In search of oncogenic drivers and mechanisms affecting therapy resistance in breast cancer, we identified Irs4, a poorly studied member of the insulin receptor substrate (IRS) family, as a 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. We show that Irs4 expression in mammary epithelial cells induces constitutive PI3K/AKT pathway hyperactivation, insulin/IGF1-independent cell proliferation, anchorage-independent growth and in vivo tumorigenesis. The constitutive PI3K/AKT 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 and that IRS4-expression confers resistance to HER2-targeted therapy. Taken together, our findings present the cellular and molecular mechanisms of IRS4-induced tumorigenesis and establish IRS4 as an oncogenic driver and biomarker for therapy resistance in breast cancer.

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The identification of oncogenes and their associated pathways is crucial for understanding therapy resistance and to develop effective treatment modalities. Insertional mutagenesis is an efficient tool to identify new oncogenes1,2. We recently discovered several novel proto-oncogenes in high throughput mouse mammary tumour virus (MMTV)-induced insertional mutagenesis screens3,4, including the insulin receptor substrate 4 (Irs4).

The 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 cancer5–7. The Irs3 gene is only found in rodents and is a pseudogene in humans8. Irs4 was first identified and characterized in the HEK293 human embryonic kidney cell line in which it was shown to undergo rapid tyrosine phosphorylation in response to insulin9,10.

Irs4s 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 (reviewed in refs 7,11). On binding ligand-activated RTKs, numerous tyrosine residues in the large C-terminal region of Irs4s 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 the activation of the PI3K/AKT and MAPK/ERK signalling pathways, respectively (reviewed in refs 5,11,12). In addition, 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, for example, p85 binding13–17.

In this study, we establish Irs4 as a novel mammary oncogene and we show that absence of negative feedback regulation in Irs4 leads to constitutive PI3K/AKT-signalling, which functionally differentiates it from Irs1 and Irs2. Next, we demonstrate that Irs4 is expressed in a subset of human breast cancers, collaborates with HER2 to drive tumorigenesis, and confers resistance to HER2-targeted therapy.

Results

Irs4 is targeted by MMTV proviral integration. We have previously performed high-throughput retroviral insertional mutagenesis screens of MMTV-induced mammary tumours in clinically relevant mouse models of breast cancer and wild-type mice3,4. From these studies and from a screen performed in mice transgenic for activated rat Erbb2 (Supplementary Data 1; GJI, MB, ERMB, JH, unpublished data), we obtained MMTV proviral insertion data from a total of 1,132 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 (Fig. 1a; Supplementary Data 1). The insertions were unlikely to activate the expression of the adjacent Col4a5 gene or the 350 kb upstream Gucy2f gene, which is also in opposite orientation to be activated. Indeed, Irs4 expression, as determined by reverse transcription–PCR (RT–PCR) analysis, highly correlated with an MMTV proviral insertion in the Irs4 locus ($P = 1.61 \times 10^{-3}$, Welch’s t-test), while Col4a5-expression did not ($P = 0.52$, Welch’s t-test; Fig. 1bc), 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 $r^2$-test; Fig. 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 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 (Fig. 1c; Supplementary Fig. 1a,b). In contrast to the other family members, Irs4 was neither expressed at any stage of post-natal mammary gland development nor in human breast tissue (Fig. 1c; Supplementary Fig. 1c). 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 Fig. 1d). This limited expression of Irs4 in human cell lines is in line with publicly available microarray data (Supplementary Fig. 1e). 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. To validate Irs4 as a genuine oncogene, we transduced full-length Irs4 complementary DNA (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 ($P = 9.25 \times 10^{-5}$, Welch’s t-test) (Fig. 2a,b), 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 cell line 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 ($P = 0.52$, Welch’s t-test; Fig. 1b,c), and in a subsequent experiment with 106 injected cells after three weeks ($P = 2.51 \times 10^{-4}$, log-rank test; Fig. 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.

Next, we transduced the full-length Irs4 cDNA into the murine mammary epithelial cell line NMuMG (NMuMG-Irs4). These cells do not express Brk (Ptk6), a breast cancer associated gene previously shown to interact with Irs4 (ref. 18). On 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 ($P = 3.13 \times 10^{-3}$, log-rank test), after which tumour growth was sporadically observed in a single flank (Fig. 2i; Supplementary Data 1; GJI, MB, ERMB, JH, unpublished data).
 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 with vector control cells (Fig. 3a–c; Supplementary Fig. 3a). 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 the removal of stimuli, in contrast to vector controls which only showed sporadic growth (Supplementary Fig. 3b). Similar observations were made for NMuMG cell proliferation (Supplementary Fig. 3c,d), 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 Fig. 3e). 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 (Fig. 3d–f; Supplementary Fig. 3f). 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.

**IRS4 induces constitutive PI3K/AKT 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 (Fig. 4a,b; Supplementary Fig. 4a). Similarly, we observed a potent growth factor-independent activation of the PI3K/AKT pathway on ectopic *Irs4* expression in MCF10A cells, but no significant effect on MAPK/ERK pathway activation (Fig. 4c,d; Supplementary Fig. 4b,c). Interestingly, MCF10A-Irs4 cells still exhibited high PI3K/AKT pathway activity under all tested conditions after 24 h, whereas the initial FBS and/or insulin/IGF-I-induced AKT phosphorylation seen after 30 min in MCF10A vector cells had returned to basal levels at the 24 h time point (Fig. 4d). Similarly, *Irs4* expression in NMuMG cells did not activate the MAPK/ERK pathway.
pathway, but did stimulate the PI3K/AKT pathway largely independent of any agonist, while NMuMG vector control cells and Irs1 and Irs2 transduced cells required stimuli to exhibit any PI3K/AKT pathway activity (Supplementary Fig. 4d–f). The strong and sustained activation of the PI3K/AKT 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 pathway in NMuMG-Irs4 cells using the PI3K-specific inhibitors ZSTK474 (ref. 19) or GDC0941 (ref. 20) reduced cell proliferation in a concentration-dependent manner (Fig. 4e; Supplementary Fig. 5a–d). Specifically, when PI3K/AKT 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 control cells (Fig. 4f; Supplementary Fig. 5e–k), consistent with the notion that Irs4 expression regulates cell proliferation through constitutive PI3K/AKT 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 (Fig. 5a). To investigate whether this could account for the IRS4-specific constitutive PI3K/AKT pathway activation, we replaced the C-terminal region of IRS4 with those of IRS1 or IRS2 containing a SHP2-binding domain (Fig. 5b; Supplementary Fig. 6a,b,c), which were fully active, as observed by the strong PI3K/AKT pathway activation under unstimulated conditions and after stimulation with 0.5% FBS for 10 min (Fig. 5c,d; Supplementary Fig. 6d,e). Over time, however, phosphorylation of PI3K/AKT pathway components clearly decreased faster in stimulated NMuMG-Irs4ASHP2-1 and NMuMG-Irs4ASHP2-2 than in NMuMG-Irs4 cells (Fig. 5e,f). In fact, the relative reduction rate and half-life of PI3K/AKT pathway signalling in the IRS4 recombinants was comparable to those in the vector controls and Irs1- and Irs2-transduced cells (Fig. 5g, Supplementary Fig. 6f). In other
words, cells expressing the chimeric IRS4 proteins harbouring the active SHP2 domain, were similarly responsive to negative feedback on 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 with cells expressing the wild-type Irs4 (Fig. 5h; Supplementary Fig. 6g,h). 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. To confirm SHP2 binding to the chimeric IRS4, but not to the wild-type IRS4, we performed co-immunoprecipitation 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 (Fig. 5i)\textsuperscript{13,21,22}. However, SHP2 was observed to bind 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 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 (Fig. 6a). Gene expression levels correlated well with protein levels, though suggested more IRS4-positive samples (Fig. 6b, Supplementary Fig. 7a), and showed a concomitant hyperactivation of the PI3K/AKT pathway (Supplementary Fig. 7b,c). To investigate whether high IRS4 expression was associated with survival, we examined a well annotated microarray data set obtained from a series of 157 human primary breast tumours from patients that developed metastatic disease\textsuperscript{23}. 10/157 (6%) of the tumours showed an elevated IRS4 microarray signal, which was associated with poor overall survival ($P = 0.0481$, log-rank test; Fig. 6c,d). 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 × 10⁻⁵, Pearson’s χ²-test; Fig. 6e; Supplementary Data 2). This subtype enrichment was also evident in the eight (5%) samples that expressed high (+++) IRS4 levels (P = 2.13 × 10⁻⁵, Pearson’s χ²-test; Supplementary Fig. 7d). 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 Fig. 7e). 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 quantitative RT–PCR (qRT–PCR). From the 30 tested HER2-positive samples, 3 (10%) were highly positive for IRS4 expression (Fig. 6f). From the 31 tested triple-negative tumours, 9 (29%) could be scored as IRS4 positive and four (13%) of these as highly positive (Fig. 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% (Fig. 6f, g).
In line with this clinical data, the two IRS4-positive human breast cancer cell lines we identified, MDA-MB-453 and HCC187 (Supplementary Fig. 1d), 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 pathway activation and cell growth (Fig. 6h–j; Supplementary Fig. 7f–i). Moreover, anchorage-independent growth of MDA-MB-453 cells was significantly less in IRS4 knockdown cells, compared with shGFP-control and parental cells \( (P = 9.09 \times 10^{-5} \text{ and } 6.47 \times 10^{-4} \text{ for shIRS4#14 and shIRS4#15, respectively, Welch’s t-test; Fig. 6k,l}) \). 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.

IRS4 synergizes with HER2 and confers therapy resistance. To assess the interaction between ERBB2 (HER2) and IRS4, we expressed the activated ErbB2 oncogene (HER2, neu) in Irs4-transduced MCF7 cells and observed a strong synergism in cell proliferation, anchorage independent growth, and in vitro tumorigenesis (Fig. 7a–c; Supplementary Fig. 8a). We did not observe this synergism between ErbB2 and overexpressed Irs1 (Fig. 7b). Also in the human HER2+ breast tumour cell line BT474, in which ESR2 is amplified, Irs4 expression had a strong effect on anchorage independent growth (P = 0.0143, Welch’s t-test; Supplementary Fig. 8b,c).

A major determinant of resistance to HER2-targeted therapy in breast cancer is hyperactivation of the PI3K/AKT pathway24,25. As we noticed IRS4 expression particularly in HER2+ tumours and found IRS4 to induce constitutive PI3K/AKT pathway activation, we investigated the involvement of IRS4 with regard to sensitivity to therapeutic agents. Ectopic expression of Irs4 in human breast tumour cell line BT474, in which ESR2 is amplified, and overexpressed IRS4 expression had a strong effect on anchorage independent growth (P = 0.0143, Welch’s t-test; Supplementary Fig. 8b,c).
HER2+ breast cancer cell lines SKBR3 and BT474, clearly induced resistance to Trastuzumab and Lapatinib (Fig. 7d,e; Supplementary Fig. 8d–f). To confirm this resistance is due to PI3K/AKT pathway hyperactivation, we treated the IRS4-transduced HER2+ breast cancer cell lines with a combination of Trastuzumab or Lapatinib and the PI3K-inhibitor GDC0941. Indeed, addition of the PI3K inhibitor abrogated IRS4-mediated resistance. In fact, combining Trastuzumab or Lapatinib with GDC0941 inhibited cell proliferation rates and anchorage-independent growth much stronger than the single-agent treatments (Fig. 7g; Supplementary Fig. 8g,h).

To assess whether IRS4-mediated resistance would occur spontaneously in HER2+ breast cancer cell lines, we cultured naive cell lines for five passages in medium containing increasing concentrations of Trastuzumab or Lapatinib. Expression of IRS4 was induced within five passages in most of the cultures, especially with Lapatinib, which was not observed when cells were cultured for five passages without the therapeutic agents (Fig. 7h,i; Supplementary Fig. 8i–k). Hence, Trastuzumab and Lapatinib exert a selective pressure in favor of resistant IRS4-positive cells. Subsequent knockdown of IRS4 in the Trastuzumab-resistant cells with the highest endogenous IRS4-expression (right-most cultures of Fig. 7i and Supplementary Fig. 8j) led to a reduced PI3K/AKT pathway activation and cell proliferation (Supplementary Fig. 9a–d). Knockdown of IRS4 additionally caused these cells to lose Lapatinib resistance (Supplementary Fig. 9e,f), further supporting the notion that Trastuzumab and Lapatinib resistance can both be IRS4 induced.

To confirm therapy resistance in vivo, we subcutaneously injected NMuMG cells, transduced with the activated ErbB2-oncogene, in one flank of 18 BALB/c nude mice and NMuMG cells additionally transduced with IRS4 in each opposite flank. After tumours were established in both flanks, nine mice were randomly assigned to receive Lapatinib treatment. Importantly, we observed a significant reduction in NMuMG-ErbB2 tumour growth (P = 8.75 * 10^-3, Welch’s t-test), whereas tumours from cells additionally expressing IRS4 were unaffected by Lapatinib treatment (Fig. 7j,k).

Together, these results confirm IRS4-induced Trastuzumab and Lapatinib resistance via hyperactivation of the PI3K/AKT pathway and suggest that IRS4 expression may also cause HER2-targeting therapy resistant relapse in patients.

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 in Moloney murine leukemia virus (MuLV) insertional mutagenesis screens in various types of lymphomas26, 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 common insertion site in any insertional mutagenesis screen reported in the Retrovirus and Transposon tagged Cancer Gene Database (RTCGD)27. This suggests that IRS4 has unique properties compared with 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 RTKs, and leads to activation of the PI3K/AKT and MAPK/ERK pathways (reviewed in refs 11,28). The importance of IRS signalling in cancer has been reported by Chang et al.29, 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 risk30 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-pathway signalling, leading to growth factor-independent cell proliferation in mammary epithelial cells (Fig. 8a–c). Although PI3K/AKT pathway activation by IRS4 has been described before31,32, 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 (reviewed in ref. 33), there is ample evidence that SHP2 can bind to IRS1 and IRS2, subsequently induces tyrosine dephosphorylation and thereby reduces signalling activity of these proteins13–17. 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 (refs 21,22), 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 replaced the SH2 and SH3 domains of IRS1 and IRS2 may no longer maintain the PI3K/AKT pathway hyperactivity as seen with wildtype IRS4 and is even as responsive
to negative feedback regulation following stimulation as IRS1 and IRS2. So, unlike IRS1 and IRS2, IRS4-mediated PI3K/AKT 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. 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 leukemia and subungual exostosis (a benign bone and cartilage producing tumour). In addition, in human hepatocellular carcinomas (HCC), IRS4 expression is frequently upregulated compared with hepatocytes, 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. 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 pathway and consequently, tumorigenesis. Indeed, several mechanisms for PI3K/AKT activation, including those triggered by mTOR, have been associated with triple-negative (basal-like) tumours. In HER2 + tumours, the MAPK/ERK pathway is activated through ERBB2, but PI3K/AKT pathway activation is additionally required, for example, via ERBB3 (HER3) upregulation (Fig. 8d). IRS3CA mutations or PTEN loss. In both triple-negative and HER2 + breast cancers, IRS4 expression is most likely an additional mechanism to activate the PI3K/AKT pathway and consequently, tumorigenesis. Indeed, we found that co-expression of IRS4 and Erbb2 in mammary epithelial cells synergistically accelerated tumour growth due to their activity in complementary oncopgenic pathways: the PI3K/AKT and MAPK/ERK pathways, respectively (Fig. 8e). Besides the identified oncogenic activities that IRS4 executes via PI3K/AKT 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 (reviewed in refs 7.50). Moreover, nuclear translocation with associated effects on, for example, DNA repair, rRNA synthesis and gene expression, has been reported for IRS proteins as well51,52,53, processes which also play a role in oncogenesis. Therefore, it may be useful to investigate these aspects in IRS4-induced tumours in the future. Hyperactivation of the PI3K/AKT pathway is frequently found to cause resistance to the ERBB2-targeting monoclonal antibody Trastuzumab (Herceptin) or the small molecule ERBB2 kinase inhibitor Lapatinib (Tykerb) in HER2 + breast cancer. This urged us to investigate whether IRS4-mediated constitutive PI3K/AKT pathway activation causes resistance to HER2-targeting drugs in HER2 + breast cancer cell lines (Fig. 8f,g). Our results clearly demonstrate that IRS4 expression induces resistance to both Trastuzumab and Lapatinib in vitro and Lapatinib resistance in vivo. In a clinically relevant setting, we moreover demonstrate that treatment of breast cancer cells with suboptimal doses of these HER2-targeting drugs consistently results in selection of cells with upregulated IRS4-levels, in particular with Lapatinib. This suggests that IRS4-induction, leading to drug resistance, may also frequently occur in patients treated with these anti-cancer drugs.

One may consider treating HER2 + tumours expressing high levels of IRS4 with drugs targeting PI3K, AKT and/or mTOR in combination with HER2-targeted therapy. However, in the recently completed BOLERO-3 randomized phase-III study of Trastuzumab-resistant advanced breast cancer, the addition of the mTOR-inhibitor Everolimus to Trastuzumab plus Vinorelbine treatment showed some clinical benefit only in hormone receptor negative (HR - ) patients. The BOLERO-1 phase-III randomized study similarly showed a prolongation of progression-free survival 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. However, the fact that both these trials did not preselect patients with tumours expressing high PI3K/AKT pathway activation may have limited the overall outcome. Indeed, patients from the BOLERO-3 trial with PTEN-low and pS6-high tumours both derived significantly more benefit from Everolimus than the PTEN-high and pS6-low groups, respectively. 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 pathway hyperactivation, including IRS4 + tumours. Altogether, we propose IRS4 as a novel clinical biomarker for HER2-targeted therapy resistance. Moreover, IRS4 may also prove to be of wider clinical relevance as biomarker for PI3K/AKT pathway-dependent breast and other cancers.

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, 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 metastasized 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 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. 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-chow (FVB background), transgenic for activated rat ErbB2 (ref. 58); MMTV-cNeu (FVB background), transgenic for activated rat ErbB2 (ref. 58); MMTV-cNeu (FVB background), a PTEN heterozygous mouse model; and MMTV-cNeu (FVB background), a p53 conditional knockout mouse model.

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/c mice (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 before and after injection. Tumour growth was measured at least twice a week in two dimensions and the animals were sacrificed when the tumour reached ~1 cm^2. In vivo Lapatinib resistance was assessed by subcutaneously injecting 1 × 10^6 NMuMG-ErbB2 in one flank and 1 × 10^6 NMuMG-ErbB2-Irs4 cells in the opposite flank, followed by random assignment of the mice to receive normal chow or chow containing Lapatinib, 4 days after observing tumours in both flanks. Lapatinib obtained from our in-house pharmacy was mixed with standard mouse chow (0.48 g kg^-1) as described before, translating to a dose equivalent to 100 mg kg^-1 per day. 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.

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. 1–2 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 cm^3 in size, cut into 1–2 mm pieces and incubated for 50 min in 0.25% Collagenase III (Invitrogen) supplemented with 10% FBS and PenStrep. SKBR3 medium was previously described by Trypan Blue staining and differed neither between cells from the test group and controls, nor before and after injection. Tumour growth was measured at least twice a week in two dimensions and the animals were sacrificed when the tumour reached ~1 cm^2. In vivo Lapatinib resistance was assessed by subcutaneously injecting 1 × 10^6 NMuMG-ErbB2 in one flank and 1 × 10^6 NMuMG-ErbB2-Irs4 cells in the opposite flank, followed by random assignment of the mice to receive normal chow or chow containing Lapatinib, 4 days after observing tumours in both flanks. Lapatinib obtained from our in-house pharmacy was mixed with standard mouse chow (0.48 g kg^-1) as described before, translating to a dose equivalent to 100 mg kg^-1 per day. 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).

**Figure 8** | Proposed model of IRS4-induced signalling and therapy resistance. (a-c) Simplified PI3K/AKT signalling cascade in normal mammary cells expressing IRS1 and IRS2, or mammary cells expressing IRS4, in presence (a, b) or absence (c) of insulin/IGF1. (d-e) Model of signalling in HER2+ (ERBB2) breast cancer cells where ERBB3 (HER3) (d) or IRS4 (e) activates the PI3K/AKT pathway. (f-g) Model of the effect of Trastuzumab or Lapatinib treatment in HER2+ breast cancer cells expressing ERBB3 (f) or IRS4 (g). Thickness of the arrows indicates strength of signalling. Red ‘X’ indicates no interaction/signalling. Dashed arrow indicates possible cross-talk.

That is all the content from the image.
Establishing 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⁻¹ Trastuzumab; 10, 20 or 40 nM Lapatinib; or equal volumes of DMSO diluent. Cells were passaged on 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 rates were comparable to the vehicle-treated cells, or kept the same when growth was noticeably slower (see Fig. 7h.; Supplementary Fig. 8j) for a schematic representation of the concentrations used.

Plasmids and gene transfection. 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: TRCN000072185, 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, shirs4#18 and shIRS4#17, respectively. Lentiviral supernatants were produced following transfection as described by the TRC (http://www.broadinstitute.org/maln/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 pore-size filter (Whatman), supplemented with 6 μg ml⁻¹ polybrene (Millipore), shirs4#20–24 and shGFP were used for transfection into our newly established P3724-R4 tumour cell line and shirs4#13–17 and shGFP for transduction in SKBR3/IR585⁻ / BT474/IR585⁻, MDA-MB-453 and HCC1187 cells. Cells expressing a shRNA were selected with 2 μg ml⁻¹ 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. The pBABE-construct with wildtype mouse Irs2 was obtained through Amersham Biosciences (Amersham BI 17171) and was originally cloned in the laboratory of Dr R. Kahan. A construct mutating rat ErbB2/Neu cDNA was a kind gift of Dr W.J. Muller (McGill Cancer Center, Montreal) and was cloned in a pMSCV vector with basicistin resistance gene. Mouse wildtype Irs4 cDNA cloned into a pMSCV vector was kindly provided by Dr A. Bera (NKI, Amsterdam). The Irs1 and Irs2 constructs were used in cotransfections of 50,000 SKBR3, 25,000 BT474, 30,000 MDA-MB-453, 30,000 HCC1187, 30,000 SKBR3/IR585⁻ or 50,000 BT474/IR585⁻ cells per well were seeded in 24-well plates (Corning) for the Trastuzumab and Lapatinib resistance experiments. 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) and imaged using a desktop scanner (Epson). The experiments with SKBR3 or BT474-cells were all performed four times, using independent stably transduced oligoclonal cell cultures. The experiments with P3724-R4, SKBR3/IR585⁻ or BT474/IR585⁻ cells were performed in triplicate.

Expression analysis. RNA isolation, RT–PCR and expression analysis of tissues and tumour material was performed as described before. 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. qPCR 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 all performed four times, using independent stably transduced oligoclonal cell cultures. The experiments with P3724-R4, SKBR3/IR585⁻ or BT474/IR585⁻ cells were performed in triplicate.

Apoptosis assays. 1.0 × 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⁻¹ insulin, 200 ng ml⁻¹ hydrocortisone and 20 ng ml⁻¹ 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 FACScan analyser.

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 agroscopic beads from Santa Cruz Biotechnology (sc-208 ACI, following manufacturer’s instructions). The immunoprecipitated proteins were solubilized in sample buffer and analysed by 4–12% Bis-Tris gel electrophoresis followed by western blotting.

Soft agar assays. Soft agar assays were performed in six-well plates. Each well contained 0.66% low-gelling temperature agarose (Agarose Aldrich) base layer on which 50,000 MCF10A, 10,000 NMU-Mg, 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 Trastuzumab, Lapatinib, GDC0941 or vehicle, where applicable). Anchorage-independent growth was assessed by counting colonies after two weeks using the GelCount instrument (Oxford Optronix), following manufacturers’ instructions. 20,000 PI-negative cells per replicate were analysed for Annexin V-staining using a Beckton Dickinson LSRII FACScan analyser.

All experiments were performed with at least two independently transduced cell cultures each.
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 rpm 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 membrane (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 normalized 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-4E-BP1, 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 4E-BP1.

Antibodies against p-AKT #473 (dil.1:2000, 1:2000), p-IRS (p-ERK) T204/T202 (49106, 1:200), p-S6 S235/236 (dil.1:850, 1:200), p-4E-BP1 T37/46 (#2855, 1:1000), total S6 (#2217, 1:1000), total 4E-BP1 (#9644, 1:1000), total MAPK (ERK) (#9112, 1:1000) and PARP (#9542) were obtained from Cell Signalling Technology. Goat anti-IRS4 antibody (#B118, 1:4000) was obtained from Everest Biotech. Antibodies against IRS1 (α-scl-599, 1:500), IRS2 (α-scl-9299, 1:500), SHP2 (α-scl-280, 1:1000) and total AKT (α-scl-9312, 1:1000) were purchased from Santa Cruz Biotechnology, α-Tubulin (#T9262, 1:5000) from Sigma-Alrich, and β-actin (#GTX26276, 1:1000) 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 included in Supplementary Fig. 11.

Statistical analyses. Statistical tests are specified in the text and were calculated using the statistical programming language R, with the ‘Biocodoctor’ and ‘survival’ packages. A P-value ≤ 0.05 was considered statistically significant.

Data availability. All data sets generated or analysed during this study are included in this published article and its Supplementary Information files.

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Author contributions
G.J.I. and J.H. conceived and designed the experiments. G.J.I. and M.B. performed the experiments. G.J.I. and E.R.M.B. interpreted the results and G.J.I. prepared the manuscript. J.H. oversaw the project.

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