida, A., Hiroko, T., Kasai, M., Furukawa, Y., Nakamura, Y., Suzuki, Y., Sugano, S. and Akiyama, T. (2004) 'DKK1, a negative regulator of Wnt signaling, is a target of the β-catenin/TCF pathway', *Oncogene,* 23(52), pp. 8520-8526.

Nishita, M., Hashimoto, M.K., Ogata, S., Laurent, M.N., Ueno, N., Shibuya, H. and Cho, K.W. (2000) 'Interaction between Wnt and TGF-β signalling pathways during formation of Spemann's organizer', *Nature,* 403(6771), pp. 781-785.

Nobes, C.D. and Hall, A. (1995) 'Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia', *Cell,* 81(1), pp. 53-62.

Nobes, C.D. and Hall, A. (1999) 'Rho GTPases control polarity, protrusion, and adhesion during cell movement', *The Journal of cell biology,* 144(6), pp. 1235-1244.

Noble, J.R., Zhong, Z., Neumann, A.A., Melki, J.R., Clark, S.J. and Reddel, R.R. (2004) 'Alterations in the p16INK4a and p53 tumor suppressor genes of hTERT-immortalized human fibroblasts', *Oncogene,* 23(17), pp. 3116-3121.

Nusse, R., Brown, A., Papkoff, J., Scambler, P., Shackleford, G., McMahon, A., Moon, R. and Varmu, H. (1991) 'A New Nomenclature for in&l and Related Genes: The Writ Gene Family', *Cell,* 64, pp. 231-232.

Nusse, R., van Ooyen, A., Cox, D., Fung, Y.K.T. and Varmus, H. (1984) 'Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15', .

Oguma, K., Oshima, H., Aoki, M., Uchio, R., Naka, K., Nakamura, S., Hirao, A., Saya, H., Taketo, M.M. and Oshima, M. (2008) 'Activated macrophages promote Wnt signalling through tumour necrosis factor-α in gastric tumour cells', *The EMBO journal,* 27(12), pp. 1671-1681.

Oka, H., Shiozaki, H., Kobayashi, K., Inoue, M., Tahara, H., Kobayashi, T., Takatsuka, Y., Matsuyoshi, N., Hirano, S. and Takeichi, M. (1993) 'Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis', *Cancer research,* 53(7), pp. 1696-1701.

Olayioye, M.A. (2001) 'Update on HER-2 as a target for cancer therapy: intracellular signaling pathways of ErbB2/HER-2 and family members', *Breast Cancer Res,* 3(6), pp. 385-389.

Olmeda, D., Moreno-Bueno, G., Flores, J.M., Fabra, A., Portillo, F. and Cano, A. (2007) 'SNAI1 is required for tumor growth and lymph node metastasis of human breast carcinoma MDA-MB-231 cells', *Cancer research,* 67(24), pp. 11721-11731.

Paget, S. (1889) 'The distribution of secondary growths in cancer of the breast.', *The Lancet,* 133(3421), pp. 571-573.

Pandur, P., Maurus, D. and Kühl, M. (2002) 'Increasingly complex: new players enter the Wnt signaling network', *Bioessays,* 24(10), pp. 881-884.

Pankov, R. and Yamada, K.M. (2002) 'Fibronectin at a glance', *Journal of cell science,* 115(Pt 20), pp. 3861-3863.

Park, J. and Schwarzbauer, J. (2013) 'Mammary epithelial cell interactions with fibronectin stimulate epithelial-mesenchymal transition', *Oncogene,* 33(13), pp. 1649-1657.

Parkin, D.M. and Fernández, L.M. (2006) 'Use of statistics to assess the global burden of breast cancer', *The breast journal,* 12(s1), pp. S70-S80.

Patel, K.J., Veronica, P., Lee, H., Corcoran, A., Thistlethwaite, F.C., Evans, M.J., Colledge, W.H., Friedman, L.S., Ponder, B.A. and Venkitaraman, A.R. (1998) 'Involvement of Brca2 in DNA repair', *Molecular cell,* 1(3), pp. 347-357.

Paterson, H.F., Self, A.J., Garrett, M.D., Just, I., Aktories, K. and Hall, A. (1990) 'Microinjection of recombinant p21rho induces rapid changes in cell morphology', *The Journal of cell biology,* 111(3), pp. 1001-1007.

Pauken, C.M. and Capco, D.G. (1999) 'Regulation of cell adhesion during embryonic compaction of mammalian embryos: Roles for PKC and β-catenin', Molecular reproduction and development, 54(2), pp. 135-144.

Pearlstein, E., Gold, L.I. and Garcia-Pardo, A. (1980) 'Fibronectin: a review of its structure and biological activity', *Molecular and cellular biochemistry,* 29(2), pp. 103-128.

Pellegrin, S. and Mellor, H. (2007) 'Actin stress fibres', *Journal of cell science,* 120(Pt 20), pp. 3491-3499.

Perou, C.M. (2011) 'Molecular stratification of triple-negative breast cancers', *The oncologist,* 16 Suppl 1, pp. 61-70.

Petersen, O.W., Ronnov-Jessen, L., Howlett, A.R. and Bissell, M.J. (1992) 'Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells', *Proceedings of the National Academy of Sciences of the United States of America,* 89(19), pp. 9064-9068.

Pharoah, P.D., Day, N.E. and Caldas, C. (1999) 'Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis', *British journal of cancer,* 80(12), pp. 1968-1973.

Pierceall, W.E., Woodard, A.S., Morrow, J.S., Rimm, D. and Fearon, E.R. (1995) 'Frequent alterations in E-cadherin and alpha- and beta-catenin expression in human breast cancer cell lines', *Oncogene,* 11(7), pp. 1319-1326.

Pike, M.C., Spicer, D.V., Dahmoush, L. and Press, M.F. (1993) 'Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk', *Epidemiologic reviews,* 15(1), pp. 17-35.

Pillé, J., Denoyelle, C., Varet, J., Bertrand, J., Soria, J., Opolon, P., Lu, H., Pritchard, L., Vannier, J. and Malvy, C. (2005) 'Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo', *Molecular Therapy,* 11(2), pp. 267-274.

Plopper, G. (2012) 'The Extracellular Matrix Is a Complex Network of Molecules That Fills the Spaces between Cells in a Multicellular Organism', in *Principles of Cell Biology.* Jones & Bartlett Publishers, pp. 193-194.

Polakis, P. (2000) 'Wnt signaling and cancer', *Genes & development,* 14(15), pp. 1837-1851.

Pollack, J.R., Sorlie, T., Perou, C.M., Rees, C.A., Jeffrey, S.S., Lonning, P.E., Tibshirani, R., Botstein, D., Borresen-Dale, A.L. and Brown, P.O. (2002) 'Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors', *Proceedings of the National Academy of Sciences of the United States of America,* 99(20), pp. 12963-12968.

Pollard, J.W. (2004) 'Tumour-educated macrophages promote tumour progression and metastasis', *Nature Reviews Cancer,* 4(1), pp. 71-78.

Pollard, T.D. and Borisy, G.G. (2003) 'Cellular motility driven by assembly and disassembly of actin filaments', *Cell,* 112(4), pp. 453-465.

Porter, P. (2008) '"Westernizing" women's risks? Breast cancer in lower-income countries', *New England Journal of Medicine,* 358(3), pp. 213-216.

Prat, A. and Perou, C.M. (2011) 'Deconstructing the molecular portraits of breast cancer', *Molecular oncology,* 5(1), pp. 5-23.

Preston-Martin, S., Pike, M.C., Ross, R.K., Jones, P.A. and Henderson, B.E. (1990) 'Increased cell division as a cause of human cancer', *Cancer research,* 50(23), pp. 7415-7421.

Proctor, R.A. (1987) 'Fibronectin: a brief overview of its structure, function, and physiology', *Review of Infectious Diseases,* 9(Supplement 4), pp. S317-S321.

Psaila, B. and Lyden, D. (2009) 'The metastatic niche: adapting the foreign soil', *Nature Reviews Cancer,* 9(4), pp. 285-293.

Pukrop, T., Klemm, F., Hagemann, T., Gradl, D., Schulz, M., Siemes, S., Trumper, L. and Binder, C. (2006) 'Wnt 5a signaling is critical for macrophage-induced invasion of breast cancer cell lines', *Proceedings of the National Academy of Sciences of the United States of America,* 103(14), pp. 5454-5459.

Qian, B. and Pollard, J.W. (2010) 'Macrophage diversity enhances tumor progression and metastasis', *Cell,* 141(1), pp. 39-51.

Rakha, E.A. and Ellis, I.O. (2009) 'Triple-negative/basal-like breast cancer: review', *Pathology,* 41(1), pp. 40-47.

Rakha, E.A., Reis-Filho, J.S. and Ellis, I.O. (2008) 'Basal-like breast cancer: a critical review', *Journal of Clinical Oncology,* 26(15), pp. 2568-2581.

Ranger, A.M., Grusby, M.J., Hodge, M.R., Gravallese, E.M., de La Brousse, Fabienne Charles, Hoey, T., Mickanin, C., Baldwin, H.S. and Glimcher, L.H. (1998) 'The transcription factor NF-ATc is essential for cardiac valve formation', Nature, 392(6672), pp. 186-190.

Rattner, A., Hsieh, J.C., Smallwood, P.M., Gilbert, D.J., Copeland, N.G., Jenkins, N.A. and Nathans, J. (1997) 'A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors', *Proceedings of the National Academy of Sciences of the United States of America,* 94(7), pp. 2859-2863.

Reis, M., Czupalla, C.J., Ziegler, N., Devraj, K., Zinke, J., Seidel, S., Heck, R., Thom, S., Macas, J., Bockamp, E., Fruttiger, M., Taketo, M.M., Dimmeler, S., Plate, K.H. and Liebner, S. (2012) 'Endothelial Wnt/beta-catenin signaling inhibits glioma angiogenesis and normalizes tumor blood vessels by inducing PDGF-B expression', *The Journal of experimental medicine,* 209(9), pp. 1611-1627.

Reis-Filho, J. and Tutt, A. (2008) 'Triple negative tumours: a critical review', *Histopathology,* 52(1), pp. 108-118.

Ren, X.D., Kiosses, W.B. and Schwartz, M.A. (1999) 'Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton', *The EMBO journal,* 18(3), pp. 578-585.

Reya, T. and Clevers, H. (2005) 'Wnt signalling in stem cells and cancer', *Nature,* 434(7035), pp. 843-850.

Reynen, K., Kockeritz, U. and Strasser, R.H. (2004) 'Metastases to the heart', *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO,* 15(3), pp. 375-381.

Richard-Parpaillon, L., Héligon, C., Chesnel, F., Boujard, D. and Philpott, A. (2002) 'The IGF Pathway Regulates Head Formation by Inhibiting Wnt Signaling in< i> Xenopus</i>', *Developmental biology,* 244(2), pp. 407-417.

Ridley, A.J. and Hall, A. (1992) 'The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors', *Cell,* 70(3), pp. 389-399.

Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T. and Horwitz, A.R. (2003) 'Cell migration: integrating signals from front to back', *Science (New York, N.Y.),* 302(5651), pp. 1704-1709.

Roarty, K. and Serra, R. (2007) 'Wnt5a is required for proper mammary gland development and TGF-beta-mediated inhibition of ductal growth', *Development (Cambridge, England),* 134(21), pp. 3929-3939.

Rodriguez, L.G., Wu, X. and Guan, J. (2005) 'Wound-healing assay', in *Cell Migration.* Springer, pp. 23-29.

Roman-Gomez, J., Jimenez-Velasco, A., Cordeu, L., Vilas-Zornoza, A., San Jose-Eneriz, E., Garate, L., Castillejo, J.A., Martin, V., Prosper, F. and Heiniger, A. (2007) 'WNT5A, a putative tumour suppressor of lymphoid malignancies, is inactivated by aberrant methylation in acute lymphoblastic leukaemia', *European journal of cancer,* 43(18), pp. 2736-2746.

Romieu, I., Scoccianti, C., Chajes, V., de Batlle, J., Biessy, C., Dossus, L., Baglietto, L., Clavel-Chapelon, F., Overvad, K. and Olsen, A. (2015) 'Alcohol intake and breast cancer in the European Prospective investigation into Cancer and Nutrition: Short title: Alcohol intake and breast cancer', *International Journal of Cancer,* .

Roszko, I., Sawada, A. and Solnica-Krezel, L. (2009) 'Regulation of convergence and extension movements during vertebrate gastrulation by the Wnt/PCP pathway', *Seminars in cell & developmental biology.* Elsevier, 986-997.

Roy, V. and Perez, E.A. (2009) 'Beyond trastuzumab: small molecule tyrosine kinase inhibitors in HER-2-positive breast cancer', The oncologist, 14(11), pp. 1061-1069.

Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E. and Polakis, P. (1997) 'Stabilization of beta-catenin by genetic defects in melanoma cell lines', *Science (New York, N.Y.),* 275(5307), pp. 1790-1792.

Ruoslahti, E. (1984) 'Fibronectin in cell adhesion and invasion', *Cancer and metastasis reviews,* 3(1), pp. 43-51.

Ruoslahti, E. (1999) 'Fibronectin and its integrin receptors in cancer', *Advances in Cancer Research,* 76, pp. 1-20.

Ryo, A., Nakamura, M., Wulf, G., Liou, Y. and Lu, K.P. (2001) 'Pin1 regulates turnover and subcellular localization of β-catenin by inhibiting its interaction with APC', *Nature cell biology,* 3(9), pp. 793-801.

Saad, S., Gottlieb, D.J., Bradstock, K.F., Overall, C.M. and Bendall, L.J. (2002) 'Cancer cell-associated fibronectin induces release of matrix metalloproteinase-2 from normal fibroblasts', *Cancer research,* 62(1), pp. 283-289.

Sabari, J., Lax, D., Connors, D., Brotman, I., Mindrebo, E., Butler, C., Entersz, I., Jia, D. and Foty, R.A. (2011) 'Fibronectin matrix assembly suppresses dispersal of glioblastoma cells', *PLoS One,* 6(9), pp. e24810.

Sahai, E. and Marshall, C.J. (2002) 'RHO–GTPases and cancer', *Nature Reviews Cancer,* 2(2), pp. 133-142.

Salehi, F., Turner, M.C., Phillips, K.P., Wigle, D.T., Krewski, D. and Aronson, K.J. (2008) 'Review of the etiology of breast cancer with special attention to organochlorines as potential endocrine disruptors', *Journal of Toxicology and Environmental Health, Part B,* 11(3-4), pp. 276-300.

Samuels, Y., Diaz, L.A., Schmidt-Kittler, O., Cummins, J.M., DeLong, L., Cheong, I., Rago, C., Huso, D.L., Lengauer, C. and Kinzler, K.W. (2005) 'Mutant PIK3CA promotes cell growth and invasion of human cancer cells', *Cancer cell,* 7(6), pp. 561-573.

Sandal, T., Valyi-Nagy, K., Spencer, V.A., Folberg, R., Bissell, M.J. and Maniotis, A.J. (2007) 'Epigenetic Reversion of Breast Carcinoma Phenotype Is Accompanied by Changes in DNA Sequestration as Measured by< i> Alu</i> I Restriction Enzyme', *The American journal of pathology,* 170(5), pp. 1739-1749.

Saneyoshi, T., Kume, S., Amasaki, Y. and Mikoshiba, K. (2002) 'The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos', Nature, 417(6886), pp. 295-299.

Saramäki, O.R., Porkka, K.P., Vessella, R.L. and Visakorpi, T. (2006) 'Genetic aberrations in prostate cancer by microarray analysis', *International journal of cancer,* 119(6), pp. 1322-1329.

Sasco, A.J., Lowenfels, A.B. and Pasker-De Jong, P. (1993) 'Review article: Epidemiology of male breast cancer. A meta-analysis of published case-control studies and discussion of selected aetiological factors', *International Journal of Cancer,* 53(4), pp. 538-549.

Sato, A., Yamamoto, H., Sakane, H., Koyama, H. and Kikuchi, A. (2010) 'Wnt5a regulates distinct signalling pathways by binding to Frizzled2', *The EMBO journal,* 29(1), pp. 41-54.

Satoh, S., Daigo, Y., Furukawa, Y., Kato, T., Miwa, N., Nishiwaki, T., Kawasoe, T., Ishiguro, H., Fujita, M. and Tokino, T. (2000) 'AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1', *Nature genetics,* 24(3), pp. 245-250.

Schaffner, F., Ray, A.M. and Dontenwill, M. (2013) 'Integrin α5β1, the fibronectin receptor, as a pertinent therapeutic target in solid tumors', *Cancers,* 5(1), pp. 27-47.

Schindl, M., Schoppmann, S.F., Samonigg, H., Hausmaninger, H., Kwasny, W., Gnant, M., Jakesz, R., Kubista, E., Birner, P., Oberhuber, G. and Austrian Breast and Colorectal Cancer Study Group (2002) 'Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer', Clinical cancer research : an official journal of the American Association for Cancer Research, 8(6), pp. 1831-1837.

Schlessinger, K., Hall, A. and Tolwinski, N. (2009) 'Wnt signaling pathways meet Rho GTPases', *Genes & development,* 23(3), pp. 265-277.

Schor, S.L., Schor, A.M. and Bazill, G.W. (1981) 'The effects of fibronectin on the migration of human foreskin fibroblasts and Syrian hamster melanoma cells into three-dimensional gels of native collagen fibres', *Journal of cell science,* 48, pp. 301-314.

Schroeder, J.A., Adriance, M.C., McConnell, E.J., Thompson, M.C., Pockaj, B. and Gendler, S.J. (2002) 'ErbB-beta-catenin complexes are associated with human infiltrating ductal breast and murine mammary tumor virus (MMTV)-Wnt-1 and MMTV-c-Neu transgenic carcinomas', *The Journal of biological chemistry,* 277(25), pp. 22692-22698.

Schulte, G. (2010) 'International Union of Basic and Clinical Pharmacology. LXXX. The class Frizzled receptors', *Pharmacological reviews,* 62(4), pp. 632-667.

Schwartz, M.A. and Shattil, S.J. (2000) 'Signaling networks linking integrins and rho family GTPases', *Trends in biochemical sciences,* 25(8), pp. 388-391.

Seifert, J.R. and Mlodzik, M. (2007) 'Frizzled/PCP signalling: a conserved mechanism regulating cell polarity and directed motility', *Nature Reviews Genetics,* 8(2), pp. 126-138.

Selzner, M., Morse, M.A., Vredenburgh, J.J., Meyers, W.C. and Clavien, P. (2000) 'Liver metastases from breast cancer: long-term survival after curative resection', *Surgery,* 127(4), pp. 383-389.

Sengupta, S. and Harris, C.C. (2005) 'p53: traffic cop at the crossroads of DNA repair and recombination', *Nature reviews Molecular cell biology,* 6(1), pp. 44-55.

Serfling, E., Berberich-Siebelt, F., Chuvpilo, S., Jankevics, E., Klein-Hessling, S., Twardzik, T. and Avots, A. (2000) 'The role of NF-AT transcription factors in T cell activation and differentiation', Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1498(1), pp. 1-18.

Shang, X., Marchioni, F., Sipes, N., Evelyn, C.R., Jerabek-Willemsen, M., Duhr, S., Seibel, W., Wortman, M. and Zheng, Y. (2012) 'Rational design of small molecule inhibitors targeting RhoA subfamily Rho GTPases', *Chemistry & biology,* 19(6), pp. 699-710.

Sharma, R. and Chopra, V. (1976) 'Effect of the wingless (wg 1) mutation on wing and haltere development in Drosophila melanogaster', *Developmental biology,* 48(2), pp. 461-465.

Shi, F., Long, X., Hendershot, A., Miano, J.M. and Sottile, J. (2014) 'Fibronectin Matrix Polymerization Regulates Smooth Muscle Cell Phenotype through a Rac1 Dependent Mechanism', *PloS one,* 9(4), pp. e94988.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999) 'The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway', *Proceedings of the National Academy of Sciences of the United States of America,* 96(10), pp. 5522-5527.

Shulewitz, M., Soloviev, I., Wu, T., Koeppen, H., Polakis, P. and Sakanaka, C. (2006) 'Repressor roles for TCF-4 and Sfrp1 in Wnt signaling in breast cancer', *Oncogene,* 25(31), pp. 4361-4369.

Siitonen, S.M., Kononen, J.T., Helin, H.J., Rantala, I.S., Holli, K.A. and Isola, J.J. (1996) 'Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer', *American Journal of Clinical Pathology,* 105(4), pp. 394-402.

Silva, J.M., Li, M.Z., Chang, K., Ge, W., Golding, M.C., Rickles, R.J., Siolas, D., Hu, G., Paddison, P.J. and Schlabach, M.R. (2005) 'Second-generation shRNA libraries covering the mouse and human genomes', *Nature genetics,* 37(11), pp. 1281-1288.

Simons, M. and Mlodzik, M. (2008) 'Planar cell polarity signaling: from fly development to human disease', *Annual Review of Genetics,* 42, pp. 517-540.

Sims, A.H., Howell, A., Howell, S.J. and Clarke, R.B. (2007) 'Origins of breast cancer subtypes and therapeutic implications', *Nature Clinical Practice Oncology,* 4(9), pp. 516-525.

Singh, A. and Settleman, J. (2010) 'EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer', *Oncogene,* 29(34), pp. 4741-4751.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A. and McGuire, W.L. (1987) 'Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene', *Science (New York, N.Y.),* 235(4785), pp. 177-182.

Slusarski, D.C., Corces, V.G. and Moon, R.T. (1997) 'Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling', *Nature,* 390(6658), pp. 410-413.

Smalley, M.J. and Dale, T.C. (2001) 'Wnt signaling and mammary tumorigenesis', *Journal of mammary gland biology and neoplasia,* 6(1), pp. 37-52.

Smid, M., Wang, Y., Zhang, Y., Sieuwerts, A.M., Yu, J., Klijn, J.G., Foekens, J.A. and Martens, J.W. (2008) 'Subtypes of breast cancer show preferential site of relapse', *Cancer research,* 68(9), pp. 3108-3114.

Smith, I.C., Heys, S.D., Hutcheon, A.W., Miller, I.D., Payne, S., Gilbert, F.J., Ah-See, A.K., Eremin, O., Walker, L.G. and Sarkar, T.K. (2002) 'Neoadjuvant chemotherapy in breast cancer: significantly enhanced response with docetaxel', *Journal of Clinical Oncology,* 20(6), pp. 1456-1466.

Smith, R.A. (2003) 'IARC handbooks of cancer prevention, volume 7: breast cancer screening', *Breast Cancer Res,* 5(4), pp. 216-217.

Sobin, L.H. and Fleming, I.D. (1997) 'TNM classification of malignant tumors, (1997)', *Cancer,* 80(9), pp. 1803-1804.

Soerjomataram, I., Louwman, M.W., Ribot, J.G., Roukema, J.A. and Coebergh, J.W.W. (2008) 'An overview of prognostic factors for long-term survivors of breast cancer', *Breast cancer research and treatment,* 107(3), pp. 309-330.

Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Lonning, P.E. and Borresen-Dale, A.L. (2001) 'Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications', *Proceedings of the National Academy of Sciences of the United States of America,* 98(19), pp. 10869-10874.

Sotiriou, C., Neo, S.Y., McShane, L.M., Korn, E.L., Long, P.M., Jazaeri, A., Martiat, P., Fox, S.B., Harris, A.L. and Liu, E.T. (2003) 'Breast cancer classification and prognosis based on gene expression profiles from a population-based study', *Proceedings of the National Academy of Sciences of the United States of America,* 100(18), pp. 10393-10398.

Soule, H.D., Vazguez, J., Long, A., Albert, S. and Brennan, M. (1973) 'A human cell line from a pleural effusion derived from a breast carcinoma', *Journal of the National Cancer Institute,* 51(5), pp. 1409-1416.

St Croix, B., Sheehan, C., Rak, J.W., Florenes, V.A., Slingerland, J.M. and Kerbel, R.S. (1998) 'E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1)', *The Journal of cell biology,* 142(2), pp. 557-571.

Stead, L.A., Lash, T.L., Sobieraj, J.E., Chi, D.D., Westrup, J.L., Charlot, M., Blanchard, R.A., Lee, J.C., King, T.C. and Rosenberg, C.L. (2009) 'Triple-negative breast cancers are increased in black women regardless of age or body mass index', *Breast Cancer Res,* 11(2), pp. R18.

Steele, B.M., Harper, M.T., Macaulay, I.C., Morrell, C.N., Perez-Tamayo, A., Foy, M., Habas, R., Poole, A.W., Fitzgerald, D.J. and Maguire, P.B. (2009) 'Canonical Wnt signaling negatively regulates platelet function', *Proceedings of the National Academy of Sciences,* 106(47), pp. 19836-19841.

Stephens, P.J., Tarpey, P.S., Davies, H., Van Loo, P., Greenman, C., Wedge, D.C., Nik-Zainal, S., Martin, S., Varela, I. and Bignell, G.R. (2012) 'The landscape of cancer genes and mutational processes in breast cancer', *Nature,* 486(7403), pp. 400-404.

Stevanovic, A., Lee, P. and Wilcken, N. (2006) 'Metastatic breast cancer', *Australian Family Physician,* 35(5), pp. 309.

Stockinger, A., Eger, A., Wolf, J., Beug, H. and Foisner, R. (2001) 'E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity', *The Journal of cell biology,* 154(6), pp. 1185-1196.

Stout, R.D. and Suttles, J. (2004) 'Functional plasticity of macrophages: reversible adaptation to changing microenvironments', *Journal of leukocyte biology,* 76(3), pp. 509-513.

Strutt, D.I., Weber, U. and Mlodzik, M. (1997) 'The role of RhoA in tissue polarity and Frizzled signalling', *Nature,* 387(6630), pp. 292-295.

Strutt, D. (2003) 'Frizzled signalling and cell polarisation in Drosophila and vertebrates', *Development (Cambridge, England),* 130(19), pp. 4501-4513.

Stuebner, S., Faus-Kessler, T., Fischer, T., Wurst, W. and Prakash, N. (2010) 'Fzd3 and Fzd6 deficiency results in a severe midbrain morphogenesis defect', *Developmental Dynamics,* 239(1), pp. 246-260.

Sugimura, R. and Li, L. (2010) 'Noncanonical Wnt signaling in vertebrate development, stem cells, and diseases', *Birth defects research.Part C, Embryo today : reviews,* 90(4), pp. 243-256.

Sun, H., Lesche, R., Li, D.M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X. and Wu, H. (1999) 'PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway', *Proceedings of the National Academy of Sciences of the United States of America,* 96(11), pp. 6199-6204.

Suzuki, H., Toyota, M., Caraway, H., Gabrielson, E., Ohmura, T., Fujikane, T., Nishikawa, N., Sogabe, Y., Nojima, M. and Sonoda, T. (2008) 'Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer', *British journal of cancer,* 98(6), pp. 1147-1156.

Symons, M. (1996) 'Rho family GTPases: the cytoskeleton and beyond', *Trends in biochemical sciences,* 21(5), pp. 178-181.

Szollosi, J., Balazs, M., Feuerstein, B.G., Benz, C.C. and Waldman, F.M. (1995) 'ERBB-2 (HER2/neu) gene copy number, p185HER-2 overexpression, and intratumor heterogeneity in human breast cancer', Cancer research, 55(22), pp. 5400-5407.

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. and He, X. (2000) 'LDL-receptor-related proteins in Wnt signal transduction', *Nature,* 407(6803), pp. 530-535.

Tanihara, H., Inoue, T., Yamamoto, T., Kuwayama, Y., Abe, H. and Araie, M. (2013) 'Phase 2 randomized clinical study of a Rho kinase inhibitor, K-115, in primary open-angle glaucoma and ocular hypertension', *American Journal of Ophthalmology,* 156(4), pp. 731-736. e2.

Tao, H., Manak, J.R., Sowers, L., Mei, X., Kiyonari, H., Abe, T., Dahdaleh, N.S., Yang, T., Wu, S. and Chen, S. (2011) 'Mutations in prickle orthologs cause seizures in flies, mice, and humans', *The American Journal of Human Genetics,* 88(2), pp. 138-149.

Tate, M.C., Shear, D.A., Hoffman, S.W., Stein, D.G., Archer, D.R. and LaPlaca, M.C. (2002) 'Fibronectin promotes survival and migration of primary neural stem cells transplanted into the traumatically injured mouse brain', *Cell transplantation,* 11(3), pp. 283-295.

Terasaki, H., Saitoh, T., Shiokawa, K. and Katoh, M. (2002) 'Frizzled-10, up-regulated in primary colorectal cancer, is a positive regulator of the WNT-β-catenin-TCF signaling pathway', *International journal of molecular medicine,* 9(2), pp. 107-112.

Tetsu, O. and McCormick, F. (1999) 'Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells', *Nature,* 398(6726), pp. 422-426.

Tian, E., Zhan, F., Walker, R., Rasmussen, E., Ma, Y., Barlogie, B. and Shaughnessy Jr, J.D. (2003) 'The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma', *New England Journal of Medicine,* 349(26), pp. 2483-2494.

Tokuhara, M., Hirai, M., Atomi, Y., Terada, M. and Katoh, M. (1998) 'Molecular Cloning of Human< i> Frizzled-6</i>', *Biochemical and biophysical research communications,* 243(2), pp. 622-627.

Torban, E., Wang, H.J., Groulx, N. and Gros, P. (2004) 'Independent mutations in mouse Vangl2 that cause neural tube defects in looptail mice impair interaction with members of the Dishevelled family', *The Journal of biological chemistry,* 279(50), pp. 52703-52713.

Tree, D.R., Shulman, J.M., Rousset, R., Scott, M.P., Gubb, D. and Axelrod, J.D. (2002) 'Prickle mediates feedback amplification to generate asymmetric planar cell polarity signaling', *Cell,* 109(3), pp. 371-381.

Tsuji, T., Ishizaki, T., Okamoto, M., Higashida, C., Kimura, K., Furuyashiki, T., Arakawa, Y., Birge, R.B., Nakamoto, T., Hirai, H. and Narumiya, S. (2002) 'ROCK and mDia1 antagonize in Rho-dependent Rac activation in Swiss 3T3 fibroblasts', *The Journal of cell biology,* 157(5), pp. 819-830.

Tuch, B.E. (2006) 'Stem cells--a clinical update', *Australian Family Physician,* 35(9), pp. 719-721.

Turner, N., Pearson, A., Sharpe, R., Lambros, M., Geyer, F., Lopez-Garcia, M.A., Natrajan, R., Marchio, C., Iorns, E., Mackay, A., Gillett, C., Grigoriadis, A., Tutt, A., Reis-Filho, J.S. and Ashworth, A. (2010) 'FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer', *Cancer research,* 70(5), pp. 2085-2094.

Ueno, K., Hazama, S., Mitomori, S., Nishioka, M., Suehiro, Y., Hirata, H., Oka, M., Imai, K., Dahiya, R. and Hinoda, Y. (2009) 'Down-regulation of frizzled-7 expression decreases survival, invasion and metastatic capabilities of colon cancer cells', *British journal of cancer,* 101(8), pp. 1374-1381.

Ueno, K., Hiura, M., Suehiro, Y., Hazama, S., Hirata, H., Oka, M., Imai, K., Dahiya, R. and Hinoda, Y. (2008) 'Frizzled-7 as a potential therapeutic target in colorectal cancer', *Neoplasia,* 10(7), pp. 697-705.

Ueno, K., Hirata, H., Majid, S., Yamamura, S., Shahryari, V., Tabatabai, Z.L., Hinoda, Y. and Dahiya, R. (2012) 'Tumor suppressor microRNA-493 decreases cell motility and migration ability in human bladder cancer cells by downregulating RhoC and FZD4', *Molecular cancer therapeutics,* 11(1), pp. 244-253.

Ugolini, F., Charafe-Jauffret, E., Bardou, V.J., Geneix, J., Adelaide, J., Labat-Moleur, F., Penault-Llorca, F., Longy, M., Jacquemier, J., Birnbaum, D. and Pebusque, M.J. (2001) 'WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene SFRP1 in most invasive carcinomas except of the medullary type', *Oncogene,* 20(41), pp. 5810-5817.

Usary, J., Llaca, V., Karaca, G., Presswala, S., Karaca, M., He, X., Langerød, A., Kåresen, R., Oh, D.S. and Dressler, L.G. (2004) 'Mutation of GATA3 in human breast tumors', *Oncogene,* 23(46), pp. 7669-7678.

van Amerongen, R. and Berns, A. (2006) 'Knockout mouse models to study Wnt signal transduction', *TRENDS in Genetics,* 22(12), pp. 678-689.

van Amerongen, R., Bowman, A.N. and Nusse, R. (2012) 'Developmental stage and time dictate the fate of Wnt/β-catenin-responsive stem cells in the mammary gland', *Cell stem cell,* 11(3), pp. 387-400.

van Amerongen, R. and Nusse, R. (2009) 'Towards an integrated view of Wnt signaling in development', *Development (Cambridge, England),* 136(19), pp. 3205-3214.

Van De Wetering, M., Sancho, E., Verweij, C., De Lau, W., Oving, I., Hurlstone, A., Van Der Horn, K., Batlle, E., Coudreuse, D. and Haramis, A. (2002) 'The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells', *Cell,* 111(2), pp. 241-250.

van Genderen, C., Okamura, R.M., Farinas, I., Quo, R.G., Parslow, T.G., Bruhn, L. and Grosschedl, R. (1994) 'Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice', *Genes & development,* 8(22), pp. 2691-2703.

van Golen, K.L., Wu, Z., Qiao, X., Bao, L. and Merajver, S.D. (2000) 'RhoC GTPase overexpression modulates induction of angiogenic factors in breast cells', *Neoplasia,* 2(5), pp. 418-425.

van Nieuw Amerongen, G.P., Koolwijk, P., Versteilen, A. and van Hinsbergh, V.W. (2003) 'Involvement of RhoA/Rho kinase signaling in VEGF-induced endothelial cell migration and angiogenesis in vitro', *Arteriosclerosis, Thrombosis, and Vascular Biology,* 23(2), pp. 211-217.

Vanhaesebroeck, B. and Waterfield, M. (1999) 'Signaling by distinct classes of phosphoinositide 3-kinases', *Experimental cell research,* 253(1), pp. 239-254.

Veeck, J. and Esteller, M. (2010) 'Breast cancer epigenetics: from DNA methylation to microRNAs', Journal of mammary gland biology and neoplasia, 15(1), pp. 5-17.

Veeck, J., Wild, P.J., Fuchs, T., Schuffler, P.J., Hartmann, A., Knuchel, R. and Dahl, E. (2009) 'Prognostic relevance of Wnt-inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) promoter methylation in human breast cancer', *BMC cancer,* 9, pp. 217-2407-9-217.

Vega, F.M., Fruhwirth, G., Ng, T. and Ridley, A.J. (2011) 'RhoA and RhoC have distinct roles in migration and invasion by acting through different targets', *The Journal of cell biology,* 193(4), pp. 655-665.

Veltmaat, J.M., Van Veelen, W., Thiery, J.P. and Bellusci, S. (2004) 'Identification of the mammary line in mouse by Wnt10b expression', *Developmental dynamics,* 229(2), pp. 349-356.

Venkitaraman, A.R. (2002) 'Cancer susceptibility and the functions of BRCA1 and BRCA2', *Cell,* 108(2), pp. 171-182.

Vermeulen, L., Felipe De Sousa, E Melo, van der Heijden, M., Cameron, K., de Jong, J.H., Borovski, T., Tuynman, J.B., Todaro, M., Merz, C. and Rodermond, H. (2010) 'Wnt activity defines colon cancer stem cells and is regulated by the microenvironment', *Nature cell biology,* 12(5), pp. 468-476.

Vincan, E. and Barker, N. (2008) 'The upstream components of the Wnt signalling pathway in the dynamic EMT and MET associated with colorectal cancer progression', *Clinical & experimental metastasis,* 25(6), pp. 657-663.

Vinson, C.R., Conover, S. and Adler, P.N. (1989) 'A Drosophila tissue polarity locus encodes a protein containing seven potential transmembrane domains', .

Visvader, J.E. and Lindeman, G.J. (2008) 'Cancer stem cells in solid tumours: accumulating evidence and unresolved questions', *Nature Reviews Cancer,* 8(10), pp. 755-768.

Vivanco, I. and Sawyers, C.L. (2002) 'The phosphatidylinositol 3-kinase–AKT pathway in human cancer', *Nature Reviews Cancer,* 2(7), pp. 489-501.

Vogel, C.L., Cobleigh, M.A., Tripathy, D., Gutheil, J.C., Harris, L.N., Fehrenbacher, L., Slamon, D.J., Murphy, M., Novotny, W.F., Burchmore, M., Shak, S., Stewart, S.J. and Press, M. (2002) 'Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-

overexpressing metastatic breast cancer', *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 20(3), pp. 719-726.

Vuoriluoto, K., Haugen, H., Kiviluoto, S., Mpindi, J., Nevo, J., Gjerdrum, C., Tiron, C., Lorens, J. and Ivaska, J. (2010) 'Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer', *Oncogene,* 30(12), pp. 1436-1448.

Wallingford, J.B. and Habas, R. (2005) 'The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity', *Development (Cambridge, England),* 132(20), pp. 4421-4436.

Wang, H., Xu, M., Ma, J., Zhang, Y. and Xie, C. (2012) 'Frizzled-8 as a putative therapeutic target in human lung cancer', *Biochemical and biophysical research communications,* 417(1), pp. 62-66.

Wang, X., Moon, J., Dodge, M.E., Pan, X., Zhang, L., Hanson, J.M., Tuladhar, R., Ma, Z., Shi, H. and Williams, N.S. (2013) 'The development of highly potent inhibitors for porcupine', *Journal of medicinal chemistry,* 56(6), pp. 2700-2704.

Wang, X., Fu, L., Li, X., Wu, X., Zhu, Z., Fu, L. and Dong, J. (2011) 'Somatic mutations of the mixed-lineage leukemia 3 (MLL3) gene in primary breast cancers', *Pathology & Oncology Research,* 17(2), pp. 429-433.

Wang, Y., Chang, H. and Nathans, J. (2010) 'When whorls collide: the development of hair patterns in frizzled 6 mutant mice', *Science Signaling,* 137(23), pp. 4091.

Wang, Y., Guo, N. and Nathans, J. (2006a) 'The role of Frizzled3 and Frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells', *The Journal of neuroscience,* 26(8), pp. 2147-2156.

Wang, J., Hamblet, N.S., Mark, S., Dickinson, M.E., Brinkman, B.C., Segil, N., Fraser, S.E., Chen, P., Wallingford, J.B. and Wynshaw-Boris, A. (2006b) 'Dishevelled genes mediate a conserved mammalian PCP pathway to regulate convergent extension during neurulation', *Development (Cambridge, England),* 133(9), pp. 1767-1778.

Wang, Y. (2009) 'Wnt/Planar cell polarity signaling: a new paradigm for cancer therapy', *Molecular cancer therapeutics,* 8(8), pp. 2103-2109.

Wang, Y., Krivtsov, A.V., Sinha, A.U., North, T.E., Goessling, W., Feng, Z., Zon, L.I. and Armstrong, S.A. (2010) 'The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML', *Science (New York, N.Y.),* 327(5973), pp. 1650-1653.

Wang, Y. and Nathans, J. (2007) 'Tissue/planar cell polarity in vertebrates: new insights and new questions', *Development (Cambridge, England),* 134(4), pp. 647-658.

Watanabe, N., Kato, T., Fujita, A., Ishizaki, T. and Narumiya, S. (1999) 'Cooperation between mDia1 and ROCK in Rho-induced actin reorganization', *Nature cell biology,* 1(3), pp. 136-143.

Weber-Hall, S.J., Phippard, D.J., Niemeyer, C.C. and Dale, T.C. (1994) 'Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland', *Differentiation,* 57(3), pp. 205-214.

Weeraratna, A.T., Jiang, Y., Hostetter, G., Rosenblatt, K., Duray, P., Bittner, M. and Trent, J.M. (2002) 'Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma', *Cancer cell,* 1(3), pp. 279-288.

Westfall, T.A., Brimeyer, R., Twedt, J., Gladon, J., Olberding, A., Furutani-Seiki, M. and Slusarski, D.C. (2003) 'Wnt-5/pipetail functions in vertebrate axis formation as a negative regulator of Wnt/{beta}-catenin activity', Science Signaling, 162(5), pp. 889.

Wieduwilt, M. and Moasser, M. (2008) 'The epidermal growth factor receptor family: biology driving targeted therapeutics', *Cellular and Molecular Life Sciences,* 65(10), pp. 1566-1584.

Willerford, D.M., Chen, J., Ferry, J.A., Davidson, L., Ma, A. and Alt, F.W. (1995) 'Interleukin-2 receptor α chain regulates the size and content of the peripheral lymphoid compartment', *Immunity,* 3(4), pp. 521-530.

Willert, K.H. (2008) 'Isolation and application of bioactive Wnt proteins', in *Wnt Signaling.* Springer, pp. 17-29.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R. and Nusse, R. (2003) 'Wnt proteins are lipid-modified and can act as stem cell growth factors', *Nature,* 423(6938), pp. 448-452.

Willett, A., Michell, M. and Lee, M. (2010) 'Best practice diagnostic guidelines for patients presenting with breast symptoms', *DOH, London,* .

Williams, C.M., Engler, A.J., Slone, R.D., Galante, L.L. and Schwarzbauer, J.E. (2008) 'Fibronectin expression modulates mammary epithelial cell proliferation during acinar differentiation', *Cancer research,* 68(9), pp. 3185-3192.

Winklbauer, R., Medina, A., Swain, R.K. and Steinbeisser, H. (2001) 'Frizzled-7 signalling controls tissue separation during Xenopus gastrulation', Nature, 413(6858), pp. 856-860.

Winn, R.A., Marek, L., Han, S.Y., Rodriguez, K., Rodriguez, N., Hammond, M., Van Scoyk, M., Acosta, H., Mirus, J., Barry, N., Bren-Mattison, Y., Van Raay, T.J., Nemenoff, R.A. and Heasley, L.E. (2005) 'Restoration of Wnt-7a expression reverses non-small cell lung cancer cellular transformation through frizzled-9-mediated growth inhibition and promotion of cell differentiation', *The Journal of biological chemistry,* 280(20), pp. 19625-19634.

Winston, J.T., Strack, P., Beer-Romero, P., Chu, C.Y., Elledge, S.J. and Harper, J.W. (1999) 'The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro', *Genes & development,* 13(3), pp. 270-283.

Wissmann, C., Wild, P.J., Kaiser, S., Roepcke, S., Stoehr, R., Woenckhaus, M., Kristiansen, G., Hsieh, J., Hofstaedter, F. and Hartmann, A. (2003) 'WIF1, a component of the Wnt pathway, is down-regulated in prostate, breast, lung, and bladder cancer', *The Journal of pathology,* 201(2), pp. 204-212.

Wodarz, A. and Nusse, R. (1998) 'Mechanisms of Wnt signaling in development', *Annual Review of Cell and Developmental Biology,* 14(1), pp. 59-88.

Wong, H., Bourdelas, A., Krauss, A., Lee, H., Shao, Y., Wu, D., Mlodzik, M., Shi, D. and Zheng, J. (2003) 'Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled', *Molecular cell,* 12(5), pp. 1251-1260.

Wong, H., Bourdelas, A., Krauss, A., Lee, H., Shao, Y., Wu, D., Mlodzik, M., Shi, D. and Zheng, J. (2003c) 'Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled', *Molecular cell,* 12(5), pp. 1251-1260.

Wood, L.D., Parsons, D.W., Jones, S., Lin, J., Sjoblom, T., Leary, R.J., Shen, D., Boca, S.M., Barber, T., Ptak, J., Silliman, N., Szabo, S., Dezso, Z., Ustyanksky, V., Nikolskaya, T., Nikolsky, Y., Karchin, R., Wilson, P.A., Kaminker, J.S., Zhang, Z., Croshaw, R., Willis, J., Dawson, D., Shipitsin, M., Willson, J.K., Sukumar, S., Polyak, K., Park, B.H., Pethiyagoda, C.L., Pant, P.V., Ballinger, D.G., Sparks, A.B., Hartigan, J., Smith, D.R., Suh, E., Papadopoulos, N., Buckhaults, P., Markowitz, S.D., Parmigiani, G., Kinzler, K.W., Velculescu, V.E. and Vogelstein, B. (2007) 'The genomic landscapes of human breast and colorectal cancers', *Science (New York, N.Y.),* 318(5853), pp. 1108-1113.

Woodburn, J. (1999) 'The epidermal growth factor receptor and its inhibition in cancer therapy', *Pharmacology & therapeutics,* 82(2), pp. 241-250.

Worthylake, R.A., Lemoine, S., Watson, J.M. and Burridge, K. (2001) 'RhoA is required for monocyte tail retraction during transendothelial migration', *The Journal of cell biology,* 154(1), pp. 147-160.

Wozniak, M.A., Modzelewska, K., Kwong, L. and Keely, P.J. (2004) 'Focal adhesion regulation of cell behavior', *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1692(2), pp. 103-119.

Wu, C., Keivenst, V.M., O'Toole, T.E., McDonald, J.A. and Ginsberg, M.H. (1995) 'Integrin activation and cytoskeletal interaction are essential for the assembly of a fibronectin matrix', *Cell,* 83(5), pp. 715-724.

Wu, Q.L., Zierold, C. and Ranheim, E.A. (2009) 'Dysregulation of Frizzled 6 is a critical component of B-cell leukemogenesis in a mouse model of chronic lymphocytic leukemia', *Blood,* 113(13), pp. 3031-3039.

Wyckoff, J.B., Jones, J.G., Condeelis, J.S. and Segall, J.E. (2000) 'A critical step in metastasis: in vivo analysis of intravasation at the primary tumor', *Cancer research,* 60(9), pp. 2504-2511.

Xu, H., Wei, Q., Liu, Y., Yang, L., Dai, S., Han, Y., Yu, J., Liu, N. and Wang, E. (2007) 'Overexpression of axin downregulates TCF-4 and inhibits the development of lung cancer', *Annals of surgical oncology,* 14(11), pp. 3251-3259.

Xu, Y.K. and Nusse, R. (1998) 'The Frizzled CRD domain is conserved in diverse proteins including several receptor tyrosine kinases', *Current biology,* 8(12), pp. R405-R406.

Xu, H.T., Wang, L., Lin, D., Liu, Y., Liu, N., Yuan, X.M. and Wang, E.H. (2006) 'Abnormal beta-catenin and reduced axin expression are associated with poor differentiation and progression in non-small cell lung cancer', *American Journal of Clinical Pathology,* 125(4), pp. 534-541.

Yamana, N., Arakawa, Y., Nishino, T., Kurokawa, K., Tanji, M., Itoh, R.E., Monypenny, J., Ishizaki, T., Bito, H., Nozaki, K., Hashimoto, N., Matsuda, M. and Narumiya, S. (2006) 'The Rho-mDia1 pathway regulates cell polarity and focal adhesion turnover in migrating cells through mobilizing Apc and c-Src', *Molecular and cellular biology,* 26(18), pp. 6844-6858.

Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S. and Nishida, E. (2002) 'JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates', *EMBO reports,* 3(1), pp. 69-75.

Yan, W., Cao, Q.J., Arenas, R.B., Bentley, B. and Shao, R. (2010) 'GATA3 inhibits breast cancer metastasis through the reversal of epithelial-mesenchymal transition', *The Journal of biological chemistry,* 285(18), pp. 14042-14051.

Yang, L., Wu, X., Wang, Y., Zhang, K., Wu, J., Yuan, Y., Deng, X., Chen, L., Kim, C. and Lau, S. (2011) 'FZD7 has a critical role in cell proliferation in triple negative breast cancer', *Oncogene,* 30(43), pp. 4437-4446.

Yarden, Y. and Sliwkowski, M.X. (2001) 'Untangling the ErbB signalling network', *Nature reviews Molecular cell biology,* 2(2), pp. 127-137.

Yee Jr, H.F., Melton, A.C. and Tran, B.N. (2001) 'RhoA/rho-associated kinase mediates fibroblast contractile force generation', *Biochemical and biophysical research communications,* 280(5), pp. 1340-1345.

Yoneda, T. and Hiraga, T. (2005) 'Crosstalk between cancer cells and bone microenvironment in bone metastasis', *Biochemical and biophysical research communications,* 328(3), pp. 679-687.

Yoon, N.K., Maresh, E.L., Shen, D., Elshimali, Y., Apple, S., Horvath, S., Mah, V., Bose, S., Chia, D. and Chang, H.R. (2010) 'Higher levels of GATA3 predict better survival in women with breast cancer', *Human pathology,* 41(12), pp. 1794-1801.

Youlden, D.R., Cramb, S.M., Dunn, N.A., Muller, J.M., Pyke, C.M. and Baade, P.D. (2012) 'The descriptive epidemiology of female breast cancer: an international comparison of screening, incidence, survival and mortality', *Cancer epidemiology,* 36(3), pp. 237-248.

Young, H.E. and Black, A.C. (2004) 'Adult stem cells', *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology,* 276(1), pp. 75-102.

Zeng, Y.A. and Nusse, R. (2010) 'Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture', *Cell stem cell,* 6(6), pp. 568-577.

Zeng, X., Huang, H., Tamai, K., Zhang, X., Harada, Y., Yokota, C., Almeida, K., Wang, J., Doble, B., Woodgett, J., Wynshaw-Boris, A., Hsieh, J.C. and He, X. (2008) 'Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions', *Development (Cambridge, England),* 135(2), pp. 367-375.

Zhang, H., Zhang, X., Wu, X., Li, W., Su, P., Cheng, H., Xiang, L., Gao, P. and Zhou, G. (2012) 'Interference of Frizzled 1 (FZD1) reverses multidrug resistance in breast cancer cells through the Wnt/β-catenin pathway', *Cancer letters,* 323(1), pp. 106-113.

Zhong, C., Chrzanowska-Wodnicka, M., Brown, J., Shaub, A., Belkin, A.M. and Burridge, K. (1998) 'Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly', *The Journal of cell biology,* 141(2), pp. 539-551.

Zhong, H., De Marzo, A.M., Laughner, E., Lim, M., Hilton, D.A., Zagzag, D., Buechler, P., Isaacs, W.B., Semenza, G.L. and Simons, J.W. (1999) 'Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases', Cancer research, 59(22), pp. 5830-5835.