

Overexpression of gankyrin in mouse hepatocytes induces hemangioma by suppressing factor inhibiting hypoxia-inducible factor-1 (FIH-1) and activating hypoxia-inducible factor-1

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Abstract

Gankyrin (also called p28 or PSMD10) is an oncoprotein commonly overexpressed in hepatocellular carcinomas. It consists of 7 ankyrin repeats and interacts with multiple proteins including Rb, Cdk4, MDM2 and NF- κ B. To assess the oncogenic activity *in vivo*, we produced transgenic mice that overexpress gankyrin specifically in the hepatocytes. Unexpectedly, 5 of 7 F2 transgenic mice overexpressing hepatitis B virus X protein (HBX) promoter-driven gankyrin, and one of 3 founder mice overexpressing serum amyloid P component (SAP) promoter-driven gankyrin developed hepatic vascular neoplasms (hemangioma/hemangiosarcomas) whereas none of the wild-type mice did. Endothelial overgrowth was more frequent in the livers of diethylnitrosamine-treated transgenic mice than wild-type mice. Mouse hepatoma Hepa1-6 cells overexpressing gankyrin formed tumors with more vascularity than parental Hepa1-6 cells in the transplanted mouse skin. We found that gankyrin binds to and sequester factor inhibiting hypoxia-inducible factor-1 (FIH-1), which results in decreased interaction between FIH-1 and hypoxia-inducible factor-1 α (HIF-1 α) and increased activity of HIF-1 to promote VEGF production. The effects of gankyrin were more prominent under 3% O₂ than 1% or 20% O₂ conditions. Thus, the present study clarified, at least partly, mechanisms of vascular tumorigenesis, and suggests that gankyrin might play a physiological role in hypoxic responses

besides its roles as an oncoprotein.

Keywords: PSMD10; HIF-1; FIH-1; oncogene; hemangioma

Abbreviations: CAD, C-terminal transactivation domain; DEN, diethylnitrosamine; FIH-1, factor inhibiting hypoxia-inducible factor-1; firefly-luciferase, F-Luc; H&E, hematoxylin and eosin; HBX, hepatitis B virus X protein; HCC, hepatocellular carcinoma; HIF, hypoxia-inducible factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SAP, serum amyloid P component.

1. Introduction

Gankyrin (also called p28, p28^{GANK} or PSMD10) was identified as an oncoprotein commonly overexpressed in hepatocellular carcinomas (HCCs) [1]. Gankyrin was also independently isolated as p28, a supposed component of the 26S proteasome, but recent studies have demonstrated that p28 associates only with free 19S particles of the 26S proteasome or their precursors and functions as a chaperone to guide their assembly [2]. As expected for a protein consisting of 7 ankyrin repeats [3], gankyrin interacts with multiple proteins and shows a variety of activities. For example, gankyrin binds to Rb and Cdk4, and accelerates phosphorylation and degradation of Rb to activate DNA synthesis genes [1]. Gankyrin binds to the E3 ubiquitin ligase MDM2, thereby facilitating ubiquitylation and degradation of p53 [4]. Gankyrin binds to NF- κ B and suppresses its activity by modulating acetylation via SIRT1 [5]. Gankyrin binds to hepatocyte nuclear factor 4 α [6] which determines hepatocyte differentiation status and enhances its degradation [6]. Gankyrin activates PI3K/AKT/mTOR/hypoxia-inducible factor-1 (HIF-1) signaling [7].

Most solid tumors contain hypoxic regions, and one of the most important cellular factors involved in the hypoxic response which promotes angiogenesis, anaerobic metabolism and resistance to apoptosis is HIF-1 [8, 9]. HIF-1 is a heterodimeric transcription factor composed

of a constitutively expressed  subunit and an inducibly expressed α subunit (HIF-1 α). Under aerobic conditions, HIF-1 α is hydroxylated by specific prolyl hydroxylases at two conserved Pro residues in a reaction requiring oxygen. Hydroxylation facilitates binding of von Hippel–Lindau protein, a component of the ubiquitin protein ligase, to HIF-1 α , leading to its proteasomal degradation. The ability of HIF-1 α to activate transcription is also prevented by factor inhibiting HIF-1 (FIH-1) [8-10]. FIH-1 hydroxylates a specific Asn residue in HIF-1 α , and disrupts interaction of HIF-1 α with the transcription co-activators p300 and CBP. Under hypoxic conditions, prolyl hydroxylase and FIH-1 activities are inhibited by substrate (O_2) deprivation, resulting in HIF-1 α stabilization and binding to the p300/CBP complex, thus allowing HIF transactivation.

Since gankyrin plays important roles in cell proliferation and apoptosis, is overexpressed in most HCCs, and confers tumorigenicity to non-malignant cells, we produced transgenic mice that overexpressed gankyrin specifically in the hepatocytes to assess its oncogenic activity *in vivo*. Unexpectedly, the mice developed vascular tumors (hemangioma/hemangiosarcomas) in the liver, and so we have tried to elucidate the underlying mechanisms for vascularization.

2. Materials and methods

2.1. Transgenic mice. To express gankyrin specifically in the liver, cDNA for the mouse wild-type gankyrin N-terminally tagged with 2x FLAG was cloned into the pBEPBgIII expression vector containing the hepatitis B virus X protein (HBX) promoter [11]. Fertilized eggs were obtained from C57BL/6J mice, and transgenic mice were produced at the Center for Animal Resources and Development, Kumamoto University, Japan. A plasmid containing the human serum amyloid P component (SAP) promoter [12] and expressing mouse wild-type gankyrin N-terminally tagged with 3x FLAG was also constructed, and transgenic mice were produced with this at the Genome Information Research Center, Osaka University, Japan, using eggs from D2B6F1 mice. For genotyping, DNA was extracted from the tail of each mouse and analyzed by Southern blotting using gankyrin cDNA as probe.

2.2. Treatment of mice. A single intraperitoneal injection of diethylnitrosamine (DEN, Sigma, 25 mg/kg of body weight) was administered to 14-day-old transgenic and control male mice. Groups of animals were euthanized at 8 months after injection, and the livers were removed, examined for visible lesions, and paraffin embedded after fixation in 10% buffered formalin.

For tumor formation, cells (2×10^6) were suspended in 0.1 ml of PBS and injected subcutaneously into the back of athymic BALB/c mice (Japan SLC Inc.). Each mouse received

Hepa1-6 cells on one side and Hepa1-6/GK cells on the other side. All experiments involving mice were approved by the Animal Research Committee of Kyoto University, and conducted in accordance with the institutional and NIH guidelines for the care and use of laboratory animals.

2.3. Human materials. Eighteen specimens of HCC were taken by needle biopsy before initiation of the treatment at Kinki University Hospital, Japan. The study protocol was approved by the institutional review boards, and written informed consent was obtained from all patients for subsequent use of their collected tissues.

2.4. Cell culture and DNA transfection. U-2 OS cells, HEK293 cells, HEK293T cells, mouse hepatoma Hepa1-6 cells and their transfectants were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO₂ as described [4]. For mild hypoxic conditions, cells were placed in a modular incubator chamber and flushed with a gas mixture containing 1 or 3% O₂, 5% CO₂, and balance N₂.

Calcium phosphate-DNA coprecipitation method was used for DNA transfection. Plasmids encoding, gankyrin, shRNA for gankyrin, HIF-1 α [14], FIH-1, and their fusion proteins have been described previously [4, 5, 10, 13].

2.5 Pathological analyses. The immunohistochemical staining was performed on 4- μ m-thick

paraffin sections of tissues fixed in 10% buffered formalin as described [14]. The sections were incubated with the primary antibodies against endothelial cell markers CD31 (dianoba GmbH) and CD34 (Abnova), followed by horseradish peroxidase–conjugated anti-rat immunoglobulin antibody (Santa Cruz Biotechnology), and were developed in Diaminobenzidine colorimetric reagent solution (DAKO). They were counterstained with hematoxylin. To assess the presence of the atypical proliferative lesion of endothelial cells, at least 1 section from 4 lobes were examined under a microscope.

2.6. Analyses of gene expression and protein interactions. Preparation of cell lysates, immunoprecipitation, and Western blot analysis were performed as described [4]. Rabbit polyclonal anti-gankyrin, anti-VEGF-A, anti-HIF-1 α , anti-FIH-1, anti- β -actin, and biotin-conjugated anti-HA antibodies (all from Santa Cruz Biotech.), anti-Myc tag antibody (MBL), anti-FLAG and biotin-conjugated anti-FLAG antibodies (Sigma), mouse monoclonal anti-HA antibody (Roche), and rabbit polyclonal antibody raised against recombinant mouse gankyrin were used as the primary antibodies in Western blotting.

For immunoprecipitation, mouse anti-HA antibody (Roche), rabbit anti-FLAG antibody, and biotin-conjugated anti-FLAG and anti-HA antibodies were used.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was done as described [14]. The relative levels of gankyrin and VEGF-A mRNAs were determined by RT-

qPCR using β -actin and GAPDH mRNA for normalization. Primer sequences used were as follows: gankyrin (human, 5'-TCTTCAAGCCATCCTGTGTG -3' and 5'-TGGTGATGTTGGACTCCTCA -3'), VEGF-A (human, 5'-AAAAGTCTGGTGTCCCAAG-3' and 5'-ATTAAACCCAGGCCACCTTT -3'; mouse, 5'-CAGGCTGCTGTAACGATGAA-3' and 5'-TATGTGCTGGCTTTGGTGAG-3'), β -actin (human, CTACGTCGCCCTGGACTTCGAGC and GATGGAGCCGCCGATCCACACGG), GAPDH (mouse, 5'-ACAACCTTTGTCAAGCTCATTCCTG-3' and 5'-TGGTCCAGGGTTTCTTACTCCTTGG-3').

2.7. Reporter assays. The reporter plasmids p2.1 and p2.4 contain wild-type and mutant copies, respectively, of the hypoxia response element (HRE) from the *ENO1* gene upstream of an SV40 promoter and firefly luciferase (F-Luc) coding sequences [13]. U-2 OS cells were cotransfected with either p2.1 or p2.4, pRL vector expressing Renilla luciferase (pRL-CMV, Promega) and plasmids expressing HA-FIH-1 and FLAG-gankyrin or gankyrin-shRNA [4].

The GAL4 reporter plasmid GAL4E1bLuc containing five GAL4-binding sites upstream of an E1b TATA sequence and the F-Luc gene, and GAL4-expressing plasmids GalA(531-826) and GalA-N803 expressing the C-terminal transactivation domain (CAD) of the wild-type and FIH-1-insensitive HIF-1 Δ , respectively, fused to the GAL4 DNA-binding domain were described

previously [15]. U-2 OS cells were cotransfected with GAL4E1bLuc, pRL-CMV, GAL4-expressing plasmids, and plasmids expressing FLAG-gankyrin or gankyrin-shRNA.

Transfected cells were exposed to mild hypoxia (1 or 3% O₂) for 48 h and harvested for dual luciferase assays (Promega) as described [5].

2.8. Statistical analysis. To determine whether the means of two groups are significantly different from each other, the Student's t-test and chi-square test were used. All statistical analyses including Fisher's exact probability test were performed using the JMP software (SAS Institute). A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Vascular neoplasms developed in the liver of gankyrin-transgenic mouse

The HBX promoter [11] was first used to direct hepatocyte-specific expression of the wild-type gankyrin in transgenic mice. Two founder (F0) mice were obtained and subsequently mated with wild-type mice to produce F1 offspring containing the transgene. F1 mice were then mated with wild-type mice to produce F2 offspring. When F0, F1 and F2 mice were sacrificed at 9 to 13 months of age, no hepatic tumor was observed (data not shown). At 22 months of age,

however, 5 of the remaining 7 male F2 developed hepatic vascular tumors, whereas no tumor was found in the control mice (Fig. 1A, B). The protein level of gankyrin in the non-tumorous liver of transgenic mice was about 1.7 fold compared with that of wild-type mice (Fig. 1C). The tumors consisted of large somewhat irregular vascular channels lined by endothelial cells. In some areas elongated or spindle-shaped endothelial cells lined vascular spaces, formed solid sheets, with an atypical nucleus, suggesting malignancy (Fig. 1B). Immunohistochemical analysis demonstrated that the tumor cells expressed the endothelial cell markers CD31 and CD34. The expression of gankyrin was less in the vascular tumors than non-tumorous liver tissues (Fig. 1D). Taken together, these results indicated that the observed tumors were hemangioma/hemangiosarcomas.

To increase the expression level of transgene in the liver, we next produced gankyrin-transgenic mice using the SAP promoter [12]. At the age of 22 months, one of the 3 F0 mice developed hemangioma/hemangiosarcomas, but none of its F1 offspring and other 2 F0 mice (Fig. 1A). In the F0 with vascular tumors, the transgene was integrated into more than one locus, resulting in inheritance of less integration sites and lower levels of gankyrin expression in the offspring (Fig. 1C and data not shown).

3.2. Increased vascularity in tumors overexpressing gankyrin

To evaluate the effect of gankyrin on angiogenesis in the liver, we used the DEN-induced

hepatocarcinogenesis model. At 8 months after DEN treatment, 100% of wild-type mice and SAP promoter-driven gankyrin-transgenic mice (F4) developed hepatocarcinomas. Multiplicity of tumors was not different between the two groups, but the incidence of microscopic vascular lesions characterized by angiectasis and atypically proliferating endothelial cells was significantly higher in the transgenic mice than wild-type mice (Fig. 2A, B).

To further examine the effect of gankyrin on neovascularization, we stably overexpressed FLAG-tagged gankyrin in mouse Hepa1-6 hepatoma cells (Hepa1-6/GK cells, Fig. 2C). Two weeks after inoculation, both Hepa1-6 and Hepa1-6/GK cells formed tumors, and tumor vascularity was grossly more prominent in the Hepa1-6/GK tumors compared with Hepa1-6 tumors in all 6 mice inoculated (Fig. 2D). Immunohistochemical staining with anti-CD31 endothelial marker antibody demonstrated increased blood vessel density in Hepa1-6/GK tumors compared with Hepa1-6 tumors. Thus, overexpression of gankyrin increased the neovascularization .

3.3 Increased VEGF expression induced by gankyrin

Since HIF-1-mediated expression of VEGF stimulates angiogenesis [8, 9], we analyzed expression of HIF-1 α and VEGF-A. As shown in Figure 3A, expression levels of VEGF-A protein and mRNA were higher in the livers of gankyrin-transgenic mice compared to wild-type mice. Overexpression of gankyrin in Hepa1-6 cells also increased protein level of VEGF-A,

although the HIF-1 α level was not increased. (Fig. 3B).

Transcriptional activation of the VEGF gene in response to hypoxia is mediated by binding of HIF-1 to HRE [13]. To examine whether gankyrin affects transcriptional activity mediated by HRE, we transfected U-2 OS cells with HRE-Luc reporter plasmid. Gankyrin enhanced the luciferase activity induced by mild hypoxia (3% O₂) by 4 fold, but only 1.5-fold or no enhancement at 1% or 20% O₂ concentration, respectively (Fig. 3C and data not shown). Conversely, suppression of gankyrin expression by shRNA reduced the luciferase activity. When HRE was mutated, the luciferase activity was not increased by hypoxia, and the enhancing effect of gankyrin was not observed, indicating that the effect was mediated by HRE.

We next examined whether gankyrin affects transcriptional activity of HIF-1 α . We employed a reporter system composed of the F-Luc gene whose expression is controlled by GAL4-binding elements (GAL4E1bLuc, Fig. 3D) and the HIF1 α -CAD fused to the GAL4 DNA-binding domain [13]. Compared with normoxia (20% O₂), luciferase activity was 2.5-fold higher at 3% O₂ concentration (Fig. 3D). Overexpression of gankyrin further increased the HIF-1 α activity by 3 fold, whereas suppression of gankyrin reduced it. At 1% or 20% O₂ concentration, however, these effects of gankyrin were not observed (data not shown). When fusion protein of HIF-1 α -CAD mutated at Asn803 was used, gankyrin showed no effect. Since Asn803 is the critical residue for FIH-1 to inhibit HIF-1 α activity, these results suggest that the

effect of gankyrin was mediated by FIH-1.

3.4. Sequestration and inhibition of FIH-1 by gankyrin

We examined whether or not gankyrin suppresses FIH-1 expression. Overexpression of gankyrin did not affect FIH-1 level (Fig. 4A). When FIH-1 was overexpressed, the gankyrin level did not change, either. Thus, we suspected that gankyrin might affect the interaction of FIH-1 and HIF-1 α . As shown in Figure 4B, binding of FIH-1 and HIF-1 α was suppressed by gankyrin. As FIH-1 binds ankryrin repeat domain proteins [16] and gankyrin contains 7 ankryrin repeats [3], we checked the possibility that gankyrin binds to FIH-1. HA-FIH-1 and FLAG-gankyrin were coimmunoprecipitated by either anti-HA or anti-FLAG antibody from cells cultured at 3% O₂, but not or only slightly at 1% or 20% O₂ concentration, respectively (Fig. 4C and data not shown). These results demonstrate that gankyrin binds to and sequester FIH-1, resulting in decreased interaction between FIH-1 and HIF-1 α and increased activity of HIF-1 under mild hypoxic conditions. When we further examined the mRNA levels of gankyrin and VEGF-A in biopsy specimens of human HCC, a moderate positive correlation ($r = 0.57$, $P < 0.02$) was found (Fig. 4D), suggesting that the gankyrin-FIH-1 interaction might have some clinical relevance.

4. Discussion

Hemangioma/hemangiosarcomas are occasionally seen in mouse liver with incidences less than 3% [17]. In the present study, 71% of the F2 transgenic mice overexpressing HBX promoter-driven gankyrin developed hepatic hemangioma/hemangiosarcomas, whereas none of the wild-type mice did. This phenotype was probably not due to random insertional mutagenesis in the transgenic mice as it was also observed in F0 mice expressing SAP promoter-driven gankyrin. In the subsequent generations of this F0, however, gankyrin levels were decreased and no hemangioma/hemangiosarcoma developed. The finding that mice with 70% increase, but none with 35% increase in the protein level of hepatic gankyrin developed hemangioma/hemangiosarcomas (Fig. 1C) suggests that there is a critical level of gankyrin to show this phenotype.

How does overexpression of gankyrin in hepatocytes induce endothelial cell-derived tumors? As gankyrin induces dedifferentiation of HCCs [6], it may also induce transdifferentiation of hepatocytes into endothelial cells. A more feasible explanation, however, would be that gankyrin facilitates a sustained release of angiogenic growth factors, providing the milieu leading to hemangiosarcoma formation [18]. Consistent with this notion, endothelial

overgrowth was more frequent in the HCCs of gankyrin-transgenic mice than wild-type mice after DEN treatment. Furthermore, mouse hepatoma transfectants overexpressing gankyrin induced more neovascularization than parental cells when subcutaneously inoculated into nude mice. VEGF-A was the first identified member of the VEGF family, and mice with transgenic VEGF-A expressed in the liver have increased vascularization and vascular permeability [19]. When myoblasts overexpressing VEGF-A are transplanted into limb or heart muscle of mice, they induce hemangiomas [20]. In the present study, VEGF-A level was higher in the liver of gankyrin-transgenic mice compared with wild-type mice, and gankyrin increased VEGF-A expression in cultured hepatoma cells. Thus, VEGF-A probably contributed to formation of hemangioma/hemangiosarcomas in the gankyrin-transgenic mice.

HIF-1 is a major factor regulating the level of VEGF, and despite induction of multiple angiogenic target genes such as adrenomedullin and placental growth factor, VEGF is essential for HIF-1 mediated neovascularization [21]. Hypoxia induces changes in the hydroxylation status of well-conserved Pro and Asn residues of HIF-1 α , resulting in protein stabilization and transcriptional activation [8, 9]. Signaling through receptor tyrosine kinases induce HIF-1 expression by increasing the rate of HIF-1 α protein synthesis via PI3K/Akt /mTOR pathway [8], and gankyrin activates this to promote VEGF expression [7]. In the present study, the HIF-1 α protein level was not increased in cells overexpressing gankyrin. Reporter assays indicated,

however, that HIF-1 transcriptional activity was increased by gankyrin, and that it was dependent on Asn803 of HIF-1 α . FIH-1 hydroxylates this residue and inhibits transcriptional activity [8-10]. In addition to HIF-1 α , proteins containing ankyrin repeat domains are common targets for hydroxylation by FIH-1, and I-B α as well as Notch-1 block the FIH-1-mediated HIF-1 α repression by sequestering FIH-1 [22]. In this case, the recognition of each substrate and their relative affinity for FIH-1 is an important determinant of FIH-1 sequestration and consequently HIF regulation. Consistent with the recent study using recombinant proteins [16], gankyrin and FIH-1 were co-immunoprecipitated from cell lysates. Furthermore, overexpression of gankyrin reduced the amount of HIF-1 α bound to FIH-1, suggesting a higher affinity for FIH-1 of gankyrin than HIF-1 α at mild hypoxia. As expected, gankyrin increased the HIF-1 transcriptional activity in reporter assays, which was dependent on FIH-1. Interestingly, the binding of gankyrin to FIH-1 and enhancement of HIF-1 activity were dependent on the O₂ concentration.

We have demonstrated in this study that sustained overexpression of gankyrin in hepatocytes, although at a low level, can induce liver hemangioma/hemangiosarcomas in mice. Gankyrin sequesters FIH-1 from HIF-1 α to activate HIF-1 and increase production of VEGF, which at least partly contributes to hemangioma/hemangiosarcoma formation. Further studies will clarify why spontaneous hemangioma/hemangiosarcomas are extremely rare in humans in

contrast to experimental animals [18], and shed light on mechanisms of vascular tumorigenesis as well as hepatocarcinogenesis. The present study also suggests that gankyrin might play a physiological role in hypoxic responses besides its roles as an oncoprotein.

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Figure legends

Fig. 1. Vascular tumors in gankyrin-transgenic mice

(A) Incidence of hepatic tumors. Gankyrin was expressed in hepatocytes by using HBX promoter (HBXp) or SAP promoter (SAPp). *, number of mice with hepatic tumors at 22 months of age / total number of mice. **, $P < 0.05$ compared with HAwT group.

- (B) Gross and microscopic appearances of hepatic vascular lesions in transgenic mice. H&E, hematoxylin and eosin staining. CD31 and CD34, immunoperoxidase staining for CD31 and CD34, respectively, using diaminobenzidine as substrate. Bar, 50 μ m
- (C) Gankyrin expression in the non-tumorous liver. Lysates prepared from indicated mice were analyzed by Western blotting and densitometry. Bars are average $\pm$ SD of total gankyrin levels normalized with actin levels, and expressed as relative to those of wild-type mice.
- (D) Expression of endogenous and exogenous (arrowhead) gankyrin in the tumor (T) and non-tumorous portion (N) of the liver from indicated F2 transgenic mice. Western blot analysis.

Fig. 2 Increased vascularity of hepatic tumors overexpressing gankyrin

- (A) Vascularity in the livers of gankyrin-transgenic (SC3ab) and wild-type (SCwt) mice with diethylnitrosamine (DEN)-induced HCCs. 8 months after DEN treatment, mice were sacrificed and tumor vascularity was evaluated microscopically. *, number of mice with endothelial overgrowth in the liver / number of mice administered DEN. $P < 0.05$ between these groups.
- (B) Typical examples of atypical proliferation of endothelial cells in (A). H&E stain. Bar, 50 μ m.
- (C) Expression of endogenous and exogenous (arrowhead) gankyrin in Hepa1-6 and Hepa1-6/GK cells analyzed by Western blotting.
- (D) Vascularity of Hepa1-6 and Hepa1-6/GK tumors in nude mouse skin. Typical gross and

microscopic (H&E and immunoperoxidase staining of CD31) appearances of formalin-fixed paraffin-embedded tumors. Bar, 50 μ m.

Fig. 3. Increased VEGF expression induced by gankyrin

(A) VEGF-A and HIF-1 α expression in the livers of wild-type (wt) and gankyrin-transgenic (tg)

mice. Western blotting (left) and RT-qPCR (right). Arrowhead, FLAG-Gankyrin. VEGF-A

transcript levels were normalized with GAPDH levels. Values are average $\pm$ SD. *, $P < 0.05$.

a.u., arbitrary unit.

(B) Effects of gankyrin on expression of VEGF-A. Hepa1-6 cells and Hepa1-6 transfectants

overexpressing FLAG-gankyrin (Hepa1-6/GK) were analyzed by Western blotting. Arrowhead,

FLAG-Gankyrin.

(C) HRE-dependent transcriptional activation. U-2 OS cells were cotransfected with *ENO1*-F-

luciferase (Luc) reporter plasmids (p2.1) or mutated reporter plasmids lacking the HIF-1

recognition sequence (p2.4), and plasmids expressing R-Luc, FLAG-gankyrin, HA-FIH-1,

gankyrin-shRNA, and scrambled-shRNA as indicated. 48 h later, some dishes were transferred

to hypoxic conditions. After further 48-h incubation, cell lysates were analyzed for Luc activity.

F-Luc activity was normalized with R-Luc activity. Values are average $\pm$ SD from 3

independent experiments. a.u., arbitrary unit.

(D) Asn803-dependent increase in HIF-1 α transcriptional activity. U-2 OS cells were

cotransfected with Gal4–F-Luc reporter plasmids (GAL4E1bLuc), plasmids expressing GAL4 DNA-binding domain (DBD) fused to wild-type (wt) or N803A mutant C-terminal half (CAD) of HIF-1 α , R-Luc, and FLAG–gankyrin and gankyrin-shRNA as indicated. After 48 h of hypoxic incubation, Luc activities were measured and expressed as in (C). GAL4-BS, GAL4-binding sites. a.u., arbitrary unit.

Fig. 4. Binding of gankyrin to FIH-1

(A) Effect of gankyrin on FIH-1 protein level. HEK293 cells were transfected with increasing amounts of plasmids expressing HA-gankyrin or HA-FIH-1, or empty vector (Mock) as indicated. 48 h later, cell lysates were analyzed by Western blotting. Arrowhead, non-specific bands.

(B) Effect of gankyrin on binding of HIF-1 α to FIH-1. HEK293T cells were cotransfected with plasmids expressing FLAG–HIF-1 α , HA–FIH-1, and Myc-tag-gankyrin, and cultured at 3% O₂ for 48 h. Cell lysates were immunoprecipitated (IP), and precipitants and inputs were analyzed by Western blotting (WB) using the indicated antibodies.

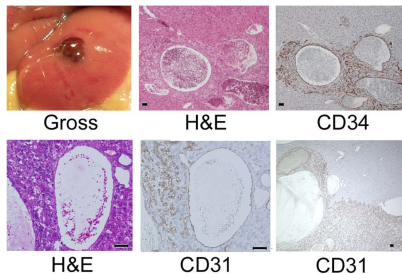
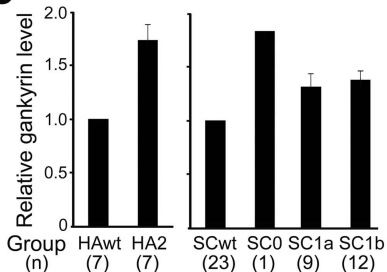
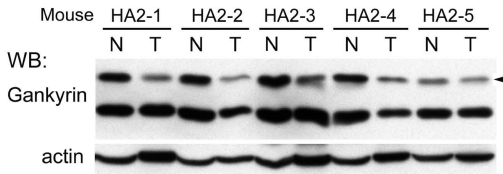
(C) Interaction of gankyrin with FIH-1. HEK293T cells were cotransfected with plasmids expressing FLAG-gankyrin and HA-FIH-1, and cultured at 3% O₂ for 48 h. Cell lysates were immunoprecipitated, and precipitants and inputs were analyzed by WB as in (B). WCL, whole

cell lysates. Experiments were repeated three times with similar results.

(D) Scatter plot of mRNA levels of gankyrin and VEGF-A in human hepatocellular carcinoma specimens. a.u., arbitrary unit.

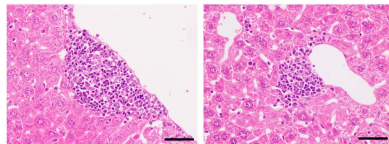
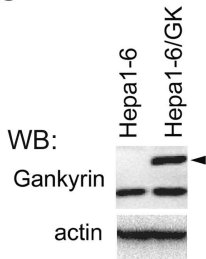
A

Group	Transgene	Generation	Tumor*
HAwt	none	F2	0 / 7
HA2	HBX _p -Gankyrin	F2	5 / 7**
SCwt	none	F1	0 / 23
SC0	SAP _p -Gankyrin	F0	1 / 1
SC1a	SAP _p -Gankyrin	F1	0 / 9
SC1b	SAP _p -Gankyrin	F1	0 / 12

B**C****D**

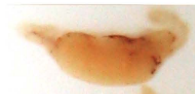
A

Group	Transgene	Endothel [*]
SCwt	none	9 / 36
SC3ab	SAP _p -Gankyrin	25 / 41

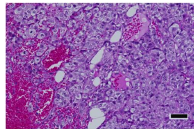
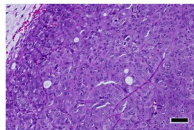
B**C****D**

Hepa1-6/GK

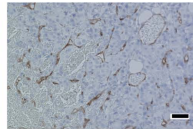
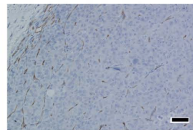
Hepa1-6



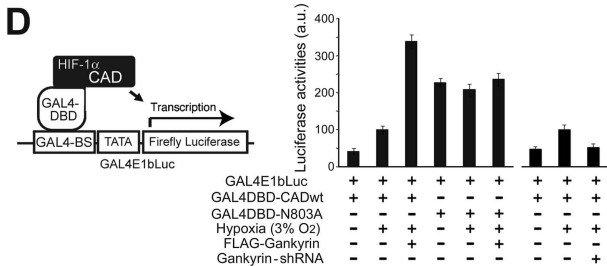
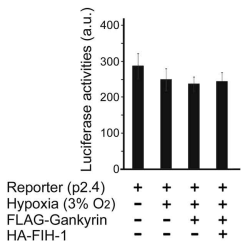
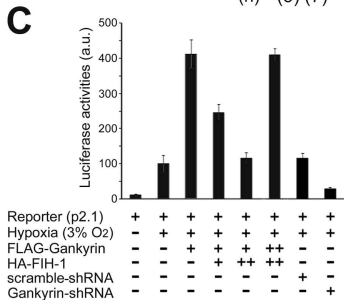
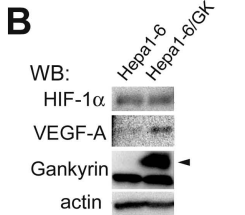
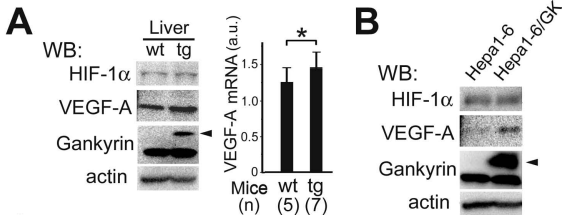
Gross

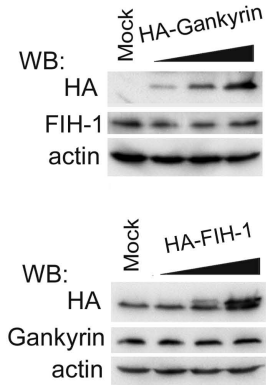
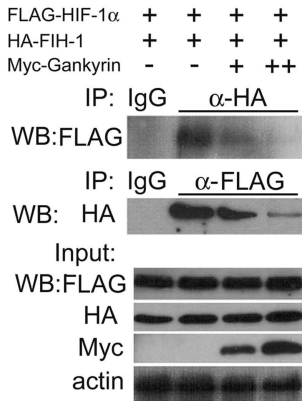
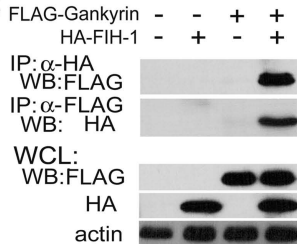


H&E



CD31



A**B****C****D**