Chapter 8

Measuring Expression Levels of Endogenous *Gli* Genes by Immunoblotting and Real-Time PCR

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Abstract

Gli proteins are transcription factors that mediate the transcriptional effects of Hedgehog signaling in vertebrates. The activities of Gli2 and Gli3 are regulated primarily by posttranslational modifications, while Gli1 is mostly regulated at the transcriptional level. Detection of endogenous Gli proteins had been hampered by lack of good antibodies, but this problem has been mostly resolved in recent years. In this chapter we describe methods of detecting expression of endogenous *Gli* genes in whole-cell lysates and in subcellular fractions and also provide protocols for the measurement of Gli mRNA levels by quantitative real-time reverse transcriptase PCR (qPCR).

Key words Gli proteins, Immunoblotting, Real-time RT-PCR, Nuclear localization, Cell fractionation

1 Introduction

Gli proteins are the final effectors of the Hedgehog pathway in vertebrates. They are the homologues of the *Drosophila* protein cubitus interruptus [1]. Of the three mammalian Gli proteins, Gli1 is present at low levels in the absence of signal and its expression at the mRNA and protein level dramatically increases in cells exposed to a Hedgehog agonist. Instead of being regulated at the transcriptional level, Gli2 and Gli3 proteins are regulated by posttranslational modifications and subcellular trafficking [2–6]. Specifically, in the basal state, Gli2 and Gli3 are phosphorylated by protein kinase A at six conserved serine residues P1–P6 and subsequently by glycogen synthase kinase 3β and casein kinase 1 [7, 8]. These phosphorylation events lead to the inhibition of the transcriptional activator function of Gli2/3 [6] and to their processing by the proteasome into truncated repressor forms (Gli2/3R) or their complete proteasomal degradation [7, 9, 10]. When the Hedgehog pathway becomes activated, the phosphorylation of P1–P6 and proteasomal processing of Gli2/3 is abrogated, and instead Gli2/3 become phosphorylated at a distinct set of sites by an unknown kinase,

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an event known as hyperphosphorylation [2–6]. Subsequently, they translocate to the tip of the primary cilium and to the nucleus, where they activate transcription [2–6, 11]. Consequently, while Gli1 expression can serve as a late, transcriptional readout of Hh signaling, Gli2 and Gli3 posttranslational modifications and subcellular localization changes can be detected <1 h after the induction of signaling [2, 3, 6]. In this protocol, we present methods to quantify Gli1 expression and assess the activation state of Gli2/3 using Western blotting.

2 Materials

2.1 Cell Culture Materials

1. NIH/3T3 cells (ATCC).
2. High-serum culture media: DMEM containing 10 % fetal bovine serum (FBS), 1× GlutaMAX, 1× nonessential amino acids, 1× sodium pyruvate, and 1× penicillin/streptomycin solution (all from Life Technologies).
3. Sterile phosphate-buffered saline (PBS), calcium- and magnesium-free.
4. 0.05 % trypsin-EDTA solution (Life Technologies or equivalent).
5. Low-serum culture media: same as high-serum media, except made with 0.5 % FBS instead of 10 %.
6. Cell culture dishes: Nunc Nunclon or equivalent tissue culture-treated 10-cm-diameter round dishes and 24-well plates or equivalent.
7. Bortezomib stock solution: 10-mM bortezomib (LC Laboratories) in DMSO, store at −20 °C.
8. Smoothened agonist (SAG) stock solution: 100-μM SAG (Axxora) in DMSO, store at −20 °C (*see* **Note 1**).

2.2 Materials for RNA Isolation and qPCR

1. RNAse removal solution (RNAseZap from Life Technologies or equivalent).
2. TRIzol (Life Technologies).
3. Chloroform.
4. RNAse-free water.
5. RNAse-free glycogen (UltraPure Glycogen from Life Technologies or equivalent).
6. 70 % ethanol solution in RNAse-free water.
7. 100 % isopropanol.
8. cDNA synthesis kit: iScript Supermix (Bio-Rad) or equivalent.
9. SYBR green qPCR mix: iTaq SYBR green Supermix (Bio-Rad) or equivalent (*see* **Note 2**).

10. qPCR plates and adhesive film (*see* **Note 3**).
11. Refrigerated tabletop microcentrifuge.
12. UV/vis spectrophotometer.
13. Thermocycler.
14. Real-time PCR instrument: Applied Biosystems 7900HT Fast or equivalent.
15. Primer stock solutions: 20-μM solutions of primers in nuclease-free water.

2.3 Materials for Cell Lysis and Protein Isolation

1. 1× PBS, calcium- and magnesium-free—precooled to 4 °C.
2. 10× RIPA salts: 500-mM Tris pH 7.4, 1.5-M NaCl.
3. RIPA buffer: 1× RIPA salts (50-mM Tris pH 7.4, 150-mM NaCl), 2 % Nonidet P-40 (or IGEPAL CA-630), 0.25 % sodium deoxycholate, 1-mM DTT, protease inhibitors (SIGMAFAST no EDTA or equivalent), and phosphatase inhibitors (1-mM NaF, 1-mM activated Na_3VO_4—*see* **Note 4**), 1-μM bortezomib (optional). Make fresh before each experiment.
4. Refrigerated tabletop microcentrifuge.
5. Protein concentration assay kit: Pierce BCA protein assay kit or equivalent (*see* **Note 5**).

2.4 Materials for Electrophoresis and Western Blotting

1. 4× Laemmli sample buffer: ready-made solution (Bio-Rad) or equivalent.
2. 1-M DTT in water: aliquot and store frozen at −20 °C.
3. 8 % SDS-PAGE gels.
4. Protein electrophoresis equipment and buffers—electrophoresis chamber, transfer apparatus, power supply, gel running and transfer buffers, and nitrocellulose.
5. Primary antibodies:
   - Rabbit anti-Gli1 (Cell Signaling, cat. # 2534S—use at 1:500 dilution)
   - Goat anti-Gli3 (R&D Systems, cat. # AF3635—use at 1:200 dilution)
   - Guinea pig anti-Gli2, made in house (*see* **Note 6**—use at 1 μg/mL)
   - Mouse anti-α-tubulin (Sigma, cat. # T-5326—use at 1:10,000 dilution)
   - Rabbit anti-lamin A (Abcam, cat. # ab26300—use at 1:1,000 dilution)
6. Reagents and equipment for chemiluminescence or fluorescence (Li-Cor Odyssey) detection, including appropriate blocking buffer and secondary antibodies.

2.5 Materials for Nuclear/Cytoplasmic Fractionation

1. 1× PBS, calcium- and magnesium-free—precooled to 4 °C.
2. 10× triethanolamine/acetic acid (TEA/AA): mix 1.99-mL TEA, 0.859-mL AA, and 150-mL H_2O, and adjust pH to 7.4 with TEA.
3. 1-M HEPES pH 7.4.
4. Prepare buffers fresh before each experiment:
   - 10-mM HEPES: dilute 1-M HEPES with ice-cold water.
   - SEAT buffer: 1× TEA/AA, 250-mM sucrose, 1× SIGMAFAST protease inhibitors (no EDTA), 1-μM bortezomib, 1-mM NaF, and 1-mM activated Na_3VO_4.
   - 5× lysis buffer: 250-mM Tris, pH 7.4, 1.35-M NaCl, 5 % NP-40, 1-mM DTT, and 1-μM bortezomib.
   - Benzonase buffer: 1× TEA/AA, 250-mM sucrose, 1× SIGMAFAST protease inhibitors (no EDTA), 1-μM bortezomib, 1-mM NaF, 1-mM activated Na_3VO_4, 1-mM $MgCl_2$, and Benzonase (EMD Millipore) 20U/mL.
5. 1-mL syringes and 25-G needles.
6. Cell lifters (Sigma, cat. #CLS3008).
7. Cooled tabletop microcentrifuge.
8. Materials for SDS-PAGE as detailed in Subheading 2.3.

3 Methods

3.1 Cell Culture

1. Culture NIH/3T3 cells in high-serum culture media. Cells should be split before they reach 80 % confluency. Split every 2–3 days at a ratio of 1:5 to 1:8, as appropriate.
2. When the cells are ready to passage, rinse with sterile PBS and cover with 2 mL per 10-cm dish of pre-warmed trypsin-EDTA solution. Incubate at 37 °C for 3–5 min until the cells detach from the surface of the dish. Tap dish on the side to dislodge remaining cells and cover with 8 mL of high-serum media. Count cells or split at a specified ratio.
3. One to two days before the experiment, split cells into dishes or well plates:
   (a) Real-time qPCR—one well from a 24-well culture plate per sample; a well from a 48- or 96-well plate also should suffice, but the quantity of RNA may be very low and RNA isolation may be problematic. Plate at 1.5×10^5 cells per well to obtain confluent cultures in 24–48 h.
   (b) Western blot from whole-cell lysates—one well from a 24-well culture plate. Plate at 1.5×10^5 cells per well to obtain confluent cultures in 24–48 h.

(c) Nuclear/cytoplasmic fractionation—10-cm dish is preferable. Plate at 4×10^6 cells per dish to obtain confluent cultures in 24–48 h.

4. After the cells have reached full confluence (look "overcrowded"), change the media to low-serum culture media for 24–40 h (*see* **Note 7**).
5. Add Hedgehog-modulating drugs as needed for 2–24 h before harvesting (*see* **Note 8**).

3.2 RNA Isolation and qPCR

RNAse contamination must be avoided at all steps. Use dedicated RNAse-free sterile microcentrifuge tubes and pipette tips and RNAse-free solutions. Wipe all work surfaces with RNAseZap or equivalent RNAse removal solution. Always wear fresh powder-free gloves—do not reuse. Avoid touching surfaces that may have had contact with the skin or hair, such as chairs, desks, door handles, etc., with your gloves. All procedures involving TRIzol and chloroform should be performed in a chemical safety cabinet. TRIzol and chloroform leftovers should be disposed as hazardous waste.

1. Rinse cells with ice-cold PBS. Remove PBS carefully.
2. Add 400-μL TRIzol per well in 24-well plates. Pipette up and down several times to thoroughly lyse cells, and leave for 5 min at room temperature.
3. Transfer the lysate to 1.5-mL microcentrifuge tubes. Add 80-μL chloroform.
4. Shake vigorously for 15 s and incubate for 2 min at room temperature.
5. Centrifuge at $12{,}000 \times g$ for 15 min at 4 °C.
6. Remove upper aqueous phase to a separate tube (*see* **Note 9**). Discard the red organic phase and the interface as hazardous waste.
7. Add 5 μg of RNAse-free glycogen to the aqueous layer.
8. Add 200-μL isopropanol and mix by vortexing.
9. Centrifuge at $12{,}000 \times g$ for 15 min at 4 °C.
10. Decant supernatant carefully but thoroughly to avoid dislodging the pellet. Add 1 mL of 70 % ethanol. Mix gently and centrifuge at $7{,}500 \times g$ for 10 min.
11. Decant supernatant and remove remaining droplets by pipetting or aspirating with a capillary tip. Take care to not touch the pellet.
12. Air-dry the pellet (*see* **Note 10**).
13. Dissolve the pellet in 20-μL RNAse-free water by pipetting up and down around 20 times (*see* **Note 11**).

Table 1
Primers for qPCR reactions

Primer	Sequence	Concentration (nM)
GAPDH forward	GGCCTTCCGTGTTCCTAC	100
GAPDH reverse	TGTCATCATACTTGGCAGGTT	100
Gli1 forward	CCAAGCCAACTTTATGTCAGGG	200
Gli1 reverse	AGCCCGCTTCTTTGTTAATTTGA	200
Gli2 forward	GTGCACAGCAGCCCCACACTCTC	50
Gli2 reverse	GGTAATAGTCTGAAGGG TTGGTGCCTGG	50

14. Measure RNA concentration of samples using a UV/vis spectrophotometer.
15. Make cDNA using iScript Supermix according to the manufacturer's instructions. Use 0.5-μg RNA per 20-μL reaction (*see* **Note 12**). Freeze the remaining RNA and store at −80 °C.
16. Perform qPCR according to the instructions of your qPCR instrument. Use the following parameters:
    (a) Primer concentration 50–200 μM (*see* Table 1)
    (b) Annealing temperature 55–60 °C
    (c) Annealing time 30–45 s
    (d) Extension time 10–15 s
    (e) 1 μL of the cDNA reaction/well

3.3 Detection of Endogenous Gli Proteins in Whole-Cell Lysates by Western Blot

1. Precool the centrifuge to 4 °C.
2. Remove media from cells and rinse briefly twice in ice-cold PBS.
3. Add 200-μL ice-cold RIPA buffer per well in a 24-well plate. Pipette up and down several times. Shake plate vigorously at 4 °C for 15–30 min to ensure complete lysis. If using larger culture surfaces, cells can be scraped off in lysis buffer and transferred to 1.5-mL microcentrifuge tubes prior to shaking.
4. Transfer lysate to a 1.5-mL microcentrifuge tube. Centrifuge at 21,000 × *g* for 30 min at 4 °C.
5. Move supernatant to a fresh tube and keep on ice.
6. Measure protein concentration using the protein concentration assay kit.

7. Transfer equal quantities of protein (20–50 μg/sample) to fresh tubes and add 4× sample buffer and DTT to 50 mM.
8. Denature protein by heating at 65 °C for 15 min in a heat block.
9. Centrifuge at room temperature for 5 min at 21,000 × *g*. Load samples on gel.
10. Run on 8 % SDS-PAGE gel at 120 V for approximately 2 h, until the dye front reaches the bottom of the gel. Transfer to nitrocellulose using wet transfer or Trans-Blot Turbo (Bio-Rad; *see* **Note 13**).
11. Detect by Western blot using chemiluminescence or fluorescence (Li-Cor Odyssey). Overnight incubation at 4 °C with primary antibodies is recommended, especially for anti-Gli3. *See* Fig. 1 for example blots (*see* **Note 14**).

3.4 Detection of Gli2/3 Nuclear Translocation and Hyperphosphorylation by Subcellular Fractionation

Perform all manipulations on ice or in the cold room at 4 °C.

1. Precool the centrifuge to 4 °C.
2. Remove media from a 10-cm dish of cells and rinse briefly twice in ice-cold PBS. Remove PBS carefully.
3. Rinse cells in ice-cold 10-mM HEPES pH 7.4. Remove the solution carefully.
4. Cover cells with ice-cold 10-mM HEPES pH 7.4 and leave on ice for 10 min.

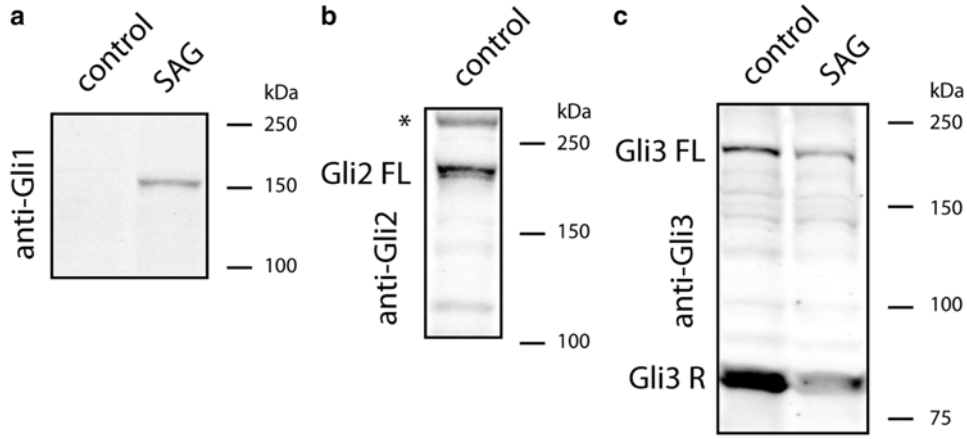

Fig. 1 Detection of endogenous Gli proteins in whole-cell lysates by immunoblotting. IrDye-coupled secondary antibodies were used, and the membranes were scanned using the Li-Cor Odyssey system. (**a**) NIH/3T3 cells were either left untreated or treated for 12 h with 100 nM SAG. Gli1 levels were measured by anti-Gli1 immunoblotting. (**b**) Anti-Gli2 immunoblot of NIH/3T3 lysate. *nonspecific band; Gli2 FL, full-length Gli2. Gli2 repressor is not detected by our antibody. (**c**) NIH/3T3 cells were treated as in **a**. Anti-Gli3 immunoblot of whole-cell lysate is shown. Gli3 FL, full-length Gli3; Gli3 R, truncated repressor form of Gli3. Please note reduced levels of Gli3 R in cells treated with SAG

5. Carefully remove all of the HEPES solution.
6. Add 500 μL of ice-cold SEAT buffer. Swirl the plate to distribute the SEAT solution over the cells.
7. Scrape cells using a cell lifter and transfer into 1.5-mL microcentrifuge tube.
8. Bring the volume up to 750 μL with SEAT buffer.
9. Shear cells with a syringe attached to a 25-G needle. For each sample make 15 up-and-down strokes. Take care to not introduce bubbles.
10. Centrifuge at 900 × *g* for 5 min. at 4 °C.
11. Transfer supernatant (600 μL) to a fresh tube and place on ice (cytoplasmic or "C" fraction). Resuspend pellet (nuclear or "N" fraction) in 1 mL of SEAT buffer by pipetting up and down with a 1-mL tip.
12. Centrifuge both C and N fractions at 900 × *g* for 5 min. at 4 °C.
13. For the N fraction:
    (a) Remove supernatant carefully without disturbing the pellet. Resuspend the pellet in 550 μL of Benzonase buffer and incubate on ice for 10 min.
    (b) Add 137.5 μL of 5× lysis buffer and pipette up and down to mix.
14. For the C fraction.
    (a) Transfer supernatant (550 μL) to a new tube. Discard the pellet.
    (b) Add 137.5 μL of 5× lysis buffer and pipette up and down to mix.
15. Lyse both fractions with shaking or rotation at 4 °C for 45 min.
16. Centrifuge at 21,000 × *g* for 30 min at 4 °C. Transfer supernatants to new tubes.
17. Optional—to increase sample concentration precipitate sample by the chloroform/methanol method or the trichloroacetic acid method. Resuspend the pellet in 1× SDS sample buffer + 50-mM DTT for 30 min. at 37 °C with vigorous mixing.
18. Transfer 70 μL of each fraction into a separate tube. Add 25 μL of 4× Laemmli sample buffer and 5 μL of 1-M DTT. Denature protein at 65 °C for 15 min in a heat block (*see* **Note 15**).
19. Detect Gli proteins by electrophoresis/immunoblotting as in Subheading 3.3 (*see* **Note 16**). To control for efficiency of separation between fractions, use anti-α-tubulin (cytoplasmic) and anti-lamin A (nuclear) antibodies as loading controls. An example immunoblot is shown in Fig. 2.

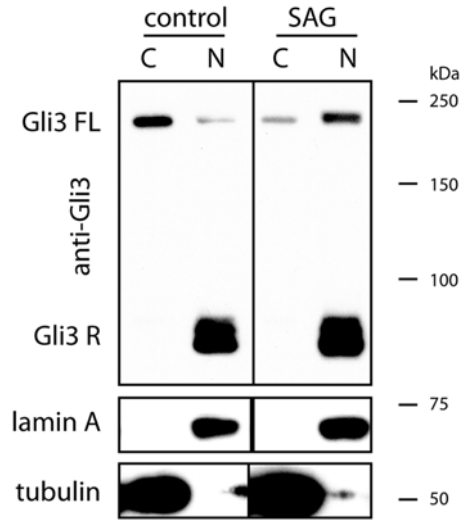

Fig. 2 Nuclear translocation of Gli3 upon Hh pathway activation. NIH/3T3 cells were treated for 2 h with 100-nM SAG and subjected to subcellular fractionation. Cytoplasmic and nuclear fractions were resolved by electrophoresis and immunoblotted with anti-Gli3, anti-lamin A, and anti-tubulin. HRP-coupled secondary antibodies and chemiluminescence-based detection were used. Please note the increased amount of full-length Gli3 (Gli3 FL) in the nuclear fraction of treated cells. Gli3 in the nuclear fraction of SAG-treated cells runs slightly higher on gel than in untreated cells, which reflects hyperphosphorylation of activated Gli3. Gli3 repressor (Gli3 R) is constitutively located at the nucleus. Lamin A was used as a nuclear marker and tubulin as cytoplasmic marker

4 Notes

1. SAG has a tendency to precipitate in aqueous solutions. When adding it to the cell culture, first dilute the stock solution in 1–2 mL of media and immediately vortex for 5–10 s, and then add the diluted solution to a dish.
2. Real-time qPCR master mix must be compatible with the available qPCR instrument. Check compatibility with your instrument on the manufacturer's website. We used iTaq SYBR Green Supermix with ROX (Bio-Rad, cat. #172-5851) with the Applied Biosystems 7900HT Fast system.
3. Plates and adhesive film must be tested for compatibility with your qPCR instrument. Contact the manufacturer for a list of compatible consumables. We used 384-well clear optical reaction plates (Applied Biosystems, cat. #4309849) and optical adhesive film (Applied Biosystems, cat. #4311971) with the Applied Biosystems 7900HT Fast equipped with a 384-well plate adapter. However, many systems are only compatible with 96-well plates.

4. Sodium orthovanadate must be activated prior to use. Prepare a 200-mM solution and adjust the pH to 10.0. Boil the solution until it loses its yellow color. Cool to room temperature. Readjust the pH to 10.0 and repeat until the solution remains colorless and the pH stabilizes at 10.0. Store aliquoted at –20 °C.
5. The BCA assay is sensitive to the presence of reducing agents such as DTT in the sample. The lysis buffer contains 1-mM DTT. To avoid errors in protein concentration measurement, add to all standard curve samples (including the negative control) a volume of lysis buffer equal to the volume of sample used for protein concentration measurement. For instance, if you add 2 μL of sample to each assay, you need to add 2 μL of lysis buffer to each of the standard curve assays and the negative control assay. If protein concentration cannot be measured immediately after lysis, the leftover lysis buffer should be stored with the samples and used in the protein concentration assay to account for DTT degradation over time.
6. The anti-Gli2 antibody we use for immunoblotting of Gli2 was custom made in guinea pigs based on Cho et al. [12]. The antigen was a his-tagged fragment containing amino acids 1053–1264 of mouse Gli2 (VQYIKAH… AKPSHLG), and the sera were affinity purified on amino-link beads coupled to the same antigen.
7. Longer serum starvation times accelerate the response and are therefore preferable in experiments where shorter drug treatments are applied (nuclear/cytoplasmic fractionation, qPCR). For 24-h treatments, serum starvation may be started at the same time as treatment.
8. Nuclear translocation and hyperphosphorylation of Gli2/3 becomes visible after 1–3 h of treatment with 100-nM SAG. Longer treatment times result in degradation of full-length Gli3 [2]. After 2–3 h of treatment, a clear increase in Gli1 mRNA becomes apparent in qPCR assays. Gli1 protein levels rise after 6–8 h of SAG treatment.
9. Take care not to touch the interface, which contains genomic DNA. Around 180 μL of aqueous phase can be withdrawn relatively safely without touching the interface.
10. The pellet should become transparent as it dries. Dissolve the pellet in water as soon as it becomes fully transparent. Do not allow the pellet to dry for too long, since it becomes more difficult to resuspend when completely dry.
11. Do not use DEPC-treated water for the resuspension of RNA pellet, since ethanol traces in the solution may interfere with subsequent reactions.
12. Using 1 μg of RNA per reaction, as recommended by the manufacturer, may result in nonlinear amplification results for the most highly expressed genes, such as GAPDH.

13. Standard-voltage semidry transfer results in poor transfer of high molecular weight proteins, such as Gli proteins. High-voltage semidry transfer (Trans-Blot Turbo) or wet transfer results in significantly better Gli protein signal on the membrane. Nitrocellulose and PVDF can both be used as membrane material, but PVDF gives high background when fluorescence scanner (Li-Cor Odyssey) is used for the detection.
14. Gli1 can be detected on the same membrane as Gli2 or Gli3 due to differences in the apparent molecular weight (Gli1 ~ 150 kDa, Gli2 and Gli3 full-length ~200 kDa, Gli3 repressor ~100 kDa). Cut the membrane into strips at 165 kDa and 120 kDa and blot the top and bottom strip with anti-Gli2 or anti-Gli3 and the middle strip with anti-Gli1. Anti-Gli2 was developed against the C-terminal part of the full-length protein and will not detect Gli2 repressor.
15. Do NOT attempt to normalize sample volumes by measuring protein concentrations. Cytoplasmic and nuclear fractions naturally have different concentrations of proteins. The method relies on careful manipulation to ensure that sample loss throughout the procedure is minimal and consistent between samples and fractions, so different samples can typically be directly compared to one another. Cytoplasmic and nuclear levels of protein can be normalized after immunoblotting to levels of loading control proteins: tubulin for cytoplasmic and lamin A for nuclear fraction.
16. After nuclear/cytoplasmic fractionation, the samples are relatively dilute. To enhance detection of low abundance proteins, such as Glis, it is recommended to concentrate the sample by precipitation. Precipitation has the added advantage of desalting the sample and making it run somewhat better on gel. High-sensitivity chemiluminescence reagents (SuperSignal West Femto—Pierce or VisiGlo Select—Amresco) may need to be used to detect Gli2/3 in dilute samples. Alternatively, fluorescence detection using the Li-Cor Odyssey system has proved in our hands to have sufficient sensitivity to detect Gli proteins, even in nuclear translocation assays.

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