Similarity in gene-regulatory networks suggests that cancer cells share characteristics of embryonic neural cells

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Cancer cells are immature cells resulting from cellular reprogramming by gene misregulation, and redifferentiation is expected to reduce malignancy. It is unclear, however, whether cancer cells can undergo terminal differentiation. Here, we show that inhibition of the epigenetic modification enzyme enhancer of zeste homolog 2 (EZH2), histone deacetylases 1 and 3 (HDAC1 and -3), lysine demethylase 1A (LSD1), or DNA methyltransferase 1 (DNMT1), which all promote cancer development and progression, leads to postmitotic neuron-like differentiation with loss of malignant features in distinct solid cancer cell lines. The regulatory effect of these enzymes in neuronal differentiation resided in their intrinsic activity in embryonic neural precursor/progenitor cells. We further found that a major part of pan-cancer-promoting genes and the signal transducers of the pan-cancer-promoting signaling pathways, including the epithelial-to-mesenchymal transition (EMT) mesenchymal marker genes, display neural specific expression during embryonic neurulation. In contrast, many tumor suppressor genes, including the EMT epithelial marker gene that encodes cadherin 1 (CDH1), exhibited non-neural or no expression. This correlation indicated that cancer cells and embryonic neural cells share a regulatory network, mediating both tumorigenesis and neural development. This observed similarity in regulatory mechanisms suggests that cancer cells might share characteristics of embryonic neural cells.

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Cancer has been proposed to be a disease of cell dedifferentiation (1, 2) resulting from gene misregulation that reprograms the terminally differentiated cells into an incomplete differentiation state, characterized by the inactivation or loss of differentiation genes and tumor suppressor genes and the activation of genes promoting cancer initiation and progression. It is expected that redifferentiation into a more mature state would be able to drive cancer cells to lose malignancy. Although cancer is heterogeneous because of extensive genetic and phenotypic variations either within a type of cancer or between different types (3), cancer cells share common malignant features, including “epithelial-to-mesenchymal transition” (EMT) (4), limited proliferation, metastasis, angio genesis, chemoresistance, reprogrammed cell metabolism, evasion of programmed cell death and immunosurveillance, etc. (2, 4). Besides, there are some common genes/signals regulating tumorigenesis of different cancer types. For example, the signaling pathways including Wnt, TGFβ, or FGF play major roles in the regulation of malignant features of different cancers (5–7). Moreover, chromatin modification enzymes are central players in neoplastic reprogramming of cells and cancer progression (8–16). These signals are also the major regulators of embryonic cell differentiation, proliferation, or migration (7, 16–19), suggesting that cancer cells and embryonic cells are similar with respect to their regulatory mechanisms. It has been unclear whether it is possible to drive redifferentiation of diverse cancer cell types to a terminal differentiation state. Moreover, most cancer studies have concentrated on the roles of individual genes or signals during cancer development and progression; a comprehensive analysis on the relatedness among cancer-related genes/signals has not been reported. We deduced that such an analysis might help to elucidate the common features of different cancer types beyond heterogeneity. Here, we show that inhibition of a few chromatin modification enzymes in distinct types of solid cancer cell lines induces postmitotic neuron-like differentiation and loss of malignant features. There is a correlation between the function/expression in cancer and in embryonic neural precursor cells. The correlation

3 The abbreviations used are: EMT, epithelial-to-mesenchymal transition; HDAC, histone deacetylase; EGFR, epidermal growth factor receptor; DNMT, DNA methyltransferase; NF, Nieuwkoop and Faber; ES, embryonic stem; TSA, trichostatin A; AZA, 5-aza-2′-deoxycytidine; TALE, trichostatin A, AZA, LSD1 inhibitor SP2509, and EPZ-6438; HCC, hepatocellular carcinoma; s-mc, S-methylcytosine; DO, disease ontology; DE, differentially expressed; GO, gene ontology; TPG, tumor-promoting gene; TSG, tumor-suppressor gene; IF, immunofluorescence; IB, immunoblotting; WCL, whole-cell lysate; WMISH, whole-mount in situ hybridization; H3K, histone H3 lysine; H4K, histone H4 lysine; m1, m2, and m3, mono-, di-, and trimethylation, respectively; ac, acetylation.
suggests that a shared regulatory network regulates both tumorigenesis and neural development.

Results

**Inhibition of chromatin modification enzymes in HepG2 cells causes postmitotic neuron-like differentiation and loss of malignancy**

To find out the factors that may regulate cancer cell differentiation, we identified the chromatin modification enzymes, primarily the histone acetyltransferases, histone deacetylases, DNA methyltransferases, methylcytosine dioxygenase, histone lysine methyltransferases, and histone lysine demethylases, by using a few restricting criteria. The factors were considered to be potential regulators of cancer cell differentiation if they 1) promote or are up-regulated in at least some major types of solid cancer; 2) are potentially involved in the earliest differentiation event during embryogenesis as reflected by early embryonic lethality of knock-out mice; 3) mediate transcriptional repression because differentiation or tumor suppressor genes are usually silenced in cancer; 4) are conserved in the basal species of multicellular organisms because conservation suggests that they might be involved in the most fundamental differentiation events during evolution; and 5) may regulate embryonic stem (ES) cell differentiation. The candidate factors include the class I histone deacetylases HDAC1 and HDAC3, lysine methyltransferase EZH2, and lysine demethylase LSD1. Although the DNA methyltransferase DNMT1 is not known in lower organisms, we included it as a candidate because it regulates one of the major types of epigenetic modifications and plays a key role in cancer development and progression (11, 20, 21).

The class I HDAC inhibitor trichostatin A (TSA), EZH2 inhibitor EPZ-6438, LSD1 inhibitor SP2509, and DNMT inhibitor 5-aza-2′-deoxycytidine (AZA) were used to block the endogenous activities of these proteins in hepatocellular carcinoma (HCC) cells line HepG2. To avoid cell death caused by high doses of the inhibitors, we determined the doses empirically so that cells would survive an extended period of treatment. HepG2 cells treated with a single inhibitor did not exhibit significant morphological change. However, cells showed morphological change gradually after the eighth day of treatment with the four chemicals together (TALE). The cells proliferated obviously slower during culture and grew with much longer processes (Fig. 1A), suggesting possibly neuron-like differentiation.

Neuron-like differentiation was supported by the presence of neuronal marker TUBB3 in cells treated with TSA and TALE (Fig. 1B). In contrast, significant expression of neural stem cell marker MS11 was detected in HepG2 cells but nearly undetectable in TALE-treated cells. Concomitantly, the HCC biomarker α-fetoprotein (AFP) was reduced in TALE-treated cells, although treatment with a single inhibitor did not cause much change. The EMT mesenchymal markers CDH2 and ZEB1 were decreased, accompanied by an increase in the epithelial marker CDH1. The pan-cancer-promoting factors, including CCND1, EGFR, FGFR1, and ERBB2, were reduced (Fig. 1B). Decreased expression was observed also for signal transducers SMAD1, SMAD2, SMAD4, STAT3 (the active form with phosphorylation at Tyr-705, pSTAT3), and β-CAT (the active form without phosphorylation at Ser-33/37/Thr-41, nonP-β-CAT), which mediate multifunctional cancer-promoting signaling pathways. Differentiation caused repression of the lysine methyltransferases SETDB1, SUV39H1, and G9A, which are aberrantly up-regulated in or promote different solid cancer types, including HCC, breast cancer, and colon cancer. Besides, the tumor suppressor TP53, also a neuronal protein, was increased. These changes imply that neuron-like differentiation occurred, and differentiated cells might lose malignancy.

Cells treated with a single inhibitor showed slightly decreased capabilities in migration and invasion. However, TALE led to a sharp reduction in cell migration and invasion (Fig. 1C). These neuron-like cells also showed a decrease in cell viability or proliferation (Fig. 1D). TSA and AZA each could efficiently inhibit cellular anchorage-independent growth, and EZH2 inhibitor and LSD1 inhibitor caused a mild reduction. The neuron-like cells showed a nearly complete growth arrest, with only tiny colonies formed (Fig. 1E and supplemental Table S1). Therefore, the inhibitor combination suppresses efficiently malignant features.

We confirmed the efficiency of these inhibitors and their effect on chromatin modification. DNMT1 methylates CpG residues of DNA, EZH2 catalyzes H3K27me3, HDACs are responsible for the lysine deacetylation of proteins, and LSD1 catalyzes the demethylation of H3K4me1/me2. LSD1 inhibitor up-regulated transcriptional active marks H3K4me1, -me2, and -me3 (supplemental Fig. S1, A–C), and EZH2 inhibitor caused a decrease in repressive mark H3K27me3 (supplemental Fig. S1E). AZA caused a slight decrease of H3K27me3 and H3K9me3 (supplemental Fig. S1, D and E), another repressive mark. TSA also induced an increment in H3K4me1, -me2, and -me3 (supplemental Fig. S1, A–C). In TALE-treated cells, H3K4me1, -me2, and -me3 were increased, whereas H3K9me and H3K27me3 were decreased (supplemental Fig. S1, A–E). The decrease in H3K9me3 corresponds with the down-regulation of enzymes responsible for H3K9 trimethylation, SETDB1 and SUV39H1 (Fig. 1B). With regard to histone acetylation, TSA or TALE caused an increase in H3K9ac (supplemental Fig. S2A), H3K27ac (supplemental Fig. S2B), and H4K16ac (supplemental Fig. S2C), all marking active transcription. Due to easy shedding of HepG2 cells from slides during IF detection of DNA methylation, 22Rv1, RKO, and A549 cells were used instead to test DNA methylation change. Indeed, the level of 5-mC was reduced strongly in TALE-treated cells (supplemental Fig. S2, D–F). Reduction in 5-mC is congruent with the fact that neuron differentiation is accompanied by DNA demethylation (22). In summary, the inhibitors could efficiently block the activities of the enzymes and establish a transcriptional active state by increasing chromatin active marks while reducing repressive marks, thereby allowing the activation of otherwise silenced genes, including tumor suppressor genes and differentiation genes, in cancer.

Gene expression profiling showed that 2977 genes were differentially expressed (DE) in HepG2 cells treated with vehicle and TALE, among which 1495 genes were up-regulated and 1482 genes were down-regulated. The genes for chromatin-
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modifying enzymes, chromatin organization, and other events of chromatin modification were the most enriched pathways among the DE genes (Fig. 2A); the most enriched GO terms are related to the cell nucleus (Fig. 2B). Hence, TALE causes significant change in chromatin modification and nuclear events. Moreover, the most enriched disease terms are associated with “liver cancer” and “cancer” (Fig. 2C), according to disease ontology (DO). Among the liver cancer-related genes, the expression of 177 genes promoting or being up-regulated in HCC was decreased in differentiated cells, whereas the expression of 102 genes of this type was increased (Fig. 2D). For genes repressing or being down-regulated in HCC, the expression of 152 genes was increased, in contrast to decreased expression of 81 genes (Fig. 2E). This tendency of expression change was in agreement with the observation that differentiation reduced malignant features. Among GE genes, 479 are neuronal genes according to GO, 297 of which were up-regulated, such as typical mature neuron marker genes BDNF, MAP2, TUBB3, SYN1, SYP, etc., and 182 showed down-regulation (Fig. 2F). When the genes for neuron projection development (GO:0031175) were focused, we detected up-regulation of 103 genes, including BDNF, CDH1, EFNA3, FEZ1, MAP2, NEFM, ROBO1, SYT1, etc., and down-regulation of 61 genes (Fig. 2G). This confirms that TALE treatment endowed cells with neuronal characteristics. Moreover, down-regulation of genes for cell cycle progression CCND1 and CCND2 (Fig. 2H), and up-regulation of cell cycle inhibition genes CDKN1A, CDKN1C, CDKN2B, and CDKN2D (Fig. 2I) confirmed that the treated cells underwent cell cycle exit, a feature of postmitotic neurons. Immunoblotting detected expression change of TUBB3, and cancer-related factors, such as CDH1, CDH2, CCND1, ZEB1, etc. (Fig. 1B), were also corroborated by the change in their transcript levels as detected in the gene expression assay, showing that the differentiation effect can be confirmed by different approaches. Taken together, TALE treatment induced a postmitotic neuron-like phenotype, leading to the loss of malignant features.

TALE induces neuron-like differentiation in cell lines of diverse types of cancer

We then tested the effect of these inhibitors on other types of cancer cell lines. When appropriate doses of the four inhibitors were added together to the breast adenocarcinoma cell line MCF7 (Fig. 3A), the prostate carcinoma cell line 22Rv1 (Fig. 3B), malignant melanoma cell line A375 (Fig. 3C), lung nonsmall cell carcinoma cell line A549 (Fig. 3D), colon carcinoma cell line RKO (Fig. 3E), or osteosarcoma cell line U2OS (Fig. 3F), all cells underwent profound morphological changes, in particular with the outgrowth of long processes (Fig. 3, A–F). Although the glioblastoma cell line U118MG did not display strong morphological changes, these cells grew with more neuritic branches (Fig. 3G). The morphological changes occurred after 5–9 days of treatment, depending on cell type. In these treated cells, the expression of proteins driving or marking neuronal differentiation, NEUROD1, TUBB3, or NF-L, was up-regulated (Fig. 3, H–N). In contrast, the pan-cancer-promoting factors, such as CCND1, PCNA, EGFR, FGFR1, ERBB2, etc., that promote cell cycle, proliferation, angiogenesis, survival, multidrug resistance, etc., were repressed (Fig. 3, H–N). The key factors involved in specific types of cancer (e.g., ESR in ESR-dependent breast cancer (Fig. 3H) and AR in castration-resistant prostate cancer (Fig. 3I)) were inhibited, whereas PGR in breast cancer cells was up-regulated (Fig. 3I), indicating a suppressive effect of malignancy. The SMAD and β-CAT signal transducers were reduced in various cell types (Fig. 3, H–N). Moreover, the protein lysine methyltransferases G9A, SETD1A, SETDB1, and SUV39H1 were down-regulated in different cancer cell lines upon differentiation (Fig. 3, H–N). Even in U118MG cells that did show strong morphological alteration, the neuron markers were increased, and a series of cancer-promoting factors were decreased (Fig. 3N). Therefore, TALE treatment induced neuron-like differentiation in other solid cancer cell lines, as in HepG2 cells.

That inhibition of a few chromatin modification enzymes in different cancer cell lines led to a similar phenotype might raise a concern about the specificity of the observed effect. As a confirmation, we tested the correlation between the expression level of these enzymes in cells and the phenotypic change. A second cell line for breast cancer, MDA-MB-231, also underwent significant neuron-like differentiation in response to TALE treatment. HDACs, EZH2, and LSD1 were significantly expressed in both MCF7 and MDA-MB-231 cells, except for relatively weak expression of DNMT1 in MCF7 (supplemental Fig. S3, A and B). In contrast, the hepatocellular carcinoma cell line Hep3B showed no or very weak morphological alteration, accompanied by weaker expression of HDAC1, EZH2, LSD1, and DNMT1 than in HepG2 cells (supplemental Fig. S3, C and D). It is known that HepG2 cells have a high metastatic potential, whereas Hep3B cells have a low one. This result demonstrates a correlation between expression level of the enzymes and cell potentials in metastasis and neuron-like differentiation. Moreover, we used immortalized cell lines of human T lymphocyte cell Jurkat and bronchial epithelium BEAS-2B, because “normal” cells usually express low levels of these epigenetic modification enzymes. Both cell lines did not show significant morphological change in response to chemical treatment. Although HDAC1/2/3, LSD1, EZH2, and DNMT1 were detected in both cell lines, they were expressed at much lower levels compared with those in HepG2 cells (supplemental Fig. S3, E and F). The result demonstrates again that expression

Figure 1. Inhibition of chromatin modifiers induces neuron-like differentiation in HepG2 cells and reduces malignant features. A, morphological changes of HepG2 after treatment for 11 days with DMSO (D), TSA (T), AZA (A), LSD1 inhibitor (L), EZH2 inhibitor (E) individually, and with the four inhibitors together (TALE). B, immunoblotting (IB) detection of cancer- and neuron-related protein expression. β-Actin was used as loading control. Whole-cell lysate (WCL) was used for IB. C, the effect of a single inhibitor and the combination on the capabilities of invasion and migration of HepG2 cells. After 8 days of treatment, equal numbers of cells were subjected to transwell assays for the indicated time periods. D, effect of inhibitor combination on cell proliferation. MITT assays were performed on cells starting on the first day of treatment (left) and starting on the eighth day of treatment (right). The experiment was performed in triplicate, and data are presented as mean ± S.E. (error bars). **, p < 0.01; ***, p < 0.001. E, single inhibitors and TALE exerted different effects on the colony formation capability of cells. After 8 days of treatment, cells were subjected to colony formation assays for another 9 days.
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A
Significantly enriched pathway terms (Top 10)
- Chromatin modifying enzymes
- Chromatin organization
- RMTs methylate histone arginines
- Signaling events mediated by HDAC Class III
- HDACs deacetylate histones
- PKMTs methylate histone lysines
- PPARA activates gene expression
- Viral carcinogenesis
- Regulation of lipid metabolism by PPARalpha
- Transferrin endocytosis and recycling

B
Significantly enriched GO terms (Top 10)
- nucleoplasm
- nuclear part
- nuclear lumen
- intracellular organelle lumen
- membrane–enclosed lumen
- organelle lumen
- negative regulation of cellular macromolecule biosynthetic process
- negative regulation of transcription, DNA–templated
- negative regulation of RNA biosynthetic process
- negative regulation of RNA metabolic process

C
Significantly enriched disease terms (Top 10)
- Liver cancer
- Cancer
- Methotrexate toxicity
- QT interval
- Lysosomal storage diseases
- Height
- Tetralogy of Fallot
- Down syndrome
- Multiple myeloma
- Eosinophils

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level of the epigenetic modification enzymes is correlated with neuronal differentiation potential of the cells and confirms the specificity of TALE-induced neuron-like differentiation in cancer cells.

The differentiated cells showed much reduced capabilities in migration and invasion (supplemental Fig. S4, A and B) and displayed a growth arrest (supplemental Fig. S4C and Table S2). In agreement with the in vitro data, intraperitoneal injection of a composite of the four inhibitors efficiently repressed tumor formation of the grafted 22Rv1 (Fig. 4, A–C) or RKO (Fig. 4, D–F) cells in immune deficient nude mice, as determined by tumor volume (Fig. 4, B and E) and weight (Fig. 4, C and F). Moreover, in the ApcMin/+ mouse model of intestinal tumor, administration of a composite of the four inhibitors in mice significantly repressed the adenoma formation in intestine (Fig. 4G).

**Knockdown of chromatin modification enzymes induces neuron-like differentiation in cancer cell lines**

To confirm that the observed differentiation was not a side effect, we examined the effect of gene knockdown on cancer cell differentiation. HepG2, RKO, MCF7, or U2OS cells displayed varied response to knockdown of a gene using shRNA (Fig. 5, A–D). In brief, shDNMT1 caused a significant neuron-like morphology in RKO (Fig. 5B) and U2OS (Fig. 5D), shEZH2 and shHDAC1 in all four cell lines (Fig. 5, A–D), shHDAC3 in RKO (Fig. 5B) and MCF7 (Fig. 5C), and shLSD1 in HepG2 (Fig. 5A) and RKO (Fig. 5B). shHDAC2 caused only minor change in RKO (Fig. 5B) and MCF7 cells (Fig. 5C).

Immunoblotting showed that each gene-specific shRNA could efficiently block its target protein expression in both HepG2 (Fig. 5, E and F) and RKO (Fig. 5, H and I) cells. Knockdown of DNMT1 or LSD1 reduced each other’s expression, and EZH2 knockdown resulted in a decrease in DNMT1, HDAC1, and LSD1 in both cell lines. HDAC1 knockdown displayed somewhat different effects on the expression of these enzymes in the two cell lines (Fig. 5, E and H). SETDB1 was also downregulated in response to blocking of DNMT1, LSD1, or EZH2 (Fig. 5, E and H). Additionally, NEUROD1 was up-regulated, whereas CDH2, FGFR1, EGFR, ERBB2, etc. and SMAD and β-CAT signal transducers were down-regulated (Fig. 5, G and J). Therefore, these enzymes regulate each other, and knockdown mimics the inhibitors in inducing a neuronal morphology and in the effect on expression of neuronal and cancer-related proteins. It is noteworthy that the effect of knockdown of a gene is cell context-dependent.

**Expression of genes for chromatin modification enzymes and genes of EMT markers is tissue-specific during embryogenesis**

We explored the rationale for why inhibition of distinct chromatin modification enzymes induces neuron-like differentiation by examining whether these enzymes have intrinsic functions in regulating neural/neuronal differentiation. During embryogenesis, the function of a gene in regulating the differentiation of a particular cell type is reflected by its tissue-specific expression. We had recourse to the embryos of *Xenopus laevis*. In neurula embryos (NF stages 13–21) (23) in which tissue or organ precursors are forming (in particular the future central and peripheral nervous systems appearing as precursor tissues or cells, including neuroectoderm, neural plate, neural fold, neural tube, and neural crest), expression of hdac1, hdac3, dnmt1, ezh2, and lsd1 was localized to neural precursor/progenitor cells. In tail bud embryos (NF stages 29–44) in which tissues and organs are formed, they were mainly transcribed in the CNS and neural crest-derived tissues (Fig. 6A). We did not use embryos earlier than neurula stages because embryonic cells at earlier stages are pluripotent or have the potential to differentiate into multiple types of tissue precursors. The expression pattern suggests that these genes have specific roles in the specification of embryonic neural cells and in the regulation of neural/neuronal differentiation. hdac2 showed no expression in neural cells in neurula embryos. However, it showed specific expression in the CNS (Fig. 6A), suggesting that it does not play a role in embryonic neural cells but plays a role in later stages of neural development. This is in agreement with the minor effect of HDAC2 knockdown on differentiation of cancer cell lines (Fig. 5, A–D).

To confirm whether the neural specific expression of these genes might be related to their function/expression in cancer or just coincidental, we detected the expression of EMT marker genes. The mesenchymal genes cdxh2, zeb2, and vim were exclusively expressed in neural cells. β-cat expression was also highly enriched in neural tissues in a background of ubiquitous expression (Fig. 6B). The expression pattern is the same as that of sox2 (Fig. 6B), a marker gene for neural precursor/progenitor cells and pluripotency and also a gene promoting various types of cancer. snai1 was localized to neural crest cells (Fig. 6B), the precursor cells for the peripheral nervous system. The combined expression domains of these genes match exactly the entire developing nervous system. We did not observe consistent expression of these genes in other embryonic cell types. The epithelial gene cdh1 was detected only in non-neural epidermis in neurula and tail bud embryos, identical to the epidermal marker gene xk81a1 in *Xenopus* (Fig. 6B). These data gave us a hint that “EMT” during tumorigenesis resembles a process of embryonic cell fate change from embryonic epidermal to neural cells.

**The function/expression of genes during tumorigenesis correlates with their tissue-specific expression during early embryogenesis**

We investigated further the relatedness between the function/expression of a gene in cancer and expression of the corresponding gene in embryos. To do this, a total of more than...
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A
DMSO
TALE

B
MCF7, day 12

22Rv1, day 12

H
MCF7
D
T
A
L
E
TALE

IB:
ESR1
PGR
TUBB3
CNP(ase)
NEUROD1
ERBB2
EGFR
CCND1
MSI1
nonP-ß-CAT
SMAD1
SMAD2
SMAD3
SMAD4
G9A
SETDB1
SETD1A
ß-ACT

C
DMSO
TALE

A375, day 8

D

A549, day 7

E

RKO, day 10

F

U2OS, day 7

G

U118MG, day 7

J
A375
D
TALE

IB:
NF-L
CDH1
RB1
AKT3
CDH2
EGFR
FGFR1
CCND1
pSTAT3
SETD1A
SETD1B
SUV39H1
G9A
ß-ACT

K
A549
D
TALE

IB:
NF-L
TUBB3
CDH1
PTEN
RBFOX3
EGFR
FGFR1
FGFR1
ABCC1
CCND1
SMAD1
SMAD2
SMAD3
SMAD2/3
nonP-ß-CAT
SETD1A
ß-ACT

L
RKO
D
TALE

IB:
TUBB3
NEUROD1
PTEN
TP53
EGFR
ERBB2
FGFR1
FGFR1
EGFR
ERBB2
FGFR1
FGFR1
ABCC1
CCND1
SMAD1
SMAD4
SMAD1
SMAD2/3
nonP-ß-CAT
SETD1A
ß-ACT

M
U2OS
D
TALE

IB:
NF-L
TUBB3
CDH1
EGFR
ERBB2
FGFR1
FGFR1
PDGFRα
PCNA
CCND1
G9A
SETD1A
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N
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IB:
NF-L
TUBB3
CDH1
EGFR
ERBB2
FGFR1
PDGFRα
PCNA
CCND1
G9A
SETD1A
ß-ACT
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3000 cancer-related genes from different databases (supplemental Table S3) were classified into four categories via literature study: 1) 943 genes promoting cancer development/progression or being up-regulated/amplified/activated in cancer, considered generally as “tumor-promoting genes” (TPGs) (supplemental Table S4); 2) 547 genes repressing cancer development/progression or expression being down-regulated/gene-deleted/silenced in cancer, considered generally as “tumor suppressor genes” (TSGs) (supplemental Table S5); 3) 1169 genes being TPGs in some cancer types while being TSGs in other(s), considered as genes with dual functions (supplemental Table S6); and 4) 372 genes with unknown function or expression in cancer (supplemental Table S7). A major part of TPGs with known expression patterns showed neural specific expression, primarily in neuroectoderm, neural plate, neural fold, neural tube, and neural crest in Xenopus neurula embryos and in the CNS later (supplemental Table S4), such as AURKA, CDK1–4, EGF, FGFR1–4, FLT1, and MTOR. By contrast, a major part of TSGs with known expression patterns showed no neural specific expression. These genes are either not expressed in the embryos (e.g. CCNA1) or are expressed in non-neural tissues, including epidermis- (e.g. SOX7), mesoderm-, or endoderm-derived tissues (e.g. CTNNB1, GADD45A, HHEX, SOX17, TPM1, TSC1, and WIF1) (supplemental Table S5). For genes with dual functions, about half of the genes with reported spatial expression showed neural specific expression (supplemental Table S6). For the genes without known expression/function in solid cancer, spatial expression of 16 genes has been described, five being expressed specifically in neural cells (supplemental Table S7). Therefore, the function/expression of genes during tumorigenesis seems to be tightly correlated with their tissue-specific expression during embryogenesis. To confirm the correlation, we detected the expression of some cancer-related genes whose embryonic expression pattern had not been known and repeated the detection of reported expression patterns of a few genes. As expected, most of the detected genes that promote or are up-regulated in multiple types of cancer displayed strict neural specific expression in neurula embryos and in CNS in tail bud embryos, including akt1, akt2, bcl9, birch5.2, cbl, cnnd1, cdk1, cd1, ckr, ddx5, dck, dot1l, dyrk1a.2, e2f1, eif3c, eif4g1, g9a, jmd1b, jmd2a, kras, msi1, mta1, nestin, nsd2, ogt, pcam, plk1, ppm1id, prmt1, prmt5, ptk2, rad21, raf1, rhb, setd1a, setdb1, smad2, sn1, stat3.2, stom1, tk1, tmem97, top1, top2a, ube2c, usp39, usp7, whsc1, xpo1, and yy1 (Fig. 1 and supplemental Table S4). Besides, WNT, TGFβ, FGF, NOTCH, and HH pathways play extensive roles during cancer development and progression. Correspondingly, transcription of the major signal transducers, such as fzd4, dvl1, β-cat, tcf7l1, smad1, smad2, smad4, fgfr1–4, et2, notch1, hes1, hey1, ptch1, smo, and gli1–3, is localized to embryonic neural cells (Figs. 6B and 7 and supplemental Fig. S4B and Table S8). Some of the pan-cancer-promoting genes are known markers for embryonic or adult neural stem/progenitor cells, such as ABCG2, ASC1L1, β-CAT, BM11, CDH2, CCXCR4, FGFR2, FGFR4, FUT4, GLUT1, HES1, ID2, JAG1, JUN, MSI1, NES, NOTCH1, PDGFR, ROR2, SNAI1, SOX2, SOX3, SOX9, STAT3, VIM, or ZIC3. It is not surprising that they regulate nearly every aspect of neural/neuronal differentiation, maturation, network formation, neuron function, neuroprotection, etc. (supplemental Table S4).

Figure 4. TALE inhibitor combination reduces tumor growth in vivo. A–C, effect of injection of TALE on tumor formation of 22Rv1 cells in athymic Nude-Foxn1nu mice, as shown by the difference in the volumes (B) and weight (C) of tumor tissues from mice injected with vehicle and TALE. D–F, effect of injection of TALE on tumor formation of RKO cells in athymic Nude-Foxn1nu mice, as shown by the difference in the volumes (D) and weight (E) of tumor tissues from mice injected with vehicle and TALE. G, effect of injection of TALE on the intestinal tumor formation in ApcMin+ mice, as shown by the difference in the numbers of adenomas from mice injected with vehicle and TALE. In B, C, E, F, and G, significance was calculated with unpaired Student’s t test. Data are presented as mean ± S.E. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 3. TALE induces neuron-like differentiation in different cancer cell lines. A–G, the morphological change in cell lines of breast cancer (A), prostate cancer (B), melanoma (C), lung cancer (D), rectal carcinoma (E), osteosarcoma (F), and glioblastoma (G) that had been treated with vehicle or TALE for the indicated time periods. H–N, IB analysis on the expression of proteins or markers involved in cancer development and progression and proteins or markers involved in neuronal differentiation in cells that were treated as in A–G. β-Actin was used as loading control. WCL was used for IB.
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**Figure 5. Stable knockdown causes neuron-like differentiation in different cancer cell lines.** A–D, shRNAs against DNMT1 (shDNMT1), EZH2 (shEZH2), HDAC1 (shHDAC1), HDAC2 (shHDAC2), HDAC3 (shHDAC3), or LSD1 (shLSD1) were stably infected into HepG2 (A), RKO (B), MCF7 (C), or U2OS (D) cells for the indicated time periods. Cells underwent morphological changes to different degrees. E–G, test of knockdown efficiency of each shRNA and their effect on protein expression in HepG2 cells. H–J, test of knockdown efficiency of each shRNA and their effect on protein expression in RKO cells. WCL was used for IB assays. Cells infected with virus packaged with empty vector (Vector) were used as control.
Among the tumor-promoting genes we detected, *hgf*, *jak2*, *pik3ca*, *pim1*, *vav2*, and *zeb1* are expressed weakly or partially in neural tissues of neurula embryos, whereas *axl* shows weak epidermal expression, and *nras*, *slc16a1*, and *tert* are not significantly expressed (supplemental Fig. S5A). We also detected the expression of some major tumor suppressor genes or the genes down-regulated in multiple cancer types. Among them, *apc*, *arid1a*, *casp9*, *hexim1*, *pbrm1*, *pten*, *st13*, and *tp53* are expressed specifically in neural cells in neurula embryos and in CNS later (Fig. 8A). *gas1* shows expression in both neural and mesodermal tissues. *pdcda4* is present in neural cells in neurula and localized to developing eye lens and midbrain-hindbrain boundary. *cdkn1b* and *dcl1* are expressed weakly in neural tissue in neurula embryos, and there was no detectable expression later on (Fig. 8A). *cdkn1a* is in the cement gland primordium in neurula and in cement gland, somites, lens, and ear vesicle. *hic1* expression is localized to the medial stripe of primary neurons and, later on, to the trigeminal nerve and notochord. *chfr* and *tet2* show no obvious transcription in neurula embryos but are transcribed in CNS in tail bud embryos. *tp53bp2* exhibits expression in the cement gland primordium. *txnip* transcription is localized strongly to somite but not detected in neurula embryos (Fig. 8A). Other genes, *axin1*, *brms1*, *cdkn2b*, *dmbt1*, *mgmt*, *perp*, *phlp1*, *tgfb1*, and *tp73*, are not expressed significantly (Fig. 8A).

Among the genes playing dual roles, *ampk*, *pkm*, *smad1*, and *smad4* were detected specifically in neural plate in neurula embryos and in CNS and other tissues in tail bud embryos. *ptgs2* was in the epidermis and the neural groove. No significant expression was detected in early embryos for *ahr1a*, *hras*, *mcam*, and *ret* (supplemental Fig. S5B).

In summary, among the TPGs, the expression patterns of 338 genes are known, 274 of which are expressed in embryonic neural tissues and 64 of which are not. The expression patterns of 105 TSGs are known, 29 of which are expressed in neural tissues and 76 of which are not. The expression patterns of 287 genes with dual functions are known, 146 of which are expressed in neural tissues and 141 of which are not (Fig. 8B and 8C).
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supplemental Tables S4–S7). Therefore, the major tumor-promoting genes or pathways are most likely to express in embryonic neural cells, whereas tumor suppressor genes are not, resembling the expression patterns of EMT genes. Solid cancer cells thus share a regulatory network with embryonic neural cells, the precursor cells for neuronal differentiation. As a further support, we found that among the 9101 expressed genes in HepG2 cells examined by a gene expression profiling assay, 792 were neural specific in *Xenopus* embryos (supplemental Table S9). This implies that cancer cells express a broader range of neural genes to specify their characteristics of embryonic neural cells.

### Discussion

We demonstrate that by inhibition of a few chromatin modification enzymes that promote tumorigenesis, cancer cell lines

![Figure 7. Neural specific expression of genes promoting or being up-regulated/activated during cancer development/progression.](image)

![Figure 8. Genes repressing or being down-regulated during cancer development/progression show varied expression patterns in *Xenopus* embryos.](image)
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can differentiate into postmitotic neuron-like cells with loss of malignant features. The neural specific expression of pan-cancer-promoting genes implies that cancer cells might have characteristics of embryonic neural precursor cells.

Cancer cells can be terminally differentiated

By inhibition of class I HDACs, DNMT1, EZH2, and LSD1, different cancer cell lines underwent a postmitotic neuron-like differentiation, as evidenced by the neuronal morphology and increased expression of mature neuron markers and neuronal differentiation factors in the differentiated cells. These chromatin modification enzymes thus serve to restrict the expression of neuron differentiation genes in cells. This function fits exactly with their presence only in neural precursor cells and suggests that these enzymes maintain the immature state of both cancer cells and embryonic neural cells. The differentiated cells showed repression of the factors promoting cell cycle progression and stimulation of cell cycle inhibitors, representing cell cycle exit. The terminal differentiation is congruent with loss of malignant traits and a tendency of general repression of tumor-promoting genes, concomitant with enhancement or activation of tumor-suppressing genes and neural specific genes. Animal experiments also demonstrated that simultaneous inhibition of HDACs, DNMT1, EZH2, and LSD1 could effectively suppress tumor growth.

Functional diversity of these enzymes, either in chromatin modifications or in the regulation of non-histone proteins, makes it complicated to decipher the exact molecular mechanisms underlying their regulation of neuron differentiation in distinct cell types. However, a common feature of these enzymes is that they are all specifically expressed in embryonic neural cells, the precursor cells for neuron differentiation. The presence of these enzymes in embryonic neural precursor cells is in agreement with their function in repressing neuron gene expression. Besides, cancer cells express numerous other embryonic neural specific genes. It is plausible that the enzymes and other neural specific gene products form a regulatory network to endow cancer cells with the properties of neural precursor cells. Considering cancer heterogeneity, it might be that cells at different stages of a cancer type or of different cancer types express different subsets of neural genes. Besides our observation, a recent work also reported neuronal differentiation of gastric cancer stem cells (24). It is likely that among other chromatin modification enzymes with neural specific expression identified in the study, some should also regulate neuronal differentiation in cancer cells.

The effect of inhibition or knockdown of a single enzyme on differentiation is cell type– or cell context– dependent. Thus, a combined inhibition of these enzymes could achieve more efficient differentiation in cells than inhibition of a single enzyme.

Cancer cells share a regulatory network with embryonic neural cells

EMT, a concept originally devised for embryonic development, is believed to be a prerequisite for tumorigenesis and endows cancer cells with malignant features, including stemness, chemoresistance, reprogrammed cellular metabolism, or dysregulated epigenetics (2, 25). However, it is unclear how EMT confers on cancer cells the malignant traits. The difference between epithelial and mesenchymal cells and the similarity between mesenchymal and cancer cells are their morphology and motility (26, 27). Although mesenchymal cell mobility features could account for cancer progression, two studies demonstrate a role of EMT in chemoresistance but not in cancer progression (28, 29). Moreover, Tarin et al. (27) emphasizes the importance of “cells at the edge of neural plate,” which correspond to neural crest cells, during EMT. The correlation between embryonic tissue-specific expression of cancer-related genes and their function/expression in cancer provides the evidence that tumorigenesis might be related to neural specification, because a major part of pan-cancer-promoting genes, including the “mesenchymal” marker genes, are only transcribed in embryonic neural cells, including neural crest cells, but not in other types of embryonic cells. Embryonic neural cells express genes that promote all features of malignancy. The genes governing pluripotency or neural stemness, including MYC, POU5F1, SOX2, NESTIN, MSI1, etc., are promoters of cancer development and progression, confer stemness to cancer cells (30), and show neural specific expression. Mesenchymal cells do not display properties of stemness except the neural crest cells (31, 32). Cancer cells are highly proliferative. Correspondingly, genes promoting cell cycle progression, such as ccc1, ccd2, cdk1, cdk2, cdk4, cdt1, e2f1, pcrea, etc., are localized to embryonic neural cells. Cancer cells are chemoresistant. The genes conferring multidrug resistance in cancer (e.g. ABCB1 and ABCG2) are markers for neural progenitor/stem cells (33). Cancer cells are characteristic of altered cell metabolism. The key genes regulating cancer metabolism, such as akt, ampk, kras, mtor, myc, ogt, pdk4, pfk2, and ptk2, show specific expression in embryonic neural cells. Moreover, the glucose transporter Glut1 shows specific expression in neural stem cells (34, 35). Dysregulated epigenetics in cancer cells corresponds with neural specific expression of epigenetic factors, such as dnm1, hdac1, hdac3, ez2h, lsd1, setd1a, setd1b, g9a, prmt1, prmt5, jmjd6, and wsc1. Furthermore, the major signal transducers of the pathways including TGFβ, WNT, FGF, HH, NOTCH, IGF, HGF, or PDGF, which are “EMT signaling pathways” (36), exhibit neural specific expression. These genes and signal transducers are not as consistently expressed in any other type of embryonic tissues or organs. The correlation between the function of a gene in cancer and in embryonic neural cells can also be exemplified by the RAS family genes. Although KRAS, NRAS, and HRAS have similar transformation activity, KRAS is much more frequently mutated in human cancer than NRAS and HRAS, suggesting that KRAS has unique properties favoring oncogenesis (37). We show that kras is specifically expressed in embryonic neural cells, demonstrating its involvement in neural development. In contrast, nras and hras are absent in embryonic neural cells.

During embryogenesis, neural induction from ectoderm requires the inhibition of Smad-mediated TGFβ signaling that drives non-neural differentiation (38, 39). Once neuroectoderm is induced, TGFβ signaling then becomes an integral part of the regulatory machinery for subsequent neural development. Cancer initiation also requires inhibition of TGFβ signaling, which promotes cancer progression later (6). Additionally,
the signaling pathways promoting neural/neural crest induction and development, mainly FGF, Wnt/β-catenin, and Notch signaling pathways (40–45), consistently play active roles in cancer. During further development of the nervous system, neural precursor and immature neuron cells undergo extensive morphological alteration, migration, and innervation of most tissues or organs, resembling the metastasis of cancer cells into other tissues. Thus, tumorigenesis might be due to the acquisition of properties of embryonic neural cells. Numerous studies have reported the importance of neural development signals during cancer development and progression, including those listed in supplemental Tables S4–S6. Many cancer types, such as gastric and colon cancer, show neurogenesis and increases in nerve density (24, 46, 47), and denervation suppresses cancer (47). Benign prostatic basal cells that are enriched for stem cell and neural/neuronal genes are associated with more aggressive potential (48). Moreover, development of melanoma is a re-emergence of the state of neural crest progenitors (49). A study also demonstrates that “cancer represents a loss-of-function driven reverse evolution back to the unicellular ‘ground state’” (50); an intrinsic link might exist between tumorigenesis and neural development.

Despite the fact that embryonic neural cells and cancer cells share a regulatory network, neural cells are per se not cancer cells. Embryonic neural cells express higher levels of genes promoting cell cycle, metabolism, epigenetic modification, cell survival, migration, etc., similar to cancer cells. Thus, they are highly proliferative, mobile, and energy-demanding and, logically, prone to errors such as gene mutations if not deliberately controlled. Actually, they express specifically the genes promoting cell apoptosis or senescence (casp3, casp9, egln3, foxo4, gadd45g, nf2, pten, and tp53), inhibiting cell cycle or proliferation (apc, arid1a, cdkn1b, gadd45g, gas1, ndrg2, nf2, phrm1, pcdh4, rb1, tes, and tp53), restricting cell migration and growth (pten), reducing cell metabolism (tp53), or maintaining genomic integrity (arid1a, pten, and tp53). The energy sensor gene ampk, originally identified as a tumor suppressor gene, is transcribed solely in neural cells during neurulation. These tumor suppressor genes are usually lost or inactivated in cancer. The disparity in expression of these genes agrees with the fact that neural development is a precisely controlled process to ensure the formation of tissues in a strict spatiotemporal pattern, whereas during tumorigenesis, the control is lost.

Many tumor suppressor genes display expression in non-neural tissues (e.g. cdkn1a, cdkn1b, ctnma2, hhox, sox17, and tsc1) or no significant expression (e.g. axin1, brms1, ccna1, cdkn2b, mgmt, or perp). During embryogenesis, genes of different tissue or cell types may antagonize each other to guarantee tissue or cell identity, establish boundaries between tissues, and ensure correct spatiotemporal pattern formation. Therefore, non-neural genes should be inactivated to allow a successful neural specification, similar to their silencing during tumorigenesis. Some tumor suppressor genes are not significantly expressed in embryos, suggesting that they might function to inhibit embryonic tissue formation, including neural tissues, for example, via inhibition of Wnt/β-catenin signaling in the case of axin1. Correspondingly, these genes are not expressed in cancer cells either. Thus, the correlation between the function/expression in cancer and their embryonic tissue-specific expression suggests that tumorigenesis might be a process of losing cell lineage identity and acquiring characteristics of embryonic neural cells.

In summary, the signals that have pan-cancer-promoting functions constitute the similarity among different cancer types. These signals work together to impart the properties of neural precursor/progenitor cells to cancer cells. Other factors or signals may function peripherally to initiate, maintain, or enhance the pan-cancer signals in the internal/external environments specific to a cell or tissue type, as reflected by cancer heterogeneity.

**Experimental procedures**

**Cell culture**

HepG2, Hep3B, MCF7, RKO, U2OS, U118MG, A375, HEK293T, and BEAS-2B cells were cultured in DMEM (Thermo Fisher Scientific, catalog no. 11965-092); 22Rv1 and Jurkat were cultured in RPMI 1640 medium (Thermo Fisher Scientific, catalog no. 11875-093); A549 was cultured in F-12K medium (Thermo Fisher Scientific, catalog no. 21127-022), and MDA-MB-231 was cultured in L-15 medium (Thermo Fisher Scientific, catalog no. 41300-039). All culture media were supplemented with 10% FBS (Gibco, catalog no. 10099141) and with 50 units/ml penicillin and 50 µg/ml streptomycin. All cells were grown at 37 °C with 5% CO₂ except for MDA-MB-231, which was cultured without CO₂. In differentiation assays using chemical inhibitors or functional knockdown, 5% FBS was added to culture medium for both control and differentiating cells. MCF7 was obtained from ATCC. 22Rv1, A375, A549, HepG2, RKO, U2OS, U118MG, HEK293T, Jurkat, Hep3B, BEAS-2B, and MDA-MB-231 were obtained from the cell bank of the Shanghai Institutes for Biological Sciences (Shanghai, China).

**Cancer cell differentiation assays using chemical inhibitor treatment**

Specific chemical inhibitors for DNMTs (AZA; Selleckchem, catalog no. S1200), EZH2 (EPZ-6438; Selleckchem, catalog no. S7128) (51), LSD1 (SP2509; Selleckchem, catalog no. S7680) (52), and class I HDACs (TSA; Selleckchem, catalog no. S1045) were used to induce the differentiation of cancer cell lines. When cells grew to 60–70% confluence, an inhibitor or inhibitors in combination were added to the culture medium. The strategy for cancer or normal cell differentiation assays is summarized in supplemental Table S10. Cells were replated, and culture medium containing inhibitor(s) was refreshed during treatment. Cells treated with vehicle (DMSO) in parallel were used as controls.

**Immunofluorescence**

HepG2 cells were treated with vehicle, an individual inhibitor, or inhibitors in combination for 10 days, and histone modification was detected using immunofluorescence (IF) exactly as described (53). To detect chromosomal DNA methylation, RKO, A549, or 22Rv1 cells were treated with a combination of the four inhibitors. After fixation and paraformaldehyde inactivation, cells were washed and treated with 2 N HCl at 37 °C for
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30 min, washed with 0.2% Tween 20 in PBS, and neutralized with 100 mM Tris-HCl (pH 8.5) for 10 min. Cells were then blocked with 0.1% Triton X-100 and 3% BSA, washed, and incubated with primary antibody against 5-mC at 4 °C overnight. The remaining steps were the same as in regular IF assays. Detailed information on antibodies and their dilutions is given in supplemental Table S11.

Whole-cell extract preparation

Cells were washed with ice-cold PBS and lysed on ice for 40 min in lysis buffer containing 150 mM NaCl, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM Tris (pH 8.0), protease inhibitor mixture (Roche Applied Science, catalog no. 04693132001), and phosphatase inhibitor mixture (Roche Applied Science, catalog no. 04906845001). Lysates were cleared via centrifugation.

Immunoblotting

Western blotting with whole cell lysates was performed using SDS-PAGE. Blots were detected with a Western blotting substrate (Tanon, catalog no. 180-501). Detailed information on antibodies and their dilutions is given in supplemental Table S10.

Soft agar colony formation assay

The bottom layer of agar was 0.7%, whereas the top layer was 0.35%, of low melting agarose (BBI, catalog no. AB0015). In each well of a 6-well culture plate, 2000 cells treated for 8–10 days with vehicle or with an inhibitor or inhibitors were plated and cultured for 1–3 weeks, depending on cell types. Colonies with a size larger than 100 μm in diameter were counted for HepG2, 22Rv1, RKO, MCF7, or U118MG cells, and colonies larger than 50 μm were counted for A549 cells because of the slow growth rate. In all colony formation assays, TALE induced differentiation caused cells to form tiny colonies much smaller than 50 μm.

Cell migration assay

Cells that had been treated with vehicle, an inhibitor, or with inhibitors in combination for 8–10 days were trypsinized. Cell migration was performed in 24-well transwell plates with 8-μm pore size inserts (Corning, catalog no. 3422). 4 × 10^4 cells in 200 μl of serum-free culture medium were added to the upper compartments. The lower compartments contained 500 μl of medium containing 10% FBS. Plates were incubated at 37 °C for different periods of time as indicated. At each time point, each 10 μl of MTT at 5 mg/ml was added per well, incubated at 37 °C for 3 h. After the removal of culture medium, 100 μl of DMSO was added per well, and the plate was placed at 37 °C for 10 min. Absorbance at 490 nm was measured by an ELISA reader (Corona SH-1000Lab microplate reader). Assays were also made for cells that had undergone an extended period of treatment. At the seventh day of treatment, 2000 control or treated cells were plated in each well. Afterward, the assays were carried out exactly as described above at four time points: the eighth, ninth, tenth, and eleventh day of treatment. All assays were performed in triplicate, and statistical significance was calculated with two-way analysis of variance.

Tumor xenograft assay and tumor model assay

All animal usage was in accordance with the guidelines of the institutional animal care and use committee at the Model Animal Research Center of Nanjing University. 5–6-week-old male athymic Nude-Foxn1nu mice were purchased from the National Resource Center for Mutant Mice (Nanjing, China) and maintained in a specific-pathogen-free facility. 2 × 10^6 of 22Rv1 or 5 × 10^6 of RKO cells suspended in 100 μl of sterile PBS were injected subcutaneously into the left flank of each mouse. Three days later, mice injected with the same type of cells were randomly divided into two groups. Each mouse in one group was injected intraperitoneally once every 2 days with a composite of TSA (1 mg/kg), AZA (0.75 mg/kg), SP2509 (0.5 mg/kg), and EPZ-6438 (1 mg/kg), and the other was injected with vehicle (10% DMSO in PBS) using the same method of injection. Tumor size was measured three times per week until the biggest tumor size reached 2 cm in diameter, when the mice were sacrificed and tumor tissues were excised. Tumor tissues were weighed, and tumor volumes were calculated using the formula, length × width^2/2. The significance of difference in tumor formation in the two groups of mice was calculated using unpaired Student’s t test.

C57BL/6-ApcMin/+ mice were obtained by crossing the male C57BL/6-ApcMin/+ mice with wild-type female C57BL/6J (both strains were purchased from the National Resource Center for Mutant Mice). After genotyping, 15 offspring C57BL/6-ApcMin/+ mice were obtained. At the age of 8 weeks, eight mice were injected intraperitoneally with a combination of TSA (1 mg/kg body weight), AZA (0.75 mg/kg), SP2509 (0.5 mg/kg), and EPZ-6438 (1 mg/kg). Seven mice that were injected with equal volumes of 10% DMSO in PBS were used as controls. Injection was repeated every 2 days, and body weight was measured every 4 days. One month later, mice were sacrificed, and intestine was dissected longitudinally, the intestinal neoplasmic
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Adenomas were counted, and the tumor size (in diameter) was measured for each mouse. The significance of difference in the number and size of adenomas between control and experimental groups was calculated by the unpaired Student’s t test.

**Gene expression profiling analysis in HepG2 cells after differentiation**

Total RNA from HepG2 cells treated with vehicle or differentiated cells was extracted with TRIzol, and first strand cDNA was synthesized from poly(A) RNA. Biotin-labeled cRNA was prepared using the MessageAmp™ Premier RNA amplification kit (Ambion). After purification, quantification, and fragmentation, cRNA probes were hybridized to the PrimeView™ human gene expression array (Affymetrix). Signals were scanned with a GeneChip® Scanner 3000 (Affymetrix, Santa Clara, CA). Microarray processing and signal analysis were performed by Beijing CapitalBio Technology Co., Ltd. (Beijing, China). Expression change was determined by TALE/DMSO -fold change of signals after normalization with the robust multarray averaging method. Expression was considered as up-regulated when the signal -fold change was >2.0 and as down-regulated when the -fold change was <0.5. Raw data were deposited in GEO under accession number GSE93409.

Enrichment and annotation of pathway, disease ontology, and gene ontology were analyzed using the software KOBAS (http://kobas.cbi.pku.edu.cn)4 (55), which contains seven pathway databases (KEGG Pathway, PID Curated, PID BioCarta, PID Reactome, BioCyc, Reactome, and Panther), five human disease databases (OMIM, KEGG Disease, FunDO, GAD, and NHGRI), and one gene ontology database (Gene Ontology). Results are shown as bar charts.

Heat maps for differentially expressed genes were generated using HeatMapImage (http://software.broadinstitute.org/cancer/software/genepattern/).4

**Analysis on the relatedness between gene expression/function in cancer and tissue-specific expression during embryogenesis**

More than 3000 cancer-related genes from different databases or sources were from the Bushman Lab website and the COMSIC cancer gene census (http://cancer.sanger.ac.uk/census)4 (56) (supplemental Table S3). By searching PubMed with key words “gene symbol or synonym or name” and “cancer”, the function/expression of each cancer-related gene was assigned to different solid cancer types according to the literature and classified into four categories (see above). Correspondingly, the spatial expression patterns of the homologous genes in *X. laevis* neurula and tail bud embryos were searched in PubMed or Xenbase (www.xenbase.org)4 and literature therein. The functions of the genes during neural/neuronal differentiation, proliferation, migration, maturation, neuroprotection, etc., were also searched. The expression/functions of cancer-related genes in cancer, the spatial expression patterns of their homologous genes in *Xenopus* embryos, and their expression/functions during neural/neuronal development and related references are listed in supplemental Tables S4 – S7.

**Knockdown assays**

shRNA-based functional knockdown was used to investigate the effect of genes of interest on cells via lentiviral infection. The sequences of shRNAs against DNMT1, EZH2, HDAC1, HDAC2, HDAC3, and LSD1 were the validated MISSION® shRNAs (Sigma-Aldrich), which were subcloned to the vector pLKO.1. The shRNAs were TRCN0000195103 (HDAC1), TRCN0000004823 (HDAC2), TRCN0000194993 (HDAC3), TRCN000021891 (DNMT1), TRCN0000040077 (EZH2), and TRCN0000046071 (LSD1). Virus containing pLKO.1 empty vector that was used as a control or containing an shRNA was packaged with HEK293T cells. The lentiviral supernatant was filtered through a 0.45-µm filter and used for infecting cells. Forty-eight hours after infection, cells were selected with puromycin at 2 µg/ml in culture for 2 days and cultured further until significant phenotype was observed.

**Construction of plasmids used for preparation of riboprobes**

cDNAs (supplemental Table S12) used for preparing antisense riboprobes were PCR-amplified from a *Xenopus* embryonic cDNA pool, ligated to pCS2+ vector, and verified with sequencing. The resulting plasmids were linearized, and riboprobes were transcribed in vitro with T7 RNA polymerase (Ambion) and cleaned up with an RNeasy kit (Qiagen).

**In situ detection of gene expression in *X. laevis* embryos**

Neurula embryos between NF stages 15 and 20 and tail bud embryos between stages 26 and 32 of *X. laevis* (23) were obtained using conventional in vitro fertilization. Spatial gene expression was detected with whole-mount in situ hybridization as described (54).

**Embryonic neural gene expression analysis in HepG2 cells**

After removal of the RefSeq genes for which signal calls were “A (absent)” from the gene expression array data, probes were averaged for a RefSeq gene if multiple probes existed for the RefSeq. Embryonic neural specific expression of the remaining RefSeq genes was determined by the spatial expression patterns in *Xenopus* neurula and tail bud embryos that are available in Xenbase (www.xenbase.org)3 or in supplemental Tables S4 – S7.

**Author contributions**—Y. C. conceived the research, performed the literature study, and wrote the manuscript. Z. Z., A. L., L. X., L. C., X. Z., and Y. G. performed cell assays; Z. Z., A. L., and L. X. performed the biochemical study; Z. Z., L. X., and Y.-L. C. performed the biochemical study; Z. Z., A. L., and L. X. performed the biochemical study; Z. Z., A. L., L. X., and Y.-L. C. performed experiments with *Xenopus* embryos and mice; L. C., X. Y., and M. Z. made plasmid constructs; Z. Z., A. L., L. X., and Y.-L. C. analyzed the results. All authors approved the final version of the manuscript.

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