miR-147b-mediated TCA cycle dysfunction and pseudohypoxia initiate drug tolerance to EGFR inhibitors in lung adenocarcinoma

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Drug tolerance is an acute defence response preceding a fully drug-resistant state and tumour relapse; however, there are few therapeutic agents targeting drug tolerance in the clinic. Here we show that miR-147b initiates a reversible state of tolerance to the epidermal growth factor receptor (EGFR) inhibitor osimertinib in non-small-cell lung cancer. With miRNA-seq analysis, we find that miR-147b is the most upregulated microRNA in osimertinib-tolerant and EGFR-mutated lung cancer cells. Whole-transcriptome analysis of single-cell-derived clones reveals a link between osimertinib tolerance and pseudohypoxia responses irrespective of oxygen levels. Further metabolomics and genetic studies demonstrate that osimertinib tolerance is driven by miR-147b-mediated repression of VHL and succinate dehydrogenase, which are linked to the tricarboxylic acid cycle and pseudohypoxia pathways. Finally, pretreatment with a miR-147b inhibitor delays osimertinib-associated drug tolerance in patient-derived 3D structures. This link between miR-147b and the tricarboxylic acid cycle may provide promising targets for preventing tumour relapse.

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Drug tolerance is an acute defence response preceding a fully drug-resistant state and tumour relapse; however, there are few therapeutic agents targeting drug tolerance in the clinic. Here we show that miR-147b initiates a reversible state of tolerance to the epidermal growth factor receptor (EGFR) inhibitor osimertinib in non-small-cell lung cancer. With miRNA-seq analysis, we find that miR-147b is the most upregulated microRNA in osimertinib-tolerant and EGFR-mutated lung cancer cells. Whole-transcriptome analysis of single-cell-derived clones reveals a link between osimertinib tolerance and pseudohypoxia responses irrespective of oxygen levels. Further metabolomics and genetic studies demonstrate that osimertinib tolerance is driven by miR-147b-mediated repression of VHL and succinate dehydrogenase, which are linked to the tricarboxylic acid cycle and pseudohypoxia pathways. Finally, pretreatment with a miR-147b inhibitor delays osimertinib-associated drug tolerance in patient-derived 3D structures. This link between miR-147b and the tricarboxylic acid cycle may provide promising targets for preventing tumour relapse.

Relapse of disease following conventional treatments remains one of the central problems in cancer management, including in EGFR-based therapy1,2. Tumour cells overcome EGFR-targeted treatment through acquisition of mutations that disrupt drug binding by EGFR and activate signalling through other protein tyrosine kinase pathways1. For example, a majority of tumours from patients with EGFR-mutant non-small-cell lung cancer (NSCLC) were shown to have acquired resistance-conferring mutations when patients were treated with EGFR tyrosine kinase inhibitors (TKIs), including EGFR<sub>T790M</sub>-positive in gefitinib treatment and EGFR<sub>C797S</sub> on combination erlotinib and osimertinib therapy3,4. Recently, it has been found that EGFR<sub>T790M</sub>-positive drug-resistant cells can emerge from EGFR<sub>T790M</sub>-negative drug-tolerant cells that survive initial drug treatment5,6. Thus, targeting drug-tolerant cells might be a new strategy to block drug resistance7. With the success in applying osimertinib in first-line treatment of EGFR<sub>T790M</sub>-positive NSCLC6, it is crucial to identify the changes driving drug tolerance. However, the molecules underlying tolerance to EGFR TKIs have not been well studied.

Aberrantly regulated metabolic pathways lead to tumourigenesis and preferential survival of tumour cells<sup>11-13</sup>. The tricarboxylic acid (TCA) cycle is a central pathway in the metabolism of sugars, lipids and amino acids<sup>14</sup>. Dysregulation of the TCA cycle can induce oncogenesis by activating pseudohypoxia responses, which result in the expression of hypoxia-associated proteins irrespective of oxygen status15-17. For example, succinate accumulation caused by functional loss of the TCA cycle enzyme succinate dehydrogenase (SDH) stabilises hypoxia-inducible factor (HIF)-1α via inhibition of prolyl hydroxylase (PHD)<sup>10,11</sup>. In addition, loss of function of the von Hippel–Lindau (VHL) protein also induces pseudohypoxia responses through decreased ubiquitination and proteasomal degradation of HIF-1α<sup>12</sup>. In comparison to other cancers, NSCLC tumours are well vascularised and tumour cells depend on high levels of the iron–sulfur cluster biosynthetic enzymes to decrease oxidative damage due to exposure to high oxygen levels12. Most recently, it was shown that drug-tolerant persistor cancer cells were vulnerable to inhibition of the GPX4 lipid hydroperoxidase, owing to a disabled antioxidant program14. However, understanding of the changes conferring drug tolerance remains limited. To address this knowledge gap, we explored which signalling pathways initiate anticancer drug tolerance and how tolerance shapes cancer metabolism and tumour relapse. In this study, we discovered that a subpopulation of tumour cells adopt a tolerance strategy to defend against EGFR-based anticancer treatments by altering miR-147b-dependent regulation of the TCA cycle and pseudohypoxia responses. We show that miR-147b, by targeting VHL and SDH, is critical for tolerance-mediated tumour relapse.

Results

Lung cancer cells adopt a tolerance strategy to EGFR inhibitors. We established 3D lung structures from immortalised AALE-tracheobronchial epithelial cells and EGFR-mutated HCC827 lung cancer cells because of the advantage of such models in visualising in vivo–like structures (Fig. 1a–c and Supplementary Fig. 1a–c). In comparison to adult lung tissues, AALE-derived 3D lung structures expressed higher levels of the ID2 gene (encoding inhibitor
of DNA binding 2), which is expressed in lung progenitor cells, on day 15 of culture, and this was followed by decreased expression on day 24, as assessed by qRT–PCR analysis (Supplementary Fig. 1 and Supplementary Table 1). In contrast, the 3D structures from AALE cells expressed lower levels of type I and II pneumocyte markers, including SFTPC (surfactant protein C), HOPX (HOPX homeobox) and NKX2.1 (NKX2 homeobox 1; also known as TTF-1 (transcription termination factor 1)), on day 15, and this was followed by increasing expression on day 24 (Supplementary Fig. 1d). Gene expression for ID2, SFTPC, HOPX and NKX2.1 in the 3D lung structures was comparable to that in adult lung tissues, in line with previous findings for 3D lung structures differentiated from pluripotent stem cells3. Similarly, 3D structures generated from a patient-derived xenograft (PDX) tumour (PDX_LU_10) obtained from a patient with lung adenocarcinoma (Supplementary Table 2) expressed tumour- and lung-relevant genes on day 25, including CEACAM5 (carcinoembryonic antigen–related cell adhesion molecule 5), LIN28B (Lin-28 homolog B), SFTPC and HOPX, in line with gene expression in the parental tumour (Supplementary Fig. 1e). Collectively, our data suggest that 3D lung structures cultured over time are relevant to clinical tissues.

In the context of both 3D structures and monolayer cultures, we treated HCC827 cells with serially diluted osimertinib for 3 d to observe acute treatment responses. We found that a subpopulation of tumour cells initially survived cytotoxic doses (0.01–2 µM) of osimertinib (Supplementary Fig. 2a). Surprisingly, a small percentage of cells could survive longer than 2–3 weeks in both the monolayer and 3D culture models when they were treated continuously with 160 nM osimertinib (Supplementary Fig. 2b). However, in contrast to drug-resistant cells, most of the surviving 3D structures disappeared when they were treated with a threefold-higher concentration of osimertinib for an additional 9 d (Supplementary Fig. 2c). This indicates that some tumour cells adopt a strategy different from that applied in drug resistance to protect themselves during the early stages of the response to EGFR-targeted therapy. To further understand the protective strategy adopted by some tumour cells, after treatment for 11 d, we withdrew osimertinib from HCC827 3D structures and found that the initially surviving 3D structures recovered and increased in size within the following 21 d. These recovered 3D structures remained similarly sensitive to osimertinib when they were again exposed to the same dose (Fig. 1b). Cells from two lung cancer cell lines with EGFR mutations, PC9 and H1975, entered a similar ‘tolerance cycle’ when gefitinib or osimertinib treatment was alternated with treatment withdrawal (Supplementary Fig. 2d,e). This suggests that a subpopulation of tumour cells enter a reversible tolerant state to defend against EGFR TKIs in the early stages of EGFR-targeted treatment.

To determine whether the tolerance observed was conferred by acquisition of the EGFR<sup>T790M</sup> mutation, we performed pyrosequencing for quantitative analysis of sequence variations in exons 19 and 20 of EGFR (Supplementary Fig. 3). We found that the exon 19 and 20 sequences of drug-tolerant cells were comparable to those of parental PC9 cells rather than EGFR<sup>T790M</sup>-positive gefitinib-resistant PC9 cells. This indicates that the tolerance strategy adopted by tumour cells against EGFR TKIs might be mediated by mechanisms other than acquisition of the EGFR<sup>T790M</sup> mutation. In further experiments, single HCC827 cells mixed with Geltrex were plated in individual wells of a 96-well plate and divided into two groups. After 24 h, half of the cells were treated with 100 nM osimertinib for 21 d (tolerant 3D structures) and the other half were treated with DMSO as a control (parental 3D structures) (Fig. 1c). We then examined the microscopic features of the resulting 3D structures by haematoxylin and eosin (H&E) staining in histology analysis. Parental 3D structures showed an adenocarcinoma-like structure. Unexpectedly, a ‘ring-like’ structure was found in the osimertinib-tolerant 3D structures (Fig. 1c). To characterise gene expression in these structures, we performed qRT–PCR analysis on parental and osimertinib-tolerant 3D structures derived from single HCC827 cells. Parental and osimertinib-tolerant 3D structures expressed comparable levels of CEACAM5 (Fig. 1d). However, osimertinib-tolerant 3D structures expressed twofold-lower levels of SFTPC and HOPX and up to twofold-higher levels of ID2 (Fig. 1d), suggesting that osimertinib-tolerant 3D structures are enriched for expression of stem cell–associated genes.

To better understand the transcriptomic changes conferring osimertinib or gefitinib tolerance in lung cancer, we generated single-cell-derived clones from the PC9 cell line (Fig. 1e). Single cells were sorted into individual wells of a 96-well plate by FACs. Beginning the following day, cells were treated with 0.1, 0.4 or 2 µM gefitinib or with vehicle for 14 d (n = 192 wells per group).
The frequency of colony formation was 8.3 ± 0.7% and 3.6 ± 0.3% in the vehicle-treated and all three gefitinib-treated groups, respectively (Fig. 1e). One parental single-cell-derived clone treated with vehicle that was sensitive to gefitinib and two drug-tolerant single-cell-derived clones treated with 0.4 μM gefitinib were randomly selected and applied in whole-transcriptome analysis by microarray. We identified genes with the greatest change in expression, finding upregulated expression of KRT17 (keratin 17), CA9 (carbonic anhydrase 9), WNT5A (Wnt family member 5A), EGLN3 (Egl-9 family hypoxia-inducible factor 3), SLC2A3 (solute carrier family 2, member 3) and LOX (lysyl oxidase), as well as downregulated expression of SPRY4 (sprouty RTK signalling antagonist 4) and IDH3A (isocitrate
Fig. 2 | miR-147b initiates drug tolerance. a, Heat map showing top upregulated and downregulated miRNAs in paired osimertinib-tolerant (OTR) and parental clones from the PC9 and HCC827 cell lines as determined by miRNA-seq analysis. b, qRT-PCR analysis of miR-147b levels in parental, recovered, and primary and secondary osimertinib-tolerant cells from the PC9 line. Parental tumour cells treated with 160 nM osimertinib for 6 d entered a drug-tolerant state (primary tolerant cells) and were subjected to brief therapy withdrawal of up to 18 d (recovered cells) followed by reinstate ment of the 160 nM dose for 11 d (secondary tolerant cells). The relative miR-147b level in parental cells was calibrated as 1. miR-423 was used as an endogenous control. n = 3 independent biological replicates. c, d, Viability of HCC827 cells expressing scrambled control (Scr) or overexpressing miR-147b after 3 d of treatment with osimertinib (Scr) or gefitinib (d). n = 3 independent biological replicates. e, Colony formation by HCC827 cells expressing scrambled control or overexpressing miR-147b and treated with osimertinib (40 nM) or gefitinib (40 nM); 20, 40 or 80 cells were plated per plate in 10-cm dishes. Colonies were stained with Glemsa on day 10 and the total number of colonies was quantified. n = 3 independent biological replicates. f, Viability of H1975 cells with miR-147b knockdown (anti-miR-147b) or expressing scrambled control (Ctrl) treated with osimertinib for 4 d. n = 3 independent biological replicates. g, Response of H1975 cells with miR-147b knockdown to treatment with 160 nM osimertinib. Left, monolayer colonies were treated for 10 d and stained with Glemsa. Right, 3D structures were treated for 14 d. –, vehicle; +, osimertinib. Scale bar, 1,000 μm. n = 4 independent biological replicates. Data are shown as the mean ± s.e.m. and were analysed by one-way ANOVA (b) or by unpaired two-tailed t test with Holm–Sidak’s correction (e,g).

We used Gene Ontology (GO) analysis to identify the signalling pathways most enriched for differentially expressed genes in gefitinib-tolerant clones in comparison to the parental clone, which included Wnt planar cell polarity (Wnt/PCP) signalling, the glutamine metabolic process, cellular response to hypoxia, cell cycle, the vascular endothelial growth factor receptor (VEGFR) signalling pathway, glutathione derivative biosynthesis, the TCA cycle, integrin-mediated signalling and PI3K signalling (Fig. 1g and Supplementary Table 3). The gene expression signatures corresponding to activated Wnt/PCP signalling and hypoxia response as well as inactivated glutamine metabolic process and TCA cycle were validated by qRT-PCR (Fig. 1h). Activated Wnt/PCP signalling has been linked to drug resistance in many studies35, but it was unexpected that activation of hypoxia responses and inactivation of metabolic processes, such as the glutamine metabolic process and the TCA cycle, were among the top signalling changes relevant to drug tolerance. Our data suggest that these pathways might cooperatively maintain a ‘tolerance signature’ in EGFR-mutant lung cancer cells when cells are exposed to EGFR TKI.

To exclude the possibility that preexisting cellular heterogeneity could be responsible for this tolerance, we generated single-cell-derived clones from the PC9 line and exposed them to 2 μM gefitinib. In parallel, as in the previous experiment, PC9 cells were cloned in the same concentration of gefitinib (parental clones treated with drug) as a control. All tested single-cell-derived clones (n = 4) had a frequency of gefitinib tolerance of approximately 1.9–2.1%, which was comparable to the frequency for the parental PC9 clone treated with the same drug (2.2 ± 0.1%) (Supplementary Fig. 4a). A similar frequency of osimertinib tolerance was found in comparison of single-cell-derived and parental clones (Supplementary Fig. 4a). In agreement with this, single-cell-derived and parental clones from the HCC827 line demonstrated comparable frequencies of osimertinib tolerance (Supplementary Fig. 4b). All our data strongly suggest that drug tolerance is spontaneously acquired rather than a reflection of preexisting cellular heterogeneity, in line with previous findings4. In addition, in comparison to PC9 cells tolerant to gefitinib (Fig. 1h), cells tolerant to osimertinib had similar expression of genes related to the hypoxia pathway and TCA cycle (Supplementary Fig. 4c,d). This suggests that lung cancer cells utilise similar strategies to protect themselves from drug-induced cytotoxicity when cells are treated with gefitinib and osimertinib. Collectively, our data have demonstrated that drug tolerance is acquired spontaneously by a small proportion of lung cancer cells.

miR-147b initiates tolerance to anticancer drugs. To test which microRNAs (miRNAs) might be linked to osimertinib tolerance, we performed miRNA-seq analysis in paired osimertinib-tolerant

1. dehydrogenase 3 (NAD+ alpha) (Fig. 1f and Supplementary Data 1).
As expected, our qRT–PCR analysis validated the up-to-fivefold upregulation of miR-147b levels in gefitinib- and osimertinib-tolerant cells as compared to parental cells in both the PC9 and HCC827 cell lines (Fig. 2b and Supplementary Fig. 5a). Furthermore, levels of miR-147b were decreased in cells from the PC9 cell line (Fig. 1f). Right, qRT–PCR analysis of levels of the predicted targets for miR-147b in H1975 cells with miR-147b knockdown as compared to parental cells in both the PC9 and HCC827 cell lines (Fig. 2b and Supplementary Fig. 5a). Furthermore, levels of miR-147b were decreased in cells from the PC9 cell line recovered upon drug tolerant cells undergoing osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d. miR-147b levels rose in the recovered cells when osimertinib withdrawal for 18 d.

Fig. 3 | A miR-147b–VHL axis mediates drug tolerance through impaired VHL activity. a, Left, gene candidates predicted as miR-147b targets by the TargetScan tool shown matched to signalling pathways enriched in the context of gefitinib tolerance in analysis of single-cell-derived clones from the PC9 cell line (Fig. 1f). Right, qRT–PCR analysis of levels of the predicted targets for miR-147b in H1975 cells with miR-147b knockdown as compared to cells receiving scrambled control. n = 3 independent biological replicates. b, Left, computational prediction of RNA duplex formation between miR-147b and the 3′ UTR of VHL mRNA. Mutations generated within the 3′ UTR for the luciferase assay are shown in red. Right, dual-luciferase reporter assay in miR-147b-overexpressing AALE cells. Firefly and Renilla luciferase activities were measured 48 h after cotransfection with miR-147b or control expression vector and a reporter construct encoding wild-type (WT) or mutant (Mut) VHL 3′ UTR. n = 3 independent biological replicates. c, Western blot analysis of the fold change in expression of hypoxia-related genes in AALE cells with miR-147b overexpression relative to cells receiving scrambled control and cells with overexpression of both miR-147b and VHL relative to cells receiving scrambled control. ACTB was used as an endogenous control. n = 3 independent biological replicates. e, Fractional viability of HCC827 cells treated with vehicle or osimertinib (20 nM) and transfected with miR-147b expression vector or VHL expression vector, alone or in combination. Cell viability was measured on day 3. The relative viability of cells treated with vehicle on day 3 was calibrated as 1. n = 7 independent biological replicates. Data are shown as the mean ± s.e.m. and were analysed by unpaired two-tailed t test (a–d) or by Kruskal–Wallis test (e).
100 nM osimertinib for 21 d. In agreement with the results in cell lines, miR-147b levels in osimertinib-tolerant PDX-tumour-derived 3D structures were up to fivefold higher than in parental 3D structures (n = 5 replicates for both the tolerant and parental structures) (Supplementary Fig. 5c). In addition, hypoxia-related genes, including ANGPTL4 (angiopoietin like 4), LOX, ENO1, LDHA (lactate dehydrogenase A), VEGEFA (vascular endothelial growth factor A) and SL2CA1 (solute carrier family member 1), were also upregulated in osimertinib-tolerant PDX-tumour-derived 3D structures (Supplementary Fig. 5d). To understand the effects of the culturing stages for 3D structures on the outcome of drug tolerance, we first generated established 3D structures (grown for 24 d) and then treated the structures with osimertinib for 21 d. We found that drug-tolerant cells derived from 3D structures on day 24 formed comparable structures and expressed similar levels of miR-147b and pseudohypoxia-related genes as cells derived from 3D structures on day 1 (Supplementary Fig. 5e-g). Thus, our data indicate that the culturing stage of 3D structures does not affect the outcome of drug tolerance. We then asked whether heterogeneity existed in the initial 3D structures with respect to expression of miR-147b and pseudohypoxia-related genes. To answer this question, we used the initial 3D structures established from single HCC827 cells on days 2, 4 and 6 to perform qRT–PCR analysis for expression of miR-147b and pseudohypoxia-related genes. We found that there was no significant difference with respect to expression of miR-147b and pseudohypoxia-related genes among the initial 3D structures (Supplementary Fig. 5h). Collectively, our data suggest that miR-147b levels are relevant to reversible drug tolerance.

EGFR and KRAS mutations are widely known to be mutually exclusive in patients with lung cancer, and mutations in KRAS are associated with a lack of sensitivity to gefitinib. EGFR-TKI-tolerant cells still responded to EGFR inhibitors at higher concentrations (Supplementary Fig. 2c), as they harboured the same EGFR-activating mutation as parental cells (Supplementary Fig. 3); thus, we hypothesised that miR-147b levels might be different in patients with mutated EGFR than in those with mutated KRAS family genes (KRAS, NRAS and HRAS). To examine this hypothesis, we performed whole-transcriptome RNA-seq analysis on a cohort of lung adenocarcinoma cell lines for miRNA profiles relevant to EGFR mutations by using a public dataset (Supplementary Table 5). We found that the top upregulated miRNAs included miR-147b, miR-936, miR-141, miR-559 and miR-200c in EGFR-mutant cell lines (n = 8) as compared to RAS-mutant cell lines (n = 17) (Supplementary Fig. 6a,b). In agreement with this, qRT–PCR analysis demonstrated that miR-147b levels in lung cancer cell lines with TKI-sensitizing EGFR mutations or EGFR mutations conferring resistance (n = 7) were higher than those in lung cancer cell lines with wild-type EGFR (n = 5) (Supplementary Fig. 6c and Supplementary Table 6). Interestingly, miR-147b levels in cancer cell lines with the EGFR<sup>770OM</sup> mutation (HCC827GR, PC9ER and H1975) were even higher than those in cell lines with TKI-sensitizing EGFR mutations (HCC827, H3255, PC9 and H1650) (Supplementary Fig. 6c).

In further analysis of lung adenocarcinoma PDXs, miR-147b levels in EGFR-mutant PDX tumours (176 ± 38) were up to fourfold higher than those in lung cancers with wild-type EGFR (54 ± 16; P < 0.05) (Supplementary Fig. 6d). This is consistent with our data for human lung cancer cell lines (Supplementary Fig. 6a). In further analysis of lung adenocarcinoma tissues in The Cancer Genome Atlas (TCGA) database<sup>12-13</sup>, the median read count for miR-147b in EGFR-mutant tumours (median = 1.16, n = 31) was 1.7-fold higher than in KRAS-mutant tumours (median = 0.68, n = 75; P = 0.2) (Supplementary Fig. 6e,f and Supplementary Table 7). The above data suggest that miR-147b might be a potent marker for EGFR-mutant lung cancers.

Furthermore, to study the functional roles of miR-147b in regulating drug tolerance, we overexpressed miR-147b from a lentiviral vector in HCC827 cells. We found that enforced overexpression of miR-147b enhanced drug tolerance by 60-fold and 30-fold when cells were treated with the half-maximal inhibitory concentration (IC<sub>50</sub>) of osimertinib and gefitinib, respectively (Fig. 2c,d). As expected, miR-147b overexpression in HCC827 cells rescued the decreased colony formation resulting from treatment with osimertinib or gefitinib (Fig. 2e). Conversely, knockdown of miR-147b with the pLenti-III-miR-Off construct by lentiviral infection of H1975 cells increased sensitivity to osimertinib by 166-fold when cells were treated at the IC<sub>50</sub> (Fig. 2f). As expected, miR-147b knockdown almost completely abolished drug-tolerant colonies and 3D structures in the presence of osimertinib within 12–21 d (Fig. 2g). This finding suggests that miR-147b is critical for regulating drug tolerance. Furthermore, in spheroid-formation assays and limiting dilution analysis, knockdown of miR-147b decreased the frequency of tumour-initiating cells (TICs) by sevenfold from 1 in 11.8 (8.5%) to 1 in 83.1 (1.2%) (Supplementary Fig. 7a-c). In line with this, miR-147b knockdown decreased the expression of stem cell–associated genes in the Wnt/PCP signalling pathway, as determined by qRT–PCR analysis, including that of WNT5A, FZD2 and FZD7<sup>24</sup> (Supplementary Fig. 7d). In addition, miR-147b knockdown also led to downregulated expression of SL2CA3 and LOX as well as upregulated expression of SPRY4 and IDH3A (Supplementary Fig. 7d). This dysregulated gene expression profile is consistent with the genes deregulated in drug-tolerant cells (Fig. 1f). Furthermore, by using a CRISPR–Cas9 approach, we knocked out MIR147B in H1975 cells (Supplementary Fig. 8a) and found that MIR147B knockout could consistently reduce cell viability in 3D structures and decrease osimertinib tolerance in H1975 cells, similarly to the earlier findings in knockdown experiments using the miR-147b lentiviral inhibitor (Supplementary Fig. 8b-d). Thus, tolerance to EGFR TKIs is conferred by miR-147b.

A miR-147b–VHL axis confers drug tolerance. To study which genes are directly repressed by miR-147b, we performed sequence-based target prediction with the TargetScan tool. The predicted targets were then analysed to determine their correspondence to the signalling pathways for drug tolerance (Fig. 3a). Among the predicted targets for miR-147b, VHL and SDHD were the top two most upregulated genes upon miR-147b knockdown in H1975 cells (Fig. 3a). These genes correspond to the cellular response to hypoxia and TCA cycle signalling pathways, respectively (Fig. 3a). However, expression of other predicted targets relevant to the tolerance gene signature, including ISCU (iron–sulfur cluster assembly enzyme) and TCEA3 (transcription elongation factor A3; involved in cellular response to hypoxia) as well as NDUFA4 (NADH dehydrogenase (ubiquinone) 1 alpha, subcomplex 4, 9 kDa; involved in the TCA cycle), was not significantly upregulated in cells with miR-147b knockdown (Fig. 3a). This indicates that VHL and SDHD are potential targets of miR-147b in the context of drug tolerance.

We next designed a dual-luciferase assay, based on the VHL 3′ UTR, that used a reporter construct with wild-type sequence as well as a reporter in which the predicted miR-147b-binding site was mutated (Fig. 3b). We found that luciferase activity from the wild-type VHL 3′ UTR was downregulated when miR-147b was overexpressed in AALE cells. However, luciferase activity from the mutant 3′ UTR did not change upon overexpression of miR-147b (Fig. 3b). We then asked whether miR-147b was more likely to be experimentally validated than the other top candidate VHL-regulating miRNAs predicted by the TargetScan tool (Supplementary Fig. 9a). We performed correlation analysis of VHL and noncoding gene expression in 60 human lung adenocarcinoma cell lines by using available RNA-seq data<sup>31</sup>. We found that miR-147b, among the miRNAs, had the strongest negative correlation between its levels and VHL expression (r = -0.34, P = 0.002), supporting our findings that miR-147b can negatively regulate VHL (Supplementary Fig. 9bc and Supplementary Table 8).
Fig. 4 | A miR-147b–SDHD axis mediates drug tolerance through SDH enzyme activity in the TCA cycle. a. Left, computational prediction of RNA duplex formation between miR-147b and the 3' UTR of SDHD mRNA. Mutations generated within the 3' UTR for the luciferase assay are shown in red. b. Principal-component analysis of parental H1975 cells, osimertinib-tolerant cells (H1975OTR) and osimertinib-tolerant cells with miR-147b knockdown. c. Levels of succinate, 2-oxoglutarate, fumarate and malate in parental H1975 cells, osimertinib-tolerant H1975OTR cells and osimertinib-tolerant H1975OTR cells with miR-147b knockdown. d. Levels of succinate, 2-oxoglutarate, fumarate and malate in parental H1975 cells, osimertinib-tolerant H1975OTR cells and osimertinib-tolerant H1975OTR cells with miR-147b knockdown. The relative level in parental H1975 cells was calibrated as 1.

b. Data are shown as the mean ± s.e.m. and were analysed by unpaired two-tailed t test (a,f) or by one-way ANOVA (d).
Furthermore, we checked VHL protein levels in miR-147b-overexpressing AALE cells, finding that VHL levels were decreased by only twofold when miR-147b was overexpressed (Fig. 3c). In the cytoplasm, an E3 ubiquitin ligase complex containing the VHL tumour-suppressor protein targets HIF-1α for destruction in the presence of oxygen36. Loss of VHL function thus leads to alterations in numerous transcriptional programs directly regulated by HIF-1α, resulting in changes in cellular metabolism and induction of angiogenesis independently of oxygen levels36. We hypothesised that the changes required for miR-147b-induced pseudohypoxia depend on the activity of VHL. To test this hypothesis, we overexpressed VHL in miR-147b-overexpressing AALE cells. As expected, gain of function of VHL decreased the expression of pseudohypoxia-related genes induced by miR-147b. Genes with perturbed expression included CA9, ANGPTL4, LOX, FOSL1 (FOS-like 1, AP-1 transcription factor subunit), PDK1 (pyruvate dehydrogenase kinase 1), COLA46 (collagen type IV alpha 6 chain), ENO1 (enolase 1), FAM83B (family with sequence similarity 83, member B), LDHA, ALDOA (aldolase, fructose-bisphosphate A), NDRG1 (N-Myc downstream regulated 1), VEGFA and SDCl (syndecan 1) (Fig. 3d). Further, functional assays showed that the enhanced osimertinib tolerance resulting from miR-147b overexpression was reduced upon VHL overexpression in HCC827 cells (Fig. 3e). Taken together, these data indicate that the activity of VHL in repressing expression of the pseudohypoxia gene signature mediates drug tolerance initiated by miR-147b.

**TCA pathways mediate drug tolerance and depend on miR-147b.** In addition to the functional roles of the VHL-mediated pseudohypoxia gene signature in drug tolerance, we hypothesised that another predicted target of miR-147b, SDHD, might also mediate the drug tolerance induced by miR-147b through its impact on the TCA cycle. To test this hypothesis, we first designed a dual-luciferase assay, based on the SDHD 3′ UTR, that used a reporter construct with wild-type sequence or a mutant reporter in which the predicted miR-147b-binding site was mutated (Fig. 4a). We found that luciferase activity from the wild-type but not the mutant 3′ UTR reporter was downregulated upon overexpression of miR-147b in AALE cells (Fig. 4a). This strongly suggests that SDHD is a direct target repressed by miR-147b. SDHD, one of the subunits of the SDH complex, catalyses the conversion of succinate to fumarate and regulates both the TCA cycle and the electron transport chain. We asked whether the drug tolerance mediated by the miR-147b–SDHD axis could have an impact on metabolite levels in metabolic pathways. To answer this question, we used the H1975 lung adenocarcinoma cell line, which harbours *EGFR*<sub>790M</sub> and *EGFR*<sub>L858R</sub> mutations, for a metabolomics study. Cells with either the *EGFR*<sub>790M</sub> or the *EGFR*<sub>L858R</sub> mutation were sensitive to osimertinib. Osimertinib-tolerant cells (H1975OTR) were derived from parental H1975 cells treated with 100 nM osimertinib for 21 d in monolayer cultures. H1975OTR cells are stable and continue to proliferate even in the presence of 100 nM osimertinib. As a control, H1975 cells were treated with vehicle for 21 d. We then performed a liquid chromatography and mass spectrometry (LC–MS) metabolomics study by using paired H1975 and H1975OTR cells (Fig. 4b). Analysis of metabolite levels demonstrated that metabolites in both the TCA cycle and electron transport chain–related redox reactions had perturbed levels in drug-tolerant cells. We observed up to a twofold increase in succinate and 2-oxoglutarate levels but up to a twofold decrease in fumarate and malate levels in drug-tolerant cells (Fig. 4c,d, Supplementary Fig. 10a and Supplementary Table 9). In addition, the levels of oxidized nicotinamide adenine dinucleotide (NAD<sup>+</sup>) were decreased by 26% in drug-tolerant cells as compared to parental cells (Supplementary Fig. 10a). This is consistent with a previous finding showing that decreased NAD<sup>+</sup> levels induced a pseudohypoxia state in ageing37. Furthermore, levels of reduced glutathione (GSH), the master antioxidant, were decreased by 86% in drug-tolerant cells as compared to parental cells (Fig. 4c). Our data suggest that the metabolic changes in the TCA cycle might be important in regulating drug tolerance. We then asked whether the perturbations in metabolite levels could be rescued by blocking miR-147b in drug-tolerant cells. To address this question, we knocked down miR-147b in drug-tolerant cells from the H1975 line and analysed metabolic changes with LC–MS. As expected, the increased levels of succinate and 2-oxoglutarate, as well as the decreased levels of metabolites such as fumarate, malate, NAD<sup>+</sup> and GSH, were partially rescued by knocking down miR-147b in osimertinib-tolerant cells (Fig. 4c–e, Supplementary Fig. 10a and Supplementary Table 9). These data validate our hypothesis that one role of miR-147b in drug tolerance is repression of the enzyme activity of the SDH complex.

We then asked whether the metabolic changes in monolayer cultures were reproducible in 3D structure models. To address this...
question, we established drug-tolerant and parental 3D structures by continuous treatment of H1975 cells with 100 nM osimertinib or vehicle for 21 d and performed an LC–MS metabolomics study. In line with our results in monolayer cultures, levels of fumarate, malate and NAD\(^+\) were reduced in osimertinib-tolerant 3D structures. Knockdown of miR-147b rescued the decreased levels of these metabolites in osimertinib-tolerant 3D structures (Supplementary Fig. 10b,c). Our data suggest that metabolic changes due to the decreased SDH activity mediated by miR-147b might underlie drug tolerance (Fig. 4e). To further examine the functional roles of SDH activity in drug tolerance, we treated H1975 cells with membrane-permeable dimethyl malonate (DMM), an inhibitor of SDH, in the presence of 100 nM osimertinib and found that DMM effectively increased tolerance to osimertinib (Fig. 4f). Collectively, our data have demonstrated that repression of SDH activity by miR-147b mediates osimertinib tolerance in lung cancer.

**Blocking miR-147b overcomes drug tolerance.** We asked whether miR-147b is a druggable target in lung cancer. First, we knocked down miR-147b with a lentivirally expressed miRNA inhibitor in H1975 cells and transplanted these cells into nude mice. Tumour growth in the cohort with miR-147b knockdown was up to twofold slower than in the control group (Supplementary Fig. 11a,b). This indicates that blocking miR-147b inhibits tumour growth in vivo.
To further understand the functional roles of miR-147b in mediating drug tolerance via regulation of pseudohypoxia signalling, we blocked miR-147b by administration of a locked nucleic acid (LNA) miRNA inhibitor and perturbed pseudohypoxia signalling with small-molecule activators and inhibitors. We found that treatment with LNA-miR-147b inhibitor increased the sensitivity of drug-tolerant 3D structures derived from the H1975 cell line to osimertinib by 30-fold in comparison to the control group (Supplementary Fig. 11c,d). The small molecule dimethylxaloylglycerine (DMOG) has been reported to activate a pseudohypoxia response by repressing the negative regulator PHD2. As expected, treatment with a single dose of 10 µM DMOG resulted in upregulated expression of pseudohypoxia-related genes in H1975 cells (Supplementary Fig. 11e). Further functional assays demonstrated that treatment with DMOG in the context of miR-147b inhibition rescued the reduced osimertinib tolerance caused by the LNA-miR-147b inhibitor (Fig. 5a).

In agreement with this functional rescue experiment, the reduced levels of expression of pseudohypoxia-related genes resulting from miR-147b knockdown were significantly reduced by simultaneous treatment with DMOG in H1975-derived 3D structures (Fig. 5b). We hypothesised that blocking the pseudohypoxia signalling pathway with small molecules might further enhance the drug sensitivity induced by the miR-147b inhibitor. To examine this possibility, we applied another small molecule, R59949, which inhibits pseudohypoxia responses through activation of PHD2. We confirmed that treatment with a single dose of 30 µM R59949 resulted in down-regulated expression of pseudohypoxia-related genes in comparison to vehicle treatment in H1975 cells (Supplementary Fig. 11f). Administration of both R59949 and LNA-miR-147b inhibitor also resulted in stronger inhibition of tolerance to EGFR TKI than treatment with LNA-miR-147b inhibitor alone (Fig. 5c). In line with this finding, the reduced expression levels of pseudohypoxia-related genes resulting from treatment with LNA-miR-147b inhibitor were reduced by simultaneous treatment with R59949 in H1975 cells (Supplementary Fig. 11g). This finding strongly supports our idea that miR-147b and miR-147b-induced pseudohypoxia signalling could be targeted with drugs to overcome osimertinib tolerance in lung cancer.

To understand the roles of HIF-1 and HIF-2 in osimertinib tolerance, we knocked down HIF1A and HIF2A (also known as EAP51 (endothelial PAS-domain protein 1)) by using lentivirally expressed short hairpin RNAs (shRNAs) in H1975 cells and investigated the effect on osimertinib response. We found that HIF1A knockdown increased cell sensitivity to osimertinib by up to 2.6-fold (Fig. 5d,e). However, HIF2A knockdown did not significantly change sensitivity to osimertinib (Supplementary Fig. 12a,b). To better understand whether gain of HIF-1 function is sufficient to induce a tolerant state, we overexpressed constitutively active HIF-1α (generated by introduction of the HIF1AASR7 mutation) in H1975 cells. As expected, overexpression of constitutively active HIF-1α increased tolerance to osimertinib by up to twofold (Fig. 5f). Thus, our results have now demonstrated that HIF-1α rather than HIF-2α activity is sufficient to induce an osimertinib-tolerant state.

Finally, we asked whether we could delay tolerance to EGFR TKIs by targeting miR-147b. To address this question, we tested 3D structures derived from PDX lung tumours. Among these tumours, the 3D structures derived from a tumour with an EGFR T790M mutation (PDX_LU_10) at passage 2 were tested in a functional study (Fig. 5g). We established 3D structures of medium size 1 week after seeding single cells into 3D cultures, recording this time point as day 0 before the administration of LNA or osimertinib. As expected, PDX-tumour-derived 3D structures in the vehicle-treated group increased in volume by up to tenfold within 14 d (Fig. 5g). With administration of 25 nM osimertinib to the 3D structures on days 1 and 4, the size of the tumour structures was decreased by 50% on day 6 and then started to recover gradually, with a 40% increase on day 14 compared with day 6. To test whether early perturbation of miR-147b could delay tolerance to osimertinib, we pretreated the 3D structures with 90 nM LNA-miR-147b inhibitor on day 0 and repeated the treatment on day 2. These pretreatments with LNA-miR-147b inhibitor decreased osimertinib tolerance on day 8 by 80% in comparison to control structures. Furthermore, the volume of these 3D structures was no more than 10% greater than that of control structures treated with the single agent osimertinib from day 8 to day 14 (Fig. 5h). Our data suggest that early treatment of EGFR-mutant lung cancer with miR-147b inhibitor might delay tolerance to EGFR TKIs in comparison to treatment with EGFR TKI alone.

Discussion
Dysregulated metabolism in cancer has recently gained attention for its potential role in promoting therapeutic resistance through a novel drug-tolerance strategy54. Here we have demonstrated that blocking miR-147b and reactivation of the TCA cycle represents a promising strategy to prevent drug-tolerance-mediated tumour relapse (Fig. 5i).

NSCLC cells have higher rates of both glycolysis and glucose oxidation than adjacent benign lung tissue46,47. Increasing evidence suggests that the metabolic enzymes of the TCA cycle and the metabolites they catalyse, such as isocitrate dehydrogenase, SDH and succinate, are involved not only in tumourigenesis but also in drug resistance48,49. In our study, the reciprocal changes we observed in TCA cycle metabolite levels when cells acquired osimertinib tolerance, including increased levels of succinate and 2-oxoglutarate and decreased levels of malate and fumarate, indicate that silencing of SDH activity is linked to drug tolerance. In addition, silencing by DMM, a small-molecule inhibitor of SDH activity, could enhance drug tolerance, in agreement with the function of miR-147b overexpression in drug-tolerant cells. It is not surprising that accumulation of succinate due to loss of function of SDH could activate pseudohypoxia signalling by repressing PHD2, as this has been reported previously50. Activation of pseudohypoxia signalling pathways by succinate is consistent with our findings that the miR-147b–SDH axis could increase expression of genes in pseudohypoxia signalling pathways. It has also been reported that miR-147b overexpression can protect cells from hypoxia-induced injuries41, and a hypoxia response has been linked to tumour cell survival and drug resistance in many cancers51,52. Other factors in our dataset might also activate pseudohypoxia responses and drug tolerance, including VHL inactivation, reduced NAD+ and decreased GSH, in line with previous findings49,53,64. In addition, these pseudohypoxia responses may further perturb the TCA cycle and cooperatively regulate drug tolerance.

Certain miRNAs regulate tumour cell growth as oncomiR46–50. We have demonstrated that higher miR-147b levels are linked to activating EGFR mutations in both cancer cell lines and lung cancer PDX tumours. This suggests that miR-147b might promote tolerance to EGFR TKIs either through reactivation of the EGFR downstream signalling pathway or by activation of another receptor tyrosine kinase that sustains downstream signalling despite inhibition of EGFR55. Another study supports our hypothesis by showing that miR-147 reverses resistance to EGFR inhibitor by inducing a mesenchymal-to-epithelial transition in KRAS-mutant cancers56.

VHL binds directly to hydroxylated AKT and inhibits AKT activity53, suggesting that the miR-147b–VHL axis might confer drug tolerance by activating AKT. In addition to noncoding RNAs, upstream transcription factors can also control VHL activity. For example, ID2, which is enriched in osimertinib-tolerant 3D structures in our study, can bind to the VHL ubiquitin ligase complex, displacing VHL-associated cullin 2, and impairing HIF-2α ubiquitination and degradation41. The cross-talk between miR-147b and transcription factors regulating VHL needs to be studied further in the future.
Articles

Our findings have shown that tumour cells protect themselves with a drug tolerance mechanism when they are treated with EGFR TKIs. These findings align with previous experimental data showing that tumour cells from lung and other cancers enter into a tolerant state when treated with TKIs. Tolerant cells may either acquire resistance-conferring mutations in EGFR or evolve into resistant cells over time by acquiring resistance-conferring mutations in EGFR. Whether cells tolerant to each generation of EGFR TKIs are derived from the same subpopulation of cells needs to be investigated in the future. In addition, we found that tolerant cells are slow cycling and are enriched for expression of stem cell–associated genes in the Wnt/PCP signalling pathway, such as Wnt5a, Fzd2 and Fzd7. These findings are conceptually similar to a recent report that drug-transition to stable resistance consists of de-differentiation. It is also not clear whether drug-tolerant cells and TICs have the same cellular origin. TICs display heterogeneous phenotypes owing to the presence of cells of different genotype in tumours. Thus, genetic background, such as mutations in EGFR and Ras, needs to be taken into consideration to better understand the association between drug-tolerant cells and TICs in the future.

In summary, we have demonstrated that cancer cells adopt a tolerance strategy to defend against EGFR inhibition that is mediated by increased levels of mRN-147b, leading to disruption of the TCA cycle and activation of pseudohypoxia responses. Targeting mRN-147b and the relevant downstream TCA cycle and/or hypoxia pathways could provide a new strategy to prevent resistance to anticancer drugs.

Methods

Cell culture. Human lung cell lines with wild-type EGFR (H358, H460, A549, H1299 and H69, ATCC) as well as EGFR-mutant cell lines (H1150, H1975, HCC827, HCC827GR, PC9, PCER and H3235; provided by S. Kobayashi, Beth Israel Deaconess Medical Center of Harvard Medical School) were cultured in DMEM (high glucose; Gibco, cat. no. 11995-065) supplemented with 10% FBS (Sigma-Aldrich, cat. no. F2442), 2 mM l-glutamine and 1% penicillin–streptomycin. Immortalised tracheobronchial epithelial cells (AALE; provided by W. C. Hahn, Dana-Farber Cancer Institute of Harvard Medical School) were derived as previously described and maintained in SAGM medium (Lonza, cat. no. CC-3118). Primary 3D cultures of a lung cancer PDX TUMOR (PDX_1U_10) were established at BIDMC of Harvard Medical School and maintained in advanced DMEM/F12 (Gibco, cat. no. 12654-010) supplemented with 1× Glutamax (Gibco, cat. no. 35050-061), 1× HEPES (Gibco, cat. no. 15630-080), 1.25 mM N-acetylcysteine (Sigma-Aldrich, cat. no. A9165), 10 mM nicotinamide (Sigma-Aldrich, cat. no. N0626), 10 µM forskolin (Sigma-Aldrich, cat. no. F6866), 1× B27 (Gibco, cat. no. 17504-044), 5 mg/ml Noggin (Sigma-Aldrich, cat. no. PHC1506), 100 ng/ml FGF10 (Gibco, cat. no. PHG0204), 20 ng/ml FGF2 (Gibco, cat. no. PHG0026), 50 ng/ml EGF (Gibco, cat. no. 10605-HNAE-250), 10 ng/ml PDGFα (Gibco, cat. no. PHG0035), 10 ng/ml FGF7 (Gibco, cat. no. PHG0094), 1% penicillin–streptomycin and 10 µM V-27632 (Selleck Chemicals, cat. no. S1049). Each cell line was maintained in a 5% CO2 atmosphere at 37°C. Cell line identities were confirmed by STR fingerprinting and all were found to be negative for mycoplasma with a MycoAlert kit (Lonza).

Ethical approval. All tissues were obtained under written informed patient consent and were de-identified. Only de-identified human tumour samples implanted in immunodeficient mice (Jackson Laboratory, strain no. NOG.Cg-Pkrdc<sup>−/−</sup> Il2rg<sup>−/−</sup>/SzJ, stock no. 005557; homozygous for both the Pkrdc<sup>−/−</sup> and Il2rg<sup>−/−</sup> alleles) were used as part of commercial production of the PDX at the Jackson Laboratory or implanted as part of this study at the Yale Cancer Center. No littermate controls were used. To generate xenograft tumours from H1975 cells with mR147b knockdown, 4–to-6-week-old female nude mice were used for subcutaneous injections at the Jackson Laboratory. For subcutaneous xenograft tumour assays, 100,000 cells in serum-free medium and growth factor reduced Matrigel (BD, cat. no. 354230; 1:1 ratio) were inoculated into the flanks of nude mice. Xenograft tumour formation was monitored with calipers twice a week by researchers blinded to mouse identity. Recipient mice were monitored and killed when tumours reached 1 cm in diameter or mice had lost 15% of their starting weight. Further details appear in the Nature Research Reporting Summary.

Patient-derived-xenograft tumour specimens. Patient tumour material was obtained through a network of collaborating cancer research centres to develop patient-derived xenograft (PDX) Resource models. All experimental protocols were reviewed and approved by the appropriate institutional animal care and use committees (IACUCs) with necessary IRB approvals and patient consents to allow tumour tissue to be used in research. These responsibilities are acknowledged in the JAX PDX Consortium Agreement, which was signed by the participating member prior to submitting the first patient specimen for model development. The JAX PDX Resource provides patient tumours as xenografts, with all personal information removed. No JAX investigator has access to patient consent forms. Successful PDX models were assigned a unique number with all reference to the donating centre and date of specimen collection removed to further protect patient privacy. Four of the six patients in the JAX PDX Resource did not receive any chemotherapy or radiotherapy before tissue collection. The other two patients received chemotherapy and radiotherapy prior to specimen collection. In addition, patients with advanced NSCLC who developed progression after initial response to EGFR TKI were consented and enrolled to a protocol approved by the Yale University IRB, in accordance with ethical guidelines, allowing the collection and analysis of clinical data, fresh tissue and the generation of PDXs. All Yale PDX models are publically available through the PDX Portal hosted by the Mouse Tumor Biology Database (MTB; http://www.tumorinformatics.jax.org/mtbw/pdxSearch.do). Tumour samples from PDXs were generated at the Jackson Laboratory and the Yale Cancer Center by subcutaneous implantation of previously passaged tumours in up to five female NSG mice. When tumour samples reached a volume of 1,000 mm<sup>3</sup>, they were shipped to the laboratory in frozen medium consisting of DMEM supplemented with 90% FBS and 10% DMSO on dry ice. Samples were washed three times with cold PBS with antibiotics (Sigma-Aldrich, cat. no. A9595), chopped with a sterile blade and incubated in 0.001% D/Nase (Sigma-Aldrich, cat. no. D4513), 1 mg/ml collagenase/disperse (Sigma-Aldrich, cat. no. 11097113001), 200 µl/µl penicillin, 200 µg/ml streptomycin and 0.5 µg/ml amphotericin B (2% antibiotics, Sigma-Aldrich, cat. no. A9595) in DMEM/F12 medium (Gibco, cat. no. 11320-033) in a 37°C water bath for 3 h with intermittent shaking. After incubation, suspensions were repeatedly triturated, passed through 70-µm and 40-µm strainers (BD Falcon, cat. no. 352355) and centrifuged at 1200×g for 5 min at 4°C. Resulting pellets were resuspended in red blood cell lysis buffer (eBioscience, cat. no. 14-4333-57) for 4 min at room temperature with intermittent shaking before resuspension in serum-free medium. After lysis, cell viability was evaluated by trypan blue exclusion. Live single cells accounted for 90% of the whole population and dead cells accounted for less than 10%. Each tumour sample yielded 1×10<sup>4</sup> to 1×10<sup>5</sup> cells, depending on the sample size.

Antibodies. For immunofluorescence staining, primary mouse anti-human ZO-1 (1:100 dilution; clone no. Z0-1A12, lot no. Q1215880, cat. no. 33-9100) was bought from Thermo Fisher Scientific. Secondary donkey anti-mouse IgG conjugated with Alexa Fluor 488 (1:500 dilution; lot no. A27037) was bought from Life Technologies. For western blot analysis, primary polyclonal rabbit anti-VHL antibody (1:100 dilution; lot no. RD2197039, cat. no. PA5-27322) was bought from Thermo Fisher Scientific. Mouse anti-β-actin antibody (1:5000 dilution; clone no. C4, lot no. H1018, cat. no. sc-77787, Santa Cruz Biotechnology) was used for a load control. Goat-anti-mouse IRDB-680D (1:1000 dilution; lot no. C7000604-06, cat. no. LI-COR826-68171, LI-COR Biosciences) and IRDB-800CW goat anti-mouse (1:20,000 dilution; lot no. C705310-02, cat. no. LI-COR827-80364, LI-COR Biosciences) antibodies were used as secondary antibodies.

Spheroids and 3D structures. For spheroid formation, single-cell suspensions (10,000 cells/well) were plated in six-well ultra-low-attachment (Corning) or non-attachment (Nunc) cell culture plates in DMEM/F12 medium (Gibco, cat. no. 11320-033) supplemented with 10% horse serum, 15 mM HEPES, 1 mg/ml NaHCO<sub>3</sub>, 0.6% glucose, 1% NEAA, 4 mg/ml BSA (Sigma-Aldrich, cat. no. A9799), ITS (0.05 mg/ml insulin-transferrin-selenium; Gibco, cat. no. A41004045), 1% antibiotics (Sigma-Aldrich), 50 mg/ml EGF and 20 ng/ml FGF2 (Invitrogen). Medium was replaced every 3 days. Spheroids were cultured for 10–14 days and then quantified. For passage, spheroids were digested with accutase (Chemicon, cat. no. SCR005) into single cells and replated into the above plates. For limiting dilution assays, 200, 600 and 1,800 cells were plated into an eppendorf tube spheroid formation.
For 3D structure formation, single-cell suspensions (2,000 cells/well in 20 μl) were plated with Geltrex (25 μl) in 96-well non-treated clear plates (Corning, cat. no. 08-772-53). Plates were incubated for 20 min at 37 °C followed by addition of 100 μl of complete growth medium. Complete growth medium consisted of advanced DMEM/F12 supplemented with 1× Glutamax, 1× HEPS, 1.25 mM N-acetylcycteine, 10 mM nicotinamide, 10 μM forskolin, 1× B27, 5 μg/ml Noggin, 100 ng/ml FGF10, 20 ng/ml FGF2, 50 ng/ml EGF, 10 ng/ml PDGF, 10 ng/ml FGF7, 1% penicillin–streptomycin and 10 μM Y-27632. Y-27632 was used for the first 3 d because Y27632 is a ROCK inhibitor that prevents apoptosis of single cells. PDGFA and FGF7 were not used until day 7 in 3D structure cultures because they are important for alveolarisation during late lung development64. FGF10 is essential for maintenance of lung progenitor cells and branching morphogenesis as well as tissue homeostasis in the adult lung65. EGF and FGF2 are mitogens for growth of epithelial cells and were previously used by us to maintain lung TBG66. Noggin and bFGF4 inhibits the hypothalamic–pituitary axis involved in development of the lungs67. Medium was changed every 3 d for 24 d. 3D structures were photographed with a microscope (Evos FL, Life Technologies) and size was measured with ImageJ 1.31s software.

Colony-formation assays in plates. Single cells were plated in 10-nm dishes in triplicate with 20, 40, 80 or 300 cells per dish. Medium was replenished every 3 d. Cells were incubated for 10–12 d followed by staining with Giemsa (Sigma–Aldrich). Plates were air dried, photographs were taken, and the total number of colonies was analysed with openCFU (http://openfcu.sourceforge.net).

Single-cell-derived clones of PC9 and HCCR827 cells. For the PC9 and HCCR827 cell lines, single cells were sorted into individual wells of a 96-well plate by FACS with a FACSaria (BD). The presence of only one cell per well was confirmed under a microscope 12 h after sorting. Gefitinib or osimertinib was administrated to both parental clones and single-cell-derived clones. For parental clones, single cells were treated immediately with 0.1, 0.4 or 2 μM gefitinib, osimertinib or vehicle for 14 d on the same day. Subsequent single-cell-derived clones were generated first and then exposed to 0.1–2 μM gefitinib, osimertinib or vehicle for 14 d. The drug response of the surviving clones was determined by measuring the IC50. The frequency of colony formation was calculated as the ratio of the total number of colonies (consisting of more than 50 cells) to the total number of wells plated with a single cell. Medium and small-molecule inhibitors were replenished every 3 d. One parent cell-derived clone was randomly selected and applied to whole-transcriptome analysis by microarray. Four single-cell-derived clones each from the PC9 and HCCR827 lines established as described above were used for drug tolerance assays.

Compounds. Osimertinib (529729) and gefitinib (S1025) were purchased from Selleck Chemicals. DMOG® (cat. no. 400091) was from Calbiochem. RS9949 (ref. 20) (cat. no. D5794) and DMM® (cat. no. 136441) were purchased from Sigma–Aldrich.

Treatment with compounds. Cell viability experiments were performed in 96-well format by using opaque white plates (Corning). For 2D monolayer cell cultures and 3D structures, cells were plated into 96-well plates with 100–2,000 cells per well in three to four replicates on day 0. Twenty-four hours after seeding, cells or 3D structures were exposed to compounds at the indicated concentrations for 72 h. Cellular ATP levels (as a surrogate for viability) were measured by using CellTiter-Glo (cat. no. G7570, Promega) or CellTiter-Glo 3D (cat. no. G9681, Promega). For co-treatment experiments, spent medium was removed 24 h after cell seeding and replaced with medium containing a single concentration of the modulator of interest (for example, osimertinib).

To establish gefitinib- or osimertinib-tolerant cells, we treated single cells from the PC9 line with 20 nM osimertinib and 40 nM gefitinib for 12–14 d. HCCR827 cell monolayers and 3D structures were treated with 20–160 nM osimertinib for 12–21 d, and H1975 cell monolayers and 3D structures were treated with 25 nM–1 μM osimertinib for 12–21 d. To study the effects of 3D structure culture stages on the outcome of drug tolerance, both single cells (grown for 1 d) and established 3D structures (grown for 24 d) from the HCCR827 line were generated, and this was followed by treatment with 100 nM osimertinib for an additional 21 d. Medium was replenished every 3 d.

RNA extraction and RT–PCR. Total RNA was extracted from solid tissues and cultured cells with the mirVana miRNA Isolation Kit (Ambion, cat. no. AM1561) according to the manufacturer’s instruction. Ten nanograms of RNA per sample was used as input for consecutive reactions including poly(A) tailing, ligation, reverse transcription and miR-Amp reaction with a TaqMan Advanced miRNA Assay (Applied Biosystems, cat. no. A28007). miRNA levels were then assessed by TaqMan Advanced miRNA Assay with TaqMan Fast Advanced miRNA master mix (Applied Biosystems, cat. no. 444457). Each PCR plate was run in an RT–PCR instrument (Roche LightCycler 480 System). The sequences for TaqMan probes for the mirVana miRNA Isolation kit (Ambion, cat. no. AM1561) were used for all samples with 200 ng of total RNA as starting material. Briefly, total RNA extraction and RT–PCR. Whole-transcriptome raw data were obtained from GenomeStudio software (version 2011.1) with subtraction of background. All mRNA raw data were normalised according to the Cross-Correlation method4. mRNA expression of mRNAs was evaluated with TaqMan probes (Applied Biosystems). The sequences for TaqMan miRNA and gene expression probes are shown in Supplementary Table 1. hsa-mir–423-5p and ACTB or GAPDH were used as endogenous controls for analyses of miRNA and gene expression, respectively.

Pyrosequencing for quantitative analysis of sequence variations. DNA was extracted from parental cells, gefitinib-tolerant cells and gefitinib-resistant cells from the PC9 line (QiAamp DNA Blood mini kit, cat. no. 51104, Qiagen) and analysed by pyrosequencing according to the manufacturer’s protocol. Ten nanograms of input DNA was amplified with the PyroMark PCR kit (Qiagen) and biotinylated primers (EGR exon 19: forward, 5′-GCACTTGCCACATCTCA-3′; reverse, 5′-AAAGGGTGGCCTGAGGTT-3′; EGR exon 20: forward, 5′-ATGGCAGCGTGGACAC-3′; reverse, 5′-TTTTGTGTTTCCGGACATAGTC-3′) in the following PCR program: 95°C for 2 min; 40 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 5 min. PCR products were incubated with streptavidin Sepharose beads (GE Healthcare) and PyroMark Binding Buffer (Qiagen) at room temperature for 30 min, and this was followed by hybridisation to sequencing primers (exon 19 deletion, 5′-ATTCGTCGTCGTCACT-3′; exon 20 T790M, 5′-GATGCCCGACGAGGCGG-3′). Pyrosequencing reactions were performed on the PyroMark Q24 platform (Qiagen) by using PyroMark Gold Q24 reagents (cat. no. 979082, Qiagen). Pyrosequencing results were analysed with PyroMark Q24 software. Samples with more than 5% mutant alleles were scored as positive.

High-throughput sequencing. Total RNA (1 μg) was processed by LC Sciences for miRNA-seq. All RNA samples were analysed for quality on an Agilent 2100 Bioanalyzer. For miRNA-seq experiments, total RNA from parental cells (treated with 20 nM osimertinib or vehicle for 14 d) from the HCCR827 and PC9 lines were applied. RNA samples were processed with binding to sample identity by using Illumina’s TruSeq small RNA sample preparation protocol for small RNA library generation (part no. 15004197 rev. F, cat. no. RS-200-90020DC). Subsequent sequencing was performed on the HiSeq 2500 platform. The resulting cDNA was used for second-strand synthesis and was cleaned to become a library suitable for in vitro transcription with T7 RNA polymerase and biotin-NTP mix. Labelled cRNA was then cleaned and 1.5 μg was hybridised to the HumanHT-12 v4 array (Illumina) for 16 h at 55°C. Following hybridisation, arrays were washed and stained with streptavidin-Cy5 (GE Healthcare). Fluorescence images were obtained with a BeadArray reader and processed with BeadScan software (version 3.5, Illumina). Whole-transcriptome raw data were obtained from GenomeStudio software (version 2011.1) with subtraction of background. All mRNA raw data were normalised according to the Cross-Correlation method4. miRNAs with significantly altered expression were identified with binding on the basis of an average fold-change cutoff of 1.5 and a P-value cutoff across all replicates of 0.05.

Two-colour western blot analysis and chemical reagents. Cells were collected and lysed with RIPA buffer supplemented with protease- and phosphatase-inhibitor cocktail (Roche). Protein concentrations of the extracts were measured by BCA assay (Pierce) and equalised with extraction buffer. Equal amounts of extract were subjected to SDS–PAGE and transferred onto Immobilon-Flex PVPD Imaging System (LI-COR Biosciences). Western blot quantification was performed with Image Studio Lite (v5.0, release 3 March 2015, LI-COR Biosciences).
Transfection with LNA in vitro. Tumour cells were plated at 2,000 cells per well in complete growth medium in a 96-well plate to obtain 30–60% confluence. 9–12 mM fluorescein-conjugated LNA-miR-147b (5’-AGACAGACAGTTTGGCCACA-3’; cat. no. A10977-011) or negative control (5’-TAACACGTCTTATAGCCCAA-3’; cat. no. 199906-011, Exonux) with PureFection reagent (System Biosciences, cat. no. LV750A-1) was applied for transfection. Transfected cells were collected after culture for 48 and 72 h.

HIF1α and EPAS1 shRNAs and cDNA transfection. H1975 cells were seeded in a six-well plate at 100,000 cells per well 1 d prior to transfection. A mixture of 2.5 μg of GFP-C-shLenti vector targeting HIF1α (Origene, cat. no. 320380) or EPAS1 (Origene, cat. no. TL131848) or encoding scrambled negative control (cat. no. TR00021), lentiviral vector encoding mutant HIF1α (HIF1Amut2); Origene, cat. no. RC025571) or control vector and 7.5 μl of PureFection was used for transfection. Transfected cells were selected and maintained in 0.5 μg/ml puromycin (for shRNAs) or 600 μg/ml neomycin (for HIF1Amut2) in DME containing 10% FBS for 9 d. Stably transfected cells were then passaged into a 96-well plate at 3,000 cells per well, and this was followed by treatment with 100 μM osimertinib for 3 d. For hsa-HIF1α, the following targeting sequences were used: shRNA1, 5’-AGCTGTCATGTCCTGCCCTTCC-3’; shRNA2, 5’-AGGCGCAATCAGCTTCTGAC-3’; shRNA3, 5’-TGACCTGATGTGCGCTTCAGTGCCATGACAAACATC-3’; shRNA4, 5’-TGAGGCCAATTGCTGCTCATGTTTTGACACACGAA-3’; shRNA5, 5’-AGCTGTTGTCGACAGAAGAAAACTGAGA-3’. Cells were then harvested and processed for LC−MS analysis. Lentiviral RNAi and knockdown for negative control (5’-CGCAC-3’→ 5’-GCGTG-3’) was substituted with 5’-CGCAC-3’. In the mutated ‘VHL construct, and the binding site 5’-CGCAC-3’→ 5’-GCGTG-3’ was substituted with 5’-CGCAC-3’→ 5’-GCGTG-3’. For lentiviral overexpression of SDHD in the mutated ‘VHL construct, and the binding site 5’-CGCAC-3’→ 5’-GCGTG-3’ was substituted with 5’-CGCAC-3’→ 5’-GCGTG-3’.

Transplantation and dual-luciferase assays. PureFection was used for transient transfection. Reporter construct with wild-type or mutant 3’ UTR sequence for VHL or SDHD (100 ng; GeneCopoeia) was cotransfected into H1975 cells with 120 nM of LNA-miR-147b or negative control (Exiqon). Firefly and Renilla luciferase activities were measured 48 h after transfection with the Dual-Luciferase Reporter System (Promega). Firefly lucinescence was normalised to Renilla lucinescence as an internal control for transfection efficiency. The miR-147b-binding site 5’-CGCAC-3’ was substituted with 5’-GGTG-3’ in the mutated ‘VHL construct, and the binding site 5’-CGCAC-3’ was substituted with 5’-GGTG-3’ in the mutated ‘SDHD construct.

Lentiviral-mediated miRNA and VHL overexpression or knockdown. For lentiviral-mediated overexpression or knockdown of miR-147b, cells (AALE, HC827, H1975 and PO89) were infected with lentiviral particles (ABM) for 48 h in the presence of 1:100 ViralPlus transduction enhancer (ABM) and 8 μg/ml polybrene (Sigma-Aldrich). Two days after infection, puromycin was added to the medium at 0.5 μg/ml, and cell populations were selected for 1–2 weeks. For lentiviral overexpression of VHL, cells (HCC827) at 70% confluence were transduced with lentiviral particles encoding VHL (1.6×10^6TU/ml; ABM) or empty control lentiviral particles (2×10^6TU/ml; ABM) in the presence of polybrene. Infected cells were then passaged and selected with puromycin (Invitrogen) at 0.5 μg/ml for 1–2 weeks.

crRNA:tracrRNA transfection. H1975–Cas9 cells were generated with plenti–EF1a–Cas9 lentiviral particles (ABM, cat. no. K003) and maintained in 0.5 μg/ml puromycin in DME. About 10% of H1975–Cas9 integrants were seeded in a 96-well plate at 3,000 cells per well 1 d prior to transfection. Edit-R synthetic crRNA (CRISPR RNA) targeting MIR147 (BE Healthcare DHarmaco, cat. no. crRNA-413428, crRNA-413429, crRNA-413430 and crRNA-413431), non-targeting control (cat. no. U-00701-01-20) and tracrRNA (trans-activating CRISPR RNA; cat. no. U-002005-20) were individually resuspended in 10 mM Tris-HCl, pH 7.5, to a concentration of 100 μM crRNA and tracrRNA were obtained at an equimolar ratio and diluted to 2.5 μM with 10 mM Tris-HCl, pH 7.5. A final concentration of 50 nM crRNA:tracrRNA complex was used for transfection. Cells were transfected with 0.4 μl/well of DharmaFECT Duo transfection reagent (BE Healthcare DHarmaco, cat. no. T-7010–02). For hsa-miR-147b, the following targeting sequences were used: crRNA 1, 5’-AGAGTACTCTATAAATCTAG-3’; crRNA 2, 5’-TTTCTGCACAAACTAGATTC-3’; crRNA 3, 5’-GCAGAAGCATTTCCGCACAC-3’; crRNA 4, 5’-GCAGAAGCATTTCCGCACA-3’. The candidate VHL mutations in lung adenocarcinoma tissues from the TCGA dataset. A Spearman correlation test was used for analysis of correlation between VHL and the candidate VHL-regulating miRNAs emerging from TargetScan (release 7.2, March 2018). TIC frequencies were estimated with ELDA software (version 4.11.0397 vom 02.01.2018). Enrichment of GO (release 2016-09-30) functional annotations in the DAVID Bioinformatics tool (v6.8, October 2016) was performed with blinding to sample identity by using online MetaboAnalyst 3.0 software (https://www.metaboanalyst.ca/3.0).

Statistical analysis. No statistical methods were used to predetermine sample size. For mouse experiments, mice were not randomised. The investigators performing tumour volume measurements were blinded to mouse group. All experiments were performed in three to seven biological replicates and independently reproduced as indicated in figure legends. Data are presented as the mean ± s.e.m. Unless otherwise stated, statistical significance was determined by a Student’s two-tailed t test in GraphPad Prism v6.02. P < 0.05 was considered to be statistically significant. For analysis of differences in the transcriptome between gefitinib- and the candidate VHL-regulating miRNAs emerging from TargetScan (release 7.2, March 2018). TIC frequencies were estimated with ELDA software (version 4.11.0.397 vom 02.01.2018). Enrichment of GO (release 2016-09-30) functional annotations in the DAVID Bioinformatics tool (v6.8, October 2016) was performed by modified Fisher’s exact test on the microarray data from single-cell-derived PC9 clones. Enrichment was based on all evidence codes.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Information and data for PDX models from the JAX PDX Resource are publicly available from the PDX Portal hosted by the Mouse Tumor Biology Database (http://www.tumorinformatics.jax.org/mtbwi/pdxSearch.do). Data from this study have been deposited in the Gene Expression Omnibus (GEO) under the following accession: GSE103155 (miRNA; single-cell-derived clones) and GSE103552 (miRNA-seq). The results shown in this manuscript were in part based on data generated by the TCGA Research Network. Genomic Data. For analyses as an association between miRNA profiles and EGFPR mutations as well as an association between VHL and miR-147b in a cohort of human lung adenocarcinoma cell lines were based on a public RNA-seq dataset (\cite{available}}
in the ArrayExpress database under accession E-MTAB-2706. The heat map for miRNA expression was generated according to the Heatmapper server (http://www2.heatmapper.ca/expression/). Genetic mutation status was confirmed by canSAR portal (v3.0 beta; https://cansar.icr.ac.uk/) and the Catalogue of Somatic Mutations In Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic/sampleOverview?id=722040). The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
W.C.Z. and F.J.S. directed the project; W.C.Z., J.M.W., K.-H.C., H.H., C.J.B. and F.J.S. wrote the manuscript; W.C.Z. performed miRNA-seq and microarray analyses, 3D structure derivation, cell culture experiments and in vivo experiments; J.M.W., K.-H.C., C.J.B., M.A.M. and K.P. established the PDX; H.H., M.Y. and J.M.A. performed metabolomics profiling; T.S. assisted with the small-molecule treatment experiments; W.C.Z. directed miRNA-147b target prediction and validation by genetic and metabolic approaches; and D.B.C. provided advice and project support.

Competing interests
Most authors declare no competing interests. DBC reports personal fees (consulting fees) and non-financial support (institutional research support) from Takeda/Millennium Pharmaceuticals, personal fees (consulting fees) and non-financial support (institutional research support) from Astrazeneca, personal fees (honoria) and non-financial support (institutional research support) from Pfizer, non-financial support (institutional research support) from Merck Sharp & Dohme Corporation and non-financial support (institutional research support) from Merrimack Pharmaceuticals, all outside the submitted work.

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Software and code

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Data collection

For targeted Mass Spectrometry, peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.1 software (AB/SCIEX). The results shown in this manuscript were part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. The genetic mutation status was confirmed by cansar portal (v3.0 beta) (https://cansar.icr.ac.uk/) and cancer Catalogue Of Somatic Mutations In Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic/sample/overview?id=722040). 

Data analysis

The TIC frequencies were estimated using ELDA software (Version 4.11.0.3973 vom 02.01.2018). For whole transcriptome analysis by microarray, fluorescent images were obtained with a Beadarray reader and processed with the BeadScan software (version: 3.5, Illumina, CA, USA). The whole transcriptome raw data were obtained from the GenomeStudio software (version: 2011.1) with the subtraction of the background. High-throughput small RNA-seq was performed on the HiSeq 2500 platform for 1 x 50-nt single-end sequencing and the sequencing adaptor was trimmed from the raw reads. To identify the known miRNAs, the remaining sequences were aligned to the miRBase (release 21.0) (http://www.mirbase.org/) using Bowtie (http://bowtie-bio.sourceforge.net/manual.shtml) (version: 1.2.1, release: 06/12/2017). Matched sequences with < 1 mismatch were known miRNAs. In addition, the unmatched sequences were used to predict the candidate novel miRNAs using miRDeep2 (Version 2.0.0.8). The hairpin RNA structures containing the unmatched sequences were predicted, complying with the criteria of pre-miRNAs in order to identify the potentially novel miRNAs. The enrichment of Gene Ontology (version: releases/2016-09-30) functional annotations was performed using DAVID Bioinformatics tool (v6.8, Oct. 2016). The heatma for miRNA expression was generated according to Heatmapper website server (http://www2.heatmapper.ca/expression/). Metabolomics analysis was performed with online MetaboAnalyst 3.0 software (https://www.metaboanalyst.ca/). To predict upstream miRNA candidates that regulate VHL, TargetScan (version: release 7.2, March 2018) was used. The pyrosequencing result was analyzed
using PyroMark Q24 software. Western blot quantification was performed by Image Studio Lite (V5.0, release/March 3, 2015, LI-COR Biosciences). GraphPad Prism v. 6.0.2 were used for the statistical analysis. The 3D structures were measured by ImageJ 1.51s software. The total number of colonies in plate was analyzed by openCFU (http://openfu.sourceforge.net).

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Information and data for PDX models from the JAX PDX Resource are publicly available from the PDX Portal hosted by Mouse Tumor Biology Database (MTB; http://www.tumor.informatics.jax.org/mtbw/pdxSearch.do). Data from this study have been deposited in the Gene Expression Omnibus (GEO) databases under the following accession: GSE103155 (microarray: single cell clones) and GSE103352 (miRNAseq). The results shown in this manuscript were part based upon data generated by the TGCA Research Network: http://cancergenome.nih.gov/. Analyses for an association between miRNA profiles and EGFR mutations as well as an association between VHL and MIR147b on a cohort of human lung adenocarcinoma cell lines were derived from a public RNA-seq dataset in the ArrayExpress database under accession number E-MTAB-2706. The heatmap for miRNA expression was generated according to Heatmapper website server (http://www2.heatmapper.ca/expression/). The genetic mutation status was confirmed by cansar portal (v3.0 beta) (https://cansar.icr.ac.uk/) and cancer Catalogue Of Somatic Mutations In Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic/sample/overview?id=722040). The data that support the findings of this study are available from the corresponding author upon request.

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| Data exclusions | No data were excluded from the analyses. |
| Replication | All experiments were performed in three to seven biological replicates, and independently reproduced as indicated in figure legends. And all attempts at replication were successful. |
| Randomization | Mice were age and sex-matched and randomized where appropriate (e.g. prior to xenograft transplantation for matched conditions). |
| Blinding | For mouse experiments, the investigators performing tumor volume measurements were blinded. For targeted Mass Spectrometry, whole transcriptome and high-throughput small RNA-seq analysis, the group allocation and data analysis were blinded. |

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---|---|
| n/a | n/a |
| ☒ Unique biological materials | ☒ Involved in the study |
| ☒ Antibodies | ☒ ChIP-seq |
| ☒ Eukaryotic cell lines | ☒ Flow cytometry |
| ☒ Palaeontology | ☒ MRI-based neuroimaging |
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Unique biological materials

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Obtaining unique materials

All unique materials used are readily available from the authors upon Material Transfer Agreement signature. DMOG (Cat #400091) was from Calbiochem. R59949 (Cat #D5794) and dimethyl malonate (DMM, Cat #136441) were purchased from Sigma-Aldrich. 3D cultures of lung patient-derived xenograft tumors (PDX_LU_10) were established at Beth Israel Deaconess Medical Center of Harvard Medical School.

Antibodies

Antibodies used

For immunofluorescence staining, primary mouse anti-human ZO-1 (1:100, clone #ZO1-1A12, lot #Q1215680, Cat #33-9100) was from Thermo Fisher Scientific. Secondary donkey anti-mouse IgG conjugated with Alexa Fluor 488 (1:500, lot #1741782, Cat #RA-21202) was from Life Technologies. For western blot, primary polyclonal rabbit anti-VHL antibody (1:100, lot #RD2197039, Cat #PAS-27322) was from Thermo Fisher Scientific. Mouse anti-β-actin (1:5,000, clone #C4, lot #I1018, Santa Cruz, sc-47778) was used as loading control. IRDye 680RD goat anti-rabbit (1: 20,000, lot #C70406-04, cat #LI-COR926-68171, LI-COR Biosciences) and IRDye 800CW goat-anti-mouse (1: 20,000, lot #C70310-02, cat #LI-COR827-08364, LI-COR Biosciences) were used as secondary antibodies.

Validation

33-9100 targets ZO-1 has been successfully used in ELISA, Immunofluorescence (IF) and Western Blot (WB) applications and shows reactivity with human and canine samples. The validation of ZO-1 antibody is demonstrated on the Thermo Fisher’s website (https://www.thermofisher.com/antibody/product/ZO-1-Antibody-clone-ZO1-1A12-Monoclonal/33-91000). PAS-27322 targets VHL in Immunohistochemistry (Paraffin) (IHC (P)), Immunoprecipitation (IP), and WB applications and shows reactivity with human and mouse samples. The PAS-27322 immunogen is recombinant fragment corresponding to a region within amino acids 1 and 213 of Human VHL. The validation of VHL antibody is demonstrated on the Thermo Fisher’s website (https://www.thermofisher.com/antibody/product/VHL-Antibody-Polyclonal/PAS-27322). sc-47778 targets β-actin in IHC (P), IP, WB and IF applications and shows reactivity with mouse, rat, human, avian, bovine, canine, porcine, rabbit, Dictostelium discoideum and Physarum polycephalum samples. The sc-47778 is raised against gizzard Actin of chicken origin. The validation of β-actin antibody is demonstrated on the Santa Cruz’s website (https://www.scbt.com/scbt/product/beta-actin-antibody-c4). A-21202 targets mouse IgG specifically in Immunocytochemistry (ICC), IF and IHC and shows minimum cross-reactivity to bovine, chicken, goat, guinea pig, hamster, horse, human, mouse, rat, and sheep serum proteins. The validation of donkey anti-mouse IgG conjugated with Alexa Fluor 488 antibody is demonstrated on the Thermo Fisher’s website (https://www.thermofisher.com/antibody/product/Donkey-anti-Mouse-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyconal/A-21202). RDye 680RD goat anti-rabbit targets rabbit IgG in WB and IHC applications. The validation of RDye 680RD goat anti-rabbit antibody is demonstrated on the LI-COR Biosciences website (https://www.licor.com/bio/products/reagents/secondary_antibodies/irdye_680rd.html). IRDye 800CW goat-anti-mouse targets mouse IgG in WB and IHC applications. The validation of IRDye 800CW goat-anti-mouse antibody is demonstrated on the LI-COR Biosciences website (https://www.licor.com/bio/products/reagents/secondary_antibodies/irdye_800cow.html).

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Human lung EGFR-wild type cell lines H358, H460, A549, H1299, and H69 were obtained from ATCC as well as EGFR-mutant cell lines H1650, H1975, HCC827, HCC827/GR, PC9, PC9ER, and H3255 were provided by Dr. Susumu Kobayashi. Immortalized tracheobronchial epithelial AALE cells were provided by Dr. William C. Hahn. 3D cultures of lung patient-derived xenograft tumor (PDX_LU_10) were established at Beth Israel Deaconess Medical Center of Harvard Medical School.

Authentication

Cell line identities were confirmed by STR fingerprinting.

Mycoplasma contamination

All cell lines were found negative for mycoplasma using the MycoAler Kit (Lonza).

Commonly misidentified lines

No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

Laboratory animals

All research involving animals complied with protocols approved by the Institutional Animal Care and Use Committees (IACUC) from BIDMC, The Jackson Laboratory and Yale University. For establishing subcutaneous tumors from PDXs, 4-6 weeks old female NSG immunodeficient mice (The Jackson Laboratory, strain # NOD.Cg-PkrdcsdclIIrgtmt1Wj/J/52), stock # D00557, homozygous for both the Prkdcscid and Il2rgtmt1Wj alleles) were used as part of commercial production of the PDX at The Jackson Laboratory or implanted as part of this study at Yale Cancer Center. No littermate controls were used. For generating xenograft tumors by transplanting H1975 cells with miR-147b knockdown, 4-6 weeks old female nude immunodeficient mice (The Jackson Laboratory, strain # NU/J, stock # 002019, homozygous for Foxn1nu) were used for subcutaneous injections at the BIDMC.
**Wild animals**  
The study did not involve wild animals.

**Field-collected samples**  
The study did not involve samples collected from the field.

## Human research participants

**Policy information about studies involving human research participants**

**Population characteristics**  
Patient characteristics are summarised in Supplementary Table 2. Information and data for PDX models from the JAX PDX Resource are publicly available from the PDX Portal hosted by Mouse Tumor Biology Database (MTB; http://tumor.informatics.jax.org/mtbwi/pdxSearch.do)

**Recruitment**  
Patient tumor material was obtained through a network of collaborating cancer research centers to develop the JAX PDX Resource. The collaborating centers were responsible for any necessary IRB approvals and patient consents to allow their tumor tissue to be used in research. These responsibilities are acknowledged in the JAX PDX Consortium Agreement, which was signed by the participating member prior to submitting their first patient specimen for model development. The JAX PDX Resource received coded patient tumor samples, with all personal identifiers removed. No JAX investigator has access to patient consent forms. Successful PDX models were assigned a unique number with all reference to the donating center and date of specimen collection removed to further protect patient privacy. Besides, patients with advanced NSCLC who developed progression after initial response to EGFR TKI were consented and enrolled to a Yale University IRB approved protocol, in accordance with ethical guidelines, allowing the collection and analysis of clinical data, fresh tissue, and the generation of patient-derived xenografts. All Yale patients received prior radiotherapy. Part of the tissues has been collected in Yale patients whose tumor have developed progression after initial response to EGFR TKI. These clinical samples are primary tissues that are tolerant or resistant to EGFR TKIs. The genetic mutations have been described in Supplementary Table 2 and these are unlikely to have an impact the results.