GLI2 IS TARGETED FOR UBIQUITINATION AND DEGRADATION BY β-TRCP UBIQUITIN LIGASE.

Neehar Bhatia1, Saravanan Thiagarajan1, Irina Elcheva1, Mohammed Saleem1, Andrzej Dlugosz2, Hasan Mukhtar1, and Vladimir S. Spiegelman1, *

RUNNING TITLE: Gli2 is a substrate of β-TrCP ubiquitin ligase.

1Department of Dermatology, University of Wisconsin Medical School, Madison, WI 53706, 2 Department of Dermatology and Comprehensive Cancer Center, University of Michigan School of Medicine, Ann Arbor, MI 48109

KEYWORDS: Gli2, Hedgehog signaling, beta-TrCP, ubiquitin, E3 ligase, prostate cancer

Address correspondence to: Vladimir S. Spiegelman, M.D., Ph.D. Department of Dermatology, University of Wisconsin Medical School, 1300 University Ave., B-25, Madison, WI 53706.
Telephone: (608) 265-8197; Fax: (608) 263-2919; Email: spiegelman@dermatology.wisc.edu

The Hedgehog (Hh) signaling pathway plays a crucial role in embryogenesis and has been linked to the development of several human malignancies. The transcription factor Gli2 plays a key role in the transduction of Hh signals by modulating transcription of some Hh target genes, yet, the mechanisms that control Gli2 protein expression are largely unknown. Here we report that β-TrCP E3 ubiquitin ligase is required for Gli2 degradation. β-TrCP2 directly binds wild type Gli2 and promotes its ubiquitination. Single amino acid substitution in Gli2 putative binding site inhibits its interaction with β-TrCP2, its ubiquitination, and stabilizes the Gli2 protein. Stable Gli2 mutant is expressed in higher levels and is more potent in the activation of Gli-dependent transcription as compared with wild type Gli2. We also found that Gli2 protein is highly expressed in prostate cancer cell lines and primary tumors, whereas the level of GLI2 mRNA is not appreciable different in normal and neoplastic prostate. These data identify β-TrCP2 as a pivotal regulator of Gli2 expression, and point to an important role for post-translational modulation of Gli2 protein levels in Hh pathway-associated human prostate cancer.

The Hedgehog (Hh) signaling plays a prominent role in embryogenesis, and its deregulation is implicated in tumorigenesis (reviewed in (1-4)). Cellular responses to the Hedgehog signal are controlled by two transmembrane proteins: the tumor suppressor Patched (PTCH) and the proto-oncogene Smoothened (SMO). In the absence of secreted Hh proteins, PTCH actively silences intracellular signaling by inactivating SMO. During physiologic signaling, Hh proteins bind and inactivate PTCH, which alleviates PTCH-mediated suppression of SMO (5). SMO activation triggers a series of intracellular events, culminating in expression of Hh target genes through the action of the Glis family of transcription factors (6,7). Gli1, Gli2 and Gli3, and their Drosophila homolog, Cubitus interruptus (Ci), are zinc finger transcription factors that are downstream effectors of Hh signaling. In the absence of Hh signaling, Ci is truncated at the carboxyl terminal domain to form a truncated repressor protein, whereas Hh activation leads to accumulation of transcriptional active, full length Ci. The situation with mammalian Gli proteins is more complex. Gli3 functions primarily as a C-terminally truncated repressor, but full-length Gli3 protein accumulates in cells responding to Hh. Gli1, on the other hand, appears to modulate gene expression by acting primarily as a transcriptional
activator, but Gli1 mutant mice are phenotypically normal, arguing against an essential function for this protein during development or postnatal life. Gli2 appears to be the major nuclear effector of Hh signaling in vivo (8-12) and functions primarily as a transcriptional activator. However, little is known about the molecular mechanisms regulating Gli2 expression at the protein level.

The Hh signaling pathway is deregulated in many human malignancies, including basal cell carcinoma (BCC), medulloblastoma, lung, prostate, breast, and some gastrointestinal cancers (13-19). Recent studies have stressed the importance of Hh signaling in human prostate cancer (13-15,20). Elevated Hh signaling pathway activity may distinguish metastatic from localized prostate cancer, and pathway manipulation can modulate invasiveness and metastasis (13). In contrast to BCCs and medulloblastomas, which are associated with inactivating mutations in PTCH or gain-of-function mutations in SMO, aberrant Hh signaling in prostate cancers appears to be the result of constitutive overexpression of Sonic hedgehog (SHH). Hence, the growth of many of the prostate cancer cells is inhibited by Hh-neutralizing antibody. Furthermore, cyclopamine, a steroidal alkaloid that interacts with SMOH directly thus inhibiting Hh signaling, was shown to induce apoptosis and inhibit proliferation of prostate cancer cells in vivo as well as in vitro.

The ubiquitin-proteasome pathway is essential for degradation of proteins regulating growth and cell cycle progression (21). Ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase (E3) sequentially tag proteins for ubiquitination and proteasomal degradation. SCF E3 ubiquitin ligases are composed of Skp1, Cul1, Roc1 and F box proteins, where F box proteins are substrate recognizing subunits (22). Beta-transducin repeat-containing F box proteins (β-TrCP) recognize substrates phosphorylated within the DSG(X)_2nS destruction motifs. SCFβ-TrCP E3 ligases ubiquitinate specifically phosphorylated substrates and play a pivotal role in the regulation of cell division and various signal transduction pathways, which, in turn, are essential for many aspects of tumorigenesis (reviewed in (23)). Genetic data have suggested that Drosophila slimb protein (orthologue of mammalian β-TrCP) is involved in proteolytic processing of Ci155 to Ci75 (24). However, there is no biochemical evidence that Slimb/β-TrCP proteins are involved in ubiquitination and degradation of Ci/Gli transcription factors.

Stabilization of the transcription factor Gli2 has been suggested as a key event in the transduction of Hh signals. The potential role of Gli2 in the development of BCC has been well documented. Gli2 is over-expressed in the majority of human BCCs, and skin-targeted over-expression of Gli2 in transgenic mice leads to the development of multiple BCCs. There is growing evidence that the transcriptional regulation of some Hh target genes, including Gli1, E2F1, Bcl2, etc., are Gli2 dependent(25-28). The promoter of one such gene, Bcl2, is regulated preferentially by Gli2 (27).

In this study we have found that SCFβ-TrCP E3 ubiquitin ligase is responsible for Gli2 degradation. β-TrCP2 directly binds wild type Gli2 and promotes its ubiquitination, which is inhibited by a single amino acid substitution in Gli2 putative binding site. We also found that full length Gli2 protein is over-expressed in prostate cancer cell lines and primary tumors. The mechanisms of Gli2 stabilization are discussed.

**Experimental Procedures**

**DNA Constructs**

The pcDNA3.1-Flag-Gli2 plasmid was generated using full-length mouse Gli2 cDNA provided by Drs. Hiroshi Sasaki and Chi-ching Hui (29). Replacement of serine 662 with alanine was carried out using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). HA-tagged β-TrCP2 and β-TrCP2ΔN encoding plasmids (30), the constructs for specific knock down of β-TrCP2 (shBTR2), β-TrCP1 (shBTR1), as well as control shRNA construct (shCON) (31) were generously provided by Dr. S.Y. Fuchs. 8x3′Gli BS-LucII [8 directly repeated copies of 3′Gli binding site from HNF3β floor plate enhancer cloned into pδ51LucII (32)] was a gift of Dr. H. Sasaki (Osaka University, Osaka, Japan), K17-luc (K17 driven luciferase reporter) was from Dr. P. Coulombe, pGL3-Bcl2 promoter luciferase plasmid (pGL3 basic vector with 1.9kb of the putative promoter and 5′ untranslated region of human Bcl2) was...
generously provided by Dr. F. Aberger (27). Plasmids for expression of renilla luciferase (pRL-TK) and β-galactosidase (pSV-40 β-gal) were purchased (Promega Corp., Madison, WI).

**Tissue Culture and Transfections**

293T human embryo kidney cells and HeLa human cervical adenocarcinoma cells were purchased from ATCC. Cells were grown in DMEM in the presence of 10% fetal bovine serum (FBS) and antibiotics at 37°C and 5% CO₂. Transfections were performed using the calcium phosphate procedure or lipofection with Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

**Antibodies and Western Blotting**

Antibodies against HA (Roche), Gli2, β-actin (Santa Cruz Biotechnology), Flag M2 (Sigma-Aldrich) were purchased. β-TrCP antibody was described previously (33). Horseradish peroxidase conjugated secondary antibodies were purchased (Cell Signaling, Santa Cruz, Jackson). Immunoprecipitation and immunoblotting procedures were performed as described elsewhere (34).

**In vivo Binding Assay**

293T cells co-transfected with FLAG-tagged Gli2 or Gli2S662A and HA-tagged β-TrCP2 were lysed in RIPA lysis buffer. Interaction between the expressed proteins was assessed by immunoprecipitation with FLAG or HA antibodies and immunoblotting with HA or FLAG antibodies, respectively. The interaction between endogenous proteins in the protein lysates from HeLa cells was analyzed by immunoprecipitation with Gli2 antibody and immunoblotting with β-TrCP antibody.

**In vitro Binding Assay**

Recombinant Gli2 proteins expressed in 293T cells were immunopurified with Flag antibody and protein A/G agarose beads, stringently washed with stripping buffer containing 20mM Tris HCl (pH 7.5), 1M NaCl, 50mM NaF, and 0.1% Nonidet P40 and equilibrated with binding buffer (20mM Tris HCl, pH 7.5, 100mM NaCl, 50mM NaF, and 0.1% Nonidet P40). For treatment with phosphatase λ, the beads were washed with the binding buffer without phosphatase inhibitors and incubated with the phosphatase λ, for 1h at 37°C followed by washes in stripping buffer and re-equilibration with binding buffer. Flag-Gli2 proteins immobilized on the beads were incubated with in vitro-translated and S³⁵S-labeled β-TrCP2 for 60 min at 4°C. The beads were extensively washed with binding buffer and associated proteins were analyzed by SDS-PAGE and autoradiography.

S³⁵S labeled β-TrCP2 was synthesized in vitro using TnT kit (Promega). Lysates of 293T cells transfected with FLAG Gli2 and Gli2S662A were incubated with S³⁵S labeled β-TrCP2 for 3 hrs at 4°C. These lysates were immunoprecipitated with FLAG antibody and associated proteins were analyzed by SDS-PAGE and autoradiography.

**In vivo Ubiquitination Assay**

293T cells were co-tranfected with HA-tagged ubiquitin, HA-tagged βTrCP2 and Flag-tagged Gli2 or Gli2S662A. Cells were lysed in RIPA lysis buffer and immunoprecipitated with FLAG antibody. Immunocomplexes were analyzed by SDS-PAGE and immunoblotting with HA antibody.

**Degradation Assay**

Pulse chase analysis was carried out on 293T cells as described elsewhere (34). Briefly, cells were grown in 100mm dishes and transfected with the indicated plasmids. Cells were starved in methionine and cysteine free DMEM followed by metabolically labeling with a ³⁵S-methionine/³⁵S-cysteine mixture (Perkin Elmer, Inc.). Chase was performed in complete DMEM (10% FBS) supplemented with 2mM unlabeled methionine and cysteine and cells were harvested at respective time points. Gli2 proteins were immunoprecipitated from RIPA lysates with FLAG antibody, separated by SDS-PAGE and analyzed by autoradiography.

**Luciferase Reporter Assays**

Hela cells were transfected with 8x3'Gli BS-LucII reporter, K17 luciferase reporter plasmid, or pGL3-Bcl2promo luciferase reporter plasmid and
respective Gli2 expression plasmids. Luciferase activity was estimated using Luciferase Reporter Assay Reagent (Promega). β-galactosidase was used for normalization and estimated using β-gal assay reagent (Pierce Biotechnology).

**Immunohistochemistry**

Prostate tissue arrays were purchased (Cybrdi, Inc., Frederick, MD). Sections were incubated at 4°C overnight with Gli-2 G20 antibody followed by donkey anti-goat biotin secondary antibody, ABC reagent (Vector Laboratories, Burlingame, CA) and developed by DAB (Sigma-Aldrich).

**RNA isolation and Real-Time RT PCR**

Real Time RT PCR for quantitative RNA measurements of Gli2 were done using SYBR Green PCR Core reagents (Applied Biosystems) as described before (Lamm 2002). GAPDH was used as reference gene.

**RESULTS AND DISCUSSION**

SCFβ-TrCP ubiquitin ligase recognizes DSG(X)2+nS destruction motif to target proteins for ubiquitination and further degradation (reviewed in (23)). Sequence analysis of Gli2 revealed the DSG/V/MEMPGTGPGS motif, which is conserved among various mammalian species (Figure 1A). We analyzed whether substrate recognizing component of SCFβ-TrCP ubiquitin ligase, F-box protein β-TrCP2 interacts with Gli2. We found that exogenously expressed Gli2 and β-TrCP2 (Figure 1B), as well as endogenous proteins (Figure 1C) interact in vivo in co-immunoprecipitation assay. Gli2 was also shown to bind in vitro translated β-TrCP2 protein (Figure 1D). To determine whether the putative β-TrCP recognition motif is responsible for this interaction we substituted potentially phosphorylated Serine 662 to Alanine in this motif of Gli2 (Figure 1A). This single amino acid substitution is predicted to disrupt Gli2 interactions with β-TrCP. Indeed, in both assays, Gli2S662A binding to β-TrCP2 was greatly diminished (Figure 1-B, D). Treatment of Flag-Gli2 with protein phosphatase λ abolished the ability of Gli2 to bind β-TrCP2 in vitro (Figure 1-D). These results demonstrate that phosphorylation of Gli2 is necessary for its recognition by β-TrCP ubiquitin ligase receptor.

Interaction of β-TrCP2 with specific substrates results in ubiquitination of these proteins (reviewed in (23)). Co-transfection of cells with β-TrCP2 construct accelerated the ubiquitination of wild type Gli2 (Figure 1E, Lane 3) as measured by in vivo ubiquitination assay. In contrast, the ubiquitination of Gli2S662A mutant that interacts poorly with β-TrCP2 was less efficient, and was not affected by β-TrCP2 over-expression (Figure 1E, Lane 6). These data demonstrate that serine 662 is critical for the interaction between Gli2 and β-TrCP2, and that binding to β-TrCP is important for Gli2 ubiquitination.

In order to analyze the role of β-TrCP in the degradation of Gli2, we inhibited β-TrCP activity by either knocking down the expression of β-TrCP1 and β-TrCP2 proteins by using short hairpin RNA (shRNA) or by expression of dominant negative mutant of β-TrCP (β-TrCP2ΔN) (30). Our results show that inhibition of β-TrCP function leads to stabilization of Gli2 protein (Figure 2-A-C). β-TrCP2ΔN extends the half-life of Gli2 from about 6 to 12 hours. Interestingly, shRNA against β-TrCP2 appeared to be more effective in the inhibition of Gli2 turnover than β-TrCP1 specific shRNA (Figure 2A). Importantly, inhibition of β-TrCP function resulted in stabilization (Figure 2C) and accumulation (Figure 2D) of endogenous Gli2 in 293T cells. These data suggest that β-TrCP is involved in degradation of Gli2 protein in mammalian cells.

Although the kinase responsible for Gli2 phosphorylation within β-TrCP recognition motif is not known, phosphorylation of Drosophila Ci by shaggy (Drosophila homologue of GSK-3β) was demonstrated to be a necessary step in Ci proteolysis (35,36). Recently Gli2 was shown to be phosphorylated by GSK3β (37). Treatment of cells with GSK3 inhibitor, LiCl substantially increased the half-life of Gli2β (Figure 2E). These data suggest that GSK3 may be involved in phosphorylation-dependent degradation of Gli2.

To further confirm the role of β-TrCP in proteolysis of Gli2, we compared the rate of degradation Gli2wt with that of Gli2S662A mutant. Gli2S662A poorly interacts with β-TrCP2 (Figure 1B, C), and is not ubiquitinated by β-TrCP2.
(Figure 1D). In comparison to Gli2wt, Gli2S662A mutant protein is more stable and exhibits a half-life of more than 9 hours (Figure 3A). Furthermore, over expression of dominant negative mutant of β-TrCP (β-TrCP2ΔN) did not affect the stability of Gli2S662A (Figure 3B). These data demonstrate that serine 662 is critical for the interaction between Gli2 and β-TrCP2. Disruption of the DSG motif by mutation of the serine residue renders it poorly interactive with β-TrCP, hence stabilizing the Gli2S662A mutant protein.

Stabilization of Gli2S662A mutant translates into the higher level of protein expression as compared to the wild type Gli2 (Figure 3C, insert). HeLa cells transfected with same amount of appropriate plasmids express higher level of Gli2S662A mutant protein as compared to the wild type protein. Figure 3C demonstrates that Gli2S662A mutant is significantly more effective than Gli2 wt in the activation of Gli-dependent transcriptional activity. 8x3′Gli BS-LucII, pGL3-Bcl2promoluciferase or K17 driven luciferase were utilized to measure Gli2 dependent transcriptional activation driven by Gli2wt or Gli2S662A. Gli2S662A is about twice as potent, than Gli2wt, in activation of transcription as measured by these 3 different reporter constructs. These results demonstrate that elevated Gli2 dependent transcriptional output is likely attributed to the higher levels of Gli2S662A protein expression. These data strongly suggest that Gli2 protein turnover is an important step in the modulation of Gli2 dependent transcription.

Gli transcription factors, including Gli1, Gli2 and Gli3, are the key modulators of Hh signaling. Gli2 and Gli3 contain both the amino terminal repressor domain as well as carboxyl terminal activator domain, whereas, Gli1 is comprised of only the carboxyl terminal activator domain. Hence, Gli1 acts as the transcriptional activator and is a secondary target, downstream of Gli2/Gli3. On the other hand, Gli3 is suggested to primarily act as the repressor, although, activator function for Gli3 has also been reported (38). Gli2, however, has been suggested to be the primary activator of Hh signaling (reviewed in (3)). Mice homozygous for Gli2 mutations exhibit developmental defects (11,39) and over expression of Gli2 results in formation of BCCs.

It has been previously shown that β-TrCP targets several essential proteins in cell transformation and signal transduction for ubiquitination and degradation, including IκBα, β-catenin and ATF4 proteins (reviewed in (23)). Genetic evidence suggest that Drosophila Slimb F box protein is involved in Hh signaling pathway in processing of full length Ci155 protein to truncated repressor form Ci75 (24). This study shows that Gli2 is the substrate of β-TrCP for ubiquitination and degradation. However, unlike Ci, there is no evidence suggesting that Gli2 undergoes proteolytic cleavage. We were unable to detect any smaller protein species of Gli2 indicating that Gli2 protein stabilization, rather than inhibition of processing, is important for downstream signaling. Future studies will delineate the functional significance of Gli2 ubiquitination and degradation by β-TrCP in the regulation of Hh signal transduction pathway.

Overexpression of Gli2 in transgenic mice induces formation of BCCs (40), and GLI2 mRNA is upregulated in the majority of human BCCs. However, there are no reports examining the expression of GLI2 protein in human cancers. GLI2 protein is dramatically induced in prostate cancer cell lines as compared to normal human prostate epithelial (NHPE) cells (Figure 4A). On the other hand, there is no significant difference in the expression of Gli2 mRNA in these cells (Figure 4B). These data are in line with previous observations that elevated expression of Gli2 message is a rare event in human prostate tumors and cell lines (14). In all, our data suggests that a novel post-transcriptional mechanism, most likely protein stabilization, is responsible for high levels of Gli2 expression in prostate cancer cells. Constitutive activation of Hh signaling in prostate cancer has been recently reported (13,14). Our data demonstrating high levels of Gli2 protein expression strongly correlate with Hh pathway activation in prostate cancer cells, suggesting that Gli2 plays an important role in mediation of Hh signaling in prostate cancer cells. Immunohistochemical analysis of an array of primary human prostate tumors using Gli2 antibody that recognizes C-terminus of Gli2 protein revealed high levels expression of Gli2 in the majority of malignant prostate epithelial cells as compared to the benign ones. Nuclear localization of Gli2 protein is a hallmark of its transcriptional activity. Importantly, 26 out of 29 prostate adenocarcinomas (90%) exhibited strong
nuclear or nuclear-cytoplasmic staining. In contrast, only one out of 5 prostate hyperplasia samples (20%) revealed nuclear accumulation of Gli2. Figure 4C shows a representative Gli2 immuno-staining of malignant and benign prostate tissues. These data further substantiate the importance of Gli2 over expression and activation in prostate cancer development, supporting our hypothesis that Gli2 may be a key component in Hh pathway activation in prostate cancer. Future studies will define the role of GLI2 in prostate tumorigenesis, and delineate the mechanisms by which prostate cancer cells achieve stabilization of GLI2.

REFERENCES

1. Mullor, J. L., Sanchez, P., and Altaba, A. R. (2002) Trends Cell Biol 12, 562-569
2. Ruiz i Altaba, A., Sanchez, P., and Dahmane, N. (2002) Nat Rev Cancer 2, 361-372
3. Matise, M. P., and Joyner, A. L. (1999) Oncogene 18, 7852-7859
4. Pasca di Magliano, M., and Hebrok, M. (2003) Nat Rev Cancer 3, 903-911
5. Cohen, M. M., Jr. (2003) Am J Med Genet A 123, 5-28
6. Lum, L., and Beachy, P. A. (2004) Science 304, 1755-1759
7. Bijlsma, M. F., Spek, C. A., and Peppelenbosch, M. P. (2004) Bioessays 26, 387-394
8. Bai, C. B., Auerbach, W., Lee, J. S., Stephen, D., and Joyner, A. L. (2002) Development 129, 4753-4761
9. Mill, P., Mo, R., Fu, H., Grachtechouk, M., Kim, P. C., Dlugosz, A. A., and Hui, C. C. (2003) Genes Dev 17, 282-294
10. Lewis, M. T., Ross, S., Strickland, P. A., Sugnet, C. W., Jimenez, E., Hui, C., and Daniel, C. W. (2001) Dev Biol 238, 133-144
11. Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. A., and Joyner, A. L. (1998) Development 125, 2759-2770
12. Roessler, E., Du, Y. Z., Mullor, J. L., Casas, E., Allen, W. P., Gillessen-Kaesbach, G., Roeder, E. R., Ming, J. E., Ruiz i Altaba, A., and Muenke, M. (2003) Proc Natl Acad Sci U S A 100, 13424-13429
13. Karhadkar, S. S., Steven Bova, G., Abdallah, N., Dhara, S., Gardner, D., Maitra, A., Isaacs, J. T., Berry, D. M., and Beachy, P. A. (2004) Nature 431, 707-712
14. Sanchez, P., Hernandez, A. M., Stecca, B., Kahler, A. J., DeGueme, A. M., Barrett, A., Beyna, M., Datta, M. W., Datta, S., and Ruiz i Altaba, A. (2004) Proc Natl Acad Sci U S A 101, 12561-12566
15. Fan, L., Pepicelli, C. V., Dibble, C. C., Catbagan, W., Zarycki, J. L., Laciak, R., Gipp, J., Shaw, A., Lamm, M. L., Munoz, A., Lipinski, R., Thrasher, J. B., and Bushman, W. (2004) Endocrinology 145, 3961-3970
16. Kubo, M., Nakamura, M., Tasaki, A., Yamanaka, N., Nakashima, H., Nomura, M., Kuroki, S., and Katano, M. (2004) Cancer Res 64, 6071-6074
17. Berman, D. M., Karhadkar, S. S., Maitra, A., Montes De Oca, R., Gerstenblith, M. R., Briggs, K., Parker, A. R., Shimada, Y., Eshleman, J. R., Watkins, D. N., and Beachy, P. A. (2003) Nature 425, 846-851
18. Thayer, S. P., di Magliano, M. P., Heiser, P. W., Nielsen, C. M., Roberts, D. J., Lauwers, G. Y., Qi, Y. P., Gysin, S., Fernandez-del Castillo, C., Yajnik, V., Antoniu, B., McMahon, M., Warshaw, A. L., and Hebrok, M. (2003) Nature 425, 851-856
19. Watkins, D. N., Berman, D. M., Burkholder, S. G., Wang, B., Beachy, P. A., and Baylin, S. B. (2003) Nature 422, 313-317
20. Sheng, T., Li, C., Zhang, X., Chi, S., He, N., Chen, K., McCormick, F., Gatalica, Z., and Xie, J. (2004) *Mol Cancer* **3**, 29
21. Nakayama, K. I., and Nakayama, K. (2005) *Semin Cell Dev Biol* **16**, 323-333
22. Deshaies, R. J. (1999) *Annu Rev Cell Dev Biol* **15**, 435-467
23. Fuchs, S. Y., Spiegelman, V. S., and Kumar, K. G. (2004) *Oncogene* **23**, 2028-2036
24. Jiang, J., and Struhl, G. (1998) *Nature* **391**, 493-496
25. Ikram, M. S., Neill, G. W., Regl, G., Eichberger, T., Frischauf, A. M., Aberger, F., Quinn, A., and Philpott, M. (2004) *J Invest Dermatol* **122**, 1503-1509
26. Regl, G., Neill, G. W., Eichberger, T., Kasper, M., Ikram, M. S., Koller, J., Hintner, H., Quinn, A. G., Frischauf, A. M., and Aberger, F. (2002) *Oncogene* **21**, 5529-5539
27. Regl, G., Kasper, M., Schnidar, H., Eichberger, T., Neill, G. W., Philpott, M. P., Esterbauer, H., Hauser-Kronberger, C., Frischauf, A. M., and Aberger, F. (2004) *Cancer Res* **64**, 7724-7731
28. Regl, G., Kasper, M., Schnidar, H., Eichberger, T., Neill, G. W., Ikram, M. S., Quinn, A. G., Philpott, M. P., Frischauf, A. M., and Aberger, F. (2002) *Oncogene* **21**, 5529-5539
29. Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., and Kondoh, H. (1999) *Development* **126**, 3915-3924
30. Fuchs, S. Y., Chen, A., Xiong, Y., Pan, Z. Q., and Ronai, Z. (1999) *Oncogene* **18**, 2039-2046
31. Kumar, K. G., Tang, W., Ravindranath, A. K., Clark, W. A., Croze, E., and Fuchs, S. Y. (2003) *Embo J* **22**, 5480-5490
32. Sasaki, H., Hui, C., Nakafuku, M., and Kondoh, H. (1997) *Development* **124**, 1313-1322
33. Spiegelman, V. S., Tang, W., Katoh, M., Slaga, T. J., and Fuchs, S. Y. (2002) *Oncogene* **21**, 856-860
34. DasGupta, R., and Fuchs, E. (1999) *Development* **126**, 4557-4568
35. Price, M. A., and Kalderon, D. (2002) *Cell* **108**, 823-835
36. Jia, J., Amanai, K., Wang, G., Tang, J., Wang, B., and Jiang, J. (2002) *Nature* **416**, 548-552
37. Riobo, N. A., Lu, K., Ai, X., Haines, G. M., and Emerson, C. P., Jr. (2006) *Proc Natl Acad Sci U S A* **103**, 4505-4510
38. Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M., and Ishii, S. (1999) *J Biol Chem* **274**, 8143-8152
39. Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J., and Hui, C. C. (1998) *Development* **125**, 2533-2543
40. Grachtchouk, M., Mo, R., Yu, S., Zhang, X., Sasaki, H., Hui, C. C., and Dlugosz, A. A. (2000) *Nat Genet* **24**, 216-217

**FOOTNOTES**

* We thank Drs. F. Aberger, P. Coulombe, S.Y. Fuchs, and H. Sasaki for their generous gifts of reagents. This work was supported by American Cancer Society Award RSG-02-140-01-CNE (to V.S.S.).

The abbreviations used are: Hh, Hedgehog; β- TrCP, beta-transducin repeats-containing protein; Ci, Cubitus interruptus; PTCH, Patched; SMO, Smoothed; PKA, protein kinase A; CK1, casein kinase 1; GSK3, glycogen synthase kinase 3.
FIGURE LEGENDS

Figure 1. β-TrCP2 interacts with Gli2, and promotes its ubiquitination in vivo.
A. Conserved β-TrCP recognition motif in Gli2 from different species.
B. 293T cells were co-transfected with plasmids for expression of HA-tagged β-TrCP2 and Flag-tagged Gli2 proteins as indicated. The interaction between the expressed proteins was assessed by immunoprecipitation followed by immunoblotting as indicated. Ig – heavy chain of immunoglobulins. A representative of three independent experiments is shown.
C. The interaction between the endogenous proteins was assessed in the protein extracts from HeLa cell by immunoprecipitation followed by immunoblotting as indicated. A representative of two independent experiments is shown.
D. Binding of in vitro-translated and 35S-labeled β-TrCP2 to Flag-Gli2 proteins expressed in 293T cells and immunopurified with Flag antibody before or after treatment with protein phosphatase λ. Aliquots of reactions were analyzed by autoradiography (upper panel) or immunoblotting with Gli2 antibody (lower panel). Input of radiolabeled β-TrCP2 is also shown. A representative of three independent experiments is shown.
E. In vivo ubiquitination of Flag-Gli2 (wild type or S662A mutant) in 293T cells co-transfected with HA-tagged ubiquitin and β-TrCP2 constructs as indicated. Immunoprecipitation reactions with Flag antibodies were analyzed by means of immunoblotting with HA antibody. Ubiquitinated Flag-Gli2 species (“Gli2~Ub”) are indicated. A representative of two independent experiments is shown.

Figure 2. Inhibition of β-TrCP function stabilizes Gli2 protein.
A. 293T cells were transfected with Flag-Gli2wt in the presence of the indicated shRNA constructs. Pulse chase analysis of Flag-Gli2 degradation in 293T cells, metabolically labeled with 35S-methionine/35S-cysteine. Cells were harvested at different time points of chase with unlabeled methionine and cysteine. Gli2 was immunoprecipitated with Flag antibody and analyzed using autoradiography. A representative of three independent experiments is shown. Data depicting percent of remaining Gli2 (compared to time point “0”) are shown below. Insert shows the levels of β-TrCP1 and β-TrCP2 expression in 293T cells transfected with the indicated shRNA analyzed by immunoblotting.
B. Pulse chase analysis of Flag-Gli2wt protein expressed in 293T cells with or without dominant negative HA-tagged β-TrCP2ΔN mutant and analyzed as in Figure 2A. A representative of two independent experiments is shown. Insert shows the levels of HA-β-TrCP2ΔN expression in 293T cells transfected analyzed by immunoblotting with HA antibody.
C. Pulse chase analysis of endogenous Gli2 protein in 293T cells with or without dominant negative HA-tagged β-TrCP2ΔN mutant. 293T cells were metabolically labeled with 35S-methionine/35S-cysteine. Cells were and harvested at different time points of chase with unlabeled methionine and cysteine. Gli2 was immunoprecipitated with Gli2 antibody (Santa Cruz Biotechnology) and analyzed using autoradiography. A representative of two independent experiments is shown. Insert shows the levels of HA-β-TrCP2ΔN expression in 293T cells transfected analyzed by immunoblotting with HA antibody.
D. Expression of endogenous Gli2 protein in 293T cells transfected with indicated shRNA constructs, analyzed by immunoblotting with Gli2 antibody. A representative of two independent experiments is shown.
E. Pulse chase analysis of Flag-Gli2wt protein expressed in 293T cells treated with LiCl was analyzed as in Figure 2A. Cells were serum starved for 12 hr and then treated with 40 mM LiCl 1 hr prior to the chase. A representative of two independent experiments is shown.

Graph data are mean ± s.d. of 2–4 separate experiments.
Figure 3. Gli2S662A is expressed in higher levels and is more potent in the activation of Gli-dependent transcription than Gli2wt.

A. The indicated Flag-Gli2 proteins (wild type or S662A mutant) were expressed in 293T cells and their degradation analyzed as in Figure 2A. A representative of two independent experiments is shown.

B. Pulse chase analysis of Flag-Gli2S662A protein expressed in 293T cells with or without dominant negative β-TrCP2ΔN mutant and analyzed as in Figure 2A. A representative of two independent experiments is shown. Insert shows the levels of HA-β-TrCP2ΔN expression in 293T cells transfected analyzed by immunoblotting with HA antibody.

C. HeLa cells were transfected with Gli-luciferase (8x3'Gli BS-LucII), pGL3-Bcl2promo luciferase, or K17 luciferase, and respective Gli2 expression plasmids as indicated. Luciferase activity was estimated using Luciferase Reporter Assay Reagent (Promega). β-galactosidase was used for normalization and estimated using β-gal assay reagent (Pierce Biotechnology). * - p<0.01 compared to cells transfected with empty vector (pcDNA3.1); ** - p<0.01 compared to cells transfected with Gli2wt, in Student’s t-test. Insert shows the levels of Flag-Gli2 expression in HeLa cells transfected with Flag-Gli2wt or Flag-Gli2S662A analyzed by immunoblotting with Gli2 G-20 antibody (protein loading was normalized by β-galactosidase activity).

Figure 4. Gli2 is over-expressed in prostate cancer cells

A. Levels of Gli2 expression in prostate cancer cell lines were analyzed using immunoblotting with Gli2 antibody (G20). A representative of two independent experiments is shown.

B. Gli2 mRNA expression was assessed by Real Time RT-PCR. GAPDH was used as an internal control. A representative of three independent experiments is shown.

C. Human prostate cancer tissue array was immunostained with Gli2 (G20) for expression of Gli2 full length protein. A representative picture of prostate hyperplasia, and prostate adenocarcinoma is shown under 400x magnification.
Prostate Hyperplasia  Prostate Adenocarcinoma (Grade III)
GLi2 is targeted for ubiquitination and degradation by β-TrCP ubiquitin ligase
Neehar Bhatia, Saravanan Thiyagarajan, Irina Elcheva, Mohammed Saleem, Andrzej Dlugosz, Hasan Mukhtar and Vladimir S. Spiegelman

J. Biol. Chem. published online May 1, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M513203200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts