AN ODYSSEY TOWARDS PERSONALISED MEDICINE IN BREAST CANCER

from discovering new cancer genes to revealing drivers of therapeutic resistance

GERJON J. IKINK
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About the cover

A human cell represented as a computer’s circuit board. The circuit tracks depict the cell’s major signalling pathways, with key signalling molecules as squares (extracellular) or circles (intracellular). The main players of this thesis are highlighted on the right. Other components are cell organelles (the ‘hardware’), including the nucleus containing the DNA (the ‘software’). In computers and cells alike, errors in signal transduction or processing – often caused by mutations – can disrupt the whole system. Cancer is a consequence of errors in cell signalling. Fortunately, computers and cells alike are programmable, so errors can be fixed. However, the system is complex and our schematic still incomplete. This thesis fills in some of the gaps, aiding our understanding of how changes in cell signalling networks affect cancer cell transformation, progression and response to therapy.

The research described in this thesis was performed at the Division of Molecular Genetics of the Netherlands Cancer Institute, Amsterdam, the Netherlands.

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An odyssey towards personalised medicine in breast cancer

From discovering new cancer genes to revealing drivers of therapeutic resistance

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General Introduction
BREAST CANCER AND PERSONALISED MEDICINE

Breast cancer is a highly heterogenous disease with regards to morphological, molecular and physiological features. This heterogeneity is also apparent in the very diverse pathological and clinical behaviours of various breast cancers, including their response to therapy (Bertos and Park, 2011; Iwamoto and Pusztai, 2010; Polyak, 2011; Pourteimoor et al., 2016; Sotiriou and Pusztai, 2009). For proper clinical decision-making, it is vital to categorise breast cancers into groups based on the above-mentioned features that have similar clinical responses. The first widely adopted way of classification, based purely on morphology, distinguishes primarily the major classes of 'ductal' and 'lobular'. Although still extensively used, this histopathological classification has proven to be incomplete and often ambiguous, resulting in limited clinical utility (Simpson et al., 2005; Viale, 2012).

Molecular genetics and advances in immunohistochemistry (IHC) have greatly improved the stratification of breast cancer. One of the first crucial discoveries was the importance of hormone receptor status, especially the oestrogen-receptor (ER), in both prognosis and prediction of response to endocrine treatment (Jensen, 2004; Simpson et al., 2005). Later, the development of the ground-breaking molecular subtype classification (also called 'intrinsic subtypes') was a major advance in the clinical stratification of breast cancer, originally distinguishing HER2-enriched, basal-like, normal-like and ER+/luminal-like tumours (Perou et al., 2000; Sørlie et al., 2001, 2003). This molecular classification was further improved by splitting the luminal-like subtype into luminal A and luminal B and the removal of the normal-like subtype, which was considered sample contamination with normal breast tissue (Hon et al., 2016; Parker et al., 2009; Peppercorn et al., 2008; Sørlie et al., 2003). An approximation of the identification into the molecular subtypes can be largely done using an IHC approach assessing the status of the oestrogen receptor (ER) and progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and cell proliferation marker Ki67 (Goldhirsch et al., 2011). This greatly aided the adoption of the molecular classification in the clinic. The IHC approach roughly designates oestrogen and/or progesterone receptor-positive (ER/PR+) with HER2-negative tumours as luminal A when Ki67 staining is low or luminal B when Ki67
staining is high; ER/PR/HER2+ (all three positive) tumours, independent of Ki67 status, also as Luminal B; ER/PR-negative, but HER2-positive tumours as HER2-enriched; and triple (i.e. ER/PR/HER2) negative tumours as basal-like (Cheang et al., 2009). Although a clinically useful surrogate, these IHC definitions do not fully overlap with the molecular subtypes, which has consequences for choosing the right treatment options (Barnard et al., 2015; Carey et al., 2010; Dowsett et al., 2013; Liu et al., 2016; Prat et al., 2015; Viale, 2012). Not much later, the PAM50 assay was developed: a quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)-based assay assessing the expression of 50 genes in formalin-fixed and paraffin-embedded (FFPE) patient material. The PAM50 assay providing a more accurate classification of breast cancers over the molecular subtypes (Nielsen et al., 2010; Parker et al., 2009; Sørlie et al., 2001).

Recently, The Cancer Genome Atlas (TCGA) Network made an integrated analysis of genomic DNA copy number arrays, DNA methylation, somatic and germline mutations analyses, mRNA expression arrays, microRNA sequencing and reverse phase protein arrays performed on over five-hundred primary breast cancers. The TCGA analysis identified four main clusters that largely overlapped with the luminal A and B, basal-like and HER2-enriched subtypes (The Cancer Genome Atlas Network, 2012), which suggests that the molecular classification captures the main heterogeneity of the disease very well. Moreover, the molecular classification clearly reflects prognosis (Blows et al., 2010; Dawood et al., 2011): luminal A breast cancer has the most favourable prognosis, whereas HER2-enriched/HER2+ and basal-like/triple-negative subtypes represent the most aggressive breast cancers with poor prognosis. Additionally, the intrinsic subtypes have high predictive value for metastatic behaviour and systemic therapy success (Blows et al., 2010; Kennecke et al., 2010; Rouzier et al., 2005). Nonetheless, considering the significant molecular heterogeneity that is still observed between tumours within each molecular subtype (The Cancer Genome Atlas Network, 2012) and the high relapse rates in all subtypes after treatment (Wang et al., 2011b), the molecular classification is clearly still too crude. Therefore, many patients may not get the optimal treatment or even a therapy that does not treat their specific tumour at all. Moreover, therapies can have strong side-effects, which patients unnecessarily suffer from whilst not benefitting from
therapy. Hence, we require a much more refined picture of the heterogeneity of breast cancer and develop more specific biomarkers to predict therapy response. Indeed, under this notion the ambition for ‘personalised medicine’ (also called ‘precision medicine’) was formed: the tailoring of clinical decision making and interventions at the level of an individual patient, instead of patient groups.

The identification of genes and genetic pathways that actually drive tumorigenesis, or collaborate with oncogenic drivers, is essential to such detailed understanding of the disease. A forward-genetic screen in murine models, where insertional mutagenesis by, for example, retroviruses is used to induce tumour formation, is a powerful tool for discovering cancer genes. Such screens allow for the discovery of oncogenes as well as the identification of collaborating genes. They can also provide insight into the step-wise progression to malignancy through cumulating mutagenic events, as they occur in human breast cancer (Mattison et al., 2009). Importantly, by insertional mutagenesis we are also able to identify genes and molecular pathways that are commonly missed by sequencing of cancer genomes, e.g., due to their low incidence (DeNicola et al., 2015), while it is relatively easier to distinguish driver from passenger mutations. Hence, insertional mutagenesis screens provide a solid starting point for the odyssey towards personalised medicine.
INSERTIONAL MUTAGENESIS

Insertional mutagenesis makes use of mobile genetic elements and retroviruses: parasitic nucleic acids that integrate their DNA into the genome of a host organism. Retroviruses occasionally carry (proto-)oncogenes that can induce polyclonal tumours within as little as a few weeks post-infection. These retroviral strains are designated 'acute retroviruses' and are obviously unsuitable for cancer gene discovery by insertional mutagenesis screens. In contrast, non-acute or slow-transforming retroviruses do not carry oncogenes, but can instead cause host cell transformation by deregulation of cellular genes in the vicinity of their integration site, specifically oncogenes and occasionally tumour suppressor genes (Mikkers and Berns, 2003; Uren et al., 2005). Specifically, these retroviruses have Long Terminal Repeats (LTRs) at both ends of their genome containing promoter and enhancer elements required for the recruitment of the transcription machinery and transcription factors driving viral gene expression. This can additionally drive host gene expression (Figure 1A). Retroviruses integrate quite randomly into the host genome (Ringold et al., 1979; Steffen and Weinberg, 1978) and the known sequence of the integrated provirus can serve as a tag to identify the location of the integration site and thus the affected gene locus. This allows high-throughput insertional mutagenesis screens for the discovery of cancer-related genes (Jonkers and Berns, 1996; Mikkers and Berns, 2003).

In mice, Murine Leukaemia Virus (MuLV) and Mouse Mammary Tumour Virus (MMTV) are commonly used for insertional mutagenesis screens. Tumours induced by MuLV and MMTV usually develop within several months to a year post-infection and are typically of monoclonal or at most of oligoclonal nature (Cohen et al., 1979; Nusse and Varmus, 1982; Peters et al., 1986). Infection of the murine hosts can easily be attained, as the retroviruses can be transmitted via the milk of infected fosters and for MuLV also via intraperitoneal injection (Duggan et al., 2006; Hainaut et al., 1985). The specific promoter and enhancer elements of these retroviruses also contribute to their tissue-specificity. Hence, MuLV and MMTV are predominantly restricted to inducing T-cell lymphomas and mammary gland carcinomas, respectively (Lewis et al., 1999; Reuss and Coffin, 2000). In this thesis, MMTV-
induced insertional mutagenesis is employed for the discovery of breast cancer genes (see Chapters 2 and 3; Klijn et al., 2013; Theodorou et al., 2007).
MECHANISM OF INSERTIONAL MUTAGENESIS

Some proviral integrations in an infected animal model will confer growth advantage to the affected cell, inducing hyperplastic outgrowth that can develop into tumour formation. This can occur when the regulatory elements in the integrated mutagen activate expression of host-endogenous proto-oncogenes neighbouring the insertion site. In the case of retroviruses, this is facilitated by the powerful promoter and enhancer elements present in the viral LTRs. Alternatively, integration can produce hypermorphic as well as hypomorphic mutations, potentially inducing tumorigenesis. For retroviruses, the polyadenylation signals or splice donor and acceptor sites present in the proviral genome can cause truncation or missplicing of cellular gene transcripts (Uren et al., 2005).

In cancer gene discovery screens using insertional mutagenesis, enhancer and promoter insertions are most commonly found. Promoter integrations are defined as insertion in or very near to the endogenous promoter of a host gene, in sense orientation. The powerful promoter elements in the integrated mutagen then takes over the function of the endogenous promoter and induces high expression of the host gene (Figure 1B). For enhancer integrations, on the other hand, the strong transcriptional enhancers in the vector increase the activity of the endogenous promoter of a gene, thus causing overexpression (Figure 1B). Enhancer integrations are predominantly found downstream in sense orientation or upstream in antisense orientation to the affected gene (Uren et al., 2005).
Intragenic integrations are also found in insertional mutagenesis screens. Such integrations within a cellular gene can lead to truncated transcripts or produce chimeric transcripts in which a stretch of host RNA sequence is combined with the vector's sequence (Figure 1B). Both can influence mRNA stability and regulation (e.g. by removing RNA interference target sites), prevent translation or alter the final protein product, affecting its activity, cellular localization and/or regulation (Kool and Berns, 2009; Ranzani et al., 2013b; Uren et al., 2005). Therefore, tumour suppressor genes can also be tagged in insertional mutagenesis, although this is much less common as this usually requires the simultaneous disruption of both alleles of the gene. Hence, retrovirus-mediated insertional mutagenesis screens are mostly found to activate oncogenes or induce gain-of-function mutations (Kool and Berns, 2009; Mikkers and Berns, 2003).

METHODS FOR RETRIEVAL OF PROVIRAL INTEGRATION SITES
In insertional mutagenesis screens for cancer gene discovery, mammary tumours from infected mice are isolated and proviral insertions in the genome are localised. This is followed by the identification of the responsible oncogenes that have been activated by proviral integration (Figure 2). The benefit of insertional mutagenesis is that the unique viral (or transposon) sequences that activate the proto-oncogenes also serve as a mark of the genetic position of the integration.

In the early days of insertional mutagenesis, identification of integration sites was a time-consuming and complex endeavour, which required genomic library screening, chromosome walking, restriction mapping and Southern blot analyses (Corcoran et al., 1984; Dickson et al., 1984; Hayward et al., 1981; Nusse and Varmus, 1982; Peters et al., 1983; Selten et al., 1984; Theo Cuypers et al., 1984; Tsichlis et al., 1983). This approach led to the identification of the most frequently tagged loci only, thus yielding limited novel oncogenes. Almost two decades later, major leaps forward were made following the completion of the first draft sequence of the mouse genome, combined with technological developments in sequencing and PCR (polymerase chain reaction) (Ranzani et al., 2013a; Uren et al., 2005), which allowed for rapid genomic localization of the sequences adjacent to the insertion.
After the isolation of tumour DNA from multiple insertional mutagenesis-induced tumours, their locations (grey triangles) are retrieved and mapped on the host reference genome (black, showing host genes as blue rectangles). The collective integration events of multiple independent tumours (green density plot) are statistically compared to background levels and/or random iterations (red density plot) to identify CISs (green rectangle).

The most commonly used techniques became Splinkerette-PCR (Devon et al., 1995) and linear amplification mediated (LAM-)PCR (Schmidt et al., 2007), of which a modified version of the former was one of the screening methods used in Chapters...
2 and 3 of this thesis (details in: Theodorou et al., 2007). Both these techniques are based on restriction enzyme digestion of tumour DNA, followed by linker-ligation, then PCR-amplification using primers against the proviral integration and the linker, and finally sequencing (Schmidt et al., 2007; Uren et al., 2008, 2009).

An issue of these methods is that restriction endonucleases have recognition sites that are unevenly distributed over the genome, leading to biases in the recovery of the integrations. This also prevents the quantitative assessment of the genetic complexity of the tumours in the screen, including the 'depth' of a specific integration (i.e. how many of the tumour cells carry the integration, a measure for clonality of the insertion), which is an indication whether a targeted gene was involved in tumour initiation or progression. To address this, fragmenting the genomic DNA by restriction enzyme digestion has typically been replaced by acoustic shearing of the DNA (Berry et al., 2012; Koudijs et al., 2011), but also a polymerase-based method that avoids breaking up the genomic DNA altogether has been developed (Paruzynski et al., 2010). DNA shearing, followed by Splinkerette-PCR ('Shear-Splink') has been utilised in the other screening method of Chapter 2 and 3 of this thesis (see also: Klijn et al., 2013).

**Statistical analyses for the identification of common insertion sites**

The identification of the target genes of the most frequent integrations in insertional mutagenesis screens, enhancer insertions, can be quite challenging as they can act from considerable distances from the affected endogenous promoter due to chromatin loops (West and Fraser, 2005). Hence, unlike promoter and intragenic integrations that are located in or directly proximal to the affected host gene, it is possible that the activated gene is not the one most near to the integration site. Moreover, each tumour will carry many integrations, most of which are merely passenger insertions that do not contribute to tumorigenesis. Extracting the oncogenic driver integrations therefore requires statistical analysis to identify genomic regions with an enrichment of integrations in multiple independent tumours, i.e. regions carrying more insertions that can be expected by chance (Figure 2). Such
integration-enriched genetic regions are referred to as common insertion sites (CISs) and are likely to harbour oncogenes or, in some cases, tumour suppressor genes. Assessing whether the integrations in CISs follow a non-random pattern can be achieved using a chi-squared goodness-of-fit test, as used in the classical Splinkerette-PCR approach of Chapters 2 and 3 of this thesis (details in: Theodorou et al., 2007). More commonly used methods to analyse insertional mutagenesis screens use Poisson distribution statistics (Mikkers et al., 2002), which in more advanced setups can also identify co-occurring or associated CISs (Bergemann et al., 2012; Sarver et al., 2012), and Monte Carlo simulation-based methods (Keng et al., 2009; Suzuki et al., 2002). Both generate repeated iterations of a random integration distribution across the genome to compare to the observed integration data (Dupuy, 2010). However, due to this random distribution, insertion biases of the integrating mutagens are not taken into account (Wu et al., 2006), although such biases do undeniably exist (Hematti et al., 2004; de Jong et al., 2014; Mitchell et al., 2004; Nielsen et al., 2005; Wu et al., 2003). Moreover, for both these methods, a fixed maximum genetic window size has to be defined to reduce the probability of finding false CISs. This is problematic in larger scale screens, as with the increase of integration data, this maximum window size has to be reduced below the biologically relevant scale representing the normal spread of integrations within a CIS, potentially leading to false-negatives. The more recently developed Kernel Convolution framework, typically using the Gaussian kernel function, overcomes these issues (De Ridder et al., 2006). The concordance between a Monte Carlo based method and a Gaussian Kernel Convolution (GKC) method used on an identical dataset is reported to be as low as 60% (March et al., 2011), emphasising the importance of the type of statistical analysis that is used. With GKC, Gaussian kernel functions are positioned on the integration sites, followed by the combining of neighbouring kernel functions to estimate the integration density for a genomic position. This thus effectively allows for the detection of CISs with varying widths, as no fixed genetic window size has to be defined (De Ridder et al., 2006). The significance of each estimated density peak on a given genomic position is determined by a threshold based on a null-distribution of insertion densities that is computed from randomly permutated data. This null-distribution can be based on a
specified background model to correct for integration bias. This Gaussian Kernel Convolution method is also used in the analysis of the Shear-Splink data in Chapters 2 and 3 of this thesis (details in: Klijn et al., 2013).

Due to technological and bioinformatical developments allowing increasingly larger scale high-throughput screens for cancer genes, it was expected that sensitivity and specificity would similarly increase. However, larger size screens detected an ever-increasing number of genes significantly associated to cancer at implausible rates, thus suggesting a profound increase in false-positive hits (Lawrence et al., 2013). The stringent Insertional Mutagenesis Database analysis pipeline, used for the screens that exploit the Shear-Splink method in Chapters 2 and 3, is designed to limit false-positive findings (Klijn et al., 2013; Koudijs et al., 2011).

**RELEVANCE OF MMTV-MEDIATED INSERTIONAL MUTAGENESIS**

Insertional mutagenesis screens utilizing MuLV have been performed far more frequently than those employing MMTV (Ranzani et al., 2013a). This is likely due to the higher integration frequency of MuLV compared to MMTV, and thus the associated likeliness of finding CISs. Moreover, MuLV allows infection via intraperitoneal injection, which may offer more control and is logistically less demanding than nursing with infected fosters. Still, the limited MMTV-induced insertional mutagenesis screens that have been performed in the past, have identified novel oncogenes and tumorigenic molecular pathways in breast cancer. The first gene identified by MMTV insertional mutagenesis was Wnt1 (wingless-type MMTV integration site family, member 1), initially named int1, referring to the first identified common integration site (Nusse and Varmus, 1982). This revealed the Wnt gene family and signalling pathway, which are heavily implicated in stem cell biology, organismal development and cancer (Nusse and Varmus, 2012). Similarly, the following common MMTV integration sites that were identified, led to the discovery of, among others, the Fgf (fibroblast growth factor), Notch and Rspo (R-spondin) gene families, each with important roles in developmental and cancer biology (Dickson and Peters, 1987; Dickson et al., 1984; Gallahan and Callahan, 1997; Gallahan et al., 1987; Roelink et al., 1990; Theodorou et al., 2007).
Although these MMTV-mediated insertional mutagenesis screens have thus substantially advanced our understanding of breast and other cancers, the yield of oncogenes has been limited. The rising popularity of other screening methods, including transposon-mediated insertional mutagenesis and short hairpin RNA (shRNA) screening, have likely contributed further to the limited use of MMTV-induced screens. However, using new approaches and more advanced techniques in MMTV-based screens may return novel leads in breast cancer.

This thesis presents the results of new MMTV-mediated insertional mutagenesis screens in Chapter 2 and follows up on the work of previously performed screens using MMTV (Klijn et al., 2013; Theodorou et al., 2007) in Chapter 3. Most MMTV-based insertional mutagenesis screens have employed wild-type mouse strains. However, in light of the notion that the most clinically relevant heterogeneity currently requiring investigation is found within and not between the currently established molecular subtypes of breast cancer (The Cancer Genome Atlas Network, 2012), we argued that it makes more sense to perform screens in mice that model these subtypes. Therefore, we performed MMTV-induced insertional mutagenesis screens in mice transgenic for the ErbB2 (erb-b2 receptor tyrosine kinase 2, also known as HER2 or neu) gene (MMTV-cNeu), a well-established model for HER2+ breast cancer (Chapter 2). In this tumour-prone MMTV-cNeu strain, mammary tumours develop with similar morphological and molecular features to human disease (Andrechek et al., 2003; Rosner et al., 2002). Moreover, in concordance with human HER2+ breast cancer cases, additional mutagenic events besides ERBB2 overexpression appear required for tumorigenesis, considering the stochastic and somewhat late tumour onset in this strain (Guy et al., 1992). Therapy-resistant cells are known to grow out in ERBB2 transgenic mouse models, as is also the case in HER2+ breast cancer patients (Knutson et al., 2004). Correspondingly, only half of HER2+ breast cancer cases respond to HER2-targeted therapies, which is also reflected in ErbB2-transgenic mouse models (Ellis and Perou, 2013; Knutson et al., 2004). With therapy resistance in mind, a cancer discovery screen could eventually also help to improve molecular classification and the development of complementary therapeutic strategies.
HER2 (ERBB2) IN BREAST CANCER

HER2 is the name often used in the clinic for the ERBB2 gene (erb-b2 receptor tyrosine kinase 2) encoding the Receptor tyrosine-protein kinase erbB-2 (ERBB2). It is one of the members of the EGF (epidermal growth factor) or ERBB receptor subfamily of receptor tyrosine protein kinases, consisting of four members: EGFR (epidermal growth factor receptor, also known as HER1 or ERBB1), ERBB2 (HER2/Neu), ERBB3 (HER3) and ERBB4 (HER4). The ERBB receptors have vital functions in embryogenesis and development by controlling cell proliferation, survival, differentiation and migration. This is also apparent from rodent (conditional) knockout studies of ERBB family members, each resulting in embryonic lethality due to severe defects in various organs (reviewed in: Olayioye et al., 2000; Wieduwilt and Moasser, 2008). ERBB receptors are also crucial in postnatal processes, especially in various stages of mammary development, where each receptor has unique expression patterns and roles (Darcy et al., 2000; Schroeder and Lee, 1998; Stern, 2003). Overexpression of ERBB genes is implicated in the formation and progression of multiple tumour types, including breast cancer (Normanno et al., 2006; Olayioye et al., 2000). ERBB2 overexpression, typically caused by gene amplification, occurs in over a quarter of breast cancer cases (Slamon et al., 1987, 1989) and defines the aggressive HER2-enriched/HER2+ breast cancer subtype (but is also possible in the Luminal B subtype).

Regulation of ERBB signalling is multi-layered and complex. ERBB signalling is regulated on the onset via the specificity, expression patterns, post-translational processing and turn-over of the numerous ligands and the extent of redundancy between them (Wieduwilt and Moasser, 2008; Yarden and Sliwkowski, 2001). EGFR is the receptor for EGF, transforming growth factor α (TGF-α) and amphiregulin, but can also function as a receptor for heparin-binding growth factor (HB-EGF), epiregulin and betacellulin that can also bind to ERBB4 (Normanno et al., 2006). Neuregulin 1 and 2 (NRG1 and NRG2) can bind ERBB3 and ERBB4, whereas NRG3, NRG4 and tomoregulin can only bind ERBB4 (Hobbs et al., 2002; Uchida et al., 1999). Most recent additions to this list are neuroglycan C ("neuregulin 6"), reported as a direct ligand of exclusively ERBB3 (Kinugasa et al., 2004), and epigen,
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a low-affinity but potent ligand of ERBB1 (Kochupurakkal et al., 2005; Strachan et al., 2001). Much effort has been invested to identify ligands of ERBB2, but none have ever been found, thus ERBB2 is generally assumed to have no ligands. Ligand redundancy is context-dependent and ligand knockout models only partly phenocopy their associated ERBB receptor knockout models (Wieduwilt and Moasser, 2008). Receptor signalling may be highly dependent on the bound ligand, as low-affinity ligands like amphiregulin, epiregulin and epigen are thought to have a sustained signalling potential due to low turn-over and inadequate receptor inactivation (Kochupurakkal et al., 2005; Tzahar et al., 1998). In contrast, signalling of the high-affinity ligands is relatively short-lived.

ERBB STRUCTURE AND SIGNALING

The ERBB receptors all consist of a large extracellular ligand-binding region, a transmembrane region and, intracellularly, a juxtamembrane segment, an ATP-binding tyrosine kinase domain and a tyrosine-rich C-terminal tail (Figure 3A). The extracellular region consists of four domains, of which the leucine-rich regions I and III (also known as L1 and L2 for leucine-rich repeats 1 and 2, respectively) facilitate ligand binding. In the ligand-free state, the extracellular regions of the ERBBs exist primarily in a closed, inaccessible, conformation, stabilised by interaction between domains II and IV (also called CR1 and CR2 for cysteine-rich 1 and 2, respectively) (Figure 3A) (Ferguson et al., 2003). ERBB2 is an exception to that, as it is permanently in an open configuration (Figure 3B) (Garrett et al., 2003). Other ERBBs occasionally open up from the tethered to an extended confirmation, which can be stabilised by ligand binding (Figure 3A). Ligands consequently shift the equilibrium to the open configuration, exposing domain II (Ferguson et al., 2003), which enables ERBB dimerisation: another layer of ERBB signalling regulation.

ERBB receptors are able to form homo- and heterodimers. Although ERBB2 has no ligands, it is the preferred dimerisation partner of all other ERBBs. Moreover, ERBB2 increases the ligand binding affinity of its binding partner, which may lead to extended ERBB signalling (Jones et al., 1999; Karunagaran et al., 1996; Tzahar et al., 1996). In contrast, ERBB3 was long thought to have an impaired tyrosine kinase
**Figure 3 | ERBB structure, signalling and targeted therapy**

(A,B) Schematic representation of the structure and domains of EGFR, ERBB3 and ERBB4 (A) and ERBB2 (B) as kinase-inactive monomer in tethered conformation (left) and in open ligand-bound conformation (right). Only in open conformation, the monomers are available for dimerisation.

(C) Schematic representation of ERBB-receptor dimerisation, resulting in the conformational change of intracellular regions that leads to the allosteric activation of the tyrosine kinase domains and subsequent phosphorylation (P) of tyrosine residues in the C-terminal tail.

(D) Simplified overview of ERBB-receptor dimers containing ERBB2 and the downstream signalling effects. Semi-transparent and dashed arrows indicate weak interactions.

(E-G) Mechanisms of action of ERBB2-targeted therapies Trastuzumab (E), Lapatinib (F) and Pertuzumab (G). Red "X" indicates no activity (E), no phosphorylation (F) and no interaction (G).
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I-IV: extracellular domains I-IV (also known as L1, CR1, L2 and CR2, respectively), TM: transmembrane region, JM: juxtamembrane segment, N/C-lobe: N-terminal and C-terminal lobes of the tyrosine kinase domain, CTT: C-terminal tail.

domain (Guy et al., 1994), requiring it to form heterodimers in order to signal. However, more recent findings established that ERBB3 has weak basal kinase activity (Shi et al., 2010), which is potently enhanced by a dimerisation partner, and is able to form (possibly productive) homodimers (Steinkamp et al., 2014). Homo- and heterodimerisation of ERBB receptors lead to asymmetric association of the intracellular regions of the binding partners. Facilitated and stabilised by the juxtamembrane segments, this results in the C-terminal lobe of one ERBB's tyrosine kinase domain (the activator) contacting the N-terminal lobe of the other ERBB's tyrosine kinase domain (the receiver), hereby allosterically activating the receiver's tyrosine kinase domain (Figure 3C) (Brewer et al., 2009; Jura et al., 2009; Zhang et al., 2006). Upon activation, tyrosine moieties on the C-terminal tails are phosphorylated by the kinase domain. These subsequently serve as docking sites for several Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain-containing adaptor and scaffolding proteins, inducing further signalling (Figure 3D) (Yarden and Sliwkowski, 2001). Formation of the specific homo- or heterodimers is determined by both the inducing ligands as well as the pool of available ERBB receptors and results in the activation of several signalling pathways (Olayioye et al., 2000).

The most prominent of these downstream signalling cascades, especially considering carcinogenesis, are the RAF/MEK/ERK/MAPK pathway, primarily controlling cell proliferation, and the PI3K/AKT/mTOR pathway, important for cell growth and survival (Figure 3D). EGFR, ERBB2 and ERBB4 predominantly activate the RAF/MEK/ERK/MAPK pathway through their many GRB2 (growth factor receptor-bound protein 2) and SHC-binding sites (Schulze et al., 2005). EGFR may signal weakly to the PI3K/AKT/mTOR pathway as well through its binding to the docking protein GAB1 (GRB2 associated binding protein 1), which interacts directly with the p85 regulatory subunit of PI3K (Mattoon et al., 2004). However, in contrast to its family members, ERBB3 contains multiple binding sites for the regulatory subunit p85 of PI3K, making it the most dominant, if not essential, binding partner.
for PI3K/AKT/mTOR pathway activation (Carraway et al., 1995; Holbro et al., 2003; Prigent and Gullick, 1994).

Overexpression of ERBB2 greatly increases the cell membrane levels of the ERBB2 protein. Due to its open conformation, which is poised to dimerise, this increases the formation of the ligand-independent ERBB2 homodimers, constitutively activating the RAF/MEK/ERK/MAPK pathway (Kraus et al., 1987; Venter et al., 1987; Yarden and Sliwkowski, 2001). However, the strong binding preference of ERBB2 to ERBB3, which potently activates PI3K/AKT/mTOR pathway, makes the ERBB2-ERBB3 heterodimer the most powerful oncogenic signalling combination (Holbro et al., 2003; Pinkas-Kramarski et al., 1996; Tzahar et al., 1996) (Figure 3D). It may therefore come as no surprise that ERBB3 expression is observed in several tumour types that overexpress ERBB2, including breast cancer (Bodey et al., 1997; Chow et al., 2001; Lemoine et al., 1992; Naidu et al., 1998; Rajkumar et al., 1996; Siegel et al., 1999). Correspondingly, breast cancer mouse models revealed selective upregulation of ErbB3 in activated ERBB2 (Neu)-induced tumours (Sieg et al., 1999). Several studies have suggested that ERBB3 is even critical to the transformation process in HER2+ breast cancer (Holbro et al., 2003; Lee-Hoeflich et al., 2008).

**HER2-TARGETING THERAPY & RESISTANCE**

Traditionally, the only treatment option for HER2+ breast cancer was chemotherapy. However, the development of the first targeted therapy in breast cancer, the monoclonal antibody Trastuzumab (Herceptin), has provided a highly successful new treatment option (Eiermann, 2001; Marty et al., 2005; Romond et al., 2005; Slamon et al., 2001; Vogel et al., 2002). Trastuzumab is a humanised murine monoclonal antibody binding domain IV of the extracellular region of the ERBB2 receptor (Figure 3E). It is thought to work by both preventing downstream signalling of ERBB2 and inducing an immune response via antibody-dependent cell-mediated cytotoxicity (ADCC) (Cooley et al., 1999; Dubská et al., 2005; Wu et al., 2008; Yakes et al., 2002), but the exact underlying mechanism of action is still unclear. Trastuzumab may be most effective against ERBB2 homodimers (Desmedt et al., 2009; Ghosh et al., 2011).
Although Trastuzumab greatly improved the prognosis of HER2+ breast cancer patients, both primary and secondary resistance are common, which sparked the development of alternative or complementing therapeutics. Among these, the tyrosine kinase inhibitor Lapatinib (Tykerb) has found its way to the clinic (Blackwell et al., 2010; Geyer et al., 2006). Lapatinib blocks signalling by targeting the intracellular kinase domain of ERBB2 as well as EGFR (Figure 3F) (Xia et al., 2002). Also the monoclonal antibody Pertuzumab (Perjeta) has passed clinical trials (Baselga et al., 2011; Swain et al., 2013). Pertuzumab works by binding domain II of ERBB2, thus blocking its dimerisation arm, consequently preventing signalling primarily by inhibiting ERBB2-ERBB3 heterodimerization (Figure 3G) (Agus et al., 2002). A conjugate of Trastuzumab with the maytansinoid (microtubule depolymerizing) and antimitotic drug emtansine (DM1) has additionally been developed (together known as T-DM1). Here the antibody functions to deliver the potent cytotoxic agent specifically to its antigen (i.e. ERBB2) expressing tumour cells, hence reducing its systemic toxicity (Lambert and Chari, 2014; Lewis Phillips et al., 2008; LoRusso et al., 2011).

Still, treatments using these therapeutic agents, usually administered in combinations, benefit only 50-80% of patients as first-line therapy and just 20%-40% in the second-line setting (i.e. when any initial treatment failed) (Santa-Maria et al., 2016). Moreover, these therapies often have strong side-effects, including febrile neutropenia and heart failure (Zhang et al., 2014). Hence, accurately predicting that a patient will not benefit from a particular therapy by using specific biomarkers will prevent unnecessary treatment and associated toxicity. The identification of molecular indicators for therapeutic failure will also aid the advance towards more personalised medicine.

Known mechanisms for resistance to HER2-targeting therapeutic agents include (1) increased ligand expression and (subsequent) activation of other ERBB receptors, (2) mutations and truncations in ERBB2 that prevent inhibition by the drugs, (3) activation of ERBB2 and its downstream pathways by other receptor tyrosine kinases, or (4) bypassing ERBB2 altogether by alterations in signalling effectors downstream of ERBB2 (Luque-Cabal et al., 2016; Rexer and Arteaga, 2012; Shi et al., 2016). In particular the downstream activation of the PI3K/AKT/mTOR pathway,
independent of ERBB2, is strongly connected to HER2-targeted therapy resistance (Appert-Collin et al., 2015; Berns et al., 2007; Chandarlapaty et al., 2012; Cizkova et al., 2013; Eichhorn et al., 2008; Esteva et al., 2010; Hanker et al., 2013; Juntila et al., 2009; Loibl et al., 2016a, 2016b; Nagata et al., 2004; Park et al., 2014; Razis et al., 2011; Serra et al., 2008; Wang et al., 2011a, 2013).
THE PI3K/AKT/mTOR PATHWAY

Signalling in the PI3K/AKT/mTOR cascade is initiated by the activation of the phosphatidylinositol 4,5-bisphosphate 3-kinases (PI3Ks) (Figure 4). The PI3K family of lipid kinases consists of several classes (IA, IB, II and III), each containing multiple isoforms, which have distinct roles (Thorpe et al., 2015). Class IA PI3Ks, consisting of heterodimers of the p85 regulatory subunit (counting five isoforms) and the p110 catalytic subunit (with three isoforms), has in particular been associated to cancer and has consequently been thoroughly investigated. In this thesis, "PI3K" mainly refers to this class IA PI3Ks.

Figure 4 | Simplified representation of the PI3K/AKT/mTOR signalling pathway

Upon activation of receptor tyrosine kinases (RTKs), the PI3K/AKT/mTOR signalling cascade is initiated. Green arrows indicate positive interactions (e.g. induction or stimulation). Red arrows indicate negative interactions (e.g. deactivation or inhibition). Red "X" indicates no activity.

Upon activation, PI3K is recruited to the membrane, where p85-induced inhibition of p110 is relieved, allowing p110 to catalyse the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂ or PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃ or PIP3). PIP3 subsequently recruits pyruvate dehydrogenase kinase 1 (PDK1) to the plasma membrane, phosphorylating the simultaneously recruited protein kinase B (PKB, commonly known as AKT) on its threonine residue 308 (T308) in its activation loop.
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(Andjelkovic et al., 1997). This allows the subsequent full activation by phosphorylation of serine 473 (S473) in the regulatory domain of AKT. This can be facilitated by several kinases, including the mechanistic target of rapamycin (mTOR) in its mTORC2 complex (Sarbassov et al., 2005), but possible also via autophosphorylation by AKT itself (Toker and Newton, 2000). Activated AKT (in this thesis primarily referring to the AKT1 isoform), being a serine/threonine kinase, facilitates the phosphorylation of numerous downstream effector molecules (Figure 4). Most prominently, the serine/threonine kinase mTOR is activated by AKT, which, in its mTORC1 complex, controls mRNA translation and protein synthesis via its substrates eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1 or 4EBP1) and S6 kinase (phosphorylating the ribosomal protein S6) (Gingras et al., 2001; Ma and Blenis, 2009). Substrates of mTORC1 can also regulate metabolism, cell cycle progression, survival, angiogenesis, autophagy and lipid synthesis for cell proliferation (Laplante and Sabatini, 2012; Perl, 2015; Wullschleger et al., 2006; Xu et al., 2014).

Phosphatase and tensin homolog (PTEN) is the major antagonist of PI3K and acts by dephosphorylating PIP3 to PIP2 (Maehama and Dixon, 1998; Stambolic et al., 1998). It is consequently known as a tumour suppressor and rivals p53 for inactivation frequency. Similarly, inositol polyphosphate-4-phosphatase type II B (INPP4B) has more recently been identified as tumour suppressor as it inhibits PI3K/AKT/mTOR pathway activity through its hydrolysis of phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P2) to phosphatidylinositol 3-phosphate (PtdIns(3)P), while PtdIns(3,4)P2 (besides PIP3) seems to be required to mediate AKT activation (Fedele et al., 2010; Gewinner et al., 2009).

Signalling pathway-activating genetic alterations in cancer most frequently affect the PI3K/AKT/mTOR pathway (Brugge et al., 2007). Also in breast cancer, including the HER2+ subtype, activation of the PI3K/AKT/mTOR signalling cascade is common. This is primarily induced by activating mutations in PIK3CA (encoding the catalytic subunit isoform p110α of PI3K; seen in ~40% of cases), INPP4B loss (~30% of cases), amplification of AKT1 (~20% of cases) and PTEN loss (~15-20% of cases) (Cornen et al., 2014; The Cancer Genome Atlas Network, 2012; Toss and Cristofanilli, 2015).
However, other (unidentified) mechanisms may influence PI3K/AKT/mTOR pathway activity and its associated critical clinical consequences, also in other cancers (Brown and Toker, 2015; Klempner et al., 2013). Hence, the characterisation of novel activators can identify vital biomarkers for therapy resistance and provide a starting point for the development of new therapeutic agents counteracting therapy escape mechanisms. Indeed, in the insertional mutagenesis screens presented in this thesis (Chapters 2 and 3) and related previous screens by our group (Klijn et al., 2013; Theodorou et al., 2007), several targets that were tagged are players in the PI3K/AKT/mTOR pathway, specifically Igf2, Eras and Irs4. The following chapters of this thesis show the association of these genes with HER2/ERBB2 and (Chapter 4) specifically addresses the relation of these genes to therapy resistance in the context of HER2+ breast cancer.

**INSULIN-LIKE GROWTH FACTOR 2 (IGF2)**

Insulin-like growth factor 2 (IGF2) is a tightly regulated secreted peptide that controls cell growth, differentiation and metabolism (O’Dell and Day, 1998). It is closely related to IGF1 with 62% amino acid homology and both IGFs have approximately 40% homology with (pro)insulin (Fürstenberger and Senn, 2002). Especially during early embryogenesis and foetal development, IGF2 plays an important role, during which the gene is most extensively expressed and IGF2 levels in circulation are also the highest (Liu et al., 1989; Sara et al., 1983). Also in neonates and adults, IGF2 is present in the serum, typically at higher concentrations than IGF1 (Bennett et al., 1983). Expectedly, aberrant IGF2 expression is linked with abnormal growth and in particular the Beckwith-Wiedemann syndrome (Ward, 1997). This growth disorder immediately associates IGF2 with tumorigenesis, as this syndrome is often accompanied with an increased tumour burden, especially in the form of Wilms’ tumours in which IGF2 is known to play a major role (Ward, 1997). Overexpression of IGF2 and increased IGF2 levels in the serum is also found in many other cancers, including breast cancer, and is associated with a poor prognosis (Espelund et al., 2008; Kalla Singh et al., 2010; Livingstone, 2013; Werner and LeRoith, 1996; Yee et al., 1988; Yu and Rohan, 2000). Besides promoting cell proliferation and survival,
IGF2 also induces angiogenesis in tumours (Heffelfinger et al., 1999; Kim et al., 1998; Pieciewicz et al., 2012).

Levels of IGF2 are stringently regulated at multiple points, but predominantly at the gene expression level (Harrela et al., 1996). The IGF2 gene is controlled by four promoters, of which three embryonic promoters are genetically imprinted so that only the paternal allele is expressed, while the maternal allele is silent (Figure 5A) (Bergman et al., 2013). In breast cancer and several other cancers, overexpression of IGF2 is commonly due to loss of imprinting (Cui, 2007; Ito et al., 2008; Murphy et al., 2006; van Roozendaal et al., 1998; Vu et al., 2003; Wu et al., 1997; Zhao et al., 2009). Interestingly, an inhibitory antisense transcript of IGF2 is transcribed from the maternal allele, while the paternal allele is epigenetically silenced, and loss of expression of this antisense transcript is associated with cancer (Figure 5A) (Okutsu et al., 2000; Vu et al., 2003).

Once secreted, IGF2 in the circulation is predominantly (>99%) associated with one of the members of a family of six high-affinity IGF-binding proteins (IGFBP) (Figure 5A) (Livingstone, 2013). IGFBPs have a dual role of inhibiting the IGFs: by sequestering the biological active free IGFs, and simultaneously by greatly extending their half-life and thus allowing systemic transport of the IGFs (Clemmons, 1997; Rajaram et al., 1997). In that regard, IGFs can act over long distances like hormones, as well as locally as tissue growth factors (Blundell et al., 1978; Sajid et al., 2011). Also dysregulation of IGFBPs has been correlated with cancer, generally with a poorer prognosis (Gianuzzi et al., 2016; Hawsawi et al., 2016; Helle et al., 2001; Hu et al., 2017; Kashyap, 2015; Livingstone, 2013; Travis et al., 2016).

Free IGF2 is able to signal via its interaction with different receptor dimers, which can also be bound by insulin and/or IGF1 (Figure 5B). Both the specific receptor as well as the ligand determine the specific downstream signals and thus cellular responses, due to distinctive recruitment and activation patterns of intracellular effectors (Belfiore et al., 2009). IGF2 action is predominantly mediated by the IGF1 receptor (IGF1R) homodimers, for which IGF2 competes with IGF1 for binding with similar affinity (De Meyts and Whittaker, 2002; Pandini et al., 2002). Upon ligand binding, IGF1R recruits various intracellular adaptor proteins, including the insulin receptor substrates (IRSs) and SHC (Scr homology 2 domain containing). This leads
to the activation of the downstream PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK signalling cascades, respectively, thus promoting cell proliferation and survival (Figure 5A). Dysregulation of IGF1R has been associated with carcinogenesis and is linked with, among others, breast cancer (Baserga et al., 1994; Farabaugh et al., 2015; Resnicoff et al., 1995; Resnik et al., 1998; Schnarr et al., 2000; Valentinis and Baserga, 2001).

**Figure 5 | IGF signalling and regulation**

(A) Schematic representation of IGF2 signalling, showing the genetic imprinting of IGF2, its inhibitory antisense transcript and IGF2R, as well as post-translational regulation. Red "X" indicates no expression. Green arrows indicate positive interactions (e.g. induction or stimulation). Red arrows indicate negative interactions (e.g. deactivation or inhibition).

(B) Overview of homodimers and heterodimers of insulin-like growth factor 1 receptor (IGF1R) and insulin receptor (IR) isoforms, including their activating ligands. Semi-transparency and dashed arrows indicate weak and/or context-dependent interactions.

IGF2 does not (or hardly) interact with the classic insulin receptor (IR or IR-B) homodimers and this receptor is primarily bound by insulin and IGF1. However, IGF2 has much higher affinity for an alternative spliced insulin receptor homodimer (isoform A; IR-A) (Denley et al., 2004; Frasca et al., 1999). IR-A expression is high
during embryogenesis, but also in several cancers, including breast cancer, as it promotes IGF2 signalling towards cell proliferation and anti-apoptosis, mostly via RAF/MEK/ERK/MAPK pathway activation (Belfiore et al., 2009; Denley et al., 2003, 2004; Frasca et al., 1999; Sciacca et al., 1999). Moreover, heterodimers (sometimes called 'hybrids') of IGF1R and either of the two IR isoforms also exist, which have varying affinity to the ligands (Figure 5B) (Belfiore et al., 2009; Frasca et al., 1999; Nakae et al., 2001). In the majority of breast cancers, hybrid receptors were found to be more prevalent than IGF1R homodimers (Pandini et al., 1999) and the heterodimers are also shown to play a role in other cancers (Belfiore et al., 2009).

Finally, IGF2 binds with high affinity to another receptor, IGF2R, which has no signalling activity. Instead, binding leads to the internalisation and subsequent lysosomal degradation of IGF2 (Figure 5A) (Brown et al., 2009a; Hassan, 2003), thereby adding an additional layer of regulation to IGF2 signalling. Moreover, IGF2R can also be cleaved from the cell membrane and released in the circulation, where it may inhibit IGF2 as well (Ellis et al., 1996; Scott and Weiss, 2000). Interestingly, IGF2R, like IGF2, is also an imprinted gene, but in contrast to IGF2, IGF2R is paternally silenced and exclusively expressed from the maternal allele (Figure 5A) (Barlow et al., 1991; Xu et al., 1993). Reduced levels of functional IGF2R induces tumorigenesis and is associated with several cancers, including breast cancer (Brown et al., 2009b; Byrd et al., 1999; Chappell et al., 1997; Cheng et al., 2009; O’Gorman et al., 1999).

Although IGF2 was a frequent target of MMTV in our screen, IGF2 signalling has been well described and is already known to be strongly implicated in breast cancer. Therefore, this gene was not further studied here. However, the effects and consequences of IGF2 on PI3K/AKT/mTOR pathway activation, and relevant comparisons with ERAS and IRS4, are discussed in this thesis.

**ES cell expressed Ras (ERAS)**

ES cell expressed Ras (ERAS; also known as Embryonic Ras) is a recently discovered member of the RAS superfamily of small GTPases. ERAS falls within the subfamily of RAS proteins as the founding members of the superfamily, Harvey Ras
(HRAS), Kirsten Ras (KRAS) and Neuroblastoma Ras (NRAS) (Colicelli, 2004), which display respectively 43%, 46% and 47% amino acid sequence identity with ERAS (Figure 6A) (Takahashi et al., 2003). RAS family members are central nodes in extensive signalling networks, transmitting extracellular signals from growth factor stimulated receptor tyrosine kinases to several cellular signalling pathways controlling essential processes such as cell cycle, differentiation, survival and metabolism. Therefore, the activity of RAS proteins is tightly regulated by a 'molecular switch system' of GTP-bound (active) and GDP-bound (inactive) states. This is controlled by the competing activating guanine nucleotide exchange factors (GEFs) and deactivating GTPase-activating proteins (GAPs) (Figure 6B) (Geyer and Wittinghofer, 1997; Hennig et al., 2015; Vetter and Wittinghofer, 2001). The responsible GTP-binding domain (G-domain) contains five highly conserved G-motifs: G1 (also called P-loop), G2 (also known as Switch I), G3 (or Switch II), G4 and G5 (Figure 6A) (Bourne et al., 1990, 1991). Although these G-motifs are also preserved in ERAS, the protein is unique in that it is almost exclusively found in GTP-bound state, i.e. constitutively active (Figure 6C), whereas other RAS proteins are predominantly in GDP-bound state (Takahashi et al., 2003). The constitutive activity of ERAS is considered to be due to a ERAS-specific serine residue in its P-loop motif (Ser50), as well as ERAS-specific alanine/aspartate (murine/human) and isoleucine residues in Switch II (Ala100/Asp100 and Ile101), which render ERAS insensitive to GAPs (Figure 6A) (Wey et al., 2016). In that sense, ERAS resembles GAP-insensitive mutants of HRAS, KRAS and NRAS that have mutations in the corresponding glycine-12 of the p-loop and glutamine-61 of Switch II (Scheffzek et al., 1997) and are well-known mutational hotspots in 3%, 22% and 8% of human cancer cases, respectively (Prior et al., 2012).

The constitutive activity of ERAS is likely the reason why its gene expression needs to be strictly regulated. Indeed, ERAS is normally not expressed in humans and the gene was initially even characterised as a pseudogene. Later studies reported that the gene contains a premature polyadenylation signal thought to produce a truncated non-coding transcript (Kameda and Thomson, 2005; Miyoshi et al., 1984; Zhan et al., 2005). This later proved incorrect, but probably did result in the unfortunate absence of a probe for the gene on most microarray platforms. In mice, Eras is only
expressed in embryonic stem cells (ESCs) and during early embryonic development in mice (Takahashi et al., 2003; Yasuda et al., 2007; Zhao et al., 2015). The underlying mechanism of its silencing is not yet clear, but occurs most likely on the transcriptional level (Nakhaei-Rad et al., 2015). Forced expression of ERAS induces malignant transformation and ERAS expression has been found in various cancer cell lines and in human gastric carcinoma tissues (Aoyama et al., 2010; Kaizaki et al., 2009; Kubota et al., 2010; Liu et al., 2013; Takahashi et al., 2003; Yashiro et al.,...
2009; Yasuda et al., 2007; Zhang et al., 2010). In Chapter 2, we present evidence that ERAS also an oncogenic driver of breast cancer.

ERAS further has a unique N-terminal extension of 38 amino acid, compared to other RAS family members (Figure 6A). The exact function of this N-terminus is still unknown, although it was recently shown to be essential for signalling activity of ERAS (Nakhaei-Rad et al., 2015), while not involved in the constitutive activity (i.e. permanent GTP-bound state) of the protein (Wey et al., 2016). As with HRAS, KRAS and NRAS, plasma membrane localization is also essential for signalling of ERAS, which is facilitated by several post-translational modifications of the C-terminus of the RAS proteins, containing the CAAX-motif and hypervariable region (HVR) (Figure 6A) (Ahearn et al., 2011; Lerner et al., 1995; Rocks et al., 2005; Schmick et al., 2014; Takahashi et al., 2003, 2005a; Willumsen et al., 1984; Yu et al., 2014).

Although generic RAS proteins are typically the main switches that activate the RAF/MEK/ERK/MAPK signalling cascade (Figure 6B), ERAS does not activate this pathway, with the possible exception of mouse ES cells during early embryonic development (Zhao et al., 2015). Instead, ERAS strongly activates the PI3K/AKT/mTOR pathway by directly interacting with the p110 catalytic subunit of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) (Figure 6C) (Chapter 2; Takahashi et al., 2003). Interactions of GTP-bound RAS proteins with their effectors are accomplished via a binding surface formed by their Switch I/II and inter-switch regions (Buhrman et al., 2011; Filchtinski et al., 2010; Milburn et al., 1990; Moodie et al., 1995; Nassar et al., 1996; Pacold et al., 2000; Wittinghofer and Nassar, 1996).

For HRAS, KRAS and NRAS, thus binding surface has high affinity for RAF/MEK/ERK/MAPK pathway effectors. Recently, the selective downstream signalling of ERAS to PI3K was determined to be caused by a few residue differences in these regions, most prominently tryptophan-79 (arginine-41 in HRAS, KRAS and NRAS) (Figure 6A) (Nakhaei-Rad et al., 2015). The PI3K/AKT/mTOR pathway is vital to ES cell proliferation, pluripotency and survival, and ERAS indeed plays an important role in somatic cell reprogramming and ES cell proliferation and differentiation (Polo et al., 2012; Takahashi et al., 2003, 2005b; Yu et al., 2014). However, ERAS is not required for ES cell pluripotency and ERAS-knockout mice
have no obvious phenotype, showing a normal development and fertility (Takahashi et al., 2003).

**INSULIN RECEPTOR SUBSTRATE 4 (IRS4)**

IRS4 is a member of the insulin receptor substrate (IRS) family, which further consists of IRS1, IRS2 and IRS3. Due to structural homology, the proteins DOK4 and DOK5 were considered members of this family as well (as IRS5 and IRS6, respectively) (Cai et al., 2003), but these molecules are more related to the downstream of kinase/docking protein (DOK) family (Hoxhaj et al., 2013). IRS1 and IRS2 have been extensively studied, whereas IRS3 and IRS4 have had very limited attention. For IRS3, this is likely because it lacks expression in humans, in which it is even considered a pseudogene (Björnholm et al., 2002). IRS4 is expressed in both humans as mice, but tissues distribution in extremely limited.

IRSs are signal-transducing docking proteins that pass on external signals from interacting receptors to intracellular signalling cascades. The IRS proteins are typified by their conserved N-termini, containing the pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, and numerous phosphorylation sites comprised of tyrosine, serine and threonine residues at the C-terminal side, which are considerably less conserved. Specifically, IRS4 has less than 30% overall amino acid residue identity with IRS1 and IRS2, whereas the PH and PTB domains of IRS4 display ~50% and over 60% identity with those of the other IRS family members (Giovannone et al., 2000). The PH and PTB domains are involved in associating the IRS protein to the cell membrane and activated transmembrane receptor tyrosine kinases, canonically the insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) (Figure 7) (Backer et al., 1997; Sawka-Verhelle et al., 1996; Voliovitch et al., 1995; Yenush et al., 1996, 1998). Upon binding, the ligand-bound (activated) receptor tyrosine kinase phosphorylates many of the tyrosine residues on the IRS protein. These phosphorylated tyrosine residues subsequently act as docking sites for downstream Src Homology 2 (SH2) containing proteins, leading to their activation and further downstream signalling (Figure 7) (Dearth et al., 2006; Taniguchi et al., 2006). IRSs have no intrinsic enzymatic (e.g. kinase) activity, but activate effectors
upon binding or by organising protein complexes (Hakuno et al., 2015; Myers and White, 1993; Skolnik et al., 1993; Sun et al., 1991). Directly downstream of IRSs are the p85 regulatory subunit of PI3K and the growth factor receptor bound protein 2 (GRB2), activating the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways, respectively (Figure 7).

Figure 7 | Structure, signalling and regulation of insulin receptor substrates (IRSs)

Schematic simplified representation of the structure of an IRS protein, showing the N-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains and the tyrosine (Y), and serine or threonine (S/T) moieties that can be phosphorylated (P). Green arrows indicate positive interactions (e.g. induction or stimulation). Red arrows indicate negative interactions (e.g. deactivation or inhibition).

Numerous threonine and (primarily) serine residues of the IRS proteins (mainly based on IRS1 and IRS2 research) can subsequently be phosphorylated by several signalling molecules further downstream. These phosphorylated serine and threonine moieties then predominantly induce negative feedback activity, among others via (1) dissociating IRSs from upstream activating receptor tyrosine kinases (Liu et al., 2001; Paz et al., 1997), (2) inhibiting docking of downstream effector proteins (Mothe and Van Obberghen, 1996), (3) proteasomal degradation of the IRS protein (Figure 7) (Pederson et al., 2001) and (4) triggering tyrosine dephosphorylation (Liu et al., 2001). For instance, specific phosphotyrosines in the C-terminus of IRS1 and IRS2 recruit protein tyrosine phosphatase, non-receptor type 11 (PTPN11; also known as Src-homology 2 domain-containing phosphatase 2, SHP2) that dephosphorylates tyrosine residues of the IRSs and thus consequently
prevents docking of downstream effectors (Hanke and Mann, 2009; Matsuo et al., 2010; Myers Jr, 1998; Pluskey et al., 1995; Sugimoto et al., 1994).

Despite the considerable structural homology among the IRS proteins, and the overlap in downstream effectors, the diverse phenotypes of IRS knockout models indicate distinctive functions. Mice deficient for *Irs1* (*Irs1*−/−) show strong embryonic and postnatal growth retardation, as well as a mild insulin resistance (primarily in muscle tissue) that does not progress to diabetes (Araki et al., 1994; Tamemoto et al., 1994). In contrast, *Irs2* knockout mice (*Irs2*−/−) do develop diabetes at young age due to insulin resistance (occurring predominantly in the liver), have defects in brain development and female fertility, but show hardly any growth defects (Burks et al., 2000; Kubota et al., 2000; Withers et al., 1998). *Irs1/Irs2* double knockouts are embryonically lethal (Withers et al., 1999), suggesting that these genes play essential and redundant roles in embryonic development. Expression of *Irs3* in mice is mainly restricted to adipose tissue (Lavan et al., 1997) and *Irs3*−/− mice are phenotypically similar to wild-type mice, with no detectable abnormalities (Liu et al., 1999). However, *Irs3* and *Irs1* may be redundant in important functions in adipogenesis and glucose homeostasis, as *Irs1*−/−/*Irs3*−/− mice have severe lipoatrophy and hyperglycaemia (Laustsen et al., 2002). In contrast, no redundancy was found between *Irs2* and *Irs3* in mice lacking both these genes (Terauchi et al., 2003). Knockout of *Irs4* (*Irs4*−/−) results in only minor defects in growth, reproduction and glucose homeostasis, thus comparable to a mild *Irs1*−/− phenotype (Fantin et al., 2000). Still, *Irs1/Irs4* double knockout mice show no clear aggravation of the *Irs1*-only knockout phenotype (Laustsen et al., 2002), indicating that although there appears a functional overlap between *Irs1* and *Irs4*, these genes have no obvious redundant roles. Double knockout mice lacking *Irs4* and *Irs2* or *Irs3* have not been generated, but the phenotypical differences of the single gene knockouts (Kubota et al., 2000; Liu et al., 1999; Withers et al., 1998) suggest that the *Irs* genes are at least in part functionally different and thus play unique roles. This is further supported by the observation that some cell types express more than one *Irs* gene.

In humans, *IRS1* and *IRS2* are quite universally expressed among tissues and coordinate cell growth, survival, migration, differentiation and metabolism (Giovannone et al., 2000). Unsurprisingly, disruptions concerning these proteins are
linked to human diseases and complications, like diabetes and even Alzheimer’s (Lavin et al., 2016; Moloney et al., 2010; Talbot et al., 2012; White, 2002). Elevated levels of \textit{IRS1} and \textit{IRS2} (i.e. overexpression) has been associated to various human cancers, although the involvement of these IRSs in tumorigenesis seems highly context-dependent (Chan and Lee, 2008; Dearth et al., 2007; Mardilovich et al., 2009). Of all mammals, IRS3 has only been identified in rodents (Björnholm et al., 2002). \textit{IRS4} appears silent in normal adult tissue, but the gene has been loosely associated to various human cancers. Specifically, increased levels of \textit{IRS4} have been found in hepatocellular carcinomas (HCC) compared to hepatocytes (Kameda and Thomson, 2005). IRS4 has also been associated with a (X;7)(q22;q34) translocation in paediatric T-cell acute lymphoblastic leukaemia (T-ALL), in which \textit{IRS4} acts as a translocation partner to the T-cell receptor beta (TCR) locus, causing high \textit{IRS4} expression (Karrman et al., 2009). In subungual exostosis, a benign bone- and cartilage-producing tumour, (X;6)(q22;q13-14) translocations have been reported that lead to upregulation of \textit{IRS4} (Mertens et al., 2011). Similarly, deletions in the COL4A5 and \textit{COL4A6} locus (located just downstream of \textit{IRS4}) were found to cause overexpression of \textit{IRS4} in uterine leiomyomas, benign smooth-muscle tumours (Mehine et al., 2014). Specific mutation in \textit{IRS4} have been found in melanomas (Shull et al., 2012) and meningiomas (Torres-Martín et al., 2015). Finally, \textit{IRS4} expression has been detected in IM-9 lymphoblastoid cells, U-2 OS osteogenic sarcoma cells, Hep-2 larynx carcinoma cells, A-431 epidermoid carcinoma cells, NCI-H720 lung atypical carcinoid cells, DMS-114 small cell lung carcinoma cells, HuNS1 multiple myeloma cells and ES-2 ovarian clear cell adenocarcinoma cells (Giovannone et al., 2000; Hoxhaj et al., 2013). However, concrete evidence that IRS4 is directly involved in the cancers these cell lines were derived from has not been reported. In chapter 2 of this thesis, IRS4 is identified as driver in breast cancer and the underlying mechanism of its oncogenesis is presented.
REFERENCES


CHAPTER 1 | General Introduction

3833.


Buhrman, G., O’Connor, C., Zerbe, B.,


Cooley, S., Burns, L.J., Repka, T., and Miller, J.S. (1999). Natural killer cell cytotoxicity of breast cancer targets is enhanced by two


Dubská, L., Anděra, L., and Sheard, M.A.


CHAPTER 1 | General Introduction


Jones, J.T., Akita, R.W., and Sliwkowski, M.X.


Kameda, T., and Thomson, J. a (2005). Human ERas gene has an upstream premature polyadenylation signal that results in a truncated, noncoding transcript. Stem Cells 23, 1535–1540.


CHAPTER 1 | General Introduction

13, 154.


Skolnik, E.Y., Batzer, A., Li, N., Lee, C.H.,


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CHAPTER 1 | General Introduction


Insertional mutagenesis in a HER2-positive breast cancer model reveals ERAS as an oncogenic driver synergistically collaborating with ERBB2


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ABSTRACT

To discover new genes and pathways contributing to oncogenesis in HER2+ breast cancer, we performed MMTV-induced insertional mutagenesis screens in ErbB2/cNeu-transgenic mouse models. The screens revealed 34 common integration sites (CISs) in mammary tumours of MMTV-infected mice, highlighting loci with multiple independent MMTV integrations in which potential oncogenes are activated, most of which had never been reported as MMTV CIS. The CIS most strongly associated with the ErbB2-transgenic genotype was the locus containing Eras (ES Cell Expressed Ras), a constitutively active RAS family GTPase. We show that upon expression, Eras acts as a potent oncogenic driver through hyperactivation of the PI3K/AKT/mTOR pathway, in contrast to other RAS proteins that signal primarily via the RAF/MEK/ERK/MAPK pathway and require upstream activation or activating mutations to induce signalling. We additionally show that ERAS synergistically enhances ERBB2/HER2-induced tumorigenesis and, in this role, can functionally replace ERBB3/HER3 by acting as a more powerful activator of PI3K/AKT/mTOR signalling. Although previously reported as pseudogene in humans, we observed ERAS mRNA and protein expression in a substantial subset of human primary breast carcinomas. Hence, ERAS is an important oncogenic driver collaborating with ERBB2/HER2 and may serve as a novel clinical biomarker for PI3K/AKT/mTOR pathway hyperactivation.
INTRODUCTION

In approximately 15-20% of human breast cancers, the \textit{ERBB2} gene (erb-b2 receptor tyrosine kinase 2, also known as \textit{HER2}, human epidermal growth factor receptor 2, or \textit{Neu}) is amplified, leading to its overexpression and subsequent constitutive activation of the downstream pathway. These tumours, referred to as HER2+ in the clinic, are highly aggressive and afflicted patients have a poor clinical outcome (Engstrøm et al., 2013; Ferrero-Poüs et al., 2000; Slamon et al., 1987).

To study HER2+ breast cancer, various transgenic mouse strains have been raised as models for the human disease, either with activated or wild-type \textit{Erbb2} under the control of the MMTV promoter (Andrechek et al., 2000; Bouchard et al., 1989; Guy et al., 1992; Muller et al., 1988). Mammary tumours arising in these strains closely recapitulate the histopathological and molecular features of HER2+ human breast carcinomas (Andrechek et al., 2003; Rosner et al., 2002). Moreover, in most \textit{Erbb2}-transgenic mouse strains, metastatic mammary tumours develop stochastically after a median latency of approximately six months, indicating that in addition to \textit{Erbb2} overexpression other genetic events are needed to induce malignant transformation of the mammary epithelial cell (Guy et al., 1992). Hence, mammary tumours in \textit{Erbb2}-transgenic mice arise through multiple genetic changes and represent \textit{bona fide} models for human HER2+ breast cancer.

Insertional mutagenesis in mouse models is an effective method to discover novel genes involved in breast cancer development. We and others have previously identified a series of novel candidate cancer genes using Mouse Mammary Tumour Virus (MMTV)-mediated insertional mutagenesis in mice (Callahan et al., 2012; Ikink et al., 2016; Kim et al., 2011; Klijn et al., 2013; Theodorou et al., 2007). MMTV causes a high incidence of murine mammary carcinomas by random insertion of its proviral DNA into the host DNA (Nusse, 1991). Proviral insertion is a mutagenic event that can activate adjacent proto-oncogenes (Callahan and Smith, 2008; Hilkens, 2006). The genomic localization of the proviral insertion can easily be determined using the technologies developed in our laboratory (Klijn et al., 2013; Theodorou et al., 2007), thus allowing the identification of candidate oncogenes.
We performed high-throughput insertional mutagenesis screens in MMTV-infected ErbB2-transgenic mice to identify genes and pathways that collaborate with ERBB2 overexpression in mammary tumorigenesis. Combined results of a screen using the classical Splinkerette PCR method (Theodorou et al., 2007) and a screen using our recently developed Shear-Splink technology followed by deep sequencing (Klijn et al., 2013; Koudijs et al., 2011), revealed 34 common integration sites (CISs) associated with putative oncogenes, of which the majority has not previously been reported as an MMTV CIS.

We found the Eras gene as the most common MMTV proviral target specific to ErbB2-induced mammary tumours. ERAS is a member of the RAS family of small GTPases, and shares 43%, 46%, and 47% amino acid sequence identity with the oncogenic HRAS, KRAS (Harvey/Kirsten rat sarcoma viral oncogene homolog), and NRAS (neuroblastoma RAS viral (v-ras) oncogene homolog) proteins, respectively. ERAS contains amino acid sequences identical to those present in constitutively active mutants of HRAS (e.g. HRasV12) and is therefore in a permanently active (GTP-bound) state (Miyoshi et al., 1984; Takahashi et al., 2003). Mouse Eras is only expressed in embryonic stem (ES) cells and appears to be responsible for the tumour-like growth properties of ES cells when growing ectopically (Takahashi et al., 2003). Additionally, ERAS was reported to be required for somatic cell reprogramming to generate induced pluripotent stem (iPS) cells and the differentiation of ES cells into specific lineage cells (Polo et al., 2012; Yu et al., 2014; Zhao et al., 2015). In all these processes, the activation of the PI3K/AKT/mTOR pathway by ERAS has been implicated, in contrast to the RAF/MEK/ERK/MAPK pathway generally activated by other RAS family members.

Here, we report that ERAS is an oncogenic driver that acts synergistically with ErbB2 in mammary tumorigenesis and that ERAS, as potent PI3K/AKT/mTOR pathway activator, can functionally replace ERBB3. Moreover, we show that ERAS expression occurs in a sizeable fraction of human HER2+ breast cancers.
RESULTS

MMTV INSERTIONAL MUTAGENESIS IN ERBB2+ PREDISPOSED BACKGROUND

We performed high-throughput sequencing of Mammary Tumour Virus (MMTV) integration sites in mammary tumours obtained from MMTV-infected FVB mice, transgenic for rat ErbB2 (neu) driven by the MMTV promoter (MMTV-cNeu strain). We employed both the classical Splinkerette PCR method and the more advanced 'Shear-Splink' technology combined with the Gaussian Kernel Convolution framework in the Insertional Mutagenesis Database (iMDB; http://imdb.nki.nl) pipeline. To discriminate MMTV insertions that activate genes driving tumorigenesis from passenger insertions, we identified the Common Integration Sites (CISs) among independent tumours. In total, the screens yielded 34 CISs, of which 23 (68%) were found in both screens (Table 1). 20 CISs have not been previously identified as an MMTV target, of which 14 have additionally not even been reported as CIS in the Retrovirus and Transposon tagged Cancer Gene Database (RTCGD; http://variation.osu.edu/rtcgd/) (Akagi et al., 2004) (Table 1).

Table 1 | Common insertion sites (CISs) found in the insertional mutagenesis screens in tumours from MMTV-cNeu mice

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chr.</th>
<th>Tumours with insertion (classical setup)</th>
<th>Tumours with insertion (Shear-Splink)</th>
<th>Known MMTV-target</th>
<th>CIS in RTCGD (retrovirus)</th>
<th>CIS in RTCGD (transposon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt1 / Wnt10b</td>
<td>15F1</td>
<td>16</td>
<td>14</td>
<td>Yes</td>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td>Eras</td>
<td>XA1.1</td>
<td>8</td>
<td>11</td>
<td>Yes</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Fgf3 / Fgf4</td>
<td>7F5</td>
<td>7</td>
<td>9</td>
<td>Yes</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>Fgf8</td>
<td>19C3</td>
<td>5</td>
<td>4</td>
<td>Yes</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>11B1.3</td>
<td>5</td>
<td>4</td>
<td>Yes</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Irs4</td>
<td>XF2</td>
<td>3</td>
<td>4</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gm26870 (lincRNA) / Mir101c</td>
<td>9A1</td>
<td>1</td>
<td>6</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Metn1 / Ptchd3</td>
<td>11E2</td>
<td>3</td>
<td>3</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
We compared the results in the *MMTV-cNeu* transgenic mice obtained with the 'Shear-Splink' setup in the present screen with the results from an identical screen.
in the parental FVB wild-type strain published earlier by our group (Klijn et al., 2013). Although the median tumour latency of the MMTV-infected \textit{MMTV-cNeu} transgenic mice (188 days) was strongly decreased when compared to wild-type FVB mice infected with MMTV (245 days) (Figure 1A), there was no significant difference in tumour latency between MMTV-infected and uninfected \textit{MMTV-cNeu} mice. However, only 39% (59/151) of the independent tumours from MMTV-infected \textit{MMTV-cNeu} mice contained an MMTV insertion belonging to a CIS in \textit{MMTV-cNeu} mice, compared to 93% in MMTV-infected FVB wild-type mice (Klijn et al., 2013). This indicates that tumorigenesis driven by the predisposed background is in strong competition with tumorigenesis driven by MMTV insertional mutagenesis. When comparing only the MMTV-infected \textit{MMTV-cNeu} mice with tumours driven by a CIS, the median latency was significantly decreased (176 days) compared to uninfected \textit{MMTV-cNeu} mice (188 days) (Figure 1B), suggesting that at least part of the CIS-associated MMTV integrations may accelerate mammary tumorigenesis in \textit{MMTV-cNeu} mice in collaboration with the \textit{ErbB2}-transgene.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{Impact of MMTV infection on tumour-free survival of \textit{MMTV-cNeu} and wild-type FVB mice}
\begin{enumerate}
\item[(A,B)] Kaplan-Meier curves of the MMTV-infected \textit{MMTV-cNeu} mice (n = 100) compared to the MMTV-infected parental FVB wild-type strain (n = 195) (A) and MMTV-infected \textit{MMTV-cNeu} mice with tumours carrying insertions in common insertion sites (CISs) (n = 49) compared to non-infected \textit{MMTV-cNeu} mice (n = 54) (B). Statistical significance of differences in latency was determined by pairwise log-rank test applying the \textit{survdiff} function in R (p-values in upper-right corner). All mice eventually developed tumours, only mice that prematurely died from other causes were excluded.
\end{enumerate}
\end{figure}
MMTV-TARGET \textit{Eras} IS ASSOCIATED WITH THE \textit{ErbB2/Neu} GENOTYPE

Loci harbouring genes of the \textit{Wnt}, \textit{Fgf} and \textit{Fgfr} family were among the most frequent CISs (Table 1), but these loci are also frequent hits in wild-type FVB, BALB/c and other mouse strains (Callahan et al., 2012; Klijn et al., 2013; Theodorou et al., 2007), and thus unlikely to be specific to the \textit{ErbB2}-transgenic (\textit{MMTV-cNeu}) genotype. Comparing the CIS-frequency in the \textit{ErbB2}-transgenic FVB wild-type strain, revealed even a significantly negative correlation of the \textit{Wnt} and \textit{Fgf/Fgfr} family CISs with the \textit{MMTV-cNeu} genotype (\( p = 7.64 \cdot 10^{-3}\) and \( p = 1.48 \cdot 10^{-3}\), respectively, Fisher’s exact test) (Figure 2A). Three CISs, however, significantly associated with the \textit{ErbB2} genotype, including the loci containing \textit{Lrfn5} (leucine rich repeat and fibronectin type III domain containing 5), the long intergenic non-coding RNA \textit{Gm26870} or microRNA 101c (\textit{Mir101c}), and especially \textit{Eras} (ES cell expressed Ras) (\( p = 3.57 \cdot 10^{-3}\), \( p = 1.06 \cdot 10^{-4}\) and \( p = 3.08 \cdot 10^{-7}\), respectively, Fisher’s exact test). More CISs may be associated with \textit{ErbB2} overexpression, considering that e.g. the \textit{Irs4} locus here only shows a trend towards enrichment in the \textit{ErbB2} genotype (Figure 2A), while we report in Chapter 3 that IRS4 collaborates with \textit{ERBB2} in tumorigenesis. When relating the MMTV integration data reported here with those of our previously published screens (Klijn et al., 2013; Theodorou et al., 2007), integrations in the \textit{Eras} locus were far more common in the \textit{MMTV-cNeu} genotype compared to the FVB wild-type strain (\( p = 1.28 \cdot 10^{-4}\), Fisher’s exact test), and also compared to the BALB/c wild-type, \textit{K14Cre;Trp53F/F} (BALB/c background) and \textit{PTEN}^{+/−} mice (FVB background) (Figure 2B). This indicates that integrations in the \textit{Eras} locus provides a selective growth advantage especially in \textit{ErbB2} transgenic mice.

Based on the genomic location and orientation of the MMTV proviral integrations in the \textit{Eras} locus, \textit{Eras} is the most likely gene candidate to be transcriptionally activated (Figure 2C), but the locus also contains the \textit{Pcsk1n} (proprotein convertase subtilisin/kexin type 1 inhibitor) and \textit{Hdac6} (histone deacetylase 6) genes that could also be transcriptionally activated by the MMTV insertions. To determine the actual MMTV target gene(s), we performed a side-by-side analysis of \textit{Pcsk1n}, \textit{Eras}, and \textit{Hdac6} mRNA expression in eight \textit{MMTV-cNeu} tumours with and 32 tumours without
**Figure 2** | *Eras* is an MMTV-target associated with the *ErbB2/Neu* genotype

(A) Enrichment of MMTV CISs in *cNeu*-transgenic FVB mice compared to FVB wild-type mice, calculated as percentage of all independent tumours in each group. The same analysis for gene families are shown in the lower-right corner. Significance of positive or negative enrichment was determined by a Fisher’s exact test and p-values are shown as *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 1·10^-4*. Source data of this analysis are available in Supplementary Table S1.
(B) Percentage of independent tumours with an MMTV insertion in the *Eras* locus for the mouse strains: FVB wild-type (n = 265), *MMTV-cNeu* (n = 177), *PTEN*<sup>−/−</sup> (n = 196), BALB/c wild-type (n = 292) and *K14Cre;Trp53<sup>F/F</sup>* (n = 202). Statistical significance of differences in relevant comparisons was determined by a Fisher's exact test: NS = not significant; **p < 0.001.

(C) Map of MMTV integrations in the *Pcsk1n/Eras/Hdac6* locus on the X-chromosome, showing the gene names (arrow heads indicate orientation), their translated exons (solid rectangles), UTRs (open rectangles) and the interspacing introns (lines). MMTV proviral insertions are indicated with triangles pointing in the orientation of integration. Green arrow heads represent tumours analysed in D.

(D) RT-PCR expression analysis of *Eras*, *Pcsk1n* and *Hdac6* in tumours with integration in the *Pcsk1n/Eras/Hdac6* locus, following the numbering of C and random tumours without integrations in this locus (the rest of the analysis is provided in Supplementary Figure S1A). PCR-products for β-actin were used as controls for RNA integrity.

(E) Percentage of *Eras* mRNA expressing independent MMTV-induced tumours from FVB wild-type mice (n = 48) and cNeu-transgenic FVB mice (n = 161), showing that *Eras* is significantly more commonly expressed in tumours from cNeu-transgenic mice; p-value = 0.01973 (*), determined by a Fisher's exact test.

(F) *Eras* mRNA expression in tissues from BALB/c wild-type mice, including tissues from various stages of mammary gland development and embryogenesis (showing days), determined by RT-PCR. A sample of murine ES-cells was used as positive control and β-actin expression was assessed as control for RNA integrity.

MMTV proviral insertion in the *Eras* locus using RT-PCR (Figure 2D and Supplementary Figure S1A). *Pcsk1n* was not expressed in any of the tumours, excluding this gene as the MMTV-target. *Hdac6* mRNA was present in all but one of the tumours from both groups, regardless of the presence of an MMTV insertion in the *Eras* locus. Moreover, also MMTV-induced mammary tumours from FVB wild-type mice and spontaneous tumours from *ErbB2* transgenic mice were almost all positive for *Hdac6* mRNA (Supplementary Figures S1B and S1C), indicating that expression of *Hdac6* is rather ubiquitous in mouse mammary tumours and not correlated to MMTV integrations in the *Eras* locus. Only *Eras* expression was clearly correlated with MMTV integration in the locus (p = 1.023·10<sup>−4</sup>, Fisher's exact test), showing expression in all but one tumour with an MMTV integration in the *Eras* locus, whereas no clear expression was observed in tumours without integration.

As our stringent method of assigning integration sites – designed to limit false-positive findings – inherently produces false-negatives in our screens, the number of integrations we report is likely an underestimation. Therefore, we compared *Eras*
gene expression in a panel of 161 tumours from MMTV-infected ErbB2 transgenic FVB mice (including those in Figure 2D and Supplementary Figure S1A) with the expression in a panel of 48 tumours from MMTV-infected wild-type FVB mice. In accordance with our previous results, we found that Eras was significantly more frequently expressed in MMTV-induced tumours from ErbB2-transgenic mice (15%) compared to wild-type mice (2%) (Figure 1E). This strongly suggests that Eras specifically collaborates with ErbB2 in mammary tumorigenesis. Importantly, we observed no Eras expression during any stage of murine mammary gland development, nor in any other mouse tissue or during embryogenesis (from ~E9 onwards). In line with previous reports (Takahashi et al., 2003), we exclusively observed expression of the gene in embryonic stem cells, indicating that Eras is strictly regulated at the transcriptional level (Figure 1F).

**ERAS STIMULATES CELL PROLIFERATION SYNERGISTICALLY WITH ERBB2**

To investigate the biological basis of the revealed oncogenic collaboration between Eras and ErbB2, we ectopically expressed ERAS in the human HER2+ (amplified wild-type ErbB2) breast cancer cell line SKBR3. We observed a strongly increased cell proliferation rate in cells transduced with ERAS compared to vector controls (Figure 3A and Supplementary Figure S2A). To assess the contribution of Eras and ErbB2, separately and in combination, we compared the proliferation rates of the spontaneously immortalised normal mammary epithelial cell line NMuMG transduced with Eras and ErbB2 individually, Eras and ErbB2 combined, or the empty vector control under various growth conditions (Figures 3B and 3C, Supplementary Figures S2B and S2C). Under all growth conditions, Eras expressing NMuMG cells showed a higher cell proliferation rate than the vector control. Moreover, cells expressing Eras appeared to be independent of insulin for growth, in contrast to cells that lacked Eras expression. NMuMG cells transduced with ErbB2 alone showed an increased cell proliferation rate, but not under low serum conditions (0.5%). However, when both Eras and ErbB2 were expressed in these cells, cell proliferation was synergistically increased under all tested conditions. Similar results, including the drastic enhancement of cell proliferation by combined expression of
ERAS and ErbB2, were obtained with the human breast epithelial cell line MCF10A, using either murine or human ERAS (Supplementary Figure S2D). Thus, coexpression of ERAS and ErbB2 synergistically enhances cell proliferation of mammary epithelial cells.

**Figure 3** | ERAS promotes cell proliferation synergistically with ERBB2

(A) Growth curves based on Crystal Violet staining of SKBR3 cells ectopically expressing ERAS compared to vector control cells, showing mean ± SD cell density, relative to day 0 (n = 3) (25,000 cells per well, 12-wells plates). Micrographs of these cells at day 9 are provided in Supplementary Figure S2A.

(B) Western blot showing human and murine ERAS protein in NMuMG cells stably transduced with human or murine ERAS (hERAS or mEras, respectively), ErbB2 alone, ErbB2 and ERAS, or the empty vector. β-actin was used as loading control. This anti-ERAS antibody did not react with HRas or KRas (see Supplementary Figure S2B). These results were confirmed with another antibody for ERAS (see Supplementary Figure S2C).

(C) Growth curves of NMuMG cells as in B under normal growth conditions (10% FBS + insulin), without insulin or under low serum conditions (0.5% FBS), based on Crystal Violet staining (30,000 cells per well, 12-wells plate). Plotted values are mean ± SD, relative to day 0 (n = 3).
ERAS AND ERBB2 COLLABORATE BY ACTIVATING COMPLEMENTARY PATHWAYS

To further investigate the observed insulin-independence of cells expressing ERAS and the mechanism of synergism with ErbB2, we grew MCF10A cells with and without these genes in near absence of serum (0.1%) and we omitted insulin and EGF from the medium, growth factors that are normally required for growth of these cells (a third factor, hydrocortisone, was always added). Empty vector control cells were unable to grow in absence of either insulin or EGF (Figures 4A and 4B), whereas expression of ERAS or ErbB2 provided the cells with the capability to grow in the absence of insulin or EGF, respectively. Co expression of ERAS and ErbB2 enabled cellular growth in medium depleted from serum, EGF and insulin. EGF primarily functions through the RAF/MEK/ERK/MAPK pathway via EGFR, whereas insulin predominantly activates the PI3K/AKT/MTOR signalling cascade through the insulin receptor (Figures 4C-4E). We therefore hypothesised that ERAS and ERBB2 collaborate through the activation of these complementary pathways. Indeed, it has been shown that ERAS expression activates the PI3K/AKT/mTOR pathway in mouse embryonic stem cells (Takahashi et al., 2003). Here, we show that also in mammary epithelial cells, expression of ERAS strongly induces PI3K/AKT/mTOR pathway activity, as determined by AKT-phosphorylation (Figures 4C and 4D). Moreover, MMTV-induced Eras expression in primary mammary tumours additionally correlates with PI3K/AKT/mTOR pathway activation (Figure 4F). Whereas ErbB2 expression alone appears to exclusively activate the RAF/MEK/ERK/MAPK pathway, its co expression with ERAS additionally enhances PI3K/AKT/mTOR pathway activity (Figures 4C and 4E; Supplementary Figure S3A). We explored possible differences in signalling downstream of ERAS compared to other oncogenic RAS family members. Although ERAS-induced potent activation of the PI3K/AKT/mTOR pathway in mouse mammary cells, it did not activate the RAF/MEK/ERK/MAPK pathway, in contrast to constitutive active mutants of canonical oncogenic RAS family members KRAS (KRasV12; Kirsten rat sarcoma viral oncogene homolog) and HRAS (HRasV12; Harvey rat sarcoma viral oncogene homolog) that primarily activated the RAF/MEK/ERK/MAPK pathway (Figure 4G: p-AKT, p-ERK and p-MEK).
Figure 4 | ERAS and ERBB2 activate complementary signalling pathways

(A) Representative Crystal Violet staining of MCF10A cells, stably transduced with ERAS and ErbB2 alone or co expressed, compared to vector control cells. 200,000 Cells per well (6-wells plates) were allowed to grow for 4 days in medium supplemented with 0.1% FCS, with and without insulin and EGF (as indicated).

(B) Quantification of cell proliferation in A, relative to the growth condition with both insulin and EGF (n = 4), plotted as mean + SD. Significant differences in cell proliferation rates upon omission of growth factors are shown and were determined by a Welch's t-test and p-values are shown as *p < 0.05; **p < 0.01; ***p < 0.001.
ERAS in HER2+ breast cancer insertional mutagenesis screens | CHAPTER 2

(C) Representative Western blots showing phosphorylated (p-) and total protein of AKT, MEK and ERK in MCF10A vector control cells in medium with and without insulin and EGF (as indicated), compared to MCF10A cells in medium devoid of those growth factors (starved), stably transduced with ERAS and ErbB2 alone or in combination (as indicated). All proteins were detected on the same blot and α-tubulin was used as loading control.

(D,E) Ratios phosphorylated over total AKT (D) and MEK (E), plotted as mean ± SD (n = 3) and quantified from the blots as in C. pERK/ERK ratios are provided in Supplementary Figure S3A.

(F) Western blots showing phosphorylated (p-)AKT (on the indicated residues) and total AKT protein in tumours with MMTV-induced Eras expression and non-MMTV-induced tumours that arose in ErbB2 transgenic mice. β-actin was used as loading control.

(G) Western blots showing PI3K/AKT/MTOR (AKT, S6, 4EBP1), RAF/MEK/ERK/MAPK (MEK, ERK) and apoptosis (PARP, caspase-3) signalling in MCF10A and NMuMG cells, stably transduced with human or murine ERAS (hERAS or mEras, respectively), KRasV12 or HRasV12, compared to vector control cells. Cells were grown under starved conditions or in complete growth medium. All proteins were detected on the same blot and α-tubulin was used as loading control.

(H) Representative micrographs showing apoptosis in green (fluorescent caspase-3/7 reagent) of 7-day starved MCF10A cells, stably transduced with human or murine ERAS (hERAS or mEras, respectively) and vector control cells (scale bar = 100 µm) (1,500 cells per well, 384-wells plates). The same analysis in NMuMG cell is provided in Supplementary Figure S3B.

(I) Quantification of percentages apoptotic cells of experiment in H, showing mean ± SD (n = 4-7). Welch's t-test showed no significant differences (p > 0.05). The same analysis in NMuMG cell is provided in Supplementary Figure S3C.

(J) Percentage of ErbB3 expression positive MMTV-induced tumours in tumours with (n = 52) or without (n = 27) Eras expression, showing a statistically significant difference with p-value = 0.0244 (*), determined by a Fisher's exact test.

ERAS could potentially also protect against apoptosis and hence in part explain the observed ERAS-induced cell expansion. However, cells expressing ERAS showed slightly more activation of the apoptosis signalling cascade, which was confirmed microscopically (Figures 4G-4I; Supplementary Figures S3B and S3C). Thus, ERAS does not protect cells from apoptosis, but does strongly induce cell proliferation via PI3K/AKT/mTOR pathway activation.

ERBB2 forms homo- or heterodimers with other ERBB family members, most notably with ERBB3 (erb-b2 receptor tyrosine kinase 3). In this complex, ERBB3 is required for PI3K/AKT/mTOR pathway signalling and thereby strongly contributes to tumorigenesis (Holbro et al., 2003; Lee-Hoeflsch et al., 2008). As we established that ERAS is a very potent activator of the PI3K/AKT/mTOR pathway, we hypothesised...
that it may functionally replace ERBB3. Indeed, we find significantly less ErbB3 expression among the Eras-positive tumours arising in MMTV-infected ErbB2-transgenic mice, compared to Eras-negative tumours (Figure 4J). Other MMTV-target genes may also be able to replace ErbB3, as we find that 42% of MMTV-induced ErbB2-driven tumours lack ErbB3 expression, while all spontaneous ErbB2-driven tumours expressed ErbB3 (Supplementary Figure S3D).

ERAS COLLABORATES SYNERGISTICALLY WITH ERBB2 IN TUMORIGENESIS

To assess if the collaboration of ERAS with ERBB2 indeed affects tumorigenic potential, we performed a soft agar colony formation assay, as anchorage-independent growth correlates well with in vivo tumorigenicity (Figures 5A and 5B; Supplementary Figures S4A and S4B). Both in MCF10A and NMuMG cells, expression of ERAS hardly enabled anchorage-independent growth, while ErbB2 expression did induce some colony formation. Combined expression of ERAS and ErbB2, however, resulted in a strong synergistic induction of anchorage-independent growth in these cells. Interestingly, this synergism was not observed between ERBB2 and constitutive active KRAS (KRasV12) or HRAS (HRasV12) (Figure 5B). Hence, the synergistic tumorigenic collaboration with ERBB2 seems unique to ERAS within the RAS family. Moreover, ERAS seems able to functionally replace ERBB3 in induction of anchorage-independent growth in collaboration with ERBB2 (Supplementary Figure S4C).

To further validate the observed oncogenic synergism between ERAS and ERBB2, we investigated whether combined expression of ERAS and ErbB2 in NMuMG cells indeed synergistically enhances tumour growth in vivo. To this end, we subcutaneously injected $1 \times 10^6$ NMuMG cells transduced with ERAS and ErbB2, alone or in combination, or vector control cells in both flanks of five female BALB/c nude mice and compared tumour latency and growth (Figure 5C). All mice injected with Eras-transduced cells developed tumours in both flanks, whereas mice injected with the vector control cells did not develop any tumours, even after a prolonged time period, implying that expression of Eras alone is already oncogenic in mammary epithelial cells. ErbB2 expressing cells were tumorigenic as expected, but combined with Eras expression, with confirmed ERAS protein expression (Figure 5D), strongly
accelerated tumour growth (Figure 5C), providing *in vivo* evidence that ERAS and ERBB2 indeed synergistically collaborate in tumorigenesis.
ERAS IS EXPRESSED IN A SUBSET OF HUMAN PRIMARY BREAST CARCINOMAS

To determine the clinical significance of ERAS, we used RT-PCR to examine the expression of ERAS mRNA in a large group of primary human breast carcinomas from patients treated in our institute (Supplementary Figure S4D and Supplementary Table S2). Five out of 51 tumours (10%) showed clear ERAS mRNA expression, with protein expression confirmed by immunohistochemistry (Supplementary Figure S4E), and an additional nine breast cancer samples exhibited lower levels of ERAS mRNA. Sequencing of the PCR-products excised from the gel confirmed that the amplified cDNA represented ERAS mRNA of human origin. Surprisingly, only few of the ERAS-positive tumours were also categorised as HER2+ (Supplementary Table S2), where we would expect an enrichment of ERAS-positive tumours, based on our
in vitro and in vivo studies. We therefore tested an independent uniform HER2+ human breast cancer series for ERAS expression by quantitative RT-PCR (Figure 5E). Eight out of the 30 tested HER2+ tumours (27%) could be scored as ERAS-positive, of which four cases (13%) were highly positive. This may even be an underestimation due to the low tumour percentage in some of the samples (Figure 5E). We did not observe samples with both high expression of ERAS and ERBB3 (Supplementary Figures S4F and S4G), strengthening our notion that ERAS may functionally replace ERBB3. The publicly available expression datasets of The Cancer Genome Atlas (TCGA) (RNA-seq) (Ciriello et al., 2015) and METABRIC (microarray) (Pereira et al., 2016) both confirm ERAS expression in a subset of human HER2+ breast cancers, albeit with somewhat lower percentages (TCGA: 12% ERAS+, 6% ERAS+++; METABRIC: 16% ERAS+, 7% ERAS+++ ) (Figures 5F and 5G). These results point at a role for this intrinsically active and oncogenic RAS-like gene in human breast cancer.
**DISCUSSION**

*ERBB2 (HER2/Neu)* is amplified and overexpressed in about one fifth of human breast cancers and is associated with a poor prognosis. To shed more light on HER2+ breast tumorigenesis, we performed high-throughput MMTV-mediated insertional mutagenesis screens in an *MMTV-cNeu* transgenic mouse model for HER2+ human breast cancer to discover genes collaborating with ERBB2. Here, we identify several novel candidate oncogenes and assess their correlation with oncogenic *ErbB2 (HER2/neu)*. We show that loci containing *Wnt* and *Fgf/Fgfr* family genes have a significant anti-correlation with the *ErbB2*-transgene, whereas the *Lrfn5* and *Gm26870/Mir101c* loci, but especially the *Eras* locus, are preferentially targeted by MMTV in the *ErbB2*-transgenic mice. The candidate target *LRFN5* (leucine rich repeat and fibronectin type III domain containing 5; also known as *SALM5*, Synaptic Adhesion-Like Molecule 5) is a poorly studied transmembrane adhesion molecule, involved in synapse formation and neurite outgrowth (Mah et al., 2010; Wang et al., 2008). Of the candidates in the other *ErbB2*-associated MMTV-targeted locus, the long intergenic non-coding RNA *Gm26870* has never been described, to our knowledge, whereas microRNA-101c (*mmu-mir-101c*) has been implicated in cancer before, specifically in gliomas generated in a Sleeping Beauty transposon-mediated insertional mutagenesis screen (Vyazunova et al., 2014). While it may be worthwhile to investigate these CISs in the context of HER2+ breast cancer, we focused on *Eras* in this study.

*Eras* is a unique RAS family member in various aspects. The gene encodes a constitutively active small GTPase and appears to be exclusively present in mammalian genomes (Colicelli, 2004). In mice, *Eras* has been reported to be expressed only in undifferentiated embryonic stem (ES) cells and teratocarcinomas derived from these cells (Takahashi et al., 2003), whilst in human ES cells, *ERAS expression* has not been detected (Kameda and Thomson, 2005). In mouse ES cells, *Eras* has been shown to activate the PI3K/AKT/mTOR pathway but not the RAF/MEK/ERK/MAPK pathway in mouse ES cells (Takahashi et al., 2003), which we also observe in both murine and human mammary cells upon ectopic expression. This is in contrast to the canonical RAS family members, KRAS, HRAS and NRAS,
which primarily activate the RAF/MEK/ERK/MAPK pathway instead. ERAS is not required for pluripotency of ES cells, but growth rate and tumorigenicity of ERAS-null ES cells is significantly reduced, which can be rescued by expression of activated PI3K. This has led to the conclusion that ERAS-induced PI3K/AKT/mTOR pathway activation is important for the tumour-like growth capacity of ES cells. In accordance, ectopic expression of Eras also confers tumorigenic properties to murine embryonic NIH-3T3 fibroblasts (Takahashi et al., 2003) and ERAS expression has been associated with human gastric cancer (Kaizaki et al., 2009; Kubota et al., 2010). In this study, we show that Eras is not expressed during any developmental stage of the adult mammary gland, but that the gene acts as a tumorigenic driver upon ectopic expression in mammary epithelial cells. We specifically demonstrate that Eras induces increased cell proliferation and insulin-independent growth.

Importantly, we show that ERAS and ERBB2 collaborate to synergistically enhance cell proliferation, anchorage-independent growth in vitro and tumorigenesis in vivo, which is not observed for KRAS, HRAS and NRAS. In human and mouse mammary tumours, ERBB2 is usually present in a heterodimer with ERBB3 (Hynes and MacDonald, 2009), in which ERBB2 activates the RAF/MEK/ERK/MAPK pathway, whereas ERBB3 activates the PI3K/AKT/mTOR pathway. We show that 42% of ErbB2-driven tumours with CIS-associated MMTV insertions no longer express ErbB3, in contrast to the spontaneous tumours in non-infected ErbB2-transgenic mice which express ErbB3 in 100% of the cases, suggesting that the activated MMTV-targets functionally replace ERBB3. Indeed, in its capacity to constitutively activate the PI3K/AKT/mTOR pathway, the MMTV-target Eras is able to adopt this role from ERBB3. Correspondingly, tumours expressing both ERAS and ERBB3 are rare or absent in both the human and murine setting.

Thus, we establish ERAS as a novel oncogenic driver that synergistically induces tumorigenesis with ERBB2, and, by hyperactivating the PI3K/AKT/mTOR pathway may functionally replace ERBB3 in this context.
CHAPTER 2 | ERAS in HER2+ breast cancer insertional mutagenesis screens

MATERIAL & METHODS

PATIENT SAMPLES

Our pathology department provided 86 random human primary breast carcinoma samples and associated clinical parameters from women treated between 2007 and 2011 at the Netherlands Cancer Institute or affiliated hospitals. Additionally, the Netherlands Cancer Institute’s Core Facility Molecular Pathology & Biobanking (CFMPB) supplied us with RNA samples of 30 randomly selected tumours from a uniform set of 129 HER2+ primary breast carcinomas, obtained from patients treated between 1989 and 2006 at our institute. All patients individually approved the use of tumour tissue for research purposes via opt-out and specific use for this study was approved by the local Translational Research Board, following positive recommendation of the Medical Ethical Committee.

INSERTIONAL MUTAGENESIS SCREENS AND TUMORIGENICITY ASSAYS

The MMTV-induced insertional mutagenesis screens followed the previously reported classical Splinkerette PCR method (Theodorou et al., 2007) or the ‘Shear-Splink’ method (Klijn et al., 2013). Briefly, new-born FVB/N-Tg(MMTVneu)202Mul/J mice (MGI ID: 1930204, here referred to as MMTV-cNeu), originally generated by William Muller (Guy et al., 1992) and bred at the Netherlands Cancer Institute, were infected with the C3H strain of Mouse Mammary Tumour Virus (C3H-MMTV) by foster nursing on BALB/c+ females harbouring the milk transmitted MMTV. These mice contain the activated rat Neu gene under an MMTV promoter, typically expressing in normal mammary epithelium, salivary gland, and lung tissue. Mice were forced bred. Individual tumours arise in hyperplastic mammary glands. When the developing mammary tumours reached 1 cm³, the tumour DNA was isolated and either enzymatically digested (classical method) or sheared (Shear-Splink method), after which the fragments were ligated to Splinkerette linkers to enable PCR amplification and sequencing. Mapping the sequences of the MMTV insertion sites to the genome and predicting the target gene(s) was either assessed manually based on the position and orientation of the integration (classical method) or using
our automated analysis platform, the Insertional Mutagenesis Database (imdb.nki.nl) (Shear-Splink method). The "survival" package in the statistical programming language R was used to plot the tumour-free survival of the mice in the insertional mutagenesis screen as Kaplan-Meier curves and to calculate log-rank tests.

Tumorigenicity was assessed by measuring two-dimensional tumour growth at least twice a week, to a maximum of 1 cm³ (end-point), following subcutaneous injection of \(1 \cdot 10^6\) viable cells in 200 µl phosphate-buffered saline (PBS) in both flanks of 4-week old female \(BALB/cABomA-nu/nu\) (BALB/c nude) mice, bred in our institute. Sample size was chosen based on previous experience in s.c. tumour growth studies. No animals were excluded in these studies, except animals that died prematurely because of causes unrelated to the experiment. The observer was blinded to the type of treatment when measuring tumour size.

All mouse experiments were approved by the local Animal Experiments Committee (DEC) and strictly followed the Dutch Code of Practice for Research with Laboratory Animals in Cancer Research.

**Expression Analysis**

RNA from cells and tumours was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) or the HighPure RNA isolation kit (Roche, Penzberg, Germany), treated with DNase and reverse transcribed to cDNA using the SuperScript III kit (Invitrogen), Tetro cDNA synthesis kit (Bioline, London, UK) or Maxima First Strand cDNA Synthesis kit for RT-qPCR (Thermo Scientific, Waltham, MA). Expression analysis was performed using the MyTaq Red DNA Polymerase kit (Bioline). All was performed according to the manufacturers’ protocols.

Murine samples were analysed for \(\text{Eras}\) expression with a primer set running from the five-prime untranslated region (5'-UTR) into the translated region, spanning the only intron in the genomic sequence (forward primer: 5'-CCCTCATCAGACTGCTACTCCTGG-3' and reverse primer: 5'-TCCTGCCCAGATGTATCCAGAACA-3'). The primer pair for murine \(\text{Hdac6}\) was designed to span multiple introns and both primers annealed to the translated region of the gene (forward primer: 5'-AACCCTGAGACAAGAGTCGC-3' and reverse
primer: 5’-GAAGCTCCCCTTCATTCATG-3’). *Pcsk1n* expression was assessed using forward primer 5’-GACGAGACTCTCTGACGTGGA-3’ and reverse primer 5’-TCCTCAGTAGCACAGCCACT-3’ and was confirmed with an independent primer pair (forward primer: 5’-CCGGCCCCCAGTGATGATGAT-3’ and reverse primer: 5’-GAGTACCCCGCAAAGTCCA-3’), both running from the translated region into the 3’-UTR and spanning an intron. β-Actin (*Actb*) expression (forward primer: 5’-TGAGACCTTTCAACACCCAG-3’ and reverse primer: 5’-GAGCCAGAGCAGTAATCTCC-3’) was used to assess cDNA quality. The human *ERAS* gene does not contain an intron. *ERAS* expression in human samples was assessed with a primer pair running from the translated region into the 3’-UTR of the gene (forward primer: 5’-GAAGGAGCCCCATGGCAAGGT-3’ and reverse primer: 5’-GGCCTGGGGAAAGGTCTACA-3’) and a pair of which both primers annealed to the translated region of the gene (forward primer: 5’-CATCCAGCTGAACCACCAGT-3’ and reverse primer: 5’-GTTTTGGCCGAGGTCTCC-3’), yielding identical results. cDNA quality was assessed by analysing human *GAPDH* (forward primer: 5’-GCCAAGGTCATCCATGACAACT-3’ and reverse primer: 5’-GAGGGGCCATCCACAGTCTT-3’) or *ACTB* (β-actin) expression (forward primer: 5’-CCAACCGCGAGAAGATGA-3’ and reverse primer: 5’-CCAGAGCCGTACAGGGATAG-3’).

**IMMUNOHISTOCHEMISTRY**

Immunohistochemistry was performed on 4 µm sections of paraffin-embedded EAF-fixed tumours using a 1:600 diluted polyclonal antibody targeting ERAS (#AP1470a; Abgent, San Diego, CA), following a heat-induced antigen retrieval using a 6.1 pH citrate buffer (Target Retrieval Solution; Dako, Carpinteria, CA).

**CELL CULTURE**

SKBR3 cells were the generous gift of Joyce Taylor-Pappadimitriou (Guy’s Hospital, London, UK) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% Foetal Bovine Serum (FBS) (Perbio,
Erembodegem, Belgium), 50 units/ml penicillin and 50 µg/ml streptomycin (Pen/Strep) (Gibco, Carlsbad, CA), and 5 µg/ml insulin (SAFC Biosciences, Lenexa, KS). NMuMG and MCF10A cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured in DMEM/F-12 mix (Invitrogen) containing 10% FBS, Pen/Strep and 5 µg/ml insulin. Additionally, 200 ng/ml hydrocortisone (Gibco) and 20 ng/ml human recombinant epidermal growth factor (EGF) (Sigma-Aldrich, St Louis, MO) was added to the medium of MCF10A cells. BT474 cells (ATCC) were cultured in DMEM supplemented with 10% FBS and Pen/Strep. All cells were cultured under an atmosphere of 5% CO₂ at 37°C.

VECTORS AND TRANSDUCTION

Constructs containing full-length human and murine ERAS-cDNA were a kind gift from Liesbeth Vredeveld (Netherlands Cancer Institute, Amsterdam, the Netherlands). These genes were cloned into pBABE-plasmids with the puromycin resistance gene, using the restriction sites of BamHI and Sall. The pcDNA3-NeuNT construct was kindly provided by Bill Muller (McGill Cancer Center, Montreal, Canada) and the activated rat Neu (ErbB2)-cDNA from this construct was cloned into a pMSCV-plasmid carrying the blasticidin resistance marker, using HindIII and EcoRI restriction sites. pBABE-vectors containing human RasV12 and KRasV12 were obtained from the labs of Daniel Peeper and René Bernhards (Netherlands Cancer Institute, Amsterdam, the Netherlands), respectively. Empty backbones of these vectors were used as controls.

Phoenix packaging cells were transfected by calcium-phosphate precipitation with the constructs above to produce ecotropic retroviruses. The culture medium was harvested 48 h post-transfection, passed through a 0.45 µm filter (Whatman, Maidstone, UK) and used for infection of target cells after adding 6 µg/ml polybrene (Millipore, Billerica, MA). Expression of the transduced genes was confirmed by RT-PCR and sequencing, following selection in medium containing 5 µg/ml blasticidin (Neu-construct) or 2 µg/ml puromycin (all other constructs) for at least 5 days. For human cells, the murine ecotropic receptor was first transduced with an amphotropic retrovirus to enable ecotropic retroviral infection.
CELL PROLIFERATION, TREATMENTS APOPTOSIS AND DOSE RESPONSE ASSAYS

After seeding (amounts of cells and type of plates used are indicated in the figure legends), cells were allowed to attach (6-16h) and were washed with medium supplemented with Pen/Strep only and cultured under the indicated conditions. The PI3K inhibitor GDC0941 (Selleck Chemicals) was dissolved DMSO. Either these drugs or equal volumes of vehicle (control) was added to the medium after the cells were allowed to attach overnight.

For cell proliferation assays, cells were fixed in 4% formaldehyde and stained with 0.1% Crystal Violet solution (Sigma-Aldrich). Apoptosis and dose response assays were analysed using the IncuCyte live cell imager (Essen BioScience, Ann Arbor, MI), which determines cell density based on phase contrast images. Apoptosis was assessed using the Kinetic Caspase-3/7 Apoptosis Assay Reagent (Essen BioScience), following manufacturer's instructions.

WESTERN BLOTTING

Cells were lysed for 30 min at 4°C in ice-cold 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 20 mM EDTA, 1% NP40, Complete Mini protease inhibitor and PhosStop phosphatase inhibitor (both from Roche). Lysates were centrifuged at 4°C and 13,000 rpm for 10 min, after which the protein concentrations of the supernatants were determined using the Micro BCA protein assay kit (Thermo Scientific, Waltham, MA) and the protein content was equalised over all samples. The Novex NuPAGE or Bolt electrophoresis systems (Life Technologies, Carlsbad, CA) were used to separate the protein samples on a 4-12% Bis-Tris gel (Life Technologies) and to perform Western blotting on a nitrocellulose membrane (Whatman). Prior to immunostaining, the membranes were blocked using the Odyssey PBS Blocking Buffer (LI-COR Biosciences, Lincoln, NE). ERAS was detected using a 1:500 diluted polyclonal antibody from Abgent (#AP1470a) and the associated blocking peptide (#BP1470a) was used for the neutralizing controls. ERAS-specificity was confirmed by staining the same samples with an independent ERAS-targeting antibody from Everest Biotech (Upper Heyford, UK) (#EB11825), diluted 1:500. Furthermore, in
semi-serial sections of subcutaneously injected NMuMG cells ectopically expressing Eras, the same regions stained positive by immunohistochemistry also stained positive by RNA in situ hybridization using an ERAS specific probe (employing the RNA-scope assay from Advanced Cell Diagnostics, Newark, CA, USA), further proving the specificity of the ERAS-antibodies. Total AKT antibody (#sc-8312) from Santa Cruz Biotechnology (Dallas, TX) was used in 1:1000 dilution, β-actin antibody (#GTX26276) from GeneTex (Irvine, CA) and α-Tubulin antibody (#T9026) from Sigma-Aldrich in 1:5000. Other antibodies used were purchased from Cell Signaling Technology (Beverly, MA) and diluted: 1:1000 p-AKT S473 (#4060), 1:2000 p-MEK Ser217/221 (#9154), 1:1000 total MEK (#4694), 1:2000 p-MAPK (p-ERK) T202/Y204 (#9106), 1:1000 total MAPK (ERK) (#9102), 1:2000 p-S6 S235/236 (#4858), 1:1000 total S6 (#2217), 1:1000 p-4EBP1 T37/46 (#2855), 1:1000 total 4EBP1 (#9644), 1:1000 PARP (#9542) and 1:1000 cleaved caspase-3 (#9661). Following binding with the appropriate IRDye 680 or IRDye 800 fluorescent dye labelled secondary antibodies (LI-COR), protein signal was imaged and quantified using the Odyssey Infrared Imaging System and Image Studio software (both from LI-COR), respectively, allowing simultaneous imaging in two channels. All imaged and quantified protein signals in a single experiment were obtained from the same blot, stripping the blot with the Newblot Nitro Stripping Buffer (LI-COR) between immunostainings, following manufacturer's recommendations, if required. Quantifications of protein signals were always normalised to the loading controls in the associated lanes.

**SOFT AGAR ASSAYS**

On a 0.6% low-gelling temperature agarose (Sigma-Aldrich) base layer in culture medium, a top layer was poured of cells suspended in 0.35% low-gelling temperature agarose in culture medium. Number of cells and used plates are indicated in figure legends. The GelCount scanner (Oxford Optronix, Milton, UK) was used to measure anchorage-independent colony formation (time-point of measurements are indicated in figure legends).
STATISTICAL ANALYSES

The number of biological replicates of the tests are provided in the legend of the figures (n=3 or more). The statistical programming language R, including the "Bioconductor" and "Survival" packages were used for statistical analyses. All the statistical tests that were used are specified in the text and/or figure legends. A p-value < 0.05 was considered statistically significant. Distributions and variances of samples (test and control groups) were determined in R and were similar.

ACKNOWLEDGEMENTS

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REFERENCES


Kameda, T., and Thomson, J. a (2005). Human ERas gene has an upstream premature polyadenylation signal that results in a truncated, noncoding transcript. Stem Cells 23, 1535–1540.


Supplementary Figure S1 | Eras expression is associated with MMTV proviral integration in the Pcsk1n/Eras/Hdac6 locus

(A) Additional part of RT-PCR expression analysis of Eras and Hdac6 in random tumours without integrations in the Pcsk1n/Eras/Hdac6 locus shown in Figure 2D. PCR-products for β-actin were used as controls for RNA integrity.

(B, C) RT-PCR expression analysis of Eras and Hdac6 in random tumours of non-MMTV-infected ErbB2 transgenic mice (B) and random tumours of MMTV-induced mammary tumours from FVB wild-type mice without integrations in the Pcsk1n/Eras/Hdac6 locus (C). PCR-products for β-actin were used as controls for RNA integrity.
Supplementary Figure S2 | ERAS in various mammary cell cultures

(A) Micrograph of SKBR3 vector controls or SKBR3 cells ectopically expressing ERAS, at day 9 of Figure 3A. Scale bar = 100 µm.

(B) Western blot showing human and murine ERAS in NMuMG cells, stably transduced with human or murine ERAS (hERAS or mEras, respectively) and HRasV12 and KRasV12 alone or co expressed with ErbB2, compared to vector control cells. The ERAS signal was abolished when the blocking peptide was added with the antibody. β-actin was used as loading control.

(C) Western blot analysis of Figure 3B, using a different ERAS-specific antibody.

(D) Crystal Violet staining of MCF10A cells, stably transduced with murine or human ERAS (mEras or hERAS, respectively) alone or co expressed with ErbB2, compared to vector control cells. 200,000 Cells per well (6-wells plate) were allowed to grow for 4 days in low serum conditions (0.5%).
Supplementary Figure S3 | ERAS and ERBB2 activate complementary signalling pathways

(A) Ratios phosphorylated over total ERK, plotted as mean + SD (n = 3) and quantified from the blots as in Figure 4C.

(B) Representative micrographs showing apoptosis in green (fluorescent caspase-3/7 reagent) of 7-day starved NMuMG cells, stably transduced with human or murine ERAS (hERAS or mEras, respectively), compared to vector control cells (scale bar = 100 µm) (3,000 cells per well, 384-wells plate).

(C) Quantification of percentages apoptotic cells of experiment in B, showing mean + SD (n = 4-7). Welch’s t-test shows no significant differences (p > 0.05).

(D) Percentage of ErbB3 expression positive tumours that developed in ErbB2-transgenic mice that were (n = 33) or were not infected by MMTV (n = 27), showing a statistically significant difference with p-value = 8.652·10^{-5} (**), determined by a Fisher’s exact test.
ERAS in HER2+ breast cancer insertional mutagenesis screens | CHAPTER 2

A

B

C

D

human primary breast tumors

ERAS

GAPDH

E

ERAS++ (09T264)

ERAS- (09T282)

F

G

ERBB3-expression

ERAS-expression

Tumor%
Supplementary Figure S4 | ERAS can functionally replace ERBB3 in collaboration with ERBB2

(A) Representative whole well scans of MCF10A cells, stably transduced with murine or human ERAS (mEras or hERAS, respectively) compared to vector controls, alone or co-expressed with ErbB2 and allowed to grow in soft agar for four weeks (20,000 cells per well, 6-wells plates).

(B) Quantification of soft agar growth of A, plotted as mean + SD (n = 4). Statistical significance of differences in relevant comparisons was determined by a Welch’s t-test and p-values are shown as **p < 0.01; ***p < 0.001.

(C) Quantification of anchorage-independent colony formation of MCF10A cells, stably transduced with ERAS, ErbB3 and/or ErbB2, allowed to grow in soft agar for two weeks (40,000 cells per well, 6-wells plates). Data are presented as mean + SD (n = 3). Statistical analysis of relevant comparisons was performed using the Welch’s t-test: **p < 0.01; NS = not significant.

(D) RT-PCR expression analysis of ERAS in 51 random human primary breast carcinomas. PCR-products for GAPDH were used as controls for RNA integrity. Pathological and clinical characteristics of these samples are provided in Supplementary Table S2.

(E) Representative micrographs of immunostaining for ERAS (brown) of human primary breast carcinomas with (left) and without (right) ERAS expression, as determined in D and Supplementary Table S2. Inserts represent enlargements of the framed areas. Scale bar low magnification 200 µm and insert 10 µm.

(F) Quantitative RT-PCR of ERBB3 mRNA expression in 30 HER2-positive primary breast carcinomas, presented in the same order as Figure 5E. The estimated tumour percentages are indicated below each sample: N/A, not available.

(G) Scatter plot of ERAS expression versus ERBB3 expression of the 30 HER2-positive primary breast carcinomas as shown in Figure 5E and Supplementary Figure S4F, respectively. None of the samples show combined high expression of both genes.
**Supplementary Table S1** | Source data of CIS-enrichment in cNeu-transgenic vs FVB wild-type mice

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<th>Locus</th>
<th>ins_FVBwt</th>
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**Potential target gene symbols of the MMTV proviral insertions (Locus), the number of independent tumours with insertion in this locus in the FVB wild-type mice (ins_FVBwt) and MMTV-cNeu mice (ins_cNeu), their respective frequencies calculated over each total of independent tumours (freq_FVBwt and freq_cNeu), the percentage of insertion frequency enriched in MMTV-cNeu compared to FVB wild-types (pref_cNeu) and the associated p-values calculated using a Fisher's exact test for count data (pVal).**
## Supplementary Table S2 | Pathological and clinical characteristics

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*Scored ERAS expression from Supplementary Figure S4D, shown as ++ (highly positive), + (positive), +/- (weak), +/- (very weak), - (negative); ER = oestrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; reg LN = regional lymph node metastasis; N/A = no data available. ER, PR and p53 status are determined by an IHC-test and are shown as % positive staining or positive/negative (pos/neg), scored by a pathologist; HER2 status is determined by an IHC-test and is shown as: 0 (negative), 1+ (negative), 2+ (borderline), 3+ (positive: HER2 protein overexpression).
IRS4 induces mammary tumorigenesis through constitutive PI3K/AKT/mTOR pathway hyperactivation

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Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Adapted from:
Ikink et al. (2016), Nature Communications, doi: 10.1038/ncomms13567
ABSTRACT

In search of oncogenic drivers and pathways in breast cancer, we identified *Irs4*, a poorly studied member of the Insulin Receptor Substrate (IRS) family, as mammary oncogene by insertional mutagenesis. Whereas normally silent in the postnatal mammary gland, *IRS4* is found to be highly expressed in a subset of breast cancers, in particular in the HER2+ and triple negative subtypes. We show that *Irs4* expression in mammary epithelial cells induces constitutive PI3K/AKT/mTOR pathway hyperactivation, insulin/IGF1-independent cell proliferation, anchorage-independent growth and *in vivo* tumorigenesis. The constitutive PI3K/AKT/mTOR pathway hyperactivation by IRS4 is unique to the IRS family and we identify the lack of a SHP2-binding domain in IRS4 as the molecular basis of this feature. Finally, we show that IRS4 and ERBB2/HER2 synergistically induce tumorigenesis *in vitro* and *in vivo*. Taken together, our findings present the cellular and molecular mechanisms of IRS4-induced tumorigenesis and establish IRS4 as an oncogenic driver and biomarker for PI3K/AKT/mTOR pathway hyperactivation in breast cancer.
INTRODUCTION

The identification of oncogenes and their associated pathways is crucial for understanding cancer. Insertional mutagenesis is an efficient tool to identify new oncogenes (Hilkens, 2006; Kool and Berns, 2009). We recently discovered several novel proto-oncogenes in high throughput Mouse Mammary Tumour Virus (MMTV)-induced insertional mutagenesis screens (Klijn et al., 2013; Theodorou et al., 2007), including the Insulin Receptor Substrate 4 (Irs4).

The Insulin Receptor Substrate (IRS) family consists of four closely related members, IRS1-IRS4, and two distant relatives, IRS5/DOK4 and IRS6/DOK5. IRS1 and IRS2 are the best studied members and are shown to have important roles in cell proliferation, survival, migration, metabolism and differentiation, and have been implicated in cancer (Chan and Lee, 2008; Dearth et al., 2007; Mardilovich et al., 2009). The Irs3 gene is only found in rodents and is a pseudogene in humans (Björnholm et al., 2002). IRS4 was first identified and characterised in the HEK293 human embryonic kidney cell line in which it was shown to undergo rapid tyrosine-phosphorylation in response to insulin (Kuhné et al., 1995; Lavan et al., 1997).

IRSs are cytoplasmic scaffolding proteins that act as signal transmitters between multiple receptor tyrosine kinases (RTK), including the IGF1 and insulin receptors, and several other Src homology 2 (SH2) domain-containing proteins (Dearth et al., 2007; Taniguchi et al., 2006). Upon binding ligand-activated RTKs, numerous tyrosine residues in the large C-terminal region of IRSs are phosphorylated. These phosphorylated tyrosine sites can subsequently serve as binding sites for downstream cytoplasmic SH2-containing effector proteins, including p85 and GRB2, leading to activation of the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK signalling pathways, respectively (Chan and Lee, 2008; Saltiel and Kahn, 2001; Taniguchi et al., 2006). Additionally, it has been reported that phosphorylation of two specific tyrosine residues in the C-terminus of IRS1 and IRS2 leads to binding of tyrosine phosphatase SHP2, providing a negative feedback loop by dephosphorylating the tyrosine residues responsible for e.g. p85-binding (Hanke and Mann, 2009; Matsuo et al., 2010; Myers Jr, 1998; Pluskey et al., 1995; Sugimoto et al., 1994).
In this study, we establish *Irs4* as a novel mammary oncogene and we show that absence of a negative feedback regulation in IRS4 leads to constitutive PI3K/AKT/mTOR signalling, which functionally differentiates it from IRS1 and IRS2. Finally, we demonstrate that *IRS4* is expressed in a subset of human breast cancers, in particular in the HER2+ and triple negative subtypes, and collaborates with ERBB2/HER2 to drive tumorigenesis.
RESULTS

IRS4 IS TARGETED BY MMTV PROVIRAL INTEGRATION

We have previously performed high-throughput retroviral insertional mutagenesis screens of Mouse Mammary Tumour Virus (MMTV)-induced mammary tumours in clinically relevant mouse models of breast cancer and wild-type mice (Klijn et al., 2013; Theodorou et al., 2007). From these studies and from a screen performed in mice transgenic for activated rat ErbB2 (Chapter 2), we obtained MMTV proviral insertion data from a total of 1132 tumours, of which 35 (3.1%) had insertions that map in the Irs4 locus on the X-chromosome. The chromosomal position and orientation of all but one of the insertions is such that either the promoter or enhancer sequences in the proviral LTR can activate Irs4 (Figure 1A and Supplementary Table S1). The insertions were unlikely to activate expression of the adjacent Col4a5 gene or the 350 kb upstream Gucy2f gene, which is also in opposite orientation to be affected. Indeed, Irs4 expression, as determined by RT-PCR analysis, highly correlated with an MMTV proviral insertion in the Irs4 locus (p = 1.61·10⁻³, Welch's t-test), while Col4a5 expression did not (p = 0.52, Welch's t-test) (Figures 1B and 1C), which confirms that Irs4 is indeed an MMTV-target and suggests that Irs4 may act as an oncogene. There was no significant difference of integration frequency in the Irs4 locus between the tested mouse genotypes (p = 0.408, Pearson's Chi-squared test) (Figure 1D), although this could be due to the low incidence of insertions near Irs4 in each individual group.

IRS4 IS THE ONLY IRS FAMILY MEMBER TARGETED BY MMTV

While Irs4 is thus frequently activated by MMTV, we did not find the closely related genes Irs1, Irs2 and Irs3 genes as targets in our insertional mutagenesis screens. This suggests that Irs4 has features that are unique in the Irs gene family. Further supporting this, we observed Irs4 expression restricted to embryonic tissues and adult brain and testis, whereas Irs1-Irs3 were found expressed quite ubiquitous, in accordance with publicly available microarray data (Figure 1E and Supplementary Figures S1A and S1B). In contrast to the other family members, Irs4 was neither
CHAPTER 3 | IRS4 drives cancer by PI3K/AKT/mTOR pathway hyperactivation

**Figure 1** | MMTV proviral insertions in the Irs4 locus and expression analysis

(A) Insertion map of a 300 Kb section of the X-chromosome comprising the Irs4 locus. The map shows the targeted Irs4 gene (blue) and non-targeted Col4a5 gene (grey). Rectangles indicate exons, where solid rectangles depict translated exons and open rectangles the UTRs. The lines interspacing the exons indicate introns. Arrowheads indicate the position and orientation (based on Ensembl build 67, NCBI m37) of MMTV proviral insertions in independent tumours, black arrowheads indicate insertions in which the MMTV-LTR putatively will act as a promoter, green or orange arrowheads (upstream or downstream, respectively) indicate insertions potentially acting as enhancer of the endogenous Irs4 promoter. One integration in this locus (white triangle) is more likely a spurious insertion improbable to activate Irs4 (but might activate Col4a5).

(B,C) Expression of Irs4 (B) and Col4a5 (C) mRNA in a random series of independent MMTV-induced mammary tumours with (n = 13) and a random series without (n = 16) an insertion in the Irs4 locus, showing a strong correlation between MMTV insertion in the Irs4 locus and Irs4 expression, but no correlation with Col4a5 expression. **p < 0.01; NS, not significant (Welch’s t-test).

(D) The percentage each genotype is contributing to the tumours with (n = 35) and tumours without (n = 1097) insertions in the Irs4 locus. No preference for Irs4 insertions in mammary tumours from any of the tested genotypes: NS, not significant (Pearson’s Chi-squared test).

(E) mRNA expression of Irs gene family members at various stages of adult mammary gland development and embryogenesis (d, days) of wild-type BALB/c mice.

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expressed at any stage of post-natal mammary gland development nor in human breast tissue (Figure 1E and Supplementary Figure S1C). Also, IRS4 expression
was only observed in two of 25 tested human breast cancer cell lines, MDA-MB-453 and HCC187 cells, and in HEK293 cells (Supplementary Figure S1D). This limited expression of IRS4 in human cell lines is in line with publicly available microarray data (Supplementary Figure S1E). Thus, Irs4 is a normally silent gene in mammary tissue with potential oncogenic properties unique to its gene family.

IRS4 ACTS AS ONCOGENIC DRIVER IN MAMMARY EPITHELIAL CELLS

In order to validate Irs4 as a genuine oncogene, we transduced full-length Irs4-cDNA into the human mammary epithelial cell line MCF10A (MCF10A-Irs4) and performed a soft agar colony formation assay. The mere expression of Irs4 in these cells induced anchorage-independent colony formation, while vector control cells did not (p = 9.25·10^{-5}, Welch's t-test) (Figures 2A and 2B), suggesting Irs4-induced tumorigenic potential.

To further substantiate the oncogenic capacity of Irs4, we derived a stable epithelial cell line (designated P3724-R4) from an MMTV-induced K14Cre;Trp53F/F mammary tumour from our insertional mutagenesis screen, with a known proviral integration in the Irs4 locus and a consequently high Irs4 expression. After confirming tumorigenicity of this P3724-R4 cell line in vivo, we used lentiviral shRNA to knockdown Irs4 levels in these cells (Figures 2C and 2D; Supplementary Figures S2A and S2B). The two P3724-R4 cultures with the most effective Irs4-knockdown grew significantly slower than the GFP-shRNA negative control, especially under low-serum (0.5%) conditions, while apoptosis rates were not affected (Figures 2E-2G; Supplementary Figure S2C). When 2·10^6 of these cells were subcutaneously injected into both flanks of five female BALB/c nude mice, none of the mice injected with either of the two Irs4-knockdowns developed tumours, whereas we observed tumour growth in both flanks of all five mice injected with 2·10^6 GFP-shRNA control cells after two weeks (p = 1.95·10^{-4}, log-rank test) and in a subsequent experiment with 1·10^6 injected cells after three weeks (p = 2.51·10^{-4}, log-rank test) (Figure 2H). Hence, knockdown of Irs4 abolished the tumorigenic potential of these mammary tumour cells, showing that the cells are dependent on IRS4 for tumour growth in vivo.
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Figure 2 | *Irs4* expression in mammary cells drives tumour growth

(A) Soft agar growth of MCF10A cells, stably transfected with *Irs4* or empty vector, allowed to grow in soft agar for two weeks. Representative whole well scans and phase-contrast micrographs of three experiments are shown (scale bar = 30 µm).

(B) Quantification of anchorage-independent growth (mean + SD) from three soft agar assays as shown in A, using independently transduced MCF10A cell cultures and each tested in duplicate. ****p < 1·10^{-4} (Welch’s t-test, compared to vector).

(C) Tumour growth in mice subcutaneously injected in both flanks with 1·10^{6} P3724-R4 tumour cells (n = 2, i.e. 4 flanks). Data are represented as mean + SD.

(D) Western blot of lysates from P3724-R4 cells transduced with the indicated shRNAs showing IRS4-knockdown efficiency at the protein-level.

(E,F) Proliferation rate of P3724-R4 cells upon knockdown of *Irs4* using shirs4#21 or shirs4#24 versus P3724-R4 control cells transfected with a short hairpin for GFP (shGFP), in 10% or 0.5% serum (FBS), showing representative Crystal Violet staining images at day 6 (E) and growth curves depicted as the mean ± SD of three experiments (F). NS, not significant; *p < 0.05, **p < 0.01 (Welch’s t-test, compared to shGFP).

(G) Percentage of apoptotic cells (Annexin V-positive) in three independent viable (propidium iodide-negative) populations of 48 h starved P3724-R4 cells as in E, determined by FACS. NS, not significant (Welch’s t-test).

(H) Tumour-free survival of groups of five mice each, subcutaneously injected with 1·10^{6} or 2·10^{6} P3724-R4 cells as in E. ****p < 0.001 (log-rank test).
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(I) Tumour-free survival of mice subcutaneously injected with $1 \cdot 10^6$ NMuMG cells ectopically expressing *Irs4* versus vector control cells (n = 5 for each group, i.e. 10 flanks). **p < 0.01 (log-rank test).

Next, we transduced the full-length *Irs4*-cDNA into the murine mammary epithelial cell line NMuMG (NMuMG-Irs4). Upon subcutaneous injection in both flanks of five female BALB/c nude mice, tumour growth was observed in all flanks injected with NMuMG-Irs4 about five weeks post-injection, whereas the mice injected with vector control cells remained tumour-free for more than 10 weeks ($p = 3.13 \cdot 10^{-3}$, log-rank test), after which tumour growth was sporadically observed in a single flank (Figure 2I and Supplementary Figure S2D). Collectively, these results confirm that *Irs4* is an oncogenic driver when expressed in mammary epithelial cells.

**IRS4 CAUSES GROWTH FACTOR-INDEPENDENT CELL PROLIFERATION**

Assessing the cellular basis of the oncogenic properties of IRS4, we found that proliferation of the MCF10A-Irs4 cells was higher primarily under conditions without insulin or IGF1, compared to vector control cells (Figures 3A-3C; Supplementary Figure S3A). The effect on proliferation was most apparent in growth medium containing low (0.5%) or no foetal bovine serum (FBS), as FBS contains these growth factors. In fact, proliferation of cells expressing *Irs4* was hardly affected by removal of stimuli, in contrast to vector controls which only showed sporadic growth (Supplementary Figure S3B). Similar observations were made for NMuMG cell proliferation (Supplementary Figures S3C and S3D), although these cells required at least 0.5% FBS for growth. This growth factor-independent proliferation capacity appears unique to *Irs4* in the *Irs* gene family as these mammary epithelial cells endogenously express *Irs1* and *Irs2* (Supplementary Figure S3E). To exclude the possible effect of expression-level differences, we stably transduced *Irs1* and *Irs2* into NMuMG cells and observed that the cell proliferation rates of these cells were still similar to vector control cells, and when cultured without addition of insulin or IGF-I only very few colonies grew out (Figures 3D-3F; Supplementary Figure S3F). Thus, the mammary epithelial cells remained insulin/IGF-I dependent even when *Irs1* or *Irs2* were overexpressed, confirming that growth factor-independent cell proliferation is exclusively induced by *Irs4* expression.
Figure 3 | Growth factor-independent cell proliferation in mammary cells expressing *Irs4*

(A) Growth curves of MCF10A cells, stably transfected with *Irs4* or empty vector, growing in the presence of the indicated serum (FBS) concentrations, supplemented with insulin (ins) or IGF1, or without supplement (-). Cell growth is represented as the local regression over quadruplicates. Results shown are representative for four independent experiments, wherein independently transduced cell cultures were used.

(B,C) Quantification of maximum growth rates (B) and times until maximum confluency was reached (C) derived from growth curves in A and determined as outlined in Supplementary Figure S3A. Data represented as mean + SD of quadruplicates. *p < 0.05, **p < 0.01, ****p < 1·10^{-4} (Welch's t-test, compared to vector in each condition).

(D) Growth curves of NMuMG cells, stably transfected with *Irs1, Irs2, Irs4* or empty vector. Conditions and data represented as outlined in A. Results shown are representative for two independent experiments.

(E,F) Quantification of maximum growth rates (E) and times until maximum confluency was reached (F) derived from growth curves in D. Data represented as mean + SD of quadruplicates. *p < 0.05 (Welch's t-test, compared to vector in each condition).

**IRS4 INDUCES CONSTITUTIVE PI3K/AKT/mTOR PATHWAY HYPERACTIVATION**

Upon knockdown of *Irs4* in the P3724-R4 cells, we observed a robust reduction in AKT-phosphorylation under all tested conditions, while no clear effect was observed on ERK-phosphorylation levels or apoptosis signalling (Figures 4A and 4B;
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Figure 4 | IRS4 constitutively stimulates PI3K/AKT/mTOR pathway signalling in mammary cell

(A) Western blot showing phosphorylated (p-) and total AKT and ERK, and (cleaved) PARP, from P3724-R4 cells transduced with the indicated shRNAs, under starved (unstimulated) conditions and after stimulation as indicated.

(B) Ratios of phosphorylated AKT over total AKT (mean + SD), quantified from three Western blots as shown in A. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 1·10^{-4} (Welch’s t-test, compared to shGFP-control in each condition).

(C) Representative Western blot of three experiments, showing phosphorylated (p-) and total-protein components in the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways in MCF10A-Irs4 cells (ectopic Irs4: +) or vector controls (-) subjected to the indicated stimuli for 30 min or 24 h.

(D) Ratios of phosphorylated AKT over total AKT (mean + SD) from three blots as shown in C. For each blot, we employed independently transduced cell cultures of each cell type shown. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 1·10^{-4} (Welch’s t-test, compared to vector in each condition).
(E) Growth curves of NMuMG-Irs4 cells in medium supplemented with 0.5% FBS and in the presence of increasing concentrations of the PI3K-specific inhibitor ZSTK474. Growth represented as the local regression over quadruplicates.

(F) Representative Western blot of two independent experiments, showing phosphorylated and total-protein of components in the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways in NMuMG-Irs4 cells cultured 48 h in medium supplemented with 0.5% FBS and 100 nM ZSTK474 or vehicle (DMSO), and vector controls.

Supplementary Figure S4A). Similarly, we observed a potent growth factor-independent activation of the PI3K/AKT/mTOR pathway upon ectopic Irs4 expression in MCF10A cells, but no significant effect on RAF/MEK/ERK/MAPK pathway activation (Figures 4C and 4D; Supplementary Figures S4B and S4C). Interestingly, MCF10A-Irs4 cells still exhibited high PI3K/AKT/mTOR pathway activity under all tested conditions after 24 h, whereas the initial FBS and/or insulin/IGF1-induced AKT-phosphorylation seen after 30 min in MCF10A-Vector cells had returned to basal levels at the 24 h time point (Figure 4D). Similarly, Irs4 expression in NMuMG cells did not activate the RAF/MEK/ERK/MAPK pathway, but did stimulate the PI3K/AKT/mTOR pathway largely independent of any agonist, while NMuMG vector control cells and Irs1 and Irs2-transduced cells required stimuli to exhibit any PI3K/AKT/mTOR pathway activity (Supplementary Figures S4D-S4F). The strong and sustained activation of the PI3K/AKT/mTOR pathway, without requiring extracellular stimuli, implies that IRS4 is a constitutive activator of this pathway, which is likely to account for the IRS4-induced growth factor-independent cell proliferation.

Inhibition of the PI3K/AKT/mTOR pathway in NMuMG-Irs4 cells using the PI3K-specific inhibitors ZSTK474 (Yaguchi et al., 2006) or GDC0941 (Folkes et al., 2008) reduced cell proliferation in a concentration-dependent manner (Figure 4E and Supplementary Figures S5A-S5D). Specifically, when PI3K/AKT/mTOR pathway activation was brought down to vector control levels, as determined by AKT-phosphorylation, NMuMG-Irs4 exhibited a proliferation rate identical to that of NMuMG-Vector cells (Figure 4F and Supplementary Figures S5E-S5K), consistent with the notion that Irs4 expression regulates cell proliferation through constitutive PI3K/AKT/mTOR pathway hyperactivation.
IRS4 LACKS NEGATIVE FEEDBACK REGULATION BY SHP2

From a structural perspective, IRS4 is a unique IRS protein in its absence of a SHP2-binding motif (Figure 5A). To investigate whether this could account for the IRS4-specific constitutive PI3K/AKT/mTOR pathway activation, we replaced the C-terminal region of IRS4 with those of IRS1 or IRS2 containing a SHP2-binding domain (Figure 5A and Supplementary Figure S6A). The resulting chimeric proteins, stably expressed in NMuMG cells (NMuMG-Irs4ΔSHP2-1 and NMuMG-Irs4ΔSHP2-2) (Figure 5B and Supplementary Figures S6B and S6C), were fully active, as observed by the strong PI3K/AKT/mTOR pathway activation under unstimulated conditions and after stimulation with 0.5% FBS for 10 min (Figures 5C and 5D; Supplementary Figures S6D and S6E). Over time, however, phosphorylation of PI3K/AKT/mTOR pathway components clearly decreased faster in stimulated NMuMG-Irs4ΔSHP2-1 and NMuMG-Irs4ΔSHP2-2 than in NMuMG-Irs4 cells (Figures 5E and 5F). In fact, the relative reduction rate and half-life of PI3K/AKT/mTOR pathway signalling in the IRS4 recombinants was comparable to those in the vector controls and Irs1 and Irs2-transduced cells (Figure 5G and Supplementary Figure S6F). In other words, cells expressing the chimeric IRS4 proteins harbouring the active SHP2-domain, were similarly responsive to negative feedback upon AKT-phosphorylation as the NMuMG-Vector, NMuMG-Irs1 and NMuMG-Irs2 cells.

In accordance with the reduction in AKT-phosphorylation, we observed a reduced proliferation of the cells expressing the Irs4 recombinants, compared to cells expressing the wild-type Irs4 (Figure 5H and Supplementary Figures S6G and S6H). Cell proliferation, however, was not completely reduced to control levels, suggesting that IRS4 also provides some basal constitutive signalling activity additional to the activity sensitive to negative feedback acting on other IRS-molecules. Still, replacement of the IRS4 C-terminal domain with the SHP2-domains of IRS1 or IRS2 provides a feedback mechanism that largely quenches IRS4 signalling, most likely due to binding of SHP2, resulting in tyrosine dephosphorylation of the chimera.
Figure 5 | Lack of functional SHP2-binding domain permits constitutive signalling of IRS4

(A) Schematic representation of the signalling domains in the IRS family proteins and recombinant IRS4 proteins containing the SHP2-domains of IRS1 (Irs4ΔSHP2-1) or IRS2 (Irs4ΔSHP2-2), showing the total amino acid length of each product. Locations of tyrosine residues (Y) in the (putative) SHP2-binding motifs and approximate locations of designated antibodies’ epitopes are shown. Start positions of the SHP2-domains in IRS1/IRS2 and the C-terminal region in IRS4 that was replaced in the recombinants are indicated. PH, Pleckstrin homology domain; PTB, phosphotyrosine-binding domain.

(B) Western blot of NMuMG cells stably transduced with the indicated wild-type Irs family members or Irs4 recombinants, and vector controls. IRS4 and IRS1 antibodies, of which the binding sites are depicted in A, also bind the differently sized recombinant proteins.

(C) Representative Western blots of three experiments, showing phosphorylated (p-) and total-protein PI3K/AKT/mTOR pathway components of the cells as in B, after 24 h starvation.

(D) Ratios phosphorylated AKT over total AKT, calculated from Western blots as shown in C. Data are represented as mean + SD from three blots using independently transduced cells. *p < 0.05, **p < 0.01 (Welch’s t-test, compared to Irs4).

(E) Representative Western blots of three experiments, showing phosphorylated (p-) and total-protein PI3K/AKT/mTOR pathway components in cells as in B after stimulation with 0.5%FBS for the indicated time periods.
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(F) Ratios phosphorylated AKT over total AKT, shown as the mean + SD from three independent blots as the one shown in E. *p < 0.05, **p < 0.01 (Welch's t-test, compared to Irs4 in each time point).

(G) Estimated half-life of PI3K/AKT/mTOR pathway signalling (pAKT) after reaching peak AKT-phosphorylation levels at 10 min stimulation, determined from three independent blots of F, calculated as shown in Supplementary Figure S6F.

(H) Growth of the cells described in B in 0.5%FBS, represented as the local regression over quadruplicates, representative of two independent experiments.

(I) Western blot of IRS1, IRS2 and chimeric IRS4 variants co-immunoprecipitated (IP) with SHP2 from lysates (input) of the cells described in B. SHP2 and α-tubulin were used as control for IP-specificity and loading control. Results are representative of three experiments using independently transduced NMuMG cells.

To confirm SHP2-binding to the chimeric IRS4, but not to the wild-type IRS4, we performed co-immunoprecipitation (Co-IP) assays using a SHP2 antibody and the transduced NMuMG cells. In accordance with previous studies, IRS1 and to a lesser extent also IRS2 co-immunoprecipitated with SHP2, whereas IRS4 did not (Figure 5I) (Fantin et al., 1998; Myers Jr, 1998; Wauman et al., 2008). However, SHP2 was observed to bind with the chimeric IRS4-variants and is thus likely to dephosphorylate the IRS4-moiety in these chimeric proteins, similar to what has been reported for IRS1 and IRS2. Collectively, these results provide a mechanism for the lack of negative feedback response of IRS4 signalling and the constitutive activation of the PI3K/AKT/mTOR pathway in cells expressing Irs4.

**IRS4-POSITIVE BREAST CANCER IS ASSOCIATED WITH POOR OUTCOME**

We examined IRS4 expression in a random set of 27 human primary breast carcinomas and found five (19%) positive samples, of which four (15%) were highly positive (Figure 6A). Gene expression levels correlated well with protein levels, though suggested more IRS4-positive samples (Figure 6B and Supplementary Figure S7A), and showed a concomitant hyperactivation of the PI3K/AKT/mTOR pathway (Supplementary Figures 7B and 7C). To investigate whether high IRS4 expression was associated with survival, we examined a well annotated microarray dataset obtained from a series of 157 human primary breast tumours from patients that developed metastatic disease (Savci-Heijink et al., 2015). 10/157 (6%) of the
Figure 6 | IRS4 in human breast cancer patient material and breast cancer cells

(A) Expression of IRS4 mRNA in 27 random human primary breast carcinomas (by qRT-PCR). Expression levels greater than median + SD (grey dashed line) were defined as positive (IRS4+) and greater than median + 2 * SD (black dashed line) as highly positive (IRS4+++).

(B) IRS4 Western blot of a subset of tumours from A. +++ are IRS4-high samples, +/- are samples at the borderline of IRS4-positive and IRS4-negative in A.
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(C) IRS4 mRNA expression levels (log_{2}-transformed) in 157 metastasised human primary breast carcinomas (microarray probe ILMN_1712774 of Illumina HumanHT-12 v4 Expression BeadChip). Log_{2}-ratios greater than 1.5 * SD (grey dashed line) were defined as positive (IRS4+) and greater than 2 * SD (black dashed line) as highly positive (IRS4+++).

(D) Overall survival of patients with IRS4-negative and IRS4-positive (combining IRS4+ and IRS4+++)) tumours using the threshold depicted in C. **p < 0.01 (log-rank test).

(E) Distribution of the IRS4-negative and IRS4-positive (combining IRS4+ and IRS4+++)) tumours using the threshold depicted in C over the indicated PAM50-based clinical subtypes. **p < 0.01 (Pearson's Chi-squared test).

(F,G) IRS4 mRNA expression in 30 HER2-positive (F) and 31 triple-negative primary breast carcinomas (G), determined by quantitative RT-PCR showing. Expression level cut-offs as in A. The tumour percentage (estimated by a pathologist) of each sample is indicated below. N/A indicates that tumour percentage was not available.

(H) Western blot showing IRS4 protein, and phosphorylated (p-) and total-AKT from MDA-MB-453 cells transduced with the indicated shRNAs.

(I) Quantified IRS4 levels plotted against ratios of phosphorylated AKT over total AKT, derived from the blot in H. Linear regression and associated R^2-values show a positive correlation.

(J) Quantified IRS4 levels, derived from the blot in H, plotted against the corresponding cell proliferation rates, determined in triplicate by the Crystal Violet staining as in Supplementary Figure S7F. Linear regression and associated R^2-values show a strong positive correlation.

(K) Representative soft agar growth scans of MDA-MB-453 cells, transduced with the indicated shRNAs, cultured for two weeks in absence of insulin, or vehicle.

(L) Anchorage-independent growth (mean + SD), relative to Parental cells, of four independent experiments as in K. ***p < 0.001, ****p < 1·10^{-4} (Welch's t-test, compared to shGFP).

tumours showed an elevated IRS4 microarray signal, which was associated with poor overall survival (p = 0.0481, log-rank test) (Figures 6C and 6D). IRS4-positive tumours were enriched among triple-negative/basal-like (6/25, 24%) and HER2-enriched tumour subtypes (2/18, 11%), whereas virtually absent in the luminal A (1/67, 1%) and B (1/47, 2%) subtypes (p = 2.68·10^{-3}, Pearson's Chi-squared test) (Figure 6E and Supplementary Table S2). This subtype enrichment was also evident in the eight (5%) samples that expressed high (+++) IRS4 levels (p = 2.13·10^{-3}, Pearson's Chi-squared test) (Supplementary Figure S7D). Moreover, one of the four highly IRS4-positive samples in the random set of 27 human primary breast carcinomas was also one of the four ERBB2-positive samples (Supplementary Figure S7E). To expand these data, we screened for IRS4 expression in an independent uniform HER2-positive and an independent uniform triple-negative
human breast cancer series by qRT-PCR. From the 30 tested HER2-positive samples, three (10%) were highly positive for IRS4 expression (Figure 6F). From the 31 tested triple-negative tumours, nine (29%) could be scored as IRS4-positive and four (13%) of these as highly positive (Figure 6G). This is likely an underestimation as the tumour percentage of the samples, as determined by a pathologist, was mostly below 60% (where known), and in some samples as low as 5-10% (Figures 6F and 6G).

In line with this clinical data, the two IRS4-positive human breast cancer cell lines we identified, MDA-MB-453 and HCC187 (Supplementary Figure S1D), are both of the triple-negative subtype. Further investigating the involvement of IRS4 in human breast cancer, we performed a knockdown of the endogenous IRS4 in these cancer cells and observed a concomitant reduction in PI3K/AKT/mTOR pathway activation and cell growth (Figures 6H-6J; Supplementary Figures S7F-S7I). Moreover, anchorage-independent growth of MDA-MB-453 cells was significantly less in IRS4-knockdown cells, compared to shGFP-control and parental cells ($p = 9.09 \cdot 10^{-5}$ and $6.47 \cdot 10^{-4}$ for shIRS4#14 and shIRS4#15, respectively, Welch’s t-test) (Figures 6K and 6L). HCC197 cells did not grow in soft agar at all, in our hands. Collectively, our data demonstrate that IRS4 is expressed in a subset of human breast tumours and human cancer cell lines. In the light of our finding that IRS4 is constitutively active, these data strongly point to a role for IRS4 as oncogene in human breast cancer, which may particularly be true in the triple-negative and HER2-positive subtypes.
DISCUSSION

In this study, we identify *Irs4* as a gene that is frequently targeted by MMTV proviral insertions and we show that *Irs4* expression confers tumorigenic properties to mammary epithelial cell lines *in vivo*. *Irs4* is also found as common insertion site (CIS) in Moloney Murine Leukaemia Virus (MuLV) insertional mutagenesis screens in various types of lymphomas (Uren et al., 2008), stressing the relevance of this gene in oncogenesis. In contrast, *Irs1*, *Irs2* and *Irs3* are not targeted, neither by MMTV nor by MuLV, and are not found as CIS in any insertional mutagenesis screen reported in the Retrovirus and Transposon tagged Cancer Gene Database (RTCGD) (Akagi et al., 2004). This suggests that IRS4 has unique properties compared to the other IRS family members, especially since all IRS proteins act primarily in the same pathways.

IRS-mediated signalling is initiated upon tyrosine phosphorylation of IRS proteins by activated receptor tyrosine kinases (RTKs) and leads to activation of the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways (Dearth et al., 2006; Taniguchi et al., 2006). The importance of IRS signalling in cancer has been reported by Chang et al. (2002), showing that IRS1 is constitutively phosphorylated in a variety of human tumours, including breast cancer, and that dominant negative IRS1 abolishes tumour cell growth *in vitro*. However, in light of our findings, IRS1 most likely still requires activation by RTKs, such as the IGF1-receptor, in these tumours. Indeed, high levels of circulating IGF1 have been associated with breast cancer risk (The Endogenous Hormones and Breast Cancer Collaborative Group, 2010) and could well be responsible for activation of IRS1. In contrast, we show that the activity of IRS4 is growth factor-independent, although its activity may be further enhanced by RTKs. Specifically, we demonstrate that IRS4 is an unconventional IRS in that it sustains a high constitutive basal activity in PI3K/AKT/mTOR pathway signalling, leading to growth factor-independent cell proliferation in mammary epithelial cells (Figures 7A-7C). Although PI3K/AKT/mTOR pathway activation by IRS4 has been described before (Hoxhaj et al., 2013; Tsuruzoe et al., 2001), the underlying mechanism thus far remained largely unclear.
Phosphorylation of specific tyrosines in the C-terminal domain of IRS1 and IRS2 enables binding of the SH2-containing tyrosine phosphatase SHP2. Despite reported conflicting and context-dependent functions of SHP2 (Siddle, 2012), there is ample evidence that SHP2 can bind to IRS1 and IRS2, subsequently induces tyrosine dephosphorylation and thereby reduces signalling activity of these proteins (Hanke and Mann, 2009; Matsuo et al., 2010; Myers Jr, 1998; Pluskey et al., 1995; Sugimoto et al., 1994). IRS4 differs from the other IRS proteins in the absence of a SHP2-binding motif and indeed, SHP2 has been shown not to bind to IRS4 (Fantin et al., 1998; Wauman et al., 2008), as confirmed by our own experiments. We predicted that the negative feedback loop through tyrosine dephosphorylation of IRS1 and IRS2 by SHP2 may not exist for IRS4 signalling. Indeed, we demonstrate here that an IRS4 protein in which we had replaced the C-terminus with the SHP2-binding domains of IRS1 or IRS2, can no longer maintain the PI3K/AKT/mTOR pathway hyperactivity as seen with wild-type IRS4 and is even as responsive to negative feedback regulation upon stimulation as IRS1 and IRS2. So, unlike IRS1 and IRS2, IRS4-mediated PI3K/AKT/mTOR signalling is not regulated at the protein-level by SHP2, explaining at least in part the constitutive activity of IRS4.

In contrast to the rather ubiquitous expression of Irs1 and Irs2, expression of Irs4 is very stringently regulated. Restricted expression of a constitutive active gene involved in cell proliferation would be expected, as such gene would render cells more prone to cancer. In fact, the inference that IRS4 is a constitutive active IRS is
also in line with our finding that the *Irs4* gene is a target for retroviral insertional mutagenesis, as this process leads with few exceptions to transcriptional deregulation of the target genes rather than to mutations affecting protein function (Jonkers and Berns, 1996). The notion that IRS4 is a constitutive active oncogenic protein primarily regulated transcriptionally also explains the recent observations that chromosomal translocation events can activate *Irs4* expression, leading to T-cell acute lymphoblastic leukaemia (T-ALL) (Kang et al., 2012; Karrman et al., 2009) and subungual exostosis (a benign bone and cartilage producing tumour) (Mertens et al., 2011). Additionally, in human hepatocellular carcinomas (HCC), IRS4 expression is frequently upregulated compared with hepatocytes (Cantarini et al., 2006), and its role in this malignancy has been further substantiated in the HEPG2 hepatoblastoma cell line where IRS4 plays an important role in cell proliferation (Cuevas et al., 2007). These findings suggest that IRS4 expression is highly relevant in various human cancers.

Our data establish *Irs4* as a potent oncogene in mouse mammary tumorigenesis and we show that IRS4 expression in human primary breast cancers is associated with poor survival. Furthermore, we observed IRS4 expression almost exclusively in the triple-negative and HER2+ subtypes, while only incidentally in the Luminal A and B subtypes. Triple-negative tumours are independent of the most common growth stimulatory signals in breast cancer, such as steroid hormones, and require other factors to activate the essential pathways in mammary carcinogenesis, like the PI3K/AKT/mTOR pathway. Indeed, several mechanisms for PI3K/AKT/mTOR pathway activation, like *PIK3CA*-mutations or copy number gain, AKT-activation and *PTEN*-loss, have been associated with triple-negative (basal-like) tumours (López-Knowles et al., 2010; Marty et al., 2008; Xu et al., 2014; Young et al., 2015). In HER2+ tumours, the RAF/MEK/ERK/MAPK pathway is activated through ERBB2, but PI3K/AKT/mTOR pathway activation is additionally required, e.g. via *ERBB3* (HER3) upregulation, *PIK3CA*-mutations or *PTEN*-loss (Arteaga and Engelman, 2014; Holbro et al., 2003; Lee-Hoeflich et al., 2008; Nagata et al., 2004; Saal et al., 2005; Stemke-Hale et al., 2008). In both triple-negative and HER2+ breast cancers, IRS4 expression is most likely an additional mechanism to activate the PI3K/AKT/mTOR pathway and consequently, tumorigenesis. Besides the identified
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Oncogenic activities that IRS4 executes via PI3K/AKT/mTOR pathway hyperactivation, IRS4 might also contribute to oncogenesis via additional mechanisms. The IRS family is known to interact with many proteins, apart from the canonical p85 and GRB2, and can even form large multiprotein complexes (Dearth et al., 2007; Hakuno et al., 2015). Moreover, nuclear translocation with associated effects on e.g. DNA repair, rRNA-synthesis and gene expression, has been reported for IRS proteins as well (Hoxhaj et al., 2013; Mardilovich et al., 2009; Reiss et al., 2012; Sun et al., 2003; Tu et al., 2002), processes which also play a role in oncogenesis. Therefore, it may be useful to investigate these aspects in IRS4-induced tumours in the future.

Altogether, we propose IRS4 as a biomarker for tumorigenesis via PI3K/AKT/mTOR pathway hyperactivation, particularly in triple negative and HER2+ breast cancer subtypes, but potentially also in other cancers.
MATERIAL & METHODS

PATIENT SAMPLES

We obtained a set of 27 random human primary breast carcinoma samples, without clinical data, from our in-house pathology department. In addition, RNA samples were obtained from 31 tumours randomly selected from a collection of triple-negative primary breast carcinomas (Kreike et al., 2007), and from 30 tumours randomly selected from a set of 129 HER2-positive primary breast carcinomas. The latter specimens were obtained from patients treated between 1989 and 2006 at the Netherlands Cancer Institute. Microarray-data of 157 primary breast tumours that had metastasised (Savci-Heijink et al., 2015) were kindly shared by the group of M.J. van de Vijver (AMC, Amsterdam) and were obtained using the Illumina HumanHT-12 v4 Expression BeadChip, followed by robust spline normalization (RSN) and ComBAT batch correction. Use of patient material was approved by the individual patients (via opt-out) and by the local Translational Research Board, following positive recommendation of the Medical Ethical Committee.

ANIMAL EXPERIMENTS

Methods used for the MMTV-induced insertional mutagenesis screens have been described previously (Klijn et al., 2013; Theodorou et al., 2007). Mouse models used for MMTV-induced insertional mutagenesis were the FVB and BALB/c wild-type mouse strains and three clinically relevant mouse models of breast cancer: MMTV-cNeu (FVB background), transgenic for rat ErbB2 (Guy et al., 1992); K14Cre;Trp53F/F (BALB/c background), a p53 conditional knockout (Liu et al., 2007); and PTEN+/− (FVB background), a PTEN heterozygous mouse model (Stambolic et al., 2000).

Tumorigenicity experiments were performed by subcutaneously injecting 1.10^6 or 2.10^6 viable cells in 200 µl PBS into each flank of three to four weeks old female BALB/cABomA-nu/nu (BALB/c nude) mice. Viability of cells was assessed by Trypan Blue staining and differed neither between cells from the test group and controls, nor prior to and after injection. Tumour growth was measured at least twice a week in
two dimensions and the animals were sacrificed when the tumour reached approximately 1 cm³. Kaplan-Meier plots of tumour-free survival were plotted using the programming language R with the "survival" package. All mouse experiments were performed in accordance with the Dutch legislation and were approved by the Animal Experiments Committee (DEC).

INSERTION SITE MAPPING
To map the MMTV insertion sites we used the protocols as previously described (Klijn et al., 2013; Theodorou et al., 2007).

CELL CULTURE AND TREATMENT
The Irs4-positive tumour cell line P3724-R4 was established from an MMTV-induced mammary tumour that developed in a K14Cre;Trp53F/F mouse and highly overexpressed Irs4 due to an MMTV proviral insertion in the Irs4 locus. 1 to 2 mm pieces of the tumour were resuspended in a small volume of PBS and subcutaneously injected in BALB/c nude mice (100 µl per flank). Tumours that developed were isolated when reaching about 1 cm³ in size, cut into 1 to 2 mm pieces and incubated for 50 min in 0.25% Collagenase III (Worthington) and 0.2% Hyaluronidase (Sigma-Aldrich) in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) at 37°C. Subsequently, the cells were filtered through a 70 µm cell strainer (BD Falcon), centrifuged at 250 g for 5 min, resuspended and cultured in DMEM supplemented with 10% Foetal Bovine Serum (FBS) (Perbio), 50 units/ml penicillin and 50 µg/ml streptomycin (PenStrep) (Gibco), 200 ng/ml hydrocortisone (Gibco) and 20 ng/ml human recombinant epidermal growth factor (EGF) (Sigma-Aldrich), on 2 µg/ml collagen-I coated Petri dishes. Medium was not supplemented with insulin in order to select for Irs4-positive cells. After selecting for epithelial cells by multiple rounds of differential trypsinisation, the immortalised epithelial cells were maintained on 10% FBS coated plastic-ware. Clones of the immortalised cell culture with a clear stable epithelial morphology were isolated and expanded. One of these clones was used for further experiments and was designated P3724-R4, which was further maintained on 10% FBS-coated Petri dishes in Dulbecco's Modified Eagle
Medium/F-12 mix (DMEM/F-12) (Invitrogen) supplemented with 10% FBS, PenStrep, 200 ng/ml hydrocortisone and 20 ng/ml EGF. Upon knockdown of Irs4, cells were maintained in medium additionally supplemented with 5 µg/ml insulin (SAFC Biosciences).

NMuMG, MCF10A, BT474, MDA-MB-453 and HCC1187 cells were obtained from ATCC. SKBR3 cells were a kind gift from J. Taylor-Pappadimitriou (Guy's Hospital, London). All cell lines were never cultured for more than eight passages upon receipt and were routinely tested for Mycoplasma (Hoechst staining and PCR).

NMuMG and MCF10A cells were cultured in Dulbecco’s Modified Eagle Medium/F-12 mix (DMEM/F-12) (Invitrogen) supplemented with Foetal Bovine Serum (FBS) (Perbio), 50 units/ml penicillin and 50 µg/ml streptomycin (PenStrep) (Gibco), and 5 µg/ml insulin (SAFC Biosciences). MCF10A-medium was additionally supplemented with 200 ng/ml hydrocortisone (Gibco) and 20 ng/ml human recombinant epidermal growth factor (EGF) (Sigma-Aldrich). BT474 and SKBR3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% FBS and PenStrep. SKBR3-medium was additionally supplemented with 5 µg/ml insulin. MDA-MB-453 and HCC1187 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS and PenStrep.

Where indicated, 5 µg/ml insulin or 100 ng/ml recombinant human IGF1 (Peprotech) was supplemented to the medium (as stimulus). PI3K-specific inhibitors GDC0941/Pictilisib (Selleck Chemicals) and ZSTK474 (Selleck Chemicals) dissolved in dimethyl sulfoxide (DMSO) were added to the culture medium at the indicated concentrations. Equal volumes of DMSO diluent were added to the medium, where mentioned, as vehicle controls.

All experiments were performed with at least two independently transduced cell cultures each.

PLASMIDS AND GENE TRANSDUCTION

Five individual shRNAs that target murine Irs4 and a shRNA targeting GFP cloned in the lentiviral vector pLKO.1 were obtained from the RNAi Consortium (TRC) (Irs4 shRNA clone IDs: TRCN0000105820, TRCN0000105821, TRCN0000105822,
TRCN0000105823, TRCN0000105824 and eGFP clone ID: TRCN0000072185, here referred to as shIrs4#20, shIrs4#21, shIrs4#22, shIrs4#23 shIrs4#24 and shGFP, respectively). Knockdown of human IRS4 was achieved using TRC shRNAs with IDs: TRCN0000063613, TRCN0000063614, TRCN0000063615, TRCN0000063616 and TRCN0000063617, here designated shIRS4#13, shIRS4#14, shIRS4#15, shIRS4#17 and shIRS4#17, respectively. Lentiviral supernatants were produced following transfection as described by the TRC (http://www.broadinstitute.org/rna/public/resources/protocols). Briefly, 48 h post-transfection, the culture medium of lentivirus 293T packaging cells (lentiviral supernatants) transfected with short hairpin constructs was passed through a 0.45 µm filter (Whatman), supplemented with 6 µg/ml polybrene (Millipore). shIrs4#20-24 and shGFP were used for transduction into our newly established P3724-R4 tumour cell line and shIRS4#13-17 and shGFP for transduction in SKBR3/RIRS4+, BT474/RIRS4+, MDA-MB-453 and HCC1187 cells. Cells expressing a shRNA were selected with 2 µg/ml puromycin, 48 h post-infection. shRNA-induced knockdown efficiency was determined by quantitative RT-PCR and immunoblotting after two weeks of puromycin selection.

The pMSCV-construct with wild-type mouse Irs1 was a kind gift of Dr. R. Baserga (D'Ambrosio et al., 1995). The pBABE-construct with wild-type mouse Irs2 was obtained through Addgene (plasmid 11371) and was originally cloned in the laboratory of Dr. R. Kahn (Tsuruzoe et al., 2001). Mouse wild-type Irs4-cDNA cloned into a pMSCV-vector was kindly provided by Dr. A. Berns (NKI, Amsterdam). The Irs1 and Irs2-constructs were used as donors of the SHP2-domains to generate the recombinant Irs4ΔSHP2-1 and Irs4ΔSHP2-2 constructs in pMSCV. The empty pMSCV backbone vector was used as control in each experiment. Ecotropic retroviruses were produced in Phoenix packaging cells, transfected with the appropriate ecotropic retroviral construct using the calcium-phosphate precipitation method. Cells were infected with the ecotropic virus as described above for lentiviral transduction. Infected cell populations expressing the introduced transgene were selected in medium containing 2 µg/ml puromycin 48 h after infection. Ecotropic retroviral infection of human MCF10A cells was facilitated by using an oligoclonal MCF10A cell pool stably expressing the murine ecotropic receptor.
All used retroviral plasmids carried the puromycin resistance gene. The integrity of the inserts in each construct was verified by sequencing and expression of the transduced constructs was confirmed by RT-PCR and Western blot analysis, following puromycin selection.

**EXPRESSION ANALYSIS**

RNA isolation, RT-PCR and expression analysis of tissues and tumour material was performed as described before (Theodorou et al., 2007). From cell lines, RNA was isolated using the HighPure RNA isolation kit (Roche), followed by DNase treatment and RT-PCR using the Tetro cDNA synthesis kit (Bioline), following manufacturers' instructions. Quantitative PCR was performed using the SensiFast SYBR Hi-ROX kit (Bioline) on a StepOnePlus Real-Time PCR system (Applied Biosystems) and expression levels were determined using the ΔΔCT-method.

MMTV-induced tumours from the insertional mutagenesis screens were assessed by quantitative RT-PCR for:

**Irs4 expression:**
Forward primer: 5'-TCCTGTACCAATGCTTCTCCG-3';
Reverse primer: 5'-CGCGAAGTATTCGTCCTGGG-3'.

**Col4a5 expression:**
Forward primer: 5'-GTCCACCAGGTACAGAAGGTC-3';
Reverse primer: 5'-CTCCTTTTCAAACCAGGTAAGCC-3'.

β-actin expression was used as a reference:
Forward primer: 5'-GGCTGTATTCCCCTCCATCG-3';
Reverse primer: 5'-CCAGTTGGTAACAATGCCATGT-3'.

Quantitative real-time PCR to assess knockdown efficiency of Irs4 was performed using the same primers for murine Irs4 and β-actin.

Expression of *Irs1-4* in BALB/c wild-type tissues was assessed by RT-PCR using the following primers:

**Irs1 expression:**
Forward primer: 5'-TCTCCAAGGAGTCGGCTCCA-3';
Reverse primer: 5'-CGTGAGGTCTGGTGTGAA-3'.
Irs2 expression:
Forward primer: 5'-TGGGTTTCCAGAACGCGCCTC-3';
Reverse primer: 5'-TTTCAACATGGCGCGCATGG-3'.

Irs3 expression:
Forward primer: 5'-GTACCGTTAGCCTGGAGGGT-3';
Reverse primer: 5'-CTTCCAGGCTTTCCGAGGAG-3'.

Irs4 expression:
Forward primer: 5'-ATTGCTGCTCCAGCTGAGGC-3';
Reverse primer: 5'-AATGGATGCAGGAGCAGTC-3'.

Expression analysis in patient samples and human breast cancer cell lines was performed with quantitative RT-PCR using human-specific primers:

IRS4 expression:
Forward primer: 5'-CGACCAAGCGACAAGAAGACT-3';
Reverse primer: 5'-GGTTCCCGAGGAAAGAAGCG-3'.

ERBB2 expression:
Forward primer: 5'-TGGCCTGTGCCCACTATAAG-3';
Reverse primer: 5'-AGGAGAGGTCAGGTTTCACAC-3'.

β-actin expression was used as a reference:
Forward primer: 5'-CCAACCGCGAGAAGATGA-3';
Reverse primer: 5'-CCAGAGGCGTACAGGGATAG-3'.

Although our RNA-isolation procedure included an integrated DNA-digestion step to avoid genomic DNA amplification, primer pairs were designed to span an intron, whenever possible.

All expression analysis results were independently confirmed by at least one alternative primer pair. Product size of PCR reactions (always 30 cycles) is indicated next to each gel. Full images of gels are provided in Supplementary Figure S8.

CELL PROLIFERATION ASSAYS AND DOSE RESPONSE

For dynamic cell proliferation analysis, 1,500 (MCF10A) or 3,000 (NMuMG) cells per well were allowed to attach overnight in a 10% FBS-coated clear-bottom black 384-well plate (BD Falcon) in DMEM/F-12 medium supplemented with PenStrep, and for
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MCF10A cells additionally supplemented with 200 ng/ml hydrocortisone and 20 ng/ml EGF. After replacing the medium with the appropriate growth medium, every well was imaged (phase-contrast) with a 4 h interval using the IncuCyte life cell imaging device (Essen BioScience). The changes in cell density over time were used by the IncuCyte software to determine the growth curves. Local regression of confluency over time, and calculations for maximum growth rates and times to reach maximum confluency were performed using the programming language R with the "cellGrowth" package. All these experiments were carried out at least in quadruplicate and were independently repeated.

For end-point cell proliferation assays, 30,000 P3724-R4, 25,000 NMuMG, 12,500 SKBR3, 25,000 BT474, 30,000 MDA-MB-453 or 30,000 HCC1187 cells per well were seeded in 24-wells plates (Corning). Cells were allowed to attach for 6 h, washed with DMEM/F-12 medium only supplemented with PenStrep and then grown under the indicated conditions. At indicated time points, the cells were fixed in 4% buffered Formaldehyde solution (Klinipath), stained with 0.1% Crystal Violet solution (Sigma-Aldrich) and imaged using a desktop scanner (Epson).

APOPTOSIS ASSAYS

1.0·10^6 P3724-R4 cells were allowed to attach overnight to a T25 flask (BD Falcon) in DMEM/F-12 medium supplemented with 10% FBS, PenStrep, 5 µg/ml insulin, 200 ng/ml hydrocortisone and 20 ng/ml EGF and were then washed and cultured for 48 h on DMEM/F-12 medium only supplemented with PenStrep (starved). Cells were released by EDTA-free trypsin-250 (Gibco) diluted in DMEM/F-12 medium and immediately stained with Annexin V and propidium iodide (PI) from the Annexin V-FITC Apoptosis Detection Kit (Abcam), following manufacturers' instructions. 20,000 PI-negative cells per replicate were analysed for Annexin V-staining using a Beckton Dickinson LSRII FACS analyser.

SOFT AGAR ASSAYS

Soft agar assays were performed in 6-wells plates. Each well contained a 2 ml 0.6% low-gelling temperature agarose (Sigma-Aldrich) base layer on which 50,000
MCF10A, 10,000 NMuMG, 10,000 BT474 or 50,000 MDA-MB-453 cells were suspended in 2.5 ml of 0.35% low-gelling temperature agarose in medium (supplemented with GDC0941 or vehicle, where applicable). Anchorage-independent growth was assessed by counting colonies after two weeks using the GelCount instrument (Oxford Optronix). All soft agar assays were performed in at least three separate experiments, using independently transduced cell cultures.

**IMMUNOPRECIPITATION**

Immunoprecipitation of SHP2 from cells lysed in NP40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 20 mM EDTA, 1% NP40, Complete Mini protease inhibitor and PhosStop phosphatase inhibitor; both from Roche) was carried out overnight at 4°C using an anti-SHP2 antibody conjugated to agarose beads from Santa Cruz Biotechnology (#sc-280 AC), following manufacturer’s instructions. The immunoprecipitated proteins were solubilised in sample buffer and analysed by 4-12% Bis-Tris gel electrophoresis followed by Western blotting.

**WESTERN BLOTTING**

Cells were washed twice with PBS and then lysed in NP40 lysis buffer for 30 min on ice. Harvested lysates were then centrifuged at 15,000 g for 10 min at 4°C and the protein concentration of the supernatants was determined by the Micro BCA protein assay kit (Thermo Scientific). Equal amounts of protein were separated on 4-12% Bis-Tris gels using the Novex NuPAGE or Bolt electrophoresis systems (Life Technologies) and subsequently transferred onto nitrocellulose membranes (Whatman). Membranes were blocked with Odyssey PBS Blocking Buffer (LI-COR) and immunostained with antibodies against the proteins of interest. Proteins were detected in either the 700 or 800 nm channel using the Odyssey Infrared Imaging System (LI-COR), after incubation with the appropriate secondary antibodies labelled with IRDye 680 or IRDye 800 fluorescent dyes (LI-COR), respectively. Results were quantified using the Image Studio software (LI-COR), where the absolute signals or ratios phosphorylated over total protein signal were always normalised over the loading control signals in each lane. In every Western blot
analysis, all proteins analysed in the same experiment were detected on the same blot. Blots were always first immunostained for IRS4, IRS1, p-AKT, p-S6, p-4EBP1, p-ERK, total ERK, SHP2 and/or loading controls (α-tubulin or β-actin), followed by stripping using the Newblot Nitro Stripping Buffer (LI-COR) as recommended by the manufacturer, and subsequently immunostained for IRS2, total AKT, total S6 and/or total 4EBP1.

Antibodies against p-AKT S473 (#4060, 1:2000), p-MAPK (p-ERK) T202/Y204 (#9106, 1:2000), p-S6 S235/236 (#4858, 1:2000), p-4EBP1 T37/46 (#2855, 1:1000), total S6 (#2217, 1:1000), total 4EBP1 (#9644, 1:1000), total MAPK (ERK) (#9102, 1:1000) and PARP (#9542) were obtained from Cell Signalling Technology. Goat anti-IRS4 antibody (#EB11828, 1:4000) was obtained from Everest Biotech. Antibodies against IRS1 (#sc-559, 1:500), IRS2 (#sc-8299, 1:500), SHP2 (#sc-280, 1:1000) and total AKT (#sc-8312, 1:1000) were purchased from Santa Cruz Biotechnology, α-Tubulin (#T9026, 1:5000) from Sigma-Aldrich, and β-actin (#GTX26276, 1:10,000) and human IRS4 (#GTX61555, 1:1000) from GeneTex.

Molecular mass (in KDa) is indicated next to each blot. Full scans of the Western blots are provided in Supplementary Figure S9.

**Statistical Analyses**

Statistical tests are specified in the text and were calculated using the statistical programming language R, with the "Bioconductor" and "survival" packages. A p-value < 0.05 was considered statistically significant.
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REFERENCES


Hanke, S., and Mann, M. (2009). The phosphotyrosine interactor of the insulin


Hanke, S., and Mann, M. (2009). The phosphotyrosine interactor of the insulin pathway hyperactivation | CHAPTER 3


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SUPPLEMENTARY FIGURES & TABLES

A

B

C

D

E
Supplementary Figure S1 | Expression pattern of Irs4 in MMTV-induced tumours, murine and human tissues and cell lines

(A) mRNA expression of Irs gene family members in various tissues from wild-type BALB/c mice, determined by RT-PCR.

(B) Irs4 expression levels in 91 normal C57BL6 mouse tissues and cells, quantified by the indicated microarray probes of the Affymetrix Mouse Genome 430 2.0 Array, using the Mouse MOE430 Gene Atlas dataset (Lattin et al., 2008) obtained from BioGPS (Wu et al., 2009). Samples discussed in the results-section on A and Figure 1E are highlighted: brain samples with high expression in green, testis in blue and mammary glands in red. Data represented as mean GCRMA-normalised log₂-transformed signal values ± SD.

(C) Expression levels of IRS4, IRS1 and IRS2 in human breast epithelium, quantified by the indicated microarray probes of the Affymetrix Human Genome U133 Plus 2.0 Array, using the Barcode on Normal Tissues dataset obtained from BioGPS (Wu et al., 2009). Expression levels shown as z-score (McCall et al., 2011), where >5 suggests expression in that tissue (indicated by grey dashed line). Data represented as mean ± SD.

(D) Expression of IRS4 mRNA in various human breast cancer cell lines, relative to IRS4 expression level in HEK-293 cells, determined by quantitative RT-PCR. Expression levels greater than median + SD (grey dashed line) were defined as positive (IRS4+) and greater than median + 2 * SD (black dashed line) as highly positive (IRS4+++).

(E) IRS4 expression levels in 84 human cell lines, quantified by microarray probe 207403_at of the Affymetrix Human Genome U133A Array, using the Human NCI60 Cell Lines dataset from BioGPS (Wu et al., 2009). Cell lines tested in D are highlighted: green for breast cancer cells, red for HEK-293. Data represented as mean GCRMA-normalised log₂-transformed signal values ± SD.
Supplementary Figure S2 | Irs4 expression in mammary cells drives tumour growth

(A) Knockdown of Irs4 expression in P3724-R4 tumour cells by the indicated short hairpin RNAs. Irs4 expression levels were determined by qRT-PCR and the results are presented relative to the expression level in the parental cell line. The most effective two Irs4-shRNAs, shIrs4 #21 and #24, caused a 95% and 87% downregulation of Irs4 expression, respectively. Data are represented as mean ± SD of triplicates.

(B) Quantification of IRS4 protein levels, corrected for loading and presented relative to the level in parental cells, derived from the Western blot shown in Figure 2D. shIrs4 #21 and #24 reduced IRS4 to respectively 2% and 3% of the levels in the parental cells.

(C) Percentage of apoptotic cells ( Annexin V-positive ) in viable ( propidium iodide-negative ) populations of P3724-R4 cells as in Figure 2G, cultured for 24 h in presence of indicated etoposide concentrations, determined by FACS.

(D) Tumour growth ( mean ± SD ) in mice shown in Figure 2I, subcutaneously injected in both flanks with 1·10^6 NMuMG cells ectopically expressing Irs4 ( n = 5 ) and vector control cells ( n = 5 ).
Supplementary Figure S3 | Effect of IRS4 on cell proliferation in mammary cells

(A) Example of local regression (black) of cell density over time of the depicted data points (grey). The maximum growth rate (blue) and maximum confluency (red) calculated from the local regression using the "cellGrowth" package in the programming language R are also shown. Representative graphical images obtained by the IncuCyte life cell imaging device and corresponding confluency are indicated on the right (scale bar = 200 µm).

(B) Representative images of unstimulated vector control and Irs4-transduced MCF10A cells, obtained by the IncuCyte imager. Time point (t, in min) and corresponding confluency (%) is indicated (scale bar = 200 µm). Vector control cells display only sporadic growth, while MCF10A-Irs4 cells grow uniformly.

(C) Representative image of Crystal Violet stained NMuMG cells, stably transfected with Irs4 or empty vector, incubated for six days under the indicated growth conditions.

(D) Growth curves of the vector control and Irs4-transduced NMuMG cells under the indicated conditions, derived from Crystal Violet stained cells as in C. Data are depicted as the mean ± SD of four experiments using independently transduced cell pools.

(E) Expression of endogenous IRS genes, ectopic Irs4 and the empty pMSCV-vector construct in transduced MCF10A cells, as determined by RT-PCR. IRS1 and IRS2, but not IRS4, are endogenously expressed in these cells. HEK-293 cDNA was used as positive control for endogenous IRS4 expression.

(F) Representative images of indicated NMuMG cells under the indicated conditions, obtained by the IncuCyte imager, with corresponding confluency (%) and time points (t, in min) indicated (scale bar = 200 µm).
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µm). Time points at ~75% differ between cell lines (see Figure 3D). NMuMG-Vector, NMuMG-Irs1 and NMuMG-Irs2 cells show only sporadic growth in 0.5%FBS, while MCF10A-Irs4 cells grow uniformly.
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▲ Supplementary Figure S4 | IRS4, not IRS1 and IRS2, constitutively stimulates PI3K/AKT/mTOR pathway signalling in mammary cells

(A) Ratios of cleaved PARP over full length PARP (mean + SD), quantified from three Western blots as shown in Figure 4A. There are no significant differences between any of the ratios within each condition (Welch’s t-test).

(B,C) Ratios of phosphorylated S6 (B) and ERK (C) over the respective total-proteins, calculated from Western blots of MCF10A cells as shown in Figure 4C. Data are represented as mean + SD and are based on three blots each loaded with lysates from independently transduced oligoclonal cell cultures. *p < 0.05, **p < 0.01 (Welch’s t-test, compared to vector in each condition). There are no significant differences between any of the p-ERK/ERK-ratios.

(D) Representative Western blots showing phosphorylated (p-) and total-protein components of the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways in NMuMG cells transduced with Irs4 or empty vector controls subjected to the indicated stimuli for 30 min or 24 h (ins = insulin).

(E,F) Ratios of phosphorylated over total AKT (E) and S6 (F) (mean + SD) from three blots as shown in D, loaded with lysates from independently transduced cell cultures. *p < 0.05, **p < 0.01, ***p < 0.001 (Welch’s t-test, compared to vector in each condition).
Supplementary Figure S5 | IRS4 induces constitutive stimulation of PI3K/AKT/mTOR pathway signalling increases cell proliferation in mammary epithelial cells

(A) Western blot showing phosphorylated (p-) and total-protein of components of the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways from NMuMG-Irs4 cells grown in medium containing 0.5%FBS for 30 h in the presence of increasing concentrations of PI3K-specific inhibitors GDC0941 or ZSTK474, or in vehicle (DMSO). Lysates from NMuMG cells transduced with empty vector were used as a control.

(B,C) Ratios of phosphorylated over total AKT (B) and S6 (C) from the blot in A.

(D) Growth curves of NMuMG-Irs4 cells in medium supplemented with 0.5%FBS and in the presence of increasing concentrations of the PI3K-specific inhibitor GDC0941, compared with vehicle treated and vector control cells. Growth was measured as cell density over time by the IncuCyte imager. Data are represented as local regression from quadruplicates.

(E) Representative Western blot of two independent experiments, showing phosphorylated and total-protein components of the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways in NMuMG-Irs4 cells cultured 48 h in medium supplemented with 0.5%FBS and 200 nM GDC0941 or vehicle (DMSO). Cells transduced with empty vector were used as vector controls.

(F,G) Ratios of phosphorylated over total AKT (F) and S6 (G) from the Western blot in E.

(H,I) Ratios of phosphorylated over total AKT (H) and S6 (I) from the blot in Figure 4F.

(J,K) Cell density of NMuMG-Irs4 cells cultured three days in medium supplemented with 0.5%FBS and 200 nM GDC0941 (J), 100 nM ZSTK474 (K) or vehicle (DMSO), and vehicle treated vector control cells. Data represented as mean ± SD of three experiments and are obtained from Crystal Violet cell staining assay. Representative images of the Crystal Violet staining are shown below the graphs.
Supplementary Figure S6 | Lack of functional SHP2-binding domain permits constitutive signalling of IRS4

(A) Schematic map of the Irs4ΔSHP2-1 and Irs4ΔSHP2-2 constructs. The 5’ translated region of Irs4 is indicated in blue and the SHP2-domain from the 3’-regions of Irs1 or Irs2 in red. Following the names of the inserted cDNAs, the nucleotide positions of the inserts are provided, based on the coding sequences of each cDNA (i.e. counting from the first A of ATG in the translational start site). Restriction sites of BglII and AvrII that were used for cloning are also shown.

(B) mRNA expression of Irs family members, Irs4 recombinants and empty vector construct transduced in NMuMG cells used in the experiments as determined by RT-PCR. For assessment of ectopic expression, the primer annealing to the vector backbone was located either upstream (5’) or downstream (3’) of the coding sequence of the insert. For specifically analysing the expression of the endogenous products, one of the primers was located in the untranslated region (UTR) of the genes, while the other was located in the translated region. Where assessing total Irs gene expression (ectopic and endogenous), we used primers pairs located in the translated region of the genes. P3724-R4 tumour cell line cDNA was used as positive control for endogenous Irs4 expression.

(C) Quantification of IRS1 protein levels in the Western blot shown in Figure 5B, corrected for loading and presented relative to the level in vector control cells. Although IRS1 is endogenously expressed in these cells, additional ectopic expression of the gene increases IRS1 levels with ~70%.

(D) Representative Western blots of three experiments, showing phosphorylated (p-) and total-protein of components in the PI3K/AKT/mTOR pathway of the same transduced NMuMG cells as in Figure 5B, after stimulation with 0.5% FBS for 10 min.
(E) Ratios phosphorylated AKT over total AKT, calculated from Western blots as those shown in D. Data are represented as mean + SD from three blots. The gels were loaded with lysates from independently transduced cells. *p < 0.05 (Welch's t-test, compared to Irs4).

(F) Linear regression of ln-transformed phosphorylated AKT over total AKT ratios vs time after stimulation, relative to peak signalling at 10 min stimulation, determined from three independent blots of Figure 5F. The transformed data points are fitted in a linear model and associated R²-values and model p-values are indicated. The 24 h time point was omitted for the linear model fit, as relative pAKT/AKT ratios increased again at this time point. For the same reason, the 8 h time point was additionally omitted to fit the linear model for NMuMG-Irs4, but was nonetheless plotted in the figure.

(G,H) Quantification of maximum growth rates (G) and times until maximum confluency was reached (H) of NMuMG cells transduced with the indicated constructs. The data were derived from growth curves represented in Figure 5H and were calculated as outlined in Supplementary Figure S3A. Data represented as mean + SD of quadruplicates. *p < 0.05, **p < 0.01, ***p < 0.001 (Welch’s t-test, compared to Irs4).
Supplementary Figure S7 | IRS4 in human breast cancer patient material and cells

(A) Quantification of IRS4 protein levels (log-scale), corrected for loading, derived from the Western blot shown in Figure 6B.

(B) Western blot analysis showing phosphorylated (p-) and total protein levels of major players in the PI3K/AKT/mTOR pathway from the primary human breast tumours shown in Figure 6B.

(C) Ratios of phosphorylated over total AKT, calculated from the blot in B, after normalization to the loading control levels (α-tubulin).

(D) Distribution of the IRS4-negative vs IRS4+++ tumours using the threshold depicted in Figure 6C over the indicated PAM50-based clinical subtypes. **p < 0.01 (Pearson's Chi-squared test).

(E) Expression of ERBB2 mRNA in the 27 random human primary breast carcinomas from Figure 6A, determined by qRT-PCR. Expression levels greater than median + SD (grey dashed line) were determined as positive (ERBB2+) and greater than median + 2* SD (black dashed line) as highly positive (ERBB2+++).

(F) Representative images of triplicates, showing Crystal Violet staining of MDA-MB-453 cells, transduced with the indicated shRNAs, allowed to grow for 10 days.
IRS4 drives cancer by PI3K/AKT/mTOR pathway hyperactivation | CHAPTER 3

(G) Western blot showing IRS4 protein, and phosphorylated (p-) and total-AKT from HCC1187 cells transduced with the indicated shRNAs.

(H) Quantified IRS4 levels plotted against ratios of phosphorylated AKT over total AKT, derived from the blot in G. Linear regression and correlated R²-values show a positive correlation.

(I) Quantified IRS4 levels, derived from the blot in G, plotted against their cell proliferation rate, determined by the Crystal Violet staining of the same cells allowed to grow for 6 days (in triplicate). Linear regression and correlated R²-values show a positive correlation.

Supplementary Figure S8 | Full size gels corresponding to Figure 1E
See the legend of Figure 1E for details.

Supplementary Figure S9 | Full sized scans of the Western blots corresponding to Figures 2D, 4A, 4C, 4F, 5B, 5C, 5E, 5I, 6B and 6G
See the legends of the respective figures for details.
CHAPTER 3 | IRS4 drives cancer by PI3K/AKT/mTOR pathway hyperactivation

**Supplementary Table S1** | Location and orientation of MMTV proviral insertions in the *Irs4* locus from the insertional mutagenesis screens, also providing tumour ID, mouse strain background and genotype

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**Supplementary Table S2** | RSN-normalized log₂-transformed IRS4 microarray expression values from 157 metastasized breast cancers and corresponding PAM50-based subtype, quantified by the microarray probe ILMN_1712774 of the Illumina HumanHT-12 v4 Expression BeadChip

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## IRS4 drives cancer by PI3K/AKT/mTOR pathway hyperactivation

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SUPPLEMENTARY MATERIAL & METHODS

SUPPLEMENTARY CELL LINES AND CULTURE CONDITIONS
184A1, BT549, CAMA1, HBL100, HCC1569, HCC1937, HCC1954, HCC38, HCC70, HEK293, HS578T, MCF7, MDA-MB-361, T47D and ZR75-30 were purchased from ATCC and the ZR75-1 cell line from Centocor. BT20, MDA-MB-134 and MDA-MB-157 were a kind gift from Dr. J. Taylor-Pappadimitriou (Guy's Hospital, London) and the MDA-MB-231 cell line was kindly provided by Dr. M.M.K. Mareel (University of Ghent). The MPL-13 cell line is an in-house established cell line from pleural effusion of a breast cancer patient. 184A1 were cultured in DMEM/F-12 medium supplemented with 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin (PenStrep). HCC1569, HCC1937, HCC1954, HCC38, HCC70 and ZR75-30 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS and PenStrep. All other cells were cultured in DMEM supplemented with 10% FBS and PenStrep. SKBR3-medium was additionally supplemented with 5 µg/ml insulin. All cell lines were never cultured for more than eight passages upon receipt and were routinely tested for Mycoplasma (Hoechst staining and PCR).

SUPPLEMENTARY APOPTOSIS ASSAYS
0.8·10⁶ P3724-R4 cells were allowed to attach overnight to a T25 flask (BD Falcon) in DMEM/F-12 medium supplemented with 10% FBS, PenStrep, 5 µg/ml insulin, 200 ng/ml hydrocortisone and 20 ng/ml EGF, after which 0.2 µM, 2 µM or 20 µM etoposide was added to the medium. After 24 h, cell exposed to 2 µM or 20 µM etoposide showed clear blebbing, upon which the cells were released by EDTA-free trypsin-250 solution and stained with Annexin V and PI using the Annexin V-FITC Apoptosis Detection Kit (Abcam), following manufacturers’ instructions, and analysed using a Beckton Dickinson LSRII FACS analyser.

SUPPLEMENTARY CELL PROLIFERATION ASSAYS
25,000 or 80,000 cells per well were seeded in 12-wells plates (Corning) for 6-8 day or 3-day experiments, respectively. Cells were allowed to attach for 6 h, washed with...
DMEM/F-12 medium only supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin (Gibco) and then grown under the indicated conditions. At the indicated time points, the cells were fixed with 4% Formaldehyde solution (Klinipath). Fixed cells of all time points were stained simultaneously with 0.1% Crystal Violet solution (Sigma-Aldrich) and imaged using a desktop scanner (Epson). Cell proliferation was quantified by extracting the dye with 10% acetic acid and measuring the absorbance at 590 nm using the Infinite plate reader (TECAN).
SUPPLEMENTARY REFERENCES


Insulin Receptor Substrate 4 (IRS4) and ES Cell Expressed Ras (ERAS) induce resistance to HER2-targeted therapy

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Division of Molecular Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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Ikink et al. (2016), Nature Communications, doi: 10.1038/ncomms13567
and:
Ikink et al. (2018), Oncogene, doi: 10.1038/s41388-017-0031-0
ABSTRACT

Personalised medicine for cancer patients requires a deep understanding of the underlying genetics that drive cancer and the subsequent identification of predictive biomarkers. The discovery of new genes and genetic pathways contributing to oncogenesis and mechanisms affecting therapy resistance are at the base of this. We previously performed MMTV-induced insertional mutagenesis screens in mouse models for breast cancer and identified IRS4 and ERAs as novel oncogenic drivers in HER2+ breast cancer. In this study, we show that IRS4 and ERBB2/HER2 synergistically induce tumorigenesis, which we have previously also showed for ERAS. Importantly, we additionally show that both ERAS and IRS4 induce primary resistance to the widely used HER2-targeting drugs Trastuzumab (Herceptin) and Lapatinib (Tykerb/Tyverb) in vivo, and are involved in acquired resistance via selective upregulation during treatment in vitro, indicating that both ERAS and IRS4 may serve as a novel clinical biomarker for PI3K/AKT/mTOR pathway hyperactivation and HER2-targeted therapy resistance.
INTRODUCTION

The HER2+ subtype of breast cancer is a highly aggressive disease with a poor prognosis (Engstrøm et al., 2013; Ferrero-Poüs et al., 2000; Slamon et al., 1987). Treatment of HER2+ breast cancer with HER2-targeted therapies such as the humanised monoclonal antibody against ERBB2, Trastuzumab (Herceptin) (Dawood et al., 2010; Romond et al., 2005; Slamon et al., 2001), or the small molecule kinase inhibitor Lapatinib (Tykerb/Tyverb) (Blackwell et al., 2010; Geyer et al., 2006; Spector et al., 2005), typically combined with chemotherapy, significantly improves the clinical outcome. However, inherent and acquired resistance to both drugs are prevalent and the underlying mechanisms leading to resistance are poorly understood (Gagliato et al. 2016; Gajria and Chandarlapaty 2011; Thery et al. 2014; D’Amato et al. 2015). Therefore, improving our knowledge about genes and pathways collaborating with ERBB2 overexpression in breast tumorigenesis is essential in designing complementary therapeutic strategies.

Using MMTV-induced insertional mutagenesis, we identified Irs4 and Eras as novel mammary oncogenic drivers, as described in Chapters 2 and 3. We additionally showed that both genes cause a potent hyperactivation of the PI3K/AKT/mTOR pathway upon expression and that both genes are normally silent in mammary tissue, but are expressed in a subset of HER2+ breast cancer. Although the collaboration of ERAS with ERBB2 has been elucidated in Chapter 2, the interplay of IRS4 and ERBB2 has so far remained unclear, but is investigated in the current study.

It is well established that a major determinant of resistance to HER2-targeted therapy in breast cancer is hyperactivation of the PI3K/AKT/mTOR pathway (Appert-Collin et al., 2015; Berns et al., 2007; Chandarlapaty et al., 2012; Cizkova et al., 2013; Eichhorn et al., 2008; Esteva et al., 2010; Hanker et al., 2013; Junttiila et al., 2009; Loibl et al., 2016a, 2016b; Nagata et al., 2004; Park et al., 2014; Razis et al., 2011; Serra et al., 2008; Wang et al., 2011, 2013). Hence, considering their expression in HER2+ tumours and their ability to induce constitutive PI3K/AKT/mTOR pathway activation, we here investigate the involvement of IRS4 and ERAS with regard to sensitivity to therapeutic agents.
CHAPTER 4 | IRS4 and ERAS induce resistance to HER2-targeted therapy

RESULTS

IRS4 SYNERGISES WITH ERBB2 TO CELL PROLIFERATION AND TUMORIGENESIS

To assess the interplay between ERBB2 (HER2) and IRS4, we expressed the ErbB2 oncogene (HER2, neu) in the murine mammary epithelial cell line NMuMG cells, transduced with Irs4 or vector control cells. Although both Irs4 and ErbB2 individually already accelerate cell proliferation, we observed a strong synergistic induction of cell proliferation rate in cells expressing both genes, notably higher than cells expressing either gene individually (Figure 1A). A significant synergistic induction was also observed for anchorage-independent growth when Irs4 and ErbB2 were co-expressed (Figures 1B and 1C). We did not observe this synergism between ErbB2 and overexpressed Irs1 (Figure 1C). Also in the human HER2+ breast tumour cell line BT474, in which ERBB2 is amplified, Irs4 expression had a strong effect on anchorage-independent growth (p = 0.0143, Welch's t-test) (Figures 1D and 1E). To assess whether this oncogenic synergistic effect of Irs4 and ErbB2 also occurs in vivo, we subcutaneously injected $1 \cdot 10^6$ NMuMG cells transduced with both genes, individually or in combination, in both flanks of five female BALB/c nude mice (Figure 1F). Indeed, co-expression of Irs4 and ErbB2 strongly induced tumour growth, confirming their synergistic collaboration towards tumorigenesis.

IRS4 AND ERAS REDUCE SENSITIVITY TO HER2-TARGETING THERAPY IN VITRO

Although HER2-targeting therapy has improved clinical outcome, therapy resistance remains a considerable problem in the clinic and the PI3K/AKT/mTOR pathway hyperactivation has been associated with therapy resistance in HER2+ breast cancer. Since we previously showed that both IRS4 and ERAS constitutively activate the PI3K/AKT/mTOR pathway, we interrogated whether expression of IRS4 or ERAS could also alter the response to HER2-targeting therapy. Indeed, we found that expression of either gene in the HER2+ breast cancer cell lines SKBR3 and BT474, leads to a marked reduction in sensitivity to both the ERBB2-targeting monoclonal antibody Trastuzumab and the small molecule ERBB2-kinase inhibitor Lapatinib,
IRS4 and ERAS induce resistance to HER2-targeted therapy

**Figure 1 | IRS4 synergises with ERBB2 (HER2)**

(A) Growth curves of NMuMG cells, stably transfected with Irs4 and/or ErbB2, or empty vector, derived from Crystal Violet staining. Cells were cultured in medium supplemented with 0.5% FBS, and with (left) or without (right) insulin. Data depicted are the mean ± SD of three experiments using independently transduced cell pools.

(B) Representative soft agar growth scans of NMuMG cells, stably transduced with Irs4, Irs1 or empty vector only or additionally with ErbB2, allowed to grow for one week.

(C) Anchorage-independent growth (mean ± SD) of three independent experiments as B. NS, not significant; ***p < 0.001, ****p < 1·10^-4 (Welch's t-test, only showing relevant comparisons).

(D) Representative whole well scans and phase-contrast micrographs of three soft agar growth experiments of BT474 cells, stably transfected with Irs4 or empty vector. The cells were allowed to grow in soft agar for two weeks. (Scale bar = 50 µm).

(E) Quantification of anchorage-independent growth by the GelCount colony counter of three independently transduced BT474 cell cultures as shown in D. Data are represented as mean ± SD. **p < 0.01 (Welch's t-test, compared to vector).

(F) Tumour growth (mean ± SD) in mice subcutaneously injected in both flanks with 1·10^6 NMuMG cells ectopically expressing Irs4 and/or ErbB2, and vector control cells (n = 5 each, i.e. 10 flanks).

enabling sustained tumour cell growth (Figures 2A-2C; Supplementary Figures S1A-S1F).

In order to confirm that the mechanism behind this apparent therapy resistance by IRS4 or ERAS is due to their potent PI3K/AKT/mTOR pathway activation, we treated HER2+ breast cancer cell lines with suboptimal concentrations of Trastuzumab or Lapatinib, in combination with the PI3K inhibitor GDC-0941 (Figures 2D-2G; Supplementary Figures S1G-S1J). Although single-agent treatments had little to no
CHAPTER 4 | IRS4 and ERAS induce resistance to HER2-targeted therapy

Figure 2 | IRS4 and ERAS induce Trastuzumab and Lapatinib resistance via PI3K/AKT/mTOR pathway hyperactivation

(A,B) Representative images of four independent experiments, showing Crystal Violet staining of SKBR3 cells, stably transfected with Irs4 (A) or ERAS (B), or empty vector, allowed to grow for 11 days in medium supplemented with the indicated concentrations Lapatinib, or vehicle (25,000 cells per well, 24-wells plates). The same experiments using BT474 cells and using Trastuzumab are shown in Supplementary Figures S1A-S1F.

(C) Lapatinib dose response curves of SKBR3 as in A and B. The data points represent the mean ± SD (n = 4) of the cell density of the Lapatinib-treated relative to vehicle control cells (1,000 cells per well were plated in 384-wells plates). The fitted curve and associated R² and IC₅₀-values are shown.

(D) Representative Crystal Violet staining of BT474 vector controls or BT474 cells stably transduced with ERAS, grown in presence of 20 nM Lapatinib (grown 13 days), 5 μg/ml Trastuzumab (grown 11 days) and/or 20 nM PI3K-inhibitor GDC-0941, or equal volumes of DMSO (vehicle) (50,000 cells per well, 12-wells plates). The same experiment using SKBR3 cells is shown in Supplementary Figure S1G.

(E) Quantification of D, showing cell proliferation relative to vehicle controls. Data plotted as mean ± SD (n = 3). A projection of the combined (i.e. additive) single agent effects of GDC-0941 (GDC) and Lapatinib
IRS4 and ERAS induce resistance to HER2-targeted therapy

(Lap) or Trastuzumab (Trast) is shown in green. The same analysis using SKBR3 cells is provided in Supplementary Figure S1H.

(F) Representative soft agar growth scans of BT474 cells, stably transduced with Irs4 or empty vector, cultured for two weeks in presence of 5 µg/ml Trastuzumab or 50 nM Lapatinib with or without 100 nM GDC0941, 100 nM GDC0941 only, or vehicle. Representative phase-contrast micrographs are provided in Supplementary Figure S1I.

(G) Quantification of anchorage-independent growth (mean + SD) of three independent experiments as in F. NS, not significant; **p < 0.01, ***p < 0.001 (Welch's t-test, only showing relevant comparisons).

influence on cell proliferation rates and anchorage-independent growth rates of breast cancer cells expression both \textit{ERBB2} and \textit{Irs4} or \textit{ERAS}, the combination of HER2 and PI3K-targeting agents potently reduced cell proliferation. In other words, addition of the PI3K inhibitor abrogated IRS4- and ERAS-mediated resistance. Thus, these data strongly suggest that it is indeed the potent PI3K/AKT/mTOR pathway activation by IRS4 or ERAS that confers resistance to HER2-targeting therapeutics.

**HER2-TARGETED TREATMENT SELECTS FOR IRS4 AND ERAS EXPRESSION IN VITRO**

In analogy to acquired therapy resistance in patients with HER2+ breast cancer, we investigated whether expression of endogenous \textit{IRS4} or \textit{ERAS} could also be attained in treatment-naive HER2+ breast cancer cell lines under selective pressure of Trastuzumab or Lapatinib. To this end, we cultured SKBR3 and BT474 cells for five passages in medium containing increasing concentrations of Trastuzumab or Lapatinib and tested expression levels of \textit{IRS4} and \textit{ERAS} after each passage. In more than half of the cultures, \textit{ERAS} expression was induced within five passages in the presence of Trastuzumab or Lapatinib, especially at higher drug concentrations (Figures 3A and 3B; Supplementary Figures S2A and S2B). Expression of \textit{IRS4} was induced within five passages in most of the cultures, especially with Lapatinib (Supplementary Figures S3A-S3D). Induction of expression of these genes was not observed when cells were cultured for five passages without the therapeutic agents (i.e. vehicle treated controls). Detailed expression analysis of each passage revealed that \textit{IRS4} and \textit{ERAS} expression increased gradually with every passage, indicating a selection for increased expression by the increasing drug concentrations (Figure 3C and Supplementary Figures S2C and S4E). Hence,
Figure 3 | HER2-targeted treatment selects for ERAS+ cells

Similar experiments for IRS4 are provided in Supplementary Figure S3.

(A,B) ERAS mRNA expression levels in BT474 cells (Parental), cultured for five passages in presence of vehicle (to aid interpretation of the graph this level is also indicated by a grey dashed line) or the indicated concentrations Lapatinib (A) or Trastuzumab (B). Expression data, as determined by quantitative RT-PCR, are represented as mean ± SD of triplicates and are shown relative to ERAS levels in vehicle controls. Lapatinib or Trastuzumab concentrations after each passage are shown below and passage number (p) is depicted in grey at the right. The same experiments using SKBR3 cells are shown in Supplementary Figures S2A and S2B.
IRS4 and ERAS induce resistance to HER2-targeted therapy

(C) Relative ERAS expression levels at each passage in the cultures that acquired the highest ERAS levels under Lapatinib or Trastuzumab selection depicted in A and B, respectively. The same experiment using SKBR3 cells is provided in Supplementary Figures S2A and S2B.

(D) Western blot, showing ERAS knockdown efficiency in the Trastuzumab-treated BT474 cell culture of C (designated: BT474/RERAS+). α-Tubulin was used as loading control. The quantification and the same analysis in SKBR3 cells are provided in Supplementary Figures S2D and S2E.

(E) Representative Western blots showing phosphorylated (p-) and total protein of AKT in a selection of the cell cultures from D. α-Tubulin was used as loading control. The same experiment using SKBR3 cells is shown in Supplementary Figure S2F.

(F) Ratios phosphorylated over total AKT, relative to shGFP controls, quantified from the blots as in E. Data represented as mean ± SD (n = 3) and statistically significant differences in PI3K/AKT/mTOR pathway activation between shGFP controls and shERAS are indicated as **p < 0.01; ***p < 0.001; ****p < 1·10^-4 (Welch’s t-test). The same analysis using SKBR3 cells is provided in Supplementary Figure S2G.

(G) Growth curves of the BT474/RERAS+ cell culture as in F in medium supplemented with 10% FBS or 0.5% FBS, based on Crystal Violet staining (50,000 cells per well, 24-wells plates). Plotted values are mean ± SD, relative to day 0 (n = 3). Statistical significance of differences in cell proliferation between shGFP controls and shERAS was determined by a Welch's t-test and p-values are shown as *p < 0.05; **p < 0.01; ***p < 0.001; NS = not significant. The same analysis using SKBR3 cells is provided in Supplementary Figure S2H.

(H) Representative Crystal Violet staining (n = 3) of the BT474/RERAS+ cells as in F, allowed to grow for 6 days in the presence of 20 nM or 40 nM Lapatinib or equal volumes of vehicle (DMSO) (50,000 cells per well, 24-wells plates). The same experiment using SKBR3 cells is shown in Supplementary Figure S2I.

Trastuzumab and Lapatinib exert a selective pressure in favour of the resistant IRS4 and ERAS-positive cells.

Subsequent knockdown of Trastuzumab-induced endogenous IRS4 or ERAS expression in BT474 and SKBR3 cultures with the highest induction (designated: BT474/IRS4+, BT474/ERAS+, SKBR3/IRS4+ and SKBR3/ERAS+, respectively) resulted in a concomitant significant reduction in PI3K/AKT/mTOR pathway activation, cell proliferation and Lapatinib resistance (Figures 3D-3H; Supplementary Figures S2D-S2I and S3F-S3K). These data provide further prove that induction of IRS4 or ERAS expression renders tumour cells insensitive to HER2-targeted therapy and as such present a new mechanism of therapy resistance.
IRS4 AND ERAS INDUCE RESISTANCE TO HER2-TARGETING THERAPY IN VIVO

To confirm therapy resistance in vivo, we subcutaneously injected $1 \times 10^6$ NMuMG cells, transduced with the ErbB2 oncogene, in one flank of 18 female BALB/c nude mice and $1 \times 10^6$ NMuMG cells additionally transduced with either Irs4 or ERAS in each opposite flank. After tumours were established in both flanks, nine mice were randomly assigned to receive Lapatinib treatment (Figures 4A and 4B; Supplementary Figures S4A and S4B). Importantly, we observed a significant reduction in NMuMG-ErbB2 tumour growth, whereas tumours from cells additionally expressing Irs4 were unaffected by Lapatinib treatment. Together, these results confirm IRS4- and ERAS-induced resistance to HER2-targeted therapy via hyperactivation of the PI3K/AKT/mTOR pathway and suggest that IRS4 or ERAS expression may also cause HER2-targeting therapy resistant relapse in patients.

**Figure 4 | ERAS induces resistance to HER2-targeted treatment**

The same experiments for IRS4 are provided in Supplementary Figure S4.

(A) Tumour growth (mean + SD) in mice subcutaneously injected with $1 \times 10^6$ ErbB2+ NMuMG cells, transduced with ERAS or empty vector, in opposite flanks, untreated or treated with 100 mg/kg/day Lapatinib (n = 9 each, i.e. 18 flanks). When tumours were established in both flanks mice were randomly allocated to either group before treatment was started (indicated by arrow). NS, not significant; *p < 0.05, **p < 0.01, ***p < 0.001 (Welch’s t-test, compared to each untreated control).

(B) Size of the tumours depicted in A at day 20, relative to the corresponding tumour’s size at start of treatment. NS, not significant; **p < 0.01 (Welch’s t-test).
DISCUSSION

Although the introduction of several HER2-targeting drugs has significantly improved clinical outcomes for HER2+ breast cancer patients, both de novo and acquired therapy resistance is common. Indeed, only half of HER2+ breast cancer cases respond to HER2-targeted therapies and this therapy resistance is also reflected in ErbB2-transgenic mouse models (Ellis and Perou, 2013; Knutson et al., 2004). Notably, PI3K/AKT/mTOR pathway activation is implicated in resistance to the commonly used HER2-targeted therapeutics, the ERBB2-targeting monoclonal antibody Trastuzumab (Herceptin) or the small molecule ERBB2-kinase inhibitor Lapatinib (Tykerb), specifically through activating mutations in PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) or loss of its antagonist PTEN (phosphatase and tensin homolog) (Berns et al., 2007; Chandarlapaty et al., 2012; Eichhorn et al., 2008; Esteva et al., 2010; Hanker et al., 2013; Majewski et al., 2015; Nagata et al., 2004; Wang et al., 2011).

In Chapters 2 and 3, we identify both IRS4 and ERAS as important genes that hyperactivate the PI3K/AKT/mTOR pathway. We here show that co-expression of Irs4 and ErbB2 in mammary epithelial cells synergistically accelerated tumour growth due to their activity in complementary oncogenic pathways: the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways, respectively, which in Chapter 2 we reported for ERAS and ErbB2 as well (Figures 5A and 5B). This urged us to investigate whether the IRS4- and ERAS-mediated constitutive PI3K/AKT/mTOR pathway activation causes resistance to HER2-targeting drugs in HER2+ breast cancer cell lines. Our results clearly demonstrate that IRS4 expression induces resistance to both Trastuzumab and Lapatinib in vitro and Lapatinib resistance in vivo (Figures 5C and 5D). Moreover, in Chapters 2 and 3 we found that IRS4 mRNA is expressed in at least 10% and ERAS mRNA in at least 13% of HER2+ human breast carcinomas, further subscibing the clinical relevance of IRS4 and ERAS. Together, these results suggest that IRS4 and ERAS expression may be a promising biomarker for de novo therapy resistance. We also demonstrate that these genes may be involved in acquired resistance. In a clinically relevant setting, we demonstrate that treatment of breast cancer cells with suboptimal doses
CHAPTER 4 | IRS4 and ERAS induce resistance to HER2-targeted therapy

of the HER2-targeting drugs Trastuzumab and Lapatinib consistently results in
selection of cells with upregulated \textit{IRS4} or \textit{ERAS} levels. This suggests that \textit{IRS4} or
\textit{ERAS} induction, leading to drug resistance, may also frequently occur in patients
treated with these anti-cancer drugs.

![Figure 5](image)

\textbf{Figure 5} | Proposed model of ERAS and IRS4-induced therapy resistance
\textbf{(A,B)} Model of signalling in HER2+ (ERBB2) breast cancer cells where ERBB3 (HER3) (A) or ERAS or
IRS4 (B) activates the PI3K/AKT/mTOR pathway.
\textbf{(C,D)} Model of the effect of Trastuzumab or Lapatinib treatment in HER2+ breast cancer cells expressing
\textit{ERBB3} (C) or \textit{ERAS} or \textit{IRS4} (D).

Thickness of the arrows indicates strength of signalling. Red 'X' indicates no interaction/signalling. Dashed
arrow indicates possible cross-talk.

The standard treatment for HER2+ breast carcinomas comprises the combination of
chemotherapy with Trastuzumab, followed by Lapatinib treatment when tumours do
not respond to Trastuzumab. Considering that ERAS was recently also implicated in
resistance to chemotherapeutic agents in neuroblastoma and gastric cancer cell
lines (Aoyama et al., 2010; Kubota et al., 2011), the clinical importance of this gene
for therapeutic response is further highlighted. Promisingly though, we observed in
a pre-clinical setting that the combination of suboptimal doses of a PI3-kinase
inhibitor with suboptimal doses of Trastuzumab or Lapatinib can abrogate both IRS4-
and ERAS-induced therapy resistance. This combination therapy may therefore offer
an important window of opportunity for treatment of HER2+ breast cancer patients
with primary or acquired resistance to HER2-targeting drugs.

One may therefore consider treating HER2+ tumours expressing high levels of \textit{IRS4}
or \textit{ERAS} with drugs targeting PI3K, AKT and/or mTOR in combination with HER2-
targeted therapy. However, in the recently completed BOLERO-3 randomised
phase-III study of Trastuzumab-resistant advanced breast cancer, the addition of the mTOR-inhibitor Everolimus to Trastuzumab and Vinorelbine treatment showed some clinical benefit only in hormone receptor negative (HR-) patients (André et al., 2014). The BOLERO-1 phase-III randomised study similarly showed a prolongation of progression-free survival (PFS) by combined treatment of Trastuzumab with Paclitaxel and Everolimus in patients with HER2+ HR- advanced breast cancer, although this did not reach protocol-specified significance (Hurvitz et al., 2015). However, the fact that both these trials did not preselect patients with tumours exhibiting high PI3K/AKT/mTOR pathway activation may have limited the overall outcome. Indeed, patients from the BOLERO-3 trail with PTEN-low and pS6-high tumours both derived significantly more benefit from Everolimus than the PTEN-high and pS6-low groups, respectively (André et al., 2014). Hence, clinical trials with selective enrolment are needed to shed more light on the clinical benefits of combined HER2 and PI3K/AKT/mTOR-targeted therapy in tumours with PI3K/AKT/mTOR pathway hyperactivation, including IRS4 or ERAS expressing tumours.

Altogether, both IRS4 and ERAS may prove useful novel clinical biomarkers for HER2-targeted therapy resistance and may also be of wider clinical relevance as biomarker for PI3K/AKT/mTOR pathway-dependency in cancer.
CHAPTER 4 | IRS4 and ERAS induce resistance to HER2-targeted therapy

MATERIAL & METHODS

IN VIVO TUMORIGENICITY ASSAYS

Tumorigenicity following subcutaneous injection of $1 \cdot 10^6$ viable cells in both flanks of 4-week old female BALB/cABomA-nu/nu (BALB/c nude) mice, bred in our institute, was assessed as described in Chapters 2 and 3. For the in vivo Lapatinib-resistance experiment, we subcutaneously injected $1 \cdot 10^6$ NMuMG cells ectopically expressing Neu alone or in combination with Irs4 or ERAS, in opposite flanks of 18 4-week old female BALB/cAnNRj-Foxn1nu mice (Janvier Labs, Le Genest-Saint-Isle, France). Upon establishment of the tumours in both flanks (4 days after first observation), the mice were randomly and evenly divided in a group provided with normal chow and a group provided with chow mixed with 0.48 g/kg Lapatinib (provided by the Netherlands Cancer Institute pharmacy). The estimated Lapatinib dose per mouse is 100 mg/kg/day, as previously reported (Seemann et al., 2013). Serum was collected by allowing blood obtained from sacrificed mice to clot at RT for ~2 h, followed by centrifugation for 12 min at 1,500g and 4°C. Lapatinib serum concentrations were analysed by HPLC-MS/MS by our in-house pharmacy laboratory. All mouse experiments were approved by the local Animal Experiments Committee (DEC) and strictly followed the Dutch Code of Practice for Research with Laboratory Animals in Cancer Research.

ESTABLISHING HER2-THERAPEUTY RESISTANT CELL CULTURES

50,000 BT474 or SKBR3 cells per well were seeded in a 6-wells plate in medium containing 0.1, 0.2 or 0.5 µg/ml Trastuzumab; 10, 20 or 40 nM Lapatinib; or equal volumes of dimethyl sulfoxide (DMSO) diluent. Cells were passaged upon reaching near-confluency and half of the cells were transferred to a new well, while the other half was used for RNA isolation. Every second passage, cells were split and transferred into two new wells instead of one. Trastuzumab and Lapatinib concentrations were increased in the next passage if cell proliferation rates were comparable to the vehicle-treated cells, or kept the same when growth was
noticeably slower (see Figures 3A and 3B as well as Supplementary Figures S2A, S2B, S3A-S3D for schematic representations of the concentrations used).

**CELL CULTURE AND **IN VITRO **EXPERIMENTS**

Details on the sources of the used cell lines, culturing conditions, constructs and vectors, transduction, *in vitro* treatments and cell proliferation, dose response and anchorage-independent growth (soft agar) assays are described in Chapters 2 and 3. Lapatinib Ditosylate (Selleck Chemicals, Houston, TX) was dissolved in DMSO, Trastuzumab/Herceptin (Roche) was dissolved in water and obtained via our outpatient clinic. GraphPad PRISM 6 software was used to calculate and plot dose response curves as nonlinear regression of cell density values, after normalization to vehicle-treated conditions, over log-transformed Lapatinib concentrations.

**STATISTICAL ANALYSES**

The statistical programming language R, including the "Bioconductor" and "Survival" packages, were used for statistical analyses. All the statistical tests are specified in the text and/or figure legends. A p-value < 0.05 was considered statistically significant.

**ACKNOWLEDGEMENTS**

See Chapters 2 and 3.
REFERENCES


IRS4 and ERAS induce resistance to HER2-targeted therapy


Supplementary Figures

Supplementary Figure S1 | IRS4 and ERAS induce Trastuzumab and Lapatinib resistance via PI3K/AKT/mTOR pathway hyperactivation

(A-F) Representative images of four independent experiments, showing Crystal Violet staining of BT474 cells (A,B,D,F) and SKBR3 cells (C,E), stably transfected with Irs4 or empty vector, allowed to grow for 11 days in presence of the indicated Lapatinib (A,B) or Trastuzumab (C-F) concentrations, or vehicle.

(G) Representative Crystal Violet staining of SKBR3 vector controls or SKBR3 cells stably transduced with ERAS, grown in presence of 50 nM Lapatinib (grown 11 days), 10 μg/ml Trastuzumab (grown 7 days) and/or 50 nM PI3K-inhibitor GDC-0941, or equal volumes of DMSO (vehicle) (at t = 0, 50,000 cells per well were seeded, 12-wells plates).

(H) Quantification of G, showing cell proliferation rate in the presence of the inhibitors relative to vehicle controls. Data plotted as mean + SD (n = 3). A projection of the combined (additive) single agent effects of GDC-0941 (GDC) and Lapatinib (Lap) or Trastuzumab (Trast) is shown in green.

(I) Representative phase-contrast micrographs of anchorage-independent growth of BT474 cells from the soft agar assay depicted in Figure 2F (scale bar = 50 μm).
CHAPTER 4 | IRS4 and ERAS induce resistance to HER2-targeted therapy

(J) Growth curves of SKBR3 cells, stably transfected with \textit{Irs4} or empty vector, treated with 50 nM Lapatinib and/or 50 nM GDC0941, or vehicle (DMSO), derived from Crystal Violet staining. Data depicted are the mean ± SD of three experiments using independently transduced cell pools.
**Supplementary Figure S2** | HER2-targeted treatment selects for ERAS+ cells

(A,B) ERAS mRNA expression levels in SKBR3 cells (Parental), cultured for five passages in presence of vehicle (this level is also indicated by grey dashed line for clarity) or the indicated concentrations Lapatinib (A) or Trastuzumab (B). Data are represented as mean ± SD of triplicates and are shown relative to ERAS levels in vehicle controls. Lapatinib (A) or Trastuzumab (B) concentrations during each passage are shown below and passage numbers (p) are depicted in grey on the right.

(C) Relative ERAS expression levels at each passage in the cultures that obtained the highest ERAS levels under Lapatinib or Trastuzumab selection depicted in A and B, respectively.

(D) Western blot, showing ERAS knockdown efficiency in the Trastuzumab-treated SKBR3 cell culture of C (designated: SKBR3/RERAS+). α-Tubulin was used as loading control.

(E) Quantification of ERAS protein levels in the SKBR3/RERAS+ and BT474/RERAS+ cells from the blots in D and Figure 3D, respectively. Levels were corrected to the loading controls of each lane (α-tubulin).

(F) Representative Western blots showing phosphorylated (p-) and total protein of AKT in a selection of cell cultures from D. α-Tubulin was used as loading control.

(G) Ratios phosphorylated over total AKT, relative to shGFP controls, quantified from the blots as in F. Data represented as mean ± SD (n = 3) and statistically significant differences in PI3K/AKT/mTOR pathway activation between shGFP controls and shERAS are indicated as *p < 0.05; **p < 0.01 (Welch's t-test).

(H) Growth curves of the SKBR3/RERAS+ cells as in G in medium supplemented with 10% FBS or 0.5% FBS, based on Crystal Violet staining (30,000 cells per well, 24-wells plates). Plotted values are mean ± SD, relative to day 0 (n = 3). Statistical significance of differences in cell proliferation between shGFP controls and shERAS was determined by a Welch's t-test and p-values are shown as *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 1·10⁻⁴.

(I) Representative Crystal Violet staining (n = 3) of the SKBR3/RERAS+ cells as in G, allowed to grow for 6 days in the presence of 30 nM or 100 nM Lapatinib or equal volumes of vehicle (DMSO) (30,000 cells per well were seeded at t = 0, 24-wells plates).
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IRS4 and ERAS induce resistance to HER2-targeted therapy | CHAPTER 4

**Supplementary Figure S3** | HER2-targeted treatment selects for IRS4+ cells

(A–D) IRS4 expression levels of BT474 cells (A,B) and SKBR3 cells (C,D) (lanes marked 'Parental') and subcultures of the same cell lines cultured for five passages in presence of increasing concentrations of Lapatinib (A,C) or Trastuzumab (B,D), relative to vehicle-treated cells (indicated by grey dashed lines). IRS4 expression levels were determined by qRT-PCR and are represented as mean ± SD of triplicates. Passage number and corresponding drug concentrations are indicated. The †-symbol indicates that the cells did not survive the increase in concentration in the fifth passage, therefore IRS4 expression of the fourth passage was measured instead. The cells were initially also cultured in the presence of 40 nM Lapatinib in passage 1, but the BT474 cells did not survive this concentration even for a single passage, while SKBR3 cells did not survive more than two passages. Hence, these data are not shown.

(E) Relative IRS4 expression levels from cultures in B, showing all five passages of a culture acquiring IRS4 expression early (left) and late (right).

(F) Western blots of IRS4 and phosphorylated (p-) and total-AKT from SKBR3/RIRS4+ and BT474/RIRS4+ cells transduced with the indicated shRNAs.

(G) Quantified IRS4 levels plotted against ratios of phosphorylated AKT over total AKT, derived from the blot in F (and duplicate). Linear regression and correlated $R^2$-values show a positive correlation.

(H,I) Growth curves of SKBR3/RIRS4+ (H) and BT474/RIRS4+ cells (I), transduced with the indicated shRNAs giving the strongest knockdown of IRS4, compared to shGFP-controls, cultured in 0.5% or 10% serum (FBS). Data depicted as the mean ± SD of three Crystal Violet staining experiments. NS, not significant; *p < 0.05, **p < 0.01, ***p < 0.001 (Welch’s t-test, compared to shGFP).

(J,K) Representative images of three experiments, showing Crystal Violet staining of SKBR3/RIRS4+ (J) and BT474/RIRS4+ cells (K) as in H and I, allowed to grow for 6 days in presence of the indicated Lapatinib concentrations, or vehicle (DMSO).

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**Supplementary Figure S4** | IRS4 induces resistance to HER2-targeted treatment

(A) Tumour growth (mean ± SD) in mice subcutaneously injected with 1·10⁶ ErbB2+ NMuMG cells (as in Figure 1F) in opposite flanks, untreated or treated with 100 mg/kg/day Lapatinib (n = 9 each, i.e. 18 flanks). Arrow indicates start of treatment. *p < 0.05, **p < 0.01, ***p < 0.001 (Welch’s t-test, compared to each untreated control).

(B) Size of the tumours depicted in A at day 20, relative to the corresponding tumour’s size at start of treatment. NS, not significant; **p < 0.01 (Welch’s t-test).
CHAPTER 5

General Discussion

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INSERTIONAL MUTAGENESIS SCREENS

RETROVIRUS (MMTV)-MEDIATED SCREENS

This thesis expands the work of MMTV insertional mutagenesis screens we previously performed (Klijn et al., 2013; Theodorou et al., 2007). Both these screens yielded predominantly CISs within gene loci belonging to the Wnt (Wingless-type MMTV integration site family), Fgf (fibroblast growth factor) and Rspo (R-spondin) gene families. This is not surprising, as these gene families have consistently been associated with MMTV in the past, independent of genetic background of the mouse model or strain of the virus. These gene loci have therefore been referred to as 'core MMTV CISs' (Callahan and Smith, 2008; Callahan et al., 2012). Even in the tumour-predisposed backgrounds carrying the mammary gland-specific deletion of the Trp53 (transformation related protein 53) gene (K14cre;Trp53F/F) or deletion of one allele of the Pten (phosphatase and tensin homolog) gene (Pten+/−), primarily these 'core MMTV CISs' were tagged and no significant CISs enrichment compared to wild-type strains was found (Klijn et al., 2013). Trp53 encodes the P53 protein, an important tumour suppressor, and is the murine equivalent to the human TP53 (tumour protein p53), a gene mutated or lost in approximately 50% of human cancers and in 30% of breast cancer cases (Bertheau et al., 2013). Pten encodes the PTEN protein, a tumour suppressor that acts as the major antagonist of PI3K/AKT/mTOR signalling pathway activity, and PTEN is deleted or carries loss-of-function mutations in many cancer types, including breast cancer (Hu et al., 2009; Saal et al., 2008; Stemke-Hale et al., 2008). Due to the lack of specific CISs in the tumour-prone mice with Pten haploinsufficiency or tissue-specific deletion of Trp53, it was suggested that MMTV is a rather inflexible insertional mutagenesis system, inducing mammary tumorigenesis through a limited number of targets (Klijn et al., 2013). However, in the screen in HER2+ transgenic mouse models (MMTV-cNeu) presented in Chapter 2, the 'core MMTV CISs' were significantly less enriched compared to wild-type mice and we identified MMTV-cNeu associated (genotype specific) CISs. This shows that MMTV insertional mutagenesis is still a useful tool to identify clinically relevant oncogenes.
Of course, it must be noticed that the value of MMTV as insertional mutagenesis tool is still dependent on its tissue-specificity, which is equally true for Murine Leukaemia Virus (MuLV). This restricted tropism of the retroviruses limits their utility for insertional mutagenesis in other tissue types, which has sparked research into other systems for cancer gene discovery screens, like transposons, lentiviral vectors and library-based screening methods.

**TRANSPOSON-MEDIATED SCREENS**

Transposons are genetic elements that can 'jump' from one genetic position to another in the genome of their hosts and their sequences are ubiquitously found in both prokaryotes and eukaryotes. The human genome is occupied for 44% with transposon-like elements of which almost all are no longer active (Mills et al., 2007). The 'resurrection' of inactive transposons in vertebrates allowed transposon-mediated insertional mutagenesis, of which Sleeping Beauty (Collier et al., 2005; Dupuy et al., 2005; Horie et al., 2001; Ivics et al., 1997) and piggyBac (Cary et al., 1989; Ding et al., 2005; Fraser et al., 1983; Wu et al., 2007) are the most prominent examples. Compared to retroviruses, transposons have the advantage that they are not restricted to a particular organism or tissue, making them much more versatile in insertional mutagenesis (Collier and Largaespada, 2005; Uren et al., 2005).

Sleeping Beauty has the disadvantages of leaving a 2-5 nucleotide footprint when excising, which can lead to frameshift mutations, and is prone to local transposon mobilisation and reintegration ('hopping') (Liang et al., 2009; Luo et al., 1998). Moreover, Sleeping Beauty is biased to TA-nucleotide containing host sequences and intragenic regions, resulting in more repressing CISs, thus more likely to identify tumour suppressors (de Jong et al., 2014; Liu et al., 2005). Conversely, piggyBac leaves no footprint upon mobilisation and shows less local hopping, but requires a genomic TTAA site to integrate and prefers insertion in transcription start sites, resulting primarily in direct gene activation (Fraser et al., 1996; de Jong et al., 2014; Liang et al., 2009; Meir et al., 2011; Wang et al., 2008).
LENTIVIRAL VECTOR-MEDIATED SCREENS

Lentiviral vectors were originally developed for use in gene therapy. They are engineered from the HIV virus genome, from which all sequences for the structural proteins required for a full viral life-cycle have been removed, while retaining integration-related sequences such as reverse transcription signals (Ranzani et al., 2013a). In an attempt to compare genotoxicity profiles of lentiviral and retroviral vectors to increase biosafety for gene therapy, lentiviral vectors engineered to carry strong promoter/enhancer sequences in their LTRs were found to have a high frequency of integrations near certain classes of growth-control and cancer-related genes (Montini et al., 2009). This allows cancer gene discovery screens by lentiviral vector-mediated insertional mutagenesis.

Lentiviral vectors have the advantage over retroviruses that they can also integrate in non-replicating cells, while retroviruses require cells to proliferate (Lewis and Emerman, 1994; Roe et al., 1993). Moreover, lentiviral vectors are also highly versatile in tissue tropism and because they are engineered to integrate in their hosts’ genome once without the ability to replicate, they can uniquely exhibit a single CIS integration in tumours (Ranzani et al., 2013b). On one hand, that results in a strong reduction of passenger integrations, easing retrieval of driver insertions and allowing detailed studying of a single mutagenic event (Ranzani et al., 2013b), but on the other hand it may greatly reduce yield and prevent the discovery of collaborating or mutually exclusive cancer genes.

ALTERNATIVE PRECLINICAL SCREENS

Alternative induced forward-genetic screening methods include knockdown screens using short hairpin RNA (shRNA) (Bernards et al., 2006), induced expression screens by employing ORF (open reading frame) cDNA libraries (Yang et al., 2011) and both loss-of-function and gain-of-function screens using the CRISPR-Cas9 genome editing technology (Chen et al., 2015; Konermann et al., 2015; Sanchez-Rivera and Jacks, 2015; Sanjana et al., 2014; Shalem et al., 2014; Wang et al., 2014). The genomic coverage and resulting bias of these methods is dictated by the used library, which is never completely genome-wide, and the delivery of the library
to the target cells is very challenging, especially in vivo (DeNicola et al., 2015). Moreover, significant off-target effects and undesired vector-induced insertional mutagenesis can occur in each of these screening methods (DeNicola et al., 2015). Nevertheless, shRNA and CRISPR-Cas9 mediated screens have a strong advantage in identifying tumour suppressors, as gene knockdown and knockout, respectively, is readily attained. This in contrast to retroviral and transposon-induced screens which requires very rare coincidental biallelic gene disruption. Moreover, the versatility of the CRISPR-Cas9 tool can additionally open doors for new forms of forward-genetic screening.

SCREENING CLINICAL SAMPLES

One could also consider screening human cancers directly, which has a great advantage with regard to immediate clinical relevance. Indeed, whole-genome, whole-exome, whole-transcriptome or gene-panel sequencing of clinical samples can offer unbiased information on tumour genetics and may discover cancer-related genes. However, mutation analysis approaches are still expensive (although increasingly cheaper), time-consuming and challenging to analyse. To acquire sufficient power, sample size and depth of sequence coverage has to be high, which is also difficult and costly to achieve (Garraway and Lander, 2017; Mwenifumbo and Marra, 2013).

RNA sequencing-based approaches can also be used to for cancer gene discovery and have the benefit of simultaneously collecting expression data, thus including the effects of epigenetic alterations, and transcriptome mutation data. This can be used to identify putative oncogenes (upregulated or gain-of-function mutation) and tumour suppressor genes (downregulated or loss-of-function mutation) as well as fusion events and splice variants when performing paired-end sequencing (Kumar et al., 2016; Wang et al., 2009). On the other hand, this combination of sequence data and expression levels also blurs cause and effect of (epi)genetic aberrations in the output, making it more difficult to identify the specific cancer genes (Mattison et al., 2009). Furthermore, RNA-seq is obviously unable to identify chromosomal rearrangement outside the transcriptome.
Cancers typically carry numerous mutations, have high intra-tumour heterogeneity and genomic instability, which is aggravated by cytotoxic therapies (Shyr and Liu, 2013). Most of these genetic alterations will not be driving the cancer, but will rather be passenger mutations. For all mentioned sequencing approaches, distinguishing the driver mutations from the passenger mutations is not trivial and is computationally demanding, especially considering that different types of genetic alterations (i.e. point mutations, indels, rearrangements, etc.) may each require different algorithms and bioinformatic pipelines (Damodaran et al., 2015). Moreover, the identification of copy number variations (CNVs), although recently possible to some extent with next generation sequencing methods, is still predominantly relying on array-based comparative genomic hybridization (CGH) and single-nucleotide polymorphism (SNP) approaches (Liu et al., 2013; Russo et al., 2014). These approaches typically have a low resolution, genome coverage and signal-to-noise ratio, making it difficult to pinpoint the specific cancer genes and to detect infrequent genetic changes. Also with these techniques, it is generally difficult to distinguish driver mutations from passenger mutations, as well as from noise (Mattison et al., 2009).

Overall, when using clinical tumour samples, the quantity and quality of the available specimens are often problematic, as these are commonlly very small, formalin-fixed paraffin-embedded (FFPE) and/or contaminated with non-tumour (i.e. 'normal') tissue (Damodaran et al., 2015; Yu et al., 2015). Moreover, clinical samples are also associated with important regulatory, ethical and privacy related considerations, especially when these are sequenced. An insertional mutagenesis screen is a preclinical functional approach and therefore does not have these issues with regard to clinical material, is relatively easier and cheaper to perform. Furthermore, these screens can also shed light on mechanisms that have not yet occurred or been detected in the clinic (e.g. predict putative resistance pathways against new therapeutic agents or genetic variations missed in a biopsy due to intra-tumour heterogeneity). Nonetheless, analyses of human tumour samples are more directly relevant to the clinic, and provide complementary insight in cancer biology as well as essential data for clinical validations. Conversely, insertional mutagenesis screens can greatly inform the translation of the correlative output of clinical screens.
to causal relationships between cancer genes and disease (i.e. distinguishing driver and passenger mutations). Hence, both preclinical functional approaches and direct analyses of clinical material are necessary to progress.

**VALUE OF MMTV AS INSERTIONAL MUTAGENESIS TOOL**

MMTV has found to be by far the least biased system with regard to integration-site preferences compared to other retroviruses and transposons (Faschinger et al., 2008; de Jong et al., 2014). MMTV is probably also less biased than the HIV-1 based lentiviral vectors, considering their strong integration bias towards (regions with) actively expressed genes (Mitchell et al., 2004; Schröder et al., 2002). Besides its highly random integration pattern, MMTV is additionally not limited by the coverage of any library design nor hindered by a technically challenging delivery. Moreover, retroviruses are naturally integrating mutagens, giving them the advantage of a long time of natural evolution for its mechanism of mutagenesis compared to engineered transposons, lentiviral vectors and library-based screening methods. Hence, in the field of breast cancer gene discovery, where its limited tropism is not an issue, MMTV-mediated insertional mutagenesis may still be the best tool to use. Considering that the most clinically relevant heterogeneity in breast cancer is found within each molecular subtype (The Cancer Genome Atlas Network, 2012), employing MMTV-induced cancer gene discovery screens would in particular be recommended in murine models for these molecular subtypes. Taking into account that therapeutic options are primarily based on the breast cancer subtype, assessing heterogeneity within the subtypes clearly also makes sense in relation to therapy resistance, as this heterogeneity is likely a defining factor in therapeutic response.
PI3K/AKT/mTOR PATHWAY-ACTIVATING SCREEN HITS

The screens presented and/or followed-up in this thesis (Chapters 2 and 3; Klijn et al., 2013; Theodorou et al., 2007) have yielded several novel candidate cancer genes. Three of these, Eras, Irs4 and Igf2, have a role in activating the PI3K/AKT/mTOR signalling cascade, of which ERAS and IRS4 are shown here to induce hyperactivation of this pathway (Chapters 2 and 3). ERAS has been known to be constitutively active, presumably due to its Ser50, Ala100/Asp100 and Ile101 residues that render it insensitive to GTPase-activating proteins (GAPs) that normally switch off RAS proteins (see Chapter 1). In Chapter 3, IRS4 is reported to have a growth factor-independent activity, in contrast to IRS1 and IRS2 (Figure 1). Specifically, IRS4 has a high basal signal transduction activity and a sustained activity upon upstream stimulation due to a lacking Src homology phosphatase 2 (SHP2)-binding site. In IRS1 and IRS2, this phosphatase is recruited by specific phosphotyrosines in their carboxyl-termini, leading to tyrosine dephosphorylation of the IRSs, consequently preventing docking and further activation of downstream effectors. Hence, IRS4 is, in contrast, unresponsive to this strong feedback regulation and hyperactivates the PI3K/AKT/mTOR pathway even upon no to very weak upstream receptor tyrosine kinase activation (Figure 1).

IGF2 is a known oncogene in breast cancer and Eras and Irs4 are also tagged in a Murine Leukaemia Virus (MuLV)-induced screen (Uren et al., 2008). Moreover, in an analysis aimed at mapping integration biases, a comparison between integrations in unselected cells and those in cells that grew out into tumours in vivo, identified Eras, Irs4 and Igf2 as significant ‘true’ as opposed to spurious MMTV CISs (p = 6.58·10^{-12}, 1.13·10^{-8} and 1.04·10^{-4}, respectively) (de Jong et al., 2014).

ROLES IN EMBRYONIC DEVELOPMENT AND STEM CELLS

Interestingly, ERAS, IRS4 and IGF2 are all mainly expressed during embryonic development and/or in embryonic stem cells (see Chapters 1-3). Moreover, their common main downstream signalling cascade, the PI3K/AKT/mTOR pathway, is linked to embryogenesis, stem cell maintenance and pluripotency (Armstrong et al.,
Figure 1 | Overview of insulin receptor substrate (IRS) signalling and regulation

Simplified IRS-induced PI3K/AKT/mTOR signalling cascade in normal cells expressing IRS1 and IRS2 (left) or cells expressing IRS4 (right). In most normal cells, either IRS1 or IRS2, or both IRSs are expressed, whereas IRS4 is rarely expressed. IRS1 and IRS2 activity is kept in check by negative feedback via SHP2-mediated tyrosine dephosphorylation. In cancer cells, various mutagenic events may activate IRS4, which is irresponsive (X) to SHP2-mediated feedback and hyperactivates the PI3K/AKT/mTOR pathway leading to tumour growth. Green arrows indicate positive interactions (e.g. induction or stimulation). Red arrows indicate negative interactions (e.g. deactivation or inhibition).

2006; Dreesen and Brivanlou, 2007; Paling et al., 2004; Riley et al., 2005; Shoni et al., 2014; Storm et al., 2009; Takahashi et al., 2005; Watanabe et al., 2006; Yang et al., 2003). Stem cells and cancer cells in general share many characteristics, including rapid clonal proliferation, anchorage-independent growth, inhibition of differentiation, indefinite cell division by bypassing replicative senescence, but also comparable gene expression signatures and biomarkers, common transcriptional and epigenetic regulators and several shared signalling pathways (Dreesen and Brivanlou, 2007; Hadjimichael et al., 2015; Hanahan and Weinberg, 2011; Monk and Holding, 2001; Oren and Smith, 2017; Reya et al., 2001; Takahashi et al., 2005). These common features have also, in part, contributed to the concept of cancer stem
cells: rare cancer cells that drive tumorigenesis in a similar fashion as normal stem cells give rise to organs (Clevers, 2011; Jordan et al., 2006; Reya et al., 2001). Indeed, cancer progression generally results in a heterogeneous collection of cells comparable, albeit less structured, to that seen in normal tissues during development (Oren and Smith, 2017; Reya et al., 2001). ERAS expression has been correlated to side-population cells (Yashiro et al., 2009), which are generally thought to be enriched for cancer stem cells (Christgen et al., 2012; Richard et al., 2013). Furthermore, a murine model of induced pluripotent stem cells (iPS)-derived cancer stem cells was also found to express Eras (Chen et al., 2012). Indications of involvement of IGF2 in cancer stem cells have recently also been reported (Tominaga et al., 2017; Zhao et al., 2016).

The theory of cancer stem cells has important clinical implications. These cells are thought to be refractory to most therapies, resulting in the reoccurrence of tumours driven by the small surviving subpopulation of such cancer stem cells after an initial successful treatment of the tumour bulk (Bütof et al., 2013; Cojoc et al., 2015; Colak and Medema, 2014; Eyler and Rich, 2008; Peitzsch et al., 2017). Effective treatment of the whole tumour would thus additionally require attacking the cancer stem cells, for which targeting the PI3K/AKT/mTOR pathway (among others) seems an appropriate strategy (Chang et al., 2013; Dubrovska et al., 2009; Francipane and Lagasse, 2016; Kolev et al., 2015; Potiron et al., 2013; Schöning et al., 2017; Sharma et al., 2015; Zhou et al., 2007). Considering all this, the link established in this thesis between ERAS and IRS4 with therapy resistance via PI3K/AKT/mTOR pathway activation and their involvement in embryonic development, justifies further studies for a potential role of these genes in (putative) cancer stem cells.

**TRANSCRIPTIONAL REGULATION OF ERAS, IRS4 AND IGF2**

In adult tissues, ERAS, IRS4 and IGF2 are all strictly regulated on transcriptional level (see Chapter 1-3). Although transcriptional control of IGF2 is thoroughly investigated, the mechanisms underlying the repression of ERAS and IRS4 are unclear. Both genes are located on the X-chromosome, theoretically resulting most likely in monoallelic expression in males, but also in females due to X-chromosome
inactivation by lyonization. *IGF2* expression is silenced by genomic imprinting, similarly resulting in monoallelic silencing (Chapter 1). Both loss-of-imprinting as well as escape from X-inactivation are mechanisms known to predispose to and cause cancer (Balaton and Brown, 2016; Chaligné and Heard, 2014; Jelinic and Shaw, 2007; Lim and Maher, 2010). However, in most to all adult human and mouse tissues, including all normal stages of the breast, *ERAS* and *IRS4* are not expressed at all (Chapters 2 and 3; Giovannone et al., 2000; Kameda and Thomson, 2005; Takahashi et al., 2003), indicating that additional epigenetic repression is present. Therefore, it is remarkable that both *ERAS* and *IRS4* were found to be expressed (independently) in a relatively small but significant subset of human breast cancers (Chapters 2 and 3).

How *ERAS* expression is activated in these tumours is not known. Previous studies first identified human *ERAS* as a pseudogene and later to produce only a truncated non-coding transcript (Kameda and Thomson, 2005; Miyoshi et al., 1984; Zhan et al., 2005). Perhaps consequently, human *ERAS* and its transcriptional regulation has not received much further attention. Very recently, high *ERAS* expression was found in one case of primary colon cancer, where the *ERAS* gene had come under control of the highly expressed housekeeping gene *USP9X*. This was caused by a *USP9X*-ERAS gene fusion resulting from a highly local chromothripsis event on chromosome X (Kloosterman et al., 2017). It is possible that *ERAS* expression could be activated via a similar mechanism in breast cancer, including a translocation event, especially considering that genomic instability is a hallmark of most cancer cells. Alternatively, earlier studies that reported *ERAS* mRNA expression in several human cancer cells, revealed some insights in its epigenetic activation that pointed to histone acetylation and promoter-localised DNA demethylation using inhibitors (Figure 2) (Yashiro et al., 2009; Yasuda et al., 2007). The involvement of epigenetic dysregulation in cancer is well-known, but predominantly entails the inverse: histone deacetylation and DNA methylation leading to the deactivation of tumour suppressors (Zahnow et al., 2016).

DNA methylation entails the addition of a methyl group to a cytosine (C) base in the DNA, generally preventing replication. In mammals, the methylated cytosine is always followed by a guanine (G), which is therefore known as a CpG dinucleotide
or simply CpG (Esteller, 2002; Herman and Baylin, 2003). Although CpG dinucleotides are overall quite rare in the genome, clusters of CpGs, known as CpG islands, are common in promoter regions. These CpG islands are predominantly unmethylated, but are known to be fully methylated by X-chromosome inactivation and genomic imprinting, leading to the stable transcriptional silencing of genes (Bird, 2002). Hypermethylation of such CpG islands, associated to the silencing of tumour suppressor genes, is also observed in many types of cancer (Baylin and Herman, 2000; Esteller, 2002).

Figure 2 | Putative epigenetic regulation of ERAS

(A) Promoter-localised DNA methylation of CpG islands is likely to cause the transcriptional silencing of ERAS, which is reinforced by the absence of acetylation of histone tails, which keeps the chromatin in a condensed, transcription impermissible conformation.

(B) Histone acetylation and promoter-localised DNA demethylation may allow ERAS expression, thus leading to the production of the constitutively active ERAS protein.

Acetylation of the DNA packaging proteins, the histones, increases gene transcription by opening up condensed chromatin to a more accessible and transcription permissible conformation (Zentner and Henikoff, 2013). The levels of histone acetylation are controlled by the counteracting activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). In many cancers, the balance between the activities of HATs and HDACs is disrupted due to various molecular aberrations, which can be germline as well as somatic and include mutations, amplifications and chromosomal translocations (Miremadi et al., 2007). Histone deacetylation and DNA methylation are interconnected and are even thought to collaborate synergistically to establish gene silencing (Cameron et al., 1999; Grewal and Moazed, 2003; Meng et al., 2011; Zahnow et al., 2016). Both mechanisms of epigenetic regulation are extensively studied in relation to tumour
suppressor silencing, which is most prevalent in cancers (Zahnow et al., 2016). The epigenetic silencing of microRNAs (miRNAs) that inhibit translation or induce mRNA degradation of oncogenes, effectively activating these oncogenes, has also been reported (Lujambio et al., 2007; Saito and Jones, 2006). Additionally, (proto-)oncogene activation through these same epigenetic alterations has been described in the case of the maternally imprinted \textit{IGF2} (see Chapter 1). \textit{IGF2} is shown to become activated after hypermethylation of the insulator region between its promoter and a distant enhancer of the paternally imprinted \textit{H19} gene (Bell and Felsenfeld, 2000; Moulton et al., 1994; Schoenherr et al., 2003; Steenman et al., 1994). Direct \textit{IGF2} upregulation after promoter-specific demethylation has been found in cases of human hepatoblastoma (Li et al., 1998). Other cases of direct epigenetic activation of oncogenes have only rarely been reported (Akiyama et al., 2003; Cho et al., 2001; Nishigaki et al., 2005; Oshimo et al., 2003; Sato et al., 2003; Toyota et al., 2000). As activation of \textit{ERAS} expression has been linked to histone acetylation and DNA demethylation, the gene may prove to be a new case of direct epigenetic upregulation of an oncogene, but this requires further investigation. This is important, as the clinical effectiveness of HDAC inhibitors (e.g. Panobinostat, Belinostat and suberoylanilide hydroxamic acid, SAHA, also known as Vorinostat) and inhibitors of DNA methylation (e.g. Azacytidine and Decitabine) could be compromised by the unintended epigenetic (re)activation of oncogenes. Histone modifications and DNA methylation status also have other profound therapeutic implications as the plasticity and reversibility of these epigenetic changes could explain acquired resistance in absence of obvious connections to genetic mutations (Brown et al., 2014; Wilting and Dannenberg, 2012). Our observations of ERAS-induced therapeutic resistance (Chapter 4) correspond with this concept.

The mechanism behind \textit{IRS4} upregulation in the \textit{IRS4}+ breast tumours has also not been elucidated, but in paediatric T-cell acute lymphoblastic leukaemia (T-ALL), strong \textit{IRS4} upregulation has been reported due to chromosomal translocation, bringing the gene under the transcriptional control of T-cell receptor β regulatory elements (Karrman et al., 2009). A somewhat related mechanism was reported recently, revealing \textit{IRS4} as a candidate pan-cancer gene that is activated due to "enhancer hijacking" in ten different tumour types, most prominently lung squamous
carcinomas and cervical squamous carcinomas (Weischenfeldt et al., 2016). Here, cis-regulatory elements such as enhancers were found to be rearranged and juxtaposed to IRS4. This resembles the activation of lrs4 by MMTV proviral integrations in our insertional mutagenesis screens (Chapter 3; Klijn et al., 2013; Theodorou et al., 2007), where the proviral transcriptional enhancers interact with the lrs4 promoter, upregulating the gene. Finally, epigenetic regulation of IRS4 expression by DNA methylation and histone modifications should also be considered, especially in the light of acquired therapy resistance (Figure 1).

**Clinical Implications: Therapy Resistance**

HER2-targeting therapy using monoclonal antibodies Trastuzumab or Pertuzumab, or the tyrosine kinase inhibitor Lapatinib, greatly improves the prognosis of HER2+ breast cancer patients (Figure 3A). However, it is well recognised that a hyperactivated PI3K/AKT/mTOR pathway can induce resistance to various therapies in cancer (Brown and Toker, 2015). Both primary and secondary resistance are common in HER2+ breast cancer and are often associated with PI3K/AKT/mTOR

**Figure 3** | ERBB2-ERBB3, ERAS and IRS4 signalling in malignant cells and therapy resistance

(A) Tumorigenesis requires continued stimulation of the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways. The ERBB2-ERBB3 heterodimer may provide both these signals in a subset of tumours, where ERBB2 provides the RAF/MEK/ERK/MAPK signal and ERBB3 the PI3K/AKT/mTOR signal.

(B) Trastuzumab (humanised monoclonal antibodies against ERBB2) and lapatinib (a tyrosine kinase inhibitor inhibiting HER2 kinase activity) prevent the oncogenic signals of ERBB2 and ERBB3, but this is circumvented by ERAS/IRS4-induced hyperactivation of the PI3K/AKT/mTOR pathway, leading to therapy resistance. Red “X” indicates no activity or signalling. Thickness of the arrows indicates strength of signalling. Semi-transparency and dashed arrows indicate weak interactions.
pathway hyperactivation (Appert-Collin et al., 2015; Berns et al., 2007; Chandarlapaty et al., 2012; Cizkova et al., 2013; Eichhorn et al., 2008; Esteva et al., 2010; Hanker et al., 2013; Juntila et al., 2009; Loibl et al., 2016a, 2016b; Nagata et al., 2004; Park et al., 2014; Razis et al., 2011; Serra et al., 2008; Wang et al., 2011, 2013). Indeed, expression of ERAS or IRS4 in various cell lines with ERBB2 overexpression greatly reduced the sensitivity to HER2-directed therapeutic agents (Chapter 4). Moreover, ERAS as well as IRS4 synergistically accelerated tumorigenesis in vitro and in vivo when co-expressed with ERBB2, most likely due to the combined potent activation of the PI3K/AKT/mTOR pathway by ERAS and IRS4 with the RAF/MEK/ERK/MAPK pathway activation facilitated by ERBB2 (Figure 3B).

As mentioned in Chapter 1, ERBB2 has a strong binding preference for ERBB3 and in this heterodimer, it is ERBB3 that is mainly responsible for PI3K/AKT/mTOR pathway activation. As such, the ERBB2-ERBB3 heterodimer is oncogenic and even suggested to be essential for HER2+ breast cancer transformation (Holbro et al., 2003; Lee-Hoeflich et al., 2008; Pinkas-Kramarski et al., 1996; Siegel et al., 1999; Tzahar et al., 1996). However, in Chapter 2, 42% of the MMTV-induced tumours from MMTV-infected ErbB2-transgenic mice lacked ErbB3 expression (whereas all tumours from non-infected mice expressed ErbB3), indicating that ERBB3 is not essential for oncogenesis in these tumours. ErbB3 was furthermore found significantly less expressed in Eras-expressing tumours compared to ERAS-negative tumours in MMTV-infected ErbB2-transgenic mice and co-expression of ERAS and ERBB3 was also not found in human breast cancer samples (Chapter 2). As ERAS and ERBB3 have the activation of PI3K/AKT/mTOR pathway in common, co-expression is indeed not expected. IRS4 is also a potent activator of the PI3K/AKT/mTOR pathway and can therefore also be expected to functionally replace ERBB3 as PI3K/AKT/mTOR pathway activator in HER2+ tumours, which would be worth investigating in the future. Replacement of ERBB3-driven activation of the PI3K/AKT/mTOR pathway by ERAS or IRS4 also fits well with the finding of ERAS- and IRS4-induced therapy resistance (Chapter 4), considering that absence of ERBB3 in HER2+ breast cancer cells and tumours has been reported to reduce sensitivity to HER2-targeted therapy (Holbro et al., 2003; Lane et al., 2000; Münster
et al., 2002; Yakes et al., 2002). As PI3K/AKT/mTOR pathway activity is essential in HER2 oncogenic signalling (Santa-Maria et al., 2016), the identification of other constitutive activators that circumvent the need for ERBB3 in activating this pathway, may reveal additional putative biomarkers for treatment options and resistance. Evidence that both ERAS and IRS4 expression may be involved in acquired resistance to HER2-targeted therapy is presented in Chapter 4. The expression of both genes could rapidly be attained in naive HER2+ breast cancer cell lines by culturing the cells for several passages in medium with increasing concentrations of Trastuzumab or Lapatinib, indicating selection for cells expressing these genes under the pressure of the drugs. Hence, Chapter 4 suggests that ERAS and IRS4 can cause both primary resistance to Trastuzumab or Lapatinib, as well as acquired resistance during treatment, and are therefore likely to play a role in relapse in breast cancer patients.
POTENTIAL TREATMENT OPTIONS

ALTERNATIVE HER2-TARGETING AGENTS

Although only Trastuzumab and Lapatinib were investigated in this thesis, treatment efficacy of other HER2-targeting drugs, and specifically Pertuzumab (monoclonal antibody inhibiting ERBB2-ERBB3 heterodimerization), is likely to be impeded by ERAS and IRS4 as well. Both ERAS and IRS4 activate the PI3K/AKT/mTOR pathway downstream of the ERBB2-ERBB3 heterodimer that Pertuzumab targets. Hence, the signalling and oncogenic effect of ERAS and IRS4 are not expected to be affected by this drug. Indeed, similar to Trastuzumab and Lapatinib, there is evidence that the effectiveness of Pertuzumab is hampered by high PI3K/AKT/mTOR pathway activity (Baselga et al., 2014; Bianchini et al., 2012; Majewski et al., 2015; Schneeweiss et al., 2014; Wuerkenbieke et al., 2015).

In contrast, another HER2-targeting therapeutic agent, the Trastuzumab-emtansine conjugate (T-DM1) may be a viable treatment option in ERAS and IRS4-positive cancers. Several studies in cancer cells, xenografts and human tumours suggest that anti-tumour and cytotoxic activity of T-DM1 is independent of PIK3CA mutation, PTEN status and ERBB3 expression (Baselga et al., 2016; Junuttila et al., 2011; Kim et al., 2016; Krop et al., 2012; Nonagase et al., 2016). Although resistance to the effects of the Trastuzumab-component of the conjugate likely still exists (Junttila et al., 2011; Nonagase et al., 2016), the emtansine-component (DM1) is hypothesised to induce cell death due to mitotic arrest and apoptosis or due to mitotic catastrophe upon cytoplasmic accumulation (Barok et al., 2011; Lewis Phillips et al., 2008). The cytoplasmic concentration of the potently cytotoxic DM1 is presumed to be increased by its Trastuzumab-mediated delivery in T-DM1 though drug-bound ERBB2 internalisation and subsequent release upon lysosomal degradation (Erickson et al., 2006). This is unlikely to be affected by PI3K/AKT/mTOR pathway activity and may thus provide a therapeutic avenue in ERAS and IRS4 expressing HER2+ breast cancers.
HER2 AND PI3K/AKT/mTOR DUAL TREATMENT

The main clinically relevant conclusion of this thesis is that both ERAS and IRS4 expression can be considered as biomarker for Trastuzumab and Lapatinib resistance, and by extrapolation also to other HER2-directed therapies. This would implicate that breast cancers expressing either of these genes should not be treated with HER2-targeted drugs, as this would only expose the patient to the toxicity of the drugs, without any expected treatment benefit.

However, considering that both ERAS and IRS4 induce this resistance through PI3K/AKT/mTOR pathway hyperactivation by acting directly on PI3K, combined treatment with a drug targeting ERBB2 and a drug targeting PI3K and/or its downstream effectors may be feasible. Currently, the only European Medicines Agency (EMA) and US Food and Drug Administration (FDA) approved PI3K/AKT/mTOR pathway inhibitor for breast cancer is the Rapamycin analogue (rapalogue) Everolimus (previously known as SDZ RAD and RAD001). Treatments with this drug in combination with HER2-targeted therapy has already been under investigation in the clinic.

In the BOLERO-1 trial, a phase-III randomised double-blind study in HER2+ advanced breast cancer, Everolimus versus placebo was tested in combination with Trastuzumab and Paclitaxel. Overall, no difference in progression-free survival was observed between the Everolimus and placebo arms (Hurvitz et al., 2015a). However, within the 43.3% of patients that were hormone receptor negative (HR-), a 7.2 month improvement in progression-free survival (PFS) was observed, although this was just outside the prespecified criteria for statistical significance (Hurvitz et al., 2015a).

The phase-III, randomised and double-blind trial BOLERO-3, tested Everolimus versus placebo, both combined with Trastuzumab plus Vinorelbine, in HER2+ Trastuzumab-resistant advanced breast cancer previously subjected to taxane treatment. Progression-free survival was slightly improved by Everolimus and, similar to the BOLERO-1 trial, mostly in HR- patients (André et al., 2014). Interestingly, patients with indicators for high PI3K/AKT/mTOR pathway activity showed more benefit with Everolimus than placebo. More specifically, both patients
with low PTEN levels and with high phosphorylated S6K levels both showed significant improvement with Everolimus and patients with PIK3CA mutations showed a trend towards a benefit from Everolimus.

Combined biomarker data from the BOLERO-1 and BOLERO-3 trials suggested that patients with tumours with a hyperactive PI3K/AKT/mTOR pathway (defined as: with known PIK3CA-activating mutations and/or low/no/mutated PTEN and/or AKT1 E17K mutation) consistently derived significant benefit from Everolimus with regard to progression-free survival (André et al., 2016). This progression-free survival benefit with Everolimus was also observed for PIK3CA mutations or PTEN loss when these were analysed separately. In contrast, patients with wild-type PIK3CA, normal levels of PTEN or normal PI3K/AKT/mTOR pathway activity, did not benefit from Everolimus (André et al., 2016). Interestingly, hormone receptor status was not associated with benefit from Everolimus treatment in this combined dataset of BOLERO-1 and BOLERO-3 (André et al., 2016). Although pooling of two independent trials with dissimilar Everolimus doses and different chemotherapy regimens has clear limitations, the conclusions deserve further clinical investigation, specifically by preselecting patients that have tumours with high PI3K/AKT/mTOR pathway activity. This especially considering the encouraging results of preclinical studies with Everolimus (Hurvitz et al., 2015b; Lu et al., 2007; Mabuchi et al., 2007; Weigelt et al., 2011). Moreover, when taking into account the strong, sometimes even lethal, toxicity associated with Everolimus (André et al., 2014), further stratification of patients is a compelling aim to improve the, so far limited, clinical efficacy of the drug.

Everolimus specifically inhibits downstream signalling of the mTORC1 complex, similarly to rapamycin by binding FKBP1A (FK506 binding protein 1A; previously FKBP12) and subsequently disrupting mTOR from interacting with RPTOR (regulatory associated protein of MTOR complex 1) (Sedrani et al., 1998). Although RPTOR is not present in the mTORC2 complex, rapamycin and rapalogues may context-dependently and after prolonged treatment also partly inhibit mTORC2 signalling by sequestering free mTOR, which subsequently prevents mTORC2 complex assembly (Barlow et al., 2012; Macaskill et al., 2011; Sarbassov et al., 2006; Zeng et al., 2007). Still, inhibition of mTORC1 but not (or only indirectly and
limited) mTORC2 signalling by rapamycin and its analogues often results in the induction of AKT phosphorylation. This among others due to the release of negative feedback loops on IRSs, primarily the phosphorylation of serine residues of IRS1 (Chapter 1; Yoon, 2017), and mTORC2, which is observed with rapamycin and its analogues (Cloughesy et al., 2008; Dibble et al., 2009; Harrington et al., 2004; Julien et al., 2010; O’Reilly et al., 2006; Shi et al., 2005; Sun et al., 2005; Tabernero et al., 2008). That could explain why the therapeutic success of rapamycin and analogues, including Everolimus, has so far been modest (Xie et al., 2016).

Inhibitors of the PI3K/AKT/mTOR pathway void of this feedback loop issue have been developed and include PI3K inhibitors (p110 catalytic subunit isoform-specific or pan-PI3K), catalytic and allosteric AKT inhibitors, catalytic mTORC1/2 dual inhibitors, and dual PI3K/mTOR inhibitors. Some of these drugs have already shown promising results in preclinical studies in HER2+ breast cancer models (Berns et al., 2007; Eichhorn et al., 2008; Hanker et al., 2013; Juntila et al., 2009; Miller et al., 2009; Nagata et al., 2004; Serra et al., 2008), including the results presented in Chapter 4. Clinical trials with numerous of these inhibitors, also in combination with Trastuzumab, Lapatinib, Pertuzumab and/or T-DM1, are currently pending approval, are ongoing or have even been completed and are in data analysis (Dey et al., 2017; Guerrero-Zotano et al., 2016; Luque-Cabal et al., 2016).

There is some evidence that treatment with PI3K/AKT/mTOR inhibitors, results in a compensatory upregulation of ERBB signalling (Chakrabarty et al., 2012; Serra et al., 2011). Targeting ERBB2/ERBB3 and PI3K/AKT/mTOR signalling simultaneously may therefore have a combined, enhanced effect. Indeed, the results in Chapters 2 and 4 suggest a synergy between ERBB2 and PI3K inhibition, in agreement with previous reports (Chakrabarty et al., 2012; Crafter et al., 2015). As early clinical studies suggest that PI3K, AKT and mTOR inhibitors can have severe adverse events (Dey et al., 2017; Guerrero-Zotano et al., 2016; Harbeck et al., 2016), such a therapeutic synergism between these and HER2-targeted drugs could offer a welcome reduction of effective dose. Still, a substantial treatment benefit will be required for these inhibitors to be incorporated into clinical practice.
IMPROVING DIAGNOSIS FOR PERSONALISED TREATMENT

BIOPSIES

In the human disease, therapy resistance may be a consequence of intratumour heterogeneity, with effectively a selection for resistant clones. However, treatment decisions are commonly based on a single sample of the primary tumour, e.g. a core needle biopsy in breast cancer. Such single biopsies do not take into account the heterogeneity within various locations of a tumour mass and between the primary bulk and in metastases (spatial heterogeneity), nor changes over time (temporal heterogeneity), e.g. due to selective pressure during treatment (Gerlinger et al., 2012; Ryška, 2016; Yates et al., 2015). Repeated multiregional biopsies before, during and after treatment, could provide highly valuable information for both clinicians and researchers. Unfortunately, such biopsies are rarely available, if done at all, which was certainly a major challenge for the research presented in this thesis. Indeed, intratumour heterogeneity can have major clinical implications. For example, it has been reported that there is a discordance in HER2-status between core needle biopsies and resection samples of the primary tumour, which in the majority of cases would have led to a change in treatment choice (Striebel et al., 2008; Wu et al., 2010). The same has been described for discordances in HER2-status between primary tumour and metastases, which also would have led to an alternative treatment decision in some cases (Amir et al., 2012; Niikura et al., 2012; Wilking et al., 2011). Hence, repeated multiregional biopsies could provide more comprehensive clinical information essential for optimal management of the disease. For the researcher, more comprehensive biopsies may provide essential data to model tumour evolution, possibly enabling the prediction of common paths that tumours take to escape therapy (Greaves, 2015; Lipinski et al., 2016), but it will also provide material to validate newly identified biomarkers. Repeated multiregional biopsies may have clear benefits, but it also carries practical issues and a burden to the patients. Much less invasive and more practical are 'liquid biopsies': the analysis on circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA; more broadly: cell-free DNA, cfDNA) in blood samples (Alix-Panabières and
Pantel, 2013; Crowley et al., 2013; Diaz and Bardelli, 2014). However, both techniques are still under development and have technical challenges, and importantly with ctDNA/cfDNA, any non-genetic information is not available. Still, CTCs and ctDNA could provide a practical avenue to follow tumour evolution and heterogeneity in real-time before, during and after treatment, knowledge which is currently sorely lacking.

**Biomarkers**

To guide personalised treatment decisions for breast cancer the need for associated biomarkers is high. Unfortunately, despite much effort, ER and HER2 status are still the only validated predictive markers in breast cancer. Although preclinical data strongly links hyperactivation of the PI3K/AKT/mTOR pathway to HER2-targetted therapy resistance, data from the clinic have not consistently supported this link, in particular for *PIK3CA*-activating mutations (Barbareschi et al., 2012; Dave et al., 2011; Esteva et al., 2010; Gianni et al., 2012; Loi et al., 2013; Pogue-Geile et al., 2015; Razis et al., 2011). This may be due to the use of different assays and analyses (Wang et al., 2013), but could also be explained by the limitations of looking only at genetic aberrations. Indeed, *PIK3CA*-activating mutations do not always lead to a concomitant activation of the pathway, based on the more accurate readout of phosphorylation status of key members of the pathway (Stemke-Hale et al., 2008; The Cancer Genome Atlas Network, 2012). The molecular and cellular effects of genetic alterations can thus be context-dependent. Moreover, the majority of mutations found in the exome of tumours may not even be expressed, considering that (in triple-negative breast cancers) only a mere 36% of genomic mutations found in the exome were observed in the transcriptome as well (Shah et al., 2012). Also, only a handful of cancer genes are commonly mutated and these explain only a part of all breast cancer cases, whereas a myriad of infrequently mutated genes causes or contributes to the substantial remainder of cases (Stephens et al., 2012). Hence, it makes sense to consider other, more comprehensive, tools that allow for looking beyond merely DNA sequences, but also take into account the effects of gene expression levels and post-translational modifications, including activation of
signalling pathway. Analysing PI3K/AKT/mTOR pathway activation directly, e.g. though the phosphorylation status of AKT, S6 and 4EBP1, may have the additional benefit of evaluating the common signalling effect of multiple genetic and epigenetic aberrations, like mutations and genomic rearrangements, altered RNA or protein degradation, changes in histone acetylation and DNA demethylation, etc., without the need of analysing all these separately.

Assessing the pathway activation status of tumours is also useful since some contributing genetic events may be very infrequent and could therefore be missed during analysis of genomic data. For example, mutations in the gene encoding the PI3K regulatory subunit p85, \textit{PIK3R1}, are detected in only 4% of HER2+ breast cancers and even less in other subtypes (The Cancer Genome Atlas Network, 2012). Conceivably therefore, \textit{PIK3R1} has only recently been identified as a significantly mutated gene in breast cancer, which was in the comprehensive analysis of the Cancer Genome Atlas Network. Both \textit{ERAS} and \textit{IRS4} expression were also not very frequently observed in breast cancers (Chapters 2 and 3) and were never implicated in breast cancer before.

Advancing research methods, technological developments and bioinformatics are likely to improve the detection of increasingly rare oncogenic drivers, but this may pose problems for the clinical validation of such rare biomarkers. If only a very low percentage of tumours e.g. carry a specific oncogenic mutation or, in the case of this thesis, express a certain oncogene, it is virtually impossible to find a clinical dataset large enough to perform any survival analysis with sufficient samples in the test group. Hence, the requirement of Kaplan-Meier plots for oncogenic validation on gene-level may not be feasible.

Similarly, clinical trials investigating treatment of infrequent oncogenic drivers would require the screening of an enormous number of patients before a meaningful population can be enrolled. It is because of this that innovative clinical trial designs, such as basket and umbrella trials, are currently being developed to cope with rare genetic alterations. Basket trials test the effectiveness of certain therapies in a pool of patients with the same genetic alteration, but which can comprise completely different tumour types. In contrast, umbrella trials are designed to guide patients of one tumour type, but with diverse genetic alterations, towards a matching (putative)
treatment. Here, assessing oncogenic pathway activation to determine treatment options may provide an additional means to address the clinical implications of infrequent oncogenic drivers and to prevent too rigid stratification based only one or a few genetic aberrations. In the case of HER2+ breast cancer, this would mean pooling all patients with tumours having PI3K/AKT/mTOR pathway hyperactivation, due to any sort of driver, and administering HER2-targeting agents combined with PI3K, AKT and/or mTOR inhibitors, when these become available and are confirmed to be clinically effective.
FURTHER CONSIDERATIONS

In Chapters 2 and 3 of this thesis, Eras and Irs4, respectively, are validated as oncogenic drivers, both in the murine *in vivo* setting and *in vitro* in human breast (cancer) cell lines. Moreover, both genes are shown to be expressed in the human disease, whereas they are normally silent in the normal adult breast. The evidence in this human material (both human cell lines and primary carcinomas) is important, as there is not always a strong overlap between mice and humans on cellular and molecular level, even if the genetics are very similar. For example, the human orthologue of the first gene identified by MMTV insertional mutagenesis, *WNT1*, is not found overexpressed human breast carcinomas, probably due to redundancy in the *WNT* family and other molecules that equally result in WNT pathway activation (Brown, 2001). As hyperactivity of the WNT pathway is associated with human breast cancer (Howe and Brown, 2004), this is exemplary for the notion that the tumorigenic effect of oncogenes and tumour suppressors may differ between mice and humans, but oncogenic pathways are often shared. Moreover, looking at pathway activity can provide a broader 'biomarker' with a more direct insight in possible therapeutic avenues. Subsequently, following basket trials, resulting therapies are more likely to be effective on many patients' tumours that share the same oncogenic pathway, but have distinct underlying oncogenic drivers. This is especially important as some of these drivers may be very rare and thus unfeasible to target individually. Chapters 2 and 3 show that the frequency of *ERAS* and *IRS4* expression in human breast cancers is quite limited, so this is likely to apply to these oncogenes as well. All this is important to take aboard for any screen that seeks to translate results from the murine setting to the clinic. ERAS, IRS4 and IGF2 seem to be oncogenic drivers in both mice and humans, but more important is the identical molecular mechanism of action in mice and humans: the activation of the PI3K/AKT/mTOR signalling cascade, which is highly relevant in (both murine and human) cancer.

Still, the most direct confirmatory model for a causal relationship between ERAS or IRS4 and human tumorigenesis, their synergism with ERBB2 and induction of therapy resistance, would be the use of patient-derived xenografts (PDX) models. But although establishment of a PDX model is already a technical feat, considering
the limited frequency of tumours presenting both ERBB2 amplification/overexpression and ERAS or IRS4 expression, it would be practically challenging to establish ERBB2+ERAS or ERBB2+IRS4 PDX models as well. Indeed, none of the existing models express one of these combinations, to our knowledge. Alternatively, assessing if there is an enrichment for ERAS or IRS4 expression in therapy resistant tumours (i.e. tumour samples obtained after failed treatment instead of the original tumour biopsy of refractory patients) would be useful and provide a strong confirmation to use the expression of these genes as a clinical biomarker. The ex vivo culturing of primary tumour cells may be very useful in the context of a direct prediction of therapeutic response for an individual patient and can take into account the influence of the tumour microenvironment (Corben et al., 2014; Majumder et al., 2015), but is still in early development and has similar logistical challenges to PDX models.

Additional directions for further research that arise from this thesis are the role of these genes in putative cancer stem cells and the exact mechanism behind their expression activation in human tumours. Importantly, the assessment of viable therapeutic options for patients with HER2+ breast cancer that expresses ERAS or IRS4 warrants investigation. In particular the clinical development of PI3K, AKT and/or mTOR inhibitors requires continued effort to hopefully fulfil their prospect to overcome HER2-targetting treatment resistance. This especially when the tumours exhibit PI3K/AKT/mTOR pathway hyperactivation due to PIK3CA mutations, PTEN loss, AKT1 amplification, ERAS or IRS4 expression or any other means.

Finally, this thesis has shown that MMTV-induced insertional mutagenesis is still a highly valuable tool for oncogene discovery and to study heterogeneity in breast cancer. Also because mammary tumours in mice resemble human breast tumours well (Dimri et al., 2005). This may inspire future screens employing MMTV again, which may especially be useful in murine models for specific molecular subtypes, potentially combined with the selective pressure of a drug used in the clinic to screen for drivers of resistance. The knowledge derived from such screens, in combination with continuing advances in the screening of clinical material, will undoubtedly contribute further to the odyssey towards personalised medicine.


Genomes . J. Virol. 82, 1360–1367.


Fraser, M.J., Ciszczon, T., Elick, T., and Bauser, C. (1996). Precise excision of TTAA-specific lepidopteran transposons piggyBac (IFP2) and tagalong (TFP3) from the baculovirus genome in cell lines from two species of Lepidoptera. Insect Mol. Biol. 5, 141–151.


Kameda, T., and Thomson, J. a (2005). Human ERas gene has an upstream premature polyadenylation signal that results in a truncated, noncoding transcript. Stem Cells 23, 1535–1540.

Karrman, K., Kjeldsen, E., Lassen, C., Isaksson, M., Davidsson, J., Andersson, A., Hasle, H., Fioretos, T., and Johansson, B. (2009). The t(X;7)(q22;q34) in paediatric T-cell acute lymphoblastic leukaemia results in overexpression of the insulin receptor substrate 4 gene through illegitimate recombination with...


CHAPTER 5 | General Discussion


Serra, V., Markman, B., Scaltriti, M., Eichhorn, P.J.A., Valero, V., Guzman, M., Botero, M.L.


Rapamycin Inhibition. Cancer Res. 65, 7052 LP-7058.


Yoon, M.-S. (2017). The Role of Mammalian Target of Rapamycin (mTOR) in Insulin Signaling. Nutrients 9, 1176.


CHAPTER 5 | General Discussion


SUMMARY
SAMENVATTING

English & Dutch Summaries
Nederlandse & Engelse Samenvattingen
ENGLISH SUMMARY

The goal of personalised medicine is to tailor therapies to the genetic information of a patient to optimise the effectiveness and safety of the treatment on an (almost) individual level. This thesis describes one of the many odysseys taken in science and medicine towards achieving that goal.

Chapter 1 explains that personalised medicine is essential for breast cancer, as this is a highly heterogeneous disease. It elaborates on the aggressive HER2+ breast cancer subtype where therapy resistance is common, which is linked to the disease’s heterogeneity. Chapter 1 then introduces the tool that was used in this thesis to identify oncogenic drivers of breast cancer: Mouse Mammary Tumour Virus (MMTV)-induced insertional mutagenesis. Several novel MMTV-targets and thus candidate (proto-)oncogenes were identified. This thesis focusses on those genes that play an activating role in the PI3K/AKT/mTOR pathway: Eras, Irs4, and to much lesser extent, Igf2.

Chapter 2 presents the results of MMTV-mediated insertional mutagenesis screens aimed at the identification of genes and genetic pathways playing a role in HER2+ breast cancer. It identifies the Eras locus as significantly more targeted by MMTV in tumours from the HER2+ breast cancer mouse models compared to wildtype mice. Chapter 2 confirms ERAS as an oncogenic driver upon expression and verifies that it is expressed in human HER2+ breast cancer (in 12-27% of cases). It also reveals that ERAS synergistically accelerates cell proliferation and tumorigenesis with ERBB2 (HER2). Additionally, chapter 2 describes that ERAS constitutively hyperactivates the PI3K/AKT/mTOR pathway and, by doing so, functionally replaces ERBB3, which is normally the activator of this pathway in HER2+ breast cancer.

Chapter 3 identifies Irs4 as an MMTV target and confirms it as a mammary oncogene. IRS4 is shown to potently activate the PI3K/AKT/mTOR pathway, independent of any upstream activation, and to be insensitive to the SHP2-mediated deactivation that is common for IRS proteins, thus leading to its sustained signalling.
Chapter 3 further identifies the absence of a SHP2-binding domain in IRS4 as the underlying mechanism of the latter. It shows that IRS4 is mainly expressed in HER2+ breast cancers, as well as triple negative subtypes, whereas only scarcely in the luminal A or B subtypes.

**Chapter 4** highlights a strong synergism towards tumorigenesis between IRS4 expression and ERBB2 overexpression, as was shown for ERAS expression in chapter 2. Chapter 4 also reveals that expression of ERAS or IRS4 greatly reduces the sensitivity to the HER2-targeting drugs Trastuzumab and Lapatinib in cancer cells *in vitro* and human xenografts *in vivo*. Evidence for a potential role for both genes in acquired therapy resistance is also presented. Chapter 4 confirms the hyperactivation of the PI3K/AKT/mTOR pathway by ERAS or IRS4 as the underlying mechanism for their induction of therapy resistance, suggesting inhibition of this pathway as a potential therapeutic avenue.

**Chapter 5** sets the findings of this thesis in a broader perspective, including considerations for personalised medicine. It discusses potential treatment options for resistant HER2+ breast cancer, in particular in the context of tumours with a hyperactivated PI3K/AKT/mTOR pathway. Chapter 5 also presents hypotheses on the mechanisms of transcriptional repression of ERAS, IRS4 and IGF2, and their upregulation in breast cancer. The roles of the three genes in embryonic development and stem cells is discussed in relation to cancer, which warrants further research. Chapter 5 further discusses the technical and practical benefits of MMTV-mediated insertional mutagenesis over other screening tools, among others that MMTV is by far the least biased tool. It concludes that this is a highly valuable tool to study the clinically relevant heterogeneity in breast cancer, to advance the next odysseys towards personalised medicine.
Kanker wordt veroorzaakt door foutjes (mutaties) in de genetische code (DNA). De mutaties in kanker zetten genen (harder) aan of ze veranderen de functie van genen, waardoor processen in de cel uit balans raken en zo kanker kunnen veroorzaken. Mutaties kunnen er ook voor zorgen dat genen uitgezet worden die als functie hebben om kankerprocessen te verhinderen. Kanker is dus een genetische ziekte, of beter gezegd: het een verzameling van allemaal verschillende genetische ziekten. Kanker kan namelijk veroorzaakt worden door een grote verscheidenheid aan mutaties in allerlei verschillende genen. Ook de verschillende soorten kanker, zoals borstkanker, bestaan elk uit verschillende ondersoorten met verschillende genetische oorzaken, wat geïntroduceerd wordt in hoofdstuk 1.

Hoofdstuk 1 legt verder uit dat verschillende patiënten meestal niet door dezelfde therapie behandeld kunnen worden doordat de genetische oorzaken verschillend zijn. Daarom is borstkanker opgedeeld in vier grote genetische ondersoorten van tumoren met vergelijkbare behandelmogelijkheden. Helaas reageren binnen deze ondersoorten niet alle tumoren even goed op de behandeling en sommigen zelfs helemaal niet (resistentie). Dat komt omdat er naast de mutaties in genen die de ondersoort bepalen, vrijwel altijd nog meer mutaties in andere genen in de tumoren voorkomen. De mutaties in deze andere genen hebben elk hun eigen effect op de tumor, waaronder bijvoorbeeld op de gevoeligheid voor een medicijn. Dit betekent vaak dat een tumor dan met meerdere verschillende therapieën zal moeten worden behandeld, elk gericht tegen een (of meerdere) van de verschillende genetische oorzaken.

De ondersoorten zullen dus nog verder opgedeeld moeten worden in kleinere groepen van tumoren met vergelijkbare behandelmogelijkheden. In sommige gevallen zullen die groepen zo klein moeten worden dat het (bijna) op het niveau van een individuele patiënt wordt. Daarom wordt er bij deze strategie ook wel gesproken van 'gepersonaliseerde therapie'. Om gepersonaliseerde therapie te kunnen bereiken, moet er goede kennis zijn over welke genen een rol kunnen spelen.
in de verschillende ondersoorten borstkanker en wat hun effecten zijn op therapieën. En als deze genen zorgen voor resistentie tegen een medicijn, dan moet bekend zijn welke andere behandelingen mogelijk zijn of hoe deze resistentie omzeild kan worden.

Een van de meest agressieve ondersoorten borstkanker is de zogenaamde 'HER2-positieve' (HER2+) borstkanker. Deze ondersoort wordt veroorzaakt door een vermeerdering van het gen \( ERBB2 \) (die ook wel \( HER2 \) wordt genoemd), waardoor deze te actief is (overexpressie) en er teveel van zijn eiwit wordt gemaakt. Het \( ERBB2 \)-eiwit staat aan het begin van een hele keten processen die de cel aansturen (signaleringsroute). Te veel van dit eiwit zorgt voor een te sterk signaal (overactivatie) van de zogenaamde \( RAF/MEK/ERK/MAPK \)-signaleringsroute, waardoor de cel uit controle raakt en zo een kankercel wordt. Momenteel worden patiënten met deze HER2+ borstkanker behandeld met een antilichaam tegen dat \( ERBB2 \)-eiwit ('Trastuzumab') of een medicijn die het signaal van het \( ERBB2 \)-eiwit remt ('Lapatinib'). Beide therapieën zijn erg effectief en hebben de prognose van patiënten met HER2+ borstkanker sterker verbeterd. Helaas komt vroeg of laat vaak resistentie voor, waarna de tumor niet meer reageert op de behandeling.

Om genen op te sporen die een rol spelen bij HER2+ borstkanker en deze resistentie, hebben we enkele screens uitgevoerd in experimentele modellen die overexpressie van het \( ERBB2 \) gen hebben. Hierbij hebben we een virus (MMTV) gebruikt om het ontstaan van mutaties in borstkanker na te bootsen, wat 'insertiumentagenese' genoemd wordt. Het MMTV-virus integreert namelijk willekeurig in het DNA, waar het genen in de buurt kan aanzetten. In sommige gevallen zijn dat genen die kanker kunnen veroorzaken (oncogenen). In het DNA van de tumoren die daardoor uitgroeien kan de locatie van het geïntegreerde MMTV-virus worden achterhaald. Als het MMTV-virus in verschillende tumoren op dezelfde locatie in het DNA wordt aangetroffen, wijst dat erop dat er op deze locatie waarschijnlijk een oncogen zit.
Hoofdstuk 2 beschrijft de resultaten van deze insertiemutagene screens. Het oncogen dat in deze screens het sterkst gecorreleerd was met de genetische oorzaak van HER2+ borstkanker is ERAS. Dit oncogen was nog nooit eerder in verband gebracht met borstkanker. Hoofdstuk 2 laat zien dat het ERAS-gen normaal gesproken uit staat in alle weefsels, inclusief borstweefsel, en alleen in embryonale cellen aan staat. Als het gen om wat voor reden dan ook (zoals een mutatie) toch aan komt te staan, dan zorgt het resulterende ERAS-eiwit voor overactivatie van de zogenaamde PI3K/AKT/mTOR-signaleringsroute. Deze signaleringsroute speelt een zeer belangrijke rol bij vele vormen van kanker. In hoofdstuk 2 wordt bevestigd dat overactivatie van deze PI3K/AKT/mTOR-signaleringsroute door ERAS zorgt voor snellere celdeling en tumorgroei. Ook wordt er aangetoond dat het ERAS-gen aan staat in ruim 10% HER2+ borsttumoren uit de kliniek.

Uit de resultaten van hoofdstuk 2 en eerdere screens in ons lab, werden ook twee andere oncogenen gevonden die de PI3K/AKT/mTOR-signaleringsroute activeren, namelijk IGF2 en IRS4. IGF2 was al een bekend oncogen in borstkanker en de vondst van IGF2 in onze screens is daarmee een bevestiging dat de screens klinisch relevante resultaten genereren. Hoofdstuk 3 laat zien dat IRS4 een nieuw oncogen is in borstkanker. Net als bij ERAS staat het IRS4-gen normaal uit in vrijwel alle weefsel, inclusief borstweefsel. Ook zorgt het aanzetten van het gen voor sterke tumorgroei door overactivatie van de PI3K/AKT/mTOR-signaleringsroute. Het IRS4-gen blijkt vrijwel alleen aan te staan in twee ondersoorten borstkanker, waaronder de HER2+ ondersoort, waar het gen in zo'n één op de tien tumoren aan blijkt te staan. Hoofdstuk 3 laat ook zien dat het IRS4-eiwit uniek is in vergelijking met zijn familieleden (IRS1 en IRS2), omdat het continu actief is. IRS1 en IRS2 moeten daarentegen eerst geactiveerd worden door signalen van hoger in de signaleringsroute en worden later weer gedeactiveerd door het enzym SHP2. Er wordt aangetoond dat het deactiverende SHP2-enzym niet kan binden met IRS4 doordat de bindingsplaats voor SHP2 zoals die op IRS1 en IRS2 niet voorkomt op IRS4. Daardoor zorgt IRS4 voor een permanente overactivatie van de PI3K/AKT/mTOR-signaleringsroute.
Zowel ERAS (hoofdstuk 2) als IRS4 (hoofdstuk 4) blijken een versterkende werking (synergie) te hebben met ERBB2 op het aandrijven van celdeling en tumorgroei. Dit is een zeer forse toename ten opzichte van ERBB2 alleen, dat van zichzelf dus al kanker veroorzaakt. De belangrijkste oorzaak daarvoor is de overactivatie van de PI3K/AKT/mTOR-signaleringsroute door ERAS of IRS4. In hoofdstuk 4 wordt bovendien aangetoond dat deze overactivatie door ERAS of IRS4 er ook voor zorgt dat cellen en tumoren met ERBB2 overexpressie resistent zijn tegen Trastuzumab en Lapatinib: de standaardtherapieën voor HER2+ borstkanker. Ook blijken cellen die normaal gevoelig zijn voor Trastuzumab en Lapatinib, na herhaalde suboptimale behandeling, snel resistent te worden door het aan zetten van het ERAS- of IRS4-gen. HER2+ borstknoten lijken dus al vanaf het begin resistent zijn of tijdens behandeling resistent te kunnen worden door ERAS en IRS4. Hoofdstuk 4 laat ook zien dat deze resistentie mogelijk tegengegaan kan worden door de activiteit van het PI3K-eiwit in de PI3K/AKT/mTOR-signaleringsroute te remmen.

In hoofdstuk 5 worden al deze resultaten in een breder perspectief geplaatst en worden mogelijke klinische oplossingen bediscussieerd. Zo wordt er besproken welke verbeteringen in diagnostiek en klinische tests kunnen bijdragen om gepersonaliseerde therapie te kunnen bereiken. Ook worden mogelijke behandelingsmogelijkheden in HER2+ borstkanker besproken die verder onderzocht zouden moeten worden. Er worden hypothesen voorgesteld over het onderliggende mechanisme waardoor de genen ERAS, IRS4 en IGF2 in tumoren van patiënten mogelijk aan komen te staan. Daarnaast wordt er opgemerkt dat ERAS, IRS4 en IGF2 alle drie een rol spelen in zowel kanker als embryonale ontwikkeling en de mogelijke link daartussen: kankerstamcellen, wat verder uitgezocht zal moeten worden.

De 'oude' techniek van insertiemutagenese met het MMTV-virus is vroeger al niet veel gebruikt en is in de afgelopen decennia steeds verder overschaduwd door nieuwere methodes voor screens. Dit proefschrift laat echter zien dat MMTV-insertiemutagenese nog een grote toegevoegde waarde heeft voor het ontdekken van oncogenen in borstkanker, zeker binnen de verschillende ondersoorten
DUTCH SUMMARY | Nederlandse Samenvatting

borstkanker. In hoofdstuk 5 worden bovendien enkele belangrijke technische en praktische voordelen van MMTV-insertiemutagenese ten opzichte van de nieuwere screening methodes besproken, waaronder dat MMTV verreweg het meest onbevooroordeeld is waar het integreert in het DNA. Het concludeert dat de kennis uit MMTV-insertiemutagenese screens ongetwijfeld zal blijven bijdragen aan het bereiken van gepersonaliseerde therapie.
Op de omslag

Een menselijke cel weergegeven als een printplaat van een computer. Het elektronisch circuit stelt de belangrijkste signaleringsroutes in de cel voor. De meest relevante signalerings-eiwitten zijn hierin weergegeven als cirkel (in de cel) en vierkant (van buiten de cel). De hoofdrolspelers van dit proefschrift staan hier rechts aangegeven. De andere componenten in het schema zijn de celorganellen (de ‘hardware’), waaronder de celkern met het DNA (de ‘software’). Zowel in computers als in cellen kunnen foutjes in de schakeling of verwerking van informatie (vaak veroorzaakt door mutaties) het hele systeem in de war sturen. Zo is kanker het gevolg van dergelijke defecten in cel signalering. Gelukkig zijn zowel computers als cellen programmeerbaar, dus kunnen foutjes weer hersteld worden. Het systeem is echter erg complex en ons overzicht ervan nog onvolledig. Dit proefschrift maakt dit schema iets completer, zodat we beter begrijpen wat voor effecten bepaalde veranderingen in signalerings-netwerken van een cel hebben op het ontstaan en bestrijden van kanker.
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DANKWOORD

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Many thanks as well to the many other people from P1 and H4, both for the nice atmosphere in (and outside) the lab and for the useful discussions and troubleshooting on the science! Andre (great to have had your medical point-of-view in the division!); Andrej (can I call you Dr. Dre already? Regardless, you’re a great scientist!); Anke; Anna-Pavлина; Bianca; Celia; Colin (thanks for being such a helpful cloning guru!); Danielle (thanks for the gezelligheid and for sharing the MvL almighty antibody arsenal at crucial moments!); Denise (thanks for providing quality music and smiles in the lab!); Ekaterina; Elisabetta; Ellen (thanks for the gezelligheid in the lab and for falling for Martijn’s jokes!); Erica; Gaurav; Geatano (thanks for the valuable research tips!); Giustina; Hester; Hilda (a good neighbour and a good neighbour! Thanks for the Lenti/Adeno virus intro and keep up the good work!); Huub (thank you for your courageous attempts to keep the chaos in the lab to a minimum); Inka; Jaco (it seems ages ago, and it is, but I still look back to some great conversations in the P1 cell culture!); Jacqueline; Jan; Jan-Hermen; Jean-Paul (keep rocking!); Jessica; Jitendra; Joanna (thanks for the shRNA advice! Especially for urging me not to go for inducability!); Joep; Johan (it was always a pleasure to have a discussion with you, even when these turned in to a passionate debate!); Johann; John Z. (thanks for the antibody staining tests!); Kate; Lona (the true local! It was great to have you in the office!); Lorenzo; Lucia (thank you for the philosophical conversations and brainstorming on the future of science: I hope we can both contribute to it, from the inside or from the outside!); Margriet; Marie-Anne; Michela; Marieke; Marij (thanks for all your friendly, quick and effective help with the stuff I couldn’t do myself, even after I left H4!); Marjon; Casare (I will definitely not miss your off-key whistling!); Martijn (thanks for the fun and the jokes! I feel sorry for all gullible people around you.); Min Chul (I will never forget your Gangnam-style); Miranda (it was always fun when you were in the office!); Monique (great to
have had such a reliable borrel-buddy and clearheaded discussion partner, whether about science or beyond!); Natalie (thanks for the nice chats and always being ready to help with emergency interventions!); Nienke (great to have shared the lab and the beers with you!); Odette; Patricia; Paul (whether talking science or anything else: it was always invigorating!); Paul-André; Rahmen (you must share the protocol for your cookies!); Rajith; Remco; Richard; Roel (too bad that we were only very briefly at the same division, but luckily we still organised a conference together!); Santiago (oddly, we met more often at the borrels than at the lab benches!); Sedef (always a joy to see you at borrels! And thank you for the PhD tips!) Sirth; Susanna (even only for a short while, thanks for the great talks and good luck with the science communication: we need more people like you!); Tanya; Waseem (you were a great scientist and person); and Yme (keep laughing and the world will laugh with you! Really)! Sorry for those that I couldn’t name at the moment: that’s the problem if you move labs two times…

Credits too to all co-authors on the papers that have not been mentioned here yet: Annabelle, Chris, Jelle, Jos and Lodewyk! I’d also like to acknowledge (again) everybody mentioned in the papers’ acknowledgements, who have not been named above: Hugo and Sabine for clinical samples and advice; Renato, William, Joyce, Liesbeth, Kathy, Katrien and René for sharing materials; Hans and Marc for sharing data; and Wouter for your useful comments on one of the manuscripts!

There are many other amazing people in the NKI that shared advice, fun, reagents, beers, cells, experiences, etc. with me. These are way too much to name, but I’ll give it a go: Ahmed, Anirudh, Arnold, Arnoud, Bram, Bruce (Liqin), Carlos, Chong, Dalila, Daniela, Donna, Eva B., Eva E., Ewald, Feline, Francesca, Fred, Gözde, Guus, Hanneke, Hans, Hellen, Ingar, Inge, Joeke, Jos, Kasia, Lennert, Lin, Linda, Marieke, Marit T., Marit v B., Metamia, Mihoko, Myrle, Nicolas, Pablo, Philip, Renée, Rik, Rui, Sach, Sander, Sanne, Sariette, Shalin, Sietske, Sjoerd K., Sjoerd v D., Sjors, Tiziana, Vera, and all others I couldn’t directly remember! Thanks for everything!

All labhelpers, thank you for sharing your advice, reagents, cell-lines, experiences, or otherwise to help a fellow labhelper! Also many thanks to all the facilities of the NKI that helped to make the research in this thesis possible (or at least much less troublesome): Minze and Erwin (for reliably keeping my cells deep-frozen in cryol!); Ben and Pasi (for making sure the robots were always ready for work!); Abdel and Roelof (for the quick DNA sequencing); Ron, Arno, Iris and Marja (for the high
quality RNAseq and support with the analysis); **Ludo** and **Guillaume** (for the statistics and bioinformatics support and for the excellent introduction to R-programming!); **Sjaak**, **Henk** and **Sido** (for taking so good care of the little ones in G1 and later in the RNC!); **Joost** and especially **Ellen** (for the many tests you did for me and the consistent staining of my histology slides) and **Yi-Jing** (for helping to assess these histology slides); **Annegien**, **Linde** and **Marcel** (for providing the essential clinical samples from the biobank!); **Lauran** and **Lenny** (for running such a smooth operation with the confocal microscopes!); **Hilde** (for the accurate bioanalytical and QC work); the **outpatient clinic staff** (for sharing the leftover Trastuzumab with me: I hope it will be of benefit to your future patients); the **glassware kitchen staff** (for the steady supply of clean lab bottles, Erlenmeyers, measuring cylinders, etc.); the **cleaning staff** (for keeping the labs and offices workable); the **Technology Transfer Office**, in particular **Koen** (for the assistance in preparing for filing the patents); and the **service centre staff**, in particular **Soenita** and **Meta** (for your always friendly help and preventing me becoming homeless!).

Much appreciated!

Also thanks to the facilities of the **WUR**, especially **Impulse**, in the final writing phase!

Het mooiste van de wetenschap vind ik dat het een wereldwijde samenwerking is. Hoewel er competitie is om beschikbare middelen (en om de eerste te zijn), is het gezamenlijke doel toch om de grenzen van onze kennis te verleggen. Geen enkele wetenschappelijke ontdekking is het werk van een enkele wetenschapper of een enkele onderzoeksgroep. Daarom dank aan alle wetenschappers die ik niet heb ontmoet, maar op wiens betrouwbare (en toegankelijk gepubliceerde) werk ik kon voortbouwen. Verder hartelijk dank voor de eer van collega’s die op mijn werk voortbouwen en voortgebouwd hebben. Ik ben ook heel erg dankbaar voor alle collega’s die tijdens congressen, meetings, social media, online fora of als reviewer bereid waren hun en mijn werk vrij te bespreken om samen tot oplossingen te komen, waar op de wereld ze ook werkten.

What I appreciate the most about science is that it is a global endeavour. Although competing for resources (and sometimes ‘fame’), scientists from across the globe collaborate to push the boundaries of scientific knowledge. No scientific discovery is based on research done by only one person or one research group, but instead follows from a long lineage of past contributions and inspirations from others. Therefore, I wish to thank all scientists whose work I could build on.

I am grateful for the reliable basis that you have laid and that you have made it (openly) accessible. Also, many thanks for the honour by all those that have built (or may build) on my work. Furthermore, I am grateful to all colleagues worldwide who dedicated their time to openly discuss their and my work at discussions at conferences, meetings, online or as reviewer.
Deze tijd was echter meer dan alleen wetenschap. Minstens net zo belangrijk waren de leerervaringen van enkele essentiële ‘afleidingen’, maar natuurlijk ook het sociale leven buiten het lab (voor zover mogelijk). Dit hield me mentaal gezond, uitgedaagd en in contact met de ‘echte wereld’. :) 

However, at least as indispensable for me and my personal growth were several essential ‘distractions’ from the research I was involved in, and of course the rare, but vital social life outside of the lab. These allowed me to recharge and develop, and kept me sane and in touch with the ‘real world’. :) 

Organising the International PhD-Student Cancer Conference in Amsterdam with Michiel, Roel and Silvia, and of course also Caroline and Henri, was an amazing experience! I really loved our, let’s say ‘revolutionary’, style of organisation! It was a year of hard work, but it was we more fun at the same time! Thank you all for that!

Henri, ook hartelijk bedankt voor het voordragen aan het JongAVL programma! En als we het daar toch over hebben, Anke, Anna, Bob, Gerke, Iris, Maarten, Marco, Nathalie, Sanne, Sharon, Steven, Suze en Sylvana: het was een fantastische tijd! Altijd erg leuk en leerzaam! Natuurlijk ook Larissa bedankt voor het opzetten van zo’n open en multidisciplinaire groep en het organiseren van zo’n stimulerend en uitdagend programma! Ook wil ik Els heel graag bedanken voor jouw uitstekende coaching-sessies en trainingen!

It was a pleasure to be in the PhD/OIO Council with you, Bianca, Cynthia, Diede, Eva, Floris, Hanneke, Harmke, Hellen, Ingar, Jeroen, Joke, Judith, Linda, Lot, Marc, Mirjam, Rik, Rui, Sander, Sandra, Sedef, Sjoerd, Sjors, Suzana, Tanja and Vincent, and of course the deans, Titia and later Hein! (I hope I did not forget anyone due to the high turnover when divisions were reshuffled!) The meetings were always very productive and efficient, which luckily always left some time to catch-up!

Ook prachtig om steeds nog een goede shot AESEC-spirit en enthousiasme te hebben gekregen in de AIESEC Raad van Advies (RAVA) met jullie, Onne, Marloes, Dorien, Rianne, Liselotte, Tjeerd, Anne en Marieke! Natuurlijk ook dank aan alle EB’ers die in die vijf jaar langsgekomen zijn en de meeste meetings organiseerden! Erg mooi dat we nog steeds een (of enkele) biertje en/of whisky’s pakken, Bart, Roel, Jurgen en Bram!

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I’m also very grateful to all colleagues in the European Commission’s Scientific Advice Mechanism (SAM) for the inspiring traineeship I had there, in particular Sigrid, Jeremy and Johannes: you confirmed that there are highly interesting other ways to have a real impact as a scientist. Great that I got the opportunity to continue this work with you all! Of course also thanks to all my fellow-trainees (really too many to name) that made these five months truly unforgettable! Very legendary that some of us from that time still meet up, wherever in the world, Ana, Alice, Arnold, François, Jason, Julien, Julius, Lucia, Michael, Oné and Seb!

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Ik ben blij dat er nog maar weinig wordt gekoehandeld, maar gelukkig zien we elkaar nog regelmatig, Anne, Marjolein en Rob! Ik vind ons maar een puur groep mensen samen! Dat er nog maar een hoop röstirondjes en m&m’s mogen sneuvelen!

Super dat we elkaar ondanks de drukke agenda’s, wereldreizen en alle verhuizingen nog regelmatig zien Babs, Marloes, Rianne en Lotte! En dat meer recentelijk zelfs ook met actieve deelname van de aanhang: Ronald, Niels, Bas en Wilco! Het bewijst elke keer weer dat je goede vrienden per definitie te weinig ziet! Laten we dat nog lang volhouden! So EB it!
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ABOUT THE AUTHOR

Curriculum Vitae
PhD Portfolio
List of Publications
Gerjon Jonathan Ikink was born in Nieuwegein, the Netherlands, on 26 February 1986. He received his pre-academic secondary education ('VWO') diploma at the Oosterlicht College, Nieuwegein, the Netherlands, in 2004.

In the same year, he started his Bachelor’s study in Biology at Wageningen University, the Netherlands, specialising in Human & Animal Biology and with a minor in Molecular & Cell Biology. His thesis was on the p53 tumour suppression pathway as a target for cancer therapy, under the supervision of Dr. Pim Zabel at the Laboratory of Molecular Biology.

After obtaining his Bachelor of Science degree in 2007, he continued his studies at Wageningen University with a Master’s study in Biology, specialising in Molecular & Cell Biology, supplemented with training in intercultural communication and scientific consultancy. Here, he completed a thesis on the identification of genes involved in pest insect resistance against host plant chemical defences in the group of Dr. Peter de Jong at the Laboratory of Entomology. He carried out most of this research at the National Institute of Agrobiological Sciences (NIAS; 農業生物資源研究所) in Tsukuba, Japan, in the laboratory of Dr. Manabu Kamimura. For this, he was supported by the 'Dr. Hendrik Muller's Vaderlandsch Fonds' talent grant and an international travel grant from the 'Integratie Landbouw' foundation. He completed another thesis, focused on visualising the role of mitochondria in the process of ageing, in the group of Dr. Fons Debets at the Laboratory of Genetics of Wageningen University. This research was part of the EU research project 'MiMage' on mitochondrial function in ageing and lifespan control of biological systems, funded by the European Commission's Sixth Framework Programme (FP6). As part of his studies, he also participated in an interdisciplinary academic consultancy project determining the feasibility of targeting regional cerebral blood flow to prevent neural energy crisis leading to Alzheimer's Disease. In addition, he was active as University Ambassador between 2005 and 2010, was mentor of a group of first year students and worked as supervisor in a course on ethics in Life Sciences in 2008. He obtained his Master of Science degree, with distinction (cum laude), in 2010.
During his studies, he worked as Executive Board member in the Wageningen office of the international student organisation AIESEC between 2008 and 2009, in which he was later active in the Board of Advisors for five years. Directly following his studies, he took an opportunity via AIESEC to work as interim director in the National Managerial Board of EMSA, a healthcare and social development NGO in Cairo, Egypt.

Later in 2010, he started working on the research presented in this thesis in the group of Dr. John Hilkens at the Division of Molecular Genetics of the Netherlands Cancer Institute, Amsterdam. During this work, he was also active in the Young Health Council (’jongGR’) of the Netherlands, providing policy advice in the field of public health, and as representative in the PhD Council of the Netherlands Cancer Institute and the Oncology Graduate School Amsterdam (OOA).

While finalising this thesis, he completed a traineeship in the European Commission’s Scientific Advice Mechanism (SAM) in Brussels, Belgium, and later worked part-time as policy advisor in the Directorate of Research and Science Policy of the Ministry of Education, Culture and Science in The Hague, the Netherlands. He will continue his career in the SAM, providing independent scientific advice to the College of European Commissioners to support policy-making.
PHD PORTFOLIO

COURSES

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<tr>
<th>Course</th>
<th>Institution</th>
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<tr>
<td>Big data and social physics</td>
<td>Massachusetts Institute of Technology, MITx</td>
<td>2014</td>
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<tr>
<td>Principles of economics with calculus</td>
<td>California Institute of Technology, CaltechX</td>
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<tr>
<td>Writing in the sciences – with distinction</td>
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<td>Symbision Academy, Amsterdam, NL</td>
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<td>Apoptosis</td>
<td>NKI &amp; OOA, Amsterdam, NL</td>
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<td>R-programming and statistics</td>
<td>NKI &amp; OOA, Amsterdam, NL</td>
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<td>Radiation protection (level 5B)</td>
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<td>Gene regulation networks</td>
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SEMINARS, WORKSHOPS AND MASTERCLASSES

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<td>Introduction to policy for scientists</td>
<td>JongGR, The Hague, NL</td>
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<td>Strategic scientific writing</td>
<td>VU &amp; OOA, Amsterdam, NL</td>
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<td>Analysis of gene expression data</td>
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<td>Deep sequencing techniques and data processing</td>
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TRAINING PROGRAMMES

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<tr>
<td>Policy traineeship (Blue Book)</td>
<td>European Commission, Brussels, BE</td>
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<td>Young talent programme (Jong NKI-AVL)</td>
<td>NKI &amp; AVL, Amsterdam, NL</td>
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<td>Clinical oncology internship for researchers</td>
<td>AVL, Amsterdam, NL</td>
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NKI  Netherlands Cancer Institute
OOA  Oncology Graduate School Amsterdam
JongGR  Young Health Council
VU  Vrije Universiteit Amsterdam
AVL  Antoni van Leeuwenhoek hospital
### MEETINGS AND CONFERENCES

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<thead>
<tr>
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<td>Euroscience Open Forum (ESOF)</td>
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<tr>
<td>8th International PhD Student Cancer Conference²</td>
<td>Heidelberg, DE</td>
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<td>PCDI Postdoc Life Sciences Retreat</td>
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<td>NKI Research Club Meetings²</td>
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<td>7th International PhD Student Cancer Conference³</td>
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<td>NKI/AVL Staff Evening²</td>
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<td>NKI Molecular Genetics site visit³</td>
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<td>KWF Tumour Cell Biology and Genetics Meeting²</td>
<td>Lunteren, NL</td>
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<td>Gordon Research Conference on Mammary Gland Biology³</td>
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<td>6th International PhD Student Cancer Conference¹</td>
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<td>28th IABCR/Breaktrough Breast Cancer Conference³</td>
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¹ organisation  
² oral presentation  
³ poster presentation

### TEACHING AND SUPERVISING

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<td>Nina Borràs Agustí – Bachelor student</td>
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<td>Kamile Pekcan – Technician student</td>
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<td>Manuela van Hoven – Technician student</td>
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<td>Fadime Gündogdu – Master student</td>
<td>Research thesis – 8 months</td>
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LIST OF PUBLICATIONS

**Gerjon J. Ikink** & John Hilkens
PI3K/AKT/mTOR pathway activators in HER2+ breast cancer therapy resistance
submitted to Oncoscience.

Insertional mutagenesis in a HER2-positive breast cancer model reveals ERAS as a driver of cancer and therapy resistance
Oncogene. 2018; 37: 1594–1609; doi: 10.1038/s41388-017-0031-0

**Gerjon J. Ikink** & John Hilkens
Insulin receptor substrate 4 (IRS4) is a constitutive active oncogenic driver collaborating with HER2 and causing therapeutic resistance

John Hilkens, Nikki C. Timmer, Mandy Boer, **Gerjon J. Ikink**, Matthias Schewe, Andrea Sacchetti, Martijn A. J. Koppens, Ji-Ying Song, and Elvira R. M. Bakker
RSPO3 expands intestinal stem cell and niche compartments and drives tumorigenesis

**Gerjon J. Ikink**, Mandy Boer, Elvira R. M. Bakker & John Hilkens
IRS4 induces mammary tumorigenesis and confers resistance to HER2-targeted therapy through constitutive PI3K/AKT-pathway hyperactivation
Nature Communications. 2016; 7: 13567; doi: 10.1038/ncomms13567

**Gerjon J. Ikink**
Het kritisch lezen van een medisch onderzoeksartikel

Anne D. van Diepeningen, Marijke Slakhorst, A. Bertha Koopmanschap, **Gerjon J. Ikink**, Alfons J. M. Debets & Rolf F. Hoekstra
Calorie restriction in the filamentous fungus Podospora anserina