Forkhead Box M1 Is Essential for Nuclear Localization of Glioma-associated Oncogene Homolog 1 in Glioblastoma Multiforme Cells by Promoting Importin-7 Expression

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*Running title: FOXM1 is essential for nuclear localization of GLI1

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Background: The transcription factors GLI1 and FOXM1 play critical roles in cancer development and progression.

Results: FOXM1 bound to the importin-7 promoter to upregulate its expression. FOXM1 deficiency inhibited importin-7 expression and nuclear localization of GLI1.

Conclusion: FOXM1 is essential for nuclear localization of GLI1 by promoting importin-7 expression.

Significance: FOXM1 and GLI1 form a positive feedback loop that contributes to glioblastoma multiforme development.

ABSTRACT

The transcription factors glioma-associated oncogene homolog 1 (GLI1), a primary marker of Hedgehog pathway activation, and Forkhead box M1 (FOXM1) are aberrantly activated in a wide range of malignancies, including glioma. However, the mechanism of nuclear localization of GLI1 and whether FOXM1 regulates the Hedgehog signaling pathway are poorly understood. Here we found that FOXM1 promotes nuclear import of GLI1 in glioblastoma multiforme cells and thus increases the expression of its target genes. Also, genetic deletion of FOXM1 in mouse embryonic fibroblasts abolished nuclear localization of GLI1. We observed that FOXM1 directly binds to the importin-7 (IPO7) promoter and increases its promoter activity. IPO7 interacted with GLI1, leading to enhanced nuclear import of GLI1. Depletion of IPO7 by IPO7 siRNA reduced nuclear accumulation of GLI1. In addition, FOXM1 induced nuclear import of GLI1 by promoting IPO7 expression. Moreover, the FOXM1/IPO7/GLI1 axis promoted cell proliferation, migration, and invasion in vitro. Finally, expression of FOXM1 was markedly correlated with that of GLI1 in human glioblastoma specimens. These data suggest that FOXM1 and GLI1 form a positive feedback loop that contributes to glioblastoma development. Furthermore, our study revealed a mechanism that controls nuclear import of GLI1 in glioblastoma multiforme cells.

The Hedgehog (Hh)8 signaling pathway is a developmental signaling pathway that controls numerous developmental processes (1). Whereas this pathway is silenced in normal adult mature cells, it is aberrantly activated in a wide range of malignancies, including glioma (2, 3). The central components of the mammalian Hh pathway
include three secreted ligands (Sonic hedgehog, Indian hedgehog, and Desert hedgehog), a negative regulatory receptor (PTCH), a positive regulatory protein (SMO), and the glioma-associated oncogene transcription factors (glioma-associated oncogene homolog 1 [GLI1], GLI2, and GLI3) (4). In particular, the zinc-finger transcription factor GLI1 is the primary marker of Hh pathway activation. Without Hh ligands, PTCH inhibits the activity of SMO to keep it from transmitting the Hh signal. Upon ligand binding, this inhibition of SMO by PTCH is relieved, and the Hh signal is activated. Activated SMO orchestrates a signaling cascade that eventually results in activation and release of the glioma-associated oncogene transcription factors from a protein complex (2-4). Activated glioma-associated oncogenes translocate into the nucleus to regulate the expression of various context-specific genes. However, the mechanism of GLI1’s nuclear localization is poorly understood.

Forkhead box M1 (FOXM1) is a member of the Forkhead family of transcription factors (5). Many studies have demonstrated that FOXM1 is involved in different aspects of tumorigenesis, including angiogenesis, invasion, and metastasis (6, 7). FOXM1 is often overexpressed in various human malignancies. For example, high expression of FOXM1 in glioblastoma multiforme (GBM) correlates with tumorigenicity of the GBM cells (8). Authors have reported FOXM1 to be a downstream target of GLI1 in the Hh pathway that contributes to tumorigenesis (9). However, how FOXM1 regulates the Hh signaling pathway remains unknown.

Given that both FOXM1 and GLI1 are activated in many cancers, including glioma, we investigated the potential relationship between these two key transcriptional factors in GBM cases. We found that FOXM1 is essential for nuclear import of GLI1 by promoting importin-7 (IPO7) expression. We also demonstrated that IPO7 binds to GLI1 and thus leads to enhanced nuclear import of GLI1. These data suggest that FOXM1 and GLI1 form a positive feedback loop that contributes to GBM development. Additionally, this study revealed a mechanism that controls nuclear import of GLI1 in GBM cells.

Experimental Procedures

Cell Culture and Transfection—FOXM1-knockout mouse embryonic fibroblasts and HEK 293T human kidney, Hs683 glioma, SW1783 anaplastic astrocytoma, HU251 glioblastoma, and U87 glioblastoma cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (HyClone, Logan, UT). HEK 293T cells were transfected with FuGENE HD (Roche Applied Science, Indianapolis, IN). FOXM1-knockout mouse embryonic fibroblasts and Hs683, HU251, and U87 cells were transfected with X-tremeGENE HP (Roche Applied Science, Indianapolis, IN). HU251 and U87 cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA).

Plasmids, siRNAs, and Reagents—Human FOXM1 and FOXM1 mutants were generated via PCR and using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Expression vectors encoding FLAG-GLI1 were obtained from Dr. Mien-Chie Hung (The University of Texas MD Anderson Cancer Center, Houston, TX). The IPO7 human promoter reporter was obtained from Dr. Moshe Oren (Weizmann Institute of Science, Rehovot, Israel) (10). Human IPO7 (plasmid #26682) was obtained from Addgene (Cambridge, MA). SMARTpool siRNA duplexes specific for FOXM1, IPO7, and a nontargeting siRNA (siControl) were purchased from Dharmacon (Lafayette, CO). Leptomycin B (LMB) was purchased from Sigma-Aldrich (St. Louis, MO).

Antibodies—An anti-FLAG tag antibody (F1804; 1:1000 immunoblot [IB], 2 µg immunoprecipitation [IP]) was obtained from Sigma-Aldrich. The GLI1 antibody (#2553; 1:1000 IB) was obtained from Cell Signaling Technology (Danvers, MA). The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): FOXM1 (sc-500; 1:1000 IB), IPO7 (sc-55235; 1:1000 IB, 2 µg IP), GLI1 (sc-20687; 1:100 immunofluorescence, 1:100 immunohistochemistry), lamin B (sc-6216; 1:1000 IB), normal mouse IgG (sc-2025; 2 µg IP), and β-actin (sc-47778; 1:10,000 IB). The following antibodies were obtained from Abcam (Cambridge, UK): FOXM1 (ab83097; 1:100 immunohistochemistry), IPO8 (ab72109; 1:1000 immunohistochemistry).
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**Subcellular Fractionation**—Cells were harvested, and nuclear protein fractions were separated using a CellLytic nuclear extraction kit (Sigma-Aldrich) according to the manufacturer’s recommended procedures.

**IP and IB Analysis**—For co-IP, cells were grown in 10-cm dishes and transfected with the appropriate plasmids. Cell lysates were incubated with 2 µg of antibody on a rotator overnight at 4°C. Protein-antibody-protein A/G agarose complexes were prepared by adding 50 µl of protein A/G-agarose beads (Amersham Biosciences, Amersham, UK) for 1 h at 4°C. After extensive washing with RIPA lysis buffer, immunoprecipitated complexes were resuspended in a reducing sample buffer and boiled for 10 min. After centrifugation to pellet the agarose beads, supernatants were subjected to SDS-PAGE and IB analysis as described previously (11).

**Immunofluorescent Analysis**—Cells were rinsed twice with PBS, fixed with 4% buffered paraformaldehyde, and permeabilized with 0.5% Triton X-100 for 15 min. Cells were then incubated with a primary antibody overnight at 4°C and then with a secondary antibody conjugated to FITC (Molecular Probes, Eugene, OR) for 1 h at room temperature. Cells were then examined using a deconvolutional microscope (Carl Zeiss, Oberkochen, Germany) as described previously (11).

**Quantitative Real-Time RT-PCR**—Quantitative real-time RT-PCR analysis was performed using total RNA and SYBR Green reagent with an ABI Prism 7000HT sequence detection system (Applied Biosystems, Waltham, MA). The sequences of the PCR primers were as follows: *Ptch1* forward, 5'-AATGGGTCCACGACAAGCCGACTA-3'; *Ptch1* reverse, 5'-TCCCGCAAGCCGTTGAGGTAGAAAG-3'; *Ptch2* forward, 5'-TCTTTCTGGGACTGTTGGCCTTTGG-3'; *Ptch2* reverse, 5'-CCTCCCCAGCTTCTCTGGTAGAAGG-3'; *CCND2* forward, 5'-CTGTGTGCCCAGCCTTTAAGTT-3'; *CCND2* reverse, 5'-GATGGCTGGCTCCACACTTC-3'; *IL-6* forward, 5'-AGACAGCCACCTCTTTCA-3'; *IL-6* reverse, 5'-TTCTGCGAGTGCTTTGCTG-3'; *GAPDH* forward, 5'-AATCCCATACCATCTCTCA-3'; and *GAPDH* reverse, 5'-TGGACTCCACGACGTACTCA-3'. The results were obtained using the comparative Ct method with the housekeeping gene *GAPDH* as a control.

**Promoter Reporters and Dual Luciferase Assay**—Cells were transfected with the IPO7 human promoter reporter plasmid together with pRL-TK and analyzed as described previously (11). Luciferase activity was measured using a dual luciferase assay system (Promega, Madison, WI).

**ChIP Assay**—U87 cells (2 × 10⁶) were prepared for a ChIP assay using a ChIP Assay Kit (Cell Signaling Technology) according to the manufacturer’s protocol. The resulting precipitated DNA specimens were analyzed using PCR to amplify a 215-bp region of the human IPO7 promoter with the primers 5'-ACTCCAAATCTCCAATCTCT-3' (forward) and 5'-ATCACCTCCTGCCCTGAGAG-3' (reverse), a 147-bp region of the human IPO7 promoter with the primers 5'-TCAAACAGGAGATTACCGCCT-3' (forward) and 5'-AATCCAGGAGGTGAGCTTGG-3' (reverse), and a 184-bp region of the human IPO7 promoter with the primers 5'-TCCGTGTATTTGCTTGGAGAG-3' (forward) and 5'-TGAGGCAGGGATCACTGCC-3' (reverse). The PCR products were resolved electrophoretically on a 2% agarose gel and visualized using ethidium bromide staining.

**Cell Proliferation and Colony Formation Assays**—For a cell proliferation assay, after transfection, U87 cells (5 × 10³) were plated in 96-well plates. Cell growth was assessed using a standard MTT assay. For a colony formation assay, after transfection, cells were trypsinized and reseeded in six-well plates at a density of 5 × 10³ cells per well. For crystal violet staining, plates were washed once each with PBS and cold methanol and then incubated in a crystal violet solution for 5 min. The plates were subsequently washed twice with double distilled water, air-dried, and scanned using an Epson (Long Beach, CA) scanner.

**Wound healing assay**—Following transfection, U87 cells were plated overnight to achieve a subconfluent cell layer in six-well plates.
The cell layer was scratched with a micropipette tip, and cultures were washed twice with a serum-free medium to remove floating cells. Wound healing was visualized by comparing photographs of the cell layer taken at 0 and 24 h and processed using the Photoshop CC software program (Adobe Systems, San Jose, CA). Differences in cell migration distances were determined using a t-test for comparing mean values.

Transwell invasion assay—An invasion assay was carried out in Transwell chambers containing polycarbonate filters (8-µm pore size; BD Biosciences, San Jose, CA) in which the upper surfaces of the filters in the invasion chambers were coated with a growth factor-reduced Matrigel matrix. Following transfection, U87 cells (5 × 10^4) in a 500-µl volume of serum-free medium were placed in the upper chambers and incubated at 37°C for 16 h for the invasion assay. The cells that penetrated through the Matrigel-coated filters were counted at a magnification of ×200 in 15 randomly selected fields, and the mean number of cells per field was recorded.

Human Tissue Specimens and Immunohistochemical Analysis—Sections of paraffin-embedded human GBM specimens were stained with antibodies against GLI1 and FOXM1. Immunohistochemical analysis of GBM tissue arrays was performed using a standard immunostaining protocol as described previously (11). The use of human brain tumor specimens was approved by the MD Anderson Institutional Review Board.

Statistical Analysis—The significance of the data on the patient specimens was determined using the Pearson correlation coefficient. The significance was determined using the Student t-test (two-tailed). P values less than 0.05 were considered significant.

RESULTS

FOXM1 Promotes Nuclear Import of GLI1 in a DNA-Binding-Dependent Manner—We analyzed the expression of nuclear FOXM1 and GLI1 protein in Hs683, SW1783, HFU251, and U87 cells. We observed markedly higher FOXM1 expression in HFU251 and U87 cells than in Hs683 and SW1783 cells (Fig. 1A). Also, the expression of nuclear GLI1 was positively correlated with the expression of nuclear FOXM1 in these cell lines. Because GLI1 is a nuclear cytoplasmic shuttling protein (12), we attempted to determine whether FOXM1 modifies the nuclear translocation of GLI1. As nuclear export of GLI1 is dependent on chromosome region maintenance homolog 1 (12), we examined GLI1 localization in the presence of LMB, a chromosome region maintenance homolog 1-dependent export inhibitor. We found that exposure to LMB increased GLI1’s nuclear localization, which was partially attenuated by knockdown of FOXM1 expression, confirming that FOXM1 is essential for nuclear import of GLI1 (Fig. 1B). Furthermore, deletion of FOXM1 in Foxm1^fl/fl mouse embryonic fibroblasts virtually abolished nuclear localization of GLI1 (Fig. 1C), further confirming that FOXM1 is required for nuclear import of GLI1.

Next, we induced overexpression of FOXM1 without a DNA-binding domain, the FOXM1 mutant R286A/H287A (which destroys the DNA-binding ability of FOXM1), and WT FOXM1 in Hs683 cells. WT FOXM1 increased endogenous nuclear GLI1 levels, whereas the other two proteins did not (Fig. 1D), suggesting that FOXM1 promotes nuclear import of GLI1 in a DNA-binding-dependent manner. Conversely, depletion of FOXM1 by siRNA in U87 cells decreased the level of nuclear GLI1 (Fig. 1E). To investigate the changes in mRNA expression of the GLI1 target genes in the presence of FOXM1, we performed quantitative real-time RT-PCR analysis of Hs683 cells overexpressing FOXM1. As shown in Fig. 1F, FOXM1 increased the expression of Ptch1, Ptch2, CCND2, and IL-6. In contrast, knockdown of FOXM1 expression decreased the expression of these genes in U87 cells (Fig. 1G). Taken together, these results suggested that FOXM1 promotes nuclear import of GLI1 in a DNA-binding-dependent manner.

IPO7 Is a Direct Transcriptional Target of FOXM1—IPO protein plays a key role in moving other proteins into the nucleus (13). The nuclear import pathway of GLI1 has yet to be fully elucidated. To understand the molecular mechanism of FOXM1 regulation in nuclear import of GLI1, we screened Hs683 cells to determine whether FOXM1 regulated the expression of several IPOs. As shown in Fig. 2A, overexpression of FOXM1 markedly increased IPO7 expression but did not change IPO8, IPO9, or IPO13 expression in Hs683 cells. Next, we
analyzed the sequence of the human IPO7 promoter using the FOXM1 consensus sequences. We identified three putative FOXM1-binding sites in the human IPO7 promoter (Fig. 2B). All of the FOXM1-binding regions of the IPO7 promoter bound to endogenous FOXM1 protein in U87 cells in our ChIP assays (Fig. 2C). Moreover, we examined whether FOXM1 transactivates the IPO7 promoter using a human IPO7 promoter luciferase reporter. Overexpressed WT FOXM1 but not FOXM1 without a DNA-binding domain or the FOXM1 mutant R286A/H287A upregulated IPO7 promoter activity in HEK 293T and Hs683 cells (Fig. 2, D and E). In comparison, IPO7 promoter activity decreased in U87 and HFU251 cells with FOXM1 siRNA (siFOXM1) (Fig. 2, F and G). Collectively, these results indicated that IPO7 is a direct transcriptional target of FOXM1 and that FOXM1 transactivates the IPO7 promoter in a DNA-binding-dependent manner.

IPO7 Binds to GLI1—To determine whether nuclear import of GLI1 by IPO7 is mediated by interaction between the two, we performed a co-IP assay. In HEK 293T cells transfected with a FLAG-GLI1 fusion construct, FLAG-GLI1 protein was coimmunoprecipitated with endogenous IPO7 (Fig. 3A). Furthermore, we examined the interaction of endogenous GLI1 and IPO7 at the physiological level. As shown in Fig. 3B, endogenous GLI1 was associated with IPO7 in U87 cells in a co-IP assay. Next, we analyzed the effect of IPO7 knockdown by siRNA on localization of endogenous GLI1 as detected via immunostaining. In contrast with control siRNA, IPO7 siRNA reduced nuclear accumulation of GLI1 (Fig. 3C). Consistent with these results, knockdown of IPO7 expression decreased GLI1 target genes expression according to quantitative real-time RT-PCR analysis (Fig. 3D). These results demonstrated that IPO7 is a key nuclear transporter for GLI1 by interacting with GLI1 protein.

FOXM1 Induces Nuclear Import of GLI1 by Promoting IPO7 Expression—We examined whether FOXM1 increases nuclear import of GLI1 via upregulation of IPO7 expression. U87 cells exhibited reduced nuclear accumulation of GLI1 in the presence of siFOXM1, but overexpression of IPO7 rescued nuclear localization of GLI1 in these cells according to immunofluorescent staining (Fig. 4A). Using Western blotting of U87 cells, we found that knockdown of FOXM1 expression resulted in decreased nuclear localization of GLI1, which was abrogated by IPO7 overexpression (Fig. 4B). Moreover, knockdown of FOXM1 expression decreased GLI1 target genes expression, and IPO7 overexpression rescued the inhibitory effect of FOXM1 depletion (Fig. 4C). Taken together, these results demonstrated that FOXM1 induces nuclear import of GLI1 by promoting IPO7 expression.

The FOXM1/IPO7/GLI1 Axis Promotes Cell Proliferation, Migration, and Invasion—Next, we examined whether the FOXM1/IPO7/GLI1 axis has a role in the proliferation, migration, and invasion of U87 cells. We found that U87 cells with FOXM1 depletion proliferated much slower than control cells did. Furthermore, the siFOXM1-transfected U87 cells were much less able to form colonies than were control cells; however, IPO7 and GLI1 overexpression rescued siFOXM1-transfected U87 cell proliferation and increased the number of colonies of these cells (Fig. 5, A and B). We also performed wound healing and Transwell invasion assays to determine whether FOXM1 affects GLI1-mediated cell migration and invasion. The results demonstrated that FOXM1 downregulation inhibited the migration of U87 cells more so than control cells. Moreover, the inhibitory effect of siFOXM1 on the migration of U87 cells was rescued by IPO7 and GLI1 overexpression (Fig. 5C). In addition, knockdown of FOXM1 expression decreased the invasion of U87 cells relative to that of control cells, and expression of IPO7 or GLI1 restored the invasion of these cells (Fig. 5D). Taken together, these results demonstrated that FOXM1 increases IPO7 expression and thus promotes nuclear import of GLI1, which results in increased cell proliferation, migration, and invasion.

FOXM1 Expression Correlates Positively with GLI1 Expression—Finally, to determine whether our findings are clinically relevant, we examined the FOXM1 and GLI1 expression levels in serial sections of 40 human primary GBM specimens via immunohistochemical analysis. The expression of FOXM1 correlated positively with that of GLI1 in the specimens (Fig. 6A). Quantification of staining demonstrated that this correlation was statistically significant in all 40 specimens ($r = 0.803; p < 0.001$) (Fig. 6B).
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results further supported a critical role for FOXM1 in induction of nuclear import of GLI1 in human GBM cells.

**DISCUSSION**

Our study demonstrated strong evidence supporting a critical role for FOXM1 in regulation of GBM development. We showed that FOXM1 enhances the growth and invasion of human glioma cells via the Hh signaling pathway. Specifically, FOXM1 stimulates transcription of IPO7 by binding directly to its promoter at three sites, which in turn leads to nuclear localization of the transcription factor GLI1, which is the most important indicator of Hh pathway activation. We also found that the level of FOXM1 expression is highly correlated with that of GLI1 expression in human GBM specimens. Because GLI1 may upregulate FOXM1 expression (9), the regulatory feedback between FOXM1 and GLI1 in our study may represent a critical mechanism of the proliferation and invasion of human brain tumors (Fig. 6C).

FOXM1 is a member of the Forkhead family of transcription factors, which promote tumorigenesis and tumor metastasis by activating a series of oncogenes (14-17). FOXM1 is involved in regulation of several signaling pathways, such as Wnt/β-catenin (18), TGF-β/Smads (19), Akt (20), and MAPK/ERK (21). However, whether cross-talk between FOXM1 and the Hh pathway exists is unclear. In the present study, we found that FOXM1 regulated nuclear localization of GLI1 in a DNA-binding-dependent manner, a crucial finding suggesting that FOXM1’s transcriptional activity is necessary for nuclear localization of GLI1. Also, we analyzed the mechanism by which GLI1 may be transported into the nucleus. Specific knockdown of IPO7 expression using siRNA resulted in reduced nuclear accumulation of GLI1, indicating the dependence of nuclear import of GLI1 on IPO7. Previous studies demonstrated that several molecules may affect nuclear localization of GLI1 (22-25). For example, nuclear entry of GLI1 was regulated by its nuclear import factor Impβ1 (importin β1) and SuFu (22); WIP1 (or PPM1D) enhanced the function of GLI1 by increasing its nuclear localization (23); Rab23 reduced the nuclear localization of GLI1 (24); SHP (small heterodimer partner) inhibited GLI1 nuclear localization (25). Thus, determining whether FOXM1 also modulates these molecules expression will be very interesting.

GLI1 is the primary nuclear effector of the Hh pathway (26), and infiltrative invasion and uncontrolled proliferation are hallmarks of GBM development (27-29). In our study, functional experiments using gain- or loss-of-function studies demonstrated that the FOXM1/IPO7/GLI1 axis promotes GBM cell proliferation, migration, and invasion. A well-known characteristic of GLI1 is that it is often overexpressed in human glioma cells (30, 31), and it directly regulates expression of oncoproteins, including cyclin D1/D2 (32), FOXM1 (13), and epithelial-to-mesenchymal transition-related proteins (33), and thus promotes the proliferation and invasion of tumor cells. We found that FOXM1 expression correlated directly with GLI1 expression in human GBM specimens, further confirming that these two important transcriptional factors have potential clinical relevance.

We also discovered that FOXM1 and GLI1 form a novel positive regulatory feedback loop. Such loops are common mechanisms of consecutive activation of factors and signaling pathways in tumor progression. Specifically, we found that FOXM1 activates the Hh pathway by stimulating nuclear localization of GLI1 via upregulation of IPO7 expression. Moreover, a previous study demonstrated that GLI1 activates FOXM1 expression (13). Therefore, this regulatory feedback loop made up of FOXM1 and GLI1 maintains consecutive activation of the Hh signaling pathway, which may be a critical mechanism of brain tumor progression.

In summary, our findings demonstrated cross-talk between FOXM1 and the Hh signaling pathway in human glioma cells. Owing to the great importance of the FOXM1/IPO7/GLI1 axis to development of human cancers, our findings strongly suggest that targeting FOXM1 is a therapeutic strategy for GBM.

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REFERENCES
1. Amakye, D., Jagani, Z., and Dorsch, M. (2013) Unraveling the therapeutic potential of the Hedgehog pathway in cancer. Nat. Med. 19, 1410-1422
2. Ng, J. M., and Curran, T. (2011) The Hedgehog's tale: developing strategies for targeting cancer. Nat. Rev. Cancer 11, 493-501
3. Rubin, L.L., and de Sauvage, F.J. (2006) Targeting the Hedgehog pathway in cancer. Nat. Rev. Drug Discov. 5, 1026-1033
4. Varjosalo, M., and Taipale, J. (2008) Hedgehog: functions and mechanisms. Genes Dev. 22, 2454-2472
5. Alvarez-Fernández, M., and Medema, R.H. (2013) Novel functions of FoxM1: from molecular mechanisms to cancer therapy. Front Oncol. 3, 30
6. Kalin, T.V., Ustiyan, V., and Kalinichenko, V.V. (2011) Multiple faces of FoxM1 transcription factor: lessons from transgenic mouse models. Cell Cycle 10, 396-405
7. Li, Y., Zhang, S., and Huang, S. (2012) FoxM1: a potential drug target for glioma. Future Oncol. 8, 223-226
8. Liu, M., Dai, B., Kang, S.H., Ban, K., Huang, F.J., Lang, F.F., Aldape, K.D., Xie, T.X., Pelloski, C.E., Xie, K., Sawaya, R., and Huang S.(2006) FoxM1B is overexpressed in human glioblastomas and critically regulates the tumorigenicity of glioma cells. Cancer Res. 66, 3593-3602
9. Teh, M.T., Wong, S.T., Neill, G.W., Ghali, L.R., Philpott, M.P., and Quinn, A.G. (2002) FOXM1 is a downstream target of Gli1 in basal cell carcinomas. Cancer Res. 62, 4773-4780
10. Golomb, L., Bublik, D.R., Wilder, S., Nevo, R., Kiss, V., Grabusic, K., Volarevic, S., and Oren, M. (2012) Importin 7 and exportin 1 link c-Myc and p53 to regulation of ribosomal biogenesis. Mol. Cell 45, 222-232
11. Xue, J., Chen, Y., Wu, Y., Wang, Z., Zhou, A., Zhang, S., Lin, K., Aldape, K., Majumder, S., Lu, Z., and Huang, S. (2015) Tumour suppressor TRIM33 targets nuclear β-catenin degradation. Nat. Commun. 6, 6156
12. Kogerman, P., Grimm, T., Kogerman, L., Krause, D., Unden, A. B., Sandstedt, B., Toftgard, R., and Zaphiropoulos, P. G. (1999) Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1. Nat. Cell Biol. 1, 312-319
13. Powers, M.A., and Forbes, D.J. (1994) Cytosolic factors in nuclear transport: what's importin? Cell 79, 931-934
14. Cai, Y., Balli, D., Ustiyan, V., Fulford, L., Hiller, A., Misetic, V., Zhang, Y., Paluch, A.M., Waltz, S.E., Kasper, S., and Kalin, T.V. (2013) Foxm1 expression in prostate epithelial cells is essential for prostate carcinogenesis. J. Biol. Chem. 288, 22527-22541
15. Dai, B., Gong, A., Jing, Z., Aldape, K.D., Kang, S.H., Sawaya, R., and Huang, S. (2013) Forkhead box M1 is regulated by heat shock factor 1 and promotes glioma cells survival under heat shock stress. J. Biol. Chem. 288, 1634-1642
16. Carr, J.R., Kiefer, M.M., Park, H.J., Li, J., Wang, Z., Fontanarosa, J., DeWaal, D., Kopanja, D., Benevolenskaya, E.V., Guzman, G., and Raychaudhuri, P. (2012) FoxM1 regulates mammary luminal cell fate. Cell Rep. 1, 715-729
17. Anders, L., Ke, N., Hydbring, P., Choi, Y.J., Widlund, H.R., Chick, J.M., Zhai, H., Vidal, M., Gygi, S.P., Braun, P., and Sicinski, P. (2011) A systematic screen for CDK4/6 substrates links FOXM1 phosphorylation to senescence suppression in cancer cells. Cancer Cell 20, 620-634
18. Zhang, N., Wei, P., Gong, A., Chiu, W.T., Lee, H.T., Colman, H., Huang, H., Xue, J., Liu, M., Wang, Y., Sawaya, R., Xie, K., Yung, W.K., Medema, R.H., He, X., and Huang, S. (2011) FoxM1 promotes β-catenin nuclear localization and controls Wnt target-gene expression and glioma tumorigenesis. *Cancer Cell* **20**, 427-442

19. Xue, J., Lin, X., Chiu, W.T., Chen, Y.H., Yu, G., Liu, M., Feng, X.H., Sawaya, R., Medema, R.H., Hung, M.C., and Huang, S. (2014) Sustained activation of SMAD3/SMAD4 by FOXM1 promotes TGF-β-dependent cancer metastasis. *J. Clin. Invest.* **124**, 564-579

20. Park, H.J., Gusarova, G., Wang, Z., Carr, J.R., Li, J., Kim, K.H., Qiu, J., Park, Y.D., Williamson, P.R., Hay, N., Tyner, A.L., Lau, L.F., Costa, R.H., and Raychaudhuri, P. (2011) Deregulation of FoxM1b leads to tumour metastasis. *EMBO Mol. Med.* **3**, 21-34

21. Wang, I.C., Snyder, J., Zhang, Y., Lander, J., Nakafuku, Y., Lin, J., Chen, G., Kalin, T.V., Whitsett, J.A., and Kalinichenko, V.V. (2012) Foxm1 mediates cross talk between Kras/mitogen-activated protein kinase and canonical Wnt pathways during development of respiratory epithelium. *Mol. Cell Biol.* **32**, 3838-3850

22. Szczepny, A., Wagstaff, K.M., Dias, M., Gajewska, K., Wang, C., Davies, R.G., Kaur, G., Ly-Huynh, J., Loveland, K.L., and Jans, D.A. (2014) Overlapping binding sites for importin β1 and suppressor of fused (SuFu) on glioma-associated oncogene homologue 1 (Gli1) regulate its nuclear localization. *Biochem. J.* **461**, 469-476

23. Pandolfi, S., Montagnani, V., Penachioni, J.Y., Vinci, M.C., Olivito, B., Borgognoni, L., and Stecca, B. (2013) WIP1 phosphatase modulates the Hedgehog signaling by enhancing GLI1 function. *Oncogene* **32**, 4737-4747

24. Chi, S., Xie, G., Liu, H., Chen, K., Zhang, X., Li, C., and Xie, J. (2012) Rab23 negatively regulates Gli1 transcriptional factor in a Su(Fu)-dependent manner. *Cell Signal* **24**, 1222-1228

25. Kim, K., Kim, K.H., Cho, H.K., Kim, H.Y., Kim, H.H., and Cheong, J. (2010) SHP (small heterodimer partner) suppresses the transcriptional activity and nuclear localization of Hedgehog signalling protein Gli1. *Biochem. J.* **427**, 413-422

26. Deng, W., Vanderbilt, D.B., Lin, C.C., Martin, K.H., Brundage, K.M., and Ruppert, J.M. (2015) SOX9 inhibits β-TrCP-mediated protein degradation to promote nuclear GLI1 expression and cancer stem cell properties. *J. Cell Sci.* **128**, 1123-1138

27. Cheng, L., Wu, Q., Guryanova, O.A., Huang, Z., Huang, Q., Rich, J.N., and Bao, S. (2011) Elevated invasive potential of glioblastoma stem cells. *Biochem. Biophys. Res. Commun.* **406**, 643-648

28. Li, X., Liu, Y., Granberg, K.J., Wang, Q., Moore, L.M., Ji, P., Gumin J., Sulman, E.P., Calin, G.A., Haapasalo, H., Nykter, M., Shmulevich, I., Fuller, G.N., Lang, F.F., and Zhang, W. (2015) Two mature products of MIR-491 coordinate to suppress key cancer hallmarks in glioblastoma. *Oncogene* **34**, 1619-1628

29. Marian, C.O., Cho, S.K., McEllin, B.M., Maher, E.A., Hatanpaa, K.J., Madden, C.J., Mickey, B.E., Wright, W.E., Shay, J.W., and Bachoo, R.M. (2010) The telomerase antagonist, imetelstat, efficiently targets glioblastoma tumor-initiating cells leading to decreased proliferation and tumor growth. *Clin. Cancer Res.* **16**, 154-163

30. Infante, P., Mori, M., Alfonsi, R., Ghirga, F., Aiello, F., Toscano, S., Ingallina, C., Siler, M., Cucchi, D., Po, A., Miele, E., D’Amico, D., Canettieri, G., De Smaele, E., Ferretti, E., Screpanti, I., Uccello Barretta, G., Bottai, M., Bottai, B., Gulino, A., and Di Marcotullio, L. (2015) Gli1/DNA interaction is a druggable target for Hedgehog-dependent tumors. *EMBO J.* **34**, 200-217

31. Carpenter, R.L., and Lo, H.W. (2012) Hedgehog pathway and GLI1 isoforms in human cancer. *Discov. Med.* **13**, 105-113

32. Yoon, J.W., Kita, Y., Frank, D.J., Majewski, R.R., Konicek, B.A., Nobrega, M.A., Jacob, H., Walterhouse, D., and Iannaccone, P. (2012) Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. *J. Biol. Chem.* **277**, 5548-5555

33. Gai, X., Lu, Z., Tu, K., Liang, Z., and Zheng, X. (2014) Caveolin-1 is up-regulated by GLI1 and contributes to GLI1-driven EMT in hepatocellular carcinoma. *PLoS One* **9**, e84551
FOXM1 is essential for nuclear localization of GLI1

FOOTNOTES
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8The abbreviations used are: Hh, Hedgehog; GLI1, glioma-associated oncogene homolog 1; FOXM1, Forkhead box M1; GBM, glioblastoma multiforme; IPO7, importin-7; LMB, leptomycin B; IB, immunoblot; IP, immunoprecipitation; siFOXM1, FOXM1 siRNA.

FIGURE LEGENDS

FIGURE 1. FOXM1 promotes nuclear import of GLI1 in a DNA-binding-dependent manner. A, IB analysis of nuclear FOXM1 and GLI1 protein expression in Hs683, SW1783, HU251, and U87 cells. B, attenuation of nuclear import of GLI1 by knockdown of FOXM1 expression. Left: U87 cells were transfected with siFOXM1 or control siRNA (siCTRL). After 24 h of transfection, cells were treated with 5 nM LMB for 8 h. Cells were then stained with an anti-GLI1 antibody and a FITC-conjugated anti-rabbit secondary antibody (green, GLI1), and the nuclei were visualized via staining with DAPI (blue). Scale bars, 20 µm. Right: results are indicated as the percentage of cells with mostly cytoplasmic fluorescence, mostly nuclear fluorescence, or both cytoplasmic and nuclear fluorescence. C, inhibition of nuclear import of GLI1 by FOXM1 deficiency. Left: Foxm1β/β and Foxm1−/− mouse embryonic fibroblasts were treated with 5 nM LMB for 8 h and then stained. Scale bars, 20 µm. Right: results are indicated as the percentage of cells with mostly cytoplasmic fluorescence, mostly nuclear fluorescence, or both cytoplasmic and nuclear fluorescence. D, promotion of nuclear import of GLI1 by FOXM1 in a DNA-binding-dependent manner. Hs683 cells were transfected with the indicated plasmids, nuclear extracts from the cells were prepared, and the GLI1 expression in the extracts was measured. Lamin B was used as a loading control for nuclear fractions. E, induction of decreased nuclear GLI1 expression by FOXM1 depletion. U87 cells were transfected with siFOXM1 or control siRNA (siCTRL). After 36 h of transfection, nuclear cell lysates were subjected to SDS-PAGE and IB analysis using anti-GLI1 or anti-FOXM1 antibodies. F, enhancement of GLI1 target genes expression by FOXM1. Expression of Ptc1, Ptc1, CCND2, and IL-6 mRNA from Hs683 cells transfected with a control vector or FLAG-FoxM1 was measured using quantitative real-time RT-PCR. The mean (± standard deviation) values for triplicate samples from a representative experiment are presented. *p < 0.05; **p < 0.01. G, induction of decreased GLI1 target genes expression by knockdown of FOXM1 expression. U87 cells were transfected with siFOXM1 or control siRNA (siCTRL). The experiment was performed as described in F. The mean (± standard deviation) values for triplicate samples from a representative experiment are presented.***p < 0.001.

FIGURE 2. IPO7 is a direct transcriptional target of FOXM1. A, increased IPO7 expression induced by FOXM1. Hs683 cells were transfected with an empty vector or FOXM1 expression vector. Cell lysates were collected at 48 h after transfection and subjected to IB analysis with the indicated antibodies. B, schematic of the human IPO7 promoter. The sequences of the FOXM1-binding elements are shown. C, results of a ChIP assay performed with U87 cells. Chromatin fragments of the cells were immunoprecipitated with an anti-FOXM1 antibody or control IgG and subjected to PCR using primers for three FOXM1-binding sites. One percent of the total cell lysates was subjected to PCR before IP as an input. D and E, HEK 293T (D) and Hs683 (E) cells were transfected with the human IPO7 promoter and
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indicated plasmids. The luciferase activity of the cells was then determined. Each error bar indicates the variation in the mean results from three independent experiments. ***p < 0.001. F and G, U87 (F) and HFU251 (G) cells were transfected with the human IPO7 promoter and siFOXM1 or a control siRNA (siCTRL). The luciferase activity of the cells was then determined. Each error bar indicates the variation in the mean results from three independent experiments. **p < 0.01; ***p < 0.001.

FIGURE 3. IPO7 is a key nuclear transporter for GLI1 by interacting with GLI1 protein. A, expression of FLAG-GLI1 in HEK 293T cells and IP of it using an anti-FLAG antibody. GLI1-bound IPO7 protein was detected in the cells using an IB with an anti-IPO7 antibody. Whole-cell lysates were directly subjected to immunoblotting with FLAG or IPO7 antibody as an input. B, co-IP of endogenous GLI1 with IPO7 in U87 cells. IPO7 was immunoprecipitated, and the amount of IPO7 bound to GLI1 was determined using an IB with an anti-GLI1 antibody. C, attenuation of nuclear import of GLI1 by knockdown of IPO7 expression. Top: U87 cells were transfected with siRNAs targeting IPO7 (siIPO7) or control siRNA (siCTRL). After 24 h of transfection, cells were treated with 5 nM LMB for 8 h. Cells were then stained with an anti-GLI1 antibody and an FITC-conjugated anti-rabbit secondary antibody (green, for GLI1), and the nuclei were visualized via staining with DAPI (blue). Scale bars, 20 µm. Bottom: results are indicated as the percentage of cells with mostly cytoplasmic fluorescence, mostly nuclear fluorescence, or both cytoplasmic and nuclear fluorescence. D, knockdown of IPO7 expression decreased GLI1 target genes expression. U87 cells were transfected with siRNAs targeting IPO7 or control siRNA. Expression of Pch1, Pch2, CCND2, and IL-6 mRNA was measured using quantitative real-time RT-PCR. The mean (± standard deviation) values for triplicate samples from a representative experiment are presented. **p < 0.01; ***p < 0.001.

FIGURE 4. FOXM1 induces nuclear import of GLI1 by promoting IPO7 expression. A, left: after 24 h of transfection with the indicated siRNA and/or plasmids, U87 cells were treated with 5 nM LMB for 8 h. Cells were then stained with an anti-GLI1 antibody and an FITC-conjugated anti-rabbit secondary antibody (green, for GLI1), and the nuclei were visualized via staining with DAPI (blue). Scale bars, 20 µm. Right: results are indicated as the percentage of cells with mostly cytoplasmic fluorescence, mostly nuclear fluorescence, or both cytoplasmic and nuclear fluorescence. B, U87 cells were transfected with the indicated siRNA and/or plasmids, nuclear extracts were prepared, and the GLI1 expression was measured. Lamin B was used as a loading control for nuclear fractions. C, U87 cells were transfected with the indicated siRNA and/or plasmids. Expression of Pch1, Pch2, CCND2, and IL-6 mRNA was measured using quantitative real-time RT-PCR. The mean (± standard deviation) values for triplicate samples from a representative experiment are presented. **p < 0.01; ***p < 0.001. NS, not significant.

FIGURE 5. The FOXM1/IPO7/GLI1 axis promotes cell proliferation, migration, and invasion. A, U87 cells were transfected with the indicated siRNA and/or plasmids, and their proliferation was analyzed using an MTT assay. Data are presented as the mean (± standard deviation) values from three independent experiments. ***p < 0.001. B, left: U87 cells were transfected with the indicated siRNA and/or plasmids, and their growth was examined using a monolayer colony formation assay. Scale bars, 10 mm. Right: results of quantitative analysis of colony numbers shown as the mean (± standard deviation) values from three independent experiments. ***p < 0.001. C, U87 cells were transfected with the indicated siRNA and/or plasmids, and their migration was detected using a wound healing assay. The cell motility was quantified by measuring the distance between the invading front of cells in six randomly selected microscopic fields for each condition and time point. The degree of motility is expressed as the percent wound closure as compared with the zero time point. The mean (± standard deviation) values from three independent experiments are presented. **p < 0.01. D, left: U87 cells were transfected with the indicated siRNA and/or plasmids and subjected to an in vitro invasion assay. Scale bars, 200 µm. Right: quantitative analysis of invasive U87 cells shown as the mean (± standard deviation) results from three independent experiments. ***p < 0.001.
FIGURE 6. FOXM1 expression positively correlates with GLI1 expression in human GBM specimens. A, immunohistochemical staining of 40 human GBM specimens with anti-FOXM1 and anti-GLI1 antibodies were carried out. Photographs of two representative specimens are shown. Scale bars, 200 µm. B, semiquantitative scoring was carried out ($r = 0.803; p < 0.001$ [Pearson correlation coefficient]) for all 40 GBM specimens. Note: some of the dots on the graph represent more than one specimen (that is, some scores overlapped). C, Model for FOXM1-mediated nuclear localization of GLI1. FOXM1 transcriptionally activates IPO7. In return, IPO7 induces nuclear localization of GLI1. Nuclear GLI1 then enhances FOXM1 protein expression, forming a positive feedback loop and promoting GBM development.
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Figure 1

A

Hs683 SW1783 HFU251 U87

FOXM1 GL1 Lamin B

D

WT DBD R268AM267A

FLAG-FOXM1 GL1 FLAG

E

siCTRL siFOX1M

GL1 FOXM1 Lamin B

F

- FOXM1 + FOXM1

Relative fold change

Ptch1 Ptch2 CCND2 IL-6

G

siCTRL siFOX1M

Relative fold change

Ptch1 Ptch2 CCND2 IL-6
Figure 2

A

CTRL
FOXM1

IPO7
IPO8
IPO9
IPO13
FOXM1
β-Actin

B

FBE1 FBE2 FBE3

IPO7

AGTTAAACATTT

TTATTTTATTTATTTATTT

C

FBE1
FBE2
FBE3

H2O input input IgG FOXM1

D

Relative Activation (Fold)

FLAG-FOXM1 WT ΔDBD R286A/H287A

E

Relative Activation (Fold)

FLAG-FOXM1 WT ΔDBD R286A/H287A

F

Relative Activation (Fold)

siCTRL siFOXN1

G

Relative Activation (Fold)

siCTRL siFOXN1

FOXM1 is essential for nuclear localization of GLI1
Figure 3

A

| IP: FLAG | Input | IB: IPO7 | IB: GLI1 |
|----------|-------|---------|---------|
| -        |       |         |         |
| +        |       |         |         |

B

| IP: IgG | Input | IB: IPO7 | GLI1 |
|---------|-------|----------|------|
| -       |       |          |      |
| +       |       |          |      |

C

GLI1

DAPI

Merged

siCTRL

siIPO7

D

Relative fold change

siCTRL

siIPO7

Ptch1

Ptch2

CCND2

IL-6

Subcellular localization %

siCTRL

siIPO7

Cytoplasmic

Nuclear

Cytoplasmic + Nuclear

Nuclear
Figure 4

A

GLI1  DAPI  Merged

siCTRL  siFOXM1  siFOXM1 + IPO7

Subcellular localization %

0  50  100

siCTRL  siFOXM1  siFOXM1 + IPO7

Cytoplasmic  Nuclear+Cytoplasmic  Nuclear

B

siFOXM1  -  +  +

IPO7  -  -  +

GLI1  FOXM1  Lamin B

C

Relative fold change

0.0  0.5  1.0  1.5

Ptch1  Ptch2  CCND2  IL-6

siCTRL  siFOXM1  siFOXM1 + IPO7

NS  NS  NS  NS

***  **  ***  ***

FOXM1 is essential for nuclear localization of GLI1
Figure 5

A

MTT assay (fold change)

siCTRL  siFOX1  siFOX1 +IPO7  siFOX1 +GLI1

C

% Wound closure

siCTRL  siFOX1  siFOX1 +IPO7  siFOX1 +GLI1

B

siCTRL  siFOX1  siFOX1 +IPO7  siFOX1 +GLI1

D

siCTRL  siFOX1  siFOX1 +IPO7  siFOX1 +GLI1

% Colonies

siCTRL  siFOX1  siFOX1 +IPO7  siFOX1 +GLI1

Invasive cells per field

siCTRL  siFOX1  siFOX1 +IPO7  siFOX1 +GLI1

FOXM1 is essential for nuclear localization of GLI1
Figure 6

A

Tumor1

GLI1

Tumor2

FOXM1

B

GLI1 level

FOXM1 level

0

5

10

15

C

GLI1 → FOXM1 → IPO7

FOXM1

IP07 promoter

Nucleus

GLI1

Transcription of target genes (Psch1, CCND2, etc.)

Tumorigenesis
Forkhead Box M1 Is Essential for Nuclear Localization of Glioma-associated Oncogene Homolog 1 in Glioblastoma Multiforme Cells by Promoting Importin-7 Expression
Jianfei Xue, Aidong Zhou, Christina Tan, Yamei Wu, Hsueh-Te Lee, Wenliang Li, Keping Xie and Suyun Huang

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