THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 25, pp. 22077–22089, June 24, 2011
Printed in the U.S.A.

Modulation of the Transactivation Function and Stability of Krüppel-like Zinc Finger Protein Gli-similar 3 (Glis3) by Suppressor of Fused*

Received for publication, January 25, 2011, and in revised form, April 4, 2011 Published, JBC Papers in Press, May 4, 2011, DOI 10.1074/jbc.M111.224964

Gary T. ZeRuth, Xiao-Ping Yang, and Anton M. Jetten[1]

From the Cell Biology Section, Division of Intramural Research, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Glis3 is a member of the Glis subfamily of Krüppel-like zinc finger transcription factors. Recently, Glis3 has been linked to both type I and type II diabetes and shown to positively regulate insulin gene expression. In this study, we have identified a region within the N terminus of Glis3 that shares high levels of homology with the *Cubitus interruptus* (Ci)/Gli family of proteins. We demonstrated that Glis3 interacts with Suppressor of Fused (SUFU), which involves a VYGHF motif located within this conserved region. We further showed that SUFU is able to inhibit the activation of the insulin promoter by Glis3 but not the activation by a Glis3 mutant deficient in its ability to bind SUFU, suggesting that the inhibitory effect is dependent on the interaction between the two proteins. Exogenous SUFU did not affect the nuclear localization of Glis3; however, Glis3 promoted the nuclear accumulation of SUFU. Additionally, we demonstrated that SUFU stabilizes Glis3 in part by antagonizing the Glis3 association with a Cullin 3-based E3 ubiquitin ligase that promotes the ubiquitination and degradation of Glis3. This is the first reported instance of Glis3 interacting with SUFU and suggests a novel role for SUFU in the modulation of Glis3 signaling. Given the critical role of Glis3 in pancreatic β-cell generation and maintenance, the elevated Glis3 expression in several cancers, and the established role of SUFU as a tumor suppressor, these data provide further insight into Glis3 regulation and its function in development and disease.

Gli-similar 1–3 (Glis1–3)[2] constitute a distinct subfamily of Krüppel-like zinc finger transcription factors that are related to members of the Gli family and the *Drosophila* homolog *Cubitus interruptus* (Ci), the downstream effectors of Hedgehog signaling, and proteins in the Zic family (1–3). Homology among Glis, Zic, and Ci/Gli proteins is limited to the five Cys_2–His_2 zinc finger motifs shared by all three subfamilies, with little or no similarity outside of the region (4). The zinc finger domain of Glis3 is required for the recognition of specific DNA-binding sites within the regulatory regions of target genes (4–8). *In vitro* studies have shown that Glis3 optimally binds the consensus sequence 5′-(G/C)TGGGGGGT(A/C)-3′, designated as the GlisBS (9). In addition to its zinc finger domain (ZFD), the Glis3 C terminus has proven indispensable for transactivation, whereas little is known currently about the role of its N terminus (4, 9).

Glis3 has been implicated in the regulation of several physiological processes, including osteoblast differentiation, pancreatic development, and the maintenance of normal renal functions (1, 5, 6, 10, 11). Genetic aberrations in the *GLIS3* gene have been linked to the development of neonatal diabetes and congenital hypothyroidism, polycystic kidney disease, and liver fibrosis, as well as type 1 and type 2 diabetes (11–16). Mice with a disrupted Glis3 function develop neonatal diabetes, hypothyroidism, and polycystic kidney disease (6, 7, 17). Recently, Glis3 has been shown to positively regulate transcription of the human proinsulin (*INS*) and rodent insulin 2 (*Ins2*) genes in pancreatic β-cells (7, 8). Transcriptional regulation and β-cell-specific expression of the insulin gene occurs via an ~600-bp upstream regulatory region (18, 19). The most notable elements within the insulin promoter are the A-, E-, and C-boxes, which bind Pdx1, β2/NeuroD1, and MafA, respectively. Glis3 induces insulin gene expression through two GlisBS within the proximal promoter region (7, 8). Thus far, little is known about the precise mechanisms that control Glis3 activation and Glis3-induced transcription.

Previous studies have shown that the DNA-binding domain and transcriptional activation domain are localized in the center and within the C terminus of Glis3, respectively (1, 4, 9); however, little is known about the function of its 500-amino acid N terminus. The objective of this study was to examine the potential role of the N terminus in modulating Glis3 activity. Analysis of a series of N-terminal deletions within Glis3 indicated the presence of several regulatory domains that influence the transcriptional activity and stability of Glis3. In addition, we identified a region within the N terminus of Glis3 that shares high homology with a corresponding region of Ci and its mammalian homologs, Gli1–3. We show that the tumor suppressor Suppressor of Fused (SUFU) interacts with Glis3 through a VYGHF motif within this N-terminal conserved region and functions as a modulator of Glis3 signaling. SUFU was able to inhibit the Glis3-mediated activation of the insulin promoter and protected Glis3 protein against proteasomal degradation. We provide evidence for a role of the E3 ubiquitin ligase scaffolding component, Cullin 3 (Cul3), in the regulation of Glis3

* This work was supported, in whole or in part, by National Institutes of Health Grant Z01-ES-100485 from the Intramural Research Program of the NIEHS.

[1] To whom correspondence should be addressed: 111 T. W. Alexander Dr., Research Triangle Park, NC 27709. Tel.: 919-541-2768; Fax: 919-541-4133; E-mail: jetten@niehs.nih.gov.

[2] The abbreviations used are: Glis, Gli-similar; GlisBS, Glis binding sequence; Ci, *Cubitus interruptus*; qRT-PCR, quantitative real-time PCR; ZFD, zinc finger domain; SUFU, Suppressor of Fused; EGFP, enhanced green fluorescent protein; Luc, luciferase.

protein stability. Cul3 associates with Glis3 at a degron located within the Glis3 N-terminal conserved region and subsequently promotes Glis3 polyubiquitination and degradation via the 26S proteasomal pathway. Our data suggest that the inhibition of the association of Cul3 with Glis3 by SUFU is in part responsible for the decreased Glis3 ubiquitination and increased protein stabilization. This is the first report identifying SUFU as an interacting partner with Glis3. Our study suggests a novel role for SUFU in the modulation of different aspects of the Glis3 signaling pathway, including the transcriptional regulation of target genes and protein stability.

MATERIALS AND METHODS

Cells and Growth Conditions—Rat insulinoma INS-1832/13 cells, a generous gift from Dr. H. Hohmeier (Duke University), were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol. HEK293 and IMCD3 cells were purchased from ATCC and cultured in DMEM containing 10% FBS and in DMEM-F12 medium (1:1) supplemented with 10% FBS, respectively.

Generation of Reporter and Expression Constructs—The generation of p3xFlag-CMV-Glis1, p3xFlag-CMV-Glis2, p3xFlag-CMV-Glis3, p3xFlagCMV-Glis3ΔN302, p3xFlag-CMV-Glis2ΔN58, p3xFlag-CMV-Glis2ΔN160, and p3xFlag-CMV-Glis2ΔC169 was described previously (6, 9, 20, 21). The plasmids p3xFlag-CMV-Glis3ΔN34, -65, -155, -288, -295, -319, -333, -355, and -388 and p3xFlag-CMV-Glis3ΔC389 were generated by PCR amplification of the respective fragments and inserting them into the EcoRI and BamHI restriction sites of the p3xFlag-CMV10 expression vector (Sigma). pCMV-Myc-SUFU was kindly provided by Rune Toftgard (Karolinska Institutet, Huddinge, Sweden) (22). The *mIP*(−696)-*Luc* luciferase reporter construct was described previously (6). pM-Glis3 was generated by inserting the full-length coding region of Glis3 into the pM expression vector (Clontech) following PCR amplification. pVP16-SUFU was created by inserting PCR-amplified full-length SUFU into the VP16 vector (Clontech). pCMV-Cul3-Myc was a generous gift from Dr. Jun Yan (University of North Carolina, Chapel Hill). pEGFP-Glis1–3 and pSUFU-DsRed were produced by cloning the full-length coding region of Glis1–3 or SUFU into pEGFP-C2 or pDsRed2-N1, respectively (Clontech). Site-directed mutagenesis of p3xFlag-CMV-Glis3, p3xFlag-CMV-Glis3ΔN302, and pM-Glis3 was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol. The primer sets utilized are as follows with mutated bases underlined: Glis3 Y349A, 5′- CAGTCAGAGGTCGCTGGGCATTTCCTGG-3′ and 5′-CCAGGAAATGCCCAGCG-ACCTCTGACTG-3′; Glis3 G350A, 5′-GTCAGAGGTCTATGCGCATTTCCTGG-GTG-3′ and 5′-CACCCAGGAAATGCGCATAGACCTCT-GAC-3′; Glis3 H351A, 5′-GTCAGAGGTCTATGGGGCTTT-CCTGGGTG-3′ and 5′-CACCCAGGAAAGCCCCATAGAC-CTCTGAC-3′; Glis3 GH → AA, 5′-GTCAGAGGTCTATGC-GGCTTTCCTGGGT-3′ and 5′-CACCCAGGAAAGCCGCA-TAGACCTCTGAC-3′; Glis3 YGH → AAA, 5′-CAGTCAGA-GGTC*GC*TGCGGCTTTCCTGG-3′ and 5′-CCAGGAAAGC-CGCAGCGACCTCTGACTG-3′. All constructs were verified by restriction enzyme analysis and DNA sequencing.

Reporter Assays—Cells were plated in 12-well dishes at 1×10^5 cells/well and incubated for 24 h at 37 °C. Cells were subsequently transfected with 1 mg of the indicated reporter, 0.3 μg of pCMV-β-galactosidase, and 0.5 μg of the indicated expression vector in Opti-MEM (Invitrogen) using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Each transfection was carried out in triplicate. Cells were harvested after 24 h by scraping them directly into 125 μl of reporter lysis buffer, and luciferase activity was measured using a luciferase assay kit (Promega). β-Galactosidase levels were measured using a luminometric β-galactosidase detection kit (Clontech) following the manufacturer's protocol. Each data point was assayed in triplicate, and each experiment was performed at least twice. Relative luciferase activity was calculated. All values underwent analysis of variance and Tukey-Kramer comparison tests using InStat software (GraphPad Software Inc.), and data are presented as mean ± S.E. Mammalian two-hybrid assays were performed with HEK293 cells plated in 12-well dishes at 1×10^5 cells/well and incubated for 24 h at 37 °C. Cells were subsequently transfected with pM or VP16 empty vector (Clontech), pVP16-SUFU, pM-Glis3 or the specified pM-Glis3 mutant, pFR-Luc, and pCMV-β-gal diluted in Opti-MEM (Invitrogen) and incubated with Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). Cells were harvested, and luciferase assays were conducted and analyzed as reported above.

Co-immunoprecipitation Assays—HEK293 cells were transiently transfected with the specified plasmids using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. 48 h after transfection, cells were harvested by scraping in radioimmune precipitation assay buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM sodium molybdate, and 0.5% Nonidet P-40) containing protease and protease inhibitor cocktails I and II (Sigma). Cell lysates were centrifuged at 16,000 × *g* for 10 min at 4 °C, and half of the supernatant was stored at −70 °C for the input fractions. The remaining half of the supernatant was incubated at 4 °C for 60 min with either 2 μg of mouse anti-Myc (Invitrogen) or 1 μg of mouse anti-FLAG M2 (Sigma-Aldrich) antibody. Protein complexes were immobilized by incubating them with protein G-Sepharose beads for 2 h at 4 °C. Beads were washed three times with 600 μl of ice-cold radioimmune precipitation assay buffer, and proteins were released from the beads by boiling for 5 min in the presence of 1× Laemmli buffer. Input and immunoprecipitated proteins were examined by Western blot analysis using mouse anti-FLAG or mouse anti-Myc antibodies.

Quantitative Reverse Transcriptase Real-time PCR Analysis—INS-1(832/13) cells were transiently transfected with p3xFlag-CMV10 empty vector, p3xFlag-CMV-Glis3ΔN302, and/or pCMV-SUFU-Myc using Lipofectamine 2000 reagent following the manufacturer's protocol (Invitrogen). RNA was isolated from the cells after 48 h using an RNeasy mini kit (Qiagen) according to the manufacturer's specifications. Equal amounts of RNA were used to generate cDNA using a high capacity cDNA kit (Applied Biosystems), and cDNA was analyzed by quantitative real-time PCR. All qRT-PCR was performed in

triplicate using a StepOnePlus real-time PCR system (Applied Biosystems). For r*Ins2*, Power SYBR Green PCR Master Mix (Applied Biosystems) was used with 25 ng of cDNA per reaction, forward primer, 5′-CAGCAAGCAGGAAGCCTATC, and reverse primer, 5′-TTGTGCCACTTGTGGGTCCT. The average Ct from triplicate samples was normalized against the average Ct of 18S rRNA.

Confocal Microscopy—IMCD3 cells were plated in 30-mm glass-bottom dishes and transfected 24 h later with pEGFP-Glis1–3 and/or pSUFU-DsRed. 48 h after transfection, cells were examined for fluorescence using a Zeiss 510 META NLO laser-scanning confocal microscope.

Western Blot Analysis and Protein Quantification—Proteins were resolved by SDS-PAGE and then transferred to PVDF membrane (Invitrogen) by electrophoresis. Immunostaining was performed with the indicated antibody at either 4 °C for 18 h or 22 °C for 2 h in BLOTTO reagent (5% nonfat dry milk dissolved in 50 mM Tris, 0.2% Tween 20, and 150 mM NaCl). Blots were subjected to three 15-min washes in TTBS (50 mM Tris, 0.2% Tween 20, and 150 mM NaCl), and bands were detected by enhanced chemiluminescence following the manufacturer's protocol (GE Healthcare).

Proteins were quantified by scanning autoradiograms into Adobe Photoshop (Adobe Systems Inc.) and measuring the mean intensity and pixel count of pertinent bands selected with the Lasso tool. The mean intensity and pixel count of experimental bands were multiplied and divided by the mean intensity multiplied by the pixel count of GAPDH bands used for normalization. All samples were run in duplicate and all experiments performed at least twice. Data shown are the average of duplicate samples ± S.E.

RESULTS

The N-terminal Region of Glis3 Influences Glis3-mediated Transcriptional Regulation—Previous studies focused predominantly on two domains within Glis3: the centrally located ZFD, which mediates the recognition of GlisBS, and a transactivation domain (TAD) within the C terminus of Glis3 that is required for its transactivation function (1, 4, 9). We reported previously that Glis3 robustly activates transcription of murine *Ins2* via its proximal promoter, which contains two functional Glis-responsive elements (7, 8). To assess the potential role of the Glis3 N terminus in modulating Glis3 transcriptional activity, we examined the effect of various N-terminal deletions on the activation of the *Ins2* promoter. Luciferase reporter assays were carried out by transfecting rat β-like INS-1(832/13) cells with *p-mIP*(−*696*)*-Luc*, a reporter under the control of the *Ins2* upstream regulatory region spanning nucleotides −696 to +8, relative to the transcriptional start site. *p-mIP*(−*696*)*-Luc* was co-transfected with either an expression vector encoding the full-length Glis3 (*p3xFlag-CMV-Glis3*) or one of a series of Glis3 mutants serially truncated at the N terminus. Progressive deletions of the Glis3 N terminus resulted in enhanced reporter gene activity that reached a maximum with Glis3ΔN302 and diminished with subsequent deletions (Fig. 1*A*). To determine whether the observed pattern of regulation was β-cell-specific, the experiment was repeated in HEK293 cells, which were used previously to demonstrate the ability of Glis3 to activate a $(GlisBS)_6$-driven reporter (9). The pattern of transcriptional activation by various Glis3 mutants observed in HEK293 cells was qualitatively very similar to that observed in INS-1 cells (Fig. 1*B*). Western blot analysis revealed that the corresponding levels of FLAG-tagged Glis3 proteins expressed in HEK293 cells varied significantly between the mutants (Fig. 1*C*) but did not correlate with their level of transactivation. Deletions within the first 155 amino acids had a relatively slight effect on Glis3-mediated activation of the *Ins2* promoter. The level of activation was significantly increased upon further deletions at the N terminus and reached a maximum with the mutant Glis3Δ302 lacking the first 302 amino acids, whereas subsequent N-terminal deletions caused a reduction in transactivation. These data indicated that several regions within the Glis3 N terminus might be involved in modulating Glis3 transactivation activity. Analysis of the level of the various mutant Glis3 proteins suggested that deletion of the first 319 amino acids might result in the stabilization of the Glis3 protein.

The Region of Glis3 between Amino Acids 296 and 354 Is Highly Conserved with a Region in the N Terminus of Ci/Gli Proteins—To ascertain whether the Glis3 N terminus contained a region exhibiting sequence homology with a recognized domain found in other proteins, the region was subjected to a BLASTp search of the NCBI database. This comparison revealed that the region between amino acids 296 and 354 not only showed high levels of homology among Glis3 proteins from various species, ranging from fishes to humans, but also to a highly conserved region within Gli1, -2, and -3 and the *Drosophila* homolog *C. interruptus*. The N termini of the Glis3 and Gli proteins exhibited little sequence homology beyond this region. A multiple sequence alignment of the conserved region revealed particularly high homology (≥70% similarity) between residues 296 and 338 of murine Glis3 and the corresponding regions within the Glis3 homologs and Gli proteins (Fig. 2*A*). This region contains a conserved basic sequence between amino acids 295 and 302. In addition, a highly conserved motif, (S/V)YGH(F/L), was found between amino acids 348 and 352. The 58-amino acid conserved region of Glis3 further contains a number of potential phosphorylation sites. In fact, the region is composed of 28% serine, threonine, and tyrosine. Interestingly, sequence analysis did not identify any significant regions of homology in the N terminus of Glis3 with that of Glis1 or Glis2, indicating that the presence of this conserved region is unique to the Glis3 member of the Glis protein subfamily. The phylogenetic relationship among the N-terminal homologous regions of Glis3, Gli proteins, and Ci is shown in Fig. 2*B*.

Glis3 Interacts with SUFU—A proteome scale yeast two-hybrid screening presented evidence that Glis3 may associate with the tumor suppressor and negative regulator of Hedgehog signaling, SUFU; however, no further characterization of this potential interaction was carried out (23). We, therefore, performed co-immunoprecipitation assays to determine whether Glis3 was indeed capable of interacting with SUFU. HEK293 cells were co-transfected with an N-terminal Myc-tagged human SUFU construct (*pCMV-SUFU-Myc*) and either *p3xFlag-CMV10* empty vector or *p3xFlag-CMV-Glis3* or *p3xFlag-CMV-GLIS3* containing full-length murine or human

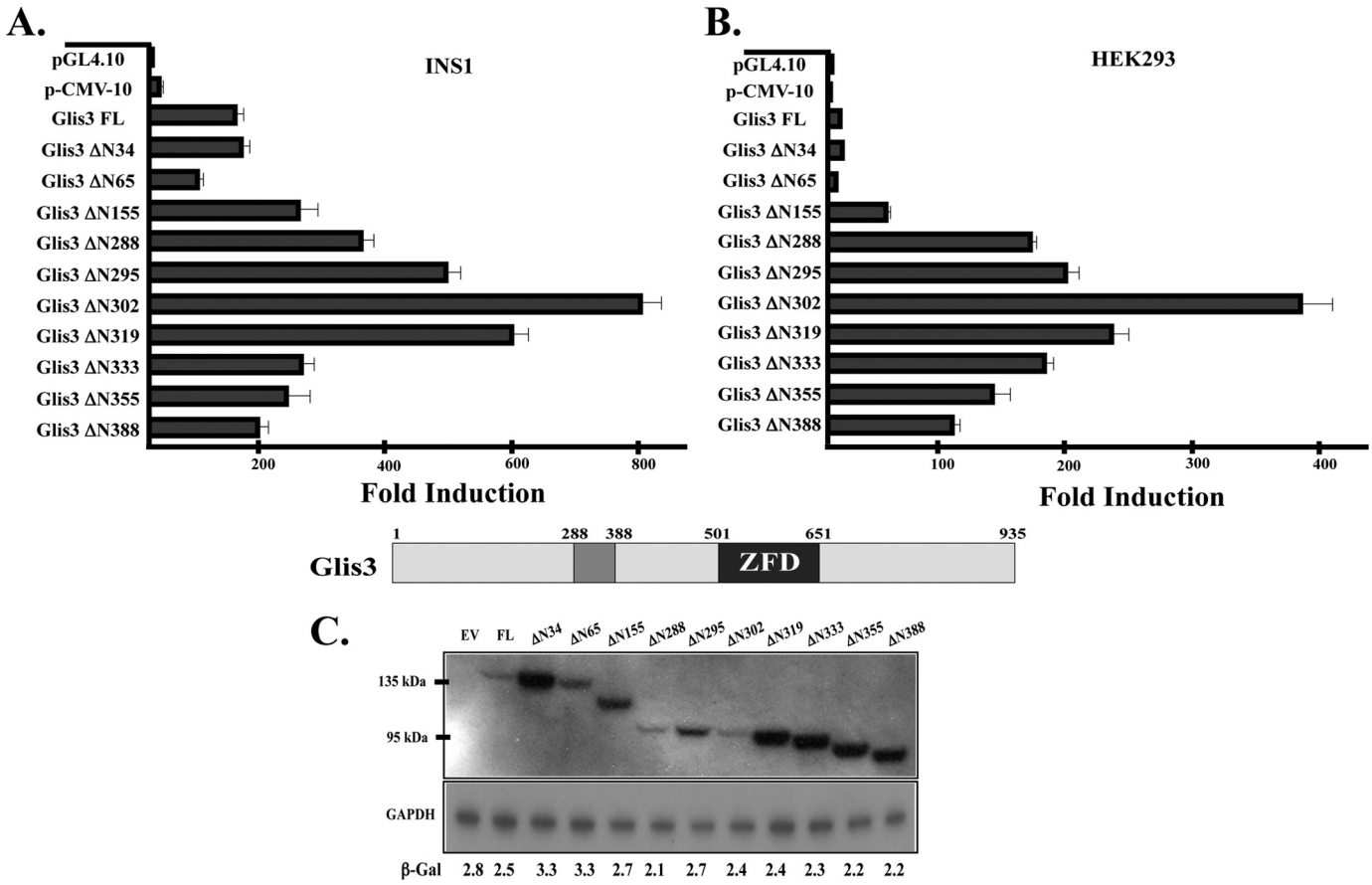

FIGURE 1. **Role of the Glis3 N terminus in Ins2 transcriptional regulation.** *A*, INS-1(832/13) cells were co-transfected with *p-mIP*(−696)-*Luc* and either *p3xFlag-CMV10*, *p3xFlag-CMV-Glis3*, or the indicated N-terminal truncated Glis3 construct. After 24 h cells were assayed for luciferase and β-galactosidase activities and the relative Luc activity calculated and plotted. Each *bar* represents mean ± S.E. *B*, HEK293 cells were transfected and assayed as detailed in *A*. *C*, *top*, a schematic representation of Glis3. The ZFD is indicated. The *dark gray shaded region* represents the N-terminal region wherein the greatest changes to Glis3 transactivation function and stability occur. *Bottom*, Western blot comparing Glis3 expression levels of the indicated Glis3 mutants. Blots were stained with HRP-conjugated mouse anti-FLAG M2 antibody or rabbit anti-GAPDH antibody. To monitor transfection efficiency, the amount of co-transfected β-galactosidase was determined by luminometry, and the values obtained are indicated.

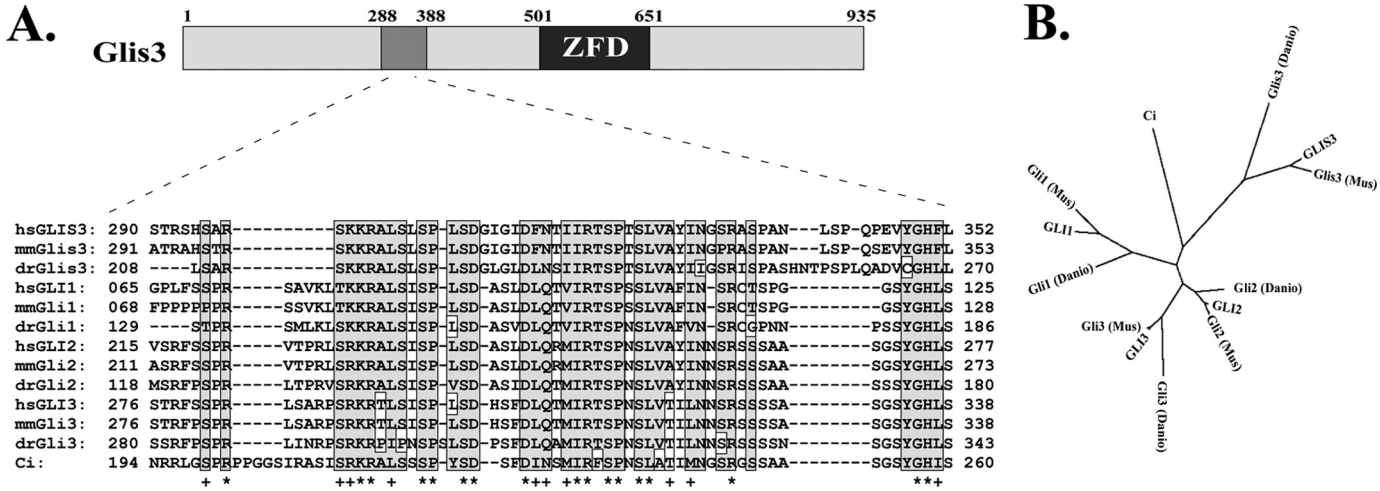

FIGURE 2. **The region of Glis3 between amino acids 296 and 354 exhibits high homology with the N termini of Ci/Gli proteins.** *A*, alignment of the conserved N-terminal regions of Glis3 and Ci/Gli proteins from representative species. *Bottom*, *shaded boxed areas* indicate conserved residues; +, represents conserved amino acid; *, represents identical amino acid. Hs, *Homo sapiens*; mm, *Mus musculus*; dr, *Danio rerio*. *B*, phylogenetic relationship between the N-terminal conserved region of Glis3 and the Ci/Gli proteins. The *unrooted tree* was obtained using the PHYLIP DrawTree program.

Glis3, respectively. As SUFU had previously been shown to interact with GLI1 (22, 24, 25), FLAG-GLI1 was used as a positive control. A representative experiment, shown in Fig. 3*A*, demonstrated that both human and murine Glis3 co-immunoprecipitated with SUFU, suggesting that Glis3 and SUFU interact. To determine the region of Glis3 involved in the interaction

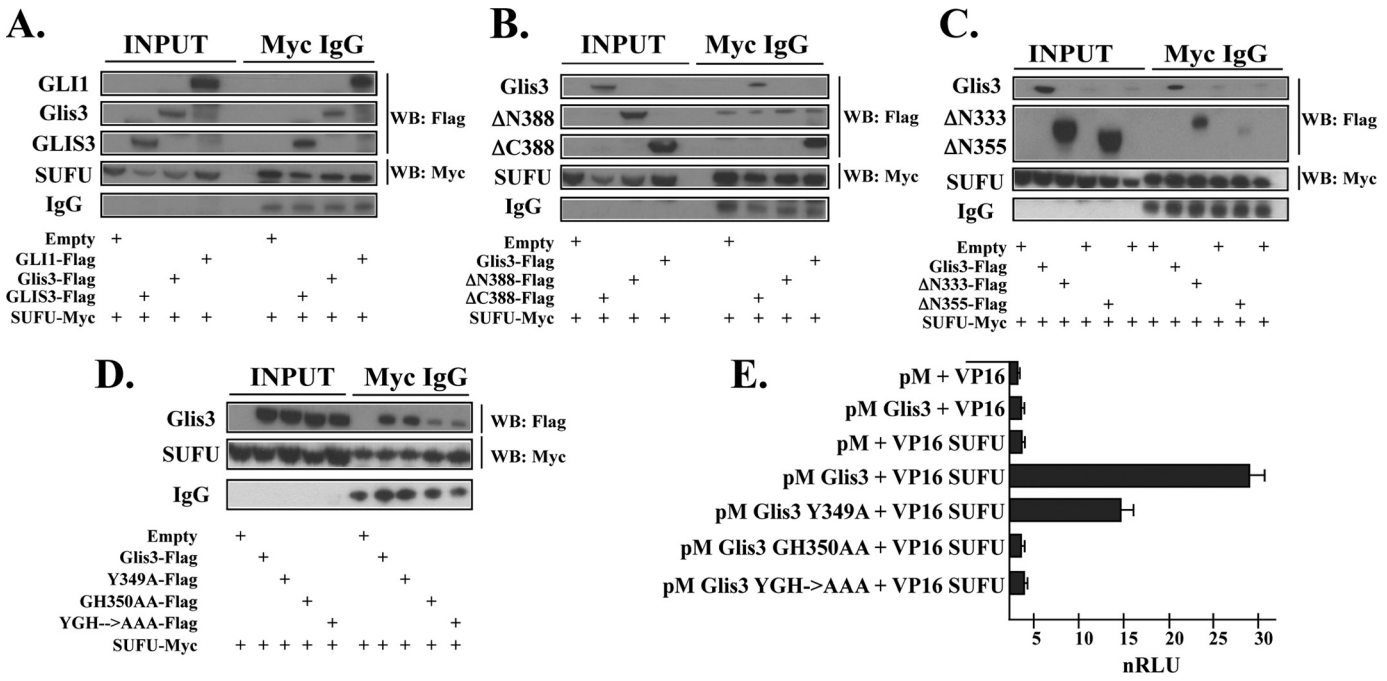

FIGURE 3. **The Glis3 N terminus interacts with SUFU.** *A*, HEK293 cells were co-transfected with pCMV-SUFU-Myc and the indicated p3xFlag-CMV mouse Glis3, human GLIS3, or GLI1 expression plasmids. Co-immunoprecipitations were carried out as described under "Materials and Methods" using a mouse anti-Myc antibody. Immunoprecipitated proteins were examined by Western blot analysis using either HRP-conjugated mouse anti-FLAG or mouse anti-Myc and an HRP-conjugated goat anti-mouse antibody. GLI1 had previously been shown to interact with SUFU and was used as a positive control. *B–D*, HEK293 cells were co-transfected with pCMV-SUFU-Myc and the indicated p3x-Flag-CMV-Glis3 plasmid. Co-immunoprecipitation and subsequent Western blot analysis were performed as described in *A*. *E*, HEK293 cells were co-transfected with the pFR-Luc reporter and the indicated pM and VP16 plasmid DNA. 24 h later cells were assayed for luciferase and β-galactosidase activities, and the relative Luc activity was calculated and plotted. Each *bar* represents mean ± S.E. *nRLU*, normalized relative luc units.

with SUFU, HEK293 cells were transfected with *pCMV-SUFU-Myc*, *p3xFlag-CMV-Glis3*, *p3xFlag-CMV-Glis3ΔN388*, or *p3xFlag-CMV-Glis3ΔC389* encoding FLAG-tagged Glis3 mutants in which, respectively, the first 388 amino acids or the zinc finger and C terminus were deleted. Both full-length Glis3 and Glis3ΔC389 co-precipitated with SUFU; however, Glis3ΔN388 was incapable of forming a complex with SUFU, indicating that the first 388 amino acids of Glis3 are required for the interaction with SUFU (Fig. 3*B*). The ability of SUFU to interact with the deletion mutant Glis3ΔN333, but not with Glis3ΔN355, further narrowed down the region of SUFU interaction to the amino acids between positions 334 and 355 (Fig. 3*C*).

A previous report showed that protein-protein interactions by SUFU involve recognition of an SYGH motif (22). Interestingly, Glis3 contains a similar motif, VYGH, located between amino acids 348 and 351 within the conserved region described above (Fig. 2*A*). To assess the importance of these residues in Glis3-SUFU binding, *in vitro* mutagenesis was utilized to mutate Tyr^{349}, Gly^{350}, and His^{351} to alanine, alone or in combination. Co-IP experiments revealed that the Y349A mutation had little effect on the interaction; however, mutation of the glycine and histidine at positions 350 and 351, respectively, dramatically reduced the association between the two proteins (Fig. 3*D*). To obtain further support for this interaction, mammalian two-hybrid analyses were performed utilizing pFR-Luc, pVP16-SUFU, and pM-Glis3 expressing Glis3 or various Glis3 YGH mutants fused to the GAL4 DNA-binding domain. The representative data shown in Fig. 3*E* were consistent with the conclusion that Glis3 and SUFU interact. Mutation of Gly^{350} and His^{351} was sufficient to virtually eliminate all interaction between the proteins. In contrast to the Co-immunoprecipitation results, the Y349A mutation significantly reduced the interaction between Glis3 and SUFU in the two-hybrid analysis.

Glis3 and SUFU Co-localized to the Nucleus—Because SUFU was capable of interacting with Glis3, it was of interest to investigate whether this interaction affected the subcellular localization of either SUFU or Glis3. Confocal microscopy was utilized to examine the subcellular localization of N-terminal EGFP-tagged Glis3 and C-terminal tagged SUFU-DsRed fusion proteins. IMCD3 cells were used because of their relatively large size and distinct cytoplasmic and nuclear compartments. EGFP-Glis3 protein was localized mainly in the nuclear compartment of IMCD3 cells both in the presence and absence of SUFU (Fig. 4). SUFU-DsRed was observed predominantly in the cytoplasm and to a lesser degree in the nucleus when expressed alone, in agreement with previous reports (22, 26). However, SUFU was largely (>80% of cells) localized to the nucleus in cells in which SUFU was co-expressed with Glis3 (Fig. 4). In contrast, SUFU-DsRed remained predominantly cytoplasmic (in >70% of transfected cells) when co-expressed with the EGFP-Glis3-YGH → AAA mutant that exhibit a reduced interaction with SUFU. Similar results were obtained in HEK293 and INS-1 cells (data not shown).

SUFU Inhibits Glis3-mediated Ins2 Transactivation—Given the apparent association between Glis3 and SUFU, it was important to determine whether SUFU had an effect on Glis3-mediated *Ins2* transcriptional regulation. To examine the effect of SUFU on the induction of *Ins2* mRNA expression, we tran-

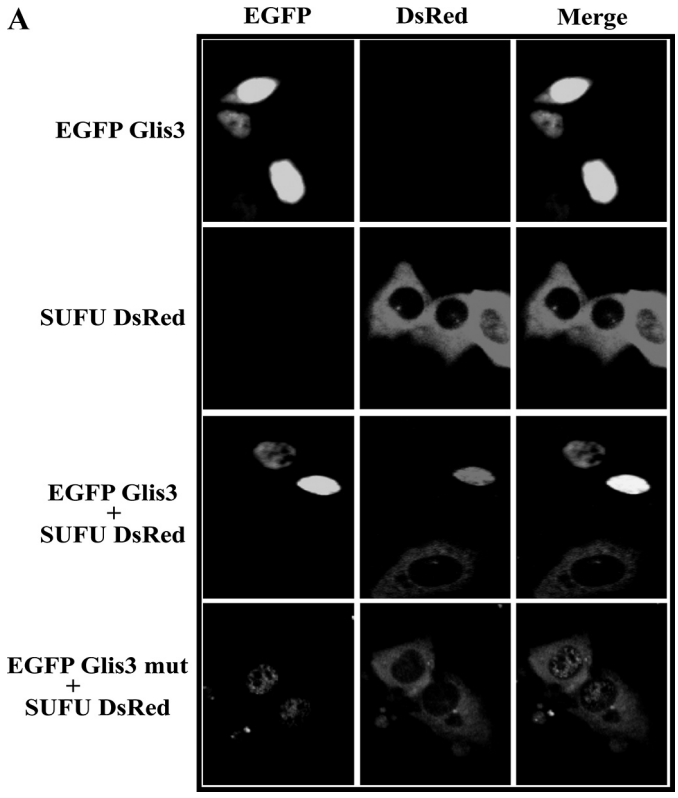

FIGURE 4. **Glis3 and SUFU co-localize to the nucleus.** IMCD3 cells were transfected with pEGFP-Glis3 or pEGFP-Glis3-YGH → AAA and pSUFU-dRed alone or in combination as indicated. Subcellular localization of the expressed proteins was examined by confocal microscopy 48 h later.

siently transfected INS-1(832/13) cells with empty vector or *p3xFlag-CMV-Glis3ΔN302* either with or without *pCMV-SUFU-Myc.* As shown in Fig. 5*A*, expression of exogenous Glis3ΔN302 increased *Ins2* mRNA levels nearly 4-fold, whereas SUFU overexpression reduced the basal level of *Ins2* mRNA by ~40%. Co-expression of *pCMV-SUFU-Myc* with *p3xFlag-CMV-Glis3ΔN302* likewise reduced *Ins2* mRNA levels by ~40% compared with expression of Glis3ΔN302 alone. To further assess the role of SUFU as a transcriptional modulator of *Ins2*, INS-1(832/13) cells were transiently transfected with *p-mIP*(−*696*)*-Luc*, *p3xFlag-CMV-Glis3*, and either *pCMV-SUFU-Myc* or empty vector. Luciferase reporter analyses indicated that co-expression with SUFU significantly reduced the activation of the *mIP*(−*696*) promoter by full-length Glis3 and the Glis3 N-terminal truncation mutants up to Glis3ΔN319 (Fig. 5, *B* and *C*), consistent with the reduction in *Ins2* mRNA levels (Fig. 5*A*). In contrast, SUFU did not inhibit the activation of the *Ins2* promoter by Glis3ΔN355, Glis3ΔN388, or Glis3-YGH → AAA, mutants that did not bind SUFU or exhibited a reduced interaction with SUFU, respectively (Fig. 5*C*). These data indicate that the inhibition of Glis3-mediated transactivation by SUFU appears to rely on the interaction with Glis3 through the YGH motif and is not related to an effect on the general transcriptional machinery. Interestingly, SUFU affected the Glis3ΔN333-induced transactivation only to a small degree, despite the ability of the two proteins to interact (Fig. 3*C*), suggesting that the region between amino acids 319 and 333 of Glis3 may also play a role in the inhibition by SUFU.

Even though SUFU repressed the transactivation mediated by Glis3 and Glis3 mutants truncated up to amino acid 319, Western blot analysis revealed that it significantly increased the level of the respective Glis3 protein (Fig. 5*D*). However, SUFU did not appear to have little effect on the protein levels of Glis3 mutants truncated beyond amino acid 319 nor did it affect the level of the full-length YGH mutant as considerably as observed with the wild type Glis3. Collectively, these data suggested that both the sequence of amino acids between positions 303 and 333 within the homologous region of Glis3 and their interaction with SUFU play an important role in modulating the transcriptional activity of Glis3 as well as in the regulation of Glis3 protein stability.

SUFU Protects Glis3 against Proteasomal Degradation—To obtain further support for our conclusion that co-expression with SUFU causes an increase in Glis3 protein levels, *p3xFlag-CMV-Glis3* was transfected into HEK293 cells with increasing concentrations of *pCMV-SUFU-Myc*, and 48 h later the level of Glis3 protein analyzed. As shown in Fig. 6*A*, the increase in Glis3 protein levels corresponded to the graded increase in SUFU. SUFU had a reduced effect on the level of the Glis3-YGH → AAA mutant, which interacted weakly with SUFU. To determine the effect of SUFU on Glis3 protein stability, HEK293 cells transfected with *p3xFlag-CMV-Glis3* and either *pCMV-SUFU-Myc* or Myc empty vector were treated with cycloheximide, and the level of Glis3 protein was determined over a period of 6 h. The results showed that Glis3 exhibited a calculated half-life of 4.5 h in cells transfected with Glis3 only (Fig. 6*B*), whereas in cells co-transfected with SUFU its half-life increased to 15.4 h, indicating that co-expression with SUFU enhanced the stability of Glis3 protein and protected Glis3 from proteolytic degradation. This was consistent with data showing that the increase in Glis3 levels by SUFU was not due to changes in the regulation of Glis3 mRNA as determined by qRT-PCR (Fig. 6*C*). Moreover, treatment with the proteasome inhibitor MG132 caused a 2.5- or 3-fold increase in Glis3 protein levels, indicating that proteolytic degradation of Glis3 is mediated by the 26S proteasome (Fig. 6*D*). In addition, co-immunoprecipitation analysis utilizing HEK293 cells co-transfected with *p3xFlag-CMV-Glis3* and *pCMV-HA-Ub* expressing HA-tagged ubiquitin demonstrated that Glis3 was ubiquitinated and that co-transfection with *pCMV-SUFU-Myc* dramatically reduced Glis3 ubiquitination (Fig. 6*E*). Collectively, these findings support a role for SUFU in the regulation of Glis3 protein stability.

SUFU Associates with Glis2—To determine whether the association with SUFU was specific to Glis3 among members of the Glis subfamily, co-immunoprecipitation analyses were carried out with HEK293 cells transiently transfected with *pCMV-SUFU-Myc* and *p3xFlag-CMV* expressing Glis1 or Glis2. Fig. 7*A* shows that SUFU co-immunoprecipitated with Glis2, albeit to a lesser degree than observed with Glis3, whereas SUFU did not co-immunoprecipitate with Glis1. These data suggested that SUFU could be part of a Glis2 protein complex, but under the experimental conditions tested, it does not interact with Glis1. Because Glis2 does not contain an YGH-like motif, its interaction with SUFU must be mediated by another mechanism. To further determine the region of Glis2 involved in this interaction, the effect of N- and C-terminal truncations on the

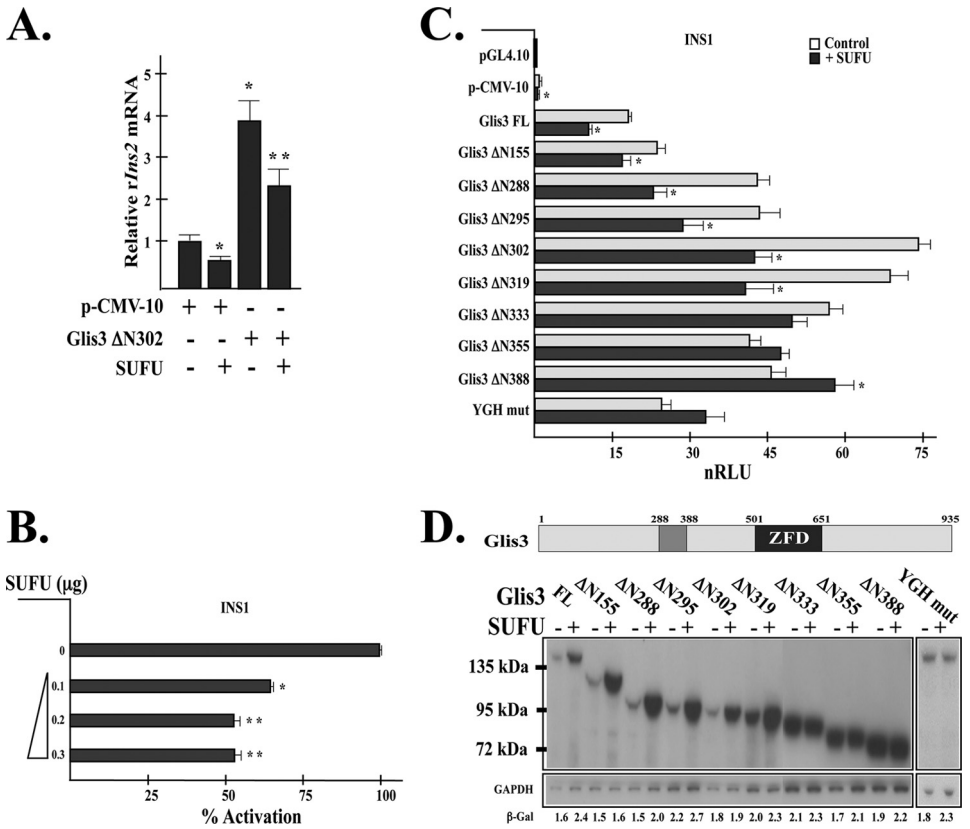

FIGURE 5. **Effect of SUFU on Glis3-mediated transcriptional regulation of Ins2.** *A*, INS-1(832/13) cells were transiently transfected with p3xFlag-CMV10 or p3xFlag-CMV-Glis3ΔN302 with or without pCMV-SUFU-Myc as indicated. After 48 h, cells were harvested, RNA was collected, and *rIns2* message was measured by qRT-PCR analysis. *Bars* represent relative *rIns2* mRNA normalized to 18S RNA ± S.E. *, statistically different from pCMV10 ($p < 0.01$); **, statistically different from Glis3ΔN302 ($p < 0.01$). *B*, INS-1(832/13) cells were transfected with *p-mIP(−696)-Luc* and co-transfected with *p3xFlag-CMV-Glis3* and the indicated quantity of *pCMV-SUFU-Myc*. After 24 h cells were assayed for luciferase and β-galactosidase activities, and the relative Luc activity was calculated and plotted. Each *bar* represents percent activation relative to control sample ± S.E. *, statistically different from 0 SUFU control ($p > 0.01$); **, statistically different from samples treated with 0.1 μg of SUFU ($p > 0.01$). *C*, INS-1(832/13) cells were transfected with pGL4.10 or p-mIP(−696)-Luc and p3xFlag-CMV-10 or the indicated *p3xFlag-CMV-Glis3* mutant and co-transfected with either *pCMV-Myc* or *pCMV-SUFU-Myc*. Cells were assayed as described in *B*. *, statistically different from paired control ($p > 0.05$). *D*, *top*, schematic representation of Glis3 as detailed in Fig. 1 legend. *Bottom*, Western blot analysis comparing the level of expression of the indicated Glis3 mutants in the presence or absence of co-expression with SUFU. Blots were stained with HRP-conjugated mouse anti-FLAG M2 antibody or rabbit anti-GAPDH antibody. To monitor transfection efficiency, the amount of co-transfected β-galactosidase was determined by luminometry, and the values obtained are indicated.

interaction was examined. Co-immunoprecipitation analyses indicated that SUFU was able to interact with Glis2ΔN160, containing the ZFD and C terminus, but not with the N-terminal region, Glis2ΔC169 (Fig. 7*B*). Previously, we reported that Glis2 was able to activate the *mIP(−696)-Luc* reporter in HEK293 cells (21). We therefore examined whether SUFU had any effect on the transactivation of the *Ins2* promoter by Glis2. In contrast to Glis3, SUFU had little effect on Glis2-mediated transactivation of the *Ins2* promoter (Fig. 7*C*). Glis1 was also able to activate the *Ins2* promoter, but this activation was slightly enhanced rather than inhibited by SUFU co-expression. Next, we examined whether SUFU affected Glis1 or Glis2 protein stability. As shown in Fig. 7*D*, SUFU did not influence the level of Glis1 or Glis2 protein. These observations indicated that in contrast to Glis3, SUFU did not affect the transactivating activity or stability of Glis1 or Glis2.

Glis3 Degradation Is Mediated by Cul3—The polyubiquitination and subsequent degradation of Glis3 by the proteasome system may involve several E3 ubiquitin ligases, including Cullin-based E3 ubiquitin ligases (27). To determine whether the E3 ubiquitin ligase scaffolding protein Cullin 3 (Cul3) (28–30) was able to promote the ubiquitination of Glis3, FLAG-tagged Glis3 was immunoprecipitated with HA-tagged ubiquitin in the presence or absence of co-expression with exogenous Cul3. Fig. 8*A* shows that co-expression of Glis3 with Cul3 resulted in a significant increase in the amount of ubiquitinated Glis3 detected, consistent with the hypothesis that Cul3 promotes Glis3 degradation. To determine whether Cul3 associates with Glis3, co-immunoprecipitation analysis was performed in HEK293 cells co-transfected with *p3xFlag-CMV-Glis3*, *p3xFlag-CMV-Glis3ΔN155*, *p3xFlag-CMV-Glis3ΔN302*, or *p3xFlag-CMV-Glis3ΔN319* and *pCMV-Cul3-Myc* as indicated. As seen in Fig. 8*B*, Cul3 co-immunoprecipitated with full-length Glis3 as well as with Glis3ΔN155 and Glis3ΔN302 lacking, respectively, the first 155 or 302 amino acids, but interacted only weakly with Glis3ΔN319, suggesting that the region between amino acids 302 and 319 is required for optimal Glis3-Cul3 interaction. Interestingly, this region was also associated with enhanced levels of Glis3 protein expression following N-terminal deletions (Figs. 1*C* and 5*D*).

To determine whether SUFU influenced the association between Cul3 and Glis3, we examined the effect of increased

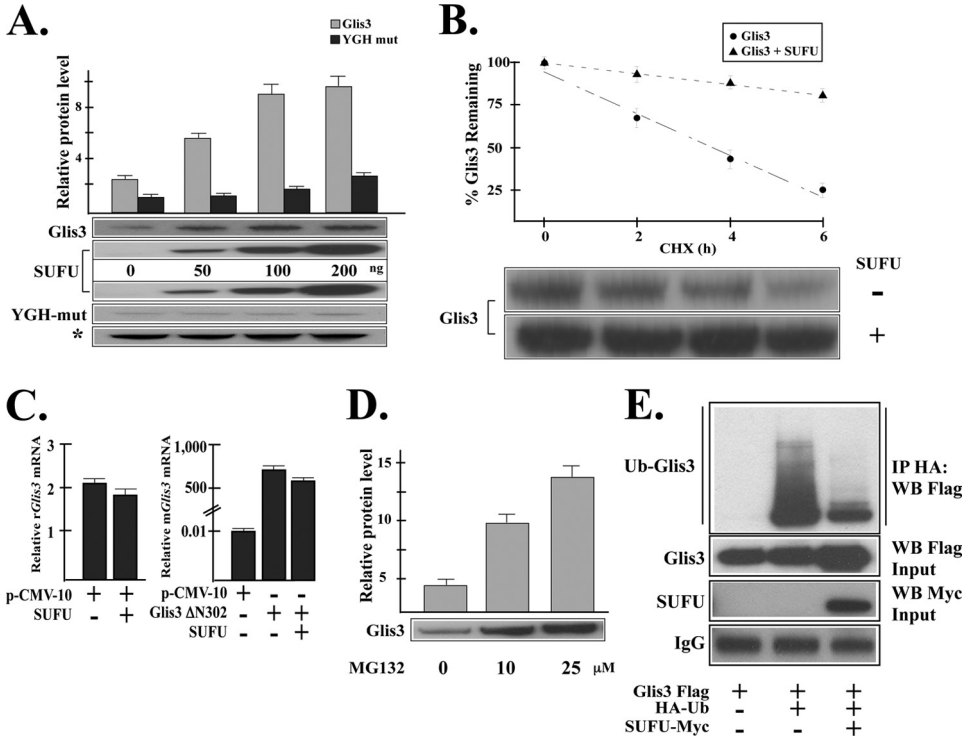

FIGURE 6. **SUFU stabilizes Glis3 expression.** *A*, HEK293 cells were transfected with either *p3xFlag-CMV-Glis3* or *p3xFlag-CMV-Glis3-YGH → AAA* and the indicated amounts of *pCMV-SUFU-Myc*. Cells were harvested after 48 h, and proteins were separated by SDS-PAGE and evaluated by Western blot analysis anti-FLAG M2 antibody. Bands were quantified and values graphed. Because of the lower levels of Glis3(YGH → AAA) expression, a longer exposure time is also shown (marked by an *asterisk*). *B*, HEK293 cells were transfected with *p3xFlag-CMV-Glis3* and *pCMV-SUFU-Myc* or Myc empty vector and 48 h later treated with 10 μg/ml cycloheximide (*CHX*) for the indicated duration. Cells were harvested and analyzed as described in *A*. Bands were quantified, points were plotted, and linear regression was applied. Quantification was normalized against GAPDH bands (not shown). *C*, INS-1(832/13) cells were transiently transfected with *p3xFlag-CMV10* or *p3xFlag-CMV-Glis3ΔN302* with or without *pCMV-SUFU-Myc* as indicated. After 48 h, cells were harvested, RNA was collected, and *mIns2* or *rIns2* message was measured by qRT-PCR analysis. *Bars* represent relative *mIns2* or *rIns2* mRNA normalized to 18S RNA ± S.E. *D*, HEK293 cells were transfected with p3xFlag-CMV-Glis3. After 48 h, cells were treated for 3 h with the indicated concentration of MG132. Cells were harvested and analyzed as described in *A*. *E*, HEK293 cells were transfected with *p3xFlag-CMV-Glis3*, *pCMV-HA-Ub*, and *pCMV-SUFU-Myc* as indicated. Cells were treated with 10 μM MG132 for 4 h prior to harvest, and co-immunoprecipitations were carried out subsequently as described under "Materials and Methods" using a rat anti-HA antibody. Immunoprecipitated proteins (*IP*) were examined by Western blot analysis (*WB*) using HRP-conjugated mouse anti-FLAG antibody or mouse anti-Myc and an HRP-conjugated goat anti-mouse antibody.

expression of SUFU on this interaction. As shown in Fig. 8*C*, increasing levels of SUFU significantly decreased the amount of Glis3 that co-immunoprecipitated with Cul3 while increasing the input level of Glis3. These results are consistent with the hypothesis that SUFU antagonizes the interaction of Cul3 with Glis3. In contrast, SUFU had little effect on the interaction of Cul3 with the Glis3-YGH → AAA mutant, which binds SUFU only weakly, and had little effect on the input level of mutant Glis3 (Fig. 8*C*). The ectopic expression of Cul3 did not cause a decrease in Glis3 protein input levels, suggesting that it did not appear to substantially enhance the overall rate of Glis3 degradation, despite increased Glis3 polyubiquitination. Possibly the endogenous level of other components of the Cul3-based E3 ubiquitin ligase complexes may be limiting, resulting in less than optimal Cul3-dependent degradation. Our data are consistent with the hypothesis that Cul3-based E3 ubiquitin ligases play a part in promoting the polyubiquitination of Glis3, whereas SUFU reduces Glis3 association with Cul3, thereby stabilizing Glis3.

DISCUSSION

Beyond a DNA-binding domain in the center of Glis3 and an activation domain at its C terminus, little is known further about the domain structure of the Glis3 protein, particularly the function of its 500-amino acid N terminus. In this study, we demonstrate for the first time that the N terminus of Glis3 plays a critical role in modulating the stability and transcriptional activity of Glis3 and that part of this regulation is mediated through interactions with SUFU and Cul3 within the N terminus.

In silico examination of the Glis3 N terminus revealed a region between amino acids 296 and 354 that is highly conserved among Glis3 homologs of different species, including that of human, mouse, and zebrafish (Fig. 2*A*). This region also exhibits high homology to a corresponding region in Gli1–3 and Ci, not found, however, in Glis1 or Glis2. The high level of homology suggests that this region may play a critical role in regulating the function of these proteins. Although most of the function of this region has yet to be established, a role for an SYGHXS consensus motif at the C-terminal end of the homologous region of Gli proteins, identified as critical for interaction with SUFU, has emerged (22, 31). In this study, we have demonstrated that SUFU interacted with Glis3 through a similar motif, ^{348}VYGHF^{352}, within this conserved region. Mutation of Gly^{350} and His^{351} to Ala greatly diminished the interaction of Glis3 with SUFU, suggesting that the VYGHF motif is impor-

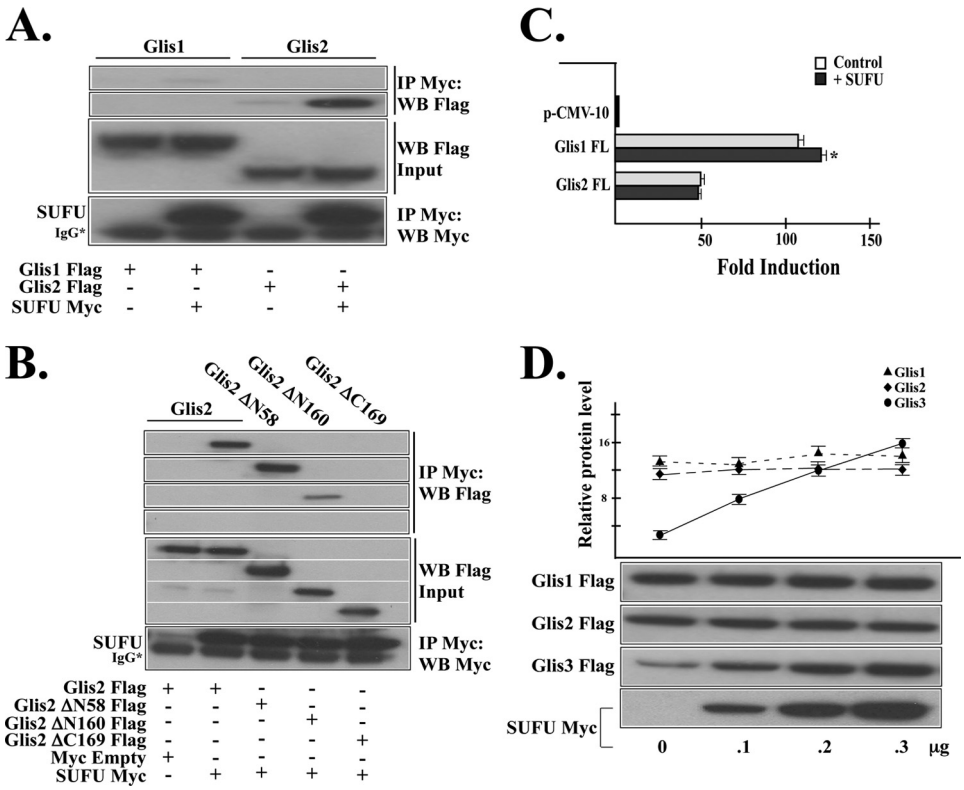

FIGURE 7. **SUFU interacts with the C terminus of Glis2.** *A* and *B*, HEK293 cells were transfected with the indicated Glis1–2 constructs and *pCMV-SUFU-Myc*. After 48 h, proteins were immunoprecipitated from isolated cell lysates with a mouse anti-Myc antibody, and immunoprecipitated proteins (*IP*) were evaluated by Western blot analysis (*WB*) with anti-FLAG M2 or anti-Myc antibodies as described under "Materials and Methods." *C*, HEK293 cells were co-transfected with *p-mIP(−696)-Luc*, *p3xFlag-CMV10*, or the indicated *p3xFlag-CMV-Glis1–2* construct and either *pCMV-Myc* or *pCMV-SUFU-Myc*. After 24 h cells were assayed for luciferase and β-galactosidase activities, and the relative Luc activity was calculated and plotted. Each *bar* represents percent activation relative to control sample ± S.E. *D*, HEK293 cells were transfected with *p3xFlag-CMV-Glis1–3* and increasing amounts of *pCMV-SUFU-Myc*. Cells were harvested after 48 h, and proteins were examined by Western blot analysis using anti-FLAG M2 antibody. Bands were quantified and values graphed.

tant for this interaction. Our observation that SUFU can interact with the N terminus of Glis3, but not its C terminus, is consistent with this conclusion. Mutation of Tyr^{349} to Ala had little effect on the interaction as determined by co-immunoprecipitation assays; however, this mutation did significantly reduce the interaction detected by mammalian two-hybrid assay. Similarly, an analogous mutation in Tyr^{121} of Gli1 reportedly does not to influence its interaction with SUFU as determined by Far Western analysis (22). Of the other Glis family members, only Glis2 was able to interact with SUFU. The association with SUFU was maintained following the deletion of the N terminus of Glis2 but was abolished with the deletion of the region including the ZFD and the C terminus. This region does not contain a SYGHXS-like, SUFU interaction motif, indicating that SUFU interacts with the Glis2 C-terminal region via a different domain or interacts indirectly with Glis2.

Our data further indicate that the N terminus of Glis3, particularly the conserved region, and its interaction with SUFU play a role in regulating the stability of Glis3 protein. Co-expression with SUFU enhanced the level of Glis3 protein without affecting Glis3 mRNA levels. This increase in Glis3 protein was found to be at least partly related to an inhibition in the proteolytic degradation of Glis3. Combined with the observation that SUFU inhibited Glis3 polyubiquitination, this suggested that the increased stability of Glis3 is due to decreased proteasomal degradation. The increase in Glis3 protein stability was at least partly dependent on its YGH motif, suggesting that SUFU interaction at this motif was important for its effect on Glis3 protein stability. However, we cannot rule out the possibility that SUFU may regulate Glis3 protein levels by other mechanisms, including translational control. Despite its interaction with Glis2, SUFU had no apparent effect on the stability of Glis2 protein. Proteasomal degradation of Glis3 may be an important mechanism in regulating Glis3 function by tightly controlling the level of Glis3 protein.

The increase in the Glis3 protein level observed after the addition of the proteasome inhibitor MG132 is consistent with proteolytic degradation of Glis3 by the proteasome system. The latter may involve a number of different E3 ubiquitin ligases. One large family of E3 ubiquitin ligases consists of multisubunit protein complexes organized by the Cullin family of scaffolding proteins (27). Our study provides evidence for a role of Cul3-based E3 ubiquitin ligases in the regulation of Glis3 protein stability and a possible connection among the actions of Cul3, SUFU, and Glis3. Cul3 typically promotes ubiquitin-dependent proteolytic degradation by binding to the BTB domain of adaptor proteins that target specific protein substrates (27); therefore, it is likely that association between Cul3 and Glis3 is mediated by a similar mechanism. We demonstrated that Cul3 is part of a Glis3 protein complex and is able to promote the polyubiquitination of Glis3. Truncation of the Glis3 N terminus up to amino acid 302 had little effect on its interaction with

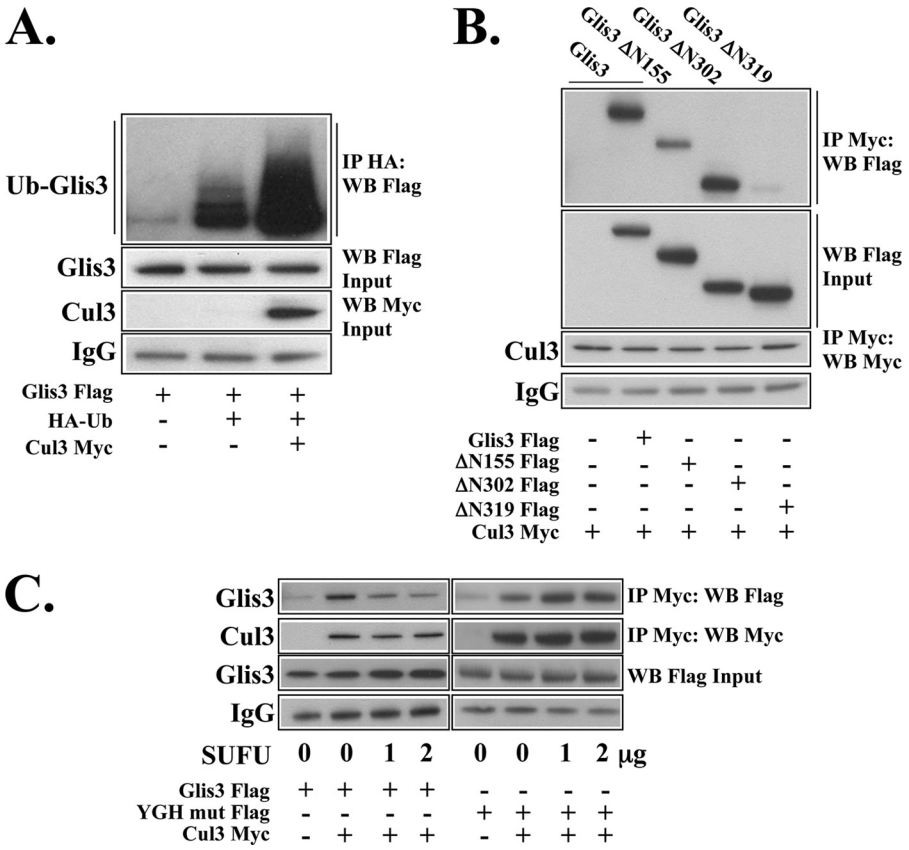

FIGURE 8. **SUFU stabilizes Glis3 by inhibiting its interaction with Cul3.** *A*, HEK293 cells were transfected with p3xFlag-CMV-Glis3, pCMV-HA-Ub, and pCMV-Cul3-Myc as indicated. Cells were treated for 3 h with MG132 before harvesting. Co-immunoprecipitations were carried out as described under "Materials and Methods" using a rat anti-HA antibody. Immunoprecipitated proteins (*IP*) were examined by Western blot analysis (*WB*) using HRP-conjugated mouse anti-FLAG antibody or mouse anti-Myc and an HRP-conjugated goat anti-mouse antibody. *B*, HEK293 cells were co-transfected with p3xFlag-CMV-Glis3, p3xFlag-CMV-Glis3ΔN155, p3xFlag-CMV-Glis3ΔN302, or p3xFlag-CMV-Glis3ΔN319 and pCMV-Cul3-Myc as indicated. After 48 h, proteins were immunoprecipitated from isolated cell lysates with a mouse anti-Myc antibody, and immunoprecipitated proteins were evaluated by Western blot analysis with anti-FLAG M2 or anti-Myc antibodies as described under "Materials and Methods." *C*, HEK293 cells were co-transfected with p3xFlag-CMV-Glis3 or p3xFlag-CMV-Glis3-YGH → AAA, pCMV-Cul3-Myc, and the indicated amount of dsRed-SUFU. Co-immunoprecipitation and subsequent analyses were carried out as described in *A*.

Cul3, whereas deletion beyond amino acid 319 dramatically reduced the interaction (Fig. 8*B*). We showed that deletion of the same region also results in a significant increase in Glis3 protein expression levels (Fig. 1*C*). This correlation is consistent with the presence of a degron located within the region between amino acids 302 and 319. Although Glis3ΔN319 appears to be stabilized relative to full-length Glis3, further stabilization was observed in the presence of SUFU, whereas SUFU had no observable effects on the stabilization of Glis3 ΔN333–388. Coupled with the significantly weakened but detectable interaction of Glis3ΔN319 with Cul3, these data indicate that the putative degron may extend as far as amino acid 333. Our data further indicated that SUFU inhibited the association between Glis3 and Cul3. Together with the observation that SUFU inhibits the level of Glis3 ubiquitination, this suggests that SUFU may stabilize Glis3 by inhibiting Cul3-Glis3 interaction and Cul3-mediated ubiquitination of Glis3. Previous studies reported that SUFU is able to stabilize Gli2 and Gli3 against proteasomal degradation by antagonizing their interaction with SPOP/Cul3 (30, 32). However, we were unable to observe any effects of SPOP on Glis3,[3] suggesting that another BTB domain-containing protein may mediate the interaction between Glis3 and Cul3.

In addition to an effect on protein stability, SUFU was shown to inhibit the transcriptional activity of Glis3. In a previous report we demonstrated that Glis3 functions as an effective activator of *Ins2* by binding two GlisBS in its promoter regulatory region (6). In the current study, we showed that SUFU was able to inhibit the induction of endogenous *Ins2* expression in β-like INS-1(832/13) cells (Fig. 5*A*). Moreover, SUFU caused up to a 50% decrease in Glis3-induced activation of the *Ins2* promoter in these cells. This inhibition by SUFU was abrogated, however, when the SUFU YGH-binding motif in Glis3 was mutated or deleted by N-terminal truncation. The latter suggests that the inhibition of Glis3-mediated *Ins2* transactivation by SUFU is dependent on the direct interaction of SUFU with Glis3 at the YGH motif. Although Glis2 was able to activate the *Ins2* promoter in HEK293 cells, this activation was not affected by co-expression with SUFU. Similarly, SUFU had no significant effect on Glis1-induced activation of the *Ins2* promoter. These results indicate that the effect of SUFU on *Ins2* activation is specific for Glis3. The mechanism by which SUFU inhibits transcriptional activation by Glis3 is currently under further investigation. Repression of Gli-activated target genes by SUFU

[3] G. T. ZeRuth, unpublished data.

has been thought to be the result of the cytoplasmic retention of Gli proteins (24, 26, 33–35) and/or by nuclear protein-protein interactions involving SAP18 and histone deactylase recruitment (24, 36–38). Our data demonstrate that the inhibition of Glis3-mediated *Ins2* activation by SUFU does not involve cytoplasmic sequestration of Glis3, as Glis3 remained localized to the nucleus in the presence of exogenous SUFU. In contrast, co-expression with Glis3 promoted the nuclear accumulation of SUFU, suggesting that SUFU-mediated inhibition of Glis3 target genes is due to a mechanism that appears to be dependent on its nuclear localization and may involve a co-repressor function of SUFU. Alternatively, several studies have linked the inhibition of proteolytic degradation of transcription factors by proteasomes to a reduction in their transcriptional activity (39, 40). Similarly, the inhibition of Glis3 ubiquitination and degradation by SUFU might be causally related to the observed repression of Glis3 transcriptional activity.

SUFU has been reported to play an important role in Hedgehog signaling wherein it interacts with and regulates the activity of Gli proteins (32, 35, 41–43). In contrast to Glis3, which promotes SUFU nuclear localization, SUFU restrains Gli1–3 to the cytoplasm, resulting in the inhibition of the transcriptional function. SUFU has been shown to co-localize to the primary cilium with Gli proteins, and although the functional consequence of their co-localization is still far from being understood, both primary cilium-dependent and -independent regulation of Gli proteins by SUFU have been reported (32, 33, 41, 42). In this context it is interesting to note that Glis2 and Glis3 have also been localized to the primary cilium (7, 10, 44). One might speculate that SUFU-Glis3 interaction might be linked functionally to the primary cilium and possibly involved in the regulation of Glis3 activity. However, future studies are required to elucidate the potential link among SUFU-Glis3 interaction, primary cilia, and Glis3 function.

Recent studies have shown that Glis3 has a critical role in both the development of pancreatic β-cells and the regulation of insulin gene transcription (1, 6, 8, 17). Interaction with SUFU and the consequent modulation of Glis3 protein stability moderate Glis3 activity and, as a consequence, are important in the regulation of *Ins* expression by Glis3. Mechanisms that stabilize and destabilize transcriptional regulators may play a critical role in the regulation of *Ins* expression in β-cells. In addition to Glis3, several other transcription factors, including Pdx1, MafA, and NeuroD1, have been implicated in the regulation of insulin gene transcription (45, 46). Glis3 co-activator complexes have been reported to interact with adjacent Pdx1, MafA, and NeuroD1 protein complexes and to act cooperatively in regulating insulin gene expression (1, 8). The association with SUFU may affect pancreas development as well as the magnitude of *Ins* expression by influencing these interactions. Furthermore, in consideration of the fact that Glis3 is overexpressed in several cancers (47, 48) and SUFU functions as a tumor suppressor (38, 49, 50), a defective interaction between these two proteins may be relevant to cancer development.

In summary, in this study we demonstrated that Glis3 interacted with SUFU through a VYGH motif within the homologous region at its N terminus and that this interaction inhibited the polyubiquitination and degradation of Glis3 but repressed its transcriptional activity. We further provided evidence showing that Cul3-based complexes can promote Glis3 polyubiquitination and proteasome-dependent degradation of Glis3. SUFU inhibits the association between Cul3 and Glis3, thereby inhibiting Glis3 polyubiquitination and degradation. These observations allow for a possible model, shown in Fig. 9, whereby SUFU and Cul3, through their interaction with Glis3, control the stability and transcriptional activity of Glis3 and may fine-tune the regulation of gene expression by Glis3. This modulation of Glis3 activity by SUFU and Cul3 may play an important role in the mechanism by which Glis3 regulates the maintenance of pancreatic β-cell function and insulin gene expression.

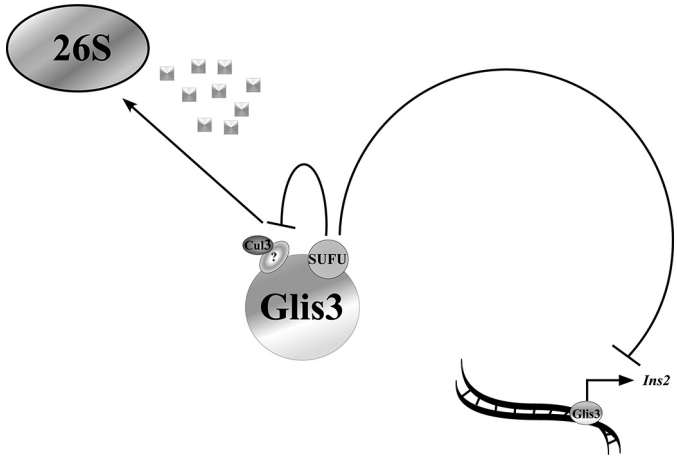

FIGURE 9. **Model of Glis3 regulation by SUFU and Cul3.** SUFU interacts with Glis3 through the YGH motif within the N-terminal conserved region of Glis3. SUFU inhibits Glis3 polyubiquitination and enhances Glis3 protein stability. Because Cul3 promotes the polyubiquitination of Glis3, Cul3-based E3 ubiquitin ligases likely play a role in the proteasome-mediated degradation of Glis3. SUFU binding hinders the interaction of Glis3 with Cul3, consequently inhibiting the proteasome-mediated degradation of Glis3. In addition, the association of SUFU with Glis3 enhances the nuclear localization of SUFU and inhibits Glis3-mediated transactivation of target genes, such as insulin. *26S*, indicates the proteasome.

Acknowledgments—We thank Drs. Kristin Lichti-Kaiser and Hong Soon Kang (National Institutes of Health, NIEHS) for their comments on the manuscript.

REFERENCES

1. Kang, H. S., ZeRuth, G., Lichti-Kaiser, K., Vasanth, S., Yin, Z., Kim, Y. S., and Jetten, A. M. (2010) *Histol. Histopathol.* **25,** 1481–1496
2. Kasper, M., Regl, G., Frischauf, A. M., and Aberger, F. (2006) *Eur. J. Cancer* **42,** 437–445
3. Merzdorf, C. S. (2007) *Dev. Dyn.* **236,** 922–940
4. Kim, Y. S., Nakanishi, G., Lewandoski, M., and Jetten, A. M. (2003) *Nucleic Acids Res.* **31,** 5513–5525
5. Beak, J. Y., Kang, H. S., Kim, Y. S., and Jetten, A. M. (2007) *J. Bone Miner. Res.* **22,** 1234–1244
6. Kang, H. S., Kim, Y. S., ZeRuth, G., Beak, J. Y., Gerrish, K., Kilic, G., Sosa-Pineda, B., Jensen, J., Pierreux, C. E., Lemaigre, F. P., Foley, J., and Jetten, A. M. (2009) *Mol. Cell. Biol.* **29,** 6366–6379
7. Kang, H. S., Beak, J. Y., Kim, Y. S., Herbert, R., and Jetten, A. M. (2009) *Mol. Cell. Biol.* **29,** 2556–2569
8. Yang, Y., Chang, B. H., Samson, S. L., Li, M. V., and Chan, L. (2009) *Nucleic Acids Res.* **37,** 2529–2538
9. Beak, J. Y., Kang, H. S., Kim, Y. S., and Jetten, A. M. (2008) *Nucleic Acids*

Res. **36,** 1690–1702
10. Kim, Y. S., Kang, H. S., Herbert, R., Beak, J. Y., Collins, J. B., Grissom, S. F., and Jetten, A. M. (2008) *Mol. Cell. Biol.* **28,** 2358–2367
11. Senée, V., Chelala, C., Duchatelet, S., Feng, D., Blanc, H., Cossec, J. C., Charon, C., Nicolino, M., Boileau, P., Cavener, D. R., Bougnères, P., Taha, D., and Julier, C. (2006) *Nat. Genet.* **38,** 682–687
12. Barrett, J. C., Clayton, D. G., Concannon, P., Akolkar, B., Cooper, J. D., Erlich, H. A., Julier, C., Morahan, G., Nerup, J., Nierras, C., Plagnol, V., Pociot, F., Schuilenburg, H., Smyth, D. J., Stevens, H., Todd, J. A., Walker, N. M., and Rich, S. S. (2009) *Nat. Genet.* **41,** 703–707
13. Dupuis, J., Langenberg, C., Prokopenko, I., Saxena, R., Soranzo, N., Jackson, A. U., Wheeler, E., Glazer, N. L., Bouatia-Naji, N., Gloyn, A. L., Lindgren, C. M., Mägi, R., Morris, A. P., Randall, J., Johnson, T., Elliott, P., Rybin, D., Thorleifsson, G., Steinthorsdottir, V., Henneman, P., Grallert, H., Dehghan, A., Hottenga, J. J., Franklin, C. S., Navarro, P., Song, K., Goel, A., Perry, J. R., Egan, J. M., Lajunen, T., Grarup, N., Sparsø, T., Doney, A., Voight, B. F., Stringham, H. M., Li, M., Kanoni, S., Shrader, P., Cavalcanti-Proença, C., Kumari, M., Qi, L., Timpson, N. J., Gieger, C., Zabena, C., Rocheleau, G., Ingelsson, E., An, P., O'Connell, J., Luan, J., Elliott, A., McCarroll, S. A., Payne, F., Roccasecca, R. M., Pattou, F., Sethupathy, P., Ardlie, K., Ariyurek, Y., Balkau, B., Barter, P., Beilby, J. P., Ben-Shlomo, Y., Benediktsson, R., Bennett, A. J., Bergmann, S., Bochud, M., Boerwinkle, E., Bonnefond, A., Bonnycastle, L. L., Borch-Johnsen, K., Böttcher, Y., Brunner, E., Bumpstead, S. J., Charpentier, G., Chen, Y. D., Chines, P., Clarke, R., Coin, L. J., Cooper, M. N., Cornelis, M., Crawford, G., Crisponi, L., Day, I. N., de Geus, E. J., Delplanque, J., Dina, C., Erdos, M. R., Fedson, A. C., Fischer-Rosinsky, A., Forouhi, N. G., Fox, C. S., Frants, R., Franzosi, M. G., Galan, P., Goodarzi, M. O., Graessler, J., Groves, C. J., Grundy, S., Gwilliam, R., Gyllensten, U., Hadjadj, S., Hallmans, G., Hammond, N., Han, X., Hartikainen, A. L., Hassanali, N., Hayward, C., Heath, S. C., Hercberg, S., Herder, C., Hicks, A. A., Hillman, D. R., Hingorani, A. D., Hofman, A., Hui, J., Hung, J., Isomaa, B., Johnson, P. R., Jørgensen, T., Jula, A., Kaakinen, M., Kaprio, J., Kesaniemi, Y. A., Kivimaki, M., Knight, B., Koskinen, S., Kovacs, P., Kyvik, K. O., Lathrop, G. M., Lawlor, D. A., Le Bacquer, O., Lecoeur, C., Li, Y., Lyssenko, V., Mahley, R., Mangino, M., Manning, A. K., Martínez-Larrad, M. T., McAteer, J. B., McCulloch, L. J., McPherson, R., Meisinger, C., Melzer, D., Meyre, D., Mitchell, B. D., Morken, M. A., Mukherjee, S., Naitza, S., Narisu, N., Neville, M. J., Oostra, B. A., Orrù, M., Pakyz, R., Palmer, C. N., Paolisso, G., Pattaro, C., Pearson, D., Peden, J. F., Pedersen, N. L., Perola, M., Pfeiffer, A. F., Pichler, I., Polasek, O., Posthuma, D., Potter, S. C., Pouta, A., Province, M. A., Psaty, B. M., Rathmann, W., Rayner, N. W., Rice, K., Ripatti, S., Rivadeneira, F., Roden, M., Rolandsson, O., Sandbaek, A., Sandhu, M., Sanna, S., Sayer, A. A., Scheet, P., Scott, L. J., Seedorf, U., Sharp, S. J., Shields, B., Sigurethsson, G., Sijbrands, E. J., Silveira, A., Simpson, L., Singleton, A., Smith, N. L., Sovio, U., Swift, A., Syddall, H., Syvänen, A. C., Tanaka, T., Thorand, B., Tichet, J., Tönjes, A., Tuomi, T., Uitterlinden, A. G., van Dijk, K. W., van Hoek, M., Varma, D., Visvikis-Siest, S., Vitart, V., Vogelzangs, N., Waeber, G., Wagner, P. J., Walley, A., Walters, G. B., Ward, K. L., Watkins, H., Weedon, M. N., Wild, S. H., Willemsen, G., Witteman, J. C., Yarnell, J. W., Zeggini, E., Zelenika, D., Zethelius, B., Zhai, G., Zhao, J. H., Zillikens, M. C., Borecki, I. B., Loos, R. J., Meneton, P., Magnusson, P. K., Nathan, D. M., Williams, G. H., Hattersley, A. T., Silander, K., Salomaa, V., Smith, G. D., Bornstein, S. R., Schwarz, P., Spranger, J., Karpe, F., Shuldiner, A. R., Cooper, C., Dedoussis, G. V., Serrano-Ríos, M., Morris, A. D., Lind, L., Palmer, L. J., Hu, F. B., Franks, P. W., Ebrahim, S., Marmot, M., Kao, W. H., Pankow, J. S., Sampson, M. J., Kuusisto, J., Laakso, M., Hansen, T., Pedersen, O., Pramstaller, P. P., Wichmann, H. E., Illig, T., Rudan, I., Wright, A. F., Stumvoll, M., Campbell, H., Wilson, J. F., Bergman, R. N., Buchanan, T. A., Collins, F. S., Mohlke, K. L., Tuomilehto, J., Valle, T. T., Altshuler, D., Rotter, J. I., Siscovick, D. S., Penninx, B. W., Boomsma, D. I., Deloukas, P., Spector, T. D., Frayling, T. M., Ferrucci, L., Kong, A., Thorsteinsdottir, U., Stefansson, K., van Duijn, C. M., Aulchenko, Y. S ., Cao, A., Scuteri, A., Schlessinger, D., Uda, M., Ruokonen, A., Jarvelin, M. R., Waterworth, D. M., Vollenweider, P., Peltonen, L., Mooser, V., Abecasis, G. R., Wareham, N. J., Sladek, R., Froguel, P., Watanabe, R. M., Meigs, J. B., Groop, L., Boehnke, M., McCarthy, M. I., Florez, J. C., and Barroso, I. (2010) *Nat. Genet.* **42,** 105–116
14. Boesgaard, T. W., Grarup, N., Jørgensen, T., Borch-Johnsen, K., Hansen, T., and Pedersen, O. (2010) *Diabetologia* **53,** 1647–1655
15. Dimitri, P., Warner, J. T., Minton, J. A., Patch, A. M., Ellard, S., Hattersley, A. T., Barr, S., Hawkes, D., Wales, J. K., and Gregory, J. W. (2011) *Eur. J. Endocrinol.* **164,** 437–443
16. Hu, C., Zhang, R., Wang, C., Wang, J., Ma, X., Hou, X., Lu, J., Yu, W., Jiang, F., Bao, Y., Xiang, K., and Jia, W. (2010) *PLoS One* **5,** e15542
17. Watanabe, N., Hiramatsu, K., Miyamoto, R., Yasuda, K., Suzuki, N., Oshima, N., Kiyonari, H., Shiba, D., Nishio, S., Mochizuki, T., Yokoyama, T., Maruyama, S., Matsuo, S., Wakamatsu, Y., and Hashimoto, H. (2009) *FEBS Lett.* **583,** 2108–2113
18. Melloul, D., Marshak, S., and Cerasi, E. (2002) *Diabetologia* **45,** 309–326
19. Ohneda, K., Ee, H., and German, M. (2000) *Semin. Cell Dev. Biol.* **11,** 227–233
20. Nakanishi, G., Kim, Y. S., Nakajima, T., and Jetten, A. M. (2006) *J. Invest. Dermatol.* **126,** 49–60
21. Vasanth, S., ZeRuth, G., Kang, H. S., and Jetten, A. M. (2011) *J. Biol. Chem.* **286,** 4749–4759
22. Dunaeva, M., Michelson, P., Kogerman, P., and Toftgard, R. (2003) *J. Biol. Chem.* **278,** 5116–5122
23. Rual, J. F., Venkatesan, K., Hao, T., Hirozane-Kishikawa, T., Dricot, A., Li, N., Berriz, G. F., Gibbons, F. D., Dreze, M., Ayivi-Guedehoussou, N., Klitgord, N., Simon, C., Boxem, M., Milstein, S., Rosenberg, J., Goldberg, D. S., Zhang, L. V., Wong, S. L., Franklin, G., Li, S., Albala, J. S., Lim, J., Fraughton, C., Llamosas, E., Cevik, S., Bex, C., Lamesch, P., Sikorski, R. S., Vandenhaute, J., Zoghbi, H. Y., Smolyar, A., Bosak, S., Sequerra, R., Doucette-Stamm, L., Cusick, M. E., Hill, D. E., Roth, F. P., and Vidal, M. (2005) *Nature* **437,** 1173–1178
24. Pearse, R. V., 2nd, Collier, L. S., Scott, M. P., and Tabin, C. J. (1999) *Dev. Biol.* **212,** 323–336
25. Ding, Q., Fukami, S., Meng, X., Nishizaki, Y., Zhang, X., Sasaki, H., Dlugosz, A., Nakafuku, M., and Hui, C. (1999) *Curr. Biol.* **9,** 1119–1122
26. Kogerman, P., Grimm, T., Kogerman, L., Krause, D., Undén, A. B., Sandstedt, B., Toftgård, R., and Zaphiropoulos, P. G. (1999) *Nat. Cell Biol.* **1,** 312–319
27. Pintard, L., Willems, A., and Peter, M. (2004) *EMBO J.* **23,** 1681–1687
28. Claiborn, K. C., Sachdeva, M. M., Cannon, C. E., Groff, D. N., Singer, J. D., and Stoffers, D. A. (2010) *J. Clin. Invest.* **120,** 3713–3721
29. Zhang, Q., Shi, Q., Chen, Y., Yue, T., Li, S., Wang, B., and Jiang, J. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 21191–21196
30. Wen, X., Lai, C. K., Evangelista, M., Hongo, J. A., de Sauvage, F. J., and Scales, S. J. (2010) *Mol. Cell. Biol.* **30,** 1910–1922
31. Croker, J. A., Ziegenhorn, S. L., and Holmgren, R. A. (2006) *Dev. Biol.* **291,** 368–381
32. Chen, M. H., Wilson, C. W., Li, Y. J., Law, K. K., Lu, C. S., Gacayan, R., Zhang, X., Hui, C. C., and Chuang, P. T. (2009) *Genes Dev.* **23,** 1910–1928
33. Jia, J., Kolterud, A., Zeng, H., Hoover, A., Teglund, S., Toftgård, R., and Liu, A. (2009) *Dev. Biol.* **330,** 452–460
34. Stone, D. M., Murone, M., Luoh, S., Ye, W., Armanini, M. P., Gurney, A., Phillips, H., Brush, J., Goddard, A., de Sauvage, F. J., and Rosenthal, A. (1999) *J. Cell Sci.* **112,** 4437–4448
35. Tukachinsky, H., Lopez, L. V., and Salic, A. (2010) *J. Cell Biol.* **191,** 415–428
36. Cheng, S. Y., and Bishop, J. M. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 5442–5447
37. Paces-Fessy, M., Boucher, D., Petit, E., Paute-Briand, S., and Blanchet-Tournier, M. F. (2004) *Biochem. J.* **378,** 353–362
38. Cheng, S. Y., and Yue, S. (2008) *Adv. Cancer Res.* **101,** 29–43
39. Leung, A., Geng, F., Daulny, A., Collins, G., Guzzardo, P., and Tansey, W. P. (2008) *Ernst Schering Found. Symp. Proc.* **1,** 75–97
40. Keppler, B. R., Archer, T. K., and Kinyamu, H. K. (2011) *Biochim. Biophys. Acta.* **1809,** 109–118
41. Humke, E. W., Dorn, K. V., Milenkovic, L., Scott, M. P., and Rohatgi, R. (2010) *Genes Dev.* **24,** 670–682
42. Zeng, H., Jia, J., and Liu, A. (2010) *PLoS One* **5,** e15900
43. Kise, Y., Morinaka, A., Teglund, S., and Miki, H. (2009) *Biochem. Biophys. Res. Commun.* **387,** 569–574
44. Attanasio, M., Uhlenhaut, N. H., Sousa, V. H., O'Toole, J. F., Otto, E.,

Anlag, K., Klugmann, C., Treier, A. C., Helou, J., Sayer, J. A., Seelow, D., Nürnberg, G., Becker, C., Chudley, A. E., Nürnberg, P., Hildebrandt, F., and Treier, M. (2007) *Nat. Genet.* **39,** 1018–1024
45. Gittes, G. K. (2009) *Dev. Biol.* **326,** 4–35
46. Oliver-Krasinski, J. M., and Stoffers, D. A. (2008) *Genes Dev.* **22,** 1998–2021
47. Yusenko, M. V., and Kovacs, G. (2009) *Histopathology* **55,** 687–695
48. Lukashova-v Zangen, I., Kneitz, S., Monoranu, C. M., Rutkowski, S., Hinkes, B., Vince, G. H., Huang, B., and Roggendorf, W. (2007) *Acta Neuropathol.* **113,** 325–337
49. Lee, Y., Kawagoe, R., Sasai, K., Li, Y., Russell, H. R., Curran, T., and McKinnon, P. J. (2007) *Oncogene* **26,** 6442–6447
50. Meng, X., Poon, R., Zhang, X., Cheah, A., Ding, Q., Hui, C. C., and Alman, B. (2001) *J. Biol. Chem.* **276,** 40113–40119