Inhibiting Tumor Protein D52 function for anti-cancer therapy application

Nuruliza Roslan

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Molecular Oncology Laboratory, Children’s Cancer Research Unit, The Children’s Hospital at Westmead
Discipline of Paediatrics and Child Health, Faculty of Medicine

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Statement of originality

I hereby declare that the contents of this thesis is my own work and that it contains no material previously published or written by another person, except where acknowledged in the text. This submission does not contain material which has been accepted for the award of another degree or diploma at the University of Sydney or at any other institution.

Nuruliza Roslan
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To them I dedicate this thesis.

Nuruliza Roslan
Abstract

Tumor protein D52 (TPD52) is known to be amplified and overexpressed in many cancer types. TPD52-amplified cancer cell lines may therefore be sensitive to reduced TPD52 expression and dependent on TPD52 for cell survival. Previously, TPD52 expression has been associated with ERBB2, suggesting that these oncogenes may be co-operating in cancer pathogenesis.

We employed RNA interference approaches to characterise the effects of reducing TPD52 expression in breast cell lines according to their TPD52 and ERBB2 amplification status. Firstly, a panel of 15 breast cell lines was selected based on their TPD52 amplification status. These cell lines were subjected to quantitative real-time (RT-) PCR to determine relative TPD52 DNA copy number and RNA transcript levels, while TPD52 protein levels were analysed by Western blotting. The results confirmed SK-BR-3 as a TPD52-amplified and overexpressing cell line. Apoptotic assays conducted after 72 hours of TPD52 gene knock-down revealed that reduced TPD52 expression was associated with increased apoptosis in the TPD52-amplified cell line SK-BR-3, relative to non-amplified cell lines (MCF-7, MDA-MB-231, MCF-10A). These results suggest that the TPD52-amplified cell line SK-BR-3 is most sensitive to reduced TPD52 expression.

Next, the effects of TPD52 knock-down were compared with those of another oncogene ERBB2, which is amplified in both SK-BR-3 (TPD52 amplified) and BT-474 (TPD52 non-amplified) cell lines. Apoptosis assay results revealed transiently reduced TPD52 expression decreased the survival of these two ERBB2-amplified breast cancer cell lines. BT-474 cells were more sensitive to reduced ERBB2 expression, whereas SK-BR-3 cells were similarly sensitive to reduced TPD52 and reduced ERBB2 expression. In SK-BR-3 cells, knock-down of both ERBB2 and
TPD52 genes led to increased apoptosis, relative to knock-down of either gene alone.

We also generated SK-BR-3 cells in which TPD52 expression was stably reduced using human TPD52 shRNA vectors, to characterise for the first time the long-term consequences of reducing TPD52 expression in breast cancer cells. Reduced TPD52 protein expression in stably shRNA-transfected cell lines was confirmed using Western blot and immunofluorescence analyses, relative to non-targeting shRNA transfected cells. Results in stably TPD52-depleted SK-BR-3 cell lines showed increased pERBB2 and pAkt levels, while MTT assays showed no changes in proliferation, indicating compensatory effects towards increased ERBB2 signaling to ensure cell survival.

In attempting to explain these findings further, we proposed a novel function of TPD52. Associations between TPD52 and ERBB2 expression, an oncogene promoting lipogenesis, led us to hypothesize that TPD52 expression may be associated with lipogenic phenotypes. Lipid droplet-associated proteins (perilipin, TIP47, adipophilin) play important roles in regulating the storage of intracellular lipid in lipid droplets. As protein-protein associations between TPD52 and both perilipin and TIP47 have been reported, we hypothesized that TPD52 overexpression might advantage cancer cells by increasing their lipid storage capacity.

TPD52-transfected 3T3 fibroblast cell lines have been previously shown to demonstrate transformed phenotypes, relative to vector control cell lines. We examined whether TPD52 expression was associated with an increase in the number and/or size of lipid droplets in these cell lines, and whether this phenotype is an isoform-specific function of TPD52. Fluorescent staining (BODIPY 493/503) of TPD52-transfected 3T3 cell lines showed increased lipid droplet numbers and sizes
relative to vector control and parental cell lines, as measured by fluorescent intensity. Immunofluorescent staining demonstrated that whereas the PAT protein adipophilin showed a diffuse cytoplasmic distribution in 3T3 parental and vector control cell lines, this was clustered into cytoplasmic structures resembling lipid droplets in TPD52-transfected 3T3 cell lines. In contrast, 3T3 cell lines expressing the related protein TPD2L1, which does not show the same oncogenic potential as TPD52, showed no alterations in lipid droplets, and similar adipophilin staining patterns as parent and vector controls.

We also investigated TPD52-expressing 3T3 cells' ability to store exogenous lipids by treating cells with oleic acid, a monounsaturated dietary fatty acid. Following oleic acid treatment, fluorescent BODIPY 493/503 staining revealed that TPD52-expressing 3T3 cells formed more lipid droplets, relative to parental and vector control cells. However, parental and vector control cells showed greater relative increases in lipid droplet numbers, than TPD52-expressing 3T3 cells. Interestingly, immunofluorescent staining demonstrated TPD52 redistribution in some TPD52-expressing 3T3 cells following oleic acid treatment, without affecting TPD52 levels as assessed by Western blot analyses. The mechanisms whereby TPD52 expressing cells accumulate more lipid droplets require further investigation.

This study therefore identified TPD52 as a survival factor in ERBB2-amplified breast cancer cell lines. For the first time, we also provide direct evidence of TPD52 involvement in regulating cellular lipid storage. TPD52 may therefore co-operate indirectly with ERBB2 by regulating lipid storage, possibly via associations with PAT proteins. This highlights TPD52 as a candidate therapeutic target in ERBB2-positive cancers, or cancers with a lipogenic phenotype.
Publications arising from this thesis


**Nuruliza Roslan**, Sarah Frost, Alvin Kamili, Laurence Cantrill, Yuyan Chen, Jennifer Byrne. Tumor Protein D52 expression increases lipid storage in cultured cells. (manuscript in preparation)
List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ACC/ACACA</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACAT 1</td>
<td>Acyl-CoA cholesterol O-acyltransferase 1</td>
</tr>
<tr>
<td>ACAT 2</td>
<td>Acyl-CoA cholesterol O-acyltransferase 2</td>
</tr>
<tr>
<td>ACLY</td>
<td>ATP citrate lyase</td>
</tr>
<tr>
<td>ADRP/Adrp</td>
<td>Adipophilin or adipose-differentiated-related-protein</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
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<tr>
<td>ATGL</td>
<td>Adipose triacylglyceride lipase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>cAMP</td>
<td>Cyclic adenosine 3', 5'-monophosphate</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>CGI-58</td>
<td>Comparative gene identification-58</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>Co-A</td>
<td>Coenzyme-A</td>
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<td>DAG</td>
<td>diacylglyceride</td>
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<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole dihydrochloride</td>
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<td>DGAT1</td>
<td>Diacylglycerol acyltransferase 1</td>
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<tr>
<td>DGAT2</td>
<td>Diacylglycerol acyltransferase 2</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<td>EEA1</td>
<td>Early endosome antigen 1</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>Epidermal growth factor receptor</td>
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<td>Estrogen receptor</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ERBB2/HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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ERBB3/HER3  Human epidermal growth factor receptor 3
ERBB4/HER4  Human epidermal growth factor receptor 4
FASN/Fasn   Fatty acid synthase
FBS         Fetal bovine serum
FITC        Fluorescein isothiocyanate
GAPDH/Gapdh Glyceraldehyde 3-phosphate dehydrogenase
G418        Geneticin
GFP         Green fluorescent protein
gp130       Glycoprotein 130
h           hour
HRP         Horse radish peroxidase
HSL         Hormone-sensitive lipase
IF          Immunofluorescence
IgG         Immunoglobulin G
IHC         Immunohistochemistry
IL-6 receptor Interleukin-6 receptor
Kb          Kilobases
kDa         Kilodaltons
LAMP1       Lysosomal-associated membrane protein 1
LD          Lipid droplet
M           Molar
MAG         monoacylglyceride
MAL2        T-cell differentiation protein 2 or Myelin and lymphocyte protein 2
MAPK        Mitogen-activated protein kinase
MED1/PBP    Mediator subunit 1 (MED1)/Peroxisome proliferator-activated receptor-binding protein (PBP)
min         minutes
mM          Millimolar
μM          Micromolar
mRNA        Messenger RNA
MTT         3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MUFA        Monounsaturated fatty acids
MW          Molecular weight
nM          Nanomolar
NADPH       Nicotinamide adenine dinucleotide phosphate-oxidase
NR1D1       Nuclear receptor subfamily 1
<table>
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<th>Abbreviation</th>
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<tr>
<td>OXPAT</td>
<td>Oxidative protein of the PAT family, or PLIN5</td>
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<td>pAkt</td>
<td>Phosphorylated Akt</td>
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<tr>
<td>PAT</td>
<td>Perilipin, ADRP, TIP47</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PDI</td>
<td>Protein disulfide isomerase</td>
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<td>pAkt</td>
<td>Phosphorylated Akt</td>
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<td>PA</td>
<td>Perilipin, ADRP, TIP47</td>
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<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<td>Protein kinase A</td>
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<td>PLIN1</td>
<td>Perilipin</td>
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<td>pMAPK</td>
<td>Phosphorylated MAPK</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<td>PrLZ</td>
<td>Prostate leucine zipper</td>
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<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted from chromosome 10</td>
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<td>Polyunsaturated fatty acids</td>
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<td>Polyvinylidene fluoride</td>
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<td>Ras-related protein 5</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<td>second</td>
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<td>PLIN4</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SE</td>
<td>Standard error</td>
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<td>siRNA</td>
<td>Short interfering RNA</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<td>SMURF1</td>
<td>SMAD specific E3 ubiquitin protein ligase 1</td>
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<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
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<td>-------------</td>
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<tr>
<td>TAG</td>
<td>Triacylglyceride/ triacylglycerol/ triglyceride</td>
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<td>TBS</td>
<td>Tris-buffered saline</td>
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<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
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<td>TIP47/Tip47</td>
<td>Tail-Interacting Protein of 47 kDa</td>
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<td>Tumor protein D52-like 3 (protein kinase NYD-SP25)</td>
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Chapter 1

Literature review
1. Introduction

This review will give a brief introduction to cancer to generate awareness of the rising numbers of cancer cases worldwide, hence the urgent need to understand how cancer develops. This will be followed by an introduction to oncogenes, and how these are involved in cancer formation. The “oncogene addiction” phenomenon will be defined and we will explain how this concept can be applied to the development of cancer treatments. The ERBB2/HER2 (Human epidermal growth factor receptor 2) oncogene will be used as an example of “oncogene addiction”, and we will describe how understanding its structure and function led to the development of the drug Herceptin for cancer treatment. We will also briefly discuss the significance of altered lipid metabolism in cancer pathogenesis, and ERBB2 will be used as an example of an oncogene which affects downstream lipid metabolism. Finally, the review will introduce the TPD52 (Tumor Protein D52) gene as the focus of this project, which is a potential oncogene identified through its overexpression in various types of cancers. It will be explained how the “oncogene addiction” concept could be applied to TPD52 in terms of developing a new anti-cancer drug specifically for TPD52 overexpressing cancers.

1.1 Cancer – A global burden of disease

Cancer is considered as one of the leading causes of death worldwide (Mathers and Loncar, 2006). In 2008, cancer resulted in 7.6 million deaths, accounting for about 13% of all human deaths worldwide (Ferlay et al., 2010). The total number of cancer cases globally is predicted to continue to rise, with WHO predicting ~13 million cancer deaths in 2030 (Ferlay et al., 2010). New cancer cases are estimated to rise to 21.4 million in 2030 compared to 12.7 million cases in 2008 (Ferlay et al., 2010).
WHO estimates that 84 million people will die of cancer between 2005 and 2015 without intervention (www.who.int/). In 2003, through the Surveillance, Epidemiology and End Results (SEER) Program cancer statistics, Gloeckler Reis et al. reported that one of every two men and one of every three women will develop cancer during their lifetime (Gloeckler Ries et al., 2003). In Australia currently, the major cancer affecting males is prostate cancer and for females the major cancer is breast cancer, as reported by the Australian Institute of Health and Welfare (AIHW) and the Australasian Association of Cancer Registries (AACR) (AIHW and AACR 2012). The four most common cancers for both men and women are colorectal cancer, melanoma of the skin, lung cancer and lymphoma (AIHW and AACR 2012). Therefore, it is very important to understand the cellular and molecular basis of cancer, in order to find ways to either prevent cancer, or cure cancer in a highly specific and effective way, at low cost. The cost of treatment is important, as current cancer treatments such as chemotherapy are expensive and therefore not always available to people in underdeveloped countries (Hanna and Kangolle, 2010).

Cancers detected early are generally associated with a better prognosis and, in many cases, can be cured (Kramer and Croswell, 2009; Peto et al., 2004). However, cures are not attempted in some advanced cancer cases, where palliative care provides better quality of life than aggressive treatment, particularly in the elderly or in patients with underlying comorbid disorders (Yancik and Ries, 2000). At present, the emerging number of cancer cases around the world is more than the number of people cured from cancer (Jemal et al., 2010). There are several factors which could improve this situation, such as generating more awareness amongst the general public as to the importance of cancer screening or vaccination (e.g. breast cancer, cervical cancer) and living a healthy lifestyle (e.g. smokers with lung cancer).
1.1.1 Causes of cancer – from alterations in a single cell to tumor formation

There are many different types of cancers affecting different parts of the body. A cancer, or tumor, can occur in any organ or tissue of the human body (Weinberg, 2007). All cancers result from mutations in cellular DNA (Weinberg, 2007). Cancers can be due to inherited factors (familial cancer) or can arise sporadically. Approximately 90-95% of all cancers are sporadic while the remaining 5-10% are familial. Such an example has been described for breast cancer, where family history is responsible for about 5 - 10% of breast cancers (De Greve et al., 2008).

Age is a major factor to consider, as the incidence of cancer increases dramatically with increased age (Suen et al., 1974). In the United States, the incidence of cancer for those aged over 65 has been reported to be 10 times greater than that of people aged below 65 (Yancik and Ries, 2000). Furthermore, the cancer death rate was 16 times greater in cancer patients over 65, compared with that of younger cancer patients (below 65) (Berger et al., 2006). More than 70% of the mortality associated with cancers including prostate, bladder, colon, uterus, pancreas, stomach, rectum and lung occurs in patients 65 and older (Yancik and Ries, 2000). A similar trend has also been observed in Australia, where the incidence rates of cancer and the risk of being diagnosed with cancer increases with age (Figure 1.1) (AIHW & AACR 2012). The association of cancer incidence and increased age is likely underpinned by the accumulation of genetic mutations over time, combined with the tendency for cellular repair mechanisms to be less effective as a person grows older (Rudolph et al., 1999; Sharpless and DePinho, 2004). Therefore, while cancer affects people of all ages, the risk for most cancer types increases with age.
Figure 1.1 Estimated incidence rates of all cancers combined by age at diagnosis, Australia, 2012. The Y-axis represents incidence rates (per 100,000 population) and the X-axis represents age group (in years). Reproduced from Australian Institute of Health and Welfare (AIHW) and Australasian Association of Cancer Registries (AACR), 2012.
Cancer has been regarded as a genetic disease, and as such, cancer-causing mutations alter the cell’s life cycle of growth, proliferation, DNA repair and death by altering the quantity or function of critical protein products (Ames et al., 1993; Bertram, 2000). This leads to the accumulation of more cancer cells and the development of a tumor mass. Mutations can arise from environmental or exogenous factors such as physical carcinogens (ionizing radiation, UV radiation), chemical carcinogens (asbestos, tobacco, aflatoxin, arsenic) or biological carcinogens (infections from viruses such as Hepatitis B virus (HBV), Hepatitis C virus (HPC), Human papilloma virus (HPV), bacteria or parasites) (Ames, 1984; Ames et al., 1993; Anand et al., 2008). Alternatively, DNA damage can also result from endogenous processes such as errors in DNA replication (Chen et al., 1999; Lavin and Khanna, 1999; van Steeg and Kraemer, 1999), the intrinsic chemical instability of certain DNA bases, or from free radicals generated during metabolism (Halliwell, 1994; Jones et al., 1992; Malkin et al., 1990). The DNA mutation may be a single nucleotide change, or a deletion or duplication of longer DNA sequences. A change in the genetic sequence can then lead to the production of a mutant protein. It is usually an accumulation of mutations in multiple genes that irreversibly transforms a normal cell into a cancerous one (Cahill et al., 1999; Cho and Vogelstein, 1992).
1.1.2 Cellular and molecular basis of cancer

Cancer is a multistage process (Grivennikov et al., 2010) (Figure 1.2) involving changes to a number of key cellular processes, known as the hallmarks of cancer, which were initially proposed in 2000 (Hanahan and Weinberg, 2000). These key processes or hallmarks are self-sufficiency in growth signals, insensitivity to growth inhibitory signals, protection against programmed cell death or apoptosis, unregulated/uncontrolled growth (division beyond normal limits), sustained angiogenesis, local tissue invasion (intrusion on and destruction of adjacent tissues), and often metastasis (spread of cancer cells to other locations in the body via the lymphatic system or blood) (Hanahan and Weinberg, 2000) (Figure 1.3). Since then, the cellular changes depicting the hallmarks of cancer have been revisited and updated. A recent review included additional hallmarks of cancer that describe the stress phenotypes of cancer cells, namely metabolic stress, proteotoxic stress, mitotic stress, oxidative stress and DNA damage stress (Luo et al., 2009) (Figure 1.3). Another hallmark has also been recently proposed, namely the evasion of immune surveillance (Kroemer and Pouyssegur, 2008). A detailed understanding of these hallmarks is important in order to develop new therapeutic strategies for treating cancer (Hanahan and Weinberg, 2011).

Two main categories of mutated genes contribute towards tumor development, namely oncogenes and tumor suppressor genes (Hanahan and Weinberg, 2000). In many cases, oncogenes promote cell proliferation whereas tumor suppressor genes inhibit this, and the activities of these two gene classes are optimally balanced under physiological conditions. Most mutations in oncogenes contribute to cancer development by allowing dominant gains of function, whereas inactivation of most tumor suppressor genes occurs through recessive loss of function mutations.
Figure 1.2 Cancer initiation and progression, from changes in a single cell through to metastatic cancer. Reproduced from Grivennikov et al. (2010)
Figure 1.3 Hallmarks of cancer. The acquired properties of a tumor cell (shown on the outside of the circle) and their cellular consequences (shown on the inside of the circle). Reproduced from Luo et al. (2009)
1.2 Oncogenes

Oncogenes are genes with the ability to induce tumor formation and malignancy when mutated or expressed at high levels (Adamson, 1987; Croce, 2008). They are the mutated forms of cellular proto-oncogenes, that encode proteins which regulate various aspects of normal cell growth and differentiation (Adamson, 1987). Proto-oncogenes can be classified into groups, based upon their normal function within cells or based upon sequence homology to other known proteins. There are five broad types of proteins encoded by proto-oncogenes: growth factors, receptors for growth factors and hormones, intracellular signal transducers, nuclear transcription factors and cell-cycle control proteins (Croce, 2008).

Oncogenes can usually act in a dominant fashion through gain of function and only one allele has to be altered for phenotypic consequences. Oncogenes can be activated via retroviral transduction or integration, point mutations (Konopka et al., 1985; Rous, 1911; Stehelin et al., 1976), gene amplification (i.e, an increase in the number of copies of an otherwise normal gene) (Press et al., 1997; Schwab et al., 1983) and/or chromosomal translocation (Finger et al., 1986; Tsujimoto et al., 1985). Activation of oncogenes may result in the direct and continuous stimulation of signaling pathways that control cellular growth and division, DNA repair, angiogenesis and other physiologic processes (Croce, 2008). Examples of well characterised oncogenes and their involvement in various types of cancers are shown in Table 1.1. As inhibiting the function of a mutant protein, or reducing the activity of a non-mutant protein is currently more feasible than restoring a function that has been lost, oncogenes are prime targets for anticancer therapies (Table 1.2).
Table 1.1 Examples of oncogenes involved in human cancers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein function</th>
<th>Examples of cancers involved</th>
<th>Nature of dysregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>Apoptosis inhibitor</td>
<td>B cell lymphomas</td>
<td>Chromosomal translocation</td>
</tr>
<tr>
<td>c-myc</td>
<td>Transcription factor, Activates other growth promoting genes</td>
<td>Leukemia, breast, stomach and lung cancers</td>
<td>Chromosomal translocation/gene amplification</td>
</tr>
<tr>
<td>K-ras</td>
<td>Small GTPase, Involved in proliferation signaling pathways</td>
<td>Lung, ovarian, colon and pancreatic cancers</td>
<td>Gene mutation</td>
</tr>
<tr>
<td>ERBB2/Her2</td>
<td>ligand-less receptor of the EGFR family</td>
<td>Breast, salivary gland and ovarian cancers</td>
<td>Gene amplification</td>
</tr>
</tbody>
</table>

Adapted from Weinstein et al. (2006)
Table 1.2 Cancer therapies that target oncogenic proteins

<table>
<thead>
<tr>
<th>Anticancer Drug Types</th>
<th>Target(s)</th>
<th>Approved cancer type(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name (Trade name, Company)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Monoclonal antibodies**

- **Trastuzumab (Herceptin, Genentech)**
  - Target: ERBB2
  - Approved cancer type: Breast

- **Cetuximab (Erbitux, ImClone)**
  - Target: EGFR
  - Approved cancer type: Colon

- **Bevacizumab (Avastin, Genentech)**
  - Target: VEGF
  - Approved cancer type: Colon, NSCLC

**Small molecules**

- **Imatinib (Gleevec, Novartis)**
  - Targets: ABL, PDGFR, KIT
  - Approved cancer type: Chronic myelogenous leukemia, gastrointestinal stromal tumors, chordoma

- **Gefitinib (Iressa, AstraZeneca)**
  - Target: EGFR
  - Approved cancer type: NSCLC

- **Erlotinib (Tarveca, Genentech)**
  - Target: EGFR
  - Approved cancer type: NSCLC

- **Sorafenib (Nexavar, Bayer/Onyx)**
  - Targets: VEGFR, PDGFR, FLT3
  - Approved cancer type: Renal cell carcinoma

- **Sunitinib (Sutent, Pfizer)**
  - Targets: VEGFR, PDGFR, FLT3
  - Approved cancer type: Gastrointestinal stromal tumors, renal cell carcinoma

ABL/ABL1 = Abelson murine leukemia viral oncogene homolog 1; EGFR = Epidermal growth factor receptor; ERBB2/HER2 = Human epidermal growth factor receptor 2; FLT3 = FMS-like tyrosine kinase 3; PDGFR = platelet-derived growth factor receptor; VEGF = vascular endothelial growth factor; NSCLC = non-small cell lung cancer.

Adapted from Croce et al. (2008)
1.2.1 The “oncogene addiction” concept

Interestingly, some tumors have been shown to be physiologically dependent on (addicted to) the uninterrupted activity of a single mutated oncogene for maintaining tumor survival and proliferation (Weinstein, 2000). Disruption of the respective oncogene leads to apoptosis, selective growth arrest, and/or differentiation of the malignant cells. This important phenomenon, termed “oncogene addiction” (Weinstein, 2002), has been documented in multiple mouse tumor models, cell culture models, and human clinical trials involving specific molecularly targeted inhibitors (Weinstein, 2000; Weinstein and Joe, 2006). These addicted oncogenes and the cellular pathways hijacked by them make attractive therapeutic targets. An example is chronic myeloid leukemia and the selective tyrosine kinase inhibitor Gleevec that preferentially and dramatically acts on tumor cells addicted to the tyrosine kinase \textit{BCR-ABL}, and causes rapid cancer regression with only limited side effects (Druker et al., 2001; Shah and Sawyers, 2003). This example indicated that tumor cells can default to an apoptotic outcome in response to oncogene inactivation, leading to complete tumor regression (Weinstein, 2000). However, the precise mechanism underlying how this phenomenon occurs is still unclear and requires further investigation. Oncogenes which are mutated or gene amplified, and not simply overexpressed are more likely targets for therapy, reflecting “hard-wiring” of cancer cells as opposed to epigenetic abnormalities (Weinstein and Joe, 2008). This “hard-wiring” reflects cancer cells being more dependent on these oncogenes for both maintaining the malignant phenotype, and for cancer cell survival.

Another example to explain the rational of the oncogene addiction concept is the \textit{ERBB2} gene that has been shown to be amplified and overexpressed in breast cancer cell lines as well as in patients (Berger et al., 1988; Press et al., 1997) and which led to the development of trastuzumab/Herceptin, a humanized monoclonal
antibody (Baselga et al., 1998; Harries and Smith, 2002; Hudis, 2007). The response of \textit{ERBB2} amplified breast cancers to Herceptin is further support for the oncogene addiction hypothesis. A better understanding of the basis of oncogene addiction is expected to identify critical predictive biomarkers for assessing patients’ suitability for therapy. Overall, a better understanding of oncogene addiction will contribute towards the development of new cancer treatments, and the best way to use these in the clinic.

\subsection*{1.3 \textit{ERBB2}: an example of the “oncogene addiction” phenomenon}

\subsubsection*{1.3.1 \textit{ERBB} receptors: structure}

\textit{ERBB2} is part of a family consisting of 4 members, namely EGFR/HER1, \textit{ERBB2}/HER2, \textit{ERBB3}/HER3, and \textit{ERBB4}/HER4. These 4 belong to the subclass 1 of the receptor tyrosine kinase (RTK) superfamily, according to their sequence homology and domain organization (Yarden and Sliwkowski, 2001) (Figure 1.4). They were named due to their homology to the viral \textit{ERBB} (\textit{v-ERBB}) oncogene (Schechter et al., 1984). These \textit{ERBB} receptors share a common structure: an extracellular ligand-binding domain containing 4 subdomains, a transmembrane domain with a single hydrophobic anchor sequence and an intracellular region, with a catalytic tyrosine kinase domain. The tyrosine kinase domain is important for the activation/induction of intracellular signaling (Figure 1.4) (Yarden and Sliwkowski, 2001). The \textit{ERBB} family members show individual differences, such as the intrinsic tyrosine kinase activity for \textit{ERBB3} being impaired (Guy et al., 1994).

\textit{ERBB2} or \textit{HER2} (\textit{Human Epidermal growth factor Receptor 2}) was cloned by homology screening with \textit{v-ERBB}, and its amino acid sequence was shown to be structurally homologous to that of EGFR (Coussens et al., 1985; Schechter et al.,
Neu, the rat homologue of ERBB2, was identified as a transforming gene activated in chemically induced rat neuroectodermal tumors (Shih et al., 1981). The ERBB2 proto-oncogene, located at chromosome 17q21-q22 (37.8 - 37.9 MB), encodes a protein of 185 kDa (Coussens et al., 1985; Schechter et al., 1984).

Figure 1.4 ERBB family receptors and their cognate ligands. Each receptor consists of three domains: a ligand-binding extracellular domain containing two cysteine-rich regions (CR1 and CR2), a transmembrane domain, and an intracellular domain containing a tyrosine kinase region. Note that ERBB2/HER2 has no known ligands and that ERBB3/HER3 has no intrinsic tyrosine kinase activity. (EGF = epidermal growth factor; EGFR = EGF receptor; HER = human epidermal receptor; HB-EGF = heparin-binding EGF; NRG = neuregulin; TGF-α = transforming growth factor-α). Reproduced from Rowinsky et al. (2004)
1.3.2 ERBB receptors: signal transduction

Signal transduction via the ERBB family receptors is initiated by ligand binding. Upon ligand binding, receptor homo/heterodimerisation occurs and each receptor subunit is activated by transphosphorylation of tyrosine residues in the receptor C-terminus (Ferguson, 2008) (Figure 1.5). Phosphorylated tyrosine residues serve as docking sites for a host of intracellular signaling molecules, leading to the activation of diverse signaling cascades such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways that mediate the physiological changes ascribed to ERBB receptors (Olayioye, 2001; Schlessinger, 2000) (Figure 1.5). These changes include regulation of cell growth, differentiation, motility and inhibition of cell death (Rowinsky, 2004). Disruption of any pathway component such as ERBB ligands, receptors, downstream signaling molecules or transcriptional targets has been reported to have profound consequences for both normal development and malignant transformation in multiple tissues (Landis et al., 2005; Rowinsky, 2004).

ERBB receptors are activated by members of the EGF family of ligands (Figure 1.4). These ligands contain an EGF-like domain comprised of a motif of ~50 amino acids, including 6 cysteine residues, with two stranded β-sheet structure (Yarden and Sliwkowski, 2001). Unlike other members of the ERBB family, ERBB2 has no known ligand and is therefore also known as an orphan receptor (Klapper et al., 1999; Rowinsky, 2004). For this reason, activation of ERBB2 is dependent on its association with other ERBB family members to form functional heterodimers (Figure 1.5). Interestingly, ERBB2 has been found to be recruited as the preferred heterodimeric partner of ERBB3 (Graus-Porta et al., 1997; Karunagaran et al., 1996; Sliwkowski et al., 1994). Of all the ERBB heterodimers, ERBB2-ERBB3 heterodimers elicit the strongest signal (Alimandi et al., 1995; Graus-Porta et al., 1997; Pinkas-
Kramarski et al., 1996; Stern, 2008). Removing ERBB3 from the cell has a drastic effect on ERBB2 mediated signaling and downstream effectors (Stern, 2008). ERBB2 has been shown to also form heterodimers with EGFR and ERBB4 as well as other non-ERBB receptors, such as the gp130 subunits of the IL-6 receptor (Rowinsky, 2004). In at least some cell types, the association between gp130 and ERBB2 is essential for ERBB2-ERBB3 phosphorylation and subsequent MAPK signaling (Grant et al., 2002). MAP kinases are important mediators of signal transduction, and activation of this pathway has been linked to cell proliferation and differentiation (Keshet and Seger, 2010). The EGFR-ERBB2 complex increases the signaling capacity of EGFR by increasing ligand affinity, as well as recycling of the heterodimer (Hendriks et al., 2003; Karunagaran et al., 1996; Wada et al., 1990). Extensive receptor–receptor interactions and the existence of numerous ligands (Figure 1.5) highlight the enormous potential for diversification of biological messages mediated by the ERBB family (Graus-Porta et al., 1997; Rowinsky, 2004).
Figure 1.5 An overview of ERBB signaling. ERBB2/HER2 receptor pairs with itself (homodimerization, when overexpressed) or with other epidermal growth factor receptors (EGFR/HER1, HER4, HER3) that have bound their respective ligands (heterodimerization), leading to activation of the intracellular tyrosine kinase domains. A phosphorylation (P) cascade is activated whereby key downstream effectors are recruited and this results in activation of multiple intracellular pathways, including mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), which promote cell proliferation, survival, cycle progression and inhibit apoptosis. (Akt/PKB = Protein kinase B; MEK = MAPK kinase; RAS = rat sarcoma; RAF = Rapidly Accelerated Fibrosarcoma; SOS = son-of-sevenless guanine nucleotide exchange factor; VEGF = Vascular endothelial growth factor). Reproduced from Hudis (2007).
1.3.3 ERBB2 amplification and cancer

ERBB2 is activated by gene amplification in some 15 - 30% of breast cancers and this is associated with increased ERBB2 protein expression (Berger et al., 1988; Slamon et al., 1987). ERBB2 gene amplification and overexpression were also found in other types of cancers including ovarian and stomach cancers (Berchuck et al., 1990; Park et al., 1989; Slamon et al., 1989). Furthermore, ERBB2 overexpression was associated with increased disease recurrence and worse prognosis in breast cancer patients (Borg et al., 1991; Guerin et al., 1988; Slamon et al., 1987; Varley et al., 1987). This led to the suggestion that ERBB2 overexpression produced by gene amplification plays an important role in tumor development.

ERBB2 overexpression is the predominant mechanism involved in cancer tumorigenesis, and it has been proposed that ERBB2 amplification overcomes repression of ERBB2 expression by sequence-specific transcription factors (Birnbaum et al., 2009). However ERBB2 mutations have been reported in breast, gastric, colorectal and lung cancers (Bekaii-Saab et al., 2006; Buttitta et al., 2006; Cohen et al., 2005; Kubo et al., 2009; Lee et al., 2006; Stephens et al., 2004). Overexpression of ERBB2 resulted in ligand-independent signaling in mouse fibroblasts and this was assumed to occur in human breast cancers as well (Hudziak et al., 1987). In vitro studies of ERBB2 overexpression showed that this produced increased cell motility, increased sensitivity to mitogenic stimuli, anchorage independence and cell transformation (Brandt et al., 1999; Chazin et al., 1992; Ignatoski et al., 1999; Spencer et al., 2000). Studies in vivo confirmed the oncogenic potential of ERBB2 in the mammary epithelia of transgenic mice (Bouchard et al., 1989; Guy et al., 1996; Muller et al., 1988). Overexpression of the unactivated rat neu protein (neu proto-oncogene) in the mammary epithelium resulted in transgenic mice stochastically developing focal mammary tumors and pulmonary metastases.
after a long latency (Guy et al., 1992). These findings all supported ERBB2 as an oncogene involved in the development of breast and other cancers.

Identification of ERBB2 amplification and overexpression in cancer led to the development of the monoclonal antibody trastuzumab (Herceptin™, manufactured by Roche-Genentech, CA, USA), that targets the extracellular domain of ERBB2 (Figure 1.6). Trastuzumab is only effective in ERBB2-overexpressing cancers. Numerous human trial observations have shown trastuzumab to be an effective treatment for patients with ERBB2-overexpressing breast cancer (Hudis, 2007). The high success rate of trastuzumab in treating breast cancer patients with ERBB2 amplification validates the importance of this receptor in sustaining breast tumors (Piccart-Gebhart et al., 2005; Romond et al., 2005). Growing evidence showing ERBB2 amplification in other types of cancers, such as ovarian, stomach and uterine cancers (Lassus et al., 2004; Santin et al., 2005; Tanner et al., 2005), have led to trials examining whether trastuzumab could also be used to treat these cancers (Fleming et al., 2010; Gravalos and Jimeno, 2008).

There have been numerous studies of the mechanism of action of trastuzumab, and even though it is still not completely known, several mechanisms have been proposed (Hudis, 2007) (Figure 1.6). This includes binding of trastuzumab to ERBB2 inhibiting the cleavage of the ERBB2 extracellular domain (Molina et al., 2001), as well as interfering with ERBB2 dimerization with other ERBB receptors (Juntila et al., 2009), and disrupting the activation of downstream signaling pathways. Increased endocytotic destruction of ERBB2 has also been observed in vitro (Raja et al., 2008) but this remains to be demonstrated in vivo (either in pre-clinical xenograft models or clinical studies) (Fiszman and Jasnis, 2011). Antibody-dependent immune mechanisms induced upon trastuzumab treatment have also been proposed. Studies using animals deficient in immune-cell-activating Fc receptors (on effector
cells) showed no response upon trastuzumab treatment, suggesting that trastuzumab recruits immune effector cells that are responsible for antibody-dependent cell-mediated cytotoxicity (Clynes et al., 2000). Trastuzumab treatment was also reported to decrease vascular endothelial growth factor (VEGF) protein levels, which was proposed to be due to trastuzumab interfering with the dimerization between ERBB-family receptors, but this was only shown in vitro (Izumi et al., 2002; Kumar and Yarmand-Bagheri, 2001). VEGF production activates angiogenesis, or the formation of new blood vessels, and is important for maintaining tumor growth (Hoeben et al., 2004). Treatment with trastuzumab has been reported to affect cell cycle regulators such as p27, an inhibitor of cell proliferation, which was shown to be induced upon trastuzumab treatment through ERBB2 binding (Le et al., 2005).
Figure 1.6 Herceptin™/Trastuzumab targeting of the ERBB2 receptor. (A) Trastuzumab is a humanized monoclonal antibody that binds to ERBB2/HER2 and inhibits tumor-cell growth through a variety of intracellular, and possibly extracellular, mechanisms. (B) Cleavage of the extracellular domain of HER2 leaves a membrane-bound phosphorylated p95, that allows the activation of downstream signal-transduction pathways. Binding of trastuzumab to a juxtamembrane domain of HER2 reduces shedding of the extracellular domain, p95, thereby reducing signal activation. (C) Trastuzumab may also reduce HER2 signaling either by homodimerization, or heterodimerization inhibition between the ERBB receptors. (D) Trastuzumab may also induce tumor-cell death via activation of antibody-dependent cell-mediated cytotoxicity. (E) HER2 down-regulation upon trastuzumab binding through endocytosis has also been proposed. Reproduced from Hudis (2007).
1.4 Lipid metabolism

1.4.1 Overview of biological functions of lipids in mammalian cells

In order for an organism to function properly, a variety of nutrients are needed, including fats or lipids, carbohydrates, proteins, and vitamins that can be obtained from the diet. Proliferating cells require nutrients, energy, and biosynthetic activity to generate biomass (proteins, lipids, nucleic acids) for cell growth (DeBerardinis et al., 2008).

Lipids are a diverse and ubiquitous group of hydrophobic compounds which play important roles in numerous biological functions in humans and other organisms (Wenk, 2005). Cells require lipids as another source of energy in addition to carbohydrates, as secondary messengers in signaling pathways, and also for building cell membrane structures (Figure 1.7) (Wenk, 2010). Lipids are the primary structural components of all cell membranes. As cells die and are degraded, there is a continuous demand for lipids for cell maintenance and growth. Lipids are the major constituents of adipose tissue, which provides insulation against heat loss, and forms protective cushions surrounding major organs (Kremmyda et al., 2011). Dietary lipids are also important for facilitating the absorption of fat-soluble vitamins (A, D, E, and K) and carotenoids (Kremmyda et al., 2011). Lipids can either be obtained from the diet, or synthesized de novo (lipogenesis). At times where lipids are in excess, they are primarily stored in fat cells or adipocytes within the organelle known as the lipid body or lipid droplet.
Figure 1.7 The cellular compartments of common biological lipids. The lipid portion of biological membranes is made up to a large extent of phospholipids (e.g. glycerophospholipids), sterols, and sphingolipids (blue box, top left). Some organelles are enriched with particular lipids (examples, cardiolipin in mitochondria and lysobisphosphatidic acid/bis(monoacylglycero)phosphate in endosomes), and lateral distribution within membranes leads to functional domains. Metabolism of membrane lipids generates highly active signaling molecules (purple box, top right). Very nonpolar lipids, such as sterol-esters and triacylglycerides, are assembled in the endoplasmic reticulum and stored in lipid bodies/droplets within cells (green box, bottom right). Reproduced from Wenk (2010).
1.4.1.1 Fatty acids – structure, types and biological functions

Many cellular lipids are built from fatty acids, which consist of a carboxyl head group followed by a hydrocarbon chain (Figure 1.8) (Evans and Hardy, 2010). Fatty acids can be saturated or unsaturated, depending on the structure of their hydrocarbon chains. Saturated fatty acids such as stearic acid and palmitic acid lack double C-C bonds, whereas unsaturated fatty acids have either one or two double bonds (Evans and Hardy, 2010) (Figure 1.8). The former cases are known as monounsaturated fatty acids (MUFA, one double bond), such as oleic acid, whereas the latter are known as polyunsaturated fatty acids (PUFA, two or more double bonds), such as linoleic (omega-6 PUFA) and linolenic (omega-3 PUFA) acids (Evans and Hardy, 2010). PUFA are also known as essential fatty acids, because humans and other mammals are not able to synthesize PUFA, and therefore they can only be obtained from the diet (Evans and Hardy, 2010; Tvrzicka et al., 2011). PUFA are important biosynthetic precursors of arachidonic and eicosapentaenoic acids which are then precursors to eicosanoids (hormone-like lipid molecules), including prostaglandins, leukotrienes and thromboxanes (Evans and Hardy, 2010). Eicosanoids are known to be involved in regulating blood clotting, inflammatory response and the reproductive system (Evans and Hardy, 2010).
Figure 1.8 Fatty acid nomenclature. (A) Fatty acids are composed of a carboxyl head group followed by a hydrocarbon-like chain. The first carbon on the head group is known as the α-carbon, whereas the second is the β-carbon, and so on. The final carbon is known as the ω-carbon. Unsaturated fatty acids are classified based on the position of the first double bond from the ω-carbon, and the number of double bonds present. B) Saturated fatty acids, such as stearate, have no double bonds. Stearate is also referred to as C18:0, as stearate has 18 carbon atoms in the hydrocarbon chain, and no double bonds. Monounsaturated fatty acids, such as oleate, have one double bond (hence C18:1), and polyunsaturated fatty acids such as linoleate (C18:2) have more than one double bond. Oleate is termed a ω-9 fatty acid, because the double bond occurs 9 carbons from the omega carbon. In contrasts, linoleate is an ω-6 fatty acid. Reproduced from Evans and Hardy (2010).
Lipid metabolism plays an important role in energy homeostasis, and involves the de novo synthesis of fatty acids (lipogenesis) and the degradation of stored lipid (lipolysis). These processes are described in detail in the following sections (Section 1.4.2.1 and 1.4.2.2). Lipogenesis results in the production of various types of lipids, from basic lipids such as palmitic and stearate acids, to more complex lipids such as phospholipids and sphingolipids. Fatty acids obtained from the diet or synthesized de novo are then used as building-blocks for more structurally complex lipids. Free fatty acids (or unesterified fatty acids) are either oxidized immediately for energy consumption through the β-oxidation pathway as an alternative pathway for energy production (Kunau et al., 1995), used as secondary messenger signaling molecules, or esterified/ re-esterified into diacylglycerides (DAGs, composed of 2 fatty acids and glycerol) or triacylglycerides (TAGs, composed of 3 fatty acids and glycerol), which are packaged into lipid droplets for energy storage (Evans and Hardy, 2010; Tvrzicka et al., 2011).

Alternatively, organic or inorganic compounds can be added to the fatty acid and glycerol backbone such as in the formation of phosphoglycerides (PG) and cholesterol esters (CE) (Figure 1.7) (Wenk, 2010). Phosphoglycerides contain a phosphate group added to the fatty acid and glycerol backbone, and form a basic component of plasma membranes (Fahy et al., 2005). Sterols are composed of four fused carbon rings with a long hydrocarbon side chain, and an example is cholesterol, another essential component of mammalian cell membranes (Fahy et al., 2005). Cholesterol is also a precursor of bile acids, Vitamin D and several steroid hormones such as corticosteroids, testosterones and oestrogens (Kremmyda et al., 2011). These steroid hormones are critical as intercellular secondary messengers. Sphingolipids such as sphingomyelins are another important structural component of the cell membrane (Kremmyda et al., 2011). Phosphoglycerides, sphingolipids, and sterols are therefore essential structural components of mammalian cell membranes,
as well as being involved in intracellular signaling and hormonal regulation (Figure 1.7) (Wenk, 2010).

Saturated fatty acids have been connected to adverse health effects, whereas unsaturated fatty acids are thought to be more beneficial for human health. Excess saturated fatty acids (due to a diet high in saturated fatty acids) have been reported to be associated with diseases such as diabetes, obesity and cardiovascular related diseases (Kremmyda et al., 2011). It is notable that palmitic acid is both the first fatty acid product once synthesized de novo, and also the most abundant saturated fatty acid in the human diet. In contrast, unsaturated fatty acids such as eicosanoids derived from linolenic acids (PUFA) have been shown to have anti-inflammatory, anti-thrombotic and anti-tumorigenic effects (Evans and Hardy, 2010; Kremmyda et al., 2011).

1.4.2 Lipid metabolism pathways

1.4.2.1 Lipogenesis or de novo fatty acid synthesis

Fatty acids can be obtained either exogenously from the diet (e.g. linoleate and linolenate) or endogenously de novo (e.g. palmitate). Lipogenesis mainly occurs in the liver and adipose tissue (Strable and Ntambi, 2010) or the lactating breast (Anderson et al., 2007). There are two main enzymes involved in de novo fatty acid synthesis or lipogenesis, acetyl-CoA carboxylase (ACC/ACACA) (Brownsey et al., 2006) and fatty acid synthase (FASN) (Asturias et al., 2005; Chirala and Wakil, 2004) (Figure 1.9). ACC is the rate limiting enzyme that converts acetyl-CoA to malonyl-CoA. Acetyl-CoA and NADPH are derived from glucose and glutamine metabolism respectively. FASN then catalyses fatty acid synthesis through condensation of acetyl-CoA (primer) and malonyl-CoA (carbon donor), in the presence of NADPH as
a reducing equivalent, to produce palmitate, a 16 carbon saturated fatty acid. Newly synthesized saturated fatty acids can be further modified to longer fatty acids by elongases, or unsaturated fatty acids by desaturases (e.g. stearoyl-CoA desaturases, such as SCD1). These processes will form more complex fatty acids such as diacylglycerides, phosphoglycerides, triacylglycerides and cholesterol esters. The elongation and desaturation of fatty acids occurs at the endoplasmic reticulum (ER) membrane (Cook and McMaster, 2002). These complex fatty acids such as phosphoglycerides, are important for maintaining the cell's membrane structure and other cellular processes (as described in Section 1.4.1.1).

Excess free fatty acids such as palmitate are toxic to cells, and induce a form of cell death known as lipotoxicity (Hardy et al., 2003; Hardy et al., 2000; Listenberger et al., 2003). One possible explanation for this is that palmitate could be used to generate ceramide, a proapoptotic secondary messenger (Obeid et al., 1993). If there is an excess of free fatty acids (i.e. during excessive food intake), these will be esterified and converted into triacylglycerides and stored in lipid droplets in adipocytes. Previously considered to be an inert organelle, lipid droplets have multiple roles in balancing lipid turnover, membrane and lipid trafficking, protein storage and degradation, and the proliferation of pathogenic viruses (Walther and Farese, 2012). Lipid droplet biology will be discussed further in Chapter 4. Adipocytes are specialized cells which can store large amounts of excess lipid in lipid droplets, in contrast to non-adipocytes which have more limited lipid storage capacity (Brasaemle, 2007). Diseases associated with excess lipid accumulation in non-adipose tissues include obesity, diabetes and cardiovascular-related diseases (Garbarino and Sturley, 2009).
Figure 1.9 Overview of the lipid metabolism pathway. Free fatty acids (FFA) are obtained either exogenously (i.e. dietary lipids, saturated or unsaturated) or synthesized de novo (lipogenesis) or through degradation of lipid stores/lipid droplets (lipolysis). Metabolism of both glutamine and glucose supplies citrate and supports the production of acetyl-CoA and NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) needed for lipogenesis. Acetyl-CoA is the precursor for both fatty acid synthesis and cholesterol synthesis. Free fatty acids are processed into complex lipids which are then utilized for cell membrane formation (phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE)) or stored as triacylglycerides (TAG) and cholesterol esters (CE) in lipid droplets. Free fatty acids are also used as an alternative source of energy through the β-oxidation pathway in mitochondria when glucose stores are limited, such as during fasting. (ACC = acetyl-CoA carboxylase, ACLY = ATP citrate lyase, FA = fatty acids, FASN = fatty acid synthase). Adapted from Kamili and Byrne (in press).
1.4.2.2 Lipolysis – degradation of lipids from lipid storage

Lipolysis occurs at times of energy deprivation, such as exercise and fasting. Specifically, lipolysis is the process whereby triacylglycerides stored in lipid droplets are hydrolyzed by lipases, releasing fatty acids. These will either be used as energy substrates through the β-oxidation pathway, or re-esterified as essential precursors for lipid and membrane synthesis, or as mediators in cell signaling processes (Figure 1.10) (Jaworski et al., 2007). Activation of lipolysis is tightly regulated by insulin and catecholamine secretion, which is dependent upon nutritional regulation. In the fed state, insulin functions to suppress lipolysis and insulin is the most potent anti-lipolytic hormone. In contrast, catecholamines are the major regulators stimulating lipolysis during fasting. Adipose triacylglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) are the major enzymes involved in lipolysis within adipocytes (Schweiger et al., 2006).

Lipolysis of stored triacylglycerides in lipid droplets is a sequential process requiring several steps. Catecholamine binding to β-adrenergic G-protein coupled receptors activates cAMP-dependent PKA (Figure 1.10). This will activate the release of CGI-58 from perilipin to interact with and activate ATGL, the primary triacylglyceride lipase for basal lipolysis (Granneman et al., 2007; Gruber et al., 2010; Subramanian et al., 2004; Zimmermann et al., 2004). ATGL is the primary lipase for hydrolysing the first fatty acid from triacylglyceride to yield diacylglyceride (DAG) (Zimmermann et al., 2004). DAG is then hydrolysed by HSL, the primary DAG lipase, to yield monoacylglyceride (MAG). Finally, monoacylglyceride lipase (MAGL) is responsible for hydrolysing MAG into the final fatty acid and glycerol molecules. Therefore, during the lipolytic process, one molecule of glycerol and three fatty acid molecules are produced by the hydrolysis of one molecule of triacylglyceride.
Figure 1.10 Regulation of lipolysis in adipocytes. Lipolysis is under tight control, being stimulated during fasting by increased catecholamines (via increased cAMP) and glucocorticoids but suppressed in the fed state by insulin. Lipolysis is catalyzed by 3 lipases. Desnutrin/ATGL predominantly performs the initial step in triacylglyceride (TAG) hydrolysis, resulting in the formation of diacylglyceride (DAG) and fatty acid (FA). Hormone sensitive lipase (HSL) catalyzes hydrolysis of TAG, DAG, and monoacylglyceride (MAG) at a ratio of 1:10:1 in vitro. Monoacylglyceride lipase (MGL/MAGL) catalyzes hydrolysis of MAG to form glycerol and FA. AC = adenylate cyclase, α AR = alpha-adrenergic receptor, ATP = adenosine triphosphate, β AR = beta adrenergic receptor, cAMP = cyclic adenosine 3’5’-monophosphate, Gαs = stimulatory GTP-binding protein alpha subunit, Gα1 = inhibitory GTP-binding protein alpha subunit, IR = insulin receptor, IRS = insulin receptor substrate, PDE3B = phosphodiesterase 3B, PKA = protein kinase A, PI3K/PKB = protein kinase B, PP1 = protein phosphatase-1. Reproduced from Jaworski et al. (2007).
Dysregulation of lipolysis has been shown to be detrimental to cell metabolism. For example, impairment of ATGL activity leads to reduced lipolysis, and increased triacylglyceride deposition in multiple tissues in both human (Fischer et al., 2007; Lefevre et al., 2001) and mouse (Haemmerle et al., 2006) is a factor leading to obesity and insulin resistance. In contrast, uncontrolled lipolysis in the context of lipodystrophy syndrome leads to the release of toxic free fatty acids into the bloodstream (Garg and Agarwal, 2009). As a consequence, an excess of free fatty acids will be imported into non-adipocytes, leading to intracellular free fatty acid accumulation which could result in lipotoxicity, as described earlier (Section 1.4.2.1).

1.4.3 Altered cellular metabolism in cancer

As discussed in section 1.1.2, cancer is a disease involving numerous altered signaling pathways (Greaves and Maley, 2012). Cancer cells are highly proliferating cells hence their bioenergetic and biosynthetic demands are higher compared to normal healthy cells. Cancer cells were initially recognized by Otto Warburg in the 1920s to exhibit differential cellular metabolism capabilities compared to non-cancerous cells. Since termed the “Warburg effect”, Otto Warburg observed that cancer cells consumed high amounts of glucose and produced high levels of lactic acid under aerobic conditions (Warburg, 1956b). He concluded possible impairment of mitochondrial regulation in cancer cells (Warburg, 1956a). However, it was reported that most highly proliferative tumor cell lines showed no defects in oxidative metabolism (Moreno-Sanchez et al., 2007). Recent reports of genomic and transcriptomic data revealed alterations of lipid-associated genes, hence providing critical evidence of altered metabolic pathways in cancer cells (Budhu et al., 2013; Hirsch et al., 2010; Kumar-Sinha et al., 2003; Wang et al., 2013). It is now being increasingly recognized that altered lipid metabolism in cancer reflects changes in upstream and downstream signaling pathways regulating cellular metabolism, most
importantly lipogenesis and lipolysis. Hence, altered metabolism has been proposed as another important hallmark in cancer pathogenesis (Hanahan and Weinberg, 2011).

1.4.3.1 Altered lipid metabolism in cancer

Generally, normal cells show low rates of de novo fatty acid synthesis, and low levels of expression of lipogenic enzymes (Weiss et al., 1986). This is in contrast to cancer cells, which often show high rates of de novo fatty acid synthesis or lipogenesis (Kuhajda, 2000; Swinnen et al., 2006). Increased lipogenesis in cancer is supported by numerous reports of increased expression of several key lipogenic enzymes such as ACLY (Bauer et al., 2005), ACC (Milgraum et al., 1997) and FASN (Swinnen et al., 2002) suggesting that increased lipogenesis plays an important role in cancer pathogenesis. Furthermore, compared to normal cells, cancer cells preferentially use free fatty acids synthesized de novo, instead of dietary free fatty acids (Kannan et al., 1980; Ookhtens et al., 1984; Vazquez-Martin et al., 2008). These synthesized fatty acids are important for fuelling membrane biogenesis and lipid-based protein modifications that serve numerous cellular functions as described earlier (Section 1.4.1), with these activities being enhanced in cancer cells.

Cancer cells such as breast cancer cell lines, have been shown to have high numbers of lipid droplets and/or increased lipid droplet storage capacity compared to that of most normal cells (Kourtidis et al., 2010). This can be explained by increased lipogenesis (Kourtidis et al., 2010), but also represents a survival strategy, preventing lipotoxicity which could otherwise lead to cell death (Listenberger et al., 2003).
1.4.3.1.1 The role of FASN

FASN was initially identified by Kuhajda et al. (1994) as OA-510, a breast cancer marker associated with poor patient prognosis. To date, FASN is one of the most widely studied lipogenic enzymes. FASN expression is usually low and/or suppressed in normal healthy cells. However, numerous reports show FASN being overexpressed in many types of cancers such as prostate (Migita et al., 2009; Swinnen et al., 2002), ovarian (Gansler et al., 1997), colon (Ogino et al., 2007), lung (Piyathilake et al., 2000), endometrial (Tsuji et al., 2004) and melanoma (Innocenzi et al., 2003). Studies from prostate (Swinnen et al., 2002) and ovarian (Piyathilake et al., 2000) cancers suggest that increased FASN expression is important for tumor initiation and progression. The mechanisms producing FASN overexpression include enhanced transcription of the FASN gene, gain of FASN gene copies, enhanced translation of FASN mRNA and increased stabilization of FASN protein (Menendez and Lupu, 2007). FASN expression may also be upregulated by growth factors receptors such as EGFR and ERBB2 via signalling pathways such as PI3K/Akt, MAPK pathways.

1.4.3.1.2 Oncogene and tumor suppressor involvement in lipogenesis

Increased lipogenesis in cancer has been linked to activated oncogenes or loss of tumor suppressor genes (Cairns et al., 2011; Koppenol et al., 2011; Levine and Puzio-Kuter, 2010; Vander Heiden et al., 2009). These oncogenes (eg. EGFR, ERBB2, Akt, Myc) and tumor suppressor genes (eg. PTEN, p53) regulate upstream signaling pathways such as RTKs, PI3K/Akt and MAPK pathways which control cellular proliferation and survival (Figure 1.12).
The PI3K/Akt signaling pathway was shown to regulate FASN expression at both the transcriptional and protein level. Treatment with PI3K inhibitor or exogenous PTEN overexpression in PTEN-null LNCaP prostate cancer cells resulted in reduced FASN expression and consequently fatty acid production (Van de Sande et al., 2002). This effect was, however, rescued by transfection of constitutively active Akt (Van de Sande et al., 2002). A similar observation was made in ovarian cancer cells upon exogenous Akt overexpression. This led to increased fatty acids and phospholipids due to activated SREBP-1c (sterol regulatory element binding protein-1c) expression that subsequently enhanced the expression of lipogenic enzymes (Porstmann et al., 2005). H-Ras transfected MCF-10A cells (non-tumorigenic human mammary cells) showed induced MAPK and PI3K activity together with increased SREBP-1c, which activated FASN expression resulting in enhanced fatty acid synthesis (Yang et al., 2002). Akt has also been shown to directly activate another lipogenic enzyme, ACLY, by phosphorylation (Berwick et al., 2002; Migita et al., 2008).

Transcriptomic and proteomic analysis revealed that proteins involved in metabolic pathways are highly expressed in ERBB2 positive breast tumors, including FASN (Kumar-Sinha et al., 2003; Zhang et al., 2005). Using Herceptin treatment, Kumar-Sinha et al. (2003) concluded that ERBB2 regulates FASN expression activity via activating the FASN promoter, through a PI3K-dependent pathway. They showed that downregulation of ERBB2 expression following either Herceptin or a PI3K-inhibitor treatment also reduced FASN expression in SK-BR-3 breast cancer cells, whereas overexpression of ERBB2 stimulated FASN promoter activity leading to increased fatty acid synthesis and treatment with Herceptin or a PI3K-inhibitor markedly decreased fatty acid synthesis (Kumar-Sinha et al., 2003). This study highlights the involvement of ERBB2 in regulating fatty acid synthesis in ERBB2-overexpressing breast cancer cells (Kumar-Sinha et al., 2003). It is also interesting to note that the key lipogenic genes FASN, ACC and ACLY are located in proximity to
ERBB2 on chromosome 17q (Figure 1.1). NR1D1 (nuclear receptor subfamily 1, group D, member 1), and PBP/MED1 (peroxisome proliferator-activated receptor-binding protein (PBP)/mediator subunit 1 (MED1)) transcription factors which regulate lipid metabolism are also located close to ERBB2 (Figure 1.11), and RNA interference studies have shown that these proteins are survival factors in ERBB2-positive breast cancer cells (Kourtidis et al., 2010). ERBB2 has been shown to regulate FASN, ACC and ACLY, while MED1 and NR1D1 have been shown to regulate these and other lipogenic genes (Kourtidis et al., 2010; Kumar-Sinha et al., 2003; Menendez and Lupu, 2007; Yoon et al., 2007; Zhang et al., 2005). Copy number increases including ERBB2 could therefore directly or indirectly affect the activity of other co-located chromosome 17q lipogenic genes. These results provide evidence to suggest that ERBB2-positive cancer cells are addicted to de novo fatty acid synthesis.
Figure 1.11 Lipid associated genes located at chromosome 17q. Gene positions are shown using hg 18 chromosome 17 co-ordinates, in MB, corresponding to the cytogenetic bands on the lower ideogram. Approximate positions of genes are shown using vertical arrows. (ACACA or ACC = Acetyl-CoA carboxylase, ACLY = ATP citrate lyase, FASN = fatty acid synthase, MED1 or PBP = peroxisome proliferator-activated receptor-binding protein (PBP)/mediator subunit 1 (MED1), NR1D1 = nuclear receptor subfamily 1, group D, member 1, STARD3 = StAR-related lipid transfer domain protein 3). Reproduced from Kamili and Byrne (in press).
1.4.3.1.3 Increased \( \beta \)-oxidation of fatty acids

As discussed earlier (Section 1.4.1.1), fatty acids also serve as an alternative source of energy through the \( \beta \)-oxidation pathway. It is presumed that in cancer cells, increased \( \beta \)-oxidation at times of limited glucose supply is another strategy to avoid cell death. \( \beta \)-oxidation is proposed to be the dominant bioenergetic pathway in prostate cancer (Liu, 2006). Prostate cancer cells were observed to have low glucose consumption (Effert et al., 1996), increased fatty acid uptake (Liu et al., 2010) as well as increased levels of \( \beta \)-oxidation enzymes (Zha et al., 2005). Ovarian cancer cells were also shown to have increased \( \beta \)-oxidation (Nieman et al., 2011). Leukemia cells have also been reported to be dependent on \( \beta \)-oxidation for cell proliferation and survival, using inhibition of \( \beta \)-oxidation by RNA interference (Samudio et al., 2010). In addition, Akt-transformed glioblastoma cells showed activation of the \( \beta \)-oxidation pathway, which rescued cells from cell death upon glucose withdrawal. This suggests that \( \beta \)-oxidation substituted for glycolysis sufficiently to maintain cell survival of these glioblastoma cells (Buzzai et al., 2005).
**Figure 1.12** Signaling pathways regulating lipid metabolism in cancer cells.

SREBP (sterol regulatory element-binding protein) regulates many lipogenic enzymes (yellow boxes) within the fatty acid (boxed in light blue) and cholesterol synthesis (boxed in orange) pathways. Oncogenic activation of the PI3K/Akt pathway increases glucose uptake and usage through SREBP activation. Mutant p53 (p53mut) activates expression of genes involved in the cholesterol synthesis pathway. Reduced cellular energy levels will activate AMPK (AMP-activated protein kinase) which will prevent lipid synthesis and activate the β-oxidation pathway by inhibiting ACC (acetyl-CoA carboxylase) activity. AMPK can also directly phosphorylate SREBP, inhibiting SREBP activity. Hypoxia activates HIF1 (hypoxia-inducible factor 1) which reduces glucose flux to acetyl-CoA through the mitochondria. Cytoplasmic citrate can be derived from the TCA (tricarboxylic acid cycle) in mitochondria or through reductive metabolism of glutamine derived α-ketoglutarate. (ACAT = acetyl-CoA acetyltransferase, ACLY = ATP citrate lyase, ACSL = acyl-CoA synthetase long-chain, ADP = adenosine diphosphate, ATP = adenosine triphosphate, CPT1 = carnitine palmitoyltransferase 1, ETC = electron transport chain, FADH$_2$ = flavin adenine dinucleotide (hydroquinone form), FASN = fatty acid synthase, GLUT = glucose transporter, HMG = 3-hydroxy-3-methylglutaryl, HMGCS = HMG coenzyme A synthase, HMGCR = HMG coenzyme A reductase, IDH = isocitrate dehydrogenase, MCT = monocarboxylate transporter, mTORC1 = mammalian target of rapamycin complex 1, NADH = nicotinamide adenine dinucleotide, PDH = pyruvate dehydrogenase, PDK = pyruvate dehydrogenase kinase, SCD = stearoyl-CoA desaturases). Reproduced from Santos and Schulze (2012).
1.4.3.2 Impact of altered lipid metabolism on cancer therapy

Altered lipid metabolism in cancer cells is produced by overexpression of key lipogenic genes (FASN, ACC, ACLY) in the lipogenesis pathway. These enzymes are therefore potential therapeutic targets in lipogenic cancers (Zhang and Du, 2012). RNA interference studies using breast cancer cell lines targeting the lipogenic genes ACC or FASN showed a decrease in palmitic acid synthesis, which was associated with induced apoptosis (Chajes et al., 2006). Supplementation of palmitate then rescued cells from apoptosis induced by ACC and FASN knock-down (Chajes et al., 2006). Similarly in prostate cancer cells, FASN or ACC knock-down led to reduced cell growth and induced apoptosis (Bandyopadhyay et al., 2005; Brusselmans et al., 2005). ACLY inhibition using RNA interference also resulted in reduced lipogenesis, which eventually reduced cell proliferation and survival in human lung adenocarcinoma A549 cells displaying aerobic glycolysis (Migita et al., 2008) and in prostate cancer PC3 cells (Hatzivassiliou et al., 2005). Both of these studies also showed that inhibition of ACLY led to reduced tumorigenesis in mice (Hatzivassiliou et al., 2005; Migita et al., 2008). A study by Bauer et al. (2005) showed siRNA knock down of ACLY significantly impaired Akt-mediated tumorigenesis in vivo (Bauer et al., 2005). Collectively, these results indicate the dependence of certain cancer cells on lipogenesis for survival and tumor formation.

These and other observations have led to the development of chemical inhibitors targeting these lipogenic enzymes. Several FASN inhibitors have been developed (eg. cerulenin, C75, orlistat) which provided promising in vitro results however, clinical trials have shown side effects such as anorexia (Menendez and Lupu, 2007). A chemical inhibitor targeting ACLY, SB-204990 was found to reduce tumor cell proliferation and survival in vitro and in vivo (Hatzivassiliou et al., 2005). Lipogenic
enzymes are therefore emerging as potential targets to treat lipogenic cancers, although adverse side-effects are currently limiting their clinical application.

1.5 Chromosome 8q21 gain in cancer

Cytogenetic analyses using fluorescent in-situ hybridization (FISH) and comparative genomic hybridization (CGH) analyses have identified that DNA copy number at chromosome 8q is frequently increased in different cancer types such as breast and prostate (Baudis, 2007; Myllykangas et al., 2006). To date, numerous genes have been proposed as chromosome 8q oncogenes, such as MYC, eukaryotic translation initiation factor 3-S3 (EIF3S3), WW domain containing E3 ubiquitin protein ligase 1 (WWP1), E2F5, elongin C or TCEB1 transcription elongation factor B (SIII), polypeptide 1(TCEB1/ElonginC), and Tumor protein D52 (TPD52), based upon the separate gain of different chromosome 8q regions, including 8q21.

Chromosome 8q21 spans about 19.5 Mb (74,000,001-93,500,000 bp) and encodes up to 91 genes (Byrne et al., 2012). Numerous studies have reported 8q21 gain in the breast cancer SK-BR-3 cell line (Kallioniemi et al., 1994), breast cancer (Fejzo et al., 1998), prostate cancer (Cher et al., 1996), bladder cancer (Kallioniemi et al., 1995; Richter et al., 1997) and osteosarcoma (Tarkkanen et al., 1995). Furthermore, it has also been shown that chromosome 8q21 gain was significantly associated with increased risk of death in a large breast cancer cohort (Rennstam et al., 2003). Collectively, these reports suggest gene(s) located at 8q21 are of importance in the initiation and/or progression of various types of cancers. A number of amplification targets within this region have been proposed, including TPD52 at 81.1–81.2 MB (Figure 1.13) (Byrne et al., 2012).
Figure 1.13 Summary of high-resolution amplicon mapping studies at chromosome 8q21 identifies TP52 amplification in cancer tissues or cell lines. Gained regions are indicated by horizontal lines according to hg18 chromosome 8 coordinates below (in MB), corresponding to the cytogenetic bands indicated on the lower ideogram. Arrows within cytogenetic bands indicate that these extend beyond the region shown. The study reporting each gained region is indicated to the left or right, with colored lines and text highlighting studies examining particular cancer types. A region of overlap between gained regions (shaded in light blue) identified as 81 to 82 Mb includes the gene TP52 (vertical arrow), supporting TP52 as a gene amplification target. Adapted from Byrne et al. (2012)
1.6 Tumor protein D52 (TPD52)

1.6.1 History - Gene identification and related genes

The human tumor protein D52 gene (TPD52) was identified by differential screening of a human breast carcinoma cDNA library (Byrne et al., 1995). Identification of other related genes identified the TPD52-like gene family, which encodes a family of adaptor proteins containing a conserved coiled-coil motif (Boutros et al., 2004; Byrne et al., 1996). The human TPD52-like family includes 4 genes: TPD52 (D52) (Byrne et al., 1995), TPD52L1 (D53) (Byrne et al., 1996; Byrne et al., 1998a), TPD52L2 (D54) (Nourse et al., 1998) and TPD52L3 (D55) (Cao et al., 2006). Phylogenetic analysis of the vertebrate TPD52-like proteins revealed that corresponding genes may have evolved from ancestral, TPD52-like sequences such as those found in Drosophila melanogaster and Caenorhabditis elegans through gene duplication events (Figure 1.14) (Boutros et al., 2004).

Orthologues of TPD52 have been reported in species such as mouse (Tpd52) (Byrne et al., 1996), rat (CRHSP-28) (Groblewski et al., 1996), Japanese quail (R10) (Proux et al., 1996), rabbit (CSPP28) (Parente et al., 1996) and Drosophila melanogaster (CG5174) (Giot et al., 2003). Human TPD52L1 and mouse Tpd52 proteins are predicted to be 52% and 86% identical to human TPD52, respectively (Byrne et al., 1996). While these sequences show significant similarity, TPD52-like sequences show little similarity to members of other protein families. This identifies the TPD52-like protein as having their own unique characteristics (Byrne et al., 1996; Shehata et al., 2008b).
Figure 1.14 Phylogenetic tree of TPD52-like protein sequences. Phylogenetic analysis based on amino-acid sequences of TPD52-like proteins (unrooted tree construction, using the *H. magnipapillata* sequence as the outgroup). Colours represent the separation of vertebrate sequence groups. D52 = TPD52, D53 = TPD52L1, D54 = TPD52L2, NYD-SP25 = TPD52L3. Reproduced from Boutros et al. (2004).
TPD52-like sequences encode hydrophilic polypeptides between 180 and 200 amino acids in length (Byrne et al., 1996; Byrne et al., 1995), that include a predicted coiled-coiled motif (40-50 amino acids) and N- and C-terminally located PEST domains (Byrne et al., 1996) (Figure 1.15). PEST domain refers to a domain which is rich in proline (P), glutamic acid (E), serine (S), and threonine (T). TPD52-like protein sequences are highly conserved in their central regions which include the coiled-coil motif (Byrne et al., 1996) (Figure 1.15), whereas the C-terminal regions are more diverse (Boutros et al., 2004; Byrne et al., 1995). TPD52 has been shown to exist as homodimers or multimers in vitro (Chen et al., 1997). A yeast two-hybrid analysis and gluthathione S-transferase pull down assays confirmed that TPD52-like proteins can bind each other (Byrne et al., 1998b). The coiled-coil motif was required for this interaction to occur (Byrne et al., 1998b; Sathasivam et al., 2001) but more C-terminal regions may be involved in facilitating and/or stabilising these interactions (Sathasivam et al., 2001). It was also proposed that interactions with itself or related proteins are important for TPD52 to exert and/or regulate its activities (Byrne et al., 1998b).

The TPD52 gene includes at least 9 exons and encodes a 184 amino acid polypeptide, with a predicted molecular mass of 19.8 kDa (Byrne et al., 1995). Alternative splicing events have been identified, generating TPD52 isoforms. Firstly, alternative use of the first exon generates TPD52 (exon 1a) and PrLZ (exon 1b) isoforms (Figure 1.15). TPD52 has been shown to have high expression levels in breast cancer cells (Byrne et al., 1996) as well as in tumor derived cell lines from multiple cancers (Chen et al., 1996), whereas PrLZ is highly elevated in prostate cancer cells (Wang et al., 2004). Generation of other isoforms is due to alternative splicing of an exon encoding a 14-3-3 binding motif (Boutrous et al., 2003). These isoforms were found to be expressed in neural tissues (Nourse et al., 1998).
Figure 1.15 **Schematic diagram of TPD52 isoforms.** Numbers represent exons, with exons 1a, 1b, 5 and 6 being subjected to alternative splicing. Exons 1a and 1b are included in TPD52 and PrLZ, respectively. TPD52 isoforms bear a single coiled-coil domain towards the N-terminus and 2 PEST domains located towards the N- and C-terminus, as indicated by a dotted line above the diagram, and solid lines below the diagram, respectively. Scale bar: 30 amino acids (aa). Adapted from Della Franca et al. (2012).
1.6.2 Understanding the physiological functions of TPD52

Numerous experimental approaches have been taken to understand TPD52 physiological functions, including the analysis of TPD52 expression and localization in cells and tissues. *TPD52* transcript and protein expression have been found within normal tissues of the prostate, pancreas, gastrointestinal tract, kidney and brain (Byrne et al., 1995; Chen et al., 1996; Chen et al., 1997; Groblewski et al., 1999; Parente et al., 1996; Wilson et al., 2001). Microscopic analysis of TPD52 subcellular localization revealed it to be a cytoplasmic protein (Balleine et al., 2000; Groblewski et al., 1999; Kaspar et al., 2003; Proux et al., 1996; Tiacci et al., 2005). TPD52 proteins have been subsequently shown to be involved in several cellular functions, such as exocytotic secretion, vesicle trafficking, calcium-mediated signal transduction, cell proliferation and apoptosis (Shehata et al., 2008b).

The rabbit TPD52 orthologue, CSPP28 was identified as being phosphorylated in response to cholinergic stimulation of gastric parietal cells, therefore CSPP28 was proposed to be involved in calcium signaling cascades in rabbit tissues (Parente et al., 1996). CRHSP-28 (the rat TPD52 orthologue) was found to be highly expressed in cultured mucosal secretory T84 cells (Kaspar et al., 2003). Specifically, they showed by confocal microscopy analysis that CRHSP-28 was detected in the cytoplasmic compartment and displayed perinuclear accentuation (Kaspar et al., 2003). Introduction of recombinant CRHSP-28 into rat pancreatic acinar cells stimulated the secretion of amylase (Thomas et al., 2001), with CRHSP-28 also undergoing a rapid translocation from supranuclear to subapical cytoplasmic compartments after stimulation of mucosal T84 and rat pancreatic acinar cells with secretagogue (Thomas et al., 2004). A secretagogue is a substance that causes another substance to be secreted. Yeast two hybrid screenings have identified partners for TPD52 which have been commonly associated with membrane
trafficking and lipid rafts, namely MAL2 (Wilson et al., 2001) and the phospholipid-binding protein annexin VI (Thomas et al., 2002). These results supported a regulatory role for TPD52 in apical membrane trafficking.

Several observations suggest TPD52 involvement in cell growth and differentiation. An early study identified R10 (the TPD52 orthologue in quail) as being encoded by a retrovirally transduced gene in proliferating neuroepithelial cells (Proux et al., 1996). R10 transcripts levels were relatively high in the neuroretina of 14 day old chick embryos compared to cultured chicken fibroblasts and remained at elevated levels in 16, 18, and 20 day old embryos and in newly hatched chicks (Proux et al., 1996). They also revealed the R10 gene product as a cytoplasmic protein in cultured avian fibroblasts. In a different study it was observed that mouse Tpd52 transcripts were highly expressed in olfactory epithelium, salivary gland and gut of 18 day old mouse embryos and in one day old mouse retina (Chen et al., 1996). TPD52 was also suggested to have an important role during B cell maturation (Tiacci et al., 2005), as TPD52 was identified as a B-cell differentiation marker, being highly expressed in all mature B cells, and maximally expressed at the plasma cell stage (Tiacci et al., 2005). TPD52 may also be involved in maintaining and/or developing an epithelial cell phenotype. The first evidence of this was observed when TPD52 overexpression in mouse NIH 3T3 fibroblast cells was associated with epithelial-like characteristics (Chen et al., 1997). TPD52 was also found to be expressed in higher levels in epithelial cells than in mesenchymal cells (Chen et al., 1997).

1.6.3 Significance of TPD52 overexpression in human cancers

Numerous studies have shown that TPD52 is overexpressed in many forms of human cancer, including ovarian (Byrne et al., 2005), breast (Balleine et al., 2000;
Byrne et al., 1995), prostate (Rubin et al., 2004; Wang et al., 2004), lung (Chen et al., 1996; Zhu et al., 2007), melanoma (Roesch et al., 2007), colon (Malek et al., 2002), B cell malignancies (Tiacci et al., 2005) and testicular germ cell tumors (Alagaratnam et al., 2009). In a proportion of these cancers, increased TPD52 expression was associated with an increase in chromosome 8q21 copy number, as found in breast (Balleine et al., 2000), prostate (Rubin et al., 2004) and ovarian cancers (Byrne et al., 2005). Shehata et al. (2008a) carried out tissue microarray analyses of breast tissue samples from breast cancer patients, and reported that high TPD52 expression was significantly associated with reduced overall patient survival (Shehata et al., 2008a). It was suggested that this finding may underpin previous associations of chromosome 8q21 gain in breast cancer and poor patient outcome (Han et al., 2006; Melchor et al., 2005; Rennstam et al., 2003). TPD52 was also included in gene expression signatures associated with adverse prognosis in breast (Adler et al., 2006; Liu et al., 2007b) and prostate cancer (Bismar et al., 2006). As a further example, TPD52 was significantly up-regulated in all 6 cases of colorectal cancer investigated, irrespective of tumor localization, pTNM (pathological Tumor-Node-Metastasis) stage or tumor grade (Petrova et al., 2008).

Collectively, the above reports suggest that TPD52 represents a cancer biomarker and therapeutic target. TPD52 overexpression can induce immunoglobulin G (IgG) antibodies in human breast cancer which suggests a lack of normal immunologic tolerance to TPD52 (Scanlan et al., 2001). TPD52 may be sufficiently immunogenic to be explored as an anticancer vaccine target, and Lewis et al. (2009) have taken this approach using mouse Tpd52 cDNA as DNA vaccine. Their data suggest that Tpd52 vaccination induced a cellular immune response that resulted in protection from murine prostate tumors that endogenously overexpress Tpd52 (Lewis et al., 2009).
It has also been reported that *TPD52* serves as a marker or regulator of cancer cell proliferation. This was initially proposed following the observation that *TPD52* levels were reduced in TPA-treated HL60 leukemic cells (Byrne et al., 1996). Further studies *in vitro* showed that increasing mouse or human *TPD52* expression by transfection in 3T3 fibroblast cell lines produced increased cellular proliferation and anchorage independent growth (Lewis et al., 2007; Shehata et al., 2008a). Overexpression of PrLZ in LnCaP prostate cancer cells also led to enhanced proliferation and tumorigenesis (Wang et al., 2004; Zhang et al., 2007a). When mouse Tpd52-expressing 3T3 cells were inoculated into naive, syngeneic, immunocompetent mice, these cells formed subcutaneous tumors and spontaneous lethal lung metastases (Lewis et al., 2007). These studies collectively support *TPD52* overexpression playing a causal role in cancer.

Loss of function RNA interference studies have been a useful tool for studying the effects of reduced *TPD52* expression in cell lines (Shehata et al., 2008a; Ummanni et al., 2008; Zhang et al., 2007a). Shehata et al. (2008a) observed that reduced *TPD52* expression resulted in significantly increased cell death or apoptosis of SK-BR-3 cells, a breast cancer cell line which is known to be *TPD52*-amplified, but not in MCF-7 cells, a breast cancer cell line that does not harbour *TPD52* amplification (Shehata et al., 2008a). This result is in agreement with previous studies where the reduced expression of other oncogenes produced increased apoptosis in oncogene-overexpressing cell lines (Kao and Pollack, 2006; Lutterbach et al., 2007; Park et al., 2006). A similar observation was obtained in LnCaP prostate cancer cells, where *TPD52* knockdown resulted in significant cell death (Ummanni et al., 2008). These results might suggest that *TPD52*-amplified or –overexpressing cell lines may be more sensitive to reduced *TPD52* expression and are “addicted” to *TPD52* expression for the cell’s survival. Interestingly, these studies indicate the possibility of developing new therapeutic strategies involving reducing *TPD52* expression or
inhibiting TPD52 function, specifically for TPD52-amplified or -overexpressing cancers.

1.6.3.1 TPD52 and ERBB2 in cancer

Previously, numerous reports have persistently linked TPD52 and ERBB2 co-expression in clinical and cell lines studies. TPD52 transcript levels were found to be increased in ERBB2-positive breast cancer cell lines (Wilson et al., 2002), followed by reports that Tpd52 transcript or protein levels were increased in mammary tissues from Erbb2 transgenic animals (Chen et al., 2010; Landis et al., 2006; Landis et al., 2005; Whiteaker et al., 2007). Kourtidis et al. (2010) also identified increased TPD52 levels in ERBB2-positive human breast cancers and cell lines, through a meta-analysis of 22 human expression studies. In addition, chromosome 8q21 amplification has been significantly associated with both ERBB2 amplification and poor patient outcome in breast cancer (Choschzick et al., 2010). As discussed earlier (Section 1.5), TPD52 has been proposed as a gene amplification target at chromosome 8q21. Thus ERBB2 and TPD52 are both amplified genes whose overexpression is reproducibly associated with poor patient outcomes in breast cancer. The significance of TPD52 and ERBB2 co-expression in breast cancer cell lines will be further examined in Chapter 3.

1.6.4 TPD52 and lipid metabolism

As mentioned in Section 1.4.3.1, increased lipid droplets has been observed in cancer cells due to increased lipogenesis. Several studies have reported links between TPD52 and lipid metabolism, specifically in lipid droplet regulation. Reduced lipid droplets in C. elegans were observed following knock-down of the TPD52 orthologue F13E6.1 (Ashrafi et al., 2003). In addition, expression microarray analysis in both human and mouse showed increased TPD52 levels in adipose tissue from
obese versus non-obese subjects (Clement et al., 2004; Keller et al., 2008; Nadler et al., 2000). The relationship between TPD52 expression and cellular lipid droplets will be explored in Chapter 4.

1.7 Project Hypotheses and Aims

Combining experimental results with frequent TPD52 overexpression in many cancer types, points to TPD52 acting as an oncogene in the development of many forms of human cancer (Section 1.6.3). This also indicates that TPD52 may represent a novel therapeutic target. However, the molecular and cellular basis of TPD52 function remains unclear. The main goal of the project presented in this thesis is to understand the function of TPD52 in promoting cancer tumorigenesis. Thus, the overall hypotheses and aims of this project are:

**Hypotheses:**

(1) TPD52 represents a potential therapeutic target in ERBB2-positive cancers

**Aim:** To employ RNA interference approaches to characterise the effects of reducing TPD52 expression in breast cell lines according to TPD52 and ERBB2 gene amplification status.

(2) TPD52 is involved in the regulation of lipid storage and therefore contributes to the lipogenic phenotype in cancer cells

**Aim:** To examine effects of lipid droplets (numbers and/or size) under basal or stimulated conditions using mouse fibroblast 3T3 cells, that either do or do not stably express TPD52.
Chapter 2

Materials and Methods
2.1 Materials

All chemicals and reagents used were of analytical grade (Table 2.1). All tissue culture materials were purchased from Life Technologies unless otherwise stated.

2.1.1 Chemicals and reagents

Table 2.1 Chemicals and reagents used

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<th>Chemical / Reagent</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>N,N,N’,N’-Tetramethylethylenediamine (TEMED)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>2-Mercaptoethanol</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>2-Propanol</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)</td>
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</tr>
<tr>
<td>4',6-diamidino-2-phenylindole (DAPI)</td>
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</tr>
<tr>
<td>30% Acrylamide/Bis solution</td>
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<td>Acetic acid, glacial</td>
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<tr>
<td>Acetone</td>
<td>Lab-Scan</td>
</tr>
<tr>
<td>Agar</td>
<td>BD</td>
</tr>
<tr>
<td>Agarose</td>
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<tr>
<td>Ammonium persulfate (APS)</td>
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<tr>
<td>BenchMark prestained protein ladder</td>
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<tr>
<td>BODIPY 493/503</td>
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<td>Sigma-Aldrich</td>
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<td>DMSO (sterile for tissue culture)</td>
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<tr>
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<td>Sigma-Aldrich</td>
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<tr>
<td>Yeast extract</td>
<td>BD</td>
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2.1.2 Buffers and Solutions

All solutions were prepared using autoclaved Milli Q water (aH\textsubscript{2}O). Unless otherwise indicated, solutions were either autoclaved or filter sterilized through 0.22 μm filters when appropriate (Table 2.2). All glassware used for cell or bacterial culture was autoclaved prior to use. All solutions were stored at room temperature (RT) unless otherwise stated (Table 2.2).

Table 2.2 Buffers and solutions composition and preparations

<table>
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<th>SOLUTION</th>
<th>COMPOSITION</th>
<th>PREPARATION AND STORAGE</th>
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<tr>
<td>10% APS</td>
<td>10% (w/v) APS</td>
<td>Prepared fresh in aH\textsubscript{2}O</td>
</tr>
<tr>
<td>Antibody diluent for immunofluorescent staining</td>
<td>1X PBS</td>
<td>Make up in aH\textsubscript{2}O to 100 ml Store at 4°C</td>
</tr>
<tr>
<td>(Section 2.6.2)</td>
<td>1mg/ml BSA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01% (w/v) saponin</td>
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<tr>
<td>Blocking buffer for immunofluorescent staining</td>
<td>1.5% (w/v) glycine</td>
<td>Make up in aH\textsubscript{2}O to 100 ml Store at 4°C</td>
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<td>(Section 2.6.2)</td>
<td>3% (w/v) BSA</td>
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<tr>
<td></td>
<td>1X PBS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01% (w/v) saponin</td>
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</tr>
<tr>
<td>1mg/ml BODIPY 493/503 stock solution</td>
<td>10 mg BODIPY 493/503</td>
<td>Divide into 500 μl aliquots, and store at -20°C</td>
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<td></td>
<td>10 ml 100% Ethanol</td>
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<td>3% BSA</td>
<td>3% (w/v) BSA</td>
<td>Make up fresh in 1X TTBS (see below)</td>
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<td>CaCl\textsubscript{2} : glycerol</td>
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<td>15% (v/v) glycerol</td>
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<td>EDTA pH 8.0</td>
<td>0.5 M EDTA</td>
<td>Adjust pH with NaOH Sterilise by autoclaving</td>
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<td>Glycerol (10%)</td>
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<td>Sterilise by autoclaving</td>
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<td>SOLUTION</td>
<td>COMPOSITION</td>
<td>PREPARATION AND STORAGE</td>
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<td>Kanamycin</td>
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<td>Store at – 20°C</td>
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<td>10X Loading dye -DNA</td>
<td>50% (v/v) Glycerol 1 mM EDTA pH 8.0</td>
<td>Sterilize glycerol by autoclaving</td>
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<td>1.0% (w/v) Bromophenol blue</td>
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<td>1.0% (w/v) Xylene cyanol</td>
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<td>Luria Bertani (LB) media</td>
<td>1% (w/v) Tryptone</td>
<td>Adjust pH to 7.5 with 10 M NaOH</td>
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<tr>
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<td>0.5% (w/v) yeast extract</td>
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<td></td>
<td>1% (w/v) NaCl</td>
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<tr>
<td>Luria Bertani (LB) plates</td>
<td>LB media as above</td>
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<tr>
<td></td>
<td>15 g/L agar</td>
<td>Add antibiotics to appropriate concentration</td>
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<td>Pour plates. Store at 4°C</td>
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<tr>
<td>MTT reagent</td>
<td>0.2% (w/v) MTT</td>
<td>Make up in 1X PBS</td>
</tr>
<tr>
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<td></td>
<td>Filter-sterilise</td>
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<tr>
<td></td>
<td></td>
<td>Store in dark at 4°C</td>
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<tr>
<td>10X PBS</td>
<td>137 mM NaCl 2.7 mM KCl 4.3 mM Na₂HPO₄</td>
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<td>1.47 mM KH₂PO₄</td>
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<tr>
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</tr>
<tr>
<td>Ponceau S stain</td>
<td>0.1% (v/v) Ponceau S 5% (v/v) Glacial acetic acid</td>
<td>Autoclaving not required</td>
</tr>
<tr>
<td>10X Running Buffer</td>
<td>25 mM Tris 250 mM Glycine 0.1% (v/v) SDS</td>
<td>Dilute in aH₂O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autoclaving not required</td>
</tr>
<tr>
<td>4X Sample buffer - Protein</td>
<td>0.5 M Tris pH 6.8 20% (v/v) Glycerol 2% (w/v) SDS 20% (v/v) β-2-mercaptoethanol 0.01% (w/v) Bromophenol blue</td>
<td>Autoclaving not required</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Store at -20°C</td>
</tr>
<tr>
<td>SOLUTION</td>
<td>COMPOSITION</td>
<td>PREPARATION AND STORAGE</td>
</tr>
<tr>
<td>-------------------------</td>
<td>--------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>0.5% Saponin</td>
<td>0.5% (w/v) Saponin</td>
<td>Make up in 1X PBS, Store at 4°C</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10% (w/v) SDS</td>
<td>Make up in autoclaved, Heat to 65°C to aid dissolution</td>
</tr>
<tr>
<td>SDS-lysis buffer</td>
<td>125 mM Tris pH 8.8, 3% (w/v) SDS, 5% (v/v) β-2-mercaptopethanol, Protease inhibitor (Roche) (1 tablet/10mL buffer)</td>
<td>Autoclaving not required</td>
</tr>
<tr>
<td>SE lysis buffer</td>
<td>75 mM NaCl, 25 mM EDTA pH 8.0</td>
<td>Dilute in aH₂O</td>
</tr>
<tr>
<td>5% Skim milk</td>
<td>5% (w/v) Skim milk powder</td>
<td>Make up fresh in 1X TTBS (see below)</td>
</tr>
<tr>
<td>SOC Broth</td>
<td>5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, 10 mM MgCl₂, 2% (w/v) dextrose</td>
<td>Sterilise by autoclaving, Add filter-sterilised dextrose, Store aliquots at – 20°C, Pre-warm to 37°C prior to use</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 M NaCl</td>
<td>Sterilise by autoclaving</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>0.5 M NaOH</td>
<td>Sterilise by autoclaving</td>
</tr>
<tr>
<td>10X TBE</td>
<td>0.9 M Tris, 0.9 M Boric acid, 20 mM EDTA pH 8.0</td>
<td>Dilute in aH₂O, Sterilize by autoclaving</td>
</tr>
<tr>
<td>10X TBS</td>
<td>100 mM Tris-HCl, 150 mM NaCl</td>
<td>Dilute in aH₂O, Adjust pH to 7.5</td>
</tr>
<tr>
<td>10X TE</td>
<td>100 mM Tris pH 7.5, 10 mM EDTA pH 8.0</td>
<td>Sterilize by autoclaving</td>
</tr>
<tr>
<td>1M Tris pH 8.8 or 7.5 or 6.8</td>
<td>1 M Tris</td>
<td>Dilute in aH₂O, Adjust pH with HCl</td>
</tr>
</tbody>
</table>
1X TTBS
1X TBS
0.1% (v/v) Tween 20
Dilute in aH2O
Make sure Tween 20 is dissolved completely

Western blot Transfer buffer
25 mM Tris
192 mM Glycine
20% (v/v) Methanol
0.005% (w/v) SDS
Prepared fresh and pre-chilled at 4°C

2.1.3 Antibodies

2.1.3.1 Primary Antibodies

All primary antibodies (Table 2.3) were kept at -80°C or -20°C for long term storage or at 4°C for short term storage (<1month), unless otherwise stated.

Table 2.3 Primary antibodies used

<table>
<thead>
<tr>
<th>Antibody (source or reference)</th>
<th>Application and dilution</th>
<th>Species</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPD52 (747) (Balleine et al., 2000)</td>
<td>WB: 1:100 IF: 1:100</td>
<td>Rabbit polyclonal</td>
<td>Human</td>
</tr>
<tr>
<td>TPD52 (Tiacci et al., 2005)</td>
<td>IF: 1:10</td>
<td>Mouse monoclonal</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TPD52 (748) (Shehata et al., 2008a)</td>
<td>WB: 1:100</td>
<td>Rabbit polyclonal</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>α-tubulin (Sigma)</td>
<td>WB: 1:1000</td>
<td>Mouse monoclonal</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>GAPDH (Ambion)</td>
<td>WB: 1:5000</td>
<td>Mouse monoclonal</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>ERBB2 (Cell signaling)</td>
<td>WB: 1:1000 IF: 1:200</td>
<td>Rabbit monoclonal</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>Phos-ERBB2 (Cell signaling)</td>
<td>WB: 1:1000</td>
<td>Rabbit monoclonal</td>
<td>Human</td>
</tr>
<tr>
<td>Antibody (source or reference)</td>
<td>Application and dilution</td>
<td>Species</td>
<td>Reactivity</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------------------</td>
<td>------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Phos-Akt (Ser473) (Cell signaling)</td>
<td>WB: 1:1000</td>
<td>Rabbit monoclonal</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>Phos-MAPK 44/42 (Cell signaling)</td>
<td>WB: 1:2000</td>
<td>Rabbit monoclonal</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>Cleaved-PARP 1 (Cell signaling)</td>
<td>WB: 1:1000</td>
<td>Rabbit polyclonal</td>
<td>Human</td>
</tr>
<tr>
<td>FASN (Santa Cruz)</td>
<td>WB: 1:200&lt;br&gt;IF: 1:50</td>
<td>Mouse monoclonal</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>ADRP (Progen Biotechnik)</td>
<td>WB: 1:2000&lt;br&gt;IF: 1:200</td>
<td>Guinea-pig polyclonal</td>
<td>Mouse</td>
</tr>
<tr>
<td>Perilipin (Progen Biotechnik)</td>
<td>WB: 1:2000&lt;br&gt;IF: 1:200</td>
<td>Guinea-pig polyclonal</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TIP47 (Progen Biotechnik)</td>
<td>WB: 1:2000&lt;br&gt;IF: 1:200</td>
<td>Guinea-pig polyclonal</td>
<td>Human</td>
</tr>
<tr>
<td>PDI (Cell signaling)</td>
<td>IF: 1:50</td>
<td>Rabbit polyclonal</td>
<td>Human, Mouse</td>
</tr>
</tbody>
</table>
2.1.3.2 Secondary antibodies

All secondary antibodies (Table 2.4) were received lyophilized and were reconstituted according to the manufacturer’s instructions. Secondary antibodies were kept at -80°C for long term storage and at 4°C for short term storage (<1month).

Table 2.4 Secondary antibodies used

<table>
<thead>
<tr>
<th>Secondary antibody (Source)</th>
<th>Application and dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-mouse-Alexa488 IgG (Molecular Probes)</td>
<td>IF: 1:1000</td>
</tr>
<tr>
<td>Donkey anti-rabbit-Alexa488 IgG (Molecular Probes)</td>
<td>IF: 1:1000</td>
</tr>
<tr>
<td>Donkey anti-mouse-Cy3 IgG (Jackson Immunoresearch Laboratories)</td>
<td>IF 1:1000</td>
</tr>
<tr>
<td>Donkey anti-rabbit-Cy3 IgG (Jackson Immunoresearch Laboratories)</td>
<td>IF 1:1000</td>
</tr>
<tr>
<td>Donkey anti-rabbit-Cy5 IgG (Jackson Immunoresearch Laboratories)</td>
<td>IF 1:1000</td>
</tr>
<tr>
<td>Donkey anti-mouse-HRP (GE Healthcare)</td>
<td>WB: 1:5000</td>
</tr>
<tr>
<td>Donkey anti-rabbit-HRP (GE Healthcare)</td>
<td>WB 1: 10 000</td>
</tr>
<tr>
<td>Anti-guinea pig-HRP (Novex®)</td>
<td>WB 1:2000</td>
</tr>
<tr>
<td>Anti-guinea pig-Alexa633 (Molecular Probes)</td>
<td>IF 1:1000</td>
</tr>
</tbody>
</table>
2.2 Mammalian cell culture

2.2.1 Breast cell lines

Breast cancer cell lines AU-565, BT-483, MDA-MB-175, HS-578T, MDA-MB-157, MDA-MB-134, DU4475, MDA-MB-468 and ZR-75-30 were kindly provided by Prof. Roger Daly from the Garvan Institute (Sydney, Australia). SK-BR-3, BT-474, MCF-7, T-47D, MDA-MB-231 and MCF-10A cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). All cell lines were subjected to short tandem repeat profiling by CellBank Australia (Westmead, Australia), which confirmed their identities. Cell lines were cultured at 37°C in a humidified incubator with 5% CO₂ in RPMI media (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 3% L-glutamine (Life Technologies), except for MCF-7 cells, where the above media were supplemented with 10 µg/ml insulin (Sigma-Aldrich, St Louis, MO, USA), and MCF-10A cells, which were cultured as described (Hope et al., 2002). Stably transfected TPD52-expressing MDA-MB-231 cell lines, as well as vector control MDA-MB-231 cell lines were derived by Rose Boutros (Boutros, 2005) and cultured in RPMI media as above, with 1 mg/ml G418 (Gibco) as described (Boutros, 2005). Cells were plated in 10 cm tissue culture dishes and allowed to grow until cells were approximately 70% confluent, after which time cells were either passaged, subjected to treatment, or harvested for analysis.

2.2.2 3T3 mouse fibroblast cell lines

The mouse Balb/c 3T3 fibroblast cell line was a gift from Robert K. Bright (Department of Microbiology and Immunology, Texas Tech University, Health Sciences Center, Lubbock, Texas, USA) and was maintained at 37°C in RPMI media (Life Technologies) supplemented with 10% FBS (Life Technologies), and 3% L-glutamine (Life Technologies). Stably transfected TPD52-expressing 3T3 fibroblasts, as well as vector-transfected 3T3 cells (Vector-3) were derived by Mona Shehata.
(Shehata et al., 2008a) and cultured in RPMI media as above, with 1 mg/ml G418 (Gibco) as described previously (Shehata et al., 2008a). Cells were plated in 10 cm tissue culture dishes and allowed to grow until cells were approximately 70% confluent, after which cells were either passaged, subjected to treatment, or harvested for analysis.

For all experiments requiring exact cell numbers, cells were trypsinized and centrifuged at 1500 rpm for 5 min in a Beckman GS-6R centrifuge at RT. The supernatant was carefully removed and the remaining cell pellet was resuspended in 10 ml media. Twenty µl of cell supernatant were added to 20 µl of trypan blue (Sigma-Aldrich) which stains dead cells blue. Cell numbers were then calculated using a hemacytometer.

2.3 Genomic DNA extraction
Genomic DNA (extracted from cultured cells) was purified using the phenol chloroform extraction method. Cells were washed twice with 1X PBS, centrifuged at 1200 rpm for 5 min, the supernatant was discarded, and 4.5 ml SE lysis buffer was added to disperse cells. Proteinase K (300 µl) was added followed by 500 µl 10% SDS, and samples were incubated in a 50°C water bath for 1 h. Samples were then rotated overnight at RT. The following day, samples were briefly centrifuged, and 5 ml phenol:chloroform:isoamyl alcohol (25:24:1) (Life Tecnologies) was added. Samples were rotated for 2-4 h at RT, and then centrifuged at 3000 rpm for 20 min. Each upper layer was transferred into a new tube containing 5 ml of phenol:chloroform:isoamyl alcohol (25:24:1), and then rotated overnight at RT. The next day, samples were centrifuged at 3000 rpm for 20 min, and each upper layer was transferred into a new tube containing 5 ml chloroform:isoamyl alcohol (24:1), rotated for 30 min at RT followed by centrifugation at 3000 rpm for 10 min at RT. This
process was repeated twice. The upper layer was transferred into a new 50 ml tube (cooled on ice) and 10 ml 100% ethanol (pre-chilled at -20°C) was slowly added. The tube was inverted several times until a white thread appeared, which was transferred into a new 1.5 ml eppendorf tube containing 500 µl 70% ethanol. The tube was inverted several times, and centrifuged at 3000 rpm for 5 min. The supernatant was discarded and the DNA was left to dry for ~30 min at RT. TE buffer was added (50 – 500 µl) to dissolve the DNA. Samples were stored overnight at 4°C, followed by DNA quantitation (Section 2.14). DNA was then stored at -20°C.

2.4 Total RNA extraction and cDNA synthesis
Total RNA was extracted using the Trizol LS reagent (Life Technologies) according to the manufacturer’s protocol, and quantitated to determine RNA concentration and purity (Section 2.14). One µg of RNA was subjected to cDNA synthesis using the SuperScript III First Strand Synthesis System for RT-PCR kit (Life Technologies), according to the manufacturer’s instructions.

2.5 Real-time PCR (genomic DNA)
The resulting DNA was amplified using Taq DNA polymerase (KAPA Biosystems) and a set of TPD52 PCR primer sequences: sense, 5’-CAG TTT AGA GCC CAG GGA AA-3’ and antisense, 5’-CGA TCA TCC AAC GTA GCA TG-3’. These target a 225 bp TPD52 fragment (ENST00000518937, transcript TPD52-001) including the whole of exon 5 (27 bp). Prior to performing real-time PCR, primers were validated by standard PCR using genomic DNA. Fifty ng/µl DNA per sample was subjected to quantitative real-time PCR performed using the Corbett Rotor-Gene 6000 (Qiagen) using the KAPA SYBR® Fast Universal qPCR kit (Kapa Biosystems). Thermal cycling conditions comprised of an initial denaturation step of 95°C for 10 min, and 40 cycles of 95°C for 15 sec, 57°C for 30 sec and 72°C for 30 sec. Relative DNA copy numbers
were calculated using Roto-Gene 6 (Version 6.1) software (Qiagen) by normalizing raw data obtained for each cell line against the MCF-10A cell line as a control. A single peak at the appropriate melting curve was used to determine that there was only one PCR product amplified.

2.6 Real-time reverse-transcriptase (RT-) PCR (cDNA)

The resulting cDNA was amplified using Taq DNA polymerase (KAPA Biosystems) and a set of TPD52 PCR primer sequences: sense, 5’- GGA AGA GGA GCA GGA AGA GC -3’ and antisense, 5’- GAT GAC TGA GCC AAC AGA CG -3’; which amplified a 264 bp region of the TPD52 coding sequence (GenBank U18914) from nt 187-451. Prior to performing real-time RT-PCR, primers were validated by standard RT-PCR using cDNA. cDNA samples were subjected to quantitative real-time RT-PCR performed using the Corbett Rotor-Gene 6000 (Qiagen) using the KAPA SYBR® Fast Universal qPCR kit (Kapa Biosystems). Thermal cycling conditions comprised an initial denaturation step of 95°C for 10 min, and 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. Relative mRNA transcript levels were calculated using the Roto-Gene 6 (Version 6.1) software (Qiagen) by normalizing the raw data obtained for each cell line against that obtained for the MCF-10A cell line as a control. A single peak at the appropriate melting curve was used to determine that there was only RT-PCR product amplified.

2.7 SDS PAGE and Western blot analyses

2.7.1 SDS-PAGE

Cells were washed twice in cold 1X PBS and lysed in SDS-lysis buffer for total protein extracts. Samples were sonicated using a Branson Sonifier 150 (ProSciTech). Protein concentrations were determined using the BCA protein assay kit as per the manufacturer’s instructions (Pierce).
Protein extracts (12-18 μg/well) were resolved using SDS-PAGE 7.5% or 12.5% polyacrylamide gels (gel components are outlined in Table 2.5 or using pre-cast 4-12% gradient polyacrylamide gels (Life Technologies). Protein samples were mixed in a 3:1 ratio with 4X Sample protein buffer and boiled for 5 min. Samples were loaded onto gels with 12 μl BenchMark prestained protein ladder (protein size range 10-200 kDa, Life Technologies) loaded into SDS-PAGE 7.5% or 12.5% polyacrylamide gels, or with 12 μl Novex® Sharp prestained protein standard (protein size range 3.5-260 kDa, Life Technologies) for 4-12% gradient gels. Gels were run at 90 V for 30 min, or until the leading dye band had traversed the stacking gel, and then at 120 V for 1.5-2 h, or until the leading dye band had run off the gel.

Table 2.5 Resolving and stacking SDS-PAGE gel compositions

<table>
<thead>
<tr>
<th>Gel component</th>
<th>Volume for 10% resolving gel</th>
<th>Volume for 12.5% resolving gel</th>
<th>Volume for 15% resolving gel</th>
<th>Volume for stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris pH 8.8</td>
<td>3.75 mL</td>
<td>3.75 mL</td>
<td>3.75 mL</td>
<td>-</td>
</tr>
<tr>
<td>1M Tris pH 6.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>500 μL</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>2.7 mL</td>
<td>1.9 mL</td>
<td>1.1 mL</td>
<td>2.9 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 μL</td>
<td>100 μL</td>
<td>100 μL</td>
<td>40 μL</td>
</tr>
<tr>
<td>30% bis acrylamide stock</td>
<td>3.3 mL</td>
<td>4.15 mL</td>
<td>5.0 mL</td>
<td>530 μL</td>
</tr>
<tr>
<td>10% APS</td>
<td>4 μL</td>
<td>4 μL</td>
<td>4 μL</td>
<td>4 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>75 μL</td>
<td>75 μL</td>
<td>75 μL</td>
<td>30 μL</td>
</tr>
</tbody>
</table>
2.7.2 Western blot transfer

The SDS-PAGE gel was then electrotransferred to a PVDF membrane as follows. Sponges and Whatman filter papers were pre-soaked in 1X transfer buffer (prepared fresh and cold) (Section 2.1.2) and the PVDF membrane was rehydrated in 100% methanol, and then rinsed in 1X transfer buffer prior to transfer assembly. Filter papers were placed on top of the pre-soaked sponges on an electrode cassette (red side) with the PVDF membrane placed on top of the filter paper. A 15 ml falcon tube was rolled over the top to remove air bubbles. The gel was carefully removed from the running apparatus and the stacking gel was detached and discarded. The gel was placed on top of the PVDF membrane, being careful to exclude air bubbles. More filter paper was laid on top of the gel, as well as the final sponge. The gel-membrane sandwich was placed into the Western transfer tank (Bio-Rad) in cold 1X transfer buffer at 80 V for 2 h on ice, or overnight at 30 V in a cold room (4°C). Once transfer was completed, membranes were rinsed once in 100% methanol, and air dried for 15 min. Membranes were rehydrated in 100% methanol, washed in water to remove all traces of methanol, and then briefly placed in Ponceau S stain to reveal protein bands. Excess stain was washed off in water, and the membranes were then scanned using an EPSON scanner (EPSON, Australia) for future reference.

2.7.3 Immunostaining of Western blots

Membranes were either blocked overnight at 4°C or at RT for 60 min with shaking in either 5% skim milk powder in 1X TTBS or 3% BSA in 1xTTBS. Blocked membranes were washed 3 times (10 min each with shaking) with 1X TTBS and incubated with the relevant primary antibodies (Section 2.1.3.1) for 1-2 h at RT or overnight at 4°C in either 1X TTBS, 5% skim milk powder or 3% BSA, with shaking. Membranes were washed 3 times in 1X TTBS (10 min each), and then incubated with the relevant horseradish peroxidase-conjugated secondary antibody (Section 2.1.3.2) for 1 h at RT in either 5% skim milk powder or 3% BSA, with shaking. Membranes were then
washed for 3 times in 1X TTBS (10 min each), and incubated for 5 min with Western Lightning Chemiluminescent reagent (Perkin Elmer). Membranes were sandwiched between 2 plastic sheets and placed in a film cassette (Kodak). Protein bands were revealed by exposing membranes to X-ray film for 30 secs to overnight, depending on signal strength. Films were developed using an automatic X-ray processor (Konica SRX-101A). Densitometry analysis using ImageJ 1.45s software was used to quantitate fold changes in protein levels, relative to loading controls.

2.9 Transient small interfering RNA transfections
The TPD52 small interfering RNA (siRNA) duplexes (Shehata et al., 2008a) siD52-1 (5’-GCAGAAACTTGGAAATCAAT-3’) and siD52-2 (5’-GAGAAGTCTTTGAAATTCCG-3’) were employed, that target the TPD52 coding sequence (GenBank U18914) at nt 295-313 and nt 566-584, respectively. ON-TARGETplus siRNA pool ERBB2 target sequences were as follows: 5’-TGG AAG AGA TCA CAG GTT A-3’, 5’-GAG ACC CGC TGA ACA ATA C-3’, 5’-GGA GGA ATG CCG AGT ACT G-3’ and 5’-GCT CAT CGC TCA CAA CCA A-3’, targeting the ERBB2 coding sequence (NM_004448.2) at four different regions from nt 1446-1464, 597-614, 1861-1879 and 490-509, respectively. Both the TPD52 and ERBB2 siRNAs were obtained from Dharmacon, whereas non-targeting siRNA (siControl) was obtained from Qiagen. Cells were seeded at 2x10^4 cells/well into 24-well plates. After 24 h, cells were transfected with 50 nM siRNA duplexes using TransIT-TKO transfection reagent (Mirus Bio) in complete media following the manufacturer’s instructions. After 24 h, transfection media were removed and replaced with complete media. Cells were harvested for analysis 48 h later.

2.10 Cell death detection assay
Cells were transfected with siRNAs and 72 h later, the level of DNA fragmentation was quantified using the Cell Death ELISAPLUS kit according to the manufacturer’s
instructions (Roche). Briefly, cells were lysed and centrifuged, and supernatants were transferred to 96-well pre-coated plates to assay cytoplasmic histone-associated DNA fragments. After incubations and washes, stop substrate was added and color development was measured at 405 nm using a Multiskan Ascent plate reader (Thermo Scientific). Samples were analysed in duplicate in 3 independent experiments. Doxorubicin (Sigma-Aldrich) was used as a positive control for apoptosis in SK-BR-3 cells (500 nM, 72 h) or BT-474 cells (1 μM, 72 h). The enrichment of mono- and oligonucleosomes released into the cytoplasm of cell lysates was detected by biotinylated anti-histone- and peroxidase-coupled anti-DNA-antibody and is calculated using the formula: absorbance of sample cells/absorbance of control cells. Enrichment factors were used as a measure of apoptosis, calculated relative to the control value for cells treated by transfection only (which was set to 1.0).

2.11 Plasmids used in generating stably TPD52-depleted cell lines

Four Hush 29mer short hairpin RNA (shRNA) constructs targeting four different regions of TPD52 coding sequence (GenBank U18914): shRNA-D52-1: 5’-AGG AGA AGA TGT TGC TGC CAC GAT CAG TG-3’, targeting nt 142-170; shRNA-D52-2: 5’-AAT TCG GCT GCA AAT GCT AGT GCC ACC AC-3’, targeting nt 578-506; shRNA-D52-3: 5’-ATC CAG ACT CTG TCT CAA GTG TTA GCA GC-3’, targeting nt 239-267; shRNA-D52-4: 5’-GCA TCT AGC AGA GAT CAA GCG GAA ACT TG-3’, targeting nt 277-305, and a non-targeting control shRNA 5’-GCA CTA CCA GTA CT-3’) were cloned into the same sites of a pGFP-V-RS expression vector which were all obtained from OriGene Technologies. The pGFP-V-RS plasmid encodes a GFP tag driven by a CMV promoter and is kanamycin resistant.
2.12 *E.coli* transformation using electrophoration

The bacterial strain *Escherichia coli* (E. coli) XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proABlacFΔM15 Tn10 (Tet’)]*) (Stratagene) was used to obtain competent cells prepared by Dr. Yuyan Chen. Approximately 50 µl competent cells were transformed with plasmid DNA in ice-cold 0.2 cm cuvettes by electrophoration (Bio-Rad Gene Pulser II) at 2.5 kV, 25 F and 200 Ω for 4-5 milliseconds. One ml pre-warmed SOC media was then added immediately, and cells were transferred to 14 ml falcon tubes and incubated with shaking at 37°C, 250 rpm for 1-1.5 h. Each cell suspension was then placed into a 1.5 ml eppendorf tube and centrifuged at 3000 rpm for 3 minutes at RT to pellet bacterial cells, and the majority of the supernatant was discarded. Cells were resuspended in the remaining supernatant and plated onto LB agar plates supplemented with 50 µg/ml kanamycin and incubated at 37°C overnight to allow colony formation.

2.13 Screening of bacterial colonies and large scale plasmid DNA preparation

Single bacterial colonies were picked and plasmid DNA was isolated using a QIAprep Miniprep Kit (Qiagen), according to manufacturer’s instructions. Mini-preparations of plasmids were quantified (Section 2.14) and sent for DNA sequencing (Section 2.15) to confirm the insert. Once sequences were confirmed, plasmid DNA was prepared in large quantities using the QIAfilter Plasmid Midi Kit (Qiagen), according to the manufacturer's instructions. DNA concentration and purity were again quantified (Section 2.14) followed by DNA sequencing for sequence verification (Section 2.15).

2.14 Spectrophotometric quantitation of DNA and RNA

DNA and RNA concentration and purity were determined using the Nanodrop spectrophotometer (Thermo Scientific) by measuring absorbance at 260 nm (A260).
The absorbance values were then calculated for DNA or RNA concentration according to the following formula:

\[
\text{Concentration of DNA or RNA (ng/μL) = A}_{260} \times \text{dilution factor} \times 50 \text{ (DNA)} = A_{260} \times \text{dilution factor} \times 40 \text{ (RNA)}
\]

The purity of DNA or RNA was also determined by measuring the A_{260}/A_{280} absorbance ratio. A ratio of 1.8-1.9 indicates a pure DNA or RNA preparation.

2.15 DNA sequencing

The plasmid DNA pellets were resuspended in 1 x TE buffer and quantitated by 260 nm absorbance readings (Section 2.14). Samples containing 200–500 ng plasmid DNA plus 5–10 pmol of the primers used. The primer sequences were sense: 5’-GAC TAT CAT ATG CTT ACC GTA ACT-3’ and antisense: 5’-CTA TGG TTG CTG ACT AAT TGA GAT-3’, which amplified the pGFP-V-RS shRNA-29 expression vector either (with or without the TPD52 shRNA sequence fragments) at nt 261-487. Plasmids were sequenced by either Sydney University Prince Alfred Molecular Analysis (SUPAMAC, Australia) or Australian Genome Research Facility (AGRF) at Westmead, Australia. The sequence traces were subjected to BLASTN analysis to confirm the identity of the inserts and inspected visually to ensure the correct reading frame.

2.16 Generation of stably TPD52 depleted SK-BR-3 cell lines

For generating stably-depleted cell lines, shRNA plasmids (Section 2.11) were transfected into SK-BR-3 cells by seeding cells at ~ 60% confluence in 10 cm dishes and transfecting 24 h later using 3 μg DNA with TransIT-LT1 transfection reagent (Mirus Bio) in serum-free media. After 3 days, cells were seeded into nine 10 cm dishes, and 2 μg/ml puromycin (Sigma-Aldrich) was added 24 h later. Puromycin
selection continued for 2 weeks, after which selected resistant clones were screened using Western blot analyses for TPD52 protein levels. Four cell lines stably depleted of TPD52 and 2 control cell lines were selected and maintained in 1μg/ml puromycin media for further analyses.

2.17 Cell proliferation assay (MTT assay)
Briefly, untreated, non-target control or stably TPD52 depleted SK-BR-3 cells were plated in triplicate in 96-well plates (3x10^3 cells/well). Cells were cultured for 24 h and then 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich) was added to each well either immediately (time 0 h) or at the designated number of days post-seeding. MTT reactions were incubated at 37°C in the presence of 5% CO₂ for 4 h. To stop each reaction, media were replaced with 100 μl DMSO (Sigma-Aldrich) per well and mixed thoroughly. Absorbances at 540 nm were read using a Multiskan Ascent plate reader (Thermo Scientific). Means of triplicate wells from 3 independent experiments were used to generate data points and SE values.

2.18 Lapatinib treatment
Lapatinib was dissolved in DMSO to produce a 54 μM stock solution. Cells were treated with vehicle control (DMSO, 0.01%) or Lapatinib (100 nM) in triplicate per treatment group. MTT cell proliferation assays were performed over 5 days as described (Section 2.17). Proteins were harvested for Western blot analysis at days 1 and 5 of Lapatinib treatment. Means of triplicate wells obtained from MTT cell proliferation assays from 3 independent experiments were used to generate data points and SE values. Cell proliferation was calculated as percentage differences in absorbance values of treated (Lapatinib) versus untreated (DMSO) cells.
2.19 *In situ* staining of cultured cells

2.19.1 Indirect immunofluorescent staining analyses

Cells were plated onto glass coverslips, and cultured overnight until reaching 70-80% confluency the next day. Cells were washed twice with 1X PBS, fixed in 2% paraformaldehyde/PBS for 15 min at RT, washed 3 times with 1X PBS, permeabilized with cold acetone:methanol (1:1) for 15 min at -20°C, washed 3 times with 1X PBS and left for at least 30 min at RT, and incubated with the relevant primary antibodies (Section 2.1.3.1) in 3% BSA in 1X PBS overnight at 4°C. Cells were washed 3 times with 1X PBS and incubated with the relevant secondary antibodies (Section 2.1.3.2) in 3% BSA in 1X PBS, for 1 h in the dark at RT. After washing (3 times in 1X PBS), cells were mounted in FluorSave mounting medium (Calbiochem) onto glass slides, and kept in a horizontal position for at least 48 h for the coverslips to stick to the slides before viewing. Cells were visualized using either a TCS SP2 or TCS SP5 confocal laser scanning microscope (Leica), using a 63X objective lens.

2.19.2 Neutral lipid staining of lipid droplets

Cells were prepared and washed as described (Section 2.19.1) and fixed in 3% paraformaldehyde/PBS for 30 min at RT, then washed 3 times with 1X PBS. BODIPY 493/503 working solution were prepared fresh by adding 10 μl of 1 mg/ml BODIPY 493/503 stock solution to 10 ml of 150 mM NaCl. Cells were stained with BODIPY 493/503 working solution for 30 min in the dark at RT. Cells were then washed 3 times with 1X PBS, and DNA was counterstained with 10 nM DAPI (Sigma-Aldrich) for 5 min. After washing (3 times in 1X PBS), cells were mounted and visualized as described (Section 2.19.1).
2.19.3 Combined cellular staining for proteins and neutral lipid

For simultaneous visualization of intracellular proteins and lipid droplets, cells were prepared, washed and fixed as described (Section 2.19.1). Cells were then incubated with blocking buffer (Section 2.1.2) for 45 min at RT, washed 3 times with 1X PBS and incubated with the relevant antibodies (Section 2.1.3.1) diluted in antibody diluent (Section 2.1.2) overnight at 4°C. Cells were left to warm at RT for 30 min before proceeding. Cells were washed 3 times (10 min each) on a rocking platform, and incubated with the relevant secondary antibodies (Section 2.1.3.2) and 1:1000 of BODIPY 493/503 stock solution diluted in antibody diluent, for 1 h in the dark at RT. After washing (3 times in 1X PBS), cells were mounted and visualized as described (Section 2.19.1).

2.19.4 Image capture and analyses

Images were taken using a Leica TCS SP2 or TCS SP5 confocal laser scanning microscope (Leica Technologies), using a Plan 63X oil objective. The 405, 488, 543 and 633 nm lasers were used to excite DAPI, Alexa488, BODIPY 493/503, Cy3, Cy5 and Alexa633, respectively. Leica confocal software and Image ProPlus 5.1 software (Media Cybernetics) were used for image analyses.

2.19.5 Lipid droplet quantitation

All image data intended for quantitative comparison were acquired at the same subsaturating exposure time. Images of at least 10 panels per cell line in each of 3 independent experiments were used to quantify lipid droplets using Image ProPlus 5.1 software. The data collected were numbers, maximum diameters of lipid droplets, with areas calculated using diameters. The 3T3 cell line, D52-2-7 was used as the BODIPY 493/503 fluorescence intensity control to set the intensity range. Further filtering was done to exclude objects with diameters smaller than 0.3 μm, to reduce noise from potential non-specific staining, based on expected lipid droplet sizes in...
non-adipocytes ranging from 0.2 – 1 μm (Suzuki et al., 2011; Walther and Farese, 2012). The same parameters were then used to quantify lipid droplets in all 3T3 cell lines.

2.20 Oleic acid treatment

2.20.1 Oleic acid/BSA complex solution preparation
A 100 mM oleic acid stock solution was prepared in 0.1 M NaOH by heating in a 70°C water bath. In an adjacent 55°C water bath, a 10% (wt/vol) FFA-free BSA solution was prepared in 1xPBS. A 5 mM FFA/10% BSA stock solution was prepared by adding 50 μL 100 mM oleic acid solution dropwise to 950 μL 10% BSA solution at 55°C, which was then vortex-mixed for 10 sec, followed by a further 10 min incubation at 55°C. The 5 mM oleic acid/10% BSA stock solution was cooled to RT filtered through 0.45 μM membrane filter, and stored at 4°C. The 5 mM oleic acid/10% BSA stock solution was then diluted to 400 μm oleic acid in growth media and used as the working solution.

2.20.2 Oleic acid treatment of 3T3 cell lines
3T3 cells were cultured as described (Section 2.2.2) in 6-well plates until ~60-70% confluency. Growth media were removed and cells were washed twice with 1XPBS. Cells were either untreated (harvested at 0 h) or treated with 400 μm oleic acid in growth media (prepared in Section 2.20.1). After 6 h or 24 h of oleic acid treatment, cells were harvested for further analyses.
2.21 Statistical analyses

The SPSS for Windows package (version 19; SPSS) was used in most analyses. The Spearman’s rank test was used for correlation tests between samples. The Mann-Whitney test was used to compare relative transcript and protein levels and other variables. Results of all cell death detection, proliferation and lipid quantitation assays were expressed as means ± SE of three independent experiments. Comparisons between groups were made using two-tailed, unequal variance Student’s t tests calculated using Excel (Microsoft).
Chapter 3

TPD52 represents a survival factor in \textit{ERBB2}-amplified breast cancer cells
3.1 Introduction

As discussed in Chapter 1, cancer incidence is predicted to continue to rise. Therefore, there is a growing need to understand cancer pathogenesis in depth, due to the multiple genetic alterations whose significance still needs to be elucidated. Studying gene amplification targets and the phenomenon of oncogene addiction has led to the successful development of molecular therapies, such as Herceptin targeting the oncoprotein ERBB2. However, not all breast cancer patients with ERBB2-positive disease respond well to therapy and some develop resistance to Herceptin (Cobleigh et al., 1999; Vogel et al., 2002). Therefore, there is an urgent need to understand the basis of drug resistance to targeted agents. Understanding the functions of proteins co-expressed with ERBB2 may identify co-operating factors which promote or enhance tumor cell phenotypes, and which may contribute to primary or secondary resistance to ERBB2-targetted therapies.

Like ERBB2 (Section 1.3.3), TPD52 is amplified or gained in breast cancer (Balleine et al., 2000), and associations between gene copy number and expression support TPD52 representing a gene amplification target in breast and other cancers (Byrne et al., 2012; Guedj et al., 2012). This was recently underscored by a large-scale genomic study identifying focal amplification of TPD52 and 3 neighbouring chromosome 8q21.13 genes in breast cancer (Cancer Genome Atlas Network, 2012). Furthermore, increased TPD52 immunohistochemical staining was associated with reduced overall survival in breast cancer patients, where this was an independent prognostic factor (Shehata et al., 2008a). TPD52 has similarly been included in gene expression (Adler et al., 2006; Liu et al., 2007b; Ross-Innes et al., 2012) or copy number (Zhang et al., 2009) signatures associated with poor prognosis in breast cancer patients. However, the functional significance of TPD52 and ERBB2 co-expression in breast cancer is not well understood. Therefore, based on
numerous reports supporting possible TPD52 and ERBB2 co-expression (Chen et al., 2010; Choschzick et al., 2010; Kourtidis et al., 2010; Landis et al., 2006; Landis et al., 2005; Whiteaker et al., 2007; Wilson et al., 2002), we proposed TPD52 may co-operate with ERBB2 in promoting cancer pathogenesis.

The Akt signaling pathway is known to be important in regulating cell proliferation and survival (Nicholson and Anderson, 2002). It is also well established that ERBB2 lies upstream of Akt within the PI3K/Akt pathway (Blume-Jensen and Hunter, 2001). In agreement with previous studies reporting ERBB2 and TPD52 co-expression as discussed in Chapter 1 (Section 1.6.3.1), TPD52 has also been shown to regulate signaling through the Akt pathway. Exogenous TPD52 expression has been reported to lead to increased cell proliferation as well as increased migration and invasion in 3T3 mouse fibroblasts (Della Franca, 2012; Shehata et al., 2008a) and LnCaP prostate cancer cell lines (Li et al., 2009; Ummanni et al., 2008; Zhang et al., 2007a). Furthermore, exogenous expression of TPD52 or its isoform PrLZ led to increased pAkt(Ser473) levels in LnCaP prostate cancer cells (Ummanni et al., 2008; Zhang et al., 2011; Zhang et al., 2007a) and 3T3 mouse fibroblast cells (Della Franca, 2012). In addition, reduced PrLZ levels in C4-2 prostate cancer cell lines was associated with reduced cell proliferation and pAkt(Ser473) levels (Zhang et al., 2011). Figure 3.1 summarizes proposed possible links between ERBB2, TPD52 and Akt that have emerged from the literature to date.
Figure 3.1 Schematic diagram showing proposed links between ERBB2, TPD52 and Akt. This is based on previous reports of TPD52 and ERBB2 co-expression in breast cancer, and of increased pAkt levels occurring in response to increased TPD52 expression in prostate cancer. The relevant studies contributing to this diagram were summarized in Section 1.6.3.1 in the case of breast cancer studies, and Section 3.1 in the case of prostate cancer studies.
3.1.1 Specific aims for Chapter 3

The specific aims for Chapter 3 were as follows:

1. To examine *TPD52* gene amplification, transcript and protein levels status in a panel of breast cell lines.

2. To employ small interfering RNA (siRNA) approaches to characterise the effects of transiently reducing *TPD52* expression in breast cell lines with different *TPD52* and *ERBB2* gene amplification status.

3. To investigate the effects of transiently reducing *TPD52* and *ERBB2* expression, both singly and in combination, in *ERBB2*-amplified breast cancer cell lines.

4. To study the long-term effects of reduced *TPD52* expression in the *TPD52*- and *ERBB2*-amplified breast cancer cell line SK-BR-3.
3.2 Results

3.2.1 TPD52 and ERBB2 copy number and expression in breast cancer cell lines

Previously, TPD52 has been reported to be significantly overexpressed in breast cancer tissue relative to normal breast (Balleine et al., 2000; Porter et al., 2003; Shehata et al., 2008a; Yu et al., 2004). However, TPD52 copy number, transcript and protein levels have not been directly examined in breast cell lines. We selected a panel of 14 breast cancer cell lines with varying TPD52 levels to compare TPD52 and ERBB2 expression, and select the appropriate cell lines for further analysis. Table 3.1 shows the known/general molecular characteristics of these 14 breast cancer cell lines. Predicted TPD52 amplification status for the 14 breast cancer cell lines (Mackay et al., 2009; Neve et al., 2006; Pollack et al., 2002) was assessed via the ROCK database (http://rock.icr.ac.uk/) (Table 3.2), and MCF-10A cells were used for comparative purposes as a non-tumorigenic breast cell line (Soule et al., 1990). Cell lines were subjected to quantitative real-time (RT)-PCR to determine TPD52 DNA copy number and transcript levels relative to those in MCF-10A cells. TPD52 and ERBB2 protein levels were analysed by Western blot analyses.

Real-time (RT-) PCR and Western blot analyses revealed differences in relative TPD52 copy number, transcript and protein levels between the 15 breast cell lines, which included 14 breast cancer cell lines and MCF-10A cells (Figure 3.2). Overall, when compared with MCF-10A cells, most (12/14) breast cancer cell lines had higher relative TPD52 copy numbers, and 13/14 breast cancer cell lines had higher relative TPD52 transcript levels (Figure 3.2A and 3.2B). A significant positive correlation was measured between relative TPD52 transcript and protein levels (Spearman’s test, \( r_s = 0.743; p = 0.002; n = 15 \)). Similarly, 11 breast cancer cell lines with increased TPD52
Table 3.1 Molecular characteristics of breast cell lines used.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ER</th>
<th>PR</th>
<th>ERBB2 amp</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-BR-3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+^M</td>
</tr>
<tr>
<td>AU-565</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+^M</td>
</tr>
<tr>
<td>BT-483</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>_del</td>
</tr>
<tr>
<td>MDA-MB-175</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+^WT</td>
</tr>
<tr>
<td>HS-578T</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+^M</td>
</tr>
<tr>
<td>BT-474</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MCF-7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+^WT</td>
</tr>
<tr>
<td>MDA-MB-157</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>_del</td>
</tr>
<tr>
<td>MDA-MB-134</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+^M</td>
</tr>
<tr>
<td>DU4475</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+^WT</td>
</tr>
<tr>
<td>T-47D</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+^4M</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+^4M</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+^M</td>
</tr>
<tr>
<td>ZR-75-30</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+^WT</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>WT</td>
</tr>
</tbody>
</table>

ER, PR positivity, ERBB2 amplification, and p53 protein levels and mutational status (^del, deletion; ^M, mutant protein; ^WT, wild-type protein) are indicated. (ER = estrogen receptor, PR = progesterone receptor, ERBB2 amp = ERBB2 amplification, p53 = tumor protein p53, + = positive, - = negative). Data obtained from Kenny et al. (2007), Lacroix et al. (2006) and Neve et al. (2006).
Table 3.2 Predicted *TPD52* amplification status from published array CGH data.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Predicted <em>TPD52</em> amplification status</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-BR-3</td>
<td>Amp (1.0036)</td>
</tr>
<tr>
<td>AU -565</td>
<td>Amp (1.0979)</td>
</tr>
<tr>
<td>BT-483</td>
<td>Amp (0.8767)</td>
</tr>
<tr>
<td>MDA-MB-175</td>
<td>Amp (0.5921)</td>
</tr>
<tr>
<td>HS-78T</td>
<td>Amp (0.5326)</td>
</tr>
<tr>
<td>MDA-MB-134</td>
<td>Gain (0.3455)</td>
</tr>
<tr>
<td>MDA-MB-157</td>
<td>Gain (0.1538)</td>
</tr>
<tr>
<td>T-47D</td>
<td>Gain (0.1214)</td>
</tr>
<tr>
<td>BT-474</td>
<td>Gain (0.1162)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Dip (0.0443)</td>
</tr>
<tr>
<td>DU4475</td>
<td>Gain (0.1269)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Loss (-0.1183)</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Dip (0.0196)</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>Dip (-0.0608)</td>
</tr>
<tr>
<td>ZR-75-30</td>
<td>Dip (0.0307)</td>
</tr>
</tbody>
</table>

Numbers in brackets represent *TPD52* array CGH ratios according to the ROCK database [http://rock.icr.ac.uk/](http://rock.icr.ac.uk/). For Pollack et al. (2002) and Neve et al. (2006) thresholds of 0.4 = amplified (Amp); 0.1 = copy number gain (Gain); -0.1 = copy number loss (Loss). In Mackay et al. (2009), the threshold for gain was 0.08 and loss was -0.08. Array CGH ratios between these ratios (< 0.1 and > -0.1, or < 0.08 and > -0.08 were considered diploid.
transcript levels also had significantly higher TPD52 protein levels than the remaining 4 cell lines (Mann-Whitney U test, p=0.037, n=15).

TPD52 copy number, transcript and protein levels were highest in SK-BR-3 and AU-565 cells (Figure 3.2), which was in broad agreement with the results of Northern blot analyses of a smaller cell line cohort that included SK-BR-3 (Balleine et al., 2000). This result also supported published microarray data indicating SK-BR-3 and AU-565 to be TPD52-amplified (Kao et al., 2009; Mackay et al., 2009; Neve et al., 2006; Pollack et al., 2002) (Table 3.2), which was also reported for SK-BR-3 cells using single cell genome sequencing (Navin et al., 2011). The SK-BR-3 and AU-565 cell lines were derived from a single patient, and both are highly ERBB2-positive (Figure 3.2C), and ERBB2-amplified (Neve et al., 2006). Interestingly, 4 ERBB2-amplified cell lines in our study (SK-BR-3, AU-565, BT-474, ZR-75-30) showed significantly higher relative TPD52 transcript and protein levels than the remaining non-ERBB2-amplified cell lines (n=11) (Mann-Whitney U test, p=0.006, n=15, TPD52 transcript levels; p=0.037, n=15, TPD52 protein levels) (Figure 3.2B-3.2D), and significant or borderline positive correlations were measured between ERBB2 protein and TPD52 transcript or protein levels, respectively (Spearman test, $r_s=0.681$; $p=0.005$; n=15, TPD52 transcript levels; $r_s=0.513$; $p=0.051$; n=15, TPD52 protein levels). Therefore, results from real-time (RT-) PCR and Western blot analyses of 15 breast cell lines confirmed SK-BR-3 and AU-565 as TPD52-amplified and overexpressing cell lines.
Figure 3.2

A

Relative TPD52 DNA copy number

B

Relative TPD52 transcript levels

C

TPD52  23kDa
ERBB2  185kDa
GAPDH  37kDa

D

Relative intensity

TPD52/GAPDH
ERBB2/GAPDH
Figure 3.2 TPD52 copy number and expression status in breast cell lines. Breast cell lines were subjected to quantitative real-time (RT)-PCR to determine relative (A) TPD52 DNA copy number and (B) TPD52 transcript levels, both normalized against levels in MCF-10A cells. Results shown are the means ± SE from 3 independent experiments performed in triplicate. (C) Representative Western Blot analysis demonstrating TPD52 and ERBB2 levels. Left, proteins analysed. Right, molecular weights of detected species. Results shown are representative of those obtained in at least 3 independent experiments. (D) Densitometric analysis of TPD52 and ERBB2 levels in breast cell lines from 3 independent experiments. Mean intensities (relative to GAPDH) of TPD52 (grey bars) or ERBB2 (black bars) are plotted on the Y axis (+/- SE), with breast cell lines shown on the X axis.
3.2.2 Optimization of transient TPD52 knock-down conditions

Our group previously has shown that transient reduction of TPD52 levels in MCF-7 and SK-BR-3 cells was associated with significantly increased apoptosis in SK-BR-3 cells only (Shehata et al., 2008a). As previously, the present study used two TPD52 siRNAs (siD52-1 and siD52-2, Figure 3.3A) which were compared with the effects of the non-targeting siRNA (siControl) used by Shehata et al. (2008a), transfection reagent (TKO)-treated cells and parental (SK-BR-3) cells. Doxorubicin treatment (100 nM for 48 h, 72 h, 96 h) of SK-BR-3 cells was used as a positive control for the induction of apoptosis. The previous transient TPD52 knock down method (Shehata et al., 2008a) was modified slightly, in that TPD52 siRNA concentrations were reduced from 100 nM to 50 nM, as similarly reduced TPD52 levels were obtained by transfecting either 50 or 100 nM TPD52 siRNAs (data not shown).

We also optimised the duration of siRNA treatment in terms of maximally reducing TPD52 expression (Figure 3.3B) and inducing apoptosis (Figure 3.3C). TPD52 levels were reduced at all time points upon TPD52 knock-down, being least reduced at 48 h and most reduced at 96 h (Figure 3.3B). Densitometry analyses indicated that upon transient TPD52 knock-down with both TPD52 siRNAs, 48 h treatment reduced TPD52 levels to ~50%, 72 h treatment reduced TPD52 levels to ~30% and 96 h treatment reduced TPD52 levels to ~20% of control levels (data not shown).

The Cell Death Detection ElisaPlus kit (Roche) detects apoptosis by quantitatively determining the formation of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes and oligonucleosomes after apoptosis (Bonfoco et al., 1995; Nagata, 2000). We aimed to choose the earliest time point where increased cell death was detected following TPD52 knock-down. Overall, reduced
Figure 3.3

A

<table>
<thead>
<tr>
<th>1a</th>
<th>1b</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
</table>

B

![Western Blot Images](image1)

- SK-BR-3
- TKO
- siControl
- siD52-1
- siD52-2
- Dox

C

![Bar Graph](image2)

- SK-BR-3
- TKO
- siControl
- siD52-1
- siD52-2
- Dox

**SK-BR-3**

- **48hrs**
- **72hrs**
- **96hrs**

**Enrichment factor**

Nuruliza Roslan
Figure 3.3 Optimization of siRNA TPD52 knock-down. (A) Schematic diagram of TPD52 coding region showing the positions of siRNA targets (siD52-1, nt 295-313 and siD52-2, nt 566-584). Numbers represent exons, with exons 1a, 1b, 5 and 6 being subject to alternative splicing. Exons 1a and 1b are included in TPD52 and PrlZ transcripts, respectively. (B) Selection of siRNA knock-down time points using SK-BR-3 cells which were subjected to transient TPD52 knock-down using 50 nM of two different siRNAs (siD52-1 and siD52-2). TPD52 protein expression levels were assessed by Western blot analyses, with GAPDH being used as a loading control. Left, proteins analysed. Right, molecular weights of detected species. (C) The effects of TPD52 knock-down 48 h, 72 h and 96 h post-transfection were assessed using a Cell Death Detection ElisaPlus kit (Roche). Untreated cells, TKO transfection reagent only and non-targeting siRNA transfected cells (siControl) were used as negative controls. Doxorubicin treatment of SK-BR-3 cells (100 nM for 48 h, 72 h, 96 h; Dox) was used as a positive control for inducing apoptosis. Assay results were expressed as enrichment factors which were normalized against TKO for all treatments. The data presented are the mean (average) values from one experiment, performed in duplicate.
TPD52 levels reflected the apoptosis enrichment factor values obtained (Figure 3.3C), as TPD52 knock-down after 48 h treatment produced the least cell death, whereas the 96 h timepoint was associated with the most cell death (Figure 3.3C). No differences in cell death were noted between cells transfected with either TPD52 siRNA compared to TKO or parental SK-BR-3 cells at 48 h. Increased cell death was noted in cells transfected with either TPD52 siRNA when compared to TKO and parental SK-BR-3 cells at 72 h and 96 h (Figure 3.3C). Only minor differences in apoptotic cell death were noted at 72 h and 96 h following TPD52 knock-down, as per the minor differences noted in TPD52 levels at these timepoints (Figure 3.3B, 3.3C, data not shown). Non-targeting control siRNA showed increased levels of cell death at 72 h and 96 h (Figure 3.3C), suggesting that this non-targeting control siRNA was not a valid control. Hence we obtained a different non-targeting control siRNA from Qiagen, which was shown to have similar effects as no treatment or TKO-treatment only in 4 breast cell lines tested (Figure 3.4). Therefore, we decided to choose the 72 h time point for all experiments, since this was the shortest time that efficiently reduced TPD52 levels and generated detectable apoptotic cell death.
Figure 3.4

A  SK-BR-3

B  MCF-7

C  MDA-MB-231

D  MCF-10A
Figure 3.4 Transient TPD52 knock-down effects in breast cell lines. Four breast cell lines (A) SK-BR-3, (B) MCF-7, (C) MDA-MB-231 and (D) MCF-10A were subjected to transient siRNA knock-down using two different siRNAs, siD52-1 and siD52-2. Untreated cells, TKO transfection reagent only and non-targeting siRNA transfected cells (siControl) were used as negative controls. Doxorubicin treatment of SK-BR-3 cells (500 nM, Dox, 72 h) was used as a positive control for inducing apoptosis. The effects of TPD52 knock-down after 72 h were assessed using a Cell Death Detection ElisaPlus kit (Roche). Assay results were expressed as enrichment factors normalized against TKO for all treatments, and means ± SE are presented for 3 independent experiments done in duplicate. (*, p<0.005, Student’s t test). TPD52 protein levels were assessed by Western blot analyses, with results shown being representative of those obtained in 3 independent experiments. GAPDH or α-tubulin was used as a loading control. Proteins analysed are indicated at the left or right of the panels, with the molecular weights of detected species shown in brackets.
3.2.3 Transient TPD52 knock-down in different TPD52-expressing cell lines

Next, we expanded the previous study of Shehata et al. (2008a) by comparing the effects of reduced TPD52 expression in 4 cell lines differing in TPD52 copy number. We chose to compare SK-BR-3 cells which are universally predicted to be TPD52-amplified and MCF-7 cells reported to show TPD52 gain in 2/3 array CGH studies (Table 3.2, Figure 3.2) with MDA-MB-231 and MCF-10A cells, which showed no apparent increased in relative TPD52 copy number (Figure 3.2). Similarly, we used the two TPD52 siRNAs (siD52-1 and siD52-2, Figure 3.3A) employed by Shehata et al. (2008a) and a different non-targeting siRNA (siControl) purchased from Qiagen, and transfection reagent (TKO)-treated cells as negative controls. Doxorubicin (500 nM, 72 h) treatment of SK-BR-3 cells was used as a positive control for the induction of apoptosis. Variable effects of Doxorubicin on TPD52 levels were obtained, indicating that Doxorubicin may indirectly affect TPD52 levels (Figures 3.3, 3.4 and 3.5).

After 72 h of TPD52 siRNA treatment, TPD52 levels were reduced in all 4 cell lines but were unaffected by non-targeting siRNA or TKO treatment (Figure 3.4). Apoptosis assays revealed that reduced TPD52 expression by TPD52 siRNA target si-D52-1 in SK-BR-3 cells was associated with significantly increased apoptosis at 72 h post-transfection, relative to siControl-treated cells (Figure 3.4A). Treatment of SK-BR-3 cells with siRNA siD52-2 reproducibly led to less efficient reductions in TPD52 levels and lower apoptosis, compared with siRNA si-D52-1 (Figure 3.4A), although these siRNAs produced similar levels of TPD52 knock-down in all other cell lines (Figure 3.4B, 3.4C, 3.4D). Despite the substantially reduced TPD52 levels produced in all 4 breast cell lines, reduced TPD52 expression with siRNA siD52-1 was associated with significantly increased apoptosis in SK-BR-3 cells only (Figure 3.4).
3.2.4 Transient TPD52 and/or ERBB2 depletion in ERBB2-amplified breast cancer cell lines

Since SK-BR-3 is amplified for both TPD52 and ERBB2 (Navin et al., 2011) and the majority of cell lines indicated to be sensitive to the effects of TPD52 knock-down were either ERBB2-amplified or positive for ERBB2 expression (Kourtidis et al., 2010; Shehata et al., 2008a) (Figure 3.4), we investigated whether the effects of reduced TPD52 expression might be associated with ERBB2 amplification status. We therefore compared the effects of TPD52 and ERBB2 knock-down, alone or in combination, in the ERBB2-amplified and overexpressing cell lines BT-474 and SK-BR-3 (Hynes et al., 1989; Kallioniemi et al., 1992). We selected BT-474 cells to compare with SK-BR-3 cells, as BT-474 cells express lower TPD52 levels (Figure 3.2), and have no apparent increase in TPD52 copy number (Figure 3.2A). While this result contrasts with previous reports of TPD52 copy number gain in BT-474 cells (Table 3.2), neither our results nor those previously reported identify TPD52 amplification in BT-474 cells, and in this sense, they clearly contrast with SK-BR-3 cells. We employed the single TPD52 target siD52-1 in these studies, based on results (Figure 3.4, data not shown) showing that siD52-1 reduced TPD52 levels to a greater extent than siD52-2 in SK-BR-3 cells.

Both SK-BR-3 and BT-474 cells showed increased apoptosis following ERBB2 knock-down relative to siControl-transfected cells, as supported by quantitative apoptosis assays and the detection of cleaved PARP-1 (Figure 3.5). Both methods indicated that higher levels of apoptosis were produced by ERBB2 knock-down in BT-474 compared with SK-BR-3 cells (Figure 3.5), consistent with previous results where ERBB2 knock-down was associated with ~73% reduced cell proliferation and
Figure 3.5

A

SK-BR-3

B

BT-474

* Enrichment factor

Enrichment factor

TPD52

23 kDa

p-ERBB2

185 kDa

(Tyr1221/1222)

ERBB2

185 kDa

cl-PARP-1

86 kDa

p-Akt(Ser473)

57 kDa

p-MAPK

44 kDa

(Thr202/Tyr204)

37 kDa

GAPDH

Nuruliza Roslan

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Figure 3.5 Transient TPD52 and ERBB2 knock-down in ERBB2-amplified cell lines. SK-BR-3 (A) or BT-474 cells (B) were treated with TPD52 (siD52-1) and/or ERBB2 siRNA (siERBB2) or non-targeting siRNA (siControl) for 72 h, and harvested for apoptosis assays and Western blot analyses. Doxorubicin treatment of BT-474 (1 μM, 72 h) (Dox) was used as a positive apoptosis control. The effects of siRNA treatment were assessed using a Cell Death Detection ElisaPlus kit (Roche). Results were expressed as enrichment factors normalized against TKO results, and means ± SE are presented for 3 independent experiments done in duplicate. Significant differences between the mean enrichment factors measured following TPD52 and/or ERBB2 knock-down versus other treatments are indicated (* p<0.05, ** p<0.001, n.s.= not significant, Student’s t test). TPD52, pERBB2, ERBB2, cleaved PARP-1, pAKT and pMAPK levels were determined using Western blot analyses. Results shown are representative of those obtained in 3 independent experiments. Left, proteins analysed. Right, molecular weights of detected species.
viability in BT-474 cells, compared with ~ 26% in SK-BR-3 cells after 4 days (Kourtidis et al., 2010). Both SK-BR-3 and BT-474 cells showed similar significant increases in apoptosis following TPD52 knock-down (Figure 3.5), despite their differing TPD52 expression levels (Figure 3.2C). Interestingly, SK-BR-3 cells showed similar sensitivity to both TPD52 and ERBB2 knock-down, and knock-down of both genes produced significantly higher levels of apoptosis, compared with the knock-down of either gene alone (Figure 3.5A). However, as BT-474 cells were more sensitive to ERBB2 knock-down than SK-BR-3 (Figure 3.5), the increased apoptosis produced by knock-down of both genes was only significant when compared with the effects of TPD52 knock-down (Figure 3.5B).

Both total ERBB2 and the pERBB2-Tyr1221/1222 phosphorylated form of ERBB2 (pERBB2) were used to examine the effects of ERBB2 and/or TPD52 knock-down. In both cell lines, TPD52 or ERBB2/pERBB2 levels were reduced after 72 h of treatment with the corresponding siRNA, either alone or in combination, but were unaffected by non-targeting siRNA or TKO treatment (Figure 3.5A and 3.5B). Transient TPD52 knock-down did not reproducibly alter ERBB2 or pERBB2 levels in both cell lines, and similarly ERBB2 knock-down did not alter TPD52 levels (Figure 3.5).

Overexpression of TPD52 or its isoform, PrLZ, in LnCaP prostate cancer cells has been reported to result in increased pAKT (pSer473) (Ummanni et al., 2008; Zhang et al., 2011; Zhang et al., 2007a). While ERBB2 knock-down was associated with reduced pAKT (pSer473) levels in both BT-474 and SK-BR-3 cells as anticipated (Yang et al., 2004), pAKT (pSer473) levels were unchanged by TPD52 knock-down (Figure 3.5). There were similarly no changes to pMAPK44/42 levels upon transient TPD52 knock-down (Figure 3.5).
On a side note, Doxorubicin was used as a positive control for assessing cell death status (ELISA method) and apoptotic status (cleaved-PARP-1 detection), but we did not intend to assess Doxorubicin’s effects on TPD52 levels. As seen in the results obtained (Section 3.2.3 and 3.2.4), variable effects of Doxorubicin on TPD52 levels were obtained, indicating that Doxorubicin may indirectly affect TPD52 levels.

3.2.5 Subcellular distributions of TPD52 and ERBB2 in SK-BR-3 cells

To further investigate the significance of TPD52 and ERBB2 co-expression in SK-BR-3 cells, we compared the distributions of both proteins using indirect immunofluorescence analyses, to investigate possible co-localization of TPD52 and ERBB2. TPD52 has been detected within the perinuclear cytoplasm of breast cancer cells (Balleine et al., 2000), whereas ERBB2 is detected mainly at the cell membrane (Hommelgaard et al., 2004). Detection of both proteins in SK-BR-3 cells reproduced these expected subcellular distributions, with TPD52 and ERBB2 co-localization being focally limited towards the cell membrane (Figure 3.6). The lack of substantial co-localization of TPD52 and ERBB2 supported the different downstream consequences of reductions in TPD52 and ERBB2 noted in ERBB2-amplified cell lines (Figure 3.5).
Figure 3.6 Limited co-localization of TPD52 and ERBB2 in SK-BR-3 cells. Immunofluorescence images of SK-BR-3 cells co-stained with TPD52 monoclonal antibody (red) and ERBB2 polyclonal antisera (green) and combined images on the right (Merge). Representative images are shown from the top and middle sections of the same cell groups. White arrows show co-localization between TPD52 and ERBB2. Images shown are representative of those obtained in 3 independent experiments. Images were taken using a TCS SP2 confocal laser scanning upright microscope (Leica Technologies) using a 63X objective. Scale bar = 10 μm.
3.2.6 Optimizing conditions for generating stably TPD52-depleted SK-BR-3 cell lines

As transiently reduced TPD52 expression led to significant apoptosis in SK-BR-3 cells, with comparable effects noted for TPD52 and ERBB2 knock-down (Figures 3.4 and 3.5), we investigated the long-term effects of reduced TPD52 levels in this cell line. We obtained 4 plasmids each containing *TPD52* shRNAs targeting different regions of *TPD52* (Figure 3.7A). A 29-mer scrambled shRNA cassette in a pGFP-V-RS plasmid vector (Origene Technologies) was employed as a non-targeting control (Figure 3.7B). Transient transfections were performed to determine whether *TPD52* shRNAs could efficiently reduce TPD52 levels, as assessed by Western blot analysis (Figure 3.7B). Using protein lysates from cells harvested 72 h post-transfection, we noted that both shRNA-D52-2 and shRNA-D52-3 were able to efficiently reduce TPD52 levels, compared to parental (parent) and non-targeting control cells (non-target) (Figure 3.7B). A total of 5 optimization attempts were made to identify the ideal amount of plasmid DNA as well as transfection reagent used (Invitrogen or Mirus Bio) that would optimally reduce TPD52 levels (Figure 3.7B, data not shown). From these experiments, 3 μg shRNA-D52-2 and shRNA-D52-3-containing plasmids and transfection reagent from Mirus Bio were used to generate stably TPD52-depleted SK-BR-3 cell lines.
Figure 3.7

A

shRNA-D52-1  shRNA-D52-3  shRNA-D52-2

1a  2  3  4  5  6  7  8

B

Parent  Non-target  shRNA-D52-1  shRNA-D52-2  shRNA-D52-3  shRNA-D52-4

TPD52  23 kDa

GAPDH  37 kDa
Figure 3.7 Optimization of shRNA TPD52 knock-down conditions. (A) Schematic diagram of the TPD52 coding region showing the positions of 4 shRNA targets (shRNA-D52-1: nt 142-170, shRNA-D52-2: nt 578-506, shRNA-D52-3: nt 239-267, shRNA-D52-4: nt 277-305) and 2 siRNA targets (siD52-1 and siD52-2, highlighted in red, as per Figures 3.3, 3.4). Regions of overlap between shRNA and siRNA targets are highlighted in light blue. Numbers represent exons, with exons 1a, 1b, 5 and 6 being subject to alternative splicing. Exons 1a and 1b are included in TPD52 and PrLZ transcripts, respectively. (B) The 4 shRNA targets were optimized for efficient TPD52 knock-down in SK-BR-3 cells. 3 μg plasmid DNA containing either TPD52 shRNA or non-targeting shRNA were transfected into SK-BR-3 cells. TPD52 protein levels were analysed 72 h after transfection using Western blot analyses. Results shown used 3 μg plasmid DNA with Mirus Bio transfection reagent and are representative from 5 optimization attempts with different amounts of plasmid DNA and transfection reagents (either from Invitrogen or Mirus Bio). GAPDH was used as a loading control. Left, proteins analysed. Right, molecular weight of detected species.
3.2.7 Generation of stably TPD52-depleted SK-BR-3 cell lines

Next, we proceeded by transfecting SK-BR-3 cells with 3μg shRNA-D52-2 or shRNA-D52-3 containing plasmids to generate stably TPD52-depleted SK-BR-3 cell lines. A schematic diagram of the strategy used for generating these stably TPD52-depleted SK-BR-3 cell lines is shown in Figure 3.8. Transfected plasmids containing TPD52 shRNA targets (shRNA-D52-2 and shRNA-D52-3, Figure 3.7A) also included GFP tags, which allowed visual assessment of transfection efficiency (Figure 3.10A). Cells were harvested during early propagation in 24 well plates (termed “early passage” cells), to identify cell lines with reduced TPD52 levels for further propagation (Figure 3.9). These cell lines and relevant controls were then re-harvested at passage 10 (Figures 3.10A, 3.10B, 3.11B).
shRNA transfection of SK-BR-3 cells in 10 cm dishes

~ 72 h

Separate transfected cells in 8 X 10 cm dishes
(add selection media containing puromycin)

~ 2 weeks

Pick single colonies, seed into 96-well plates

Propagate into 24-well plates

Assess TPD52 protein levels using Western blot

Further propagation into 6-well plates, followed by 10 cm dishes

Harvest (“Early passage”)

Harvest after 10 passages (“Passage 10”)

Figure 3.8 Generation of stably TPD52-depleted SK-BR-3 cell lines using puromycin selection. SK-BR-3 cells were transfected with 3 μg plasmid DNA containing shRNA targets (shRNA-D52-2, shRNA-D52-3 or non-target control shRNA). Total protein lysates harvested from single cell colonies propagated into 24-well plates (termed “early passage” cells), were assessed for reduced TPD52 levels using Western blot analyses. Cell colonies identified with reduced TPD52 levels were further propagated. Total protein lysates were harvested after 10 passages (termed “passage 10” cells) and assessed for stably reduced TPD52 levels using Western blot analyses.
Total protein lysates were harvested from cells during early propagation and used in Western blot analyses to assess TPD52 expression. We screened a total of 9 cell colonies transfected with the non-targeting control shRNA plasmid. All 9 colonies showed similar TPD52 levels when compared to parental SK-BR-3 cells (Figure 3.9A). A total of 26 cell colonies were screened for reduced TPD52 expression (14 cell colonies for shRNA-D52-2, 12 cell colonies for shRNA-D52-3) (Figure 3.9B and 3.9C). Western blot analyses identified 6 colonies (3 for shRNA-D52-2, 3 for shRNA-D52-3) with the most reduced TPD52 levels compared to parental SK-BR-3 cells, and these were further propagated until passage 10 under puromycin selection (Figure 3.9B and 3.9C).

Immunofluorescence (Figure 3.10A, data not shown) and Western blot analyses (Figures 3.10B, 3.11A, 3.11B) confirmed stably reduced TPD52 expression in shRNAD52-2 and shRNAD52-3-transfected cell lines after 10 passages, compared with non-targeting control and parental cell lines. shRNA-D52-3 transfected cells reproducibly showed greater reductions in TPD52 expression than shRNA-D52-2 transfected cells (Figures 3.10, 3.11A, 3.11B, data not shown). Similarly, whereas 3 shRNA-D52-3 cell lines maintained stably reduced TPD52 levels at both early passage and passage 10 (Figure 3.11A and 3.11B), only 1/3 shRNA-D52-2 cell lines retained reduced TPD52 expression (Figure 3.11A, 3.11B, data not shown). The remaining 2 shRNA-D52-2 cell lines regained parental SK-BR-3 levels of TPD52 expression by passage 6, and were not analysed further (data not shown).
Figure 3.9

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shRNA-D52-2

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shRNA-D52-3
Figure 3.9 Screening of stably TPD52-depleted SK-BR-3 cell colonies. SK-BR-3 cells were transfected with plasmid DNA containing (A) non-target control shRNA, (B) shRNA-D52-2, (C) shRNA-D52-3. Protein lysates from SK-BR-3 parental cells (parent) were used as a positive control for comparing TPD52 levels. Total protein lysates were harvested from single colonies propagated in 24-well plates under puromycin selection, and assessed for TPD52 levels using Western blot analyses. Numbers identify the cell colonies screened. Two non-target colonies and 6 TPD52 shRNA target colonies that showed most reduced TPD52 levels were selected for further propagation and analysis (numbers shown in red or green). Two shRNA-D52-2 colonies subsequently regained parental TPD52 levels after passage 6 (numbers in green, data not shown). The remaining 4 shRNA-D52 colonies (shown in red) were renamed as shRNA-D52-2-5 = shRNA-D52-2-A, shRNA-D52-3-1 = shRNA-D52-3-B, shRNA-D52-3-4 = shRNA-D52-3-C, shRNA-D52-3-5 = shRNA-D52-3-D. Left, proteins analysed. Right, molecular weights of detected species.
Figure 3.10

A

Non-target  shRNA-D52-2-A  shRNA-D52-3-D

TPD52

GFP

B

Non-target  shRNA-D52-2-A  shRNA-D52-3-D

TPD52  23 kDa

GAPDH  37 kDa
Figure 3.10 Generation of stably TPD52-depleted SK-BR-3 cell lines. (A) Immunofluorescence images of SK-BR-3 cells stably transfected with shRNA-D52-2 (shRNA-D52-2-A) or shRNA-D52-3 (shRNA-D52-3-D) or non-target shRNA and harvested at passage 10. Cells were stained with human TPD52 monoclonal antibody (top row, red). shRNA constructs were also GFP-tagged (bottom row, green). Images were taken using a TCS SP2 confocal laser scanning upright microscope (Leica Technologies) using a 63X objective. Scale bar = 10 μm. (B) TPD52 levels in SK-BR-3 cell lines stably transfected with shRNA-D52-2 (shRNA-D52-2-A) and shRNA-D52-3 (shRNA-D52-3-D) and non-target shRNA, as assessed by Western blot analyses at passage 10. Left, proteins analysed. Right, molecular weights of detected species.
3.2.8 Protein expression analyses of stably TPD52-depleted SK-BR-3 cell lines

The consequences of stably depleted TPD52 levels were compared in early passage and passage 10 cells, relative to controls. Increased cleaved PARP-1 levels were detected in early passage TPD52-depleted cells (Figure 3.11A), in accordance with the results of transient TPDS2 knock-down in SK-BR-3 cells (Figure 3.5A). Cleaved PARP-1 levels were lower in TPD52-depleted cell lines harvested at passage 10 (Figure 3.11B). Depletion of TPD52 by 40-90% in early passage cells was associated with increased pERBB2 but not total ERBB2 levels in all 4 cell lines, relative to controls (Figure 3.11A and 3.11C). When cell lines were grown to passage 10, TPD52 levels remained reduced by 50-90% in TPD52-depleted cell lines, and pERBB2 expression remained increased by ~2-fold (Figure 3.11B and 3.11C). Passage 10 cell lines with lower TPD52 levels showed higher pERBB2 levels (Figure 3.11B, cell lines B and D), whereas cell lines which had partially recovered TPD52 expression showed lower pERBB2 levels (Figure 3.11B, cell lines A and C). Significant negative correlations were measured between mean TPD52 and pERBB2 levels (obtained through densitometric analysis of 3 replicate Western blots) in early passage and passage 10 cells (Spearman rank correlation, $r_s$=-0.886, $p=0.019$, n=6, early passage; $r_s$=-0.943, $p=0.005$, n=6, passage 10), and in the combined populations (Spearman rank correlation, $r_s$=-0.846, $p=0.001$, n=12) (Figure 3.11C). While no changes in pAKT (Ser473) or pMAPK44/42 levels were noted in early passage cell lines (Figure 3.11A), increased pERBB2 levels were accompanied by ~2-4-fold increases in pAKT (Ser473) levels in 3/4 TPD52-depleted cell lines analysed (Figure 3.11B), with pMAPK44/42 levels remaining unchanged harvested at passage 10. Taken together, these results suggest that SK-BR-3 cells compensate for the deleterious effects of TPD52 depletion by increasing ERBB2 signaling.
Figure 3.11

Early passage

A  

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Passage 10

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C  

Early passage  

Passage 10

Nuruliza Roslan
Figure 3.11 Analysis of stably TPD52-depleted SK-BR-3 cell lines. Western blot analyses of total protein extracts from parental SK-BR-3 cells and SK-BR-3 cell lines stably transfected with non-targeting control, shRNA-D52-2 (cell line A) or shRNA-D52-3 shRNAs (cell lines B-D) and harvested at early passage (A) or at passage 10 (B). Results shown are representative of those obtained in 3 independent experiments. *Left*, proteins analysed. *Right*, molecular weights of detected species. (C) Scatter plot comparing mean TPD52 (X axis) and pERBB2 levels (Y axis) measured in parental, non-target control and stably TPD52-depleted SK-BR-3 cell lines. Open circles represent values obtained for early passage cells, whereas black circles represent values obtained for cell lines at passage 10. Densitometric values were obtained through the analysis of 3 replicate Western blots, normalized to the loading control GAPDH, and then to levels measured in parental cells, set at 1.0.
3.2.9 Cell proliferation effects in stably TPD52 depleted SK-BR-3 cell lines

Previously, TPD52 overexpression in 3T3 fibroblast and prostate cancer cell lines led to increased cell proliferation (Shehata et al., 2008a; Ummanni et al., 2008), whereas stably reduced TPD52 in prostate cancer cell lines was associated with reduced cell proliferation (Ummanni et al., 2008; Zhang et al., 2007a). Cell proliferation was therefore assessed using MTT assays in stably TPD52 depleted cell lines harvested after passage 10, relative to the parental SK-BR-3 and non-targeted control cell line. This revealed that all 4 stably TPD52 depleted cell lines proliferated at similar rates as parental or non-targeting control cells over 3 days (Figure 3.12).

3.2.10 Lapatinib sensitivity in stably TPD52 depleted SK-BR-3 cell lines

Since stably reduced TPD52 expression led to increased pERBB2 levels (Figure 3.11), we determined whether stably reduced TPD52 expression was associated with altered sensitivity to Lapatinib (Diermeier-Daucher et al., 2011). Lapatinib (GW572016) is a small molecule inhibitor of EGFR and ERBB2 tyrosine kinases that causes apoptosis in ERBB2-overexpressing cells (Xia et al., 2002). Lapatinib concentration (100 nM) was based on the previously reported lapatinib IC$_{50}$ values (ie, the concentration of lapatinib necessary to reduce cell viability by 50%), which ranged between 50 – 100 nM for SK-BR-3 cells (O’Brien et al., 2010; O’Neill et al., 2012; Zhang et al., 2008). MTT assays indicated all cell lines showed comparable viability when cultured in the presence of 100 nM Lapatinib over 5 days (Figure 3.13A). Western blot analysis of proteins harvested on day 1 revealed that Lapatinib treatment reduced pERBB2 levels as expected (Rusnak et al., 2001) (Figure 3.13B).
Figure 3.12 Proliferation of stably TPD52-depleted SK-BR-3 cell lines. Cell proliferation was quantified using MTT assays, in SK-BR-3 cells stably depleted of TPD52 (using shRNA-D52-2 or shRNA-D52-3 shRNAs), and in parental or non-targeting (non-target) control cells. Values plotted are means ± SE for 3 independent experiments done in triplicate.
**Figure 3.13**

**A**

**Lapatinib treatment (100nM)**

- **Parent**
- **Non-target**
- **shD52-2-A**
- **shD52-3-B**
- **shD52-3-C**
- **shD52-3-D**

% Cell proliferation vs. Day 0, Day 1, Day 2, Day 3, Day 4, Day 5.

**B**

Lapatinib Day 1 (100nM)

- **Non-target**
- **shD52-2-A**
- **shD52-3-B**
- **shD52-3-C**
- **shD52-3-D**

- **p-ERBB2 (Tyr1221/1222)**
- **GAPDH**

- 185 kDa
- 37 kDa
Figure 3.13 Lapatinib sensitivity of TPD52 depleted SK-BR-3 cell lines. (A) SK-BR-3 cells stably depleted of TPD52, parental and non-target control cells were treated with 100 nM Lapatinib and subjected to MTT assays over 5 days. Means ± SE are shown for 3 independent experiments done in triplicate. The percentage of cell proliferation was calculated as the percentage difference between the A₅₄₀ values for treated (Lapatinib) and vehicle control (DMSO, 0.01%) cells at each time-point. (B) Western blot analyses of cells with or without lapatinib treatment (100 nM Lapatinib, + or 0.01% DMSO, -). Cell lines stably transfected with non-target control or shRNA-D52-2 (shRNAD52-2-A) or shRNA-D52-3 (shRNAD52-3-B, -C, D) were analysed after 1 day of 100 nM Lapatinib or 0.01% vehicle control treatment. Levels of pERBB2 were determined by Western blot analysis and GAPDH was used as a loading control. Right, proteins analysed. Left, molecular weights of detected species.
3.2.11 Transient ERBB2 knock-down effects in stably TPD52-depleted SK-BR-3 cells

Lapatinib is a dual ERBB2/EGFR inhibitor (Rusnak et al., 2001), however it has been shown to predominantly target ERBB2 (Zhang et al., 2008). As lapatinib treatment did not show any altered lapatinib sensitivity in stably TPD52-depleted SK-BR-3 cells, we decided to extend this result by directly comparing the effects of transiently reducing ERBB2 in TPD52-depleted and non-targeting control cells using ERBB2 siRNA knock-down. Transient TPD52 (using siD52-1 and siD52-2) and ERBB2 knock-down was performed in the non-targeting control cell line in parallel. The TPD52, pERBB2, cleaved-PARP-1, pAkt (Ser473) and pMAPK44/42 levels obtained (Figure 3.14A) were similar to those resulting from transient TPD52 and/or ERBB2 knock-down in parental SK-BR-3 cells (Figure 3.5A).

In contrast, transient ERBB2 knock-down in 4 stably TPD52-depleted SK-BR-3 cell lines was not associated with increased cleaved PARP-1 detection (Figure 3.14B) compared to increased cleaved PARP-1 in non-targeting control cells (Figure 3.14A). Furthermore, ERBB2 knock-down did not reduce pERBB2 levels to those of non-targeting control cells in 3/4 stably TPD52-depleted SK-BR-3 cell lines (Figure 3.14B). This could in part reflect the high pERBB2 levels in stably TPD52-depleted SK-BR-3 cells (Figures 3.11 and 3.14). In addition, transient ERBB2 knock-down showed markedly reduced pAkt (Ser473) levels in non-targeting control cells (Figure 3.14A), similar to previous results (Figure 3.5A), whereas pAkt (Ser473) levels were only marginally reduced upon ERBB2 knock-down in stably depleted TPD52 cell lines (Figure 3.14B). This agrees with the relatively high pERBB2 levels in these cell lines following ERBB2 knock-down and the fact that baseline pAkt levels were also increased in stably TPD52-depleted cell lines (Figures 3.11 and 3.14).
**Figure 3.14**

<table>
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<th>siControl</th>
<th>siD52-1</th>
<th>siD52-2</th>
<th>siD52-1 + siERBB2</th>
<th>siD52-2 + siERBB2</th>
<th>Dox</th>
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<tbody>
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<td></td>
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Figure 3.14. Transient ERBB2 knock-down effects in stably depleted TPD52 SK-BR-3 cell lines. (A) Non-targeting control cell line or (B) stably depleted TPD52 SK-BR-3 cell lines (cultured at > 10 passages) were treated with TPD52 siRNA (siD52-1 or siD52-2) and/or ERBB2 siRNA (siERBB2) or non-targeting siRNA (siControl) for 72 h, and harvested for Western blot analyses. Doxorubicin treatment of SK-BR-3 (500 nM, 72 h) (Dox) was used as a positive apoptosis control. TPD52, pERBB2, ERBB2, cleaved PARP-1, pAKT and pMAPK levels were determined using Western blot analyses. Results shown are representative of those obtained from 2 independent experiments. *Left*, proteins analysed. *Right*, molecular weights of detected species.
Transient ERBB2 knock-down showed reduced pMAPK44/42 levels in both non-targeting control and stably TPD52-depleted cells (Figure 3.14), as in parental SK-BR-3 cells (Figure 3.5A), although reductions in stably TPD52-depleted cell lines (Figure 3.14B) were less than those noted in the non-targeting control (Figure 3.14A). While total ERBB2, Akt and MAPK levels were not examined, based on previous results (Fig. 3.5, 3.11 and data not shown), we expect that these would have remained unchanged. Taken together, these results revealed that transient ERBB2 knock-down was relatively ineffective on a background of existing TPD52 knock-down and did not lead to increased apoptosis. These results therefore contrasted with those of lapatinib treatment of these cell lines, as will be discussed in the following section (Section 3.3.3).
3.3 Discussion

The work in this chapter explored the significance of TPD52 and ERBB2 co-expression in breast cancer cell lines since these two gene amplification targets have been reported to be co-expressed in numerous breast cancer studies (Chen et al., 2010; Kourtidis et al., 2010; Landis et al., 2006; Landis et al., 2005; Whiteaker et al., 2007; Wilson et al., 2002). Data presented in this chapter demonstrate TPD52 as a survival factor in ERBB2-amplified breast cancer cells and suggesting a possible cooperation between TPD52 and ERBB2 in cancer pathogenesis.

3.3.1 TPD52 and ERBB2 co-expression in breast cancer cell lines

The present study used a cell line cohort that was relatively small for statistical analyses. However, significant or borderline correlations between TPD52 transcript or protein levels and ERBB2 levels were still measured in the panel of 15 breast cell lines examined. We also demonstrated significantly higher TPD52 levels in 4 ERBB2-amplified breast cancer cell lines than in the larger group of 11 non-amplified ERBB2 breast cell lines. Nevertheless, these associations were likely driven by the inclusion of the TPD52-amplified SK-BR-3 and AU-565 cell lines within this cohort, thus these data were further supported by significant positive associations between relative TPD52 and ERBB2 transcript levels using RT-PCR analysis of breast cancer patient samples (Roslan et al., 2013, Appendix 2). These analyses were performed by an independent group in France, and were therefore not described in this thesis.
3.3.2 TPD52 transient knock-down effects

Previous studies using breast cancer cell lines have shown differing sensitivities to TPD52 knock-down, but the reasons for this have not been directly examined (Kourtidis et al., 2010; Shehata et al., 2008a). Results from our laboratory indicated that SK-BR-3 but not MCF-7 cells showed significant apoptotic cell death in response to transient TPD52 knock-down (Shehata et al., 2008a). Having demonstrated differential TPD52 expression and gene copy number across a panel of 15 breast cell lines, we expanded our previous study (Shehata et al., 2008a) to determine the effects of transiently reducing TPD52 expression in additional cell lines. Despite achieving substantial TPD52 depletion in 4 breast cancer cell lines and MCF-10A cells, only the ERBB2-amplified SK-BR-3 and BT-474 cell lines showed significant apoptosis in response to reduced TPD52 expression (Figures 3.4 and 3.5).

TPD52 knock-down produced very similar increases in apoptosis in SK-BR-3 and BT-474 cells, despite their marked differences in TPD52 expression and TPD52 amplification status (Figure 3.2). These observations concur with those obtained by Kourtidis et al. (2010) who studied the effects of TPD52 gene knock-down using high through-put analyses of cell viability, and reported higher reductions in cell viability in the ERBB2-amplified cell lines BT-474, MDA-MB-453, MDA-MB-361, and SK-BR-3, compared with MCF-7 and HMEC cells. BT-474 cells were more sensitive than SK-BR-3 cells to reduced ERBB2 expression, suggesting that BT-474 cells are highly dependent on ERBB2 for survival (Wang et al., 2011). Similarly, it has been reported that BT-474 cells contain a higher ERBB2 copy number than SK-BR-3 cells (Kallioniemi et al., 1992; Yeh et al., 2008). In contrast, SK-BR-3 cells showed similar levels of apoptotic cell death in response to TPD52 or ERBB2 knock-down, suggesting that these two oncogenes may be of similar importance in maintaining SK-BR-3 cell viability. A study by Navin et al. (2011) showed that TPD52 and ERBB2
are amplified to very similar extents in SK-BR-3 cells. It was interesting to note that when both genes were knocked down in SK-BR-3 cells, this resulted in significantly higher apoptosis compared to single knock-down of either gene, which could possibly suggest an additive effect. However, since knocking down ERBB2 did not affect TPD52 levels, or vice versa, in both SK-BR-3 and BT-474 cells, this suggests that ERBB2 does not directly regulate TPD52 levels, and vice versa (Figure 3.5). In summary, the effects of TPD52 knock-down may be determined by the ERBB2 status, as opposed to TPD52 amplification or expression status as previously proposed (Shehata et al., 2008a).

Transient TPD52 knock-down leading to increased cell death has been repeatedly reported in androgen-dependent LnCaP cells (Ummanni et al., 2008), and in the androgen-independent derivative cell line C4-2 (Zhang et al., 2011; Zhang et al., 2007a). Interestingly, both LnCaP and C4-2 cells detectably express ERBB2 (Cortes et al., 2012; Misra et al., 2006; Pignon et al., 2009), and thus the consequences of TPD52 knock-down in LnCaP and derivative cell lines are consistent with TPD52 promoting the survival of ERBB2-positive cancer cells. However, Kourtidis et al. (2010) also reported that the ERBB2-negative breast cancer cell line MDA-MB-468 showed similar reductions in viability to TPD52 knock-down as did ERBB2-amplified and overexpressing cell lines. It is known that the MDA-MB-468 cell line is an EGFR-amplified cell line (Gasch et al., 2013), thus it would appear that ERBB2 amplification or overexpression, and possibly other amplified oncogenes confer sensitivity to loss of TPD52 expression in cancer cells.

Other consequences of TPD52 knock-down were different from those which would have been predicted by previous studies. Ectopic TPD52 overexpression resulted in increased pAKT levels (Ummanni et al., 2008; Zhang et al., 2011), and reduced TPD52 expression in C4-2 prostate cancer cells led to reduced pAKT levels (Zhang
et al., 2011). However, in the present study, transient TPD52 knock-down did not alter pAKT levels in either SK-BR-3 or BT-474 cells. The siRNA targets employed in both the present study (Figure 3.5) and that of Zhang et al. (2011) bind sequences that are common to PrLZ and TPD52 transcripts, and therefore the different results obtained are unlikely to reflect differential targeting of PrLZ versus TPD52. These could however highlight functional differences between PrLZ and TPD52 in prostate versus breast cancer cells, or other differences between the particular cell lines examined. The fact that ERBB2 but not TPD52 knock-down resulted in reduced pAKT levels, while knock-down of both genes produced increased apoptosis, indicate that TPD52 regulates cell survival independently of ERBB2 and AKT signaling in ERBB2-amplified breast cancer cells.

3.3.3 TPD52 long-term knock-down effects

Whereas transient gene knock-down analyses examine the acute consequences of reduced gene activity, longer term gene knock-down analyses allow the assessment of whether and how cells can adapt to reduced gene function. We therefore examined the longer term effects of TPD52 knock-down in SK-BR-3 cells, as these cells have similar TPD52 and ERBB2 copy numbers (Navin et al., 2011), and were similarly sensitive to transient TPD52 and ERBB2 knock-down (Figure 3.5A). Even though both TPD52 siRNA targets reduced TPD52 levels in all breast cell lines tested, only TPD52 siRNA target (siD52-1) resulted in significant cell death in SK-BR-3 cells, although target siD52-2 showed a similar trend towards increased cell death (Figure 3.4A). We also noted that siD52-2 was less efficient in reducing TPD52 in SK-BR-3 cells, which also might explain the reduced cell death produced by this siRNA. SK-BR-3 cells show a high degree of TPD52 amplification, which could disrupt the siD52-2 binding site on some gene copies. The derivation of multiple SK-BR-3 cell lines with multiple shRNAs also allowed the possibility of extending the
results obtained with siD52-1 using additional shRNA targets. The differences in residual TPD52 levels (Figures 3.10 and 3.11) were likely due to the different TPD52 shRNA vectors used. However, even though there were clear differences between the shRNA vectors’ performance, both TPD52 shRNAs effectively and stably reduced TPD52 levels.

While TPD52 depletion led to increased cell death in the short term, these cells simultaneously increased ERBB2 signaling, which was stably maintained over 10 cell culture passages and beyond. By 10 passages, most TPD52-depleted cells also showed increased pAKT levels, as well as increased pERBB2 levels. Increased pAKT levels temporally followed increased pERBB2, in that this was detected by passage 10, but not in early passage cells. An inverse relationship between TPD52 and pERBB2 levels emerged in stably TPD52-depleted versus control SK-BR-3 cell lines. As cells harvested at passage 10 showed very similar proliferation as parental cells, increased pERBB2 followed by increased pAKT levels are likely to represent compensatory mechanisms to overcome the consequences of reduced TPD52 levels. It seems unlikely that PrLZ upregulation occurred in response to TPD52 depletion, as the shRNA targets employed bind sequences common to PrLZ and TPD52 transcripts, and the TPD52 antisera employed detect an epitope shared by TPD52 and PrLZ (Shehata et al., 2008b).

The effects of TPD52 depletion in SK-BR-3 cells contrast with the effects of stably reducing PrLZ/TPD52 expression in C4-2 cells, which was associated with reduced cell proliferation and colony-forming capacity (Zhang et al., 2011). Given the many differences between SK-BR-3 and C4-2 cells, it is not unexpected that stable TPD52 depletion produced different responses in these cell lines over time. It is possible that reducing TPD52 levels in SK-BR-3 cells requires adaptive changes for cells to remain viable, and/or that ERBB2 amplification may allow SK-BR-3 cells to more
readily adapt to reduced TPD52 levels. It may be significant that next generation sequencing of SK-BR-3 genomic DNA has revealed that 3 regions of chromosome 8q, one of which includes *TPD52*, are co-amplified with chromosome 17q (Hillmer et al., 2011) which agrees with the similar copy numbers reported for *TPD52* and *ERBB2* in SK-BR-3 in another next generation sequencing study (Navin et al., 2011). Hence, co-amplification of *TPD52* and *ERBB2* in SK-BR-3 cells may partially explain the similar effects of transient knock down of TPD52 and ERBB2 in this cell line, and therefore the long-term effects of reduced TPD52 provides additional evidence of SK-BR-3 cells relying upon both proteins for survival.

A limitation in the current study was the use of one cell line (SK-BR-3 cells) to generate stably-depleted TPD52 cell lines. The generation of stably-depleted SK-BR-3 cell lines required multiple screenings and propagation as described (Figure 3.8) which took ~ 7 months to successfully obtain. The fact that knocking down TPD52 in three non-*ERBB2* amplified cell lines did not result in increased cell death would predict that stable knock-down of TPD52 in a non *ERBB2*-amplified cell line would probably generate no obvious phenotype. Given the time constraints on the completion of this thesis, we did not place a high priority on conducting these additional experiments.

Ideally, we would like to extend our results by examining cancer cell lines that are *TPD52*-amplified but not *ERBB2*-amplified. To this end, we obtained the HCC2157 suspension cell line, that was predicted to be *TPD52*-amplified (Neve et al., 2006) but initial transient TPD52 knock-downs failed to reduce TPD52 levels, likely due to cell clustering that might have led to reduced transfection efficiency (data not shown). Western blot analyses revealed that HCC2157 cells showed high levels of TPD52, similar to those in SK-BR-3, but these cells were also positive for ERBB2 expression (data not shown). Therefore, future studies will seek to examine the effects of TPD52
knock-down in cell lines with high level TPD52 expression that lack similarly high ERBB2 levels.

Our collaborators in France analysed *TPD52* and *ERBB2* transcript levels in a diagnostic breast cancer cohort and found a significant positive correlation (Roslan et al., 2013, Appendix 2). High-level *TPD52* expression was associated with significantly poorer metastasis-free survival, both in the overall cohort, the large HR+/ERBB2- sub-group, and the smaller HR+/ERBB2+ subgroup in which *TPD52* expression was highest (Roslan et al., 2013, Appendix 2). Furthermore, Shehata et al., (2008a) previously reported that increased TPD52 immunohistochemical staining was associated with reduced overall survival. These combined results suggest that TPD52 and ERBB2 co-expression in breast cancer is both functionally and clinically significant.

The poorer metastasis-free survival of patients with tumors with high-level *TPD52* expression (Roslan et al., 2013, Appendix 2), combined with the demonstration that *ERBB2*-amplified cell lines rely upon TPD52 for survival (Figures 3.5 and 3.11), indicates that TPD52 overexpression could conceivably promote tumor resistance towards ERBB2-targetted therapies, particularly as TPD52 was indicated to regulate cell survival independently of ERBB2. Since transient reduction of both TPD52 and ERBB2 led to significantly higher apoptosis than knock-down of either gene alone, and stably TPD52 reduced SK-BR-3 cells showed increased pERBB2 levels, we hypothesised that the latter cell lines would be sensitized to ERBB2 inhibitors.

Lapatinib works intracellularly by reversibly binding to the intracellular domain of the tyrosine kinase receptor domain of both EGFR and ERBB2, which then inhibits receptor phosphorylation and activation of the downstream signaling pathways (Vazquez-Martin et al., 2011). Initially, 50 nM lapatinib was used to examine lapatinib
sensitivity in the stably TPD52-depleted SK-BR-3 cells (as this concentration was also in the IC$_{50}$ range (O'Brien et al., 2010; O'Neill et al., 2012; Zhang et al., 2008)). The experiment was repeated twice and showed no obvious differential lapatinib sensitivity (data not shown). Therefore, we increased the lapatinib concentration to 100 nM in order to see any differential sensitivity. However, stable reduction of TPD52 expression did not lead to significant sensitization to 100 nM Lapatinib treatment and pERBB2 levels were reduced in comparable levels as in control cells (Figure 3.13), similar to 50 nM Lapatinib treatment (data not shown). Hence, it might be useful to use a wider range of Lapatinib concentrations (0.01 μM – 10 μM) to extend these current results.

In contrast, transient ERBB2 knock-down in stably TPD52-depleted SK-BR-3 cells revealed pERRB2 levels were not reduced as in non-targeting control cells (Figure 3.14). This could mean that ERBB2 depletion using siRNA was ineffective in these stably TPD52-depleted SK-BR-3 cells, but this might not be the case since ERBB2 depletion in control cells showed pERBB2 levels were markedly reduced (Figure 3.14A). Lapatinib treatment and ERBB2 siRNA treatment of stably TPD52-depleted SK-BR-3 cells are two different experiments using different strategies to reduce pERBB2 levels. Lapatinib acts at the protein level, while ERBB2 siRNA targets ERBB2 mRNA. Initially, pEGFR, pPTEN and pERBB3 levels were assessed using Western blot analyses in the stably TPD52-depleted SK-BR-3 cells, but all protein levels assessed were unchanged compared to non-targeting control and parental cells (data not shown). Taken together, it is possible that transient ERBB2 knock-down results reflect other adaptive changes occurring in stably TPD52-depleted SK-BR-3 cells, such as acquired genetic changes and/or the up-regulation of independent pathways, which are yet to be determined.
3.4 Summary and conclusion

We have shown that TPD52 and ERBB2 are significantly co-expressed in breast cell lines, and that both genes independently contribute to the survival of ERBB2-amplified breast cancer cells. Short-term TPD52 knock-down produced significant cell death in ERBB2 amplified cell lines, via a mechanism independent of AKT signaling. However, long-term TPD52 depletion in TPD52- and ERBB2-amplified SK-BR-3 cell lines was associated with significant inverse correlations between TPD52 and pERBB2 levels, indicating that ERBB2-amplified cells may compensate for reduced TPD52 levels by increasing ERBB2 signaling. These results combined with the lack of substantial co-localisation between TPD52 and ERBB2 in SK-BR-3 cells indicate that TPD52 promotes the survival of ERBB2-positive cancer cells through indirect co-operation with ERBB2. Further studies were carried out in Chapter 4 to determine the basis of this co-operation.
Chapter 4

A possible role for TPD52 in regulating lipid storage
4.1 Introduction

In the previous results chapter, we examined the significance of TPD52 and ERBB2 co-expression in breast cancer cells, by studying the effects of transiently or stably reducing TPD52 levels (Chapter 3). So far, we have shown that TPD52 is important for maintaining ERBB2-amplified cancer cell survival, although TPD52 may act independently of ERBB2 signaling via PI3K/Akt or MAPK pathways. In this chapter we therefore examined other mechanisms by which TPD52 might co-operate with ERBB2.

Results from Chapter 3 have shown significant associations between TPD52 and ERBB2 expression in breast cancer cell lines and this is of interest to us, as ERBB2 expression has been associated with increased expression of multiple key lipogenic genes, such as fatty acid synthase (FASN), Acetyl-CoA carboxylase (ACC), mediator complex subunit 1 (PBP/MED1) and nuclear receptor subfamily 1, group D, member 1 (NR1D1) (Kourtidis et al., 2010; Kumar-Sinha et al., 2003; Yoon et al., 2007; Zhang et al., 2005). These genes are located on chromosome 17 together with ERBB2 (Figure 1.11) (Menendez and Lupu, 2007). ERBB2-amplified cancer cells such as SK-BR-3 and BT-474 have been shown to have high levels of intracellular lipid (Kourtidis et al., 2010). Studies have also shown that targeting ERBB2 using ERBB2 inhibitors (e.g. Herceptin) affects these lipid-regulating proteins, leading to reduced FASN and ACC levels (Kumar-Sinha et al., 2003; Yoon et al., 2007; Zhang et al., 2005). This suggests that lipid-associated proteins such as FASN are candidates for therapeutic targets in ERBB2-amplified cancers (Vazquez-Martin et al., 2007a; Vazquez-Martin et al., 2007b). Kourtidis et al. (2010) reported that knocking down lipid-associated genes reduced the viability of ERBB2-positive breast cancer cells (Kourtidis et al., 2010). This study used an RNAi screen which identified several genes which significantly affected cell viability according to ERBB2 amplification.
status. These genes included the lipid-associated genes MED1 and NR1D1, and also TPD52 (Kourtidis et al., 2010). This study therefore suggested that TPD52 may cooperate with ERBB2 by regulating lipid storage.

4.1.1 Lipid droplets – a major lipid storage organelle

Until the 1990s, lipid droplets were described as inert organelles (Murphy, 2001). However, more recent investigations have discovered that lipid droplets provide more than just storage of excess fatty acids and cholesterol esters, as will be described in the following Section 4.1.3 (Walther and Farese, 2009). Lipid droplet biogenesis and lipolysis are known to be tightly regulated by multiple proteins and enzymes (Section 1.4.2). Regulation of stored lipids is important for maintaining cell viability, since excess free fatty acids are known to be toxic to normal cells (Listenberger et al., 2003). Hence, highly proliferative cancer cells have evolved to overcome this limitation by altering lipid metabolism, in part by increasing their lipid storage capacity. This process helps support the increased biosynthetic and bioenergetic demands of the cancer cell.

4.1.1.1 Lipid droplet structure and size

Even though lipid droplets in white adipocytes have been extensively studied, it is now known that lipid droplets exist ubiquitously in eukaryotic (Kurat et al., 2009) and prokaryotic cells (Alvarez and Steinbuchel, 2002; Ashrafi, 2007; Hsieh and Huang, 2005; Law and Wells, 1989; Packter and Olukoshi, 1995). Lipid droplets, also known as lipid bodies, fat bodies, oil bodies, spherosomes or adiposomes, are cellular storage sites for neutral lipids, as described in a recent review (Walther and Farese, 2012). Lipid droplet structure is highly conserved between lipid droplets in different
cell types and species (Yang et al., 2012b). The lipid droplet hydrophobic core consists mainly of triacylglycerides and cholesterol esters, and is enclosed by a phospholipid monolayer consisting mostly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Bartz et al., 2007; Tauchi-Sato et al., 2002) (Figure 4.1). The phospholipid monolayer surrounding lipid droplets has been confirmed using cryoelectron microscopy (Tauchi-Sato et al., 2002). The lipid droplet surface is coated with various proteins (Tauchi-Sato et al., 2002) that regulate aspects of lipid droplet biology such as droplet synthesis, mobilization, interaction with other organelles and movement (Wan et al., 2007).

Lipid droplet numbers and sizes may differ between cell types or individual cells within a population. Non-adipocytes usually have many small lipid droplets, with diameters ranging from 100 nm to 1 µm, whereas adipocyte lipid droplets can have diameters up to 100 µm, mostly in the form of one lipid droplet per adipocyte (Suzuki et al., 2011). Due to their small sizes, lipid droplets in non-adipocytes often appear to be randomly distributed throughout the cell in cultured systems (Suzuki et al., 2011).

Lipid droplets also have the ability to expand either in size and/or numbers (Yang et al., 2012a), although this process could be cell type-dependent. For example, lipid droplets in adipocytes can expand up to 100 µm, whereas Drosophila S2 cell lipid droplets did not increase in size but increased in numbers, resembling clusters of grapes, in response to oleate treatment (Guo et al., 2008; Krahmer et al., 2011). Lipid droplets can also increase in size under pathological conditions, such as lipid droplets in liver cells during hepatic steatosis (Matsusue et al., 2008). Most cell types other than adipocytes increase lipid droplet numbers but not size, such as lipid droplets in yeast cells upon entering stationary growth phase (Fei et al., 2011). Increasing lipid droplet numbers has been suggested to provide more surface area for lipases for more efficient lipolysis (Marcinkiewicz et al., 2006). Phospholipids such
as phosphotidylcholine (Krahmer et al., 2011) and lipid-associated proteins such as FSP27 (fat specific protein 27) (Gong et al., 2011) and perilipin (Brasaemle and Wolins, 2012) which localize on the lipid droplet surface, have been shown to be involved in regulating the size of lipid droplets (Yang et al., 2012a).

Figure 4.1 Schematic overview of lipid droplet structure. Lipid droplets are composed of a core of nonpolar/neutral lipids (mainly triacylglycerides and cholesterol/sterol esters), which are enclosed by a monolayer of polar lipids (e.g. phospholipids and sterols) and associated proteins which include DGAT2 (diacylglycerol acyltransferase 2), Rab18, perilipin, and CCT (CTP:phosphocholine cytidylyltransferase; the rate-limiting enzyme in phosphatidylcholine synthesis). Reproduced from Krahmer et al. (2009).
Lipid droplets form rapidly in response to increased free fatty acid levels in cells, as reported in studies using cultured cells and in vivo (Pol et al., 2004). Several hypotheses or models regarding lipid droplet formation have been proposed (Walther and Farese, 2012), which commonly propose that lipid droplets form within the endoplasmic reticulum (ER). Lipid droplets have been proposed to originate from the ER in what has been called the “budding-off” model (Figure 4.2). This model proposes that the lipid droplet core originates as a lens of neutral lipid between the leaflets of the ER (Murphy and Vance, 1999). This eventually expands, and buds off into the cytoplasm, with the surrounding phospholipid monolayer derived from the outer ER monolayer (Murphy and Vance, 1999). Several lines of evidence support this model. Ultrastructural studies using electron microscopy revealed lipid droplets to be in close proximity with the ER, or to remain attached to the ER, using 3T3-L1 adipocyte cells (Blanchette-Mackie et al., 1995), HepG2 cells (Tauchi-Sato et al., 2002), 3T3 cells (Ozeki et al., 2005) and U937 monocytic cells (Wan et al., 2007). In addition, proteomic studies revealed the presence of ER proteins isolated from lipid droplets from 3T3-L1 adipocytes (Brasaemle et al., 2004), CHO cells (Liu et al., 2004), and monocytic cells (Wan et al., 2007). Alternatively, an “egg-cup” model has been proposed, where lipid droplets grow or expand in apposition to the ER, as opposed to within it, which was observed in human monocytes using electron microscopy (Robenek et al., 2006).

In mammalian cells, diacylglycerol acyltransferase 1 (DGAT1) and DGAT2 catalyse the final step in TAG synthesis (Yen et al., 2008), whilst acyl-coenzyme A cholesterol O-acyltransferase 1 (ACAT1) and ACAT2 play major roles in catalyzing the synthesis of cholesterol esters (Chang et al., 2009). These enzymes reside primarily in the ER (Kuerschner et al., 2008), although DGAT2 can also be found at the LD surface.
Collectively, the ER localization of these enzymes crucial for the final steps of lipid droplet formation supports the biogenesis of lipid droplets within the ER.

Figure 4.2 Lipid droplet formation from the ER into the cytoplasm according to the “budding-off” model. In this model, neutral lipids (triacylglycerols and cholesterol esters) are proposed to be synthesized between the leaflets of the ER membrane. The nascent LD then buds off from the ER membrane, forming an independent organelle enclosed by a monolayer of phospholipids and LD-associated proteins such as the members of the PAT-domain family of proteins (perilipin, adipophilin and TIP47). ER = endoplasmic membrane, LD = lipid droplet. Reproduced from Martin and Parton (2006).
4.1.2 Proteins regulating lipid droplet function

4.1.2.1 PAT family proteins: Main players in lipid droplet regulation

The coordination of lipid storage and utilization is mainly regulated by the PAT family of proteins. The PAT protein family was originally named after the members of perilipin (PLIN1), adipophilin or adipose-differentiated-related-protein (ADRP or PLIN2) and TIP47 (for Tail-Interacting Protein of 47 kDa or PLIN3) (Brasaemle, 2007). Other proteins also important for lipid droplet regulation and included in the PAT protein family are S3-12 (PLIN4) and OXPAT (for oxidative protein of the PAT family, or PLIN5) (Brasaemle and Wolins, 2012). Most PAT proteins contain a conserved lipid-droplet binding motif known as the PAT domain (Miura et al., 2002) (Figure 4.3). The types of PAT proteins that associate with lipid droplets vary according to cell type, and the maturity or stage of lipid droplet formation. A study using 3T3-L1 adipocytes stimulated with oleic acid observed different localizations for PAT proteins (Wolins et al., 2005). TIP47 and S3-12 localized to the smallest lipid droplets, whereas ADRP localized to intermediate-sized lipid droplets, and perilipin was detected at the largest lipid droplets (Wolins et al., 2005).

Perilipin mainly regulates lipid storage in adipocytes (Greenberg et al., 1991), while ADRP mainly regulates lipid storage in non-adipocytes (Brasaemle et al., 1997). In contrast, TIP47 is involved in trafficking lipids between the cytoplasm and lipid droplets (Brasaemle, 2007; Wolins et al., 2005). Perilipin is highly expressed in adipocytes, while adipophilin and TIP47 are mainly expressed in non-adipocytes (Brasaemle, 2007). Perilipin acts as the main gatekeeper regulating lipolysis in adipocytes, and reduces lipolysis by binding and sequestering CGI-58 (Granneman et al., 2007; Miyoshi et al., 2006; Subramanian et al., 2004). Perilipin and ADRP are unstable when not bound to lipid droplets, whereas DAG synthesis activates
recruitment of TIP47 from the cytoplasm to lipid droplets (Skinner et al., 2009). Several studies have shown that ADRP binds fatty acids and cholesterol (Atshaves et al., 2001; Serrero et al., 2000), promoting triacylglyceride accumulation (Imamura et al., 2002) and stimulating fatty acid uptake (Gao and Serrero, 1999). Therefore, PAT proteins control the flux of lipids to and from lipid droplets, resulting in either increased lipid storage or increased lipolysis.

Figure 4.3 Schematic diagram of the structural features of perilipin and related proteins. The positions of structural features that are shared by mouse perilipin-related proteins are shown; greater intensity of color represents higher similarity of sequences between family members, whereas lighter color represents reduced sequence similarity. The N-termini of perilipin, adipophilin, TIP47, and OXPAT, but not S3-12, contain 100 amino acid (aa) sequences termed the PAT or PAT1 domain that are highly conserved between 4 members of the family (green). Reproduced from Brasaemle (2007).
4.1.2.2 Other proteins found to be involved in lipid droplet regulation

A number of proteomic studies have analyzed isolated lipid droplets to identify lipid droplet-resident or -associated proteins (Bartz et al., 2007; Beller et al., 2006; Binns et al., 2006). Apart from reproducibly finding known lipid droplet proteins such as ATGL, CGI-58 and the PAT proteins, these studies also reported other proteins that provide valuable clues to further understand lipid droplet function and regulation, such as ER resident proteins, mitochondrial proteins, cytoskeletal proteins and even histones. Rab family proteins, particularly Rab5, Rab7, Rab11 and Rab18, have been frequently identified (Brasaemle et al., 2004; Fujimoto et al., 2004; Liu et al., 2004), as have caveolins which have been shown to traffic lipids from the Golgi apparatus to the plasma membrane (Cohen et al., 2004; Le Lay et al., 2006; Pol et al., 2004). Many of these proteins still warrant further investigation, to confirm their involvement in lipid droplet regulation.

Rab proteins have been previously shown to be important in regulating exocytic and endocytic pathways (Corbeel and Freson, 2008; Grosshans et al., 2006). The detection of Rab5 on purified lipid droplets implies the interaction of early endosomes with lipid droplets (Liu et al., 2007a). Of most interest is Rab 18, as this appears to associate with lipid droplets late in adipogenesis, replacing ADRP (Ozeki et al., 2005). Another study reported that stimulating lypolysis led to increased Rab18 association with lipid droplets (Martin et al., 2005). The varied functions of Rab proteins that associate with lipid droplets highlight the different functions of lipid droplets within the cell, which will be further discussed in the following section.
4.1.3 Lipid droplet function

Lipid droplets' most important physiological function is as an energy reservoir, storing TAGs and upon lipolysis, providing fatty acids as building blocks for membranes or substrates for energy metabolism. Generally, adipocytes supply energy needs for the whole organism, whereas lipid storage in non-adipocytes is usually for the cell's own consumption. Lipid droplets also provide a protective role, acting as scavengers for free fatty acids in order to avoid lipotoxicity and lipoapoptosis (Schaffer, 2003). Increased cellular free fatty acids have been shown to be toxic, and impairment of TAG synthesis and storage has also been shown to be detrimental to the cell (Listenberger et al., 2003; Schaffer, 2003).

Lipid droplets have also been proposed as organizing centres. They act as sites for synthesizing and metabolizing a wide range of signaling lipids such as eicosanoids, 2-arachidonoylglycerol and N-acylethanolamines (Kaczocha et al., 2010; Zechner et al., 2009). In addition, several enzymes for eicosanoid synthesis have been found in leukocyte lipid droplets (Bozza et al., 2009). Lipid droplets may also serve as protein storage sites, as for example histones were found in the lipid droplets of Drosophila oocytes (Cermelli et al., 2006). These histones were localized to lipid droplets until needed for rapid nuclear division associated with embryo segmentation. Hence, whereas histones were once considered as a contaminating protein in isolated lipid droplets, they are now known to be a major lipid droplet protein in Drosophila embryos (Cermelli et al., 2006). Lipid droplets may also provide temporary storage of other proteins for future use, or sequestration prior to proteosomal degradation (Ohsaki et al., 2006).

Lipid droplets have also been shown to assist in the replication of intracellular pathogens such as the Hepatitis C and Dengue (Samsa et al., 2009) viruses, and
Chlamydia trachomatis (Kumar et al., 2006). It was reported that the core D2 protein of Hepatitis C virus associates with lipid droplets (Hope and McLauchlan, 2000; Miyanari et al., 2007) and this process is known to be important in virus assembly (Shavinskaya et al., 2007). Another example was observed when HeLa cells were infected with Chlamydia trachomatis. This parasite tags lipid droplets with a bacterial protein, Lda3 (lipid droplet-associated protein 3), leading to lipid droplet binding and sequesteration in the bacteria-containing vacuole, hence utilizing the lipids for metabolism (Cocchiaro et al., 2008; Kumar et al., 2006). The authors concluded that lipid droplets play a role in the intracellular replication of Chlamydia trachomatis. Targeting lipid droplets may therefore provide new therapeutic strategies for treating these infections.

4.1.4 3T3 fibroblast cells as a model for investigating TPD52-like function

As described in Section 4.1.2.1, the main regulators of lipid droplet formation are members of the PAT protein family which includes perilipin, adipophilin (ADRP) and TIP47 (Brasaemle, 2007). PAT protein expression has been examined using samples from various types of cancer (Straub et al., 2010), showing that PAT proteins are frequently and differentially expressed. TIP47 has also been proposed as a biomarker for cervical cancer using blood from cervical carcinoma patients (Szigeti et al., 2009). Increased expression of PAT proteins in cancer further supports deregulated lipid storage within cancer cells. Several interaction analyses have reported that TPD52 binds PAT proteins. TPD52 was identified as a binding partner in a yeast two-hybrid screen using a perilipin bait (Yamaguchi et al., 2006). In another study, TPD52 co-immunoprecipitated with TIP47 and other proteins, including Rab5 (Zhang et al., 2007b). These studies suggest the possibility of TPD52 associating with PAT proteins in regulating lipid storage.
Collectively, based on several studies (Section 1.6.4) reporting links between TPD52 and lipid droplets and interactions with PAT proteins as mentioned above, we hypothesized that TPD52 is involved in regulating lipid storage and therefore contributes to the lipogenic phenotype in cancer cells. To date there has been no direct demonstration of TPD52 overexpression affecting lipid storage in any cell type. Therefore, we sought to study the effects of TPD52 expression on lipid droplets and PAT proteins using 3T3 mouse fibroblast cell lines that had been stably transfected with TPD52 or TPD52L1 (Shehata et al., 2008a) (Figure 4.4). The 3T3 cell line has been a reliable model in studying TPD52 function (Lewis et al., 2007; Shehata et al., 2008a) as well as being a classic cell line model for studying lipid storage (Bostrom et al., 2005; Imamura et al., 2002; Murphy et al., 2010). Previously, Shehata et al. (2008a) identified different non-redundant functions for TPD52 and TPD52L1, as 3T3 cells showed increased cell proliferation and anchorage-independent growth upon exogenous TPD52 expression only. This highlights the oncogenic potential of TPD52 and we decided to extend this finding further by investigating TPD52 effects on lipid storage, as another factor which may contribute to TPD52’s oncogenicity.
4.1.5 Specific aims for Chapter 4

Experiments described in this chapter aimed to determine whether:

1. Stable TPD52 expression led to an increase in the size and/or numbers of lipid droplets in 3T3 cells.

2. Changes in PAT protein localization and/or expression occurred in stably TPD52-expressing 3T3 cells, relative to controls.

3. TPD52L1, which does not share the oncogenic functions of TPD52, alters lipid droplet sizes and/or numbers, and/or alters PAT protein localisation or expression in 3T3 cells.

4. TPD52-expressing 3T3 cells have an enhanced ability to take up exogenous lipid, relative to controls.
4.2 Results

The parental, vector-transfected and stably TPD52- and TPD52L1-expressing 3T3 mouse fibroblast cell lines have been previously described (Shehata et al., 2008a) (Figure 4.4A). Western blot analysis confirmed that D52-2-1 and D52-2-7 cells maintained TPD52 expression (Figure 4.4B), D52L1-4 and D52L1-6 cells maintained TPD52L1 expression (Figure 4.4C), whereas the parental and vector (vector-3) control cells did not detectably express TPD52 or TPD52L1. These results confirm that stably transfected cells retained exogenous expression of TPD52 or TPD52L1, as anticipated (Shehata et al., 2008a).

4.2.1 Increased lipid droplets in TPD52- but not TPD52L1-expressing 3T3 cell lines

Firstly, we investigated the effects of TPD52 or TPD52L1 expression in 3T3 cells on lipid droplets. BODIPY 493/503, a lipid soluble dye, was used to stain lipid droplets and was visualized using confocal microscopy. Increased lipid droplet staining was shown in the TPD52-expressing 3T3 cell lines D52-2-1 and D52-2-7 (Figure 4.5B) compared to parental and vector-3 control cell lines, where smaller and fewer lipid droplets were detected (Figure 4.5A). Interestingly, the TPD52L1-expressing 3T3 cell lines D52L1-4 and D52L1-6 (Figure 4.5C) showed similar lipid droplet staining as parental and vector-3 control cell lines (Figure 4.5A).
Figure 4.4

A

3T3 cell line

DNA

+ Vector only

3T3 cell line

Increased cell proliferation

(Shehata et al., 2008a)

3T3 cell line

No change in cell proliferation

(Shehata et al., 2008a)

Good model for TPD52 function

Classic model for lipid storage function

B

parent-3T3
Vector-3
D52-2-1
D52-2-7

TPD52

23 kDa

Gapdh

37 kDa

C

parent-3T3
Vector-3
D52L1-4
D52L1-6

TPD52L1

23 kDa

Gapdh

37 kDa
Figure 4.4 3T3 mouse fibroblast cell line model. (A) Graphic representation of the TPD52- and TPD52L1-expressing 3T3 fibroblast cell lines used in this study. Vector only (vector-3) and parent 3T3 (not shown) fibroblast cell lines were used as control cells. Western blot analysis of (B) TPD52 and (C) TPD52L1 levels in TPD52- or TPD52L1-expressing 3T3 cell lines, compared to parent and vector-3 control 3T3 cells. Results shown are representative of those obtained from 3 independent experiments. Left, proteins analysed. Right, molecular weights of detected species.
Figure 4.5

(A) Control cells (parent, vector-3), (B) TPD52-expressing (D52-2-1, D52-2-7) and (C) TPD52L1-expressing (D52L1-4, D52L1-6) 3T3 cells were stained with BODIPY 493/503 (green) for lipid droplets and DAPI (blue) for nuclear staining and visualised using confocal microscopy with a X63 objective. Images are representative of 3 independent experiments. Scale bar = 15μm.

Figure 4.5 Increased lipid droplets in TPD52- but not TPD52L1-expressing 3T3 cells.
Lipid droplets in these and other BODIPY images (Figure 4.5) were quantified using the Image Pro Plus 5.1 software. The image analysis results confirmed that there was a significant increase in mean lipid droplet numbers per cell in the two TPD52-expressing 3T3 cell lines, but not in the two TPD52L1-expressing 3T3 cell lines, compared to vector-3 control cells (Figure 4.6A). There was a ~10-fold increase in lipid droplet numbers per cell in D52-2-7 cells and a ~4-fold increase in D52-2-1 cells, when compared to vector-3 control cells (Figure 4.6A). There were also smaller but significant increases in the mean lipid droplet diameter (μm) and area (μm²) in D52-2-7 cells compared to vector-3 control cells (Figures 4.6B and 4.6C). In addition, Western blot analyses clearly showed that D52-2-7 cells expressed higher TPD52 levels than D52-2-1 cells (Figure 4.4B). Taken together, our results provide direct evidence for the first time that TPD52 expression leads to significant increases in lipid droplet numbers and sizes in 3T3 fibroblasts.

4.2.2 Increased lipid droplets in TPD52-expressing MDA-MB-231 cells

Since TPD52-expressing 3T3 cells revealed significant increased lipid droplet numbers, we examined a second stably TPD52-expressing cell line, derived from the breast cancer cell line MDA-MB-231. These cell lines were derived as previously described (Boutros, 2005) and were chosen for TPD52 overexpression since MDA-MB-231 cells showed no apparent increase in TPD52 copy number (Figure 3.2A, Table 3.2), and hence express lower TPD52 levels than many breast cancer cell lines (Figure 3.2). BODIPY staining revealed increased lipid droplets in the stably TPD52-expressing MDA-MB-231 cells compared to parent and vector control cells (Figure 4.7). This provided evidence of possible involvement of TPD52 in lipid droplet regulation in a second cell line.
Figure 4.6

A  Mean lipid droplets/cell

B  Mean lipid droplet diameter (μm)

C  Mean lipid droplet area (μm²)
Figure 4.6 BODIPY quantitation analyses of TPD52- and TPD52L1-expressing 3T3 cell lines. (A) Mean lipid droplet number per cell, (B) mean lipid droplet diameter (µm) and (C) mean lipid droplet area (µm²) were quantified from 10 random fields of view per cell line using images such as those shown in Figure 4.5, using Image-Pro Plus 5.1 software. Data are presented as means ± SE (y-axis) from 3 independent experiments. Comparisons which were significantly different from vector-3 control cells are shown with their corresponding p values (Student’s t test).
Figure 4.7 Increased lipid droplets in TPD52-expressing MDA-MB-231 cells. (A, B) Control cells (parent, vector) and (C) TPD52-expressing (H1D2) MDA-MB-231 cells were stained with BODIPY 493/503 (green) for lipid droplets and visualised using confocal microscopy using X63 magnification. Images were derived from one experiment. Scale bar = 10 µm.
4.2.3 Altered distribution of adipophilin (Adrp) in TPD52- but not TPD52L1-expressing 3T3 cell lines

Next, we examined the effects of stable TPD52 or TPD52L1 expression on PAT protein expression and distribution in 3T3 cells. Since ADRP is the major PAT protein that regulates lipid droplets in non-adipocytes (Brasaemle et al., 1997), we analysed Adrp expression and sub-cellular localization in TPD52- and TPD52L1-expressing 3T3 cell lines, compared to parental and vector-3 control cell lines. Results from immunofluorescent staining of Adrp in two TPD52-expressing 3T3 cell lines, D52-2-7 and D52-2-8, revealed that whereas TPD52 was mainly detected in the perinuclear region, Adrp staining appeared as clustered ring structures (Figure 4.8A and 4.8B, long arrows). These Adrp-stained ring structures resembled lipid droplets (Imamura et al., 2002). In contrast, the parental and vector-3 control cell lines showed more diffuse cytoplasmic Adrp staining (Figure 4.8C and 4.8D). Enlarged images of a D52-2-7 cell (Figure 4.8A), showed detectable but limited co-localization between Adrp and TPD52 within ring structures (short arrows).

Interestingly, the TPD52L1-expressing 3T3 cell lines D52L1-4 and D52L1-6 showed similar Adrp staining patterns to the parental and vector-3 control cell lines (Figure 4.9). The altered distribution of Adrp in TPD52-expressing cells supports increased lipid droplet numbers and sizes in these cell lines (Figures 4.5 and 4.6).
Figure 4.8

A  D52-2-7

   ADRP  TPD52  Merge

B  D52-2-8

C  parent

D  vector-3
Figure 4.8 Altered distribution of adipophilin (Adrp) in TPD52-expressing 3T3 cells. Immunofluorescent staining visualized using confocal microscopy with a X63 objective, demonstrated that Adrp staining (green) was clustered (long arrows) in two TPD52-expressing 3T3 cells (A, B), compared to a more diffuse cytoplasmic distribution of Adrp staining in the parent and vector control cells (C, D, long arrows). Higher magnification (white boxed region, images below A) revealed Adrp structures resembling lipid droplets (long arrows), and co-localization of Adrp and TPD52 (merge, yellow) (short arrows). Cells were stained with guinea-pig polyclonal Adrp (green) and rabbit polyclonal TPD52 (red) antisera, whereas nuclei were stained with DAPI (blue) in combined images on the right (merge). Images shown are representative of those obtained in 3 independent experiments. Scale bar = 20 µm.
Figure 4.9

A  D52L1-4

ADRP

Merge

B  D52L1-6

C  parent

D  vector-3

20 µm
Figure 4.9 Adipophilin (Adrp) distribution in TPD5L1-expressing 3T3 cells is similar to that of parent and vector control cells. Immunofluorescent staining visualized using confocal microscopy with a X63 objective demonstrated that two TPD52L1-expressing 3T3 cells showed similar diffuse cytoplasmic Adrp (green) staining patterns (A, B, long arrows) as 3T3 parent and vector controls (C, D, long arrows). Cells were stained with guinea-pig polyclonal Adrp antisera (green, left panels), whereas nuclei were stained with DAPI (blue) combined images on the right (merge). Images shown are representative of those obtained in 3 independent experiments. Scale bar = 20 μm.
4.2.4 Localization of TPD52 relative to adipophilin (Adrp) and lipid droplets in TPD52-expressing 3T3 cells

The altered distribution of Adrp around structures resembling lipid droplets (Figure 4.8A and 4.8B) in TPD52-expressing 3T3 cells needed to be confirmed. ADRP co-localization with lipid droplets, specifically at the surface of lipid droplets has been shown previously in many different cell types (Brasaemle et al., 1997). We also wanted to compare the localization of TPD52 to that of lipid droplets. Therefore, triple staining of TPD52 (red or pseudo-coloured green), ADRP (green) and lipid droplets (pseudo-coloured magenta) was performed in TPD52-expressing 3T3 cells compared to parental and vector control cells. This was attempted on several occasions, but triple-staining of cells was successful in only one experiment. Microscopy analyses in the D52-2-7 cell line revealed that Adrp-clustered ring structures in the cytoplasm surrounded lipid droplets (Figure 4.10C, long arrows), as also reported in an Adrp-expressing 3T3 cell line (Imamura et al., 2002). Limited TPD52 labelling co-localized with Adrp and also surrounded some lipid droplets (Figure 4.10A and 4.10B, short arrows). The perinuclear staining of TPD52 did not co-localize with Adrp or lipid droplets (Figure 4.10A and 4.10B, open arrows). Therefore, we confirmed that Adrp co-localized to lipid droplets in TPD52-expressing 3T3 cells, which agrees with these cells having increased lipid droplets when compared to vector control cells (Figure 4.10D).
Figure 4.10

(A) D52-2-7

(B) BODIPY

(C) Agrp

(D) vector-3

Nuruliza Roslan
Figure 4.10 TPD52 localization relative to that of adipophilin (Adrp) and lipid droplets in TPD52-expressing 3T3 cells. Immunofluorescent images of the TPD52-expressing 3T3 cells D52-2-7 (A, B, C) and vector-3 cells (D) triple stained with rabbit polyclonal TPD52 (red or pseudo-coloured for green), guinea pig polyclonal Adrp (green) antisera and BODIPY 493/503 (pseudo-coloured for magenta) for lipid droplets. Cells were also treated with DAPI to stain the nuclei (blue). Combined images are shown on the right (merge). Images were visualized using confocal microscopy with a X63 objective. Below each image (A, B) are enlarged images of boxed regions showing limited co-localization between TPD52, Adrp and lipid droplets (small arrowheads), or no co-localization between TPD52 and Adrp or lipid droplets seen at the perinuclear region (A, B; open arrowheads). The expected co-localization of Adrp and lipid droplets was detected in D52-2-7 cells (C, arrows). Adrp was detected as clusters in D52-2-7 cells (A, C) compared to a more diffuse cytoplasmic distribution in vector-3 cells (D). Images shown were from a single experiment. Scale bar = 20 μm.
4.2.5 PAT protein and Fasn levels in TPD52-expressing 3T3 cells, relative to controls

Since there was a significant increase in lipid droplet numbers in the TPD52-expressing 3T3 cells (Figure 4.6A), we investigated PAT protein levels in these cells. Protein lysates of differentiated 3T3-L1 cells (provided by Ms. Sarah Frost) were used as a positive control for perilipin and Adrp expression (Brasaemle et al., 1997). Western blot analyses revealed that perilipin was not detected in the TPD52-expressing 3T3 cells, parental or vector control cells, with the possible exception of D52-2-7 cells (Figure 4.11A). In contrast, perilipin was abundantly detected in differentiated 3T3-L1 cells (Figure 4.11A). Western blot analyses revealed that there were no obvious differences in Adrp levels in either TPD52- or TPD52L1-expressing 3T3 cells, when compared with parent and vector control cells (Figure 4.11A, data not shown). This suggests that Adrp levels are similar in all 3T3 cells examined, and that increased lipid storage in TPD52-expressing cells led to Adrp being re-located to lipid droplets.

We also analysed fatty acid synthase (Fasn) levels in the 3T3 cell lines. Fasn is the major rate-limiting enzyme involved in synthesis of de novo free fatty acids (Menendez and Lupu, 2007). Increased lipid droplet numbers could indicate an increase in available free fatty acids, requiring increased lipid storage to overcome lipotoxicity. However, Western blot analyses revealed that Fasn levels were similar in TPD52-expressing 3T3 cells and the vector control cells (Figure 4.11B), indicating that the increased lipid storage in TPD52-expressing cells was not accompanied by altered Fasn expression.
Figure 4.11 Western blot analyses of PAT protein and Fasn levels in 3T3 cell lines. Total protein extracts from parent, vector only and TPD52-expressing 3T3 cell lines were subjected to Western blot analyses for (A) perilipin, adipophilin (Adrp), TPD52 and (B) fatty acid synthase (Fasn) levels. Gapdh was used as loading control. Differentiated 3T3-L1 adipocytes were used as a positive control for perilipin and Adrp expression. Left, proteins analysed. Right, molecular weights of detected species. Results shown are representative of those from 3 independent experiments.
4.2.6 PAT protein and FASN levels in stably TPD52-expressed MDA-MB-231 cells, relative to controls

We wanted to further confirm our results obtained in Figure 4.11 by investigating PAT protein and FASN levels in TPD52-expressing MDA-MB-231 cells. Protein lysates of differentiated 3T3-L1 cells (provided by Ms. Sarah Frost) were used as a positive control for perilipin (Brasaemle et al., 1997). Western blot results again revealed perilipin was not detected, except in 3T3-L1 cells (Figure 4.12A). ADRP was not detected in TPD52-expressing MDA-MB-231 cells (Figure 4.12A), although it was subsequently confirmed using transient ADRP expression in SK-BR-3 breast cancer cells that Adrp antisera only detect mouse Adrp (S. Frost, unpublished results) and the bands detected in protein lysates of MDA-MB-231 cells were likely non-specific (Figure 4.12A). In contrast, transient expression of perilipin in SK-BR-3 cells confirmed that perilipin antisera detect human perilipin as well (S. Frost, unpublished results). Therefore, we were unable to examine ADRP expression in TPD52-expressing MDA-MB-231 cells. TIP47 and FASN levels were similar in all MDA-MB-231 cell lines examined (Figure 4.12B). The high FASN levels in SK-BR-3 cells (Figure 4.12B) are consistent with previous results (Kumar-Sinha et al., 2003; Yoon et al., 2007; Zhang et al., 2005). Thus, these results together with those shown in Figure 4.11, indicate that increased lipid storage in TPD52-expressing cells is not accompanied by altered perilipin, TIP47 or FASN expression.
Figure 4.12 Western blot analyses of PAT protein and Fasn levels in stably TPD52-expressing MDA-MB-231 cell lines. Total protein extracts from SK-BR-3, vector only and stably TPD52-expressing MDA-MB-231 cell lines (H1D2 and H2D2) were subjected to Western blot analyses for (A) perilipin, adipophilin (Adrp/ADRP), TPD52 and (B) TIP47 and fatty acid synthase (FASN) levels. Gapdh/GAPDH was used as loading control. Differentiated 3T3-L1 adipocytes were used as a positive control for perilipin and Agrp expression. Left, proteins analysed. Right, molecular weights of detected species. Results shown are from a single experiment.
4.2.7 FASN expression and localization in breast cell lines

It has been shown that ERBB2-amplified breast cancer cells have high intracellular lipid content (Kourtidis et al., 2010). Furthermore, in ERBB2-amplified breast cancer cell lines, FASN was found to be associated with ERBB2 expression (Kumar-Sinha et al., 2003). Reduced FASN expression was shown using ERBB2 inhibitors such as Herceptin in the SK-BR-3 breast cancer cell line (Kumar-Sinha et al., 2003). Based on our results showing increased intracellular lipid droplets in TPD52-expressing 3T3 cells (Figure 4.5) and together with significant positive associations between TPD52 and ERBB2 expression in breast cancer cell lines (Chapter 3), we decided to compare TPD52 and FASN expression in a panel of 15 breast cell lines. Western blot results revealed frequent co-expression of TPD52 and FASN (Figure 4.13A), although statistical analysis revealed no significant correlation between FASN and TPD52 levels (Spearman test, \( r_s = 0.418 \); \( p = 0.121 \); \( n = 15 \)). Therefore, TPD52 involvement in lipid droplet regulation does not seem to be associated with FASN expression in this breast cell line cohort, in agreement with results shown in Figures 4.11 and 4.12.

Next, we compared the localizations of FASN and TPD52 in the ERBB2- and TPD52-amplified cell line SK-BR-3 using indirect immunofluorescent staining. FASN was mainly detected in the cytoplasm and at the plasma membrane (Figure 4.13B) as reported previously (Jin et al., 2010), whereas TPD52 showed cytoplasmic, plasma membrane and perinuclear staining (Figure 4.13B). Co-localization of FASN and TPD52 was detected at the plasma membrane, and to a limited extent within the cytoplasm, but FASN did not co-localize with TPD52 within the perinuclear cytoplasm (Figure 4.13B). Together, these results provide preliminary evidence that TPD52 and FASN may be co-localized in breast cancer cell lines, however a larger cell line cohort is needed to confirm this observation.
Figure 4.13

A

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<th>ERBB2</th>
<th>GAPDH</th>
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<tr>
<td>MCF-10A</td>
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</table>

B

FASN  TPD52  Merge

20 µm
Figure 4.13 FASN expression in breast cell lines. (A) FASN levels were compared with those of ERBB2 and TPD52 in a panel of human breast cell lines. Total protein lysates were subjected to Western blot analyses. GAPDH was used as a loading control. Images shown are representative from 3 independent experiments. Western blot images of TPD52, ERBB2 and GAPDH were also shown in Figure 3.2C. Left, proteins analysed. Right, molecular weights of detected species. (B) Immunofluorescent staining of FASN (green) and TPD52 (red) in SK-BR-3 cells showing cytoplasmic and plasma membrane distributions with focal areas of colocalization (arrows). Cells were co-stained with mouse monoclonal FASN antibody (green) and rabbit polyclonal TPD52 antisera (red), with combined images shown on the right (merge). Images were visualized using confocal microscopy using a X63 objective. The results from a single experiment are shown. Scale bar = 20 μm.
4.2.8 TIP47 expression and localization in breast cell lines

TIP47 has been reported to be involved in the trafficking of lipids (Wolins et al., 2001). To date, no study has compared TIP47 and TPD52 expression in any cell line. Initially, we wished to examine Tip47 expression and sub-cellular distribution in 3T3 cells, but the TIP47 antisera proved to only detect human TIP47 (data not shown). Therefore, Western blot analyses of TIP47 compared to TPD52 levels in 8 breast cell lines was performed by Ms Sarah Frost. TIP47 was detected in all cell lines, with no obvious association between TIP47 and TPD52 levels (Figure 4.14A). Interestingly, indirect immunofluorescent staining showed co-localization of TPD52 and TIP47 in distinct ring structures in SK-BR-3 cells (Figure 4.14B, arrows), which resembled lipid droplets previously detected in HeLa cells (Smirnova et al., 2006; Wolins et al., 2001). Either rabbit polyclonal TPD52 antisera or mouse monoclonal TPD52 antibody were used in 2 independent experiments. One experiment used rabbit polyclonal TPD52 and one experiment used mouse monoclonal TPD52, with guinea pig polyclonal TIP47 antisera, and both TPD52 antisera produced the same co-localization result (Figure 4.14B, data not shown).
Figure 4.14

A

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<th>BT-483</th>
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47kDa

23kDa

37kDa

B

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10 µm
Figure 4.14 TIP47 expression in breast cell lines. (A) Total protein lysates were subjected to Western blot analyses. GAPDH was used as a loading control. Images shown are representative from 3 independent experiments. Left, proteins analysed. Right, molecular weights of detected species. (B) Immunofluorescent staining of TIP47 (green) and TPD52 (red) in the TPD52-amplified cell line SK-BR-3 showing distinct co-localization of TIP47 with TPD52 in ring structures (arrows, merge). Cells were co-stained with guinea-pig polyclonal TIP47 (green) and rabbit polyclonal TPD52 (red) antisera, with combined images shown on the right (merge). Images were visualized using confocal microscopy using a X63 objective. Images shown are representative from a total of 2 independent experiments, in which rabbit polyclonal TPD52 antisera and mouse monoclonal TPD52 antibody were each used once. Scale bar = 20 μm.
4.2.9 Oleic acid uptake by TPD52-expressing versus control 3T3 cells

Since we demonstrated that TPD52-expressing 3T3 cells showed increased numbers of lipid droplets per cell compared with parent and vector control cells (Figure 4.6A), we queried whether these cells would also show an increased capacity to take up and store lipid, compared with parent and vector control cells. We decided to treat 3T3 cell lines with oleic acid, an unsaturated fatty acid which is not toxic to cultured cells over short incubation periods (< 24 h). The chosen concentration of 400 µM oleic acid has been used in numerous reports using different cell types such as mouse 3T3-L1 cells (Beller et al., 2008; Brasaemle et al., 2000), CHO cells (Tansey et al., 2003), HEK 293, MCF-7, HeLa, and BHK cells (Listenberger et al., 2007). We therefore treated 3T3 cell lines with 400 µM oleic acid for 24 h, and compared these with cells cultured in media only and harvested at 0 h to obtain a baseline control. Cells were stained with BODIPY 493/503 to visualize lipid droplets using confocal microscopy.

Confocal microscopy analyses revealed that 24 h oleic acid treatment produced increased lipid droplets in all cell lines, relative to those detectable prior to oleic acid treatment (Figure 4.15). However, more lipid droplets were detected in all TPD52-expressing 3T3 cells, relative to parent and vector control cells (Figure 4.15). This was confirmed by quantitation of BODIPY staining. There was a significant ~2-fold increase in the mean lipid droplet number per cell in the 3 TPD52-expressing 3T3 cells, compared with the parent and vector control cells (Figure 4.16A). Interestingly, all 3 TPD52-expressing 3T3 cells showed very similar mean lipid droplet numbers per cell after oleic acid treatment, despite different basal numbers of lipid droplets per cell (Figure 4.16A). The mean lipid droplet numbers per cell in parent and vector control cells were very similar before and after oleic acid treatment.
Figure 4.15

A

Media only (0 h)

400 µM oleic acid (24 h)

B

D52-2-7

20 µm

20 µm

D52-2-1

20 µm

20 µm

D52-1-12

20 µm

20 µm

vector-3

20 µm

20 µm

parent

20 µm

20 µm

Nuruliza Roslan
Figure 4.15 Lipid droplets following oleic acid treatment of 3T3 cell lines. (A-C) TPD52-expressing, (D) vector or (E) parent 3T3 cell lines were incubated with 400 µM oleic acid for 24 h (right panel), and compared with the same cell lines harvested at 0 h, grown in media only (left panel). Cells were stained for lipid droplets using BODIPY 493/503 (green) and visualized by confocal microscopy using a X63 objective. (A) An enlarged image of a D52-2-7 cell (white boxed region, right hand panel) following incubation with 400 µM oleic acid for 24 h is shown at the far right. Images shown are representative of those obtained in 3 independent experiments. Scale bar = 20 µm.
Figure 4.16

A. Mean lipid droplets/cell

B. Relative fold change
Mean lipid droplets/cell

C. Mean lipid droplet diameter (µm)

D. Relative fold change
Mean lipid droplet diameter (µm)

E. Mean lipid droplet area (µm²)

F. Relative fold change
Mean lipid droplet area (µm²)
Figure 4.16 BODIPY staining quantitation before and after oleic acid treatment of 3T3 cell lines. Confocal images of 10 random fields of view were taken of all cell lines before (0 h) or after 400 μM oleic acid (24 h), that were stained with BODIPY 495/503 as per Figure 4.14. Images were analysed using Image Pro Plus 5.1 for mean lipid droplet numbers per cell (A), mean lipid droplet diameter (C) and mean lipid droplet area (E). Relative fold changes in mean lipid droplet numbers per cell (B), mean lipid droplet diameter (D) or mean lipid droplet area (F) were calculated from the results shown in A, C and E respectively. Data are presented as means ± SE, from 3 independent experiments. Significantly different comparisons between groups (TPD52-expressing 3T3 cells were grouped together, compared to parent and vector-3 control cells) are shown with the corresponding p values (Student’s t test).
We then analysed the relative fold change of mean lipid droplet numbers per cell before and after oleic acid treatment. There were ~18-fold and ~14-fold increases in mean lipid droplet numbers per cell in parent and vector control cells, respectively, after 24 h oleic acid treatment (Figure 4.16B). In contrast, TPD52-expressing 3T3 cells showed a ~4-fold increase (D52-1-12 and D52-2-1 cells), and a ~2-fold increase (D52-2-7 cells) in mean lipid droplet numbers per cell (Figure 4.16B). This meant that even though oleic acid-treated TPD52-expressing 3T3 cells formed more lipid droplets then controls (Figure 4.16A), parent and vector control cells showed significantly greater relative increases in lipid droplet numbers (Figure 4.16B).

Under basal conditions, D52-2-7 cells showed the highest lipid droplet numbers per cell and also showed the highest TPD52 levels (Figure 4.16A and 4.17A). D52-2-7 cells also showed the lowest relative fold increase in lipid droplet numbers per cell of any cell line examined (Figure 4.16B). Interestingly, while the relative increases in lipid droplet numbers varied between the 3 TPD52-expressing 3T3 cell lines, they all showed very similar lipid droplet numbers per cell after oleic acid treatment (Figure 4.16A).

Increased lipid droplet numbers per cell after 24 h oleic acid treatment were accompanied by increased lipid droplet diameters and areas (Figure 4.16C and 4.16E). The apparent increase in lipid droplet diameter and area might be due to lipid droplets being in clusters (Fig 4.9), and this will be discussed further (Section 4.3). An enlarged image of a D52-2-7 cell after 24 h oleic acid treatment showed that droplets were relatively uniform in size (Figure 4.15A, far right), and this is supported by the small error bars obtained from the analysis of mean lipid droplet diameter (Figure 4.16C). Although BODIPY quantitation revealed that there were significant increases in lipid droplet diameter and area in TPD52-expressing cells when compared to controls (Figure 4.16C and 4.16E), relative fold changes of these
parameters were similar in all 3T3 cell lines analysed (Figure 4.16D and 4.16F). The significant increases in lipid droplet diameter and size in TPD52-expressing cells were noted to be largely driven by D52-2-7 cells (Figure 4.16C and 4.16E). In summary, all cell lines showed larger lipid droplets following oleic acid treatment, and this was not primarily determined by TPD52 expression status.

4.2.10 PAT protein, Fasn and TPD52 levels remained unchanged in TPD52-expressing 3T3 cells after oleic acid treatment

We wanted to determine whether oleic acid treatment could affect PAT protein and Fasn levels in 3T3 cell lines. Therefore, we harvested total protein lysates from these cell lines after 0 h, 6 h, and 24 h of 400 μM oleic acid treatment (Figure 4.17A). Western blot and densitometry analyses revealed that Adrp levels were increased after 6 h and 24 h oleic acid treatment in all 3T3 cell lines (Figure 4.17A, data not shown), as expected (de Wilde et al., 2010; Fan et al., 2009; Masuda et al., 2006), with similar increases noted in all 3T3 cell lines. In contrast, Fasn, TPD52 and perilipin levels were not obviously affected by oleic acid treatment in any cell line (Figure 4.17, data not shown). Collectively, Western blot and densitometry results revealed no significant changes in perilipin, Fasn or TPD52 levels in TPD52-expressing 3T3 cells after 400 μM oleic acid treatment for 6 h or 24 h.
Figure 4.17 Adrp, Fasn, TPD52 and perilipin levels after oleic acid treatment of 3T3 cell lines. Western blot analyses of (A) Adrp, Fasn and TPD52 levels (A) and (B) perilipin and TPD52 levels in total protein lysates from the indicated 3T3 cell lines treated with 400 µM oleic acid and harvested at 0 h, 6 h or 24 h. A representative Western blot image is shown from 3 independent experiments. Differentiated 3T3-L1 adipocytes were used as a positive control for perilipin (B). Gapdh was used as a loading control. (*) Non-specific staining. Left, proteins analysed. Right, molecular weights of detected species.
4.2.11 TPD52 localization relative to that of Adrp, lipid droplets, and Pdi (ER marker) in TPD52-expressing 3T3 cells following oleic acid treatment

In Section 4.2.3, we described altered Adrp distribution in TPD52-expressing 3T3 cells. We therefore compared the localizations of TPD52, Adrp and lipid droplets in TPD52-expressing 3T3 cells compared with control cells after oleic acid treatment, using indirect immunofluorescence microscopy. Immunofluorescent staining revealed changes in TPD52 distribution in TPD52-expressing 3T3 cells after oleic acid treatment (Figure 4.18B), compared with TPD52 localization prior to treatment (Figure 4.18A). TPD52 staining increased within the perinuclear region, relative to untreated cells (Figure 4.18A and 4.18B). In addition, there were also more “spider-web”-like projections within the perinuclear region that were revealed by TPD52 staining (Figure 4.18B, inset). Lipid droplets were either detected between or in close proximity to these structures (Figure 4.18B).

Immunofluorescent images of vector control cells showed more pronounced Adrp staining after oleic acid treatment (Figure 4.18G and 4.18H), in agreement with the results of Western blot analyses (Figure 4.17A). TPD52-expressing 3T3 cells also showed an increase in Adrp staining, in agreement with the results of Western blot analyses (Figure 4.17A) and increased Adrp-associated lipid droplets (Figure 4.18E and 4.18F). Adrp was detected near lipid droplets before and after oleic acid treatment in both cell lines (Figure 4.18E-4.18H). Limited co-localization of TPD52 and Adrp was detected before or after oleic acid treatment of D52-2-7 cells (Figure 4.18I-4.18J). Similarly, TPD52 showed limited co-localization with lipid droplets (Figure 4.18A and 4.18B).
Figure 4.18

D52-2-7 (Media only – 0 h)

A

BODIPY | TPD52 | merge

D52-2-7 (400 µM oleic acid – 24 h)

B

BODIPY | TPD52 | merge

vector-3 (Media only – 0 h)

C

BODIPY | TPD52 | merge

vector-3 (400 µM oleic acid – 24 h)

D

BODIPY | TPD52 | merge
Figure 4.18 (continued)

D52-2-7 (Media only – 0 h)

E  

D52-2-7 (400 µM oleic acid – 24 h)

F  

vector-3 (Media only – 0 h)

G  

vector-3 (400 µM oleic acid – 24 h)

H
Figure 4.18 (continued)

I. D52-2-7 (Media only – 0 h)

J. D52-2-7 (400 µM oleic acid – 24 h)

K. vector-3 (Media only – 0 h)

L. vector-3 (400 µM oleic acid – 24 h)
Figure 4.18 TPD52 localization relative to that of Adrp and lipid droplets before and after oleic acid treatment. 3T3 cell lines were treated with media only (0 h) or with 400 μM oleic acid, and harvested after 24 h. Immunofluorescent images of D52-2-7 cells (A, B, E, F, I, J) and vector-3 control cells (C, D, G, H, K, L) triple stained with rabbit polyclonal TPD52 antisera (red or pseudo-coloured as green), guinea pig polyclonal ADRP antisera (green) and BODIPY 493/503 (pseudo-coloured as magenta), and analyzed with confocal microscopy using a X63 objective. Cells were also treated with DAPI (blue) to stain the nuclei, shown in combined images on the right (merge). Below images of D52-2-7 cells (A, B, E, F, I, J) are enlarged images of boxed regions showing co-localization between TPD52, Adrp and/or lipid droplets (small arrowheads), a lack of co-localization between TPD52 and lipid droplets (A, B) or Adrp (I, J) within the perinuclear region, and enhanced TPD52 staining after oleic acid treatment (A, B, I, J; open arrowheads). Adrp and lipid droplets co-localized in D52-2-7 and vector-3 cells as anticipated (E, F, H; shown with arrows). Without oleic acid treatment, Adrp was detected in clusters in D52-2-7 cells (E) and showed a more diffuse cytoplasmic distribution in vector control cells (G). A single experiment was performed. BODIPY staining in (A, E, I, C, G, K) was also shown in Figure 4.10. Images in (A, B, C, D) are repeated in (E, F, G, H) respectively. TPD52 staining shown in (A, B, C, D) is repeated in (I, J, K, L), respectively. Adrp staining shown in (E, F, G, H) is repeated in (I, J, K, L) respectively. Scale bar = 20 μm.
Studies have reported that lipid droplets can be formed by budding from the endoplasmic reticulum (ER) membrane and have been demonstrated to be in close proximity with the ER membrane (Blanchette-Mackie et al., 1995; Ozeki et al., 2005; Tauchi-Sato et al., 2002; Wan et al., 2007). Since we noticed perinuclear projections stained for TPD52 in D52-2-7 cells after 24 h 400 µM oleic acid treatment (Figure 4.18A, 4.18B, 4.18l, 4.18J), we examined whether these structures could be associated with the ER. Protein disulfide isomerase (PDI) protein was previously used as an ER marker which stains the ER membrane in a lace-like pattern (Ohsaki et al., 2008), and we therefore compared this with TPD52 in D52-2-7 cells. Pdi staining was detected throughout the cytoplasm in both D52-2-7 and vector control cells, before and after 24 h 400 µM oleic acid treatment (Figure 4.19). TPD52 and Pdi did not obviously co-localize in D52-2-7 cells, either before or after oleic acid treatment (Figure 4.19A and 4.19B). The perinuclear TPD52 projections noted following 24 h 400 µM oleic acid treatment therefore did not co-localize with the ER marker Pdi (Figure 4.19B).
Figure 4.19

A. D52-2-7 (Media only – 0 h)

B. D52-2-7 (400 µM oleic acid – 24 h)

C. vector-3 (Media only – 0 h)

D. vector-3 (400 µM oleic acid – 24 h)
Figure 4.19 TPD52 localization relative to that of Pdi in TPD52-expressing 3T3 cells. 3T3 cell lines were treated (A, C) with media only (0 h) or (B, D) with 400 μM oleic acid and harvested after 24 h. (A, B) D52-2-7 and (C, D) vector control cells were stained with mouse monoclonal TPD52 antibody (red) and rabbit polyclonal PDI antisera (green) and analyzed by confocal microscopy using a X63 objective. Combined images are shown on the right (merge). Below images of D52-2-7 cells (A, B) are enlarged images of boxed regions showing the lack of co-localization between TPD52 and Pdi (arrows) in cells treated or not treated with oleic acid. A single experiment was performed. Scale bar = 20 μm.
4.3 Discussion

The work in this chapter explored a novel function for TPD52 in the regulation of lipid storage. Previous studies suggest TPD52 as a potential oncogene (Adler et al., 2006; Balleine et al., 2000; Byrne et al., 2012; Liu et al., 2007b; Shehata et al., 2008a). However, the underlying mechanisms are still elusive. Data presented in this chapter suggest that TPD52 may promote tumorigenesis via regulation of lipid storage, possibly through interactions with PAT proteins (Yamaguchi et al., 2006; Zhang et al., 2007b). The increase in intracellular lipid droplets produced in response to TPD52 expression may assist to meet the high energy demands of tumor cells.

4.3.1 A lipogenic phenotype in TPD52-expressing 3T3 cells

To date, a number of studies have suggested links between TPD52 and lipid droplet regulation (Ashrafi et al., 2003; Yamaguchi et al., 2006; Zhang et al., 2007b). For the first time, data presented in this chapter revealed that TPD52 expression increased lipid droplet numbers in 3T3 cells. This was based on the analyses of images of BODIPY stained lipid droplets (Figures 4.5 and 4.6), supported by electron microscopy images (Appendix 1). In addition, increased lipid content, specifically triacylglyceride in TPD52-expressing 3T3 cells has been recently confirmed by Dr. Alvin Kamili detected using an enzymatic triacylglyceride assay (A. Kamili, unpublished results). A similar result was obtained when mouse Adrp was overexpressed in 3T3 cell lines, resulting in increased lipid droplets as revealed by Oil Red O staining, without induction of other adipocyte-specific or lipogenic genes (Imamura et al., 2002).

TPD52 expression in 3T3 cells led to increased proliferation and anchorage-independent growth which are characteristic of transformed cells (Shehata et al.,
The increased proliferation of TPD52-expressing 3T3 cells might indicate an increase in energy demand which subsequently might increase utilization of lipids from lipid storage through the β-oxidation pathway. As mentioned before, increased β-oxidation has been reported to occur in prostate cancer and glioblastoma cells (Section 1.4.3.1.3). Therefore, it would be of interest for future studies to investigate the rate of β-oxidation as well as the levels and activities of the enzymes involved, such as carnithine palmitoyl (CPT) and acetyl-CoA oxidase (ACO).

TPD52-expressing cell lines showed increased lipid droplet numbers per cell, with a mean diameter of ~1 μm (Figure 4.6). Most lipid droplets in non-adipocytes are smaller than those in adipocytes (Suzuki et al., 2011). Lipid droplets are usually ~1 μm or smaller in non-adipocytes, compared with ~100 μm in size for adipocytes. Therefore, in TPD52-expressing 3T3 cells the greater increase in lipid droplet numbers, as opposed to size, suggests that TPD52 regulates lipid droplet formation as opposed to the growth and/or fusion of lipid droplets. This observation was also reported in Drosophila S2 cells, which upon oleic acid treatment, formed numerous clusters of lipid droplets which were smaller in size than the large lipid droplets seen in mature adipocytes (Guo et al., 2008).

Analyses of TPD52 expression in a human cell line model may be more informative in terms of understanding the role of TPD52 in human cancer pathogenesis. Therefore, we provided preliminary results of increased lipid droplets in exogenously TPD52-expressing MDA-MB-231 breast cancer cell lines (Figure 4.7). It will be important to repeat the lipid droplet staining using both TPD52- and TPD52L1-expressing MDA-MB-231 breast cancer cell lines for further confirmation. Overall, these results provide the first direct evidence that TPD52 expression produces increased lipid droplet numbers in fibroblasts (Figure 4.5) and the breast cancer cell
line MDA-MB-231 (Figure 4.7). Ideally, these findings should be confirmed upon transient TPD52 overexpression to exclude aberrant lipid metabolism due to cell selection following stable and constitutive TPD52 overexpression. However, preliminary findings of transient TPD52 expression (for 72 h) in breast cell lines (BT-474, 2 experiments; MCF-7, HS-578T, and MCF-10A, a single experiment each) showed that FASN levels remained unchanged.

Following oleic acid treatment, TPD52 expression allowed 3T3 cells to form more lipid droplets than either vector control or parental cells (Figure 4.15). However, TPD52-expressing 3T3 cells showed lesser relative increases in lipid droplet numbers relative to either vector control or parental cells (Figure 4.16A). The fact that the three TPD52-expressing 3T3 cell lines showed very similar mean lipid droplet numbers per cell after oleic acid treatment might indicate that these cells had reached a maximal capacity to store lipid. Vector control and parental cells also demonstrated very similar lipid droplet numbers per cell after oleic acid treatment. Further investigations such as more prolonged incubations with oleic acid, as well as investigating the cell proliferation effects of oleic acid treatment would extend the findings of this study. Nevertheless, the fact that parental and vector control cell lines showed greater relative increases in lipid droplet numbers than TPD52-expressing cells might suggest that TPD52 does not function primarily by regulating lipid uptake (Figure 4.16B). Radiolabelled fatty acids could be used as a more accurate way to measure the level and rate of fatty acid uptake in future studies.

We acknowledge that there were limitations with the software used to quantitate BODIPY stained lipid droplets, which may have led to an underestimation of lipid droplet numbers. This was most likely in TPD52-expressing 3T3 cells after oleic acid treatment which showed many lipid droplets clustered together (Figure 4.15). Lipid droplets could be distinguished as individuals visually (Figure 4.15A), but this was not
always possible using Image ProPlus 5.1 software. Therefore, numbers of individual lipid droplets in TPD52 expressing 3T3 cells after 24 h oleic acid treatment might actually be higher than presented (Figure 4.16A). However, these analyses still demonstrated a significant increase in lipid droplet numbers in TPD52-expressing 3T3 cell lines compared to parent and vector controls, both at baseline and after 24 h oleic acid treatment (Figure 4.16A). Other approaches are required to confirm this result, and currently this is on-going, by measuring total lipid content in these cell lines using an enzymatic triacylglyceride assay. We are also optimizing conditions for further analysing the lipid content and specifically identifying lipid species using mass spectrometry.

In non-adipocytes, ADRP is known to coat lipid droplets and to be the major PAT protein regulating their formation (Brasaemle et al., 1997) and lipolysis (Listenberger et al., 2007). ADRP has been found to surround smaller lipid droplets in 3T3-L1 pre-adipocytes and during early adipocyte differentiation, but not in mature adipocytes where larger lipid droplets are surrounded by perilipin (Brasaemle et al., 1997). The uniform size of lipid droplets seen post oleic acid treatment was increased in TPD52-expressing 3T3 cells, compared to parental and vector control cells (Figure 4.16C and 4.16E). However, the relative fold change in lipid droplet size was comparable for all cells (Figure 4.16D and 4.16F). This suggests that lipid droplets in these cells were commonly restricted in their capacity to increase in size, following oleic acid treatment. Furthermore, this could possibly be attributed to Adrp and not perilipin, being the major PAT protein expressed in these 3T3 cells which also co-localized with lipid droplets (Figure 4.18E, F, H). As mentioned previously, Adrp has been shown to be involved in lipolysis (Listenberger et al., 2007), where Adrp overexpression was shown to reduce the association of ATGL and TIP47 with lipid droplets. Therefore, in our 3T3 cells, once the lipid droplets have reached their maximal storage capacity, Adrp might also activate lipolysis and this will increase the
intracellular free fatty acid content. In response to this, the cells will then activate lipid
droplet formation in order to prevent lipotoxicity due to intracellular free fatty acids.
However, these are only assumptions that warrant further investigations. The smaller
size lipid droplets in most non-adipocytes are assumed to be more efficient as an
intracellular energy source (Suzuki et al., 2011).

Electron microscopy results processed and imaged by our collaborator Dr. Dongwei
Wang (Appendix 1), further confirmed our BODIPY staining results, which showed
more lipid droplets in D52-2-7 cells (Appendix 1B) when compared with vector
controls (Appendix 1A). Lipid droplets were noted to be in close proximity as well as
attached to the ER (Appendix 1B). Lipid droplet formation from the ER has been
proposed by numerous investigators (Brasaemle et al., 2004; Liu et al., 2004; Murphy
and Vance, 1999; Robenek et al., 2006; Wan et al., 2007). Interestingly, we also
noted numerous whorled, laminated structures in D52-2-7 cells (Appendix 1B) when
compared to vector controls. These structures resembled phospholipid inclusions
(O’Farrell et al., 2001) although this would need further confirmation. Alternatively,
these structures may be lysosome intermediates, possibly caused by lipid overload
(Sathasivam et al., 2003). This is interesting as exogenous TPD52 expression using
Chinese Hamster ovary (CHO-K1) cells was shown to co-localize with lysosome-like
secretory organelles and with Rab27, vesicle-associated membrane protein 7
(VAMP7) and lysosomal-associated membrane protein LAMP1 (Thomas et al.,
2009). Recently, we also detected partial TPD52 co-localization with LAMP1 in
TPD52-expressing 3T3 cells (data not shown). Therefore, it will be interesting to
determine whether TPD52 localization with lysosomes reflects phospholipid
inclusions within the TPD52-expressing 3T3 cells.

Electron microscopy results also indicated that the ER of D52-2-7 cells was swollen
compared to vector control cells (Appendix 1). Studies have shown that ER stress
plays an important role in regulating lipid metabolism and homeostasis (Fu et al., 2011; Gentile et al., 2011; Guo et al., 2007; Wei et al., 2006). ER-stress-dependent dysregulation of lipid metabolism has been reported to cause diseases such as dyslipidemia, insulin resistance, cardiovascular disease, type 2 diabetes, and obesity (Basseri and Austin, 2012). Saturated fatty acids such as palmitate and stearate are known to induce ER stress in various cell types, as well as modulating intracellular signaling such as survival and apoptosis (Guo et al., 2007; Wei et al., 2006). These preliminary electron microscopy observations suggest that ER stress could be occurring in TPD52-overexpressing 3T3 cells, due to the accumulation of lipids. This could be further investigated using ER stress markers such as Grp78 (glucose-regulated protein-78), Grp94 (glucose-regulated protein-94), calreticulin, and CHOP (CAAT/enhancer binding protein (C/EBP) homologous protein) (Samali et al., 2010).

We are currently optimizing conditions for electron immunogold labelling of TPD52 in 3T3 cell lines, which has never been achieved previously. This would give more detailed information as to where exactly TPD52 is localizing within the cell. Previously, EM images have revealed that labelling of endogenous TPD52 localized to vesicular structures in pancreatic acinar cells (Thomas et al., 2004). This result was further confirmed with EM images showing dual labelling of TPD52 and EEA-1 (an early endosomal marker) in these acinar cells. However it is not known whether these cells also show a lipogenic phenotype, and so the localization of TPD52 in pancreatic acinar cells may not resemble that of TPD52 when expressed in 3T3 cells.
4.3.2 How the lipogenic phenotype in TPD52-expressing 3T3 cell lines may be produced

4.3.2.1 Fatty acid synthase – FASN

As explained previously, high levels of FASN have been found in breast cancer cell lines (Kumar-Sinha et al., 2003; Zhang et al., 2005) which also showed high intracellular lipid content (Kourtidis et al., 2010; Kourtidis et al., 2009). Although we observed the focal co-localization of TPD52 and FASN proteins towards the plasma membrane of SK-BR-3 cells (Figure 4.13B), there was no significant correlation between TPD52 and FASN levels in breast cell lines, but this needs to be confirmed in a larger cell line cohort. Nevertheless, Fasn/FASN levels were comparable in both TPD52-expressing 3T3 and MDA-MB-231 cells, compared to relevant vector control cells (Figure 4.11B and 4.12B). Hence, these results indicate that TPD52 may not directly affect FASN expression, and may instead affect other enzymes involved in de novo fatty acid synthesis. The precursor for de novo fatty acid synthesis is acetyl-CoA, which can be obtained through glycolysis or glutaminolysis (Section 1.4.2.1). Cancer cells have been reported to have high glycolysis rates leading to high intracellular lipid content from de novo fatty acid synthesis (Lunt and Vander Heiden, 2011). Thus, it would be worth comparing the levels of enzymes in glycolytic and glutaminolytic pathways, which provide the precursor for de novo fatty acid synthesis.

4.3.2.2 PAT proteins – Perilipin, Adipophilin and TIP47

Initially, TPD52 has been found in the lipid droplet fraction of Drosophila (Cermelli S. et al, 2006). However, to date, there is no biochemical evidence showing direct interactions between TPD52 and lipid droplets. However, previous results (Yamaguchi et al., 2006; Zhang et al., 2007b) and those presented in this chapter
suggest that TPD52 might regulate lipid storage via associating with PAT proteins, specifically ADRP and TIP47. Interactions between TPD52 with TIP47 and ADRP were also supported by direct interaction testing in the yeast-two-hybrid system (S. Frost, unpublished results).

Immunofluorescent staining showed altered Adrp distribution in TPD52-expressing 3T3 cells relative to controls, with Adrp surrounding lipid droplets in TPD52-expressing 3T3 cells (Figure 4.8 and 4.10C). Ectopic Adrp expression is known to stimulate lipid droplet formation and accumulation (Imamura et al., 2002). As Adrp levels remained unchanged in both TPD52-expressing and vector control 3T3 cells (Figure 4.11A), TPD52 may not regulate Adrp levels, and instead Adrp could re-locate to lipid droplets as a consequence of increased lipid droplet formation. It would be valuable to analyze ADRP expression in the panel of breast cell lines used in this study, but the available ADRP antisera did not detect human ADRP (Figure 4.12).

4.3.2.3 Prominent perinuclear TPD52 staining post oleic acid treatment of 3T3 cells

We noted more prominent and enhanced staining of TPD52 at the perinuclear region after oleic acid treatment of TPD52-expressing 3T3 cells. That this staining had a spider-web-like appearance in some cells suggested that this might reveal the ER. Lipid droplets have been proposed to be synthesized in and bud off from the ER (Murphy and Vance, 1999). However, the ER marker Pdi did not co-localize with TPD52, including within the perinuclear region (Figure 4.19A and 4.19B). It was nonetheless interesting to note that TPD52 was detected in close proximity to and/or surrounding lipid droplets, although their co-localization was not frequently noted. Knowing TPD52’s involvement with vesicular trafficking (Thomas et al., 2010; Thomas et al., 2002; Thomas et al., 2009; Thomas et al., 2004) and its interaction
with TIP47 (S. Frost, unpublished results), the TPD52-positive structures might function as passages for lipid transfer/trafficking/delivery. Lipid droplets are also known to be transported on microtubules (Welte et al., 1998), therefore co-staining 3T3 cells with TPD52 and with microtubule markers such as dynein (Bostrom et al., 2005) would also be worth considering. These structures could also be associated with the Golgi apparatus, which has been shown to be involved in lipid trafficking (Surma et al., 2012). Regardless of the underlying mechanism, the re-distribution of TPD52 after oleic acid treatment warrants further investigation.

### 4.3.3 Increased lipid storage is an isoform-specific function of TPD52

As described in Chapter 1, TPD52L1 is 52% identical/66% conserved with respect to TPD52 at the amino acid level (Byrne et al., 1996), which includes differences in alternatively spliced regions (Byrne et al., 1998b). However, Y2H and other studies have found both TPD52 and TPD52L1 to have common binding partners (Byrne et al., 1998b; Shahheydari, 2013; Wilson et al., 2001). The results presented in this chapter highlight the possibility of a novel, isoform-specific function for TPD52 in regulating lipid storage, potentially mediated via interactions with PAT proteins.

Unlike TPD52, TPD52L1 did not promote proliferation and anchorage-dependent growth in 3T3 cells, and transient TPD52L1 knock down did not induce apoptotic cell death in SK-BR-3 cells (Shehata et al 2008a). Using 3T3 cells as a model, we discovered that TPD52 but not TPD52L1 expression led to increased intracellular lipid droplets (Figure 4.5). TPD52L1-expressing 3T3 cells also showed similar Adrp staining patterns as parent and vector control cells (Figure 4.9). Similarly, TIP47 and ADRP were found to interact with TPD52 but not TPD52L1 in the yeast-two-hybrid system respectively (S. Frost, unpublished results). The differential interaction of TPD52 and TPD52L1 with TIP47/ADRP could therefore for the first time provide a
molecular explanation for the isoform-specific oncogenic functions of TPD52. TPD52 has been proposed to represent a signaling intermediate and regulator of vesicle trafficking (Thomas et al., 2009; Thomas et al., 2004). Thus, TPD52 may also function as an adaptor protein that works in parallel with TIP47 in trafficking lipids throughout the cytoplasm.

### 4.4 Summary and conclusions

In summary, results from this chapter demonstrate that TPD52 expression led to markedly increased lipid droplet numbers in 3T3 cells. In contrast, this was not observed in TPD52L1-expressing 3T3 cells. In addition, after oleic acid treatment, TPD52-expressing cells formed increased numbers of lipid droplets, although these relative increases were less than those measured in vector control cells. The mechanisms whereby TPD52-expressing cells form or accumulate more lipid droplets need further investigation. These may be underpinned by interactions with lipid droplet-associated protein, such as TIP47 and ADRP which may account for the isoform-specific oncogenic functions of TPD52. Taken together, the results in this chapter highlight the importance of TPD52 in regulating intracellular lipid storage, and identify a potentially important functional difference between TPD52 and TPD52L1.
Chapter 5

General Discussion
5.1 Introduction

Despite the increasing recognition of the significance of TPD52 overexpression in various cancer types, including breast, prostate, ovarian, lung and colorectal cancers (discussed in Chapter 1, Section 1.6.3), little is known about how increased TPD52 expression advantages cancer cells. Studies of TPD52-transfected fibroblasts revealed TPD52 regulation of cell proliferation and anchorage-independent growth in vitro (Shehata et al., 2008a) as well as formation of subcutaneous tumors and lung metastases in vivo (Lewis et al., 2007). However, the molecular functions of TPD52 and related proteins remain obscure. The current project aimed to understand the molecular basis of TPD52 oncogenic functions. Data presented in this thesis demonstrate TPD52 as a survival factor in ERBB2-amplified breast cancer cell lines (Chapter 3) and propose a novel function for TPD52 in regulating lipid storage (Chapter 4).

5.2 TPD52 promotes the survival of ERBB2-positive cancer cells

Findings from Shehata et al. (2008a) and others (Ummanni et al., 2008; Zhang et al., 2011; Zhang et al., 2007a) reported that reduced TPD52 expression in vitro leads to cell death. Combined with frequent reports of TPD52 amplification and overexpression in various cancers, this suggested that TPD52-amplified cancer cell lines may be more sensitive to reduced TPD52 expression, which would in turn highlight TPD52 as a potential therapeutic target in TPD52-amplified cancers. Reports of TPD52 and ERBB2 co-expression in cancers (Chapter 1, Section 1.6.3.1) also led us to hypothesise that TPD52 may co-operate with ERBB2 in cancer pathogenesis and that TPD52 could represent a potential therapeutic target in ERBB2-positive cancers. Through RNA interference approaches, we sought to examine the transient and long-term effects of reduced TPD52 expression. To date,
no study had directly characterised TPD52 expression status in breast cancer cell lines, and no-long term study of TPD52 knock-down had been performed in breast cancer cell lines.

Firstly, our breast cell line cohort showed that TPD52 and ERBB2 were significantly co-expressed, and this was supported by RT-PCR data from breast cancer patient samples (Roslan et al., 2013; Appendix 2). Secondly, we showed that transient reduction of TPD52 in two ERBB2-amplified cell lines did not affect pERBB2 and pAkt or pMAPK levels, but still induced significant cell death. Hence, TPD52 may act independently of Akt and MAPK signaling in regulating cell survival and TPD52 and ERBB2 may function independently of each other. Thirdly, long-term depletion of TPD52 in a TPD52- and ERBB2-amplified cell line revealed an inverse relationship between TPD52 and pERBB2 levels, and that cells may compensate for reduced TPD52 levels by increasing ERBB2 signaling to maintain cell survival. A more detailed understanding of changes in stably TPD52-depleted SK-BR-3 cell lines could be pursued at the genetic level, by performing expression microarray analysis. This might help to identify key genes and signaling pathways altered in response to long-term TPD52 knock-down.

Knock-down of oncogenes has been reported to affect phenotypes other than cell proliferation or survival. Knock-down of human pituitary tumor-transforming gene 1 (PTTG1), an oncogenic transcription factor, did not affect cell proliferation but reduced breast cancer cell invasion and metastasis by regulating GEF-H1/RhoA signaling (Liao et al., 2012). Knock-down of a new amplification target SMURF1 also did not affect cell proliferation, but reduced invasion in pancreatic cancer cells (Kwei et al., 2011). Data from our group have shown that TPD52 overexpression in 3T3 fibroblasts led to increased migration and invasion (Della Franca, 2012). Therefore, future experiments could investigate the migration and invasion capability of stably...
TPD52-depleted SK-BR-3 cells. Additionally, it would be worth assessing cell migration and/or invasion upon Lapatinib treatment or ERBB2 siRNA knock-down. Due to time constraints, these experiments were not able to be performed.

It is important to understand the effects of reduced TPD52 expression both in the short and long-term, as this will be valuable for the future development of chemical inhibitors of TPD52. The in vitro approach of reducing TPD52 expression provided in this thesis will be valuable for comparison with TpD52 knock-out mice or similar in vivo approaches which are yet to be delivered. Based on the results in Chapter 3, TPD52 inhibition may be less successful in patients with ERBB2-positive cancers, unless TPD52 and ERBB2 were inhibited together. Given the emerging possibility that TPD52 function is important in the context of ERBB2 expression, it seems important to examine the effects of TPD52 knock-down in TPD52-amplified cell lines that are not also ERBB2-amplified. Although attempts have been made, we are yet to identify a TPD52-amplified cell line that does not also detectably express ERBB2. As TPD52 is known to be amplified and overexpressed in various cancer types, it would be worth identifying TPD52-amplified cell lines from cancer types other than breast cancer, and this is being investigated. We are currently assembling a larger cohort of cancer cell lines predicted to be TPD52-amplified using online database tools (canSAR, cBioPortal), and are examining whether these cell lines are or are not ERBB2-amplified. Once cell lines are identified, long-term knock-down models in a carefully chosen subset will be derived, to compare these results with those obtained for SK-BR-3. Additionally, future work could also examine if TPD52 levels are upregulated in stably ERBB2-depleted SK-BR-3 cells.

Collectively, the findings presented in Chapter 3 could have implications for future therapeutic strategies, as both TPD52 and ERBB2 expression have been associated with poor patient outcomes (Adler et al., 2006; Borg et al., 1991; Choschzick et al.,
The poorer metastasis-free survival of patients with breast tumors with high level TPD52 expression, both in the overall cohort and the HR+/ERBB2+ subgroup, suggests that TPD52 overexpression is clinically significant in ERBB2-positive cancers (Roslan et al., 2013; Appendix 2). TPD52 overexpression could possibly promote tumor resistance towards ERBB2-targetted therapies, since TPD52 was indicated to regulate cell survival independently of ERBB2 expression. That SK-BR-3 cells may at least partially escape the effects of reduced TPD52 levels by increasing signaling through the amplified oncogene ERBB2 shows complementary functions for these genes, although different signaling mechanisms likely underpin these. These results might also suggest that pathways regulated by highly amplified oncogenes such as ERBB2 and TPD52 might represent obvious escape routes from the effects of a given targeted therapy, and tumors with many highly amplified genes may be afforded more escape choices. Further research into oncogene co-amplification may therefore help to understand these escape mechanisms, and to translate such results to the clinic.

5.3 TPD52 involvement in regulating lipid storage

Known TPD52 binding partners are not able to fully explain TPD52’s oncogenicity, and TPD52 reports of binding partners are not exclusive for TPD52, but also bind other TPD52-related proteins such as TPD52L1 (Byrne et al., 1998b). We addressed this issue by proposing a novel function for TPD52 in Chapter 4.

We revealed for the first time that ectopic TPD52 but not TPD52L1 expression in mouse 3T3 fibroblasts led to increased lipid droplets, with droplets numbers being more significantly increased than size. This provided direct evidence for TPD52
involvement in regulating lipid storage, as well as evidence of an isoform-specific function for TPD52 which might explain TPD52’s oncogenicity. However, it remains uncertain whether the lipogenic phenotype observed in TPD52-expressing 3T3 cells is due to TPD52 increasing the uptake and/or synthesis of free fatty acids, and/or the inhibition of fatty acid export from lipid droplets. Upon oleic acid treatment, TPD52-expressing 3T3 cells formed more lipid droplets, but to lesser relative extent compared to control cells. It is also unknown how TPD52 overexpression leads to increased lipid droplet formation. As discussed earlier (Section 4.3), there are many signaling pathways and proteins involved in regulating lipid metabolism, and TPD52’s role in this regulation is currently being pursued by other group members. Future studies could also examine whether TPD52 amplification is broadly associated with increased lipid storage/lipogenesis, by identifying other TPD52-amplified cancer cell lines, beyond SK-BR-3 and AU-565 breast cancer cell lines.

Our results support TPD52 regulating lipid storage via interactions with PAT proteins such as TIP47 and/or ADRP, possibly by acting as a lipid carrier. TPD52 has been previously localized to vesicular structures, and to interact with membrane trafficking proteins such as EEA-1, LAMP1 and RAB5 (Shahheydari, 2013; Thomas et al., 2009; Thomas et al., 2004). Lipid droplets also contain trafficking proteins such as RAB proteins (Liu et al., 2007a; Martin et al., 2005; Ozeki et al., 2005). Therefore, TPD52 could be associating with these trafficking proteins for transporting lipids between membrane compartments. Additionally, it will also be of interest to determine whether TPD52 is associated with PAT protein expression in clinical cancer samples. If so, TPD52 could be useful as a biomarker for cancers with a lipogenic phenotype.

Another interesting future direction would be to determine whether TPD52 expression could protect cells from lipotoxicity. The current study used oleic acid, a
monounsaturated fatty acid known to be non-toxic to cells (Hardy et al., 2000; Listenberger et al., 2003). In future experiments, palmitate could be used, since it has been shown to be toxic to various cell types such as human endothelial cells (Zhang et al., 1992), human skin fibroblasts (Rosenthal, 1981), rat cardiomyocytes (de Vries et al., 1997) and CHO cells (Listenberger et al., 2003). Kourtidis et al. (2009) have revealed that using breast cancer cell lines, MCF-7 cells (ERBB2-negative, low intracellular lipid content) are resistant to high doses of palmitate supplementation, whereas exogenous administration of palmitate was toxic to ERBB2-positive BT-474 cells which contained high intracellular lipid content (Kourtidis et al., 2009). Therefore, it would be interesting to examine the effects of palmitate treatment in TPD52-amplified cancer cells. As we have shown that TPD52-expressing 3T3 cells contain high intracellular lipid content, TPD52 inhibitors might also be useful in targeting TPD52-overexpressing cancers by reducing lipid storage, which might then lead to reduced cell proliferation and cell death. Hence, TPD52 could be useful as a biomarker for cancers sensitive to drugs targeting lipid storage.

5.4 Significance of lipid metabolism and cancer

ERBB2-overexpressing cancer cells have been shown to display a lipogenic phenotype, supported by enhanced activation (direct or indirect) of metabolic signaling (Kourtidis et al., 2010; Kumar-Sinha et al., 2003; Zhang et al., 2005). As high levels of intracellular free fatty acids lead to cytotoxicity and cell death (Kourtidis et al., 2009; Listenberger et al., 2003), interference with lipid storage may therefore be a potent means of killing cancers that are dependent upon high lipid levels for survival, through gene amplification and/or overexpression of ERBB2. This highlights the potential for novel metabolic signaling strategies to target ERBB2-positive cancers (Menendez and Lupu, 2007).
We have shown that TPD52 promotes the survival of ERBB2-positive cancer cells, which is supported by persistent associations between TPD52 and ERBB2 expression (Chen et al., 2010; Kourtidis et al., 2010; Landis et al., 2006; Landis et al., 2005; Whiteaker et al., 2007; Wilson et al., 2002). Our collective observations suggest that TPD52 may co-operate indirectly with ERBB2, through increasing lipid storage, possibly mediated by associations with PAT proteins. This suggests that TPD52 represents a potential therapeutic target in ERBB2-positive cancers. Since reduced TPD52 expression did not induce significant cell death in breast cancer cell lines other than ERBB2-amplified cell lines, inhibiting TPD52 function in normal tissues may not provoke widespread toxicity. Unlike agents targeting more broadly expressed lipogenic proteins, TPD52-targetted cancer therapies may also avoid the limiting anorexic side effects of agents such as cerulenin targeting FASN (Menendez and Lupu, 2007). The data presented in this thesis, supports inhibiting TPD52 function or expression as a potential method for treating lipogenic cancers. In addition, the differential expression of TPD52 in obese versus non-obese adipose tissue (Clement et al., 2004; Keller et al., 2008; Nadler et al., 2000) also suggests that TPD52 inhibitors could be applied to treat obesity.
5.5 Final conclusions

TPD52 is a known amplification target in various cancer types. The data presented in this thesis have revealed that TPD52 is important for the survival of ERBB2-positive breast cancer cells, and that TPD52 may co-operate with ERBB2 in promoting tumorigenesis and/or progression. However, TPD52 appears to regulate cell survival independently of ERBB2 signaling. We also revealed for the first time that ectopic TPD52 expression in mouse fibroblast cells leads to increased lipid droplets, and therefore proposed a novel function for TPD52 in regulating intracellular lipid storage. This lipogenic phenotype was only observed in TPD52-expressing but not TPD52L1-expressing cells, which may partially explain the isoform-specific oncogenic function of TPD52. TPD52 may co-operate indirectly with ERBB2 by regulating lipid storage, possibly via associations with PAT proteins, but this requires further validation.
Chapter 6

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Nuruliza Roslan


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Appendix 1
Appendix 1. Electron microscopy confirmed increased lipid droplets in TPD52-expressing 3T3 cells compared with vector controls. Ultrastructural analysis using electron microscopy (CM12 BioTwin transmission electron microscope, Philips; X135000 objective) of (A) vector-3 cells compared with (B) D52-2-7 cells. Lipid droplets are indicated by red dots and in some cases, red arrows. Endoplasmic reticulum (ER) membranes are indicated by blue arrows, phospholipid inclusions are indicated by black arrows and lipid droplet-associated-ER structures are indicated by yellow arrows. Images were derived from one experiment. Cells were provided by Nuruliza Roslan, which were fixed, embedded, sectioned and stained for electron microscopy and imaged by Dr. Dongwei Wang, Kids Research Institute, Westmead. Scale bar = 2 µm.
Appendix 2
TPD52 Represents a Survival Factor in ERBB2-Amplified Breast Cancer Cells

Nuruliza Roslan,1,2 Ivan Bièche,3,4 Robert K. Bright,5 Rosette Lidereau,4 Yuyan Chen,1 and Jennifer A. Byrne1,2*

1Molecular Oncology Laboratory, Children’s Cancer Research Unit, Kids Research Institute, The Children’s Hospital at Westmead, Westmead, NSW, Australia
2The University of Sydney Discipline of Paediatrics and Child Health, The Children’s Hospital at Westmead, Westmead, NSW, Australia
3Laboratoire d’Oncogénétique, Institut Curie - Hôpital René Huguenin, FNCLCC, Saint-Cloud, France
4Laboratoire de Génétique Moléculaire—INSERM U745, Faculté des Sciences Pharmaceutiques et Biologiques, Université René Descartes, Paris, France
5Department of Immunology and Molecular Microbiology and TTUHSC Cancer Center, Texas Tech University Health Sciences Center, Lubbock, Texas

TPD52 and ERBB2 co-expression has been persistently reported in human breast cancer and animal models of this disease, but the significance of this is unknown. We identified significant positive associations between relative TPD52 and ERBB2 transcript levels in human diagnostic breast cancer samples, and maximal TPD52 expression in the hormone receptor (HR)- and ERBB2-positive sub-group. High-level TPD52 expression was associated with significantly reduced metastasis-free survival, within the overall cohort (log rank test, \(P = 8.6 \times 10^{-4}, n = 375\)) where this was an independent predictor of metastasis-free survival (hazard ratio, 2.69, 95% confidence interval 1.59–4.54, \(P = 2.2 \times 10^{-4}, n = 359\)), and the HR- and ERBB2-positive sub-group (log rank test, \(P = 0.035, n = 47\)). Transient TPD52 knock-down in the ERBB2-amplified breast cancer cell lines SK-BR-3 and BT-474 produced significant apoptosis, both singly and in combination with transient ERBB2 knock-down. Unlike ERBB2 knock-down, transient TPD52 knock-down produced no reduction in pAKT levels in SK-BR-3 or BT-474 cells. We then derived multiple SK-BR-3 cell lines in which TPD52 levels were stably reduced, and measured significant inverse correlations between pERBB2 and TPD52 levels in viable TPD52-depleted and control cell lines, all of which showed similar proliferative capacities. Our results therefore identify TPD52 as a survival factor in ERBB2-amplified breast cancer cells, and suggest complementary cellular functions for TPD52 and ERBB2. © 2013 Wiley Periodicals, Inc.

Key words: HER2; Neu; tumor protein D52; PrL2; gene amplification

INTRODUCTION

Targeted therapies are increasingly employed for cancer treatment, yet predicting patient response remains problematic. This situation is exemplified by considering the oncogene ERBB2 (human epidermal growth factor-2, Neu or HER2), whose amplification occurs in approximately 15% of breast cancer cases [1]. The association between ERBB2 amplification and/or overexpression and poor prognosis led to the development of ERBB2-targetted therapies for the treatment of breast and other cancers [2,3]. However, not all patients with ERBB2-positive disease respond to ERBB2-targetted therapies, and many develop resistance [2,3]. Understanding the functions of proteins co-expressed with ERBB2 may identify co-operating factors which promote or enhance tumor cell phenotypes, and which may contribute to primary or secondary resistance to ERBB2-targetted therapies.

A gene/protein that has been persistently linked with ERBB2 expression is the chromosome 8q21.13 oncogene and adaptor protein TPD52. TPD52 was originally identified from an ERBB2-expressing breast tumor [4]. TPD52 transcript levels were subsequently found to be increased in ERBB2-positive breast cancer cell lines [5], followed by reports that Tpd52 transcript or protein levels were increased in mammary tissues from Erb2 transgenic animals [6–9]. Kourtidis et al. [10] also identified increased TPD52 levels in ERBB2-amplified breast cancer cells, and suggest complementary cellular functions for TPD52 and ERBB2.© 2013 Wiley Periodicals, Inc.
expression support

ERBB2 is amplified or gained in breast cancer [11], and associations between gene copy number and expression support TPDS2 representing a gene amplification target in breast and other cancers [12,13]. This was recently underscored by a large-scale genomic study identifying focal amplification of TPDS2 and three neighboring chromosome 8q21.13 genes in breast cancer [14]. Chromosome 8q21 amplification has been significantly associated with both ERBB2 amplification and poor patient outcome in breast cancer [15], and increased TPDS2 immunohistochemical staining was associated with reduced overall survival in breast cancer patients, where this was an independent prognostic factor [16]. TPDS2 has similarly been included in gene expression [17–19] or copy number [20] signatures associated with poor prognosis in breast cancer patients. Thus ERBB2 and TPDS2 are both amplifications whose overexpression is reproducibly associated with poor patient outcomes in breast cancer. The present study examined the significance of TPDS2 and ERBB2 co-expression, by defining the expression of TPDS2 relative to ERBB2 in primary breast cancer samples and breast cell lines, and the effects of depleting TPDS2 and ERBB2 levels in breast cancer cell lines with differing TPDS2 and ERBB2 gene amplification and/or expression status.

MATERIALS AND METHODS

Patients and Samples

Samples of 375 primary invasive breast tumors excised from women managed at Institut Curie—Hôpital René Huguenin (Saint-Cloud, France) from 1978 to 2008 were immediately stored in liquid nitrogen until RNA extraction. All patients who entered the institution before 2007 were informed that their tumor samples might be used for scientific purposes and had the opportunity to decline. Since 2007, patients entering the institution have given their approval by signed informed consent. This study was approved by the local ethics committee (Breast Group of Hôpital René Huguenin). Tumor samples were included if the proportion of tumor cells exceeded 70%. Normal breast tissue from women undergoing cosmetic breast surgery (n = 8) were used as a source of control RNA. Breast cancer patients (mean age 62.5 yr, range 31–91 yr) met the following criteria: primary unilateral non-metastatic breast carcinoma for which complete clinical, histological and biological data were available; no radiotherapy or chemotherapy before surgery; and full follow-up at Institut Curie—Hôpital René Huguenin. The histological type, number of positive axillary nodes and macroscopic tumor size (Table 1) were established at the time of surgery. The malignancy of infiltrating carcinomas was scored according to Scarff Bloom Richardson’s (SBR) histoprognostic system (Table 1).

Treatment consisted of modified radical mastectomy (253 cases, 67.4%) and breast conserving surgery plus locoregional radiotherapy (122 cases, 32.6%). Patients had a physical examination and routine chest radiography every 3 mo for 2 yr, then annually. Mammograms were done annually. Adjuvant therapy was administered to 298 patients, consisting of chemotherapy alone in 73 cases, hormone therapy alone in 168 cases, and both treatments in 57 cases. During a median follow-up of 7.9 yr (range 6 mo to 29 yr), 157 patients developed distant metastasis.

Hormone receptor (HR), estrogen receptor α (ERα), progesterone receptor (PR), and ERBB2 status were determined at the protein level using biochemical methods (dextran-coated charcoal method, enzyme immunoassay, or immunohistochemistry) and confirmed by real-time quantitative RT-PCR assays [21,22]. The population was divided into groups according to HR (ERα and PR) and ERBB2 status: two luminal subtypes [HR + (ERα+ or PR +)/ERBB2+ (n = 47)] and [HR + (ERα+ or PR +)/ERBB2− (n = 224)]; an ERBB2+ subtype [HR− (ERα− and PR−)/ERBB2+ (n = 46)]; and a triple-negative subtype [HR− (ERα− and PR−)/ERBB2− (n = 58)].

Cell Lines and Cell Culture

Breast cancer cell lines AU-565, BT-483, MDA-MB-175, HS-578T, MDA-MB-157, MDA-MB-134, DU4475, MDA-MB-468, ZR-75-30 were kindly provided by Prof. Roger Daly (Garvan Institute, Australia). SK-BR-3, BT-474, MCF-7, T-47D, MDA-MB-231, and MCF-10A cell lines were obtained from the American Type Culture Collection (Rockville, MD). All cell line identities were confirmed through short tandem repeat profiling by CellBank Australia (Westmead, Australia). Cell lines were cultured in a humidified incubator at 37 °C/5% CO2 in RPMI media (Life Technologies, Inc. Ltd, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 3% l-glutamine (Life Technologies), except for MCF-7 cells, where the above media were supplemented with 10μg/mL insulin (Sigma–Aldrich, St. Louis, MO), and MCF-10A cells, which were cultured as described [23].

Total RNA and Genomic DNA Extraction, cDNA Synthesis and Real-Time (Reverse-Transcriptase) PCR

Total RNA was extracted from breast tumor samples by using acid–phenol guanidium, as described [24]. RNA quality was determined by electrophoresis through agarose gels and staining with ethidium bromide. Real time RT-PCR analyses were performed as described [25]. Briefly, reactions were performed using an ABI Prism 7900 Sequence Detection System using the SYBR® Green PCR Core Reagents kit (Perkin–Elmer Applied Biosystems, Carlsbad, CA). Thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 65°C for 1 min. Quantitative
values were obtained from Ct values using the Perkin–Elmer Biosystems analysis software, according to the manufacturer’s instructions. Results were expressed as fold differences in TPD52 expression relative to the endogenous RNA control TBP. Gene expression values were then expressed relative to the median value obtained in normal breast samples, which was set at 1.0.

Genomic DNA was extracted from cultured cells using the phenol/chloroform method. TPD52 PCR primer sequences were sense: 5’-CAG TTT AGA GCC CAG GGA AA-3’ and antisense: 5’-CGA TCA TCC AAC GTA GCA TG-3’. Quantitative real-time PCR was performed using the Corbett Rotor-Gene 6000 (Qiagen, Valencia, CA) using the KAPA SYBR fast Universal qPCR kit (Kapa Biosystems). Thermal cycling conditions comprised an initial denaturation step of 95°C for 10 min and 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Relative DNA copy numbers were calculated by normalizing raw data for each cell line against MCF-10A.

Total RNA was extracted from breast cell lines using Trizol LS reagent (Life Technologies) according to the manufacturer’s protocol. One microgram of RNA was subjected to cDNA synthesis using the SuperScript III First Strand Synthesis System for RT-PCR kit (Life Technologies), according to the manufacturer’s instructions. TPD52 RT-PCR primer sequences were sense: 5’-GGA AGA GGA GCA GGA AGA GC-3’ and antisense 5’-GAT GAC TGA GCC AAC AGA CG-3’. Quantitative real-time RT-PCR was performed using the Corbett Rotor-Gene 6000 (Qiagen) using the KAPA SYBR fast Universal qPCR kit (Kapa Biosystems). Thermal cycling conditions comprised an initial denaturation step of 95°C for 10 min and 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Relative gene transcript levels were calculated by normalizing raw data obtained for each cell line against that obtained for MCF-10A.

**Western Blot Analyses**

Cells were lysed in 3% SDS lysis buffer as described [26]. 12–18 μg total protein extracts were resolved using SDS-PAGE on 12.5% polyacrylamide gels or 4–12% gradient gels (Life Technologies) and electrotransferred to nitrocellulose PVDF membranes (Millipore, Billerica, MA). Membranes were blocked overnight at 4°C in 5% skim milk powder in 0.1% Tween 20 in TBS, washed twice with 0.1% Tween 20 in TBS and incubated with one or more of the following primary antibodies: affinity-purified rabbit polyclonal TPD52 antisera (1:100) [11], rabbit monoclonal

**Table 1. Characteristics of the 375 Primary Breast Cancers Analyzed**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients</th>
<th>Number of metastases (%)</th>
<th>P-valuea</th>
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<tr>
<td><strong>Age</strong></td>
<td></td>
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<tr>
<td>≤50</td>
<td>74</td>
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<tr>
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<tr>
<td>&gt;25 mm</td>
<td>202</td>
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<td>117 (41.5)</td>
<td>NS (0.39)</td>
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<tr>
<td>Pos</td>
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<td>HR−/ERBB2−</td>
<td>58</td>
<td>25 (43.1)</td>
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*Log-rank test. NS, not significant. bScarff Bloom Richardson classification.
ERBB2 (1:1000, Cell Signalling Technology, Inc., Beverly, MA); rabbit monoclonal pERBB2(Tyr1221/1222) (1:1000, Cell Signalling); rabbit monoclonal Pan-AKT (1:1000, Cell Signalling); rabbit monoclonal pAKT(Ser473; 1:1000, Cell Signalling); rabbit polyclonal cleaved PARP-1 (1:1000, Cell Signalling); rabbit monoclonal pMAPK(44/42) (1:1000, Cell Signalling); mouse monoclonal α-tubulin (1:1000; Sigma–Aldrich); mouse monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000; Ambion, Austin, TX). Primary antibody incubations were performed in 0.1% Tween 20 in TBS for 1–2 h at room temperature (RT) or overnight at 4°C. Membranes were washed three times and then incubated with a horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse secondary antibody (1:10 000 or 1:5000 respectively; GE Healthcare, Uppsala, Sweden) for 1 h at RT. Membranes were then washed and visualized by Western Lightening chemiluminescent reagent (Perkin Elmer Life Science, Boston, MA). Densitometry analysis using ImageJ 1.45s software was used to quantitate fold changes in protein levels relative to loading controls.

Indirect Immunofluorescence Analyses

Cells were plated onto glass coverslips, cultured overnight, washed twice with PBS, fixed in 2% paraformaldehyde/PBS for 15 min, washed twice, permeabilized with cold acetone:methanol (1:1) for 15 min, washed twice, and incubated with TFDS2 mouse monoclonal antibody (1:10) [27] and ERBB2 rabbit monoclonal (1:400, Cell Signalling Technology) in 3% bovine serum albumin in PBS overnight at 4°C. Cells were washed twice and incubated with secondary Alexa488 anti-mouse (1:1000, Life Technologies) and Cy3-conjugated anti-rabbit (1:1000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) antibodies in 3% bovine serum albumin in PBS for 1 h in the dark at RT. After washing, cells were mounted in FluorSave mounting medium (Calbiochem, San Diego, CA) and visualized using a TCS SP2 confocal laser scanning upright microscope (Leica, Heidelberg, Germany), using a 63× objective lens.

Transient Small Interfering RNA Transfections

1. TFDS2 small interfering RNA (siRNA) duplexes [16] and an ON-TARGETplus pool targeting ERBB2 (5'-TGG AAG AGA TCA CAG GTT A-3', 5'-GAG ACC CGC TGA ACA ATA C-3', 5'-GGA GGA ATG CCG AGT ACT G-3' and 5'-GCT CAT CGC TCA CAA CCA A-3') were obtained from Dharmacon (Lafayett, CO), whereas non-targeting siRNA (siControl) was obtained from Qiagen. Cells were seeded at 2 × 10^4 cells/well into 24-well plates. After 24 h, cells were transfected with 50 nmol/L siRNA duplexes using TransIT-TKO transfection reagent (Mirus Bio, Madison, WI) in complete media following the manufacturer’s instructions. After 24 h, transfection media were removed and replaced with complete media. Cells were harvested for analysis 48 h later.

2. MTT assay: 10^3 cells were seeded in 96-well plates (3 × 10^5 cells/well), cultured for 24 h and then 50 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma–Aldrich) was added either immediately (0 h) or at the designated number of days post-seeding. MTT reactions were incubated at 37°C in the presence of 5% CO_2 for 4 h. To stop each reaction, media were replaced with 100 μL DMSO (Sigma–Aldrich) per well. Absorbances at 540 nm were read using a Multiskan Ascent plate reader. Means of triplicate wells from three independent experiments were used to generate data points and SE values.

3. Western blotting: To generate data points and SE values.

4. Statistical Analysis:

The SPSS for Windows package (version 19; SPSS) was used in most cell line analyses. Spearman rank correlation was used for correlation tests between samples. The Mann–Whitney U test was used to...
proliferation assays were expressed as means ± SE of three independent experiments. Comparisons between groups were made using two-tailed, unequal variance Student’s t test calculated using Excel (Microsoft, Redmond, WA).

Relationships between transcript levels of different genes, and transcript levels and clinical parameters were identified by using chi-square, Mann–Whitney U test, and Spearman rank correlation tests. Metastasis-free survival was determined as the interval between initial diagnosis and detection of the first metastasis. To visualize the efficacy of a molecular marker (TPDS2 level) to discriminate two populations (patients that developed/did not develop metastases) in the absence of an arbitrary cut-off value, data were summarized in an ROC (receiver operating characteristic) curve [28]. The AUC (area under curve) was calculated as a single measure for discriminate efficacy. Survival distributions were estimated by the Kaplan–Meier method, and significance of differences between survival rates was ascertained with the log-rank test. Best-fitting multiple Cox proportional hazards models with backward step-wise selection were used to identify independent predictors of survival from potential risk factors [29].

RESULTS

TPDS2 Expression Relative to ERBB2 in Primary Breast Cancer Tissues

Quantitative RT-PCR was employed to study TPDS2 expression in 375 invasive breast carcinomas (Table 1), relative to eight normal breast samples. TPDS2 was overexpressed more than threefold, relative to normal breast, in 311/375 (82.9%) of breast carcinomas, and more than 10-fold in 65/375 (17.3%) cases (Table 2). The tumor cohort was divided into 4 subgroups based upon HR and ERBB2 status. TPDS2 was significantly differentially expressed in the four subgroups compared (Mann–Whitney test, \(P < 1 \times 10^{-7}, n = 375\)), with TPDS2 expression being highest in HR+/-ERBB2+ cases (Table 2, Table 3). TPDS2 was significantly differentially expressed according to ERα, PR, and ERBB2 status, but not according to patient age, SBR histological grade, lymph node status, macroscopic tumor size, PIK3CA mutation status, or KI67 transcript levels (Table 3). A significant positive correlation between relative TPDS2 and ERBB2 levels (Spearman rank correlation, \(r_s = 0.273, P = 5.3 \times 10^{-8}, n = 375\)) contrasted with a significant inverse correlation between TPDS2 and EGF levels (Table 3).

Area under the curve analyses were performed to identify a cut-point by which to divide the cohort into relevant TPDS2 expression subgroups. We therefore compared tumors with relative TPDS2 expression \(\leq 16.68\) (\(n = 353, 94.1\%\)), with those with relative...
TPD52 expression >16.68 (n = 22, 5.9%). Interestingly, the percentage of cases in the high TPD52 expression group is very similar to the percentages of breast cancer cases estimated to be TPD52-amplified from TCGA cohorts (5.8% of 484 cases, 6.2% of 482 cases [14], data available through the cBio Cancer Genomics Portal, http://www.cbioportal.org/public-portal/). A significantly greater proportion of patients with tumors in the high TPD52 expression group relapsed with metastases (16/22 cases, 72.7%), compared with patients with tumors in the lower TPD52 expression group (141/353 cases, 39.9%; chi-square test, \( P = 0.0025, n = 375 \)). Metastasis-free survival was significantly poorer for patients with tumors with high TPD52 expression, in the overall cohort (log rank test, \( P = 8.6 \times 10^{-4}, n = 375 \); Figure 1A), the HR+/ERBB2+ subgroup (log rank test, \( P = 0.035, n = 47 \); Figure 1B) and the HR+/ERBB2– subgroup (log rank test, \( P = 0.0059, n = 224 \)), whereas the HR–/ERBB2– (n = 58) and HR–/ERBB2+ (n = 46) subgroups included only one or two tumors in the high TPD52 expression category, respectively. Multivariate analyses indicated that high TPD52 levels were an independent predictor of metastasis-free survival (hazard ratio, 2.69, 95% confidence interval 1.59–4.54, \( P = 2.2 \times 10^{-4}, n = 359 \)), following adjustment for lymph node, tumor size, SBR, ERα, and PR status (Table 4).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients (%)</th>
<th>TPD52 transcript levels, relative to normal breast median (range)</th>
<th>( P )-value*</th>
</tr>
</thead>
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<tr>
<td>Total</td>
<td>375 (100)</td>
<td>5.20 (0.73–115)</td>
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<tr>
<td>Age ≤50</td>
<td>74 (19.7)</td>
<td>4.79 (1.07–36.9)</td>
<td>0.098 (NS)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>301 (80.3)</td>
<td>5.26 (0.73–115)</td>
<td></td>
</tr>
<tr>
<td>SBR histological grade*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>37 (10.1)</td>
<td>4.78 (1.64–15.1)</td>
<td>0.14 (NS)</td>
</tr>
<tr>
<td>II</td>
<td>190 (51.9)</td>
<td>5.35 (1.20–81.8)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>139 (38)</td>
<td>4.93 (0.73–115)</td>
<td></td>
</tr>
<tr>
<td>Lymph node status*</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>103 (27.5)</td>
<td>4.89 (0.73–81.8)</td>
<td>0.30 (NS)</td>
</tr>
<tr>
<td>1–3</td>
<td>186 (49.7)</td>
<td>5.15 (1.07–87.6)</td>
<td></td>
</tr>
<tr>
<td>&gt;3</td>
<td>85 (22.7)</td>
<td>5.40 (1.45–115)</td>
<td></td>
</tr>
<tr>
<td>Macroscopic tumor size**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤25 mm</td>
<td>166 (45.1)</td>
<td>5.07 (1.07–36.9)</td>
<td>0.67 (NS)</td>
</tr>
<tr>
<td>&gt;25 mm</td>
<td>202 (54.9)</td>
<td>5.26 (0.73–115)</td>
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<tr>
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<td>3.92 (0.73–36.9)</td>
<td>&lt;0.00000124</td>
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<tr>
<td>Positive</td>
<td>270 (72)</td>
<td>5.64 (1.20–115)</td>
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<td>PR status</td>
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<td>0.012</td>
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<td>Positive</td>
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<td>5.34 (1.20–115)</td>
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<td>ERBB2 status</td>
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<td>5.48 (1.40–87.6)</td>
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<td>5.14 (1.40–36.9)</td>
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<td>224 (59.7)</td>
<td>5.34 (1.20–115)</td>
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<td>HR+/ERBB2+</td>
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<td>7.40 (2.0–87.6)</td>
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<td>PIK3CA mutation status</td>
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<td>5.26 (1.07–115)</td>
<td>0.51 (NS)</td>
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<td>KI67 mRNA expression</td>
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<td>( r_s = 0.038 )</td>
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<tr>
<td>( P = 0.47 ) (NS)*</td>
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<td>EGFR mRNA expression</td>
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<tr>
<td>( P = 0.0000012^* )</td>
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</table>

*Mann–Whitney U-Test.

**Scarff Bloom Richardson classification, information available for 366 patients.

*Information available for 374 patients.

**Information available for 368 patients.

*Spearman rank correlation.
We selected a panel of 14 breast cancer cell lines according to their predicted TPD52 amplification status [30–33], assessed via the ROCK database (http://rock.icr.ac.uk/), and MCF-10A cells as a non-tumorigenic breast cell line, to compare TPD52 and ERBB2 expression in cell lines with varying TPD52 levels, and select cell lines for further analysis. Real-time (RT-) PCR and Western blot analyses revealed differences in relative TPD52 copy number, transcript and protein levels between the 15 breast cell lines (Figure 2). Although the cell line cohort was small for statistical analyses, four ERBB2-amplified cell lines (SK-BR-3, AU-S65, BT-474, ZR-75-30) showed significantly higher relative TPD52 transcript and protein

<table>
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<th>Variables</th>
<th>Metastasis-free survival HR (95% CI)a</th>
<th>P-value</th>
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<td>0</td>
<td>1</td>
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</tr>
<tr>
<td>1–3</td>
<td>1.58 (1.25–1.99)</td>
<td>0.00013</td>
</tr>
<tr>
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<td>2.49 (1.56–3.98)</td>
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<tr>
<td>Relative TPD52 expression</td>
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<tr>
<td>&gt;16.68</td>
<td>2.69 (1.59–4.54)</td>
<td>0.00022</td>
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<td>Macroscopic tumor size</td>
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<tr>
<td>≤25 mm</td>
<td>1</td>
<td></td>
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<tr>
<td>&gt;25 mm</td>
<td>1.58 (1.13–2.21)</td>
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</tr>
<tr>
<td>positive</td>
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</tbody>
</table>

aHazard ratio (HR) and 95% confidence interval (CI).
levels than non-ERBB2-amplified cell lines ($n = 11$; Mann–Whitney test, $P = 0.006$, $n = 15$, TPDS2 transcript levels; $P = 0.037$, $n = 15$, TPDS2 protein levels; Figure 2B–D), and significant or borderline positive correlations were measured between ERBB2 protein and TPDS2 transcript or protein levels, respectively (Spearman test, $r_s = 0.681$; $P = 0.005$; $n = 15$, TPDS2 transcript levels; $r_s = 0.513$; $P = 0.051$; $n = 15$, TPDS2 protein levels). These associations were likely driven by the inclusion of the TPDS2-amplified SK-BR-3 and AU-565 cell lines within this cohort.

Transient TPDS2 and ERBB2 Depletion in Breast Cell Lines

We previously reported that transient TPDS2 knock-down using three siRNA targets produced significantly increased apoptotic cell death in SK-BR-3 cells, but not MCF-7 cells [16]. SK-BR-3 cells show high TPDS2 copy number and expression, relative to MCF-7 cells (Figure 2). We subsequently examined the effects of transient TPDS2 knock-down in the non-TPDS2-amplified cell lines MDA-MB-231 and MCF-10A (Figure 2), using two TPDS2 siRNAs previously employed [16]. TPDS2 depletion was not associated with significantly increased apoptosis in either cell line (data not shown).

As the majority of cell lines indicated to be sensitive to the effects of TPDS2 knock-down were either ERBB2-amplified or positive for ERBB2 expression [10,16], we directly compared the effects of TPDS2 and ERBB2 knock-down, alone or in combination, in the ERBB2-amplified and overexpressing cell lines BT-474 and SK-BR-3, the latter cell line also being TPDS2-amplified (Figure 2A) [34]. Both SK-BR-3 and BT-474 cells showed increased apoptosis following ERBB2 knock-down, relative to non-targeting siRNA-transfected cells (Figure 3). Quantitative apoptosis assays and detection of cleaved PARP-1 indicated that higher levels of apoptosis were produced by ERBB2 knock-down in BT-474 than SK-BR-3 cells (Figure 3), which agrees with the reported effects of ERBB2 knock-down on the viability of these cell lines [10]. SK-BR-3 and BT-474 cells showed similar significant increases in apoptosis following TPDS2 knock-down (Figure 3), despite their differing TPDS2 levels.
SK-BR-3 cells showed similar sensitivity to both TPD52 and ERBB2 knock-down, and knock-down of both genes produced significantly higher levels of apoptosis, compared with the knock-down of either gene alone (Figure 3A). Transient TPD52 knock-down did not reproducibly alter total or pERBB2 levels in either cell line, and similarly ERBB2 knock-down did not alter TPD52 levels (Figure 3).

Overexpression of TPD52 isoforms in LnCaP prostate cancer cells has been reported to result in increased pAKT (pSer473) [35–37]. While ERBB2 knock-down was associated with reduced pAKT levels in both BT-474 and SK-BR-3 cells as anticipated [38], pAKT levels were unchanged by TPD52 knock-down (Figure 3). There were similarly no changes to pMAPK44/42 levels upon transient TPD52 knock-down (Figure 3).

**Subcellular Distributions of TPD52 and ERBB2 in SK-BR-3 Cells**

To further investigate the significance of TPD52 and ERBB2 co-expression in SK-BR-3 cells, we compared the distributions of both proteins using indirect immunofluorescence analyses. Detection of both proteins reproduced their expected subcellular distributions [11,39], with TPD52 and ERBB2 co-localization being focally limited towards the cell membrane (Figure 4). The lack of substantial co-localization of TPD52 and ERBB2 in SK-BR-3 cells supports the non-overlapping signaling effects of TPD52 and ERBB2 knock-down in ERBB2-amplified cell lines (Figure 3).

**Generation and Analysis of Stably TPD52-Depleted SK-BR-3 Cell Lines**

As transient TPD52 and ERBB2 knock-down had comparable effects in SK-BR-3 cells, we investigated the long-term effects of TPD52 depletion in this cell line using TPD52 shRNA targets (Additional file 1). Cell lines were harvested during early propagation in 24 wells (termed early passage cells, Figure 5A) and re-harvested at passage 10 (Additional file 1, Figure 5B). Immunofluorescence (Additional file 1, data not shown) and Western blot analyses (Additional file 1, Figure 5A and B, data not shown) confirmed reduced TPD52 expression in shRNA-D52-2 and shRNA-D52-3-transfected cell lines, compared with non-targeting control and parental cells. shRNA-D52-3 transfected cells reproducibly showed greater, more temporally stable reductions in TPD52 expression than shRNA-D52-2 transfected cells (Additional file 1, Figure 5A and B, data not shown). Increased cleaved PARP-1 levels were detected in early passage TPD52-depleted cells (Figure 5A), in accordance with the results of transient TPD52 knock-down in SK-BR-3 cells (Figure 3A).

Depletion of TPD52 by 40–90% in early passage cells was associated with increased pERBB2 but not...
total ERBB2 levels, relative to controls (Figure 5A and C). At passage 10, TPD52 levels remained reduced by 50–90% in TPD52-depleted cell lines, and pERBB2 expression remained increased by ~2-fold (Figure 5B and C). Passage 10 cell lines with lower TPD52 levels showed higher pERBB2 levels (Figure 5B, cell lines B and D), whereas cell lines which had partially recovered TPD52 expression showed lower pERBB2 levels (Figure 5B, cell lines A and C). Overall, significant inverse correlations were measured between mean TPD52 and pERBB2 levels, relative to levels measured in untreated cells, both in early passage and passage 10 cells, and in these groups combined (Spearman test, \( r_s = -0.886, P = 0.019 \), \( n = 6 \), early passage; \( r_s = -0.943, P = 0.005 \), \( n = 6 \), passage 10, \( r_s = -0.846, P = 0.001 \), \( n = 12 \), combined groups; Figure 5C). While no changes in pAKT or pMAPK levels were noted in early passage cell lines (Figure 5A), increased pERBB2 levels were accompanied by 2–4-fold increased pAKT but not pMAPK levels in 3/4 TPD52-depleted cell lines harvested at passage 10 (Figure 5B). Previously, stably reduced TPD52 in C4-2 prostate cancer cells was associated with reduced cell proliferation [37]. In contrast, MTT assays performed over 3 d revealed that all stably TPD52-depleted SK-BR-3 cell lines proliferated at similar rates as untreated or non-targeting control cells (Figure 6).

**DISCUSSION**

The oncogene addiction hypothesis predicts that tumors bearing amplification of a given oncogene will be usually sensitive to the removal of that gene’s expression or function [40]. Demonstrating that this is the case identifies an oncogene as a driver of tumorigenesis, whose disruption might play a major role in stabilizing or removing disease. However, the experimental study of amplification targets is complicated by the fact that many genes may be co-amplified, mutated or otherwise inactivated within a given tumor system [1], and thus a single gene’s effects may be enhanced or diluted by the presence of other alterations. It seems clear that these factors could also influence responses to targeted therapies, but how this occurs is only beginning to be explored.

Numerous studies have reported co-expression of TPD52 and ERBB2 [5–10], two gene amplification targets in breast cancer. In the present study, we demonstrate that TPD52 and ERBB2 co-expression is both functionally and clinically significant. Firstly, ERBB2-positive diagnostic breast tumors expressed significantly higher TPD52 levels than ERBB2-negative tumors, and high-level TPD52 expression was associated with significantly poorer metastasis-free survival, both in the overall cohort, the large HR + / ERBB2 sub-group, and the smaller HR + /ERBB2+ subgroup in which TPD52 expression was highest. Secondly, acute TPD52 knock-down produced significant cell death in cell lines amplified for ERBB2 via a mechanism independent of AKT signaling. Finally, stable TPD52 depletion in TPD52- and ERBB2-amplified SK-BR-3 cell lines was associated with significant inverse correlations between TPD52 and pERBB2 levels, indicating that ERBB2-amplified cells may compensate for reduced TPD52 levels by increasing ERBB2 signaling. These combined results indicate that TPD52 may co-operate with ERBB2 in promoting tumorigenesis and/or progression, by enhancing breast cancer cell survival.

Cancer cells have previously shown differing sensitivities to TPD52 knock-down, although the reasons for this have not been directly examined [10,16]. In the present study, the ERBB2-amplified SK-BR-3 and BT-474 cell lines showed very similar increases in apoptosis in response to reduced TPD52 expression, despite their marked differences in TPD52 expression and relative TPD52 copy numbers. This indicates that cellular responses to TPD52 knock-down may be partly determined by ERBB2 status. Transient TPD52 isoform knock-down also produced increased cell death in LnCaP prostate cancer cells [36], and in the androgen-independent derivative cell line C4-2 [35,37]. As both LnCaP and C4-2 cells express ERBB2 [41–43] the consequences of TPD52 knock-down in these cell lines are consistent with TPD52 promoting the survival of ERBB2-positive cancer cells. However, Kourtidis et al. [10] also reported that the ERBB2-negative breast cancer cell line MDA-MB-468 showed similar reductions in viability to TPD52 knock-down as ERBB2-amplified and/or -expressing cell lines. Thus it would appear that ERBB2 amplification or expression, and other factors yet to be determined, confer sensitivity to TPD52 depletion in cancer cells.

Other consequences of TPD52 knock-down were different from those which would have been predicted by previous results. TPD52 isoform overexpression in LnCaP prostate cancer cells resulted in

**Figure 6.** Proliferation of stably TPD52-depleted SK-BR-3 cell lines. Cell proliferation was quantified using MTT assays, in SK-BR-3 cells stably depleted of TPD52 (using shRNA-D52-2 or shRNA-D52-3 shRNA), and in untreated or non-targeting (non-target) control cells. Values plotted are means ± SE for three independent experiments done in triplicate.

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increased pAKT levels [35–37] and reduced TPD52 expression in C4-2 cells led to reduced pAKT levels [37]. However, in the present study, transient TPD52 knock-down did not alter pAKT levels in SK-BR-3 or BT-474 cells. The siRNA targets employed in both the present study and that of Zhang et al. [37] bind sequences that are common to PrLZ and TPD52 transcripts, and therefore the different results obtained are unlikely to reflect differential targeting of PrLZ versus TPD52. These could however highlight functional differences between PrLZ and TPD52 in prostate versus breast cancer cells, or other differences between the particular cell lines examined. The fact that ERBB2 but not TPD52 knock-down resulted in reduced pAKT levels indicates that TPD52 regulates cell survival independently of AKT signaling in ERBB2-amplified breast cancer cells.

Longer term gene knock-down experiments can highlight whether and how cells adapt to reduced gene function. We examined the longer term effects of TPD52 knock-down in SK-BR-3 cells, as these cells have similar TPD52 and ERBB2 copy numbers [34], and were similarly sensitive to transient TPD52 and ERBB2 knock-down. While TPD52 depletion led to increased cell death in the short term, TPD52-depleted cell lines that remained viable over 10 passages showed increased ERBB2 signaling, with most also showing increased pAKT levels. As TPD52-depleted cell lines showed very similar proliferation to controls, increased pERBB2 and pAKT levels likely represent compensatory mechanisms to escape the consequences of reduced TPD52 levels. It seems unlikely that PrLZ upregulation occurred in response to TPD52 depletion, as the shRNA targets employed bind sequences common to PrLZ and TPD52 transcripts, and the TPD52 antisera employed detect an epitope shared by TPD52 and PrLZ [44]. This contrasts with the reported effects of stably reducing PrLZ/TPD52 expression in C4-2 cells, which was associated with reduced cell proliferation and colony-forming capacity [37]. Given the many differences between SK-BR-3 and C4-2 cells, it is not unexpected that stable TPD52 depletion produced different responses in these cell lines over time. It is possible that reducing TPD52 levels in SK-BR-3 cells requires adaptive changes for cells to remain viable, and/or that ERBB2 amplification may allow SK-BR-3 cells to more readily adapt to reduced TPD52 levels. Sequencing of the SK-BR-3 genome revealed that 3 regions of chromosome 8q, one of which includes TPD52, are co-amplified with chromosome 17q [45], in agreement with the similar copy numbers reported for TPD52 and ERBB2 in SK-BR-3 cells using next-generation sequencing [34]. Co-amplification of TPD52 and ERBB2 in SK-BR-3 cells may partially explain the similar effects of transient TPD52 or ERBB2 knock down in this cell line, and is consistent with SK-BR-3 cells relying upon both proteins for survival. It will be important to examine the effects of stable TPD52 depletion in a broader range of TPD52-amplified cancer cell lines with and without ERBB2 amplification, but these need to be identified for such work to proceed.

It is tempting to speculate as to how TPD52 and ERBB2 co-operate in cancer cells, although the current poor understanding of TPD52’s molecular functions renders this difficult. The significant positive correlation between relative TPD52 and ERBB2 levels in breast cancer samples measured in the present study, combined with previous reports of TPD52 and ERBB2 co-expression [5–10], suggest that TPD52 might regulate ERBB2 expression, or vice versa. However, neither transient nor stable TPD52 depletion reproducibly altered total ERBB2 levels, and transient ERBB2 knock-down did not measurably affect TPD52 levels in BT-474 or SK-BR-3 cells. Similarly, there was no evidence that short-term TPD52 depletion altered pERBB2 levels, indicating that TPD52 does not directly regulate ERBB2 signaling, although an inverse relationship between TPD52 and pERBB2 levels emerged in stably TPD52-depleted versus control SK-BR-3 cell lines. The latter association, combined with the lack of substantial co-localization between TPD52 and ERBB2 in SK-BR-3 cells, and the different signaling consequences of transient TPD52 and ERBB2 knock-down, suggest that TPD52 and ERBB2 co-operation occurs indirectly. ERBB2 is a powerful oncogene on account of its pleiotropic effects on cellular proliferation and survival [3]. It is therefore likely that TPD52 promotes similar phenotypes through independent mechanisms, which are yet to be identified.

Increased TPD52 immunohistochemical staining has been associated with reduced overall survival in breast cancer patients [16], and TPD52 has been included in gene expression signatures associated with poor prognosis [17–19]. In the present study, a >16.68 relative TPD52 expression cut-point maximally discriminated patients that did or did not develop metastases, identifying a 5.9% subgroup with high-level TPD52 expression and reduced metastasis-free survival. This proportion of patients is very similar to the 5.8–6.2% of breast cancers in TCGA cohorts that are TPD52-amplified (http://www.cbioportal.org/public-portal/). This suggests that TPD52-amplified breast cancers may be a clinically relevant subgroup which show reduced metastasis-free survival, which is also supported by TPD52 gain being included in a copy number signature that identified breast cancer patients with increased probability of distant metastasis [20]. The poorer metastasis-free survival of patients with tumors with high-level TPD52 expression, combined with the demonstration that ERBB2-amplified cell lines rely upon TPD52 for survival, indicates that TPD52 overexpression could conceivably promote tumor resistance towards ERBB2-targeted therapies, particularly as TPD52 was indicated to regulate cell survival independently of ERBB2. In a
broader sense, our results also suggest that pathways regulated by highly amplified oncogenes represent obvious escape routes from the effects of targeted therapies. Further research into oncogene co-amplification may therefore help to understand these escape mechanisms, and to translate such results to the clinic.

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REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web-site.

Additional file 1 Generation of stably TPD52-depleted SK-BR-3 cell lines. (A) Schematic diagram of TPD52 coding region showing the positions of shRNA targets (shRNA-DS2-2 and shRNA-DS2-3). Numbers represent exons, with exons 1a, 1b, 5 and 6 being subject to alternative splicing. Exons 1a and 1b are included in TPD52 and PrLZ transcripts, respectively. (B) Immunofluorescence images of SK-BR-3 cells stably transfected with shRNA-D52 (shRNA-D52-2-A or shRNA-D52-3-D) or non-target shRNA cultured at passage 10. Cells were stained with human TPD52 monoclonal antibody (top row, red), shRNA constructs were also GFP-tagged (bottom row, green). Images were taken using a TCS SP2 confocal laser scanning upright microscope (Leica Technologies) using a 63X objective. (C) TPD52 levels in SK-BR-3 cell lines stably transfected with shRNA-DS2 (shRNA-DS2-2-A or shRNA-DS2-3-D) or non-target shRNA cultured at passage 10. Left, proteins analysed. Right, molecular weights of detected species.