Supporting Information

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SI Methods

Antibodies. Antibodies used in these studies correspond to the following: CDK8 (sc-1521), MED1 (sc-5334), transcription factor (TF) IIEβ p34 (sc-238), TFIIH p89 (sc-293), and peroxisome proliferator-activated receptor (PPAR)γ (sc-7196) were purchased from Santa Cruz Biotechnology; FLAG (F3165) was purchased from Sigma-Aldrich; MED12 (A300-774A) was purchased from Bethyl Laboratories; MED23 (550429) was purchased from BD Pharmingen; MED6 (ab35432) and TATA box-binding protein (TBP) (ab62126) were purchased from Abcam; and MED18 (NB100-2360) was purchased from Novus Biologicals. Production and purification of MED30 and MED4 polyclonal antibodies have been described (1).

Expression and Reporter Plasmids. Expression plasmids pM-GLI3-MBD, pCMV-GAL4-CMV-β-cat, pact-FLAG-Gli3, pJT4-Shh, and reporter plasmids pG5-E1B-Luc, 8 × 3′ Gli-BS Luc, and pact–β-galactosidase have been described previously (1, 2). Plasmids p3XFLAG-CMV-hMED12 WT/R961W/N1007S expressing FLAG-tagged human WT/R961W/N1007S derivatives have been described previously (3). Plasmids p3XFLAG-CMV-hMED12r WT/R961W/N1007S expressing siRNA-resistant FLAG-tagged human WT/R961W/N1007S derivatives were generated from their corresponding non–siRNA-resistant counterparts by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis kit (Stratagene). Two nucleotide changes were introduced into MED12 cDNA sequence corresponding to the region in MED12 RNA targeted by MED12-specific siRNA. These two nucleotide changes (T > G at nucleotide 957 and A > C at nucleotide 960) are silent mutations that do not alter corresponding amino acids 319 and 320 of the encoded MED12 protein, but nonetheless render the MED12-encoded RNA siRNA resistant. Plasmid pcDNA3.1-FLAG CDK8 WT was generated by PCR-based amplification of the CDK8 cDNA sequence using primers carrying an encoded FLAG epitope followed by subcloning of PCR-amplified DNA into the HindIII and XhoI sites of pcDNA3.1. Plasmid pcDNA3.1-FLAG CDK8 D173A was generated by site-directed mutagenesis of pcDNA3.1-FLAG CDK8 WT using the QuikChange II Site-Directed Mutagenesis kit (Stratagene).

Transfection, Reporter Assays, and RNAi. For MED12 knockdown/rescue assays in HeLa cells, cells were seeded into 10-cm dishes 24 h before transfection with control (D-001210-01-05) or MED12-specific (M-009092-00) siRNAs (Dharmacon) using Transit SiQuest transfection reagent (Mirus Bio). siRNA-treated cells were reseeded at 1.0×10^5 cells per well in 12-well culture plates, and 24 h later transfected with plasmids pG5-E1B-Luc, pM, pM-GLI3-MBD, p3XFLAG-CMV-hMED12r WT/R961W/LN1007S, and pact–β-galactosidase using Fugene 6 (Promega). Forty-eight hours following DNA transfections, cells were harvested and processed for luciferase (Promega) and β-galactosidase (Applied Biosystems) activities. For MED12 knockdown/rescue assays in CH310T1/2 cells, cells were seeded into 10-cm dishes 24 h before nucleofection with control siRNA (D-001210-20) or Med12-specific (M-048744-00) siRNAs (Dharmacon) using a Nucleofector II and Nucleofector Kit R (Amaxa Biosystems). Nucleofected cells were seeded at 1.0×10^5 cells per well in 12-well culture plates, and 24 h later transfected with plasmids pact-FLAG-GLI3, p8 × 3′ Gli-BS Luc, p3XFLAG-CMV-hMED12r WT/R961W/LN1007S, and pact–β-galactosidase using Fugene 6 (Promega). Twenty-four hours following DNA transfections, cell culture medium was replaced with control or Shh-conditioned medium, and cells were cultured for an additional 24 h before cell harvest and processing for luciferase (Promega) and β-galactosidase (Applied Biosystems) activities as described previously (2).

For CDK8 knockdown experiments in HeLa cells, cells were seeded into 10-cm culture dishes 24 h before transfection with control (D-001210-01-05), MED23-specific (M-013220-00), MED12-specific (M-009092-00), or CDK8-specific (J-003242-12) siRNAs (Dharmacon) using Transit SiQuest transfection reagent (Mirus Bio). siRNA-treated cells were reseeded at 1.0×10^5 cells per well in 12-well culture plates, and 24 h later transfected with plasmids pG5-E1B-Luc, pM, pM-GLI3-MBD, and pact–β-galactosidase using Fugene 6 (Promega). Forty-eight hours following DNA transfections, cells were harvested and processed for luciferase (Promega) and β-galactosidase (Applied Biosystems) activities. For CDK8 knockdown experiments in CH310T1/2 cells, cells were seeded into 10-cm culture dishes 24 h before transfection with control (D-001210-20), Med12 (M-048744-00), Cdk8 (J-003242-12), or Med23 (M-013220-00) specific siRNAs (Dharmacon) using a Nucleofector II and Nucleofector Kit R. Electroporated cells were seeded at 1.0×10^5 cells per well in 12-well culture plates, and 24 h later transfected with plasmids pact-FLAG-GLI3, p8 × 3′ Gli-BS Luc, and pact–β-galactosidase using Fugene 6 (Promega). Twenty-four hours posttransfection, cell culture medium was replaced with control or Shh-conditioned medium, and cells were cultured for an additional 24 h before cell harvest and processing of luciferase (Promega) and β-galactosidase (Applied Biosystems) activities as described previously (2).

For CDK8 knockdown/rescue assays in HeLa cells, cells were seeded into 10-cm culture dishes 24 h before infection with lentiviruses encoding either nonspecific shRNA, or shRNA specific for CDK8 (3′ untranslated region). Cells were infected for 4 h in the presence of polybrene (8 mg/mL). Twenty-four hours postinfection, cells were selected with puromycin (2,500 ng/mL) for 48 h. After puromycin selection, cells were seeded at 1×10^5 cells per well (12-well format) in the presence of 2,500 ng/mL puromycin. Twenty-four hours postseeding, cells were transfected with plasmids pG5-E1B-Luc, pM, pM-GLI3-MBD, pCDNA3.1-FLAG CDK8 (WT or D173A), and pact–β-galactosidase using FuGene6 (Promega). Forty-eight hours post-transfection, cells were harvested and processed for luciferase (Promega) and β-galactosidase (Applied Biosystems) activities. For generation of shRNA-expressing lentivirus, 293T cells were cotransfected with 4 μg pLKO.1 shRNA transfer vector (Sigma; shCDK8, TRCN0000000489 and shControl, SHC002), 4 μg pMD2.G (AddGene), and 4 μg pSPAX2 (AddGene) using FuGene6 reagent (Roche) under serum-free conditions. After 16 h, medium was changed, and recombinant lentiviruses were harvested 24 and 48 h later. Lentivirus was subjected to concentration by ultracentrifugation at 120,000 × *g* for 2 h.

For cyclopamine treatment experiments, control, FG, and Lujan patient-derived cells were treated with DMSO alone or DMSO/cyclopamine (5 μM) for 24 h before cell harvest and mRNA isolation. For shGLI3 knockdown experiments, control, FG, and Lujan patient-derived cells were nucleofected with control or shGLI3-specific knockdown vectors (RHS4533-NM_000168; Open Biosystems) using a Nucleofector II and Nucleofector Kit R (Amaxa Biosystems). At 48 h postnucleofection, cells were harvested for mRNA isolation.

For GW9662 treatment, control, FG, and Lujan patient-derived cells were treated with DMSO alone or DMSO/GW9662 (5 μM) for 24 h before cell harvest and mRNA isolation. For shPPARγ knockdown experiments, control, FG, and Lujan patient-derived cells were nucleofected with control or shPPARγ-

specific knockdown vectors (RHS4533-NM_138712; Open Biosystems) using a Nucleofector II and Nucleofector Kit R (Amaxa Biosystems). At 48 h postnucleofection, cells were harvested for mRNA and protein isolation.

Baculovirus-Based Protein Expression. A C-terminal hemagglutinin (HA) tag was appended to full-length MED12 by PCR followed by subcloning into a pFASTBAC1 baculovirus transfer vector. MED12 mutant derivatives FG/R961W and Lujan/N1007S were generated by site-directed mutagenesis of WT MED12-HA in pFASTBAC1. C-terminal FLAG-tagged CDK8 (WT and D173A) and N-terminal 6-His CyclinC were PCR amplified and cloned into pFASTBAC1. An N-terminal calmodulin binding peptide epitope tag (CBP tag; RRWKKNFIAVSAANRFKKISSSGAL) was first inserted into a $pCS2^{+}$ expression vector by primer annealing. MED13 was then PCR amplified in multiple steps from an expression vector from Origene (SC126930) and placed into the $pCS2^{+}$-CBP vector. CBP-MED13 was then subcloned into pFASTBAC1. pFASTBAC1 donor plasmids with the full-length MED12-HA, CDK8-FLAG, 6His-CyclinC, and CBP-MED13 were transformed into DH10Bac competent cells (Invitrogen). Isolated recombinant bacmid DNAs were confirmed by PCR and sequencing and subsequently used for transfection of Sf9 insect cells. After three rounds of viral amplification, high titer baculovirus stocks were used for infection of High Five insect cells (Invitrogen). At 48 h postinfection, High Five cells were resuspended and lysed in 0.5 M D buffer [500 mM NaCl, 0.1% Nonidet P-40, 0.1 mM EDTA pH 8.0, 10% (vol/vol) glycerol, 20 mM Hepes pH 7.9] using a dounce homogenizer at 4 °C. Lysates were clarified by centrifugation at 26,000 × *g* for 30 min.

Immunoprecipitation/Kinase Assays. Whole cell lysates from insect High Five cells expressing CBP-MED13, 6His-CyclinC, CDK8-FLAG-WT or D173A, and MED12-HA- WT, R961W, or N1007S derivatives were subjected to FLAG immunoprecipitation for 1 h at 4 °C in 0.2 M NaCl D buffer. Note that because CDK8 expression is limiting relative to other kinase module subunits during viral expression, WT and mutant MED12 derivatives are thus present in excess levels at the input concentrations used in these immunoprecipitation reactions. Immune complexes were washed in 0.2 M NaCl D buffer and either eluted in Laemmli sample buffer, and processed by SDS-10% (wt/vol) PAGE for immunoblot analysis, or subjected to in vitro kinase assay in 25 mM Tris pH 7.5, 20 mM $MgCl_2$, 2.5 mCi [γ-^{32}P]ATP and 2 μg of purified GST or GST-3xCTD sustrate. ^{32}P-labeled GST-3xCTD was resolved by SDS-12% PAGE, stained with Coomassie blue, and visualized by PhosphorImager analysis. GST-3xCTD was cloned by first annealing primers encoding three heptapeptide repeats (TSPTSPS) from the C-terminal domain (CTD) within the RNA polymerase II large subunit, and subsequently introducing the annealed double-stranded product into pGEX6P-1. Clones were confirmed by enzyme digestion and transformed into BL21 *Escherichia coli* competent cells, grown to an OD_{600} of 0.6 and induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside for 4 h at 30 °C. Cells were pelleted, resuspended in Lysis250 buffer (50 mM Tris pH 7.5, 5 mM EDTA, 250 mM NaCl), and lysed using a sonicator at 4 °C. GST-3xCTD was purified from clarified lysates (centrifugation at 26,000 × *g* for 30 min) on glutathione sepharose 4B (GE Healthcare; 17–0756-01) following washing with Lysis250 and elution reducing buffer (25 mM Tris pH 8.0, 20 mM reduced glutathione (Sigma; G4251).

GST Pull-Down Assays. GST and GST-GLI3 MBD were expressed in *E. coli* strain BL21 CodonPlus (Stratagene). Soluble lysates were prepared in Lysis250 buffer (50 mM Tris·HCl, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40) supplemented with protease inhibitors. Resuspended cells were subjected to one freeze–thaw cycle followed by sonication and clarification by centrifugation at 35,000 × *g* for 30 min at 4 °C. For GST pull-down assays using radiolabeled recombinant WT MED12 and mutants (FG/Lujan), 20 μg each of GST or GST-GLI3 MBD was immobilized on glutathione sepharose beads (GE Healthcare Life Sciences) and washed extensively with Lysis250 buffer containing 0.1% Nonidet P-40. Bead-immobilized GST or GST-GLI3 MBD were washed once with 0.1 M KCl D buffer containing 0.1% Nonidet P-40 and subsequently incubated with either radiolabeled recombinant WT or mutant MED12 derivatives. Beads then were washed five times with 0.2 M KCl D buffer (0.1% Nonidet P-40). Bound proteins were eluted with Laemmli sample buffer, resolved by SDS-10% PAGE, and processed by PhosphorImager analysis.

Immunoprecipitation Assays. For immunoprecipitation experiments involving FLAG-MED12/R961W/N1007S and HA-GLI3, HeLa cells were cotransfected with p3XFLAG-CMV-hMED12 WT/R961W/N1007S (4 μg) and pCDNA3.1-HA-Gli3 (4 μg). At 48 h posttransfection, nuclear extracts were prepared and subjected to immunoprecipitation using FLAG-specific antibody as described previously (2). For immunoprecipitations from control, FG, and Lujan patient cells, nuclear extracts (1 mg; adjusted to 0.3 M KCl and 0.1% Nonidet P-40) were incubated for 12 h at 4 °C with either MED30-specific rabbit polyclonal antibodies, rabbit IgG, CDK8-specific goat polyclonal antibodies, or goat IgG followed by addition Protein A-sepharose beads (20 μL). Immune complexes were washed five times for 5 min each at 4 °C with 0.2 M KCl D buffer (0.1% Nonidet P-40). Immunoprecipitated proteins were subsequently eluted with Laemmli sample buffer, resolved by SDS-10% (wt/vol) PAGE, and processed by immunoblot analysis for the presence of Mediator subunits.

RT-qPCR. RNA isolated from CH310T1/2 as well as control, FG, and Lujan patient cell lines was reverse transcribed using oligo(dT) and SuperScript III (Invitrogen) following standard procedures, and subjected to reverse transcription-quantitative PCR (RT-qPCR) using an Applied Biosystems 7300 Real-Time PCR system and Absolute SYBR Green mix (Applied Biosystems). Primer sequences used for RT-qPCR are provided in the table below.

Primers.

	Forward	Reverse
ChIP primers		
GLI1	ACGGCAAGAGGGAGGGAAATAGAA	ACCCGCGAGAAGCGCAAACTT
GLI1-up	AACCTGGGCTGGCATACCTTAACT	TCGGTTGTCCAGTAACTGTGCTGT
ASCL1	AGCCCAGCACTCTCTCACTTCT	ATTTCCTTCTTTCACTCGCCCTCC
ASCL1-UP	GCTGAGCCAAGCCAAATACATGGT	TAGACACTGTCCTACCCAGGGAAA
AKT2	TCTAACCCACACAGGCCCAAAGTT	TGACGTACAAAGAGCCAGAGACGA
AKT2-up	TCCCAAAGTGCTGGGATTACAGGT	TTGAATCTTGTGGCAGGGCAACAC
KLF10	GATTGTGTACACGCTCCACTGACA	CCAATCAACGGCAAAGGTGTGTGA
KLF10-up	AGTTCAAGACCAGCCTGACCAACA	TGGGTTCAAGTGATTCTCCTGCCT

Cont.

	Forward	Reverse
mRNA primers		
GLI1	TTTGGACCCAACTTGCCCAATCAC	TGCTGCCCTATGTGAAGCCCTATT
b-ACTIN	AATGTGGCCGAGGACTTTGATTGC	AGGATGGCAAGGGACTTCCTGTAA
SHH	AGTCCAAGGCACATATCCACTGCT	GGAAAGTGAGGAAGTCGCTGTAGA
NGN2	AGGGCAGGTGTAGCCTTTCTGATT	CGCCACCCTTGGCTTTGACAATAA
ASCL1	AAGAGCAACTGGGACCTGAGTCAA	AGCAAGAACTTTCAGCTGTGCGTG
BMP4	TGCAGACCCTGGTCAATTCTGTCA	AGCATGGAGATGGCACTCAGTTCA
CREB5	CGTGCCTCCTTGAAACAAGCCATT	ATGAAACACCAGCACCTGCCTAGA
PPARγ	CTGTTTGCCAAGCTGCTCCAGAAA	AAGAAGGGAAATGTTGGCAGTGGC
AKT2	ACTGGCCTTTCTCCTCGTCTTCAT	TTGCTGAAGGGAACGGCTAGTACA
KLF10	TGGCAGATGGAAGTGAGCAAGCTA	GCTTCTGCTTTAAGCCCACGTTGT

1. Kim S, Xu X, Hecht A, Boyer TG (2006) Mediator is a transducer of Wnt/beta-catenin signling. *J Biol Chem* 281(20):14066–14075.
2. Zhou H, Kim S, Ishii S, Boyer TG (2006) Mediator modulates Gli3-dependent Sonic hedgehog signaling. *Mol Cell Biol* 26(23):8667–8682.
3. Ding N, et al. (2008) Mediator links epigenetic silencing of neuronal gene expression with x-linked mental retardation. *Mol Cell* 31(3):347–359.

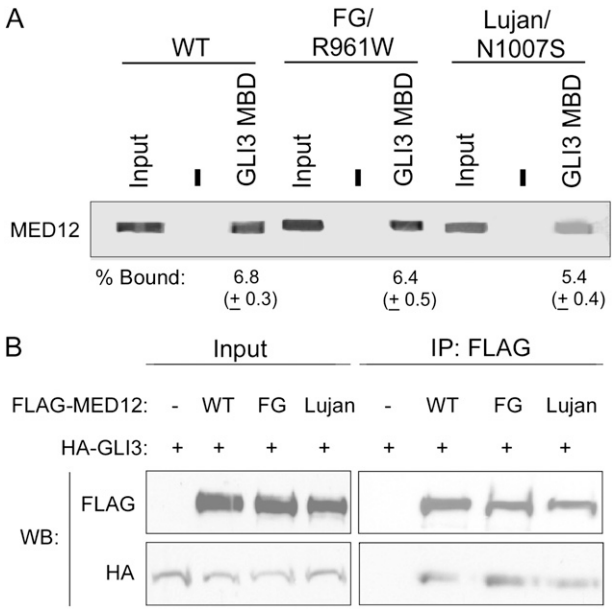

Fig. S1. MED12 WT, R961W, and N1007S derivatives bind comparably to GLI3. (*A*) Recombinant full-length MED12 WT, FG/R961W, or Lujan/N1007S derivatives were expressed and radiolabeled with [^{35}S]methionine by translation in vitro before incubation with glutathione-sepharose-immobilized GST (–) or GST-GLI3 MBD (GLI3 MBD) as indicated. Bound proteins were eluted with Laemmli sample buffer, resolved by SDS/PAGE, and visualized by phosphorimager analysis. Input was 10% of each in vitro translated protein used in binding reactions. The amount of each radiolabeled MED12 derivative retained by GST-GLI3 MBD (% bound) was quantified and is expressed as a percentage of the total input. Values represent the average of at least three binding experiments ± SEM. (*B*) HA-GLI3 was expressed without or with FLAG-MED12 WT, FG/R961W, or Lujan/N1007S derivatives in HeLa cells before processing of nuclear extracts by immunoprecipitation (IP) using antibodies specific for the FLAG epitope as indicated. Immunoprecipitates were resolved by SDS/PAGE and processed by Western blot (WB) analysis using FLAG- or HA-specific antibodies as indicated. Input was 10% of the nuclear extract used for IP reactions.

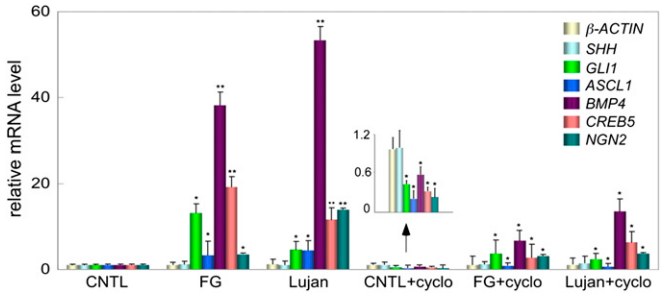

Fig. S2. Cyclopamine inhibits enhanced expression of GLI3-target genes in FG and Lujan patient-derived cells. RNA from control (CNTL), FG/R961W (FG), and Lujan/N1007S (Lujan) patient-derived cell lines treated without or with 5 μM cyclopamine (cyclo) was used for RT-qPCR. mRNA levels for each gene were normalized to β-actin mRNA and expressed relative to their corresponding mRNA levels in control (CNTL) patient-derived cell lines without cyclopamine treatment. Data represent the mean ± SEM of at least three independent experiments performed in duplicate. Asterisks denote statistically significant differences (Student *t* test, $*P < 0.05$, $**P < 0.01$). For cells treated without cyclopamine, asterisks denote statistically significant differences compared with relative mRNA levels in CNTL cells. For cells treated with cyclopamine, asterisks denote statistically significant differences compared with relative mRNA levels in each corresponding cell line treated without cyclopamine.

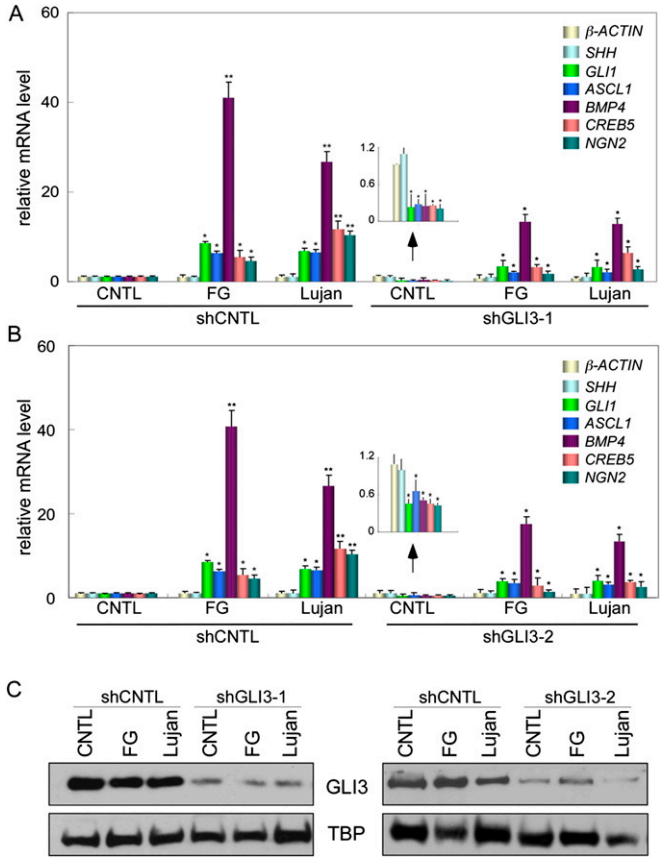

Fig. S3. GLI3-specific shRNA inhibits enhanced expression of SHH-responsive genes in FG and Lujan patient-derived cells. (*A* and *B*) RNA from control (CNTL), FG/R961W (FG), and Lujan/N1007S (Lujan) patient-derived cell lines treated with CNTL or two different GLI3-specific shRNAs (shGLI3-1 and shGLI3-2 targeting distinct regions in the GLI3 mRNA) was used for RT-qPCR. mRNA levels for each gene were normalized to β-actin mRNA and expressed relative to their corresponding mRNA levels in control (CNTL) patient-derived cell lines treated with CNTL shRNA. Data represent the mean ± SEM of at least three independent experiments performed in duplicate. Asterisks denote statistically significant differences (Student *t* test, $*P < 0.05$, $**P < 0.01$). For cells treated with shCNTL, asterisks denote statistically significant differences compared with mRNA levels in CNTL cells. For cells treated with shGLI-1 and shGLI3-2, asterisks denote statistically significant differences compared with each corresponding cell line treated with shCNTL. (*C*) Nuclear extracts from representative knockdown assays in *A* and *B* were resolved by SDS/PAGE and processed by Western blot analysis using antibodies specific for GLI3 or TBP as an internal loading control.

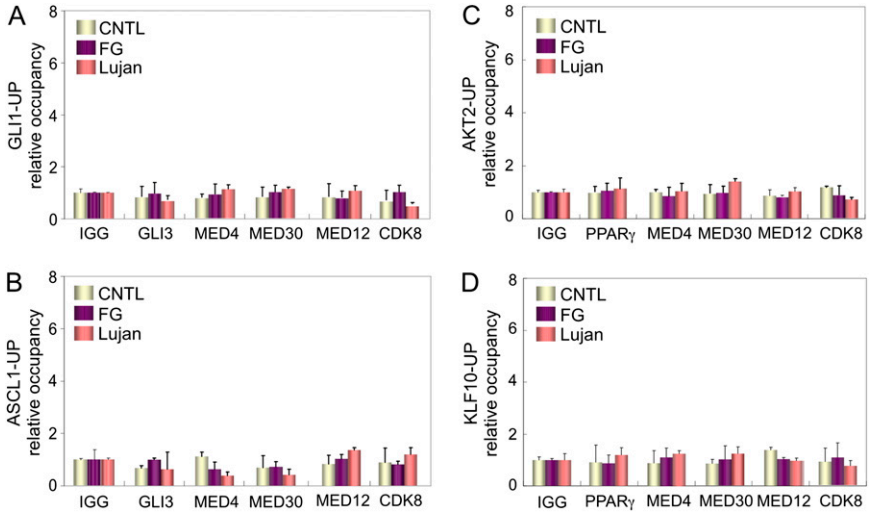

Fig. S4. GLI3, PPARγ, and Mediator do not bind to DNA sequences upstream of *GLI1*, *ASCL1*, *AKT2*, and *KLF10* genes. (*A–D*) Soluble chromatin prepared from control (CNTL), FG/R961W (FG), and Lujan/N1007S (Lujan) patient-derived cell lines was subjected to IP using the indicated antibodies. Immunoprecipitated chromatin was analyzed by quantitative PCR using primers located far upstream of the *GLI1* and *ASCL1* genes (*A* and *B*) or the *AKT2* and *KLF10* genes (*C* and *D*). DNA occupancy for each protein is expressed relative to control IgG, which was arbitrarily assigned a value of 1.

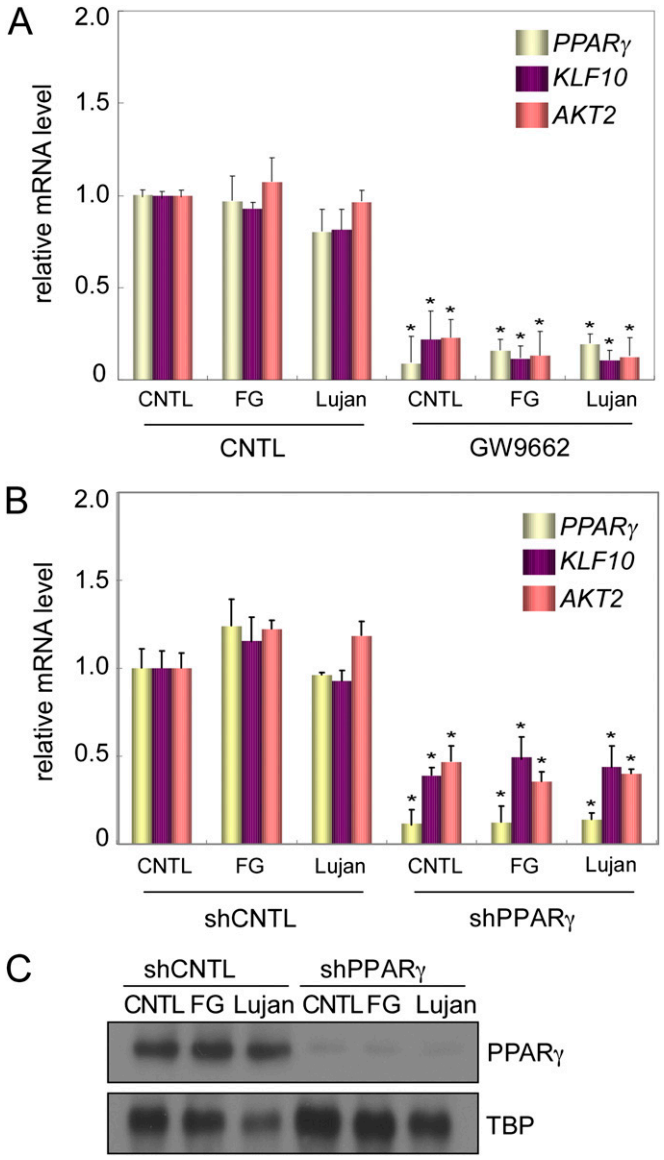

Fig. S5. PPARγ-specific shRNA and PPARγ antagonist GW9662 inhibit *AKT2* and *KLF10* expression in patient-derived cells. (*A* and *B*) RNA from control (CNTL), FG/R961W (FG), and Lujan/N1007S (Lujan) patient-derived cell lines treated without or with 5 μM GW9662 (*A*) or with CNTL or PPARγ-specific shRNAs (*B*) was used for RT-qPCR. mRNA levels for each gene were normalized to β-actin mRNA and expressed relative to their corresponding mRNA levels in control (CNTL) patient-derived cell lines treated without GW9662 (*A*) or with CNTL shRNA (*B*). Data represent the mean ± SEM of at least three independent experiments performed in duplicate. Asterisks denote statistically significant differences compared with relative mRNA levels in each corresponding cell line treated without cyclopamine (*A*) or with shCNTL (*B*) (Student *t* test, $*P < 0.05$). (*C*) Nuclear extracts from a representative knockdown assay were resolved by SDS/PAGE and processed by Western blot analysis using antibodies specific for PPARγ or TBP as an internal loading control.