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Oncogenic PIK3CA induces centrosome amplification and tolerance to genome doubling

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Mutations in PIK3CA are very frequent in cancer and lead to sustained PI3K pathway activation. The impact of acute expression of mutant PIK3CA during early stages of malignancy is unknown. Using a mouse model to activate the Pik3caH1047R hotspot mutation in the heterozygous state from its endogenous locus, we here report that mutant Pik3ca induces centrosome amplification in cultured cells (through a pathway involving AKT, ROCK and CDK2/Cyclin E-nucleophosmin) and in mouse tissues, and increased in vitro cellular tolerance to spontaneous genome doubling. We also present evidence that the majority of PIK3CAH1047R mutations in the TCGA breast cancer cohort precede genome doubling. These previously unappreciated roles of PIK3CA mutation show that PI3K signalling can contribute to the generation of irreversible genomic changes in cancer. While this can limit the impact of PI3K-targeted therapies, these findings also open the opportunity for therapeutic approaches aimed at limiting tumour heterogeneity and evolution.
O f the eight genes encoding catalytic PI3K subunits in mammals, only PIK3CA, which encodes the ubiquitously expressed p110α catalytic subunit, is frequently mutated in solid tumours. PIK3CA mutations cluster in so-called hot-spots, and give rise to a more active p110α protein that stimulates the PI3K pathway. Thus far, the oncogenic potential of PI3K has largely been attributed to its role in stimulating processes such as cell survival and proliferation, spurring the development of inhibitors of the PI3K pathway as anti-cancer agents such as cell survival and proliferation, spurring the development of inhibitors of the PI3K pathway as anti-cancer agents.

Several Cre recombinase-based mouse models have been created to explore the role of mutated PIK3CA. Interestingly, whereas transgenic overexpression of mutant PIK3CA has been found to be an effective inducer of cancer, other models, in which mutated PIK3CA is expressed from its endogenous locus, demonstrate that mutant PIK3CA, on its own, is a weak oncogene (see for example ref. 9), with cancer arising only after long latency periods (>1 year; reviewed in ref. 10).

In the current study, we created an Flp recombinase-based knock-in mouse model of inducible expression of mutant PIK3CA from its endogenous locus. Using this model, we show that mutated PIK3CA is a weak oncogene on its own, but that it can cooperate with other oncogenic lesions, such as heterozygous loss of the Apc tumour suppressor. We also show that systemic induction of heterozygous mutant PIK3CA at embryonic or adult stages can have dramatic organisational consequences and leads to lethality.

We assessed signalling and cell biological changes induced early upon heterozygous expression of mutant PIK3CA, which allowed us to uncover two previously unappreciated roles of PI3K signalling, namely the induction of centrosome amplification and increased tolerance to tetraploidization, both of which have been implicated in tumourigenesis and tumour evolution.

**Results**

A mouse model of inducible endogenous p110αH1047R expression. As a model of physiological expression of mutant PIK3CA from its endogenous locus, we generated a mouse line in which one of the two wild-type (WT) PIK3CAWT alleles is converted to PIK3CAH1047R (Fig. 1a). Due to the presence of a neomycin (Neo) selection cassette in the targeted PIK3CA locus, the expression of the mutant p110αH1047R protein was dampened, as shown in embryonic stem (ES) cells (Fig. 1b) and PIK3CAH1047RWT mice (Supplementary Fig. 1a). A more detailed analysis of PI3K pathway activation in the inducible PIK3CAH1047RWT ES cells is reported in ref. 18.

In order to rescue the dampened expression of the p110αH1047R protein, we removed the Neo cassette through recombination via its flanking frt sites. This was achieved by crossing PIK3CAH1047RWT mice with mice that express a germline Flp recombinase which is either constitutively active (hACTB::FlpeERT2 mice19) or inducible by tamoxifen (or its derivative 4-hydroxytamoxifen (4-OHT)) (CAG::FlpeERT2 mice20). 4-OHT treatment of primary MEFs, isolated from PIK3CAH1047RWT mice crossed onto CAG::FlpeERT2 mice, resulted in the removal of the Neo cassette (Supplementary Fig. 1b, restored p110αH1047R expression levels similar to that of endogenous p110αWT (Fig. 1c).
and led to PI3K pathway activation (Fig. 1c). Enhanced Akt phosphorylation was also observed in primary fibroblasts from human fibro-adipose overgrowth syndrome patients with mosaic, heterozygous expression of the PIK3CA H1047L mutation21 (Supplementary Fig. 1c).

Organismal impact of heterozygous p110α H1047R expression.

Heterozygous expression of p110α H1047R induced by constitutive Flp expression from day 0 post coitum19 resulted in mutant embryos which developed normally until embryonic day (E) 8.5 (Supplementary Fig. 2a). At E9.5, however, p110α H1047R embryos had not grown in size compared to E8.5 and showed increased PI3K/Akt pathway activation, with a dramatic collapse in overall tissue integrity, a lack of vascular remodelling and absence of hierarchical organization of vessels, widespread apoptosis and increased levels of p53 (Supplementary Fig. 2a–e). No live embryos were recovered at E10.5. These observations are similar to those of a previous report on embryonic p110α H1047R expression using a Cre-inducible Pik3ca H1047R mouse line22.

Tamoxifen-mediated Flp activation20 in adult mice at 8 weeks of age, led to efficient recombination of the Pik3ca H1047R locus (Supplementary Fig. 3a) and PI3K pathway activation in all tissues investigated (Supplementary Fig. 3b). These mice appeared to be in good health, but over time died suddenly, for reasons that are unclear, or had to be humanely terminated either because of displaying sudden ataxia and seizures or becoming acutely moribund without prior or accompanying disease symptoms. Most mice died within one year of age (median survival time 220 days post recombination of the mutated allele; Supplementary Fig. 3c). At the time of death, there was evidence for a weight increase in several organs, including the brain (Supplementary Fig. 3d). Importantly, Pik3ca H1047R mice did not develop neoplastic lesions or cancer, as assessed by histopathology of multiple organs (Supplementary Tables 1 and 2).

Tamoxifen-induced expression of Pik3ca H1047R together with intestine-specific heterozygous deletion of the Apc tumour suppressor gene (Apcflox/+ mice) accelerated colon cancer progression (median survival time of 290 and 134 days in

**Fig. 2** p110α H1047R expression leads to centrosome amplification in cells and tissues. a Frequency of cells with centrosome amplification (n > 2) in WT and Pik3ca H1047R primary E13.5 MEFs (72 h after addition of 4-OHT or vehicle; 200 cells were scored per genotype and per time point; values = mean ± SD) and mouse keratinocytes (isolated from 8-week-old Pik3ca WT;Flpe-ER T2 or Pik3ca H1047R+neo;Flpe-ER T2 mice, cultured for 7 days of which the last 48 h were in the presence of 4-OHT or vehicle; 400 cells were scored per genotype and per condition). Statistically significant differences are indicated by *(P < 0.05), as determined by non-parametric Mann–Whitney t test (one-tailed). b Whole-mount of E8.5 embryos stained for pericentrin. Dashed lines contour single-cell nuclei. White arrows point towards individual centrosomes in the WT cells. Mutant embryos show enlarged and amplified number of centrosomes per cell. c Cryosections of skin and colon of 8-week-old Pik3ca WT;Flpe-ER T2 and Pik3ca H1047R+neo;Flpe-ER T2 mice treated for 5 consecutive days with tamoxifen (80 mg kg−1 by gavage), stained for γ-tubulin (red) and actin (green). White arrows point towards centrosomes. Dashed lines contour single-cell nuclei. White boxes surround areas that are magnified underneath.
Apc\textsuperscript{fl}ox/+ and Pik3ca\textsuperscript{H1047R}; Apc\textsuperscript{fl}ox/+ mice, respectively; Supplementary Fig. 4a–c). In summary, these observations corroborate that heterozygously expressed mutant Pik3ca can have a major impact on the animal, both in adult life and during embryonic development. Our results also reinforce the concept that mutant Pik3ca is not efficient at initiating tumour formation on its own, but cooperates with other tumour-promoting genetic lesions\textsuperscript{9,23–25}. p110\textalpha\textsuperscript{H1047R} expression leads to centrosome amplification. We next sought to understand the early cellular impact of endogenous p110\textalpha\textsuperscript{H1047R} expression, using primary MEFs as the main model. Pik3ca\textsuperscript{H1047R} induction in these cells led to Akt activation (Fig. 1c), an increase in cell number, loss of contact inhibition and a low level of colony formation, without obvious changes in cell death (Supplementary Fig. 5a–d).

Further characterization of p110\textalpha\textsuperscript{H1047R} MEFs revealed that these cells frequently displayed extra centrosomes (Fig. 2a). Centrosomes are the main microtubule-organizing centre in animal cells and critical for the formation of the mitotic spindle. Immediately after cell division, each cell contains one centrosome which is then duplicated, in a cell cycle-dependent manner and in parallel with DNA replication\textsuperscript{26}, in preparation for the next round of cell division. Centrosomes are composed of two centrioles surrounded by pericentriolar matrix which can be detected by antibodies against pericentrin or \gamma\textsuperscript{-}tubulin. p110\textalpha\textsuperscript{H1047R} expression in MEFs led to a significant increase in the number of cells with supernumerary centrosomes, with >30\% of the cells showing more than two centrosomes 5 days after treatment with 4-OHT (Fig. 2a and Supplementary Fig. 6a and experimental controls shown in Supplementary Fig. 6b).

Supernumerary centrosomes were also more frequent in fibroblasts from a PIK3CA\textsuperscript{H1047L}-driven human overgrowth syndrome patient\textsuperscript{21} (Supplementary Fig. 6c) and in MCF-10A human mammary epithelial cells transiently transfected with p110\textalpha\textsuperscript{H1047R} (Supplementary Fig. 6d). In MCF-10A cells, other cancer-associated modes of PI3K pathway activation, namely Ap\textgamma\textsuperscript{fl}ox/\textgamma and Pik3ca\textsuperscript{H1047R};Ap\textgamma\textsuperscript{fl}ox/\textgamma mice, respectively; Supplementary Fig. 4a–c).
**Fig. 4** p110α<sub>H1047R</sub> expression leads to centrosome overduplication through RhoA and ROCK pathway activation. a Assessment of RhoA activity in Pik3ca<sup>WT</sup>/Flpe-ERT<sup>T2</sup> and Pik3ca<sup>H1047R+/neo</sup>/Flpe-ERT<sup>T2</sup> MEFs, 3 days after treatment with 4-OHT. The experiment was performed three times using three independent Pik3ca<sup>WT</sup>/Flpe-ERT<sup>T2</sup> and Pik3ca<sup>H1047R+/neo</sup>/Flpe-ERT<sup>T2</sup> MEFs. A representative experiment is shown. b Cyclin E protein levels in Pik3ca<sup>WT</sup>/Flpe-ERT<sup>T2</sup> and Pik3ca<sup>H1047R+/neo</sup>/Flpe-ERT<sup>T2</sup> MEFs, 3 days after treatment with 4-OHT. Cell extracts from three independent MEF lines per genotype are shown. c Activation of signalling pathways involved in centrosome duplication by p110α<sup>H1047R</sup> expression, and effect of p110α inhibition (by 24 h of treatment with 3 μM A66). Cell extracts from two independent MEF lines per genotype are shown. Each experiment was performed three times. d Immunofluorescence analysis revealed that the extra centrosomes seen in p110α<sup>H1047R</sup> MEFs were positive for exogenously expressed Cent2-GFP and composed of two centrioles, demonstrating that p110α<sup>H1047R</sup> induction did not lead to pericentriolar matrix fragmentation or premature centriole splitting (representative images shown in Fig. 3a and Supplementary Fig. 6f). In addition, the extra centrosomes were found to co-localize with mitotic spindle microtubules (representative images shown in Fig. 3b), and to always contribute to the establishment of the mitotic spindle.

**Discussion**

**p110α<sup>H1047R</sup> expression leads to centrosome overduplication.**

Compared to WT cells, p110α<sup>H1047R</sup> MEFs did not show any obvious increase in the number of senescent cells (Supplementary Fig. 7a), DNA damage (Supplementary Fig. 7b, c) or alterations in cell cycle profiles (i.e. prolonged G1/S or G2/M; Supplementary Fig. 7d), all of which are known causes of centrosome number deregulation<sup>11,27</sup>.

Expression of the helical E545K mutant of PIK3CA, the D560Y mutant of PIK3R1 (the p85 regulatory subunit of PIK3CA) as well as overexpression of PIK3CA<sup>WT</sup> (reflecting PIK3CA amplification in cancer), all displayed more centrosomes than parental cells (Supplementary Fig. 6d).

Interestingly, evidence for in situ centrosome amplification was also observed in E8.5 p110α<sup>H1047R</sup> embryos (Fig. 2b) and in adult skin and colon tissue, 2 weeks after the induction of p110α<sup>H1047R</sup> (Fig. 2c). In line with this, keratinocytes explanted from adult mice, following a 2-week in vivo induction of p110α<sup>H1047R</sup>, also showed extra centrosomes (Fig. 2a and Supplementary Fig. 6e).

Immunofluorescence analysis revealed that the extra centrosomes seen in p110α<sup>H1047R</sup> MEFs were positive for exogenously expressed Cent2-GFP and composed of two centrioles, demonstrating that p110α<sup>H1047R</sup> induction did not lead to pericentriolar matrix fragmentation or premature centriole splitting (representative images shown in Fig. 3a and Supplementary Fig. 6f). In addition, the extra centrosomes were found to co-localize with mitotic spindle microtubules (representative images shown in Fig. 3b), and to always contribute to the establishment of the mitotic spindle.

Alternative mechanisms for generating extra centrosomes include cytokinesis failure or multiple cycles of centrosome duplication prior to cell division<sup>11</sup>. After induction of p110α<sup>H1047R</sup> in MEFs that were growth-arrested by serum-deprivation, centrosome amplification emerged in parallel with the initiation of DNA synthesis (as measured by BrdU incorporation) in the G1/S transition, and before the cells had gone through cytokinesis (Fig. 3c). These data suggest that early after induction of p110α<sup>H1047R</sup> centrosome amplification occurs during the first round of cell division, before the cells have
determined. Our data are consistent with p110α interplay and timing of these signalling events remains to be determined. Akt inhibitor (Fig. 4d and Supplementary Fig. 8a). The precise enhanced activation of the centriole duplication machinery. and CDK2/Cyclin E-Nucleophosmin, most likely leading to overduplication in S-phase, which then interacts with ROCKII allowing its activation and initiation of centrosome duplication.28,29. In line with our conclusion that p110αH1047R expression leads to centrosome overduplication in S-phase, the activation of ROCK (as measured by the phosphorylation of MLC on Ser19) and the phosphorylation of Npm on T199 (a phosphorylation site of CDK2)30 were enhanced in p110α mutant cells (Fig. 4c). Both these signalling events were inhibited either by a CDK2 inhibitor (Fig. 4d), and at longer time points, by a p110α-selective inhibitor (Fig. 4c) or an Akt inhibitor (Fig. 4d and Supplementary Fig. 8a). The precise interplay and timing of these signalling events remains to be determined. Our data are consistent with p110αH1047R signalling to the centrosome at the time of initiation of duplication at the G1/S transition, through a mechanism involving AKT, ROCKII and CDK2/Cyclin E-Nucleophosmin, most likely leading to enhanced activation of the centriole duplication machinery.

We next tested the impact of pharmacological intervention with PI3K/Akt or Rho/ROCK signalling on p110αH1047R-induced centrosomal abnormalities, either before their genesis in primary cells or once they had been established in a tumour context.

Exposing primary MEFs to inhibitors of p110α, Akt or ROCK during the induction phase of p110αH1047R expression prevented centrosome amplification (Fig. 4e), with no significant impact on cell proliferation (Supplementary Fig. 8b). A similar experiment to the one shown in Fig. 3c but in the presence of a ROCK inhibitor, revealed that blocking ROCK activity prevented centrosome amplification before cells had gone through cytokinesis (Supplementary Fig. 9a), suggesting that centrosome amplification occurs during the first round of cell division upon p110αH1047R induction, in a ROCK-dependent manner.

We next created tumour-derived cell lines, by immortalizing primary p110αH1047R MEFs in vitro using p53 inactivation and inoculating them in nude mice. Once the injected cells had given rise to tumours, cell lines derived thereof were established in cell culture (further referred to as Nutu cells). A high fraction of Nutu cells had centrosome amplification (Fig. 4f). A similar result in primary MEFs, treatment of Nutu cells with inhibitors of Akt and especially of ROCK reduced the fraction of cells with centrosome amplification (Fig. 4f), underscoring the importance of Akt and ROCK signalling in centrosome amplification. These data indicate that Pik3caH1047R-induced centrosomal abnormalities, once established, can still be partially reverted pharmacologically. The overall importance of ROCK in the biology of oncogenic Pik3CA is illustrated by the observation that treatment with two different ROCK inhibitors (Y27632 and H1152) clearly decreases pik3caH1047R-induced cell transformation of primary MEFs (Supplementary Fig. 9b).

Efficient centrosome clustering upon p110αH1047R expression. Centrosome amplification can lead to the formation of multipolar spindles during mitosis and therefore compromise cell fitness, as the progeny derived from cells that do not cluster the extra centrosomes can die or undergo cell cycle arrest after undergoing a multipolar division. Since an enhanced level of cell death was not observed in p110αH1047R MEFs (Supplementary Fig. 5d), we speculated that these cells clustered their extra centrosomes efficiently. Indeed, live imaging by time-lapse microscopy of MEFs expressing centrin2 (a component of the centriole) tagged with GFP revealed that, of the cells with multiple centrosomes (30% in p110αH1047R MEFs vs. 10% in WT cells; Fig. 2a), most of the p110αH1047R cells were able to exit mitosis efficiently, with only 6% of mutant cells failing to do so, compared to a 35% failure rate in WT cells (n = 152 and 161 mitoses counted for WT and p110αH1047R MEFs, respectively; Fig. 5a and Supplementary Fig. 10 and Supplementary Movies 1, 2 and 3). These data show that, compared to WT cells, cells expressing p110αH1047R are more proficient at completing mitosis with extra centrosomes.

Lack of detectable p110αH1047R-induced chromosome segregation errors. It has previously been shown that cells with extra centrosomes acquire chromosome segregation errors after passing through a transient ‘multipolar spindle intermediate’ in which merotic kinetochore microtubule attachment errors accumulate before centrosome clustering and anaphase. The assessment of chromosome segregation errors during anaphase did not reveal differences in the frequency of segregation errors (~15% in each genotype; including acentric and centric chromosomes, and anaphase bridges) in MEFs, 72 h after treatment with 4-OHT (Fig. 5b). To better understand the observed lack of segregation errors upon p110αH1047R induction, we performed a more detailed analysis of the different spindle configurations observed in mitotic cells. We focused on cells with centrosome amplification going through mitosis (note that we observed ~10% centrosome amplification in WT and ~30% in p110αH1047R populations). Analysis of mitotic spindles in prometaphase and metaphase in these cells revealed a lower incidence of multipolar configurations in p110αH1047R than in WT cells (Fig. 5c). This suggests that even at earlier stages of mitosis, when erroneous kinetochore microtubule attachments are established (merotic attachments), there is already a difference in the clustering efficiency of extra centrosomes between WT and p110αH1047R. The total number of the multipolar spindles in early mitosis were slightly higher in the mutant population (~8 WT cells with multipolar spindles vs. ~14 p110αH1047R cells with multipolar spindles in every 100 cells), yet we did not observe higher frequencies of single chromosome segregation errors in the mutant cells.

Altogether these findings point toward a higher clustering efficiency of extra centrosomes in p110αH1047R-expressing cells and a lower number of multipolar intermediates (see Discussion for further details).

p110αH1047R expression allows tetraploid cell propagation. Analysis of metaphase chromosome spreads revealed an increase in aneuploid cells in p110αH1047R MEFs populations (Fig. 6a). Indeed, a range of chromosome number alterations was observed 3 and 5 days after p110αH1047R induction, when cells had undergone multiple cell divisions (average doubling time (DT) 26.2 h and 20.9 h for WT and p110αH1047R MEFs, respectively) (Fig. 6b; Supplementary Fig. 11 shows the individual experiments performed 3 and 5 days after 4-OHT treatment; experimental controls are shown in Supplementary Fig. 12—an independent experiment in which MEFs were subjected to parallel FACS and metaphase spread analysis is shown in Supplementary Fig. 13).
Aneuploidy in p110α<sup>H1047R</sup> MEFs was characterized by a preponderance of cells with a near-tetraploid chromosome number (around 80 chromosomes) (Supplementary Figs. 11 and 13). The observation that a large fraction of aneuploidy cells have a near-tetraploid content with very few cells with 41–59 chromosomes suggests that these cells have become aneuploid following a genome doubling event (tetraploidization). Keratinocytes explanted from Pik3ca<sup>H1047R</sup> mice also showed this tendency (Fig. 6c). Given the lack of cytokinesis failure early upon induction of p110α<sup>H1047R</sup> in MEFs (Fig. 3d), these observations indicate that p110α<sup>H1047R</sup> expression facilitates the propagation of tetraploid cells that arise stochastically. This could be explained by an increased tolerance to tetraploidization<sup>33,34</sup>, reflected by the fact that cells did not arrest as G1 tetraploids (4 N) but reached 8 N DNA content. Alternatively or in addition, the greater centrosome clustering efficiency observed earlier (Fig. 5a) would enable newly formed tetraploid cells to avoid catastrophic multipolar divisions.

In order to test whether p110α<sup>H1047R</sup> expression could provide tolerance to tetraploidization, primary WT and p110α<sup>H1047R</sup> MEFs were treated with dihydrocytochalasin B (DCB), an agent that blocks cytokinesis, to experimentally increase the fraction of tetraploid cells in the cultures. Cells were filmed for 30 h after DCB washout to assess cell division of binucleated cells resulting from cytokinesis failure. Strikingly, the number of p110α<sup>H1047R</sup> cells dividing after DCB washout was much higher than in WT cells (60% vs. 20%, respectively; Fig. 6d and Supplementary Movie 4), indicating that p110α<sup>H1047R</sup> cells have a less strict G1 tetraploidy checkpoint than WT cells, and display a higher tolerance to tetraploidy.

Inactivation of p53 is known to be an important mechanism leading to tolerance to genome doubling<sup>35,36</sup>. Both WT and p110α<sup>H1047R</sup> MEFs showed a proliferative arrest upon doxorubicin treatment (Supplementary Fig. 14a), with concomitant upregulation of p53 (Supplementary Fig. 14b), indicative of a functional p53 response to DNA damage. This suggests...
alternative tolerance routes to genome doubling in the presence of p110α<sup>H1047R</sup>.

**PIK3CA mutations and genome doubling in human cancer.** Emerging evidence suggests that PIK3CA mutations can occur late in the evolution of some cancers, being present in only a subset of cancer cells within the tumour<sup>37,38</sup>. However, in breast cancers (see Methods for breast cancer subtypes analyzed), mutations in PIK3CA showed a significant tendency to be clonal, i.e. to be present in all tumour cells (Fig. 7a; <i>P < 0.0001</i>), indicative of PIK3CA mutation being an early event in the evolution of this tumour type. In genome-doubled breast cancers, the majority of PIK3CA mutations were found to precede the genome duplication event (Fig. 7b), with a frequency significantly higher than would be expected by chance (<i>P < 0.0001</i>).

PIK3CA mutations, which are particularly enriched in estrogen receptor (ER)-positive and luminal subtype cancers (Supplementary Fig. 15a, b), showed a tendency to be mutually exclusive with mutations in TP53 (Supplementary Fig. 15c; <i>P < 0.0001</i>, Fisher’s exact test), which occur more frequently in basal-like tumours. In contrast to mutations in TP53, a known tolerance mechanism towards genome doubling<sup>35-37</sup>, we did not observe an enrichment of PIK3CA mutations in genome-doubled breast tumours. All together, these data are consistent with a role of PIK3CA mutation as a potential tolerance mechanism for genome doubling in breast cancer, conceivably independent of the p53 pathway, and also suggest that additional genomic events may be required to facilitate such tolerance in TP53 WT tumours. It remains to be demonstrated that PIK3CA mutation can provide such activities in vivo.

The significant enrichment for clonal mutations and the significant tendency for mutations in PIK3CA to precede genome doubling remained significant when focusing on ER-positive breast cancers or luminal breast cancers (Supplementary Fig. 16a, b). However, in other subtypes, there were insufficient numbers of mutations in PIK3CA to reliably assess clonality and timing.
Impact of PI3K pathway blockade on tetraploidization.

Treatment with inhibitors of p110α, Akt or ROCK during the induction phase of p110αH1047R expression in primary MEFs prevented tetraploidization (shown for p110α and ROCK inhibitors in Fig. 8a).

We next tested the impact of these inhibitors on spontaneously in vitro transformed p110αH1047R MEFs that differ in the percentage of diploid/tetraploid cells in the cell population (Fig. 8b; showing 2 clones—top and bottom graphs) and on Nutu cells, which are near-tetraploid (Fig. 8c (showing clone 1) and Supplementary Fig. 17 (showing clone 2)). In none of these chromosomal configurations, could these agents rescue the normal ploidy (Fig. 8b, c; Supplementary Fig. 17). This is in contrast to what was observed for centrosome amplification, which could be normalized pharmacologically (Fig. 4e, f).

Moreover, stably transformed diploid and tetraploid p110αH1047R MEFs established after long-term culture, were found to be equally sensitive to inhibition of p110α with A66 (Supplementary Fig. 18 a, b).

Taken together, these data indicate that Pik3caH1047R-induced chromosomal abnormalities, once established, cannot be reverted pharmacologically, with p110αH1047R expression providing tolerance, in a ROCK-dependent manner, to the early stages of tetraploidization but, once this genomic state is stably established, does not provide enhanced sensitivity to PI3K pathway inhibition.

Discussion

In this study, we report on previously unappreciated biological consequences of expression of mutated p110α, namely centrosome amplification, tolerance to tetraploidization and induction of aneuploidy (Fig. 9). At present, the functional connection between these biological phenomena remains unclear but the observation that each of these responses can be blocked by inhibition of the ROCK kinase strongly suggests a functional interrelationship downstream of signalling by oncogenic p110α PI3K.

We show here that p110αH1047R expression leads to supernumerary centrosomes in a variety of primary mouse and human cells, as well as in embryonic and adult mouse tissues. Indirect evidence for a role of PI3K in centrosome biology was previously found in human HeLa and HCT116 cancer cells, in which stable transfection of an oncogenic Met tyrosine kinase receptor was documented to induce centrosome amplification in a PI3K-dependent manner. Recent data indicate that centrosome amplification on its own is not a clear driver of cancer development but can cooperate with p53 loss to accelerate cancer development. Similar observations were made with our mutant mice, where Pik3caH1047R expression did not lead to spontaneous cancer within the time frame analyzed, but accelerated the onset of cancer upon concomitant heterozygous loss of the Apc tumour suppressor gene; as shown previously, or in the presence of other oncogenic lesions, such as loss of p53 or PTEN. However, the specific contribution of Pik3caH1047R-driven centrosome amplification in interaction of mutant Pik3ca with other cancer-promoting genetic lesions remains unknown.

Centrosome amplification can give rise to chromosome segregation errors, as a result of multipolar spindle formation during the clustering process, resulting in aneuploidy. Despite the presence of centrosome amplification and increased centrosome clustering efficiency, the frequency of chromosome segregation errors appeared to be unaltered early after p110αH1047R expression in MEFs and less multipolar spindles were observed. Given the observation that all extra centrosomes contribute to the formation of the mitotic spindle (Fig. 3b), we speculate that p110αH1047R expression allows extra centrosome clustering without the formation of multipolar spindles and/or enables cells
chromosome missegregation between WT and Pik3caH1047R cell model is too low to observe differences in chromosome numbers of two independent stably transformed p110αH1047R MEF clones with a different degree of tetraploidy. c) Impact of inhibition of Akt (by 1 μM Akti X) or ROCK (by 10 μM Y27632) for 21 days on chromosome numbers of p110αH1047R Nutu clone 1 (shRNA p53-immortalized p110αH1047R MEF clone passed through nude mice as tumour). Chromosome counting was performed on DAPI-stained metaphase spreads. Fifty spreads were counted per line and condition, unless otherwise stated.

Fig. 8 Impact of pharmacological blockade of PI3K pathway components on tetraploidization in primary and tumour-derived MEFs. a) Impact of inhibition of p110α (by 3 μM A66) or ROCK (by 10 μM Y27632) on p110αH1047R-induced chromosomal abnormalities in primary MEFs. One Pik3caH1047R-neo, Flpe-ERT2 MEF line was analyzed and at least 50 metaphase spreads were counted per condition. Inhibitors were added together with the 4-OHT to induce p110αH1047R expression. b) Impact of 14 day inhibition of p110α (by 3 μM A66), Akt (by 1 μM MK-2206) or ROCK (by 10 μM Y27632) on chromosome numbers of p110αH1047R transformed p110αH1047R MEF (clone 1) or ROCK inhibitor (Y27632) (n = 61). c) Impact of inhibition of Akt (by 1 μM Akti X) or ROCK (by 10 μM Y27632) for 21 days on chromosome numbers of p110αH1047R Nutu clone 1 (shRNA p53-immortalized p110αH1047R MEF clone passed through nude mice as tumour). Chromosome counting was performed on DAPI-stained metaphase spreads. Fifty spreads were counted per line and condition, unless otherwise stated.
intervention with PI3K in cancer is currently aimed at targeting cancer cell proliferation and survival. Our data show that pharmacological inhibition of the PI3K/ROCK pathway can reduce the fraction of cells that has centrosome amplification within a population, but cannot revert the tetraploidy-derived aneuploidy facilitated by the presence of Pik3ca\(^{H1047R}\), once it has been established. This could help to explain the currently limited therapeutic impact of PI3K-targeted therapies in cancer and the observation that PIK3CA mutation is not, on its own, always a clear predictor of sensitivity to PI3K pathway inhibitors\(^{31}\). On the other hand, our findings also suggest that pharmacological intervention with PI3K signalling in cancer might reduce tumour heterogeneity and evolution by limiting the perpetuation of genetically unstable tetraploid cells. Genomic analyses of PIK3CA-mutant positive tumour samples from patients treated with PI3K pathway inhibitors might uncover evidence for such a new role for PI3K in a clinical context. The timing of intervention with PI3K pathway inhibitors in cancer might therefore be of critical importance for maximizing their therapeutic impact.

### Methods

#### Reagents

In case the concentration of antibody stocks was known, we used 1 μg of antibody per immunoprecipitate, 1–2.5 μg ml\(^{-1}\) for western blotting and 1–5 μg ml\(^{-1}\) for immunohistochemistry. When the concentration of commercial antibody stocks was unknown, we used 10 μl per immunoprecipitate, 1/1000–5000 for western blotting and 1/50–500 for immunohistochemistry. Antibodies used for Western blotting were from the following commercial providers (catalogue numbers are in brackets): Cell Signaling Technologies: Akt/PKB (9272), pAkt-T308 (9275), pAkt-S473 (9271), pNP-TPN-19 (3541), NP-NP (3542), pMLC-S19 (3675), pMLC (3672), Cdc14 (4129), vinculin (4650), p53 (9282); Sigma: β-actin (A1978), α-tubulin (T6074) and α-actinin (A5044); BD Biosciences: p110α (611400); Merck: β-actin (A1978), α-tubulin (A5044), and GAP (ab1218); Sigma: α-tubulin (T6074); Cell Signaling Technologies: cleaved-caspase-3 (9664); Merck Millipore: pHistone H2AX-S139 (05–636); Santa Cruz Biotechnology: RhoA (sc-418). Antibodies used for immunofluorescence were from the following providers: Abcam: γ-tubulin (ab11316), pericentrin (ab4448), β-tubulin (ab6046) and GFP (ab1218); Sigma: α-tubulin (T6074); and Cell Signaling Technologies: cleaved-caspase-3 (9664); Merck Millipore: pHistone H2AX-S139 (05–636); Santa Cruz Biotechnology: Endomucin (sc-65495); 53BP1 (sc-22760); Fitzgerald: human Cetn (96–CS1058). Rhodamine Phalloidin (R415, Thermo-Fisher Scientific) was used to detect F-actin. Secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or Cy3 were from Jackson ImmunoResearch Labs, and secondary antibodies conjugated to AlexaFluor 594 (A11012) and AlexaFluor 647 (A21445) were from ThermoFisher Scientific. Other reagents were sourced as follows: from Selleck Chemicals: T27632 (S1049) and MK-2230 (S1078); Merck: Akt-X (124020); Symansis: A66 (sy-a66); Tocris: H1152 (2414) and NU6140 (3301); Sigma: Tamoxifen (H7904) and dihydroxyoctochalin B (D1641).

#### Mice

Mice were kept in individually ventilated cages and cared for according to UK Home Office regulations, with procedures approved by the Ethics Committees of Queen Mary University London, UK and University College London, UK. Unless otherwise mentioned, the mice used were backcrossed to the C57BL/6 background for 10 generations, and littermates were used as controls.

#### Generation of Pik3ca\(^{H1047R,neo}\) mice

Gene targeting was carried out by Taconic (Cologne, Germany) and shown schematically in Fig. 1a. Genomic DNA covering the last 6 exons of the coding region of Pik3ca was isolated and a point mutation, resulting in conversion of CAT (H) to CGT (R) in the C-terminal p110α kinase domain, was introduced by site-directed mutagenesis. In addition, a neo selection cassette flanked by fr sites was inserted into exons 8 and 9 of the targeting vector using standard restriction enzyme- and PCR-based cloning techniques. The linearized targeting construct was electroporated into C57BL/6 ES cells. G418-resistant clones with targeted integration of the vector were identified by Southern blot analysis of HindIII-digested, XmnI-digested or BspGI-digested genomic DNA using different probes homologous to genomic DNA sequences flanking the selection cassette. The presence of the introduced SGT sequence was confirmed by DNA sequencing. Chimeras were generated from clones with the G418 mutation and founders identified by PCR analysis of tail DNA. Male chimeras carrying the PI3K mutation (germline transmitters) were bred with C57BL/6 females to generate the Pik3ca\(^{H1047R,neo}\) heterozygous mice. The presence of the Pik3ca\(^{H1047R,neo}\) kypomyosm was the mouse. The presence of the Pik3ca\(^{H1047R,neo}\) mutant allele was monitored by PCR using the following primers (shown schematically in Fig. 1a): primer 1 = onco-p110α-F: 5’-GTTTACAGCTGGAAATAAA GCC-3’; primer 2 = onco-p110α-R: 5’-AGATCCAGGCTTCGTTCC-3’, giving rise to an expected PCR fragment of 307 bp (Supplementary Fig. 1b). In each amplification cycle, denaturation was performed for 30 s at 95°C, annealing for 30 s at 60°C and extension for 1 min at 72°C; the number of cycles was 35 followed by a final extension of 10 min at 72°C.
Pik3caH1047R neo-hypomorph mice were bred with mice that express a germine FLP recombinase, which was either constitutively active (hACTR-Flpe mice) or inducible by 4-hydroxytamoxifen (4-OHT) treatment (CAG-FlpeERT2 mice). In order to induce Flpe-mediated deletion of the neo selection cassette, Flpe-mediated or Flpe-ERT2–mediated recombination of the neo selection cassette was monitored by PCR for the presence of the primers (Supplementary Fig. 1b); primer 1 = 5′-TCTTTCCTTCTCACCATGCC-3′, primer 2 = 5′-TCCTTCTGTATTTGGGATCTG-3′.

Isolation of primary MEFs. Embryos were obtained from timed pregnant females. The day of the presence of a copulation plug was considered as day E0.5. E13.5 embryos were minced, dissociated using trypsin and cells allowed to adhere on tissue culture dishes in DMEM, 10% FBS, penicillin and streptomycin, as described in ref. 32. Unless otherwise indicated, early passage (P2 to P3) MEFs were used in experiments. The presence of a copulation plug was considered as day E0.5. E13.5 embryos were minced, dissociated using trypsin and cells allowed to adhere on tissue culture dishes in DMEM, 10% FBS, penicillin and streptomycin, as described in ref. 32. Unless otherwise indicated, early passage (P2 to P3) MEFs were used in experiments.

Cell culture. MEFs and human dermal fibroblasts were cultured in DMEM with 10% FBS, penicillin and streptomycin. Primary keratinocytes were cultured in Keratinocyte-SFM 1x (Invitrogen, 37010–022), 20 μM CaCl2, and 20 mM HEPES. MCF-10A cells (ATCC CRL-10317) were cultured in DMEM/F12 with 5% horse serum, 20 ng ml−1 EGF, 0.5 μg ml−1 hydrocortisone, 100 mg ml−1 cholesta toxin, 10 μg ml−1 insulin, penicillin and streptomycin. MEFs were cultured in standard culture medium and treated with 4-OHT. Cells were allowed to reach confluence and kept in culture for 2 weeks until they were treated with 4-OHT in vitro and injected subcutaneously into the upper and lower parts of each flank of hemisected nude mice (n = 8, Harlan UK). Only p53 shRNA-immortalized Pik3caH1047R MEFs gave rise to tumours and with low penetrance (three out of eight tumours injected) after a latency period of 2–3 months. Tumours were excised, minced, trypsinized and propagated as cell lines in culture, further referred as Nutu1, Nutu2 and Nutu3.

In vitro induction of Pik3caH1047R in mice. For postnatal removal of the neo cassette by tamoxifen-inducible FLP-mediated recombination, Pik3caH1047R-FlpeER T2 and Pik3caH1047Rneo-FlpeER T2 mice at the age of 8 weeks were dosed with tamoxifen in corn oil (80 mg kg−1) by gavage for 5 consecutive days. Animals were followed over time and culled when they became apparently ill. A healthy control animal was cultured in parallel.

In vitro induction of Pik3caH1047R in MEFs. Pik3caH1047R-FlpeER T2 and Pik3caH1047Rneo-FlpeER T2 MEFs cultured in standard growth medium were treated for 72 h (unless stated otherwise) with 1 μM 4-OHT solubilized in ethanol (vehicule). Culture medium was replenished every 48 h with fresh 4-OHT. Flpe-ERT2–mediated recombination of the Neo selection cassette was monitored by PCR.

Isolation and induction of Pik3caH1047R in mouse skin keratinocytes. Adult mouse dorsal epidermal keratinocytes were isolated as described in ref. 33. For uses in experiments to determine aneuploidy, 8-week-old Pik3caH1047R-FlpeER T2 or Pik3caH1047Rneo-FlpeER T2 mice were treated with tamoxifen (80 mg kg−1) by gavage for 5 consecutive days. Two weeks after the last day of treatment, the dorsal skin was shaved and isolated, cut into small pieces, washed once with PBS and twice with 70% ethanol, submerged in trypsin (T4424, Sigma) and incubated for 30 min at 37 °C. The epidermis was detached and minced and cells were plated on 10 cm dishes. After 24 h, cells were washed once with Hank’s solution, incubated with culture medium which was replenished every other day until the cultures reached confluence (6–7 days after seeding). Coating of culture dishes for keratinoctye cultures was prepared by incubation of 10 μl of collagen (354236, BD Biosciences) and 10 μl fibronectin (354008, BD Biosciences) in 980 μl of EMEM (06-174-1, Lonza) + 5 mM HEPES (13600–056, Gibco) to obtain a final concentration of 30 μg ml−1 collagen and 10 μg ml−1 fibronectin, incubated with coating solution at 4 °C overnight on a rocker platform. Primary cultures were then washed 3 times with Hank’s solution (14175–129, Gibco). For centrosome analysis in cultured cells, cells grown on coverslips treated with poly-l-lysine (P9820, Sigma) or on collagen I coated glass culture dishes in the case of MCF-10A cells (Corning, 354225), cells were incubated for 1 h with ice-cold methanol for 10 min, blocked with PBS containing 3% BSA for 40 min and incubated overnight at 4 °C with primary antibodies. All primary antibodies were detected using species-specific fluorescein isothiocyanate or rhodamine secondary antibodies (Molecular Probes) and DNA was detected with 0.2 μg ml−1 DAPI (D9542, Sigma). Secondary antibodies were mounted in mounting medium with DAPI (4′,6-diamidino-2-phenylindole, Calbiochem). Confocal laser scanning microscopy was performed with an LSM 710 (Zeiss) confocal microscope.

For centrosome analysis in cultured cells, adult tissues, 8-week-old Pik3caH1047R-FlpeER T2 or Pik3caH1047Rneo-FlpeER T2 mice were treated with tamoxifen (80 mg kg−1) by gavage for 5 consecutive days. Two weeks after the last day of treatment, tissues were snap-frozen in OCT freezing medium (TF5M, Electron Microscopy Sciences) and 10 μm cryosections affixed to poly-l-lysine coated slides. Sections were thawed at room temperature for 30 min before staining, fixed for 15 min in 4% PFA in PBS at room temperature, washed 3 times for 5 min with PBS, blocked with mouse IgG blocking reagent (Millipore) for 1 h, washed times for 5 min with PBS and blocked for 1 h in 5% donkey serum in PBS-0.3% Tween (PBS-T). Slides were then incubated overnight at 4 °C with antibodies to pericentrin (1:500 in PBS-T), washed 3 times in PBS, incubated with anti-rabbit-Cy3 antibody (1:500 in PBS-T) and AlexaFluo 488 phallodin (1:500 in PBS-T) for 1–2 h in the dark at room temperature, washed three times with PBS and mounted with Vectashield mounting media containing DAPI (H-1200, Vector Labs).

For centrosome analysis in embryos, whole-mount embryo immunofluorescence was performed. E8.5 embryos were isolated, transferred to 1.5 ml Eppendorf tubes and fixed in 4% PFA in PBS at 4°C overnight on a rocker platform, followed by washes with 0.1% Triton X-100 in PBS (PBT). Embryos were incubated in 0.25% Triton X-100 in PBS for 15 min and washed three times with PBT before blocking with 2.5% normal donkey serum (D9663, Sigma), 2.5% normal goat serum (G9023, Sigma) and 3% bovine serum albumin (A7906, Sigma) in PBT for 3 h. Following removal of the blocking solution, anti-pericentrin antibody in 200 μl PBT was added to the Eppendorf tube containing the embryos and incubated overnight at 4°C. The following day, 1 ml PBT was added to the solution containing the embryos, embryos washed 5 times in PBT and incubated in PBT for an extra 20 min. Then, 200 μl PBT solution containing the secondary Cy3–anti-rabbit antibody was added and incubated overnight at room temperature. Embryos were then washed 5 times for 5 min in PBT and once for 15 min. Finally, embryos were mounted on depression slides (S175201, Fisher scientific) in Vectashield mounting media containing DAPI (H-1200, Vector Labs) for 2 days.

Analysis of chromosome segregation errors. Cells cultured on poly-l-lysine-treated coverslips were fixed in 10% Triton X-100, 1 M PIPES, 0.5 M EGTA, 1 M MgCl2, and 4% formaldehyde, blocked with PBS containing 3% BSA for 40 min and incubated for 1 h with primary antibodies to hACTR-FlpeER T2 (1:300) and β-tubulin (1:600). Secondary antibodies (1:500) goat anti-rabbit conjugated to AlexaFluo 594 and goat anti-human AlexaFluo 647 were added for 1.5 h.
h at room temperature. DNA was stained with 1 μg mL\(^{-1}\) DAPI (10236276001, Roche) and coverslips mounted in Vectashield (Vector H-1000). Images were acquired using an Applied DeltaVision RT microscope (Applied Precision, LLC) equipped with a CoolSnap HQ camera.

**Transient transfection.** MCF-10A cells were transfected using Lipofectamine 3000 Transfection Reagent (ThermoFisher Scientific; LI000008) according to the manufacturer's instructions. The plasmids used were pff-pha-HA-PK3CA\(^{1,7}\), pff-pha-HA-PK3CA\(^{1,7}\) with GST-Cent2 MEFs. Cells were seeded in µ-Plate 96 wells (Ibidi; IB-8962) in DMEM medium 48 h after treatment with 4-OHT, followed by filming of the cells for 30 h at 37 °C. To study the efficiency of the G1 checkpoint, an assay of DCB-induced tetraploidy was used. Briefly, MEFs were seeded on ibidi µ-Plate 96 wells in DMEM medium with 4-OHT for 24 h, followed by overnight incubation in the presence of 10 μM DCB followed by washing out of this agent in fresh culture and by fixation of the cells for 30 h at 37 °C. Images were collected with a high-content analysis microscope (ImageXpress Micro XLS Widefield High-Content Analysis System/Molecular Devices) every 15 min using MetaXpress High-Content Image Acquisition and Analysis Software (Molecular Devices). Time-lapse images from the whole duration of the experiment were then converted into a movie using Image J, and cells with more than two centrosomes or binucleated cells entering mitosis were tracked.

**Measurement of RhoA activity.** The capacity of RhoA–GTP to bind to GST–rhotein–RBD was used to determine the cellular levels of active GTPase. Cells were lysed in extraction buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM MgCl\(_2\), 1% NP-40, 10% glycerol, 1 mM DTT, protease and phosphatase inhibitor cocktail) and the RhoA pull-down assay was performed by incubation of cleared lysates with GST–rhotein–RBD (Cytoskeleton Inc.) for 45 min at 4 °C, followed by four washes of the beads in wash buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM MgCl\(_2\), 1% NP-40, 10% glycerol, 1 mM DTT, protease and phosphatase inhibitor cocktail). Bound proteins were solubilized by the addition of 25 μL of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) Laemmli loading buffer, followed by separation on 12.5% SDS-PAGE gels and western blotting for RhoA.

**MEF proliferation assay.** Early passage (P2–P4) MEFs were incubated at 2 × 10^4 cells per well in 96-well plates in 100 μL medium (DMEM, 10% FBS, penicillin and streptomycin) in the presence of the indicated drugs for 72 h. MTS reagent (G5421, Promega) was added and absorbance at 492 nm was read 3 h later and analyzed using Magellan software. For cell counting experiments, 2.25 × 10^5 cells were seeded in six-well plates in the presence of 4-OHT. Cells were collected every 24 h and counted by Easy Cell Counter (Roche). The population doubling time was calculated using the following formula: DT = T ln(Inf/Xb). T is the incubation time in hours, Xb is the cell number at the beginning of the incubation time and X is the cell number at the end of the incubation time (ATCC animal cell culture guide).

**BrDU incorporation assay.** MEFs were serum-starved for 48 h in the presence of 4-OHT followed by incubation in FBS-containing medium for the indicated times. One hour before collection, cells were incubated in labelling medium containing 10 μM 5-bromo-2′-deoxyuridine (BS502, Sigma) followed by trypsinization, washing with PBS and fixation in 70% ethanol. Following two further washes in PBS, cells were incubated with 2 M HCl for 20 min and probed with anti-BrD-U monoclonal antibody (5292, Cell Signaling Technologies) and detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG2a (Jackson Immunoresearch Labs). Cells were resuspended in 500 μl propidium iodide solution (50 mg mL\(^{-1}\); P4864, Sigma) and RNase A (100 μg mL\(^{-1}\); R5503, Sigma) and analyzed using a BD Accuri C6 Flow cytometer (BD Bioscience) and FlowJo software.

**Cell cycle analysis.** Experiments were carried out in synchronized or non-synchronized MEF cell populations. For experiments with synchronized cells, MEFs were incubated with 4-OHT for 72 h. For the last 24 h of this culture, cells were incubated in the presence of 12 μM Aphidicolin (178273, Merck) followed by washing out of this agent (release) in culture medium for the times indicated. After washing, MEFs were detached by trypsinization, collected and fixed in ice-cold 70% ethanol. For experiments with asynchronous cells, MEFs incubated in culture medium were incubated with 4-OHT on day 0, and further incubated for 3 and 5 days. After washing with PBS, cells were detached, collected and fixed in ice-cold 70% ethanol. To determine the mitotic index, cells were probed with pH3-L176 (Ab5176, Abcam) and detected with FITC-labelled goat anti-rabbit IgG antibody (5292, Cell Signaling Technologies) and detected with HA-PIK3CA\(^{1,7}\) transgenic mice to generate HA-PIK3CA\(^{1,7}\) H1047R+neo; H1047R−neo; PIK3CA WT, pBabe-HA–PK3CA cells were used. Briefly, 5 days after incubation in the presence of 4-OHT, MEFs were detached by trypsinization, washed with cold PBS and resuspended in 1× binding buffer at a final concentration of 1 × 10^6 cells mL\(^{-1}\). A total of 100 μL of this suspension was incubated with FITC-Annexin V and PI solution for 15 min at room temperature in the dark. After incubation, 400 μL of binding buffer was added and the cells were analyzed using a FACS Calibur (BD Bioscience) and FlowJo software.

**Apoptosis assay.** The assay was performed using a FITC-Annexin V Apoptosis Detection Kit II (BD Pharmingen) according to manufacturer’s instructions. Briefly, 5 days after incubation in the presence of 4-OHT, MEFs were detached by trypsinization, washed with cold PBS and resuspended in 1× binding buffer at a final concentration of 1 × 10^6 cells mL\(^{-1}\). A total of 100 μL of this suspension was incubated with FITC-Annexin V and PI solution for 15 min at room temperature in the dark. After incubation, 400 μL of binding buffer was added and the cells were analyzed using a FACS Calibur (BD Bioscience) and FlowJo software.

**Senescence assay.** The assay was performed using a Senescence Cells Histological Staining Kit (CS3003, Sigma) according to manufacturer’s instructions. Briefly, 72 h after incubation in the presence of 4-OHT, MEFs were fixed for 7 min in fixative buffer, washed in PBS and stained with staining mixture overnight at 37 °C without CO\(_2\). The cells were observed under a bright field microscope (DM II, LED, Leica). The percentage of β-galactosidase-positive cells was calculated by counting blue-stained cells and the total number of cells.

**DNA damage checkpoint experiments.** For cell counting, MEFs were seeded in 12-well plates in the presence of 4-OHT. The day after, cells were treated with 0.2 μg mL\(^{-1}\) doxorubicin (D1515, Sigma) for 20 h. After the incubation time, the drug was washed out and cells allowed to proliferate. Cells were collected and manually counted for the indicated times. For p53 Western blotting, MEFs were seeded in 6 cm plates in the presence of 4-OHT. 48 h after induction, cells were treated with 0.2 μg mL\(^{-1}\) doxorubicin for 18 h, after which cells were lysed and total protein isolated.

**Endomucin staining.** Freshly isolated E9.5 mouse embryos were fixed in 4% paraformaldehyde overnight at 4 °C. After washing in PBS, embryos were dehydrated in increasing concentrations of methanol (50%, 80% methanol/PBT, 100% methanol), rehydrated in decreasing concentrations of methanol, followed by a 30 min wash in Pblec buffer (0.2 mM CaCl\(_2\), 0.2 mM MgCl\(_2\), 0.2 mM MnCl\(_2\) and 2% Triton X-100 in PBS) and overnight incubation at 4 °C with antibody to endomucin (Ab3950, Abcam) for 12 h. After five washes in PBTE, embryos were incubated with Cy5-labelled secondary antibody diluted 1:100 in PBS, 0.5% BSA, 0.25% Tween-20. Finally, embryos were washed three times in PBS, post-fixed for 1 min in 4% paraformaldehyde and mounted.

**Immunoblotting.** Cells were lysed in lysis buffer (10 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 2 mM CaCl\(_2\), 0.1 mM Na\(_3\)VO\(_4\), protease inhibitors (Calbiochem), 1 mM PMSF). The lysates were incubated for 20 min and cleared by centrifugation at 4 °C. The samples were denatured in sample buffer (10% SDS, 20% glycerol, 200 mM Tris-HCl pH 6.8, 10 mM DTT, 0.05% bromophenol blue), resolved by SDS-PAGE, and transferred onto an Immobilon-P PVDF membrane (Millipore). The blots were incubated in blocking buffer (5% w/v) nonfat dry milk in Tris-buffered saline plus Tween 20 (1:1) and incubated with primary antibodies for 16 h at 4 °C. The blots were incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for 1 h at room temperature. The antigen–antigen complex was visualized by Pierce ECL Western Blotting Substrate (Thermo Scientific).

The endomucin immunoblots and larger areas are presented in Supplementary Fig. 19.

**Mutation and copy number analysis.** TCGA breast cancer mutation and copy number analysis was performed as described in ref. 37, with genome doubling assessed as outlined in ref. 34. In brief, mutations were classified as clonal or subclonal based on the 95% confidence interval of the posterior distribution of the clonal fraction. Mutations with the 95% confidence interval overlapped with the 1 were grouped as clonal, with all other mutations classified as subclonal. To assess whether H1047R mutations in PK3CA were significantly more often clonal than
expected by chance in breast cancer, a permutation test was used, as outlined in ref. 37. In brief, the same number of mutations as observed at H1047R sites in PIK3CA were randomly sampled from the entire cohort of mutations in TCGA breast cancer 10,000 times, obtaining a background distribution, representing the expected proportion of clonal/subclonal mutations. A P-value was then obtained by comparing the observed proportion of clonal/subclonal mutations to this background distribution. The same technique was used to assess whether PIK3CA mutations occurred more often before or after genome doubling. In this case only genome-doubled breast tumours were used and mutations were timed relative to copy number events. Timed mutations were restricted to those occurring in regions with at least two copies of the major allele. Given that a genome doubling event will double mutations preceding the event, all mutations present at multiple copies were classified as preceding doubling, while those only present at one copy were classified as occurring after the duplication.

Breast cancer subtype classification was performed using the gene expression in R, grouping breast tumours into five subtypes: luminal B-like: Her2-like: basal-like: luminal A-like: and normal. ER status was assessed by immunohistochemistry and obtained from clinical data from TCGA portal. Tendency towards mutual exclusivity between mutations in PIK3CA and TP53 was assessed using a Fisher’s exact test, using all breast cancer tumours and using either all non-silent mutations or the ten most frequent hotspots mutations. This mutual exclusivity remained significant when focusing on only genome-doubled tumours (P = 0.04). A two-tailed Fisher’s exact test was used to assess significance.

Data availability. All relevant data are available from the authors.

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Author contributions

I.M.B., R.P. and B.V. designed research; I.M.B., R.P., S.D.C., S.M.D., V.M., N.K., W.P., K. G.M., E.L., L.S., D.M., M.G. and L.S.M. performed research; V.M. and R.K.S. contributed new reagents and/or analytic tools; I.M.B., R.P. and B.V. analyzed data; I.M.B., R.P. and B.V. wrote the paper with input from L.S. and C.S. All authors discussed the results and commented on the manuscript.

Additional information

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