SETDB1-mediated methylation of Akt promotes its K63-linked ubiquitination and activation leading to tumorigenesis

Guihua Wang1,2, Jie Long2,3,8, Yuan Gao4,8, Weina Zhang2, Fei Han2, Chuan Xu2, Li Sun1,2, Shun-Chin Yang2, Jingqin Lan1, Zhenlin Hou1, Zhen Cai2, Guoxiang Jin3, Che-Chia Hsu2, Yu-Hui Wang2, Junbo Hu1, Tsai-Yu Chen4, Hongyu Li5, Min Gyu Lee4 and Hui-Kuan Lin6,7*

The serine/threonine kinase Akt plays a central role in cell proliferation, survival and metabolism, and its hyperactivation is linked to cancer progression. Here we report that Akt undergoes K64 methylation by SETDB1, which is crucial for cell membrane recruitment, phosphorylation and activation of Akt following growth factor stimulation. Furthermore, we reveal an adaptor function of histone demethylase JMJD2A, which is important for recognizing Akt K64 methylation and recruits E3 ligase TRAF6 and Skp2-SCF to the Akt complex, independently of its demethylase activity, thereby initiating K63-linked ubiquitination, cell membrane recruitment and activation of Akt. Notably, the cancer-associated Akt mutant E17K displays enhanced K64 methylation, leading to its hyper-phosphorylation and activation. SETDB1-mediated Akt K64 methylation is upregulated and correlated with Akt hyperactivation in non-small-cell lung carcinoma (NSCLC), promotes tumour development and predicts poor outcome. Collectively, these findings reveal complicated layers of Akt activation regulation coordinated by SETDB1-mediated Akt K64 methylation to drive tumorigenesis.

Akt kinase serves as a central node for cell proliferation, survival and cell metabolism important for tumorigenesis1,2. Recent studies reveal K63-linked ubiquitination of Akt as a critical event for cell membrane translocation, T308 phosphorylation and activation of Akt (apart from PI3K-mediated PIP3 production)3,4. TRAF6 and Skp2-SCF are identified as two E3 ubiquitin ligases mediating K63-linked ubiquitination and activation of Akt in response to growth factor insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF), respectively5,6. Growth factors trigger the association of E3 ligases with Akt, thereby promoting K63-linked ubiquitination of Akt7,8. While K63-linked ubiquitination is required for Akt cell membrane recruitment and activation, it does not affect Akt-PPIP3 binding9,10. Thus, Akt-PPIP3 binding and K63-linked ubiquitination appear to be two distinct and early events crucial for Akt membrane recruitment and activation. However, it remains unclear how growth factors trigger the interaction of Akt with its E3 ligase to elicit K63-linked ubiquitination.

Lysine methylation of non-histone proteins is involved in numerous molecular events including protein–protein interaction, protein stability, protein subcellular localization, and transcription11–13. Although a substantial number of the lysine methyltransferases (KMTs) have been identified in the human genome, only a few non-histone proteins are known to be methylated by a limited number of KMTs14,15. Whether or not Akt methylation occurs and plays an important role in Akt signalling and tumorigenesis remains to be determined. In this study, we identify SETDB1 (also known as ESET or KMT1E) as an Akt-interacting protein that methylates Akt at K64 to elicit Akt ubiquitination, cell membrane recruitment, phosphorylation and activation following stimulation with growth factors. We demonstrate that SETDB1-mediated K64 methylation of Akt serves as a scaffold to recruit histone demethylase JMJD2A (also known as KDM4A), which then brings Akt’s E3 ligases (such as TRAF6 and Skp2-SCF) to the Akt complex, thereby promoting Akt K63-linked ubiquitination, cell membrane recruitment and activation as well as tumorigenesis. This study therefore identifies SETDB1-mediated Akt K64 methylation as an essential step for K63-linked ubiquitination and activation of Akt in response to stimulation with growth factors.

Results
SETDB1 interacts with Akt and is required for Akt activation. To better understand regulatory modes for Akt phosphorylation and activation, we conducted a systematic mass spectrometry analysis to identify novel Akt-interacting proteins by using 293T cells stably expressing HA-Akt1. Interestingly, one candidate Akt1 interacting protein was SETDB1, belonging to the SET-domain proteins and serving as a histone H3 lysine-9-specific methyltransferase (Supplementary Fig. 1a and Supplementary Table 1)16. We confirmed the interaction between endogenous Akt and SETDB1 by the co-immunoprecipitation assay (Fig. 1a) and demonstrated the direct binding between Akt and SETDB1 using an in vitro binding assay (Fig. 1b). However, SETDB1 was not a substrate of Akt, as the in vitro kinase assay showed that recombinant active Akt1 could directly phosphorylate GSK3β (a known Akt substrate) but not SETDB1 (Fig. 1c).

*These authors contributed equally: Jie Long and Yuan Gao. *e-mail: hulin@wakehealth.edu

| VOL 21 | FEBRUARY 2019 | 214–225 | www.nature.com/naturecellbiology

There are amendments to this paper
Fig. 1 | SETDB1 interacts with Akt and is required for Akt activation. a, Whole-cell extracts (WCE) of HEK293 cells were collected and subjected to co-immunoprecipitation (Co-IP) assays and immunoblotting (IB). b, Immunoprecipitated Flag-SETDB1 from HEK293 cells transfected with Flag-SETDB1 were incubated with GST-Akt1 WT or GST purified from engineered bacteria for the in vitro binding assay, followed by immunoprecipitation (IP) of Flag-SETDB1 and IB analysis. Line 1 is GST-Akt1 input control and lines 2–4 show in vitro binding assay followed by immunoprecipitation (IP) Flag-SETDB1 and IB analysis. c, An in vitro kinase assay shows that Akt phosphorylates GSK3β but not SETDB1, as determined by a phospho-serine/threonine (p-S/T) antibody. d, HEK293 cells were serum-starved for 1 day, treated with 50 ng ml⁻¹ IGF-1 for various times, and whole-cell extracts were collected for immunoprecipitation (IP) with SETDB1, followed by IB analysis. e, Setdb1⁺/− and Setdb1⁻/⁻ MEFs were serum-starved, treated with IGF-1 for 15 min, and whole-cell extracts were collected for IB analysis. f, HEK293 cells silenced with control (shLuc) or SETDB1 shRNA (nos. 1 and 2) were serum-starved, treated with IGF-1 for 15 min, and whole-cell extracts were collected for IB analysis. g, HEK293 cells silenced with control (shLuc) or SETDB1 shRNA (nos. 1) were serum-starved, treated with EGF for 5 min, and whole-cell extracts were collected for IB analysis. h, HEK293 cells transfected with vector or SETDB1 were serum-starved, treated with IGF-1 for 15 min, and whole-cell extracts were collected for IB analysis. i, HEK293 cells transfected with vector, Flag-SETDB1 (WT, 1 μg, 3 μg and 5 μg) and Flag-SETDB1 H1224K (H1224K) were harvested for IB analysis. IB data represent results from 3 independent experiments. Unprocessed blots are shown in Supplementary Fig. 8.
Notably, the interaction of endogenous Akt and SETDB1 was promoted by IGF-1 stimulation (Fig. 1d). As SETDB1 interacts with Akt following growth factor stimulation, we determined whether SETDB1 regulates Akt activation by loss- and gain-of-function approaches. Setd1bΔC mouse embryonic fibroblasts (MEFs) and SETDB1-knockdown cells displayed impairment of AktT308 phosphorylation, an essential phosphorylation event for Akt activation, following IGF-1 and EGF stimulation (Fig. 1e–g and Supplementary Fig. 1b). However, AktT308 phosphorylation was only slightly affected by SETDB1 knockdown (Fig. 1g). Moreover, overexpression of WT SETDB1, but not the SETDB1 H1224K mutant, deficient for methyltransferase activity, enhanced basal and IGF-1-induced AktT308 phosphorylation (Fig. 1h,i and Supplementary Fig. 1c). Collectively, these data suggest that SETDB1 is required for AktT308 phosphorylation in response to growth factor stimulation.

**SETDB1 triggers Akt K64 methylation to mediate Akt phosphorylation.** As SETDB1 is known to induce di-methylation of p5313 and interacts with Akt, we investigated whether Akt is a substrate of SETDB1. Notably, both IGF-1 and EGF treatment induced Akt tri-methylation, correlating with Akt phosphorylation and activation (Fig. 2a,b, Supplementary Fig. 1d and Supplementary Table 2). Among the three Akt isoforms15,16, basal and STEDB1-mediated tri-methylation of Akt were much more pronounced in Akt1 than Akt2 and Akt3 (Fig. 2c). An in vitro methylation assay revealed that SETDB1, but not the SETDB1 H1224K mutant, could tri-methylate or di-methylate Akt1 directly (Fig. 2d). Moreover, Setdb1ΔMEFs and SETDB1-knockdown cells displayed impairment of Akt tri-methylation following IGF-1 and EGF stimulation (Fig. 2e–g), while overexpression of SETDB1 enhanced Akt tri-methylation following IGF-1 stimulation (Fig. 2h). These data along with above findings suggest that SETDB1 is an Akt methyltransferase responsible for Akt tri-methylation in response to growth factor stimulation.

To identify Akt1 methylation sites, mass spectrometry analysis was used to find multiple lysine residues on Akt (including K64, K112, K154, K183 and K400) that could be methylated (Supplementary Fig. 1e). However, by comparing control cells with SETDB1-knockdown cells, only Akt K64 methylation was SETDB1 dependent (Supplementary Fig. 1f). Akt K64 is located in the pleckstrin homology (PH) domain, which is essential for Akt-PIP3 lipid binding and protein–protein interaction, is highly conserved from *Xenopus* to human (Fig. 2i). We therefore focused on Akt K64 methylation and examined its role in Akt activation. We generated a K64-specific methylation antibody of Akt that recognized K64 di-methylation and tri-methylation of Akt (anti-Me2,3-Akt K64) (Fig. 2j–l). Using both lysine tri-methylation antibody and anti-Me2,3-Akt K64 antibody, we found that Akt K64 methylation was induced by both IGF-1 and EGF, and mutation of K64 to arginine (R) impaired Akt K64 methylation (Fig. 2k,l). Consistent with the results that SETDB1-deficient cells exhibited defects in AktT308 phosphorylation and activation, loss of Akt K64 methylation also impaired AktT308 phosphorylation (Fig. 2k,l). An in vitro methylation assay revealed that SETDB1 could tri-methylate or di-methylate Akt1 at K64 (Fig. 2m). Therefore, SETDB1-mediated K64 methylation of Akt is induced by growth factor stimulation and plays a crucial role in AktT308 phosphorylation.

**Akt K64 methylation is critical for Akt activation and cell membrane localization.** To corroborate the role of Akt K64 methylation in Akt phosphorylation, we employed DLD Akt1/2−/− cells and found that Akt1 K64 methylation, Akt1T308 phosphorylation and Akt1 substrate Foxo3a phosphorylation were induced in DLD-1 Akt1/2−/− cells restored with Akt1, but not with Akt1 K64R (Fig. 3a). E17K is the most common Akt mutation identified in numerous human cancers17,18, correlating with constitutive AktT308 phosphorylation and activation, as well as greater oncogenic potential19. Interestingly, we found that the Akt1 E17K mutant displayed not only hyper-phosphorylation of Akt atT308, but also higher Akt K64 methylation compared to WT Akt, probably due to the enhancement of the interaction between Akt and SETDB1 (Fig. 3b). Conversely, loss of Akt K64 methylation impaired heightened Akt tri-methylation and T308 phosphorylation in the Akt1 E17K mutant (Fig. 3b). Collectively, Akt methylation was reduced in the Akt1 E17K/K64R mutant (Fig. 3c). Furthermore, we obtained the same result in NIH3T3 cells with stable expression of Akt1 WT, Akt1 E17K or the Akt1 E17K/K64R mutant (Fig. 3d). Hence, K64 hyper-methylation of Akt contributes to hyper-phosphorylation of the Akt1 E17K mutant.

We then sought to understand how SETDB1-mediated Akt K64 methylation regulates AktT308 phosphorylation and activation in response to growth factor stimulation. We detected Akt K64 methylation in the cytosol (Fig. 3f) and confirmed the K64 methylated Akt was recruited to the cell membrane following growth factor treatment using biochemical fractionations/western blot analysis and immunofluorescence assays (Fig. 3e and Supplementary Fig. 2a). SETDB1 was detected mainly in the cytosolic fraction, but only a weak signal of SETDB1 could be detected in the cell membrane fraction (Fig. 3f), and SETDB1 deficiency impaired IGF-mediated Akt cell membrane recruitment and T308 phosphorylation (Fig. 3f and Supplementary Fig. 2b). These results indicate that SETDB1-mediated Akt methylation at K64 is likely to have occurred in the cytosol, and is crucial for Akt activation and cell membrane localization following growth factor stimulation.

**SETDB1-mediated Akt K64 methylation promotes tumorigenesis.** To determine the oncological consequences of SETDB1-mediated Akt K64 methylation, we used an NSCLC A549 cell model. Knockdown of SETDB1 reduced IGF-1-induced Akt K64 methylation and T308 phosphorylation, and T308 phosphorylation was rescued by restoration of constitutively active of Akt (Myr-Akt) (Fig. 4a,b). Accordingly, cell proliferation and in vitro colony formation were reduced in SETDB1-knockdown cells, and restoration of Akt activity by Myr-Akt rescued these defects (Fig. 4c,d). Moreover, glucose uptake and lactate production were reduced in SETDB1-knockdown cells compared with control cells (Fig. 4e,f). In xenograft tumour models, SETDB1-knockdown cells exhibited much lower tumorigenic potential than control cells (Fig. 4g and Supplementary Fig. 3a,b), correlating with reduced Akt K64 methylation and T308 phosphorylation in xenograft tumour tissues (Fig. 4b). Moreover, restoration of Akt activation in SETDB1-knockdown cells by Myr-Akt partially rescued the defect in tumorigenic potential (Fig. 4i and Supplementary Fig. 3b). Thus, SETDB1 appears to promote tumorigenesis by inducing Akt K64 methylation and activation.

To determine the role of Akt K64 methylation in oncogenic Akt-mediated tumorigenic potential, we performed anchorage-independent soft agar assays using NIH3T3 cells with stable overexpression of either Akt, the cancer-associated mutant E17K or the E17K/K64R double mutant. While overexpression of Akt was insufficient to drive NIH3T3 cell transformation, overexpression of the Akt E17K mutant could drive cell transformation. Remarkably, loss of Akt K64 methylation impaired the transformation potential of the E17K mutant (Supplementary Fig. 3c). These results suggest that SETDB1-mediated Akt K64 methylation contributes to the oncogenic activity of the Akt E17K cancer-associated mutant.

**SETDB1-mediated Akt K64 methylation correlates with Akt activation and predicts poor survival of NSCLC patients.** By examining cBioPortal (http://www.cbioportal.org) and Oncomine databases, we found that SETDB1 is not only upregulated in diverse human cancers, but also overexpressed in different types of lung cancers (Supplementary Fig. 4a,b). The KM-PLOT database revealed that higher expression of SETDB1 was associated with poor survival in patients with NSCLC. These results suggest that SETDB1-mediated Akt K64 methylation contributes to the poor survival of NSCLC patients.
Fig. 2 | SETDB1 triggers Akt K64 methylation leading to Akt phosphorylation. a, b, HEK293 or A549 cells were serum-starved, treated with IGF-1 (a) or EGF (b) for various times, and whole-cell extracts were collected for IP with Akt antibody, followed by IB analysis. c, HEK293 cells were transfected with the indicated plasmids and whole-cell extracts were collected for IP with HA antibody, followed by IB analysis. d, Immunoprecipitated Flag-SETDB1 or Flag-SETDB1 H1224K from HEK293 cells transfected with Flag-SETDB1 or Flag-SETDB1 H1224K were incubated with S-adenosyl-L-methionine along with Akt protein for in vitro methylation of Akt. The methylated proteins were separated by SDS-PAGE, and Akt methylation was analysed by IB analysis. e, Flag-SETDB1 H1224K from HEK293 cells transfected with Flag-SETDB1 or Flag-SETDB1 H1224K were incubated with S-adenosyl-L-methionine along with Akt WT peptide, K64-Me1 peptide, K64-Me2 peptide and K64-Me3 peptide were synthesized by LifeT ein Lab. f, g, h, i, HEK293 cells silenced with control (shLuc) or SETDB1 shRNA (nos. 1 and 2) were serum-starved, treated with IGF-1 for 15 min (f) or EGF for 5 min (g), and whole-cell extracts were collected for IP with Flag-SETDB1 were immunoprecipitated, treated with IGF-1 for 15 min, and whole-cell extracts were collected for IP with Akt antibody, followed by IB analysis. h, HEK293 cells transfected with vector or SETDB1 were serum-starved, treated with IGF-1 for 15 min, whole-cell extracts were collected for IP with Akt antibody, followed by IB analysis. i, The Akt K64 site amino acid in different species. j, Dot blot validates Akt K64 di-/tri-methylation antibody. PEP00021 affinity-purified antibody was a non-methyl antibody that recognizes the peptide N-CQLIKTERPRP. WT Akt peptide, K64-Me1 peptide, K64-Me2 peptide and K64-Me3 peptide were synthesized by LifeT ein Lab. k, l, HEK293 cells transfected with indicated plasmids were serum-starved, treated with IGF-1 for 15 min (k) or EGF for 5 min (l), and whole-cell extracts were collected for IP with HA antibody, followed by IB analysis. m, Immunoprecipitated Flag-SETDB1 from HEK293 cells transfected with Flag-SETDB1 were incubated with S-adenosyl-L-methionine along with GST-Akt1 WT or GST-Akt1 K64R purified from engineered bacteria for in vitro methylation of Akt. The methylated proteins were separated by SDS-PAGE, and Akt methylation was analysed by IB analysis. All IB data represent results from three independent experiments. Unprocessed blots are shown in Supplementary Fig. 8.
with poorer survival of NSCLC patients (Supplementary Fig. 4c). Moreover, using the Human Protein Atlas database, higher expression of SETDB1 was also associated with poor survival in other cancer types (Supplementary Fig. 4d). We detected SETDB1 expression and Akt K64 methylation and T308 phosphorylation in fresh NSCLC specimens, and found that SETDB1 was
Fig. 4 | SETDB1-mediated Akt K64 methylation promotes tumorigenesis, correlates with Akt activation and predicts poor survival of cancer patients.

a, A549 cells silenced with control (shLuc) or SETDB1 shRNA (nos. 1 and 2) were serum-starved, treated with IGF-1 for 15 min, and whole-cell extracts were collected for IP with Akt antibody, followed by IB analysis. b, A549 cells were silenced with control (shLuc) or SETDB1 shRNA (nos. 1 and 2) with or without Myr-Akt transfection, and whole-cell extracts were collected for IB analysis. c, Colony formation assay in A549 cells with shLuc, SETDB1 knockdown, or SETDB1 knockdown plus Myr-Akt overexpression. d, Cell number was counted in A549 cells with control (shLuc), SETDB1 shRNA (no. 1), or SETDB1 shRNA (no. 1) plus Myr-Akt overexpression. e, Glucose uptake was measured in A549 cells silenced with control (shLuc) or SETDB1 shRNA (no. 1). f, Cell number was counted in A549 cells with control (shLuc), SETDB1 shRNA (nos. 1 and 2), or SETDB1 knockdown plus Myr-Akt overexpression. g, Cell number was counted in A549 cells with control (shLuc), SETDB1 shRNA (nos. 1 and 2), or SETDB1 knockdown plus Myr-Akt overexpression. h, Lactate production was measured in A549 cells silenced with control (shLuc) or SETDB1 shRNA (no. 1 and 2). In d–e, data represent mean ± s.d. (n = 3 independent experiments); in f, data represent mean ± s.d. (n = 4 independent experiments). g, Tumour volume of xenograft tumours derived from A549 cells silenced with control (shLuc) or SETDB1 shRNA (no. 1); data represent mean ± s.d. (n = 7 mice for each group, ***P < 0.0001). h, IB of lysed xenograft tumours derived from A549 cells silenced with control (shLuc) or SETDB1 shRNA (no. 1 and 2) with or without Myr-Akt transfection, followed by IB analysis. i, Kaplan–Meier plot analysis of 111 cases of NSCLC patients with low or high expression of SETDB1. Red box is the enlarged view and scale bar represents 100 μm. j, Kaplan–Meier plot analysis of 111 cases of NSCLC patients with low or high expression of Akt K64 methylation. IB data in a,b,h,j represent results from three independent experiments. Statistical significance in d–g,i,l was assessed using Student’s two-tailed t-test. Statistics source data in d–g,i,k,l are shown in Supplementary Table. 4. Unprocessed blots are shown in Supplementary Fig. 8.
Akt K64 methylation is essential for Akt-E3 ligase interaction and Akt K63-linked ubiquitination. We sought to explore the mechanism by which Akt K64 methylation regulates Akt recruitment to the cell membrane. Consistent with the finding that Akt K64 methylation is critical for this recruitment, co-immunoprecipitation assays revealed that endogenous PDK1 interacted with Akt1, but not with Akt1 K64R (Fig. 5a). However, there was no difference in the in vitro binding of recombinant PDK1 with Akt1 WT and Akt1 K64R isolated from cells transfected with Flag-Akt1 WT or Flag-Akt1 K64R. Furthermore, in vitro PIP3-binding assays revealed that Flag-Akt1 WT or Flag-Akt1 K64R isolated from cells bind to PIP3 with the same efficiency (Fig. 5b,c). These data indicate that the defect in cell membrane recruitment of Akt K64R is likely to contribute to its reduction in PDK1 binding.

We then determined the crosstalk between Akt tri-methylation and Akt K63-linked ubiquitination. Of note, K63-linked ubiquitination of Akt was markedly reduced in the Akt K64R mutant (Fig. 5d). Akt ubiquitination was significantly increased in the Akt1 E17K mutation (Fig. 5e). Consistently, K63-linked ubiquitination was markedly reduced in SETDB1-knockdown cells and Setdb1−/− MEFs (Fig. 5f−h), whereas the Akt K8R mutant (which is defective in Akt K63-linked ubiquitination) did not affect Akt K64 methylation (Supplementary Fig. 5a), suggesting that SETDB1-mediated Akt tri-methylation is an upstream event of K63-linked ubiquitination of Akt. To further validate this, the kinetics and chronological sequence of endogenous SETDB1-Akt binding, K64 methylation, ubiquitination, membrane translocation and phosphorylation of Akt1 were examined (Fig. 5i,j). Specifically, we detected SETDB1-Akt interaction and Akt K64 methylation 5 min after IGF-1 treatment, followed by Akt K63-linked ubiquitination and T308 phosphorylation (Fig. 5i), indicating that Akt K64 methylation is the earlier event induced by growth factor. Moreover, Akt K64 methylation was correlated with T308 phosphorylation and cell membrane localization of Akt (Fig. 5j). Hence, SETDB1-mediated Akt K64 methylation is indispensable for K63-linked ubiquitination of Akt.

To elucidate the mechanism by which Akt K64 methylation regulates K63-linked ubiquitination and phosphorylation of Akt by growth factor stimulation, we determined whether Akt K64 methylation orchestrates the interaction of Akt with its E3 ligases TRAF6 and Skp2-SCF. Although Akt readily interacted with TRAF6 and Skp2, the Akt1 K64R mutant showed marked reduction in interacting with TRAF6 and Skp2 under basal conditions as well as with IGF-1 or EGF treatment (Fig. 5k,l and Supplementary Fig. 5b,c). These data underscore the crucial role of Akt K64 methylation in the recruitment of E3 ligases to Akt, thereby triggering K63-linked ubiquitination of Akt.

JMJ2A is important for recognizing K64 methylated Akt and facilities the Akt-E3 ligase interaction. As TRAF6 or Skp2 do not contain any domain that can recognize methylated proteins, we reasoned that they may require an adaptor to bridge the methylated form of Akt and TRAF6/Skp2. We hypothesized that methylation of Akt at K64 may serve as a scaffold to recruit an adaptor of TRAF6/Skp2 to recognize methylated Akt and in turn facilitates the recruitment of TRAF6 or Skp2 to Akt for eliciting K63-linked ubiquitination of Akt. To test this hypothesis, we conducted unbiased mass spectrometry analysis to identify factors that bridge K64-methylated Akt and TRAF6/Skp2. We identified more than 300 proteins that interacted with Akt1 WT, but not with the Akt1 K64R mutant. Of note, Skp2 and TRAF6 were detected among them (Fig. 6a and Supplementary Table 2), further supporting the notion that Akt K64 methylation acts to facilitate the recruitment of E3 ligases Skp2 and/or TRAF6 to Akt for eliciting Akt ubiquitination.

Interestingly, we found that JMJ2A (a KDM family protein that recognizes and demethylates methylated proteins) was a potential interacting protein of WT Akt1, but not of Akt1 K64R (Fig. 6a, Supplementary Fig. 1a, and Supplementary Tables 1 and 2). We confirmed the endogenous interaction of JMJ2A with Akt and TRAF6/Skp2 by co-immunoprecipitation assay (Fig. 6b,c). Notably, we observed marked reduction of Akt interaction with TRAF6 or Skp2 in JMJ2A-knockdown cells (Fig. 6d,e), suggesting that JMJ2A mediates the interaction of Akt with its E3 ligases.

We then determined whether SETDB1-mediated Akt K64 methylation regulates the interaction between Akt and JMJ2A. JMJ2A interacted with Akt, but not with the Akt K64 mutant, both with IGF-1 treatment and in serum culture conditions (Fig. 6f,g). While the interaction of JMJ2A with TRAF6 remained unchanged with IGF-1 treatment, its interaction with Akt was markedly impaired with SETDB1 knockdown (Fig. 6h). In vitro binding assays showed that recombinant JMJ2A could interact with Akt, but not the Akt K64R mutant, isolated from HEK293 cells transfected with HA-Akt or HA-Akt K64R (Fig. 6h). Moreover, the interaction of Akt with Skp2 was reduced in SETDB1-knockdown cells with EGF treatment (Fig. 6i). Thus, SETDB1-mediated Akt K64 methylation is critical for the interaction of Akt with JMJ2A, thereby recruiting TRAF6 or Skp2 to trigger K63-linked ubiquitination of Akt following growth factor stimulation.

JMJ2A non-demethylase function contributes to Akt ubiquitination and activation. JMJ2A emerged as a novel player in cancer development, largely dependent on its demethylase activity23. We then determined whether JMJ2A acts through its demethylase activity to orchestrate Akt activity. JMJ2A and the JMJ2A H188A mutant deficient in demethylase activity increased Akt1 T308 phosphorylation to a similar degree (Fig. 7a), indicating that JMJ2A promotes Akt activity independently of its demethylase activity. Following IGF-1 treatment, overexpression of JMJ2A led to enhanced Akt activation, while knockdown of JMJ2A reduced Akt phosphorylation and its substrate phosphorylation (Fig. 7b). However, IGF-1-mediated Akt K64 methylation remained unchanged in JMJ2A-knockdown cells (Fig. 7c), suggesting that JMJ2A regulates Akt activity downstream of Akt K64 methylation.

As JMJ2A is critical for Akt ubiquitination and activation by facilitating E3 ligase-Akt interaction following growth factor stimulation, we expect that JMJ2A would regulate the recruitment of K64-methylated Akt and Akt to the cell membrane. Indeed, JMJ2A knockdown impaired the recruitment of K64-methylated Akt and Akt to the cell membrane following growth factor treatment (Fig. 7d). In JMJ2A knockout primary MEFs, knockout of JMJ2A by Cre recombinase expression also decreased Akt ubiquitination and T308 phosphorylation (Fig. 7e and Supplementary Fig. 6a). In JMJ2A-knockdown cells, IGF-1-induced Akt ubiquitination...
Fig. 5 | Akt K64 methylation is essential for Akt K63-linked ubiquitination and Akt-E3 ligase interaction. a, HEK293 cells were transfected with the indicated plasmids and whole-cell extracts were collected for IP with PDK1 antibody, followed by IB analysis. b, c, Immunoprecipitated Flag-Akt1 WT or Flag-Akt1 K64R from HEK293 cell transfected with Flag-Akt1 WT or Flag-Akt1 K64R were incubated with PDK1 (b) or PIP3 (c) beads in vitro, followed by IB analysis. d, e, IB of lysed HEK293 cells transfected with the indicated plasmids along with His-ubiquitin (His-Ub), Ni-nitrilotriacetic acid (Ni-NTA), nickel bead pulldown. f, HEK293 cells were silenced with control (shLuc) or SETDB1 shRNA (no. 1) along with HA-Akt and His-ubiquitin, and whole-cell extracts were collected for nickel bead pulldown and IB analysis. g, HEK293 cells silenced with control (shLuc) or SETDB1 shRNA (nos. 1 and 2) were transfected with His-ubiquitin, serum-starved, treated with IGF-1 for 30 min, and whole-cell extracts were collected for IP with Akt antibody, followed by IB analysis. h, Setdb1−/− and Setdb1+/− MEFs cells were serum-starved, treated with IGF-1 for 30 min, and whole-cell extracts were collected for IP with Akt or SETDB1 antibody, followed by IB analysis. i, Akt ubiquitination band intensity was adjusted by IP Akt; Akt band intensity was adjusted by IP SETDB1; Akt K64 methylation and Akt1T308 phosphorylation band intensity was adjusted by whole-cell extract Akt; and the average intensity data were obtained from three replicate experiments and the relative ratio is shown. j, HEK293 cells were serum-starved for 12 h and treated with IGF-1 at different time points before harvesting to separate cell fractionations for IB analysis. k, HEK293 cells transfected with indicated plasmids were serum-starved, treated with IGF-1 for 30 min, and whole-cell extracts were collected for IP with HA antibody, followed by IB analysis. l, HEK293 cells transfected with indicated plasmids were serum-starved, treated with EGF for 5 min and 10 min, and whole-cell extracts were collected for IP with HA antibody, followed by IB analysis. IB data represent results from three independent experiments. Unprocessed blots are shown in Supplementary Fig. 8.
**Fig. 6 | JMJD2A is important for recognizing K64-methylated Akt and facilities the Akt-E3 ligase interaction.**

**a.** HEK293 cells were transfected with HA-Akt1 WT or HA-Akt1 K64R for 48h. Whole-cell extracts were then collected for IP with HA antibody, followed by mass spectrometry analysis for detecting binding proteins. Some proteins that bind only to Akt1 WT are listed, such as JMJD2A, Skp2 and TRAF6. **b.** HEK293 cells were transfected with vector or Flag-JMJD2A, and whole-cell extracts were collected for co-IP with Flag antibody, followed by IB analysis of the indicated proteins. **c.** HEK293 cells were transfected with vector or HA-Akt, and whole-cell extracts were collected for co-IP with HA antibody, followed by IB analysis of the indicated proteins. **d.** HEK293 cells were silenced with control (shLuc) or JMJD2A shRNA (no. 1), and whole-cell extracts were collected for co-IP with Akt antibody, followed by IB analysis indicated proteins. **e.** HEK293 cells silenced with control (shLuc) or SETDB1 shRNA (no. 1) were serum-starved, treated with IGF-1 for 30 min, and whole-cell extracts were collected for IP with JMJD2A antibody, followed by IB analysis. **f.** HEK293 cells transfected with vector, HA-Akt1 WT or HA-Akt1 K64R were serum-starved for 1 day, treated with 50 ng ml⁻¹ IGF-1 for various times, and whole-cell extracts were collected for IP with HA antibody, followed by IB analysis indicated proteins. **g.** HEK293 cells were transfected with vector, HA-Akt1 WT or HA-Akt1 K64R (K64R), whole-cell extracts were collected for IP with JMJD2A antibody, followed by IB analysis. **h.** HEK293 cells silenced with control (shLuc) or SETDB1 shRNA (no. 1) were serum-starved, treated with IGF-1 for 30 min, and whole-cell extracts were collected for IP with JMJD2A antibody, followed by IB analysis. **i.** Immunoprecipitated HA-Akt1 WT and HA-Akt1 K64R from HEK293 cells transfected with HA-Akt1 WT or HA-Akt1 K64R were incubated with recombinant JMJD2A purified from engineered bacteria for in vitro binding assays, followed by IP with HA and IB analysis. **j.** HEK293 cells silenced with control (shLuc) or SETDB1 shRNA (no. 1) were serum-starved, treated with EGF for 10 min, and whole-cell extracts were collected for IP with Akt antibody, followed by IB analysis. IB data shown in **b-j** represent results from 3 independent experiments. Unprocessed blots are shown in Supplementary Fig. 8.
Fig. 7 | JMJD2A non-demethylase function contributes to Akt ubiquitination and activation. a, HEK293 cells were transfected with vector, pBabe-JMJD2A or pBabe-JMJD2A H188A, and whole-cell extracts were collected, followed by IB analysis. b, HEK293 cells transfected with vector or Flag-JMJD2A were serum-starved, treated with IGF-1 for various times, and whole-cell extracts were collected, followed by IB analysis. c, HEK293 cells silenced with control (shLuc) or JMJD2A shRNA (nos. 1 and 2) were serum-starved, treated with IGF-1 for 15 min, and whole-cell extracts were collected, followed by IB analysis. d, HEK293 cells silenced with control (shLuc) or JMJD2A shRNA (nos. 1 and 2) were serum-starved, treated with IGF-1 for 15 min or 30 min, and cell membrane fractionations were collected for IB analysis. e, Primary JMJD2Alox/lox MEF cells were infected with retrovirus Cre, and whole-cell extracts were collected for IP with Akt antibody, followed by IB analysis. f, IB of lysed HEK293 cells silenced with control (shLuc) or JMJD2A shRNA (nos. 1 and 2) were serum-starved, treated with IGF-1 for 15 min or 30 min, and cell membrane fractionations were collected for IB analysis.

i, Cell number was counted in A549 cells with shLuc, SETDB1 knockdown, or JMJD2A knockdown plus Myr-Akt overexpression. j, Colony formation assay in A549 cells with shLuc, SETDB1 knockdown, or JMJD2A knockdown plus Myr-Akt overexpression. Data represent mean ± s.d. (n = 3 independent experiments, **** P < 0.0001, Student’s two-tailed t-test). k, Colony formation assay in A549 cells with shLuc, JMJD2A shRNA (no. 1), or JMJD2A shRNA (no. 1) plus Myr-Akt overexpression. Data represent mean ± s.d. (n = 3 independent experiments, Student’s two-tailed t-test). l, The working model of SETDB1-mediated Akt K64 methylation in growth-factor-mediated Akt ubiquitination, cell membrane recruitment and activation. IB data in a–f represent results from three independent experiments. Statistics source data for g,i are shown in Supplementary Table 4. Unprocessed blots are shown in Supplementary Fig. 8.
and T308 phosphorylation were impaired (Fig. 7f), and JMJD2A restoration in JMJD2A knockdown cells rescued Akt phosphorylation (Supplementary Fig. 6b).

Finally, we determined the oncogenic role of JMJD2A and its association with Akt activation. In A549 cells with JMJD2A knockdown, Akt phosphorylation was reduced (Supplementary Fig. 6c). Accordingly, the glucose uptake was decreased in JMJD2A knockdown A549 cells compared with control cells (Fig. 7g). While JMJD2A knockdown reduced colony formation and cell proliferation, the restoration of Akt activity by Myr-Akt rescued these defects (Fig. 7h,i). In xenograft tumour models, we found that JMJD2A knockdown cells exhibited much lower tumorigenic potential than control cells (Supplementary Fig. 6d,e). Remarkably, restoration of Akt activity in JMJD2A-knockdown cells by Myr-Akt partially rescued the defect (Supplementary Fig. 6d,e). We assessed the expression of JMJD2A in patient tissue microarrays (TMAs) and showed that the expression of JMJD2A was positively correlated with Akt1 T308 phosphorylation (Supplementary Table 3). Taken together, these results indicate that JMJD2A acts as an adaptor to promote Akt activation and oncogenic activity.

**Discussion**

Our current study provides insight into the regulation of K63-linked ubiquitination of Akt by revealing that Akt K64 methylation is an essential event for the interaction of Akt and its E3 ligases. We show that SETDB1-dependent K64 methylation of Akt following growth factor stimulation is recognized by JMJD2A, resulting in subsequent recruitment of E3 ligases to elicit K63-linked ubiquitination and activation of Akt. It should be noted that JMJD2A interacts with the Skp2 and TRAF6 E3 ligases independently of growth factor stimulation and Akt K64 methylation. Therefore, SETDB1-mediated K64 methylation of Akt represents a sensing signal to drive the interaction of Akt with its E3 ligase in response to growth factor stimulation. Although PI3K connects growth factor receptors to Akt activation, we found that PI3K is not required for SETDB1-mediated Akt K64 methylation (Supplementary Fig. 7a). Moreover, Akt R25C mutant, deficient for PIP3 binding and cell membrane localization, displayed the same level of Akt K64 methylation as WT Akt (Supplementary Fig. 7b). Thus, PI3K activity, Akt-PI3P binding and Akt cell membrane localization appear to be dispensable for SETDB1-mediated Akt K64 methylation.

The methylation has emerged as a prevalent posttranslational modification that regulates a variety of non-histone proteins, while little is known about SETDB1 non-histone substrates. In this study, we identify Akt as a novel non-histone substrate of SETDB1 and demonstrate that SETDB1-mediated Akt K64 methylation is a key event for Akt ubiquitination and activation in response to growth factor stimulation. This study expands our understanding of the substrates and function of SETDB1, which is amplified and overexpressed in many cancer types. Consistently, we show that SETDB1 is overexpressed in diverse cancers including NSCLC, and its overexpression predicts a poor survival outcome. Notably, we found that SETDB1-mediated Akt K64 methylation is upregulated in NSCLC and correlated with Akt activation. Moreover, increased levels of Akt K64 methylation confer poor prognosis in NSCLC patients. The transcriptional regulation by SETDB1 via the induction of histone H3K9 methylation is thought to be a primary action for SETDB1-mediated biological functions. However, we demonstrate that activation of Akt through SETDB1-mediated Akt K64 methylation also contributes to SETDB1-mediated oncogenic activity. Thus, SETDB1 appears to act through direct transcriptional and non-transcriptional regulation to impact its oncogenic processes. Our study therefore reveals that oncogenic activity of SETDB1 is partly due to activation of Akt through SETDB1-mediated Akt K64 methylation.

In a recent independent study by Wei and his colleagues, K140/K142 was identified as the SETDB1-mediated Akt methylation site, which is required for AktT308 and Akt473 phosphorylation and activation. Their mass spectrometry analysis also identified K64 as the Akt methylation site, consistent with our findings. Although our results in Fig. 2k revealed K64 as the major site for SETDB1-mediated Akt methylation, SETDB1-dependent methylation could also occur at other sites on Akt since there is still a weak tri-methylation signal detected in the Akt K64R mutant using the tri-methylation antibody. To provide a comprehensive understanding of Akt methylation and activation, we also examined Akt K140/K142 methylation and confirmed that K140 and K142 were Akt methylation sites (Supplementary Fig. 7c), and Akt K140/K142 methylation was required for AktT308 and Akt473 phosphorylation and activation (Supplementary Fig. 7d). Interestingly, it seems that Akt K64 and Akt K140/142 methylation occur independently and do not affect each other (Supplementary Fig. 7d). Wei and our groups reveal that methylation of Akt is critical for Akt activation, although it involves different lysine residues (K64 versus K140/K142), probably through distinct molecular mechanisms. Our study shows that Akt K64 methylation is crucial for ubiquitination and T308 phosphorylation of Akt but not Akt-PI3P binding and Akt473 phosphorylation, while the other study indicates that Akt K140/K142 methylation is required for both AktT308 and Akt473 phosphorylation and Akt-PI3P binding.

JMJD2A is upregulated in human cancers and displays oncogenic activity in mouse tumour models through promoting YAP1 expression and repressing CHD5. While most studies reveal that JMJD2A acts though its demethylase activity to orchestrate gene expression and oncogenic activity, our study defines a novel adaptor function of JMJD2A in Akt signalling activation independent of its demethylase activity. JMJD2A bridges the interaction of Akt with its E3 ligase to facilitate Akt ubiquitination and activation. In addition, we demonstrate that JMJD2A displays oncogenic activity partly through promoting Akt activation. Notably, an earlier study revealed that JMJD2A regulates DNA damage signalling independently of its enzymatic activity. Our study, together with this report, suggests that JMJD2A may regulate various biological processes through its role as an adaptor independently of its enzymatic activity.

In summary, we identify Akt K64 methylation by SETDB1 following growth factor stimulation as a crucial initiation event for subsequent K63-linked ubiquitination of Akt by recruiting JMJD2A and E3 ligases to the Akt complex, leading to cell membrane recruitment, T308 phosphorylation and activation of Akt (Fig. 7j). Importantly, our study reveals that SETDB1-mediated Akt K64 methylation not only serves as a key signal to drive Akt hyper-activation and promote cancer progression, but also represents a poor prognosis marker for NSCLC patients. Therefore, targeting SETDB1-mediated Akt K64 methylation may be a promising strategy for the treatment of cancers such as NSCLC.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41556-018-0266-1.

Received: 21 December 2017; Accepted: 12 December 2018; Published online: 28 January 2019

**References**

1. Cheung, M. & Testa, J. R. Diverse mechanisms of AKT pathway activation in human malignancy. Curr. Cancer Drug Targets 13, 234–244 (2013).
2. Manning, B. D. & Cantley, L. C. AKT/PKB signaling: navigating downstream. Cell 129, 1261–1274 (2007).
3. Yang, W. L. et al. The E3 ligase TRAF6 regulates Akt ubiquitination and activation. Science 325, 1134–1139 (2009).
4. Chan, C. H. et al. The Skp2-SCF E3 ligase regulates Akt ubiquitination, glycolysis, herceptin sensitivity, and tumorigenesis. Cell 149, 1098–1111 (2012).
5. Yang, W. L., Wu, C. Y., Wu, J. & Lin, H. K. Regulation of Akt signaling activation by ubiquitination. Cell Cycle 9, 487–497 (2010).
6. Hamamoto, R., Saloura, V. & Nakamura, Y. Critical roles of non-histone protein lysine methylation in human tumorigenesis. Nat. Rev. Cancer 15, 110–124 (2015).
7. Campaner, S. et al. The methyltransferase Set7/9 (Setd7) is dispensable for the p53-mediated DNA damage response in vivo. Mol. Cell 43, 681–688 (2011).
8. Kunizaki, M. et al. The lysine 831 of vascular endothelial growth factor receptor 1 is a novel target of methylation by SMYD3. Cancer Res. 67, 10759–10765 (2007).
9. Mazur, P. K. et al. SMYD3 links lysine methylation of MAPKJ2 to Ras-driven cancer. Nature 510, 283–287 (2014).
10. Dasgupta, M., Dermawan, K. J., Willard, B. & Stark, G. R. STAT3-driven transcription depends upon the dimethylation of K49 by EZH2. Proc. Natl Acad. Sci. USA 112, 3985–3990 (2015).
11. Kim, E. et al. Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. Cancer Cell 23, 839–852 (2013).
12. Zhang, X. & Bruce, T. C. Enzymatic mechanism and product specificity of SET-domain protein lysine methyltransferases. Proc. Natl Acad. Sci. USA 105, 5728–5732 (2008).
13. Jacob, Y. et al. Regulation of heterochromatin DNA replication by histone H3 lysine 27 methyltransferases. Nature 466, 987–991 (2010).
14. Schultz, D. C. et al. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes Dev. 16, 919–932 (2002).
15. Fei, Q. et al. Histone methyltransferase SETDB1 regulates liver cancer cell growth through methylation of p53. Nat. Commun. 6, 8651 (2015).
16. Kim, M. S., Jeong, E. G., Yoo, N. J. & Lee, S. H. Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukemias. Br. J. Cancer 98, 1533–1535 (2008).
17. Bleeker, F. E. et al. AKT1(E17K) in human solid tumours. Oncogene 27, 5648–5650 (2008).
18. Carpten, J. D. et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature 448, 439–444 (2007).
19. Gao, J. et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Science Signal. 6, pl1 (2013).
20. Cerami, E. et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2, 401–404 (2012).
21. Györffy, B., Surowiak, P., Budcziés, J. & Lancezyk, A. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. PLoS ONE 8, e82241 (2013).
22. Uhlen, M. et al. A pathology atlas of the human cancer transcriptome. Nature 507, 455–460 (2014).
23. Mallette, F. A. & Richard, S. JMJD2A promotes cellular transformation by activating DNMT3A. Mol. Cell 67, 1432–1442 (2017).
24. Wu, F. Y., Hsu, W. C. & Rose, J. A. J. L. Oncogenic AKT E17K mutation in common solid cancers and acute leukemias. Mol. Cell 67, 1432–1442 (2017).
25. Mallette, F. A. et al. The JMJD2A histone demethylase in breast cancer. Int. J. Oncol. 41, 1701–1706 (2012).
26. Kim, T. D. et al. Histone demethylase JMJD2A drives prostate tumorigenesis through transcription factor ETV1. J. Clin. Invest. 126, 706–720 (2016).
27. Mallette, F. A. & Richard, S. JMJD2A promotes cellular transformation by blocking cellular senescence through transcriptional repression of the tumor suppressor CHD5. Cell Rep. 2, 1233–1243 (2012).
28. Berry, W. L., Shin, S., Lightfoot, S. A. & Janknecht, R. Oncogenic features of the JMJD2A histone demethylase in breast cancer. Nat. J. Oncol. 41, 1701–1706 (2012).
29. Mallette, F. A. et al. JMJD2A-mediated H3K4me3 epigenetic repression in tumor suppressor CHD5. Cancer Res. 78, 5487–5497 (2018).
30. Yang, W. L., Wu, C. Y., Wu, J. & Lin, H. K. Regulation of Akt signaling activation by ubiquitination. Cell Cycle 9, 487–497 (2010).
31. Rodriguez-Paredes, M. et al. Gene amplification of the histone methyltransferase SETDB1 contributes to human lung tumorigenesis. Oncogene 33, 2807–2813 (2014).
32. Sarraf, S. A. & Starchev, I. Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin remodelling. Mol. Cell 15, 595–605 (2004).
33. Fritsch, L. et al. A subset of the histone H3 lysine 9 methyltransferases Suv39h1, G9a, GLP, and SETDB1 participate in a multi-plexer complex. Mol. Cell 37, 46–56 (2010).
34. Guo, X. D. et al. Methylation by SETDB1 promotes Akt kinase activity and oncogenic functions. Nat. Cell Biol. (2018).
35. Kim, T. D. et al. Histone demethylase JMJD2A drives prostate tumorigenesis through transcription factor ETV1. J. Clin. Invest. 126, 706–720 (2016).
36. Mallette, F. A. & Richard, S. JMJD2A promotes cellular transformation by blocking cellular senescence through transcriptional repression of the tumor suppressor CHD5. Cell Rep. 2, 1233–1243 (2012).
37. Berry, W. L., Shin, S., Lightfoot, S. A. & Janknecht, R. Oncogenic features of the JMJD2A histone demethylase in breast cancer. Nat. J. Oncol. 41, 1701–1706 (2012).
38. Mallette, F. A. et al. The JMJD2A histone demethylase in breast cancer. Int. J. Oncol. 41, 1701–1706 (2012).
39. Mallette, F. A. et al. RNF8- and RNF168-dependent degradation of KDM4A/JMJD2A triggers 53BP1 recruitment to DNA damage sites. EMBO J. 31, 1865–1878 (2012).

Acknowledgements
We are grateful to the members of Lin’s lab for critical inputs and suggestions. We thank Z.-P. Liu, Y. Shinkai, F. J. Rauscher III, B. Vogelstein, D. Bohmann, M.C. Hung and R. Janknecht for providing mice, cell lines or plasmids. We thank E. Spooner for performing mass spectrometry assays. We acknowledge the support of Cellular Imaging & flow cytometry Shared of Resource, the Wake Forest Baptist Comprehensive Cancer Center, supported by the National Cancer Institute’s Cancer Center Support Grant (P30CA012971). This work is supported by start-ups from Wake Forest School of Medicine, NIH grants (R01CA182424 and R01CA193813) to H.K.L., NIH grants (R01CA194904 and R01CA197178) to H.L. and NIH grants (R01CA207098 and R01CA207109) to M.G.L.

Author contributions
G.W. and H.K.L. designed the research and analysed the data. G.W. carried out the major experiments. J.L. performed the immunohistological characterization. J.L., Y.G., W.Z., F.H., C.X., L.S., Z.H. and Z.C. assisted with the experiments. T.Y.C. and M.G.L. assisted with in vitro methylation assay, G.W. and W.Z. developed the work models. M.G.L. and H.L. provided scientific inputs. G.W. and H.K.L. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41556-018-0266-1.
Reprints and permissions information is available at www.nature.com/reprints.
Correspondence and requests for materials should be addressed to H.-K.L.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature Limited 2019, corrected publication 2021.
JMJD2A was sub-cloned from Flag-JMJD2A using blunt end cloning method. MD Anderson Cancer Center), respectively. The mammalian expression vector (The Wistar Institute), (His)6-ubiquitin and HA-Akt1 were gifts from D. and confirmed negative. All cell lines were subjected to routine fingerprinting (pHelper) and envelope plasmid (pEnv) using the calcium phosphate transfection procedures were conducted under the approved protocol of Institutional Animal Care and Use Committee (IACUC). JMJD2A n. 1: 5′-CGCCGGTACCTACAGAGTAT-ACCTGTACGATAGAATAGGATACACTGAGCTTTT-3′; shJMJD2A no. 2: 5′-CGGCGCTGATGAC TTTCTGAACATCTGCAAGATCACGAGGCTT-3′. For retroviral infection, MSCV and MSCV-Cre were transduced with VSV-G and Gag-Pol retroviral packaged plasmids into the HEK290B7 cells. Medium was replaced 6 h later. After 48 h, the virus particles were collected to infect MEFs for another 48 h. The cells were selected by 1 µg/mL puromycin for 4–5 days. For transient transfection, plasmids were transfected using either the calcium phosphate method or Lipofectamine 2000 (Invitrogen) reagent following the manufacturer’s standard procedures, with pcDNA-HA-Akt as the template. Flag-SETDB1 and Flag-SETDB1 H1224K were obtained from F. J. Rauscher III (The Wistar Institute), (His)6-ubiquitin and HA-Akt1 were gifts from D. Bohmann (University of Rochester) and M. C. Hung (The University of Texas MD Anderson Cancer Center), respectively. The mammalian expression vector pEV35S-Flag-JMJD2A was a gift from R. Janknecht (Oakland University), pBabe-MJD2A was sub-cloned from Flag-MJD2A using blunt end cloning method. Enzyme dead mutants (H188A) of MJD2A were generated using a site-directed mutagenesis kit (Agilent Technologies, 200518) according to the manufacturer’s standard procedures, with pcDNA-HA-Akt as the template. Recombinant human IGF-1 protein (291-G1) and recombinant human EGF protein (236-EG) were obtained from R&D Systems.

In vivo methylation assays, in vitro binding assays and ubiquitination assays. In vivo methylation assays were performed as described previously14. Each experiment was successfully carried out two to three times, as indicated. Antibodies were validated by using positive and negative control tissues and cells. For protein–protein interactions, cells were lysed by RIPA lysis buffer (150 mM NaCl, 0.5% NP-40, 5 mM EDTA, protease inhibitor cocktail (Roche)). The following antibodies were used for IP and IB: anti-Akt antibody (IP, 1:200; IB, 1:1,000, Cell Signaling, cat. no. 9275), anti-phospho (S473)-Akt antibody (IB, 1:2,000, Cell Signaling, cat. no. 4060), anti-phospho (T308)-Akt (IP, 1:1,000, Cell Signaling, cat. no. 9275), anti-MJD2A antibody (IP, 1:100; IB, 1:1,000, Cell Signaling, cat. no. 5328), anti-SETDB1 antibody (IP, 1:100; IB, 1:1,000, Cell Signaling, cat. no. 2196), anti-K63 ubiquitin antibody (IB, 1:1,000, Cell Signaling, cat. no. 12930), anti-N-cadherin (IB, 1:1,000, Cell Signaling, cat. no. 4212), anti- phospho (S9)-GSK3β antibody (IB, 1:1,000, Cell Signaling, cat. no. 9322), anti-GSK3β antibody (IB, 1:3,000, Cell Signaling, cat. no. 5676), anti-tri-methyl lysine (IB, 1:1,000, Cell Signaling, cat. no. 14680), anti-di-methyl-lysine (IB, 1:1,000, Cell Signaling, cat. no. 14117), anti-monoo-methyllysine (IB, 1:1,000, Cell Signaling, cat. no. 14679), anti-Akt K64 di/- tri-methylation rabbit polyclonal antibody generated by Proteintech using the peptide CQLM-K(Me3)-TERPRP anti-phospho-Akt antibody (IB, 1:1,000, Sigma, cat. no. A3441), anti-HA antibody (IB, 1:1,000, Sigma, cat. no. H3663), anti-Flag antibody (M2, 1:200; IB, 1:3,000, Sigma, cat. no. F3165). The western blot band intensity analysis was done by ImageJ (NIH, Image J).

Viral infection and transfection. For lentiviral short hairpin RNA (shRNA) infection, 293T cells were prepared at 50–60% confluency and co-transfected with either luciferase (shLuc) or target gene shRNA with packaging plasmid (pHelper) and envelope plasmid (pEnv) using the calcium phosphate transfection method. Medium was changed 6 h later. After 48 h, the virus particles were harvested and used to infect parental cells for another 48 h. The stably infected cells were then selected for 2 µg/mL puromycin. 293T cells were isolated at 15–30 days after marking. Embryos were digested and the resulting cells were cultured in complete DMEM. All procedures were conducted under the approved protocol of Institutional Animal Care and Use Committee (IACUC).JMJD2A n. 1: 5′-CGCCGGTACCTACAGAGTAT-ACCTGTACGATAGAATAGGATACACTGAGCTTTT-3′; shJMJD2A no. 2: 5′-CGGCGCTGATGAC TTTCTGAACATCTGCAAGATCACGAGGCTT-3′. For retroviral infection, MSCV and MSCV-Cre were transduced with VSV-G and Gag-Pol retroviral packaged plasmids into the HEK290B7 cells. Medium was replaced 6 h later. After 48 h, the virus particles were collected to infect MEFs for another 48 h. The cells were selected by 1 µg/mL puromycin for 4–5 days. For transient transfection, plasmids were transfected using either the calcium phosphate method or Lipofectamine 2000 (Invitrogen) reagent following the manufacturer’s standard procedures. For site-directed mutagenesis, endogenous Akt by IP assay and subjected to mass spectrometry analysis. The mass spectrum shows a trypsic digest of Akt. For Supplementary Fig. 1, we pulled down endogenous Akt by IP assay in shSETDB1 HEK293 cells and subjected it to time-of-flight mass spectrometry (TOFMS) analysis. The investigators were blinded to allocation during experiments or outcome assessment. After the mass spectrometry analysis, we pick up the data with the 3-fold-change cutoff as the potential binding proteins or modifications, which we then subject to co-immunoprecipitation to further confirm protein–protein binding or modifications.

Patient materials. 156 NSCLC patients without systemic drug therapy before surgery were surgically resected and histologically diagnosed at the third affiliated hospital of Sun Yat-sen University, China during the period 1999–2011. Follow-up information is available for 111 patients until 30 December 2011. Information was obtained from patients before surgery for paraffin-embedded (FFPE) lung cancer and fresh lung cancer specimens to be used for this research and approved by the Institutional Review Board of the Institute of Biophysics, Sun Yat-sen University. All samples were reevaluated by an expert pathologist according to the World Health Organization (WHO) grading system and the General Rules for Clinical Lung Cancer. The study is compliant with all relevant ethical regulations involving human participants.

Animal study. We complied with the regulations of the ethical committee of Institute of Biophysics, Huazhong University of Science and Technology. The Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology has approved our animal studies. All animal experiments were carried out in accordance with the Animal Study Guidelines of Huazhong University of Science and Technology. Female 5-week-old BALB/c nude mice were used for xenograft study. For xenograft study, the tumors were treated as indicated cells were subcutaneously injected into athymic nude mice (5–8 mice for each group). Tumour size was measured weekly with a caliper, and tumour volume was determined with the standard formula L x W^2/2, where L is the longest diameter and W the shortest diameter. For all xenograft studies, mice were euthanized at the ethical endpoint when they failed to meet the predefined IACUC quality-of-life guidelines. This study is compliant with all relevant ethical regulations regarding animal research.

Immunohistochemical (IHC) staining and evaluation. Formalin-fixed, paraffin-embedded tissue sections (4 µm thick) obtained from 156 primary NSCLC tumours as well as 50 healthy lung tissues were used. Sections were deparaffinized and dehydrated through graded alcohols and xylene, endogenous peroxidase activity and non-specific antigen were blocked with 3% hydrogen peroxide. The sections were heated for 5 min twice at 100°C with Tris EDTA (pH 9.0) in...
a microwave oven for antigen retrieval. All sections were incubated with anti-Akt K64 Me3 rabbit polyclonal antibody (1:200), anti-SETDB1 rabbit polyclonal antibody (1:100, Cell Signaling), anti-Akt T308 rabbit polyclonal antibody (1:200, Cell Signaling), anti-JMJD2A rabbit polyclonal antibody (1:100, Cell Signaling), at 37 °C for 1 h. After washing, the sections were incubated with the Chem-Mate Envision /HRP, Rabbit (ENV) reagent at 37 °C for 30 min. Rinsed gently with PBS, the sections were visualized by the Chem-Mate TM DAB + Chromogen, counterstained with hematoxylin, mounted in neutral gum, and analysed using a bright field microscope.

The immunohistochemically stained tissue sections were reviewed and scored by two independent experienced pathologists blinded to the clinicopathological variables. For each section, five high-power fields were randomly selected and the staining was evaluated by the extensity (the percentage of positive tumour cells or normal epithelial cells in relation to the whole tissue area: negative, 0; ≤10%, 1; 10–50%, 2; ≥51%, 3) and the intensity (absent, 0; weak, 1; moderate, 2; strong, 3). The combined score (extensity × intensity) of 0–4 and 5–9 were defined as low and high expression levels of Akt K64 methylation, AktT308, JMJD2A and SETDB1, respectively.

Statistics and reproducibility. All co-IP and immunostaining were repeated 3 times with similar results obtained. All statistical analyses were processed using SPSS 16.0 statistical software. Spearman’s correlation coefficient was calculated to evaluate the correlation between Akt K64 Me3 and SETDB1 expression levels. Survival analysis was performed using the Kaplan–Meier method and log-rank test. Overall survival was measured from the date of surgery to the date of death or the date on which the patient was last known to be alive, and the data were censored if patients were alive or lost to follow-up. The investigators were blinded to allocation for IHC analyses. P values were generated using a two-sided t-test to calculate statistical significance, with P < 0.05 representing a statistically significant difference. No statistical method was used to predetermine sample size. No mice that completed in vivo studies were excluded from analyses.

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

Data availability
Mass spectrometry data have been deposited in ProteomeXchange with the primary accession code code PXD011966. The human lung adenocarcinoma data were derived from the TCGA Research Network: http://cancergenome.nih.gov/. The dataset derived from this resource that supports the findings of this study is available in http://www.cbioportal.org. The human cancer SETDB1 protein expression data were derived from the Human Protein Atlas Network and the supports the findings of this study is available in https://www.proteinatlas.org/ENSG00000143379-SETDB1/pathology. Source data for Figs. 4 and 7 and Supplementary Figs. 3 and 6 have been provided in Supplementary Table 4. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

References
37. Cederquist, C. T. et al. Systemic insulin sensitivity is regulated by GPS2 inhibition of AKT ubiquitination and activation in adipose tissue. Mol. Metab. 6, 125–137 (2017).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
| ☑   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☑   | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | The statistical test(s) used AND whether they are one- or two-sided |
| ☑   | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ☑   | A description of all covariates tested |
| ☑   | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☑   | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☑   | For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted |
| ☑   | Give P values as exact values whenever suitable. |
| ☑   | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☑   | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☑   | Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated |
| ☑   | Clearly defined error bars |
| ☑   | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection | No software was used

Data analysis | All statistical analyses were processed using SPSS 16.0 statistical software. MS/MS data were searched against the Uniprot Human protein database (version 20151209 containing 21,024 entries) using Mascot 2.5.1 (Matrix Science) and data analysis was performed using the Scaffold 4.4.8 software (Proteome Software).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No human or animal subjects necessitating sample size calculations were used in this study |
|-------------|----------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded |
| Replication | All experiments were performed in at least \( n=2 \) biologically independent experiments. For proliferation curve experiments, \( n=3 \) independent biological samples were used, enabling statistical calculations. No replicates were excluded from analyses presented, and all attempts at replication were successful. |
| Randomization | For each immunohistochemically stained tissuesection, 5 high power fields were randomly selected and the staining was evaluated by the extensity. |
| Blinding | Mass Spec samples were blinded before analysis. The immunohistochemically stained tissue sections were reviewed and scored by two independent experienced pathologists blinded to the clinicopathological variables. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| x   | Unique biological materials |
| x   | Antibodies |
| x   | Eukaryotic cell lines |
| x   | Palaeontology |
| x   | Animals and other organisms |
| x   | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| x   | ChIP-seq |
| x   | Flow cytometry |
| x   | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Jmjd2aflox/flox MEFs were prepared from Jmjd2aflox/flox mice as previously described 1, 2. In short, female pregnant mice were sacrificed, and embryos were isolated at 13.5 days after mating. Embryos were digested and the resulting cells were cultured in complete DMEM. All procedures were conducted under the approved protocol of Institutional Animal Care and Use Committee (IACUC). Jmjd2aflox/flox mice were provided by Dr. Zhi-Ping Liu (The University of Texas Southwestern).

Antibodies

Antibodies used

- anti-Akt antibody (IP: 1:200; IB: 1:1000, Cell Signaling, Cat # 9275), anti-phospho (S473)-Akt antibody (IB: 1:2000, Cell Signaling, Cat # 4691), anti-junD antibody (IB: 1:1000, Cell Signaling, Cat # 4060), anti-phospho (T338)-Akt antibody (IB: 1:1000, Cell Signaling, Cat # 9275), anti-JMJD2A antibody (IP: 1:100; IB: 1:1000, Cell Signaling, Cat # 5328), anti-SETDB1 antibody (IP: 1:100; IB: 1:1000, Cell Signaling, Cat # 2196), anti-K63 ubiquitin antibody (IB: 1:1000, Cell Signaling, Cat # 12930), anti-N-cadherin antibody (IB: 1:1000, Cell Signaling, Cat # 14215), anti-phospho (S9)-GSK3β antibody (IB: 1:1000, Cell Signaling, Cat # 9322), anti-GSK3β antibody (IB: 1:1000, Cell Signaling, Cat # 5876), anti-tri-methyl Lysine (IB: 1:1000, Cell Signaling, Cat # 14680), anti-Di-methyl Lysine (IB: 1:1000, Cell Signaling, Cat # 14117), anti-mono-methyl Lysine (IB: 1:1000, Cell Signaling, Cat # 14679), anti-Akt K64 Di-/tri-methylation antibody (IB: 1:100, Sigma, Cat # A6441), anti-HA antibody (IB: 1:1000, Sigma, Cat # H3663), anti-Flag antibody (M2, IP: 1:200; IB: 1:3000, Sigma, Cat # F3165). 

Validation

The Akt K64 methylation antibody is validated and the data was listed in the figure 2j. anti-Akt antibody (Cell Signaling, Cat # 4691), validation: https://www.cellsignal.com/products/primary-antibodies/akt-pan-
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  Setdb1+/− and Setdb1−/− mouse embryonic fibroblasts (MEFs) were gifts from Pro. Yoichi Shinkai. Jmjd2aflox/flox MEFs were prepared from Jmjd2aflox/flox mice as previously described in method, NIH3T3, A549, HEK293 and 293T cells were got from ATCC and cultured in DMEM containing 10% FBS. DLD-1Akt1/2−/− cell line was gifted by Dr. Bert Vogelstein.

Authentication  All cell lines were routinely checked for mycoplasma contamination and confirmed negative. All cell lines were subjected to routine fingerprinting analyses to confirm identity.

Mycoplasma contamination  All cell lines tested negative for mycoplasma contamination.

Commonly misidentified lines  No cell lines were used that are listed in the ICLAC register.

Animals and other organisms

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

Laboratory animals  Jmjd2aflox/flox MEFs were prepared, female pregnant mice (Jmjd2aflox/flox mice, B6, JAX stock #029424) were sacrificed, and embryos were isolated at 13.5 days after mating. Embryos were digested and the resulting cells were cultured in complete DMEM. All procedures were conducted under the approved protocol of Institutional Animal Care and Use Committee (IACUC). Jmjd2aflox/flox mice were provided by Dr. Zhi-Ping Liu (The University of Texas Southwestern). For xenograft studies, nude mice (BALB/c, 5 Weeks old, Female) were used for xenograft studies. For in vivo tumorigenesis assays, 5×10^6 of A549 treated as indicated cells were subcutaneously injected into athymic nude mice ((BALB/c, 5 Weeks old, Female, 5-8 mice for each group).

Wild animals  This study did not include wild animals.

Field-collected samples  This study did not include field-collected samples.

Human research participants

Policy information about studies involving human research participants

Population characteristics  All samples were reevaluated by experienced pathologist according to the World Health Organization (WHO) grading system and the General Rules for Clinical Lung Cancer.

Recruitment  156 NSCLC patients without systemic drug therapy before surgery were surgically resected and histologically diagnosed at the
Recruitment

Recruitment was performed at the third affiliated hospital of SUN Yat-sen University, China during the period 1999-2011. Follow-up information is available for 111 patients until 30th December, 2011. Informed consent was obtained from patients before surgery for paraffin-embedded (FFPE) lung cancer and fresh lung cancer specimens to be used for this research and approved by the Institutional Review Board of the Institute of Biophysics, SUN Yat-sen University.