Dual blockade of the lipid kinase PIP4Ks and mitotic pathways leads to cancer-selective lethality

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Achieving robust cancer-specific lethality is the ultimate clinical goal. Here, we identify a compound with dual-inhibitory properties, named a131, that selectively kills cancer cells, while protecting normal cells. Through an unbiased CETSA screen, we identify the PIP4K lipid kinases as the target of a131. Ablation of the PIP4Ks generates a phenocopy of the pharmacological effects of PIP4K inhibition by a131. Notably, PIP4Ks inhibition by a131 causes reversible growth arrest in normal cells by transcriptionally upregulating PIK3IP1, a suppressor of the PI3K/Akt/mTOR pathway. Strikingly, Ras activation overrides a131-induced PIK3IP1 upregulation and activates the PI3K/Akt/mTOR pathway. Consequently, Ras-transformed cells override a131-induced growth arrest and enter mitosis where a131’s ability to de-cluster supernumerary centrosomes in cancer cells eliminates Ras-activated cells through mitotic catastrophe. Our discovery of drugs with a dual-inhibitory mechanism provides a unique pharmacological strategy against cancer and evidence of cross-activation between the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways via a Ras-PIK3IP1-PI3K signaling network.

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The Ras/Raf/MEK/ERK and PI3K/Akt/mTOR signaling pathways are essential for cell survival and proliferation in response to external cues. Mutation of proteins within these pathways are among the most common oncogenic targets in human cancers\(^1\), and this has spawned a longstanding effort to develop selective inhibitors of these pathways for cancer therapy. Unfortunately, there is ample evidence that cross-talk or cross-amplification of signaling events occurs between these pathways, which both positively and negatively regulate downstream cellular growth events\(^2\). Moreover, the antitumor activities of single-agent targeted therapies directed to block these signaling pathways has generally been disappointing with an unintended pathway activation leading to drug resistance\(^3\). This has prompted the testing of multiple targeted therapies in combination in order to inhibit multiple oncogenic dependencies\(^4\), however, combined treatment with drugs that target the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR signaling pathways has met with marginal clinical success\(^5\). Thus, there remains the ultimate goal of identifying targets that mediate resistance and cross-talk between these two central pathways.

Here we show a novel compound with dual-inhibitory properties, named a131, that effectively eliminates Ras-activated cancer cells through mitotic catastrophe, while protecting normal cells and allowing them to retain their proliferative capacity. Notably, we have identified the PIP4K lipid kinase family\(^6\),\(^10\) as the target of a131 inhibition and delineated a critical role for PIP4K lipid kinases that differently regulate the cell cycle entry between normal and Ras-activated cancer cells. Furthermore, we provide evidence of a mechanism for cross-activation between the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways via Ras-suppressing PIK3IP1, a suppressor of the PI3K/Akt/mTOR pathway, in Ras-pathway activated cancer cells as well as in clinical samples from patients with colorectal and lung adenocarcinomas. Consequently, Ras-activated cancer cells override a131-induced growth arrest and enter mitosis where a131 effectively eliminates Ras-activated cancer cells through mitotic catastrophe. Together, our results provide novel pharmacological strategies against Ras-pathway activated cancers and a mechanism for cross-activation between the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways via a Ras\(^+/+\)PIK3IP1\(^/-\)PI3K signaling network, which promises further insight into the role of this signaling network in regulating cross-talk known to drive response and resistance to clinically relevant targeted therapies.

**Results**

**a131 causes selective killing effects in cancer cells.** We undertook a small-molecule screen to investigate the specific signaling networks needed for the proliferation and survival of transformed cells using isogenic human BJ foreskin fibroblasts either immortalized with only hTert (hereafter named as normal BJ) or fully transformed with hTert, small t, shRNAs against p53 and p16 and H-RasV12-ER (estrogen receptor-fused H-Ras bearing the activating G12V mutation) (hereafter named as transformed BJ)\(^1\). In contrast, we found that 4-OHT-induced acute activation of H-RasV12-ER alone was sufficient to sensitize normal BJ cells to a131-induced cell death. In contrast, we found that 4-OHT-induced acute activation of H-RasV12-ER alone was sufficient to sensitize normal BJ cells to a131-induced cell death, and this effect was further enhanced in the context of transformed BJ cells (Fig. 1f). Together, these data indicate that a131 displays a strong selective lethality against Ras-activated or Ras-transformed cells.

Consistent with a131-induced aneuploidy in transformed cells (Fig. 1c, panel d’), time-lapse analysis revealed that a131 treatment immediately induced mitotic arrest in transformed BJ cells (Supplementary Fig. 2c) concomitant with massively misaligned chromosomes (Fig. 1g; Supplementary Fig. 2d), which subsequently missegregated into daughter cells often with catastrophic multipolar division, leading to cell death (Supplementary Movies 1 & 2). In contrast, such catastrophic cell division rarely occurred in a131-treated normal BJ cells (Supplementary Movies 3 & 4) and demonstrated significantly fewer mitotic defects than those in transformed cells (Supplementary Fig. 2c & d). Detailed analysis using high-resolution immunofluorescence microscopy revealed that a131 caused de-clustered centrosomes and multipolar mitotic-spindles in transformed BJ (Fig. 1g, h) and other cancer cells (Supplementary Fig. 2e), since most cancer cells contain supernumerary centrosomes clustered in a bipolar manner before division\(^1\). In contrast, functional bipolar spindle formed in a majority of a131-treated normal BJ cells that were able to enter mitosis (Fig. 1h; Supplementary Fig. 2f; Supplementary Movie 4) despite a slight decrease in metaphase spindle length (Supplementary Fig. 2g). Together, these data suggest a131 as a potent antimitotic agent that preferentially kills cancer and transformed cells by inducing immediate cancer-selective mitotic catastrophe in vitro, while it arrests normal cells at the G1/S phase of the cell cycle in a reversible manner, which explains a broad-spectrum of its anticaner effects (Supplementary Fig. 2a & b).

The antitumor activities of a131 and of b5, a derivative of a131 designed to improve aqueous solubility, were further determined in mouse xenograft models derived from both HCT-15 human colon adenocarcinoma cells and MDA-MB-231 human breast tumor cells harboring mutant K-RasG13D. As expected, paclitaxel did not show significant antitumor activity against HCT-15 (Fig. 1i). Whereas, both oral and intraperitoneal injections of a131 arrested normal BJ cells at the G1/S phase of the cell cycle with few BrdU incorporation (Fig. 1c, panel b’), which was also confirmed with gene set enrichment analysis (GSEA) of genes promoting the cell cycle (Supplementary Fig. 1c). Importantly, this a131-induced growth arrest in normal BJ cells was transient and reversible after a131 removal (Supplementary Fig. 1d). Further, unlike the DNA-damaging Topo II inhibitor etoposide, a131-induced growth arrest occurred in the absence of genotoxic stresses (Supplementary Fig. 1e). This cancer-selective lethality of a131 was further confirmed using a panel of human normal and cancer cell lines (GI\(_{50}\) = 6.5 vs. 1.7 \(\mu\)M (normal vs. cancer)) (Fig. 1e; Supplementary Fig. 2a; Supplementary Data 1). Of note, the difference in GI\(_{50}\) values between normal and cancer cells using MITT assay that measures cell proliferation rate (Fig. 1b, e) is likely underestimated, since a131 preferentially induced cell death in transformed and cancer cells, but not normal cells (Supplementary Fig. 2b), whereas it only arrested normal cells at the G1/S phase of the cell cycle in a transient and reversible manner (Supplementary Fig. 1d). Nonetheless, these data suggest that a131 is a potent antiproliferative agent with a clear selectivity toward cancer cells killing.
a131 and b5 demonstrated marked antitumor efficacies without any body weight loss (Fig. 1i) and cancer cell death as determined by TUNEL staining (Fig. 1j). As observed in in vitro tissue culture, b5 treatment caused massively misaligned chromosomes with multipolar spindles in tumor sections (Fig. 1k). Moreover, in a tumor spheroid culture or orthotopically implanted ex vivo model, a131 treatment significantly suppressed growth of Ras-driven glioma-initiating cells (GICs) (Supplementary Fig. 3a & b). In addition, a131-induced apoptosis only in tumors, but not surrounding normal tissues in ex vivo model (Supplementary Fig. 3c). Taken together, a131 is a unique compound with a potent and broad anticancer efficacy by inducing cancer-selective mitotic catastrophe in vitro, ex vivo, and in vivo.

**a131 eliminates cancer cells via a dual-inhibitory mechanism.**

Using various derivatives of a131, we found that the properties of a131 can be separated pharmacologically into two distinct pharmacophores (experimental details and a summary of the results presented in Supplementary Fig. 4a–c and Supplementary Data 2). Through the analysis of a131 structure-and-activity relationship, we classified these compounds into four groups: Group 1 compounds, which possess the dual-inhibitory properties of both causing the arrest of normal BJ cells at the G1/S phase and also causing mitotic arrest/catastrophe in transformed BJ cells (e.g., a131, b5); Group 2 compounds, which only cause the arrest of normal BJ cells at the G1/S phase (e.g., a166); Group 3 compounds, which cause mitotic arrest/catastrophe in
transformed BJ cells, but do not arrest normal BJ cells at the G1/S phase (e.g., a159); and Group 4 compounds, which are inactive or weakly active (e.g., a132). Importantly, only Group 1 compounds retain the ability to selectively kill transformed BJ cells (Supplementary Fig. 4c). In contrast, Group 2 and Group 4 compounds failed to kill either normal or transformed cell lines, while compounds in Group 3 killed both normal and transformed cell lines with much less selectivity than those in Group 1 (Supplementary Fig. 4c & d; Fig. 1b). Notably, a131-like cancer-selective lethality was recapitulated by combining compounds in Groups 2 and 3 (Supplementary Fig. 4e). Moreover, while paclitaxel and etoposide treatment alone showed a minimal selectivity against transformed BJ cells, pretreatment with a166 in Group 2 markedly augmented such selectivity by protecting normal BJ cells from chemotherapeutic toxicity (Supplementary Fig. 4f & g). Together, these data suggest that the dual-inhibitory property of compounds in Group 1 (e.g., a131) is essential to achieve cancer-selective lethality. Furthermore, compounds in Group 2 (e.g., a166) and Group 3 (e.g., a159) can be classified as chemoprotective and chemotherapeutic agents, respectively.

Identification of PIP4Ks as target of a131. To identify cellular targets and signaling pathways of a131 that are responsible for arresting only normal BJ cells at the G1/S phase of the cell cycle, we explored the mass spectrometry implementation of the cellular thermal shift assay (MS-CETSA) for target identification on the proteome level. To increase confidence in target identification, both a131 and a166 were applied for CETSA analysis to find common target proteins. After collecting data covering >8000 proteins in lysates of normal BJ cells, >4000 proteins were used for each compound in the final analyses. Using ranking based on Euclidian distances and thermal shift size, we selected 16 and 11 proteins as potential significant hits for a131 and a166, respectively (Supplementary Fig. 5; Supplementary Data 3; ranking strategies are discussed in Methods). Ferrochelatase in a131 and coproporphyrinogen-III oxidase in a166 were identified as prominent hits. These two proteins of the heme synthesis pathway, however, have previously been identified as promiscuous binders of multiple drugs indicating their inhibition is unlikely to give the observed phenotypes of a131 and a166. Instead, the members of PIP4Ks (phosphatidylinositol 5-phosphate 4-kinases) stood out as the most prominent common hits, which could constitute candidates for the pharmacological targets; two out of the three family members (PIP4K2A and 2C) in a131 and all three family members (PIP4K2A, 2B, and 2C) in a166 were identified as CETSA hits (Fig. 2a, b). Indeed, a131 was able to inhibit the kinase activity of purified PIP4K2A in vitro as well as PIP4Ks activity with IC₅₀ of 1.9 µM and 0.6 µM, respectively (Fig. 2c, d). Likewise, both a166 and I-Ome-AG-538, previously reported to show PIP4KA inhibition, also inhibited the PIP4K2A activity with IC₅₀ of 1.8 and 2.1 µM, respectively (Fig. 2c). a166 also inhibited in vitro PIP4Ks activity, although somewhat less than than a131 did (Fig. 2c, d). Of note, a132 in Group 4 compounds, which failed to kill either normal or transformed BJ cell lines (Supplementary Fig. 4c), exerted ~10% inhibition of PIP5Kα enzyme activity at both ~2 and ~5 µM, and ~40% inhibition at the high concentration of >10µM, although this inhibition was significantly less than that of a131, a166 or I-Ome-AG-538 (Fig. 2c). It is also worth noting that a159 in Group 3 compounds with antimitic activity exhibited no inhibitory activity against PIP5Kα in vitro, but it did exhibit some measurable inhibitory activity against the endogenous PIP4Ks (Fig. 2c, d), suggesting a possibility that some of the other proteins in the hit list correspond to proteins that make less direct physical interactions with the compounds leading to additional off-targets or a result of poly pharmacological effects. Importantly, knockdown of all PIP4K isoforms using three different sets of siRNAs (Supplementary Fig. 6a) induced growth arrest only in normal BJ cells (Fig. 2e, f), a phenocopy of a131 and a166 treatment (Supplementary Fig. 4c). Of note, siRNA-mediated knockdown of other CETSA hits including adenosine kinase (ADK) and pyridoxal kinase (PDKX) did not show a significant growth inhibition in normal BJ cells (Supplementary Fig. 6b, c). Moreover, GSEA and KEgg pathway analysis revealed that PIP4Ks knockdown in normal BJ cells downregulated the set of genes promoting cell cycle (Fig. 2f) with a significant number of comparably upregulated or downregulated common cellular pathways as similar to a131 and a166 treatment (Supplementary Fig. 6d, e). Of note, siRNA-mediated knockdown of other CETSA hits including adenosine kinase (ADK) and pyridoxal kinase (PDKX) did not show a significant growth inhibition in normal BJ cells (Supplementary Fig. 7a, b), demonstrating specificity of PIP4Ks as the cellular targets of a131 and a166. Furthermore, similar to a166 treatment (Supplementary Fig. 4c–g) PIP4Ks knockdown also showed significant chemoprotective effects only in normal BJ cells from paclitaxel, etoposide and a159 treatment (Supplementary Fig. 7c–e). Similar to a166 treatment alone (Supplementary Fig. 4c), PIP4Ks knockdown did not induce a significant apoptosis (<2N) in normal BJ cells (Supplementary Fig. 7f)
Fig. 2 Identification of PIP4Ks as targets of a131 responsible for selective growth arrest in normal cells. a, b Target identification using CETSA. All experiments were performed in two fully independent replicates. a Venn diagrams of positive hits from a131 and a166 with the list of commonly targeted hits. Individual hits were ranked by distances (Methods). b Melting curves for PIP4K isoforms in duplicated experiments of a131 vs. DMSO (top) or a166 vs. DMSO (bottom) treatment. c, d PIP4Ks enzyme activity assays using PI5P as substrate (Methods). Inhibition curves ± S.D. (n = 4) of the indicated compounds are shown. c In vitro PIP4K2A enzyme activity pre-incubated with the indicated compounds. d In vitro PIP4Ks activity using HeLa cells in order to verify cell type-independent inhibition by a131 or its indicated derivatives. The compounds were further added to cell lysates at the indicated concentrations during the measurement of PIP4K activity. e BrdU incorporation assay as in Fig. 1c. Normal and transformed BJ cells were transfected with control or three different sets of PIP4Ks siRNAs (mixture of PIP4K2A, PIP4K2B, PIP4K2C) for 48 h. The percentage of cells with BrdU-positive populations is shown with mean values ± S.D. (n = 3). Two-tailed unpaired t tests were performed to determine statistical significance. Note that the differences in the percentage of BrdU-positive cells between normal and transformed BJ cells were due to a relatively slower growth of normal BJ cells as compared to transformed cells, and not due to a significant growth arrest with the control siRNA. f GSEA enrichment plot and heatmap of KEGG cell cycle pathway genes. Normal BJ cells were treated with a131 and a166 at 5 μM for 24 h or transfected with indicated siRNAs for 48 h. The per-sample expression profiles of these genes are depicted in the heatmap using an intensity-based, row-normalized color scale from blue to red, with blue indicating lower expression.
Likewise, PIP4Ks knockdown in transformed BJ cells only caused a marginal increase in apoptosis (Supplementary Fig 7e), indicating that the dual-inhibitory property of a131 is essential to achieve cancer-selective lethality. Together, these data suggest that PIP4Ks are the cellular targets of a131 and a166. This is to the best of our knowledge the first published study where MS-CETSA has been used to unravel pharmaceutical targets for hits from a phenotypic screen.

**Ras overrides PI3K pathway suppression by PIP4Ks inhibition.**

Recent studies using cancer cells and knockout mouse models of individual PIP4K isoforms indicate the involvement of PIP4Ks in controlling the PI3K/Akt/mTOR pathway, well known to promote the G1/S phase transition. Interestingly, PIP4K loss-of-function mutants in Drosophila possessing only one isoform of PIP4K show inhibition of the PI3K/Akt/mTOR pathway23. Importantly, a131 treatment or PIP4Ks knockdown using three different sets of siRNAs also consistently caused inhibition of the PI3K/Akt/mTOR pathway only in normal BJ cells, but not in transformed counterparts (Fig. 3a, b). Likewise, 4-OHT–induced H-RasV12-ER activation was sufficient to reactivate the PI3K/Akt/mTOR pathway in normal BJ cells even after a131 and a166 treatment or PIP4Ks knockdown (Fig. 3c), which correlates with activated Ras overriding a131-induced, a166-induced, or PIP4Ks knockdown-induced growth arrest in normal BJ cells (Fig. 3d). Together, these data suggest a role of PIP4Ks in promoting the PI3K/Akt/mTOR signaling pathway in a Ras-dependent manner.

**Ras-PI3K pathway cross-talk via Ras/PIK3IP1:PI3K network.**

The molecular components that control the interactions between the Ras/Raf/MEK/ERK and the PI3K/Akt/mTOR pathways is not

![Fig. 3](image-url) a131 and Ras antagonistically control the PI3K/Akt/mTOR pathway. a-c Immunoblot analysis of normal and transformed BJ cells treated with a131 or a166 for 24 h a, c or transfected with indicated siRNAs for 48 h b, c. Relative ratios of phosphorylated/total levels of Akt and p70S6K are shown in a. d BrdU incorporation assay as in Fig 1c. 4-OHT (4HT(+)) was added in normal BJ cells for 24 h. Subsequently, cells were treated with a131 or a166 at 5 μM for 48 h (top). Normal BJ cells were transfected with indicated siRNAs for 48 h and treated with 4-OHT for 24 h (bottom). The percentage of cells with BrdU-positive population in comparison with DMSO is shown with mean values and ± S.D. (n = 3).
fully understood\(^3\). Neither a131 and a166 treatment nor PI4Ks knockdown inhibited the Ras/Raf/MEK/ERK pathway in normal BJ cells, as determined by ERK phosphorylation (Fig. 3a–c). Thus, to determine how a131 controls the PI3K/Akt/mTOR pathway in a Ras-dependent manner, we interrogated differences in the gene expression levels of known regulators and effectors associated with PI3K in normal and transformed BJ cells upon a131 treatment. Strikingly, among these genes, the PI3K-interacting protein 1 gene (PIK3IP1) was significantly upregulated only in a131-treated normal BJ cells (Fig. 4a). Indeed, qRT-PCR and immunoblot analysis confirmed upregulation of PIK3IP1 at both the mRNA and protein levels in either a131-treated and a166-treated or PI4Ks knockdown normal BJ cells (Fig. 4b, c). Conversely, PIK3IP1 mRNA expression in transformed BJ cells was not only significantly lower, but also was unresponsive to a131 and a166 treatment (Fig. 4b). Moreover, 4-OHT-induced H-RasV12-ER activation was sufficient to downregulate mRNA and protein levels of PIK3IP1 in a131-treated normal BJ cells (Fig. 4c) and to dissociate RNA polymerase II (Pol II) from the PIK3IP1 promoter (Fig. 4d). In contrast, pharmacological inhibition of MEK-attenuated H-RasV12-ER-induced PIK3IP1 suppression (Fig. 4e), suggesting the molecular basis for positive cross-talk between the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways is mediated by negative transcriptional regulation of PIK3IP1.

**High MAPK activity suppresses PIK3IP1 in human cancers.** PIK3IP1 binds the p110 catalytic subunit of PI3K heterodimers and inhibits PI3K catalytic activity, which leads to inhibition of the PI3K/Akt/mTOR pathway, and PIK3IP1 dysregulation contributes to carcinogenesis\(^{24-27}\). Therefore, we determined whether a131-mediated upregulation of PIK3IP1 was indeed responsible for the observed inhibition of the PI3K/Akt/mTOR pathway and the G1/S phase transition in normal BJ cells. Indeed, PIK3IP1 knockdown in normal BJ cells significantly restored activation of the PI3K/Akt/mTOR pathway and rescued the population of cells in the G1/S phase transition (Fig. 4d, e).

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**Fig. 4** Identification of positive cross-talk between Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathway via Ras-PIK3IP1-PI3K signaling network. a, The PI3K network analysis using gene expression of PI3K regulators in normal (middle) and transformed BJ cells (right) treated with a131 at 5 μM for 24 h. PI3K regulators, including PIK3IP1, were identified to interact with PI3K with experimental support and high confidence from STRING. The per-sample expression profiles of PIK3IP1 are depicted in the heatmap (left). Color: negative log FDR (false discovery rate), coded from white to red in a scale from 0.15 to 5.37. Size: log ratio; Border: upregulation (red) or downregulation (blue); Shape: upstream (square), parallel (diamond) or downstream (circle). b, qRT-PCR analysis of PIK3IP1 expression in BJ cell lines treated with a131 or a166 at 5 μM for 24 h. Mean values ± S.D. (n = 3). c, Immunoblot analysis of normal BJ cells treated with 4-OHT (+) for 24 h to activate H-RasV12-ER and subsequently with a131 (left) or transfected with indicated siRNAs for 48 h (right). Representative immunoblots shown (n = 3). d, ChIP analysis of normal BJ cells treated with a131 using antibodies against RNA polymerase II (Pol II) for the PIK3IP1 gene promoter (n = 3). e, Normal BJ cells treated with a131 were subsequently treated with MEK inhibitor U0126 (10 μM) for additional 2 h. Then, 4-OHT added to activate H-RasV12-ER for various time points. Where indicated, two-tailed unpaired t tests were performed to determine statistical significance.
BrdU-positive proliferative cells, which were suppressed by a131 treatment (Fig. 5a, b). Together, these data reveal positive cross-talk between the Ras and PI3K pathways via the Ras-PIK3IP1-PI3K signaling network. Moreover, PIK3IP1 mRNA levels were not only considerably lower in Ras-mutant and Raf-mutant cancer cells compared with normal cells (Supplementary Fig. 8a), but a131-mediated and a166-mediated induction of PIK3IP1 was also significantly attenuated in these cancer cells, unlike normal cells (Fig. 5c; Supplementary Fig. 8b). Similarly, analysis of an Oncomine dataset derived from patient samples reveals that...
PIK3IP1 expression was significantly lower in human colorectal and lung adenocarcinomas where Ras mutations and activation of Ras signaling pathways are common compared with their corresponding normal tissues or squamous cell lung carcinoma where Ras mutations are uncommon (Supplementary Fig. 8c). Indeed, a negative correlation between PIK3IP1 expression and Ras mutation status in human colorectal and lung adenocarcinomas were observed (Supplementary Fig. 8d). Conversely, pharmacological inhibition of MEK and ERK significantly increased PIK3IP1 expression in many Ras-mutant and Raf-mutant cancer cells (Fig. 5d; Supplementary Fig. 8e), while this observed de-repression of PIK3IP1 was much prominent in most of Raf-mutant cancer cells (Supplementary Fig. 8e), indicating the high MAPK activity is responsible for the suppression of PIK3IP1. Furthermore, this de-repression of PIK3IP1 correlated with concomitant inhibition of the PI3K/Akt/mTOR pathway in HCT116, A549 and transformed BJ cells, but not in those cells unable to de-repress PIK3IP1 (Fig. 5d). Conversely, PIK3IP1 knockdown significantly restored activation of the PI3K/Akt/mTOR pathway and suppressed cell death induced by inhibition of MEK and ERK (Fig. 5e), further indicating positive cross-talk between the Ras and PI3K pathways via the Ras-PIK3IP1-PI3K signaling network for cancer cell proliferation and survival (Fig. 5f).

Discussion

The detailed mechanism of how a131 and activated Ras antagonistically regulate PIK3IP1 expression requires further investigation. PI(3)P is known to interact with its receptors possessing PHD fingers (e.g., ING2, TAF3) in order to control selective gene expression9,23,29. Interestingly, 20-fold increase in nuclear PI(3)P was observed during the G1/S phase of the cell cycle29. Although it remains to be determined, given that a131 treatment or PIP4Ks knockdown also arrested normal cells at the G1/S phase, it is tempting to speculate that a131 treatment or PIP4Ks knockdown may increase PI(3)P levels to activate a nuclear receptor that promotes transcriptional upregulation of PIK3IP1. Conversely, activated Ras may signal to inhibit such nuclear receptors or transcription factors to suppress PIK3IP1 expression, thereby establishing positive cross-talk with the PI3K pathway in Ras-pathway mutated/activated cancers for proliferation. Given that MEK and ERK inhibitors attenuated Ras-mediated suppression of PIK3IP1, the Raf/MEK/ERK cascade is likely involved in inhibiting such transcriptional receptor, which is an important topic for further detailed investigation.

The requirement of PIP4Ks for tumorigenesis, especially in the absence of p53, has been demonstrated10. Although the mitotic targets of a131 responsible for de-clustering supernumerary centrosomes in cancer cells remain to be determined, together with a131’s ability to inhibit PI(3)P, its potent and broad anticancer efficacy by inducing cancer-selective mitotic catastrophe (Fig. 6) provides novel pharmacological strategies against not only Ras-pathway mutated/activated cancers, but more broadly applicable to a vast majority of human cancers. Furthermore, our discovery of a mechanism for cross-activation between the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways via a Ras/PIK3IP1-PI3K signaling network promises further insight into the role of this signaling network in regulating cross-talk known to drive response and resistance to clinically relevant targeted therapies.

Methods

Cell lines and culture and reagents. Isogenic BJ human foreskin fibroblast cell lines, including non-transformed (normal) and transformed BJ cells and all gastric cancer cell lines, were kind gifts from Dr. Mathijs Voorhoeve12 and Dr. Patrick Tan (Duke-NUS)38, respectively, and tested for mycoplasma infection. The culture media for the cell lines used in this study are summarized in Supplementary Data 1. All other human cancer cell lines used in this study were purchased from ATCC and cultured in accordance with ATCC’s instructions. H-RasV12-ER was activated by exposing the B-derivided fibroblasts to 4-OHT (100 nM, Sigma-Aldrich). Three different sets of siRNAs were used in this study to target PIK3IP4 isotypes: pool1: PIP4K2A (5′-CTGGCCAGATGTCCCTGGTA-3′), PIP4K2B (5′-CACGATCAA TGCCTGAGCAA-3′), and PIP4K2C (5′-CCCGAGGATGTCACTGTTGGA-3′), pool2: PIP4K2A (5′-CGCCTTAATGGTGTGAGGT-3′), PIP4K2B (5′-CCCG TGCATATTTCCTCCT-3′), and PIP4K2C (5′-CCCGAGGATGTCACTGTTA-3′); pool3: PIP4K2A (5′-CTGGCCAGATGTCCCTGGTA-3′), and PIP4K2B (5′-CCCGAGGATGTCACTGTTA-3′). To knockdown PHD, siRNAs 1-3 were used. To knockdown PI3K, siRNAs 4-6 were used. Non-silencing control siRNA was purchased from Dharmacon. For siRNA transfection, Lipofectamine 2000 (Invitrogen) or Dharmafect (Dharmacon) was used according to the manufacturer’s instructions.

Cytotoxicity test. Cells were plated in 96-well microplates on day 0, and a131 was added to each well on day 1 at a range of different concentrations (from 0.1 to 40 μM) in triplicate. After 3 days of culture, the number of viable cells was determined using MTT cell proliferation assays by adding thiazolyl blue tetrazolium bromide (MTT reagent, Invitrogen) at a concentration of 0.5 mg/ml to each well and incubating for 4 h at 37 °C. The medium was then removed, and the blue dye remaining in each well was dissolved in DMSO by mixing with a microplate mixer. The absorbance of each well was measured at 540 and 660 nm using a microplate reader (Benchmark plus, Bio-Rad). Optical density (OD) values were calculated by subtracting the absorbance at 660 nm from the absorbance at 540 nm. Mean OD values from control cells containing only DMDS-treated wells were obtained as 100% viable. The concentration of drug that reduced cell viability by 50% (GI50) was calculated by non-linear regression fit using GraphPad Prism.

Crystal violet staining. Cells were washed twice in 1× PBS and stained with 0.5% crystal violet dye (in methanol:deionized water = 1:5) for 10 min. Excess crystal violet dye was removed by washing five times (10 min per wash) with deionized water on a shaker, and the culture plates were dried overnight.

Analysis of cell death and cell growth arrest. Cell death was assessed via Annexin V and/or PI (propidium iodide) staining according to the manufacturer’s instructions (ebioscience). Cell growth arrest was assessed by direct measurement of DNA synthesis through incorporation of the nucleoside analog bromodeoxyuridine (BrdU). Briefly, BrdU (30 μM, Sigma-Aldrich) was added for 2 h before harvesting cells. Cells were subsequently stained with Pacific Blue-conjugated BrdU antibody (Invitrogen) for 1 h followed by PI staining. Stained cells were analyzed by MACSQuant (MACS). Three independent experiments were performed in triplicate. The percentage of Annexin V/PI-positive or BrdU-positive cells was quantified using FlowJo software (Becton Dickinson). Where indicated, the combined activity of caspase-3/7 was determined using the caspase-Glo 3/7 Assay Kit (Promega) and normalized to the number of viable cells as determined by MTT assay.

In vivo study. BALB/c athymic female nude mice (nu/nu, 5–7 weeks) (InVivos) were kept under specific pathogen-free conditions. The care and use of mice was approved by the Duke-NUS IACUC in accordance with protocol 2015/SHS/1030. HCT-15 human colon cancer cells (3 × 106) or MDA-MB-231 human breast cancer cells (4 × 106) with Matrigel were subcutaneously injected into the flanks of mice. When the mean tumor volume reached 100–300 mm3 (Day 1), the mice were randomly divided into experimental groups of 6 mice by an algorithm that moves animals around to achieve the best case distribution to assure that each treatment group has an equal tumor burden and standard deviation. No statistical method was used to predetermine sample size. The animals were treated with drug (a131) injection (i.p) or control (s.c.) injection of a131 (5 mg/ kg) or vehicle control twice per day for 12 days (HCT15) or 15 days (MDA-MB-231). a131 and b5 were dissolved in DMDS followed by the addition of PEG400 and deionized water (pH 5.0) (final concentrations, 10% DMSO, 50% PEG400). Paclitaxel (Cayman Chemical) was dissolved in ethanol:Tween 80 = 1:3 (v/v) solution and followed by the addition of a 5% glucose solution (4% ethanol:Tween 80:5% glucose). Paclitaxel (10%), salmeterol Xinafoate (3%) and a131 (10%) were dissolved in 7:5:80 (v/v/v) Tween 80:n-ace- tocitic acid:5% glucose solution and stored in 70% ethanol. For immunostaining, antigens were retrieved from formaldehyde-fixed, paraffin-embedded tissue sections for 30 min by boiling in sodium citrate buffer (pH 6.0) using a microwave histoprocessor (Milestone). Endogenous peroxidase activity in tissue sections was depleted by treatment with 3% hydrogen peroxide (H2O2 in 1:10 tris-buffered saline solution (TBS) for 20 min at room temperature. Tumor tissue sections were incubated overnight with anti-β-tubulin antibody (Abcam; 1:100 in 3% BSA/TBS-Tween 20) at 4 °C followed by incubation with anti-goat-rabbit FITC-conjugated secondary
antibody (Invitrogen; 1:200 in 3% BSA/TBS) for 1 h at 25 °C. After dehydration treatment, coverslips were mounted using DAPI mounting medium (Vector).

Images were acquired in 3D-SIM mode using a super resolution microscope (Nikon) and the number of cells with either 2 or ≥3 mitotic-spindles was quantified (n > 50 cells per section, 6–7 sections per treatment). For detection of apoptosis using the TUNEL method, the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore) was used for formaldehyde-fixed, paraffin-embedded tumor tissue sections treated with b5 (80 mg/kg, IP) or control vehicle for 12 days. Slide scans were acquired using a MetaSystems Metafer built on a Zeiss AxioImager Z.2 upright microscope. The system is equipped with a CoolCube1.

**Fig. 6** Proposed model. Inhibition of PIP4Ks by a131 or a166 arrests normal cells at the G1/S phase of the cell cycle by suppressing the PI3K/Akt/mTOR signaling pathway via transcriptional upregulation of PIK3IP1 (a, top). This cell cycle arrest is reversible after drug removal (a, bottom). In contrast, mutation or activation of the Ras/Raf/MEK/ERK pathway in cancer cells promotes positive cross-talk with the PI3K/Akt/mTOR pathway by negative transcriptional regulation of PIK3IP1, which allows cancer cells to bypass a131-induced growth arrest at the G1/S phase of the cell cycle, but subsequently leads cancer cells to a131-induced mitotic catastrophe and cell death. However, it is important to note that the mitotic targets of a131 remain unidentified. Pretreatment with a166 protects normal cells from chemotherapeutic toxicity by arresting normal cells at the G1/S phase of the cell cycle. In contrast, mutation or activation of the Ras/Raf/MEK/ERK pathway in cancer cells bypasses the growth arrest, leading to cell death caused by chemotherapeutic drugs (Eto, etoposide; PTX, paclitaxel) and a159. Of note, this Ras→PIK3IP1→PI3K signaling network identified in this study may contribute to “oncogene collaboration” of the Ras and PI3K pathways for cancer cell growth and proliferation.
expressed genes were identified and the number of heat producing mRNA. In all cases, no data or animals were excluded and results are expressed as mean and standard deviation of the mean.

Spheroid formation assay. Murine GICs were established and cultured as described previously. Briefly, Inka/Arf-null neural stem/progenitor cells were transduced with human H-RasV12 and DsRed and propagated in serum-free Dulbecco modified Eagle medium/F12 (Sigma-Aldrich) supplemented with recombinant human epidermal growth factor (PeproTech) and basic fibroblast growth factor (PeproTech) at 20 ng/mL, heparan sulfate (Sigma-Aldrich) at 200 ng/mL and B2 supplement without vitamin A (Invitrogen, Carlsbad, CA). GICs were dissociated and plated in 96-well plates at a density of 100 cells/well. Vehicle (DMSO), temozolomide (Sigma-Aldrich) at 100 μM or α131 at 5 μM were added and sphere formation and size evaluated 7 days after plating. Three plates were prepared for each treatment group, and 30 wells were quantified per plate. Images were acquired on a BX-Z700 inverted fluorescence microscope (Keyence). Quantification was performed by Nikon NIS-element software (n = 90).

Brain slice explants and drug treatment. Fifty thousand GICs were orthotopically implanted into the forebrain of wild-type mice, and at 7 days post-implantation, brain slice explants were established as previously described. Coronal slices (200 μm) were cultured on Millicell-CM culture plate inserts (Millipore) and treated with vehicle or α131 for 4 days. Images were acquired on an FV10i Olympus confocal microscope (Olympus) and tumor area was quantified by Nikon NIS-elements software. Experiments were performed in triplicate. At the end of the experiment (Day 4), slices were fixed overnight in 4% paraformaldehyde, embedded in paraffin and then sectioned at a thickness of 4 μm. Deparaffinized sections were stained with rabbit polyclonal antibody against cleaved caspase-3 (Cell Signaling). Immune complexes were detected using Histo Mark Immuno HRP secondary antibody (Bio-Rad) and anti-HRP secondary antibody (DAKO). All animal experiments were performed in accordance with the animal care guidelines of Keio University. 

qRT-PCR. Total RNA was isolated from cultured cells using the RNeasy mini kit (Qiagen). cDNA was synthesized from 1 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR analysis was performed using the iQ SYBR Green Super mix (Bio-Rad) using the following gene-specific primers: human PIP4K2A 5′-AAAGAAGGACGCATCTGGGAC-3′, 5′-ATGGCCTGACTCCATCT-3′, GATGGAG-3′, human PIP4K2B 5′-CCACAGCATGAATGGCCTGAG-3′, 5′-TCCTTAAATCAATGGCGGCTGG-3′, human PIP4K2C 5′-CCGGGAAGCCAGGGTGATGATG-3′, 5′-AGCCGCTACGAGACTCCACA-3′, and human PIP4K2D 5′-CAACCTCACTCTCCATTTCTGTAG-3′, 5′-CAAGAGACGACTGTTA-3′. The TATA-binding protein gene was used for normalization. All PCR reactions were performed in triplicate.

Microarray data analysis. Biotin-labeled cRNA was prepared from 250–500 ng of total RNA using the Illumina TotalPrep RNA Amplification Kit (Ambion Inc.). cRNA yields were quantified with a Agilent Bioanalyzer, and 750 ng of biotin-labeled cRNA was hybridized to the Illumina Human HT-12 v4.0 Expression Beadchip according to manufacturer’s instructions (Illumina, Inc.). Following hybridization, bead chips were washed and stained with Cy3-labeled streptavidin according to the manufacturer’s protocol. Dried bead chips were scanned on the Illumina BeadArray Reader confocal scanner (Illumina, Inc.). Gene expression signals obtained after chip scanning were quantified normalized in Partek Genomics Suite v6.6 (Partek Inc.). Genes with a normalized maximum signal >1000 in all groups were considered similar to background and removed from further analysis. Sample outliers were detected using principal component analysis in Partek. Differentially expressed genes were identified via 1-way ANOVA with post hoc contrasts specifying the desired pair-wise comparisons. The magnitude of differential gene expression between groups was expressed as fold change and the statistical significance of differences in gene expression were ascertained by the false discovery rate (FDR). For most analyses, genes with an absolute log fold change >0.58 and FDR <5% were considered significantly differentially expressed. Gene expression profiles across comparator groups were visualized through heat maps generated via the gplots library in R 3.2.3 using the gplots and RColorBrewer packages, with genes in rows and treatments in columns. Enrichment graph plots for each gene (represented as bars), which are ranked ordered by their signal-to-noise metric between the control and treated compounds or PIP4Ks knockdown samples. Gene expression values were row-normalized and mapped along the y-axis, representing an ascending scale of expression signals. In some analyses, the gene expression matrix was subjected to hierarchical clustering according to Ward’s algorithm prior to the generation of heatmaps. To evaluate the effects of differential gene expression on biological mechanisms, we performed GSEA using a customized version of the KEGG pathway repository obtained from the Molecular Signatures Database (MSigDB). Biological pathways containing 10–20 genes were considered for analysis, and pathways with FDR <10% were considered statistically significant.

**Immunoblot analysis.** Total cell lysates were prepared with 1% Triton X-100 (25 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% Triton-X100, 1 mM diethiothreitol (DTT), protease inhibitor mix (Complete Mini, Roche) and phosphatase inhibitor (PhosphoStop, Roche)) and subjected to SDS-PAGE. The following antibodies were used: a 1:5000 dilution of PBS-conjugated α-H-actin (Sigma-Aldrich), α-\(\gamma\)-tubulin (Sigma-Aldrich, #T6557; 1:0000) and α-\(\beta\)-tubulin (Abcam, #ab18207; 1:2000), IsoType-specific secondary antibodies (1:500 dilution) coupled to Alexa Fluor 488, 594, or Cy5 ( Molecular Probes) were used. Cells were counterstained with DAPI (Thermo Scientific). Images were acquired at RT with 3-D SIM mode using confocal microscope (Nikon) equipped with a 10× NIKOR objective, a 63×1.4 Plan Apo objective and a 100× Plan Apo objective. Protein expression levels were normalized to DAPI-stained nuclei using a customized version of the KEGG pathway repository obtained from the Molecular Signatures Database (MSigDB). Biological pathways containing 10–20 genes were considered for analysis, and pathways with FDR <10% were considered statistically significant.
give false positives in a shift analysis). Euclidean distance (ED) score of thermal shifts of all the proteins with complete replicates were then calculated as follows:

$$\text{ED score} = \frac{\sum_{\text{protein}} |d_{\text{protein}} - d_{\text{control}}|}{102}$$

where $\sum_{\text{protein}} |d_{\text{protein}} - d_{\text{control}}|$ is the sum of inter-treatment ED indicating the noise in similarity of protein melting curves from the same treatment in replicate runs. By definition, larger ED score corresponds to the proteins reproducibly showing a significant shift when compared to control, thus they are potential protein hits targeted by the compound or treatment. ED hit lists were generated for a131 and a166 using a cutoff at median +2.75*MAD (median absolute deviation). The ED hits were applied to the respective normalization factors vector to each vector of normalization factors. Finally, the data normalization was achieved by applying the respective normalization factors in the vector to the protein fold-change vectors of each of the element in the vector of normalization factors, thereby normalizing the data.

The compounds were further added to cell lysates at the indicated protocol.

**PIPK4 enzyme assay.** HEK293 cells were treated with DMSO or compounds for 24 h. Cells were lysed with RIPA buffer (Sigma-Aldrich) and total protein concentrations were measured using a bicinchoninic acid protein assay kit (Thermo Scientific). Tissue samples were further added to cell lysates at the indicated concentrations during the measurement of PIP4K activity. Next, 10 μg of cell lysate was incubated with 1 μM P1(5)P and 380 nM ATP for 1 h at 37 °C. PIP4K activity was measured by recording luminescent signals (Tecan) using a PIP4K Activity Assay Kit (Echelon) according to the manufacturer’s instructions. For cell-free PIP4K2A activity assays, serial dilutions of compounds were pre-incubated with 1 ng of PIP4K2A (kind gift from Daichi Sanyo Co., Ltd. and Daichi Sanyo RD Novare Co., Ltd.) in reaction buffer (50 mM HEPES (pH 7.0), 13 mM MgCl2, 0.05% CHAPS, 0.01% BSA, 2.5 mM DTT) for 1 h at 25 °C. DOPS (80 μM, Avanti polar lipids), P1(5)P (20 μM, Echelon) and ATP (10 μM, Sigma-Aldrich) were added and further incubated for 90 μM at room temperature. PIP4K2A activity was measured by recording luminescent signals (Tecan) using an ADP-Glo Kinase Assay (Promega) according to the manufacturer's protocol.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChiP) assays were carried out using the Magna ChiP A/G Kit (Millipore) according to the manufacturer’s instructions. Enrichment of Pol II binding to PIK3IP1 was evaluated by qPCR using 1/10 of the immunoprecipitated chromatin as template and iQ SYBR Green Super mix (Bio-Rad). Primer sequences are available upon request.

**Data availability.** Microarray data that support the findings of this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE104301. All other relevant data are available from the corresponding author on reasonable request.

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Author contributions
M.K. and S.H.L. conceived and led the functional studies with the technical assistants from P.-J.L., K.H.L., and J.W. S.C.S. and B.W.D. conceived and led the generation of small molecules. N.M. and O.S. performed the tumor spheroid and ex vivo model experiments under the supervision of H.S. D.L., N. P., G.K.D., R.S., and A. L. performed MS-CETSA under the supervision of P. N. S.G. performed GSEA and KEGG pathway analysis. M.K., F.M., D.M.E., and S.H.L. wrote the manuscript and all authors commented on the manuscript.

Additional information
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Competing interests: S.H.L., M.K., S.C.S., and B.W.D. are inventors on a filed patent application covering the discoveries of small molecules presented in this manuscript. The remaining authors declare no competing financial interests.

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