In vivo reprogramming drives Kras-induced cancer development

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The faithful shutdown of the somatic program occurs in the early stage of reprogramming. Here, we examined the effect of in vivo reprogramming on Kras-induced cancer development. We show that the transient expression of reprogramming factors (1–3 days) in pancreatic acinar cells results in the transient repression of acinar cell enhancers, which are similarly observed in pancreatitis. We next demonstrate that Kras and p53 mutations are insufficient to induce ERK signaling in the pancreas. Notably, the transient expression of reprogramming factors in Kras mutant mice is sufficient to induce the robust and persistent activation of ERK signaling in acinar cells and rapid formation of pancreatic ductal adenocarcinoma. In contrast, the forced expression of acinar cell-related transcription factors inhibits the pancreatitis-induced activation of ERK signaling and development of precancerous lesions in Kras-mutated acinar cells. These results underscore a crucial role of dedifferentiation-associated epigenetic regulations in the initiation of pancreatic cancers.

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-induced pluripotent stem cells (iPSCs) can be established from somatic cells by the transient expression of pluripotency-related transcription factors (TFs). Given that epigenetic regulation plays a central role in cell fate determination and maintenance, this technology allows for the reorganization of epigenetic regulation from the differentiated somatic cell state into the pluripotent state without affecting genetic information. Recent studies revealed the stepwise epigenetic changes during iPSC derivation. In early-stage reprogramming, the faithful shutdown of the somatic program occurs through the silencing of somatic cell-specific enhancers, which is associated with a loss of original cell identity. Upon prolonged expression of the reprogramming factors, gradual activation of the pluripotency-related transcriptional network is observed in late-stage reprogramming.

Notably, such events in cellular reprogramming are similarly observed in cancer development. Cancer cells often lack terminal differentiation ability, suggesting that repression of the somatic cell program is involved in cancer development. In contrast, poorly differentiated cancers that lose most differentiation features sometimes exhibit an activation of pluripotency-related genes such as targets of Nanog, Oct3/4, and Sox2. Moreover, poorly differentiated cancers with pluripotency-related gene expressions are associated with high malignancy, especially in the mammary glands. Collectively, these findings suggest that some aspects of cellular reprogramming are associated with cancer development.

Indeed, our previous study demonstrated that premature termination of in vivo reprogramming leads to kidney cancer development through altered epigenetic regulation. Consistent with the partial reprogramming state, these cancer cells lose kidney cell-specific molecular signatures while they partially acquire the trait of embryonic stem cells (ESCs) including self-renewing capacity. Notably, these cancers resemble Wilms' tumor, which is the most common childhood kidney cancer. Furthermore, these cancer cells were readily reprogrammable into iPSCs that are capable of differentiating into non-cancerous kidney cells. These results raised the possibility that reprogramming-associated epigenetic regulation has a significant impact on childhood cancer development, which is also in agreement with recent observations that childhood cancers harbor relatively few genetic mutations. However, the functional significance of epigenetic regulation related to cellular reprogramming remains largely unclear in adult cancer development.

Pancreatic cancer is one of the most common causes of cancer mortalities in adults in developed countries. The median survival period is less than 6 months and the 5-year survival rate is 3–5%. The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC). PDAC is one of the well-characterized cancers for multistep cancer progression models that have “Big 4” mutations (mutations in KRAS, CDKN2A, TP53, and SMAD4). Histologically, PDAC is thought to arise from a spectrum of preneoplastic lesions with ductal morphology that are designated as pancreatic intraepithelial neoplasia (PanIN). Since early PanIN lesions harbor mutations or amplification of the KRAS at high frequency (over 90%), it has been proposed that KRAS mutation is responsible for PanIN formation and thus is an initial event during pancreatic cancer development. In contrast to premalignant lesions, PDAC often harbors additional mutations such as a loss of CDKN2A and inactivating mutations at TP53 and SMAD4. Together, current consensus holds that pancreatic cancer arises through a stepwise progression with characteristic genetic mutations for each histologically distinguishable stage.

In the present study, based on the shared properties between cellular reprogramming and cancer development, we examined the effect of in vivo reprogramming in the development of Kas-induced cancer. We show that reprogramming-mediated repression of somatic cell enhancers, which is associated with early stage of reprogramming, in conjunction with KRAS mutation results in rapid development of PDAC. We also show that partial acquisition of the ESC signature, which occurs later stage of reprogramming, causes the development of cancers that resemble human α-fetoprotein (AFP)-producing cancer. These results highlight the crucial role of reprogramming-related epigenetic regulation in Kas-induced cancer in vivo.

**Results**

**Kras and p53 mutations are insufficient for ERK activation.** We first generated Pdx1-ires-Cre knocked-in ESCs by homologous recombination (Fig. 1a and Supplementary Fig. 1A), and then Pdx1-ires-Cre knocked-in mice to induce pancreas-specific Cre-LoxP recombination. Lineage tracing analysis of Pdx1-ires-Cre mouse using Rosa26 LacZ reporter allele confirmed that the Cre-LoxP recombination occurs in almost all pancreatic cells (Supplementary Fig. 1B, C), which was consistent with a previous report using transgenic mice expressing Pdx1-Cre. Similarly, we knocked-in LoxP-Stop-loxP (LSL)-HA tag-KrasG12D allele into endogenous Kras locus in ESCs (Supplementary Fig. 1D) and generated knocked-in mice. We then generated LSL-HA tag-KrasG12D and Pdx1-ires-Cre (hereafter HA-KC) compound mice to examine the expression of KrasG12D oncprotein (Fig. 1a). HA immunostaining exhibited membranous expression of KrasG12D oncprotein in the pancreatic cells of HA-KC mice at 6 weeks of age (Fig. 1b). However, despite the expression of KrasG12D oncprotein, most pancreatic cells were histologically normal (Fig. 1b) except for the focal formation of early PanIN, indicating that Kras mutation alone cannot transform pancreatic acinar cells.

To further investigate the effect of oncogenic mutations on the ERK signaling pathway and aberrant proliferation, we next generated LSL-KrasG12D, Pdx1-ires-Cre (KC) mice and LSL-KrasG12D, LSL-p53R172H, Pdx1-ires-Cre (KPC) compound mice (Fig. 1c) using well-established Kras and p53 mutant alleles. In accordance with the results in HA-KC mice, most pancreatic cells exhibited recombination in both KrasG12D and p53R172H alleles in KPC mouse (Fig. 1d, Supplementary Fig. 9). However, the majority of pancreatic cells were histologically normal in both KC and KPC mice at 6 weeks of age, except for the spotted formation of early PanIN and the dysplastic area (Fig. 1e). Although PDAC development was observed in KPC mice at 8 weeks of age, the affected area was still limited, and most pancreatic cells remained histologically normal (Fig. 1e). Consistently, pERK immunostaining showed positive staining only in PanIN and PDAC lesions, while the majority of pancreatic cells were negative for pERK (Fig. 1e), demonstrating that compound mutations of Kras and p53 are insufficient for robust activation of the ERK pathway. Similarly, the majority of pancreatic cells in KPC mice were negative for Ki67, a cell proliferation marker (Fig. 1e), which indicates that compound mutations of Kras and p53 gene are insufficient for inducing the abnormal proliferation of pancreatic cells. These results suggest that aberrations beyond Kras and p53 mutations are required for PDAC development.

**Reversible ductal metaplasia by in vivo reprogramming.** In order to investigate the influence of in vivo reprogramming in the pancreas, we next generated mice in which reprogramming factors can be induced specifically in the pancreas upon doxycycline (Dox) treatment. We established mice harboring Pdx1-ires-Cre, Rosa LSL-rtTA3, and Colla1:tetO-Oct3/4, Sox2, Klf4, c-Myc-ires-mCherry (hereafter C-OSKM) (Fig. 2a). After Dox treatment, we confirmed mCherry fluorescence in the pancreas of these mice.
**Fig. 1** Kras/p53 compound mutations are insufficient for PDAC development.  

**a** A schematic illustration of the genetic construct to activate HA-KrasG12D in the pancreas.  

**b** Representative histology and immunostaining for HA-KrasG12D. Scale bars, 50 μm.  

**c** Genetic strategy to study the influence of somatic activation of Kras and p53 mutations in the pancreas.  

**d** Southern blotting of LSL-KrasG12D and LSL-p53R172H allele. ESCs containing Pdx1 ires-Cre, LSL-KrasG12D and LSL-p53R172H alleles were used as control. Note that majority of two LoxP alleles are converted into one LoxP allele in the pancreas of KPC mouse (Cont control, Panc pancreas).  

**e** Immunostaining for pERK and Ki67 in the pancreas of 6-week-old wild-type mice, KC mice, and KPC mice. KPC mice at 8 weeks of age showed a focal PDAC area with pERK staining (bottom). Scale bars: HE and pERK (low magnification) staining, 200 μm; pERK (high magnification) staining, 50 μm; and Ki67 staining, 100 μm.
(Supplementary Fig. 2A). We also performed histological analysis of 4-week-old mice given Dox for 3 days (Fig. 2b).

It has been proposed that the transition of acinar cells into a duct-like state (acinar to ductal metaplasia (ADM)) is an early event of pancreatic cancer development. Notably, Dox-treated mice often exhibited dilatation of the acinar glands and expression of CK19, a ductal marker (Fig. 2c). CK19 expression was sometimes observed in amylase-expressing cells (Fig. 2c), suggesting that ADM occurs by the short induction of reprogramming factors. Consistent with this notion, Oct3/4 expression was observed in newly formed ductal cells (Fig. 2d). Notably, after 1 week of Dox withdrawal, the histology of the pancreas was almost normal, and CK19-expressing ADM lesions were hardly detected (Fig. 2e, f), presumably reflecting the

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epigenetic memory of acinar cells. Taken together, these results show that the 3-day expression of reprogramming factors causes reversible ADM phenotype.

A recent study demonstrated that continuous Klf4 overexpression induces ADM. To further investigate whether cellular reprogramming is indeed related to ADM formation, we next examined the effect of another reprogramming cocktail that lacks Klf4. For this purpose, we established ESCs with Dox-inducible polycistronic cassette containing Sall4, Nanog, Esrrb, and Lin28 (hereafter SNEL), which was reported as an alternative reprogramming factor cocktail, using the KH2-ESC system (Fig. 2g)⁹,¹⁰. To investigate the effect of SNEL expressions on reprogramming, mouse embryonic fibroblasts (MEFs) containing Dox-inducible SNEL alleles were established from E13.5 embryos. After 2 weeks of Dox exposure to SNEL-MEFs, iPSC-like colonies were observed (Supplementary Fig. 2B, C). Although these iPSC-like colonies could not be expanded, they exhibited increased alkaline phosphatase activity (Supplementary Fig. 2D), reflecting the partial reprogramming state. We then examined the in vivo effect of SNEL expression in the pancreas. After Dox treatment, SNEL-inducible chimeric mice exhibited mCherry fluorescence in various organs, including the pancreas (Supplementary Fig. 2E, F). Notably, Dox treatment longer than 2 weeks resulted in the formation of ADM lesions in the pancreas (Fig. 2h, i). Consistently, CK19-positive regions often co-expressed amylase (Fig. 2j), indicating that ADM was similarly induced by SNEL expression. Moreover, the ADM lesions were hardly detectable at 1 week after Dox withdrawal, suggesting that the SNEL-induced ADM phenotype is reversible (Supplementary Fig. 2G). Collectively, these results indicate that the transient expression of reprogramming factor cocktails induces reversible ADM phenotype.

In vivo reprogramming represses acinar cell enhancers. To investigate the molecular basis of reprogramming-mediated ADM formation, we first examined transcriptional changes of the pancreas during in vivo reprogramming. The pancreas of C-OSKM mice given Dox for 3 days was analyzed for global gene expressions by microarray. In accordance with histological evidence of ADM formation, the expression of acinar cell-related genes were upregulated in these samples (Fig. 3b), suggesting that the silencing of acinar cell-related genes is coupled with the induction of ADM-related genes.

Previous studies demonstrated that silencing of the somatic cell-specific enhancers is an initial epigenetic event during reprogramming. We next examined the effect of reprogramming factor expressions on the enhancers of pancreatic cells. ChiP-seq analysis for H3K27ac was performed using the pancreas of C-OSKM mice given Dox for 3 days. A perfusion fixation protocol was employed for cross-linking chromatin in vivo. Consistent with reduced expressions, H3K27ac deposition was significantly reduced at the enhancers of several acinar cell-related genes including Ptf1a, Mist1 (Bilha15), Nr5a2, and Cpa1 (Fig. 3c and Supplementary Fig. 3A) after Dox treatment, but it was not altered at the control Actb gene (Supplementary Fig. 3B). In contrast, H3K27ac deposition was not increased in upregulated genes, such as CK19 or in ADM-related genes (Supplementary Fig. 3C), which supports the notion that the repression of acinar cell enhancers is an initial epigenetic regulation step after reprogramming factor expression. Similarly, Sox9, a key TF in ductal cells, did not exhibit an enrichment of H3K27ac deposition (Supplementary Fig. 3C).

Furthermore, genome-wide analysis revealed that enhancers including super enhancers (SEs)⁹,¹⁰, which are significantly repressed after Dox treatment for 3 days (Fig. 3d and Supplementary Fig. 3D, E). Consistently, the number of enhancers and SEs were substantially decreased by Dox treatment for 3 days (Fig. 3d and Supplementary Fig. 3E). Moreover, the expressions of genes linked with Dox OFF-specific SEs were decreased by Dox treatment, while genes linked with common-SEs or Dox ON-specific SEs were not repressed (Fig. 3e). Ptf1a promoter and enhancer remained unmethylated in the pancreas after 3 days of Dox treatment (Supplementary Fig. 3F), indicating that DNA methylation was not involved in the Ptf1a repression. Notably, the Dox-induced reduction of H3K27ac deposition was not observed at 2 weeks after Dox withdrawal, indicating the repression of SEs is reversible (Fig. 3d). Collectively, these results suggest that reprogramming factor expression causes transient epigenetic repression of acinar cell enhancers and loss of acinar cell identity, which lead to ADM formation. To further connect the loss of acinar cell enhancer with ADM formation, we induced acinar-cell-related TFs (Mist1 or Ptf1a) together with OSKM in the pancreas (Supplementary Fig. 4A–C). Notably, the co-expression of Ptf1a or Mist1 with OSKM attenuated the OSKM-induced expression of CK19 and ADM formation (Fig. 3f and Supplementary Fig. 4D). Together, these results suggest that the silencing of acinar cell program causes the induction of ADM.

Finally, to connect OSKM-induced ADM formation with pathological conditions, we examined the effect of caerulein treatment, which is well-established pancreatitis stimuli and induces transient ADM formation, on pancreatic SEs. We...
performed intraperitoneal injection of caerulein eight times for 2 days (2 μg/injection every consecutive hour for 7 h) and performed ChIP-seq analysis for H3K27ac after 24 h at Day 3. Histological analysis revealed that OSKM-expressing pancreatic lesions phenocopies caerulein-induced ADM lesions (Supplementary Fig. 4E). Consistent with previous studies, we confirmed that the caerulein treatment causes reduced expression of acinar cell-related genes such as Ptf1a and Mist1 (Supplementary Fig. 4F). Of particular note, enhancers that are altered by OSKM expression were similarly altered by caerulein treatment.
In vivo reprogramming activates ERK in Kras mutant pancreas. To elucidate the influence of the loss of acinar cell identity on Kras-mutated pancreatic cells, we next established ESCs containing LSL-Kras^{G12D}, Pdx1-ires-Cre, Rosa LSL-rTA3, and Colla1:teto-OSKM-ires-mCherry (hereafter KC-OSKM) and generated chimeric mice to induce Kras mutation and reprogramming factors simultaneously in the pancreas (Fig. 4a). The experimental protocol of Dox treatment for OSKM induction is shown in Fig. 4b. Consistent with our findings that OSKM expression induces ADM, increased expression of CK19 was observed in both C-OSKM mice and KC-OSKM mice (Fig. 4c). Notably, pERK was strongly induced in CK19-positive area of KC-OSKM mice (Fig. 4c), suggesting that the increased EGFR expression was sustained in reduced acinar cell-related transcription factors (Kras mutation cooperatively activates the ERK pathway in conjunction with reprogramming factor expression (Fig. 4c). In sharp contrast to reversible ductal metaplasia in C-OSKM mice, CK19-expressing ducts were observed even at 1 week after Dox withdrawal in KC-OSKM mice (Fig. 4d), suggesting that the ADM phenotype is sustained in the presence of Kras mutation. Consistently, increased expression of pERK was sustained in the ductal area at 1 week after Dox withdrawal in KC-OSKM mice (Fig. 4e, f). Furthermore, ductal lesions were histologically displayed PanIN/PDAC (Fig. 4d, e, Supplementary Fig. 5A). The affected lesions sometimes displayed positive staining for Alcian blue, a PanIN marker, and often exhibited widespread Sirius red staining, which showed that massive fibrosis simultaneously occurs in PanIN/PDAC lesions within 10 days of Dox exposure (Fig. 4e, f, Supplementary Fig. 5B).

Previous studies have demonstrated that increased expression of endogenous EGFR activates the MAPK pathway in human and mouse PanIN/PDAC area. To unveil the molecular mechanism for persistent activation of the ERK signaling pathway in KC-OSKM mice, we examined EGFR expression in Dox-treated C-OSKM and KC-OSKM mice. Although Dox treatment for 3 days increased EGFR expression in both C-OSKM and KC-OSKM mice, the increased EGFR levels were pronounced in KC-OSKM mice, while only modest expression was observed in C-OSKM mice (Fig. 4g and Supplementary Fig. 5C). Furthermore, the increased EGFR expression was sustained in KC-OSKM mice even after Dox withdrawal, whereas C-OSKM mice showed almost no EGFR staining (Fig. 4g and Supplementary Fig. 5C, 5D), suggesting that Kras mutation is involved in robust and continuous EGFR expression through positive feedback regulation. Western blot confirmed the sustained activation of the ERK signaling pathway and increased EGFR expression in the pancreas of KC-OSKM mice even after Dox withdrawal (Fig. 4h, Supplementary Fig. 9). These results suggest that maintenance of the acinar cell trait prevents ERK signaling activation in the pancreas of Kras mutant mice.
Dox for 1 week, however, the p21- and γH2AX-positive area was substantially reduced in proliferating lesions of both KC-OSKM and KPC-OSKM mice (Supplementary Fig. 6A–C), suggesting that escape from senescence occurs regardless of the p53 status. Consistently, there was no significant difference in the survival rate between Dox-treated KC-OSKM mice and Dox-treated KPC-OSKM mice (Supplementary Fig. 6D), suggesting that Kras mutation alone together with transient repression of acinar cell enhancers cause the escape from senescence and the rapid PDAC development.

Taken together, our results demonstrate that the repression of acinar cell enhancers results in ADM formation and the...
subsequent cancer growth in Kras-mutated acinar cells of the pancreas. Finally, we tried to maintain acinar cell enhancers by expressing acinar cell-specific TFs (Ptf1a or Mist1) in caerulein-treated KC mice, in which caerulein-induced inflammation initiates ADM/PanIN formation and activates ERK signaling (Fig. 5f and Supplementary Fig. 4E). Notably, forced expression of either Ptf1a or Mist1, but not the control mCherry, strongly inhibited PanIN formation (Fig. 5g and Supplementary Fig. 7A). Consistently, pERK staining was significantly reduced by the expression of acinar cell-related TFs (Fig. 5h). These results provide additional evidence that acinar cell enhancers safeguard Kras-induced cancer initiation.

Undifferentiated cancer development by in vivo reprogramming. It has been shown that the acquisition of the pluripotency-related epigenetic landscape occurs at late-stage reprogramming. We next extended the duration of reprogramming factor expression in KC-OSKM mice to examine the effect of late epigenetic regulation of reprogramming on cancer development. KC-OSKM mice were given Dox for 1–2 weeks, followed by Dox withdrawal for 1–2 weeks. In contrast to the PanIN/PDAC formation in KC-OSKM mice given Dox for 3 days, prolonged treatment of Dox caused the development of histologically undifferentiated cancer with higher proliferative activity in the pancreas of KC-OSKM mice (Fig. 6a). Notably, these cancers exhibited the expression of pluripotency-related proteins, Sall4 and Lin28a, independently of OSKM transgene expressions (Fig. 6a).

We found that KC-OSKM mice given Dox for 1–2 weeks often developed undifferentiated cancers in the stomach (Fig. 6b and Supplementary Table 1), which is consistent with the observation that Cre-LoxP recombination also occurs in the pyloric glands of the stomach with Pdx1-ires-Cre allele (Supplementary Fig. 1B, C). These gastric cancers similarly exhibited the expression of Sall4 and Lin28a (Fig. 6c). In agreement with the acquisition of pluripotency-related epigenetic regulation, partial activation of ESC-Core and ESC-Myc modules was observed in gastric cancers in KC-OSKM mice (Supplementary Fig. 8A). C-OSKM mice treated with Dox for 1–2 weeks also developed undifferentiated cancer in the stomach (Supplementary Fig. 8B, C), albeit at lower frequency than KC-OSKM mice (p < 0.01, Fisher’s exact test, Supplementary Table 1), suggesting that the Kras mutation promotes but is not essential for the development of undifferentiated cancers.

In vivo reprogramming induces AFP-producing cancers. Human AFP-producing cancers predominantly arise in the stomach and display poorly differentiated histology and show dismal prognosis. Notably, we observed focal expression of AFP in gastric cancers in KC-OSKM mice given Dox for 1–2 weeks (Fig. 6c, d), which raised the possibility that the acquisition of pluripotency-related epigenetic regulation is associated with the development of human AFP-producing cancers. Therefore, we next investigated the expression of pluripotency-related proteins in human AFP-producing gastric cancers. Twenty-five gastric cancers that were diagnosed as AFP-producing gastric cancers, were examined for the expression of SALL4, LIN28A, and LIN28B by immunostaining. Notably, as observed in gastric cancers in KC-OSKM mice, SALL4, LIN28A, and LIN28B were frequently co-expressed in human AFP-producing gastric cancers (Fig. 6e, f and Supplementary Table 2), suggesting that these cancers exhibit activation of the pluripotency-related regulatory network.

Finally, we re-examined the microarray data previously used to investigate 200 cases of human gastric cancers. We found that AFP, LIN28B, and SALL4 were overexpressed in seven, eight, and nine cases of the 200 human gastric cancers, respectively. Consistent with the frequent co-expression of pluripotency-related proteins in human AFP-producing gastric cancers, partial activation of ESC-Core and ESC-Myc modules was observed in AFP-, LIN28B-, and SALL4-expressing human gastric cancers (Fig. 6g and Supplementary Fig. 8D), while such activations were not obvious in EBRB2-highly expressed gastric cancers (n = 12) (Supplementary Fig. 8D), which recapitulated our observations of reprogramming-induced gastric cancers in KC-OSKM mice.

Discussion
Cancer is believed to arise through an accumulation of genetic mutations. Indeed, genome-wide sequencing analyses have revealed a number of driver mutations in multiple cancer types. The functional significance of such driver mutations has been demonstrated by reverse genetic approaches where rodents in which these mutations were introduced develop the same types of cancer observed in human patients. However, the genetically engineered cancer models always exhibit a substantial latency period for cancer development, indicating that additional events are required for the development of full-blown cancer. Therefore, which sets of genetic mutations are sufficient for cancer development remains unclear in most cancers. Indeed, in the present study we show that most pancreatic cells with compound mutations at Kras and p53 display no obvious activation of the ERK signaling pathway and exhibit normal histology at 6 weeks of age, which is consistent with previous findings that oncogenic KRAS mutations are detectable in human disease-free pancreas.

Notably, we demonstrate that short, transient expression of reprogramming factors in Kras mutant mice at 4 weeks of age results in persistent ERK activation and widespread PDAC development within 10 days, indicating that cellular reprogramming is sufficient for the initiation of cancer growth in pancreatic cells with Kras mutation.

Alteration of the epigenetic modifications is one of the most common aberrations detected in human cancers. Decades of studies have demonstrated that epigenetic regulation plays a significant role in cancer development both in vitro and in vivo.
However, recent sequencing analysis identified that most cancer types harbor mutations at epigenetic modifier genes at varying frequencies, which has raised the possibility that genetic abnormalities are the primary cause of the dysfunction or dysregulation of epigenetic modifiers that result in secondary alterations in the cancer epigenome. Therefore, direct evidence of bona fide epigenetic alterations that drive cancer development is still limited, especially in vivo.

iPSC derivation is mediated through epigenetic reorganization without affecting genomic information. In the initial stage of
reprogramming in vitro, reprogramming factors repress somatic enhancers, which causes the repression of cell-type specific gene expressions. Consistently, the gradual repression of somatic cell-specific gene expressions is similarly observed during in vivo reprogramming. Notably, the early reprogramming process in vivo is reversible up to 7 days, which reflects epigenetic memory of the cells at early-stage reprogramming. In the present study, we show that in vivo reprogramming primarily represses acinar cell enhancers and induces remarkable down-regulation of acinar cell-related genes such as Ptf1a and Mist1, suggesting that in vivo reprogramming induces the loss of acinar identity. The fact that this loss is reversible further supports the notion that the process is an epigenetic event. Together, the rapid and widespread development of PDAC by 3-day expression of reprogramming factors provide in vivo evidence that transient alteration of epigenetic regulation plays a critical role on cancer initiation. Our results also highlight the significance of dedifferentiation-related epigenetic regulation in cancer initiation, which may provide mechanistic insight into how dedifferentiation is involved in cancer development.

Previous studies using genetically engineered mouse models show that adult ductal cells are refractory to Kras-mediated oncogenic transformation. In contrast, adult acinar cells are competent to give rise to PDAC in the presence of Kras mutation-mediated oncogenic transformation. As diagrammed in the pancreas, the fact that this loss is reversible further supports the notion that the process is an epigenetic event. Together, the rapid and widespread development of PDAC by 3-day expression of reprogramming factors provide in vivo evidence that transient alteration of epigenetic regulation plays a critical role on cancer initiation. Our results also highlight the significance of dedifferentiation-related epigenetic regulation in cancer initiation, which may provide mechanistic insight into how dedifferentiation is involved in cancer development.

In summary, taking advantage of in vivo reprogramming systems, we show that reprogramming-related epigenetic regulation has a profound impact on Kras-induced cancer. We propose that epigenetic fluctuations provoked by environmental factors may cause a transient dedifferentiation state, which is sufficient for continuous ERK signaling activation and cancer growth of quiescent pancreatic cells with Kras mutation.

Methods

Vectors. Pdx1-ires-Cre vector: The cDNA fragment (3.8 kb) of Pdx1-ire-Cre-pA-PGK-Puro-pA with 50 bp homologous arms was established using KAPA HiFi Hotstart ReadyMix (KAPA Biosystems). This fragment was recombined into the 3′ side of the exon 2 of Pdx1 BAC (BACPAC Resources Center) using the Red/ET BAC recombination system. The fragment of Pdx1-ires-Cre-pA-PGK-Puro-pA sequence with 3.8 (5′ arm)/2.5 kb (5′ arm) homologous arm was retrieved and used as a targeting vector. LSL-HA tag-KrasG12D vector: LoxP-PGK-Bsd-pA-LoxP-HA tag-KrasG12D fragment (1.9 kb) with 350/750 bp homologous arms was generated and recombined into the 5′ side of the untranslated region of the Kras BAC (BACPAC Resources Center), retrieved with 4.7 (5′ arm)/4.7 kb (3′ arm) homologous arm, and used as a targeting vector. Rosa LSL-rtTA3 vector: rtTA3 cDNA construct with Kozak sequence (753 bp) was generated and inserted into pROSA26-DEST vector (Addgene) using Gateway technology (Thermo Fisher Scientific) and as used as a targeting vector. pCR8-GW-SNEL vector: A 6.4 kb fragment containing Sall4-P2A-Nanog-T2A-Envb-E2A-Lin28a cDNA was generated using In-Fusion HD Cloning Kit (Takara Bio). The cloning primers are shown in Supplementary Table 3. The fragment of Sall4-P2A-Nanog-T2A-Envb-E2A-Lin28a cDNA (717 bp) was cloned using Life Technologies Gateway technology and inserted into pCR8-GW-TOPO using Topo cloning technology (Invitrogen) and inserted into Col1a1 locus of KH2 ESCs using the Flp in recombination system. Rosa:teto-mCherry vector: mCherry cDNA (717 bp) was cloned using the primers described in Supplementary Table 3. The mCherry cDNA construct was added to the 5′ side of Sall4 cDNA. This fragment was inserted into pCR8-GW-TOPO vector using Topo cloning technology (Invitrogen) and inserted into Rosa:teto-attr1-R2-mCherry targeting vector as described previously using Gateway technology. Rosa:teto-OSKM-mCherry vector: Mist1 cDNA (600 bp) was cloned using the primers described in Supplementary Table 3. The Mist1 cDNA construct was added to the 5′ side of Mist1 cDNA. This fragment was inserted into pCR8-GW-TOPO vector using Topo cloning technology (Invitrogen) and inserted into pCR8-GW-TOPO vector using Topo cloning technology (Invitrogen) and inserted into Rosa:teto-attr1-R2-mCherry targeting vector as described previously using Gateway technology.

Experimental protocol for Dox treatment in mice. a schematic illustration of the genetic construct of Pdx1-ire-Cre, LSL-KrasG12D, LSL-p53R172H, Rosa LSL-rtTA3, Col1a1::tetO-OSKM-ires-mCherry (KPC-OSKM) mice to induce pancreas-specific Kras-p53 compound mutations and in vivo reprogramming. b Experimental protocol for Dox treatment in KPC-OSKM chimeric mice. c Representative images of the pancreases of C-OSKM, KC-OSKM, KPC-OSKM mice given Dox for 3 days, followed by Dox withdrawal for 1 week. Scale bars, 500 μm. d pERK and Sirius red staining of the pancreas in KPC-OSKM mice. Scale bars, 100 μm. e Quantification of the PDAC area in the pancreas of KC-OSKM and KPC-OSKM chimeric mice. Scale bars, 500 μm. f Quantification of the PDAC area in the pancreas of KC-OSKM and KPC-OSKM chimeric mice. Scale bars, 500 μm. g Quantiﬁcation of the pERK-positive area/mCherry-positive area in the pancreas of KPC-mCherry, KC-Ptf1a, and KC-Mist1 mice. Scale bars, 100 μm. h Quantiﬁcation of the pERK-positive area/mCherry-positive area in the pancreas of KPC-mCherry, KC-Ptf1a, and KC-Mist1 mice. Scale bars, 100 μm. i Quantiﬁcation of the pERK-positive area/mCherry-positive area in the pancreas of KPC-mCherry, KC-Ptf1a, and KC-Mist1 mice. Scale bars, 100 μm. j Quantiﬁcation of the pERK-positive area/mCherry-positive area in the pancreas of KPC-mCherry, KC-Ptf1a, and KC-Mist1 mice. Scale bars, 100 μm. k Quantiﬁcation of the pERK-positive area/mCherry-positive area in the pancreas of KPC-mCherry, KC-Ptf1a, and KC-Mist1 mice. Scale bars, 100 μm. l Quantiﬁcation of the pERK-positive area/mCherry-positive area in the pancreas of KPC-mCherry, KC-Ptf1a, and KC-Mist1 mice. Scale bars, 100 μm. m Quantiﬁcation of the pERK-positive area/mCherry-positive area in the pancreas of KPC-mCherry, KC-Ptf1a, and KC-Mist1 mice. Scale bars, 100 μm. n Quantiﬁcation of the pERK-positive area/mCherry-positive area in the pancreas of KPC-mCherry, KC-Ptf1a, and KC-Mist1 mice. Scale bars, 100 μm.
Fig. 6 Prolonged expression of reprogramming factors induces poorly differentiated cancers in KC-OSKM chimeric mice. 

a Sall4, Lin28a, and Ki67 staining in undifferentiated pancreatic cancer induced by Dox treatment for 1 week followed by Dox withdrawal for 1 week in KC-OSKM chimeric mice. Scale bar, 50 μm. 

b Representative macroscopic image, histology, and Ki67 immunostaining of gastric cancer induced by prolonged transient expression of reprogramming factors in KC-OSKM chimeric mice (Dox On for 2 weeks and then Dox Off for 2 weeks). Scale bars, 100 μm. 

c Immunostaining for Sall4, Lin28a, and Afp in poorly differentiated gastric cancers in KC-OSKM chimeric mice. Scale bars, 100 μm. 

d Incidence of Afp, Sall4, and Lin28a expression in poorly differentiated gastric cancers in KC-OSKM chimeric mice. 

e Histology and immunostaining for SALL4, LIN28A, and LIN28B in human AFP-producing gastric cancers. Scale bar, 100 μm. 

f Incidence of SALL4-, LIN28A-, and LIN28B-positive cases in human AFP-producing gastric cancers. 

g Partial activation of ESC-Core and ESC-Myc modules in human gastric cancers expressing Afp or SALL4.
inserted into Rosa::tetO-attR1-cdLB-attR2-tres-mCherry targeting vector by Gate-
way technology. Rosa::tetO-Ptf1a vector: Ptf1a cDNA was synthesized using a commercial service (Eurofins genomics). The Kozak sequence was added to the 5′ side of Ptf1a cDNA. The cDNA fragment which was cut by EcoRI and purified with Gel/PCR Extraction Kit (FastGene), inserted into pCR8-GW-TOPO vector, and recombinated into Rosa::tetO-attR1-cdLB-attR2-tres-mCherry targeting vector by Gateway technology. Piggybac tetO-EGFP::ires-EGFP vector: The pCR8-GW-TOPO vector described above was inserted into Piggybac tetO-attR1-cdLB-attR2-tres-mEGFP vector (KW509) using Gateway technology. Piggybac tetO-Mist1-tres-EGFP vector: The Mist1 cDNA from pCR8-Ptf1a-tres-vector described above was inserted into Piggybac tetO-attR1-cdLB-attR2-tres-mEGFP vector (KW509) using Gateway technology.

Homologous recombination into ES cells. The ESC lines V6.5 and KH2 were previously described.24 Twenty micrograms of each targeting vector were linearized using specific restriction enzymes (PciI-tres-Cre targeting vector: Scal, LSL-HA tag-Krai3) and inserted into 100 μl of high plasmid DNA (Nacalai tesque) containing 25 mM HEPEs. A mixture of the targeting vector and ESCs (4.0 × 106) was transfected with Qiagen IluX-I'TraX targeting vector: AsSiL, Rosa LSL-rTA3 targeting vector: PcnL, RosA::tetOnCreC (Gibco), and 1000 U/mL human LIF (Wako)). After 24 h of electroporation, we started the selection with the specific antibiotics. The concentrations of antibiotics were puromycin (3 μg/mL, and hygromycin B (Roche) 150 μg/mL. For quantitative PCR (qPCR) and ChIP-seq of the pancreas of E13.5 of KH2-SNEZ mice, we started Dox administration to induce Pdx1-ires-Cre, Rosa LSL-LacZ and Mice. Rosa LSL-LacZ mice16, LSL-Kra41215 mice16, LSL-p53R172H mice19, and Col1a1::tetO-OSKM-ires-mCherry mice60 have been described previously. All mice were maintained on a C57BL/6 and 129X1/Sv mixed background. For germline transmission, 8-week-old male chimeric mice were mated with 8-week-old C57BL/6 female mice (Japan SLC) to obtain transgenic mice. All animal studies were conducted in compliance with ethical regulations in Kyoto University and were approved by Center for iPS Cell Research and Application (CiRA), Kyoto University. To obtain the unbiased and reliable results, more than five mice were used in each group. Sample size was determined in each figure. A few mice had been excluded because of the mortality before the evaluation. The coat color chimera of chimeric mice was evaluated before experiments (Supplementary Fig. 5B). Mice that had no chimera contribution had been excluded in each experiment.

Mice genotype. Ear tips of 3-week-old mice were collected and dissolved in 500 μL of DNA elution buffer (containing 100 mM Tris HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 1% Protein kinase) at 65 °C. After centrifugation at 4 °C and 15,000 rpm for 5 min, 300 μL of supernatant was collected and mixed with equivalent isopropanol and vortexed. After centrifugation at 4 °C, the pellets were washed 70% ethanol and then dried. DNA was dissolved in 10 μL of TE buffer.

Alkaline phosphatase staining of Dox-treated KH2-SNEZ MEFs. MEFs from E13.5 of KH2-SNEZ mice were cultured with 2.0 μg/mL of Dox in ESC medium for 2 weeks. ALP staining was performed according to the manufacturer’s instructions using the ALP stain Kit (Muto Pure Chemicals).

X-gal staining. The precise procedure was described previously.24 Whole mount staining: Mice under anesthesia were perfused through the vascular system with 10 mL of phosphate-buffered saline (PBS) to clear the blood. Perfusion was performed with 10 mL of PBS containing 10% FPA, 200 mM NaCl, and 1% Protein kinase at 4 °C for 10 min. The tissues were embedded in Tissue-Tek O.C.T compound (Sakura) and frozen tissue sections were cut serially into 10-μm-thick sections and stained with the procedure described above. Counterstaining was performed using Nuclear Fast Red (Merck Millipore).

Blastocyst collection and microinjection. Blastocysts collection: 8-week-old ICR female mice (Japan SLC) were treated with 7.5 U of serotonin (ASKA Animal Health) by intraperitoneal injection. After 48 h of serotonin treatment, mice were injected with 7.5 U of gonadotropin (ASKA Pharmaceutical). The mice were then mated with ICR male mice (Japan SLC). Plague was checked the next morning. Two days later, these female mice were sacrificed by cervical dislocation and operated to pick out oviducts. Two-cell stage fertilized eggs were collected by perfusion with M2 medium (Sigma) and maintained in KSOM medium. Two days later, the obtained blastocysts were used for microinjection. For microinjection, 25 μL of ESCs were treated with trypsin and pipetted 15 times to dissociate them into single cells. The single cells were cultured in a gelatin-coated 10-cm dish with 10 mL ESC medium for 30 min to attach MEFs onto the dish. Three milliliters of supernatant containing ESCs was collected. Three to five ESCs were injected into the ICR blastocysts under an OLYMPUS BX51 microscope. Twenty to 25 injected blastocysts were transplanted into the uterus of pseudopregnant ICR female mice (Japan SLC).

Establishment of ESCs. Collected blastocysts were placed on the MEFs in a 24-well dish and cultured with 500 μL of 21-containing ESC medium (1 μM of PD0325901 (STEMGENT) and 3 μM of CHIR99021 (STEMGENT) in ESC medium)22. One hundred microliters of 2i-ESC medium was added into each well every day. One week later, the colonies were passaged and expanded in ESC medium on the MEFs. Male ESCs were selected by genotyping. Sprague-Dawley using Go 

Caerulein treatment. The precise procedure was described previously.25 We performed intraperitoneal injection of caerulein (Sigma) eight times for 2 days (2 μg/injection every consecutive hour for 7 h). Caerulein was dissolved in PBS.
RNA extraction from tissues. The tissues of mice were freshly collected and dipped into 1 mL of RNA later (Introntein) overnight at 4 °C. Subsequently, the tissues were poured into 1 mL of Sepasol (Nacalai tesque) and homogenized with a sonicator (Quonica) for 5 s. After centrifugation at 4 °C at 12,000 g for 10 min, supernatants were carefully collected and mixed with 200 μL of chloroform (Wako). After shaking for 15 s, the mixtures were left to stand for 2 min at room temperature and centrifuged at 4 °C and 12,000 g for 15 min. Three hundred microliters of supernatant was carefully collected and mixed with 300 μL of 70% ethanol. The mixtures were injected into RNeasy spin column (Qiagen), and RNA was extracted according to the manufacturer’s instructions.

Quantitative RT-PCR. RNA was extracted using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA was quantified with NanoDrop (Thermo Scientific). Five-hundred nanograms of RNA was used for the reverse transcription reaction into cDNA by using PrimerScript RT reagent Kit (Takara Bio). Real-time qPCR was performed by using Go Taq qPCR Master Mix (Promega). Transcript levels were normalized by β-actin. The PCR primers are shown in Supplementary Table 6.

Western blotting from the pancreatic tissue. The pancreatic tissues were sectioned into 5 × 5 mm and shattered with a sonicator (Quonisa) for 5 s in RIPA buffer (10 mM of Tris HCl (pH 8.0), 150 mM NaCl, 1% Triton, 1% DOC, and 0.1% SDS) containing 0.5% Protease inhibitor, 1% DTT and 100× phosphate buffer inhibitor (Nacalai tesque) on ice. After centrifugation at 20,000 g for 10 min, the 100 μL of supernatants were collected. The protein was quantified with a Molecular Probes (Life Technologies) Quick start protein assay Kit (BIO-RAD). The samples were processed in 30 μg, and denature at 95 °C for 5 min. Electrophoresis was performed in 10% SDS-PAGE gel, 100 for 15 and 200 V for 35 min with the use of PowerPac HC (BIO-RAD). Next, the protein in the gel was transferred onto the Amersham Hybond-P PVDF Membrane (GE Healthcare). Membranes were blocked for 60 min with 4% Skim Milk (Nacala Tesque) in 1× TBS with 0.05% Tween-20 (TBST) and then incubated with primary antibodies in blocking buffer (4% Skim Milk in TBST) overnight at 4 °C. The lists of antibodies and dilutions are shown in Supplementary Table 7. The membrane was washed in TBST and incubated with secondary antibodies in blocking buffer for 2 h at room temperature. The membrane was washed in TBST again, then incubated with Pierce ECL plus Western Blotting Substrate (Thermo Scientific) for visualization. The bands were detected with LAS4000 (GE HealthCare).

Paraffin-embedded specimen preparation. The dissected tissue samples were fixed in 4% PFA (Wako) for overnight at room temperature. The next day, the samples were poured into 70% ethanol (Wako) for several hours, and then poured into 80% ethanol, 90% ethanol, 100% ethanol, and xylene (Wako), and embedded in paraffin using a spin tissue processor (STP120; Thermo Scientific). Sections were sliced into 3–4 μm thickness, and stained with hematoxylin and eosin (Muto Pure Chemicals). Serial sections were used for the immunohistochemical analyses.

Immunostaining. The samples were soaked in xylene for 5 min three times to remove paraffin and 100% ethanol for 5 min three times to hydrophilize. After washing with water for several min, the samples were soaked in epitope retrieval buffer (Nichirei Bioscience) and microwaved at 100 W for 10 min. The samples were then soaked in 1× PBS for several min, and incubated with 150 μL primary antibodies in PBS with 2% bovine serum albumin (BSA) (MP Biomedicals) for 1 h at room temperature. The lists of antibodies and dilutions are shown in Supplementary Table 7. After washing with PBS for 5 min two times, the samples were processed with secondary antibodies for 30 min at room temperature. The samples were washed in PBS for 5 min two times, and nuclear staining was performed with hematoxylin for 10–30 s. After washing with water for 10 min, the samples were poured into 100% ethanol three times and placed in xylene three times. The samples were evaluated under a microscope (Olympus BX41).

Immunofluorescent staining. The same procedure as immunostaining described above was performed until primary antibodies treatment. The samples were processed with 500× DAPI (Introntein) and 150× fluorescence-labeled secondary antibodies diluted with 0.5% BSA PBS, for 90 min at room temperature. The lists of antibodies and dilutions are shown in Supplementary Table 7. After washing in PBS for 5 min two times, the samples were mounted and evaluated with a confocal laser scanning microscope (Zeiss LSM700).

Alcian blue staining. Tissue samples were soaked in xylene for 5 min three times, 100% ethanol for 5 min three times, washed with water for several minutes, and finally stained with Alcian blue 8GX solution (Merck Millipore) and nuclear fast red (Merck Millipore), according to the manufacturer’s protocol.

Sirius red staining. Tissue samples were soaked in xylene for 5 min three times and 100% ethanol for 5 min three times, washed with water for several minutes, and stained with Picro-Sirius Red Stain Kit (PSI).

Microarray analysis. Two hundred nanograms of total RNA prepared from the pancreatic tissues was subjected to cDNA synthesis with a WT Expression Kit (Ambion), and the resultant cDNA was fragmented and hybridized to a Mouse Gene 1.0 ST Array (Affymetrix). After hybridization, GeneChip arrays were washed and stained using GeneChip Fluidics Station 450 (Affymetrix) and detected with the Scanner 3000 TG system (Affymetrix) following the manufacturer’s standard protocols. The data were analyzed using GeneSpring GX software (version 14.8; Agilent Technologies).

Chromatin immunoprecipitation from pancreatic tissue. Perfusion fixation with ice-cold 4% PFA (Wako) was employed for fixation of the pancreatic tissue of C57BL/6 mice. The pancreas was dissected after 5 min of fixation and treated with glycine to quench the formaldehyde. ChiP was performed as described previously. H3K27Ac antibody (Monoclonal Antibody Lab) was used for the ChiP analysis, and mouse IgG antibody (Abcam) was used as a control.

ChiP-seq analysis. Purified ChiP DNA (5 ng per sample) was used for the generation of sequencing libraries with TruSeq ChiP Sample Prep Kit (Illumina). The resultant DNA libraries were assessed on Agilent Bioanalyzer and quantified with KAPA Library Quantitation Kits (KAPA BIOSYSTEMS). The libraries were sequenced to generate single-end 80 bp reads using NextSeq 500 (Illumina).

ChIP-seq data analysis. ChiP-Seq Peak calls were made as previously described. Briefly, the low-quality bases at the 3′ end reads and the adaptors were trimmed using cutadapt-1.9.1 (ref. 63). Peaks longer than 14 nucleotide were mapped to mouse genome (mm10) with BWA 0.7.12 (ref. 64). Duplicate reads and low mapping quality reads (MAPQ < 20) were removed using Picard (http://broadinstitute.github.io/picard/) and SAMtools65 for further analysis. Mapped reads were visualized using igv-2.3 (ref. 66). Peaks were called using MACS2 (ref. 67) plus IDR framework developed by the ENCODE project52. The reads mapped to the blacklisted regions (https://sites.google.com/site/anshulkundaje/projects/blacklists) and sex chromosomes were excluded for the peak calling. The heatmaps for ChiP-Seq data were drawn using the ngsplot-2.47 program52. Super enhancers were identified by HSK27ac with ROSE pipelines52,60. Super enhancers identified in this study were assigned to the transcripts whose TSS were within the SEs.

Bisulfite sequencing from pancreatic tissue. Freshly collected pancreatic tissues were frozen in liquid nitrogen and grounded into powder using mortar. Genome DNA of the pancreas was extracted using PureLink Kit (Invitrogen) according to the manufacturer’s protocol. Bisulfite treatment was performed using EZ DNA Methylation-Gold Kit (Zymo Research). The target sequence was cloned by Ex Taq HS (Takara Bio) and ligated into pCR4-Topo vector using Topo cloning technology (Invitrogen). These plasmids were transformed into DH5α at 37 °C, overnight. Colony PCR was performed using Go taq master Mix (Promega) and ascertained ligation of the plasmids. After cleavage of the PCR products by exonuclease I (New England Biolabs) and shrimp alkaline phosphate (Takara Bio), the sequence reaction was performed using M13 8v primer (5′-CAGGAAAA-CAGCTATGAC-3′) and was sequenced with ABI 3500 xl (Applied Biosystems). Methylation rates were measured using Quantification tool for Methylation Analysis (QUMA) software (Riken).

Quantification analysis. To quantify the immunostained tissue samples, immunostained section was randomly photographed at ×200 magnification three times using each mouse. The pictures were processed with ImageJ software (NIH) to evaluate the positive area of each CK19, phospho-ERK, Alcian blue, Sirius red, and EGFR staining. The positive area was determined as the mean of positive area in three independent histological images. The results were evaluated with Graphpad Prism 7 software for quantification. To assess H2AX, p21, Ki67, Caspase 3-positive rates, we randomly photographed epithelial area of the pancreas at ×200 magnification. The positive nucleus of epithelial cells was counted. The number of each positive nucleus was divided by the number of total nucleus of epithelial cells. For quantification of immunofluorescent samples, section was randomly photographed at ×200 magnification. The ratio of CK19-positive cells in EGFP-expressing cells was determined in ten independent immunofluorescent images for each cohort.

Statistical analysis of tissue samples. Statistical parameters including statistical significance and n values are described in the figures. Statistical analyses were carried out using Prism 7 Software (GraphPad). For statistical comparison of two groups, we performed Mann–Whitney U-test (two-tailed, 95% confidence intervals). A box-and-whisker plot is used to display information about the range, the median and the quartiles. Solid lines in each box indicate the median. Bottom and top of the box are lower and upper quartiles, respectively. Whiskers extend to ±1.5 interquartile range (IQR). Numbers in parentheses indicate the number of mice examined. *p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant.
Patients-derived paraffin-embedded tissue samples. Human patients-derived paraffin-embedded tissue samples were used in compliance with ethical regulations in Kyoto University Hospital.

Statistical analysis of survival. Kaplan–Meier survival curves were calculated using the survival time for each mouse. The Log-rank test was used to test for significant differences between the groups using Prism 7 Software (GraphPad).

Data availability. ChiP-seq and microarray data reported in this study have been deposited to the Gene Expression Omnibus (GEO) under accession GSE100842.

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Author contributions

H.S., N.S., A.T., T.U., M.K., S.S., B.K., K.W., Y.I., Y.K., T.Y. and Yasuhiro Y. conceived the ideas and designed the experiments, acquired the data and performed the analyses and interpretations. S.K., Yosuke Y., N.S., A.T., T.U., T.Y., and Yasuhiro Y. provided technical assistance. S.K. and A.T. assisted with mouse experiments. Yosuke Y. and H.H. provided clinical samples and contributed to clinical data analyses. N.S., K.W., and Y.K. provided materials. N.S., Y.Y., H.H., and Yosuke Y. contributed to pathological examination. H.S. and Yasuhiro Y. wrote the manuscript.

Additional information

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