LETTER

PAK signalling drives acquired drug resistance to MAPK inhibitors in BRAF-mutant melanomas

Hezhe Liu1, Shujing Liu2, Gao Zhan3, Bin Wu1, Yueyao Zhu1, Dennie T. Frederick4, Yi Hu5, Wengqun Zhong6, Sergio Randell7, Norah Sadek8, Wei Zhang1, Gang Chen, Chaoxian Cheng, Jingwen Zeng1, Lawrence W. Wu1, Jie Zhang6, Xiaoming Liu2, Wei Xu7, Clemens Krepler1, Katrin Sproesser1, Min Xiao1, Benchun Miao4, Jianglan Liu1, Claire D. Song1, Jephrey Y. Liu1, Giorgos C. Karakousis8, Lynn M. Schuchter7, Yiling Lu9, Gordon Mills9, Yusheng Cong10, Jonathan Chernoff11, Jun Guo12, Genevieve M. Boland13, Ryan J. Sullivan8, Zhi Wei9, Jeffrey Field14, Ravi K. Amaravadi8, Keith T. Flaherty1, Meenhard Herlyn5, Xiaowei Xu2, § & Wei Guo8, §

Targeted BRAF inhibition (BRAFi) and combined BRAF and MEK inhibition (BRAFi and MEKi) therapies have markedly improved the clinical outcomes of patients with metastatic melanoma. Unfortunately, the efficacy of these treatments is often countered by the acquisition of drug resistance1–6. Here we investigated the molecular mechanisms that underlie acquired resistance to BRAFi and to the combined therapy. Consistent with previous studies, we show that resistance to BRAFi is mediated by ERK pathway reactivation. Resistance to the combined therapy, however, is mediated by mechanisms independent of reactivation of ERK in many resistant cell lines and clinical samples. p21-activated kinases (PAKs) become activated in cells with acquired drug resistance and have a pivotal role in mediating resistance. Our screening, using a reverse-phase protein array, revealed distinct mechanisms by which PAKs mediate resistance to BRAFi and the combined therapy. In BRAFi-resistant cells, PAKs phosphorylate CRAF and MEK to reactivate ERK. In cells that are resistant to the combined therapy, PAKs regulate JNK and β-catenin phosphorylation and mTOR pathway activation, and inhibit apoptosis, thereby bypassing ERK. Together, our results provide insights into the molecular mechanisms underlying acquired drug resistance to current targeted therapies, and may help to direct novel drug development efforts to overcome acquired drug resistance.

Several mechanisms, including ERK reactivation7–9, upregulation of the mTOR10 and WNT–β-catenin pathways9, and modulation of apoptosis11 have been reported to mediate acquired drug resistance to BRAFi (BR). However, the molecular mechanisms that underlie resistance to the combined therapy of BRAFi and MEKi (CR), remain elusive.

In some patients, CR is mediated by mutations that augment mechanisms of BRAFi resistance, leading to the activation of downstream effectors of the MAPK and PI3K signalling axes5,12,13. We examined the phosphorylation of ERK (p-ERK(T202/Y204)) in both BR and CR cell lines. Consistent with previous findings, our immunoblotting analysis and immunohistochemistry (IHC) staining showed that the level of p-ERK(T202/Y204) in BR cells was similar to, or higher than, the level expressed in their respective parental cells14 (Fig. 1a and Extended Data Fig. 1a). In CR, however, p-ERK(T202/Y204) was greatly reduced in five out of six cell lines compared to their respective parental cell lines (Fig. 1b). This observation was further corroborated by the IHC staining of p-ERK(T202/Y204) in paired pre- and post-treatment tumour biopsy specimens from eight patients on BRAFi and MEKi combination therapy. p-ERK(T202/Y204) was increased in one out of eight post-treatment tumour biopsy specimens, but was reduced or remained low in the remaining specimens (Fig. 1c, Extended Data Fig. 1b and Supplementary Tables 1, 2). We also analysed p-ERK activity in tumour samples of combination therapy-resistant patient-derived xenografts (CRPDX) that were implanted in different mice. ERK was not reactivated when these mice were treated with BRAF and MEK inhibitors (Extended Data Fig. 1c). The data suggest that the mechanisms underlying CR are different from those that induce BR in many patients.

We detected increased levels of phosphorylated CRAF (p-CRAF(S338)) in most of the acquired drug-resistant cell lines, similar to previous studies13 (Fig. 1d, e). CRAF is directly phosphorylated by PAKs at serine 338 (S338)15,16. We found that PAKs were activated in most of the resistant cells and CRPDX tumour samples (Fig. 1d, e and Extended Data Fig. 1c, d). PAKs are serine/threonine protein kinases that function downstream of the small GTPases CDC42 and RAC1, and are involved in many tumorigenic pathways17. CDC42 and RAC1 show increased expression in some BR and CR cell lines (Extended Data Fig. 1e). Quantitative real-time PCR with reverse transcription (RT–qPCR) analysis shows that the expression of PAK1, PAK2, RAC1 and CDC42 was increased in post-treatment tumour biopsies that were derived from eight patients with metastatic melanoma treated with either BRAFi or BRAFi and MEKi (Fig. 1f). In addition, gene set enrichment analysis of RNA-sequencing data derived from paired pre- and post-treatment tumour biopsy specimens of six patients and the public database18 showed PAK signalling activation in most of the tumour biopsies with acquired resistance to MAPK inhibitors (Extended Data Fig. 1f–1k and Supplementary Table 3).

It has been previously reported that parental melanoma cells with the BRAFV600E mutation are insensitive to the inhibition of PAKs19. Here we found that, unlike parental cells, both BR and CR cells became sensitive to the PAK inhibitor PF-3758309 (ref. 20; Fig. 2a and Extended Data Fig. 2a). Fluorescence-activated cell sorting (FACS) analyses showed...
Conversely, ectopic expression of the constitutively active mutant parental, BR (as indicated by ‘-BR’) (with acquired drug resistance. a

PF-3758309 and profiled them using a reverse-phase protein array established from BR patient-derived xenografts (BRPDX) with

upon PAK inhibition, we treated BR cell lines, including those standing of the signalling pathways that were altered in BR cell lines and 1205Lu-BR xenografts (Fig. 3c). To gain a comprehensive under-

PF-3758309 downregulated MAPK signalling in BR cell lines (Fig. 3b)

S338 and MEK at S298 (refs 15, 16). We found that both p-CRAF(S338)
survival of therapy-resistant melanoma when the MAPK pathway is

Extended Data Fig. 6), suggesting that PAKs have a pivotal role in the
der PAK1 function by RNA interference (RNAi), expression of the

bitors in parental 1205Lu cells (Fig. 2c). In addition to targeting

PAK1, RNAi-mediated silencing of PAK4, a member of the group II

PAKs, also inhibited the growth of BR and CR cells (Extended Data

stream targets of ERK, p-ELK1(S383) and p-RSK(T359/S363). In

addition, mTOR pathway activity was mostly inhibited as indicated

by the decrease in both p-4E-BP1 and p-S6 (Fig. 4 and Extended Data

Fig. 7b, c). Expression of the active PAK1(1079/423E) had limited effect

on cells in the absence of PLX4720, but blunted the inhibitory effect of

PLX4720 on p-MEK1(S217/S221), p-ERK(T202/Y204), p-S6(S235/S236)
on cells in the absence of PLX4720, but blunted the inhibitory effect of

and p-S6(S240/S244), p-4E-BP1(S65) and p-4E-BP1

downstream target p-c-JUN(S73) (also known as p-JUN(S73)); (2) inhibition of cell-cycle progression as shown by the decrease in FOXM1, cyclin B1 and CDK1, concurrent with the decrease in p-Rb(S807/S811); (3) inhibition of mTOR signalling as indicated by the decrease in p-S6(S235/S236), p-S6(S240/S244), p-4E-BP1(S65) and p-4E-BP1

(T37/T46). We also examined the effect of expressing constitutively active PAK1 in the parental cell line 1205Lu using a reverse-phase protein array and immunoblotting. PAK1(1079/423E) had limited effect on cells in the absence of PLX4720, but blunted the inhibitory effect of

PLX4720 on p-MEK1(S217/S221), p-ERK(T202/Y204), p-S6(S235/S236) and p-S6(S240/S244) (Fig. 3e, f).

For CR cells, PF-3758309 did not significantly affect the phosphorylation of ERK as was observed in BR cells, but inhibited the downstream targets of ERK, p-ELK1(S383) and p-ERK(T359/S363). In addition, mTOR pathway activity was mostly inhibited as indicated by the decrease in both p-4E-BP1 and p-S6 (Fig. 4 and Extended Data Fig. 7b, c). Expression of the active PAK1(1079/423E) in 1205Lu parental cells did not lead to ERK reactivation in response to PLX4720 and PD0325901, but instead promoted cell-cycle progression, as indicated by the increase in p-Rb and p-cyclin B1. Furthermore, the decrease in BIM and BAX in cells expressing constitutively active PAK1 indicated that apoptosis was inhibited (Extended Data Fig. 7d). Together, our analyses showed that PAKs mediate CR through at least four pathways: (1) PAK activation is required for sustained JNK activity. Because JNK and ERK have common downstream targets such as ELK1, JUN and FOX3,12, JNK activity may partially compensate for the lack of ERK activation. (2) Activated PAKs and CRAF phosphorylate BAD, which blocks
apoptotic signalling. (3) PAKs regulate the phosphorylation of β-catenin. (4) PAKs probably activate the mTOR pathway (Fig. 4 and Extended Data Fig. 7). Compared with parental and BR cells, CR cells were more sensitive to JNK and S6K inhibitors, but less responsive to the ERK inhibitor, which is consistent with a recent study (Extended Data Fig. 8f). This study highlights PAKs provide insight into the rewiring of the signalling networks in BR and CR cells (Extended Data Fig. 8f). This study highlights PAKs as pivotal mediators of drug resistance, and potential therapeutic targets for treating patients whose tumours progress on these targeted therapies.

Our study reveals mechanisms that underlie acquired drug resistance to both BRAFi and combined BRAFi and MEKi therapies, and provide insight into the rewiring of the signalling networks in BR and CR cells (Extended Data Fig. 8f). This study highlights PAKs as pivotal mediators of drug resistance, and potential therapeutic targets for treating patients whose tumours progress on these targeted therapies.
Figure 4 | Signalling pathways in CR melanoma cells with PAK inhibition. 

**a.** Heat map analysis of indicated proteins in CR cells treated with PF-3758309. 

**b.** Immunoblot analysis of 1205Lu and UACC903 parental and CR cells after the indicated treatments. The levels of MAPK-pathway-related proteins, cell-cycle-related proteins, and apoptosis-related proteins were analysed.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 22 October 2015; accepted 24 August 2017.

Published online 27 September 2017.

1. Chapman, P. B. et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N. Engl. J. Med.* **364**, 2507–2516 (2011).
2. Shi, H. et al. Acquired resistance and clonal evolution in melanoma during BRAF inhibitor therapy. *Cancer Discov.* **4**, 80–93 (2014).
3. Boussermart, L. et al. eIF4F is a nexus of resistance to anti-BRAF and anti-MEK cancer therapies. *Nature* **513**, 105–109 (2014).
4. Larkin, J. et al. Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. *N. Engl. J. Med.* **371**, 1867–1876 (2014).
5. Long, G. V. et al. Increased MAPK reactivation in early resistance to dabrafenib/trametinib combination therapy of BRAF-mutant metastatic melanoma. *Nat. Commun.* **5**, 5694 (2014).
6. Long, G. V. et al. Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *N. Engl. J. Med.* **371**, 1877–1888 (2014).
7. Nazarian, R. et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* **468**, 973–977 (2010).
8. Johannessen, C. M. et al. A melanocyte lineage program confers resistance to MAP kinase pathway inhibition. *Nature* **504**, 138–142 (2013).
9. Villanueva, J. et al. Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* **18**, 683–695 (2010).
10. Anastas, J. N. et al. WNT5A enhances resistance of melanoma cells to targeted BRAF inhibitors. *J. Clin. Invest.* **124**, 2877–2890 (2014).
11. Tentori, L., Lascal, P. M. & Graziani, G. Challenging resistance mechanisms to therapies for metastatic melanoma. *Trends Pharmacol. Sci.* **34**, 656–666 (2013).
12. Wagle, N. et al. MAP kinase pathway alterations in BRAF-mutant melanoma patients with acquired resistance to combined RAF/MEK inhibition. *Cancer Discov.* **4**, 61–68 (2014).
13. Moriceau, G. et al. Tumoral-combinatorial mechanisms of acquired resistance limit the efficacy of BRAF/MEK cotargeting but result in melanoma drug addiction. *Cancer Cell* **27**, 240–256 (2015).
14. Van Allen, E. M. et al. The genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. *Cancer Discov.* **4**, 94–109 (2014).
15. King, A. J. et al. Acquired resistance and clonal evolution in melanoma during BRAF V600E mutation. *Cancer Discov.* **4**, 80–93 (2014).
16. Tran, N. H. & Frost, J. A. Phosphorylation of Raf-1 by p21-activated kinase 1 and Src regulates Raf-1 autophosphorylation. *J. Biol. Chem.* **278**, 11221–11226 (2003).
17. Rudin, M. C., Semenova, G., Kosoff, R. & Chernoff, J. PAK signalling during the development and progression of cancer. *Nat. Rev. Cancer* **14**, 13–23 (2014).
18. Hugo, W. et al. Non-genomic and immune evolution of melanoma acquiring MAPK Resistance. *Cell* **162**, 1271–1285 (2015).
19. Long, G. V. et al. P21-activated kinase 1 (PAK1) as a therapeutic target in BRAF wild-type melanoma. *J. Natl Cancer Inst.* **105**, 606–607 (2013).
20. Murray, B. W. et al. Small-molecule p21-activated kinase inhibitor PF-3758309 is a potent inhibitor of oncogenic signaling and tumor growth. *Proc. Natl Acad. Sci. USA* **107**, 9446–9451 (2010).
21. Cavaglini, G., Dolfi, F., Claret, F. X. & Karin, M. Induction of c-fos expression through phosphorylation of serine 338. *Nature* **396**, 180–183 (1998).
22. Bacigalupo, S., Dériard, B., Davis, R. J. & Cerione, R. A. Cdc42 and PAK-mediated apoptosis inhibition in metastatic melanoma. *Nat. Commun.* **5**, 5694 (2014).
23. Alavi, A., Hood, J., Frausto, R., Stupack, D. G. & Cheresh, D. A. Role of Raf in vascular protection from distinct apoptotic stimuli. *Science* **301**, 94–96 (2003).
24. Higuchi, M., Onishi, K., Kikuchi, C. & Gotoh, Y. Scaffolding function of PAK in the PDK1–Akt pathway. *Nat. Cell Biol.* **10**, 1356–1364 (2008).

**Supplementary Information** is available in the online version of the paper.

**Acknowledgements** We thank Pfizer, Inc. for providing PF-3758309, and Plexxikon, Inc. for providing PLX4720 and PD0325901. This work is supported by NIH grants R01-GM085145, U54-CA193417 and CA174523, single grant to W.G., CA114046 and CA114046 and CA174523 to X.J., the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation and NCI CA025874, CA114046 and CA174523 to M.H, and CA142928 to J.C.

**Author Contributions** H.L., W.G., X.X. and M.H. conceived and designed the experiments. S.L., Y.Z. and X.L. performed the experiments. H.L., S.L. and G.Z. performed the reverse-phase protein array experiments. D.T.F., B.M., R.J.S., W.X., J.Y.L., G.C.K., M.H. and R.K.A. performed bioinformatics and statistical analyses. Y.L. and G.M. performed the sequencing. L.M.S., G.M.B., J.C., J.F., R.K.A. and K.T.F. provided melanoma specimens, key constructs or associated clinical data. H.L., S.L., G.Z., M.H., X.X. and W.G. wrote the manuscript.

**Author Information** Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. Correspondence and requests for materials should be addressed to W.G. (gouwei@sas.upenn.edu). X.X. (xug@mail.med.upenn.edu) and M.H. (Herlynm@wistar.org).

**Reviewer Information** Nature thanks A. A. Qutub, C. Wells and the other anonymous reviewer(s) for their contribution to the peer review of this work.
METHODS

Cell culture, reagents, plasmids and antibodies. All human metastatic melanoma cell lines were established at The Wistar Institute as previously described25. They were authenticated by DNA fingerprinting and were tested regularly before assays to avoid mycoplasma contamination. All melanoma cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with 5% FBS. Resistant cells were maintained with PLX4720 at 3 μM for BR cells and BR/PLX cells or the combination of PLX4720 at 3 μM plus PD0325901 at 300 nM for CR cells throughout the experiment. 1205Lu cells stably expressing the vector control or PAK1L107F/T423E mutant were selected using 1 μg ml⁻¹ puromycin for 10 days. DNA and RNA transfection were conducted using Lipofectamine2000 (Invitrogen), Fugene 6 (Roche) or Lipofectamine RNAiMAX (Invitrogen). The human PAK1 siRNA sequence was 5'-GAAAGAATTATACTACCGTTT-3’; the PAK2 siRNA sequence was 5'-AGAAGAGAGACTGATCATATTAA-3’ (ref. 26) and the control luciferase GL2 siRNA sequence was 5'-AACGTACGCCGAATACCTTGCA-3’. Short hairpin RNA (shRNA) targeting JNK1, JNK1/2 and CTNNB1 (β-catenin) were provided by S. Andreadis (University of Buffalo) and Z. Lu (M.D. Anderson)27. The shRNA clones targeting PAK4 were ordered from Sigma-Aldrich: 5’-GAGGCCAAGGGCTATCCCAT-3’, 5’-GGAATGTGGGAGAGATGTGA-3’ and 5’-GACGTACCTGTCCTGACC-3’. PLX4720 and PD0325901 were provided by Plexikon. PF-3758309 was provided by Pfizer. MEK162, LGX818, GSK1120212, GDC0973 and PLX4032 were purchased from Selleckchem. Human kinase-dead PAK1/2/3/7 mutant and constitutively active PAK1L107F/T423E mutant were cloned into the pCMV6 or pBabe PURO vector. All constructs were confirmed by sequencing. All information about the primary antibodies is included in Supplementary Table 5. Secondary antibodies were purchased from Invitrogen or GE Healthcare.

RNA extraction and RT-qPCR. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s instructions. cDNA synthesis was performed with the M-MLV Reverse Transcriptase Kit (Promega). RT-qPCR was performed using the Fast SYBR Green Master Mix Kit (Life Technologies). Amplifications were performed using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies). All experiments were performed in triplicate. Expression ratios of controls were normalized to 1. Oligonucleotide primers used in RT-qPCR: PAK1, GCTGTTCTGGATGTGTTGGA and TTCTGAAACTGGTGGCACTG; PAK2, ACAGAAGACCCCCAGCAGGTAGT and AAAGACTTGCCAGACCAC; PAK4, CAGGGAGAGGGCCACGCA and CCTGTAACTGGCACCAC; RAC1, CAATGGGTCTCCTTGAGAGTACA and ACGTCTGTTTGCGGGTAGGAGAG; CDC42, TAACTCCACCTGTCCTCAAGATCC and CCTCTACAACACATTCTTCAGACC; GAPDH, GAAGGTGAAGGTCGGAGTC and GAAGATGGTGTAGGGTATTTC.

Cell viability, cell cycle and apoptosis assay. Cell viability was assayed by Giemsa staining. In brief, equal numbers of cells were seeded at approximately 40% confluency in six-well plates and treated with DMSO or the indicated inhibitors. The cells were then washed with PBS and fixed with methanol and acetone (1:1), and stained with Giemsa solution (Millipore) for 30 min. The cell density was measured using Adobe Photoshop or ImageJ. The values after background subtraction were normalized to the control group. The MTT assay was performed using the Cell Proliferation Kit I (Roche) according to the manufacturer’s instructions. The IC₅₀ values were calculated from dose–response curves using Graphpad Prism 5.

For the apoptosis assay, BR and CR cells were treated with DMSO or PF-3758309 for 72 h. Cells were resuspended in 0.1 ml PBS after centrifugation, and then co-stained with propidum iodide (Sigma-Aldrich) at 1 μg ml⁻¹ and PSV64 3 (Molecular Targeting Technologies) at 5 μM. Cell suspensions were kept in the dark at room temperature for 5 min. Subsequently, 0.2 ml PBS with 10% FBS was added to each cell suspension (multiplication–comparison correction) in at least four BR cell lines out of the nine BR cell lines after PF-3758309 treatment versus DMSO. Similarly, in Extended Data Fig. 7b, we show the proteins with significant changes in at least two CR cell lines out of the three CR cell lines after PF-3758309 treatment versus DMSO.

For the cell-cycle analysis, BR and CR cells were treated with DMSO or 1 μM PF-3758309 for 48 h. Control or treated cells were fixed with ice-cold 100% EtOH for 20 min, stained with propidium iodide and subsequently analysed using a FACScan. FSC and SSC gating was used to select single cells for the cell-cycle analysis. The G0/G1 peak was used to select single cells for the cell-cycle analysis. The G0/G1 peak was used to select single cells for the cell-cycle analysis.

Reverse-phase protein array. The reverse-phase protein array (RPAA) assay was performed by the M.D. Anderson Cancer Center core facility using 50 μg protein per sample. Antibodies were validated by western blotting20. The RPAA data have been submitted to the Gene Expression Omnibus public database at the National Center for Biotechnology Information, following the information about RPAA experiment guidelines. The accession codes are GSE96902 and GSE96753.

IHC staining. IHC staining was performed on formalin-fixed, paraffin-embedded sections. Antigen retrieval was performed by steaming the slides in citrate buffer for 5 min (pH 6.0). Sections were incubated with anti-p-ERK (1:100; Cell Signaling) antibody overnight at 4°C, followed by incubation with a biotinylated secondary antibody (1:200; Jackson Immuno Research) for 30 min. Detection was performed using Nova Red or DAB (Fisher Scientific). The pathologist who examined the tissue sections was blinded to the clinical treatment information. There were no identifiable images of human research participants.

Patient specimen collection and generation of PDX. Clinical data and tissue collection from patients with Stage IV melanoma was approved with informed consent of the patient at the University of Pennsylvania Abramson Cancer Center or Massachusetts General Hospital, in accordance with the Institutional Review Boards of both institutions. Data collection was performed in compliance with all relevant ethical regulations for human research participants. The collection of tumour tissues for the generation of PDX was approved by the University of Pennsylvania Institutional Review Board21. PDX tumours derived from patients with metastatic melanoma who progressed on the combinatorial targeted therapies were expanded in vivo using NOD/SCID/IL-2Rγ−/- (NSG) mice before the therapy experiments. The expansion phase was under continuous drug pressure at approximately clinical plasma levels.

Xenograft tumour model. The animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania and The Wistar Institute. Drug-resistant cells (5 × 10⁶ cells per animal) were injected into flanks of eight-week-old female athymic nude mice, and mice were then allocated randomly to each treatment group. Daily oral administration of PLX4720 (200 p.p.m.), PD0325901 (7 p.p.m.) and PF-3758309 (25 p.p.m.) individually or in combination, was started when the tumour volume in mice reached ~100 mm³. Tumour volume was calculated using the formula: tumour volume = length × width × height/2. The mice were subjected to continued treatment until the tumour reached 10% of the body weight or reached the limit of the allowed tumour dimensions. For single tumours, growth was limited to a diameter of 2.0 cm (or a volume of 4.2 cm³) at the widest point as long as the mouse remained healthy. For multiple tumours in a mouse, tumour growth was limited to a total diameter of 3.0 cm (the limit for any of the single tumour was still 2.0 cm). Throughout the experiments, all of the mice were carefully monitored at least twice a week for signs of distress, and were euthanized when they were determined to have poor body condition scores. Mice that died or with tumour sizes that reached the dimension limit set by Penn IACUC guidelines shortly after the start of the procedure were excluded from statistical analysis. No statistical methods were used to predetermine sample size.

Statistical analyses and reproducibility. RPAA data analysis was performed according to the protocol from the M.D. Anderson Cancer Center. Specifically, relative protein levels for each sample were determined by interpolation of each dilution curve from the standard curve (supercurve) of the slide (antibody). Supercurve is constructed by a script in R written by the RPAA core facility. These values are defined as the Supercurve log₂ value. All data points were normalized for protein loading and transformed to a linear value, designated as ‘normalized linear’. The normalized linear value was transformed to a log₂ value, and then median-centred for further analysis. Median-centred values were centred by subtracting the median of all samples in a given protein. All of the above-mentioned procedures were performed by the RPAA core facility. The normalized data provided by the RPAA core facility were analysed using the LIMMA package in R. In Extended Data Fig. 7a, we show proteins with significant changes (false discovery rate is controlled at the 0.01 level by using the methods including P values, RPPA, R and using multiple–comparison correction) in at least four BR cell lines out of the nine BR cell lines after PF-3758309 treatment versus DMSO. Similarly, in Extended Data Fig. 7b, we show the proteins with significant changes in at least two CR cell lines out of the three CR cell lines after PF-3758309 treatment versus DMSO.

The microarray data were analysed with the lumi package in the R/Bioconductor environment2. The probes were filtered to include genes with a detection value of P < 0.05 in all samples for use in further analyses. The data was transformed by variance-stabilizing transformation (VST) and then normalized by quantile normalization using the lumi package. The microarray data of genes of interest, including PAKs, RAC1 and MEK162, were extracted and subsequently visualized in heat maps using the heat map.2 program within the gplots package of R.

The enrichment score (Extended Data Fig. 1g, i, k) was generated using the single-sample GSEA (ssGSEA) method22. The enrichment score analysis was carried out using GSVapackage in R33. The enrichment score in the ssGSEA
method essentially is the weighted sum of the difference between the empirical cumulative distribution functions of the genes within the gene signature and the remaining genes.

All other statistical analyses were performed using R, version 2.14. Two-way ANOVA (mouse tumour volumes) and log-rank test (mouse survival) were used to compare data between PLX4720 or PLX4720 with PD0325901 and all other groups, no multiple comparisons. Two-sided Student’s t-tests were used for pairwise comparison of the remaining datasets.

Data availability. RPPA data are available from the NCBI Gene Expression Omnibus (GEO) under accession numbers GSE96902 and GSE96753. Patient information is provided in Supplementary Table 1. Information for p-ERK IHC staining is provided in Supplementary Table 2. Information for patients with PAK-pathway activation is provided in Supplementary Table 3. IC_{50} values for MTT assays are provided in Supplementary Table 4. Antibody information is provided in Supplementary Table 5. All mouse data are provided in Supplementary Table 6. The number of times the experiments have been completed independently with similar results is listed in Supplementary Table 7. For immunoblot source data, see Supplementary Fig. 1. Source Data are provided for all graphs. All of the data are available from the authors upon reasonable request.

25. Zhang, G. et al. Targeting mitochondrial biogenesis to overcome drug resistance to MAPK inhibitors. J. Clin. Invest. 126, 1834–1856 (2016).
26. Coniglio, S. J., Zavarella, S. & Symons, M. H. Pak1 and Pak2 mediate tumor cell invasion through distinct signaling mechanisms. Mol. Cell. Biol. 28, 4162–4172 (2008).
27. Arias-Romero, L. E., Villamar-Cruz, O., Huang, M., Hoeflisch, K. P. & Chernoff, J. Pak1 kinase links ErbB2 to β-catenin in transformation of breast epithelial cells. Cancer Res. 73, 3671–3682 (2013).
28. You, H. et al. JNK regulates compliance-induced adherens junctions formation in epithelial cells and tissues. J. Cell Sci. 126, 2718–2729 (2013).
29. Ji, H., Wang, J., Fang, B., Fang, X. & Lu, Z. α-Catenin inhibits glioma cell migration, invasion, and proliferation by suppression of β-catenin transactivation. J. Neurooncol. 103, 445–451 (2011).
30. Tibes, R. et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. Mol. Cancer Ther. 5, 2512–2521 (2006).
31. Krepler, C. et al. Personalized preclinical trials in BRAF inhibitor-resistant patient-derived xenograft models identify second-line combination therapies. Clin. Cancer Res. 22, 1592–1602 (2016).
32. Barbie, D. A. et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. Nature 462, 108–112 (2009).
33. Hänzelmann, S., Castelo, R. & Guinney, J. GSVA: gene set variation analysis for microarray and RNA-seq data. BMC Bioinformatics 14, 7 (2013).
Extended Data Figure 1 | See next page for caption.
**Extended Data Figure 1 | ERK and PAK activity in BR and CR melanoma.** Related to Fig. 1. a, b, IHC staining of p-ERK(T202/Y204) in paired pre- and post-treatment tumour biopsy specimens acquired from patients who relapsed on BRAFi (a) or combined BRAFi and MEKi (b) treatment. Note that some of the strongly positive-stained cells are macrophages rather than tumour cells. Scale bars, 50 μm. The tissues were stained with Nova Red. c, Western blotting analysis of the levels of p-ERK^T202/Y204 and p-PAK1(S199/S204)/PAK2(S192/S197) in WM3939 CRPDX tumour samples. Tumours from mice treated with vehicle control or with BRAFi and MEKi are shown. WM9-CR was used for comparison. d, Western blotting of PAK phosphorylation in matching parental, BR and CR cells. e, Western blotting using a polyclonal antibody that recognizes both CDC42 and RAC1 in matching parental, BR and CR cells. CDC42 and RAC1 could not be separated by SDS–PAGE owing to their similar molecular weights. f, h, j, Heat maps of expression levels of PAKs, RAC1 and CDC42 in paired pre- and post-treatment tumour biopsy specimens acquired from patients with metastatic melanoma who progressed on MAPK inhibitors. Data were analysed using the LIMMA package in R. The fold change of expression levels in a paired post-treatment tumour biopsy specimen over the pre-treatment tumour biopsy specimen is shown in the heat map. Colour scale, the log_2-transformed expression of each gene was normalized to the mean value of all samples. g, i, k, Heat maps of the enrichment scores of two PAK signalling-related gene sets in paired pre- and post-treatment tumour biopsy specimens acquired from patients with metastatic melanoma who relapsed on MAPK inhibitors. The value for each entry is the difference in enrichment score of post-treatment over pre-treatment specimens. Gene expression microarray or RNA-sequencing data were downloaded from EGAD00001001306, GSE65184, GSE65185 and GSE61992.
Extended Data Figure 2 | CR cells resistant to the combination of PLX4720 and PD0325901 exhibit cross-resistance to other combinations of BRAF and MEK inhibitors, and are sensitive to PAK inhibitor PF-3758309. Related to Fig. 2. a, Paired parental and CR cells were treated with a combination of three different sets of BRAF and MEK inhibitors, separately, for four days and then fixed and stained with Giemsa. b, Quantification of cell survival (n = 3 biologically independent samples). c, d, Relative survival of matching parental, BR and CR cells treated with increasing concentrations of PF-3758309 (n = 3 or 4 biologically independent samples, as indicated). All IC50 values are listed in Supplementary Table 4. Two-sided Student's t-tests were used for statistical analyses of the IC50 values. e–h, Seven BR (e) and six CR cell lines (f) were treated with DMSO, 1 μM or 3 μM PF-3758309 for 72 h, and then fixed and stained with Giemsa. The data are shown in g for BR cells and in h for CR cells (n = 3 biologically independent samples). The cell density was measured using ImageJ software (NIH). The values after background subtraction were normalized to cells treated with DMSO. Two-sided Student's t-tests were used for statistical analyses (b–d, g, h). Data are plotted as mean ± s.e.m.
Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | Inhibition of PAKs by PF-3758309 decreased the viability of drug-resistant melanoma cells. Related to Fig. 2. 

a, b, Relative survival of BR and CR cells treated with increasing concentrations of the PF-3758309 (PF), PLX4720 (PLX) and PD0325901 (PD) for 48 h. Cell viability was analysed by MTT assay. The data were normalized to cells treated with DMSO ($n = 4$ biologically independent samples). c, FACS analysis of BR cells and CR cells treated with PF-3758309. All cells were labelled with propidium iodide and PSVue 643, and analysed using a BD LSRII. d, Quantification of cell apoptosis. The percentage of apoptosis cells after PF-3758309 treatment was compared to cells treated with DMSO ($n = 3$ or 4 biologically independent samples as indicated). e, g, Giemsa staining of BRPDX cell lines WM3936 and WM3903 that were treated with DMSO, or different concentrations of PF-3758309 for three days. The quantification of the staining is shown in g ($n = 3$ biologically independent samples). f, h, Anchorage-independent growth assay of WM3936 cells. A total of 2,000 cells were seeded in medium with soft agar in six-well plates. Scale bar, 200 μm. The number of colonies in each field is quantified in h ($n = 6$ biologically independent samples). i, WM3936 cells were treated with DMSO or different concentrations of PF-3758309 for three days. All cells were labelled with propidium iodide and PSVue 643, and then analysed using a BD LSRII. j, Quantification of cell apoptosis. The percentage of apoptosis cells after PF-3758309 treatment was compared to cells treated with DMSO ($n = 5$ biologically independent samples). Two-sided Student’s t-tests (a, b, d, g, h, j) were used for statistical analyses. Data are plotted as mean ± s.e.m.
Extended Data Figure 4 | Cell-cycle analysis of BR and CR cells treated with PF-3758309. Related to Fig. 2. 

a, Immunofluorescence staining of Ki-67 (red) in indicated cells, which were treated with DMSO or 1 μM PF-3758309. The nuclei were stained with DAPI (blue). 

b, Quantification of cells with Ki-67 staining (n > 70 cells per assay, three independent experiments).

c, Flow cytometric analysis (10,000 cells were analysed per assay). Cells were fixed, stained with propidium iodide, and then analysed by a FACscan flow cytometer and ModFit LT (Verity Software).

d, Histograms of propidium iodide staining (n = 3 biologically independent samples). Two-sided Student’s t-tests (b, d) were used for statistical analyses. Data are plotted as mean ± s.e.m.
Extended Data Figure 5 | Inhibition of PAKs by siRNA, kinase-dead dominant-negative PAK1K299R mutant or PAK inhibitor IPA-3 decreased the viability of drug-resistant melanoma cells. Related to Fig. 2. a, b, Relative survival of BR or CR cells transfected with PAK1K299R or PAK1KD, or siRNA against PAK1 and PAK2. Cells were then cultured with PLX4720 or PLX4720 and PD0325901 at different concentrations for 48 h. Cell viability was analysed by MTT assays (n = 4 biologically independent samples). Two-sided Student’s t-tests (for IC50 values) were used for statistical analysis. c, d, PAK1 and actin levels in cells were analysed by western blotting. e, BR and CR cells were treated with DMSO, 10μM or 20μM IPA-3 for 72 h, and then processed for Giemsa staining. f, Quantification of the staining in e (n = 3 biologically independent samples). g, RT–PCR analysis of the expression of PAK4 in indicated cells. h, Giemsa staining of indicated cells. i, Quantification of the staining in h (n = 3 biologically independent samples). Two-sided Student’s t-tests (a, b, f, i) were used for statistical analysis. Data are plotted as mean ± s.e.m.
Combined inhibition of MAPK and PAK pathways significantly inhibited BR and CR tumour proliferation in mice and improved survival. Related to Fig. 2. a, b, Tumour growth curves. Mice were injected with 1205Lu-BR (n = 9 mice per group) or WM9-BR (n = 9 mice per group) cells (a), WM9-CR (n = 5 mice per group) or A2058-CR (control n = 8, other n = 9 mice per group) cells (b), and proceeded for MAPK or PAK inhibition for the indicated number of days. c, Survival curves of mice bearing 1205Lu-BR and WM9-CR xenografts (n = 5 mice per group). All groups were compared to the PLX or PLX and PD group; no multiple comparisons. Two-way ANOVA (a, b) or log-rank test (c) were used for statistical analyses. Individual tumour volume data points can be found in the Source Data. Data are plotted as mean ± s.e.m. For mouse survival, the function survdiff from the survival R package was used.
Extended Data Figure 7 | RPPA and immunoblotting analyses of signalling proteins in melanoma cells treated with MAPK or PAK inhibitor. Related to Figs 3, 4. a, BR cell lines and BRPDX cell lines were treated with DMSO or PF-3758309 for 48 h. Protein lysates from these cells were then analysed by RPPA. Data were analysed using the LIMMA package in R. The levels of identified proteins (that displayed significant changes in at least four BR cell lines after PF-3758309 treatment versus DMSO, $P < 0.01$) are shown in the heat map. Colour scale, log$_2$-transformed expression (red, high; blue, low) for each protein was normalized to the mean value of all samples. b, 1205Lu-CR, UACC903-CR and WM164-CR cells were treated with DMSO or PF-3758309 for 48 h. Cell lysates were analysed by RPPA. Data were analysed using the LIMMA package in R. The levels of identified proteins (that displayed significant changes in at least two CR cell lines after PF-3758309 treatment versus DMSO, $P < 0.01$) are shown in the heat map. Colour scale, log$_2$-transformed expression (red, high; blue, low) for each protein was normalized to the mean value of all samples. c, 1205Lu and UACC903 parental and CR cells were treated as indicated (Fig. 4b). Protein levels were analysed in three independent assays, and the staining was measured by ImageJ. ($n = 2$ for p-ELK1(S383) and p-BAD(S112), $n = 3$ for all other proteins). To minimize variation caused by different exposure time in each independent assay, the staining was normalized to the mean of all samples from the same group before statistical analyses. Two-sided Student’s $t$-tests were used for statistical analyses. Data are plotted as mean ± s.e.m. d, 1205Lu cells stably expressing PAK1T107N/423E or vector control were treated with 1 μM PLX4720 and 100 nM PD0325901 for 48 h. The levels of MAPK pathway-related proteins, cell-cycle-related proteins and apoptosis-related proteins were analysed by western blotting.
Extended Data Figure 8 | Inhibition of JNK, S6K or β-catenin inhibited BR and CR cell viability. Related to Fig. 4. a, Giemsa staining of 1205Lu and UACC903 parental, BR and CR cells that were treated with DMSO, the ERK inhibitor SCH772984 (3 μM), the JNK inhibitor SP600125 (3 μM) or the S6K inhibitor PF-4708671 (3 μM) for three days. b, Quantification of cell survival (n = 3 biologically independent samples). Cell density was quantified with ImageJ. The values were normalized to those of parental cells treated with 1 μM of the respective inhibitor. c, Giemsa staining of indicated cells that were infected with luciferase shRNA, JNK1 shRNA, JNK1/2 shRNA and two different β-catenin shRNA. The quantification of the staining is shown in d (n = 3 biologically independent samples). e, RT–PCR analysis of the expression of JNK1 and β-catenin in the indicated cells. GAPDH was used as a loading control. f, Schematic diagrams showing the molecular mechanisms by which PAKs mediate acquired drug resistance of BRAFV600E melanoma cells to BRAFi (left) and BRAFi and MEKi (right). Two-sided Student’s t-tests (b, d) were used for statistical analyses. Data are plotted as mean ± s.e.m.
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

 Experimental design

1. Sample size
   Describe how sample size was determined.
   The sample size was based on what effect sizes other researchers in similar fields have found. We use this as an estimate of sample size. Throughout this study, at least three biological replicates were included in each experiment. Each experiment was repeated at least twice.

2. Data exclusions
   Describe any data exclusions.
   In the mouse studies, the mice that died shortly after procedure or those with tumor burden reached U. Penn IACUC dimension guideline at early stage of treatment were excluded. For other experiments, all data were included.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All attempts to replicate the experiments were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Mice were allocated randomly to each treatment group. For the other experiments, all data were included.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   The investigators were not blinded to the group allocation during the experiments as different drug treatments need to be administered. However, the pathologist who examine the tumor was blinded to the treatment groups.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).
   n/a  Confirmed
   □  The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   □  A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   □  A statement indicating how many times each experiment was replicated
   □  The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   □  A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   □  The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted
   □  A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   □  Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

software R, version 2.14

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Except for the chemicals that were obtained from certain companies under agreements, all unique materials are available for distribution from the authors or companies.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Antibody information including catalog numbers and clone numbers are shown. More information is provided in the company websites.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

All cell lines presented in this study were established in Dr. Meenhard Herlyn's lab at The Wistar Institute.

b. Describe the method of cell line authentication used.

All cell lines presented in this study were authenticated by DNA fingerprinting.

c. Report whether the cell lines were tested for mycoplasma contamination.

All the cell lines presented in this study were tested for mycoplasma contamination and they were free of mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

None.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Mouse, nude mice (Foxninu) and NSG (NOD. Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice; female/male, 12 weeks;

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

N/A
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

- 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- 3. All plots are contour plots with outliers or pseudocolor plots.
- 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation. For the apoptosis and cell death assay, BR and CR cells were treated with DMSO, 3μM or 5μM PF3758309 for 72 hr. All cells were labeled with propidium iodide and PSVue 643, and then analyzed by BD LSRII. Cells used in cell cycle analysis assays, BR and CR cells, were treated with DMSO or 1μM PF3758309 for 48 hr. Treated cells and controls were then stained with propidium iodide, and subsequently analyzed using a FACSCalibur.

6. Identify the instrument used for data collection. A BD LSRII was utilized for analysis of apoptosis and cell death. A FACSCalibur was used for cell cycle analysis.

7. Describe the software used to collect and analyze the flow cytometry data. DIVA software was used for collecting events on the LSRII. Apoptosis and cell death assay flow cytometric data was analyzed using FloJo software. We used CellQuest software to collect cell cycle data on the FACSCalibur. We used ModFit software for post collection analysis of FCS files.

8. Describe the abundance of the relevant cell populations within post-sort fractions. N/A.

9. Describe the gating strategy used. FSC and SSC gating was used to select the cells for PI analysis for cell cycle analysis. The G0/G1 peak was centered at the PI/FL-2H Channel=200 using cells that were within the FSC/SSC gate and within the specified FL2-A/FL-W gate. FCS files were then analyzed post-collection using ModFit software. Events that were in both the FSC/SSC and within the FL-2A/FL-2W gates were analyzed to determine the % of cells in each stage of the cell cycle(Pi/FL-2H).

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☑
Author Correction: PAK signalling drives acquired drug resistance to MAPK inhibitors in BRAF–mutant melanomas

Hezhe Lu, Shujing Liu, Gao Zhang, Bin Wu, Yueyao Zhu, Dennie T. Frederick, Yi Hu, Wenqin Zhong, Sergio Randell, Norah Sadek, Wei Zhang, Gang Chen, Chaoran Cheng, Jingwen Zeng, Lawrence W. Wu, Jie Zhang, Xiaoming Liu, Wei Xu, Clemens Krepler, Katrin Sproesser, Min Xiao, Benchun Miao, Jianglan Liu, Claire D. Song, Jephrey Y. Liu, Giorgos C. Karakousis, Lynn M. Schuchter, Yiling Lu, Gordon Mills, Yusheng Cong, Jonathan Chernoff, Jun Guo, Genevieve M. Boland, Ryan J. Sullivan, Zhi Wei, Jeffrey Field, Ravi K. Amaravadi, Keith T. Flaherty, Meenhard Herlyn, Xiaowei Xu & Wei Guo

Correction to: Nature https://doi.org/10.1038/nature24040, published online 27 September 2017.

In this Letter, two relevant references were omitted. The first1 shows that exogenous expression of the dominant-negative form of PAK1 sensitizes BRAF–mutated, previously untreated melanoma cells to BRAF inhibition (BRAFi) or MEK inhibition (MEKi). This reference, along with several others, was removed from our original submission owing to length limitations. The second2 was published during the later stages of the resubmission of our Letter. It shows that inhibition of PAK1 causes re-sensitization to BRAFi of previously untreated cells that ectopically express activated Rac1. Ectopic expression of constitutively active PAK1 increased the tolerance of cells to BRAFi or MEKi. These studies used treatment-naive cells and did not address the issue of acquired drug resistance (that is, cells that showed an initial response but gained resistance over the course of treatment with BRAFi or combined BRAFi and MEKi), which is the central topic of our Letter. Nevertheless, the data support the general notion that PAK1 is involved in regulating resistance to BRAFi and MEKi. We regret not citing these two papers to highlight their relevance and the distinctions between their findings and those of our Letter. The original Letter has not been corrected.

1. Singhal, R. & Kandel, E. S. The response to PAK1 inhibitor IPA3 distinguishes between cancer cells with mutations in BRAF and Ras oncogenes. Oncotarget 3, 700–708 (2012).
2. Babagana, M. et al. P21-activated kinase 1 regulates resistance to BRAF inhibition in human cancer cells. Mol. Carcinog. 56, 1515–1525 (2017).