The American Journal of Human Genetics, Volume *89*

Supplemental Data

Mutations in *CDON*, Encoding a Hedgehog Receptor, Result in Holoprosencephaly and Defective Interactions with Other Hedgehog Receptors

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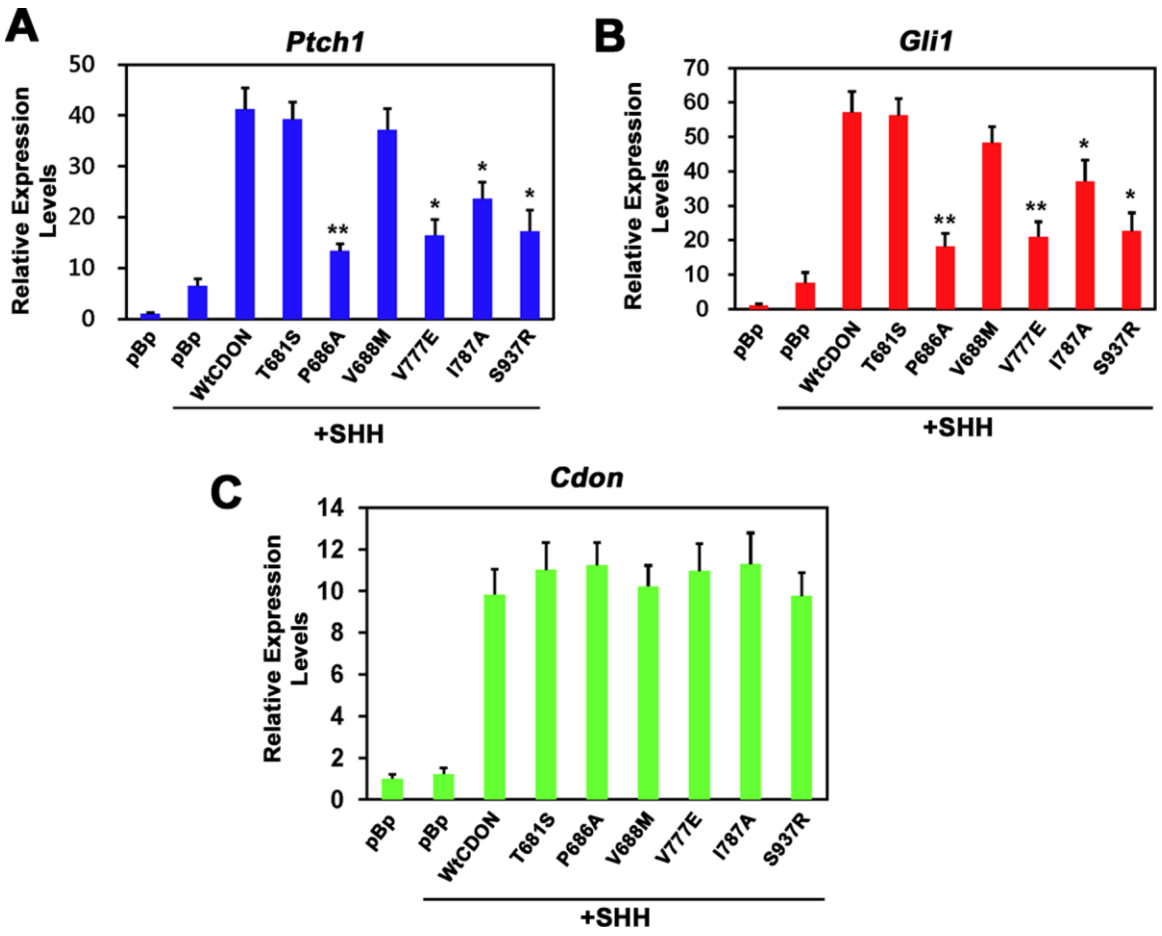


Figure S1. CDON Variants Do Not Support SHH-Dependent Gene Expression
(A-C) qRT-PCR analysis of *Ptch1* (A), *Gli1* (B) and *Cdo* (C) expression in 10T1/2 cells transfected with 2 μg of the indicated CDON vectors, plus or minus treatment with SHH. Expression was normalized to *Gapdh*. Error bars represent the means of triplicate determinations ± SD. **, $p < 0.01$, *, $p < 0.05$ as compared to wild-type CDON. CDON variants are designated as in Figure 1.

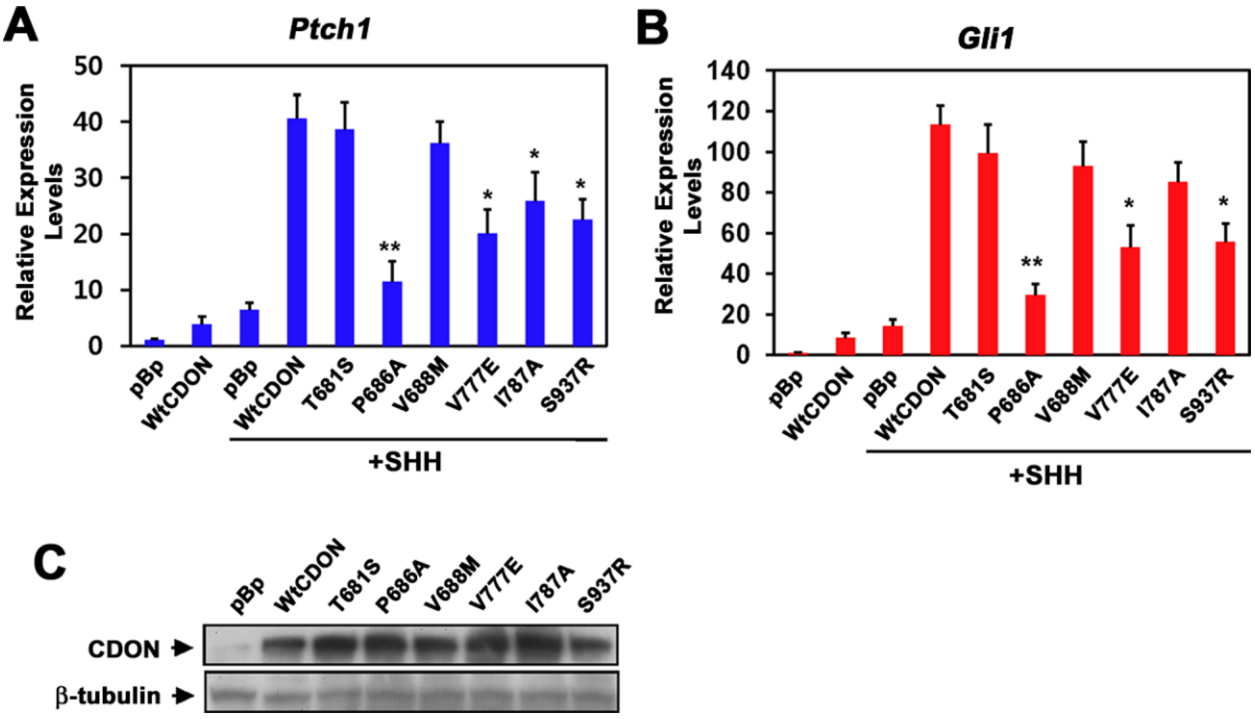


Figure S2. Overexpression Reveals Partial Loss of Function in Some CDON Variants
(A, B) qRT-PCR analysis of *Ptch1* (A) and *Gli1* (B) expression in 10T1/2 cells transfected with 5 μg of the indicated CDON vectors, plus or minus treatment with SHH. Expression was normalized to *Gapdh*. Error bars represent the means of triplicate determinations ± SD. **, $p < 0.01$, *, $p < 0.05$ as compared to wild-type CDON.
(C) Western blot analysis of CDON expression in 10T1/2 cells under the conditions used in (A, B). CDON variants are designated as in Figure 1.

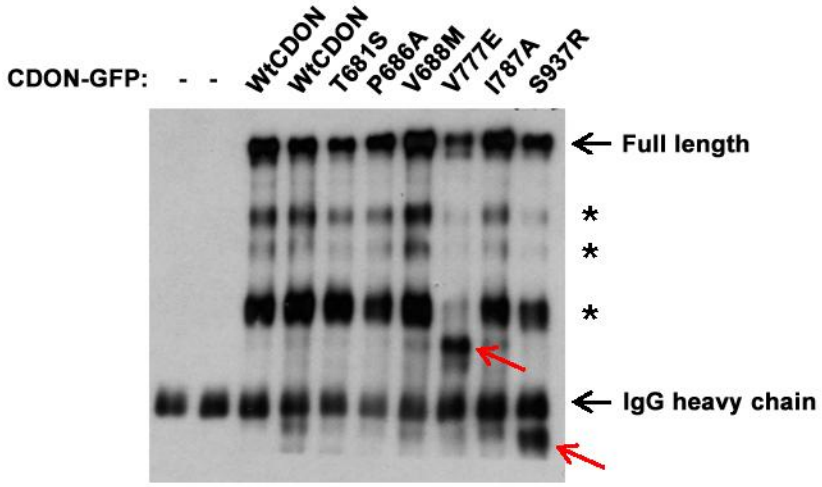


Figure S3. CDO p.Val777Glu (V777E) and p.Ser937Arg (S937R) Display Different or Additional Products Than Wt CDON upon Limited Proteolysis

Wt CDON and HPE-associated variants with a C-terminal GFP tag were expressed in 293T cells and immunoprecipitated with anti-CDON antibodies. Immunoprecipitates were incubated at 4^{o} C prior to Western blot analysis with anti-GFP antibodies. Protelolytic degradation products are indicated by asterisks; the red arrows indicate the presence of a different (V777E) or additional (S937R) proteolytic product not seen with Wt CDON or the other variants. Note that V777E and S937R also display reduced half-lives (Table 1). CDON variants are designated as in Figure 1.

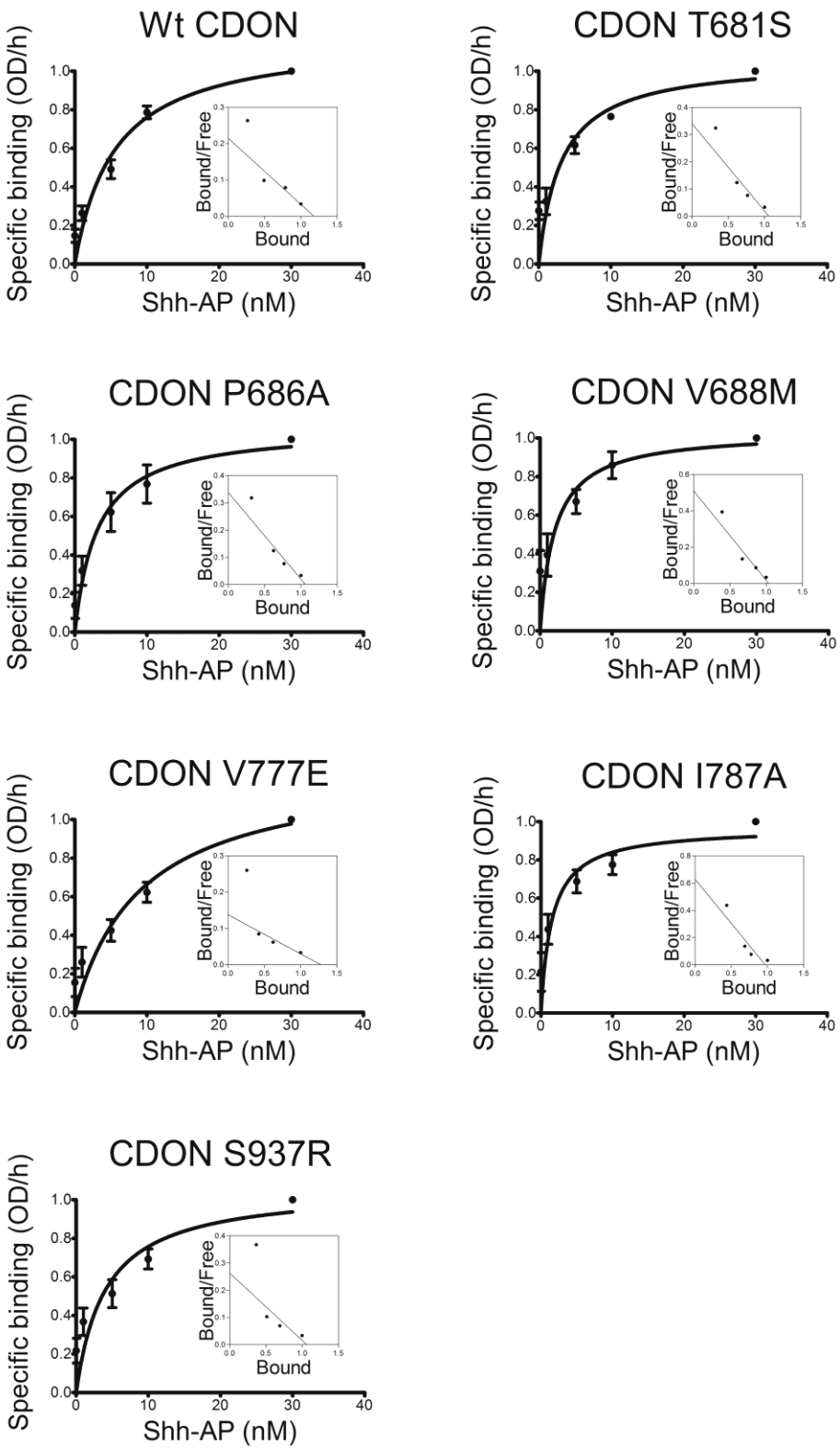
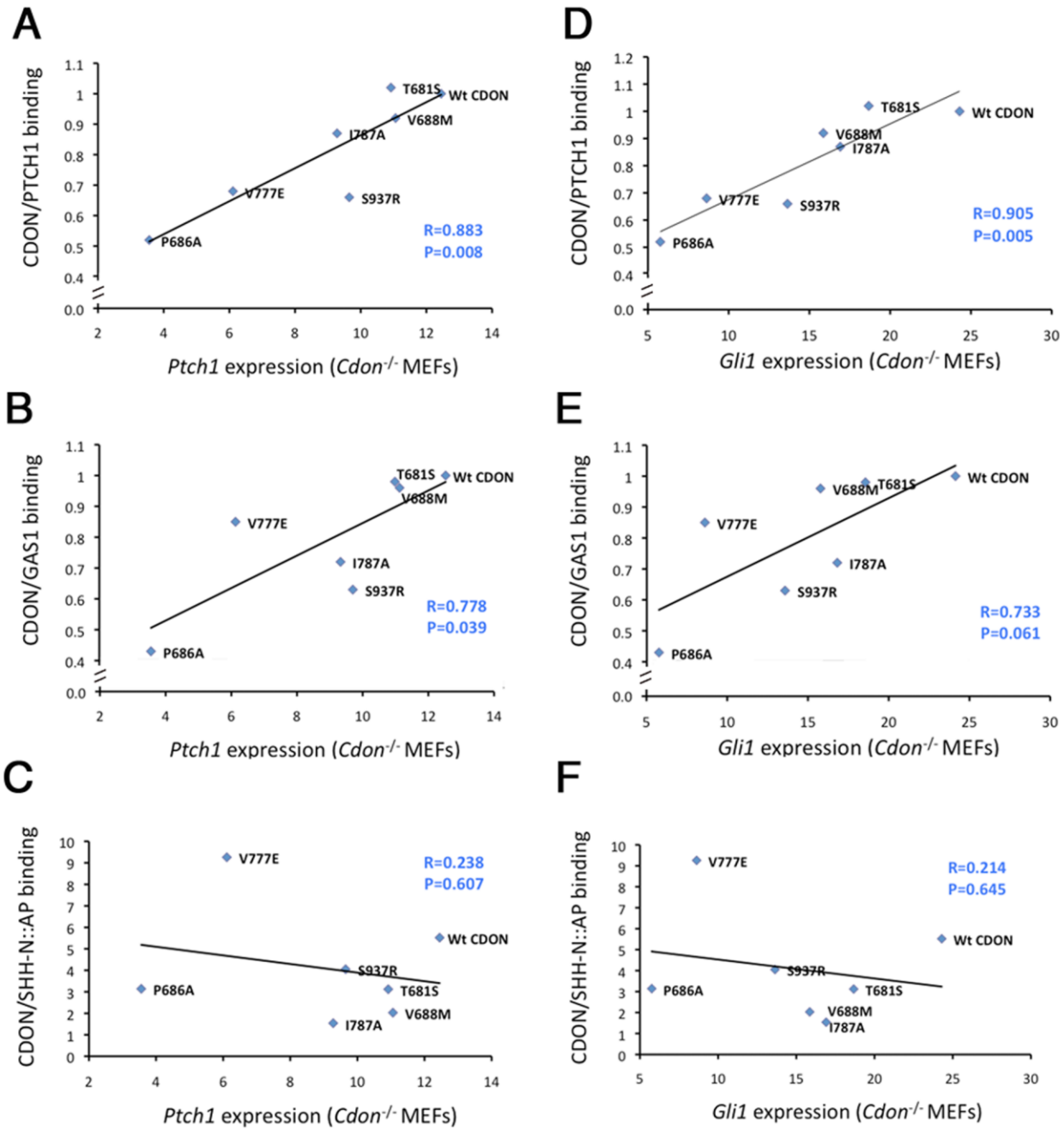


Figure S4. Measurement of Dissociation Constants for SHH-N::AP Binding to CDON Variants

Saturation binding curves and Scatchard analysis plots (inset) for CDON and the indicated variants. Points on the curves represent means of triplicate determinations ± SD. CDON variants are designated as in Figure 1.


A
CDON/PTCH1 binding
T681S
Wt CDON
V688M
I787A
V777E
S937R
P686A
R=0.883
P=0.008
Ptch1 expression (Cdon-/- MEFs)
D
CDON/PTCH1 binding
T681S
Wt CDON
V688M
I787A
V777E
S937R
P686A
R=0.905
P=0.005
Gli1 expression (Cdon-/- MEFs)
B
CDON/GAS1 binding
T681S
Wt CDON
V688M
V777E
I787A
S937R
P686A
R=0.778
P=0.039
Ptch1 expression (Cdon-/- MEFs)
E
CDON/GAS1 binding
V688M
T681S
Wt CDON
V777E
I787A
S937R
P686A
R=0.733
P=0.061
Gli1 expression (Cdon-/- MEFs)
C
CDON/SHH-N::AP binding
V777E
R=0.238
P=0.607
Wt CDON
S937R
P686A
T681S
V688M
I787A
Ptch1 expression (Cdon-/- MEFs)
F
CDON/SHH-N::AP binding
V777E
R=0.214
P=0.645
Wt CDON
S937R
P686A
T681S
V688M
I787A
Gli1 expression (Cdon-/- MEFs)

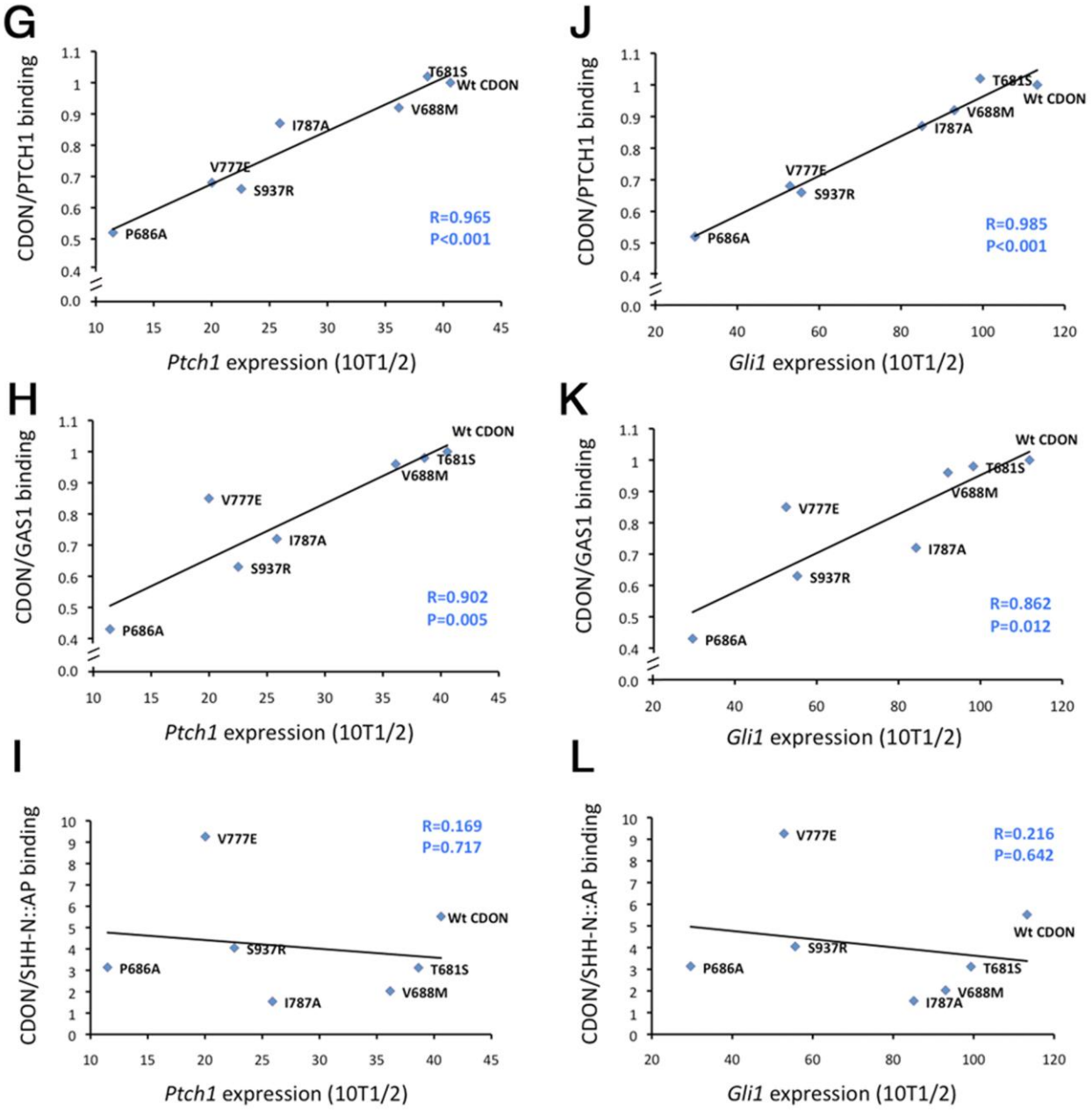


Figure S5. Correlations between CDON Variant Activity in Cell-Based Assays and Binding to PTCH1, GAS1, and SHH

Gli1 and *Ptch1* expression in *Cdon*$^{-/-}$ MEFs and in 10T1/2 cells (Figure S2A-D) were plotted against quantification of CDON/PTCH1 binding, CDON/GAS1 binding and CDON/SHH-N::AP binding (Figure 2B, Figure 2G and Table 2, respectively). Correlation coefficients (R) are shown for each; P values were determined with Student's t-test.

(A) CDON/PTCH1 binding vs. *Ptch1* expression in *Cdon*$^{-/-}$ MEFs.

(B) CDON/GAS1 binding vs. *Ptch1* expression in *Cdon*$^{-/-}$ MEFs.

(C) CDON/SHH-N::AP binding vs. *Ptch1* expression *Cdon*$^{-/-}$ MEFs.

(D) CDON/PTCH1 binding vs. *Gli1* expression in *Cdon*$^{-/-}$ MEFs.

(E) CDON/GAS1 binding vs. *Gli1* expression in *Cdon*$^{-/-}$ MEFs.
(F) CDON/SHH-N::AP binding vs. *Gli1* expression in *Cdon*$^{-/-}$ MEFs.
(G) CDON/PTCH1 binding vs. *Ptch1* expression in 10T1/2 cells.
(H) CDON/GAS1 binding vs. *Ptch1* expression in 10T1/2 cells.
(I) CDON/SHH-N::AP binding vs. *Ptch1* expression in 10T1/2 cells.
(J) CDON/PTCH1 binding vs. *Gli1* expression in 10T1/2 cells.
(K) CDON/GAS1 binding vs. *Gli1* expression in 10T1/2 cells.
(L) CDON/SHH-N::AP binding vs. *Gli1* expression in 10T1/2 cells.
CDON variants are designated as in Figure 1.

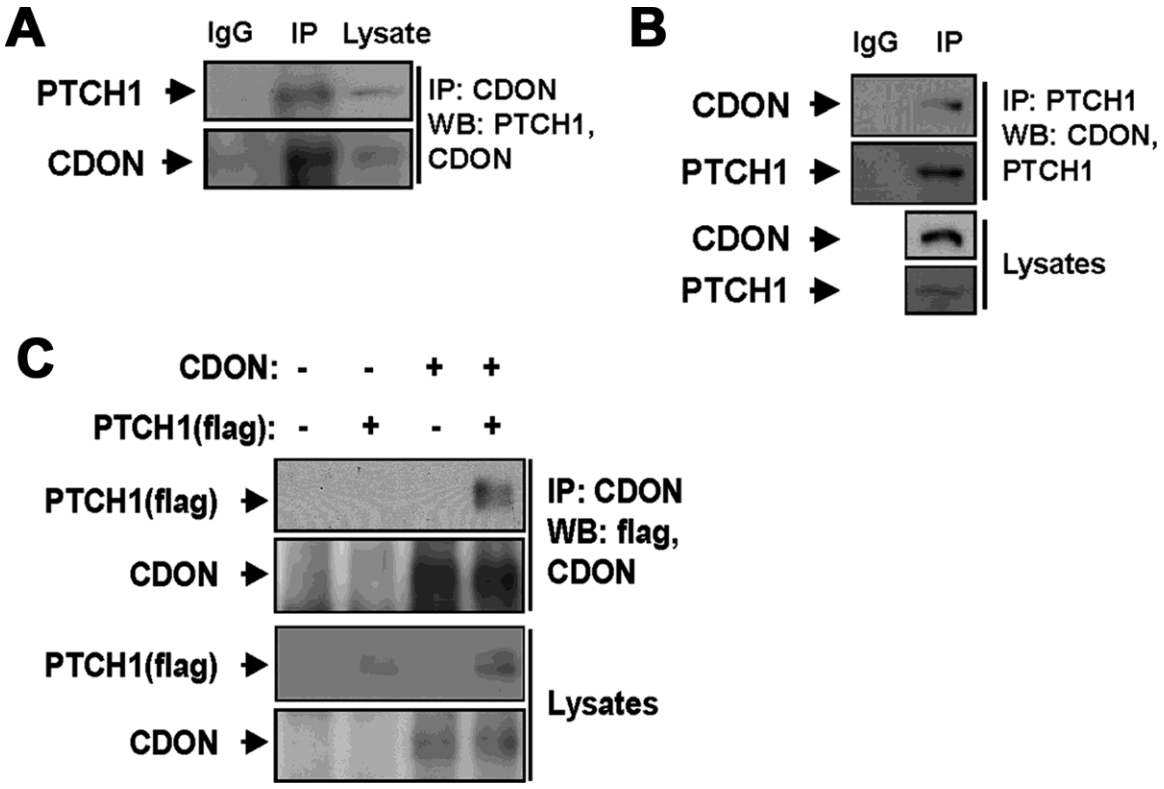


Figure S6. CDON Interacts with PTCH1
(A) RD cell lysates were immunoprecipitated with antibodies to CDON or control IgG and immunoblotted with antibodies to PTCH1 and CDON. Lysates were immunoblotted as a control. (B) RD cell lysates were immunoprecipitated with antibodies to PTCH1 or control IgG and immunoblotted with antibodies to CDON and PTCH1. Lysates were immunoblotted as a control. (C) 293T cells were transfected with expression vectors encoding CDON and/or flag-tagged PTCH1 (+) or control vectors (-) as indicated. Lysates were immunoprecipitated with antibodies to CDON and Western blotted with antibodies to flag epitope or CDON. Lysates were also blotted as a control. This experiment is a reciprocal co-immunoprecipitation to that shown in Figure 3A.

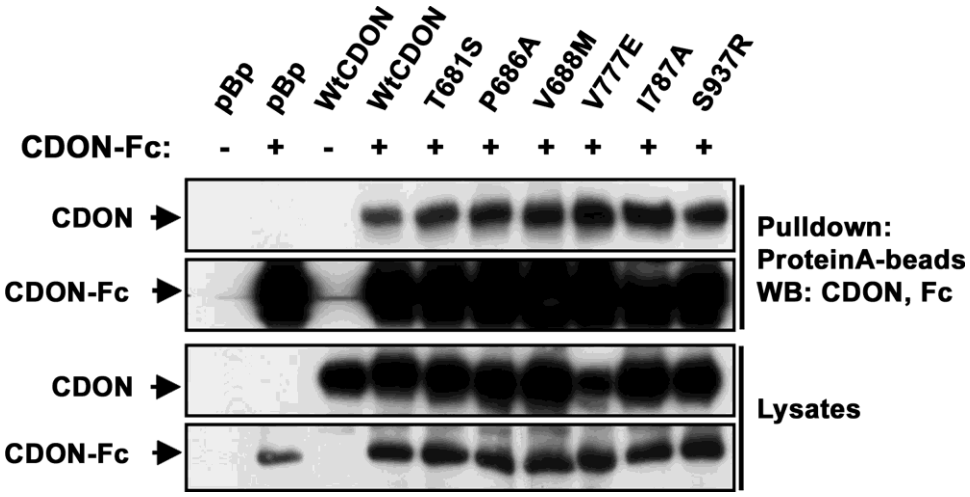


Figure S7. CDON Variants Do Not Display Defects in Interaction with Wt CDO
293T cells were transfected with plasmids encoding CDON ectodomain-Fc fusion protein and CDON variants as indicated, lysates were pulled down with protein A-agarose and Western blotted with antibodies to Fc or CDON intracellular region (CDON). Lysates were probed as a control. CDON variants are designated as in Figure 1.

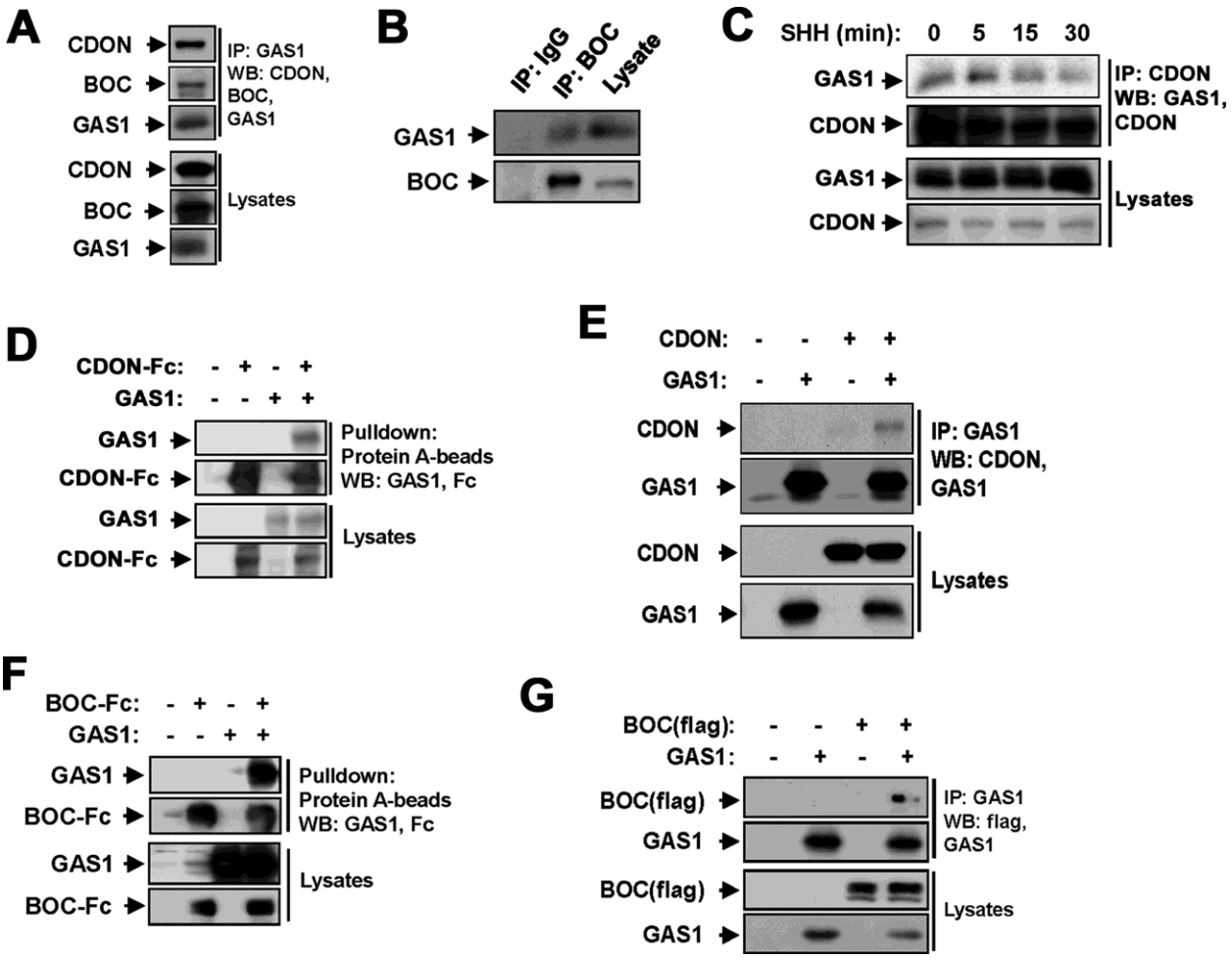


Figure S8. CDON and BOC Interact with GAS1

(A) C2C12 cell lysates were immunoprecipitated with antibodies to GAS1 and immunoblotted with antibodies to CDON, BOC and GAS1. Lysates were immunoblotted as a control. This experiment is a reciprocal co-immunoprecipitation to that shown in Figure 3E.

(B) 10T1/2 cell lysates were immunoprecipitated with antibodies to BOC or with control IgG and immunoblotted with antibodies to BOC and GAS1. Lysates were probed as a control.

(C) 10T1/2 cells were treated with recombinant SHH for the indicated times and cell lysates were immunoprecipitated with antibodies to CDON then Western blotted with antibodies to CDO and GAS1. Lysates were immunoblotted as a control.

(D) 293T cells were transfected with expression vectors encoding CDON ectodomain-Fc fusion protein and GAS1 as indicated, lysates were pulled down with protein A-agarose and Western blotted with antibodies to GAS1 or Fc as indicated. Lysates were probed as a control.

(E) 293T cells were transfected with expression vectors encoding GAS1 and/or CDON (+) or control vectors (-) as indicated. Lysates were immunoprecipitated with antibodies to GAS1 and Western blotted with antibodies to CDON or GAS1. Lysates were also blotted as a control.

(F) 293T cells were transfected with expression vectors encoding BOC ectodomain-Fc fusion protein and GAS1 as indicated, lysates were pulled down with protein A-agarose and Western blotted with antibodies to GAS1 or Fc as indicated. Lysates were probed as a control.

(G) 293T cells were transfected with expression vectors encoding GAS1 and/or flag-tagged BOC (+) or control vectors (-) as indicated. Lysates were immunoprecipitated with antibodies to GAS1 and Western blotted with antibodies to flag epitope or GAS1. Lysates were also blotted as a control.

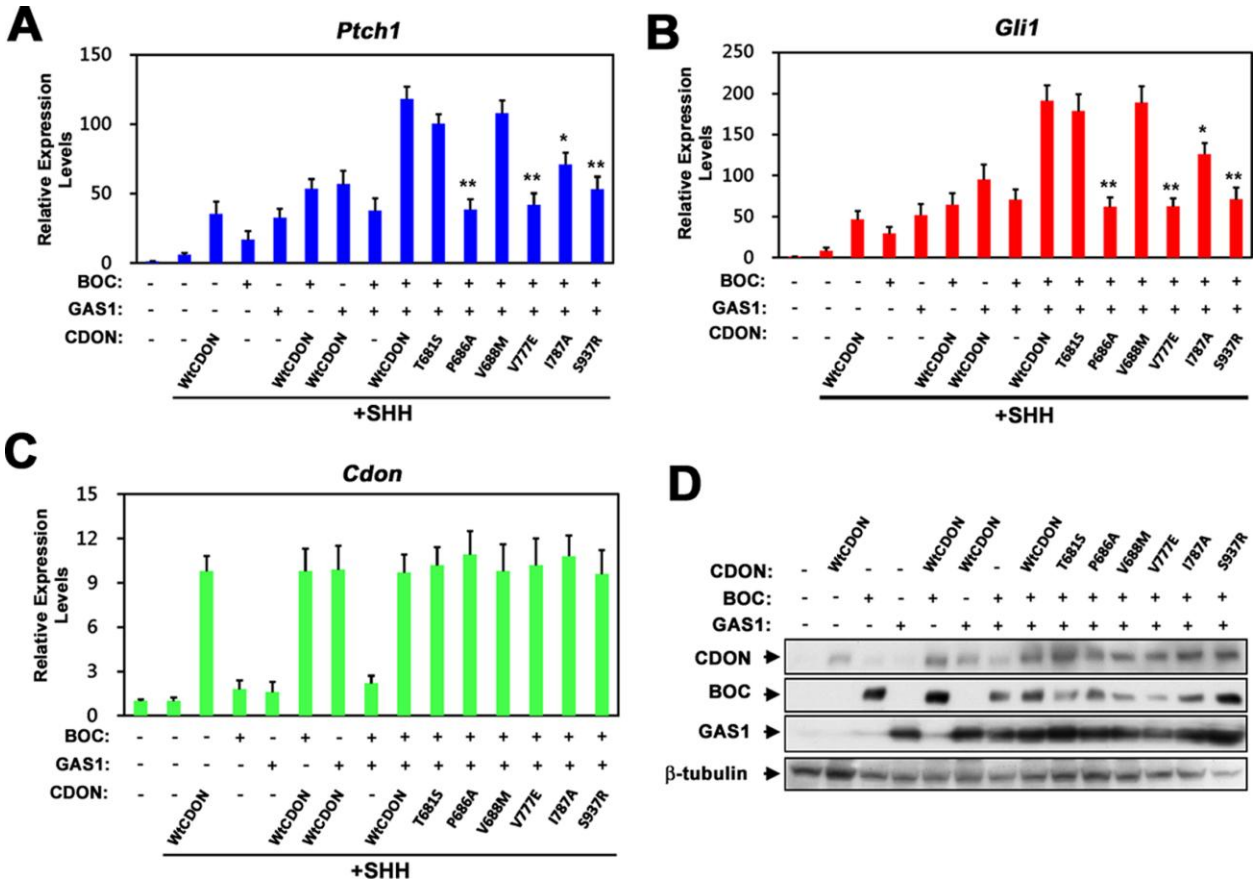


Figure S9. CDON, BOC and GAS1 Have Roughly Additive Activity in Promoting SHH-Dependent Gene Expression

(A, B) qRT-PCR analysis of *Gli1* and *Ptch1* expression in 10T1/2 cells transfected with 2 μg of the indicated CDON, BOC and GAS1 expression vectors, plus or minus treatment with SHH. Expression was normalized to *Gapdh*. Error bars represent the means of triplicate determinations ± SD. **, $p < 0.01$, *, $p < 0.05$ as compared to cells transfected with Wt CDON, BOC and GAS1.

(C) qRT-PCR analysis of expression of CDON mutants in *Cdon*$^{-/-}$ MEFs from same RNA samples analyzed in (A, B).

(D) Western blot analysis of CDON, BOC ands GAS1 expression in 10T1/2 cells transfected with 5 μg of CDON, BOC and GAS1 expression vectors.

Table S1. Genetic Variation of the *CDON* Coding Region

Variation	Amino acid	dbSNP	Hetero dbSNP [a]	MAF [b] subjects (n =282)	MAF controls (n = 96)	MAF (n = 44)
c.76+21G>A	N/A	rs1939890	0.236	0.00175	-	-
c.197A>G	p.Lys66Arg	rs7122277	0.065	0.0175	0.0104	0.18
c.223G>A	p.Val75Ile	rs3740912	0.49	0.657	0.646	0.81
c.330T>C	p.Pro110Pro	rs35131477	0.053	0.0684	-	0.14
c.349+39_40insT	N/A		-	0.189	N.D.	-
c.350-11A>G	N/A		-	0.00175	-	-
c.484G>A	p.Glu162Lys	rs3740909	0.197	0.0930	0.0677	0.28
c.497-20G>A	N/A		-	0.00175	-	-
c.640+12G>A	N/A	rs4426144	0.202	0.105	0.0677	-
c.1144G>C	p.Ala382Pro		-	-	0.0052	-
c.1167G>A	p.Met389Ile		-	-	0.0052	-
c.1263T>C	p.Phe421Phe		-	-	0.0052	-
c.1296G>A	p.Pro432Pro	rs11220313	0.044	-	0.0104	-
c.1500C>T	p.Cys500Cys		-	0.00175	-	-
c.1603G>A	p.Ala535Thr	rs76247998	0.295	0.00526	0.0208	-
c.1671G>A	p.Lys557Lys	rs35884952	0.025	0.0175	0.0156	0.06
c.1818G>T	p.Leu606Leu		-	0.00175	0.0052	-
c.1842G>A	p.Lys614Lys	rs35705696	0.025	-	0.0052	-
c.1847G>A	p.Arg616Gln		-	-	0.0052	-
c.1851+14G>A	N/A		-	0.0035	0.0156	-
c.2037A>G	p.Ala679Ala	rs516664	0.416	0.498	0.515	0.48
c.2051C>G	p.Thr684Ser		-	0.00175	-	0.06
c.2057C>T	p.Ala686Val	rs12274923	0.101	0.377	0.505	0.44
c.2065C>G	p.Pro689Ala		-	0.00175	-	-
c.2071G>A	p.Val691Met		-	0.00175	-	-
c.2362+48T>G	N/A		-	-	0.0052	-
c.2339T>A	p.Val780Glu		-	0.00175	-	-

Table S1. Genetic variation of the *CDON* coding region (continued)

c.2392A>G	p.Ile798Val		-	-	0.0052	-
c.2368A>G	p.Thr790Ala		-	0.00175	-	-
c.2623A>G	p.Ser875Gly	rs115533243	N.D.	-	0.0052	-
c.2818A>C	p.Ser940Arg		-	0.00175	-	-
c.2859G>T	p.Gly953Gly		-	0.00175	-	-
c.3039C>T	p.Asn1013Asn	rs684805	0.369	0.166	0.187	0.65
c.3156G>A	p.Lys1052Lys		-	0.00175	-	-
c.3165T>C	p.Asn1055Asn	rs564214	0.366	0.166	0.187	0.65
c.3294G>A	p.Thr1098Thr	rs3740904	0.50	0.072	0.265	0.55
c.3297C>A	p.Ala1099Ala		-	0.00175	-	-
c.3393C>T	p.Ser1131Ser		-	0.00175	-	-
c.3526G>A	p.Val1176Ile	rs78304400	0.180	0.008	0.010	0.06
c.3549C>T	p.Val1183Val	rs2276061	0.397	0.063	0.469	0.49
c.3559C>T	p.Arg1187Cys		-	0.00175	0.010	-
c.3588C>T	p.Asp1196Asp		-	0.00175	0.0208	-
c.3662T>A	p.Ile1221Asn	rs684535	0.274	0.372	0.802	0.87
c.3794*15C>T	N/A		-	0.0052	0.0052	-

[a] Heterozygosity measurements of human *CDON* (NM_016952.4) variations based on up to 51 different populations from dbSNP (www.ncbi.nlm.nih.gov/SNP). [b] MAF = minor allele frequency based on dHPLC profiles or direct sequencing (Jehee *et al*., 2006).

Table S2. Primers and Amplification Conditions

Exon	Size (pb)	T. *annealing* PCR (ºC)	Primer Sequences 5'→3'	Elution Temp (ºC)	*Buffer* B (%)
1	175	54	F: CTCTGGAAGCCTGTCCTGATTGCT R: GTTTTATAGGATTAAGATGAGTAAAGG	58	52
2	401	50	F: CTTTTCTCCCCTGCTTTCTTTGC R: GGTCTTTCCCCTCTTCAGAAATATAAG	54;59	60;58
3	236	54	F: TGCATGTATTTATTTATCTGTTTC R: TAATGAGCATTTGTTCTGTGTGTG	55;59	55;52
4	249	58	F: AGTTGTGCTGTGGTGTCCTTCATAAAG R: GGACTCTTCCTCTTCCAGTTACCATT	56	55
5	398	54	F: ATCGAAGACTTACTAATAATCTCTC R: CCTTCAGGTATCAGTTAGACCAGA	53.5;59	60;58
6	395	54	F: GAAGTACATTTTGAGGGCTAGAGTTT R: TAATTTCATTTTTGACCACAACAA	54.5;59	59;57
7	473	54	F: CATCGTAGGTACTACAATGTTCTTC R: CACTTTATCTTCTATTATAAAAGGA	50;60	61;59
8	388	54	F: AATGAGGAGTATCTTCAAGCTGTCTCC R: CGTTCAGGTGTGAGCCGAGAAAGATGA	59;62	58;57
9	268	58	F: CCTCCATCTCCTGCTTTTCTTTTCTG R: GACATCAGGGCAGCCAGCTCATTCC	61	57
10	256	54	F: GTACTCTTTTTTTTCCACTTTACATACA R: TCTGAAGCAGGTCAGTTATATTTGT	53;58	57;53
11	306	54	F: TTGTCTCTGACATGGTGGGTTATT R: TATATGTATCTAAAGTTCACATCG	55;59	60;57
12	361	58	F: GTCCTATTATAGCTTTTCTTAGTTATG R: ACATGACCAAACCACAAAATCTCAT	55;59	58;57
13	210	50	F: TAATTTTTCTCTTTGACAAGTCTT R: ATATTCATAAACCTTCTTTCTCCA	56;58	55;54
14	232	58	F: CATTTAGGCTGTATGATGTGTAAA R: TCAGAAGCAGTAATCCAGGGTTGG	58	57
15	314	58	F: TCTGTATTCACTCTGACATCATGTT R: CTACCCACCTTTAGAAAAAGAGAAG	55;59	59;56
16	361	58	F: TGTGTGCGTGTGTGTCTTTTTTGT R: CCTGAGGGACAGAGGGGCTTTTAT	57;60	60;57
17	186	58	F: TGTGAGTACCTTCAGCATTCCTTC R: ATATGCCTTATTCACAACAGCTTG	58;62	54;47
18	446	58	F: GTCTGTGAATACAGAATACCAAATG R: CCTTCCCATACACAGCTCTTAGCT	56;61	60;59
19	265	58	F: AGACTGCTTTGTAAAAATCAGCCT R: GCTTGAAGTTGGAACATGACTGGT	59.5;62	58;54

Table S3. Genetic Variation of the *SHH*, *ZIC2*, *SIX3* and *TGIF* Loci in Individuals with *CDON* Mutations

Patient number	*CDON* variation	Amino acid	*SHH* NM_000193.2	*ZIC2* NM_007129.2	*SIX3* NM_005413.2	*TGIF* NM_003244.2	Other[a]
5410	c.2051C>G	p.Thr684Ser	rs1233555 c.301-49G>A	-	-	-	none
7190	c.2065C>G	p.Pro689Ala	rs1233555 c.301-49G>A	-	rs78018362 c.90G>A (p.Ala30Ala)	-	none
5308	c.2071G>A	p.Val691Met	-	c.1377-1406dup; p.Ala461-470dup	-	-	none
6864	c.2339T>A	p.Val780Glu	rs1233555 c.301-49G>A	-	-	-	none
5288	c.2368A>G	p.Thr790Ala	-	-	-	rs2229337 c.420A>G (p.Pro140Pro); common variant c.488C>T (p.Pro163Leu)[b]	none
7321	c.2818A>C	p.Ser940Arg	rs1233555 c.301-49G>A	-	-	-	none

[a] Research analysis of multiple candidate gene coding and non-coding elements (reviewed in Roessler and Muenke, 2010).
[b] unpublished CLIA laboratory observations.

Table S4. Primers for Construction of Rat *Cdon* Site-Directed Mutants

Rat CDON mutant	Primer[1]
p.Thr681Ser	5’-catcatcaaaaaac**aGc**caAgcTtcctttccacctgtg-3’
p.Pro686Ala	5’-acccaggcgtcctttt**Gca**cctgtgggGatccctaagcggcct-3’
p.Val688Met	5’-gcgtcctttccacct**Atg**ggGatccctaagcggcct-3’
p.Val777Glu	5’-cttccaaactctct**gAg**gaagtccgAagCttagagccaggttcg-3’
p.Ile787Ala	5’-gagtttagagccaggAtcC**GCa**tacaaatttaggg-3’
p.Ser937Arg	5’-cccatgaaagagttg**CgT**acGcctcccagttcttca-3’

[1]Capitalized letters are changes from wild-type rat sequence. Bold letters designate the altered codon in the mutant. Bold capitals produce the amino acid substitution, capitals not in bold are silent changes that create a new restriction site used for identification of the site-directed mutant.