Cell cycle deregulation in breast cancer subgroups and effects on proliferation, migration and tamoxifen resistance

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Cell cycle deregulation in breast cancer subgroups and effects on proliferation, migration and tamoxifen resistance

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LUND UNIVERSITY
Faculty of Medicine

Academic dissertation
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Abstract

Breast cancer is a heterogenous disease which can be divided in subgroups of distinct biology with disparate prognosis and response to treatment. The overall aim of this thesis was to delineate subgroup specific behaviour in breast cancer cells with focus on proliferation, migration and stem-like cell activity. In addition, we have studied the functional retinoblastoma tumour suppressor (RB) protein pathway and associations to tamoxifen response. Furthermore, the expression of yes-associated protein (YAP1), reported to have both oncogenic and tumour suppressive functions, was investigated in breast cancer subgroups and related to tamoxifen response.

Two of the key processes in malignant behaviour, proliferation and migration, have previously been reported to act as two opposing events in a cancer cell. We found that siRNA-mediated reduction of cyclin D1, a protein expressed in the active cell cycle, resulted in a migratory increase in cell lines negative of estrogen receptor (ER) expression. Conversely, in ER positive cell lines, downregulation of cyclin D1 resulted in decreased migratory capacity and a reduced number of stem-like cells, as measured by the mammosphere assay. Two agents inhibiting the cell cycle machinery, currently undergoing clinical trials, were further evaluated. Results showed that use of these agents in ER negative cell lines increased the number of stem-like cells, whereas a decrease was observed in the ER positive cells. These results point to the disparate effects of cell cycle-targeting treatments on breast cancer cells and highlight the importance of subgroup analysis.

The breast cancer therapy tamoxifen is widely used in patients with ER positive breast cancer; however resistance occurs in approximately one-third of patients. By analysing a breast cancer tumour material from a patient cohort randomised to receive tamoxifen or control treatment, we have found that a non-functional RB pathway in ER positive breast tumours was associated with tamoxifen insensitivity. The non-functional RB pathway was however not correlated to prognosis, indicating that status of RB pathway holds purely treatment predictive information.

In addition, YAP1 was analysed in the tamoxifen randomised patient cohort and tumours lacking YAP1 protein expression were correlated with resistance to tamoxifen. Furthermore, in ER negative breast tumours, higher YAP1 expression was associated with increased proliferation whereas in ER positive tumours, YAP1 was negatively correlated to proliferation and grade. In vitro experiments downregulating YAP1 resulted in increased levels of ER and progesterone receptor (PgR), indicating aberrant signalling of the ER pathway.

Taken together, we have shown that the consequences of targeting cell cycle proteins may differ depending on ER expression. Furthermore, we have linked a non-functional pathway of the cell-cycle regulator RB and absent YAP1 protein expression to impaired tamoxifen response, identifying two potential biomarkers for predicting tamoxifen insensitivity.

Key words: Breast cancer, breast cancer subgroups, proliferation, migration, stem cells, tamoxifen, predictive marker, cyclin D1, CDK4/6, RB, YAP1

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List of Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.

Paper I

Lehn S*, Tobin NP*, Berglund P, Nilsson K, Sims AH, Jirström K, Härkönen P, Lamb R, Landberg G.
Downregulation of the oncogene cyclin D1 increases migratory capacity in breast cancer and is linked to unfavorable prognostic features.
Am J Pathol 2010, **177**:2886-2897

Paper II

Lamb R, Rogerson L, **Lehn S**, Landberg G.
Cell cycle regulators cyclin D1 and CDK4/6 have estrogen receptor dependent divergent functions in breast cancer migration and stem-like cell activity.
Submitted

Paper III

Lehn S, Fernö M, Jirström K, Rydén L, Landberg G.
A non-functional retinoblastoma tumor suppressor (RB) pathway in premenopausal breast cancer is associated with resistance to tamoxifen.
Cell Cycle 2011, **10**(6):956-962

Paper IV

Lehn S, Tobin NP, Sims AH, Stål O, Jirström K, Axelson H, Landberg G.
Decreased expression of Yes-associated protein is associated with outcome in the luminal A breast cancer subgroup and to an impaired tamoxifen response.
Manuscript

* Authors contributed equally.

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Abbreviations

4-OH-tam	4-hydroxitamoxifen
aCGH	array Comparative Genomic Hybridisation
AF-1, 2	Activation function-1, 2
AIs	Aromatase Inhibitors
AIB1	Amplified in breast cancer 1
AKT/PKB	AKT/Protein kinase B
ALDH	Aldehyde dehydrogenase
AP-1	Activator protein-1
AR	Androgen receptor
CASP8	Caspase-8 gene
CCND1	Cyclin D1 gene
CDK	Cyclin-dependent kinase
CDKN1A	Cyclin-dependent kinase inhibitor 1A gene (p21)
CDKN1B	Cyclin-dependent kinase inhibitor 1B gene (p27)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)
CDKN2B	Cyclin-dependent kinase inhibitor gene 2B (p15)
CI	Confidence interval
CKIs	CDK inhibitors
CSC	Cancer stem cell
CTGF	Connective tissue growth factor
DBD	DNA-binding domain
DCIS	Ductal carcinoma *in situ*
DMP-1	Cyclin D1-interacting myb-like protein-1
E2	17β-estradiol
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor α
ER-	ER negative
ER+	ER positive
ERE	Estrogen response element
FACS	Fluorescent activated cell sorter
FGFR	Fibroblast growth factor receptor
HDAC	Histone deacetylase
HER2/ ERBB2/Neu	Human epidermal growth factor receptor 2
ICC	Immunocytochemistry
IHC	Immunohistochemistry
LBD	Ligand-binding domain
LCIS	Lobular carcinoma *in situ*
LOH	Loss of heterozygosity
MEF	Mouse embryo fibroblast
MISS	Membrane-initiated steroid signalling
MLC	Myosin light-chain
MLCK	Myosin light-chain kinase
MS	Mammosphere
NCOR	Nuclear receptor corepressor gene
N-CoR	Nuclear receptor corepressor protein
NHG	Nottingham Histological Grade
NISS	Nuclear-initiated steroid signalling
NLS	Nuclear localisation signal
P/CAF	p300/CBP-associated factor

PgR	Progesterone receptor
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol 3-kinase gene (alpha)
RB	Retinoblastoma tumour suppressor protein
RB	Retinoblastoma tumour suppressor gene
RFS	Recurrence-free survival
ROCK	Rho kinase
SERD	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator
SMRT	Silencing mediator for retinoid and thyroid receptors
SP-1	Specificity protein-1
SRC-1	Steroid receptor co-activator-1
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
TAZ	Transcriptional co-activator with PDZ-binding motif
TDLU	Terminal duct lobular unit
TGF-β	Transforming growth factor beta
TMA	Tissue microarray
TransATAC	Translational research cohort of Arimidex, Tamoxifen, Alone or in Combination
YAP1	Yes-associated protein

Introduction

In 2011, 8382 Swedish women were diagnosed with breast cancer [1]. It is by far the most common cancer diagnosis among Swedish women, constituting 30% of all newly diagnosed cancers [2]. Several subgroups of breast cancer exist both on a histological as well as a molecular level, and prognosis and treatment options may differ widely between subgroups. The presence or absence of the estrogen receptor (ER) has long been indicative of two distinct biological entities of breast cancer, and research over the last decade based on gene expression analysis in breast tumours have revealed additional molecular subgroups with prognostic implications [3-5].

In this thesis, the overall aims have been to identify and delineate subgroup specific behaviours relating to proliferation, migration and stem-like cell properties in breast cancer cells, and to try to identify factors important for response to the commonly used endocrine therapy tamoxifen. Both *in vitro* methods and clinical tumour materials from patient cohorts have been used in the studies. Our focus has mainly been on cell cycle proteins that are frequently deregulated in breast cancer.

We have explored a contrasting link between proliferative and migratory behaviour in cancer cells, linking the cell-cycle proteins to migration. Furthermore, we have investigated how treatments affecting the cell cycle may increase or deplete the number of cells with stem-like cell properties. We also identified a functional retinoblastoma tumour suppressor protein (RB) pathway as important for predicting response to tamoxifen, and finally we determined the significance of yes-associated protein (YAP1) in breast cancer molecular subgroups and its relationship to tamoxifen response.

Taken as a whole, this thesis aims to underline both the importance of the cell cycle in breast cancer pathogenesis, and the necessity to analyse biomarkers in the context of specific, well-defined patient subgroups.

The normal and malignant breast

Normal development of the breast

The rudimentary ductal tree of the human breast is already present in the foetus and is identical in the two sexes until the onset of puberty [6]. The glands of the breast consist of two cellular compartments; the epithelium and the surrounding stroma. During puberty, the epithelial compartment develops into a more mature branched ductal system termed 'terminal duct lobular units' (TDLUs) and following pregnancy, the lobuloalveolar compartment required for lactation is further expanded and differentiated (Fig. 1). Subsequent to lactation, the glands undergo involution by massive cell death, resulting in reinstatement of the ductal architecture resembling that before pregnancy. The very dynamic changes to the breast tissue occurring after the foetal stadium are largely dependent on hormonal signalling, primarily executed by estrogen- and progesterone receptor pathways [7].

There are two major cell types in the breast, the luminal epithelial and myoepithelial cell type. During lactation, the luminal epithelial cells of the alveoli are responsible for the secretion of milk into the lumen of the ducts, whereas the myoepithelial cells contract in response to the hormone oxytocin, creating a flow of milk through the ducts towards the nipple [8]. The two cell types are suggested to arise from a common progenitor, a breast stem cell, situated suprabasally in the TDLUs (Fig. 1) [9].

Several of the signalling molecules important for normal breast development have been implicated in breast cancer, including cyclin D1 and the estrogen receptor (ER). The signalling pathways involving these proteins will be further addressed in later sections.

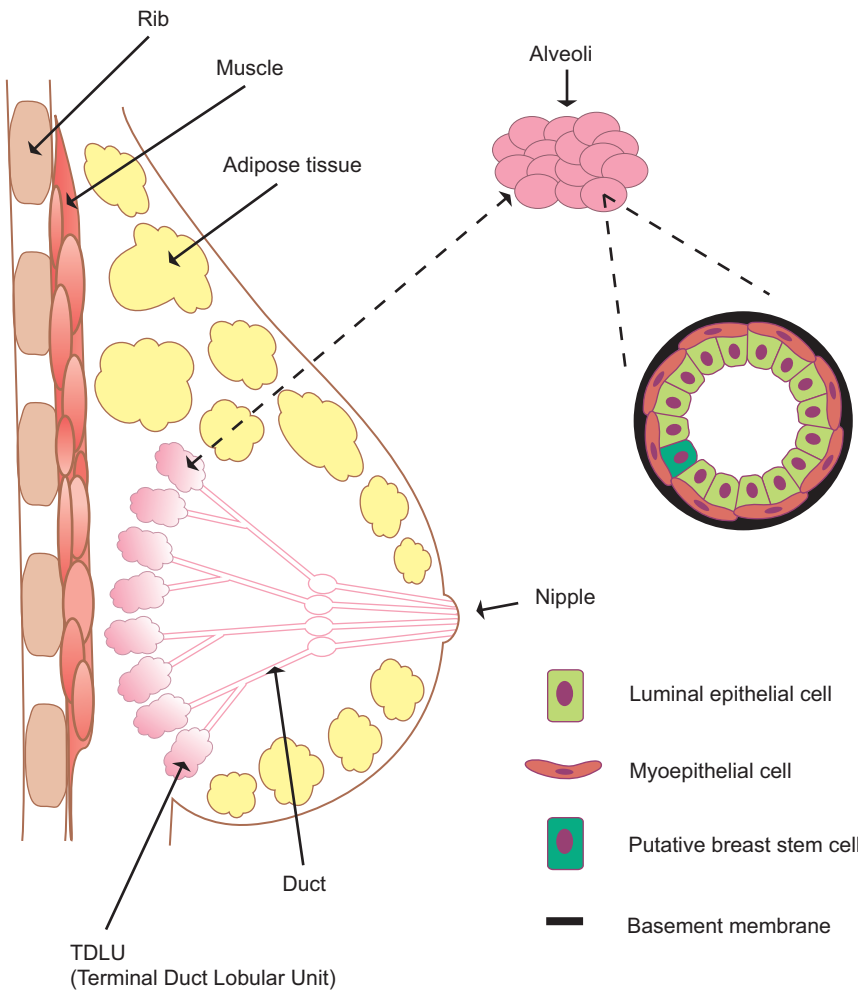

Figure 1. Schematic illustration of the normal breast.
The ducts and TDLUs (Terminal Duct Lobular Units) constitute the functional units of the breast. During lactation, milk is produced and secreted by the luminal epithelial cells of the alveoli and transported to the nipple through the ducts. A cross section of the alveoli shows the luminal epithelial and myoepithelial cells; cell types which are also present in the structure of the ducts.

Breast cancer

Epidemiology and aetiology

Breast cancer is the most common cancer diagnosis among women in the Western world with approximately 700 000 new cases every year, of which 8000 are diagnosed in Sweden [1, 10]. Despite an increase in breast cancer incidence over the last 20 years, mortality rates are decreasing and the 5-year and 10-year survival rates in Sweden are 87.8% and 78.8%, respectively [1, 2]. The majority of breast cancers are sporadic and non-familial. Germ-line mutations of *BRCA1* and *BRCA2*, associated with a high risk of developing breast cancer, are only detected in 15-20% of cases in families with a history of breast cancer. This implies additional genes of low to medium penetrance contributing to breast cancer risk, that have not yet been identified [11].

There are multiple factors of different significance likely to contribute to an individual's overall risk of developing breast cancer. Established risk factors are early menarche and late menopause, as well as nulliparity or late age at first child-birth, age and geographical location [12]. Risk factors exerting a modest impact on breast cancer risk are hormone replacement therapy and life style factors such as body mass index, alcohol consumption and smoking, whereas a family history of breast cancer and previous history of breast cancer or benign breast disease are high risk factors [12-14].

A meta-analysis of approximately 35 500 breast cancer patients examining different risk factors and the risk of developing a specific breast tumour subtype revealed that reproductive factors and body mass index was mainly associated to hormone-receptor positive tumours. This suggests that breast tumours negative of hormone receptors and HER2 (human epidermal growth factor 2), also referred to as triple-negative tumours, might have a distinct aetiology separate from the risk factors of hormone-dependent tumours [15].

Breast cancer initiation, progression and cell of origin

Breast cancer is believed to arise mainly in the TDLUs (Fig. 1). The progression of breast cancer has been proposed to follow a linear multi-step process of aberrant proliferation. Morphologically it may be described as starting with flat epithelial atypia followed by atypical ductal hyperplasia and ductal carcinoma *in situ* (DCIS), which eventually may progress into an invasive ductal carcinoma with potential metastatic properties (Fig. 2) [16, 17]. Corresponding stages for lobular invasive carcinoma would be atypical lobular hyperplasia and lobular

carcinoma *in situ* (LCIS). There are molecular data on both the genomic and transcriptomic level supporting this linear process, however all steps are not required for invasive cancer to develop [16].

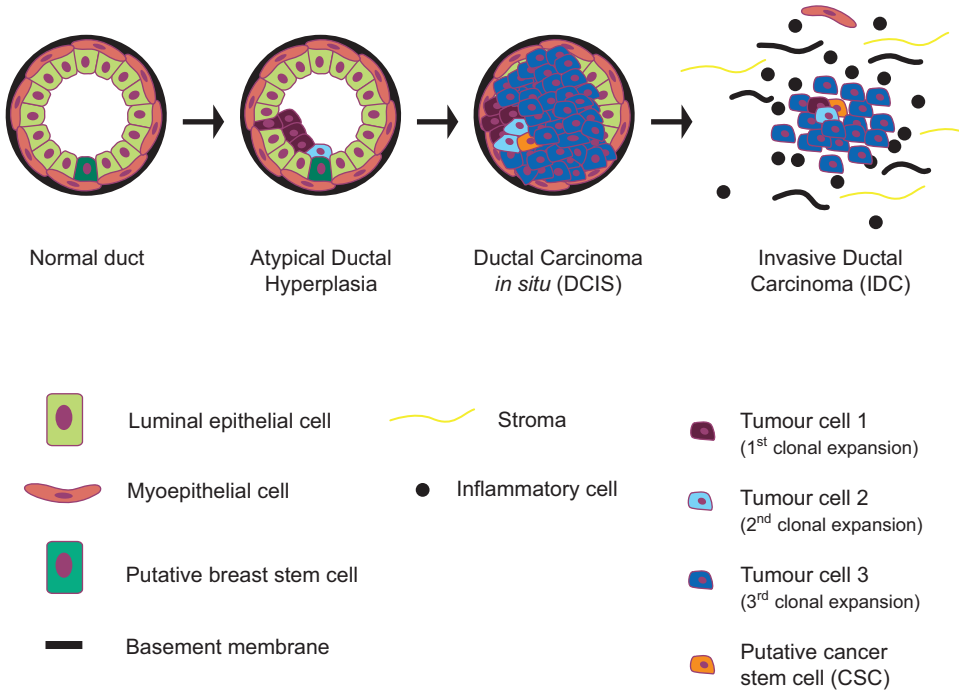

Figure 2. Illustration of the main steps of breast cancer progression.
Aberrant proliferation leads to atypical ductal hyperplasia which may subsequently develop into ductal carcinoma *in situ*, in which the basement membrane is still intact. When cancer cells break through the basement membrane the cancer becomes invasive, depicted in the last step. The cell types of the duct are indicated and the heterogeneity of cancer cells is illustrated by different tumour cell populations.

There are two proposed models attempting to explain the evolution and initiation of breast cancer; the sporadic clonal evolution model and the cancer stem cell model. The first non-hierarchical model proposes that any breast epithelial cell may acquire mutations eventually leading to cell transformation. Selection of cells experiencing advantageous genetic and epigenetic alterations, "survival of the fittest", will subsequently lead to tumour progression. The second, hierarchical model, often referred to as the cancer stem cell (CSC) model, suggests that there exists a pool of tumour stem cells responsible for initiation and maintenance of tumour growth. The current view of the field tends to favour a model encompassing both scenarios, where for example the proposed CSCs are subjected to clonal evolution during tumour progression (Fig. 2) [16, 18, 19].

Furthermore, to explain the heterogeneity of breast cancer, two hypothetical models have been proposed. The first model suggests that a different cell of origin is accountable for the different subtypes observed, and this cell is generally assumed to be either a stem- or progenitor cell. The second hypothesis proposes that the cell of origin may be the same for different subtypes but depending on the acquired specific genetic and epigenetic events, different phenotypes arise resulting in heterogenic subtype classifications [20]. Recently, a luminal progenitor cell was identified as the cell of origin in basal-like breast tumours contradicting the general belief that this molecular tumour subtype arise from basal stem cells [21].

Common genetic alterations in breast cancer

Activation of proto-oncogenes by for example point mutations or DNA amplification combined with inactivation of tumour suppressor genes by mutations, promoter methylation or deletions, all contribute to breast cancer development [22]. In a study analysing the genomes of 100 human breast tumours, mutations defined as driver mutations were found in at least 40 cancer genes, and 73 different combinations of mutated genes were found [23]. These observations highlight the notion of breast cancer as a disease with great genetic diversity. In the following section, a number of oncogenes and tumour suppressors implicated in breast cancer will be addressed.

The *PIK3CA* gene encoding the p110 catalytic α-subunit of the heterodimeric PI 3-kinase is considered an oncogene, targeted by point mutations in 30-36% of all breast cancers [23, 24]. Mutations of *PIK3CA* is reported to be enriched in ER^+ breast cancers and in the HER2 breast cancer subgroup [24, 25]. Another implicated oncogene in breast cancer is *CCND1* (encoding cyclin D1) located at chromosome 11q13 and amplified in 8-15% of all breast cancers [26, 27]. The *ERBB2* gene (17q12) encoding HER2 is amplified in 10-34% of breast cancers, and *MYC* (8q24) is amplified in approximately 11% [28, 29]. The *AIB1* gene (Amplified in breast cancer-1) on chromosome 20q13 functions as a co-activator of ER and is amplified in 5-10 % of breast cancers [30, 31].

Genetic alterations such as the hereditary germ-line mutations of *BRCA1* and *BRCA2* are examples of tumour suppressor genes where loss of function contributes to malignant cell behaviour. Additional examples of tumour suppressor genes are the *TP53* and *RB* genes, both shown to be deleted or inactivated by somatic point mutations in 15-34% and 39% of all breast cancers, respectively [32, 33]. Point mutations or promoter gene methylation of the *CDH1* gene encoding E-cadherin is reported in up to 85% of lobular invasive carcinomas [34]. Additional genes identified as tumour suppressors in breast cancer are *PTEN* (antagonising

the PI 3-kinase pathway), *NCOR1*, *CASP8*, *MAP3K1* and *CDKN1B*. Their overall frequency of alteration is however low; ranging from 1-6% [23]. The transcription factor *GATA3* has been shown to harbour point mutations in approximately 18% of ER^+ tumours [23].

There are still an unknown number of tumour suppressor genes to be discovered, most likely residing in gene regions commonly subjected to deletion during breast cancer progression. Examples of chromosomal regions frequently deleted or displaying loss of heterozygosity (LOH) in breast cancer are 1p, 1q, 3p, 6q, 8p, 11q, 13q, 16q, 17p, 17q and 22q, to mention a few [33].

Prognostics and treatment

Prognostics

Histologic classification

Histologic classification refers to the growth pattern of a tumour. A large histologic diversity exists in breast tumours, whereby 17 different special types make up 25% of all diagnosed cases. The most common histological type is the invasive ductal carcinoma which is diagnosed in about 50-80% of all breast carcinomas, followed by invasive lobular carcinoma, prevalent in 5-15% [35, 36]. Histologic type does in most cases not confer much prognostic information with the exception of medullary carcinoma, which despite a high histological grade has a relatively good prognosis [35].

The Nottingham Histological Grade and TNM staging

Grading tumours according to the Nottingham Histological Grade (NHG) has proven very useful in terms of predicting disease aggressiveness. Tumours are assigned a grade from I (well differentiated) to III (poorly differentiated), by evaluating morphological features of the tumour such as tubule formation, nuclear polymorphism and mitotic count [37]. The prognostic implication of NHG has been validated in several independent patient cohorts [38].

An additional measure of classifying breast tumours to obtain prognostic information is the use of TNM staging, where tumour size (T), lymph node involvement (N) and distant metastasis (M) are taken into account. By combining three factors all holding prognostic information, an estimate of the clinical stage of the disease is obtained [39].

Immunohistochemical markers

Several immunohistochemical markers are assessed in the clinic to provide both prognostic, and perhaps most importantly, treatment predictive information. The estrogen receptor (ER) and progesterone receptor (PgR) are both evaluated to determine possible benefit of endocrine targeted treatments, and a cut-off level at 10% positive nuclei is used for both markers. The growth factor receptor HER2 is evaluated initially by immunohistochemistry, followed by a FISH/CISH analysis (fluorescent or chromogenic *in situ* hybridisation) to determine amplification status of the *ERBB2* gene. The evaluation of HER2 is used in guiding treatment with the HER2-targeted antibody trastuzumab, and also serves as a prognostic factor indicative of aggressive disease [40]. The proliferative marker Ki-67 is evaluated immunohistochemically to determine the proliferative activity of a tumour. However, consensus regarding its clinical use has not been reached, partly due to lack of a clearly defined cut-off value and difficulties in standardising the immunohistochemical method [40, 41].

Molecular subgroups of breast cancer

Research over the last decade utilising gene expression profiling of breast cancer tissue have provided new insights into the heterogenic molecular composition of breast cancer, and has led to the identification of several molecular subgroups with prognostic, and possibly also treatment predictive, implications. Initially proposed by Perou *et al.* in the year of 2000, four molecular subgroups were identified by hierarchical clustering of gene expression data from 38 invasive breast cancers; the luminal, normal breast-like, HER2 and basal-like subgroups [3]. This work was soon followed by a report from the same group where the luminal subgroup was further divided into luminal A and luminal B subgroups. Most importantly, the subtype classification was now also demonstrated to hold prognostic information, with luminal A and basal-like breast cancers displaying the best and worst prognosis, respectively [5].

Both luminal A and B subgroups are characterised by hormone receptor positivity, but luminal B cancers display increased proliferation and are more often of higher histological grade. Luminal A breast cancers are usually of lower grade and present with a low proliferation index (Table 1). In the luminal B subgroup, chromosomal aberrations such as amplification of the 11q13 gene *CCND1* as well as amplification of *FGFR1* at 8p11 are frequently found [42, 43]. Tumours harbouring functional loss of retinoblastoma tumour suppressor protein (RB) may also be found in the luminal B subgroup, although this specific molecular event is more common in the basal-like subgroup [32]. The basal-like subgroup also comprises the so-called triple-negative tumours, negative for immunohistochemical markers ER, PgR and HER2 [44]. The HER2 subgroup is, as the name implies, enriched for tumours with amplification of the *ERBB2* gene,

however tumours may fall in this subgroup regardless of presence of the specific amplification event. One study reported the normal-like subgroup to be enriched for patients currently using HRT (hormone replacement therapy) and these tumours were generally smaller and of low grade [45].

Table 1. Summary of molecular subgroup characteristics

Subgroup	ER+	PgR+	HER2+	Histological grade*	Proliferation Ki67	Outcome
Luminal A	91-100%	70-74%	8-11%	I/II: 70-87% III: 13-30%	Low	Good
Luminal B	91-100%	41-53%	15-24%	I/II: 38-59% III: 41-62%	High	Intermediate or poor
HER2	29-59%	25-30%	66-71%	I/II: 11-45% III: 55-89%	High	Poor
Basal-like	0-19%	6-13%	9-13%	I/II: 7-12% III: 88-93%	High	Poor
Normal-like	44-100%	22-63%	0-13%	I/II: 37-80% III: 20-63%	Low or intermediate	Intermediate

*Nottingham histological grade I,II or III. ER=estrogen receptor α, PgR= progesterone receptor, HER2=human epidermal growth factor 2. Information obtained from reference [46].

Molecular subgroup classification was initially thought to be indicative of cell of origin, hence luminal and basal subgroups were suggested to originate from luminal and basal progenitor cells, respectively [5]. However, it has been reported that deletion of *Brca1* (loss of which is closely associated to a basal-like phenotype in human breast cancer) in the mouse mammary epithelial luminal progenitors, and not in the basal cells, give rise to the basal-like phenotype [21]. In addition, carriers of *BRCA1* mutations have been shown to harbour an aberrant luminal progenitor cell population [47]. This suggests a luminal progenitor to be the cell of origin for basal-like breast cancers, contrary to the initial belief.

Gene expression profiling is a dynamic research area and recently an integrated genomic and transcriptomic analysis of 2000 breast tumours identified 10 novel subgroups with prognostic implications. One of the subgroups identified was a high-risk ER^+ subgroup characterised by 11q13/14 amplification, highlighting the importance of this specific genetic aberration in breast cancer [48]. Additional subgroups identified are the claudin-low, characterised by enrichment in markers of epithelial-to-mesenchymal transition (EMT), immune response genes and

features reminiscent of cancer stem cells, and the androgen receptor (AR) positive molecular apocrine subgroup, symptomatic of increased androgen signalling [46, 49, 50].

Gene expression assays which have been approved for clinical use are the Oncotype DX® and MammaPrint® assays [51, 52]. The Oncotype DX® test measures the expression of 21 genes of which 5 are reference genes, and may be used in patients with ER^+ node-negative breast cancer. The MammaPrint® signature is based on 70 genes and is approved for determining the prognosis in patients with node-negative, stage I or II invasive breast cancer with a tumour size of less than 5 cm. However, these tests are not currently employed in the Swedish treatment guidelines [40].

To conclude, the importance of adapting a subgroup perspective even in basic research is now apparent. Placing results from the lab bench in a molecularly relevant breast cancer subgroup context is necessary in order to further our understanding of the complexity of breast cancer.

Treatment

Surgery and radiotherapy

Surgery has a prominent role in breast cancer treatment. The primary tumour may be removed either by performing breast-conserving surgery or mastectomy [40]. Several adjuvant therapies may subsequently be added, guided by tumour characteristics such as expression of ER and PgR, HER2 status and overall prognostics described previously. During the surgical procedure, a sentinel lymph node biopsy is performed to determine the potential presence of malignant cells. In the case of a negative biopsy, further resection of the lymph nodes is not necessary, sparing the patient of side effects associated with lymph node removal [53].

Chemotherapy

Chemotherapy may be offered after surgical removal of the primary tumour as a means of eradicating dormant micrometastases. In case of an inoperable primary tumour, neoadjuvant or preoperative chemotherapy may be given to render it surgically removable. The more common chemotherapy treatment regimes used are the anthracyclin-based polychemotherapies FAC (fluorouracil, doxorubicin, cyclophosphamide) and FEC (fluorouracil, epirubicin, cyclophosphamide) as well as the combination therapy CMF (cyclophosphamide, methotrexate, fluorouracil) [54]. Chemotherapy is associated with severe side effects and toxicity, hence identifying patients who would do equally well on e.g. only endocrine therapy is an extremely important and ongoing research area.

Endocrine therapy

Several types of endocrine therapy which target the estrogen receptor (ER) may be allocated to patients diagnosed with ER^+ breast cancer. Tamoxifen is a non-steroidal drug categorised as a 'selective estrogen receptor modulator' (SERM) widely used in the adjuvant setting. Its functional mechanisms are more closely addressed in the section of *Tamoxifen and resistance mechanisms*. Raloxifene, another compound from the SERM family, has been approved as a chemopreventive drug in women experiencing a high risk for developing breast cancer but has failed as an alternative to tamoxifen in breast cancer treatment [55].

The Aromatase Inhibitors (AIs) are designed to attenuate estrogen synthesis by targeting the enzyme aromatase, involved in the process of converting androgens into estrogens. AIs have proven to be more efficient in ER^+ postmenopausal patients compared to treatment with tamoxifen, and are now the recommended treatment for this patient group [56].

Fulvestrant, a compound belonging to the group of 'selective estrogen receptor downregulators' (SERDs) functions by binding the ER and inducing rapid degradation of the receptor. It is considered to have a more "pure" anti-estrogenic effect compared to tamoxifen, which has estrogenic effects (i.e. induction of proliferation) in for instance uterine tissue [57, 58]. At present, fulvestrant is indicated for use as a second-line treatment in postmenopausal patients with advanced ER^+ disease who have progressed on adjuvant endocrine therapies [40]. A clinical trial evaluating tamoxifen and fulvestrant in postmenopausal patients with advanced ER^+ disease, previously untreated with endocrine therapy, showed no significant differences in time to progression between the two treatment arms [59].

HER2-targeted therapy

The monoclonal antibody trastuzumab (Herceptin®) was designed to target and inhibit HER2 which is amplified or overexpressed in 20-30% of all breast cancers [60]. HER2 functions both as a prognostic and treatment predictive factor, and patients with HER2-positive tumours experience a worse prognosis despite specific HER2-targeting treatments. Recently, evidence has emerged indicating a possible clinical benefit of adjuvant trastuzumab in patients with HER2-negative breast cancer, and a prospective clinical trial (NSABP B-47) has been initiated to clarify these observations [61]. HER2 signalling has been suggested to regulate the mammary stem- or progenitor population of a breast tumour, and this is an attractive explanation to why adjuvant HER2 targeting might be efficient in patients with tumours not overexpressing HER2 [62].

Key characteristics of breast cancer:
Proliferation, migration and breast cancer stem cells

The cell cycle

The fundamental process of cell growth and division is a tightly regulated course of actions, carried out by oscillating levels of a family of proteins known as the cyclins. The cyclins associate to and activate members of the serine/threonine cyclin-dependent kinase (CDK) family in a cell cycle phase specific manner [63]. Their order of expression is illustrated in Fig. 3.

The main phases of the cell cycle are:

G_0 (G stands for Gap phase) in which the cell is residing in a quiescent, resting state awaiting an external stimuli to trigger the start of a cell cycle. The majority of the cells in the body reside in this phase.

G_1 (Gap 1 phase) is entered once the cell receives an external mitogenic signal and involves preparation of DNA duplication.

S phase (Synthetic phase) during which the cell replicates its DNA.

G_2 phase (Gap 2 phase) in which the cell prepares for M phase and cell division.

M phase (Mitosis) involves the separation of the replicated chromosomes into two daughter cells. This phase consists of several distinct subphases namely prophase, metaphase, anaphase and telophase. M phase is ended by cytokinesis, the actual cell division.

In the normal cell, several so-called 'checkpoints' are present throughout the different phases to ensure proper execution of the various steps towards cell duplication [64]. Cancer cells may be described as cells with acquired features which allow them to override such checkpoints, leading to uncontrolled proliferation. The restriction point was initially described in yeast as a checkpoint exerting its control of cell cycle progression in late G_1 phase, but is no longer defined as a checkpoint *per se* [65]. Instead, the restriction point is described as a point of no return for cell cycle progression, and it denotes the point in time of the cell cycle in which the cell may switch from dependence on external mitogenic stimuli to independence of them. One of the hallmarks of cancerous cells is the

independence of external signals for cell proliferation, and events involved in the regulation of the restriction point are frequently altered in cancer [66, 67]. The following section will address cell cycle proteins mainly involved in the transition of the restriction point in late G_1/S phase.

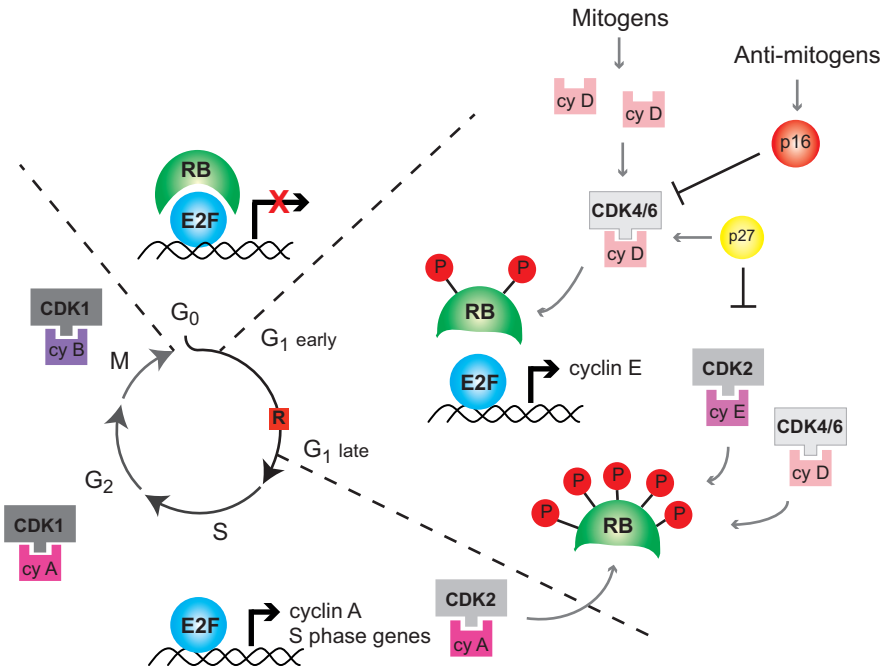

Figure 3. The cell cycle and regulation of G_1 and G_1/S transition.
The main phases of the cell cycle and corresponding cyclin-CDK complexes are indicated. In a resting cell (G_0), RB actively represses E2F-mediated transcription. Upon mitogenic signalling, cyclin D is synthesised. Cyclin D-CDK4/6 complexes phosphorylate RB which becomes partly inactive, resulting in cyclin E transcription. Cyclin E-CDK2 complexes continue to phosphorylate RB which becomes completely inactivated, leading to transcription of S phase genes. p27 acts as an assembly factor for cyclin D-CDK4/6 complexes, but inhibits cyclin E-CDK2. R denotes the restriction point and indicates the transition from early G_1 mitogen-dependence to late G_1 independence. Partly modified from [67] and [68].

Cell cycle molecules of the G_1/S phase

The first cyclins to be expressed once the cell receives an external mitogenic signal are the D-type family of cyclins, such as cyclin D1 [69]. Several signalling cascades have been described to converge on cyclin D1 expression, including the Ras/Raf/MAPK, ER and PI 3-kinase pathways [70, 71]. Various cytokines may

induce cyclin D1 expression through STAT3 and STAT5 [72, 73]. Binding of Wnt to its receptor Frizzled leads to β-catenin-mediated cyclin D1 transcription [74].

Cyclin D forms a complex with the catalytic subunits CDK4 and CDK6, allowing for phosphorylation and thereby inactivation of the retinoblastoma tumour suppressor protein (RB). The conformational change inflicted on RB by phosphorylation leads to the release of transcription factors belonging to the E2F transcription factor family, triggering expression of target genes such as cyclin E and cyclin A. In addition to E2Fs, the RB complex includes transcriptional repressors such as histone deacetylases and chromosomal remodelling SWI/SNF complexes, and these interactions are also disrupted by the phosphorylation event (reviewed in [75]). The subsequent cyclin E-CDK2 and cyclin D-CDK4/6 complexes continue to phosphorylate RB, creating a positive feed-back loop increasing cyclin E and cyclin A transcription levels. The cell is pushed further in G_1 towards S phase. If no negative signals (e.g. withdrawal of mitogens) are received inhibiting further phosphorylation of RB, the restriction point is passed and progression of the cell cycle is now completely reliant on intrinsic signalling, for the moment exerted by cyclin E-CDK2 complexes. As the cell enters S phase, cyclin A becomes the dominant cyclin bound to CDK2 (Fig. 3) [75].

The family of cyclins has been described as proto-oncogenes due to their proliferation-promoting capabilities, and overexpression or deregulation of cyclin D1 and cyclin E is frequently observed in breast cancer [76]. The tumour suppressor RB is recurrently reported to be either lost or functionally inactivated in the cancer setting [67].

Cell cycle inhibitors

CDK inhibitors (CKIs) such as $p16^{INK4a}$ (encoded by *CDKN2A*), $p15^{INK4b}$ (*CDKN2B*), $p21^{WAF1}$ (*CDKN1A*) and $p27^{KIP1}$ (*CDKN1B*) exert another level of cell cycle control, mainly by hindering the formation of active cyclin-CDK complexes. $p27^{KIP1}$ inhibits the actions of CDK2 but also functions as an assembly factor for cyclin D-CDK4/6 complexes. In an actively cycling cell, practically all $p27^{KIP1}$ molecules are bound to cyclin D-CDK4/6 complexes. This leaves insufficient amounts of $p27^{KIP1}$ to inhibit cyclin E-CDK2 complexes, thereby facilitating cell cycle progression. When cyclin E-CDK2 becomes the prevailing complex in late G_1 it may actually antagonise its own inhibitor by phosphorylating $p27^{KIP1}$, targeting it for degradation [77]. These events all contribute to transition through the restriction point (Fig. 3). Another CKI, $p15^{INK4b}$, has been shown to be induced upon signalling of the growth arresting cytokine TGF-β (transforming growth factor beta) resulting in $p15^{INK4b}$ binding of CDK4 and CDK6, and subsequent inhibition of cyclin D-dependent kinase activity [78]. $p16^{INK4a}$ is also an inhibitor

of CDK4/6 whereas $p21^{WAF1}$ binds and inhibits CDK2. $p21^{WAF1}$ is reported to be one of the main effectors of the cell cycle arrest resulting from p53 signalling, initiated by the sensing of DNA damage [79, 80]. Proteins of the CKI family are considered tumour suppressors and loss of CKI expression is frequently observed in human cancers, including breast cancer [67].

Concluding remarks

It should be underlined that the proliferative status in combination with grade of a breast tumour is repeatedly demonstrated to hold great prognostic implication. Several of the more advanced gene expression signatures with prognostic value have later been shown to merely mirror the tumour grade and proliferation, despite measuring expression of unique sets of gene transcripts [81, 82]. Patterns of cell cycle aberrations have been shown to correlate to specific tumour subtypes and outcome, and could potentially add prognostic as well as predictive information [26, 83-85].

Migration

The ability of a cell to acquire a migratory phenotype is essential for processes such as developmental morphogenesis and tissue repair. However, initiation of a migratory programme in cancerous cells can lead to invasion into the surrounding tissue and vasculature, constituting the initial step of tumour metastasis [66].

The process of migration involves a very complex network of signalling resulting in cell polarisation and membrane extension, followed by contraction and thereby movement. Several comprehensive reviews have been published describing the events of a migratory cycle [86-88]. A brief overview of the main steps of migration and associated molecules are outlined below.

Polarisation

The first step in the onset of migration is cell polarisation. Upon sensing a migratory-promoting agent, the cell takes on a polarised morphology, involving the establishment of a distinction between the cell front and back. Examples of molecules involved in this process are Cdc42 and Rac of the Rho GTPase family, and PIP_3 which is produced locally in the cell front by action of PI 3-kinase. The formation of activated integrin receptor complexes are also of importance in the polarisation process [88].

Membrane extension

Second, cell protrusions are formed which bind ECM (extracellular matrix) or adjacent cells to subsequently allow for the cell to pull its way forward. There are several types of protrusions with differences in their morphology, for example the broad lammelipodia, spike-like filopodia and cylindrical finger-like pseudopods. These protrusion events are driven by activated Cdc42 and Rac, resulting in activation of the Arp2/3 complex which in turn leads to actin polymerisation [89]. Rac activation has also been associated to microtubule polymerisation [90]. The protrusions are stabilised by the formation of adhesion complexes binding to the ECM or surrounding cells. The adhesion complexes consist of molecules belonging to the transmembrane integrin receptor family which cluster in the cell membrane to form focal contacts. The focal contacts are of a dynamic nature and may both adhere stably to the substrate or loosen their grip as the cell moves along a path. Signalling through the focal contacts involves for example the focal adhesion kinase (FAK), which binds the integrin cytoplasmic tail and subsequently recruits both actin-binding proteins and regulatory molecules such as PI3-kinase and Rho-family GTPases [86].

Cell body contraction and rear release

In the last step of migration, a contractile force is required to regulate the actual movement of the cell [87]. This event involves the motor protein myosin II which interacts with and pulls on the actin filaments. Myosin II is activated through phosphorylation of myosin light-chain (MLC) which in turn is regulated by the kinases MLCK (myosin light-chain kinase) and ROCK (Rho kinase). As the cell moves forward, adhesion disassembly and retraction occur at the rear of the cell. Myosin II and FAK have been shown to be important for retraction, and the tension itself, coming from the pulling forward of the cell, has been shown to contribute to detachment [87, 88].

Breast cancer stem cells

The concept of breast cancer stem cells (CSCs) has attracted a lot of attention during the last decade. Cancer stem cells are in general defined as cells with tumour-initiating properties, capable of self-renewal and differentiation [19]. Breast cancer stem cells may be propagated *in vitro* by use of the mammosphere assay, which enrich for cells with stem-like properties [91].

The first CSC identified from human solid tumours was derived from primary human breast cancers [92]. Breast CSCs (or tumour initiating cells) were identified by a cell surface expression phenotype of $CD44^{+}CD24^{-/low}$ in flow cytometry experiments. As few as 100 cells of this particular phenotype were required for tumour formation in mice, and the defined cell population was capable of re-generating the heterogenic phenotype present in the initial tumour. Thousands of cells with alternate phenotypes were on the other hand unsuccessful in establishing tumours [92].

Breast cancer cell subpopulations defined by high ALDH (aldehyde dehydrogenase) activity have also been suggested to be enriched in stem/progenitor properties [93]. Identified by use of the ALDEFLUOR® assay, only ALDH positive breast cancer cells were able to form tumours in mice even when transplanted in numbers as low as 500. The $CD44^{+}CD24^{-/low}$ phenotype and ALDH activity was shown to overlap in a small fraction (1%) of the investigated cancer cell populations. This double phenotype appeared to be highly enriched in cancer stem cells and a mere of 20 cells from this subpopulation were able to initiate tumour growth [93].

Combinations of phenotypes reported to be enriched for cancer stem cells have further proven useful in predicting outcome in breast cancer patients [94, 95]. As cancer stem cells have been postulated to be involved in drug resistance and tumour persistence, increased frequencies of cancer stem cell subpopulations in a tumour have been hypothesised to correlate to a worse prognosis [96, 97]. Some attempts have been made to use immunohistochemistry for CSC identification instead of flow cytometry. In two recent studies, primary breast tumours were stained with specific antibodies for CD44, CD24 and ALDH1 among others. The combination of multiple markers had greater prognostic value compared to individual marker expression [94, 95].

Epithelial-to-mesenchymal transition (EMT) has also been linked to CSCs. When EMT was induced in an immortalised human mammary epithelial cell line, cells acquired stem cell-like features such as the ability to form mammospheres. This suggests involvement of the EMT process in the self-renewal feature of CSCs [98].

CSC theories remain controversial and there is debate regarding both theoretical and experimental issues. Take for example the xenograft model, in which CSCs are tested for their tumour-initiating properties. This model does not allow for evaluation of the importance of the tumour microenvironment and the immune system, which is a notable drawback given that CSCs may arise as a consequence of microenvironmental signals or other external cues [99]. In solid tumour models, the need to dissociate tumour tissue into single cells may affect viability and behaviour of the cancer stem cells. In addition, parameters including the duration

of the experiment and the type of mouse model used may have a critical impact on model outcome [99, 100].

Cell cycle associated proteins

The focus in this thesis primarily lies on the well-known cell cycle associated proteins cyclin D1 and RB. This section will describe these two molecules in further detail, both from a cell cycle- and breast cancer perspective. YAP1, the protein of interest in paper IV, has been closely linked to proliferation but its precise role in the cell cycle has not been as thoroughly defined as for cyclin D1 and RB. Hence, YAP1 will be addressed from a more general cancer perspective.

Cyclin D1

The cyclin D family

CCND1 was identified in 1991 as a gene involved in chromosomal rearrangements in a subset of benign parathyroid tumours [101]. Cyclin D1 was soon recognised as a protein with oncogenic growth-promoting properties, and its overexpression and amplification is reported in various cancer types [67]. Cyclin D1 consists of 295 amino acids and has several functional domains. The N-terminal of cyclin D1 holds an RB-binding domain, and a conserved domain termed the cyclin box which is important for the binding of CDKs, is found in the central part. A sequence present in the C-terminal has proven to be important for CDK-independent functions of cyclin D1 [102].

CCND1 is located at chromosome 11q13, whereas the loci of *CCND2* and *CCND3* map to chromosomes 12p13 and 6p21, respectively [103]. The three D-type cyclins constitute a subfamily within the cyclin family and share a similar exon-intron organisation [103]. They display an average of 57% identity in their coding regions and the cyclin box harbours 78% identity between the three family members. This should be compared to the 39% identity seen when compared to cyclin A, 36% compared to cyclin E and 29% identity when compared to cyclin B [104]. During mouse embryonic development, the different cyclin D proteins are expressed in a dynamic and often highly exclusive expression pattern although co-expression of all D-type cyclins may be observed as well [105]. Apart from the growth-promoting activities of the D cyclins they are also implicated in promoting

cell differentiation of specific cellular compartments [106]. Mice lacking all D-type cyclins have been shown to develop normally until mid/late gestation, after which they die [107]. This implies that normal proliferation in the developing mouse embryo may occur to some extent despite lack of all D-type cyclins.

Single cyclin D knock-outs as well as combinations of knock-outs of the D-type cyclins in mouse development have also been analysed [106]. All single cyclin D knock-outs were viable but presenting with varying phenotypes. Cyclin D2 knock-out lead to female sterility and impaired proliferation of peripheral B-lymphocytes [108]. Disrupting the cyclin D3 gene resulted in a phenotype of hypoplastic thymus with loss of T-cell maturation [109]. Mice lacking cyclin D1 were of small body size and suffered from underdeveloped retinas. The cyclin D1 null phenotype was also characterised by a defective development of the lobuloalveolar compartment during pregnancy, and lactation could not occur [110]. This observation together with several studies on primary human breast cancers identifying frequent overexpression specifically of cyclin D1, constitute the background to why cyclin D1 is the most extensively studied cyclin in the breast cancer setting [111, 112].

Cyclin D1 in models of breast cancer

Specific overexpression of cyclin D1 in the virgin mammary gland is reported to lead to increased proliferation and development of the lobuloalveolar compartment, reminiscent of early pregnancy. Around the age of 18 months, MMTV-*cyclin D1* mice developed multiple independent adenocarcinomas [113]. These experiments manifested the oncogenic capacity of cyclin D1 but also indicated cyclin D1 as a rather weak oncogene, considering the long time-frame before tumour onset. In comparison, MMTV-*neu* mice have tumour onset within an average of 3 months [114].

Cyclin D1 has been shown to be of importance for the development of mammary cancers induced by c-*neu* and v-Ha-*ras*, but not for those induced by c-*myc* or *Wnt-1* [115]. This suggests that not all oncogenic pathways are reliant on the induction of cyclin D1 for initiation of mammary neoplasia. Further studies have revealed that in the MMTV-*neu* mouse model, cyclin D1 is mediating its oncogenic effects by the activation of CDK4 [116].

CDK4 and CDK6

Cyclin D1 interacts specifically with two CDKs, CDK4 and CDK6 [117]. They are exclusive CDK-partners of the cyclin D family. *CDK4* gain and amplification

has been reported in 14%, 25% and 24% in the luminal A, B and HER2 molecular breast cancer subgroups, respectively [24]. CDK4 null mice survive embryogenesis but display a phenotype reminiscent of the small body sized cyclin D1 knock-out mice. In addition the mice are sterile, a phenotype observed in the cyclin D2 knock-out mice [108, 110, 118]. Amplification of *CDK4* has been associated to increased proliferation in breast cancer [119].

CDK6 null mice are viable and only display minor defects in hematopoietic cell populations, whereas the double CDK4/CDK6 knock-out results in late embryonic or postnatal lethality [120]. CDK6 has so far not been extensively studied in breast cancer. One study reported the protein to be downregulated in breast cancer and suggested that CDK6 might restrain rather than stimulate breast epithelial cell proliferation [121]. In addition, CDK6 has been implicated in blocking differentiation, a feature not shared with CDK4 [122].

CDK-independent effects of cyclin D1

Cyclin D1 may also be involved in activities unrelated to its function as a CDK regulatory subunit [123]. By interacting with the ER and SRC-family co-activators, cyclin D1 has been reported to activate ER in a ligand-independent manner [124, 125]. In breast tumours there is a strong correlation between overexpression of cyclin D1 and ER positivity, possibly supporting activation of ER mediated by cyclin D1 [126, 127]. Other transcription factors known to be affected by cyclin D1 in a CDK-independent fashion are DMP-1 (cyclin D1-interacting myb-like protein 1), androgen receptor, STAT3 (signal transducer and activator of transcription 3) and SP-1 [128-131]. However in contrast to the activating effect of cyclin D1 on ER transcription, in these examples cyclin D1 functions as a transcriptional co-repressor.

Furthermore, cyclin D1 has been implicated to function in a CDK-independent manner during DNA repair [132]. One of the first reports of a functional role for cyclin D1 in DNA repair came from Li *et al.* in 2010 [133]. Upon DNA damage cyclin D1 was shown to tether to the chromatin which subsequently resulted in recruitment of RAD51, known to play a critical role in the recombination repair of double-strand breaks. Further studies have shown that depletion of cyclin D1 results in increased sensitivity to ionizing radiation in both *in vitro* and *in vivo* experimental systems [134].

Cyclin D1 in breast cancer: amplification and overexpression

CCND1 amplification of chromosome 11q13 is frequently reported in breast cancer with numbers ranging from 8-15%. Protein overexpression is observed in 19-67% of all breast cancers and the event of *CCND1* amplification is in general linked to protein overexpression [26, 27, 112, 126, 135-140].

Several reports conclude that high cyclin D1 expression is associated to a better outcome [27, 126, 135] whereas some report the opposite or no difference [136, 140, 141]. The amplification of *CCND1* is more consistently reported to correlate to a worse outcome [26, 27, 135, 136], implying that cyclin D1 protein overexpression and amplification of *CCND1* are in fact two separate events in breast cancer, despite involvement of the same protein. It is reported that the amplification of chromosome 11q13 is accompanied by deletion of the distal part of 11q in up to 70 % of cases [42, 142]. Hence, the amplification of *CCND1* is frequently associated to loss of several putative tumour suppressor genes of unknown importance. One such gene located at 11q22 and thus often deleted upon *CCND1* amplification is *YAP1*, the main focus of paper IV which will be addressed below.

Yes-associated protein (YAP1)

Introduction to YAP1

Deletions of the distal part of chromosome 11q is a common event in breast cancer and reports of loss of heterozygosity (LOH) range from 37-63 % depending on the patient cohort studied [143-147]. The *YAP1* gene is located at 11q22 and functions as a transcriptional co-regulator [148].

There are eight different isoforms of YAP1, resulting from alternative splicing [149]. YAP1 contains several functional domains, of which the WW domain involved in protein-protein interactions has been most extensively studied [150]. Depending on the isoform, YAP1 contains one or two WW-domains which are characterised by presence of two highly conserved tryptophane (W) residues. The WW domains may bind and interact with proteins containing a proline rich sequence (PPXY, also known as PY-motif, where P stands for proline, Y designates tyrosine and X any amino acid). Since identified in YAP1, WW domains have been found in many proteins [151].

YAP1 was initially identified in 1994 when shown to bind the SH3 domain of the yes proto-oncogene through a proline-rich sequence [152]. Two proteins were

shortly thereafter identified and characterised as putative ligands of YAP1, binding with high specificity to the WW domain. These proteins were named WBP-1 and WBP-2 (WW domain binding protein 1 and 2) [153]. WBP-2 has later been identified as a co-regulator of ER, constituting a possible link between YAP1 and estrogen receptor signalling [154, 155].

In mice, disruption of *Yap1* results in lethality at embryonic day 9.5. TAZ (transcriptional co-activator with PDZ-binding motif), which displays approximately 50 % amino acid identity with YAP1 and is implicated in exerting functions similar to YAP1, can not compensate for the lost YAP1 expression in the developing mouse embryo [156, 157].

Cell cycle function of YAP1

YAP1 is implicated as one of the key players in the conserved tumour suppressive Hippo signalling network. This network is important for organ size control through regulation of cell growth, proliferation and apoptosis [158]. When there are no signals relayed through the Hippo pathway, nuclear YAP1 binds and activates TEAD transcription factors which result in transcription of e.g. CTGF (connective tissue growth factor). This leads to stimulation of cell growth and proliferation [159]. Active signalling through the Hippo network results in phosphorylation of YAP1 at serine residue 127, ultimately resulting in YAP1 inactivation by sequestering of the protein in the cytoplasm [160]. The growth-promoting activities of YAP1 are thereby inhibited. Inhibitory signalling may be initiated for example by cell-cell contacts, and is important for mediating prevention of tissue overgrowth. If signalling through the Hippo pathway on the other hand is lost, YAP1 remains constitutively active in the nucleus which may result in improper oncogenic signalling [160].

YAP1 as an oncogene

In hepatocellular carcinoma, YAP1 has been identified together with the inhibitor of apoptosis cIAP1 (gene name *BIRC2*) as key oncogenes of the 11q22 amplicon [161]. In addition it was established that YAP1 and cIAP1 drive oncogenesis in a cooperative fashion. YAP1 has further been implicated as an oncogene in several cancer types such as esophageal squamous cell carcinoma, non-small-cell lung carcinoma, ovarian clear cell carcinoma, colorectal carcinoma and medulloblastoma [162-166].

YAP1 in breast cancer – a subgroup specific tumour suppressor?

In breast cancer, reports of YAP1 function have been contradictory. In 2008, Yuan and co-workers published a report suggesting YAP1 to function as a tumour suppressor in breast cancer [167]. They reported downregulation of YAP1 to result in increased migration and invasion, suppression of anoikis, and enhancement of tumour growth in nude mice. However, several reports suggest YAP1 to be oncogenic in breast cancer and expression of constitutively active YAP1 in murine mammary epithelial cells rendered these untransformed cells highly metastatic [168-171]. These conflicting results could possibly be explained by YAP1 exerting separate functions in different cell types. Possibly, YAP1 might have oncogenic or tumour suppressive features dependent on breast cancer subgroup context.

Retinoblastoma tumour suppressor protein (RB)

RB – the first tumour suppressor protein identified

In 1971, after observing the incidence rate of sporadic and hereditary retinoblastoma (a childhood tumour arising from retinal cells), Alfred Knudson proposed his now famous "two-hit hypothesis" [172]. The paper concluded that both copies of the gene associated with retinoblastoma had to be disrupted for the tumour to develop. The hypothesis later became the ground for the concept of tumour suppressor genes, and when the *RB* gene was identified in 1986 and found associated with retinoblastoma, the first tumour suppressor gene had been identified [173].

Cell cycle function of RB

RB is located at chromosome 13q14 and belongs to a gene family of "pocket proteins", also including p107 and p130 [174]. The pocket domain of RB consists of domains A and B and contains a recognition site for histone deacetylases (HDACs) and proteins related to the SWI/SNF nucleosome remodelling complex [175, 176]. A second binding site in the pocket domain allows for RB to bind members of the transcription factor family E2F, with strong preference for E2F-1, E2F-2 and E2F-3 [177]. The resulting complex of RB, HDACs and E2F sits on the DNA, thereby inhibiting transcription of genes required for cell cycle progression [178]. In the nucleus, RB also binds and represses the function of the non-receptor

tyrosine kinase c-Abl. Upon phosphorylation of RB, c-Abl is released and activated, contributing to cell cycle progression [179].

The sequential phosphorylation of RB by different cyclin-CDK complexes is fundamental to cell cycle progression. Sixteen phosphorylation sites have been identified in RB. During progression of the cell cycle, RB is initially unphosphorylated (active repression), followed by hypo- (partial repression) and hyperphosphorylated (inactive repression) forms of the protein (Fig. 3) [180, 181]. A model has been proposed in which cyclin D-CDK4/6 phosphorylate RB in the carboxy-terminal region resulting in the displacement of HDACs from the pocket region. This event is sufficient to relieve the transcriptional inhibition of the cyclin E gene, required for further cell cycle progression. In addition, the cyclin D-CDK4/6 mediated phosphorylation of RB appears to function in the recruitment of cyclin E-CDK2 complexes. This interaction facilitates the phosphorylation of Ser-567 by cyclin E-CDK2, resulting in a conformational change of the RB pocket domain and complete disruption of E2F inhibition [182]. The free E2F complex is ultimately able to initiate transcription of genes driving cell cycle progression.

RB and apoptosis

RB has been reported to be involved in mediating apoptosis through a p53-dependent pathway. Unbound, free E2F-1 complexes are implicated in the transcription of the *ARF* gene. The *ARF* gene product can in turn inhibit the MDM2-mediated turnover of p53. Accordingly, loss of RB function may trigger apoptosis through the accumulation of p53, acting as an intrinsic protective mechanism in cells where the RB pathway is deregulated [183, 184]. Reports indicate that sufficient accumulation of free E2F only occurs if RB function is completely abolished. Consequently, in a proliferating cell under the control of RB, the ARF/p53 pathway is not activated due to insufficient levels of free E2F complexes [185].

RB alterations in cancer

Many of the germ-line mutations of the *RB* gene are confined to the pocket region, and most tumour-derived mutations also result in a disrupted pocket function [186, 187]. Besides the close association of *RB* germ-line mutations and retinoblastoma, inherited *RB* mutations also predispose to small-cell lung cancer and melanoma [188].

RB loss or mutation is reported in various cancer forms. Germ-line mutations of *RB* associate to cancer initiation, whereas loss or mutational inactivation of *RB* in

sporadic cancers is associated to tumour progression [188]. In breast cancer, *RB* loss or mutations are enriched in the luminal B and basal-like subtypes [32].

Several studies have linked RB loss to chemotherapy sensitivity, reviewed in [68]. One study reported RB loss of protein expression to be an independent factor in predicting response to chemotherapy in ER-negative patients [189].

The Estrogen receptor pathway and tamoxifen resistance

The benefit of estrogen withdrawal by ovariectomy, which resulted in tumour regression in breast cancer patients, was observed as early as 1899 by the surgeon Stanley Boyd [190]. Preceding his study, the British physician Beatson had made similar observations when removing the ovaries in premenopausal women with breast cancer [191]. The recognition of an estrogen receptor was first described in the 1960s by Jensen and Jacobson, when tissue uptake and retention of radio-labelled estradiol was detected in the uterus of rats [192]. A description of the basic biochemical function of the ER soon followed and by the end of the 1970s it was established that patients with ER-rich tumours were more likely to respond to endocrine treatment compared to patients with ER-negative tumours [193, 194]. ER is still today a critical predictive marker for the response to endocrine treatments. Nevertheless, a number of patients with ER-positive breast cancer will eventually present with recurrent disease, and many researchers are focusing on finding additional markers for predicting endocrine response. Below is an introduction to the structure and function of ER followed by a brief overview of what is known thus far of the mechanism by which tamoxifen functions, and of why certain tumours are resistant to the drug.

Estrogen receptor signalling

The estrogen receptor

ERα and ERβ isoforms

The ER is a nuclear hormone receptor belonging to the family of hormone-activated transcription factors [195]. ER exists in two isoforms, ERα and ERβ, transcribed from two distinct genes located on separate chromosomes [196, 197]. ERα is the predominant isoform expressed in the uterus, mammary gland, testis, pituitary, liver, kidney, heart and skeletal muscle. The ERβ transcript on the other hand is significantly expressed in the ovary and prostate, as determined by studies

in mice and rats [198, 199]. Co-expression of the receptors may be seen in a number of tissues, however the two transcripts are rarely expressed within the same cell type, implying disparate functions of the two isoforms [198, 200]. ERα knock-out mice display impaired development of the mammary gland and the architecture of the gland resembles that of newborn mice throughout the mice's lifespan [201]. Conversely, ERβ knock-out mice have mammary glands with normal ductal structures which appear to undergo normal differentiation during pregnancy and lactation, suggesting ERα to be the predominant receptor in normal mammary gland development and regulation [198, 202]. In this thesis, ER will refer to ERα if not otherwise specified.

ER protein structure

The ER consists of six functional domains, schematically depicted in Fig. 4. Located within domains A and B is the activation function-1 (AF-1) which in conjunction with the activation function-2 (AF-2) of domain E is involved in mediating transcription. The ligand-binding domain (LBD) is located in the same region as AF-2 and in between the AFs is the DNA binding domain of region C, required for the activated receptor to bind to specific DNA elements for transcription initiation. Domain D functions as a flexible hinge between regions C and E and contains one of several nuclear localisation signals (NLS) [203, 204]. Specific regions of the F domain have proven to be important for the response to ligand stimulation and for the binding of receptor co-activator proteins [205, 206].

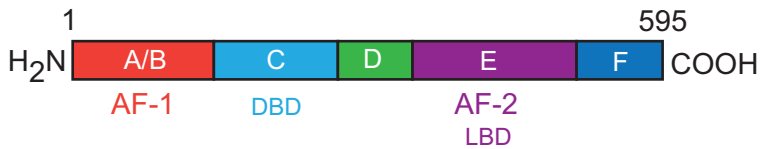

Figure 4. Protein structure of ER.
The ER consists of six functional domains termed A, B, C, D, E and F, further described in the text. The N-terminal AF-1 domain is regulated by phosphorylation and is constitutively active, wheras AF-2 is activated upon hormone binding.
ER=estrogen receptor, AF-1=activation function-1, AF-2=activation function-2, DBD=DNA-binding domain, LBD=ligand-binding domain

Activation of ER signalling

Estrogen and the estrogen receptor are key regulators of complex biological networks regulating diverse functions within a cell such as proliferation, apoptosis, invasion and angiogenesis [207-209]. The ER is activated by the female hormone estrogen which exists in three major forms in the human body, namely estrone, estradiol and estriol. In premenopausal women, ovaries produce between 70 and

500 μg of an estradiol known as 17β-estradiol (E2) daily and although the different estrogens all have a high affinity for ER, E2 is the more potent activating ligand [199, 210]. Upon binding of E2 to the ligand-binding domain, the ER undergoes a transformational change into a dimerised active receptor complex which translocates into the nucleus. Several co-factors such as the steroid receptor co-activators (SRCs), and p300/CBP (CREB-binding protein) are also recruited to the complex [211]. The various pathways through which ER may activate transcription are outlined below and illustrated in Fig. 5.

Nuclear-initiated steroid signalling pathway (NISS)

The ER nuclear-initiated steroid signalling pathway may be divided in three categories; A) the classical ligand-dependent, B) the non-classical ligand-dependent and C) the ligand-independent pathway of genomic signalling. In the classical pathway, the ligand-activated ER complex binds directly to DNA motifs knows as Estrogen Response Elements (EREs) located in the proximity of target gene promoters (Fig. 5A). In the non-classical pathway, the activated ER complex tethers to already bound transcription factors such as the AP-1 (activator protein-1) or SP-1 (specificity protein-1) complexes, acting as a co-regulator (Fig. 5B) [212-214]. In addition, the ER may be activated in a ligand-independent manner by direct phosphorylation of key residues (serine 106/107, 118, 167 and 305) primarily in the receptor AF-1 function by kinases such as extracellular regulated kinase (ERK) 1/2, p38 mitogen-activated protein kinases (MAPKs), epidermal growth factor receptor (EGFR) and AKT/PKB (Fig. 5C) [215-219].

Membrane-initiated steroid signalling pathway (MISS)

In addition to acting as a nuclear transcription factor, the ER has also been suggested to be activated near the plasma membrane where it may modulate and interact with several different pathways (Fig. 5D) in a rapid, non-genomic mode referred to as the MISS pathway [220]. ER may in this fashion alter the expression of genes normally regulated by growth factor receptors such as the PI3K/AKT and Ras/ERK1/2/MAPK pathways [221]. HER2 has also been suggested to interact with ER in this manner. This interaction is most likely a factor contributing to the resistance to tamoxifen frequently observed in ER^+ and HER2-overexpressing tumours [222, 223].

Transcriptional output of ER signalling

Up to 1000 genes have been suggested to be regulated by ER [224]. Gene expression profiling of E2 stimulated breast cancer cells have shown that the majority of the affected transcripts are in fact downregulated, yet the net result is still an increase in proliferation-associated processes and suppression of apoptosis [208]. Some of the well-known upregulated transcripts upon E2 stimulation are Myc, cyclin D1 and the progesterone receptor (PgR) [225-227].

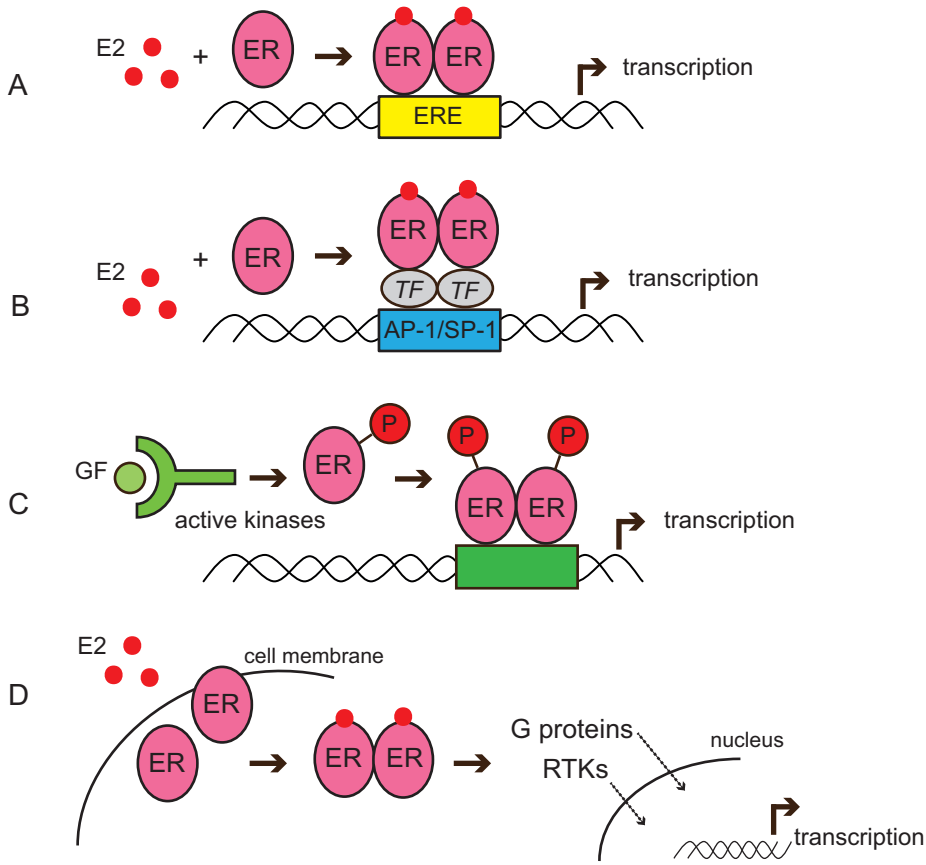

Figure 5. Activation of ER signalling pathways.
A. The classical ligand-dependent pathway. Upon ER binding of E2, the receptors dimerise and bind to ERE elements in the DNA. **B**. In the non-classical ligand-dependent pathway, E2 activates ER which dimerise and bind to already tethered transcription factors in the nucleus. **C**. In the ligand-independent ER pathway, the ER is phosphorylated by activated growth factor receptors, leading to nuclear transcription. **D**. ER is activated by E2 near the plasma membrane and may subsequently activate growth factor signalling pathways.
ER=estrogen receptor, E2=estrogen, ERE=estrogen response element, TF=transcription factor, AP-1=activator protein-1, SP-1=specificity protein-1, GF=growth factor, P=phosphorylation, RTKs=receptor tyrosine kinases

Tamoxifen and resistance mechanisms

Tamoxifen is widely used in both the treatment of breast cancer and in the preventive setting for patients with a high risk of developing breast cancer. The use of tamoxifen has reduced breast cancer recurrences and increased survival rates significantly [54, 228]. However, one-third of women treated with the recommended 5-year course of tamoxifen will relapse within 15 years, pointing to the necessity of identifying new and better biomarkers predicting response to tamoxifen [54].

Mechanism of tamoxifen

AF-1 and AF-2 in ER signalling – the basis for understanding the mechanism of tamoxifen

The two activation function domains of ER, AF-1 and AF-2, have been reported to promote transcription both independently and through functional cooperation. Their relative contribution to the transcriptional output varies in a promoter- and cell type-specific fashion [229-232]. The disparate transcriptional activities of AF-1 and AF-2 have been shown to be important for treatment using the class of drugs known as selective estrogen receptor modulators (SERMs) to which tamoxifen belongs, as these drugs may antagonise the AF-2 function of ER but concurrently activate AF-1. Hence, tamoxifen may have opposite effects in different tissues depending on the transcriptional impact of AF-1 and AF-2 in the specific cell type.

Tamoxifen is an orally administered drug converted in the body to its active metabolites, mainly 4-hydroxitamoxifen (4-OH-tam) and endoxifen [233, 234]. The converted molecules bind the ligand-binding domain of ER in the proximity of AF-2 in an estrogen competitive manner [204, 235]. Instead of recruitment of co-activator proteins, the co-repressors N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid receptors) have been reported to associate to AF-2 upon tamoxifen binding resulting in transcriptional repression [236, 237]. In tissues dependent on AF-2 mediated transcription such as the breast, the binding of tamoxifen results in an antagonistic response and transcription of ER target genes is inhibited. However, in tissues dependent on AF-1 activation, the binding of tamoxifen is interpreted by the cell as if estrogen has activated the receptor and ER target genes are upregulated. This is the case in bone and uterine tissues [238]. Clinically, these selectively modulating effects of tamoxifen are manifested in beneficial effects on bone density where tamoxifen acts as an estrogen, but also results in an increased risk for the patient of developing endometrial cancer due to the estrogenic (i.e. proliferative) effects of tamoxifen in this tissue type [239, 240].

Mechanisms of tamoxifen resistance

Resistance to tamoxifen can be described as either intrinsic or acquired, where the intrinsic or *de novo* resistance is primarily mediated by lack of ER expression. Conversely, the acquired resistance which develops during the course of tamoxifen administration is not as easily defined and a number of different mechanisms have been suggested to contribute to resistance, comprehensively reviewed in references [241-243]. There are several markers originating from clinical observations which have hinted on the mechanisms underlying acquired resistance, such as decreased or lost ER and PgR expression and upregulation of HER2 in patients treated with tamoxifen [243]. A brief overview of some of the suggested resistance mechanisms, summarised in Fig. 6, will be addressed in this section.

Co-regulators

First, co-regulators of ER constitute a group of proteins repeatedly associated to tamoxifen resistance. The ER co-activator protein SRC-3 (also known as AIB1, amplified in breast cancer-1 or NCoA3/TRAM-1), is frequently amplified and overexpressed in breast cancer [30, 31]. Studies of this co-activator both *in vitro* and in xenograft models have linked its overexpression to tamoxifen resistance. High SRC-3 levels have been associated to an impaired tamoxifen response in patients [244]. Expression of an additional ER co-activator SRC-1 (alternative name NCoA1) has also been clinically implicated in mediating tamoxifen resistance [245]. In a paper by Redmond *et al.*, SRC-1 and ER co-association were shown to be increased in a tamoxifen resistant cell line and SRC-1 was further shown to be a strong independent factor of reduced disease-free survival (Fig. 6) [246].

Co-repressors recruited to the tamoxifen-bound receptor, such as N-CoR, have been suggested to be important in mediating the inhibitory effect of tamoxifen. Accordingly, low N-CoR mRNA expression was significantly associated to decreased relapse-free survival in a patient cohort exclusively treated with tamoxifen, and supporting results have been obtained using a xenograft mouse model [236, 247].

Growth factor receptor signalling

Secondly, another suggested resistance mechanism is cross-talk between ER and growth factor receptor signalling pathways, providing alternative pathways to proliferation and survival of tumour cells in the presence of tamoxifen. Overexpression of HER2 as well as excessive EGFR, PI3K/AKT and Erk signalling may lead to improper activation of ER, suggested to contribute to tamoxifen insensitivity [216, 248, 249]. The mechanism by which HER2

overexpression contributes to tamoxifen resistance has been well characterised [215] and results from a clinical trial co-targeting ER and HER2 in a metastatic breast cancer setting showed improvements in progression-free survival [250]. Members of the FGFR (fibroblast growth factor receptor) family are also implicated in resistance to tamoxifen. *FGFR-1* amplification and overexpression have been associated to endocrine resistance both in patient cohorts and in cell lines [43]. One report recently demonstrated that expression of FGFR-3 was increased in breast tumours insensitive to tamoxifen, and that direct activation of FGFR-3 promotes proliferation in tamoxifen-resistant breast cancer cell lines (Fig. 6) [251].

Several studies have linked specific phosphorylation of ER to tamoxifen response. Phosphorylation of ER at serine-118 has been associated to increased tamoxifen sensitivity whereas phosphorylation of serine-305 has been correlated to tamoxifen resistance [252, 253].

Cell cycle regulators

The third category of pathways implicated in tamoxifen resistance comprises cell cycle regulatory proteins, where cyclin D1 has been extensively studied. Notably, overexpression of cyclin D1 has been implicated in mediating tamoxifen resistance in patients [141, 254]. *In vitro* studies have suggested the events underlying resistance to be the association of ER and cyclin D1, leading to recruitment of SRC-1 and P/CAF (p300/CBP-associated factor). This interaction results in a hormone-independent transcriptional activation of ER, shown to be insensitive to 4-OH-tam [124, 125, 255]. However, results have not been consistent. Neuman and co-authors have reported efficient inhibition of cyclin D1-activated ER by 4-OH-tam [256]. A recent report from the TransATAC (Translational research cohort of Arimidex, Tamoxifen, Alone or in Combination) trial found no difference in outcome in the tamoxifen treated arm when stratifying patients for cyclin D1 expression [27].

The role of cyclin D1 in tamoxifen resistance remains unclear. Lack of subgroup stratification for patients with *CCND1* amplified tumours in some analyses renders interpretation of the role of cyclin D1 in endocrine resistance difficult. The *CCND1* amplification event clearly signifies a subgroup with a worse prognosis and poorer tamoxifen response, and it is therefore advisable to analyse this subgroup separately [48, 257].

As the third paper reports in this thesis (Lehn *et al.* 2011) inactivation of the RB pathway has been suggested to lead to tamoxifen insensitivity. Studies of cell lines and xenograft models, as well as patient tumour materials, all indicate that a non-functional RB pathway is associated with tamoxifen resistance [83, 84, 258]. The exact molecular mechanisms underlying these observations are not known, but

deregulation of RB/E2F target genes have been proposed to be one of the functional consequences of RB inactivation mediating resistance [83].

The role of additional cell cycle regulators such as cyclin E and the CDK inhibitors p21 and p27 (encoded by the genes *CDKN1A* and *CDKN1B*, respectively) have not been as extensively studied as cyclin D1. Regarding cyclin E, overexpression has been associated with endocrine resistance [259]. However, in a study including an untreated control group in the experimental design, no significant impact of cyclin E expression on tamoxifen response was found [260]. High expression of p27 has been associated to predict response to tamoxifen, whereas exclusive cytoplasmic expression of p21 was associated to AKT activation and tamoxifen resistance in patients [85, 261, 262].

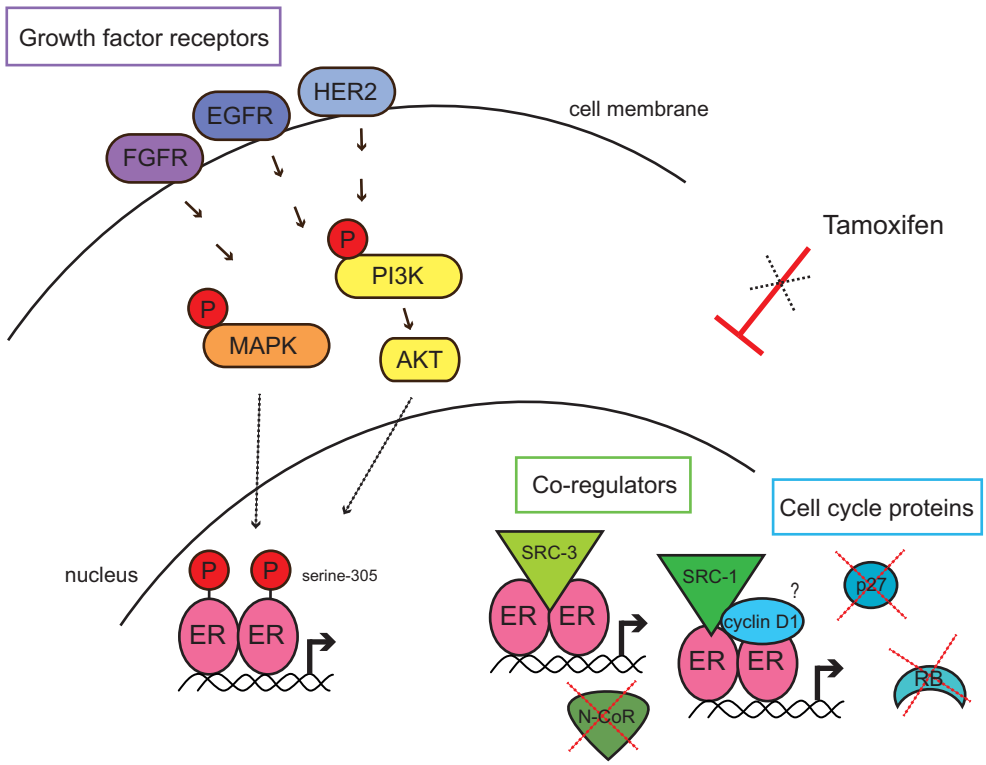

Figure 6. A simplified illustration of mechanisms implicated in tamoxifen resistance. Overexpression of co-activators such as SRC-1 and SRC-3, and loss of the co-repressor N-CoR have been suggested to confer tamoxifen insensitivity. Aberrant growth factor receptor signalling results in phosphorylation of ER and mediates transcription despite presence of tamoxifen. Loss of cell cycle proteins p27 and RB have been reported to abolish the tamoxifen inhibitory effect. Overexpression of cyclin D1 might be involved in tamoxifen resistance by acting as an ER co-activator, however reports are inconsistent. For references, please see the text.

Clinical impact of research on tamoxifen resistance

At present, immunohistochemical analysis of ER with a cut-off level at 10% positive nuclei is the only marker used in the clinic to decide whether or not to recommend endocrine treatment [40]. Gene signatures such as the commercially available Oncotype DX® and MammaPrint® have proven useful in adding prognostic information and guiding overall treatment of breast cancer patients.

In many instances, endocrine treatment will be given in combination with chemotherapy. However, overtreatment of patients with harsh chemotherapy regimes is a major problem and several clinical trials have been initiated to identify the subgroup of ER^+ patients who will do equally well without adjuvant chemotherapy. The MINDACT trial (Microarray In Node-negative and 1 to 3 positive lymph node Disease may Avoid ChemoTherapy) [263] and TAILORx (Trial Assigning Individualized Options for Treatment (Rx)) [264] will make use of the Oncotype DX® and MammaPrint® gene signatures for stratifying patients with the aim of identifying the right patient for the right treatment. Both these trial designs include randomised arms for endocrine therapy and hopefully new clinically validated markers or gene expression signatures for predicting tamoxifen response will emerge from these trials.

The present investigation

Aims

The general aim of this thesis was to study the relationship between proliferation, migration and stem-like cell activity in breast cancer cells, focusing on cyclin D1 and associated proteins in breast cancer subgroups. An additional main objective was to study the cell cycle protein retinoblastoma tumour suppressor (RB) and the chromosome 11q yes-associated protein (YAP1), and their role in tamoxifen resistance.

Specific aims

- To examine the influence of cell proliferation on migratory capacity in breast cancer cells
- To analyse the role of cyclin D1 and CDK4/6 in migratory- and stem-like cell activities in ER^+ and ER^- breast cancer subgroups
- To determine the prognostic and tamoxifen predictive value of a functional RB signalling pathway in premenopausal breast cancer patients
- To examine the function of YAP1 in breast cancer molecular subgroups in regards to recurrence-free survival and tamoxifen resistance

Results and discussion

Cell cycle proteins cyclin D1 and CDK4/6, and associations to proliferative, migratory and stem-like cell activities (paper I and II)

Previous studies have implied a contrasting relationship between the proliferative and migratory activities of cancerous cells, also referred to as the *go-or-grow hypothesis* [265-269]. This concept suggests that an actively dividing cell cannot simultaneously execute efficient migration. To increase the understanding of the very complex behaviours of tumour cells, we wanted to investigate both in general and in detail, the relationship between proliferation and migration in breast cancer cells. **Paper I** was initiated using this contrasting theory as a starting-point with specific focus on cyclin D1, known to have a prominent role in breast cancer biology as previously addressed. In **paper II**, results from paper I were further validated and a subgroup perspective in terms of ER^+ and ER^- disease was added. Also, consequences of cell cycle inhibitory treatment using two different drugs currently undergoing testing in clinical trials were investigated and related to migratory and stem-like cell activities.

*Proliferation and migration are two contrasting events in the MDA-MB-231 breast cancer cell line (**paper I**)*

We wanted to investigate in detail how migratory behaviour in breast cancer cells relates to proliferation, as these processes are critical in the progression of breast cancer. The breast cancer cell line MDA-MB-231 was synchronised by serum starvation, resulting in approximately 90% of the cell population residing in the G_0/G_1 cell cycle phase. Following addition of serum medium, cells progressed through the cell cycle in a synchronised manner, enabling assessment of the migratory ability of cells in different cell cycle phases. Cell populations in which the majority of cells were present in the G_0/G_1 phase displayed significantly increased migration compared to later time points when cells had progressed to S- and G_2/M phases, a result which supports a contrasting relationship between migration and proliferation in breast cancer cells. This result was verified by sorting actively cycling cells according to cell cycle phase using a Fluorescent Activated Cell Sorter (FACS) which allows for assessment of migratory ability in separate cell cycle phases independently of serum stimulation. Cells present in G_0/G_1 phase displayed a significantly increased migratory capacity compared to cells in S- or G_2/M-phases, supporting the result obtained from the synchronised cell population experiments.

*Decreased cyclin D1 expression correlates to increased migration in an actively cycling cell population (**paper I**)*

To test for a possible role for the G_1-cyclin cyclin D1 in the migratory differences of cell cycle phases, siRNA was employed to attenuate cyclin D1 expression. Migration was measured in an actively cycling MDA-MB-231 cell population, consisting of approximately 60% of cells in G_0/G_1-, 30% in S- and 10% in G_2/M-phase. Upon cyclin D1 siRNA treatment, cell cycle phase distributions were changed; the G_0/G_1 fraction was increased whereas S- and G_2/M fractions decreased. A significant migratory increase was observed upon cyclin D1 silencing, implying a role for cyclin D1 in regulating migratory behaviour in breast cancer cells. A similar G_0/G_1 phase accumulation was achieved by silencing CDK4 and CDK6, known to associate with cyclin D1 to form an active kinase complex required for cell cycle progression [270]. However, the silencing of CDK4 and CDK6 did not confer any differences in migratory capacity despite changes in cell cycle phase distribution, pointing to cyclin D1 modulating migratory behaviour independently of CDK4 and CDK6.

There was a relatively modest increase in migration upon silencing cyclin D1 in actively cycling cells (1.7-fold) compared to migratory changes in the synchronised cell population (3-fold). This could partly be explained by the fact that the effect of cyclin D1 downregulation was most prominent in S- and G_2/M-phases, which in our study were identified to have a lower basal migratory activity. This result stresses the complexity of the interplay between the proliferative and migratory processes. We might conclude that cells in S- and G_2/M-phases do retain some migratory capacity, although it is decreased, which in addition may be modulated by cyclin D1. Furthermore, as these cell cycle phases constitute the lesser fraction of an actively cycling cell population, it is likely that the cyclin D1-mediated increase in migration is partly subdued by the larger G_0/G_1 population, in which cyclin D1 silencing had no significant effect on migration.

To further validate the result of cyclin D1 modulating migration, cyclin D1 was re-introduced in MDA-MB-231 cells silenced for cyclin D1 to rescue the original phenotype. When reintroducing cyclin D1, cells migrated less, in support of cyclin D1 inhibiting migration.

The two assays used to study migration (modified Boyden chamber Transwell assay and Time lapse microscopy) measure different aspects of migration. In the Transwell assay, cells suspended in serum-free medium migrate towards a serum gradient, assessing ability for directional migration as a response to chemotactic signals. In contrast, time lapse microscopy measures random migration, and the level of intrinsic migratory cues of cells is calculated by tracking cell motility during a defined time frame. Decreased cyclin D1 resulted in increased migration in both systems, implying that chemotactic signals are not critical for the increased

cell movement. Proliferation may in some cases be a confounding factor when assessing migration. However, migration was in general assessed after or during 3 to 5 hours; hence proliferation should not be a critical parameter in our experiments as it takes significantly longer (approximately 24 h) for a cell to complete one cell cycle.

Low cyclin D1 protein expression is independently associated to decreased recurrence-free survival ***(paper I)***

In an attempt to translate our results to an *in vivo* situation, a premenopausal primary breast cancer tumour material was analysed for cyclin D1 expression. It should be stressed that in premenopausal breast cancer, aggressive tumour subtypes such as the basal-like are generally over-represented. This may be due to *BRCA1* and to some extent *BRCA2* hereditary gene mutations which are associated to early onset of breast cancer and to the mentioned subtype [271, 272].

Interestingly, strong cyclin D1 nuclear intensity was significantly associated to a less infiltrative growth pattern of primary breast tumours, and to a smaller tumour size. Considering the previous associations of downregulated cyclin D1 and increased migration in cell lines, this implied that cyclin D1 might have a function in restraining infiltrative tumour cell behaviour *in vivo*. Furthermore, when combining subgroups of high/low cyclin D1 and high/low expression of the proliferative marker Ki-67, the subgroup of low cyclin D1/high Ki-67 expression displayed a significantly decreased recurrence-free survival, compared to the high cyclin D1/high Ki-67 subgroup in an untreated (only surgery) patient cohort. This suggests that decreased expression of cyclin D1 in combination with increased proliferation is associated with a more aggressive progression in ER^+ breast cancer. The impact on recurrence could potentially be explained by the observed *in vitro* effect of cyclin D1 on restraining migratory behaviour.

Cyclin D1 was in addition found to be an independent prognostic factor in the ER^+ untreated patient cohort, after adjustment of known prognostic factors including grade, node involvement, tumour size and Ki-67. These results further emphasise the complexity of how proliferating cells relate to migration, and suggests that a variety of factors most likely are contributing to the observed correlations.

Microarray analysis to identify gene candidates mediating the migratory increase upon cyclin D1 silencing ***(paper I)***

In search of a more specific mechanism involved in the CDK-independent, cyclin D1 associated migratory changes, a microarray analysis was performed. Transcripts significantly changed upon cyclin D1 silencing, but not CDK4/6 knockdown, were further analysed. Using a GoMiner approach [273], cellular processes associated with the cytoskeleton and microtubules were shown to be

significantly more altered in cyclin D1- over CDK4/6 silenced cells. In addition, a number of genes were identified as plausible mediators of the migratory effect. Another study from our group has identified one of the upregulated genes, *ID1* (Inhibitor of differentiation), as partly mediating the migratory effect of cyclin D1 silencing through upregulation of EMT markers [274].

In conclusion, we have illustrated a new function for the cell cycle protein cyclin D1 in regulating breast cancer migration, where decreased expression is associated to an increased migratory activity. The paper also indicates that high cyclin D1 expression correlates to a better prognosis in an ER^+ untreated patient cohort, independently of other known prognostic factors such as histological grade, lymph node involvement and proliferation.

Cell cycle modulation of cyclin D1 and CDK4/6 – associations to migratory and stem-like cell activities in ER^+ and ER^- subgroups ***(paper II)***

To date, several clinical trials are evaluating proliferation inhibiting treatment regimes. From a clinical perspective, arresting cell proliferation is one main endpoint manifested in shrinkage of a tumour mass. However by affecting proliferation, other important processes such as migration and stem-like cell activities might be altered. A worst-case scenario would be that by inhibiting proliferation, migratory or invasive and stem-like cell activities would be concurrently increased. We have examined the consequences of downregulating cyclin D1 and CDK4/6, and also overexpressing cyclin D1 in a panel of breast cancer cell lines and primary tumours of both ER^+ and ER^- origin, with specific focus on migration and stem-like features.

Decreasing cyclin D1 expression in the ER^- cell lines MDA-MB-231 and MDA-MB-468 resulted as previously observed in increased migration. In two ER^+ cell lines tested, results were the opposite and migration was decreased upon cyclin D1 and CDK4/6 silencing. A mammosphere formation assay was used as a measure of stem-like cell activity. This assay measures the capability of a cell to self-renew and form spheres in non-adherent culture, a measure of anoikis resistance. Hence, the assay is a way to evaluate stem-like cell characteristics. The mammosphere experiment resulted in changes analogous to those found in the migration assay. Decreasing cyclin D1 in ER^- cell lines led to increased mammosphere formation, whereas in ER^+ cell lines, a decrease was observed. Primary breast cancer samples were also included and the same trends as for cell lines were noted.

Overexpression of cyclin D1 was able to reverse the observed phenotypes. ER^- cell lines and primary samples displayed decreased migratory activities and mammosphere formation, whereas overexpression in ER^+ cells resulted in an increase of these features.

To examine the implied dependence of our results on expression of ER, ER$^-$ cell lines were transfected with an ER vector. In line with our previous observations, the ER converted cell lines now mimicked the response to cyclin D1 silencing or overexpression observed in ER$^+$ cell lines, with decreased and increased migratory and stem-like cell activities, respectively.

In conclusion, the expression of ER appears to determine the type of response to cell cycle inhibition by cyclin D1 and CDK4/6 siRNA. ER negative cells adopted a more aggressive phenotype with increased stem-like cell activity and migration, whereas ER$^+$ cells displayed less aggressive features with decreased mammosphere formation and migration upon cyclin D1 and CDK4/6 downregulation. In addition, these results suggest that ER$^-$ cells may respond positively to cyclin D1 and CDK4/6 silencing (decreasing migratory and stem-like cell activities) in a fashion similar to ER$^+$ cells, simply by the re-expression of ER.

*Cell cycle inhibitors undergoing clinical trials have opposing effects dependant on ER expression in cell lines and primary cells (**paper II**)*

In order to evaluate our observations in a clinical context, two agents currently tested in clinical trials of breast cancer (www.Clinicaltrials.gov 2012-11-29) were tested, namely Flavopiridol and PD0332991. The flavone Flavopiridol is synthetically derived from an alkaloid found in the leaves and stems of an Indian plant, and it has been reported to function in inhibiting transcription and CDKs, causing cell cycle arrest [275, 276]. Specific inhibition of cyclin D1 by Flavopiridol has also been reported [277, 278], and as such is referred to as a cyclin D1 inhibitor in paper II, although a diverse cross-section of proteins are affected. PD0332991 is a small molecule inhibitor, specifically inhibiting CDK4/6 activity [279]. Treatment with these two compounds mirrored that of siRNAs and resulted in increased migratory and stem-like cell activity in ER$^-$ cell lines and primary breast cancer samples, whereas treatment in ER$^+$ equivalents resulted in decreased migration and mammosphere formation. Re-expression of ER could reverse the effect of the two drugs in ER$^-$ cell lines, in support of our previous results.

To summarise, we have confirmed the previously reported effect of cyclin D1 in modulating migration (**paper I**). However, in all cell lines tested except MDA-MB-231, CDK4/6 silencing yielded results similar to cyclin D1 downregulation. This implies a more general cell cycle-associated effect in all cell lines but MDA-MB-231. Our results suggest that treatment with Flavopiridol or PD0332991 in a breast cancer setting possibly should be limited to patients presenting with ER$^+$ breast cancer, as both migratory and stem-like cell activities were increased in ER$^-$ cell lines and primary samples upon treatment with the two drugs. The importance of identifying subgroups which may benefit from cell cycle modulating treatments have recently been addressed in a comprehensive review discussing cyclin D1 as a

therapeutic target in cancer [280]. Our results add to the notion of subgroup importance in breast cancer and in treatment modulating cell cycle activities.

Studies of tamoxifen resistance in subgroups of breast cancer (paper III and IV)

Tamoxifen is a widely prescribed drug for ER^+ breast cancers; however one-third of patients treated with tamoxifen will eventually present with recurrent disease [54]. An important area within breast cancer research is therefore the identification of biomarkers that may provide additional information on whether or not a patient will respond to tamoxifen. Subgroups of patients with ER^+ tumours predicted not to respond to tamoxifen could then be considered for treatments with therapies based on other mechanisms of action than tamoxifen, for example aromatase inhibitors.

In **paper III** and **paper IV**, two markers were tested of their ability to predict response to tamoxifen. In both studies, tumour material from a patient cohort originating from a tamoxifen randomised clinical trial was analysed. This trial was initiated in 1986 to examine the benefit of tamoxifen treatment in premenopausal breast cancer patients. The randomised setting with one treated and a corresponding untreated control group renders this tumour material unique in character, and enables the analysis of a true tamoxifen response. Only patients presenting with stage II disease were included, irrespective of ER status, and a total of 564 patients were recruited [281]. This patient cohort will be referred to as the tamoxifen randomised cohort in the following text. In **paper IV**, an additional tumour material was analysed, originating from a patient cohort used for the screening of antibodies for the Human Atlas Protein project, referred to as the screening cohort [282, 283]. All patients diagnosed with primary invasive breast cancer at Skåne University Hospital in Malmö during the years of 2001 and 2002 were eligible for inclusion. Both pre- and postmenopausal patients were included and all patients underwent surgery followed by treatment according to guidelines.

To study protein expression, tumours were arranged in so-called tissue microarrays (TMAs), a technique used to simultaneously study tumours from many different patients. Development of the TMA technique in the late nineties has made it possible to retrospectively study large tumour materials in a high-throughput manner [284]. To construct a TMA, cylindrical tumour core "biopsies" are taken from formalin-fixed, paraffin-embedded tumour tissues from a number of patients (the donor block, Fig. 7A). The "biopsies" are then placed in a defined order in a new paraffin block (receiving block, Fig. 7B). The receiving block is subsequently sectioned and stained immunohistochemically (Fig. 7C). For each section, a different antibody may be used for the staining process, and this allows

for analysis of hundreds of markers in the same patient set. Studies have shown that although the tissue cores taken from the tumours are of small size, they adequately represent the whole-section staining pattern in 95% of cases [285].

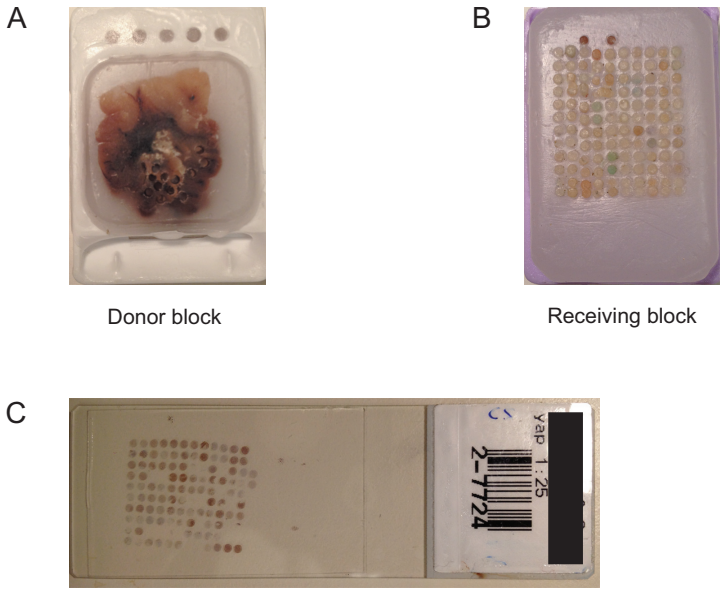

Figure 7. Basic description of tissue microarray construction.
A. Cylindrical tumour core "biopsies" are taken from one single tumour embedded in paraffin (donor block). This step is repeated for e.g. 100 individual tumours. **B**. The cylindrical tumour biopsies are placed in the receiving paraffin block. The receiving block shown in the figure contains tumour samples from 60 patients (two biopsies for each tumour). **C**. The donor paraffin block is sectioned, mounted on a glass slide and stained with a specific antibody. The protein expression is evaluated and subsequently correlated to clinical patient data. Please note: tumours shown are not necessarily of breast origin.

Status of the cell cycle regulator RB is important for tamoxifen response ***(paper III)***

RB plays a key role in controlling cell cycle progression, and we aimed to determine the importance of a functional RB pathway in tamoxifen response.

*Definition of a non-functional RB signalling pathway (**paper III**)*

Phosphorylated RB (phos-RB) protein was immunohistochemically stained and scored in the tamoxifen randomised tumour material in groups of 0-10%, 11-25%, 26-50% and >50% of positive nuclei. Phos-RB expression was subsequently compared to the proliferative marker Ki-67 to determine which tumours presented with an aberrant RB signalling pathway. A normal and functional RB pathway can be described as a linear relationship between the degree of RB phosphorylation and Ki-67 staining, illustrated by the grey line in Fig. 8. Unphosphorylated RB normally inhibits cell cycle progression, reflected by lack of Ki-67 expression. In the actively dividing cell, different cyclin/CDK complexes phosphorylate RB, resulting in conformational changes which lead to inactivation of the protein. This ultimately results in the release of E2F transcription factors, driving the proliferative cycle [270]. Hence, tumours displaying a low fraction of phosphorylated RB, yet concurrently had an elevated Ki-67 expression, were considered to harbour a non-functional RB signalling pathway (illustrated by red dots in Fig. 8).

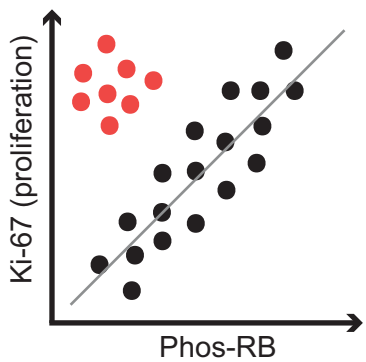

Figure 8. Schematical plot over phosphorylated RB and Ki-67 parameters illustrating the definition of a functional vs. non-functional RB pathway.
In patients with tumours harbouring a functional RB pathway, the degree of phosphorylated RB is followed by an increase of Ki-67 staining (black dots). A low degree of RB phosphorylation accompanied by a high fraction of Ki-67 positive cells is indicative of a non-functional RB pathway (red dots).
Dots are merely illustrative and are not representative of number of patients.

Phosphorylation of the two RB serine residues (807/811) scored in this study have previously been reported to be crucial for efficient RB phosphorylation and cell cycle progression. By mutating the Ser-807/811 sites of the RB protein, efficient phosphorylation of RB was prevented in the SAOS-2 cell line, and even overexpression of cyclin A could not overcome the growth suppressing activity of the mutated RB Ser-807/811 protein [181]. Cells negative of phosphorylated Ser-807/811 staining but highly positive for Ki-67 staining clearly proliferate independently of RB, rendering these two sites in combination with a proliferative marker well suited for identifying tumours with a non-functional RB signalling pathway.

*A non-functional RB pathway is associated to aggressive tumour features, but holds no prognostic value (**paper III**)*

The two identified patient groups with either a functional (n=273) or a non-functional (n=57) RB pathway was further studied. Correlations to clinicopathological parameters revealed associations of a functional RB pathway and a less aggressive disease, i.e. smaller tumours of lower histological grade that were ER and PgR positive. There was a larger fraction of lymph node positive patients in the RB functional group compared to the RB non-functional (75% vs. 53%) however lymph node involvement was not linked to recurrence-free survival in the RB functional subgroup (data not shown). Furthermore, a non-functional RB pathway was correlated to high cyclin E/low cyclin D1 expression. Conversely, a functional RB pathway was associated to high cyclin D1/low cyclin E expression. For a tumour cell with a functional RB pathway to be able to proliferate, the repressive function of RB has to be inactivated by phosphorylation. This may be achieved by an increased cyclin D1 expression, which together with CDK4/6 phosphorylate and thereby inactivate RB. However, a cell harbouring a non-functional RB pathway is not reliant on the cyclin D1-CDK4/6 axis. Instead, loss of RB function results in constitutive activity of the E2F transcription factors, ultimately yielding high cyclin E expression stimulating cell proliferation [286, 287].

RB pathway status was not correlated to outcome in the untreated patient group, despite associations to more aggressive tumours. This has been reported previously, however in some cases treated patients have been included in the analyses and as such do not represent the true prognostic value of RB [288-290].

*Loss of a functional RB pathway is associated with an impaired tamoxifen response (**paper III**)*

To determine the importance of RB pathway status in relation to tamoxifen response, recurrence-free survival was examined in the ER^+ patient cohort. Patients with a defined functional RB pathway who received tamoxifen had a

significantly increased recurrence-free survival compared to the corresponding untreated patient group (p=0.003). There was however no difference in outcome when comparing treatment in patients with a non-functional RB pathway (p=0.270). An interaction analysis further demonstrated a significant difference in tamoxifen response between the RB groups.

Notably, gene expression signatures of RB deregulation have also been used to study endocrine resistance in breast cancer, resulting in conclusions similar to ours [83, 291]. Analysis of RB pathway disruption is likely to be one of future predictive tools in guiding treatment options, but it remains to be determined how to uniformly identify the unresponsive subgroup.

We conclude that a non-functional RB pathway predicts resistance to tamoxifen treatment. Our study has confirmed and built upon the pre-existing literature regarding RB loss of function and its importance in endocrine resistance [83, 258, 291]. Our approach in combination with gene expression analyses has the potential to identify an ER^+ subgroup of patients with a non-functional RB pathway who would benefit from treatment options other than tamoxifen.

*Studies of yes-associated protein in breast cancer and links to tamoxifen resistance (**paper IV**)*

Previous studies have implicated YAP1 as an oncogenic driver of proliferation in several cancer forms [164-166, 292-294]. In breast cancer, YAP1 has been associated to both oncogenic and tumour suppressive features [167-171, 295]. Given that the *YAP1* gene is located in the 11q22 region, reported to be frequently deleted in breast cancer and previously implicated in tamoxifen resistance, we wanted to investigate YAP1 further [296]. Whilst YAP1 was one of the transcripts identified as specifically downregulated by cyclin D1 silencing in **paper I**, attempts to confirm its ability to mediate cyclin D1's effect on migration were unsuccessful. Hence, studies of YAP1 were recommenced from a general breast cancer perspective with specific focus on tamoxifen response.

*YAP1 is associated to both less and more aggressive features in breast cancer subgroups, in an ER-dependent manner (**paper IV**)*

YAP1 overall protein intensity was scored as either absent, weak, intermediate or strong in the tamoxifen randomised- and screening patient cohorts. In the ER^+ subgroup of the tamoxifen randomised cohort YAP1 was negatively correlated to grade and proliferation, whereas in the ER^- subgroup YAP1 was positively associated to proliferation and a borderline significance for histological grade was also observed (p=0.062). In the screening patient cohort, absent YAP1 expression correlated to lymph node positivity in the ER^+ subgroup, and in this subgroup a borderline significance was again observed where YAP1 correlated negatively to histological grade (p=0.060). YAP1 mRNA expression was further analysed in a

gene expression meta-dataset consisting of six previously published gene expression datasets with clinical follow-up, including data from 1107 breast cancer patients. YAP1 mRNA was not associated to clinical parameters such as histological grade, lymph node status or tumour size in the ER^+ subgroup, however in the ER^- subgroup there was a positive correlation of YAP1 mRNA and larger tumour size (p=0.037).

In conclusion, while the correlations of YAP1 in the patient cohorts are not consistent, the trends are that decreased YAP1 in the ER^+ subgroup is linked to more aggressive features, whereas in the ER^- subgroup, increased YAP1 correlates with aggressiveness.

YAP1 protein intensity and YAP1 *copy number are negatively correlated to amplification of 11q13* CCND1 *gene (**paper IV**)*

A persistent inverse correlation between cyclin D1 and YAP1 expression on both protein and mRNA level was observed in all ER^+ patient subgroups of the cohorts studied. The *YAP1* gene is located at 11q22 and the distal part of 11q is frequently lost upon amplification of the known oncogene *CCND1* located at 11q13 [42, 142]. Analysis of copy number changes in an aCGH (array Comparative Genomic Hybridisation) patient cohort (n=171) revealed significant associations of *CCND1* copy number gain and *YAP1* loss, although there were cases present where *YAP1* was lost without concomitant *CCND1* amplification. Hence, we reasoned that the recurrent inverse correlation of YAP1 and cyclin D1 protein and mRNA could be the result of chromosomal gains coupled with concurrent losses.

In the randomised patient cohort, *CCND1* status was known for 209 patients of which YAP1 intensity had been successfully scored. The inverse correlation of YAP1 and cyclin D1 protein expression persisted after removing all known *CCND1*-amplified tumours from the analysis (n=33), possibly indicating additional mechanisms other than chromosomal aberrations for maintaining the inverse relationship. However, it should be noted that cyclin D1 is reported to be overexpressed in up to 44% of breast cancers, whereas YAP1 expression is repeatedly reported to be decreased [112, 126, 135-138, 140, 167, 295]. Hence, it cannot be excluded that the inverse correlation could be occurring randomly.

*Absent YAP1 protein expression correlates to an impaired tamoxifen response **(paper IV)***

To examine the impact of YAP1 expression on patient outcome, dichotomised YAP1 mRNA expression (divided by the median) was correlated to recurrence-free survival in the different molecular subgroups of the gene expression dataset. In the luminal A subgroup (n=286) low YAP1 expression was significantly correlated to a decreased recurrence-free survival (p<0.001). YAP1 expression was not associated to outcome in any of the other subgroups (luminal B, HER2,

basal- and normal-like). This result led us to hypothesise that decreased YAP1 expression could be linked to tamoxifen resistance. The greater part of the luminal A subgroup would most likely receive endocrine targeted treatment since tumours falling within this category are often low-proliferative, and ER and/or PgR positive. Subsequent analysis of the tamoxifen randomised patient cohort demonstrated that patients with tumours scored as weak, intermediate or strong YAP1 intensity had a significantly increased recurrence-free survival (p=0.001) when treated with tamoxifen. There was however no response to tamoxifen in patients with tumours lacking YAP1 expression (p=0.522) and an interaction analysis proved there to be a significant difference in tamoxifen response between weak, intermediate or strong, and absent YAP1 expressing tumours (p=0.042). Further analyses showed that in the control patient group (no adjuvant tamoxifen), YAP1 was not correlated to recurrence-free survival. In conclusion, YAP1 is not a prognostic factor in ER^+ breast cancer, but is important in predicting response to tamoxifen therapy.

On a side note, YAP1 was dichotomised by the median in the gene expression dataset, which on protein level would translate into two groups where absent/weak and intermediate/strong YAP1 intensity constitutes the cut-offs. However, the tumours in the group of absent YAP1 intensity clearly represented a subgroup with a distinctive biology, providing adequate evidence for tumours lacking YAP1 intensity to be analysed against the remaining YAP1 intensity scores.

*YAP1 is an independent predictor of outcome in the luminal A subgroup compared to a selection of 11q22 genes (**paper IV**)*

As previously mentioned, the chromosomal region of *YAP1* is frequently deleted in breast cancers, indicating that *YAP1* may not directly explain the observed results but merely act as a marker for 11q chromosomal aberrations. Utilising the gene expression meta-dataset, we analysed a selection of genes in the proximity of *YAP1* (two genes centromeric and six distal to *YAP1*) in an attempt to rule out the possibility of co-deletions mediating the prominent negative effect of decreased *YAP1* expression on recurrence-free survival. The genes found to correlate the strongest to *YAP1* in the luminal A subgroup (*BIRC2* and *TMEM123*), possibly due to co-deletions, were further analysed in a multivariate analysis. Low *YAP1* expression remained the only factor indicative of outcome (p=0.019) after adjustment of the two 11q22 genes, cyclin D1 expression, grade, tumour size and lymph node involvement.

In conclusion, YAP1 predicts outcome in the luminal A subgroup independently of a selection of 11q22 gene products. This points to YAP1 specifically modulating outcome in luminal A breast cancers.

*The tamoxifen response is delayed and decreased upon YAP1 downregulation in the breast cancer cell line T47D (**paper IV**)*

WST-1 analysis measuring cell viability was further employed to measure tamoxifen response *in vitro*, in the presence or absence of YAP1. A cell line classified as luminal A (T47D) was transfected with two different siRNAs targeting YAP1 mRNA and treated with ethanol (control), 17β-estradiol (E2) or E2 combined with 4-hydroxi-tamoxifen (4-OH-tam). After four days of treatment, viability was analysed. The two siRNAs did not yield a completely identical result. The decrease in viability upon tamoxifen treatment was not evident in siYAP #8 downregulated cells until concentrations reached 1 μM, whereas siCtr cells responded to a tamoxifen concentration 10-fold less. By utilising a luciferase assay, the activity of the Estrogen Response Element (ERE) to which estrogen-activated ER can bind and induce transcription, was measured to assess the influence of YAP1 on the activity of this particular DNA element. Results showed that the inhibitory action by tamoxifen on ERE activity was not as efficient when YAP1 was downregulated. However, the differences were not major and it is likely that YAP1 may affect other DNA elements involved in mediating estrogen and tamoxifen response, such as the cAMP response-like DNA element implicated in transcription of the *CCND1* gene upon estrogen stimulation [70].

The T47D cell line was further examined for changes in protein expression using immunocytochemistry. YAP1 was downregulated using siRNA and cells were treated for four days with control, E2 or combined E2 and 4-OH-tam treatment. Interestingly, YAP1 downregulation resulted in a marked increase of PgR protein expression, particularly in control treated and E2/4-OH-tam treated cells. Treatment with E2/4-OH-tam in siCtr cells resulted in a distinct decrease of PgR expression whereas no difference was detected in siYAP1 cells, implying an aberrant activation of the ER pathway upon YAP1 downregulation. The PgR promoter is reported not to contain any classical EREs [297] explaining why the ERE luciferase construct did not reflect the distinct effects on PgR expression observed on immunocytochemistry.

In conclusion, YAP1 downregulation results in a delayed and decreased tamoxifen response *in vitro*, possibly due to an aberrant activation of the ER signalling pathway as illustrated by increased PgR and ER protein levels.

Conclusions

The studies have identified cell cycle regulators cyclin D1 and CDK4/6 as of importance for migratory and stem-like cell activities, and we have shown RB and YAP1 to be important factors for tamoxifen response.

We may conclude that:

- Actively dividing MDA-MB-231 breast cancer cells display impaired migration whereas cells in G_0/G_1 phase are highly migratory (**paper I**)
- Downregulating cyclin D1 in MDA-MB-231 cells results in increased migration, an effect most prominent when cells are in S-phase (**paper I**)
- Downregulation of cyclin D1 and CDK4/6 results in increased migration and mammosphere formation in ER^- breast cancer cell lines, but results in a decrease in ER^+ cell lines (**paper I** and **paper II**)
- Treatment with Flavopiridol and PD0332991 in ER^- breast cancer cell lines results in increased migration and mammosphere formation, but in ER^+ cell lines, these activities are decreased (**paper II**)
- A non-functional RB pathway is not a prognostic factor in premenopausal breast cancer patients but confers tamoxifen resistance (**paper III**)
- YAP1 correlates negatively to aggressiveness in ER^+ breast cancer, but positively in ER^- breast cancer (**paper IV**)
- Absent YAP1 protein expression is associated with an impaired tamoxifen response (**paper IV**)
- Downregulation of YAP1 *in vitro* results in increased ER and PgR protein levels, possibly contributing to the decreased tamoxifen sensitivity (**paper IV**)

Populärvetenskaplig sammanfattning

Bröstcancer är den vanligaste cancerdiagnosen hos kvinnor, och under år 2011 fick över 8000 svenska kvinnor denna diagnos. Termen bröstcancer är egentligen ett samlingsnamn för alla de olika typer av cancer som kan uppstå i bröstet. En del typer av bröstcancer har en bra prognos medan andra trots aggressiv behandling alltjämt har en dålig prognos.

Cancer är en sjukdom där kroppens egna celler felprogrammeras genom att skador uppstår på cellens DNA. Detta kan leda till att celldelningsprocessen, som vanligtvis är strikt kontrollerad i cellen, initieras felaktigt. Cellen förökar sig utan att det finns ett behov av fler celler. Samtidigt fallerar andra cellulära säkerhetssystem som ska förhindra att skadade celler förökar sig, t.ex. slutar cellen reparera skadat DNA och förlorar förmågan att självdö. De felaktigt nyproducerade cellerna innehåller alla det muterade DNA:t, som kopierats under celldelningen. På så sätt ökar de felaktiga cellerna i antal.

Med tiden ackumuleras fler DNA-skador i cancercellerna vilket leder till att de får nya oönskade egenskaper, som t.ex. förmågan att invadera omkringliggande vävnad. I värsta fall kan cancercellerna nå fram till blodkärl eller ta sig in i lymfsystemet. Om detta sker kan cancern spridas till andra organ i kroppen och bilda nya dottertumörer. Denna process kallas metastasering, och det är först när cancern sprider sig vidare i kroppen som den kan bli livshotande.

För att cancerceller ska nå ett blodkärl måste de kunna röra på sig, migrera. Vi har undersökt hur en cancercells rörlighet förhåller sig till dess förmåga att dela sig. Experimentella försök har tidigare visat att en cell som migrerar sällan förökar sig just när den befinner sig i rörelse. Detta resonemang brukar kallas the *go-or-grow hypothesis*. Vi har försökt identifiera proteiner i cellen som skulle kunna ha inverkan på både celldelning och migration, för att bättre förstå hur dessa två processer hänger samman. I delarbete I har vi studerat ett protein som kallas cyklin D1 och som tillverkas i cellen i början på den cykel av händelser som resulterar i att en cell delar sig. Vi kunde visa att när cyklin D1 blockerades i cancerceller delade de sig i mindre utsträckning, samtidigt som deras rörlighet ökade. När cyklin D1 istället aktiverades, minskade cellernas rörlighet. I patientstudier har man tidigare noterat att höga nivåer av cyklin D1 i en tumör ofta är associerat till en bättre prognos för patienten, trots att cyklin D1 är intimt kopplat till celldelning. Våra resultat tyder på en ny migrationshämmande funktion för cyklin

D1, som delvis skulle kunna förklara kopplingen mellan cyklin D1 och bättre prognos.

Inom cancerforskning finns en teori att cellerna som utgör kärnan av en tumör är av en särskild sort, som man benämner cancerstamceller. En stor del av de behandlingar man använder mot cancer slår mot celler som aktivt delar sig. Cancerstamceller befinner sig ofta i ett vilande stadium och det är därför svårt att få behandlingen att slå ut denna celltyp. Konsekvensen kan bli att patienten får återfall i sin cancersjukdom trots att behandlingen varit framgångsrik i övrigt. I delarbete II har vi med hjälp av laborativa metoder undersökt hur olika typer av behandlingar som blockerar cellcykel-associerade proteiner, som t.ex. cyklin D1, påverkar andelen cancerstamceller. Vi fann att olika typer av bröstcancerceller reagerade olika på samma behandling. I den ena celltypen minskade andelen cancerstamceller, medan andelen ökade i den andra. Våra resultat understryker vikten av att definiera vilken typ av cancerceller som en tumör består av för att bättre kunna förutse effekten av behandlingar som inhiberar cellcykeln.

Bröstcancertumörer karaktäriseras främst utifrån förekomsten av ett protein som kallas östrogenreceptorn. Antingen klassas tumören som positiv eller negativ i förhållande till denna receptor. När det kvinnliga könshormonet östrogen, som cirkulerar i kroppen, kopplas till östrogenreceptorn aktiveras celldelning. Denna möjlighet till aktivering utnyttjar vissa bröstcancerceller genom onormalt höga nivåer av östrogenreceptorn. Man brukar säga att dessa tumörer är hormonberoende. Ett vanligt läkemedel som används vid hormonberoende bröstcancer är tamoxifen. Tamoxifen kan, liksom östrogen, binda till östrogenreceptorn med skillnaden att tamoxifen blockerar istället för att aktivera celldelningsprocessen. Tamoxifen är en mycket effektiv behandling i många fall, men ibland uppstår okänslighet, resistens, mot läkemedlet. Om man kan förutse hos vilka patienter tamoxifen-resistens kommer att uppstå, kan man ersätta tamoxifen med annan förhoppningsvis mer verksam behandling. I delarbete III och IV har vi undersökt betydelsen av två proteiner, retinoblastomprotein (RB) och yes-associerat protein (YAP1), och om de kan ge information om huruvida en patient kommer att ha nytta av tamoxifen eller inte. Vi studerade proteinnivåer av RB och YAP1 i en stor samling av bröstcancertumörer från patienter där vi även har tillgång till klinisk uppföljningsdata och information som t.ex. vilken typ av behandling som använts och om, och i så fall när, återfall i bröstcancer har skett. Med hjälp av detta material kunde vi konstatera att för att en patient ska ha nytta av tamoxifen, bör RB-proteinet kunna detekteras i tumören. Resultaten indikerade också att YAP1 är viktigt för att tamoxifenbehandling ska fungera. Nu fortsätter vårt arbete med att försöka förstå de molekylära mekanismerna som ligger bakom våra resultat.

Sammanfattningsvis har vi kommit fram till att cellcykel-proteinet cyklin D1 kan påverka cellers rörlighet, vilket i förlängningen kan ha betydelse för cancercellens

förmåga att metastasera. Vi har påvisat att cellcykel-inhiberande behandling kan öka andelen cancerstamceller i vissa typer av bröstcancerceller. Slutligen har vi identifierat två proteiner, RB och YAP1 som viktiga faktorer för tamoxifenbehandlingens blockerande effekt.

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