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Several *PATCHED1* Missense Mutations Display Activity in *patched1*-Deficient Fibroblasts*

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Mutations in mouse and human *patched1* (*ptc1*) genes are associated with birth defects and cancer. Ptc1 is a receptor for Hedgehog (Hh) signaling proteins. Hh proteins activate transcription of target genes, including *ptc1,* and Ptc1 represses those genes, both by regulating the activity of Gli transcription factors. We have established mammalian cell lines with reduced Ptc1 function and a *lacZ* reporter to investigate Hh signal transduction. Embryonic fibroblasts were derived from mice, heterozygous or homozygous for a *ptc1* mutation that inserts *lacZ* under the control of the *ptc1* promoter (*ptc1-lacZ*). In heterozygous *ptc1* cells, *ptc1-lacZ* was expressed at low levels but could be induced by Sonic Hedgehog (Shh) and Gli-1. Homozygous *ptc1* cells expressed high levels of *ptc1-lacZ* without Hh stimulation. *ptc1-lacZ* expression was dependent on cell density in *ptc1* homozygotes and Hh-stimulated heterozygotes but was independent of density when Gli1 was used to activate *ptc1-lacZ*. A wild-type *ptc1* transgene introduced into homozygous *ptc1* cells greatly reduced *ptc1-lacZ* expression. Expression of either half of Ptc1 alone resulted in improper maturation of the protein and a failure to complement the $ptc1^{-/-}$ cells. When co-expressed, both Ptc1 halves matured and had an activity similar to that of the intact protein. Three missense *PTCH1* mutations exhibited significant functions in homozygous *ptc1* cells. The missense mutants retained activity when expressed at about 10-fold lower levels and appeared as stable as wild-type Ptc1. These studies suggest that some tumors and disease phenotypes may arise from small reductions in PTCH1 activity.

The *patched* gene (*ptc*) controls cell growth and specification in the developing and postnatal tissues of many animals (reviewed in Refs. 1 and 2). The basal cell nevus syndrome (BCNS)[1] is associated with mutations in a human *ptc* homolog, *PTCH1* (3, 4). BCNS individuals who are $PTCH1^{+/-}$ have developmental malformations such as polydactlyly and spina bifida and develop a variety of tumors including basal cell carcinoma (BCC) and medulloblastoma (5). *PTCH1* mutations also arise at high frequency in sporadic BCCs (6) and medulloblastomas from normal individuals (7–9). In human skin, *PTCH1* is thought to behave as a tumor suppressor gene where tumors arise when both copies of the gene are mutated (6, 8, 9). In mice, however, medulloblastomas can arise by haploinsufficiency of *ptc1* (10, 11).

ptc is a critical regulator of signaling by the Hh family of secreted proteins (reviewed in Refs. 12–14). In mice, three *hh* homologs have been identified, of which *Shh* has been best characterized (15, 16). *ptc* encodes a multiple transmembrane-domain protein (17–19) and is a receptor for Hh proteins (20–22). In the absence of ligand, Ptc blocks the transcription of specific genes. In vertebrates such targets include the signaling protein *BMP2* (23, 24), the transcription factor *Gli1* (25, 26), and the *ptc* gene itself (19, 20, 27). Upon reception of a Hh signal, Ptc activity is blocked, thereby relieving the inhibition of target gene transcription. Ptc accomplishes its repressive effects by somehow inactivating Smoothened (Smo), a seven transmembrane domain protein required for Hh signaling (28, 29).

In vertebrates, the transcription of many Hh-regulated targets is controlled through the activity of the *Gli* genes. *Gli* family members encode zinc finger transcription factors (30, 31) and are homologous to *Cubitus interruptus* in *Drosophila* (32). Gli proteins appear to mediate their effects directly. Gli consensus-binding sites have been identified in the promoter sequences of *HNF3β* (33) in mice and *ptc* in *Drosophila* (34). Furthermore, these binding sites are required for induction by Hh ligands. Gli proteins have different effects on Hh-regulated genes. In mice, Gli1 and Gli2 are transcriptional activators, whereas Gli3 is a repressor of Hh targets (35–37). The transcription of *Gli* genes is also regulated, with Shh inducing *Gli1* and *Gli2* and repressing *Gli3* (25, 38).

ptc1-deficient mice illustrate the role of this gene in proper development and cell division. *ptc1* null mutations cause multiple abnormalities in the developing limb, gut, and nervous system and are lethal at embryonic day 9 (39, 40). Mice heterozygous for *ptc1* are viable but develop a similar spectrum of tumors as BCNS patients, including BCC-like lesions, medulloblastoma, and rhabdomyosarcoma (39–41). Both BCNS patients and mice heterozygous for *ptc1* have generalized over-

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[1] The abbreviations used are: BCNS, basal cell nevus syndrome; BCC, basal cell carcinoma; BFA, Brefeldin A; CBP, CREB-binding protein; Endo H, endoglycosidase H; FACS, fluorescence activated cell sorting; GFP, green fluorescent protein; HA, hemagglutinin; Hh, Hedgehog; IRES, internal ribosome entry site; LTR, long terminal repeat; MSCV, murine stem cell leukemia virus; PNGase F, peptide-N[4]-(acetyl-*beta*-glucosaminyl)-asparagine amidase; Ptc1 or PTCH1, Patched1; Shh, Sonic Hedgehog; Smo, Smoothened; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; DMEM, Dulbecco's modified Eagle's medium; RIPA, radioimmune precipitation buffer.

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growth as seen by macrocephaly and a large body size (5, 39, 40). A decrease in body size of mice correlates with an increase of *ptc1* expression (42).

Despite its critical role as a regulator of the Hh signals, little is known about how PTCH1 controls signaling or how *PTCH1* mutations cause tumors. Numerous truncation and missense mutations in *PTCH1* have been identified, and these mutations are dispersed over much of the coding sequence (reviewed in Ref. 66). To understand how human *PTCH1* mutations alter Ptc1 function, we established a Hh- and Ptc1-responsive cell culture system. Cell lines were derived from embryonic fibroblasts from heterozygous and homozygous *ptc1* mice in which part of the *ptc1* gene was deleted and replaced with the *lacZ* gene (40). In these cells, *lacZ* transcription is controlled by the endogenous *ptc1* promoter (*ptc1-lacZ*) and is regulated by Ptc1 activity. Here we show that $ptc1^{-/-}$ cells have strongly increased the transcription of Hh-regulated genes. Expression of a wild-type *ptc1* transgene complemented the expression of *ptc1-lacZ*, but *ptc1* transgenes containing *PTCH1* missense mutations retained significant activity. These data suggest that small reductions of PTCH1 activity may lead to tumor formation and developmental abnormalities.

EXPERIMENTAL PROCEDURES

Cell Establishment and Maintenance—Embryonic fibroblasts were generated from $ptc1^{+/-}$ and $ptc1^{-/-}$ embryos at embryonic day 8.5 (40). Dissected embryos were minced, trypsinized, and individually plated on 24-well plates. Cells were grown in high glucose DMEM with 15% fetal calf serum, L-glutamine, 0.1 mM β-mercaptoethanol, 1× non-essential amino acids (Mediatech), and 1 mM pyruvate in a humidified incubator with 5% CO_2. The cells were genotyped by PCR as described (40). Viral supernatants were isolated following transient transfection of Bosc 23 cells with either the MSCV retroviral plasmid alone (43) or in combination with pVPack-GP and pVPack-Eco plasmids (Stratagene) to increase viral titers. Transduced cells were selected for 10–14 days in media containing 2 μg/ml puromycin. Because of the tendency of confluent cells to detach from the surface, the media were not changed during the experiments.

β-Galactosidase Activity Determination—For *in situ* staining, cells were grown on cover slips, fixed for 15 min in 1% glutaraldehyde in phosphate-buffered saline, and stained for 24 h at 37 °C with either 0.04% ($ptc1^{-/-}$ cells) or 0.16% X-gal ($ptc1^{+/-}$ cells). For enzymatic assays following 5–8 days of culture, cells were first plated at 2.1×10^4 cells/cm^2 (about 30% confluent) in 35-mm dishes. Then at different time points cells were trypsinized, counted, and lysed in 0.4 ml β-galactosidase lysis buffer (Promega). 50 μl of cell extracts were assayed after 45 min ($ptc1^{-/-}$ cells) or 24 h ($ptc1^{+/-}$ cells) at 37 °C. For enzymatic assays following 1 day of culture, cells were plated at either 2.1×10^4 cells/cm^2 in 35-mm dishes or 8.4×10^4 cells/cm^2 (about 100% confluent) in 24-well plates, and 24 h later they were trypsinized, resuspended in 0.25 ml of β-galactosidase lysis buffer, and 50 μl of cell extracts were assayed for 24 h at 37 °C. β-galactosidase activity was calculated by (OD420)/(μg of protein) (extinction coefficient of 2×10^4) and was normalized to total cellular protein using Bio-Rad protein assay reagent. For each time point, duplicate wells were plated, and each well was assayed in triplicate. Values shown are averages of these six values with the associated experimental error.

Shh Induction—To make conditioned media, Bosc 23 cells were transfected with either Shh-N (44) or a control vector, and after 60 h the supernatants were isolated and filtered through a 0.45-μm filter. To induce *ptc-lacZ,* cells were plated at 8.4×10^4 cells/cm^2, the media was replaced 2 h later with control or Shh-N supernatants, and the cells were assayed 48 h later.

Vectors—A hemagglutinin (HA) epitope was incorporated at the C-terminal of a mouse *ptc1* cDNA (19) using PCR, then restriction-digested to remove part of the epitope including the stop codon, and ligated in-frame into a vector containing a C-terminal triple HA epitope (a gift from D. Kingsley). This tagged form of Ptc was detected by anti-HA antibodies and was fully functional. Full-length cDNAs for mouse *ptc1*, human *Gli1* (30), and mouse *Shh* (15) were cloned into MSCV-pac (45). MSCV-IRES-enhanced GFP (a gift from C. Klug) was modified by cloning pGK-puromycin from MSCV-pac distal to IRES-GFP. All *ptc1* constructs were cloned proximal to IRES-GFP. Site-directed mutants of *ptc1* were made using QuikChange™ (Stratagene), and two independent clones were analyzed for each mutant. To produce the N- and C-terminal halves of mouse *ptc1*, full-length *ptc1* was digested with *Bgl*II. The N-terminal half was ligated in-frame into a vector containing a C-terminal triple HA epitope, and the C-terminal half was ligated in-frame in a vector containing an N-terminal triple HA epitope (a gift from D. Kingsley). The *ptc1* halves were then separately cloned into MSCV.

mRNA Analysis—$ptc1^{+/-}$ and $ptc1^{-/-}$ cells were plated at 6.7×10^3 cells/cm^2 in 225 cm^2 flasks. After 7.5 days, β-galactosidase levels were measured, and total RNA was isolated using acid-guanidinium (46). Poly(A) mRNA was isolated from total RNA using PolyATract (Promega). *lacZ* mRNA was detected using 10 μg of total RNA, whereas all other transcripts were detected using 1 μg of poly(A) mRNA. Full-length mouse cDNA probes were used unless otherwise stated: *ptc1* (19), *smo* (22), *lacZ* (47), *Hip* (48), *Shh* (15), 1.7 kb of *Gli1*, 1.0 kb of *Gli2*, 0.8 kb of *Gli3* (49), 0.6 kb of *ptc2* (a gift of C.C. Hui), and a 0.9-kb β-actin probe amplified from mouse genomic DNA with the following primers: CATTGCTCGAGGTGACGAGGCCCAGAGCAAGAG and CAAGTCTCGAGAGGGGCCGGACTCATCGTACTC.

Immunoblot and Glycosidase Treatment—Cells were lysed in RIPA buffer with Complete protease inhibitors (Roche) for 45 min on ice. For glycosidase treatment, Nonidet P-40 was added to the lysates to a 1.5% final concentration and then incubated for 45 min at 37 °C with either 1000 units of Endo H_f or 500 units of PNGase F (New England Biolabs) in a 50-μl volume. Lysates were solubilized in an equal volume of 2× Laemmeli buffer at room temperature, size-fractionated on 7.5% polyacrylamide gels, and transferred to Protran (Schleicher & Schuell). The transfers were incubated with HA.11 (Convance), GM130 (Transduction Laboratories), or actin (Sigma) antibodies and visualized using ECL reagent (Amersham Biosciences).

Fluorescence-activated Cell Sorting (FACS) Analysis—Cells were sorted on a FACSVantage SE (BD Pharmingen), and spectra were collected and analyzed using Cell Quest, version 3.2. At least 1×10^5 cells were isolated for each GFP level and were cultured for analysis. After sorting, cells were maintained in media containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1 mg/ml amphotericin B. Each cell line was analyzed by FACS before and after the complementation assays to ensure that the GFP distribution remained unchanged during the course of the experiment.

Metabolic Labeling and Immunoprecipitation—Cells were plated at 8×10^5 cells per 6-cm plate, cultured for 24 h, grown in high glucose DMEM lacking cysteine and methionine (−cys/met) for 30 min, and incubated with 0.16 mCi/ml [^{35}S]methionine and [^{35}S]cysteine (TRAN^{35}S LABEL, ICN) in DMEM−cys/met for 75 min. Cells were washed once and incubated in normal medium for varying times. For brefeldin A treatment, cells were incubated with 20 μg/ml brefeldin A (Sigma) during both the starvation and label period.

At each time point, cells were washed with phosphate-buffered saline, scraped into PBS plus Complete, washed again, and lysed in 0.7 ml of RIPA plus Complete for 15 min on ice. At 4 °C, the extracts were pelleted at 16,000 × *g*, and the supernatant was precleared with 50 μl of Pansorbin (Calbiochem) for 10 min and immunoprecipitated for 1 h with 5 μg of mouse anti-HA (Convance) or an isotype-matched control antibody. 30 μl of protein-G agarose (Roche) was added for 30 min, and the immunoprecipitates were washed twice each with RIPA plus 5% bovine serum albumin and Complete, RIPA plus 500 mM NaCl, and RIPA. The immunoprecipitates were solubilized in 20 μl of 2× Laemmeli buffer, heated at 37 °C for 5 min, and resolved on an 8% polyacrylamide gel. Gels were fixed, dried, and exposed to a phosphorimager screen (Amersham Biosciences). Decay curves were calculated using ImageQuant (Amersham Biosciences). Experiments were performed three times for wild-type Ptc1 and G495V and twice for all other constructs.

RESULTS

Isolation of $ptc1^{+/-}$ and $ptc1^{-/-}$ Cells—Cell lines were derived from mice in which part of the *ptc1* gene was deleted and replaced with a *lacZ* gene (*ptc1-lacZ*) (40). As a result, Ptc1 function is eliminated and *lacZ* is placed under the control of the endogenous *ptc1* promoter. In these mice, the expression pattern of *ptc1-lacZ,* as determined by β-galactosidase staining, reflects endogenous *ptc1* transcription (40). In heterozygous mice, Ptc1 inhibits *ptc1-lacZ* transcription except in areas adjacent to sources of Hh where inactivation of Ptc1 by Hh permits β-galactosidase accumulation. Homozygous null *ptc1* mice die at embryonic day 9. At this stage, the absence of Ptc1

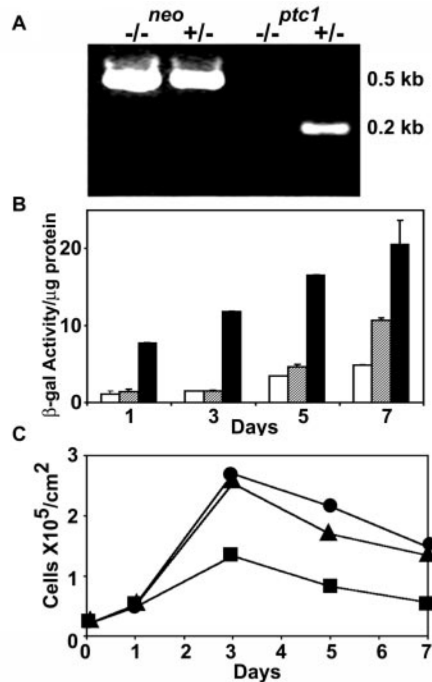

FIG. 1. ***Shh* or *Gli1* stimulates β-galactosidase activity in the *ptc1* heterozygous cells.** *A*, genomic DNA was isolated from $ptc1^{+/-}$ and $ptc1^{-/-}$ cells and PCR amplified to detect the wild-type *ptc1* allele and neomycin (*neo*) in the gene-targeted *ptc1* allele. *B*, MSCV infection of the $ptc1^{+/-}$ cells (*open bars*) did not induce *ptc1-lacZ*, whereas *Shh* (*hatched bars*) or *Gli1* (*filled bars*) infection did. Induction of β-galactosidase by *Shh*, but not *Gli1,* required culturing at high densities. *C*, *Gli1* (*circles*) and *Shh* (*triangles*) infected $ptc1^{+/-}$ cells grew faster initially compared with MSCV infected cells (*squares*), but all declined in number after day 3.

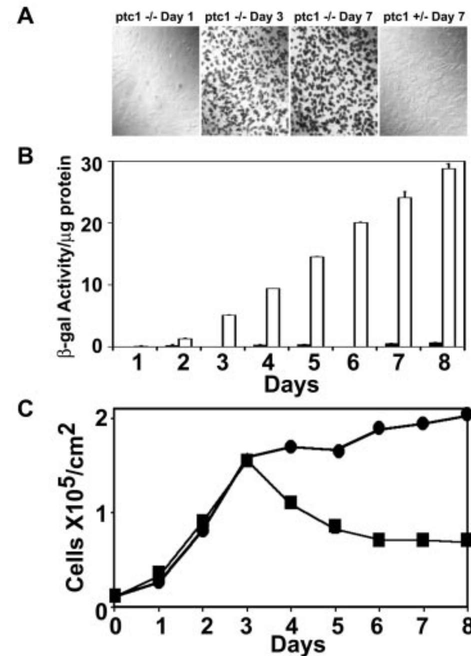

FIG. 2. **Homozygous and heterozygous *ptc1* cells require culturing at high densities to activate *ptc1-lacZ*.** X-gal staining of fixed cells (*A*) and enzymatic measurement of β-galactosidase activity (*B*) show that $ptc1^{-/-}$ cells did not induce high levels of β-galactosidase activity until reaching high densities on day 3 and after. *B*, a linear increase of enzymatic activity was detected over time in the $ptc1^{-/-}$ cells (*open bars*) but not in the $ptc1^{+/-}$ cells (*filled bars*). *C*, the $ptc1^{-/-}$ cells (*circles*) increased in number after reaching confluence, whereas the $ptc1^{+/-}$ cells (*squares*) declined.

function allows persistent Hh target gene transcription, and β-galactosidase staining is detected in all tissues except the endoderm (40). Heterozygous and homozygous *ptc1* cells were obtained from a single litter of embryos from interbred heterozygous *ptc1* mice. The cells were genotyped by PCR for the presence of the *neomycin* transgene and for the presence or absence of the wild-type allele of *ptc1* (Fig. 1*A*).

$ptc1^{+/-}$ Cells Respond to Shh and Gli1—In heterozygous *ptc1* cells, Ptc1 inhibits the transcription of Hh-activated genes (40). Stimulation of this pathway, either by ligand inactivation of Ptc1 or overexpression of Gli1, should induce *ptc1-lacZ* transcription in these cells. To test this idea, we expressed Shh or Gli1 under the control of a retroviral LTR using the MSCV retrovirus (45). $ptc1^{+/-}$ cells were infected with MSCV, MSCV-*Shh* (Shh cells), or MSCV-*Gli1* (Gli1 cells), and stable integrants were selected for resistance to puromycin. Cells were plated at low density (~30% confluence), and β-galactosidase enzymatic activity was determined over a course of 7 days (Fig. 1*B*).

Gli1 expression immediately induced *ptc1-lacZ* in the heterozygous *ptc1* cells even prior to plating (data not shown), whereas the response by Shh was slower and less robust. After the first day of plating, the Gli1 cells had approximately 6× the β-galactosidase activity of the MSCV and Shh cells (Fig. 1*B*). By day five, the Shh cells began to accumulate β-galactosidase activity, and by day seven, the Shh and Gli1 cells had 2 and 4× higher levels of *ptc1-lacZ*, respectively, than the MSCV cells. Shh and Gli1 cells grew to twice the density of the MCSV cells, suggesting that pathway activation leads to increased cell division. After reaching their highest density, however, all three types of cells suffered similar declines in cell number (Fig. 1*C*). Stimulation of signaling by a constitutively active form of Smoothened, Smo-M2 (50), also induced *ptc1-lacZ* in $ptc1^{+/-}$ cells (data not shown).

$ptc1^{-/-}$ Cells Express High Levels of ptc1-lacZ in a Density-dependent Manner—Unlike $ptc1^{+/-}$ cells, $ptc1^{-/-}$ cells lack functional Ptc1 and should transcribe *ptc1-lacZ* in the absence of Shh. We compared *ptc1-lacZ* expression in the $ptc1^{+/-}$ and $ptc1^{-/-}$ cells by plating cells at low density and assaying enzymatic activity over 8 days. Both cell types had little enzymatic activity during the first 2 days. However, by day three the cells reached confluence, and β-galactosidase activity increased in the $ptc1^{-/-}$ cells (Fig. 2, *A–C*). Eight days after plating, the $ptc1^{-/-}$ cells showed a 40-fold increase over the $ptc1^{+/-}$ cells (Fig. 2*B*). Histochemical staining of fixed cells showed that after 7 days of plating, all $ptc1^{-/-}$ cells were *lacZ*-positive, whereas all $ptc1^{+/-}$ cells were negative (Fig. 2*A*).

The homozygous *ptc1* cells differed from stimulated heterozygous *ptc1* cells in two ways: they expressed *ptc1-lacZ* at much higher levels and remained viable after reaching confluence. After a week of growth, the $ptc1^{-/-}$ cells had 28- and 19-fold higher enzymatic activity than similarly plated $ptc1^{+/-}$ cells expressing either Shh or Gli1, respectively (data not shown). With respect to confluence, the density of the $ptc1^{-/-}$ cells continued to increase even after reaching confluence (Fig. 2*C*), whereas the $ptc1^{+/-}$ cells decreased in number, irrespective of Shh or Gli1 expression (Figs. 1*C* and 2*C*). The homozygous *ptc1* cells grew to even higher densities if the culture medium were replenished after confluence, whereas the heterozygous *ptc1* cells did not (data not shown). This suggests that the $ptc1^{-/-}$ cells are not contact-inhibited.

Shh-expressing $ptc1^{+/-}$ constitutively express Shh and should respond by always expressing *ptc1-lacZ*, as should $ptc1^{-/-}$ cells, which lack Ptc1 activity. However, both $ptc1^{-/-}$ and Shh-stimulated $ptc1^{+/-}$ cells required an additional condition, culturing at high density, before they expressed *ptc1-lacZ*.

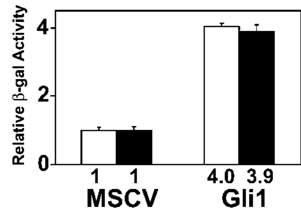

FIG. 3. **Gli1 induces *ptc1-lacZ* in subconfluent homozygous and heterozygous *ptc1* cells.** Subconfluent $ptc1^{+/-}$ (*open bars*) and $ptc1^{-/-}$ cells (*solid bars*) infected with MSCV had relatively low β-galactosidase activity, whereas infection with Gli1 increased levels. *Numbers beneath bars* indicate fold levels relative to MSCV cells.

In contrast, $ptc1^{+/-}$ cells that expressed Gli1 induced *ptc1-lacZ* at low densities (Fig. 1, *B* and *C*). The ability of Gli1 to induce a Hh target gene in subconfluent *ptc1* heterozygous cells prompted us to see if Gli1 would have the same effect in *ptc1* null cells. Both $ptc1^{+/-}$ and $ptc1^{-/-}$ cells were stably infected with either MSCV or MSCV-*Gli1* retrovirus and assayed for *ptc1-lacZ* activity after 24 h of culture at low cell densities. Under these conditions, the $ptc1^{+/-}$ and $ptc1^{-/-}$ cells infected with MSCV alone had low levels of activity, whereas both cell types transduced with *Gli1* had ~4-fold higher levels (Fig. 3). Hence, in both heterozygous and homozygous *ptc1* cells, Gli-1 can bypass the need for high densities to induce *ptc1-lacZ* expression.

Transcriptional Regulation of Hh Components in $ptc1^{+/-}$ and $ptc1^{-/-}$ Cells—In the $ptc1^{-/-}$ cells, the increase in β-galactosidase activity over time could be caused by the accumulation of *ptc1-lacZ* transcripts or by increased translation of previously transcribed mRNA. To distinguish between these possibilities, we examined *ptc1-lacZ* expression in $ptc1^{-/-}$ and $ptc1^{+/-}$ cells that were cultured at high density (~100% confluence) for either 12 h or 6 days. The *ptc1-lacZ* transcript could not be detected in the $ptc1^{+/-}$ cells isolated at either time point. In the $ptc1^{-/-}$ cells, a 4-kb transcript present at low levels initially was induced about 10-fold on day six (Fig. 4*A*). The blot was subsequently probed with β-actin (Fig. 4*A*) and glyceraldehyde-3-phosphate dehydrogenase (data not shown), and both transcripts decreased after 6 days in both cell types. Transcripts for two other Hh pathway components, CREB-binding protein (CBP) and *smo*, were unchanged by culturing at high density in both cell types (data not shown). These data indicate that the induction of *ptc1-lacZ* in homozygous *ptc1* cells in response to culturing at high density is specific and not a general transcriptional property in these cells.

The transcriptional regulation of other Hh signaling components was examined in $ptc1^{-/-}$ and $ptc1^{+/-}$ cells. Cells were cultured for 7.5 days and used as a source of mRNA to determine the expression of *Shh*, *smo*, and the three *Gli* genes (Fig. 4*B*). Neither the $ptc1^{+/-}$ nor $ptc1^{-/-}$ cells expressed *Shh* (data not shown), but both expressed similar levels of *smo*. *Gli1* and *Gli2* were expressed in the $ptc1^{-/-}$ cells, but only *Gli2* was detected in the $ptc1^{+/-}$ cells, at about 4-fold lower levels. *Gli3* was regulated in the opposite manner, with 3-fold lower expression in the $ptc1^{-/-}$ cells compared with the $ptc1^{+/-}$ cells. The Hh receptors, *Hip* (48) and *ptc2* (51–53), were detectable in the $ptc1^{-/-}$ cells but not in the $ptc1^{+/-}$ cells (Fig. 4*B*). However, *ptc2* expression was detected in the $ptc1^{+/-}$ cells by RT-PCR, suggesting that this mRNA is present at very low levels (data not shown). These Shh targets are regulated in the *ptc1* cells like they are *in vivo*, indicating that multiple aspects of Hh signaling are reproduced in this cell culture model.

Complementation of the $ptc1^{-/-}$ Cells by a ptc1 Transgene—To test whether a *ptc1* transgene can reduce the high levels of *ptc1-lacZ* in the $ptc1^{-/-}$ cells, mouse Ptc1 containing

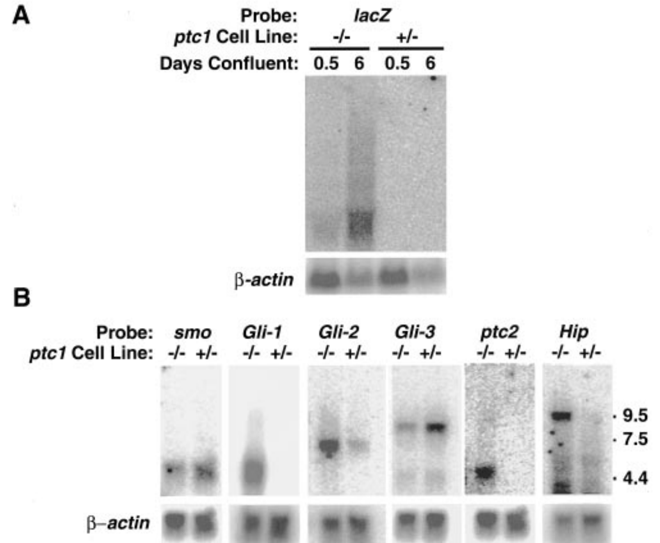

FIG. 4. **Regulation of Hh target genes in *ptc1* heterozygous and null cells.** *A*, $ptc1^{-/-}$ but not $ptc1^{+/-}$ cells induced *ptc1*-*lacZ* transcription after culturing at high densities. *B*, although both $ptc1^{+/-}$ and $ptc1^{-/-}$ cells expressed *smo*, the $ptc1^{-/-}$ cells induced *Gli1*, *Gli2*, *Hip*, and *ptc2* and repressed *Gli3* relative to $ptc1^{+/-}$ cells. *Gli1*, *Hip*, and *ptc2* expression were not detected in the $ptc1^{+/-}$ cells.

an HA epitope at the C-terminal was expressed using the MSCV retrovirus. $ptc1^{-/-}$ cells were infected with either MSCV (MSCV cells) or MSCV-*ptc1* (Ptc1 cells), plated at high density for 24 h, and assayed for β-galactosidase activity. These conditions considerably shortened the length of time required to assay *ptc1-lacZ* activity and yielded results comparable with those from longer culture times. Following 24 h of culture, the MSCV cells had high levels of β-galactosidase activity, whereas the Ptc1 cells had about 7-fold lower levels (Fig. 5*A*). The HA epitope did not appear to alter Ptc1 function as both a tagged and an untagged version complemented the $ptc1^{-/-}$ cells equally well (data not shown). Although *ptc1-lacZ* expression was largely complemented in this assay, the β-galactosidase levels were not quite as low as in similarly plated $ptc1^{+/-}$ cells (data not shown).

The complemented $ptc1^{-/-}$ cells responded to Shh by inducing *ptc1-lacZ*. The MSCV and Ptc1 cells were grown for 2 days in control or Shh-containing conditioned media and assayed for β-galactosidase activity. Although neither condition altered β-galactosidase levels in the MSCV cells, Shh treatment of the Ptc1 cells induced β-galactosidase activity to a level comparable with the MSCV cells (Fig. 5*B*). These experiments indicate that the *ptc1* transgene is fully functional because it inhibits *ptc1-lacZ* transcription and reactivates this target gene in response to ligand.

Both Halves of Ptc1 Are Required for Ptc1 Function—In *Drosophila*, expression of either the N-terminal or C-terminal half of Ptc alone results in no activity, whereas co-expression of both largely reconstitutes function (54). A number of *PTCH1* mutations found in human tumors are predicted to produce truncated proteins similar to the N-terminal half of fly Ptc (55–57). To understand the effect of these human mutations, we tested whether the two halves of mouse Ptc1 had activity when expressed alone or together in $ptc1^{-/-}$ cells. Homozygous *ptc1* cells were stably infected with a retrovirus expressing an HA-tagged N-terminal (Ptc1-N) or C-terminal half of Ptc1 (Ptc1-C). Immunoblots of total cell extracts indicated that Ptc1-N and Ptc1-C migrated as doublets of ~90 and 100 kDa, respectively, about 10% larger than their predicted sizes of 80 and 90 kDa, respectively (Fig. 6*A*). The steady-state level of Ptc1-C was ~5-fold higher than that of Ptc1-N when compared

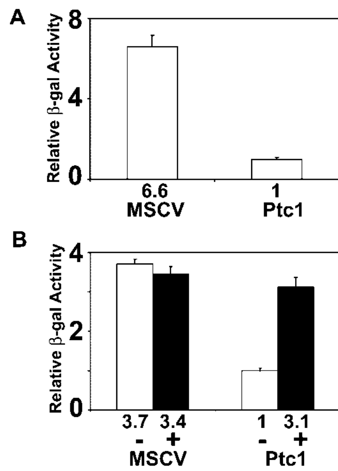

FIG. 5. **A *ptc1* transgene in $ptc1^{-/-}$ cells complements *ptc1-lacZ* and responds to Shh.** *A*, Ptc1 expression in the homozygous *ptc1* cells reduced β-galactosidase activity 7-fold relative to the control. *B*, incubation of the complemented $ptc1^{-/-}$ cells with Shh-containing media (*filled bars*) but not control-conditioned media (*open bars*) induced β-galactosidase activity to levels near that of uncomplemented cells. *Numbers beneath bars* indicate fold levels relative to unstimulated Ptc1 cells.

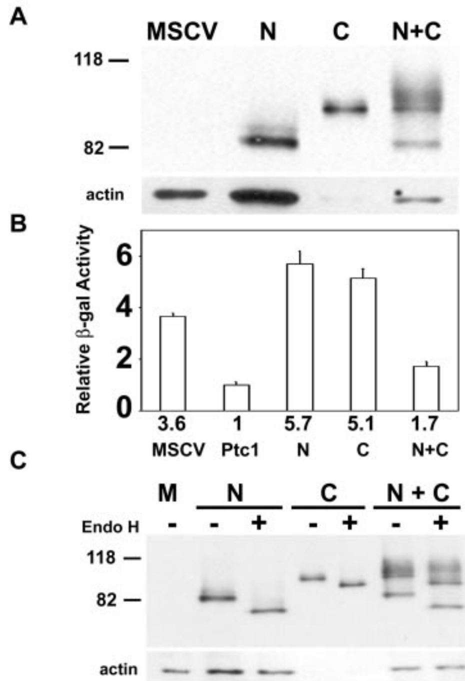

FIG. 6. **Both halves of Ptc1 are required for proper function and glycosylation.** $ptc1^{-/-}$ cells infected with control (*MSCV*), Ptc1-N (*N*), Ptc1-C (*C*), or both Ptc1-N + Ptc1-C (*N+C*) retrovirus were tested for protein production (*A*), β-galactosidase activity (*B*), and sensitivity to Endo H (*C*). *B*, when expressed separately in the $ptc1^{-/-}$ cells, Ptc1-N or Ptc1-C was not functional, whereas co-expression of both halves reconstituted Ptc1 activity to a level similar to the intact protein. *Numbers beneath bars* indicate fold levels relative to Ptc1 cells. *C*, when treated in the absence (-) or presence (+) of Endo H, Ptc1-N or Ptc1-C glycosylation was Endo H-sensitive when expressed separately but partially resistant when co-expressed. Actin was a loading control for the immunoblots.

with actin levels that were used as a control for sample loading. Either half of Ptc1 alone had no function in the $ptc1^{-/-}$ cells because β-galactosidase levels were similar to the MSCV control (Fig. 6*B*).

Co-expression of the Ptc1 halves reconstituted function almost as well as the intact protein. To express both halves, the Ptc1-N cells were superinfected with the Ptc1-C retrovirus to generate Ptc1-N+C cells. Although either Ptcl half alone was nonfunctional, co-expression of both reduced β-galactosidase levels by 3-fold (Fig. 6*B*). When treated with Shh supernatants, both Ptc1 and Ptc1-N+C cells responded similarly with a marked increase in β-galactosidase activity (data not shown). These results indicate that mouse Ptc1 requires both halves for activity and that the halves can function even when produced as two separate proteins.

Both Halves of Ptc1 Are Required for Ptc1 Maturation—Co-expression of both Ptc1 halves resulted in higher molecular-weight proteins than when either half was expressed alone (Fig. 6*A*). Because Ptc1 is a glycoprotein (58), we examined whether either half was glycosylated. When treated with either PNGase F (data not shown) or endoglycosidase H (Endo H), the mobility of both Ptc1-N and Ptc1-C increased, so they migrated near their predicted sizes (Fig. 6*C*). Both halves, therefore, contain *N*-linked glycosylation sites. Glycoproteins in the endoplasmic reticulum and *cis*-Golgi are Endo H-sensitive, but upon entering the medial Golgi the carbohydrates are modified into an Endo H-resistant form (59). Although both Ptc1-N and Ptc1-C were completely Endo H-sensitive when expressed separately, the halves were partially Endo H-resistant when expressed together (Fig. 6*C*). This suggests that both halves of Ptc1 are required for proper maturation of the protein during biosynthesis.

PTCH1 Missense Mutations Modestly Reduce Ptc1 Function—*PTCH1* mutations have been proposed to inactivate the function of the protein in tumors, thereby causing inappropriate Hh signaling and excess cell division (3, 4, 6). Indeed, numerous truncating mutations have been identified, mutations that presumably eliminate *PTCH1* function (57). It is not clear, however, how missense mutations affect protein function. We tested three missense mutations in *PTCH1* that were identified in either BCNS individuals or sporadic BCCs. These mutations, G509V (60), Q816L (4), and R1114W (61) are unlikely to be benign polymorphisms because they alter highly conserved residues. Furthermore, at positions 509 and 816, mutations have been identified in several independent tumors or BCNS individuals (4, 60).

Using site-directed mutagenesis, we incorporated these mutations at the corresponding positions in mouse *ptc1* (G495V, Q802L, and R1100W), cloned each into MSCV, and stably infected the $ptc1^{-/-}$ cells. All three Ptc1 mutants were expressed at levels comparable with the wild-type protein (Fig. 7*A*). When assayed for β-galactosidase activity, the mutants retained significant, though reduced, activity (Fig. 7*B*). Compared with normal Ptc1, the G495V, R1100W, and Q802L mutations reduced function by 3.7-, 2.3-, and 1.8-fold, respectively.

The missense mutants may have activity in the $ptc1^{-/-}$ cells because the proteins are produced at artificially high levels. Overexpression of the mutants could overcome a modest folding or structural defect and complement $ptc1^{-/-}$ cells. To address this concern, we isolated cells that expressed lower levels of transgene-derived Ptc1. A modified MSCV retrovirus was used that expresses transgenes as a bicistronic message proximal to an IRES followed by GFP. Because the expression level of the gene proximal to the IRES correlates well with the expression level of GFP (62), cells producing low levels of Ptc1 could be purified on the basis of GFP expression using FACS.

The correlation between the level of GFP fluorescence, Ptc1 production, and the ability to complement $ptc1^{-/-}$ cells was first analyzed for the wild-type protein. $ptc1^{-/-}$ cells were stably infected with Ptc1 IRES-GFP (Fig. 8, *A* and *B*), and cells expressing high or low levels of GFP were isolated by FACS (Fig. 8*A*). When grown in culture, both sets of cells stably

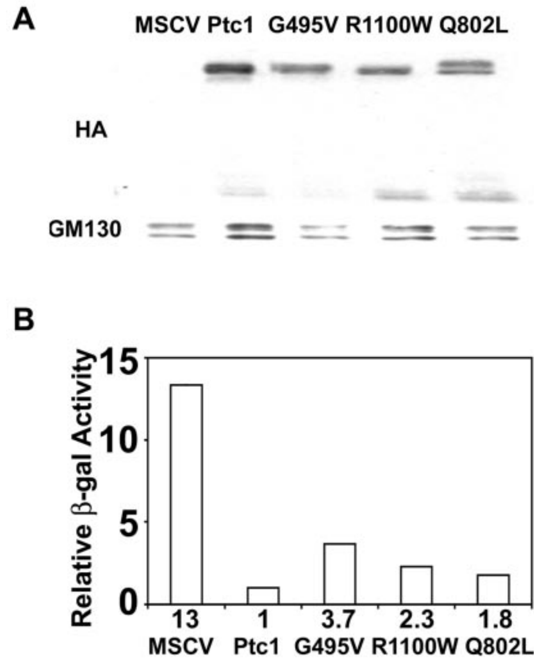

FIG. 7. **Human *PTCH1* missense mutations do not substantially alter Ptc1 function.** $ptc1^{-/-}$ cells expressing missense mutations G495V, R1100W, or Q802L were examined for transgene production (*A*) and regulation of *ptc1-lacZ* activity (*B*). *A*, even though the missense mutants were produced at levels comparable with the normal protein (*B*), R1100W- and G495V-expressing cells had 2–4-fold higher β-galactosidase activity as cells expressing wild-type Ptc1.

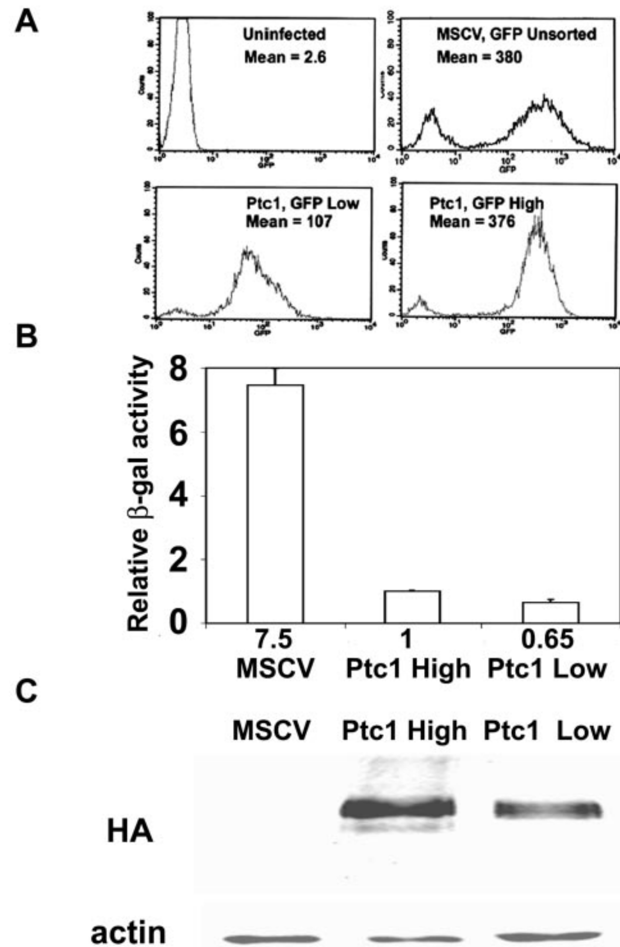

FIG. 8. **Isolation and characterization of $ptc1^{-/-}$ cells expressing high or low levels of Ptc1.** A heterogeneous population of $ptc1^{-/-}$ cells expressing Ptc1 IRES-GFP were sorted for high or low GFP by FACS (*A*) and assayed for *ptc1-lacZ* activity (*B*) and Ptc1 protein (*C*). *A*, FACS analysis of uninfected $ptc1^{-/-}$ cells showed very low fluorescence (*top left*), whereas cells infected with a control IRES-GFP retrovirus had a broad distribution (*top right*). Cells expressing high and low GFP were sorted, grown, and when analyzed again by FACS, maintained stable GFP levels during culture (*lower panels*). *B*, Ptc1 IRES-GFP-infected cells sorted for high or low GFP fluorescence had about 8-fold less β-gal activity compared with unsorted MSCV control cells and (*C*) showed correspondingly high or low Ptc1 protein expression as detected by immunoblot.

maintained their GFP expression levels over time as determined by FACS (data not shown). The Ptc1 IRES-GFP cells had about 8× less β-galactosidase activity than the unsorted IRES-GFP control, independent of whether they expressed high or low levels of GFP (Fig. 8*B*). The 3.5-fold difference in GFP fluorescence between the high and low populations corresponded to a similar difference in Ptc1 protein as detected by immunoblot (Fig. 8*C*). Hence, over the range tested, the level of complementation was independent of the amount of Ptc1 produced. Both high and low levels of Ptc1 functioned equally well.

The Ptc1 missense mutants were cloned into the bicistronic MSCV retrovirus, and for each low, and high GFP-expressing populations were isolated by FACS. The mean fluorescence intensity for the high GFP-expressing populations for all constructs was about 7–10-fold the mean fluorescence of the low expressing populations (data not shown). An immunoblot of cell lysates confirmed the correlation between GFP fluorescence and Ptc1 protein levels (Fig. 9*A*). Both wild-type and Q802L Ptc1 reduced β-galactosidase activity by 10–12-fold at both high and low expression levels (Fig. 9*B*). When selected for high expression, R1100W and G495V reduced β-galactosidase activity by about 7-fold (Fig. 9*B*), similar to that seen with unselected populations (Fig. 8*B*). However, when selected for low expression, G495V, but not R1100W, had further reduced activity; β-galactosidase levels were lowered only 4-fold. Thus, even with an approximate 10-fold reduction in protein production, all three mutants retained most or all of their activity.

The Stability of PTCH1 Mutants Is Not Substantially Altered—To determine whether the reduced activity of the Ptc1 mutants was from decreased protein stability, we measured the half-life of wild-type Ptc1 and each of the missense mutants. $ptc1^{-/-}$ cells expressing wild-type or mutant Ptc1 were sorted for high levels of GFP fluorescence and grown in culture. Cells were metabolically labeled with ^{35}S-containing amino acids and then incubated with excess unlabelled amino acids for various lengths of time. Ptc1 proteins were immunoprecipitated and size-separated by SDS-PAGE. Cells expressing wild-type Ptc1 were also treated with brefeldin A during the pulse period to induce a block in endoplasmic reticulum to Golgi trafficking (63). The mobility of Ptc1 under this condition indicates the mobility of the newly synthesized, immature form of Ptc1 (Fig. 10*A*, *arrowhead*). The immature form of Ptc1, with an apparent molecular mass of ~170 kDa, is only faintly visible in the absence of brefeldin A (BFA). Glycosylation of Ptc1 during maturation causes the protein to migrate diffusely in the range of 175–185 kDa (Fig. 10*A*, *bracket*). Wild-type Ptc1 had a half-life of 42 ± 6.7 min. The half-lives for G495V, R1100W, and Q802L were 43 ± 20 min, 64 ± 9.9 min, and 25 ± 2.6 min, respectively. Because these half-lives were within a factor of two of that of wild-type Ptc1, it appears that the missense mutations do not substantially alter the stability of the protein.

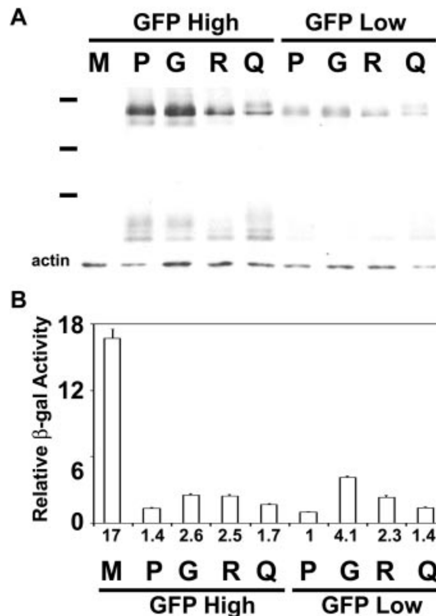

FIG. 9. ***ptc1$^{-/-}$* cells expressing reduced levels of mutant Ptc1 proteins still retain significant activity.** *ptc1$^{-/-}$* cells infected with the parental IRES-GFP retrovirus (*M*) or wild-type (*P*), G495V (*G*), R1100W (*R*), or Q802L (*Q*). Ptc1 IRES-GFP retrovirus were sorted for high or low GFP levels and analyzed for protein expression (*A*) and β-galactosidase activity (*B*). *A*, high and low GFP-expressing cells were sorted for a 7–10-fold difference in fluorescence and showed a similar range of protein expression for each form of Ptc1 as seen by immunoblot. *B*, the R1100W and Q802L mutations showed similar activity at either expression level, whereas G495V had even less activity when expressed at the lowest levels.

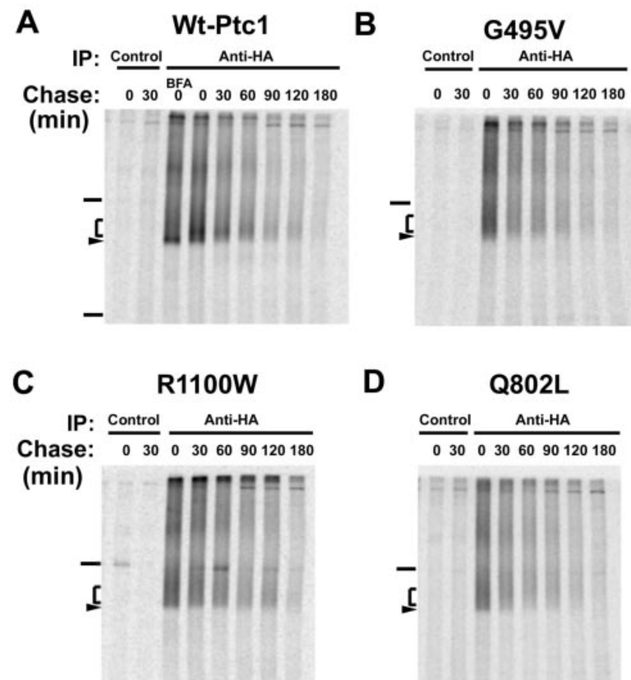

FIG. 10. ***PTCH1* mutations do not alter protein stability.** *ptc1$^{-/-}$* cells expressing wild-type (*A*), G495V (*B*), R1100W (*C*), and Q802L (*D*) Ptc1 were metabolically labeled and then incubated with excess unlabelled amino acids over time. Ptc1 proteins were immunoprecipitated with isotype control (0 and 30 min only) or anti-HA antibody. The half-life of wild-type Ptc1 (~40 min) was not substantially different from that of the missense mutants (25–60 min). Wild-type Ptc1 was also treated with BFA during the pulse period to indicate the mobility of immature Ptc1 at ~170 kDa (*arrowhead*). In the absence of BFA, the immature form is faintly visible, whereas the mature forms of Ptc1 are glycosylated and migrate in the 175–185 kDa size range (*bracket*). The *bars* indicate molecular mass standards of 206 and 119 kDa.

DISCUSSION

lacZ Is an Endogenous Reporter for Hh Signaling—We have used a *lacZ* gene inserted into a Hh target gene to study the activities of Hh pathway components. *ptc1-lacZ* is a sensitive and specific indicator of Hh signaling and offers several unique advantages. Since *ptc1-lacZ* is present in cells in one or two copies, this avoids problems caused by high copy reporter plasmids such as titering out limiting transcription factors (squelching). Although most Hh-regulated target genes are induced in only a subset of responsive tissues, almost all developing cells that can or do respond to Hh signals include *ptc1* induction in the response (40). Hence *ptc1-lacZ* is a faithful indicator of signal transduction by the Hh pathway. In addition to *ptc1-lacZ*, other Shh targets, such as *Hip*, *ptc2*, *Gli1*, *Gli2*, and *Gli3*, are appropriately modulated in the *ptc1$^{+/-}$* and *ptc1$^{-/-}$* cells.

High Densities Are Required for Induction of ptc1-lacZ—According to current models of Hh signaling, both *ptc1$^{-/-}$* cells and Shh-stimulated *ptc1$^{+/-}$* cells should persistently express *ptc1-lacZ*. However, neither expresses *ptc1-lacZ* until they have been cultured at high density (Figs. 1*B* and 2*B* and Ref. 64). The density-dependence of Hh signaling has also been observed in NIH-3T3 cells (64). Culturing at high density appears to be permissive rather than instructive because in the absence of other stimuli, high density alone does not induce *ptc1-lacZ* in the *ptc1* heterozygous cells (Fig. 2*A*). The density-dependence does not appear to be caused by the accumulation or depletion of a factor in the culture medium because exposure of cells to conditioned media from confluent *ptc1$^{-/-}$* cells does not induce *ptc1-lacZ* in subconfluent *ptc1$^{-/-}$* cells (data not shown). High densities may cause the induction of a component required for Hh signal transduction. *smo* and CBP are not such targets, because their expression is not modulated by changes in cell-density in either the *ptc1$^{+/-}$* or *ptc1$^{-/-}$* cells (data not shown).

The density requirement of both heterozygous and homozygous *ptc1* cells can be bypassed by *Gli1* expression. Gli1 may be limiting in cells plated at low density, and the effect of culturing at high densities may be to induce *Gli1*, after which *ptc1* and other genes regulated by Hh are transcriptionally activated. Interestingly, *ptc1$^{+/-}$* cells expressing Gli1 had a further 2-fold increase in β-galactosidase levels after being cultured at high densities (Fig. 1*B*). Hence, expression of Gli1 does not appear to account for all of the effects of density. At present, the signals and mechanisms mediating the density-dependence of Hh signaling are not known.

The density-dependence for Hh signaling may provide insight into the low metastatic potential of BCC (5). Metastasis requires local invasion and systemic distribution of tumor cells. Hh signaling induces expression of the anti-apoptotic gene *bcl-2* in human keratinocytes and promotes growth by making these cells refractory to cell cycle inhibitors (65). If *ptc1$^{-/-}$* cells require high cell densities to acquire these features, isolated metastatic cells would lose their growth and anti-apoptotic advantages once they have separated from the primary tumor focus. Such a tumor type would be more likely to spread by local expansion, consistent with the normal course for BCC.

A Requirement for Both Halves of Ptc1 for Maturation and Function—Ptc homologs from a variety of animals appear to have a duplicated structure composed of six putative transmembrane domains and a major extracellular loop in a tandem repeat (66). These characteristics are similar to that of transporter family members and suggest that either half of Ptc alone

might be functional as has been tested for several transporters. In *Drosophila,* expression of either Ptc half alone has no function, but co-expression of both halves reconstitutes activity (54). Similarly, neither half of mouse Ptc1 was functional in the *ptc1*$^{-/-}$ cells. Co-expression of both halves reconstituted activity comparable with intact Ptc1 (Fig. 6*B*).

Both halves of Ptc1 are required for the proper maturation of the protein during biosynthesis. When expressed alone, each half was blocked in the secretory pathway prior to the medial-Golgi, as detected by Endo H sensitivity. When the two halves were co-expressed, a subpopulation became Endo H resistant and function was detected (Fig. 6, *B* and *C*). Ptc1 appears to require self-association to fully mature and become functional. Because both halves contained an HA epitope, we could not determine what portion of the Endo H resistant population represented Ptc1-N or Ptc1-C.

Some PTCH1 Mutation Retain Significant Function—We have analyzed three missense *PTCH1* mutations identified from BCNS patients or BCCs. In addition we have tested Ptc1-N, a deletion that is similar to a number of truncating mutations found in sporadic tumors or BCNS patients (55–57). With the exception of Ptc1-N and Ptc1-C, the other Ptc1 mutants had some activity in *ptc1*$^{-/-}$ cells. The Q802L mutant worked as well as the wild-type protein, whereas the G495V and R1100W mutants had 2 to 4-fold reduced activity. Why these mutations retain significant function could be explained in several ways.

One possibility is that the mutations are benign polymorphisms rather than functionally significant alterations. Several observations make this unlikely. Sequence alignments show that mouse Ptc1 residues Q802 and G495 are identical, and R1100 is highly conserved among 12 Ptc1 and Ptc2 homologs (data not shown), arguing that these residues are functionally important. Additionally, mutations at residues G509 and Q816 of human PTCH1 have each been found in multiple BCNS or BCC patients (4, 60, 67). For these reasons these changes are unlikely to be benign polymorphisms.

The Ptc1 mutants may show substantial activity in the *ptc1*$^{-/-}$ cells because they are probably produced at artificially high levels by the retrovirus. It is possible that expression of the mutants at levels like that of endogenous Ptc1 may reveal profound functional defects. Because of the lack of high affinity Ptc1 antibodies, it has not been possible for us to detect endogenous Ptc1 in either unstimulated or Shh-stimulated cells. Thus we do not know how the expression levels of transgene-derived Ptc1 compare with the endogenous protein. However, wild-type Ptc1 can largely complement the *ptc1*$^{-/-}$ cells at levels barely detectable by immunoblot (data not shown), suggesting that the retrovirus overexpresses Ptc1 transgenes many fold over what is required for complementation. To address the issue of overexpression, we isolated *ptc1*$^{-/-}$ cells that produced lower amounts of exogenous Ptc1 using a retrovirus that coupled transgene expression with GFP production via an IRES. Cells containing high or low levels of GFP were isolated by FACS and contained correspondingly high or low levels of Ptc1 (Fig. 8*C*), similar to that observed with other transgenes (62). For the missense mutants, cells were isolated that contained 7- to 10-fold differences in GFP levels. In only G495V was activity titrated by lowering expression levels. Q802L had near wild-type function and R1100W had twice the β-galactosidase levels compared with the normal protein, irrespective of its expression level (Fig. 9, *A* and *B*). These results suggest, contrary to our initial expectations, that PTCH1 missense mutants are compromised but not devoid of function.

The partial loss of activity of the PTCH1 mutants could be due to destabilization of the protein. We performed metabolic labeling experiments to measure the half-lives of wild-type and mutant Ptc1 proteins. As measured in the *ptc1*$^{-/-}$ cells, normal Ptc1 was degraded rapidly, with a half-life of about 40 min. For comparison, the immature form of the transmembrane protein cystic fibrosis transmembrane conductance regulator (CFTR) is similarly unstable, with an initial half-life of 20–40 min. Within 60 min, a portion of CFTR acquires a mature conformation, after which it has an ~20-fold increase in stability (68, 69). Ptc1, however, does not acquire a more stable conformation but appears to be degraded with a half-life similar to that of immature CFTR. The Ptc1 missense mutants were degraded with similar kinetics, each with half-lives that were within a factor of two of wild-type Ptc1. Because of the difficulty of accurately measuring such brief half lives, we believe that a 2-fold difference in turnover is not significantly different. However, we cannot rule out subtle differences in half-life between the missense mutants and the normal protein. Overall, we believe that these missense mutations do not alter Ptc1 stability, consistent with their retention of significant function in the *ptc1*$^{-/-}$ cells.

Environmental and genetic factors in conjunction with *PTCH1* mutations contribute to the severity of clinical features in BCNS families (5, 57, 70). Given these extragenic influences, it is still reasonable to expect different BCNS phenotypes to correlate with the type of Ptc1 mutation, similar to what has been seen with other tumor suppressors (71, 72). Previous work (57) has not revealed a phenotype-genotype correlation between *PTCH1* mutations and the spectrum of anomalies in BCNS patients; however, this study only analyzed truncating mutations. Recent analysis of a similar series of truncating mutations in *Drosophila* revealed that most Ptc truncations have no activity (73). In this report, we have analyzed a truncating mutation (Ptc1-N) and missense mutations. Our finding of a spectrum of activity in these PTCH1 mutants, and the presence of activity in all the missense mutants, may merit an extension of the earlier study of phenotype-genotype correlations to include missense alterations. It is possible that *PTCH1* truncation mutations may have little or no activity and might correlate with more severe presentations, whereas mutations that alter single amino acids may retain activity and associate with milder clinical outcomes.

It seems likely that even slight reductions in Ptc1 activity may cause inappropriate Hh target gene activity and tumor formation. In mice, medulloblastomas and certain developmental defects arise from *ptc1* haploinsufficiency (10, 11). Some developmental defects in BCNS patients also arise from loss of a single copy of *PTCH1* (5). The *ptc1*$^{-/-}$ cells provide a useful system for analyzing the effects of tumor-derived mutations in *PTCH1* and for studying other steps in Hh signal transduction.

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