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Clinical potential of the mTOR effectors S6K1, S6K2 and 4EBP1 in breast cancer

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FÖRORD OCH FÖRFATTARENS TACK

Bröstcancer är i dag en av våra största folksjukdomar. Tack vare intensiv forskning och klinisk utveckling överlever nu allt fler denna sjukdom, men ännu behöver mycket mer göras. Jag är mycket tacksam över att ha fått möjlighet att delta i denna kamp mot cancer under dessa år som denna avhandling sammanställts, och förhoppningen är att denna studie tillsammans med andra studier, kan vara en användbar pusselbit och ett steg i rätt riktning.

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Elin Karlsson, Adelöv 2014

SAMMANFATTNING

Klinisk potential för cellernas nav mTOR och dess måltavlor S6K1, S6K2 och 4EBP1 för en bättre individanpassad behandling av bröstcancer

Tumörsjukdomar, för kvinnor främst bröst-och lungcancer, utgör en av de främsta dödsorsakerna i västvärlden i dag. Tack vare utveckling av metoder för diagnos och behandling, liksom upptäckt på allt tidigare stadium har prognosen för bröstcancerpatienter förbättrats radikalt de senaste 25-30 åren. Dock diagnosticeras i Sverige ca 8000 nya fall av bröstcancer varje år, och omkring 1500 kvinnor dör av sjukdomen.

Ett stort problem är att brösttumörer är väldigt olikartade, och kanske egentligen borde betraktas som flera olika sjukdomar. Ett mål med dagens forskning är därför att hitta vägar för att kunna individanpassa behandling och därmed förbättra överlevnaden i bröstcancer, men också minska bieffekterna av onödiga behandlingar. En viktig del i detta är att öka kunskaperna om bröstcancers biologi och förstå vad som egentligen händer i cancercellerna i olika former av tumörer. Därmed skulle man också kunna identifiera nya markörer i tumörerna som skulle kunna användas som ett tillägg till dagens ganska grova bedömningskriterier i kliniken.

Utveckling och tillväxt av den normala bröstkörteln från speciella stamceller, sker gradvis och i olika stadier av livet. Detta styrs genom ett komplext samspel mellan hormoner, främst östrogen och progesteron, samt olika tillväxtfaktorer mellan cellerna, och deras målmolekyler inne i cellerna. Man tror att brösttumörer uppkommer och tillväxer p.g.a. att detta samspel går överstyr och man får en överaktivering av tillväxtsignaler i körtelcellerna. En välkänd orsak är att cellerna får förhöjda nivåer av östrogenreceptorer, målmolekylerna för östrogen. I dag behandlas patienter vars tumörer har höga nivåer av östrogenreceptorer, med antiöstrogener.

På senare år har man identifierat faktorn mTOR, som en annan viktig målmolekyl och ett nav för integrering av olika tillväxtsignaler inne i normala celler, bl.a. från östrogen och insulin (**se Figur nedan**). Det har visats att människor som har medfödda mutationer som leder till att mTOR blir mindre aktivt i cellerna, i princip aldrig utvecklar cancer eller diabetes. Däremot leder detta till andra komplikationer, som dvärgväxt och annan underutveckling. mTOR är ofta överaktivt i tumörceller jämfört med normala celler. Sedan 2012 behandlas vissa patienter med spridd bröstcancer med en kombination av antiöstrogener och mTOR-hämmare. Långt ifrån alla svarar dock på denna behandling och mer kunskaper om mTOR och dess målmolekyler i cellerna behövs för att kunna optimera behandling och förstå när den gör nytta.

I denna studie har vi tittat på de tre mest kända av mTORs målmolekyler; S6K1 S6K2 och 4EBP1. Det var tidigare känt att S6K1 kunde finnas i höga nivåer i upp till 20% av alla brösttumörer och detta var associerat med sämre överlevnad hos dessa patienter. Nu har vi kunnat visa att även nivåerna av S6K2 är förhöjda i ungefär lika stor andel tumörer, dock sällan samtidigt som S6K1. Däremot verkar nivåerna av S6K2 och 4EBP1 ofta vara förhöjda i samma tumörer. Höga tumörnivåer av S6K2 och 4EBP1 samtidigt kunde kopplas till ökad risk att dö i bröstcancer. Vi kunde också visa att

tumörnivåerna av S6K1, S6K2 och 4EBP1 kan användas för att förutsäga om en bröstcancerpatient bör ha nytta av antiöstrogenbehandling eller ej.

Vi har också tittat på möjligheten att utveckla nya, specifika behandlingar för att hämma mTORs målmolekyler mer specifikt. S6K1 och S6K2 är 70% identiska till utseendet och har länge betraktats som biologiskt likvärdiga. Våra studier, tillsammans med andra nya studier, pekar dock på att S6K1 och S6K2 verkar kunna ha delvis olika roller, både i normala vävnader och i olika tumörtyper. I odlade cancerceller i provrör, har vi kunnat släcka ner aktiviteten av S6K1 och S6K2 individuellt och sett att detta verkar kunna ge olika effekter på nivåerna av andra faktorer i cellerna och även påverka celltillväxt. Genom att i detalj jämföra utseendet och strukturen på S6K1 och S6K2 har vi hittat mönster som skiljer sig åt så mycket att det borde vara möjligt att utforma specifika hämmare mot dessa faktorer.

Sammantaget tyder denna studie på att tumörnivåerna av S6K1, S6K2 och 4EBP1 skulle kunna fungera som biologiska markörer för att kunna bedöma bröstcancerpatientens prognos och förutsäga när antiöstrogen behandling är verksamt eller när ytterligare behandling behövs. Genom att utveckla specifika hämmare mot S6K1 respektive S6K2 skulle det vara möjligt att få en mer skräddarsydd behandling än med dagens mTOR-hämmare. Kunskap om mTORs målmolekyler S6K1, S6K2 och 4EBP1 skulle därmed kunna vara ett nytt verktyg för mer individanpassad diagnostik och behandling och därmed förbättrad bröstcanceröverlevnad i framtiden.

Figur Det komplexa samspillet mellan hormoner och olika tillväxtfaktorer som styr normal bröstkörtelutveckling går överstyr vid bröstcancer, vilket leder till okontrollerad celltillväxt. I cellerna tros mTOR och dess målmolekyler S6K1, S6K2 och 4EBP1 kunna utgöra ett nav i detta samspel.

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ABSTRACT

The prognosis of patients diagnosed with breast cancer has been considerably improved in the latest 25 years, as a result of continuous development of diagnostics and treatment regimens. Though, tumour diseases, for woman mainly lung cancer and breast cancer, still constitute one of the most common causes of death in developed countries, following heart diseases. A future utopia is to develop more individualised therapy strategies, to further increase breast cancer survival, but also to decrease the risk of severe side-effects of unnecessary treatments.

Normal mammary gland development is regulated by a complex interplay between growth factors and hormones, mainly oestrogen and progesterone, in different cell types. Breast cancer origin and progression is assumed to result from an imbalance in this interplay, leading to the so called “Hallmarks of cancer”, including unlimited cellular proliferation. A central hub in the regulation of proliferation is the intracellular mTOR signalling pathway. Antioestrogen therapy is widely used in breast cancer clinics, however resistance towards this treatment is a remaining problem, and overactivation of mTOR may be one reason behind. A new treatment regimen constituting a combination of mTOR inhibitors with endocrine therapy was recently clinically approved for advanced breast cancers. Although significant benefit for this combination treatment is evident for some patients, counteracting feedback mechanisms are assumed to diminish the effects.

The work presented in this thesis focuses on the genes S6K1, S6K2 and 4EBP1 which are main effectors of the intracellular mTOR signalling pathway and thereby secondary targets of the mTOR inhibitors. Our results suggests that the gene amplification status, expression levels of the corresponding mRNA and protein of S6K1, S6K2 and 4EBP1 as well as their cellular localisation may be used to predict breast cancer outcome and the benefit from antioestrogen treatments. These factors are indicated to play separate roles in different subtypes of breast cancer, and specific targeting of S6K1 and S6K2 may be valuable in different tumour subtypes, and in comparison to present day’s mTOR inhibitors, further promote individualised therapies, and thereby increase breast cancer survival.

ORIGINAL PUBLICATIONS

This thesis was based on the following original publications:

Study 1

Gizeh Pérez-Tenorio, **Elin Karlsson**, Marie Ahnström-Waltersson, Birgit Olsson, Birgitta Holmlund, Bo Nordenskjöld, Tommy Fornander, Lambert Skoog, and Olle Stål, *Clinical potential of the mTOR targets S6K1 and S6K2 in breast cancer*. Breast Cancer Res Treat, 2011. **128**(3): p. 713-23. (GPT and EK contributed equally).

Study 2

Elin Karlsson, Marie Ahnström-Waltersson, Josefine Bostner, Gizeh Pérez-Tenorio, Birgit Olsson, Anna-Lotta Hallbeck, and Olle Stål, *High-resolution genomic analysis of the 11q13 amplicon in breast cancers identifies synergy with 8p12 amplification, involving the mTOR targets S6K2 and 4EBP1*. Genes Chromosomes Cancer, 2011. 50(10): p. 775-87.

Study 3

Elin Karlsson, Gizeh Pérez-Tenorio, Risul Amin, Josefine Bostner, Lambert Skoog, Tommy Fornander, Dennis C Sgroi, Bo Nordenskjöld, Anna-Lotta Hallbeck and Olle Stål; *The mTOR effectors 4EBP1 and S6K2 are frequently coexpressed, and associated with a poor prognosis and endocrine resistance in breast cancer: a retrospective study including patients from the randomised Stockholm tamoxifen trials*. Breast Cancer Res. 2013 Oct 17;15(5):R96

Study 4

Elin Karlsson, Ivana Magić, Josefine Bostner, Christine Dyrager, Fredrik Lysholm, Anna-Lotta Hallbeck, Olle Stål and Patrik Lundström, *Revealing potentially different roles of the mTOR-targets S6K1 and S6K2 in breast cancer by expression profiling and structural analysis*. 2014, Manuscript. (OS and PL contributed equally).

BACKGROUND

Breast cancer is despite advances in diagnostics and treatments still a leading cause of malignancy associated death among women worldwide. A high heterogeneity, encompassing a wide variety of pathological and clinical behaviours, as well as problems with identifying risk factors predisposing for breast cancer may explain the difficulties in controlling the disease. The future utopia would be further strategies for protecting against cancer development, for earlier detection by screening, and for a more individualised therapy, to increase breast cancer survival, and to decrease the risk of severe side-effects. For this purpose, additional tumour specific clinical markers and treatment targets are needed. Our understanding of breast cancer origin and progression can hopefully be improved by seeking to characterise the complex patterns of underlying genetic alterations and subsequent deregulations of signalling pathways.

The present study encompasses the mTOR signalling pathway, which is commonly deregulated in breast cancer and has recently emerged as a promising new therapeutic target. The focus was laid on the downstream effectors of mTOR; S6K1, S6K2 and 4EBP1, with an effort to evaluate their specific roles in carcinogenesis and to characterise their potential as clinical markers and as therapeutic targets. This introduction will give an overview of both the clinical and molecular aspects of breast cancer, concluding into what so far is known about the role of mTOR signalling within this context, and describe the specific aims of the present study.

Breast cancer: clinical aspects

The incidence of breast cancer in the western countries is increasing, a trend that is estimated to be real and not only a consequence of the aging of the population [1]. In Sweden, there are at present approximately 8000 new cases registered every year, with a median patient age at diagnosis of 65 years. Breast cancer mortality rates have yet remained relatively constant at 1500 deaths per year, even slowly decreasing, likely as a result of improved detection and treatment strategies [2].

In general, the aetiology of breast cancer remains unknown. Studies on inhabitants emigrating from low to high risk countries have indicated that the western life style and environment significantly increases the risk. Yet, apart from increased age, no specific factors in this sense have been pointed out. Hormonal features, in terms of early menarche, late menopause and late or few pregnancies have been suggested as predisposing, as well as diagnostic radiation against the mammary gland [1]. The most well-known risk factor is a family history of breast cancer, which is estimated to account for up to 10% of the cases. Germline mutations in the DNA-repair genes *BRCA1* and *BRCA2* are identified as responsible in a major part of cases, though other susceptibility genes are actively sought after (reviewed in [3]). At present, studies are evaluating the role of the *TP53*, *STK11*, *PTEN*, *CDH1* and *CHEK2* genes in familial breast cancer [4].

Breast cancer histology

The great majority of breast cancers are defined as carcinomas, arising in the epithelium of the lobules or the ducts of the mammary gland (for an overview of breast anatomy, see **Figure 1**). Initiation of malignancy is extremely rare in the stromal, vascular or fatty components of the breast [1]. To date, pathologists have defined 18 different histopathological subtypes of breast cancer [5]. Mainly, breast carcinomas are histologically classified as non-invasive (*in situ*) or invasive. Non-invasive lesions are further subdivided into lobular (LCIS) or ductal (DCIS) carcinoma *in situ*, based on the site of origin. LCIS does not form a palpable tumour and is not visible on mammography. This lesion is therefore a purely histological diagnosis, considered as a marker of increased risk for the development of invasive cancer. DCIS is defined as the precursor of invasive ductal carcinoma (IDC), which in turn is the most common form of breast cancer including 70-80% of all cases. Invasive lobular carcinoma (ILC) represents 5-15% of all breast cancers, whereas other rare, though specified invasive cancers can be exemplified by tubular, medullar, mucinous and papillary carcinomas.

Tumour staging, prognostics and treatments

The high heterogeneity of breast cancer proposes the importance of improved individualised prognostics and therapy strategies. Various prognostic and predictive factors are at present utilised, and additional markers are continuously evaluated. The staging and prognostic tools, as well as treatment strategies briefly covered in this section, are the mainly recommended in clinical routine by the Swedish Breast Cancer Group (**Table 1**) [6].

In general, the prognosis and treatment of a breast cancer patient is determined by the tumour stage at the time of diagnosis. The TNM classification system, stated by the Union for International Cancer Control (UICC), is widely used in clinical routine. Here, staging is based on the size of the primary tumour (T), axillary lymph node involvement (N) and presence of metastases (M). In addition to TNM classification, several other markers are used for assessing the risk of systemic recurrence and for determining adjuvant therapy. Histological grade describes tumour cell differentiation and is defined based on tubule formation, mitotic count and nuclear pleomorphism. Cell proliferation is commonly evaluated as the presence of Ki-67 antigen, evaluated with immunohistochemistry (IHC).

The primary treatment serves the purpose to achieve local control of the tumour. This mainly implies mastectomy or breast conserving surgery with complete local excision of the tumour tissue. Postoperative radiotherapy reduce the risk of local recurrence for all tumours, but the choice of this treatment is dependent on many factors, including possible side effects, the age and health condition of the patient. Radiotherapy is recommended following breast conserving surgery and in case of high grade tumours, as well as after resection of axillary lymph node metastasis. This commonly involves an absorbed dose of 50 Gy, divided into 25 fractions, during 5 weeks.

Systemic adjuvant treatments are offered when there is risk of recurrent disease. Postoperative chemotherapy is commonly given in 4-6 cycles during 5 months after primary surgery. Today, this usually involves a combination treatment of 3 cycles fluorouracil/5FU, epirubicin and cyclophosphamide (FEC), followed by 3 cycles of taxanes. Steroid hormones, mainly oestrogen and progesterone, are known to play essential roles in breast cancer development and progression. The presence of oestrogen- (ER) and progesterone receptors (PgR), as evaluated by IHC, is the decisive

factor for the choice of endocrine treatment. About 75 % of breast tumours are diagnosed as ER positive, and since the late 1970s, the ER inhibitor tamoxifen has been the golden standard treatment for this group of patients. In recent years aromatase inhibitors, suppressing oestrogen synthesis, have been established as a second possible endocrine treatment for postmenopausal patients. Premenopausal woman may receive gonadotropin-releasing hormone (GnRH) analogues as a complement to tamoxifen. In addition, fulvestrant which targets the ER for degradation, is a second-line treatment for postmenopausal woman with ER-positive disease, relapsing on previous endocrine therapies.

In the last decade, the human epidermal growth factor receptor 2 (HER2, *ERBB2*) has emerged as an important predictive factor, shown to be amplified and overexpressed in approximately 15% of breast cancers. In clinical routine, HER2 status is initially evaluated by IHC. Uncertain cases, as well as tumours classified as 2+ or 3+ (moderate or strong expression of HER2 protein) are further assessed for amplification of the corresponding gene, by *in situ* hybridisation (ISH). Patients whose tumours are judged as HER2 positive by ISH are since 2006 offered treatment with the anti-HER2 antibody trastuzumab (Herceptin) in addition to chemotherapy.

In general, patients with ER/PgR positive tumours with certain risk factors are offered endocrine treatment, whereas the choice of chemotherapy is based on the stage and aggressiveness of the tumour as determined by the factors mentioned above. Recurrence with distant metastases, generalised disease, is so far considered as incurable. In these cases, systemic treatments may still be able to restrain disease progression and stabilise the disease, equivalent to a chronic disease.

In addition to ER and HER2 inhibitors, several other targeted therapies have been, or are currently evaluated in clinical trials and a few are at present used in combination with chemotherapy regimens, for treatment of advanced breast cancer. These include bevacizumab (VEGF inhibitor), lapatinib (HER1 and HER2 inhibitor) and pertuzumab (prevent HER2 dimerisation). In 2012, the mTOR inhibitor everolimus was clinically approved in combination with the aromatase inhibitor exemestane, for the treatment of recurrent ER-positive breast cancer [7].

Table 1 Overview of the present clinically used prognostic and treatment predictive factors for breast cancer in Sweden (IHC: immunohistochemistry, ISH: in situ hybridisation)

Clinical factor	Grading				
TNM stage	<i>stage 0</i>	<i>stage I</i>	<i>stage II</i>	<i>stage III</i>	<i>stage IV</i>
<i>tumour size (T)</i>	<i>In situ</i>	T<2.0 cm	T 2.0-5.0 cm	T >5.0 cm	any
<i>nodal status (N)</i>	N0	N0	and/or N+	any	any
<i>distant metastases (M)</i>	M0	M0	M0	M0	M+
Nottingham grade	<i>grade 1</i>		<i>grade 2</i>		<i>grade 3</i>
<i>tubule formation (1-3)</i>	score 3-5,		score 6-7,		score 8-9,
<i>mitotic count (1-3)</i>	well		moderately		poorly
<i>nuclear pleomorphism (1-3)</i>	differentiated		differentiated		differentiated
Proliferation Ki-67	<i>low</i>		<i>high</i>		
	≤25% positive nuclei		>25% positive nuclei		
ER status	<i>negative</i>		<i>positive</i>		
	≤ 10% positive nuclei		>10% positive nuclei		
PgR status	<i>negative</i>		<i>positive</i>		
	≤ 10% positive nuclei		>10% positive nuclei		
HER2 status	<i>negative</i>		<i>positive</i>		
	IHC 0-1+		IHC 2+ or 3+ and ISH ≥5 gene copies, or ratio HER2/chromosome enumeration probe (CEP)17 in 20 to 60 cells, mean ratio >2.0		
Patients age	<i>low risk</i>		<i>high risk</i>		
	age > 40 years		age ≤40 years		

The need for further clinical markers of prognosis and prediction of treatment benefit

Despite advances in prognostics and treatment, the present clinicopathological tools are still rather limited. Prognostic markers, indicating the natural progression of disease with or without treatment, as well as predictive markers of treatment benefit and new therapeutic targets for resistant tumours are highly needed. This would enable further patient specific therapies, lowering side-effects as a result of unnecessary treatments, as well as allow identification of patients in need for additional or prolonged therapies.

New prognostic and treatment predictive markers evaluated in clinical trials

Breast cancer is, as mentioned earlier, a highly heterogeneous disease, exhibiting a wide variety of genetic alterations, cellular features and clinical behaviours. Traditionally, breast cancers have been classified into prognostically meaningful groups at the clinical and histological level, however, it is increasingly evident that cellular and molecular features are of equal importance [8]. At present, several new indicators and markers for prognosis and treatment prediction are evaluated in clinical trials.

In addition to age, other physiological factors such as body mass index (BMI) and physical activity are suggested as valuable factors in prognostics. Regarding tumour specific markers, presence of angiogenesis, as well as high proliferation are indicators of poor prognosis. In addition to Ki-67 and mitotic index, the expression of proliferation cell nuclear antigen (PCNA), as well as cyclin A and phosphohistone H3 (PPH3) are evaluated as new proliferative markers [6]. The urokinase plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1) are potential predictive markers for tumour invasion.

Even in the case of well-established clinical markers, essential issues are the significance of thresholds for positivity, as well as differential expression in different parts of the tumours and even more between primary tumour and metastasis. At present, Swedish recommendations for the definition of ER positivity is >10% positive nuclei [6]. Some studies, though, have shown a benefit from endocrine treatment for patients with tumours harbouring <10% ER-positive cells, therefore St. Gallen and American Society of Clinical Oncology (ASCO) guidelines since 2009 recommend >1% ER positivity as a standard. There are also ongoing discussions proposing that ER status should not be stated in discrete values, rather on a continuous scale (oral presentation, Åke Borg, Villa Aske 2013). The predictive role of PgR expression has been debated in the light of contradictory studies, possibly resulting from methodological problems. Recently, the St. Gallen and ASCO guidelines stated that PgR has predictive value in addition to ER, for endocrine treatment benefit [9, 10].

In recent years, it has been shown that the expression of common prognostic and predictive markers alters between primary tumour and metastasis in a significant number of cases, and re-evaluation of ER, PgR and HER2 status in biopsies from metastases are now recommended when possible [6, 11].

Treatment resistance, with focus on endocrine therapies

A main issue in breast cancer clinics is the occurrence of *de novo* or acquired treatment resistance. Underlying mechanisms of resistance as well as predictive markers of treatment benefit are actively sought. Anthracyclin-based chemotherapy acts by blocking replication through intercalation with DNA and inhibition of topoisomerase IIa, and in turn topoisomerase IIa expression is suggested as a marker of benefit from this therapy. The presence of p53 mutations has been related to less response to anthracyclins, and good benefit from taxanes, disrupting microtubule formation [6]. To monitor the effect of treatments on metastatic breast cancer, a new method for detection of circulating tumour cells (CTCs) in serum and plasma was recently approved [6].

For ER positive breast cancers, tamoxifen significantly reduces the recurrence and death rates among these patients. In the mammary gland, tamoxifen acts as an anti-oestrogen by competitively inhibiting the ER. In other tissues, the drug can also act as an agonist of the ER, and has been shown to reduce cholesterol levels and prevent coronary heart diseases. Even if most ER positive tumours respond well to tamoxifen, still about 30 % of tumours remain resistant to endocrine therapy, either *de novo* or acquired during the treatment. Cross-talk between ER and growth factors signalling pathways, mainly the receptor tyrosine kinase (RTK)/PI3K/AKT/mTOR axis, is suggested as a mechanism of endocrine resistance [12, 13]. As mentioned above, mTOR inhibitors in combination with endocrine therapies are promising, indicating the importance of this pathway in this context. Other suggested mechanisms of endocrine resistance include loss of ER expression or expression of truncated isoforms, posttranslational modification of the ER, deregulations of cofactors or the downstream CDK4/CDK6/pRb/E2F cell cycle axis, as well as changes in tamoxifen metabolism, which may also result from overstimulation of growth signalling pathways [12, 13].

Whole genome breast cancer classification

In recent years, promising trials have been initiated in portraying tumours on a genome wide scale. A breakthrough in breast cancer classification was done by researchers at the Norwegian Radium Hospital and Stanford University, who revealed five subtypes of breast cancer based on their gene expression profiles [14]. These five subtypes, referred to as luminal A, luminal B, HER2+, basal-like and normal breast-like, have been shown to be conserved across ethnical groups and are already evident at pre-cancerous stages [15]. This apparent internal homogeneity, together with the heterogeneity between tumours indicate specific tumour progression pathways for each tumour subtype. One hypothesis is that the different subtypes originate from different types of stem or progenitor cells [15]. The subdivision of tumours into these groups has also been shown relevant from a prognostic point of view [16].

Based on genome-wide gene expression data, several multigene assays have been developed and shown able to predict outcome in certain patient groups [17]. The Real-Time polymerase chain reaction (PCR) based Oncotype Dx profile involving 21 genes, can be used to predict overall survival among patients with node-negative ER-positive breast cancers treated with tamoxifen. The MammaPrint signature, encompassing 70 genes derived from expression arrays, is a useful prognostic indicator in the ER positive as well as the ER negative subgroup. A similar profile, encompassing 50 genes constitute the PAM50 gene array, which is mainly used to detect high risk patients among ER

positive, tamoxifen treated patients. The ratio of the two ER-regulated genes HoxB13 and IL17BR has also been used in the H/I index to predict prognosis in endocrine treated as well as untreated patients with ER positive disease.

In a large and comprehensive study, Wood and colleagues have given their view of breast- and colorectal cancers based on genome wide mutation profiles [18]. When sequencing all, known to date, protein coding genes in eleven breast- and eleven colorectal cancers, approximately 80 point mutations leading to amino acid exchanges were found in each tumour. Overall, the sites of mutations appear to be very infrequent, with only a few commonly mutated genes. Statistical analyses have indicated that in each tumour, less than 15 of the mutations are responsible for driving tumourigenesis, whereas the majority of alterations are harmless and a result of genomic instability. A large challenge is therefore to distinguish tumour “drivers” from “passengers” in that sense. The number of potential driver genes is large, however, it is suggested that all changes together actually reflect alterations in a limited number of signalling pathways. This is in agreement with the fact that different genotypes can result in the same phenotype [18]. Thus, targeting pathways rather than genes may be an important clinical consideration.

Another aspect regarding genomic classification of tumours is alterations in DNA copy number, which as with the transcriptome, now is possible to study on a whole-genome scale. It is indicated that the overall pattern of gene amplifications is quite concordant with elevated gene expression and further studies are ongoing with the aim to investigate the global impact of DNA copy number changes on the five gene expression patterns in breast tumours [16]. Indeed, certain patterns of DNA copy number alterations have been associated with the different expression profiles [19]. Comparisons between histological phenotypes and the patterns of genetic alterations have revealed new insight in breast cancer progression [8]. The molecular profiles differ significantly between tumours with different histological grade, suggesting that low grade carcinoma rarely is a precursor of high grade and that this classification rather reflects diverse ways of tumour progression. On the other hand, no evident associations between histological types and specific patterns of genetic alterations have been elucidated, raising the question whether these designations actually are appropriate [8].

Comprehensive studies of breast cancer on a molecular level have significantly increased our understanding of the disease. However, an integration of the clinical, histological, cellular and molecular definitions of breast cancer is certainly needed for a better management of the disease and for finally being able to translate all findings into clinical practice. An improved understanding of the biology of breast cancer would facilitate this integration.

The biology of breast cancer

Tumourigenesis is proposed to be a multistep process, with an underlying mechanism analogue to Darwinian evolution. Through stepwise genetic and epigenetic changes, progenitor cells and stromal cells receive defects in the systems controlling normal cell proliferation and homeostasis. This promotes selection of characteristics, by Hanahan and Weinberg referred to as the “Hallmarks of cancer” [20, 21].

Breast cancer is a highly heterogeneous disease, at the genetic as well as the clinicopathological level. However, based on gene expression profiling, five main tumour subtypes have been distinguished [14]. These subtypes were suggested to be derived from different stages in the normal mammary gland development, from gland stem cells to differentiated myoepithelial and luminal cells [22, 23]. These findings support the hypothesis of malignancy arising in certain cancer stem cells or progenitor cells and propose a new dimension on the view of breast tumour development, as cells on certain stages may give rise to different subtypes of breast cancer. This section will summarise the current achievements in the efforts to characterise the biology of breast cancer, starting with the cellular mechanisms regulating normal gland development.

Normal mammary gland maturation

The mammary gland is a highly complex organ, with the ability to undergo dynamic morphological changes during development and maintain the capability of remodelling and regeneration in a cyclical fashion during pregnancy [24]. The ductal tree is embedded within the fat pad, constituting a complex stroma including adipocytes, fibroblasts, immune cells, blood vessels and nerve cells (**Figure 1 a**). The mammary duct consists of a hollow lumen surrounded by an inner layer of luminal epithelial cells, a middle layer of myoepithelial cells and an outer basement membrane (**Figure 1 b**). The ability of a constant remodelling process requires the presence of a population of mammary stem cells constitutively able to differentiate into mature myoepithelial and luminal cells. The number and regenerative abilities of mammary stem cells are tightly regulated by oestrogen and progesterone levels, although the cells do not express ER or PgR. It is estimated that ER and PgR are expressed in 30-50% of luminal epithelial cells, though not in myoepithelial or basal cells, in the normal mammary gland. The ER+/PgR+ population of cells is non-proliferative, yet stimulates proliferation of ER-/PgR- cells, including the clone of mammary gland stem cells, through paracrine signalling. The balance between ER+/PgR+ and ER-/PgR- cells is suggested to be partly regulated by the levels of TGFβ1 and the transcription factor C/EBPβ, and the number of ER+/PgR+ cells are increasing with age, simultaneously with an increased breast cancer risk.

Figure 1 Mammary gland anatomy (a) and cells of the ducts and lobules [24] (b).

Briefly, the first step of mammary gland development occurs during mid-gestation with the formation of five pairs of placodes in the epithelial cell layer, which invaginate into the mesenchyme leading to formation of mammary buds. Through proliferation steps, the mammary buds extend into 10-20 sprouts. This rudimentary ductal structure has formed by embryonic day 18.5, and remains quiescent until puberty when increasing levels of hormones and growth factors promote proliferation of the ductal ends into terminal end buds (**Figure 2 a**). These in turn continue to develop into an epithelial branching structure, eventually filling the space of the fat pad. During the cycles of pregnancy, the luminal epithelium proliferates and differentiates into secretory alveoli, producing milk into the lumen. At the end of lactation, involution of the secretory units occurs, involving removal of 80% of the epithelium through an apoptotic process.

The development and remodelling steps are orchestrated through a tightly regulated and time coordinated process requiring signalling between the several cell types and stromal components. The process is initiated by endocrine signalling from the ovaries and pituitary, mainly involving oestrogen, progesterone and growth hormone (somatotrophin), respectively. These hormones signal to both epithelial and stromal cells (**Figure 2 b**). Growth hormone binds to its receptors in stromal cells, leading to production of IGF1, in turn binding IGF1R at the epithelial cells, promoting their proliferation.

Oestrogen signals through ER in both stromal and epithelial cells, stimulating production of HGF and AREG, respectively. HGF stimulates epithelial proliferation by binding to the HGFR, while AREG binds the EGFR on the stromal cells. Progesterone signals through PgRs on the epithelial cells, stimulating expression of mainly AREG and RANKL, stimulating proliferation of epithelial cells in a paracrine manner. Stromal cells also produce various other growth factors e.g. TGF α and FGFs and the epithelial cells respond to additional receptors including EPHA2, FGFR1 and HER2/ERBB2. In addition, matrix metalloproteases (MMPs) secreted in a tightly controlled pattern during gland development, plays important roles by degrading of ECM, as well as in controlling cell migration and survival (**Figure 2 b**).

As will be shown below, many of the factors essential for normal gland maturation are deregulated during malignant transformation and are suggested as drivers of tumourigenesis and therefore potential treatment targets.

Figure 2 Mammary gland development; developmental stages of the ductal tree (a), endocrine and paracrine signalling regulates mammary gland differentiation (b). (GnRH: gonadotrophin-releasing hormone, FSH: follicle-stimulating hormone, LH: luteinising hormone; GH: growth hormone, GHRH: GH releasing hormone, ECM: extra-cellular matrix).

Breast cancer: development and progression

Cancer development and progression is considered a multistep process, driven by epigenetic and genetic changes, as well as aberrant interactions within the microenvironment (reviewed in [15]). According to the clonal evolution theory, the transition of normal breast epithelial cells to a pre-malignant state involves genetic and subsequent phenotypic alterations in the myoepithelial cell layer [15]. Concurrently, the number of stromal cells increases. The carcinoma becomes invasive as a result of loss of myoepithelial cells and a subsequent degradation of the basement membrane. At this stage, cells are prone to invade surrounding tissues, enter the vasculature and migrate to distant organs, eventually giving rise to metastases. The whole tumour entity usually consists of both invasive and *in situ* components. Pre-invasive lesions are suggested to be quite common among the whole population, nevertheless, the mechanisms protecting some people from the transition to an invasive carcinoma remain unknown [15].

In recent years, a theory of cancer arising in certain types of stem cells has emerged. This so called cancer stem cell (CSC) theory has been intensively debated, in relation to the clonal evolution theory [25]. Proponents argue that for cancer cells to acquire the wide range of hallmarks as described below, the ability of the high plasticity of stem cells is needed. The high heterogeneity of tumours may also be explained by the CSC theory. Breast CSCs are suggested to originate from normal mammary gland stem cells, or from malignant epithelial cells going through epithelial to mesenchymal transition (EMT) [26]. To date, no available methods can with certainty distinguish normal stem cells from CSCs, obstructing the proving of their existence. CSCs are at present defined by the expression of certain stem cell markers, mainly CD44, CD24 and ALDH1, their properties of forming mammospheres *in vitro*, and the ability of a few CSCs to form a tumour after xenograft transplantation. Whether the phenotypic markers of CSCs also are functional remains to be investigated. Breast cancer originating from stem cells may be more aggressive than tumours arising from differentiated cells, and a high number of CSCs in tumours are connected to a poor outcome. However, a tumour may contain several different types of cells, all possessing stem cell characteristics, and identifying the cell-of-origin for a tumour is difficult. It has been suggested that CSC characteristics may result from mutations in specific signalling pathways.

Overall, it is likely that cancer development and progression may be best explained by a combination of the above theories, where stem cell characteristics facilitate clonal evolution leading to the hallmarks of cancer. As mentioned above, recent data propose that different molecular subtypes of breast cancer may arise from cells in different steps of the normal mammary gland development. According to this, differentiated luminal or myoepithelial cells may give rise to luminal, hormone responsive breast cancers, whereas mammary stem cells are the source of claudin-low and basal-like subtypes, associated with a more mesenchymal expression signature and more aggressive tumours [22, 23], (**Figure 3**).

Figure 3 Subpopulations of cells in normal breast tissue are potential cells of origin for the different subtypes of breast cancer.

Importance of oestrogen and progesterone signalling in breast cancer

Steroid hormones have long been known to play important roles in the development and progression of breast cancer. This was first shown by George Beatson, who in 1896 published the results of an improved breast cancer outcome and decrease in tumour size for premenopausal patients after surgical removal of the ovaries [27]. Subsequent studies revealed that not all patients had benefit from ovarian removal, and no method to predict response to the treatment was able until the discovery of the ER in the 1960s [28, 29]. It is now known that about 75% of breast cancer tumours exhibit ERs, and in these cases oestrogen facilitates growth of the tumour. Oestrogen and progesterone are synthesised from cholesterol in several enzymatic steps. Oestrogen exists in many shapes, where oestradiol (E2) is the most biological active form in breast tissue. Premenopausally, the ovaries are the main oestrogen producers, whereas circulating androgens secreted from the adrenal gland are the primary oestrogen source after menopause. In general, oestrogen diffuses through the plasma membrane of the cell and binds to ERs in the nucleus, though also oestrogen action via membrane bound ERs are observed. ER α , encoded by *ESR1* at 6q25, is the classical prognostic and predictive marker in breast cancer, whereas the importance of the second ER isoform ER β , encoded by *ESR2* at 14q23, remains unclear. The ER-oestrogen complex mediates its biological function by increasing or decreasing the transcriptional activity of certain genes, foremost involved in cell cycle regulation, thus promoting proliferation [30]. In the classical mechanism, E2 binds nuclear ER, causing a conformational change in the receptor, promoting posttranscriptional modifications, coactivator recruiting and receptor dimerisation. Subsequently, the ER-complex binds oestrogen-responsive elements (EREs) in the promoter region of its targets genes, including among others the *MYC* gene. Approximately 1/3 of ER targeted genes are regulated through the non-classical mechanism, where ER affects transcription indirectly, by binding other transcription factors, e.g., the AP1 complex including C-fos/C-jun. These genes involve e.g. *CCND1*, *IGF1* and *SERPINE1*. ER may also directly bind ERE-halvesites or binding sites of SP-1, as is the case in regulation of the *PR* and also reported for *CCND1*.

PgR, encoded by the *PR* gene at 11q13 exists in two isoforms, PgRA and PgRB, where PgRA has a shorter amino terminal domain [31]. *In vitro*, PgRB is a stronger transcriptional activator than PgRA. The two isoforms are partly differentially expressed, e.g. PgRA is mainly expressed in the ovaries and uterine whereas PgRB is foremost involved in development and proliferation of the mammary gland epithelium. Like ER, the PgR is a transcription factor acting through *cis*-acting progesterone responsive elements (PREs) or through binding to other transcription factors. PgR regulated genes involve among others RANKL. The PgR also has non-transcriptional roles in the cytoplasm, activating phosphorylation cascades within minutes *in vitro*. The activity of PgR is regulated by ligand binding, as well as phosphorylation by among others MAPK, CDK2/cyclin A and CK2. In general, sustained high levels of oestrogen and progesterone are predisposing for breast cancer, but as clinical markers ER and PgR are associated with low grade tumours, a favourable short-term prognosis and response to endocrine therapies [32]. Alterations in the balance between PgRA and PgRB have been observed in tumour cells, and a high PgRA/PgRB has been associated with poor prognosis and less response to endocrine therapies [31]. In the majority of neoplasms, the population of proliferating ER+/PgR+ luminal epithelial cells is increasing, likely as a result of a switch from paracrine to autocrine steroid hormone signalling through RANKL. Cyclin D1 is required for the proliferation of ER+/PgR+ cells, and in line with this cyclin D1 is commonly amplified and overexpressed in ER/PgR+ breast tumours [32]. Paracrine signalling may be more important in ER-/PgR- breast cancer, and progesterone has been shown to stimulate proliferation of ER-/PgR- cells only in the presence of RANKL [31].

Features of tumourigenesis: the hallmarks of cancer

In a highly attentive article in 2000, Hanahan and Weinberg proposed that the wide range of genetic alterations found in malignant cells often result in a few essential capabilities common for most tumours [20]. This global view of cancer has been very well accepted among the majority of cancer researchers, and recently an updated and refined description of the so called “hallmarks of cancer” was presented [21]. This section will briefly cover each of these basic capabilities and strategies by which it can be acquired in human cancers in general, and breast cancer in particular (**Figure 4**).

The hallmarks of cancer

-
- Overactivation of growth signalling
 - Deregulation of antigrowth signals
 - Apoptosis resistance
 - Metabolic reprogramming
 - Limitless replication
 - Angiogenesis
 - Metastasis
 - Evading immune destruction
- Genomic instability
 - Tumour microenvironment
 - Chronic inflammation

Figure 4 *The proposed hallmarks of cancer [20, 21].*

Activation of growth signalling

All normal cells are dependent on mitogenic stimulatory signals for their ability to grow and proliferate. These signals are mediated by diffusible growth factors binding to transmembrane receptors which transmit the signals into the cells, and further to intracellular circuits, finally translating the signals into action (**Figure 5**). Growth is also stimulated by the interaction between cell adhesion molecules and extracellular matrix (ECM) components. During tumour development, cells acquire the capability to generate these signalling cascades more or less independent of exogenous growth stimulation [20, 21]. The release of mitogenic signals are often increased in cancer tissue and may be mediated through autocrine signalling by the cancerous cells, or through malignant cells stimulating stromal cells to release growth factors in a paracrine manner. As previously mentioned, oestrogen signalling is a main determinant of cell proliferation in breast cancer, mediating a proliferative effect primarily by increasing the transcriptional activity of cell cycle promoting genes [33], and also through cross-talk to several growth signalling pathways [34] (**Figure 5**). Upregulation of steroid converting enzymes leading to increased local production of oestrogen in breast cancer tissue is also reported [35].

Intracellular growth signalling pathways are shown to be deregulated in most tumours. The PI3K/AKT pathway [36] regulates several cellular processes, including growth and proliferation, after activation by RTKs (**Figure 5**). Another intensively studied pathway is the Ras/MAP kinase pathway (**Figure 5**), which is frequently overactivated in many cancers. The growth signalling autonomy of cancer cells appears even more complicated, when considering that several intracellular pathways are

cross-connected in a complex circuit, which is far from unravelled, enabling extracellular signals to mediate multiple effects (**Figure 5**).

Deregulation of antigrowth signals

To maintain tissue homeostasis in normal cells, proliferative and antiproliferative signals counterbalance each other in synergy. Antigrowth signals act mainly through the Gap1 (G1)-checkpoint of the cell cycle (**Figure 5**). During the G1-phase, the cell monitors the internal and external environment to ensure that conditions are suitable before entering the DNA-replicative synthesis (S)-phase. Antigrowth signals can block proliferation by forcing the cell into a resting Gap0 (G0)-state or a postmitotic state. This is mainly mediated through TGF β , which prevents phosphorylation of the retinoblastoma protein (pRb) through activation of p21 and p27 (**Figure 5**). Unphosphorylated pRb blocks proliferation by inactivation of the E2F transcription factor that controls expression of genes involved in G1 to S-phase progression. Myc is a transcription factor that among many other functions has been shown to regulate the G1 cell cycle checkpoint. In normal cells, stimulation of E2F1 and Myc induces a negative feedback on proliferation by inducing expression p53 (**Figure 5**). The signalling circuit of the G1-checkpoint can be disrupted by a variety of mechanisms during carcinogenesis. p53 is probably the most well-known and studied tumour suppressor and inactivating mutations are reported in 20-30% of breast cancers. Overexpression of the cell cycle promoting proteins cyclin D1 and cyclin E has been reported in 40-50% respective 20-30% of invasive breast cancers [37]. The *Myc* gene has been reported amplified and overexpressed in 15-25% of breast tumours and is associated with a poor prognosis [37].

Metabolic reprogramming

To enable the high proliferation and growth, cancer cells require an increased amount of energy. The metabolic switch of cancer cells from normal glycolysis followed by oxidative phosphorylation, to an aerobic glycolysis, was proposed by Otto Warburg already in the 1930s [38, 39]. The Warburg effect includes an increased glucose uptake by upregulation of GLUT1 receptors, to compensate for the least efficient way of producing ATP. The increased lactate production may in some tumours be utilised by a special tumour cell population adapted to usage of lactate as an energy source [20, 21]. Recent findings suggest upregulation of common cancer pathways, including PI3K/AKT/mTOR to play a key role in the metabolic switch [40, 41]. The AMPK/mTOR axis constitutes a central hub for the regulation of cellular growth and proliferation in response to nutrient, energy and oxygen availability, as well as growth factors including IGFs and insulin. In addition to an increased energy consumption, cancer cells also require cellular building blocks including nucleotides, fatty acids and proteins, which among others may be acquired through mTOR upregulation of the translational machinery [40].

Apoptosis resistance

The apoptotic programme is suggested to be present in latent form in all cell types, where it can be triggered by a variety of signals, including DNA damage, withdrawal of growth factors, viral infections or p53 expression [1]. The ability to avoid apoptosis is shared by most if not all tumour cells. Normally, cell surface receptors and intracellular sensors, monitoring the environment, are responsible for triggering the apoptotic machinery [42]. The extrinsic pathway regulates apoptosis positively or negatively through extracellular signals. Mainly, the TNF family members TNF α and FAS mediate death signals via TNF-R1 and the FAS receptor respectively. This can be balanced with antiapoptotic signals via growth factor signalling. The intrinsic programme may be induced in response to e.g. DNA damage, where the proapoptotic Bcl-2 members Bak, Bid, Bim, Bad and Bax (the last mentioned upregulated by p53) stimulate mitochondria to release cytochrome C (**Figure 5**). Cytochrome C in turn activates Apaf1 which initiates an effector caspase cascade finally leading to apoptosis. On the other hand, the antiapoptotic proteins Bcl-2, Bcl-XL and Bcl-W inhibit Cytochrome C release in a counteracting mechanism [20, 21].

In tumour cells, the most commonly used strategy for evading apoptosis, is probably through loss of p53 function by mutations, as mentioned in a previous section. Overstimulation of the PI3K/AKT pathway, which transmits antiapoptotic signals, is another causing factor. The apoptosis suppressor *BCL2* is a well-known oncogene, which has attracted much attention in the context of breast cancer since its regulation seems to be hormone dependent. Expression of the protein in human breast tissue has been shown to vary dramatically throughout the menstrual cycle, and the levels are increased in ER positive tumours [1]. In addition, ER-signalling in breast tumours was recently shown to mediate antiapoptotic responses by suppressing the expression of p53 targeted genes [43].

Limitless replication

Studies based on cell culturing have revealed that mammalian cells carry an intrinsic program that restricts the number of cell divisions, independently of the growth signalling pathways mentioned above [44]. The limiting factors are the telomeres; several thousands of short tandem repeats situated at the ends of all chromosomes. During each cell cycle, 50-100 bp of telomeric DNA are lost from each chromosome. When telomeres shorten to a critical length, normal cells in culture enter a non-growing state called senescence. Disruption of the pRb signalling described above can circumvent this process and instead force the cells into a state of massive cell death and karyotypic disarray referred to as crisis. A few cells reach a state of immortalisation, the ability to multiply without limit. It is suggested that most cancer cells are immortalised, and achieve this state by maintaining the telomeres. Upregulated expression of telomerase, an enzyme capable of replicating telomeres and adding nucleotides to chromosome ends, is the most well-known strategy. In a few cases, a mechanism called alternate lengthening of telomeres (ALT), which is based on intrachromosomal changes, has been shown to serve the same purpose [20, 21].

The role of telomeres and telomerase in tumourigenesis may be considered as a double-edged sword, since telomere shortening, foremost as a result of normal ageing, is a key driver of genomic instability in combination with dysfunctional p53, promoting development of epithelial cancers [45].

Figure 5 Overview of the main intracellular signalling circuits regulating growth, proliferation and apoptosis in breast cancer cells.

Role of the tumour microenvironment

It is now accepted that tumours not solely constitute malignant cells, and may rather be considered complex organs consisting of several types of interplaying cells [20, 21]. In addition to transformed epithelial cells, these are mainly endothelial cells in vasculature and lymphatic vessels, as well as vasculature supporting pericytes, cancer-associated fibroblasts (CAFs), and various types of immune cells. Inflammatory cells may secrete proinflammatory cytokines and chemokines e.g. IL-1, IL-6 and CXCL12 involved in the angiogenetic switch. These can also stimulate recruitment of bone marrow-derived stem cells, in turn enhancing proliferation of cancer stem cells. In breast cancer, the extracellular TGF β is one of the most studied mediators of stromal-epithelial interactions. The cytokine is implicated to have a dual role, where epithelial TGF β , as previously mentioned, is considered a tumour suppressor due to its role in positively regulating apoptosis. However, stromal derived TGF β , possibly regulated by oestrogen, has been shown to promote EMT and thereby metastasis in later stage cancers [46, 47]. Increased local synthesis of oestrogen in breast tissue, mainly in stromal cells and adipocytes, is also assumed to stimulate malignant growth, among others through cross-talk to cytokine and chemokine signalling (**Figure 6**) [48, 49]. Thus, the complex cross-talk between different cells present in the developing normal gland also contributes in the tumourigenesis.

Evading immune destruction

The role for the immune system in cancer prevention is largely unknown, however it is suggested that the majority of arising tumour cells are immunologically detected and destroyed before further development [21]. In line with this, immunosuppressive treatment during organ transplantation increases the risk of certain cancer types e.g. skin and lung cancer. One newly suggested hallmark of cancer is the ability to avoid immune destruction. In breast cancer, mechanisms include downregulation of the surface markers HLA-1 and MICB (**Figure 6**), upregulation of antiapoptotic factors, production of proteinase inhibitors, or active induce of apoptosis in immune cells [50]. As described above, emerging evidence also show that the immune system may have a role not only in preventing, also in promoting cancer, through the secretion of proinflammatory cytokines and chemokines.

Angiogenesis

An essential factor for maintaining cell function and survival is the vasculature and its ability to supply the cells with oxygen and nutrients. Angiogenesis, the growth of blood vessels, is carefully and accurately regulated by several mechanisms. The angiogenetic switch is promoted by upregulation of VEGF, FGF and their respective receptors (**Figure 6**), as well as downregulation of the endogenous inhibitor thrombospondin-1. Intracellularly, increased Ras expression and loss of p53 function has been shown to be two of several underlying mechanisms. The bioavailability of angiogenesis activators and inhibitors can also be regulated by extracellular proteases e.g. by releasing FGF stored in the ECM [20]. In addition, a variety of stromal cells are involved in the angiogenesis [21].

Recently, it has been clear that the angiogenic switch occurs relatively early in the development of cancer and also in microscopic, premalignant tumours [21].

Figure 6 Overview of the main cell signalling circuits regulating angiogenesis, migration, metastasis and avoidance of immune destruction in cancer cells.

Metastasis

It has been proposed that 90% of all cancer deaths are caused by metastases, rather than by the primary tumour [51]. By leaving the primary tumour, cells can form new colonies where space and nutrients are more accessible. The mechanisms behind metastasis are far from unravelled, however, it is suggested to involve changes in the adhesion of cells to their environments, as well as activation of ECM degrading proteases [20, 21] (**Figure 6**). E-cadherins are ubiquitously expressed on epithelial cells and mediate mechanical attachment between cells, as well as transmission of antigrowth signals, via β -catenin. The majority of cancers, including breast cancers have reduced or lost E-cadherin function by one of several mechanisms.

Matrix degrading proteases are believed to facilitate metastasis by clearing the way for cancer cells through the stroma, across blood vessel walls and epithelial cell layers [1]. Elevated expression of e.g. the uPA family of serine proteases is often detectable in tumour tissue and it is indicated that stromal and inflammatory cells are the main protease producers, yet the tumour cells probably induce this secretion [20, 21].

Emerging evidence suggests that the process by which epithelial cells can develop migratory properties may occur through EMT [20, 21]. During EMT, cells regress to a more undifferentiated state and acquire the migratory properties of mesenchymal cells, by upregulating expression of the embryonic transcription factors Snail, Slug, Twist and ZEB1/2, in turn down-regulating e.g. E-cadherin (**Figure 6**). The role of EMT for metastatic progression is still under debate [52]. However, increasing efforts are made in understanding the mechanisms behind the metastatic progress, which may be the most essential step to target in cancer progression.

Genomic instability

The tumour progression pathway can highly differ between cancers and the above mentioned capabilities can be developed at different times during the progression. However, it is clear that genomic changes play a major role for cells to develop malignancy. In normal cells, the advanced control-and repair systems are effective enough to make these multiple genomic changes unlikely to occur. It is therefore likely that in the early stage of cancer, the cells develop genomic instability, leading to increased mutability [20, 21]. Genomic instability is suggested to result from primary mutations affecting the systems of replication and DNA repair. Defects in the DNA damage sensor p53 and its pathway, as mentioned above, is evident in most cancers. BRCA 1 and 2 are suggested to be involved in regulation of DNA transcription and repair (**Figure 5**), although the exact mechanisms are unclear. Genetic instability is in all probability one of the earliest hallmarks of cancer and may therefore be present even in tissue appearing to be histologically normal. However, whether inherited or sporadic, genomic instability leads to increased potential of developing further genetic changes, such as point mutations, chromosomal translocations, gene loss or amplifications [37].

Gene amplification

Genetic alterations are fundamental characteristics in the development of malignancy. Copy number aberrations of certain chromosomal regions are frequently found in solid tumours and are, with its subsequent changes in gene expression, expected to be essential for tumourigenesis. Gene amplification is a non-random event that refers to an increase in copy numbers of a restricted chromosomal region [53]. The amplicon is usually 0.5-10 Mb in length and shall by definition not exceed 20 Mb [54]. Amplification of larger chromosomal regions by aneuploidy or translocations, as well as a low copy number increase (<4), is usually referred to as gain rather than amplification. Oncogenes can be amplified to a copy number up to 500 in tumour cells [55], yet it is well accepted that also deletions and low-level gains are of importance in cancer pathology [54]. Gene amplification often leads to enlarged quantity of the corresponding mRNA and protein [56, 57] and seems to occur non-randomly, suggesting that amplicons harbour genes essential for tumour progression. Identifying patterns of gene amplification in tumours therefore has prognostic as well as diagnostic usefulness and could be utilised as a guide for treatment design [53]. Gene amplifications usually appear in advanced cancers, probably as a result of genomic instability, and could therefore be indicators of high grade and poor prognosis [54]. Increasing numbers of amplifications in a tumour have been associated with worse prognosis [58, 59]. In addition, certain patterns of gene amplification seem to be cancer type specific [54], and might therefore be valuable as independent classification parameters.

The value of gene amplification analysis in clinical practice is becoming more and more evident. Numerous specific amplicons, exemplified by these harbouring the *ERBB2/HER2* (17q12), *MYC* (8q24), *CCND1* (11q13), *AIB1* (20q12), *FGFR1* (8p12), *EGFR* (7p12) and *MDM2* (12q14) genes in breast cancer, have been shown prognostically significant [58]. Genes in the centre of amplicons are candidate drivers of tumourigenesis, and since the tumour therefore could be addicted to their enhanced expression, they are interesting therapeutic targets [53]. So far, there are few available drugs directed towards amplification-activated oncogenes, nonetheless, many possible targets are under investigation.

Of importance for the present project, the genes encoding the mTOR targets S6K1, S6K2 and 4EBP1 are localised in amplification hotspots at 17q23, 11q13 and 8p12, respectively. The *RPS6KB1* gene has earlier been shown amplified in breast cancer, associated with a poor prognosis [60-63]. In this project, we also report clinical significance of amplification of the *RPS6KB2* gene at 11q13, as well as *EIF4EBP1* at 8p12. The following section will describe discovery and clinical relevance of the 11q13 amplicon in breast cancer, concluding with its relations to 8p12 amplification.

Mechanisms of gene amplification

Amplicons can appear intrachromosomally at the site of origin, or as extrachromosomal DNA-fragments that lack a centromere and replicate independent of the chromosomes. Intrachromosomal amplicons were first observed cytogenetically by FISH and are therefore referred to as homogeneously staining regions (HSR). When sufficiently large, extrachromosomal amplicons can be visualised by FISH as double minutes (DMs), whereas molecular biology tools are needed to recognise the submicroscopic DNA fragments designed episomes [54]. Amplicons might contain mixtures of DNA fragments from different chromosomal locations and can be distributed in various forms throughout the genome [53].

The mechanisms behind amplicon formation are not fully established, however, several models explaining the amplification process are suggested [64]. The breakage-fusion-bridge (BFB) model proposed by McClintock is the main explanation for HSR formation [65]. The process is initiated by telomere loss or other double-strand breakages (**Figure 7**), which promotes fusion of the sister chromatids after the following DNA-replication. The result is a dicentric chromosome that forms a bridge during anaphase and is prone to break again, thereby initiating a new BFB cycle. The process can repeat in several subsequent divisions, until the telomeres are stabled by e.g. translocation to another chromosome, resulting in an amplicon with amplification units arranged as inverted repeats. At the same time, the DNA sequences distal to the amplicons are commonly deleted or translocated [53].

Figure 7 The breakage-fusion-bridge (BFB) model explaining the mechanism of gene amplification. Following the loss of a telomere, sister chromatids fuse. During anaphase, the resulting dicentric chromosome forms a bridge, which might break again, initiating a new BFB cycle.

According to the excision-segregation model, extrachromosomal fragments are formed by looping out from the chromosomes. This happens through breakage of replication bubbles. The fragments can also be formed secondary, during breakdown of HRSs whereas HRSs in some cases can be composed of DMs inserted into a regular chromosome [64].

The genomic site-specificity of structural chromosomal alterations has also been suggested to be influenced by fragile sites; chromosomal regions that are damage sensitive to chemicals that affect replication [54]. Statistically, it has been shown that fragile sites often appear at the ends of amplicons, however, at the same time these sites seem to be distributed equally on amplified and non-amplified regions [66]. Fragile sites in the proximity of the gene under selection may be necessary, nevertheless the genomic localisations of amplicons are likely also determined by the sites of oncogenes, as described in the next section.

Amplicons as indicators of tumour driving oncogenes

It is increasingly evident that specific amplicon formation is a result of selective pressure during tumour development. This was first discovered by *in vitro* studies, where cell lines were shown to develop cytotoxic resistance by upregulating drug target genes through amplification [67]. Another strong argument is the observation that the locations of gene amplifications are not randomly distributed across the genome, and that known oncogenes often colocalise with amplification hotspots [54]. Defining and mapping amplicons are therefore of great interest in the search for new oncogenes promoting growth advantage and other tumour characteristics. Recently developed array based techniques are now available for whole-genome analysis of gene amplification patterns, allowing a comprehensive view of amplicon boundaries as well as identification of recurrent regions of amplifications. Amplicons usually harbour several genes, and thus pinpointing a specific driver gene is a big challenge. It is assumed that under selective pressure, the amplicon can be narrower with focus on the driver gene, why it is suggested that the genes of interest should be situated in the region of maximal amplitude. However, subsequent investigations on whether candidate oncogenes actually are expressed in the tumour, if amplification is coherent with overexpression and in the long run, if its amplification status has prognostic and predictive value for clinical outcome, are also necessary [53].

The 11q13 amplicon in breast cancer

Amplification of the 11q13 chromosomal region was discovered and connected to breast cancer for almost two decades ago, and has thereafter been extensively studied. A heterogeneous amplification pattern, several amplification cores and an extremely gene dense region make the search for candidate oncogenes a complicated process. The following section will describe the discovery of the 11q13 amplicon in breast cancer, comparisons with its amplification patterns in other cancers, associations with breast cancer subtypes, candidate oncogenes in the region and its coamplifications with 8p12.

The discovery of 11q13 amplification

In the late 1980s, studies on mice indicated that a virus, now referred to as mouse mammary tumour virus (MMTV), could give rise to mouse mammary tumours by activating the fibroblast growth factors *fgf3* and *fgf4* (reviewed in [68]). Further studies confirmed a role of the genes in mouse mammary tumourigenesis and this prompted interest in linking these findings to human breast cancer. The human *FGF3* was subsequently mapped to 11q13 and in connection to this, it was revealed that the gene is amplified in a significant proportion of breast carcinomas. Shortly afterwards, the *FGF4* gene was shown to be situated at the same chromosomal region and coamplified with *FGF3* in breast and oesophageal tumours. However, when corresponding mRNA levels were measured, it turned out that the genes were more or less transcriptionally inactive in breast tissue and an increase in DNA copy number did not change this fact. Thus, *FGF3* and *FGF4* seemed to be passenger genes, and the search for a new driver oncogene in the 11q13 region was initiated. The 11q13 region had also in other contexts been implicated in tumourigenesis. In B-cell lymphomas, this region was shown involved in translocation with chromosome 14, t(11;14) and a hypothetical oncogene at 11q13 was referred to as B-cell leukaemia 1 (*BCL1*) [69]. In rare cases of parathyroid adenoma, a chromosomal inversion places 11q13 adjacent to 11p15, leading to increased expression of an 11q13 located gene referred to as Parathyroid Adenomatosis 1 (*PRAD1*) [70]. In the early 1990s, several groups studying cell cycle regulation simultaneously identified and isolated a gene whose corresponding protein was shown to promote progression through the G1 phase of the cell cycle. The gene, mapped to 11q13, was referred to as *CCND1* and its corresponding protein; cyclin D1 ([68] and references therein). It soon became clear that *BCL1*, *PRAD1* and *CCND1* were the same gene, and a new candidate oncogene in the 11q13 region was introduced. Hitherto, all markers used for studies of 11q13 amplification had been placed in the proximity to the *CCND1* gene. However, when the region of study was extended, it turned out that the amplicon actually spanned a much larger area. Subsequently, Shuurin *et al.* investigated the expression of genes within the area and clarified the *CCND1* gene and also the *EMS1* gene as being overexpressed as a consequence of amplification [71]. To date, the *CCND1* gene is still the far most extensively studied candidate oncogene in the 11q13 region in breast cancer, though studies have revealed several additional amplicons, suggesting further driver oncogenes in the area [72].

11q13 amplification in different cancer types

It is now well established that the chromosome band 11q13 is amplified in a significant proportion of primary breast tumours. Observed frequencies range from 10% up to 25%, depending on selection of patient cohorts, applied methodology and choice of markers [58, 73-78]. Amplification of this region has been reported to the same extent in DCIS and benign breast tumours [79-81], suggesting that this is an early event, connected to initiation of carcinogenesis, rather than a secondary effect of genomic instability. This event is not restricted to breast cancer and has also been found in other cancers, mainly ovarian cancer, oral- (OSCC) and head-and-neck squamous cell carcinoma (HNSCC), where up to 36% has been reported [82]. The patterns of amplification seem to vary between different cancers. Whereas *CCND1* and the area in its proximity constitutes the main amplicon in this region in breast cancer, this gene is rarely amplified in ovarian cancer, where amplicons usually arise more distally [83].

Amplification patterns and candidate oncogenes at 11q13

Originally, it was thought that one single amplicon, spanning numerous megabases (Mb), represented all 11q13 amplifications. However, it is now evident that the region harbours several amplicon cores that can be amplified independently or together in different combinations. Ormandy and colleagues describe four core regions [84], whereas Albertson suggests up to eight independent amplicons [53]. Consequently, this implies that the region contains several genes with the potential to drive tumourigenesis. The nature of the amplicons vary a lot from case to case and may consist of one or several specific amplicons, or a large amplicon spanning the whole region. In addition, many 11q13 amplifications are accompanied by a deletion of the distal part of the q-arm, which is interesting in several aspects. This area of 11q harbours several potential tumour suppressor genes, whose deletion could facilitate cancer progression. Furthermore, the occurrence of telomere loss suggests that BFB cycles could underlie the formation of these amplicons, and the amplicons usually occurs as inverted duplicated segments [85]. 11q13 is an extremely gene dense region and up to date approximately 500 genes are known to be situated in the area. Therefore, distinguishing potential amplicon drivers remains a challenge.

Implicated associations with subtypes and amplification patterns

It is now more or less stated that 11q13 amplification is connected to positive ER status. A strong correlation between 11q13 amplification and Luminal subtype, as well as an inverse correlation to basal-like expression type have also been shown [77, 86]. Although cyclin D1 may be the prime candidate for this connection, the same tendency is seen for several independent amplification cores in this region, making the mechanisms behind this connection yet unclear. Due to the availability of anti-oestrogen therapies, ER positivity is at present considered as a marker of good prognosis. However, among patients with ER positive tumours, these also harbouring 11q13 amplification seem to constitute a subgroup with higher grade and a relatively poor prognosis [58, 74]. When considering histological subtypes, 11q13 amplifications have been shown most prevalent in lobular breast carcinomas [87, 88].

The presence of one amplicon increases the likelihood of developing additional amplifications, probably partly as a result of genomic instability [58]. These multiple amplicons can be associated with each other, both physically and statistically. Several studies have demonstrated that the 11q13 region often is coamplified with regions on 8p11-12 [59, 77]. The presence of both of these amplifications seems to predict a worse patient outcome than either amplicon alone, suggesting a synergy between them [59]. 8p12 is frequently amplified in breast cancers and a main candidate driver oncogene for this region has been *FGFR1* [89]. However, as in the 11q13 case, 8p12 harbour several amplicons that can be amplified independent or together [90], indicating more than one driving oncogene. Paterson *et al.* investigated the physical structure of the 11q13-8p12 coamplifications in some breast tumours and breast cancer cell lines and demonstrated that a variety of arrangements can occur. This includes amplifications of a translocation junction, separate amplifications of the regions on different chromosomes and also in a few cases on the same chromosome, further strengthening the probability of multiple independent targets on the two regions [91].

In the present project, results suggest the mTOR targets S6K2 and 4EBP1 as candidate oncogenes for the 8p12/11q13 synergy. The following sections will describe the implicated roles of the mTOR/S6K/4EBP1 pathway in breast cancer.

The mTOR/S6K/4EBP1 pathway in breast cancer

The mTOR is assumed to be a critical effector in several cellular functions commonly deregulated in cancer [92]. The PI3K/AKT signalling pathway, a major regulator of mTOR function, is the far most altered pathway in breast cancer and is estimated to be constitutively activated in > 70 % of cases [93]. In response to growth factors, hormones, nutrients, hypoxia and energy (ATP) the PI3K/AKT/mTOR pathway regulates cell growth, proliferation and metabolism through translational control of essential proteins. Recently, it was shown that humans carrying mutations in the growth hormone receptor leading to reduced IGF/mTOR signalling are resistant to development of malignant tumours, suggesting this pathway as one major factor behind the origin of cancer [94]. The following section describes the molecular basis of RTK/PI3K/AKT/mTOR signalling, what is known about its role in breast cancer, and its implicated role in endocrine resistance.

Receptor tyrosine kinases as main regulators of PI3K/AKT/mTOR signalling

The human kinome encompasses 518 known protein kinases, divided into ten families, with a well-conserved kinase domain structure [95, 96] (**Table 2, Figure 8**). Kinases are estimated to regulate the function of >50% of all cellular proteins. The first crystal structure of a kinase domain was represented by PKA in 1991. The RTKs are highly conserved from *C. elegans* to human and have been shown as key regulators of many cellular functions [97]. To date, 58 human RTKs have been described, and divided into 20 subfamilies (**Figure 9**). This section will shortly describe the known mechanisms of RTK signalling, with focus on the EGFR, FGFR, IGFR and IR families, which are the major RTKs implicated in breast cancer and regulators of mTOR signalling.

Table 2 Overview of the human kinome.

Protein kinase families	
-AGC: cAMP, cGMP-dependent and protein kinase C e.g. AKT, PDK, S6K	-RGC: receptor guanylate cyclases
-CAMK: calcium/calmodulin dependent	-STE: sterile 7, 11 and 20 kinases
-CK1: casein kinases	-TK: tyrosine kinase e.g. RTKs
-CMGC: CDK, MAPK, GSK3, CLK	-TKL: tyrosine kinase like
-PKL: protein kinase like PIK: PI-3 kinases PIKK: PI-3 kinase like, e.g. mTOR	-Atypical/other

Figure 8 Overview of the conserved kinase domain structure exemplified by protein kinase A (PKA).

Receptor tyrosine kinase structure and regulation

All RTKs possess a common structure with an extracellular ligand binding domain, a single transmembrane helix and a cytoplasmic tyrosine kinase domain including additional C-terminal and juxtamembrane regulatory regions (**Figure 9**). Several different mechanisms of ligand binding and activation have been evident [98]. In general, ligand binding to the extracellular domain of the receptor facilitates dimerisation and subsequent transphosphorylation at intracellular tyrosine residues, in turn allowing recruitment and activation of downstream intracellular signalling pathways.

After activation, a second phase of transphosphorylation occurs, forming several phosphotyrosines as docking sites for downstream molecules. For IGFR, IRSs are the sites for the second phase phosphorylation. In turn, phosphorylation of IRS can be regulated through several positive and negative feedback mechanisms. Primarily, docking of Src homology 2 (SH2) and phosphotyrosine binding proteins occurs, including IRS (IGFR and IR), FRS2 (FGFR and NGRs) or Gab1 (several RTKs). These may in turn be phosphorylated by the tyrosine kinase, facilitating recruitment and activation of additional proteins, several containing SH3, WW, PDZ, PH, PV, C1, C2 or FYVE domains.

RTK signalling constitutes extremely complex networks, with numerous feedback mechanisms, where multiple factors and conditions determine the final outcome, including the amount of different ligands and receptors and downstream factors. This is also balanced with negatively regulating mechanisms, mainly RTK dephosphorylation by protein tyrosine phosphatases (PTPs), receptor internalisation through clathrin-mediated endocytosis, downregulation by ubiquitination as well as downstream cellular feedback mechanisms.

Figure 9 Domain structures of the 20 known receptor tyrosine kinase families [98]. (For further explanations see list of abbreviations).

PI3K/AKT signalling is the most overstimulated pathway in breast cancer

The PI3K was first discovered in the 1980s by Lewis Cantley and colleagues, searching for kinases with activity towards phosphoinositides, in mouse 3F3 cells [99, 100]. In humans, Vollerich *et al.* cloned and mapped the *PIK3CA* gene to 3q26 in 1994 [101]. To date, three classes of PI3Ks are known, where class I is the most well-studied and foremost implicated in oncogenesis [102], and will therefore primarily be considered in the context of this project. The class I PI3Ks constitute a heterodimer with one catalytic subunit, p110, encoded by the *PIK3CA*, *PIK3CB*, *PIK3CG* or *PIK3CD* genes and one regulatory subunit, p85, encoded by *PIK3R1*, *PIK3R2*, or *PIK3R3*.

The p85 regulatory subunit contains SH2 and SH3 domains, facilitating interactions with phosphotyrosines at activated RTKs. Subsequently, PI3K reaches close proximity with the plasma membrane, and after activation by conformational changes, PI3K phosphorylates the inositol ring 3'-OH group of inositol phospholipids in the plasma membrane, generating the turnover of phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinositol-3,4,5-trisphosphate (PIP₃). The reverse reaction is generated by PTEN. PIP₃ becomes docking site for other signalling molecules, including the serine/threonine kinases AKT and PDK1 (**Figure 10**).

Three members of the PKB/AKT family, with 80% sequence homology have been characterised to date, named PKB α (AKT1, 14q32), PKB β (AKT2, 19q13) and PKB γ (AKT3, 1q44). In most tissues, AKT1 is the predominant isoform, whereas AKT2 mainly is located in insulin responsive tissues and AKT3 is foremost expressed in neuronal tissue (reviewed in [103]).

AKT and PDK1 bind PIP₃ via their pleckstrin homology (PH) domains. Activation of AKT is then mediated by PDK1 phosphorylation of T308. A second phosphorylation on S473, mediated by mTORC2 is required for complete AKT activation. Activated AKT may be translocated to the nucleus, or remain in the cytoplasm for activation of numerous downstream actors, regulating several key cellular mechanisms. These include cell cycle progression, apoptosis, transcription and translation [104], the latter in which mTOR plays a key role (**Figure 10**).

mTOR signalling from the two complexes mTORC1 and mTORC2

The serine/threonine kinase mTOR was initially identified in the early 1990s as the cellular target of the immunosuppressor and antitumour agent Rapamycin [105]. Ever since, multiple cellular functions of mTOR and its complex signalling circuit have been uncovered. The gene encoding mTOR is located at the chromosomal region 1p36. Recently, the crystal structure of the mTOR kinase domain was reported [106]. It is now known that mTOR exists in two cellular complexes, referred to as mTORC1 [107] and mTORC2 [108], both possessing essential cellular functions.

The mTORC1 is a main integrator of tissue homeostasis

mTORC1 is composed of mTOR, Raptor, mLST8, PRAS 40, and FKBP38 [109]. In response to growth factors, hormones, nutrients, hypoxia and energy (ATP) mTORC1 regulates cell growth, proliferation and metabolism through translational control of essential proteins (**Figure 10**). As mentioned previously, two well-known mitogenic stimulators of mTOR function are the PI3K/AKT and Ras/MAPK cell signalling pathways, which regulate mTORC1 via phosphorylation and inhibition of the TSC1-TSC2 complex. TSC1-TSC2 in turn inhibits the GTPase Rheb. Active GTP bound Rheb activates mTORC1 in a partly unknown mechanism, by binding FKBP38 and the mTOR kinase domain, inducing an active conformation. mTORC1 activation is also promoted by phosphorylations of the mTOR kinase domain at several sites. Efforts in characterisation of responsible kinases for mTOR regulation have suggested AKT, S6K as well as autophosphorylation by mTOR itself, however, information is scarce ([110, 111] and references therein). The Wnt signalling pathway has also been implicated as an upstream regulator of mTOR function, possibly through inactivation of GSK-3 (**Figure 10**) [112]. During hypoxia, mTORC1 function is diminished by Redd1/Redd2, in turn upregulated by hypoxia inducible factor (HIF1 α) [109]. The availability of energy and nutrients is also critical for mTORC1 function. A low ATP/AMP ratio results in activation of AMPK via LKB1 phosphorylation. AMPK in turn, phosphorylates TSC2 resulting in mTORC1 inactivation. The mechanisms behind nutrient stimulation of mTOR are far from unravelled, though it is suggested that a high level of branched amino acids, in particular Leucine, activates mTORC1 by upregulating among others the Rag GTPases. The most well-known substrates of mTORC1 are the p70 ribosomal S6 kinases S6K1 and S6K2, and 4EBP1, which are involved in regulation of the translational machinery [113].

mTORC2 regulates cytoskeletal dynamics as well as AKT

Whereas numerous upstream regulators of mTORC1 function have been identified, the mechanisms behind mTORC2 activation are less known. mTORC2 is shown phosphorylated and activated in response to growth factors, though the intracellular pathways are still unravelled [92]. In addition to mTOR, mTORC2 consists of mLST8, mSIN1, PROTOR and Rictor. mSIN1, in turn exists in three different isoforms, therefore three distinct mTORC2s can be defined, possibly with divergent properties. The complex has been implicated in regulation of cytoskeletal dynamics, through activation of Rho GTPases and PKC α . mTORC2 has now been revealed as the earlier unknown kinase responsible for phosphorylating AKT at S473, thereby promoting its activation [114].

Figure 10 Overview of the RTK/PI3K/AKT/mTOR/S6K/4EBP1 signalling pathway. Suggested substrates and interaction partners of S6K1 and S6K2, respectively, are shown [115-121].

The mTOR targets S6K1, S6K2 and 4EBP1

Increased cellular proliferation is one of the hallmarks of cancer. To maintain a high proliferation, transformed cells commonly upregulate the translational machinery, resulting in an increased rate of protein synthesis, enabling the cells to double in size before division. As a result, histological examinations of highly transformed cells show enlarged nucleoli, where the ribosome biogenesis occurs [122]. The process of ribosome biogenesis is highly complex and energy consuming and it has been shown that in actively growing cells, ribosome biogenesis require >70% of their total gene transcription. This involves coordinated regulation of the three RNA polymerases (I-III) for production and processing of four ribosomal RNAs, 80 ribosomal proteins as well as more than 200 additional proteins and non-coding RNAs involved in the process ([123] and references therein).

In normal cells, the mTORC1 pathway is a major regulator of protein synthesis by phosphorylating several factors in the translational initiation complex [124]. The next section describes the discovery of S6K1, S6K2 and 4EBP1, which are the main targets of mTORC1 phosphorylation, its cellular roles and implications in breast cancer.

Discovery of S6K1 and S6K2, homologous AGC kinases

Ribosomes, essential for the translational machinery, are in higher eukaryotes composed of the 60S (large) and 40S (small) subunits. 60S consists of the three RNA molecules 5S, 5.8S and 28S together with approximately 45 proteins, whereas 40S comprises the 18S rRNA and 33 proteins. The ribosomal protein S6 (rpS6) in the 40S subunit was the first discovered ribosomal protein to be regulated by phosphorylation [125]. The phosphorylation of rpS6 is now a well-established response to mitogens, and the mechanisms of phosphorylation have been studied thoroughly [115]. The first identified S6 enzymes were the p90 ribosomal S6 kinases (in mammals RSK1-4), responsible for S6 phosphorylation in *Xenopus* oocytes. However, subsequent studies led to the discovery of the p70 ribosomal S6 kinase, as the major S6 kinase in somatic cells [126, 127]. The *RPS6KB1* gene, encoding S6K1, was first cloned in rat and rabbit [128-130] and shortly after the human orthologue located at the chromosomal region 17q23, was discovered and shown to encode two splicing variants, p70 and p85S6K1 [131]. At the end of the 1990s, a close paralogue of S6K1, termed S6K2, was cloned by several groups [132-135]. The *RPS6KB2* gene, encoding S6K2 is located at the chromosomal region 11q13. To date, three main S6K1 isoforms and two S6K2 isoforms are known, and the proteins are together referred to as the p70 ribosomal S6 kinases [115], (**Figure 11**).

The serine/threonine kinases S6K1 and S6K2 belong to the cAMP-dependent, cGMP-dependent and protein kinase C (AGC) family of kinases, also including among others PKA, AKT, PDK1, SGK and p90S6K. The AGC family members have a high homology in their catalytic domains and become active after phosphorylation by PDK1 at a Tyrosine residue in the activation loop (**Figure 8**) [115].

Figure 11 Domain structures and motifs of the known S6K1 and S6K2 isoforms.

S6K1 and S6K2 share 70 % overall amino acid identity, whereas the catalytic domains have even higher sequence homology with > 83 % overlapping residues. Also the kinase extension and pseudosubstrate domains are very similar. Regarding S6K1, p70 is the predominant form, which is expressed ubiquitously among different tissues, localised mainly in the cytoplasm. The p85 isoform, resulting from an alternative translational starting site, contains an additional 23 amino acid nuclear localisation sequence (NLS), suggesting targeting of this isoform mainly to the nucleus [115]. Recently, a novel S6K1 isoform, p31S6K1 was discovered. The p31S6K1 contains a severely truncated kinase domain, nonetheless, it has been shown to possess transforming activity, which is probably kinase-independent [136]. Also S6K2 can be translated from alternative starting sites, and two different isoforms have been detected, termed p54 and p56S6K2, where p54 is the predominant form. Both S6K2 isoforms contain a C-terminal NLS, mainly localising them to the nucleus, and p56S6K2 contains an additional 13 amino acid N-terminal NLS [115].

Differences between S6K1 and S6K2 are mainly found in the N and C-terminal domains, which are probably important for localisation, regulation and function of the proteins. S6K1 contains a C-terminal PDZ binding domain, facilitating recruitment of S6K1 to the cytoskeleton via neurabin [137]. S6K2, in turns, harbours a C-terminal proline-rich domain allowing interactions with proteins containing SH3 or WW domains [138]. The domain structure and the several phosphorylation sites are well conserved and are found in the corresponding *drosophila* dS6K, indicating that the two S6K isoforms present in mammals result from gene duplication [139, 140]. Phylogenetic studies have shown that S6K1 likely is the ancestral kinase and that S6K2 has arisen at a later time [141].

Recently, the crystal structure of the S6K1 kinase domain was reported [142, 143]. Activated S6K1, like all known AGC kinases, show a more ordered structure than its inactive state, resulting from a salt bridge between Glu-143 and Lys-123 in the ATP-binding pocket. Characterisation of both the phosphorylated and non-phosphorylated states gives further insights in conformations important for S6K1 activation, and gives the opportunity for design of drugs targeting S6K. Shortly after the release of the 3D structure, the first specific S6K1 inhibitor PF-4708671 was developed [144].

Regulation of S6 kinase function through PDK1/mTORC1 phosphorylation and PP2A dephosphorylation

Soon after its discovery, it was shown that the activity of S6K could be inhibited by the immunosuppressor and antitumour agent Rapamycin [145-147], concluding mTOR as the major regulator of S6K function [137, 148] (**Figure 10**). mTORC1 phosphorylates and activates the S6 kinases at the conserved hydrophobic motif. Raptor, in the mTORC1 complex, binds directly to the TOR signalling (TOS) motif in the N-terminal part of S6Ks, mediating interaction between mTOR and S6K [115]. The full kinase activity of S6K1 and S6K2 also requires phosphorylations at other residues. Phosphorylation of the activation loop residue by PDK1 is preceded by several phosphorylations in the C-terminal region. Responsible kinases *in vivo* have not been fully elucidated, though several kinases have been suggested *in vitro*, among others ERK1/2, JNK1/2 and CDK1 [115]. Although several phosphorylation sites are conserved between S6K1 and S6K2 (**Figure 11**), some differences in the regulation are reported.

Regarding S6K1, a model was recently proposed where in a first step, the interaction between the C-terminal autoinhibitory domain and the N-terminal is released by a Ca²⁺-dependent mechanism. This allows phosphorylation of S411, S418, T421 and S424 by mTORC1, which gives access to further

phosphorylation of S371 and T389 by the same kinase. Finally, PDK1 can phosphorylate T229 in the activation loop, leading to an open conformation of S6K [139].

In S6K2, exchanging the mTORC1 target residue T388 to Glutamate (mimicking phosphorylation) renders a constitutively active kinase, insensitive to rapamycin and wortmannin, which is not the case for S6K1. However, probably phosphorylation of T388 is not enough for full activation, since T388 is not phosphorylated in S370 mutants, suggesting that phosphorylation of this site need to precede T388 phosphorylation. A model of S6K2 activation was earlier suggested where MEK dependent pathways phosphorylate Serine residues in the C-terminal domain, followed by S370 phosphorylation and mTORC1-dependent phosphorylation of T388. Phosphorylation of T388 in turn, allows phosphorylation of T228 by PDK1 [149].

Dephosphorylation and inactivation of S6K1/2 is mediated primarily by the protein phosphatase PP2A, in response to rapamycin or cellular stresses [115].

Subcellular localisation and expression levels of the S6 kinases may determine their activity

Posttranslational modifications of the S6 kinases, as well as their domain structures and interactions, allow regulation of their activity and subcellular localisation. P85 S6K1 is shown to be mainly localised to the nucleus, whereas P70 S6K1 is preferably seen in the cytoplasm, though it can be shuttled between the nucleus and the cytoplasm, despite not having an NLS. However, phosphorylation of Serine 17 by CK2, enhances its nuclear export back to the cytoplasm, ensuring a cytoplasmic fraction always to be present [150]. A recent study has shown, however, that in primary fibroblasts, p85 is cytoplasmic, p31 is exclusively nuclear and p70 can be shuttled between the nucleus and cytoplasm upon mTORC1 phosphorylation at T389. The nuclear-cytoplasmic shuttling of p70S6K1 is not dependent on its kinase activity [151]. As mentioned above, p56S6K2 contains two NLS motifs and is thus mainly found in the nucleus. The predominant S6K2 isoform p54S6K2 is shuttled between cytoplasm and nucleus, and in response to growth factors, phosphorylation of S473 in the C-terminal domain by PKC inactivates the NLS, rendering S6K2 in the cytoplasm.

The cellular functions of S6Ks are also due to the overall expression levels of the protein. The mechanisms regulating ubiquitination and proteasomal degradation of S6Ks are not well known, however it is shown that Roc1 ubiquitin ligase interacts with S6K1, but not S6K2, suggesting a further mechanism for different regulation of the kinases [152]. In addition, acetylation of Lysine residues of S6K2 seems to stabilise the protein, suggesting that this may oppose ubiquitination, in a similar mechanism as for p53 [153]. MDM2 has been shown to interact with S6K1 and S6K2 and induce its ubiquitination in response to serum and growth factors. Ubiquitination seems to be independent of phosphorylation and activation of the kinases [154].

Discovery of the 4EBPs

The rate limiting step in the translational apparatus is the binding of mRNA to the ribosomes, which is mediated by eukaryotic translation initiation factor 4E (eIF4E), directing the ribosomes to the 5' cap structure of mRNA. The *EIF4E* gene, located at 4q23, was first cloned in 1987 [155] and eIF4E is a part of the large eIF4F complex, also containing eIF4A and eIF4G. The activity of eIF4E is considered mainly regulated by the 4E binding proteins (4EBPs). 4EBP1 and its close homologue 4EBP2 were initially cloned in 1994 and identified as interactors of eIF4E [156]. Later, these were mapped to the chromosomal regions 8p12 and 10q22, respectively [157]. In 1998, a third homologous eIF4E interacting protein named 4EBP3 was detected [158], and mapped to chromosome 5q31 [159]. The 4EBP1 protein is a rather small protein with 118 amino acids, encoded by three exons. The 120 amino acid sequence of 4EBP2 is 56% homologous with 4EBP1, also encoded by three exons, whereas 4EBP3, 57% and 59 % homologous to 4EBP1 and 4EBP2, respectively, is composed of 100 amino acids where the third exon is non-coding. 4EBP1 is the best characterised 4EBP [160]. The protein contains six known Serine/Threonine phosphorylation sites, whereof two are directly phosphorylated by mTORC1. 4EBP1 is foremost expressed in tissues involved in glucose and lipid homeostasis, mainly pancreas, muscle, liver and adipose tissue, whereas 4EBP2 is ubiquitously expressed. 4EBP3 lacks an N-terminal regulatory motif necessary for phosphorylation by mTORC1, and is assumed to possess different functions from 4EBP1 and 4EBP2.

S6Ks and 4EBP1 in regulation of protein synthesis

In higher eukaryotes, pre-mRNAs are commonly spliced, removing introns before translocation to the cytoplasm. During splicing, several protein complexes are bound to the mRNA, mainly TREX, CBP80/20 and the exon junction complex (EJC). The protein complexes facilitate nuclear export of mRNA and are involved in a pioneer round of translation, where mRNAs with premature termination codons are eliminated. During this step, EJCs dissociate from the complex and CBP80/20 is exchanged to eIF4E, facilitating recruiting of a translational initiation complex (**Figure 12**). EIF4E recruits eIF3 and the 40S ribosomal subunit to 5'-mRNA. mTORC1 binds eIF3 in the complex and phosphorylates 4EBP1 and S6K1. Phosphorylation of 4EBP1 leads to its dissociation from eIF4E, promoting recruiting of proteins to the complex, including the scaffolding protein eIF4G, the RNA helicase eIF4A and PAPB1. Activated S6K1 phosphorylates rpS6 in the 40S ribosomal subunit, and also the eIF4A cofactor eIF4B, recruiting it to eIF4A [125]. S6K1 also phosphorylates and promotes degradation of PDCD4, a tumour suppressor that binds and inhibits eIF4A. S6K1 also increases the rate of translation by phosphorylating eEF2K. The eEF2K kinase is a negative regulator of translational elongation by phosphorylation and inactivation of eEF2. S6K1 phosphorylation of eEF2K at S366 inactivates the kinase, promoting translation [161] (**Figure 12**). Altogether, these steps lead to activation of eIF4A helicase, facilitating 40S scanning for the start codon and translational initiation.

Figure 12 Roles of S6K1 and 4EBP1 in the translational machinery [121, 124, 125].

mTOR/S6K1 has been shown to facilitate translation of spliced mRNA over unspliced, in a process dependent of the Aly/REF family homologue SKAR. Activated nuclear S6K1 is recruited to newly synthesised mRNA by SKAR at the exon-junction complex during splicing. Knockdown of S6K1, SKAR or EJC components leads to a diminished translation of spliced mRNA. SKAR is phosphorylated by S6K1, but not S6K2, and knockdown of S6K2 has no impact on the translational rate of spliced mRNA, suggesting that this function is isoforms specific [124].

Most studies regarding S6K in regulation of protein synthesis is restricted to S6K1. However, also S6K2 has been shown involved in the translational machinery, and may possess functions independent of S6K1. *In vitro*, FGF2 activation results in increased levels of S6K2 [162], and formation of a complex involving S6K2, B-raf and PKC ϵ . PKC ϵ activates S6K2 by phosphorylation, leading to upregulation of synthesis of antiapoptotic proteins; a mechanism which is probably involved in chemoresistance [117, 162].

RpS6 is the most well-known substrate for S6 kinases and its phosphorylations have been investigated thoroughly [125]. The five phosphorylation sites are situated in a well conserved region in the C-terminal domain and are suggested to be phosphorylated in the ordered manner, S236>S235>S240>S244>S247. S236 is the primary substrate and the only residue within a consensus recognition sequence of S6K. Knockout studies in mice have shown that the levels of phosphorylated rpS6 are significantly reduced when S6K2 is deleted, whereas the levels are almost restored in S6K1-knockout mice, indicating that S6K2 may be the primary S6 kinase [163]. However, knockout of the rpS6 phosphorylation sites result in a phenotype similar to that of S6K1^{-/-}, suggesting that S6K1 may be essential for specific pools of rpS6, e.g. during different times of the development or in different cellular compartments [164]. Phosphorylated rpS6 has been detected both in the nucleus and the cytoplasm of the cells [163]. The rpS6 protein is conserved and can be found in yeast, however yeast does not possess any homologue to S6K1 and S6K2 [125].

RpS6 is localised to the interface between the 60S and 40S ribosomal subunits where it interacts with tRNA, mRNA and translational initiation factors, suggesting a key role in regulation of protein synthesis [125]. However, studies regarding the relationship between rpS6 phosphorylation and efficacy of protein synthesis have resulted in contradictory findings. Knock-in of rpS6^{P-/-} in mice has shown no difference in the proportion of ribosomes engaged in polysomes in liver cells and the rate of global protein synthesis increases in mouse embryonic fibroblasts (MEFs) from pS6^{P-/-} mice [164]. Thus, rpS6 phosphorylation may in some cell types result in downregulation of protein synthesis, suggesting S6Ks to be responsible for fine tuning of protein synthesis [125]. Early studies of rpS6 phosphorylation have demonstrated a strong correlation between S6K expression, rpS6 phosphorylation and the translation of 5' terminal oligopyrimidine tract (TOP) mRNA, encoding many components of the translational machinery. However, subsequent studies have failed to show this relationship [125], and the theory was more or less discarded when it was shown that S6K1-/S6K2-mice exhibited complete 5'TOP mRNA translation [163]. Though, the translational efficacy of 5'TOP mRNAs has in some cell types been shown rapamycin dependent, and a role for the mTOR/S6K pathway in 5'TOP mRNA translation cannot be totally ruled out [125].

Nuclear substrates of S6K1 and S6K2: involvement in transcriptional regulation and cell cycle control

Studies have shown that S6K1 has nuclear substrates, suggesting a role in regulation of transcription, in addition to its functions in translation. One nuclear S6K1 target is the transcription factor CREM τ . CREM τ modulates gene expression by binding to cAMP-responsive elements, commonly activated by the adenylate cyclase signalling pathway. The role of S6K1 phosphorylation of CREM τ for gene expression is still unknown [125]. ER α was recently shown to be a substrate of S6K1 [165]. S6K1 phosphorylates ER at S167, leading to transcriptional activation and subsequent increased cell proliferation, probably independent of ligand binding. In a recent study, phosphoproteomic screening identified four new nuclear S6K1 substrates *in vitro*; GRP75, CCT β , PGK1 and RACK1. GRP75 and CCT β are involved in chaperone function, whereas PGK1 is an enzyme in the glycolytic pathway and RACK1 is a scaffold protein involved in vesicle trafficking [166] (**Figure 10**).

Whether S6K2 is involved in regulation of transcription remains unknown. However, SH3-binding motifs, which can be found in S6K2, are shown to facilitate interaction of cofactors with ER α [138], allowing to speculate that S6K2 also has the potential to act as a cofactor of the receptor. Nuclear S6K2, but not S6K1 has been shown to associate with several RNA-binding proteins, including the hnRNPs [120]. HnRNPs are chromatin-associated nuclear proteins that bind specifically to different RNA sequences and are involved in several steps of RNA processing, including splicing, polyadenylation, mRNA stability and translational regulation [167]. Upon serum stimulation, mTOR associates with the hnRNP-S6K2 complex and activates S6K2 which promotes cell proliferation through unknown mechanisms [120]. Recently, it was also shown that S6K2, but not S6K1, can bind to the transcription factor YY1 in serum stimulated cells [116] (**Figure 10**).

The S6 kinases have also been implicated in cytoskeletal dynamics in the context of cell cycle regulation. *In vitro*, both S6K1 and S6K2 have been shown active throughout the cell cycle, where S6K1 peaks in the mitotic (M) phase and S6K2 in both gap2 (G2) and M [168]. However, in mid G1 phase p70S6K1 becomes phosphorylated by mTOR at T389 and translocated to the nucleus [169]. S6K2, but not S6K1, has been reported to localise to the centrosome during all steps of the cell cycle, suggesting that it may be involved in cytoskeletal regulation through centrosome signalling [170]. S6K has also been shown to regulate the cell cycle by activating Myc function, through phosphorylation and inactivation of the MYC suppressor Mad [171]. mTORC1/S6K1 may also stimulate cellular proliferation by upregulating pyrimidine synthesis [172, 173].

The S6 kinases in regulation of cell growth: implications from knock-out studies

The expression of the S6 kinases in different mouse and human tissues has recently been investigated [141]. A similar mRNA expression of both the kinases could be detected in all tissues examined. The levels of S6K1 protein were uniform in all tissues, whereas S6K2 varied profoundly, suggesting that the levels of S6K2 can be regulated at posttranscriptional level in a tissue-specific manner. Specifically, in the adrenal gland, S6K2 protein levels were found very low, whereas the expression was totally absent in the kidney.

The S6 kinases are believed to have overlapping functions, however there are also data indicating divergence in their biological activities. Knock-out of *S6K1* in mice, and *dS6K* in *drosophila* have

resulted in a reduction in animal body size during embryogenesis, as a result of a decrease in individual cell size [140, 174]. In contrast, *S6K2*^{-/-} mice had normal or slightly increased body size. Of note however, *S6K1*-deficient mice showed a significant upregulation of *S6K2* protein in several tissues, suggesting a compensatory mechanism, which may explain why the phenotype of size reduction was mostly overcome by adulthood [163]. Deletion of both *S6K1* and *S6K2* in mice, as well as *dS6K* in *drosophila* have been shown semilethal, severely reducing the viability. In contrast, no differences in lethality of *S6K1* or *S6K2* deficient mice have been seen, supporting compensatory and essential roles for the kinases in normal development [163, 174]. The reduced viability of *S6K* deficient mice and *drosophila* may partly be explained by its ability to phosphorylate and inactivate the proapoptotic factor BAD [175].

In addition to a reduction of animal body size, *S6K1* knockout mice exhibited a phenotype comparable to type 2 diabetes mellitus, with hypoinsulinemia and glucose intolerance, due to a selective decrease in size of the pancreatic β -cells [176]. It was also shown that the mice were resistant to developing diet-induced obesity, as a result of inactivation of a negative feedback-loop, where chronic exposure to nutrients causes desensitisation of the insulin receptor [177].

Activation of the PI3K/AKT/mTOR pathway as a clinical marker and target in cancer therapeutics

Deregulated metabolic pathways as a result of the western lifestyle with excess energy intake is suggested to be predisposing for cancer as well as other well-fare diseases such as diabetes. Humans carrying mutations in GHR leading to reduced IGF production and subsequent mTOR signalling are resistant to development of type II diabetes and malignant tumours, implicating an important connection between the origin of these diseases, where this pathway may be a major hub [94]. The PI3K/AKT/mTOR pathway is upregulated in > 70% of breast cancers. Aberrant activation of RTK signalling is common, mainly through increased autocrine ligand activation, receptor amplification/overexpression, chromosomal translocations or mutations producing a constitutively active kinase. As mentioned previously, *ERBB2* at 17q23 is amplified and overexpressed in approximately 15% of breast cancers. Also, *FGFR1* at 8p12 is found amplified and overexpressed in about 10% of cases. Furthermore, commonly appearing aberrations are *PIK3CA* mutations, *AKT* gene amplification/mutations as well as overexpression and activation of several upstream growth factors and RTKs. Loss of function of negative mTOR regulators e.g. PTEN, TSC1/2 and LKB1 is also frequently found in breast tumours [178] (**Table 3**). The most common aberration is mutations in *PIK3CA*, foremost in the helical domain (E542K or E545K) or the kinase domain (H1047R), leading to a constitutively active kinase. The highest frequency of *PIK3CA* mutations are found in the ER positive subgroup [178].

Table 3 Common alterations in the PI3K/AKT/mTOR pathway in breast cancer [60, 61, 178, 179].

Factor	Alteration	Frequency of alterations		
		Luminal (ER+)	HER2+	Basal (Triple Negative)
<i>ERBB2 (HER2)</i>	Amplification or overexpression	10%	~100%	0%
<i>EGFR</i>	Amplification		0.8% of all cases	
<i>IGF1R and INSR</i>	Receptor activation, <i>IGF1R</i> amplification	41-48%	18-64%	42%
<i>FGFR1</i>	Amplification, activating mutation	8.6-11.6%	5.4%	5.6%
<i>PTEN</i>	Loss-of-function mutation or reduced expression	29-44%	22%	67%
<i>PIK3CA</i> (<i>p110α/PI3K</i>)	Activating mutation	28-47%	23-33%	8-25%
<i>PIK3CB</i> (<i>p110β/PI3K</i>)	Amplification		5% of all cases	
<i>PIK3R1</i> (<i>p85α/PI3K</i>)	Inactivating mutation		2% of all cases	
<i>KRAS</i>	Activating mutation		4-6% of all cases	
<i>INPP4B</i>	Reduced expression or genomic loss	10-33%	54%	53%
<i>LKB1</i>	Loss-of-function mutation or reduced expression		?	
<i>TSC1/2</i>	Loss-of-function mutation or reduced expression		?	
<i>PDK1</i>	Amplification or overexpression	22%	22%	38%
<i>AKT1</i>	Activating mutation	2.6-3.8%	0%	0%
<i>AKT2</i>	Amplification		2.8% of all cases	
<i>RPS6KB1</i>	Amplification or overexpression		3.8-12.5% of all cases	

Implications of S6K1, S6K2 and 4EBP1 in cancer

Evidence for involvement of S6K kinases in cancer development are increasing. *RPS6KB1* amplification and S6K1 protein overexpression has earlier been associated with a worse outcome in breast cancer [60-63, 180]. Since S6K2 was discovered much later than S6K1, very little is yet known about its oncogenic properties. However, S6K2 overexpression in breast cancer cells has been reported [181]. Strong accumulations of S6K2 in the nuclei of breast cancer cells have been detected in 80% of tumours, whereas the same result for S6K1 was 25%. The increase in S6K2 expression is mainly found in the nuclei of cancer cells in peripheral areas of the tumours. In addition, the accumulation of S6K2 was correlated with expression of the proliferation markers PCNA and Ki-67. *RPS6KB2*, the gene encoding S6K2 is located very adjacent to the *CCND1* gene, in the chromosomal region 11q13, which is frequently amplified in many breast cancers.

A hallmark of cancer is the ability of cells to leave the primary tumour site and migrate to distant areas where space and nutrients are more accessible [20, 21]. Recent studies have indicated that the S6 kinases may have a role in this process. In ovarian cancer cells, it was shown that S6K1 can act as an actin filament cross-linking protein and also activate Rac1, cdc42 and their downstream effector PAK1, promoting cytoskeletal reorganisation and migration [182]. S6K1 has also been shown able to upregulate mRNA expression of the embryonic transcription factor SNAIL in ovarian cancer cells, inducing morphologic changes consistent with EMT characteristics e.g. downregulation of E-cadherin [183].

4EBP1 is generally considered as a tumour suppressor, due to the ability of unphosphorylated protein to inhibit the translational machinery, upon negative regulation by mTOR. However, several findings point towards a dual role for 4EBP1, possessing both tumour suppressive and oncogenic functions. Tumour suppressor genes are commonly deleted in cancer, though the gene encoding 4EBP1 is located in the chromosomal region 8p12, which is frequently amplified in breast cancer and several studies show a high correlation between 4EBP1 amplification and increased mRNA expression [184-187]. In addition to gene amplification, another suggested mechanism of 4EBP1 overexpression is through Myc dependent transcription. Amplification or induced expression of Myc have been shown to promote Myc binding to the 4EBP1 gene and increase its expression. This in turn leads to inhibition of autophagy and rapamycin resistance [188].

High levels of phosphorylated 4EBP1 have been associated with a high grade and worse outcome in several malignancies [189, 190], which is generally considered as a result of its role as a marker of activated mTOR signalling. However, in large tumours, overexpression of unphosphorylated 4EBP1, and eIF4G in response to hypoxia has been shown to switch the ribosomal machinery from cap-dependent to cap-independent translation [191]. This enables tumour cells to increase selective translation of mRNAs containing internal ribosome entry sites (IRESs), including VEGF, promoting angiogenesis. 4EBP1 has also recently been implicated in a positive feedback loop by binding and stabilising mTORC1, thereby promoting its activation [192]. The dual role of 4EBP1 in cancer may be explained by its emerging divergent roles in different cellular compartments. Approximately 30% of the 4EBP1 expressed in cells is located in the nucleus, where it has a role in regulating the availability of eIF4E for the cytoplasmic translational machinery, by retaining eIF4E in the nucleus [193]. High nuclear levels of 4EBP1 would thus inhibit translation and subsequent proliferation.

mTOR at the cross-road between diabetes and cancer

Epidemiologic studies have shown that obesity and type 2 diabetes are risk factors for the development of several cancer types, including breast cancer [194]. In addition, breast cancer patients with underlying type 2 diabetes are shown to have more advanced breast cancers and a poorer prognosis compared to patients without diabetes [195, 196]. A complex interplay between insulin, the IGF system, and glucose metabolism in different tissues and during different time points may underlie the connection between diabetes and cancer. Under normal circumstances, rising blood glucose levels following a meal triggers insulin release from beta-cells of the pancreas (**Figure 13**). In addition, GH release from the pituitary stimulates IGF1 release from the liver. Insulin acts on most tissues, though mainly liver, muscle and adipose tissues. In muscle and adipose tissue insulin stimulates glucose uptake by controlling GLUT4 glucose transporters through the PI3K/AKT pathway. IGF1 is, as previously mentioned, a master regulator of whole body cellular growth. Type 2 diabetes is generally defined by decreased insulin sensitivity, mainly in liver, muscle and adipose tissues [197]. This is suggested to result from among others downregulation of PI3K/AKT/mTOR signalling in these tissues. In response to enhanced blood glucose levels, pancreatic release of insulin increases to compensate for the insulin resistance. Eventually the ability of the beta-cells to produce insulin often fails to compensate the insulin resistance, with hyperglycaemia as a result, and type 2 diabetes can be diagnosed [198, 199].

Hyperinsulinemia may stimulate cancerous development and growth directly by acting on epithelial tissues that commonly are not insulin resistant. Indirectly, rising insulin levels in turn lead to increased levels of free circulating IGF1 as well as oestrogens, by influencing the expression of binding proteins, e.g. globulins. Also hyperglycaemia has been associated with increased risk of developing cancer, among others breast cancer [200], as well as a poor breast cancer outcome [201].

IGF-signalling may play divergent roles in different stages of mammary gland development, and could therefore also have different impact on breast cancer susceptibility in different ages [202]. In the mammary gland from prepubertal mice, IGF1 was shown to promote proliferation mainly through PI3K/AKT/mTOR-signalling increasing the cyclin D1 protein levels. In the postpubertal gland, IGF1 instead promoted proliferation through ERK/MAPK signalling, independent on cyclin D1, although still via upregulation of mTOR. ER-levels are suggested to determine the proliferative route of IGF1. The levels of ER change through development stages, and are high in prepubertal but lowered in postpubertal stages as a result of increased levels of circulating oestrogens. In the postmenopausal gland, ER levels are again elevated, which in combination with high levels of circulating IGF1 as a result of dietary factors is a potential risk factor for breast cancer in this group. However, IGF1 levels in the prepubertal stage where ER-levels are also high may indeed be a predisposing factor for breast cancer development. This is supported by the fact that young African American girls often develop early onset, aggressive, breast cancer and also have higher levels of circulating IGF1, in comparison with European girls in the same ages.

As a consequence of the importance of the insulin/IGF1 axis for both diabetes and cancer, therapies aiming to maintain the energy homeostatic balance in diabetes may also be promising in the prevention and treatment of cancer. To date, the biguanide metformin is the most widely prescribed drug to treat hyperglycaemia in diabetes [203]. Several prospective studies have found an association between metformin use in diabetic patients and a decreased risk of cancer [204, 205], as well as a favourable outcome among cancer patients [206]. Metformin was clinically introduced already in the 1950s as an antihyperglycaemic agent, however, the exact cellular and molecular mechanisms of

metformin action are yet to be elucidated. Physiologically, metformin has shown favourable effects on several aspects of type 2 diabetes, leading to stabilised levels of blood glucose, reduced levels of circulating insulin and IGF1, as well as increased insulin sensitivity in peripheral tissue (reviewed in [203, 207]). It has been suggested that many of these effects are primarily mediated through an increased LKB1/AMPK activity, resulting from metformin-induced increase in AMP/ATP ratio [207]. Recent data have demonstrated that metformin may act on hepatocytes by specifically and mildly inhibit mitochondrial respiratory chain complex I, leading to reduced gluconeogenesis [203]. The anti-cancer effects of metformin are suggested to result from lowered blood glucose, insulin and IGF1, and also by direct inhibition of tumour growth and proliferation mediated by LKB1/AMPK activation in turn inhibiting mTOR signalling [203, 208].

Figure 13 Insulin/IGF signalling and its effects on mammary gland epithelial proliferation under normal circumstances.

PI3K/AKT/mTOR and its effectors as potential targets in cancer therapy-feedback regulation a bottle neck

Upregulation of the mTOR signalling pathway facilitates several hallmarks of cancer. Interplay between ER and PI3K/AKT/mTOR signalling is suggested to promote cellular growth and proliferation, and also diminish the effects of ER targeted therapies. The cross-talk between ER and growth-signalling pathways can occur on multiple levels in a bidirectional manner. Several RTKs, as well as ligands and adaptor proteins are transcriptional targets of the ER. In addition, ER can rapidly activate PI3K/AKT through non-genomic mechanisms. AKT, S6K1 as well as ERK are able to activate ER in a ligand-independent manner by phosphorylating the receptor at S167 [165, 209, 210]. Also, AKT/mTOR regulates translation, as well as transcription of the ER transcriptional targets Myc and cyclin D1, promoting cell cycle progression [211]. Endocrine resistant cells show upregulation of IGF1R, IR, HER2, EGFR, FGFR1 and subsequent PI3K/AKT/mTOR activation [13], and activation of the pathway has been shown a predictive marker for poor response to endocrine therapies [212–217]. Concomitant targeting of ER and HER2 is used clinically for tumours expressing both receptors. *In vitro*, combining ER targeted therapies with IGF1R inhibitors has been shown useful [213], but dual targeting in clinical phase III studies has so far failed to show any benefit ([218] and references therein). As mentioned above, the use of mTOR inhibitors in combination with aromatase inhibitors has been shown successful [7] and is today clinically approved. The clinical benefit of mTOR inhibitors, though, seems to vary between different tumour types and so far the effectiveness has mainly been restricted to disease stabilisation rather than tumour regression.

An arising problem when using PI3K/AKT/mTOR antagonists is the phenomenon of counteracting feedback mechanisms, where the most well-known involves S6K1 diminishing AKT signalling through inhibition of IRS1 and IRS2 (**Figure 10**) [125]. The IRS1/2 adaptor proteins activate PI3K/AKT signalling upon stimulation of IGF and other growth factors through its receptors. Activated S6K1 phosphorylates IRS1 at S307, leading to dissociation from the receptor [219]. S6K1 can also phosphorylate IRS1 at S1101, blocking its phosphorylation and activation by the RTK [220]. S6K1 and S6K2 also seem to regulate the stability of IRS1 mRNA [219], and S6K1 is involved in a positive feedback mechanism by phosphorylating mTOR at T2446/S2448 [110, 111]. However, the effect of mTOR phosphorylation at these sites is not fully elucidated. The S6 kinases have also been shown to possess negative feedback by regulating the subcellular localisation of PTEN, a negative regulator of PI3K/AKT signalling [221]. PTEN is localised to the nucleus of differentiated and cell cycle arrested cells (G0/G1 phase), but is exported to the cytoplasm during G1-S transition, a localisation where it often can be found in tumour cells. S6K1/2 was able to bind PTEN and promote its nuclear export, facilitating cell cycle progression. Whereas S6K1 is involved in negative feedback loops of AKT/mTOR signalling, S6K2 has been shown to activate AKT and promote cell survival in MCF7 breast cancer cells [222]. This suggests that S6K1 and S6K2 may have different roles on AKT activation.

Studies have indicated additional roles of 4EBP1, independent of mTORC1. Rapalogs, mainly targeting mTORC1, have been shown to completely inhibit pS6K but only partially p4EBP1 [223]. In bladder cancer, 4EBP1 was shown to be regulated by PI3K, but not through mTORC1 [224] and mTOR-independent 4EBP1 phosphorylation has been associated with resistance to mTOR kinase inhibitors [225]. Additional kinases for 4EBP1 regulation remain to be identified, where upstream factors of the PI3K/AKT pathway are likely candidates.

Overall, the mTOR pathway has emerged as a critical effector in the interplay between nutrients, growth factors and hormones regulating normal physiology, and consequently a key factor in the cellular deregulations occurring in cancerous states. Findings point towards significant differences in regulation and effects of its downstream factors S6K1 and S6K2 in normal physiology, which may also be relevant in the clinical setting. In addition, an oncogenic role of 4EBP1 is emerging. An increased knowledge of the intracellular pathways involved in the mTOR signalling circuit, and its role in different tissues and during different conditions and states, would be potentially helpful in the future prevention and treatment of cancer.

AIM OF THE PROJECT

The purpose of this project was to evaluate the role of alterations downstream the mTOR signalling pathway for breast cancer development, prognosis and treatment response, specifically:

Study 1: Due to the location of S6K2 in the 11q13 chromosomal region commonly amplified in malignancies, and the high homology between S6K1 and S6K2, investigate whether S6K2 is amplified and overexpressed in breast cancer and evaluate its prognostic and endocrine treatment predictive role, in comparison with S6K1 amplification.

Study 2: Look further into the 11q13 amplicon and study S6K2 amplification in relation to other candidate oncogenes in the region as well as to genome wide copy number alterations, and to evaluate the 11q13/8p12 coamplification and its prognostic value.

Study 3: Investigate the S6K2/4EBP1 (11q13/8p12) coamplification/coexpression identified in study 2 in larger cohorts, as well as the prognostic and endocrine treatment predictive role of 4EBP1 at the protein level.

Study 4: Investigate different roles of S6K1 and S6K2 and their individual potentials as new clinical targets in breast cancer.

MATERIALS AND METHODS USED IN THE STUDIES

This part contains a brief description and comments on the different patient materials and methods used in the studies. Further details can be found in the respective publication.

Breast cancer patient materials and models

Study 1-3 encompass two cohorts from the randomised adjuvant Stockholm tamoxifen trials, referred to as Stockholm 2 and Stockholm 3. In *Study 3 and 4*, three publically available datasets were used to confirm some of the results. In addition, an *in vitro* model of breast cancer was used in *Study 4*. This section will describe the different materials used in the studies, discuss their original designs and clinical value.

The treatment randomised Stockholm trial

To determine the value of a potential new treatment, clinical procedure or biological marker, large randomised controlled trials are considered the golden standard [226, 227]. Randomisation serves the purpose to balance the groups to avoid selection biases and thereby factors confounding the results. It also permits the use of probability theory and subsequent statistical tests. For correct randomisation, no prior knowledge about assignment groups should be known that could influence participants or the researchers. Patient materials from large randomised clinical studies are rather rarely available, and therefore highly valuable.

The Stockholm 2 and Stockholm 3 cohorts used in the present study are derived from a large, randomised trial conducted by the Stockholm breast cancer group between November 1976 and April 1990, mainly aiming to evaluate the benefit from adjuvant tamoxifen treatment. All patients were randomised to receive tamoxifen for two years, or no endocrine treatment. Patients in the Stockholm 2 cohort were further randomised, using a 2 x 2 factorial study design, to postoperative radiotherapy (RT) or cyclophosphamide-methotrexate-5-fluorouracil (CMF)-based chemotherapy. Most of the patients in the tamoxifen arm, if disease-free after two years, were then randomised to receive tamoxifen for three more years, or no further adjuvant treatment. Detailed information about study designs and long-term follow-up data have been previously reported in detail [228, 229]. Patients in the Stockholm 2 cohort were postmenopausal and had positive lymph nodes and/or a tumour diameter exceeding 30 mm, whereas the Stockholm 3 cohort consisted of postmenopausal breast cancer patients with a tumour diameter ≤ 30 mm and no lymph node involvement. Patient flow through the studies is presented in **Figure 14** and clinicopathological data can be found in **Table 4**. Retrospective studies of biomarkers were approved by the local ethics board at the Karolinska Institute, Stockholm, Sweden, and at the time, no further consent from the patients was needed.

ER expression was determined at the time of diagnosis, before 1988 this was done using isoelectric focusing and after this year, quantitative enzyme immunoassay (EIA) was used [228, 229]. In the

Stockholm 3 cohort, where tissue microarrays (TMAs) were available, ER and PgR status were further determined retrospectively by IHC [230]. Cut-off levels for ER and PgR positivity were >10% stained nuclei or, when IHC data was not available, 0.05 fmol/μg DNA. Isoelectric focusing/EIA and IHC data have been shown to be comparable [231]. In the Stockholm 2 cohort, HER2 protein was quantified retrospectively by flow cytometry [232] and HER2 amplification was determined with quantitative Real-Time PCR [233]. HER2 protein expression in the Stockholm 3 cohort was evaluated with IHC as described elsewhere [234], whereas tumour grade was evaluated retrospectively according to the Nottingham system [230]. In the Stockholm 2 cohort, S-phase fraction (SPF) was previously determined by flow cytometry [235], and mutations in *PIK3CA* exon 9 or 20 was assessed by single-strand conformational polymorphism (SSCP) [179]. Breast cancer subtypes in Stockholm 3 were defined according to St Gallen 2011 [9] as follows: Luminal A (ER+ and/or PgR+, Her2-, grade I-II), Luminal B1 (ER+ and/or PR+, HER2-, grade III), Luminal B2 (ER+ and/or PR+, HER2+), HER2-like (ER- and PR- and HER2+), and Basal-like (ER- and PR- and HER2-).

From the Stockholm 2 cohort, fresh-frozen tumour tissues were available for DNA and RNA preparation, from 207 (*Study 1*) and 93 samples (*Study 3*), respectively. For *Study 2*, DNA was available from 29 breast tumours with 11q13 amplification, and 11q13 amplification was defined as four or more gene copies of *CCND1* and/or *PAK1* and/or *RPS6KB2* ([74] and *Study 1*), whereof 23 was available for RNA extraction and Real-Time PCR. From the Stockholm 3 cohort, 912 paraffin-embedded samples were available for TMA preparation (*Study 1* and *Study 3*). As a result of routine mammographic screening, breast tumours are at present detected at a very early stage, and today 75-80% of diagnosed tumours are node-negative. The Stockholm 3 cohort is therefore representative for the present clinical situation, whereas the Stockholm 2 cohort represent higher stage cancers.

Figure 14 Patient flow through the studies (Tam: tamoxifen 2 or 5 years, RT: radiotherapy, CMF: cyclophosphamide methotrexate 5-fluorouracil chemotherapy, TMA: tissue microarray, IHC: immunohistochemistry).

Table 4 Patient characteristics of the cohorts used in the studies.

	<i>Stockholm 2</i> (n=207)	<i>Stockholm 3</i> (n=912)	<i>Van de Vijver</i> (n=295)	<i>Uppsala</i> (n=251)	<i>Karolinska</i> (n=159)
	n (%)				
<i>Tumour size</i>					
< 20 mm	83 (40.1)	697 (76.4)	155 (53.0)	127 (50.6)	97 (61.0)
>20 mm	124 (59.9)	189 (20.7)	140 (47.0)	124 (49.4)	60 (37.7)
missing	0 (0)	26 (2.9)	0 (0)	0 (0)	2 (1.3)
<i>Lymph node status</i>					
-	22 (10.6)	912 (100)	151 (51.2)	158 (62.9)	94 (59.1)
+	185 (89.4)	0 (0)	144 (48.8)	84 (33.5)	60 (37.7)
missing	0 (0)	0 (0)	0 (0)	9 (3.6)	5 (3.1)
<i>Grade</i>	N/A				
1		142 (15.6)	75 (25.4)	67 (26.7)	28 (17.6)
2		438 (48.0)	101 (34.2)	128 (51.0)	58 (36.5)
3		169 (18.5)	119 (40.3)	54 (21.5)	61 (38.4)
missing		163 (17.9)	0 (0)	2 (0.8)	12 (7.5)
<i>ER</i>					
-	61 (29.5)	195 (21.4)	69 (23.4)	34 (13.5)	29 (18.2)
+	144 (69.6)	689 (75.5)	226 (76.6)	213 (84.9)	130 (81.8)
missing	2 (1.0)	28 (3.1)	0 (0)	4 (1.6)	0 (0)
<i>PgR</i>	N/A		N/A		N/A
-		380 (41.7)		61 (24.3)	
+		415 (45.5)		190 (75.7)	
missing		117 (12.8)		0 (0)	
<i>HER2</i>			N/A	N/A	N/A
-	146 (70.5)	739 (81.0)			
+	56 (27.1)	93 (10.2)			
missing	5 (2.4)	80 (8.8)			

Cell culture and RNA interference as an *in vitro* model for breast cancer

In the late 1940s, intense research was ongoing in developing approaches enabling culturing of cancer cells in test tubes, which would revolutionise the studies of cancer origin, growth and drug testing [236]. In 1951, George O Gay at John Hopkins, achieved in establishing the first immortal human cell line [237]. The cell line, now referred to as HeLa, named from the patient Henrietta Lacks, was derived from a cervix adenocarcinoma and is today the most wide-spread human cell line. It has to date been used in a countless number of research studies as an *in vitro* model of human cancer, but also in studies of other diseases. Since 1962, the American Type Culture Collection (ATCC), a private, non-profit biological resource centre established in 1925, has been the main resource of storing and distributing cell lines to the research community. The use of human cell lines as models of cancer allows studies of mechanisms of drug responses and evaluation of causal relationships at the cellular level. However, the accuracy of *in vitro* models for cancer *in vivo* is continuously debated.

The phenomenon of RNA interference was first described by Fire and Mello in 1998, who showed that double-stranded RNA (dsRNA) can silence gene expression in *C. Elegance* [238]. RNA interference occurs when the enzyme Dicer cleaves dsRNA to short fragments, 21-23 nucleotides, referred to as small interfering RNA (siRNA). Today, siRNA is synthetically produced and are frequently used to study gene functions *in vitro*. Research is also continuously ongoing in using siRNA as a new therapeutic method *in vivo* [239].

In *Study 4*, the human breast cancer cell line ZR751, expressing high levels of both S6K1 and S6K2 (Cancer cell line encyclopaedia [240] and unpublished observations), was used to explore global gene expression alterations after knock-down of S6K1, S6K2 or both S6K1 and S6K2 simultaneously. In addition, several cell lines were used in *Study 1-3* as sources of RNA, DNA and protein, for use as calibrator samples in Real-Time PCR, and for evaluation of antibody specificity using western blot before IHC staining.

Preparation of tumour tissue

Appropriate collection of tumour tissue is essential for histopathological evaluation in the clinic, but also to allow research on new potential markers and targets. Today, formalin-fixed paraffin embedded (FFPE) tissues are routinely collected for these purposes, which allow histological studies, mainly by IHC, as described below. However, several molecular biological methods, including microarray and Real-Time PCR still require fresh-frozen tissues to preserve high quality DNA and RNA, although these methods are continuously optimised to allow usage of FFPE tissues. According to the routines recommended by the Swedish breast cancer group [6], all tumour tissues to a possible extent, after informed consent from the patients, should be collected and stored, including blood, fine-needle biopsies as well as tissues from metastases. The latter would improve targeted treatment strategies for metastatic disease.

Tissue microarray (TMA) construction

Analyses of biomarkers in large materials have increased the need for high throughput techniques. The use of TMAs allows studies of up to 1000 samples in a single experiment, on one microscope slide [241, 242]. Briefly, for TMA preparation core needle biopsies from paraffin embedded tissues are reembedded in new paraffin blocks and the blocks are cut in sections (in our case of 4 µm) and mounted on frost-coated slides.

In the present studies, the protein expressions of S6K1, S6K2, S6K1/2 phosphorylated at T389 (pS6K), 4EBP1 and 4EBP1 phosphorylated at Serine 65 (p4EBP1) were evaluated in the Stockholm 3 cohort by IHC staining of TMAs, which is further described below.

Extraction of genomic DNA and total RNA from fresh-frozen tissue

Careful extraction of DNA and RNA is essential to receive maximum yield, purity and integrity of biomolecules for downstream molecular biological analyses [243]. In general, the main steps include disruption of cells or tissues, inactivation of nucleases, denaturation of nucleoprotein complexes and extraction of nucleotides through several steps of filtering and precipitation. At present, several kits are commercially available for quick nucleotide extraction, requiring a minimum of harmful chemicals. DNA samples are in general stable, and can be stored at room-temperature during use, whereas RNA samples, due to composition of the molecules, are highly sensitive for degradation and, where possible, should be stored in -70°C. In *Study 1-4*, DNA and total RNA were extracted from fresh-frozen tumour tissue estimated to contain >50% cancer cells, as well as from cell lines where mentioned.

For DNA, the Puregene® DNA purification kit was used. Briefly, the tissue was digested in proteinase-K solution, and the cell lysate was treated with RNase. Proteins were precipitated using protein precipitation solution included in the kit and pelleted by centrifugation. The supernatant was collected and DNA was precipitated with isopropanol and pelleted by centrifugation. Finally, the DNA was washed with 70% ethanol, air-dried and dissolved in sterile water. DNA concentration was

calculated by the Lambert-Beer law, $Abs = \varepsilon \cdot l \cdot c$ [244, 245], where the absorbance at 260nm was measured using a NanoDrop ND-1000 Spectrophotometer. For subsequent analyses, the DNA was long term stored in -70°C .

For RNA preparations, fresh-frozen tumour tissue was homogenised with a microdismembrator or a TissueLyser and total RNA was isolated with the mirVana™ miRNA isolation kit according to instructions provided by the manufacturer. Purified RNA was dissolved in nuclease-free water with addition of RNAsin ribonuclease inhibitor and stored at -70°C . RNA integrity numbers (RIN) [246] and concentrations were assessed with an Agilent 2100 Bioanalyzer. Only samples with RIN values ≥ 5 were included in the analysis.

Quantitative Real-Time PCR

One of the most important contributions to present days molecular biology was the invention of the PCR by Kary Mullis in the 1980s [247]. PCR is a highly sensitive method for efficient amplification of small amounts of specific nucleic acid sequences. Quantitative Real-Time PCR uses the PCR technique for quantification of gDNA or complementary DNA (cDNA). It involves a fluorogenic probe to enable detection of the specific PCR-product in real time, as it accumulates during PCR cycles. The probe is labelled with a reporter dye on the 5' end and a quencher dye on the 3' end (**Figure 15 a**). As with usual PCR, the reaction mixture is transferred between three temperatures during each PCR-cycle. At the first temperature $\approx 95^{\circ}\text{C}$, double-stranded nucleic acids denature and become single stranded. At the annealing temperature $\approx 55^{\circ}\text{C}$, primers and probes attach to the templates. When bound to the target sequence, the probe reporter dye is quenched. DNA-synthesis is initiated when the temperature is set just below the optimum for Taq polymerase, usually $\approx 74^{\circ}\text{C}$ (**Figure 15 b**). During the extension phase of the PCR, the probe will be degraded by the DNA polymerase 5' nuclease activity. This results in separation of the reporter dye from the quencher dye and a subsequent increase in fluorescence (**Figure 15 c**). During the PCR cycles, the fluorescence is constantly measured, and the increase is proportional to the quantity of the nucleic acid product in each sample. Thus, the higher the original quantity of nucleic acid target in the sample, the earlier (in PCR-cycles), the reporter signal reaches a detectable level (threshold cycle, Ct-value). The fluorescence signal is usually displayed as an amplification plot (**Figure 15 d**).

For the present studies, reverse transcription of RNA was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems) with 200 ng total RNA in reactions of 20 μl according to manufacturer's instructions. DNA or RNA quantity of each sample was estimated by using the standard curve method (*Study 1*), or the $\Delta\Delta\text{Ct}$ method (*Study 2-4*). To adjust for differences in total nucleic acid input, the Ct-values were normalised against the copy number or expression levels of endogenous control genes in each sample. To enable comparison between different runs, a calibrator sample from the same cell line was added on each PCR plate.

Figure 15 Principles of Real-Time PCR. Primers and probe anneal to the target sequence (a). DNA polymerase extends the primers (b). The polymerase exonuclease activity cleaves the probe and separates the reporter from the quencher, allowing the reporter fluorescence signal to increase (c). Example of a Real-Time PCR amplification plot: x-axis= PCR cycle, y-axis: fluorescence intensity (logarithmic) (d) (R=reporter dye, Q=quencher dye).

Immunostaining

Since the first description by Albert Coons in 1941 of an antibody-based method for specific detection of a protein in tissues or cells [248], the immunostaining methods have developed extensively and are now routinely used for clinical purposes as well as in research. In this project, immunohistochemistry was used to evaluate the expression of S6K2 (*Study 1*), 4EBP1 and p4EBP1 (*Study 3*), as well as S6K1 and pS6K [249] in the Stockholm 3 breast cancer material. In addition, western blot was used for validation of antibody specificity in both studies. The following sections shortly describe these methods.

Western blot

In 1975, Edwin Southern invented a method for detection of specific DNA sequences in samples, subsequently named Southern blot [250]. This was soon followed by similar methods for RNA and protein detection, acronyms named Northern and Western blot [251], respectively.

The western blot method includes separation of molecules according to size using gel electrophoresis, followed by transfer of molecules to a nitrocellulose membrane allowing detection with the use of protein specific antibodies. This method is commonly used for studies of *in vitro* alterations in protein expression or phosphorylation levels during different circumstances. Size separation allows evaluation of antibody specificity towards an epitope or protein with known size, however the sensitivity is rather low and large differences in expression levels are needed to qualitatively distinguish between protein amounts. Samples are denatured before electrophoresis, thus native epitopes may not always be detected, sometimes obstacle a comparison with immunohistochemistry. However, when possible, western blot is a method recommended for evaluating antibody specificity, which is used in *Study 1* and *Study 3*. Western blot running procedure was described in the respective publications.

Immunohistochemistry (IHC)

Due to its simplicity, including low time consuming and availability in using FFPE tissues, IHC [252] is routinely used by pathologists for clinical evaluation of protein expression in tissue, e.g. assessment of ER, PgR, HER2 and Ki-67 status. A large advantage is also the possibility to distinguish expression levels between different cell types in the tissues, as well as intracellular localisation of the protein. Though, drawbacks under discussion include problems with reproducibility due to different handling of tissues and sometimes lack of antibody specificity, as well as the subjective judgement and absence of possibility to absolute quantification.

In general, the IHC procedure includes tissue fixation, followed by antigen retrieval, blocking of endogenous peroxidases, and blocking of unspecific binding before adding a primary antibody. Thereafter, a secondary antibody commonly conjugated to horseradish peroxidase (HRP) is used for colour development and detection. Finally counterstaining and glass slide mounting is performed. Evaluation of staining should be done by at least two independent observers to increase objectiveness.

In the present work, the protein expressions of S6K2 (*Study 1*), 4EBP1 and p4EBP1 (*Study 3*), as well as S6K1 and pS6K [249] were evaluated in the Stockholm 3 cohort by IHC stainings of TMAs, as further described in the respective publication.

Microarray

In the last two decades a genomic revolution has taken place. Scientists have been able to complete the sequences of a variety of organisms and this culminated in 2001 with the release of the human genome [253]. This has led to the beginning of a new genomic era, where this enormous amount of sequence information can be utilised in a wide variety of research fields to increase the understanding of biological mechanisms. In the light of this, the microarray technology has evolved, and appeared to be a valuable tool.

The microarray technology was invented in the late 1980s by a team of scientists led by Stephen P.A. Fodor [254]. The principle was taken over by the Affymetrix Company in 1992, and in less than a decade commercialised to what today is called the GeneChip® platform [255]. The use of microarrays has expanded a lot during the last decade, and to date the major approaches are genome wide analysis of gene expression, single nucleotide polymorphism (SNP) genotyping, resequencing (analysis of sequence variation) and DNA methylation profiling. In addition to Affymetrix there are today several companies offering different microarray technologies.

In *Study 2*, Affymetrix SNP Gene Copy number arrays were used to analyse whole genome amplification profiles of 11q13 amplified breast tumours. Furthermore, in *Study 4*, Affymetrix expression arrays were used for evaluation of global mRNA expression alterations after *in vitro* silencing of S6K1 and S6K2.

DNA and mRNA arrays for tumour genome screening

Expression profiling has recently been shown a clinically useful tool for global molecular evaluation of breast cancers [16]. However, a disadvantage with this strategy is that RNA molecules in general are very unstable, making extractions and standardisations difficult. Furthermore, mRNA expression could be highly variable between tissues and within cells during different times, and finding a standard definition of overexpression is therefore challenging. DNA is more stable than mRNA and since specific copy number aberrations seem to be key drivers of tumourigenesis, direct analysis of copy number alterations on a genome wide scale may have a potential to be an alternative or complementary approach for subtype classification, diagnosis and prognosis of breast tumours [256]. An obstructing factor worth mentioning however, is the recently discovered phenomenon of copy-number polymorphism; that is low-level differences in copy numbers of certain chromosomal fragments between normal individuals [257, 258]. To date, the extent of this genetic variation remains unknown, but it is a factor worth taken into consideration when studying copy number alterations in tumours.

GeneChip® array technology

Microarrays are miniature chips covered by multiple oligonucleotides representing a number of selected SNPs or transcripts. The principles of Affymetrix GeneChip® arrays are reviewed in [255]. For DNA sequence analysis, the most recent generation of GeneChip® microarrays are the SNP arrays. This array was initially designed for genotyping and genetic association studies, but was soon shown to be useful for estimation of DNA copy number variations [259]. For global gene expression analysis, several types of arrays are available, depending on the suitable coverage of transcripts, splicing variants etc.

The arrays are usually composed of a 1.28*1.28 cm solid quartz wafer, where 25-mer DNA oligonucleotides are successively synthesised by photolithography. Each specific oligonucleotide exists in more than one million copies on a specific location on the array, defined as one *feature* (**Figure 16**). In turn, each array includes millions of features. On the 250K DNA arrays used in *Study 2*, each specific feature exists in a probe pair comprised of a so called perfect match (PM) feature that is complete complementary to its target sequence, and a mismatch (MM) feature, containing a single base substitution in the centre. The MM probes are used as internal controls of non-specific binding and by subtracting the signal intensity of the MM probe from the corresponding PM probe, background signal variations can be reduced. On a SNP array, any given SNP is represented by a set of different features, defined as a probeset. A probeset includes features for both the sense and the antisense strand. Additionally, there are probes with a slight variation in the location of the SNP within the nucleotide sequence. For CN arrays, there are usually five probe pairs (2*5 probes) for each strand of each allele, giving a total number of $2*5*2*2=40$ features representing each SNP.

Microarray running procedures and data analyses are described in the respective publication.

Figure 16 Dissection of a GeneChip array. Each array (a) contains millions of features (b). A feature is a location on the array containing more than one million copies of a specific nucleotide (c, d). Each specific sequence is represented on the chip by several features spread on the array (photo: Tomas Tollwé).

Bioinformatics databases and analysis tools

The modern biology with rapidly evolving techniques allowing whole-genome studies, has resulted in a need for large libraries storing and integrating the enormous volumes of produced data, as well as available tools for different types of data analysis. The bioinformatics field is continuously and rapidly expanding. In the following section, an overview of the largest databases will be presented, and a description of tools used in our present analyses.

Genomic and protein databases

There are today three large main facilities in the US, Europe and Japan for storage and integration of genome sequence information, represented by the National Center for Biotechnology (NCBI) at the National Library of Medicine (NLM), the European Bioinformatics Institute (EMBL-EBI) and DNA database of Japan (DDBJ) [260]. These databases allow all published genomic data to be freely available and they work tightly in cooperation for sharing information between each other. These databases are also linked with corresponding mRNA and protein databases, where NCBI/protein and Uniprot/Swissprot are the largest. For 3D structures of large biological molecules, including proteins and nucleic acids, the Protein Data Bank (PDB) is the only worldwide database of information and coordinates. For development and maintenance of a standardised terminology, the Gene Ontology (GO) bioinformatics initiative was started in 1998 [261]. The GO annotations also classify proteins into its molecular functions, involvement in biological processes and cellular compartments, further promoting data analysis and interpretation. The public web server g:Profiler [262, 263] was used to predict pathways and cellular functions enriched in the array data in *Study 4*.

Tools for browsing genomic data, sequence alignments and PCR primer design

Bioinformatics tools for browsing genomic data, as well as sequence alignments of DNA and protein are utilised in many biological fields and several genomic browsers for easy viewing of genomic data have been developed. In *Study 2*, the NCBI tool MapViewer was applied for whole-genome mapping of known genes within regions of gains and losses based on SNP array data, whereby positions of genes were received from the Human genome build 36, ver3. For sequence alignments, three main algorithms are at present used for this purpose; basic local alignment search tool (BLAST), Fast All (FASTA) and ClustalW [260]. In the present project, the BLAST tool was used in *Study 1*, whereas both BLAST and ClustalW were used in *Study 4*. Primers and probes for S6K1 and S6K2 gene amplification analysis were designed (*Study 1*) using the software Primer Express version 1.5a (Applied Biosystems) and specificity was controlled by comparison to sequences in the Genebank using BLAST. Protein sequence alignment of S6K1 and S6K2 (*Study 4*) was performed in order to pinpoint similarities and differences between the primary structures of the proteins.

Homology modelling of protein 3D structures

For a protein that is homologous to >50% to another protein with known 3D structure, it is possible to predict its structure with a high degree of confidence using *in silico* homology modelling [264]. Basically, the method is built on the assumption that native proteins form the most stable conformation in a state where the free-energy is minimised [265, 266]. In addition, tertiary structures are often more conserved than primary structures, thus similarities at the sequence level are often indicative of higher homologous three-dimensional structures. As previously mentioned, in the S6K1 and S6K2 kinase domains approximately 80 % of the residues are conserved. In *Study 4*, the three-dimensional structure of the S6K2 kinase domain was modelled according to standard procedures with the two different S6K1 crystal structures, (PDB ID: 3A62 and 4L3J) as templates, using ICM (Molsoft LLC, San Diego, CA, USA) and Prime (Prime version 3.1, Schrödinger LLC, New York, NY, UA, 2012), respectively. Briefly, the sequences of the templates and S6K2 were initially aligned using Prime and the alignment was then refined using ClustalW. Then a secondary structure prediction was performed and subsequently the three-dimensional model was built using an energy-based algorithm. The last step was refinement of loops that lacked electron density in the template (one in this case). The ligands that were co-crystallised with S6K1 were removed prior to homology modelling. The proteins were visualised using PyMol (Schrödinger LLC) and the pairwise root-mean-square-deviation (RMSD) between the template (S6K1) and model (S6K2) was calculated using the SuperPose webserver [267].

Public microarray datasets

The intervention of microarrays, allowing whole genome studies, resulted in a need for large libraries storing the enormous volumes of produced data. Subsequently, in 2000, NCBI at NLM established the Gene Expression Omnibus (GEO) database [268]. This was followed by the ArrayExpress database, founded by the EMBL-EBI [269, 270]. The use of microarrays soon became routine in medical research, and to standardise the data for publication, the Minimum Information About a Microarray Experiment (MIAME) guidelines were proposed [270]. These guidelines were in 2002 accepted by the Nature journals, proposing that all microarray data should be deposited in either the GEO or ArrayExpress databases where they should be publically available. This allows critical evaluation of published microarray data, as well as free accession for use in other studies. To date, GEO and Array Express are the largest microarray data archives, containing more than 40000 experiments each.

In *Study 3 and 4*, public available datasets encompassing preprocessed mRNA expression data were used for three cohorts, further referred to as van de Vijver cohort (n=295) (available at: <http://bioinformatics.nki.nl/data.php>), Uppsala cohort (n=236) (NCBI/GEO: GSE3494) and Karolinska Institute cohort (n=159) (NCBI/GEO: GSE1456) [271-273]. The van de Vijver cohort comprises 295 consecutive patients, primary stage I or stage II breast cancers from the fresh-frozen-tissue bank of the Netherlands Cancer Institute, and were initially used for evaluation of the 70-gene profile for predicting prognosis [273]. The Uppsala cohort included 236 consecutive patients mainly from the Uppsala County, Sweden, 1987-1989 [271]. The original study aimed to investigate the transcriptional fingerprint of p53 mutated tumours. The 159 samples available in the Karolinska cohort, derived from a larger cohort including all breast cancers operated at the Karolinska Hospital

1994-1996, where the aim was to identify gene expression profiles of poor prognosis for the addition of adjuvant therapies [272].

Statistics

For estimation of relationships between different variables in two groups, Fishers exact test [274], or Chi2 tests were used (*Study 1*). For ranked variables, Spearman's Rank order correlation [275] was applied (*Study 1-4*). Multiple regression analysis was utilised in cases where one dependent variable was correlated to multiple independent variables (*Study 3*). The Kaplan-Meier product limit method [276] was used to estimate the cumulative probabilities of local recurrence-free survival (LRFS), distant recurrence-free survival (DRFS) or breast cancer survival (BCS) (*Study 1-4*). Differences between the curves were evaluated with the Log-rank (Mantel-Cox) test [277] (*Study 1-4*) or Gehan's test for multiple groups (*Study 3*). For univariate and multivariate analysis of event rates, as well as interaction analysis, Cox proportional hazard regression was used [278] (*Study 1-4*).

To give a comprehensive view of the 11q13 amplification patterns across all samples and identify regions of significant aberrations, the principles of a statistical approach referred to as Genomic Identification of Significant Targets in Cancer (GISTIC) was applied [279] (*Study 2*). GISTIC methodology is based on the assumption that both the frequencies of occurrence, and the amplitude of the aberrations, are corner stones in the development of functionally important genomic changes. In this case, the mean value of the smoothed log2ratio of each SNP was calculated across all samples and plotted against genomic location. Resulting peaks of maximal amplitude are indicative of regions harbouring driving oncogenes.

Student's t-test was utilised when comparing differences in gene expression levels in public datasets between different patient groups (*Study 4*). To evaluate differences in gene expression after *in vitro* siRNA knock-down of S6K1 and/or S6K2 (*Study 4*), cell intensity data was processed by the software Genespring (Agilent). Raw data was normalised to the median of the control samples using the Robust Multi-array Average (RMA) summarisation algorithm [280]. Paired t-test was used to estimate significant differences in gene expression after silencing of S6K1 and/or S6K2 in comparison to scrambled siRNA.

RESULTS AND DISCUSSION

The prognosis of patients diagnosed with breast cancer has been considerably improved in the latest 25 years, as a result of continuous development of diagnostics and treatment regimens. However, tumour diseases, for woman mainly lung cancer and breast cancer, still constitute the second most common causes of death in developed countries, following heart diseases. A future utopia is to develop more individualised therapy strategies, to further increase breast cancer survival, but also to decrease the risk of severe side-effects of unnecessary treatments. As previously mentioned, humans carrying mutations leading to reduced IGF1/mTOR signalling have been shown resistant to development of malignant tumours as well as diabetes, implicating this pathway as one major factor at the cross-road of these diseases [94]. A new treatment regimen constituting a combination of mTOR inhibitor with endocrine therapy was recently clinically approved for advanced breast cancers [7]. Although significant benefit for this combination treatment is evident, counteracting feedback mechanisms are assumed to diminish the effects of the present day's rapalogues. An increased knowledge of the intracellular pathways involved in the mTOR signalling circuit, and its role in different tissues and during different conditions and states, would be potentially helpful in the future prevention and treatment of cancer as well as diabetes. In the present project, we have evaluated downstream effectors of mTOR signalling, mainly S6K1, S6K2 and 4EBP1, with the purpose of finding new potential disease-specific clinical markers and treatment targets for breast cancer.

The mTOR effector S6K2 is a new candidate oncogene in the 11q13 amplicon in breast cancer

When the present project was initiated, previous investigations focusing on S6K1 had showed its corresponding gene *RPS6KB1*, located at the chromosomal region 17q in the neighbourhood of *ERBB2*, to be amplified in breast cancer [61, 62, 281]. *RPS6KB1* amplification and high protein expression had also been associated with an adverse prognosis [61-63, 281], indicating the mTOR target as a new candidate driver oncogene in the 17q amplicon. Regarding the homologous protein S6K2, few studies had been conducted, but two reports indicated that the protein levels of S6K2 may be increased in breast tumours [181, 282]. The location of the corresponding gene *RPS6KB2* in the 11q13 area, harbouring several known oncogenes amplified in breast cancer, prompted us to investigate the possibility of this gene to be amplified in breast cancer (*Study 1*). In this project involving 207 stage II breast cancers, *RPS6KB2* gain was found in 21.3% (44/207) of the tumours, whereof amplification defined as at least four gene copies was detected in 4.3% (9/207) of the tumours. Univariate and multivariate analyses indicated a connection between *RPS6KB2* amplification and poor outcome in the patient group, independently of other clinicopathological factors and amplification of the neighbouring oncogene *CCND1* (**Figure 17**). In a small cohort of 23 patients, the correlation between *RPS6KB2* gene copy number and mRNA expression levels was also confirmed. Consequently, *RPS6KB2* may be considered a new candidate oncogene in the 11q13 amplicon in breast cancer. In a recent publication, similar results have been shown in gastric cancer, where *RPS6KB2* amplification was detected and associated with a poor prognosis [283].

Previous results regarding *RPS6KB1* in breast cancer were also confirmed in our study, showing *RPS6KB1* gain in 21.4% (44/206) of the tumours, including amplification in 10.7% (22/206), where the former was also predictive of a poor prognosis. Notably, *RPS6KB1* and *RPS6KB2* gene amplification were mutually exclusive events, suggesting compensatory tumourigenic roles. The combination variable *RPS6KB1* or *RPS6KB2* amplification was inversely correlated to the presence of mutations in the *PIK3CA* (p110 α) gene, E545K, E542K (exon 9) or H1047R (exon 20).

Figure 17 Distant recurrence-free survival among all patients in the Stockholm 2 cohort, in relation to RPS6KB1 amplification (a), RPS6KB1 gain (b), RPS6KB2 amplification (c), RPS6KB2 gain (d), the combination variables RPS6KB1 or RPS6KB2 amplification (e) and RPS6KB1 and/or RPS6KB2 gain (f). (amplification ≥ 4 gene copies, gain ≥ 3 gene copies)

RTK/PI3K signalling is tightly regulated by several feedback mechanisms among others acting on IRS1 [284]. Downstream AKT activation induces a positive loop, enhancing IRS signalling through phosphorylations at certain Serine residues. On the contrary, activated mTOR and S6K1, as well as IKK and JNK diminishes further IRS signalling through phosphorylations at other sites, by reducing RTK and PI3K binding and increasing IRS1 degradation. A new study has investigated the consequences of mutations at different sites of *PIK3CA*, showing that the E545K mutation in exon 9 leads to downstream signalling through IRS1 independent of upstream receptor activation [285]. When the *RPS6KB1* and *RPS6KB2* gene amplifications were correlated to the E545K and H1047R mutations, separately, *RPS6KB1* was inversely correlated to E545K only (**Table 5**). This was not evident for *RPS6KB2*. S6K1, but not S6K2, has been shown involved in the negative feedback described above, inhibiting AKT-signalling by phosphorylating IRS1 [222], supporting this finding and indicating separate cellular functions for S6K1 and S6K2.

Possibly partly due to its chromosomal locations, *RPS6KB1* gene amplification was associated with HER2-amplification and protein expression, whereas *RPS6KB2* was rather connected to ER-positive breast cancer. PI3K/AKT/mTOR signalling has been shown to interfere with ER pathways, affecting the response to ER-targeted therapies in breast cancer [286]. Consequently, the predictive value of *RPS6KB1* and *RPS6KB2* gene amplification for the outcome of tamoxifen treatment was investigated in the cohort. Benefit from tamoxifen was seen in the group of patients with tumours harbouring *RPS6KB2* gene gain, whereas no significant response was seen in the *RPS6KB2* negative group (**Figure 18**). For *RPS6KB1* amplification or gain, no connection to benefit from endocrine treatment was evident (**Figure 18**), further supporting a clinical relevant difference between S6K1 and S6K2.

Table 5 Correlations between S6K1 and S6K2 amplification, and mutations in *PIK3CA* exon 9 or exon 20.

<i>PIK3CA</i> mutation	<i>RPS6KB1</i> gain n (%)			<i>RPS6KB2</i> gain n (%)		
	0	1		0	1	
Exon 9						
0	137 (86.2)	44 (100.0)		142 (88.2)	40 (93.0)	
1	22 (13.8)	0 (0)	p=0.0090	19 (11.8)	3 (7.0)	P=0.36
Exon 20						
0	137 (86.2)	38 (86.4)		139 (86.3)	37 (86.1)	
1	22 (13.8)	6 (13.6)	p=0.97	22 (13.7)	6 (14.0)	P=0.96

Figure 18 Distant recurrence-free survival among patients treated (Tam) or not treated (no Tam) with tamoxifen, in the groups of patients with ER-positive tumours harbouring normal RPS6KB1 gene copy number (a), RPS6KB1 gain (b), normal RPS6KB2 gene copy number (c) or RPS6KB2 gain (d). (Tests for interaction: RPS6KB1gain vs. tam: $p=0.63$, RPS6KB2 vs. tam: $p=0.065$).

S6K2/4EBP1 coamplification and mRNA coexpression is associated with a poor outcome, indicating a synergy between mTOR targets

Gene amplification of the 11q13 chromosomal region has been shown clinically relevant in breast cancer, connected to ER positive status, poor prognosis and diverse response to endocrine treatment [58, 72-74, 76, 78, 87]. Numerous studies of 11q13-amplified tumours have revealed a complex and heterogeneous amplification pattern, likely as a result of several driving oncogenes. In a following project, we aimed to further investigate the clinical relevance of *RPS6KB2* amplification in the context of the 11q13 amplicon, as well as its relations to genome-wide copy number alterations (*Study 2*). Whole-genome SNP arrays were used in a screen of 29 breast tumours with 11q13 amplification, representing a cohort of approximately 200 patients. Assuming that the frequencies of occurrence as well as the amplitude of the aberrations are essential, the statistical method GISTIC [279] allowed estimation of important amplicon cores in the region. Resulting data showed three main amplicon cores in the 11q13 area of these tumours (**Figure 19 a**). Depending on methodology, selection of tumour samples or cell lines, several different cores in the 11q13 have been suggested [76, 85, 87, 287-290], but a recent review summing up previous high-resolution studies of the amplicon, confirms our present findings of these three cores, but also an additional one between core 2 and 3 [72]. The selection on tumours harbouring amplification of *RPS6KB2*, *CCND1* and/or *PAK1* may explain the absence of the fourth core in our study.

Previous connections between positive ER status and 11q13 amplification were suggested to be restricted to the core including among others *CCND1*, *FGF3*, *FGF4* and *FGF19* in *Study 2*, using multivariate analysis. The indicated role of cyclin D1 as a cofactor of the ER [291], and *CCND1* as an ER-targeted gene [292] support these findings. This also confirmed the previous observation of 11q-ter loss, including among others the PgR gene along with 11q13 amplification, likely resulting from the breakage-fusion-bridge cycles suggested to form the amplicon [85].

Intriguingly, amplifications at 11q13 are commonly cooccurring with amplifications at 8p12, and the 11q13/8p12 coamplification has been associated with a worse outcome than either amplicon alone, suggesting a tumourigenic synergy [59, 77]. In line with this, 8p12 gain or amplifications were detected in 55.2% (16/29) of the tumours in the present study (**Figure 19 b**). Interestingly, when restricting the GISTIC analysis of the 11q13 amplicon to tumours with or without 8p12 gain/amplification, the most proximal amplicon, containing among other genes *RPS6KB2*, mainly appeared in the 8p12 positive tumours (**Figure 20**). Multivariate analysis confirmed the 11q13/8p12 coamplification to mainly include the proximal 11q13 core.

Figure 19 Comprehensive view of the 11q13 amplicon (a) and the 8p12 amplicon (b) in 29 breast cancers and identification of the most significant amplifications, using the principles of GISTIC methodology.

Figure 20 GISTIC view of the 11q13 amplicon in the 29 tumours divided into two groups based on 8p12 status: 8p12+ ≥ 3 DNA copies (a), 8p12- < 3 DNA copies (b).

Previous suggested drivers of the 8p12 amplicon formation are mainly the *FGFR1* and *PPAPDC1B* genes [184, 293]. Due to the location of the mTOR target *RPS6KB2* in the 11q13 core coamplified with 8p12, one may also deem the *EIF4EBP1* gene at 8p12, encoding the mTOR effector 4EBP1, as interesting. Consequently, 4EBP1 mRNA expression was evaluated along with *FGFR1* and *PPAPDC1B* in the cohort of 11q13 amplified tumours. Correlations between gene copy number and mRNA levels were confirmed for these three genes, as also reported previously [184, 185, 187]. In addition, these were also significantly correlated to S6K2 mRNA. Interestingly, *FGFR1* mRNA was also correlated to *FGF3*, *FGF4* and *FGF19* mRNA. 8p12 gene amplification, as well as high *FGFR1* or 4EBP1 mRNA expression, but not *PPAPDC1B* was predictive of a poor prognosis in the cohort of patients with 11q13 positive tumours (**Figure 21**). As previously discussed [72], this confirmed several genes in the 11q13/8p12 amplicons as potential drivers, but also adds the mTOR targets S6K2 and 4EBP1 to this list.

Figure 21 Distant recurrence-free survival among patients with 11q13 positive tumours in relation to; 8p12 gain/amplification (8p12+: ≥ 3 DNA copies, 8p12-: < 3 DNA copies) (a), high mRNA expression of *PPAPDC1B* (b), *FGFR1* (c) and *4EBP1* (d). The limit for high mRNA levels was defined by the median for each gene.

To further evaluate the clinical implications of the S6K2/4EBP1 coexpression, as well as possible divergence between S6K1 and S6K2 in this sense, the mRNA expression levels of S6K1, S6K2 and 4EBP1 were assessed in a larger cohort from the Stockholm 2 material, consisting of 93 available patient samples (*Study 3*). Here, the strong correlation between S6K2 and 4EBP1 mRNA levels was confirmed, whereas this could not be seen for S6K1 and 4EBP1 (**Figure 22**).

High mRNA levels of S6K2 and/or 4EBP1 were also associated with an adverse outcome in the patient group (**Figure 23**). As a validation, the same analyses were performed in three publicly available datasets, where the correlations as well as the prognostic value of S6K2/4EBP1 could be confirmed (**Figure 22** and **Figure 23**). For S6K1 mRNA, no prognostic value was evident.

Figure 22 Spearman's rank order correlation evaluating associations between S6K1, S6K2 and 4EBP1 mRNA expression (continuous values) in four breast cancer cohorts (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Consequently, the results of these studies suggest an essential distinction between S6K1 and S6K2 as clinical markers, which may result from partly functional differences between these kinases. The role of 4EBP1 in breast cancer may seem contradictory. Phosphorylation of 4EBP1 by mTORC1 promotes dissociation of 4EBP1 from eIF4E, enabling eIF4E to induce protein translation, consequently, 4EBP1 has been considered as a tumour suppressor. However, phosphorylated 4EBP1 has been generally accepted as a marker of activated mTOR signalling and high levels in tumours have been associated with a worse outcome in several malignancies [189, 190, 294, 295]. Results from our studies regarding *EIF4EBP1* gene amplification and high mRNA levels as associated with a poor prognosis, indicate a dual role for 4EBP1. Supporting this finding, 4EBP1 has been shown able to bind and stabilise mTORC1, promoting activation of the signalling pathway [192].

Figure 23 Breast cancer survival (BCS) in relation to high S6K2 and/or 4EBP1 mRNA in four cohorts; Stockholm 2 (a); van de Vijver (b); Uppsala (c); and Karolinska (d).

Whole-genome mRNA profiles of S6K2 and 4EBP1 positive tumours are highly overlapping, revealing associations with cell cycle regulators and IGF signalling

To further evaluate the implicated differences between S6K1 and S6K2 at the transcriptional level, as well as the association between S6K2 and 4EBP1, whole genome-transcriptional profiles of breast tumours expressing high levels of these genes, respectively were investigated (*Study 4*). The whole genome-profiles of S6K2 and 4EBP1 high tumours showed significant overlaps, whereas few similarities were seen between these profiles and the profiles for S6K1 high tumours. Correlations, positive or negative, of expression levels for certain genes may have several reasons [296, 297]. A causal relationship may be present where one of the factors regulates the expression levels of the other. Coamplification, perhaps as a result of chromosomal colocalisation may be present as well as coregulation by the same transcription factors. In malignancies, coexpression of genes involved in the same biological processes may be a result of an evolutionary tumour promoting advantage, whereas an inverse relationship could result from mutually exclusive roles of the factors. Overlapping expression profiles of S6K2 and 4EBP1, included several factors involved in cell cycle promotion and proliferation, among these CCNB1, CCNB2, CENPA, CDC20 and CDC25B. Notably, the transcription factor E2F1 and several of its targets were strongly associated with S6K2/4EBP1, but not with S6K1, and this could be confirmed in two additional data sets (**Figure 24**).

Figure 24 Spearman's rank order correlation evaluating associations between S6K1, S6K2, 4EBP1 and E2F1 mRNA expression (continuous values) in three breast cancer cohorts (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Loss of function of the tumour suppressor pRb, leads to aberrant activity of E2F1, promoting cellular proliferation. The pRb was the first identified tumour suppressor and its function is lost in many cancers, including 20-35% of breast cancers [298]. pRb associates directly with the transcription factor E2F1, regulating its activity, through pRb phosphorylation by cyclin-dependent kinases (CDKs), mainly associated to cyclin D and cyclin E (**Figure 5**). However, the role of E2F1 in cell cycle regulation and tumourigenesis has long been under debate [299-301] and E2F1 has also been shown a trigger of apoptosis in response to DNA damage. High E2F1 mRNA levels have been associated with a poor outcome in breast cancer [302]. E2F1 activity is regulated by upstream growth factor signalling pathways, and Ras/MAPK as well as PI3K/AKT pathways have been shown to inhibit E2F1 proapoptotic gene transcription [301]. In addition, AKT has been shown a transcriptional target of E2F1, suggesting a positive feedback inhibiting apoptosis. A recent study has linked E2F1 to mTOR signalling, showing that E2F1 can activate mTORC1 and downstream signalling probably by recruiting mTORC1 to endosomes, independent of AKT and insulin signalling [303].

One of the main genes inversely correlated to both S6K2 and 4EBP1 was IGF1. The inverse relationship with several factors in the IGF signalling pathway could be confirmed in three datasets (**Table 6**). The IGF/IGFR axis is a main activator of AKT/mTOR signalling [304], indicating that the inverse correlation may reflect a compensatory relationship and mutually exclusive IGF1 or S6K2/4EBP1 levels. IGF1 and IGF2 can signal in an endocrine, paracrine or autocrine manner [304]. IGF1 and IGF2 bind and activate IGF1R, leading to homodimerisation as well as heterodimerisation with the INSR, promoting IRS1 recruiting and downstream PI3K/AKT/mTOR activation. IGF2R, in turn, is considered an inhibitor of IGF signalling by binding IGF2, promoting its degradation. As mentioned in the introduction, absence of systemic IGF signalling seems to protect from cancer as well as diabetes, highlighting this pathway as an interesting therapeutic target. However, side-effects on the normal physiology have so far impeded the use of IGFR inhibitors in the clinic.

The cellular metabolic control performed by the IGF system in normal cells, is directly linked to mTOR activity. Upregulation of intracellular pathways may render the tumour cells insensitive to systemic proliferative and metabolic control through IGF, supporting the inverse correlation between S6K2/4EBP1 and IGF levels. However, in the present study, the earlier implicated prognostic value of S6K2/4EBP1 was shown restricted to tumours expressing high levels of IGFRs or HER2, indicating an important role in growth factor driven tumours (**Figure 25**). The prognostic value was also confirmed in a multivariate analysis, including several clinicopathological factors as well as E2F1.

Table 6 Spearman's rank order correlation evaluating associations between S6K1, S6K2, 4EBP1 and mRNA expression of factors in the IGFR signalling pathway (continuous values) in three breast cancer cohorts (R-values for $p < 0.01$ highlighted, positive correlations in grey).

		IRS1	IRS2	IRS4	IGF1	IGF2	IGF1R	IGF2R	INSR
S6K1	<i>van de Vijver</i>	-0.09	-0.05	0.09	0.02	-0.23	0.02	-0.08	0.03
	<i>Karolinska</i>	0.12	-0.11	-0.21	-0.13	-0.18	0.05	0.08	0.01
	<i>Uppsala</i>	-0.05	-0.10	-0.10	-0.13	-0.14	0.03	0.25	-0.04
S6K2	<i>van de Vijver</i>	-0.20	0.22	0.10	-0.43	-0.28	-0.06	0.02	0.14
	<i>Karolinska</i>	-0.12	-0.16	0.13	-0.30	-0.29	-0.23	0.18	-0.04
	<i>Uppsala</i>	-0.31	-0.19	0.03	-0.27	-0.24	-0.22	-0.06	-0.20
4EBP1	<i>van de Vijver</i>	-0.32	0.08	0.14	-0.36	-0.27	-0.18	0.08	0.03
	<i>Karolinska</i>	-0.25	-0.36	0.00	-0.49	-0.46	-0.30	0.24	-0.11
	<i>Uppsala</i>	-0.19	-0.14	-0.03	-0.48	-0.43	-0.26	-0.06	-0.14

Figure 25 Breast cancer survival in relation to S6K2 and/or 4EBP1 mRNA, in the van de Vijver, Karolinska and Uppsala patient cohorts respectively; IGF1R and/or IGF2R and/or HER2 high (a, c, e); IGF1R and IGF2R and HER2 low (b, d, f).

For S6K1 mRNA, no prognostic value in the HER2/IGFR positive patient group was seen. IGF1R and IGF2R are thought to have opposite roles in IGFR signalling, but addition of IGF2R to the IGF1R and HER2 positive subgroup improved the prognostic value of S6K2/4EBP1, through reasons that need to be further evaluated. Likely, autocrine or paracrine growth factor signalling is still essential for the activation of intracellular signalling via S6K2/4EBP1. S6K1, in turn, may inhibit RTK signalling via the feedback regulation of IRS1.

Differences between S6K2 and 4EBP1 profiles could also be seen. One interesting finding was the strong inverse correlation between 4EBP1 and ESR1 mRNA, encoding the ER α (*Study 4*). When restricting the analysis to ER negative tumours, 4EBP1 was positively associated with mRNA expression of oestrogen-related receptor alpha (ESRRA). ESRRA was identified as a result of its close homology to the ER, and is preferably expressed in ER negative breast cancers [305, 306]. It is considered an orphan receptor, and its activity has been shown regulated by the PI3K/AKT signalling pathway. The 4EBP1 correlation to ESRRA could also be confirmed in two additional datasets, preferably in the ER negative subcohort. These results may suggest an alternative signalling through ESRRA, independent of the ER, in tumours expressing high levels of 4EBP1.

Analyses of mRNA levels in tumour tissue in general reflect the combined mRNA expression in several types of cells in addition to the tumour cells, including stromal and normal epithelial cells. The mRNA-levels are in turn a result of a complex gene regulation on multiple levels, intracellular as well as determined by a cross-talk between different cells. It has been shown that cancer stem cells may go through a metamorphosis, allowing a differentiation to several cell types [21], suggesting that all cells in a tumour may stem from the same progenitor. In the light of this, analysing the combined mRNA expression of tumours may be defensible.

Intracellular localisation and expression levels of S6K1, S6K2 and 4EBP1 protein determine associations with clinicopathological factors and predictive role for endocrine treatment benefit

Studies on gene copy number and mRNA levels of the mTOR targets S6K1, S6K2 and 4EBP1 have resulted in data pointing towards a clinical potential of these factors in breast cancer, and indicated partly divergent roles for the homologous S6 kinases in this context. The relationship between gene copy number/mRNA and corresponding protein expression levels and functions is debated. A recent review on this issue conclude that the abundance of mRNA in general highly reflects the ability to detect protein expression in cells [307]. Unfortunately, we have not been able to investigate the possible relation between gene/mRNA levels and its corresponding protein expression for S6K1, S6K2 and 4EBP1. However, access to material from the large randomised Stockholm 3 trial allowed us to explore the clinical potential of these factors on the protein level.

S6K1, S6K2 and 4EBP1 protein expression could be ubiquitously detected in the nucleus and cytoplasm of breast tumours (*Study 1, 3 and [249]*). Furthermore, S6K phosphorylated at the mTORC1 activation site T389 (pS6K) and 4EBP1 phosphorylated at S65 were evaluated in the material (*Study 3 and [249]*). Earlier implicated associations between S6K1, S6K2 and 4EBP1 and clinicopathological factors as well as prognosis seem to be dependent on the cellular localisation of the protein. In line

with the findings for *RPS6KB1* amplification, cytoplasmic S6K1 (S6K1cyt) was also associated with HER2 positivity. S6K1 in the nucleus (S6K1nucleus) could be weakly connected to both HER2 and ER positivity. On the contrary, S6K2, especially nuclear, was strongly correlated to ER and PgR expression. Nuclear pS6K was also weakly associated to ER. Cytoplasmic 4EBP1 and p4EBP1 was inversely connected to ER-positivity, as earlier shown for 4EBP1 mRNA, and positively associated to high grade. **Table 7** shows the correlations of S6K and 4EBP1 to breast cancer subtypes.

S6K1 in the cytoplasm was associated with high grade and a poor prognosis, whereas no prognostic value was evident for S6K2 or pS6K, regardless of cellular location (*Study 1* and [249]). High cytoplasmic levels of p4EBP1 have been shown to predict a poor prognosis in several cancer types [189, 190, 294, 295] and this could also be confirmed in the present study (**Figure 26**). Interestingly, 4EBP1 independent of phosphorylation status was also indicated to associate with prognosis, and the variable 4EBP1cytoplasm>nucleus, indicating higher cytoplasmic than nuclear levels of 4EBP1, was significantly predictive of an adverse outcome among systemically untreated breast cancer patients (**Figure 26**).

Table 7 Protein expression of S6K1, S6K2, pS6K, 4EBP1 and p4EBP1 in relation to Breast Cancer Subtypes, assessed by Spearman's Rank order correlation. Breast cancer subtypes in Stockholm 3 were defined according to St Gallen 2011 [9] as follows: Luminal A (ER+ and/or PgR+, Her2-, grade I-II), Luminal B1 (ER+ and/or PR+, HER2-, grade III), Luminal B2 (ER+ and/or PR+, HER2+), HER2-like (ER- and PR- and HER2+), and Basal-like (ER- and PR- and HER2-) (R-values for $p<0.01$ highlighted, positive correlations in grey).

	Breast cancer subtype				
	Luminal A	Luminal B1	Luminal B2	HER2-like	Triple negative
	n=457 (63.0%)	n=54 (7.4%)	n=37 (5.1%)	n=55 (7.6%)	n=123 (16.9%)
<i>S6K1cyt</i>	-0.09	0.09	0.16	0.04	-0.07
<i>S6K1nucleus</i>	0.01	0.02	0.17	0.00	-0.12
<i>S6K2cyt</i>	0.09	-0.04	0.02	-0.02	-0.08
<i>S6K2nucleus</i>	0.32	-0.16	-0.04	-0.08	-0.22
<i>pS6Kcyt</i>	-0.01	0.05	0.00	-0.01	-0.03
<i>pS6Knucleus</i>	0.07	0.00	0.06	-0.03	-0.13
<i>4EBP1cyt</i>	-0.23	0.17	0.06	0.10	0.04
<i>4EBP1nucleus</i>	-0.11	0.11	0.04	0.03	0.01
<i>p4EBP1cyt</i>	-0.28	0.15	0.08	0.06	0.13
<i>p4EBP1nucleus</i>	0.08	-0.05	-0.09	-0.04	0.01

Figure 26 Distant recurrence-free survival among systemically untreated patients in the Stockholm 3 cohort, in relation to p4EBP1 cytoplasmic protein (a), p4EBP1 nuclear protein (b), 4EBP1 cytoplasmic protein (c), 4EBP1 nuclear protein (d), 4EBP1cyt $\geq$ nucleus (e).

New clinical markers predicting the benefit from different treatments are essential to improve the possibilities to further individualised therapies. As mentioned above, *RPS6KB2* gene gain was shown able to predict benefit from tamoxifen treatment in the studied cohort. In line with this, high levels of nuclear S6K2 protein was also associated with tamoxifen benefit, although restricted to the ER/PgR positive subgroup (**Figure 27**). On the other hand, in the ER positive/PgR negative cohort, nuclear S6K2 with borderline significance predicted a poor benefit from tamoxifen. Also nuclear S6K1 was associated with less tamoxifen benefit, and this reached significance for the variable S6K1nucleus>cyt in the ER positive subgroup. A similar trend was seen for pS6K, but here the variable pS6Kcyt and/or nucleus was needed to reach significance in the analysis [249]. The associations of S6K1, S6K2 and pS6K with endocrine resistance, for S6K2 restricted to the ER positive/PgR negative subgroup, allow for the speculation that ER signalling in this subgroup may be driven in a hormone-independent manner, via cross-talk to intracellular signalling pathways including mTOR/S6K. *In vitro* studies have shown that S6K1 may phosphorylate ER at S167, resulting in ligand-independent activation of the receptor [165]. Accordingly, nuclear pS6K, S6K1 as well as S6K2 was correlated with pER_S167, whereas the latter was also associated with pER_S305 in the material (unpublished data).

The correlation between S6K2 and 4EBP1 could be confirmed at the protein level, for both the cytoplasmic and nuclear compartments. In addition S6K1 and 4EBP1 was associated, and pS6K correlated to S6K1 and S6K2 in both compartments. Cytoplasmic 4EBP1 was predictive of a poor benefit from tamoxifen in the ER positive/PgR positive subgroup (**Figure 27**) and a similar trend could be seen for p4EBP1.

Figure 27 Distant recurrence-free survival among patients in the Stockholm 3 cohort treated or not treated with tamoxifen, in the groups of patients with ER-positive tumours harbouring: low S6K2nucleus, PgR+ (a), high S6K2nucleus, PgR+ (b), low S6K2nucleus, PgR- (c), high S6K2nucleus, PgR- (d), low 4EBP1cyt, PgR+ (e), high 4EBP1cyt, PgR+. Tests for interaction: PgR+ S6K2nucleus vs. tam $p=0.034$, PgR- S6K2nucleus vs. tam: $p=0.13$, PgR+ 4EBP1cytoplasm: $p=0.034$.

4EBP1 is regulated by phosphorylation at multiple sites, and the roles for the different sites are not totally established. The 4EBP1 antibody was raised towards a sequence surrounding S112, thus at the very C-terminus of 4EBP1, and recognises both unphosphorylated as well as 4EBP1 phosphorylated at different sites. In addition, the 4EBP1 and p4EBP1_S65 stainings were highly correlated, especially for the cytoplasmic pools of the proteins, indicating that to some extent, the same proteins are detected. This may also reflect that an increase in total protein expression often is accompanied with an increased phosphorylation and activation of the proteins. Therefore, it is possible that 4EBP1 activation (by foremost mTOR) may be the reason behind its role in endocrine resistance. Interestingly, in a recent publication, both phosphorylated and total 4EBP1 was related to a poor outcome among patients with ER positive breast cancers, treated with tamoxifen [308], in keeping with our findings. In that study, protein expression was determined by reverse-phase protein arrays, ruling out the possibility to distinguish between cytoplasmic and nuclear expression.

In the present study, the endocrine treatment predictive value for 4EBP1, as well as S6K2, was especially evident in the ER/PgR expressing subgroup. In addition, the prognostic significance of 4EBP1 was most prominent in combination with PgR expression, suggesting a possible cross-talk to nuclear receptors. The role of progesterone signalling in breast cancer remains controversial. In general, circulating progesterone is considered as a risk factor for breast cancer development by promoting cellular proliferation. However, in primary breast cancer, PgR expression is associated with differentiated, less aggressive tumours and a favourable prognosis [31]. Upregulation of the IGF/PI3K/AKT/mTOR pathway is one suggested mechanism behind PgR downregulation in breast cancer, despite a functional ER. In agreement, our study showed an inverse association between S6K2/4EBP1 and PgR mRNA levels, in the three available cohorts. Furthermore, the gene encoding PgR is located at the proximal part of the 11q chromosomal arm, which is commonly deleted in 11q13/8p12 amplified tumours (*Study 2*). However, 4EBP1 was recently described as a possible target gene for PgR[309], suggesting the presence of a negative feedback loop downregulating PgR after growth factor pathway stimulation. The function of PgR can be regulated by receptor phosphorylation at multiple sites, through growth factor receptor signalling pathways, and a subpopulation of cytoplasmic PgR has also been shown able to activate kinase cascades, including PI3K/AKT [31]. It is tempting to speculate that a coordinated expression of PgR and cytoplasmic growth signalling factors including S6K2/4EBP1 may facilitate the proliferative and oncogenic role of PgR, promoting tumour progression and therapy resistance. In addition, PgR may in the long run be downregulated through PI3K/AKT/mTOR pathway stimulation and subsequent aberrant ER signalling, leading to acquired endocrine resistance among patients with initially ER/PgR positive breast cancers.

Altogether, the expression levels and possibly the phosphorylation status of the mTOR effectors S6K1, S6K2 and 4EBP1 may be potential candidates as markers for prediction of benefit from tamoxifen treatment. In combination with their role in prognosis, or with the addition of other prognostic markers, these factors may consequently enable a more secure judgement on whether tamoxifen therapy is needed, when this therapy only may be useful, or when a combination of endocrine treatment with other therapies may be essential. A similar role for the prediction of other endocrine therapies may be conceivable, however, this needs further investigation. Studies have also shown several other factors, among others in the PI3K/AKT pathway, to be useful in the prediction of endocrine treatment benefit [286, 310, 311]. Meta-studies comparing the usefulness, functionality and reproducibility of different markers, are needed to pin-point the optimal factor or combination of markers for this important purpose.

Structural comparison and in vitro silencing of S6K1 and S6K2 suggest a possibility to develop isoform specific inhibitors

The presented findings indicate clinically relevant differences between the mTOR effectors S6K1 and S6K2, and that amplification and overexpression may be present in partly different breast cancer subtypes. A recent review also highlights these indications [118]. In the light of this, it may be conceivable that isoform-specific targeting could be useful, allowing further tumour-specific therapies. Consequently, a study was performed using RNA interference for *in vitro* silencing of S6K1 and S6K2 separate or along, with subsequent transcriptional analysis to explore their effects on global mRNA expression profiles (*Study 4*). The breast cancer cell line ZR751 was used as a model due to the relatively high expression levels of both S6K1 and S6K2 ([240] and unpublished observations). Data indicated that silencing of S6K1 and S6K2 results in a partly compensatory upregulation of the other isoform, respectively. A feedback upregulation of mTOR, Raptor and Rictor in the two mTOR-complexes was evident, especially during simultaneously silencing of S6K1 and S6K2. Also an upregulation of p90 S6 kinases could be seen, likely compensating for the loss in rpS6 phosphorylation.

Isoform-specific silencing of S6K1 and S6K2, respectively, also resulted in significant differences in global expression profiles. The genes upregulated by S6K1siRNA, but not S6K2siRNA, included *EIF4E2*, *CCND1*, *FGFR1*, *INSRR* and the mitogen activated kinases *MAP3K4* and *MAPK3*. Downregulated genes after S6K1 knock-down included *EIF4EBP1* and *EIF4G2*, as well as the adaptor *GAB3*. Enriched upregulated genes were coupled to membrane-bounded organelle, targets of miR144 and the transcription factors USF and CDPCR1.

S6K2 silencing resulted in upregulation of genes enriched in several cellular processes. These represented cell cycle/mitosis; among others *CCNE1*, *CCNE2*, *CCNG2*, *CCNL1*, *CCNT1*, *CDK12* and *CDKN3*, prostate cancer; including the androgen receptor (AR), metabolism as well as targets of the transcription factors *E2F*, *AP2*, *SPI*, *ETF* and *Pax3*. Other upregulated genes upon S6K2 knock-down included the PI3K/AKT associated genes *IGF2R*, *PTEN*, *PIK3R3*, *EIF2AK2* and *EIF5B*, the oncogenes *ABL1* and *MDM2*, and the tumour suppressor candidates *DGDHS*, *TSSC1* and *TUSC3*. Downregulated genes after S6K2siRNA included genes in ion and monoamine transport as well as ClassA1 Rhodopsin-like receptors. Downregulated genes of note included *ABL2* and *PTPN22*. Eight genes were downregulated with S6K1 knock-down, but upregulated after S6K2 silencing (*Study 4*). In line with previous data, these results support the indices of compensatory as well as specific roles for S6K1 and S6K2. We and others ([222] and unpublished data) have noticed that the compensatory upregulation of pAKT via mTORC1/S6K1 silencing, seems not to occur after S6K2 silencing, further supporting this. Additional studies are needed to explore the effect of S6K1 and S6K2 silencing/inhibition on compensatory mechanisms on the protein level, as well as possible anti-tumourigenic effects under different circumstances *in vitro* and *in vivo*.

Whole-genome profiling of S6K1 and S6K2 high tumours, as well as after *in vitro* knock-down of the two kinases both resulted in significant differences, suggesting that isoform specific targeting of S6K1 or S6K2 may be valuable to allow further individualised breast cancer treatment regimens. The development of specific kinase inhibitors for therapeutic purposes is desirable [312]. However, the conserved homology between kinases has obstructed this process. The high similarity between the S6K1 and S6K2 primary structures allowed us to use the crystal structures of S6K1 for *in silico*

predictions of the S6K2 kinase structure (*Study 4*). The resulting models point towards some essential differences in the ATP-binding site of the two kinases, possibly allowing design of isoform-specific inhibitors (**Figure 28**).

Initially, to evaluate structural differences between S6K1 and S6K2, the primary structures were aligned using BLAST. S6K1 and S6K2 are both composed of 15 exons, where 1-14 are homologous, resulting from gene duplication. In contrast, exon 15 is different between the two kinases; S6K1 has a C-terminal PDZ-binding motif whereas S6K2 contains a proline-rich domain (*Study 4*). The amino acid sequences in the domains are highly similar. The residues in the active site, substrate binding site and hydrophobic motif are identical between the two kinases. The ATP binding site contains one amino acid shift, S6K1-S6K2: Y151C (S6K1 residue numbering), resulting in a slightly different size of the ATP-binding pockets between the two kinases (**Figure 28**). The corresponding residue in other members of the AGC family are commonly aromatic (Tyrosine or Phenylalanine) and to our knowledge, S6K2 and the 35% homologous SGK494 are the only members of the AGC family of kinases to have a non-aromatic residue at this site, facilitating development of S6K2-specific inhibitors. Two amino acid shifts are also present in the activation loop S6K1-S6K2: D223E and T225A, possibly allowing differences in the mechanisms of kinase activation.

The S6K1 kinase structure bound to staurosporin (**Figure 28 a**) showed relatively low electron density in the α C-helix as well as the activation loop both regulating kinase activity, indicating highly dynamic structures, although the phosphorylation at T229 partly stabilised the structure. When S6K2 was modelled against this structure, the α C-helix was assumed to be even shorter in S6K2 (residues R120-S125) than in S6K1 (residues A111-E126) (**Figure 28 b**), possibly as a result of differences in neighbouring amino acids, leading to an even more dynamic structure of S6K2.

The S6K1 kinase structure bound to the specific inhibitor PF-4708671 (**Figure 28 c**) showed significantly higher electron density in the α C-helix compared to the staurosporin bound structure, suggesting that the inhibitor stabilises the kinase domain in a non-activating conformation. Consequently, when using this structure as a template, also the S6K2 showed a longer, apparently less dynamic, α C-helix (**Figure 28 d**). Altogether, the structure comparisons between S6K1 and S6K2 indicate highly similar secondary and tertiary structures, but also dynamic structures. The similarities are likely more pronounced than in reality and the modelled structures are clearly biased towards the used template. It is for instance noteworthy that the two different S6K1 structures that were used as templates deviate more from each other with an RMSD of 1.25 Å (backbone), 1.35 Å (all heavy atoms) than the respective templates and the models. However, even if the structures of S6K1 and S6K2 kinase domains are as similar as the models suggest, there are differences that can be exploited for development of S6K2 specific inhibitors. The unique presence of the amino acid Cysteine in S6K2 at a position occupied by Tyrosine in S6K1, creates a significantly different environment in the ATP-binding domain, suggesting an opportunity for future development of isoform-specific inhibitors.

This study sheds further light on downstream mTOR signalling in breast cancer, supporting that S6K1 and S6K2 signalling may in part possess different roles in tumourigenesis. A structural comparison of the S6K1 and S6K2 kinase domains revealed significant differences that could be of importance for differences in regulation and function of the two kinases and also useful for future development of isoform-specific inhibitors. At the global tissue level, the expressions of S6K1 and S6K2 have been shown inversely correlated [118]. S6K1 are ubiquitously expressed, whereas the levels of S6K2 vary between tissue and the protein is mainly found in the central nervous system, lungs and gastrointestinal tracts. S6K2 levels in tumour tissue are in general high compared with corresponding

normal tissue. The emerging different roles of S6K1 and S6K2 suggest that specific targeting of either isoform may be valuable in different tumour subtypes, and in comparison to present day's mTOR inhibitors, further promote individualised therapies.

Figure 28 Structural comparison of S6K1 PDB ID 3A62 (a) and S6K1 PDB ID 4L3J (c) and overlaid with the corresponding S6K2 homology models (b) and (d).

CONCLUSIONS

In summary, the results of the present project suggest that:

- The mTOR effector S6K2 is a new candidate oncogene in the 11q13 amplicon in breast cancer (*Study 1*).
- S6K2/4EBP1 coamplification and mRNA coexpression is associated with a poor outcome, indicating a synergy between mTOR targets (*Study 2 and 3*).
- Whole-genome profiles of S6K2 and 4EBP1 positive tumours are highly overlapping and associated with cell cycle regulators and IGF signalling, indicating different roles of S6K1 and S6K2 (*Study 4*).
- Intracellular localisation and expression levels of S6K1, S6K2 and 4EBP1 determines associations with clinicopathological factors and indicate possible predictive roles for endocrine treatment benefit (*Study 1 and 3*).
- Structural comparison and *in vitro* silencing of S6K1 and S6K2 suggest a possibility to develop isoform specific inhibitors, which may facilitate further individualised therapies for breast cancer (*Study 4*).

PERSPECTIVES

“A good gene in one time and place may be a bad gene in another time or place”

This quotation proposed by Woolhouse *et al.* (2002) in the context of spread of viruses, may actually be well applied to conclude the riddle of cancer. In one way or another, this may explain the main questions; why does cancer arise? How can it be prevented? And, when developed, how can it be treated? No doubt, cancer arises as a result of a sustained long-term complex interplay between the outer and inner environment and an extensive number of genetic and epigenetic factors, throughout all tissues and cells of the body. These comprehensive interactions also determine the faith of the malignant progression, as well as the quality of response to applied treatment, shortly and long-term. To distinguish the underlying patterns and mechanisms explaining which gene is good or bad in which time and place would possibly be essential to find the answers.

During the last years, cancer research has moved the first, trembling steps towards this future utopia. For a number of cancer diseases, specific environmental features have been identified as risk factors, to a lower or higher degree, for malignant development. Also, certain genetic variants have been shown predisposing for cancer, or protective against the disease. The extensive numbers of alterations found in tumours have been proposed to result in a few underlying capabilities, or “hallmarks” of cancer. For breast cancer, whole genome-studies have been able to distinguish a limited number of tumour subtypes. These have in turn been possible to relate to different states of the normal gland development, giving a further dimension of breast cancer development.

An integration of the comprehensive amount of present data resulting from studies in different fields of cancer research and a large common effort in objective analysis, is certainly needed in continuation towards the utopia proposed by Hanahan and Weinberg that cancer biology will develop into a logical science where the complexities of the disease can be described in a few, underlying principles, finally allowing prevention of malignant development, patients specific diagnosis and tailored treatment strategies. Our hope is, that in the light of other findings, this present study may be able to be a contribution in some way to this comprehensive puzzle and its future solution.

ABBREVIATIONS

The lists of abbreviations contains two parts, the first one including genes and gene products which are not spelt out in the text, and a second part of other scientific terms, which are as normally explained with full names in the text.

Genes and gene products

4EBP1	EIF4E-binding protein 1
ABL	Abelson tyrosine-protein kinase
ACTB	beta-actin
AIB1	amplified in breast cancer 1
AKT	protein kinase B/v-akt murine thymoma viral oncogene homologue
ALDH1	aldehyde dehydrogenase
ALK	Anaplastic lymphoma kinase
AMPK	AMP-activated protein kinase
API	activator protein 1(transcription factor)
Apaf1	apoptosis activating factor 1
AR	androgen receptor
AREG	amphiregulin
Axl	tyrosine-protein kinase receptor UFO
BCL1	B-cell lymphoma 1 (CCND1)
BCL2	B-cell lymphoma 2
BRCA 1 and 2	Breast Cancer gene 1 and 2.
C11orf30	chr 11 open reading frame 30, encoding the EMSY protein
C/EBP β .	CCAAT/ enhancer transcription factor
C1, C2	PKC activating domains 1 and 2
CBP	cap binding protein
CCN	cyclin gene
CCNA	cyclin A
CCNB	cyclin B gene
CCND1 (BCL1, PRAD1)	cyclin D1 gene
CCT β	chaperonin containing T-complex protein
CD24	cluster of differentiation 24
CD44	cluster of differentiation 44
CDC42, CDC25B, CDC20	cell division cycle
CDH1	cadherin 1, e-cadherin
Cdk	cyclin-dependent kinase
CENPA	Centromere protein A
CHEK2	CHK2 checkpoint homolog
CK2	casein kinase 2
c-Myc (Myc)	cellular myocytosis gene
CREM τ	cAMP responsive element modulator
CTTN (EMS1)	cortactin

CXCL	C-X-C motif chemokine
DDR	discoidin domain receptor tyrosine kinase
E2F	E2-promoter binding factor, transcription factor family
eEF2	eukaryotic elongation factor 2
eEF2K	eukaryotic elongation factor 2 kinase
EGF-domain	epidermal growth factor domain
EGFR/HER1	epidermal growth factor receptor
EIF	eukaryotic translation initiation factor
EIF4E	eukaryotic initiation factor 4E
EJC	exon junction complex
EMS1	excess microsporocytes 1
EPHA2	ephrin receptor A2
ER	oestrogen receptor
ERBB (HER)	v-erb-b2 erythroblastic leukemia viral oncogene homolog
ERK	External regulated kinase
ESR1	ER α gene
ESR2	ER β gene
ESRRA	oestrogen-related receptor alpha
ETF	EGFR-specific transcription factor
FADD	Fas-Associated protein with Death Domain
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FKBP38	FK506 binding protein 8
FKHR	forkhead family member
FRS	fibroblast growth factor receptor substrate
FYVE	Fab 1, YOTB, Vac 1, <u>EEA1</u> domain
Fz	frizzled domain
GAB	GRB2-associated binding protein
GHR	growth hormone receptor
GLUT1	glucose transporter 1
GRP75	glucose-regulated protein; heat shock 70kD protein 9
GSK3	glycogene synthase kinase 3
HER	Human Epidermal Growth Factor Receptor
HFG	hepatocyte growth factor
HGFR/Met	hepatocyte growth factor receptor
Hif1	hypoxia inducible factor
HLA1	human leucocyte antigen
HoxB13	homeobox B13, transcription factor
Id4	inhibitor of DNA binding 4
Ig	immunoglobulin domain
IGF	insulin-like growth factor
IGFR	Insulin-like growth factor receptor
IKK	I κ B kinase,
IL	interleukin
IL17BR	interleukin 17 receptor B
INPP4B	inositol polyphosphate-4-phosphatase, type II
INSR/IR	insulin receptor

INSRR	insulin-related receptor 1
IRS	insulin-receptor substrate
JNK	c-Jun N-terminal kinase
Ki-67	Antigen identified by monoclonal antibody Ki-67
LdlA	Low Density Lipoprotein Receptor Class A domain
LKB1 (STK11)	Serine/threonine kinase 11
LMR	Lemur
Mam	meprin/A5-protein/PTPmu
MAPK	mitogen activated protein kinase
MAPKAP	Target of rapamycin complex 2 subunit
MDM2	Mouse double minute 2 homolog
MICB	MHC class I polypeptide-related sequence B
mLST8	MTOR associated protein, lethal with SEC13 protein 8 homolog
MMP	matrix metalloprotease
mTOR	mammalian/mechanistic target of rapamycin
mTORC	mTOR complex
Musk	Muscle-Specific Kinase
MYEOV	myeloma overexpressed
p4EBP1	phosphorylated 4EBP1
P53	Tumour protein 53 (p53)
PAI-1	plasminogen activator inhibitor type 1
PAK1	p21-activated kinase 1
PAPB1	polyA binding protein complex
PAX	paired box transcription factor
PCNA	proliferating cell nuclear antigen
PDCD4	programmed cell death 4
PDK1	phosphoinositide dependent protein kinase 1
PDZ	post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) domain
PGK1	Phosphoglycerate kinase 1
PgR	progesterone receptor
PH	pleckstrin homology
PHLPP	PH domain and Leucine rich repeat Protein Phosphatase
PI3K	phosphatidyl inositol 3-kinase
PIK3C	PI3K subunit p110 genes
PIK3R	PI3K subunit p85 genes
PIKK	phosphoinositide kinase related kinase
PIP ₂	phosphatidylinositol-4,5-bisphosphate
PIP ₃	phosphatidylinositol-3,4,5,-trisphosphate
PKA	protein kinase A
PKB (AKT)	protein kinase B
PP2A	protein phosphatase 2A
PPAPDC1B	Phosphatidic acid phosphatase type 2 domain-containing protein 1B
PPFIA1	Protein Tyrosine Phosphatase, Receptor Type, F Polypeptide (PTPRF), Interacting Protein (Liprin), Alpha 1
PPH3	phosphohistone H3
PPIA	peptidylprolyl isomerase A (cyclophilin A)

PRAD1	parathyroid Adenomatosis 1 (CCND1)
PRAS40	proline-rich AKT substrate, 40 kDa
pRb	retinoblastoma tumor suppressor
PRE	progesterone responsive elements
PROTOR	Protein observed with Rictor
pS6K	S6K1 and/or S6K2 phosphorylated at T389
Psi	plexins, semaphorins, integrin domains
PTEN	phosphatase and tensin homologue
PTP	protein tyrosine phosphatase
RACK	Receptor for Activated C-Kinase
RAD9A	RAD9 homolog A
RANK	receptor activator of NF-kappa-B
RANKL	receptor activator of NF-kappa-B ligand
Raptor	regulatory associated protein on mTOR
RAS	rat sarcoma oncogene
RET	rearranged during transfection
RHEB	Ras homologue enriched in brain
Rictor	RPTOR independent companion of MTOR, complex 2
Ror	Receptor tyrosine kinase-like orphan receptor
Ros	C-ros oncogene, receptor tyrosine kinase
rpS6	ribosomal protein subunit 6
RPS6KB1	ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (S6K1)
RPS6KB2	ribosomal protein S6 kinase, 70 kDa, polypeptide 2 (S6K2)
RSK	p90 ribosomal S6 kinase
RYK	related to receptor tyrosine kinase
S6K1	p70 ribosomal S6 kinase 1
S6K2	p70 ribosomal S6 kinase 2
SAM	Sterile alpha motif
Sema	semaphorin domain
SERPINB	ovalbumin gene
SH	Src homology
SKAR	S6K1 Aly/REF-like target
SP1	specificity protein 1 (transcription factor)
STAT5	signal transducer and activator of transcription 5
STK11 (LKB1)	serine/threonine kinase 11, liver kinase B1
STYK1	serine/threonine/tyrosine kinase 1
TGF	transforming growth factor
Tie	tyrosine kinase with immunoglobulin-like and EGF-like domains 1, angiopoietin receptor.
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
Tp53	tumour protein 53 (p53)
Trk	tropomyosin-receptor-kinase
TSC1 and 2	Tuberous sclerosis complex 1 and 2
uPA	urokinase plasminogene activator
USF	Upstream stimulatory factor 1
VEGF	vascular endothelial growth factor

WIF	Wnt-inhibitory factor
Wnt	wingless-type MMTV integration site family
WW	Trp-Trp repeat motif domain
YWTD	Tyr-Trp-Thr-Asp repeat
YY1	Yin Yang 1
ZEB	zinc finger E-box binding homeobox 1

Scientific terms

AGC	cAMP-dependent, cGMP dependent and protein kinase C family
ASCO	American society of clinical oncology
ATCC	American Type Culture Collection
BCS	breast cancer survival
BLAST	Basic local alignment search tool
BMI	body mass index
BRLMM	Bayesian Robust Linear Model with Mahalanobis distance classifier
CAF	cancer associated fibroblast
cDNA	complementary DNA
CI	confidence interval
CISH	chromogenic <i>in situ</i> hybridisation
CMF	cyclophosphamide methotrexate 5-fluorouracil chemotherapy
CR	C-terminal regulatory region
CSC	cancer stem cell
Ct	threshold cycle
CTC	circulating tumour cell
DAB	3,3'-diaminobenzidine hydrochloride
DCIS	ductal carcinoma <i>in situ</i>
DDBJ	DNA database of Japan
DM	double minutes
DRFS	distant recurrence-free survival
E2	oestradiol
ECM	extracellular matrix
EIA	enzyme immunoassay
EMBL-EBI	European Bioinformatics Institute
EMT	epithelial to mesenchymal transition
ERE	oestrogen-responsive elements
FASTA	fast all
FEC	fluorouracil/5FU, epirubicin cyclophosphamide
FFPE	formalin fixated paraffin embedded
FISH	fluorescent <i>in situ</i> hybridization
G1	gap1 phase of cell cycle
G2	gap2 phase of cell cycle
gDNA	genomic DNA
GEO	gene expression omnibus
GISTIC	genomic identification of significant targets in cancer
GnRH	gonadotropin-releasing hormone
GO	gene ontology
Gy	Gray (unit)
HeLa	cervix carcinoma cell line, from the patient Henrietta Lacks
HMM	Hidden Markov Model
hnRNP	heterogeneous ribonucleoprotein
HNSCC	head and neck squamous cell carcinoma
HR	hazard ratio
HSR	Homogeneously staining regions

ICM	Internal Coordinate Mechanics (software)
IDC	Invasive Ductal Carcinoma
IHC	immunohistochemistry
ILC	Invasive Lobular Carcinomas
IRES	internal ribosomal entry sites
KD	kinase domain
kDa	kilodalton (unit)
KE	kinase extension domain
LCIS	Lobular carcinoma <i>in situ</i>
LRFS	local recurrence-free survival
Mb	megabase (million base pairs)
MCF7	Michigan Cancer Foundation-7, breast cancer cell line
MEF	mouse embryonic fibroblast
MIAME	minimum information about a microarray experiment
miR	microRNA
MM	mismatch
M-phase	mitotic phase of cell cycle
NCBI	National Center for Biotechnology
NLM	National Library of Medicine
NLS	nuclear localisation sequence
NR	N-terminal regulatory region
NTC	no template control
OSCC	oral squamous cell carcinoma
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDB	protein databank
PH	plekstrin homology
PM	perfect match
PyMOL	<i>Python</i> -enhanced <i>molecular</i> graphics tool (software)
REMARK	reporting recommendations for tumour marker prognostic studies
RIN	RNA integrity number
RMA	robust multiarray average
RT	radiation therapy
RTK	receptor tyrosine kinase
S	Svedberg Unit, sedimentation
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SPF	s-phase fraction
SSCP	single-strand conformational polymorphism
TMA	tissue microarray
TNM	tumour, node, metastasis
TOP	5' terminal oligopyrimidine tract
TOS	tor signalling motif
UICC	Union for International Cancer Control
ZR751	breast cancer cell line

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