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Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma.
NATURE (2014) 508
DOI: 10.1038/nature13121

Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma.

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Treatment of *BRAF(V600E)* mutant melanoma by small molecule drugs that target the BRAF or MEK kinases can be effective, but resistance develops invariably[1,2]. In contrast, colon cancers that harbour the same *BRAF(V600E)* mutation are intrinsically resistant to BRAF inhibitors, due to feedback activation of the Epidermal Growth Factor Receptor (EGFR)[3,4]. We show here that 6 out of 16 melanoma tumours analysed acquired *EGFR* expression after the development of resistance to BRAF or MEK inhibitors. Using a chromatin regulator-focused shRNA library, we find that suppression of sex determining region Y-box 10 (*SOX10*) in melanoma causes activation of TGFβ signalling, thus leading to upregulation of *EGFR* and Platelet Derived Growth Factor Receptor β *(PDGFRB)*, which confer resistance to BRAF and MEK inhibitors. Expression of *EGFR* in melanoma or treatment with TGFβ results in a slow-growth phenotype with cells displaying hallmarks of oncogene-induced senescence. However *EGFR* expression or exposure to TGFβ becomes beneficial for proliferation in the presence of BRAF or MEK inhibitors. In a heterogeneous population of melanoma cells having varying levels of *SOX10* suppression, cells with low *SOX10* and consequently high *EGFR* expression are rapidly enriched in the presence of drug, but this is reversed when the drug treatment is discontinued. We find evidence for *SOX10* loss and/or activation of TGFβ signalling in 4 of the 6 EGFR-positive drug-resistant melanoma patient samples. Our findings provide a rationale for why some BRAF or MEK inhibitor resistant melanoma patients may regain sensitivity to these drugs after a drug holiday and identify patients with EGFR-positive melanoma as a group that may benefit from re-treatment after a drug holiday.

Activating mutations in the *BRAF* oncogene are found in over half of the patients with advanced melanoma[5,6]. Inhibition of the oncogenic BRAF protein with the small molecule inhibitor PLX4032 (vemurafenib) or its downstream effector MEK with GSK1120212 (trametinib) have shown impressive initial responses in patients with *BRAF* mutant melanoma[1,2]. However, single agent therapies for advanced cancers are rarely curative, due to the rapid development of resistance. To date, several drug resistance mechanisms have been identified in melanomas treated with vemurafenib, including increased expression of the gene encoding the COT kinase, mutation of downstream *MEK1* kinase, *NRAS* mutations and amplification or alternative splicing of the *BRAF* gene[7-11]. Moreover, increased expression of receptor tyrosine kinases (RTKs) has been observed as a mechanism of BRAF inhibitor resistance[11-13].

It has been shown recently that intrinsic resistance of *BRAF* mutant colon cancers to vemurafenib is the result of feedback activation of EGFR when BRAF is inhibited[3,4]. To investigate whether *BRAF(V600E)* mutant melanoma patients frequently develop resistance to BRAF or MEK inhibitors through acquired expression of *EGFR* in their tumours, we obtained biopsies from *BRAF(V600E)* mutant melanomas from sixteen patients treated with either the MEK inhibitor trametinib (n=1) or the BRAF inhibitors dabrafenib (n=3) or vemurafenib (n=12). Tumour biopsies collected both before treatment initiation and after the development of drug resistance were stained for EGFR expression. We found that 6 out of 16 post treatment biopsies gained significant EGFR expression as judged by immunohistochemistry (Figure 1a, b Table S1).

Melanomas are derived from the neural crest and in general do not express *EGFR*[14]. Hence, acquired *EGFR* expression during drug selection may represent a stress response that is not favoured in the absence of drug treatment. Indeed, the proliferation rate of A375 melanoma cell lines engineered to express *EGFR* decreased as the concentration of EGFR ligand increased (Figure 1c, ref[3]). Moreover, A375 cells that express *EGFR* also proliferate slower compared to parental control cells in nude mouse xenografts, but are resistant to

trametinib (Fig 1d). To investigate the cause of this slow-growth phenotype, we performed western blotting for a number of cell cycle-associated proteins on parental A375 cells and *EGFR*-expressing derivatives. *EGFR* expression resulted in hypophosphorylated pRB protein, induction of the CDK inhibitors CDKN1A ($p21^{cip1}$) and CDKN1B ($p27^{kip1}$) and acidic β-galactosidase (Figure 1e, f), markers that have been associated with oncogene-induced senescence[15,16]. These markers were also induced upon expression of oncogenic versions of *BRAF* or *MEK*, but much less when activated mutants of *AKT1* or *PIK3CA* were expressed in A375 cells (Extended Data Fig. 1). We conclude that *EGFR* expression is disadvantageous for *BRAF(V600E)* melanoma cells in the absence of BRAF or MEK inhibitor drugs, but it confers a selective advantage in the presence of these drugs.

Acquired *EGFR* expression may be the result of an adaptive response of the cancer cell population during drug selection. To ask in an unbiased way which factors might modulate *EGFR* expression in melanoma cells, we compiled a "chromatin regulator" library of shRNAs targeting 661 genes, including the KATs (lysine acetyltransferases), KMTs (lysine methyltransferases), KDACs (lysine deacetylases), KDMs (lysine demethylases), chromatin remodelling complexes and proteins that harbour chromatin binding/associated domains (Table S2). A375 melanoma cells, which express very low levels of *EGFR*, were infected with the chromatin regulator library and selected with vemurafenib for 3 weeks. After this, the vemurafenib-resistant cells were harvested and strongly EGFR-positive cells ($EGFR^{high}$) were isolated from the drug-resistant population by Fluorescence-Activated Cell Sorting (Figure 2a). Treatment of cells with either the chromatin regulator library or vemurafenib alone did not increase the fraction of $EGFR^{high}$ cells. In contrast, a significant fraction of $EGFR^{high}$ cells could be retrieved when cells were infected with the chromatin regulator library *and* were selected for vemurafenib resistance (Figure 2b). We conclude that $EGFR^{high}$ melanoma cells do not merely appear as a consequence of silencing of certain chromatin regulators, but that these cells only emerge when the population is placed under drug-

selection pressure. This suggests that silencing of the gene(s) that induce *EGFR* expression is not favoured in the absence of vemurafenib.

To identify which gene(s) in the chromatin regulator library can induce EGFR expression, we isolated genomic DNA from the EGFR^{high} cells and non-drug treated control cells and determined the abundance of the shRNA vectors in each cell population by deep sequencing, as described previously[3]. shRNAs that confer resistance to vemurafenib through upregulation of EGFR should be enriched in the EGFR^{high} fraction. shRNA screens are notorious for yielding false positive results. Therefore, in principle only those genes that are represented by multiple shRNAs should be followed up in a genetic screen[17]. However, in this screen we did not identify any genes for which multiple shRNAs were enriched (Table S3). We therefore focused on the top 10 most strongly enriched genes for follow up experiments. We tested multiple additional shRNA vectors for each of these 10 genes for their ability to increase *EGFR* expression, as this was a selection criterion in the genetic screen (Extended Data Fig. 2a, b). Only suppression of the SRY (sex determining region Y)-box 10 (*SOX10*) gene induced prominent *EGFR* expression when multiple *SOX10* shRNAs (sh*SOX10*) were used in four melanoma cell line models (Figures 2c, 2d, Extended Data Fig. 2c, 4c, 5c). *SOX10* knockdown (*SOX10*^{KD}) induced a slow-growth phenotype and also displayed the hallmarks of oncogene-induced senescence in multiple melanoma models (Figure 2e, Extended Data Fig. 2e, f, g, 4b, e, f, 5b, e, f).

Next we confirmed that *SOX10*^{KD} indeed induced vemurafenib resistance in melanoma. We infected A375 cells with sh*SOX10* and cultured cells in the presence of vemurafenib. *SOX10*^{KD} slowed down proliferation of A375 cells in the absence of drug, but in the presence of vemurafenib *SOX10*^{KD} conferred drug resistance, both in short-term and long-term assays (Figure 2e, Extended Data Fig. 2d, e). Moreover, under vemurafenib selective pressure, cells having a higher degree of *SOX10*^{KD} were selected, which consequently also expressed higher levels of *EGFR*, consistent with the notion that increased *EGFR* levels drive drug resistance (Extended Data Fig. 2h). Vemurafenib resistance through *SOX10* suppression was also seen in additional melanoma cell lines (Extended Data Fig. 4a, 5a). Note that a low

concentration of vemurafenib actually increased proliferation rate of *SOX10*^{KD} cells, consistent with the model that hyperactive BRAF-MEK signalling induces senescence markers, which is inhibited by vemurafenib (Extended Data Fig. 4a, g).

To study how *SOX10* suppression induces *EGFR* expression, we performed transcriptome sequencing (RNAseq) of both parental A375 and A375-*SOX10*^{KD} cells (Table S4). Gene set enrichment analysis of the SOX10-upregulated genes revealed an enrichment of genes with SMAD2/3 (downstream mediators of TGFβ signalling) and JUN binding sites in their promoters (Table S5). Consistent with this, *SOX10* suppression induced TGFβ receptor 2 (*TGFBR2*) expression as well as a number of *bona fide* TGFβ target genes, including *JUN,* in multiple melanoma cell models (Figure 3a, b, Extended Data Fig. 4d, 5d). Levels of active JUN (pJUN) were also increased by *SOX10*^{KD} (Figure 3a). That treatment of melanoma cells with recombinant TGFβ causes resistance to vemurafenib further supports a role for TGFβ signalling in vemurafenib resistance (Figure 3c and ref[18]). TGF-β1 □□□□□□□□□ not only caused induction of *EGFR* expression, but also of Platelet Derived Growth Factor Receptor β (*PDGFRB*, Figure 3d, e) and also resulted in induction of senescence-associated β-galactosidase (Figure 3f). Consistently, *SOX10* suppression also induced PDGFRB expression (Extended Data Fig. 3c, 4c, 5c). Moreover, suppression of *TGFBR2* inhibited *EGFR* and *PDGFRB* induction in *SOX10*^{KD} cells (Figures 3g, h), whereas ectopic expression of *TGFBR2* induced pJUN*, EGFR* and *PDGFRB* expression (Figure 3i). JUN is a regulator of *EGFR* expression and TGFβ regulates *PDGFRB* [19-21]. Moreover, SMADs and JUN cooperate in activation of *EGFR* expression[22,23]. SOX10 is known to regulate the melanocyte transcription factor MITF[24]. Indeed, A375 cells with sh*SOX10* also had reduced *MITF* expression, but *MITF* suppression alone did not change *EGFR* or *PDGFRB* expression and did not cause vemurafenib resistance (Extended Data Fig. 7c, d, e). In summary, our data provide support for a model in which activation of TGFβ signalling by *SOX10* loss leads to increased *EGFR* and *PDGFRB* expression and vemurafenib resistance.

Treatment of A375-*SOX10*^{KD} cells with a combination of both vemurafenib and the EGFR inhibitor gefitinib did not lead to proliferation arrest, indicating that EGFR was not the sole driver of drug resistance in *SOX10*^{KD} cells (Extended Data Fig. 3a). Indeed, an unbiased survey of RTKs revealed that *SOX10*^{KD} activated not only EGFR, but also PDGFRB and ERBB3 (Extended Data Fig. 3b, 3c). A similar pattern of RTK activation was observed following TGF-β1 treatment, highlighting the similarity between *SOX10* suppression and acquired TGFβ signalling (Extended Data Fig. 3b, d). Many RTKs share two major downstream signalling pathways (RAS-MEK-ERK and PI3K-AKT). Consistent with this, we found that combined inhibition of these two downstream pathways using BRAF and PI3K inhibitors could restore growth inhibition in *SOX10*^{KD} cells (Extended Data Fig. 3a).

Our data are consistent with a model in which cells with low *SOX10* and high *EGFR* and *PDGFRB* expression are positively selected in the presence of drug, but that such cells are counter-selected in the absence of drug. To test this model directly, we infected A375 cells with sh*SOX10* and subjected this heterogeneous population of *SOX10*^{KD} cells to vemurafenib selection for one week. At this point, we harvested part of this population and determined *EGFR* expression by FACS analysis. Under vemurafenib selection, an increased level of *EGFR* and a markedly decreased level of *SOX10* were observed. When these cells were subsequently cultured for one more week in the absence of vemurafenib, the *EGFR*^{high}/SOX10^{low} population was depleted (Figure 4a, Extended Data Fig. 6a). These data indicate that acquired *EGFR* expression is only advantageous to melanoma cells in the presence of drug selection, but is counter-selected in the absence of drug.

Consistent with a role for SOX10 in regulation of *EGFR* expression in melanoma, we found an inverse correlation between *SOX10* and *EGFR* expression in a panel of 34 melanoma cell lines[25] (Figure 4b) and a similar inverse relation between *SOX10* and *PDGFRB* (Extended Data Fig. 6b). The most extreme cell line in this panel, LOXIMVI, completely lacked *SOX10* expression and had the highest *EGFR* expression. When we expressed *SOX10* in this cell line, EGFR and PDGFRB were reduced and TGFBR2 and

TGFBR3 as well as JUN and pJUN levels were also downregulated, consistent with the notion that SOX10 regulates these RTKs through an effect on TGFβ signalling (Extended Data Fig. 6c, d). Consistently, expression of *SOX10* in LOXIMVI cells increased their sensitivity to vemurafenib (Extended Data Fig. 6e).

To ask directly whether *SOX10* is involved in EGFR-associated drug resistance in *BRAF(V600E)* melanoma patients, we isolated RNA from the six patients studied above that had gained *EGFR* expression after acquisition of trametinib, dabrafenib or vemurafenib resistance (Table S1). We performed RNAseq analysis to determine changes in transcriptome upon drug resistance. In two patients the levels of *SOX10* mRNA were reduced (Figures 4c, Extended Data Fig. 6f). *EGFR* and *PDGFRB* mRNA were greatly increased in patient 5, whereas no evidence was found in this patient of alternative *BRAF* splicing[7] or *BRAF* over-expression (Extended Data Fig. 7a, b). Patient 3 has strong induction of EGFR protein post resistance (Figure 1a), but at first glance, *EGFR* mRNA levels appear only minimally induced. However, scrutiny of the RNAseq data reveals that the apparent lack of induction of *EGFR* in this tumour sample pair is caused by the abnormally high *EGFR* transcript abundance in the pre-treatment sample and not the lack of *EGFR* expression in the post-treatment sample (Extended Data Fig. 6g). This is most likely due to the contamination of this sample with the strongly EGFR positive skin material (see Figure 1a). These tumours also manifested increased TGFβ signalling (Figure 4c, Extended Data Fig. 6h). Two further pairs of tumour samples showed induction of *EGFR* and *PDGFRB* without significant loss of *SOX10* after drug resistance emerged. These tumours displayed induction of TGFβ receptor expression and induction of a number of *bona fide* TGFβ targets, suggesting that these tumours somehow had acquired TGFβ signalling (and subsequent induction of *EGFR* and *PDGFRB* expression) in a *SOX10*-independent fashion (Figure 4c).

Clinical evidence indicates that melanoma patients that have developed vemurafenib resistance can regain sensitivity to the drug after a drug holiday, suggesting a reversible and

adaptive transcriptional response to the drug[26]. That drug resistance is reversed in the absence of drug indicates that this adaptive response is not favoured in the absence of drug. Our data provide a molecular underpinning for the concept that drug resistance may arise at a fitness cost in the absence of drug (Figure 4d). Melanoma patients whose tumours acquire *EGFR* expression as a result of drug resistance development may be candidates to be re-treated with drug after a drug holiday.

Acknowledgements.

We thank the NKI Core Facilities for Genomics and Molecular Pathology & Biobanking for tumour tissue and support in DNA sequencing. We thank Severine Roy for collecting clinical data and Nyam Kamsu Kom for tissue preparation. This work was supported by grants from the European Research Council (ERC), the Dutch Cancer Society (KWF), the EU COLTHERES project and grants by the Netherlands Organization for Scientific Research (NWO) to Cancer Genomics Netherlands (CGC.NL). Additional support was provided by Fondazione Piemontese per la Ricerca sul Cancro – ONLUS grant 'Farmacogenomica – 5 per mille 2009 MIUR' (F.D.N.); AIRC MFAG 11349 (F.D.N.); AIRC IG grant n. 12812 (A.B.) and Canadian Institutes of Health Research (CIHR) grant MOP-130540 (S.H.).

FIGURE LEGENDS

Figure 1 | Acquired *EGFR* expression in *BRAF(V600E)* mutant melanoma after vemurafenib resistance.

a, b, Immunohistochemical (IHC) analysis (a, brown staining; b, pink staining) showing increased EGFR expression in formalin-fixed paraffin embedded (FFPE) (Patient #1, #2, #3, #4 and #5) and frozen (Patient #6) melanoma tissue sections from *BRAF(V600E)* mutant melanoma patients who developed resistance to vemurafenib, dabrafenib or trametinib as indicated. For each patient, the first biopsy is from the pre-treatment tumour; the second biopsy was performed after the tumour had progressed under treatment. For patient #4, the first biopsy was performed when the patient was in partial response, but rapidly developed secondary resistance. 4.5 months later, the second biopsy was taken. **c,** EGFR expression confers growth-disadvantage to *BRAF(V600E)* mutant melanoma cells and EGFR ligand potentiates the growth deficiency *in vitro*. A375 *BRAF(V600E)* melanoma cells transduced with control lentiviral vectors (Ctrl. , **PLX304-GFP**) or vectors expressing EGFR (EGFR, **PLX304-EGFR**) were seeded at the same density and cultured in the presence of EGF at indicated concentration for 2 weeks. The cells were fixed, stained and photographed. **d,** EGFR expression confers growth-disadvantage to *BRAF(V600E)* mutant melanoma, but induces trametinib resistance *in vivo*. CD1 nude mice were inoculated with *BRAF(V600E)* mutant melanoma A375 cells transduced with control retroviral vectors or vectors expressing *EGFR*. Once tumours were established, animals were treated with vehicle, trametinib. Relative tumour volume is shown. Error bars represent SEM (n=5). * $p < 0.05$, single-sided Wilcoxon–Mann–Whitney test. **e,** Western blot analysis of RB protein, CDK inhibitors CDKN1A ($p21^{cip1}$) and CDKN1B ($p27^{kip1}$) in EGFR expressing A375 cells. HSP90 served as a loading control. **f,** EGFR expression induces senescence. Senescence was detected by staining of β-galactosidase activity. All experiments shown except the ones that involve clinical samples and animals were performed independently at least 3 times.

Figure 2 | FACS-assisted shRNA genetic screen identifies SOX10 as a determinant of vemurafenib resistance and EGFR expression.

a, Schematic outline of the of the FACS-assisted shRNA screen. Human "Chromatin Regulator" shRNA library polyclonal virus was generated to infect A375 cells, which were then left untreated (control) or treated with 0.5μM vemurafenib. After 12 days, the untreated cells were harvested. The cells that survived from 21 days of vemurafenib treatment were FACS sorted for EGFR expression. Subsequently, shRNA inserts from both samples were recovered by polymerase chin reaction (PCR) and identified by massive parallel sequencing. **b,** $EGFR^{high}$ cells result from the combination of infection with chromatin regulator library and vemurafenib selection. A375 cells infected with "chromatin regulator" library (Chr Lib) were cultured in the presence of 0.5 μM vemurafenib for 21 days (right lower panel). Cells were harvested with 2 mM EDTA, stained with anti-EGFR antibody and analysed for $EGFR^{high}$ cells by flow cytometry. A375 cells cultured with or without vemurafenib, and A375 cells infected by Chr Lib without vemurafenib treatment served as controls. **c, d,** Suppression of *SOX10* induces *EGFR* expression. (c) Western blot analysis of EGFR and SOX10 levels in cells targeted by two independent shSOX10 vectors. HSP90 served as a loading control. (d) The level of *EGFR* induction was determined by qRT-PCR analysis of the relative mRNA level of EGFR. **pLKO.1** empty vector served as a control vector (Ctrl.). Error bars represent S.D. of measurement replicates (n=3). **e,** Two independent shRNAs targeting *SOX10* confer a proliferation-disadvantage in the absence of drug, but induce vemurafenib resistance. A375 cells expressing shRNAs (as shown in figure 2c) targeting *SOX10* were seeded at the same density in 6-well plates and cultured in the absence (for 2 weeks) or presence of vemurafenib (for 4 weeks) at the indicated concentrations. The cells were fixed, stained and photographed. All experiments shown except shRNA screen were performed independently at least 3 times.

Figure 3 | Activation of TGFβ signalling leads to increased *EGFR and PDGFRB*

expression

a, Suppression of *SOX10* activates TGFBR/JUN signalling. Two independent shRNAs targeting *SOX10* were individually introduced into A375 cells by lentiviral transduction. The levels of TGFBR2, p-JUN and JUN were determined by western blot analysis. HSP90 served as a loading control. **b,** *SOX10* loss leads upregulation of TGFβ receptors and its *bona fide* target genes. Relative mRNA level of *ANGPTL4, TAGLN, CYR61, CTGF, TGFBR3, TGFBR2 and JUN* were determined by transcriptome sequencing. **pLKO.1** empty vector served as a control vector (Ctrl.). **c,** TGFβ activation confers a growth disadvantage but vemurafenib resistance. A375 cells were seeded at the same density in 6-well plates and cultured in the absence or presence of recombinant TGFβ or vemurafenib at the indicated concentrations. The cells were fixed, stained and photographed. **d, e,** Recombinant TGF-β1 treatment activates JUN and upregulates EGFR and PDGFRβ expression. A375 cells were cultured in the absence or presence of 200pM recombinant TGF-β1 for 7 days before harvested for western blot or qRT-PCR analysis. Error bars represent S.D. of measurement replicates (n=3). **f,** Recombinant TGF-β1 treatment induces senescence. A375 cells were cultured in the presence of 200pM recombinant TGFβ for 14 days. Senescence was detected by staining of β-galactosidase activity. **g, h,** SOX10 loss induced EGFR and PDGFRβ upregulation is TGFBR2-dependent. A375 cells were infected with lentiviral shRNA vectors as indicated. Relative mRNA levels of *EGFR* and *PDGFRB* were determined by qRT-PCR analysis; EGFR, PDGFRβ, TGFBR2 and SOX10 levels were determined by Western blot analysis. Error bars represent S.D. of replicate measurements (n=3). **i,** TGFBR2 overexpression is sufficient to upregulate EGFR and PDGFRβ. TGFBR2 was introduced to A375 cells by lentiviral transduction (TGFBR2, **PLX304-TGFBR2**). **PLX304-GFP** serves as a control vector (Ctrl.). The levels of EGFR, PDGFRβ, TGFBR2, p-JUN and JUN were determined by Western blot analysis. All experiments shown except RNA-seq were performed independently at least 3 times.

Figure 4 | Inverse relationship between *SOX10* and *RTKs* expression in melanoma.

a. Intermittent drug dosing alters relative proportions of $EGFR^{high}$ and $EGFR^{low}$ cell populations. A375 cells were infected with shSOX10-1 to generate a polyclonal cell population of $SOX10^{KD}$ cells. The infected cells were seeded in 6-well plates, harvested and stained with antibody against EGFR for flow cytometry analysis at day 0, day 7 and day 14 (0.5μM vemurafenib treatment started on day 0 and stopped on day 7). **PLKO.1** (Ctrl.) vector served as a control. **b,** Inverse correlation between *SOX10* and *EGFR* in a panel of human *BRAF* mutant melanoma cell lines. Relative gene expression levels of *SOX10* and *EGFR* were acquired from Cancer Cell Line Encyclopedia (CCLE). R stands for Pearson product-moment correlation coefficient. **c,** Differential gene expression of *SOX10*, *EGFR, PDGFRB,* TGFβ receptors and TGFβ target genes in pre- and post-treatment patient tumour biopsies. Total RNA was isolated from FFPE specimens derived from tumour biopsies of patient #5, #2 and #6 both before and after development of drug resistance. After reverse transcription, gene expression levels were determined by transcriptome sequencing (patient #5 and patient #2) or qRT-PCR analysis (patient #6). Error bars represent S.D. of measurement replicates (n=3). **d**, Model for senescence induction after development of vemurafenib resistance. Upregulation of RTKs leads to enhanced signalling through the RAS-BRAF-MEK pathway. Consequently, vemurafenib is no longer able to fully silence the signalling to MEK and drug resistance is seen. When the drug is removed, supra-physiological levels of BRAF-MEK signalling induced a state of oncogene-induced senescence, which subsequently leads to negative selection of the RTKs and restores drug responsiveness. All experiments shown except the ones that involve clinical samples were performed independently at least 3 times.

METHODS SUMMARY

A detailed description of the methods is available in the Methods section.

METHODS

Cell Lines

A375 melanoma cell line was obtained from ATCC. SK-MEL-28 and COLO679 were kind gifts from Dr D. Peeper (Amsterdam, The Netherlands). WM266-4 cell line was kindly provided by Dr. Richard Marias. A375 and WM266-4 cells were cultured in DMEM medium supplemented with 8% FBS, 1% penicillin/streptomycin and 2mM L-glutamine. COLO679 cell was cultured in RPMI medium supplemented with 8% FBS, 1% penicillin/streptomycin and 2mM L-glutamine.

Compounds and antibodies

Trametinib (# S2673), vemurafenib (# S1267), gefitinib (# S1025) and GDC0941 (# S1065) were purchased from Selleck Chemicals (Houston, Texas, US). TGF-β1 was purchased from R&D (#240-B-010).

Antibody against HSP90 (H-114), p21 (C-19), TGFBR2 (C-16), p-c-Jun (KM-1) and c-Jun (N) were from Santa Cruz Biotechnology anti-EGFR for FACS application (GR01L) was from Millipore; anti-EGFR for western blot analysis (610017), Rb (554136) and p27 (610242) antibodies were from BD Biosciences; Antibody against TGFBR3(#2519), p-Rb (#9307), p-MEK(#9154), MEK (4694) and PDGFRB(#4564, #3166) antibodies were from Cell Signaling; Antibody against SOX10 (ab155279) was from Abcam.

Plasmids

Individual shRNA vectors used were collected from the TRC library (Table S6).

The following plasmids were purchased from Addgene to generate **PLX304-EGFP, PLX301-SOX10, PLX304-EGFR, PLX301-EGFR** and **PLX304-TGFBR2** constructs by Gataway cloning[8,27,28].

Plasmid 24749**: pDONR221-hSOX10**

Plasmid 25890: **pLX304**

Plasmid 25895: **pLX301**

Plasmid 25899: **pDONR221_EGFP**

Plasmid 23935: **pDONR223-EGFR**

Plasmid 23623: **pDONR223-TGFBR2**

FACS-assisted shRNA screen with a customized library

Lentiviral vectors (**PLKO.1**) encoding shRNAs that target chromatin regulator genes are listed in Table S2. The chromatin regulator library contains six plasmids pools. Lentiviral supernatants of the plasmids were produced as described at http://www.broadinstitute.org/rnai/public/resources/protocols. A375 cells were infected independently by the six virus pools (multiplicity of infection <1) and selected with puromycin (2μg/ml) for cells containing integrated shRNA. Cells were then pooled and seeded at 350.000 cells per 15cm dish in the absence or presence of 0.5μM vemurafenib (8 dishes for each condition) for 21 days. The medium was refreshed every 3 days. The cells without vemurafenib treatment were harvested at day 12. At day 21, the cells treated with vemurafenib were collected using 2mM EDTA (# E4884, Sigma-Aldrich). Then, the cells were stained with mouse anti-human EGFR antibody primarily (#GR01L, Clone 528, Millipore) followed by secondary staining with Alexa Fluor 647 conjugated goat anti-mouse IgG antibody (#A-21236, Invitrogen), after which the cells were washed and suspended in D-MEM medium containing 2% FBS. BD FACSAria™ III (BD Bioscience) was used to sort out $EGFR^{High}$ cells. The FACS data was analysed by FlowJo programme version 7.6.3 (Tree Star). The genomic DNA was isolated from non-drug treated control cells and drug treated $EGFR^{high}$ cells using DNeasy® Blood and Tissue Kit (#69506 Qiagen). shRNA inserts were recovered from 500ng genomic DNA following by the experimental steps of PCR

amplification (PCR1 and PCR2) as described[3]. PCR product purification was performed using High Pure PCR Product Purification Kit according to manufactures' instruction (#11732676001, Roche). Purified PCR products were subjected to deep sequencing to identify the shRNA inserts.

Staining of β-galactosidase activity

For Figure 1f, Extended Data Fig. 2f and Extended Data Fig. 4e, the staining method is as follows:

Cells were washed with PBS and fixed with 0.5% glutaraldehyde solution (in PBS pH7.4) for 15 min at room temperature (RT). Then the cells were washed with PBS for 5 min and with PBS/$MgCl_2$ pH 6.0 twice for 5 min at RT. X-Gal staining solution (freshly prepared) was added to the cells and the incubate was performed at 37°C for 8 hours to overnight. Cells were washed again with PBS for 5 min at RT for 3 times before the pictures are taken.

For Figure 3f and Extended Data Fig. 5e, Senescence Cells Histochemical Staining Kit (CS0030-1KT) from Sigma was applied according to the manufacturer's instructions.

Long-term Cell Proliferation Assays

Cells were seeded into 6-well plates (3×10^4 cells/well) and cultured both in the absence and presence of drugs as indicated. For details, see [29].

Protein lysate preparation and Immunoblots

Cells were seeded in medium containing 8% fetal bovine serum (FBS) for 24 h, and then washed with PBS and lysed with RIPA buffer supplemented with protease inhibitor (cOmplete, Roche) and Phosphatase Inhibitor Cocktails II and III (Sigma). All lysates were freshly prepared and processed with Novex® NuPAGE® Gel Electrophoresis Systems (Invitrogen).

Mouse xenografts

Retroviral vector–transduced A375 cells (5×10^6 cells/mouse) were injected subcutaneously into the right posterior flanks of 7-week-old immunodeficient CD1 nude female mice (6 mice/group; Charles River Laboratories, Calco, Italy). Tumour formation was monitored twice a week, and tumour volume based on calliper measurements was calculated by the modified ellipsoidal formula (tumour volume = 1/2(length × width^2)). When tumours reached a volume of approximately 0.3 cm^3, mice were randomized into treatment arms and treated for a 21-day period. Trametinib was formulated in 0.5% hydroxypropylmethylcellulose (Sigma) and 0.2% Tween-80 in distilled water pH 8.0, and it was dosed at 0.15 mg/Kg daily by oral gavage. All animal procedures were approved by the Ethical Commission of the University of Turin and by the Italian Ministry of Health and they were performed in accordance with institutional guidelines.

Melanoma patient tumour samples

Permission was granted by the NKI or IGR ethical committee to take biopsies from *BRAF(V600E)* mutant patients before and after vemurafenib, dabrafenib or trametinib treatment. All patients consented to participate in the study. *BRAF(V600E)* mutation were determined by Department of Pathology at NKI or IGR.

Immunohistochemistry

EGFR staining, FFPE samples

Immunohistochemistry was performed on a BenchMark Ultra autostainer (Ventana Medical Systems, Inc.) Briefly, paraffin sections were cut at 4 µm, heated at 75 degrees for 28 minutes and deparaffinized in the instrument with EZ prep solution (Ventana Medical Systems) Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems). EGFR was detected by incubating sections with antibody clone 5B7 (5278457001;

Roche (Ventana)) for 16 minutes. Specific reactions were detected using UltraView Universal Alkaline Phosphatase Red Detection or DAB Kit (Ventana Medical Systems), and slides were counterstained with Hematoxylin.

EGFR staining, fresh frozen samples

Fresh frozen sections (4-um-thick) were mounted on 3-aminopropylethoxysilane (Sigma, St. Louis, MO, USA) and glutaraldehyde coated slides. After 10 minutes fixation with ethanol, slides were incubated with anti-EGFR using clone 31G7 (1:50; Life technologies, Zymed) using standard procedures, followed by incubation with the PowerVision Poly-HRPanti-Mouse IgG (ImmunoLogic, Duiven, The Netherlands). Sections were counterstained with haematoxylin.

RNA isolation, qRT-PCR and RNA sequencing

FFPE samples

Method of total RNA isolation from FFPE samples is as described[18]. cDNA was obtained by reverse transcription using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, AB) according to manufacturer's manual. EGFR expression assay (Hs01076078_m1), SOX10 expression assay (Hs00366918_m1), PDGFRB expression assay (Hs01019589_m1), TGFBR3 expression assay (Hs01114253_m1), TGFBR2 expression assay (Hs00234253_m1), CTGF expression assay (Hs01026927_g1), TAGLN expression assay (Hs01038777_g1), CYR61 expression assay (Hs00998500_g1), JUN expression assay (Hs01103582_s1) and ACTB expression assay (Hs01060665_g1) were used to detect the gene expression on the AB 7500 Fast Real-time PCR system following the manufacturer's instructions.

Cell line samples

RNA isolation from cell lines harvested with TRIzol® reagent (Invitrogen) according to the manufacture's instruction. cDNA synthesis was performed with Maxima Universal First Strand cDNA Synthesis Kit (# K1661, Thermo scientific) according to manufacturer's instruction. The primers were used for QRT-PCR were described in Table S7.

For RNA sequencing, the library was prepared using TruSeq RNA sample prep kit according to the manufacturer's protocol (Illumina). RNA sequencing data is available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50535

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Author Contributions:

R.B., A.B., F.D.N., L.W., C.R., RL. B., and A.E. supervised all research. R.B., and C.S. wrote the manuscript. C.S., L.W., S.H., G.H., A.P., D.Z., S.H., P.B., C.L., C.M., S.V., J.W., W.G., I.H., A.S. designed and performed experiments and J.H., C.B., C.R., S.V., A.E. provided clinical samples and gave advice.

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Extended data Figure 1 | Ectopic expression of oncogenic version of EGFR effectors induces senescence at different levels.

Oncogenic BRAF(V600E), MEK (MED-DD), PIK3CA(H1047R), or AKT (Myr-AKT) were introduced to A375 cells by retroviral transduction. pBabe-empty vector served as a control vector (Ctrl.). Senescence was detected by staining of β-galactosidase activity. All experiments shown were performed independently at least three times.

Extended data Figure 2 | Effects of SOX10 suppression in melanoma.

a, Suppression of *SOX10* strongly induces *EGFR* expression. Multiple independent shRNA vectors (5 vectors per gene) targeting the top 10 gene candidates were individually introduced to A375 cells by lentiviral transduction. The level of *EGFR* induction was determined by qRT-PCR analysis of the relative mRNA level of EGFR. **pLKO.1** empty vector served as a control vector (Ctrl.). **b,** Knockdown efficiency of the shRNA vectors targeting the top 10 gene candidates from the genetic screen. Multiple independent shRNA vectors targeting the top 10 candidate genes were individually introduced to A375 cells by lentiviral transduction. The knockdown efficiency of the shRNA vectors was determined by qRT-PCR analysis of the mRNA levels of the corresponding genes. Means of duplicate measurements are shown. **c,** *SOX10* suppression leads to *EGFR* upregulation in a second *BRAF(V600E)* mutant melanoma cell line SK-MEL-28. Error bars represent S.D. of measurement replicates (n=3). **d,** Two independent shRNAs targeting *SOX10* confer vemurafenib resistance. A375 cells expressing shRNAs against *SOX10* were seeded at the same density in 96-well plate and treated with vemurafenib at indicated concentrations for 6 days. Cell viability was determined by CellTiter-Blue® assay according to the manufacturer's instruction. Relative survival is presented as the ratio of cell viability in the presence of vemurafenib to that in the absence of drug treatment. Error bars represent S.D. of triplicate independent experiments. **e,** *SOX10* suppression is a disadvantage for melanoma cell proliferation. shRNAs targeting *SOX10* were introduced into A375 cells by lentiviral transduction. **pLKO.1** empty vector served as a control vector (Ctrl.). After puromycin selection, cells were seeded in 384-well and cell

confluence was measured by IncuCyte imaging system. Error bars represent S.D. of triplicate independent experiments. **f,** *SOX10* suppression induces senescence. Senescence was detected by staining of β-galactosidase activity. **g,** Western blot analysis of RB protein, CDK inhibitors CDKN1A ($p21^{cip1}$) and CDKN1B ($p27^{kip1}$) in SOX10 knockdown A375 cells. HSP90 served as a loading control. **h,** Vemurafenib treatment selects for cells that have higher level of EGFR *and* lower level of *SOX10*. A375 cells expressing shRNAs targeting SOX10 as described above were cultured in the absence or in the presence of 1 μM vemurafenib for 10 days before the harvest for qRT-PCR analysis. Error bars represent S.D. of measurement replicates (n=3). All experiments shown except panel a and b were performed independently at least three times.

Extended data Figure 3 | SOX10 loss and TGFβ activation induce multiple RTKs.

a, EGFR inhibition (gefinitib) is not sufficient to restore vemurafenib sensitivity of *SOX10*-loss cells; Targeting PI3K, a common downstream effector of RTKs, with a selective inhibitor (GDC0941) sensitizes *SOX10*-loss cells to vemurafenib. shRNAs targeting *SOX10* were introduced into A375 cells by lentiviral transduction. **pLKO.1** empty vector served as a control vector (Ctrl.). Cells were seeded in 6-well plates at the same density in the presence or absence of drug(s) at indicated concentration. Cells were cultured for 2 weeks in the absence of vemurafenib or 4 weeks in the presence of vemurafenib before fixing and staining. Figure 2e is shown again as a reference. **b,** Increased RTKs activation in *SOX10*-knockdown cells by long-term vemurafenib treatment. A375 cells infected by shSOX10-1 vector or the **PLKO.1** empty vector (Ctrl.) were cultured in the absence or presence of 1 μM vemurafenib for the indicated number of days and processed with Human Phospho-Receptor Tyrosine Kinase Array Kit (R&D) according to the manufacturer's instructions. **c**, SOX10 knockdown upregulates both EGFR and PDGFRβQuantification of protein and mRNA were accomplished by Western blot and qRT-PCR analysis. Error bars represent S.D. of measurement replicates (n=3). **d**, Increased RTKs activation in A375 cells by long-term

treatment with recombinant TGFβ (200 pM) and vemurafenib (1 μM). A375 cells were cultured in the presence of vemurafenib (1μM), recombinant TGFβ (200pM) or their combination for indicated number of days and processed with Human Phospho-Receptor Tyrosine Kinase Array Kit (R&D) according to the manufacturer's instructions. All experiments shown except RTK array analysis were performed independently at least two times.

Extended data Figure 4 | SOX10 loss activates TGFβ signalling and induces senescence in WM266-4 cells.

a, SOX10 loss confers vemurafenib resistance in BRAF(V600D) melanoma cell line WM266-4. Cells expressing empty vector PLKO.1 (Ctrl.) or shRNAs targeting *SOX10* transduced by lentivirus were treated with increasing concentrations of vemurafenib for 6 days. Cell viability was determined by CellTiter-Blue® assay according to the instruction of manufacturer. Relative survival is represented as the ratio of cell viability in the presence of vemurafenib to that in the absence of drug treatment. Error bars represent S.D. of triplicate independent experiments. **b,** SOX10 downregulation leads to growth deficit in WM266-4 cells. Cells expressing the control vector **PLKO.1** (Ctrl.) or shRNAs against *SOX10* were seeded at the same density in 96-well plates and cultured for 6 days. Cell viability was determined by CellTiter-Blue® assay. Error bars represent S.D. of triplicate independent experiments. **c,** *SOX10* suppression results in *EGFR* and *PDGFRB* upregulation in WM266-4 cells. Error bars represent S.D. of measurement replicates (n=3). **d,** *SOX10* loss upregulates TGFβ receptor and its *bona fide* target genes. Relative mRNA level of *EGFR, PDGFRB, SOX10, ANGPTL4, TAGLN, CYR61, CTGF, TGFBR2 and JUN* were determined by qRT-PCR analysis. **pLKO.1** empty vector served as a control vector (Ctrl.). Error bars represent S.D. of measurement replicates (n=3). **e,** *SOX10* suppression induces senescence in WM266-4 cells. Senescence was detected by staining of β-galactosidase activity. **f,** Western blot analysis

of RB protein, p-RB (S780), and CDK inhibitor CDKN1B ($p27^{kip1}$) in *SOX10* knockdown cells. HSP90 served as a loading control. **g,** Vemurafenib treatment compromises oncogene induced senescence in SOX10 knockdown cells. WM266-4 cells expressing PLKO.1 (Ctrl.) or shSOX10-1 were seeded at the same density in 6-well plates and cultured in the absence or presence of vemurafenib at indicated concentration for 72 hours before the harvest for western blot analysis. All experiments shown were performed independently at least three times.

Extended data Figure 5 | SOX10 loss activates TGFβ signalling and induces senescence in COLO679 cells.

a, SOX10 loss confers vemurafenib resistance in BRAF(V600E) melanoma cell line COLO679. Cells expressing empty vector **PLKO.1** (Ctrl.) or shRNAs targeting *SOX10* transduced by lentivirus were treated with increasing concentrations of vemurafenib for 6 days. Cell viability was determined using CellTiter-Blue® according to the instruction of manufacturer. Relative survival is represented as the ratio of cell viability in the presence of vemurafenib to that in the absence of drug treatment. Error bars represent S.D. of triplicate independent experiments. **b,** SOX10 downregulation leads to growth deficit in COLO679 cells. Cells expressing the control vector **PLKO.1** (Ctrl.) or shRNAs targeting *SOX10* were seeded at the same density in 96-well plates and cultured for 6 days. Cell viability was determined using CellTiter-Blue® assay. Error bars represent S.D. of triplicate independent experiments. **c,** *SOX10* suppression results in *EGFR* and *PDGFRB* upregulation in COLO679 cells. Error bars represent S.D. of measurement replicates (n=3). **d,** *SOX10* loss upregulates of TGFβ receptor and its *bona fide* target genes in COLO679 cells. Relative mRNA level of *EGFR, PDGFRB, SOX10*, *ANGPTL4, TAGLN, CYR61, CTGF, TGFBR2 and JUN* were determined by qRT-PCR analysis. **pLKO.1** empty vector served as a control vector (Ctrl.). Error bars represent S.D. of measurement replicates (n=3). **e,** *SOX10* suppression induces

senescence in COLO679 cells. Senescence was detected by staining of β-galactosidase activity. **f,** Western blot analysis of RB protein, p-RB (S780) and CDK inhibitor CDKN1B ($p27^{kip1}$) in SOX10 knockdown cells. HSP90 served as a loading control. All experiments shown were performed independently at least three times.

Extended data Figure 6 | *EGFR* and *SOX10* expression are inversely correlated in melanoma

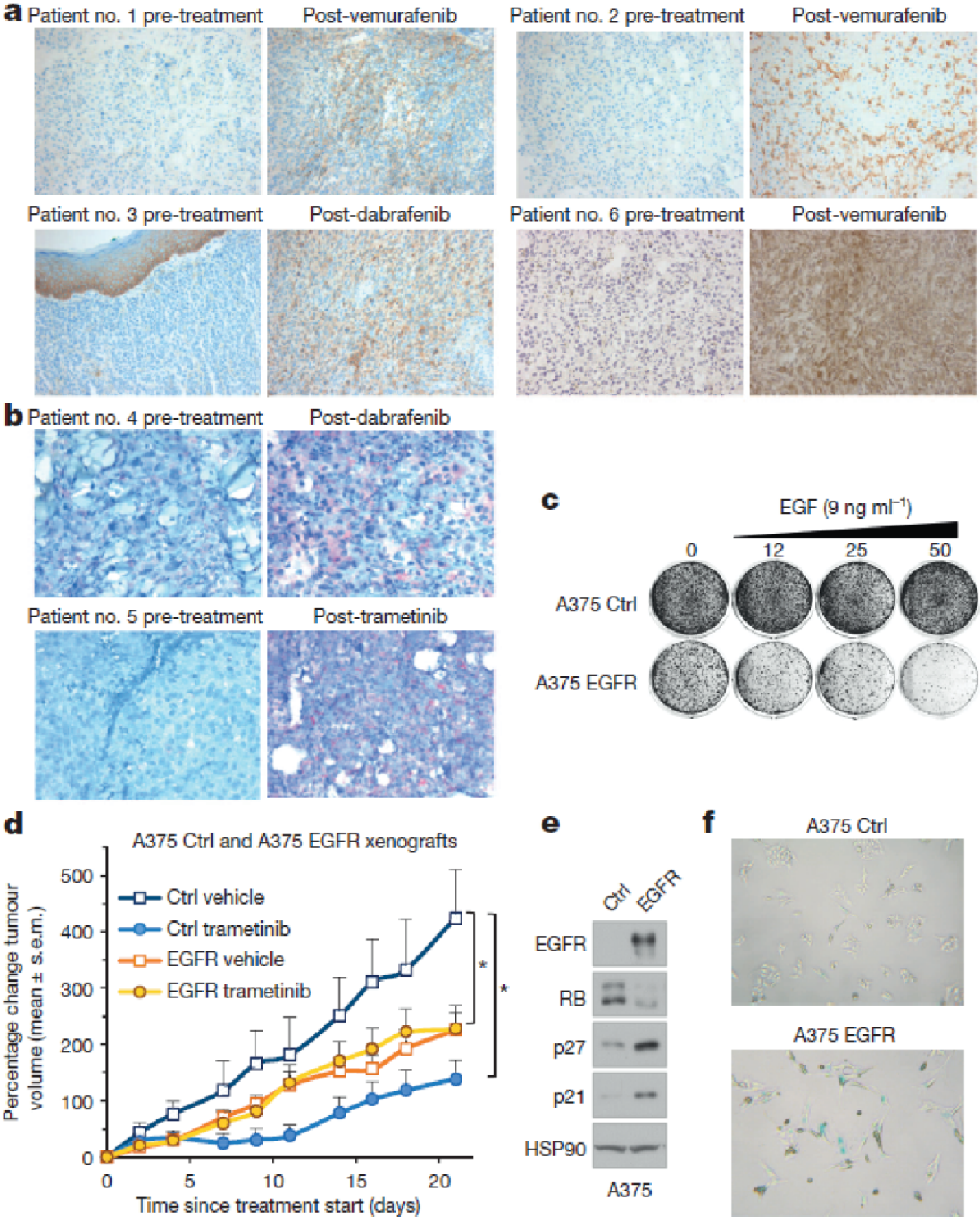
a, A375 cells infected by two independent non-overlapping shSOX10 vectors or the **PLKO.1** empty vector (Ctrl.) were cultured in the absence or presence of 1 μM vemurafenib for the indicated number of days. The last two samples (labelled in blue) were first treated with 1 μM vemurafenib for 10 days and subsequently cultured in the absence of vemurafenib for the indicated number of days. Means of duplicate measurements are shown. **b,** Inverse correlation between *SOX10* and *PDGFRB* in panel of human *BRAF* mutant melanoma cell lines. Relative gene expression levels of *SOX10* and *PDGFRB* were acquired from Cancer Cell Line Encyclopedia (CCLE). R stands for Pearson product-moment correlation coefficient. **c, d,** Ectopic expression of SOX10 suppresses TGFβ signalling and downregulates EGFR and PDGFRB in LOXIMVI cell line. SOX10 was introduced to LOXIMVI cells by lentiviral transduction (SOX10, **PLX301-SOX10**). **PLX301-GFP** served as a control vector (Ctrl.). Protein levels were determined by Western blot analysis and mRNA levels were determined by qRT-PCR analysis. Error bars represent S.D. of measurement replicates (n=3). **e,** Ectopic expression of SOX10 sensitizes LOXIMVI cell to vemurafenib. Cells expressing GFP or SOX10 transduced by lentivirus were treated with increasing concentrations of vemurafenib for 6 days. Cell viability was determined using CellTiter-Blue® assay. Relative survival is represented as the ratio of cell viability in the presence of vemurafenib to that in the absence of drug treatment. Error bars represent S.D. of triplicate independent experiments. **f**, *SOX10*, *EGFR* and *PDGFRB* expression levels in tumour biopsies from patient #3. **g,** *EGFR* expression levels in patient tumour samples (patient #2, #3 and #5), represented as percentage

of *EGFR* transcript reads of the total number of transcript reads obtained through RNAseq analysis. **h,** Gene expression level of TGFβ receptors and target genes in tumour biopsies from patient #3. (f-h), Total RNA was isolated from FFPE specimens derived from tumour biopsies of patient as indicated both before and after development of drug resistance (figure 1a,b). After reverse transcription, gene expression levels were determined by transcriptome sequencing. All experiments shown except the ones that involve clinical samples were performed independently at least two times.

Extended data Figure 7 |Role of BRAF and MITF in *SOX10*-induced drug resistance.

a, PCR analysis of *BRAF* splicing variant in cDNA from patient #5. PCR primers flanking the junction of exon #3 and exon #9 was used to detect the 61-kDa BRAF variant identified by ref[7]. cDNA derived from C4 clone of SKMEL-239 cells served as a positive control. **b,** Differential gene expression of BRAF and neural cell markers in patient biopsies. Total RNA was isolated from FFPE specimens derived from tumour biopsies of patient #5 before and after development of drug resistance (figure 1b). After reverse transcription, gene expression levels were determined by transcriptome sequencing. **c,** *SOX10* suppression leads to *MITF* downregulation. The mRNA levels of *MITF* and *SOX10* were determined by qRT-PCR analysis. **pLKO.1** empty vector served as a control vector (Ctrl.). Error bars represent S.D. of measurement replicates (n=3). **d,** Suppression of *MITF* does NOT induce *EGFR* or *PDGFRB*. shRNAs targeting MITF were introduced to A375 cells by lentiviral transduction. Relative mRNA level of *SOX10, MITF, EGFR, PDGFRB* and *DCT* were determined by qRT-PCR analysis. Error bars represent S.D. of measurement replicates (n=3). **e,** *MITF* knockdown does NOT affect vemurafenib sensitivity. shRNAs targeting MITF were introduced to A375 cells by lentiviral transduction. Cells were seeded at the same density in 6-well plates and cultured in the absence or presence of vemurafenib (for 3 weeks) at the indicated concentrations. The

cells were fixed, stained and photographed. All experiments shown except the ones that involve clinical samples were performed independently at least two times.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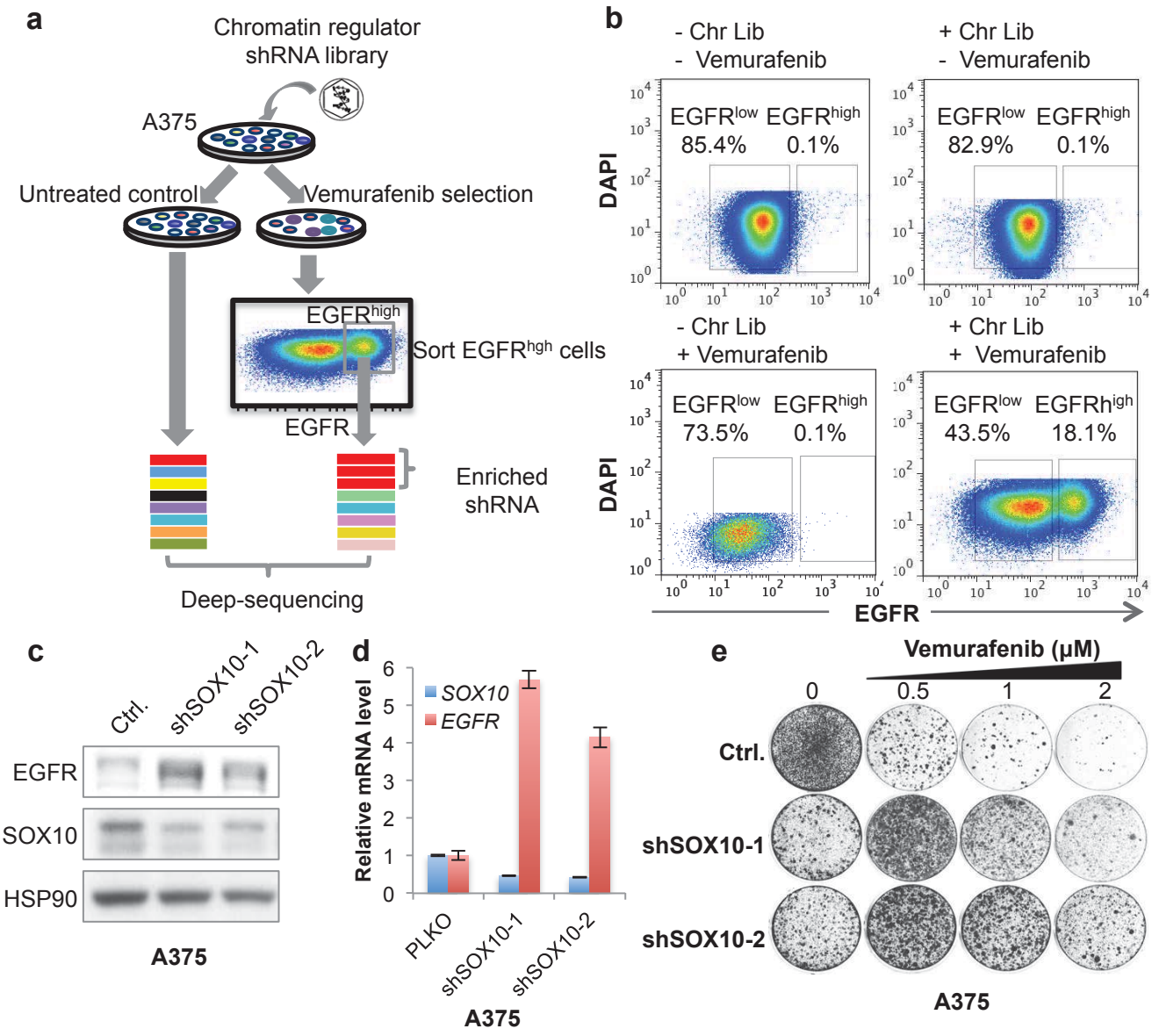
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+ Chr Lib
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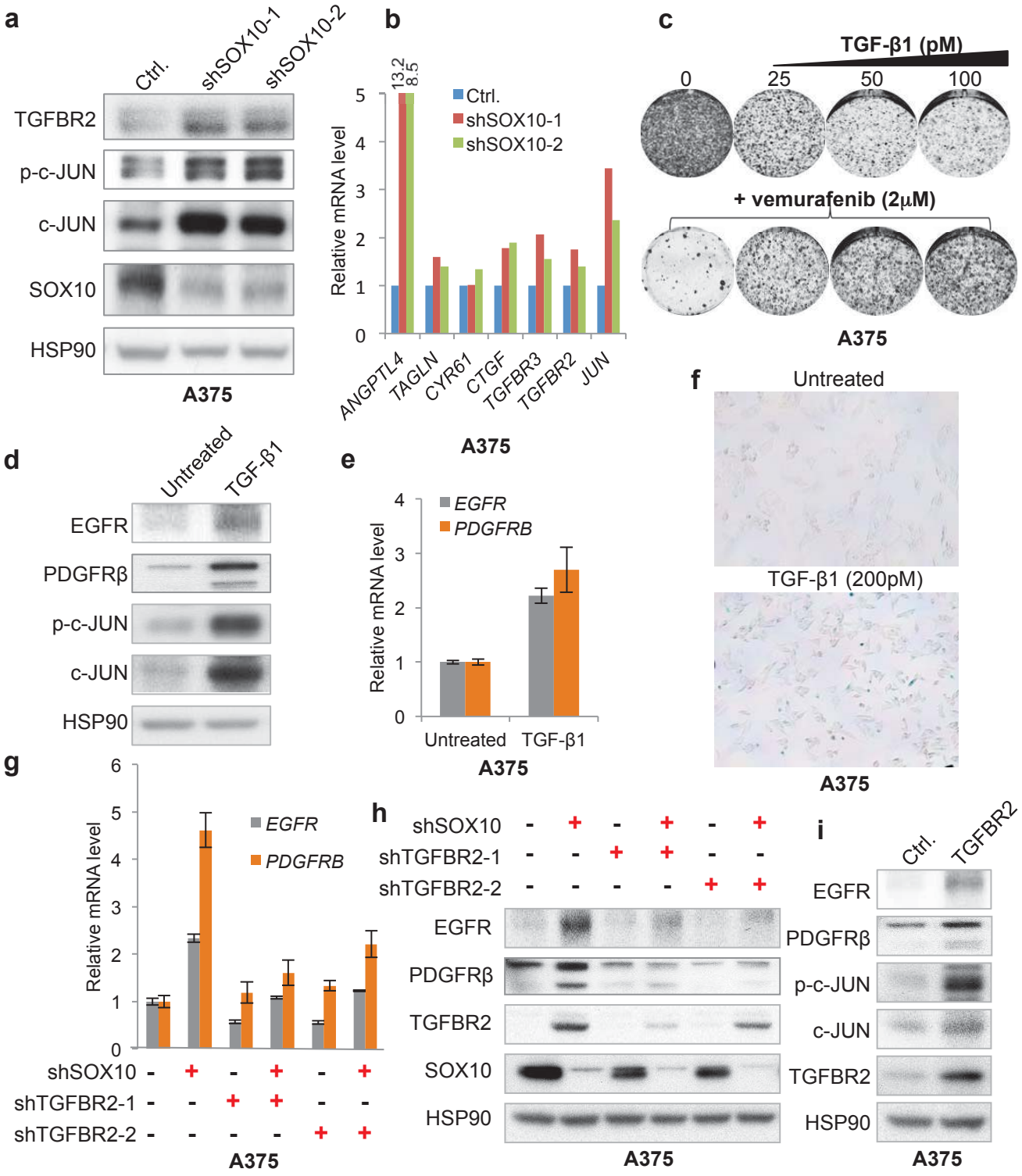
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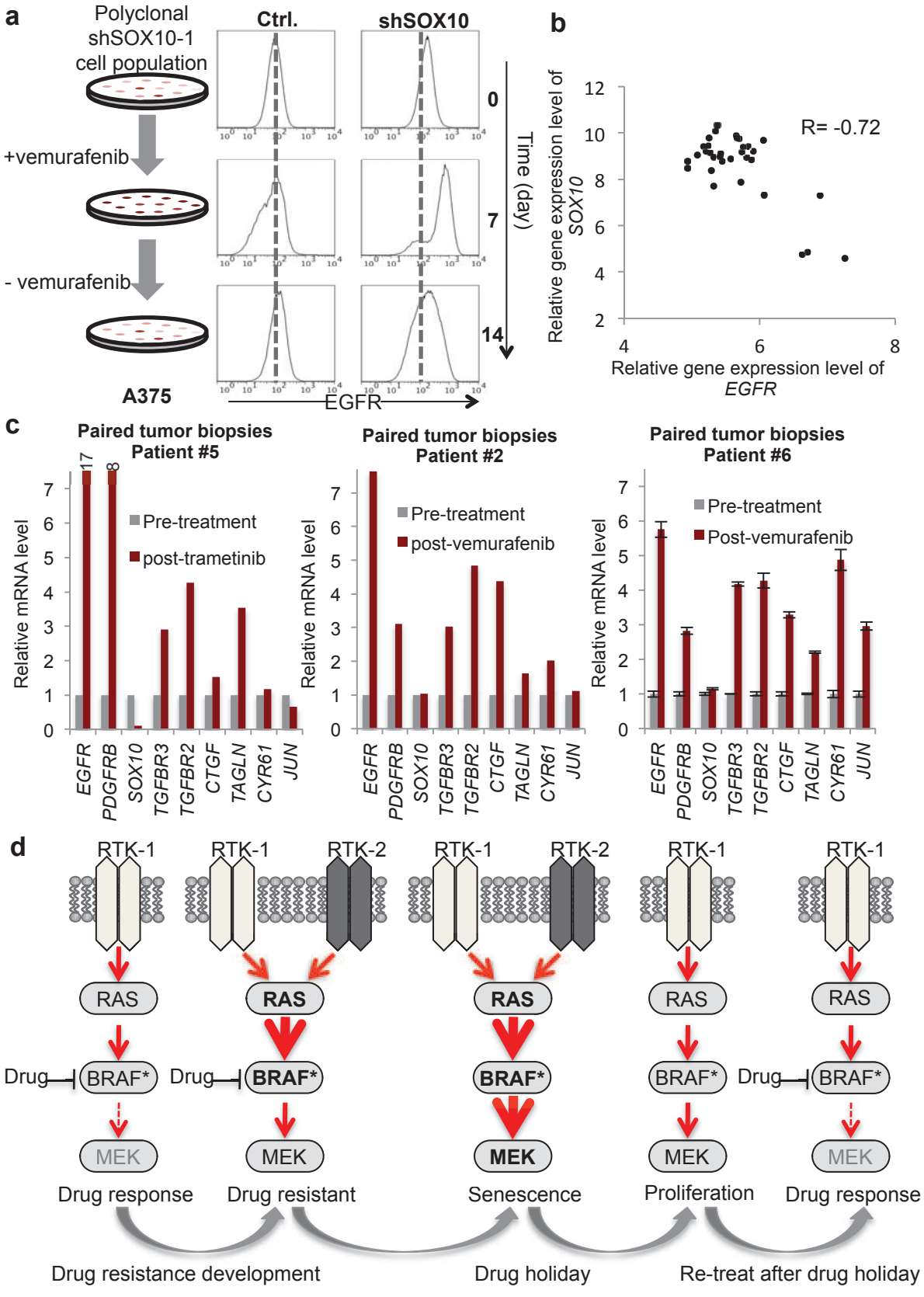
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c
Paired tumor biopsies Patient #5
Paired tumor biopsies Patient #2
Paired tumor biopsies Patient #6
Relative mRNA level
Pre-treatment
post-trametinib
post-vemurafenib
Post-vemurafenib
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