High-dose oncogenic PIK3CA drives constitutive cellular stemness through self-sustained TGFβ pathway activation

Ralitsa R. Madsen1,2,3,* , James Longden4,5, Rachel G. Knox2,3, Xavier Robin4, Franziska Völlmy4, Kenneth G. Macleod6, Larissa Moniz7, Neil O. Carragher6, Nicholas McGranahan7, Rune Linding4,5, Bart Vanhaesebroeck7, Robert K. Semple1,2,3,*

1Centre for Cardiovascular Science, Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, UK.
2Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, University of Cambridge, Cambridge, UK.
3The National Institute for Health Research Cambridge Biomedical Research Centre, Cambridge, UK.
4Biotech Research and Innovation Centre, University of Copenhagen, Copenhagen, Denmark.
5Humboldt-Universität zu Berlin, Berlin, Germany.
6Edinburgh Cancer Research UK Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Crewe Road South, Edinburgh, UK.
7University College London Cancer Institute, Paul O’Gorman Building, University College London, London, UK.

*To whom correspondence should be addressed: rmadsen@ed.ac.uk or rsemple@ed.ac.uk

One-sentence summary: Biallelic genetic PI3Kα activation rewire signaling and induces constitutive stemness downstream from self-sustained TGFβ pathway activation.
Oncogenic PIK3CA mutations activate phosphoinositide 3-kinase-alpha (PI3Kα) and are among the commonest somatic mutations in cancer. We recently demonstrated that the “hotspot” variant PIK3CA<sup>H1047R</sup> exerts striking allele dose-dependent effects on stemness in human pluripotent stem cells (hPSCs), and found multiple oncogenic PIK3CA copies in a substantial proportion of human cancers. This suggested that the consequences of oncogenic PI3K signaling may differ according to the strength of genetic PIK3CA activation. Here, to identify the stemness-promoting mechanism, we profiled isogenic wild-type, PIK3CA<sup>WT/H1047R</sup> and PIK3CA<sup>H1047R/H1047R</sup> iPSCs by high-depth transcriptomics, proteomics and reverse-phase protein arrays (RPPA). We report that the phenotypic switch in homozygous PIK3CA<sup>H1047R</sup> hPSCs occurs downstream of signaling “rewiring” towards self-sustained TGFβ pathway activation and increased NODAL expression, which was no longer reversible by pharmacological PI3Kα inhibition. Gene expression analysis of PIK3CA-associated human breast cancers in The Cancer Genome Atlas revealed increased expression of NODAL according to tumor stage and PIK3CA<sup>H1047R</sup> allele dosage. Together with the emerging link between NODAL re-expression and cancer aggressiveness, our data suggest that TGFβ pathway inhibitors warrant investigation in breast tumors stratified by PIK3CA<sup>H1047R</sup> allele dosage.
Introduction

Class IA phosphoinositide 3-kinases (PI3Ks) are evolutionarily conserved enzymes that catalyze the formation of the membrane-bound second lipid messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). PI3Ks are activated downstream of many receptor tyrosine kinases, with the ensuing increase in PIP3 and its byproduct PI(3,4,5)P3 activating the serine/threonine kinases AKT and mTORC1. PI3K activation is best known for promoting cell survival, glucose uptake, anabolic metabolism, cell proliferation and cell migration (1). Among the class IA PI3K isoforms (PI3Kα, PI3Kβ, PI3Kδ), the ubiquitously-expressed PI3Kα, encoded by the PIK3CA gene in humans, is the main regulator of organismal growth, development and survival (2).

Activating mutations in PIK3CA are among the most common somatic point mutations in cancer, together with alterations in the tumor suppressors PTEN and TP53 (cBioPortal, accessed August 2019) (3). Many PIK3CA-associated cancers harbor multiple cancer-promoting mutations within the pathway (4, 5), consistent with experimental evidence that heterozygous expression of a strongly activating PIK3CA mutation alone is not sufficient to trigger cellular transformation in vitro or to accelerate tumorigenesis in vivo (reviewed in Ref. (6)). This is further supported by observations of people with disorders in the PIK3CA-related overgrowth spectrum (PROS). These are caused by postzygotic acquisition of an activating PIK3CA mutation, and are not known to be associated with excess adult malignancy (6).

We recently reported that human induced pluripotent stem cells (iPSCs) with homozygous knock-in of the cancer “hotspot” mutation PIK3CA\(^{H1047R}\) exhibit striking phenotypic differences to isogenic cells that are heterozygous for the same variant (5). This suggested that major cellular consequences of genetic PI3K activation are exquisitely sensitive to the strength of the pathological PI3K signal in the context of endogenous expression. Human iPSCs, like embryonic stem cells from the inner cell mass of the pre-implantation blastocyst (collectively human pluripotent stem cells, hPSCs), can differentiate into derivatives of all three embryonic germ layers (7). They may also indefinitely self-renew (7), although this requires carefully controlled culture conditions (8). In the presence of homozygous PIK3CA\(^{H1047R}\) expression, however, these cells exhibited robust, self-sustained stemness in vitro and in vivo (5).

The core hPSC pluripotency gene regulatory network features a feedforward, autoregulatory circuit comprising three transcription factors, namely SRY box 2 (SOX2), Octamer-binding transcription factor 3/4
(OCT3/4; encoded by *POU5F1*), and the homeobox transcription factor NANOG (9–11). SOX2 helps sustain OCT3/4 expression, which is required for establishment and maintenance of the pluripotent state (12). However, even modest overexpression of OCT3/4 destabilizes the pluripotency network and triggers differentiation (13, 14). In contrast, NANOG, while dispensable for maintenance of pluripotency (15), stabilizes the pluripotency gene regulatory network. Overexpression of NANOG by as little as 1.5-fold leads to sustained self-renewal (or “stemness”) of murine and human PSCs (16–19). In hPSCs, *NANOG* expression is activated by the transcription factors SMAD2/3 (20), which in turn are activated by receptors binding TGFβ, Activin or NODAL (21). Overexpression of NODAL thus results in self-sustained stemness of hPSCs even in differentiation-promoting conditions (22, 23).

NODAL expression is not only critical in embryogenesis, but is also more rarely seen in adult organs during extensive tissue remodeling, as in placenta, endometrium and the lactating mammary gland (24). There has also been growing awareness in recent years that aggressive cancers often re-express NODAL, “hijacking” its ability to promote cellular plasticity and epithelial-mesenchymal-transition (EMT), and recapitulating in a corrupted form some of its effects in embryonic stem cells (24). However, the mechanism(s) driving NODAL re-expression in neoplastic conditions and a possible relationship to known oncogenes remain unclear.

In this study, we used transcriptomics, proteomics and reverse phase protein arrays (RPPA) to investigate further the PI3K signaling threshold revealed by our prior studies, confirming near-binary changes in gene and protein expression around a sharp PI3K activity threshold defined by heterozygosity and homozygosity for the same strongly activating *PIK3CA* allele. We further demonstrate that the stemness phenotype of *PIK3CA*<sup>H1047R/H1047R</sup> iPSCs is maintained by self-sustained TGFβ signaling, in line with increased *PIK3CA*-mediated NODAL expression. Finally, we show that the allele dose-dependent link between *PIK3CA*<sup>H1047R</sup>, NODAL expression and stemness applies to human breast cancers. By deploying pathway and network topology analysis and algorithms to identify the signaling rewiring underlying the ‘locked’ stemness induced by increased *PIK3CA*<sup>H1047R</sup> allele dosage, we raise the possibility that pharmacological targeting of TGFβ signaling in breast cancers stratified by *PIK3CA*<sup>H1047R</sup> allele dosage may limit cancer stem cell emergence and be a valuable therapeutic strategy.

**Results**
A sharp PI3K activity threshold determines gene expression changes in PIK3CA<sup>H1047R</sup> iPSCs

We previously generated isogenic human iPSCs with heterozygous or homozygous knock-in of the “hotspot” PIK3CA<sup>H1047R</sup> mutation. Surprisingly, in heterozygous cells we found few discernible phenotypic changes and few differentially expressed protein-coding transcripts, despite the strong associations of PIK3CA<sup>H1047R</sup> heterozygosity with cancer and PROS. In contrast, homozygous PIK3CA<sup>H1047R/H1047R</sup> cells exhibited marked morphological changes and altered gene expression, with strong enrichment for cancer-associated pathways (5).

To substantiate the apparent PI3K activity threshold manifest in PIK3CA<sup>H1047R</sup>-driven gene expression changes, we undertook RNA sequencing at substantially greater depth, increasing the sample size to four previously unstudied, independently-derived iPSC cultures for each PIK3CA genotype. Homozygous mutant cells clearly separated from heterozygous and wild-type cells, which overlapped with each other on multidimensional scaling (Fig. 1A). Consistent with improved statistical power, we now detected reduced levels of 451 transcripts and increased levels of 710 transcripts in PIK3CA<sup>WT/H1047R</sup> hPSCs (Fig. 1B). This dropped to 149 and 343 transcripts, respectively, after applying an absolute fold-change cut-off of minimum 1.3 (Fig. 1B and table S1), consistent with the small magnitude of significant expression changes (fig. S1A). Use of the same cut-off, in sharp distinction, yielded 2873 and 2771 transcripts of decreased or increased abundance, respectively, in homozygous iPSC mutants (Fig. 1B and table S2). Not only was the number of gene expression changes higher by an order of magnitude in homozygous cells, but many expression changes were also large compared to wild-type controls (fig. S1A). The fold-changes in gene expression seen in PIK3CA<sup>H1047R/H1047R</sup> cells were highly correlated with our previously reported findings (Spearman’s rho = 0.74) (fig. S1B), whereas the correlation was low (Spearman’s rho = 0.1) for PIK3CA<sup>WT/H1047R</sup> iPSCs (fig. S1C).

Given previous reports that heterozygosity for PIK3CA<sup>H1047R</sup> in breast epithelial cells extensively remodels gene expression (25, 26), we undertook further transcriptional profiling in two unrelated cellular models of genetic PIK3CA activation. First, we examined iPSCs derived from a woman with clinically obvious but relatively mild PROS due to mosaicism for PIK3CA<sup>E418K</sup> (Fig. 1C) (27). Heterozygous iPSCs were compared to wild-type lines established simultaneously from dermal fibroblasts derived from the same skin biopsy, which was possible due to genetic mosaicism of the sampled area of skin. Like PIK3CA<sup>WT/H1047R</sup>iPSCs, PIK3CA<sup>WT/E418K</sup>iPSCs closely clustered with the isogenic wild-type controls on MDS plotting (Fig. 1D), with only 30 differentially-expressed genes (table S3). We also studied previously reported Pik3ca<sup>WT/H1047R</sup> mouse embryonic fibroblasts (MEFs) 48 h after Cre-
mediated Pik3ca<sup>HHo47R</sup> induction (28). Wild-type and Pik3ca<sup>Wild/Ho47R</sup> MEFs were superimposable on an MDS plot (Fig. 1E), with only 192 downregulated and 77 upregulated genes (table S4).

Collectively, these findings corroborate the existence of a threshold of PI3K pathway activity which determines the large majority of gene expression changes in PIK3CA<sup>HHo47R/Ho47R</sup> iPSCs in a near-binary manner. While higher depth of sequencing did reveal statistically significant gene expression changes in heterozygous iPSCs, effect sizes were modest and more variable. Similar findings in heterozygous MEFs suggest that this may be generalizable to differentiated cell types, irrespective of species.

To assess whether transcriptional changes observed in iPSCs were mirrored in the proteome, we applied label-free proteomics to the iPSC lines used in our first study (5). Around 4,600 protein ratios were obtained for both heterozygous versus wild-type and homozygous versus wild-type iPSC comparisons, as estimated using a novel Bayesian approach based on the Markov Chain Monte Carlo (MCMC) method (29). In contrast to other algorithms, the MCMC method generates an error estimate alongside each protein concentration which permits more confident determination of proteins with the most robust differential expression. The number of differentially expressed proteins correlated with PIK3CA<sup>HHo47R</sup> allele dosage, with 54 and 258 differentially-expressed proteins in PIK3CA<sup>Wild/Ho47R</sup> and PIK3CA<sup>HHo47R/Ho47R</sup> cells, respectively (Fig. 1F, table S5 and S6). Of these, 27 proteins were differentially expressed in both heterozygous and homozygous PIK3CA<sup>HHo47R</sup> iPSCs (table S7), with 16 changing in opposite directions (Fig. 1F). There was a good correlation between differentially-expressed proteins and corresponding transcripts in PIK3CA<sup>HHo47R/Ho47R</sup> iPSCs (fig. S2A, S2B), but not in heterozygous mutants (fig. S2C, S2D). As for the relatively poor correlation seen between transcriptomic experiments for heterozygous cells, this likely reflects the small magnitude of gene expression changes induced by heterozygous PIK3CA<sup>HHo47R</sup>.

Altogether, these data consolidate the view that only homozygosity for PIK3CA<sup>HHo47R</sup> results in robust and widespread transcriptional changes in otherwise normal, diploid cells, arguing against the “butterfly” effect of heterozygosity suggested in prior studies using a genetically abnormal breast epithelial cell line (25, 26).

**PIK3CA<sup>HHo47R/Ho47R</sup> iPSCs show evidence for signaling rewiring**

We previously demonstrated a graded increase in AKT (S473) phosphorylation across heterozygous and homozygous PIK3CA<sup>HHo47R</sup> iPSCs (5). To assess in more detail whether the near-binary gene expression difference between heterozygous and homozygous PIK3CA<sup>HHo47R</sup> cells is underpinned by corresponding differences in indices.
of PI3K pathway activation, we profiled phosphorylation of a wider repertoire of pathway components using reverse phase phosphoprotein arrays (RPPA). To mimic the physiological environment of the pluripotent epiblast, we studied cells in growth factor-replete conditions.

Observed changes in protein phosphorylation were surprisingly modest, with the maximal change a two-fold increase in AKT phosphorylation (on S473 and T308) in \( \text{PIK3CA}^{H1047R/H1047R} \) cells. Contrasting with the near-binary response seen at the transcriptional level, heterozygous and homozygous \( \text{PIK3CA}^{H1047R} \) expression generally produced graded phosphorylation of PI3K pathway components, with slightly higher levels in homozygous iPSCs (Fig. 2A).

None of the mutant genotypes showed consistently increased mTORC1-dependent phosphorylation of P70S6K or its downstream substrate S6 (fig. S3A), perhaps reflecting saturation of this level of the pathway due to the presence of the additional stimuli in the medium (e.g. amino acids). When deprived of growth factors for 1 h prior to RPPA profiling, both heterozygous and homozygous mutant did exhibit increased P70S6K phosphorylation, whereas S6 phosphorylation remained similar to wild-type cells (Fig. 2B). Inhibition of PI3K\( \alpha \) activity with low-dose BYL719 for 24 hours fully reversed canonical PI3K signaling-related changes in phosphorylation of downstream proteins including AKT, GSK3, FOXO1, TSC2 and P70S6K (Fig. 2B). Other changes in protein phosphorylation remained unaffected in \( \text{PIK3CA}^{H1047R/H1047R} \) hPSCs, including increased SMAD2 and ERK1/2 phosphorylation as well as increased expression of c-MYC and IGF1R (Fig. 2B, fig. S3B). This suggests signaling rewiring that is, unexpectedly, resistant to acute inhibition of the primary inducing event.

Pathway and network analyses implicate TGF\( \beta \) signaling in \( \text{PIK3CA}^{H1047R} \) dose-dependent stemness

Pathway and network analyses were next applied to proteomic and transcriptomic data in an attempt to identify candidate mechanism(s) mediating \( \text{PIK3CA}^{H1047R} \) dose-dependent stemness. Consistent with our previous study, in which we showed strong induction of the TGF\( \beta \) family member \( \text{NODAL} \) (5), TGF\( \beta 1 \) was again the most significant predicted upstream activator according to Ingenuity\textsuperscript{®} Pathway Analysis (IPA) of the top 2000 upregulated and top 2000 downregulated transcripts in \( \text{PIK3CA}^{H1047R/H1047R} \) iPSCs (Fig. 3A). TGF\( \beta 1 \) was also the most significant predicted upstream activator revealed by analysis of the \( \text{PIK3CA}^{H1047R/H1047R} \) proteomic dataset (Fig. 3B). These data strongly suggest activation of the TGF\( \beta \) pathway in homozygous \( \text{PIK3CA}^{H1047R} \) iPSCs.
Although \(PIK3CA^{WT/H1047R}\) iPSCs showed around 10-fold fewer differentially-expressed genes than homozygous iPSC cells, IPA for upstream activators in the heterozygous iPSCs also revealed multiple TGF\(\beta\) pathway-related stimuli among the most significant predicted activators (Fig. 3C). Moreover TGF\(\beta\)1 was predicted as one of only two differential upstream activators when analysis was performed on genes that were concordantly differentially expressed (\(n = 180\)) in \(PIK3CA^{H1047R}\) mutant iPSCs versus wild-type controls (Fig. 3C and table S8). The other significant upstream regulator common to both heterozygous and homozygous \(PIK3CA^{H1047R}\) was MAPK1 (encoding ERK2), which is consistent with RPPA as well as immunoblot evidence of increased ERK kinase phosphorylation in \(PIK3CA^{H1047R}\) mutant iPSCs (Ref. (5) and schematic in Fig. 2A). Although TGF\(\beta\) signaling-related predictions held in heterozygous \(PIK3CA^{H1047R}\) iPSCs, the significance of the effect (overlap p-value = 1.74e-05) was much lower than in homozygous (overlap p-value = 4.25e-21) mutants. This points towards a critical role for the TGF\(\beta\) pathway in mediating the allele dose-dependent effect of \(PIK3CA^{H1047R}\) in human iPSCs.

To complement the IPA analysis, which is based on highly curated, proprietary datasets, we undertook non-hypothesis-based Weighted Gene Correlation Network Analysis (WGCNA) – a network-based data reduction method that seeks to determine gene correlation patterns across multiple samples, irrespective of the function of individual genes (30). Using all transcripts expressed across wild-type and both heterozygous and homozygous \(PIK3CA^{H1047R}\) iPSCs (Fig. 4A), this analysis returned a total of 43 clusters (or modules) of highly interconnected genes (Fig. 4B). Of the two modules with the highest correlation to the homozygous trait, one showed enrichment for several KEGG pathway terms of relevance to the stemness phenotype of \(PIK3CA^{H1047R/H1047R}\) iPSCs – most notably “Signaling pathways regulating pluripotency in stem cells” (Fig. 4C). Given prior predictions of strong activation of TGF\(\beta\) signaling in homozygous mutants, we next constructed the minimal network of differentially expressed genes in \(PIK3CA^{H1047R/H1047R}\) iPSCs that linked the pluripotency, PI3K and TGF\(\beta\) signaling pathways within this network module (Fig. 4D). The resulting network exhibited high interconnectivity, with multiple shared nodes across all three pathways, suggesting close crosstalk between PI3K and TGF\(\beta\) signaling in stemness regulation. Finally, the fact that most of the network nodes represented genes with increased expression in homozygous mutants strengthens the notion that strong oncogenic PI3K\(\alpha\) activation stabilizes the pluripotency network in human iPSCs.
Inhibition of TGFβ signaling destabilizes the pluripotency gene network in PIK3CA<sup>H1047R/H1047R</sup> iPSCs

TGFβ signaling plays a critical and well-established role in pluripotency regulation (20, 23, 31), and a differentiation-resistant phenotype has previously been reported in NODAL-overexpressing hPSCs (22). These observations, together with increased NODAL expression in homozygous PIK3CA<sup>H1047R</sup> iPSCs and computational identification of enhanced TGFβ pathway activity in PI3K-driven “constitutive” stemness, led us to hypothesize that strong PI3Kα-dependent activation of NODAL expression underlies the establishment of the differentiation-resistant phenotype of homozygous mutants through stabilization of the pluripotency gene network. Specifically, we hypothesized that NODAL elicits autocrine enhancement of TGFβ signaling in PIK3CA<sup>H1047R/H1047R</sup> iPSCs, with downstream promotion of NANOG expression ultimately “locking” the cells in a state of perpetual stemness.

Testing this hypothesis in hPSCs is challenging for both biological and technical reasons. These include lack of specific pharmacological NODAL inhibitors and the difficulty in detecting the subtle early phenotypic consequences of partial destabilization of the hPSC pluripotency gene regulatory network. Moreover, the widely-adopted maintenance medium and coating substrate we used for cell culture both contain TGFβ ligands (32, 33), which may mask effects of NODAL repression by PI3Kα-specific inhibition. To minimize these confounders, we prepared maintenance medium with and without recombinant NODAL supplementation, and assessed expression of NODAL itself and NANOG as surrogate markers of stemness over 72 h of culture.

Within 48 h, exclusion of NODAL from the medium resulted in the expected downregulation of NODAL and NANOG expression in wild-type iPSCs, and this was greater still at 72 h (Fig. 5 and fig. S5A). In contrast, NODAL removal had no effect on the increased NODAL and NANOG expression in PIK3CA<sup>H1047R/H1047R</sup> iPSCs (Fig. 5 and fig. S5A), in line with a self-sustained stemness phenotype. Exposure of NODAL-free homozygous cultures to 250 nM BYL719 reduced NODAL expression within 24 h, and this continued to decrease subsequently (Fig. 5), consistent with NODAL’s known ability to control its own expression through a feedforward loop (34). Despite a 55% reduction in NODAL mRNA after 72 h, however, little effect on NANOG expression was seen (Fig. 5). The lack of NANOG decrease may reflect the short time course studied, or the exquisite sensitivity of hPSCs to residual upregulation of NODAL in homozygous PIK3CA<sup>H1047R</sup> iPSCs. This may further be compounded by residual low levels of TGFβ-like ligands in the coating substrate, or possibly by the increased expression of two other TGFβ
superfamily ligands, GDF3 and TFGB2, which were detected in the transcriptome analysis of homozygous mutant cells.

Thus, to confirm that TGFβ signaling is required for maintenance of stemness in PIK3CA^{H1047R/H1047R} iPSCs, we treated cells with SB431542 – a potent and specific inhibitor of TGFβ and NODAL type I receptors (35). This resulted in acute and complete repression of NODAL expression within 24 h, accompanied by downregulation of NANOG expression (Fig. 5). A similar effect was observed on POU5F1 (OCT3/4) expression, consistent with destabilization of the pluripotency gene regulatory network in PIK3CA^{H1047R/H1047R} iPSCs (Fig. 5). Confirming this, we used a lineage-specific gene expression array to demonstrate a similar reduction in the expression of several other well-established stemness markers (MYC, FGF4, GDF3) with increased expression in PIK3CA^{H1047R/H1047R} iPSCs, performing the analysis after 48 h of TGFβ pathway inhibition (Fig. S4B). Despite the short treatment duration, we also found evidence for the expected neuroectoderm induction upon inhibition of the TGFβ pathway (22, 36), reflected by increased expression of CDH9, MAP2, OLFM3 and PAPLN (Fig. S4B).

Collectively, these data strongly suggest that the stemness phenotype of PIK3CA^{H1047R/H1047R} iPSCs is mediated by self-sustained TGFβ signaling, most likely through PI3K dose-dependent increase in NODAL expression, and is amenable to reversal through pharmacological inhibition of the TGFβ pathway but not of PI3Kα.

Stage II human breast cancers with multiple PIK3CA^{H1047R} alleles exhibit increased NODAL expression

It has long been known that dedifferentiating tumor cells re-express embryonic markers (37), and that the TGFβ pathway promotes cancer stem cell maintenance, metastasis and drug resistance (38–40). The strong link between homozygosity for PIK3CA^{H1047R} and TGFβ signaling in human iPSCs prompted the question of whether this mechanism may be of relevance in human PIK3CA-associated cancers. Specifically, NODAL re-expression has previously been linked to aggressive features such as invasion and metastasis in a range of cancers (reviewed in Refs. (24, 41)), and associated with poor prognosis in breast cancer (42–45). Given the high prevalence of PIK3CA^{H1047R} in breast cancers (35.4 %; cBioPortal non-overlapping breast cancer studies, accessed August 2019) (3), we next stratified the breast invasive carcinoma (BRCA) dataset in The Cancer Genome Atlas (TCGA) according to PIK3CA^{H1047R} allele dosage and conducted targeted analysis of NODAL, NANOG, POU5F1 and MYC expression, comparing tumors with multiple PIK3CA^{H1047R} copies to those with a single copy.
PIK3CA<sup>H1047R</sup> was detected in 108 BRCA samples, 57 of which had more than one copy of the mutant allele. Of the remaining 51 samples with a single PIK3CA<sup>H1047R</sup> allele, 10 harbored a second PIK3CA mutation and were thus grouped with cancers with multiple PIK3CA<sup>H1047R</sup> copies (giving rise to a total of 67 samples classified as ‘multiple’). MYC mRNA levels were robustly detected in both sample groups (Fig. 6A), consistent with ubiquitous expression. In contrast, mRNA expression of the embryonic markers NODAL, NANOG and POU5F1 was low (<1 count per million) (Fig. 6A), as expected if breast cancer stem cell-like cells only comprise a small proportion of the bulk tumor tissue (46). The low expression notwithstanding, there was evidence of increased expression of MYC, POU5F1 and NODAL in several samples with multiple PIK3CA<sup>H1047R</sup> copies (Fig. 6A).

Given that re-expression of NODAL has been linked with aggressive breast cancer (42–45), we next stratified the samples according to tumor stage and assessed the expression of the four stemness markers (Fig. 6B). Stage I denotes breast cancer that has not spread outside the breast and exhibits little or no involvement of proximal lymph nodes, whereas stage II and III tumors exhibit progressively higher spreading to lymph nodes (47). Stage IV denotes breast cancer that has spread to other organs (47). We excluded stage IV samples from the analysis due to low sample size (2 and 1 in the “single” and “multiple” PIK3CA<sup>H1047R</sup> groups, respectively). Notably, in stage II breast tumors, for which the sample size was sufficient, NODAL expression increased as a function of PIK3CA<sup>H1047R</sup> allele dosage (Fig. 6B). This trend was apparent despite the genetically heterogeneous nature of the samples and suggests that the allele dose-dependent signaling rewiring connecting PIK3CA<sup>H1047R</sup>, NODAL expression and self-sustained stemness in human iPSCs may be relevant to breast cancer progression (Fig. 6C), with implications for therapeutic targeting of tumors with multiple PIK3CA<sup>H1047R</sup> copies.

Discussion

The so-called “hotspot” variant PIK3CA<sup>H1047R</sup> is the most common activating PIK3CA mutation in human cancers and in a group of largely benign overgrowth disorders known as PROS (6). We recently found that PIK3CA-associated cancers often harbor multiple mutant PIK3CA copies, and demonstrated that homozygosity but not heterozygosity for PIK3CA<sup>H1047R</sup> leads to self-sustained stemness in human pluripotent stem cells (hPSCs) (5). Using a combination of computational analyses and targeted experiments, the current study adds further support for the existence of a PI3K signaling threshold in cells with oncogenic PI3Kα activation. We provide evidence for self-
sustained TGFβ pathway activation as the main mechanism through which PIK3CA^H1047R homozygosity “locks” hPSCs in a differentiation-resistant state that becomes independent of the driver mutation and the associated PI3K pathway activation.

High-depth transcriptomics confirmed that heterozygosity for PIK3CA^H1047R fails to induce widespread substantial transcriptional remodeling, whether chronically modelled in CRISPR-edited iPSCs or acutely induced in mouse embryonic fibroblasts (MEFs) by Cre expression, despite induction of canonical PI3K pathway activation in both cases (current study and Ref. (5, 28, 48)). Similarly, iPSCs with heterozygous expression of PIK3CA^E418K, a “non-hotspot” mutation, were transcriptionally indistinguishable from their isogenic wild-type controls. In contrast to the mild transcriptional consequences of heterozygous PIK3CA^H1047R expression, homozygosity for PIK3CA^H1047R was associated with differential expression of nearly 1/3 of the hPSC transcriptome. This observed near-binary response is not a consequence of a similar quantitative difference in PI3K pathway activation assessed by phosphoprotein profiling, which instead showed a relatively modest and graded increase in homozygous versus heterozygous PIK3CA^H1047R iPSCs. This suggests that the apparent sharp PI3K signaling threshold that determines the cellular response in hPSCs is “decoded” distal to the canonical pathway activation.

The increased expression of several proteins in homozygous PIK3CA^H1047R iPSCs, and their resistance to downregulation in response PI3Kα-specific inhibition with BYL719, further suggested that the phenotype of these cells may have become partially uncoupled from the oncogenic trigger event. In systems biology terms, the near-binary response observed with two versus one copy of PIK3CA^H1047R, and the inability to reverse the phenotype upon inhibition of the primary PI3K signaling defect, is consistent with a non-linear network topology characterized by bistability and an “all-or-nothing” response. This is usually mediated by indirect or direct positive feedback loops (49) such as those known to occur between the TGFβ pathway and the pluripotency network (9, 21, 34).

The well-known dose-dependent effects of TGFβ signaling in a developmental context (34) helps explain our previous observations of allele dose-dependent stemness effects of PIK3CA^H1047R in hPSCs (5). Specifically, our data suggest a model in which homozygosity but not heterozygosity for PIK3CA^H1047R promotes NODAL expression and thus increases TGFβ pathway activity to a level that is sufficient for increased NANOG expression and stabilization of the stem cell state (Fig. 6C), but insufficient to tip the balance towards mesendoderm differentiation (5). Such an indirect effect of PI3K activation on the pluripotency network in hPSCs is in line with the lack of
NANOG downregulation in response to PI3Kα-specific inhibition (this study and Ref. (5)). Future studies are warranted to determine whether PI3K-dependent regulation of NODAL expression contributes to its developmental functions in vivo, which include roles in gastrulation and establishment of the body axes (34). It is notable that patients with a germline activating AKT2 mutation exhibit overgrowth that is predominantly left-sided (50), yet it remains unclear whether this is linked to differential regulation of NODAL expression.

Since specific pharmacological inhibition of NODAL is not possible, our strongest evidence for its role in the stemness phenotype of PIK3CA<sup>H1047R</sup>/H1047R<sup>H1047R</sup> hPSCs comes from the ability of these cells to sustain increased expression of key stemness genes without exogenous NODAL supplementation. Moreover, the ability of SB431512 to collapse the stemness gene signature in PIK3CA<sup>H1047R/H1047R</sup> hPSCs unequivocally demonstrates that sustained TGFβ pathway activation is required to maintain their stemness phenotype. Exactly how PI3K activation regulates NODAL expression remains to be determined. A potential mechanism involves increased expression of the stem cell reprogramming factor MYC, which was observed both at the mRNA and protein level in homozygous but not heterozygous PIK3CA<sup>H1047R</sup> iPSCs. Furthermore, MYC was the only node in the WGCNA-based network of pluripotency, PI3K and TGFβ pathway components that was classified as a member of all three pathways. Finally, increased MYC expression has previously been shown to exert oncogenic effects that depend on a sharp threshold of MYC expression, reminiscent of the effects we observe for PIK3CA activation (51). As the first specific pharmacological inhibitor of MYC was recently reported (52), its use in the PIK3CA<sup>H1047R</sup> hPSC system may provide additional insight into the potential role of MYC as a molecular link between oncogenic PI3Kα activation, NODAL expression and stemness.

The close relationship between PI3K and TGFβ pathway-driven stemness has potentially important implications for understanding PIK3CA-driven cancer. Breast cancers are enriched for PIK3CA mutations, and studies in mice have revealed that PIK3CA<sup>H1047R</sup> induces multipotency in differentiated mammary cells (53, 54). Conversely, advanced cancers typically exhibit increased phenotypic plasticity and stemness, which is closely linked to drug resistance and the ability of cancer cells to undergo epithelial-mesenchymal-transition (EMT) and metastasize (40, 55). Prolonged exposure of human mammary epithelial cells to TGFβ was recently shown to trigger irreversible EMT and PI3K/AKT-dependent stemness (56), illustrating the existence of a reciprocal relationship between the two signaling networks in the context of pluripotent and cancer stem cell regulation. A previous study...
reported increased AKT-mediated phosphorylation of the pro-metastatic factor TWIST1 in invasive cancer cells and advanced human breast tumors, further demonstrating a positive feedback loop characterized by increased TGFβ2 expression, autocrine TGFβ signaling and potentiation of the PI3K signaling pathway (57). Overexpression of PIK3CA<sup>H1047R</sup> in MCF10A breast epithelial cells has also been linked to induction of TGFβ1 expression, EMT and stem cell-like properties (58, 59).

We now report that increased NODAL expression is discernible even in the context of cellularly heterogeneous human breast cancers, with the degree of upregulation related to the number of oncogenic PIK3CA<sup>H1047R</sup> alleles present. This suggests that our findings in hPSCs have potential clinical relevance (Figure 6c). Monotherapy with pan- or isoform-specific PI3K inhibitors has had modest success in cancer (60), and it is thus noteworthy that PI3Kα-specific inhibition with BYL719 was unable to reverse the stemness phenotype of PIK3CA<sup>H1047R/H1047R</sup> iPSCs. Systematic studies in a larger number of breast cancer samples and breast cancer cell lines with defined PIK3CA<sup>H1047R</sup> copy number are needed to confirm the phenomenon that we have observed in human iPSCs. Nevertheless, based on our current findings of increased NODAL expression in some human breast cancers with multiple PIK3CA<sup>H1047R</sup> copies, we believe that TGFβ pathway inhibition is worthy of further exploration in this setting. Moreover, as induction of pro-tumorigenic TGFβ signaling has been reported in a range of human cancers (24, 61), the allele dose-dependent effects of PIK3CA<sup>H1047R</sup> may extend beyond breast cancer. Accordingly, it has previously been reported that PIK3CA amplification leads to TGFβ pathway-dependent EMT in a mouse model of oral carcinogenesis, with additional evidence that the degree of PIK3CA amplification correlates with tumor stage in human head and neck squamous cell carcinoma (62).

Finally, our study demonstrates the power of network-based approaches to study the highly context-dependent complexity of cell signaling and to identify potential therapeutic targets beyond those suggested by simply considering the presence/absence of a specific genetic defect (63–65). Given the dose-dependent effects of both TGFβ and PI3K pathway activation, a comprehensive understanding of their concerted actions will hinge upon adoption of network-based analyses and mathematical modeling capable of capturing context-dependent relationships in response to qualitative and quantitative signaling differences. This may result in tailored treatment strategies that take into account PIK3CA allele dose-dependent signaling reconfiguration in individual tumor cells.
Materials and Methods

Unless stated otherwise, standard chemicals were acquired from Sigma Aldrich, with details for the remaining reagents included in Supplementary Key Resources Table.

Induced pluripotent stem cell (iPSC) culture and treatments

Maintenance

The derivation of the iPSC lines, including associated ethics statements, has been described previously (5). All lines were grown at 37°C and 5% CO₂ in Essential 8 Flex (E8/F) medium on Geltrex-coated plates, in the absence of antibiotics. For maintenance, cells at 70-90% confluency were passaged as aggregates with ReLeSR, using E8 supplemented with RevitaCell (E8/F+R) during the first 24 h to promote survival. A detailed version of this protocol is available via protocols.io (doi: dx.doi.org/10.17504/protocols.io.4rtgv6n).

All cell lines were tested negative for mycoplasma and genotyped routinely to rule out cross-contamination during prolonged culture. Short tandem repeat profiling was not performed. All experiments were performed on cells within 10 passages since thawing.

Collection for RNA sequencing and total proteomics

For RNA sequencing and total proteomics, subconfluent cells were fed fresh E8/F 3 h prior to snap-freezing on dry ice and subsequent RNA or protein extraction. Relative to the results in Ref. (5), the current transcriptomic data of PIK3CA^H1047R were obtained more than 6 months following the first study, with cells at different passages, and were thus independent from one another. Moreover, sample collection for the second transcriptomics experiment was conducted over three days according to a block design, thus allowing us to determine transcriptional differences that are robust to biological variability.

Cell lysate collection for RPPA
For RPPA in growth factor-replete conditions, cells were fed fresh E8/F 3 h before collection. To assess variability due to differences in collection timing, clones from each iPSC genotype were collected on each one of three days according to a block design, giving rise to a total of 22 cultures. To test the effect of the PI3Kα-specific inhibitor BYL719, cells were treated with 100 nM drug (or DMSO only as control treatment) for 24 h and exposed to growth factor removal within the last hour before collection. All cells were washed in DPBS prior to collection to rinse off residual proteins and cell debris.

TGFβ/NODAL signaling studies

Wild-type or homozygous PIK3CA<sup>H1047R</sup> iPSCs were seeded in 12-well plates all coated with Geltrex from the same lot (#2052962; diluted in DMEM/F12 lot #RNBH0692). Cells were processed for seeding at a ratio of 1:15 according to the standard maintenance protocol. One day after seeding, individual treatments were applied to triplicate wells. Briefly, cells were first washed twice with 2 and 1 ml of Dulbecco’s PBS (DPBS) to remove residual growth factors. The base medium for individual treatments was Essential 6 supplemented with 10 ng/ml heat-stable FGF2. This was combined with one of the following reagents or their diluent equivalents: 100 ng/ml NODAL (diluent: 4 mM HCl), 250 nM BYL719 (diluent: DMSO), 5 μM SB431542 (diluent: DMSO). Cells were snap-frozen on dry ice after 24, 48 and 72 h following a single DPBS wash. Individual treatments were replenished daily at the same time of day to limit temporal confounders.

Mouse embryonic fibroblast (MEF) culture

The derivation and culture of the wild-type and PIK3CA<sup>WT/H1047R</sup> MEFs used in this study have been reported previously (28). Cell pellets were collected on dry ice 48 h after induction of heterozygous PIK3CA<sup>H1047R</sup> expression, without prior starvation.

RNA sequencing

Induced pluripotent stem cell lysates were collected in QIAzol and processed for RNA extraction with the DirectZol Kit as per the manufacturer’s instructions. The final RNA was subjected
to quantification and quality assessment on an Agilent Bioanalyzer using the RNA 6000 Nano Kit, confirming that all samples had a RIN score of 10. For PIK3CA$^{H1047R}$ iPSCs and corresponding wild-types, an Illumina TruSeq Stranded mRNA Library Prep Kit was used to synthesize 150-bp-long paired-end mRNA libraries, followed by sequencing on an Illumina HiSeq 4000, with average depth of 70 million reads per sample. PIK3CA$^{WT/E418K}$ and isogenic control iPSCs were subjected to 50-bp-long single-end RNA sequencing (RNA-seq) at an average depth of 20 million reads per sample.

MEF RNA was extracted using Qiagen’s RNAeasy miniprep (with QIAshredder). All samples had a confirmed Agilent Bioanalyzer RIN score of 10. An Illumina TruSeq Unstranded mRNA kit was used to prepare 100-bp-long paired-end libraries, followed by Illumina HiSeq 2000 sequencing.

Data analysis

The raw reads were mapped to the human genome build GRCh38 (for iPSC RNA-seq) or the mouse genome build GRCm38 (for MEF RNA-seq), and gene level counts were performed using Spliced Transcripts Alignment to a Reference (STAR) v2.5 (66). Subsequent data processing was performed using the open-source R software according to the limma-voom method (67). Briefly, raw counts were converted to counts per million (cpm) using the cpm() function in edgeR (68), followed by normalization according the trimmed mean of M (TMM) method (69). The mean-variance relationship was modelled with voom(), followed by linear modelling and computation of moderated t-statistics using the lmFit() and eBayes() functions in the limma package (67). The associated p-value for assessment of differential gene expression was adjusted for multiple comparisons with the Benjamini-Hochberg method at false-discovery rate (FDR) $\leq 5\%$ (70). The function duplicateCorrelation() was applied to correct for the use of replicate iPSC clones.

Correlations between corresponding transcriptomics and/or proteomics data were calculated using Spearman’s rank-order correlation test for non-normally distributed data.

Label-free total proteomics

Sample preparation
Cells were cultured to subconfluence in Geltrex-coated T175 flasks, and protein was harvested by lysis in 3 ml modified RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA) supplemented with phosphatase inhibitors (5 mM β-glycerophosphate, 5 mM NaF, 1 mM Na3VO4) and protease inhibitors (Roche cOmplete ULTRA Tablets, EDTA-free). The lysates were sonicated on ice (4x 10s bursts, amplitude = 60%; Bandelin Sonopuls HD2070 sonicator) and spun down for 20 min at 4300g. Ice-cold acetone was added to the supernatant to achieve a final concentration of 80% acetone, and protein was left to precipitate overnight at -20°C. Precipitated protein was pelleted by centrifugation at 2000g for 5 min and solubilized in 6 M urea, 2 M thiourea, 10 mM HEPES pH 8.0. Protein was quantified using the Bradford assay and 8 mg of each sample were reduced with 1 mM dithiothreitol, alkylated with 5 mM chloroacetamide and digested with endopeptidase Lys-C (1:200 v/v) for 3 h. Samples were diluted to 1 mg/ml protein using 50 mM ammonium bicarbonate and incubated overnight with trypsin (1:200 v/v). Digested samples were acidified and urea removed using SepPak C18 cartridges. Peptides were eluted, and an aliquot of 100 μg set aside for total proteome analysis. The peptides were quantified using the Pierce quantitative colorimetric peptide assay. The equalized peptide amounts were lyophilized and resolubilized in 2% acetonitrile and 1% trifluoroacetic acid in order to achieve a final 2 μg on-column peptide load.

**Mass spectrometry (MS) data acquisition**

All spectra were acquired on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) operated in data-dependent mode coupled to an EASY-nLC 1200 liquid chromatography pump (Thermo Fisher Scientific) and separated on a 50 cm reversed phase column (Thermo Fisher Scientific, PepMap RSLC C18, 2 μM, 100A, 75 μm x 50 cm). Proteome samples (non-enriched) were eluted over a linear gradient ranging from 0-11% acetonitrile over 70 min, 11-20% acetonitrile for 80 min, 21-30% acetonitrile for 50 min, 31-48% acetonitrile for 30 min, followed by 76% acetonitrile for the final 10 min with a flow rate of 250 nl/min.

Survey-full scan MS spectra were acquired in the Orbitrap at a resolution of 120,000 from m/z 350-2000, automated gain control (AGC) target of 4x10^5 ions, and maximum injection time of 20 ms.
Precursors were filtered based on charge state (≥2) and monoisotopic peak assignment, and dynamic exclusion was applied for 45s. A decision tree method allowed fragmentation for ITMS2 via electron transfer dissociation (ETD) or higher-energy collision dissociation (HCD), depending on charge state and m/z. Precursor ions were isolated with the quadrupole set to an isolation width of 1.6 m/z. MS2 spectra fragmented by ETD and HCD (35% collision energy) were acquired in the ion trap with an AGC target of 1e4. Maximum injection time for HCD and ETD was 80 ms for proteome samples.

Whole-exome sequencing (WES) and FASTA file generation

WES was performed on a single clone per genotype to generate cell-specific databases for downstream mass spectrometry searchers. Genomic DNA was extracted with Qiagen’s QIAamp DNA Micro Kit according to the manufacturer’s instructions, followed by quantification using the Qubit dsDNA High Sensitivity Assay Kit and by dilution to 5 ng/μl in the supplied TE buffer. The samples were submitted for library preparation and sequencing by the SMCL Next Generation Sequencing Hub (Academic Laboratory of Medical Genetics, Cambridge). Sequencing was performed on an Illumina HiSeq 4000 with 50X coverage across more than 60% of the exome in each sample. Raw reads were filtered with Trimmomatic (71) using the following parameters: headcrop = 3, minlen = 30, trailing = 3. The trimmed reads were aligned to the human reference genome (hg19 build) with BWA (72), followed by application of GATK base quality score recalibration, indel realignment, duplicate removal and SNP/indel discovery with genotyping (73). GATK Best Practices standard hard filtering parameters were used throughout (74).

In order to find non-reference, mutated peptides in the MS data, we increased the search FASTA file with mutations affecting the protein sequence, as detected by WES with a high sensitivity filter: QD ≤1.5, FS ≥ 60, MQ ≥ 40, MQRankSum ≤ -12.5, ReadPosRankSum ≤ -8.0, and average DP ≥ 5 per sample. The Ensembl Variant Effect Predictor (VEP) with Ensembl v88 was used to predict the effect of the mutations on the protein sequence (75). For every variant with an effect on the protein sequence we added the predicted mutated tryptic peptide at the end of the protein sequence.

Mass spectrometry searches
Raw files were processed using MaxQuant 1.5.0.2 (76) with all searches conducted using cell-specific databases (see Whole-exome sequencing and FASTA file generation), where all protein sequence variants were included in addition to the reference (Ensemble v68 human FASTA). Methionine oxidation, protein N-terminal acetylation and serine/threonine/tyrosine phosphorylation were set as variable modifications and cysteine carbamidomethylation was set as a fixed modification. False discovery rates were set to 1% and the “match between runs” functionality was activated. We filtered out peptides that were associated with multiple identifications in the MaxQuant msms.txt file, had a score < 40, were identified in the reverse database or came from known contaminants. Analysis of the observed peptides passing these filters was performed using a Monte Carlo Markov Chain model as described previously (29). Briefly, the model predicted the average ratio (sample versus control) of a peptide as a function of the observed protein concentration (obtained from the MaxQuant evidence.txt file). Combined with a noise model, a distribution of likely values for the parameters was obtained. The mean and standard deviation of this resulting distribution was used to calculate a z-score which was used together with the fold-change (FC) for subsequent filtering for differentially expressed proteins ($|z| > 1.2;|\ln(FC)| > \ln(1.2)$).

**Reverse phase protein array (RPPA)**

For RPPA, snap-frozen cells were lysed in ice-cold protein lysis buffer containing: 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl$_2$, 10% (v/v) glycerol, 1% (v/v) TritonX-100, 1 mM EGTA, 100 mM NaF, 10 mM Na$_3$P$_2$O$_7$, 2 mM Na$_3$VO$_4$ (added fresh), 1X EDTA-free protease inhibitor tablet, 1X PhosStop tablet. Protein concentrations were measured using BioRad’s DC protein assay, and all concentrations were adjusted to 1 mg/ml with lysis buffer and 1X SDS sample buffer (10% glycerol, 2% SDS, 62.5 mM Tris-HCl pH 6.8) supplemented with 2.5% β-mercaptoethanol.

The protein lysates were processed for slide spotting and antibody incubations as described previously (77). Briefly, a four-point dilution series was prepared for each sample and printed in triplicate on single pad Avid Nitrocellulose slides (Grace Biolabs) consisting of 8 arrays with 36x12 spots each. Next, slides were blocked and incubated in primary and secondary antibodies. The processed arrays were imaged using an Innopsys 710 slide scanner. Non-specific signals were determined for each
slide by omitting primary antibody incubation step. For normalization, sample loading on each array was determined by staining with Fast Green dye and recording the corresponding signal at 800 nm. Details for all primary and secondary RPPA antibodies are included in Supplementary Key Resources Table.

Data analysis

Slide images were analyzed using Mapix software (Innopsys), with the spot diameter of the grid set to 270 μm. Background signal intensity was determined for each spot individually and subtracted from the sample spot signal. A test for linearity was performed from the four-point dilution series, according to a flag system where $R^2 > 0.9$ was deemed good, $R^2 > 0.8$ was deemed acceptable and $R^2 < 0.8$ was poor (excluded from subsequent analyses). Median values from the four-point dilution series were calculated for each technical replicate and normalized to the corresponding Fast Green value to account for differences in protein loading. For each sample and protein target, a mean expression value was calculated from the remaining technical replicates and normalized to the corresponding mean of the wild-type group. All phosphoprotein signals were also normalized to the corresponding total protein values.

A statistical test for differential expression was performed on datasets with more than three samples per group, using the limma package to apply the limma-trend method with lmFit() and eBayes(), specifying collection time as blocking factor (67). Phosphoprotein and total protein lists were processed separately. The associated p-value for assessment of differential gene expression was adjusted for multiple comparisons with the Benjamini-Hochberg method at FDR ≤ 5% (70). The function duplicateCorrelation() was applied to correct for the use of replicate iPSC clones on the same day. Heatmaps were generated using the heatmap.2() function within the gplots package in R, using target-wise correlation for dendrogram construction.

Reverse transcription-quantitative PCR (RT-qPCR)
Cellular RNA was extracted as described above for RNA Sequencing, and 200 ng used for complementary DNA (cDNA) synthesis with Thermo Fisher’s High-Capacity cDNA Reverse Transcription Kit. Subsequent SYBR Green-based qPCRs were performed on 2.5 ng total cDNA. A 5-fold cDNA dilution series was prepared and used as standard curve for relative quantitation of gene expression. TBP was used as normalizer following confirmation that its gene expression remained unaffected by the tested conditions. Melt curve analyses were used to confirm amplification of a single product by each primer. All primers had amplification efficiencies 95%-105%. Individual samples were loaded in duplicate in 384-well plates.

TaqMan hPSC Scorecards (384-well) were used according to the manufacturer’s instructions with the following modifications. From each sample diluted to 20 ng/μl, two 50 μl RT reactions were set up, with 500 ng RNA sample in each. Next, the two RT replicates were combined to obtain 1 μg cDNA in a total volume of 100 μl (final concentration: 10 ng/μl). This was subsequently diluted to 0.715 ng/μl and 10 μl loaded into each Scorecard well. All Ct values were mapped to their corresponding genes using the TaqMan hPSC Scorecard analysis software provided by the manufacturer. Genes with Ct values < 15 were excluded from further analyses. To be considered for downstream processing, genes were also required to have Ct values < 30 in at least two out of the eight samples. Next, Ct values were linearized (antilog) under the assumption of 100 % primer amplification efficiency. The geometric expression mean of the control gene assays was used for subsequent normalization of individual gene expression values.

All qPCR data were acquired on a Quant Studio™ 5 Real-Time PCR System (Thermo Fisher Scientific). The thermocycling conditions (SYBR Green reactions) were as follows (ramp rate 1.6°C/s for all): 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min, followed by melt curve analysis (95°C for 15 sec, 60°C for 1 min, and 95°C for 15 min with ramp rate 0.075°C/sec). The TaqMan hPSC Scorecard thermocycling conditions were as specified by the manufacturer in the accompanying template.

All relevant primer sequences are included in Supplementary Key Resource Table.

Bioinformatic analyses
The list of differentially expressed total proteins ($PIK3CA^{H1047R/H1047R} \text{ vs wild-type}$) was subjected to IPA (build version: 448560M; content version: 36601845) against the Ingenuity Knowledge Base, considering only relationships where confidence was classified as “Experimentally Observed”. Following exclusion of chemicals and drugs, the Upstream Regulators list was used for generation of Volcano plots of the respective activation z-scores and overlap p-values.

IPA was also used to analyze the lists of differentially expressed genes in both heterozygous and homozygous $PIK3CA^{H1047R}$ iPSCs (IPA build version: 484108M; content version: 45868156) and MEFs (IPA build version: 486617M; content version: 46901286), using the Ingenuity Knowledge Base and considering only relationships where confidence was classified as “Experimentally Observed”. Chemicals and diseases were excluded from Node Types. For the iPSC datasets, differentially expressed genes were only considered for IPA analysis if having an absolute log2(fold-change) $\geq$ log2(1.3). The choice of this relatively permissive log fold-change choice was guided by the *limma*() tutorial for RNAseq (67), and the assumption that small fold-changes in the expression of genes that act within the same pathway may be sufficient to elicit a functionally important response and thus should not be omitted. This consideration is of particular relevance for transcriptomic data from heterozygous $PIK3CA^{H1047R}$ iPSCs where fold-changes were relatively small. Due to the high number of differentially expressed transcripts in $PIK3CA^{H1047R/H1047R}$ iPSCs, the analysis was conducted using the top 2000 up- and top 2000 downregulated transcripts.

The IPA Upstream Regulator Analysis is based on the proprietary Ingenuity Knowledge Base which is used to compute two scores based on user-specified data: an enrichment score (Fisher’s exact test p-value) that measures overlap between observed and predicted regulated gene sets; a z-score that assesses the match between observed and predicted up/down regulation patterns (78). The results of the Upstream Regulators Analysis were extracted for downstream Volcano plotting of overlap p-values and associated activation z-scores. Note that for heterozygous $PIK3CA^{H1047R}$ iPSCs, a bias-corrected activation z-score was used for plotting to take into account any bias arising from a larger number of upregulated vs downregulated genes in these cells.
Weighted Gene Correlation Network Analysis (WGCNA)

RNA sequencing counts from all 12 samples were converted to reads per kilobase million (RPKM). A threshold of 10 RPKM was used to filter out low-expression genes, followed by removing any genes with missing values caused by this filtering. The RPKM values for the remaining 16,823 genes were log2 transformed and taken forward for network analysis using the WGCNA R package (30, 79), with a soft power threshold of 28 (chosen to maximize scale independence and minimize mean connectivity) and a minimum module size of 30 genes.

To identify modules associated with homozygosity for \( PIK3CA^{H1047R} \), we used the correlation between a gene’s module membership (eigengene) and significance for differential expression in homozygous \( PIK3CA^{H1047R/H1047R} \) iPSCs. The top two most significant modules for the homozygocity trait were selected for functional enrichment analysis. CytoScape plugin ClueGO (version 2.5.4) (80) was used to perform pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) ontology (build 27.02.19) (81). All settings were kept at default values. Only pathways with \( p \)-value \(< 0.05 \) were selected, and a custom reference gene set was used as background (the 16,823 genes analysed using WGCNA). Network visualization was performed using Cytoscape (82).

TCGA Breast Cancer Data Analysis

The number of \( PIK3CA^{H1047R} \) copies in The Cancer Genome Atlas (TCGA) breast invasive carcinoma (BRCA) samples was retrieved by filtering previously published copy number information from this dataset (5). Samples were classified as having multiple \( PIK3CA^{H1047R} \) copies (“multiple”) when the mutant allele dosage (mut.multi) \( \geq 1.5 \). Samples with a single \( PIK3CA^{H1047R} \) copy and an additional \( PIK3CA \) variant were also classified as “multiple”. The associated RNA sequencing and clinical data were retrieved using the R package TCGAbiolinks (version: 2.12.3) according to the accompanying vignette (83). The raw sequencing counts were converted to cpm and normalized as described for iPSC/MEF RNAseq analysis above. A filtering step was applied, requiring \( \geq 1 \) cpm in more than one tumor sample for a gene to be considered expressed. Copy number data, RNAseq and clinical information were merged based on the TCGA sample barcode and filtered according to genes
of interest. Candidate gene expression data were plotted either as a function of the number of
\( PIK3CA^{H1047R} \) allele or upon additional stratification according to tumor stage.

**Statistical analyses**

Bespoke statistical analyses are specified in the relevant sections above. Statistical analyses were restricted to -omics datasets to correct appropriately for multiple comparisons and to take advantage of target-wise variances as outlined in Ref. (67). In line with recent (ATOMIC) recommendations by the American Statistical Association (84), we have avoided arbitrary use of “statistical significance” applied to data from small-scale cell culture experiments which violate assumptions of the most widely used statistical tests. Instead, we present all data from multiple orthogonal experiments, alongside complete information on experimental replicates, independent clones and replicate cultures.

The same reasoning applies to our analysis of TCGA breast cancer data, where frequentist tests do not take into account the prior hypothesis that \( NODAL \) may have higher expression in breast cancer samples with multiple \( PIK3CA^{H1047R} \) copies. A Bayesian approach would be more appropriate, but it remains unclear what the prior distribution should be in the absence of data that complement our human iPSC studies. We acknowledge the relatively small sample size used in Discussion and note that systematic testing in future studies with large sample size and bespoke breast cancer cell models is needed. Our communication of this analysis in its current format is guided by the possibility of a potentially clinically relevant finding worthy of further studies.
Supplementary Materials:

Fig. S1, related to Fig. 1. Fold-change distribution and transcriptome correlations.

Fig. S2, related to Fig. 1. Transcriptome-proteome correlations.

Fig. S3, related to Fig. 2. Additional RPPA data.

Fig. S4, related to Fig. 5. Alternative representation of the experimental data in Fig. 5A and additional RT-qPCR-based profiling of lineage-specific markers.

Table S1. List of differentially expressed genes in PIK3CA<sup>WT/H1047R</sup> vs wild-type hPSCs after applying an absolute fold-change cut-off of minimum 1.3.

Table S2. List of differentially expressed genes in PIK3CA<sup>H1047R/H1047R</sup> vs wild-type hPSCs after applying an absolute fold-change cut-off of minimum 1.3.

Table S3. List of differentially expressed genes in PIK3CA<sup>WT/E418K</sup> vs wild-type hPSCs.

Table S4. List of differentially expressed genes in PIK3CA<sup>WT/H1047R</sup> vs wild-type MEFs.

Table S5. List of differentially expressed proteins in PIK3CA<sup>WT/H1047R</sup> vs wild-type hPSCs.

Table S6. List of differentially expressed proteins in PIK3CA<sup>H1047R/H1047R</sup> vs wild-type hPSCs.

Table S7. List of differentially expressed proteins in both heterozygous and homozygous PIK3CA<sup>H1047R</sup> hPSCs vs wild-type controls.

Key Resources Table.
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design and sample processing, and R.R.M carried out statistical analysis. R.R.M. performed all RNA sequencing
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Figure Legends

Fig. 1. Transcriptomic analyses of human and mouse cell lines with endogenous expression of oncogenic PIK3CA. (A) Multidimensional scaling (MDS) plot of the transcriptomes of wild-type (WT), PIK3CA<sub>WT/H1047R</sub> (HET) and PIK3CA<sub>H1047R/H1047R</sub> (HOM) human iPSCs. The numbers in brackets indicate the presence of two closely overlapping samples. (B) The number of differentially expressed genes in iPSCs heterozygous or homozygous for PIK3CA<sub>H1047R</sub> before and after application of an absolute fold-change cut-off ≥ 1.3 (FDR ≤ 0.05, Benjamini-Hochberg). The data are based on four iPSCs cultures from minimum two clones per genotype. See also fig. S1. (C) Woman with asymmetric overgrowth caused by mosaicism for cells with heterozygous expression of PIK3CA<sub>E418K</sub>. Skin biopsies obtained from unaffected and affected tissues were used to obtain otherwise isogenic dermal fibroblasts for subsequent reprogramming into iPSCs. This image was reproduced from Ref. (27) (D) MDS plot of the transcriptomes of wild-type (WT) and PIK3CA<sub>WT/E418K</sub> iPSCs (based on 3 independent mutant clones and 3 wild-type cultures from 2 independent clones). (E) MDS plot of the transcriptomes of wild-type (WT) and PIK3CA<sub>WT/H1047R</sub> (HET) mouse embryonic fibroblasts (MEFs) following 48 h of mutant induction (n = 4 independent clones per genotype). (F) Venn diagram showing the number of differentially expressed proteins in PIK3CA<sub>H1047R/H1047R</sub> (HOM) and PIK3CA<sub>WT/H1047R</sub> (HET) iPSCs relative to wild-type controls, profiled by label-free total proteomics on three clones per genotype. An absolute fold-change and z-score ≥ 1.2 were used to classify proteins as differentially expressed. The number of discordant and concordant changes in the expression of total proteins detected in both comparisons are indicated. See also fig. S2.

Fig. 2. Reverse Phase Protein Array (RPPA) of PIK3CA<sub>WT/H1047R</sub> (HET) and PIK3CA<sub>H1047R/H1047R</sub> (HOM) human iPSCs. (A) Left: Diagram of PI3K pathway-related phosphorylated proteins, with color code used to signify differentially expressed targets in PIK3CA<sub>H1047R</sub> mutant iPSCs versus isogenic wild-type controls. Color-coded targets were significant at FDR ≤ 0.05 (Benjamini-Hochberg). Right: Barplots show representative examples of differentially expressed phosphorylated protein targets, revealing relatively modest quantitative changes. Phosphorylated proteins were normalized to the corresponding total protein when available. The data are based on 10 wild-type cultures (3 clones), 5 PIK3CA<sub>WT/H1047R</sub> cultures (3 clones) and 7 PIK3CA<sub>H1047R/H1047R</sub> cultures (2 clones) as indicated. See also fig. S3A. (B) Unsupervised hierarchical clustering based on target-wise correlations of RPPA
data from wild-type (WT), \(\text{PIK3CA}^{\text{WT/H1047R}}\) (HET) and \(\text{PIK3CA}^{\text{H1047R/H1047R}}\) (HOM) iPSCs following short-term growth factor removal (1 h, +/- 100 nM BYL719 (PI3K\(\alpha\) inhibitor) for 24 h. The data are from two independent experiments, each performed using independent clones. For each row, the colors correspond to Fast Green-normalized expression values in units of standard deviation (z-score) from the mean (centered at 0) across all samples (columns). Groups of phosphorylated proteins exhibiting a consistent expression pattern in BYL719-treated \(\text{PIK3CA}^{\text{H1047R/H1047R}}\) iPSCs are specified. See also fig. S3B.

**Fig. 3.** Ingenuity® pathway analyses (IPA) predict activation of TGF\(\beta\) signaling in heterozygous and homozygous \(\text{PIK3CA}^{\text{H1047R}}\) iPSCs. (A) IPA of upstream regulators using the list of top 2000 upregulated and top 2000 downregulated mRNA transcripts in \(\text{PIK3CA}^{\text{H1047R/H1047R}}\) iPSCs (for RNAseq details, see Fig. 1B). Red points signify transcripts with absolute predicted activation z-score > 2 and overlap p-value < 0.001 (Fisher’s Exact Test). The red rectangle highlights the most significant upstream regulator, TGF\(\beta\)1. (B) As in (A), but using the list of differentially-expressed proteins identified by total proteomics and red-coloring targets with predicted activation z-score > 2 and overlap p-value < 0.05 (Fisher’s Exact Test). (C) As in (A), but using the list of differentially expressed total proteins in \(\text{PIK3CA}^{\text{WT/H1047R}}\) iPSCs and red-coloring upstream regulators with absolute predicted bias-corrected z score > 2 and overlap p-value < 0.05 (Fisher’s Exact Test). Red rectangles highlight the two upstream regulators (TGF\(\beta\)1 and MAPK1) with absolute predicted bias-corrected z score > 2 that remained significant (overlap p-value < 0.05) when the analysis was repeated using the list of shared and concordant differentially expressed genes (n = 180) in heterozygous and homozygous \(\text{PIK3CA}^{\text{H1047R}}\) iPSCs vs wild-type controls.

**Fig. 4.** Weighted gene correlation network analysis (WGCNA) identifies links among pluripotency components, TGF\(\beta\) and PI3K signaling. (A) Schematic of the WGCNA workflow and subsequent data selection for visualization. (B) Unsigned WGCNA modules identified using the list of transcripts expressed in wild-type, \(\text{PIK3CA}^{\text{WT/H1047R}}\) and \(\text{PIK3CA}^{\text{H1047R/H1047R}}\) iPSCs (for RNAseq details, see Fig. 1B). (C) The two gene network modules with genes whose module membership correlated strongest with differential expression in homozygous \(\text{PIK3CA}^{\text{H1047R}}\) iPSCs. The color of each module corresponds to its color in the module dendrogram in (B). Representative KEGG pathways with significant enrichment in each gene network module are listed.
(hypergeometric test with two-sided uncorrected p < 0.05). (D) The minimal network connecting KEGG pluripotency, PI3K/AKT and TGFβ pathway components within the turquoise gene network module. Fill color and shape are used to specify direction of differential mRNA expression in PIK3CA<sup>H1047R/H1047R</sup> iPSCs and pathway membership, respectively. Fill color saturation represents gene expression fold-change (FC; log2) in PIK3CA<sup>H1047R/H1047R</sup> (HOM) vs wild-type (WT) iPSCs.

Fig. 5. TGFβ signaling-dependent regulation of stemness in PIK3CA<sup>H1047R/H1047R</sup> iPSCs. Gene expression time course of NODAL, NANOG and POU5F1 in wild-type (WT) or PIK3CA<sup>H1047R/H1047R</sup> iPSCs following the indicated treatments for 24 h, 48 h or 72 h. B250: 250 nM BYL719 (PI3Kα-selective inhibitor); E6/FGF2: Essential 6 medium supplemented with 10 ng/ml basic fibroblast growth factor 2 (FGF2). SB431542 is a specific inhibitor of the NODAL type I receptors ALK4/7 and the TGFβ type I receptor ALK5; used at 5 μM. When indicated, cultures were supplemented with 100 ng/ml NODAL. The data are from two independent experiments, with each treatment applied to triplicate cultures of three wild-type and two homozygous iPSC clones. To aid interpretation, gene expression values are normalized to the E6/FGF2 condition within each genotype and time-point. An alternative visualization that illustrates the differential expression of NODAL and NANOG between mutant and wild-type cells is shown in fig. S4A. For analysis of additional lineage markers, see fig. S4B. A.U., arbitrary units.

Fig. 6. Stage II human primary breast tumors with multiple PIK3CA<sup>H1047R</sup> copies have increased NODAL gene expression. (A) Violin plots of NODAL, POU5F1, NANOG and MYC mRNA expression in counts per million (cpm) in PIK3CA-associated human primary breast tumors (n = 108) stratified according to the presence of a single (41 tumors) or multiple copies (67 tumors) of PIK3CA<sup>H1047R</sup> and/or a second PIK3CA mutation. (B) Boxplots of the data in (a) stratified according to tumor stage information if available. The number of samples containing a single PIK3CA<sup>H1047R</sup> allele and tumor stage information were: 9 (stage i), 21 (stage ii) and 5 (stage iii); the corresponding numbers in samples with multiple copies were: 9 (stage i), 42 (stage ii) and 14 (stage iii).
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High-dose oncogenic PIK3CA drives constitutive cellular stemness through self-sustained TGFβ pathway activation

Ralitsa R. Madsen*, James Longden, Rachel G. Knox, Xavier Robin, Franziska Völlmy, Kenneth G. Macleod, Larissa Moniz, Neil O. Carragher, Nicholas McGranahan, Rune Linding, Bart Vanhaesebroeck, Robert K. Semple*

* Corresponding author. Email: rmadsen@ed.ac.uk (R.R.M.); rsemble@ed.ac.uk (R.K.S.)

This document includes:

- Fig. S1, related to Fig. 1. Fold-change distribution and transcriptome correlations.
- Fig. S2, related to Fig. 1. Transcriptome-proteome correlations.
- Fig. S3, related to Fig. 2. Additional RPPA data.
- Fig. S4, related to Fig. 5. Alternative representation of the experimental data in Fig. 5A and additional RT-qPCR-based profiling of lineage-specific markers.
**Fig. S1**, related to Fig. 1. **Fold-change distribution and transcriptome correlations.** (A) Combined violin-boxplot representation of the fold-change (log2) distribution of gene expression changes between heterozygous and homozygous $PIK3CA^{H1047R}$ and wild-type iPSCs as indicated. (B) and (C) Correlation plot of the log2 expression fold-changes (FC) of significant total proteins vs the corresponding mRNA transcripts in $PIK3CA^{H1047R}/H1047R$ (HOM) (B) and $PIK3CA^{WT}/H1047R$ (HET) (C) iPSCs. Spearman’s rho and the corresponding $p$-values are indicated on all plots. The first transcriptomic study (RNAseq1) was based on three independent cultures from three different clones per genotype; the second transcriptomic study (RNAseq2) used four independent cultures per genotype, from minimum two independent clones each.
Fig. S2, related to Fig. 1. Transcriptome-proteome correlations. (A) Correlation plot of the log2 expression fold-changes (FC) of differentially expressed total proteins (|z| ≥ 1.2 and |ln(FC)| ≥ 1.2) in PIK3CA\textsuperscript{H1047R/H1047R} iPSCs and the corresponding mRNA transcripts in an independent set of cultures. If identified as differentially expressed following statistical analysis (FDR < 0.05), mRNA transcripts are highlighted in red irrespective of absolute fold-change. (B) As in (A), but starting with all differentially expressed mRNA transcripts (FDR < 0.05 irrespective of fold-change magnitude) and plotting them to the corresponding protein identified by total proteomics. If differentially expressed (see (a)), the matched proteins are highlighted in red. (C) and (D) As in (A) and (B), respectively, but using the data for PIK3CA\textsuperscript{WT/H1047R} iPSCs. All proteomic data were obtained from 3 independent clones per genotype using cultures at passages P47-P52, corresponding to the cultures used in our previous study (Madsen et al., 2019). The high-depth transcriptomic data were obtained from 4 independent cultures (minimum 2 independent clones) per genotype using cultures at passages P55-P59. Spearman’s rho and the corresponding p-values are indicated on all plots.
Fig. S3, related to Fig. 2. Additional RPPA data. (A) Venn-diagrams specifying differentially expressed phosphorylated and total proteins in PIK3CA<sub>WT/H1047R</sub> (HET) and PIK3CA<sub>H1047R/H1047R</sub> (HOM) iPSCs relative to wild-type controls, based on RPPA profiling of cells cultured in growth factor-replete conditions. The data are based on a total of 10 wild-type cultures, 5 PIK3CA<sub>WT/H1047R</sub> cultures and 7 PIK3CA<sub>H1047R/H1047R</sub> cultures, and all shown targets were differentially expressed at a false-discovery rate (FDR) ≤ 0.05. Following quality checks (see Materials and Methods), the RPPA data included 21 phosphorylated and 21 total proteins. (B) Heatmap of total proteins from
RPPA profiling of wild-type (WT), PIK3CA^{WT/H1047R} (HET) and PIK3CA^{H1047R/H1047R} (HOM) iPSCs following short-term growth factor removal (1 h), +/- 100 nM BYL719 (PI3Kα inhibitor) for 24 h. Groups of total proteins exhibiting a consistent expression pattern in BYL719-treated PIK3CA^{H1047R/H1047R} iPSCs are specified.
**Fig. S4**, related to Fig. 5. Alternative representation of the experimental data in Fig. 5A and additional RT-qPCR-based profiling of lineage-specific markers. (A) As in Fig. 5A, but representing individual gene expression values for *NODAL* and *NANOG* scaled to the mean expression in *PIK3CA*\(^{H1047R/H1047R}\) after 24 h in E6 medium supplemented with FGF2 and NODAL. A.U., arbitrary units. (B) TaqMan hPSC Scorecards were used to profile a set of stemness and (neuro)ectoderm markers in *PIK3CA*\(^{H1047R/H1047R}\) iPSCs following the indicated treatments for 48 h. Each bar corresponds to a single sample, with colors specifying the use of two independent clones per treatment. For each gene and clone, values are scaled to the expression value in cells cultured in E6 medium with FGF2 and NODAL.
Table S1. List of differentially expressed genes in $PIK3CA^{WT/H1047R}$ vs wild-type hPSCs after applying an absolute fold-change cut-off of minimum 1.3.

Table S2. List of differentially expressed genes in $PIK3CA^{H1047R/H1047R}$ vs wild-type hPSCs after applying an absolute fold-change cut-off of minimum 1.3.

Table S3. List of differentially expressed genes in $PIK3CA^{WT/E418K}$ vs wild-type hPSCs.

Table S4. List of differentially expressed genes in $PIK3CA^{WT/H1047R}$ vs wild-type MEFs.

Table S5. List of differentially expressed proteins in $PIK3CA^{WT/H1047R}$ vs wild-type hPSCs.

Table S6. List of differentially expressed proteins in $PIK3CA^{H1047R/H1047R}$ vs wild-type hPSCs.

Table S7. List of differentially expressed proteins in both heterozygous and homozygous $PIK3CA^{H1047R}$ hPSCs vs wild-type controls.

Key Resources Table.