SMYD3 links lysine methylation of MAP3K2 to Ras–driven cancer

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Deregulation of lysine methylation signalling has emerged as a common aetiologic factor in cancer pathogenesis, with inhibitors of several histone lysine methyltransferases (KMTs) being developed as chemotherapeutics.1 The largely cytoplasmic KMT SMYD3 (SET and MYND domain containing protein 3) is overexpressed in numerous human tumours2–4. However, the molecular mechanism by which SMYD3 regulates cancer pathways and its relationship to tumourigenesis in vivo are largely unknown. Here we show that methylation of MAP3K2 by SMYD3 increases MAP kinase signalling and promotes the formation of Ras-driven carcinomas. Using mouse models for pancreatic ductal adenocarcinoma and lung adenocarcinoma, we found that abrogating SMYD3 catalytic activity inhibits tumour development in response to oncogenic Ras. We used protein array technology to identify the MAP3K2 kinase as a target of SMYD3. In cancer cell lines, SMYD3-mediated methylation of MAP3K2 at lysine 260 potentiates activation of the Ras/Raf/MEK/ERK signalling module and SMYD3 depletion synergizes with a MEK inhibitor to block Ras-driven tumorigenesis. Finally, the PP2A phosphatase complex, a key negative regulator of the MAP kinase pathway, binds to MAP3K2 and this interaction is blocked by methylation. Together, our results elucidate a new role for lysine methylation in integrating cytoplasmic kinase-signalling cascades and establish a pivotal role for SMYD3 in the regulation of oncogenic Ras signalling.

The Ras family of oncoproteins is activated in a large fraction of human cancers. To explore possible connections between KMTs and human cancers driven by activation of Ras, we surveyed the level of expression for 54 known and candidate human KMT genes in pancreas ductal adenocarcinoma (PDAC), a cancer nearly universally initiated by oncogenic Ras mutations. We found that five KMT-encoding genes (SMYD3, MLL5 (also known as KMT2E), EZH2, SETD5 and WHSC1L1) were consistently upregulated in human data sets (Extended Data Fig. 1a–c). SMYD3, which showed the most significant correlation with PDAC in our meta-analysis, is reported to be overexpressed in several cancers with elevated histone H3 lysine 4 trimethylation (H3K4me3)10,11, including the lung and the pancreas12. We therefore asked whether SMYD3 might have a role in Ras-driven tumorigenesis.

Little is known regarding SMYD3 cancer-related function in vivo. We previously generated Smyd3 mutant mice, which develop normally, and are viable and fertile (Extended Data Fig. 2d; data not shown; ref. 6). To investigate the role of Smyd3 in Ras-driven cancers, Smyd3 mutant mice were crossed with mice harbouring a loxP-Stop-loxP KrasG12D knock-in allele (Kras+/LSL-G12D), which allows for the controlled induction of oncogenic K-Ras and the initiation of tumours in distinct organs, including the lung and the pancreas13,14. PDAC is thought to arise from the transdifferentiation of acinar cells into duct-like cells upon activation of Ras signalling15,16. We observed induction of SMYD3 expression during this process in mice with pancreas-specific activation of K-Ras (Fig. 1a) and in an ex vivo acinar-to-ductal metaplasia (ADM) assay11 (Fig. 1b). In this assay, Smyd3 was required for efficient duct formation from acinar cells (Fig. 1c, d). In vivo, ADM and PDAC initiation are triggered in young Kras mutant mice by inducing severe acute pancreatitis via repeated injections of caerulein11 (Fig. 1e). In this system, Smyd3 deletion reduced the appearance of pancreatic intra-epithelial neoplasia (PanIN) brought on by Kras activation, as determined by histopathological analysis and decreased signal for both phosphorylated ERK1/2 (pERK1/2, a downstream marker of Ras activity) and MUC5AC (a marker of PanINs) (Fig. 1f, g). In the absence of a pancreatitis trigger, PanIN lesions develop by 6 months in p48Cre;Kras+/LSL-G12D mice, a process that was attenuated by Smyd3 loss (Fig. 1h; Extended Data Fig. 3a). Next, to study PDAC growth and to perform survival studies, we used the p48Cre;Kras+/LSL-G12D; p53fl/fl (Krasp53) mutant model (p48 and p53 are also known as Ptf1a and Tp53, respectively), which is characterized by rapid PanIN-to-PDAC progression and malignant transformation with 100% penetrance in a relatively short latency (~50–60 days)17. At autopsy, the pancreatic tissue from Krasp53 mutant mice was entirely occupied by transformed cells, whereas areas of normal pancreatic tissue remained in Krasp53; Smyd3 mutant mice (Extended Data Fig. 3b, c). Furthermore, loss of Smyd3 extended the lifespan of the animals (Fig. 1i) and resulted in reduced levels of the PDAC biomarker pERK1/2 in biopsy samples (Fig. 1j; Extended Data Fig. 3b). Notably, K-Ras expression was not affected by Smyd3 deletion (Extended Data Fig. 3d). Based on these data, we conclude that Smyd3 is required for efficient initiation of pancreatic cancer by oncogenic K-Ras.

Oncogenic activation of the Ras pathway is a frequent event in lung adenocarcinoma, a cancer that also shows high SMYD3 expression (Extended Data Fig. 1d). Intratracheal injection of an adenovirus expressing the Cre recombinase (Ad-Cre) in adult KrasG12D mice led to the development of atypical adenomatous hyperplasia (AAH) and adenomas in the lungs within 12 weeks, irrespective of Smyd3 status (Fig. 2a, b; Extended Data Fig. 3d). In contrast, at 16 and 20 weeks or more after Ad-Cre infection, mice lacking Smyd3 showed significantly smaller and less advanced tumours than control mice (Fig. 2a, c, d; Extended Data Fig. 3d; data not shown). Specifically, quantification of tumour grade indicated that Smyd3 loss impeded the critical transition from adenoma to adenocarcinoma (Fig. 2c), which was also observable at the whole-organ level (Fig. 2d). Moreover, the lifespan of KrasG12D mice expressing p53 was 20% longer if they were mutant for Smyd3 (Fig. 2e). Progression of lung cancer to carcinoma correlates with amplification of Ras/MEK/ERK signalling18,19. Smyd3 deletion resulted in lower detection of pERK1/2 relative to control tumours without an overall change.
in total levels of Ras (Fig. 2f; Extended Data Figs 1a and 3f). Together, these observations indicate that SMYD3 promotes Ras-driven cancer development and progression in vivo.

Depletion of SMYD3 by RNA-mediated interference (RNAi) using a short hairpin RNA (shRNA) strategy in LKR10 mouse cells (a LAC-derived cell line\(^a\)), A549 (a human LAC cell line) and CFPac1 (a human PDAC cell line) reduced the proliferation rates for all three cell types, and inhibited their ability to grow in anchorage-independent condition (Extended Data Fig. 1a–c). Furthermore, knockdown of SMYD3 in CFPac1 cells inhibited tumour growth in mouse xenograft experiments (Extended Data Fig. 1d–f). Thus, SMYD3 acts to maintain a number of tumorigenic characteristics in mouse and human cancer cell lines driven by oncogenic Ras.

Next, wild-type SMYD3, a catalytically inactive form (SMYD3(F183A))\(^d\), or vector control were co-expressed with the Cre recombinase in the lungs of Kras;Smyd3 mutant mice by lentiviral transduction (Extended Data Fig. 5a). Complementation of wild-type SMYD3 into the lungs of Kras;Smyd3 mutant mice resulted in a higher tumour burden and pERK1/2 signal relative to the control Cre-alone infection and expression of mutant SMYD3(F183A) (Fig. 3a–c). Reconstitution experiments also demonstrated that SMYD3 catalytic activity is required for pancreatic ADM (Extended Data Fig. 5b, c). We previously reported that SMYD3 methylates histone H4 at lysine 5 (H4K5) and not at other lysines on histones\(^e\). However, in LAC and PDAC cells and tumours, virtually all SMYD3 is present in the cytoplasm (Fig. 1a and Extended Data Figs 2a, 3e and 6b), indicating that the cancer-relevant substrate in these cell types is unlikely to be nuclear H4K5 but rather a cytoplasmic protein.

In a biochemical screen for SMYD3 targets on a protein array platform containing more than 9,000 potential substrates\(^f\), the only candidate to be methylated by SMYD3 in three independent experiments was the MAP kinase pathway component MAP3K2 (Extended Data Fig. 6a). As shown in Fig. 3d, recombinant wild-type SMYD3, but not the catalytically dead SMYD3(F183A) mutant, methylated recombinant MAP3K2 in vitro. Using a mutagenesis approach, we identified lysine 260 of MAP3K2 as the single site of methylation catalysed by SMYD3 (Fig. 3e; data not shown). The immediate sequence surrounding K260 of MAP3K2 and K5 of H4 is identical (GKGG), although the catalytic efficiency (\(k_{\text{cat}}/K_{\text{m}}\)) of SMYD3 for MAP3K2 is nearly two orders of magnitude greater than it is for H4 (Extended Data Fig. 6c). We also did not detect any methylation of H3 at lysine 4 by SMYD3, a previously reported activity\(^g\) (Extended Data Fig. 6d, e). In addition, SMYD3 was the only KMT of the eleven tested that could methylate MAP3K2 (Fig. 3f, g; Extended Data Fig. 6f). Furthermore, whereas SMYD3 methylated MAP3K2, it had no detectable activity on a dozen other members of the MAP kinase signalling cascade (Fig. 3h).

In vitro methylation assays on MAP3K2 peptides spanning K260 (amino acids 249 to 273) with K260 either unmethylated, mono-, di- or tri-methylated showed that SMYD3 can use all lower states of methylation as substrates to generate the fully saturated trimethyl state at K260 (Extended Data Fig. 6g, h). Thus, SMYD3 mono-, di- and tri-methylates MAP3K2 at lysine 260 (MAP3K2-K260me) with high specificity in vitro.

To investigate MAP3K2 methylation in cells, we raised methyl-specific antibodies against the different states of methylation at K260 (Extended Data Fig. 6i–l). Both the mono- and di-methyl specific antibodies recognized MAP3K2-K260me in normal and tumour cell lines, as well as in tumour lysates from Kras;Smyd3 mutant mice.

**Figure 1 | SMYD3 loss inhibits Ras-driven pancreatic tumorigenesis.** a, Representative immunohistochemistry (IHC) images showing SMYD3 expression in cells undergoing acinar-to-ductal metaplasia (ADM, arrowheads) but not in acini (asterisk) in p48\(^{Cre}\); Kras\(^{LSL-G12D}\); Smyd3\(^{-}\)(Kras) mice. b, Smyd3 expression increases during ADM formation. Quantitative PCR with reverse transcription (qRT–PCR) analysis of Smyd3 expression at the indicated times from control- and EGF-induced ADM ex vivo samples (four independent biological replicates). c, Smyd3 depletion inhibits ADM. Wild-type (WT), p48\(^{Cre}\)-positive acinar clusters (asterisk) undergo ADM and form ducts (arrowhead) ex vivo, whereas Smyd3 mutant acini explants inefficiently form ducts. d, Quantification of acinar and ductal clusters on day 3 of culture as in c (four independent biological replicates with three technical replicates each). e, Schematic of the caerulein pancreatitis-induced tumorigenesis protocol\(^2\). f, Representative haematoxylin and eosin (HE) staining and IHC for pERK1/2, a marker of Ras activity, and MUC5, a marker of PanIN lesions (arrowheads). g, Quantification of MUC5-positive lesions in caerulein-treated pancreata from Kras\(^{LSL-G12D}\) and Kras;Smyd3\(^{-}\) (n = 6) mutant mice. h, Quantification of spontaneous PanIN lesions formed in 6-month-old Kras\(^{LSL-G12D}\) (n = 8) and Kras;Smyd3\(^{-}\) (n = 8) mutant mice. The grade of lesions is indicated. i, Kaplan–Meier survival of Krasp53 mutant mice (p48\(^{Cre}\); Kras\(^{LSL-G12D}\); p53\(^{lox/lox}\) (n = 33, median survival = 56 days) and Krasp53;Smyd3\(^{-}\) mutant mice (n = 21, median survival = 68.5 days). P = 0.005 by log-rank test for significance. j, Immunoblots with the indicated antibodies of Krasp53 and Krasp53;Smyd3\(^{-}\) mutant pancreatic tumour lysates. Loss of SMYD3 was also confirmed by immunostaining of pancreatic sections (Extended Data Fig. 4d). All scale bars, 50 μm. *P value < 0.05; **P value < 0.01; n.s., not significant (two-tailed unpaired Student’s t-test). Data are represented as mean ± s.e.m.
Figure 3 | SMYD3 methylates MAP3K2 in cancer cells. a, Analysis of lung cancer development in Kras;Smyd3 mutant mice following infection with a lentivirus expressing Cre only or simultaneously Cre and WT SMYD3 or inactive SMYD3(F183A). Histological analysis (HE staining) and IHC for pERK1/2 were performed 24 weeks post lentiviral infection. IHC confirmed lentiviral-mediated expression of SMYD3. Scale bars, 50 μm. b, c, Quantification of total tumour area per lung and pERK1/2 positive area per lung, respectively (n = 4 for each experimental group). Data are represented as mean ± s.e.m. *P value < 0.05 (two-tailed unpaired Student’s t-test). d, SMYD3 directly methylates MAP3K2. In vitro methylation assay on full-length recombinant MAP3K2 with recombinant wild-type SMYD3, catalytic-dead SMYD3(F183A), or glutathione-S-transferase (GST) control. Top panel, autoradiogram of methylation assay. Bottom panel, Coomassie stain of proteins in the reaction. e, SMYD3 methylates MAP3K2 at K260. In vitro methylation assay as in d with the indicated proteins on MAP3K2 amino acids 1–350, MAP3K2 amino acids 1–350 with a K260A substitution, and MAP3K2 amino acids 351–619. Arrow indicates GST, which is a stable breakdown product of recombinant proteins. f, g, MAP3K2 is a specific substrate of SMYD3. In vitro methylation assays as in d on MAP3K2 using the indicated KMTs (positive controls for the known KMTs shown in Extended Data Fig. 6). h, SMYD3 specifically methylates MAP3K2. In vitro SMYD3 methylation assay as in d on the indicated MAP kinase pathway proteins. Asterisk indicates SMYD3 knockdown breakdown product. Arrow indicates GST, which is a stable breakdown product for many of the screened substrates. i, Immunoblots with the indicated antibodies from input (cytoplasmic extract) or the indicated IPs (immunoprecipitations) from LKR10 cells stably expressing control or Smyd3 shRNA. j, Immunoblots with the indicated antibodies and samples as in i of lung tumour biopsy lysates isolated from Kras and Kras;Smyd3 mutant mice. Asterisks represent detection of IgG. For experiments e–k representative data based on three or more independent biological replica are shown.

Data Fig. 6i). In co-transfection experiments in human 293T cells, over-expressed MAP3K2 was methylated at K260 upon SMYD3 overexpression (Extended Data Fig. 6i). Endogenous methylation at MAP3K2-K260 was observed in LKR10 cells and RNAi-mediated depletion of SMYD3 in these cells resulted in loss of this signal (Fig. 3i). Finally, the MAP3K2-K260me2/3 signal was significantly reduced in tumour tissue micro-dissected from Kras versus Kras;Smyd3 mutant mice (Fig. 3j). Thus, SMYD3 is required for maintenance of physiological levels of MAP3K2-K260 methylation in cancer tissue and cells.

The cytoplasmic kinase MAP3K2 is activated in response to a variety of stress and mitogenic stimuli, including epidermal growth factor (EGF), and relays signals to downstream MAP kinase components such as ERK1/2a and ERK5b. Given that the pERK1/2 and pERK5 signals are reduced in LAC and PDAC samples from Smyd3 mutant mice (Figs 1 and 2; Extended Data Fig. 3), we reasoned that SMYD3-mediated methylation of MAP3K2 may regulate signalling within the Ras/ERK pathway. We therefore examined the relationship between SMYD3 and EGF-stimulated ERK1/2 activation. First, endogenous SMYD3 was depleted in LKR10 cells by shRNA targeting the 3′ untranslated region of Smyd3 and then we reconstituted the depleted cells with RNAi-resistant wild-type SMYD3 or catalytically inactive SMYD3(F183A). In control cells, EGF treatment triggered ERK1/2 phosphorylation, and this response was greatly reduced by SMYD3 depletion (Fig. 4a). Complementation with wild-type SMYD3 re-established the EGF-mediated ERK1/2 phosphorylation response, whereas complementation with SMYD3(F183A) failed to do so (Fig. 4a). SMYD3 was also required when serum was used to activate ERK1/2 activation in LKR10 cells (Extended Data Fig. 7b) and when EGF was used to activate ERK1/2 in human lung and pancreatic cancer cells (Extended Data Fig. 7c, d). Finally, we established a MAP3K2 complementation system to investigate the role of K260 of MAP3K2. In these experiments, wild-type MAP3K2, but not the SMYD3-resistant MAP3K2(K260A) mutant, reconstituted the EGF-mediated ERK1/2 phosphorylation response (Fig. 4b).

To characterize how the SMYD3–MAP3K2 axis impinges on the overall MAP kinase network, the level of EGF-induced activation for several kinases was determined in control, SMYD3 knockdown and MAP3K2 knockdown LKR10, A549 and CPAC1 cells (Fig. 4c; Extended Data Fig. 7a–d). SMYD3 and MAP3K2 were both required for full activation of ERK5, ERK1/2, MEK1/2 and JNK (known downstream targets of MAP3K2; refs 18–22), but dispensable for activation of AKT and Craf (RAF-1) (p38 was not activated in the cell lines tested; Extended Data Fig. 7a–d). Notably, MEK1/2 activation was impaired in SMYD3 and MAP3K2 knockdown cell lines. As the canonical ERK1/2 activation pathway consists of Raf-MEK1/2-ERK1/2 and both SMYD3 and MAP3K2 were required for MEK1/2 and ERK1/2 activation but not Craf, we postulated that SMYD3 methylation of MAP3K2 directly influences MEK1/2 phosphorylation. Indeed, pMEK1/2 signal increased in response to overexpression of wild-type MAP3K2 and a SMYD3-resistant MAP3K2(K260A) mutant, but not a catalytically dead MAP3K2 (K385M) mutant (Fig. 4d, e; Extended Data Fig. 7e). SMYD3 expression alone had no effect on MEK1/2 activation (Extended Data Fig. 7e), but resulted in increased MEK1/2 phosphorylation when co-expressed with wild-type MAP3K2 relative to co-expression with MAP3K2(K260A) (Fig. 4d, e). Notably, MAP3K2 phosphorylates MEK1 in vitro kinase assays a, and this activity was unchanged if SMYD3-methylated MAP3K2 or MAP3K2(K260A) were used in the kinase assays rather than wild-type, unmethylated MAP3K2 (Extended Data Fig. 7f). These results indicate that the molecular mode of action linking MAP3K2 methylation to MEK1/2 activation is not due to changes in the intrinsic kinase activity of MAP3K2 but rather another mechanism.

Given the role of SMYD3–MAP3K2 in activating MEK1/2, we tested whether SMYD3 depletion augments the effects of the MEK1/2 inhibitor trametinib, which is currently being investigated to treat NSCLC and PDAC (http://clinicaltrials.gov/). Administration of Kras and Kras;Smyd3 mutant mice with a normal dose of trametinib blocked tumorigenesis in both strains, although phosphorylation of ERK1/2 was still lower in mice depleted of SMYD3 (Extended Data Fig. 8). Notably, a low-dose trametinib regimen, which only partially inhibited pERK1/2 levels and the formation of neoplastic lesions in Kras mutant mice, was sufficient to block tumorigenesis and ERK1/2 activation in Smyd3 knockouts (Extended Data Fig. 8). Trametinib was also more potent in cancer cell lines when coupled with SMYD3 depletion (Extended Data Fig. 9a). These data indicate that SMYD3 may act in concert with MEK1/2 signalling...
in tumorigenesis. Indeed, overexpression of activated MEK1(S218D, S222D) (MEK1-DD) rescued the effects of SMYD3 depletion in lung cancer cells (Extended Data Fig. 9b).

Because the intrinsic kinase activity of MAP3K2 is not directly altered by methylation, we postulated that this modification event was involved in modulating a key protein–protein interaction. To identify candidate methyl-sensitive binding partners of MAP3K2, a SILAC (stable isotope labelling by amino acids in cell culture)-based quantitative proteomic screen was performed with cytoplasmic extracts to isolate proteins that bound differentially to MAP3K2-K260me3 peptides versus MAP3K2-K260me0 peptides. Although this analysis did not show enrichment of any K260me3-binding proteins, it did reveal six candidates that bind to the unmethylated peptide but are blocked by K260 trimethylation. Of these six proteins, three are members of the PP2A serine/threonine phosphatase complex (Fig. 4f). In our proteomics experiment we identified the catalytic PPP2CA protein, the structural PPP2R1A protein and the regulatory PPP2R2A protein, three classes of subunits that comprise the typical heterotrimERIC PP2A complex. This complex is a major cellular phosphatase that inactivates key members of the MAP kinase signalling cascade (reviewed in refs 25, 26). We found that the interaction between the PP2A complex and MAP3K2 is direct, as recombinant PPP2R2A, but not PPP2R2A, bound to MAP3K2-K260me0 peptides and not to MAP3K2-K260me3 peptides in in vitro peptide pull-down experiments (Fig. 4g; top panel). Moreover, PPP2R2A was specifically recovered from cytoplasmic extracts using MAP3K2-K260me0 peptides but not by MAP3K2-K260me3 peptides (Fig. 4g; middle panel). Thus, amino acids 249–273 of MAP3K2 are sufficient for binding directly to the PP2A complex, via PPP2R2A, and methylation at K260 inhibits this interaction.

We next tested the ability of the PP2A inhibitor cantharidin to ‘phenocopy’ SMYD3 function (Fig. 4h; Extended Data Fig. 10a–d). Cantharidin treatment had no gross effect on tumour formation in p48<sup>+/cre</sup> Kras mutant mice relative to vehicle treatment; in contrast, administration of cantharidin to p48<sup>+/cre</sup> Smymd3 mutant mice restored tumour formation to the level seen with wild-type SMYD3 in the p48<sup>+/cre</sup> Kras mutant mice. These data suggest an in vivo functional connection between the Ras pathway, SMYD3 and PP2A.

We have identified SMYD3-catalysed methylation of MAP3K2 as a key event regulating Ras signalling in cancer cells. Although MAP3K2 was shown to phosphorylate MEK1<sup>1/2</sup> directly, a complete understanding of how MAP3K2 functions in Ras signalling remains to be determined. Our data suggest a model in which increased SMYD3 activity generates a population of methylated MAP3K2, which—via mechanisms such as blocking the association of the PP2A phosphatase with components of the MAP kinase network—intensifies the output of this pathway in response to oncopgenic Ras (Extended Data Fig. 10e). In addition, the cytoplasmic localization of the SMYD3–MAP3K2 dynamic suggests a paradigm for how signalling through lysine methylation and phosphorylation can be integrated to regulate key signal transduction cascades. A clinical implication of this work is the identification of SMYD3 as a candidate therapeutic target for pharmacologic intervention to treat pancreatic and lung cancers, as well as potentially other Ras-driven tumours. The complete loss of SMYD3 function has no visible phenotype in mice, suggesting that SMYD3 inhibitors would have minimal collateral toxicity as chemotherapeutics. Thus, one could envision a therapeutic strategy comprising inhibitors of Raf or MEK that are currently used in the clinic with a SMYD3 inhibitory agent, which could mitigate potential drug toxicity by lowering the overall dose needed for each medicine and combat the development of resistance. Together, our findings reveal a new function for lysine methylation signalling in the cytoplasm in the regulation of cancer pathways.

**METHODS SUMMARY**

Animal studies were performed according to practices prescribed by the NIH at Stanford’s Research Animal Facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The Smymd3 mutant mice used in lung and pancreatic cancer experiments were initially obtained from the KOMP Repository. SILAC was performed using HeLa cells extracts grown in either normal amino acids culture condition (‘light’) or using modified amino acids culture condition (‘heavy’). Full methods and data analysis are described in detail in Methods.
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Author Contributions N.R. and P.K.M. contributed equally to this work and are listed alphabetically. They were responsible for the experimental design, execution, data analysis and manuscript preparation. P.K. and A.J.B. performed the bioinformatics meta-analysis. P.W.T.C.J. and M.V. performed the SILAC experiments. S.L. and B.A.G. performed the bioinformatics analysis and manuscript preparation. P.K. and A.J.B. performed the bioinformatics analysis and manuscript preparation.

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METHODS

Ethics statement. Mice were maintained according to practices prescribed by the NIH at Stanford’s Research Animal Facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Mouse strains. Kras<sup>LSLG12D</sup>/p53<sup>fl/fl</sup>, and p48<sup>−/−</sup>/p48<sup>−/−</sup>;Kras<sup>LSLG12D</sup>/p53<sup>fl/fl</sup> mice have been described before<sup>28,29</sup>. Smyd3<sup>flm/lm</sup> (KOMP) mice were obtained from the KOMP Repository. Details on the targeted allele are available on the KOMP website<sup>1</sup>. Briefly, mice were constructed using the ‘knockout first’ strategy. In this allele, insertion of a LacZ cassette with strong splice acceptor in intron 2 of the Smyd3 gene creates a knock-out allele serving additionally as a reporter. Expression of the Cre recombinase in cells removes the LacZ cassette and further deletes several Smyd3 exons, resulting in a null allele (Extended data Fig. 2f). Mice were of mixed C57BL/6;129SV background and we systematically used randomly picked littermates as controls in all the experiments (sex ratio per cohort balanced).

Pancreatic cancer mouse models. Pancreatic intraepithelial neoplasia (PanIN) development. PanIN progression was analysed in p48<sup>−/−</sup>;Kras<sup>LSLG12D</sup> (Kras) and p48<sup>−/−</sup>;Kras<sup>LSLG12D</sup>/Smyd3<sup>flm/flm</sup> (Kras;Smyd3) mice by administration of 8-hourly intraperitoneal injections of caerulein (125 μg per kg body weight), (Sigma-Aldrich) over 2 consecutive days as described previously<sup>30</sup>. Mice were treated as indicated with the PPARα inhibitor carnitidrin (0.15 mg kg<sup>−1</sup> BID, IP), (Sigma-Aldrich) or the MEK inhibitor trametinib (Selleckchem) (1 mg kg<sup>−1</sup> or 0.1 mg kg<sup>−1</sup> intraperitoneally daily) or vehicle 10% cyclodextran. Pancreatic lesions were analysed 7 days after the last injection.

Spontaneous model of pancreatic intraepithelial neoplasia (PanIN) development. PanIN progression was analysed in p48<sup>−/−</sup>;Kras<sup>LSLG12D</sup>/p53<sup>−/−</sup> (Kras;p53) and p48<sup>−/−</sup>;Kras<sup>LSLG12D</sup>/Smyd3<sup>flm/flm</sup> (Kras;p53:Smyd3) mutant mice. Mice were followed for signs of disease progression. At endpoint, tumours were processed for histological and immunohistochemical analysis. To calculate relative normal acinar area Kras;p53:Smyd3 tumour sections were stained for amylase. Positive regions on six random, non-overlapping, 100 images were collected from 3 mice per genotype. For each image positive amylase area was normalized to total pancreas tissue area using ImageJ software. Error bars represent means ± standard error of the mean (s.e.m.).

Lung cancer mouse models

Adenovirus-induced lung adenocarcinoma (LAC). Kras<sup>LSLG12D</sup>/p53<sup>−/−</sup> (Kras) and Kras<sup>LSLG12D</sup>/Smyd3<sup>flm/flm</sup> (Kras;Smyd3) mice were treated with 5 × 10<sup>6</sup> plaque-forming units of adenovirus expressing Cre (University of Iowa adenovirus core) by intratracheal injection as previously described<sup>31</sup>. Tumours were analysed and quantified at 12, 16 and 20 weeks post-infection, n = 6 for each group.

Lentivirus-induced lung adenocarcinoma. Generation of a dual promoter lentiviral vector for Cre and cDNA expression was described before<sup>32</sup>. To determine the effect of reconstitution of exogenous Smyd3<sup>−/−</sup> and Smyd3<sup>flm/flm</sup> expression on lung tumour progression a lentiviral vector was developed that expressed both the Smyd3 complementary DNA and Cre. A lentivirus expressing Cre alone was used as a control. Virus was produced and titred as described previously<sup>33</sup>. Briefly, the whole virus was recovered by ultracentrifugation at 25,000 r.p.m. for 90 min and resuspended in calcium-phosphate. The supernatant was collected at 48 and 72 h. Concentrated virus was diluted in a null allele (Extended data Fig. 2d). Mice were of mixed C57BL/6;129SV background and we systematically used randomly picked littermates as controls in all the experiments (sex ratio per cohort balanced).

Preparation of pancreatic epithelial explants. Pancreatic epithelial explants from 4- to 6-week-old p48<sup>−/−</sup>;p53<sup>−/−</sup> (WT) and p48<sup>−/−</sup>;p53<sup>−/−</sup>;Smyd3<sup>−/−</sup> (Smyd3) were established by modification of previously published protocols<sup>34</sup>. In brief, the whole pancreas was collected and treated twice with 1.2 mg ml<sup>−1</sup> collagenase VIII (Sigma-Aldrich), Following multiple wash steps with McCoy’s medium 25,000 r.p.m. for 90 min and suspended in PBS. Cohorts of Kras<sup>LSLG12D</sup>/p53<sup>−/−</sup> (Kras;Smyd3) mice were infected with each lentiviral vector. Tumour burden was analysed 24 weeks after lentiviral infection, n = 4 for each treatment.

Histology, immunohistochemistry and X-gal staining. Tissue specimens were fixed in 4% buffered formalin for 24 h and stored in 70% ethanol until paraffin embedding. 3-μm sections were stained with haematoxylin and eosin (HE) or used for immunohistochemical studies.

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed, paraffin embedded mouse and human tissue sections using a biotin–avidin method as described before<sup>35</sup>. The following antibodies were used: rabbit anti-Amylase (Sigma-Aldrich), pERK1/2 (Cell Signalling), MUC5 (NeoMarkers) and Smyd3 (Abcam). Sections were developed with DAB and counterstained with haematoxylin. Pictures were taken using a Zeiss microscope equipped with the Axiovision software. Analysis of the tumour area and IHC analysis was done using ImageJ software by measuring pixel units.

X-gal staining. Staining of cryosections (8 μm) was carried out as described previously<sup>36</sup>, slides were counterstained with nuclear fast red.

qRT–PCR. RNA was isolated using the Qiagen RNeasy Isolation Kit followed by cDNA synthesis (SuperScript II, Invitrogen). Real-time PCR was performed with 800 nM primers diluted in a final volume of 20 μl in SYBR Green Reaction Mix (Applied Biosystems). RT–PCRs were performed as follows: 95°C for 10 min, 35 cycles of 95°C for 15 s and 60°C for 1 min. qRT–PCR data are representative of 4 independent mouse pancreatic epithelial explants isolations per treatment. All samples were analysed in triplicate. Gapdh expression was used for normalization. The following primers were used:

| Primer                  | Sequence                        |
|------------------------|--------------------------------|
| Smyd3-F                  | 5′-TGGGACCATTTAGGCGGTAAC      |
| Smyd3-R                  | 5′-CTCAAGGCTTGGCAGTCACTCTTCT      |
| Gapdh-F                  | 5′-CCACCTTAAATCTACGAGGGG       |
| Gapdh-R                  | 5′-CCTTCACCATGGCGAAAAGTT        |

Meta-analysis of public PDAC and NSCLC data sets. We downloaded raw data for gene expression studies (7 pancreatic cancer, 6 NSCLC) from the NCBI GEO and EBI ArrayExpress. After re-annotating the probes, each data set was normalized separately using gcRMA. We applied two meta-analyses approaches to the normalized data. The meta-analysis approach has been recently described<sup>36</sup>. Briefly, the first approach combines effect sizes from each data set into a meta-effect size to estimate the amount of change in expression across all data sets. For each gene in each data set, an effect size was computed using Hedges’ adjusted g. If multiple probes mapped to a gene, the effect size for each gene was summarized using the fixed effect inverse-variance model. We combined study-specific effect sizes to obtain the pooled effect size and its standard error using the random effects inverse-variance technique. We computed z-statistics as a ratio of the pooled effect size to its standard error for each gene, and compared the result to a standard normal distribution to obtain a nominal P value. P values were corrected for multiple hypotheses testing using false discovery rate (FDR)<sup>37</sup>. We used a second non-parametric meta-analysis that combines P values from individual experiments to identify genes with a large effect size in all data sets. Briefly, we calculated a r-statistic for each gene in each study. After computing one-tail P values for each gene, they were corrected for multiple hypotheses using FDR. Next, we used Fisher’s sum of logs method, which sums the logarithm of corrected P values across all data sets for each gene, and compares the sum against a chi-square distribution with k degrees of freedom, where k is the number of data sets used in the analysis.
Plasmids. Bacterial expression plasmids were created using pGEX-6P1 vector. Transient mammalian expression plasmids were created using pCDNA3.1 HA, pCDNA3.1 Myc and pCagFlag vectors. The different inserts were amplified by PCR using either cDNA or specific clones from the human ORFeome library as template. Single-point mutations of SMPD3 and MAP3K2 were generated using the QuickChange site-directed mutagenesis protocol (Stratagene), and clones were confirmed by DNA sequencing. SMPD3 and MAP3K2 shRNA targeting UTR regions were cloned in a pSiCOR vector carrying a puromycin resistance gene.

Human SMPD3 shRNA sequence directed against the 3′ UTR TGCTGGTTGTC GTTCTGGAAATTCGGAATGACTGAAACACACGACGTTTTC.

Mouse Map3k2 shRNA sequence directed against the 3′ UTR TTAGTGA TTTGCATGAGCCTTTT TATAGTAAATCCAAGGATTTGTAAGTGGTCTGCCTTTC.

Cancer cells, reagents and transfections. K-Ras mutant lung and pancreatic carcinomas (LKB1/LKB1 (mouse lung), A549 (human lung) and CFPAC (human pancreas) were used (all these cell lines are wild-type for EGFR, see below and the COSMIC database for the human cell lines). 293T, LKR10, A549 and CFPAC 1 cells were grown in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO), 100 units/ml penicillin/streptomycin and 2 mM L-glutamine. All cells were cultured at 37 °C in a humidified incubator with 5% CO2. For transient expression, cells were transfected using Mirus 293T transfection reagent and collected 24 to 36 h later. For stable knockdown, cells were transfected with lentiviral shRNA constructs using the packaging vectors pGagpol and pERK5 and pAKT and AKT and pCRaf and CRaf, PPP2R2A (4370, 4695, 9121, 9122, 9125, 9252, 9211, 3371, 2965, 4685, 9427, 9422, 5689, Cell Signaling); HA and Myc (26183, 23161 Pierce). Immunoblots signal intensity was measured using ImageJ software. Quantification data are expressed as e.m.

Expression and purification of recombinant proteins. For expression of GST-tagged recombinant proteins, transformed BL21 cells were induced with 0.1 mM IPTG overnight at 20 °C, and proteins were purified using glutathione Sepharose beads (GE Healthcare) and eluted in 10 mM reduced glutathione (Sigma) or cleaved from the GST tag using purified Precision enzyme. Recombinant human histone H3 and H3K4* mutant (first thirteen lysines mutated to arginine except lysine K4) titrated as purifier and purified as described above.

ProtoArray, methylation and kinase assays. In vitro methylation assays were performed using 1 to 2 μg of recombinant proteins or peptides incubated with 1 μg of recombinant methyltransferases and 0.1 mM of S-adenosyl-methionine (SAM, Sigma) or 2 μM H3-AdoMet (American Radiolabeled Chemicals) in buffer containing 50 mM Tris-HCl (pH 8.0), 10% glycerol, 20 mM KCl, and 1 mM MgCl2, and 1 mM PMSF at 30 °C overnight. The reaction mixture was resolved by SDS–PAGE, followed by autoradiography, Coomassie stain (Pierce) or mass spectrometry analysis.

Kinetic constants $K_{\text{m}}$ and $V_{\text{max}}$ were determined using Grafit (Erithacus software) from SMYD3 methylation activity on histone H4 (EMD Millipore, USA) and MAP3K2 (Origene, USA) was assessed by radiometric assays using [3H-SAM with specific product capture on arginine binding SPA beads for assays using histone substrates or RNA binding SPA beads when MAP3K2 was the substrate. (PerkinElmer, USA). Assay conditions were 20 mM Tris pH 8, 3 mM DTT, 50 μM ZnCl2, 0.005% Tween-20, 1.2 μM unlabeled SAM, 0.2 μM [3H-SAM and 25 mM SMPD3 final. Reactions were quenched using 2 μM unlabeled SAM. SPA signal was quantified in a Microbeta scintillation counter (PerkinElmer, USA). Data were fit to the Michaelis–Menten equation, where the slope was plotted as a function of the concentration of substrate.

To verify the activity of the other lysine methyltransferases tested, in vitro methylation assays were performed using known substrates, as previously reported13–18. In vitro kinase assays were performed by incubating 1 μg of recombinant MAP3K2 WT or mutants with 1 μg of recombinant MEK1 in kinase buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM diithiothreitol (DTT), 0.1 mM Na3VO4, 10 mM MgCl2, and 200 μM ATP (Cell Signalling) at 37 °C during 30 min.

Peptide pull-down and SILAC. MAP3K2 peptides were generated by 21st Century Biotechnologies and are based on the following sequence: DYDPNPEFKKGKGGTYP RRYHYSVHY[L-Biotin]-amidine.

For peptide pull-downs, 5 to 10 μl of streptavidin Sepharose beads (GE Healthcare) were saturated with 10 μg of specific biotinylated peptides for 2 h at 4 °C under rotation in peptide buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP–40), then washed 3 times in the same buffer. Beads were then incubated with either 1 μg of recombinant proteins or 1 μg of HeLa cytoplasmic extract for 4 h at 4 °C under rotation in peptide buffer. Beads were then washed 3 times in the same buffer and resuspended in Laemmli buffer for immunoblot analysis.

For SILAC peptide pull-down, HeLa cytoplasmic extracts were prepared from cells cultivated in either normal amino acids culture condition (‘light’) or using modified amino acids culture condition (‘heavy’). A 2-way experiments was performed, the ‘forward’ condition combining MAP3K2-K260me0 peptide with light extract and MAP3K2-K260me3 peptide with heavy extract, the ‘reverse’ condition combining MAP3K2-K260me0 peptide with light extract and MAP3K2-K260me3 peptide with heavy extract. Beads of each pair of peptide pull-down were then pooled together, washed and extracts were resuspended in Laemmli buffer and resolved by SDS–PAGE. In-gel trypsin digestion was performed and peptides purified using C18 stage tips (Fisher) before mass spectrometry analysis to quantify the ratio of each potential binders for K260me0 and K260me3 peptides, in forward and reverse condition. To identify outliers in both the forward and the reverse experiment, boxplot statistics was applied (cut off = 1.5× interquartile range). Proteins identified as outlier in both experiments are assigned as significant interactors. Amino acid complements used for SILAC are l-lysine-2HCl (Thermo Scientific 88429), l-arginine-2HCl (Thermo Scientific 88427), l-lysine-2HCl (Thermo Scientific 88438), l-arginine-2HCl (Thermo Scientific 88433), l-proline (Thermo Scientific 88430).

Active Ras pull-down and detection. Ras activity refers to the level of guanosine triphosphate-bound Ras, which is able to bind Ras binding domain (RBD) of RAF-1 as measured using a RBD-domain pull-down assay kit as recommended by the manufacturer (The Active Ras Pull-Down and Detection Kit, Thermo Scientific). Briefly, tumour biopsies were homogenized on ice in lysis buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 1% Igepal CA-630, 150 mM NaCl, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 10 μg ml−1 apotinin, 10 μg ml−1 leupeptin and 1 mM sodium orthovandate. These samples were sonicated and centrifuged at 15,000g for 10 min at 4 °C to remove cellular debris. Protein concentration was measured. Equal amounts

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of lysate were incubated for 30 min at 4 °C with agarose beads coated with RBD. The beads were then washed three times with ice-cold lysis buffer, boiled for 5 min at 95 °C, and active Ras was analysed by immunoblotting following standard protocol using Ras-specific antibodies (Thermo Scientific). For comparison to total Ras protein, 2% of total lysates used for pull-down was analysed by immunoblot.

**Cancer xenografts.** For xenograft analysis, 500,000 CFPac1 cells were injected into the flank of NSG mice with Matrigel (BD Bioscience). Tumour volume was measured at the times indicated and calculated using the ellipsoid formula (length × width)².

**Cell assays.** Anchorage-independent growth was assessed in soft agar assays. Cell proliferation was assessed by counting cell number at indicated days and expressed relative to the control as previously described. Cell viability in response to treatment with the MEK inhibitor trametinib (Selleckchem) was measured by an MTT assay (Roche) according to the manufacturer’s instructions.

**Statistics.** Kaplan–Meier survival curves were calculated using the survival time for each mouse from all littermate groups. The log-rank test was used to test for significant differences between the groups. For image quantification and gene expression analysis statistical significance was assayed by Student’s t-test with the Prism GraphPad software (two-tailed unpaired and paired t-test depending on the experiment; variance was first systematically examined using an F-test). *P value < 0.05; **P value < 0.01; ***P value < 0.001; ns, not significant. Data are represented as mean ± standard error of the mean (s.e.m.).
Extended Data Figure 1 | SMYD3 is a highly overexpressed KMT in Ras-associated cancers. a, Analysis of seven publically available human pancreatic ductal adenocarcinoma (PDAC) gene expression studies from the NCBI GEO and EBI ArrayExpress for SMYD3 levels. The red line indicates expression of SMYD3 in pancreatic cancer biopsies (n = 203); the blue line marks normal pancreas samples (n = 91). The scale shows relative expression levels (log2).

b, A bioinformatics meta-analysis identified 5 lysine methyltransferase overexpressed in human pancreatic ductal adenocarcinoma (PDAC). Meta-effect size and statistical tools are described in the Methods. FDR, false discovery rate.

c, d, Summary of SMYD3 expression levels in seven (n = 294 independent samples) publicly-available expression data sets of PDAC and six data sets (n = 319 tumours and n = 147 normal independent samples) of non-small cell lung cancer (NSCLC), respectively. Detailed statistical description in the Methods section.
Extended Data Figure 2 | Analysis of SMYD3 expression in human and mouse PDAC and lung adenocarcinoma (LAC). a, Immunohistochemical analysis of SMYD3 expression in mouse and human WT pancreas, PanIN lesions, and PDAC. The expression pattern was further analysed using a Smyd3LacZ reporter knock-in strain. Smyd3LacZ mice were crossed to p48;KrasG12D (Kras) mice and studied at progressing stages of disease. Analysis of LacZ activity by X-gal staining as a surrogate for Smyd3 expression is shown (lower panel) (see Extended Data Fig. 3 for a cartoon of the knock-in allele). b, Immunoblot analysis with the indicated antibodies on tumour biopsy lysates from wild-type pancreas and from the pancreas of Kras mutant mice at 4.5 and 9 months of age when mice develop PanIN and PDAC, respectively (each time point represents two biological replicates). c, IHC analysis of SMYD3 expression in normal lung, atypical adenomatous hyperplasia (AAH), and lung adenocarcinoma (LAC). X-gal analysis of LacZ activity in Kras-driven tumours with the Smyd3LacZ reporter strain (lower panel). All images shown are representative. Arrowheads indicate nuclear localization of SMYD3. Scale bars, 50 μm. d, Smyd3 knockout allele diagram. In this allele, insertion of a LacZ cassette with a strong splice acceptor in intron 2 of the Smyd3 gene creates a mutant allele serving additionally as a reporter (Smyd3LacZ). Expression of the Cre recombinase in cells removes the LacZ cassette and further deletes Smyd3 exon 2, resulting in a null allele Smyd3KO. SA, splice acceptor; pA, polyadenylation signal.
Extended Data Figure 3 | Smyd3 deletion inhibits pancreatic tumorigenesis. a, Analysis of pancreatic tumorigenesis at 6 months in Kras and Kras;Smyd3 mutant mice. Representative serial histology section (HE), IHC for pERK1/2 and the PanIN marker MUC5. b, Pancreatic cancer phenotypes in Kras;p53 and Kras;p53;Smyd3 mutant mice. Representative IHC for pERK1/2. Arrowheads indicate areas with intact acinar cells. c, Quantification of intact normal acinar area (amylase-positive area) in Kras;p53 and Kras;p53;Smyd3 mutant mice. Data are represented as mean ± s.e.m. ***P value < 0.001 (two-tailed unpaired Student’s t-test). d, Representative HE and pERK1/2 IHC images of lung sections from Kras and Kras;Smyd3 mutant mice 12, 16 and 20 weeks after Ad-Cre infection. pERK1/2 is a marker of Ras activity and advanced tumours. e, f, IHC analysis of SMYD3 expression in the PDAC (e) and LAC (h) mouse models. Arrowheads indicate cytoplasmic localization of SMYD3. Scale bars, 50 μm. g, h, Immunoblot analysis with the indicated antibodies probing pancreatic adenocarcinoma (g) or lung adenocarcinoma tumour lysates (h) dissected from Kras and Kras;Smyd3 mutant mice. Active Ras corresponds to Ras protein in the GTP-bound state pulled down with the RAF Ras-binding domain (RBD) (see Methods). *Tubulin loading control as in Fig. 1j and 2f, respectively.
Extended Data Figure 4 | SMYD3 functions to maintain the tumorigenic characteristics of human and murine cancer cells. a–c, Cell proliferation rates (top panels) and colony formation in soft agar assays (bottom panels) of murine LAC cell line LKR10 (a), human LAC cell line A549 (b), or human PDAC cell line CFPac1 (c) with or without SMYD3 depletion by stable shRNA (respective immunoblot in middle panels). d–f, SMYD3 depletion in CFPac1 attenuates tumour growth in mouse xenografts. d, Macroscopic picture of xenografts from control and SMYD3 knock-down tumours at the end of the experiment. Scale bar, 1 cm. e, Volume analysis shows that shSMYD3 significantly inhibits the expansion of pancreatic tumours (n = 6 for each group). f, HE of the tumours and IHC confirmation of SMYD3 expression and knock-down. All scale bars, 50 μm. *P value < 0.05; **P value < 0.01; ***P value < 0.001 (two-tailed unpaired Student’s t-test). Data are represented as mean ± s.e.m.
Extended Data Figure 5 | Lentiviral reconstitution of SMYD3 in pancreatic acinar-to-ductal-metaplasia (ADM) assays and in lung cancer cells in vivo.

a, IHC analysis of SMYD3 reconstitution in the lung (from Fig. 3a). b, Immunofluorescent detection of SMYD3 expression in wild-type and transduced acinar clusters (left panel). Acini (asterisk) transduced with lenti-Cre carrying wild-type SMYD3 but not catalytically inactive SMYD3(F183A) undergo ADM and form ducts (arrowhead) ex vivo. c, Quantification of acinar and ductal clusters after lentiviral infection (each treatment represents four independent biological replicates). Data are represented as mean ± s.e.m. *P value < 0.05 (two-tailed unpaired Student’s t-test).
Extended Data Figure 6 | SMYD3 specifically methylates MAP3K2 at lysine 260 in vitro. a, SMYD3 methylates MAP3K2 on protein arrays. Representative image ($n = 3$ independent experiments) showing a SMYD3 methylation assay on a ProteinArray. The close-up shows the two independent MAP3K2 spots on the array being methylated. b, SMYD3 is detected in the cytoplasm and not the nucleus in LKR10 cells. Immunoblot analysis with the indicated antibodies of LKR10 cell lysates biochemically separated into cytoplasmic, nuclear and chromatin fractions (see Methods). c, SMYD3 catalytic efficiency is two orders of magnitude greater on MAP3K2 than on H4. $k_{\text{cat}}$, $K_M$, and $k_{\text{cat}}/K_M$ values of SMYD3 activity on recombinant H4 and MAP3K2 as substrates are shown. d, Schematic of the H3K4* mutant form used in e. Note that the only lysine available to be methylated in H3 is present at K4. e, In vitro methylation assay on full-length recombinant MAP3K2, H3 or H3K4* with recombinant SMYD3 and PRDM9. Top panels, short and long exposure autoradiograms of the methylation assay. No signal was detected for SMYD3 on H3 and H3K4* after long exposures. The asterisk and line indicate breakdown products of MAP3K2 that contain K260 and can be detected in this methylation assay upon long exposure. Bottom panel, Coomassie stain of proteins in the reaction. f, Positive control of activity for enzymes used in Fig. 3f, g on their known respective substrates (MAP3K2, histone H3, nucleosome or RelA as indicated). g, In vitro methylation assays using MAP3K2-K260meo, me1, me2 or me3 peptides as SMYD3 substrates. Dot blot is shown as control of peptide’s comparable concentration used for the methylation assay. h, Mass spectrometry analysis of SMYD3 methylation activity on unmodified MAP3K2-K260 peptide. i, Specificity of the indicated MAP3K2-K260me antibodies in dot blot assays using MAP3K2-K260meo, me1, me2 or me3 peptides. j, MAP3K2 is methylated in cells upon SMYD3 overexpression. Immunoblot analysis with the indicated antibodies from 293T cells lysates after Flag immunoprecipitation in cells overexpressing Flag–MAP3K2 and/or HA–SMYD3.
Extended Data Figure 7 | SMYD3 and MAP3K2 knockdown both impair MAP kinase signalling. a–d, Immunoblot analysis with the indicated antibodies of LKR10 (a, b), A549 (e), and CFPac1 (d) lysates. Asterisk indicates a slower migrating ERK5 species that is phosphorylated. Stimulation, 10% serum-complemented media for 15 min (b) or EGF for 15 min at 25 ng ml−1 (a, c, d). Immunoblots are representative of 3 independent biological replicates. e, f, SMYD3 methylation of MAP3K2 does not alter the intrinsic kinase activity of MAP3K2. e, Immunoblot analysis with the indicated antibodies from lysates of 293T cells transfected with control vector, wild-type SMYD3, catalytically dead SMYD3(F183A), wild-type MAP3K2, MAP3K2(K260A), or kinase dead MAP3K2(K385M). f, Methylation of MAP3K2 does not alter its in vitro kinase activity. In vitro kinase assays were performed with the indicated recombinant versions of MAP3K2 (wild-type, SMYD3-resistant K260A mutant, or kinase dead K385 mutant) pre-methylated with wild-type SMYD3 or as a control, inactive SMYD3, using MEK1 as a substrate. MEK1 phosphorylation was detected by immunoblot analysis with the indicated antibody.
Extended Data Figure 8 | SMYD3 knockout augments the effects of the MEK1/2 inhibitor trametinib (GSK1120212) in vivo. a, Schematic of the caerulein pancreatitis-induced tumorigenesis protocol. Mice were treated with a normal dose of trametinib (1 mg per kg intraperitoneally daily) or a low dose (0.1 mg per kg intraperitoneally daily) or vehicle control. b, Immunoblot analysis with indicated antibodies of two independent pancreas biopsies per treatment group. c, Quantification of MUC5-positive lesions in caerulein-treated pancreata from Kras and Kras;Smyd3 mutant mice treated with trametinib or vehicle control (n = 5, each treatment). *P value < 0.05; **P value < 0.001 (two-tailed unpaired Student’s t-test). Data are represented as mean ± s.e.m. d, Representative macroscopic pictures of pancreata from each treatment group. Scale bar, 1 cm. e, Representative serial HE staining and IHC for pERK1/2, a marker of Ras activity, and MUC5, a marker of PanIN lesions. All scale bars, 50 µm.
Extended Data Figure 9 | SMYD3 depletion augments the effects of the MEK1/2 inhibitor trametinib (GSK1120212) in Ras-driven cancer cells. 

a–c, Relative cell proliferation rates (bottom panel) of murine LAC cell line LKR10 (a), human LAC cell line A549 (b), or human PDAC cell line CFPac1 (c) with or without SMYD3 depletion by stable shRNA (SMYD3 proteins levels are shown in top panel) in response to the indicated doses of trametinib. Experiments shown represent an average of 3 independent experiments performed in triplicates for each cancer line. Values represent the number of cells relative to control shRNA cells without treatment at 48 h.

b, Constitutively active MEK1 (MEK1-DD) increases EGF-mediated ERK1/2 activation in SMYD3 depleted-cells. Immunoblot analysis with the indicated antibodies using lysates from A549 cells stably expressing shControl or shSMYD3 and transfected with HA–MEK1-DD. Stimulation: EGF treatment for 15 min at 25 ng μl⁻¹.
Extended Data Figure 10 | Treatment with the PP2A inhibitor cantharidin phenocopies SMYD3 function in vivo. a, Schematic of the caerulein pancreatitis-induced tumorigenesis protocol. Mice were treated with the PP2A inhibitor cantharidin (iPP2A, 0.15 mg kg⁻¹ intraperitoneally twice a day) or vehicle control. b, Immunoblot analysis with indicated antibodies on two independent pancreas biopsies per treatment group. c, Macroscopic pictures of WT and Kras;Smyd3 mutant pancreata. Note that treatment with the PP2A inhibitor leads to the development of enlarged, ‘hard’ pancreata characteristic of tumorigenic development even in Kras;Smyd3 mutant mice. Scale bar, 1 cm. d, Representative serial haematoxylin and eosin (HE) staining and IHC for Erk1/2, a marker of Ras activity, and MUC5, a marker of PanIN lesions. All scale bars, 50 μm. e, Summary model for SMYD3 regulation of MAP kinase signalling after MAP3K2 methylation. Oncogenic Ras activates several kinase cascades that play important roles in pancreas and lung cancer development, including four major MAPK pathways (ERK1/2, ERK5, JNK, and p38) as well as AKT signalling. SMYD3 is frequently overexpressed in pancreatic and lung cancers, two cancer types that are commonly driven by oncogenic Ras signalling. Overexpression of SMYD3 and the resulting methylation of MAP3K2 at K260 potentiate activation of kinases like ERK1/2 and ERK5 in response to stimuli like oncogenic Ras. We postulate a mechanism in which the PP2A complex is unable to bind methylated MAP3K2, which decreases the ability of this enzyme to terminate activating phosphorylation events on MAP3K2 and/or MAP3K2 downstream targets. Under conditions with excessive SMYD3 protein, the physiological relationship between PP2A and MAP3K2 is disrupted and results in an increased pathological MAP3K2 signalling, which cooperates with Ras to promote tumorigenesis.