Supporting Information

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SI Experimental Procedures

Site-Directed Mutagenesis and Plasmid Constructs. The pRK5-Ptc, pRK5-Ptc-HA, constructs encoding Patched (Ptc) deleted of its last intracellular domain (pRK5-Ptc1-1165) or after caspase cleavage site (D1392) (pRK5-Ptc1-1392) pRK5-Sonic Hedgehog (SHH) and pcDNA3-DRAL-3xflagM2 were described in ref. 1. The pcDNA3-Bax-HA, the pcDNA3-Nedd4-myc, and the pCMV-Mdm2 were, respectively, a kind gift from A. Arrigo (Research Cancer Center of Lyon, Lyon, France), G. Melino (University of Leicester, Leicester, UK), and L. Corbo (Research Cancer Center of Lyon, Lyon, France). For direct two-hybrid, the coding sequence of the four WW domains of Nedd4 was amplified from pcDNA3-Nedd4-myc by PCR using the primers Nedd4WW-F and Nedd4WW-R (sequences given in Table S1) and then inserted into pGADT7 plasmid by digestion through EcoRI-XhoI. The pGBKT7-Ptc1165-1392 has been described previously (1). For immunoblot experiments to detect ubiquitination, either pRK5-Ubiquitin-HA or pcDNA3-Ubiquitin-3xflagM2 was used. Both constructs encode human ubiquitin (Ub) B. The pcDNA3-Nedd4C1286S-myc was obtained by directed mutagenesis via Quickchange strategy (Stratagene) on pcDNA3-Nedd4-myc with the primers indicated in Table S1 (Nedd4C1286S-F and Nedd4C1286S-R). For coimmunoprecipitations and immunoblots, the pcDNA3-caspase-9 dominant-negative [pcDNA3-Casp9(DN)] was used for transfection (2). For caspase activity assay pcDNA3-wild-type caspase-9 was used (pcDNA3-Casp9) (2). pcDNA3-Casp9 was used as templates to generate the pcDNA3-noncleavable caspase-9 (pcDNA3-NC Casp9) constructs by inserting two mutations (D315/330A) via Quickchange strategy (primers are indicated in Table S1). The pcDNA3-Casp9(DN), pcDNA3-Casp9 and pcDNA3-NC Casp9 were then used as templates to generate the mutation of five lysines into arginine located at the C-terminus part of the caspase-9 (K394/398/409/410/414R) (caspase-9 5KR). pcDNA3-Casp9 was also used as templates to generate each single mutation K394R, K398R, K409R, K410R, and K414R by directed mutagenesis via Quickchange strategy (Table S1). ShRNA constructs used to silence Nedd4 (ShNedd4) were made with pSilencer 1.0-U6 siRNA expression vector (Ambion) according to the manufacturer's instructions.

Cell Cultures, Transfection Procedures, Reagents. Transient transfection of HEK293T was performed with calcium phosphate for coimmunoprecipitation or with Jetprime (Polyplus) for cell death assay and immunoblot according to the manufacturer's instructions. Caspase-9 null mouse embryonic fibroblasts (MEF *Casp9*$^{-/-}$) were a kind gift from D. Green (St. Jude Children's Research Hospital, Memphis, TN). MEF cells were cultured in DMEM media supplemented with 10% calf fetal serum and 2 μL of β-mercaptoethanol. MEF cells were transfected with Jetprime (Polyplus) for cell death assay. Recombinant SHH-N was from R&D Systems and were added at the time of transfection at 600 ng/mL. MG132 was used at 0.5 μg/mL for 2 h and was purchased from Sigma. For siRNA experiments, cells were transfected with 60 pmols siRNA using Jetprime reagent. Nedd4 and control siRNAs were from Sigma.

Two-Hybrid Analysis. Matchmaker two-hybrid system III (Clontech) was used according to the manufacturer's instructions using AH109 yeast cotransformed with pGBKT7-DNA binding domain GAL4 fused to Ptc 1165–1392 (pGBKT7-Ptc7IC) and the pGADT7-GAL4 transcriptional activation domain AD fused to four WW domains of Nedd4 (pGADT7-WWNedd4) (Clontech). Cells were then allowed to grow in the absence of leucine, tryptophane, adenine, and histidine, and in the presence of X-α Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) (10 mg/mL) and 3-amino-1,2,4-triazole (55 mM). As a negative control, yeast cells were cotransformed with pGBKT7-Ptc7IC and empty pGADT7 vector, or with pGADT7-WWNedd4 and empty pGBKT7 vector.

Coimmunoprecipitation and Immunoblotting Analysis. Coimmunoprecipitation were carried out on endogenous proteins in embryonic day (E) 14 mouse brain or in MiaPaca-2 human pancreatic cell line and on overexpressed proteins in HEK293T cells, as described previously (1). To assay shRNA efficiency on Nedd4 protein, HEK293T were transfected using both Nedd4-encoding vector and ShRNA control or ShNedd4. For experiments requiring SHH, in addition to transfection with SHH-encoding vector, recombinant SHH was added at a final concentration of 600 ng/mL 24 h before harvesting the cells. E14 mouse brain were mechanically dissociated, frozen at −80 °C, and lysed in 50 mM Hepes pH 7.6, 150 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40 in the presence of protease inhibitor mixture (Roche) by 40 strokes in a Dounce homogenizer. HEK293T cells were lysed in the same buffer and solubilized proteins were further incubated with anti–Ptc-1 (Santa Cruz Biotechnology; 0.8 μg/mL), anti-FlagM2 (Sigma; 2.4 μg/mL), anti-Myc (Sigma; 2.4 μg/mL), anti-Nedd4 (Santa Cruz Biotechnology; 1 μg/mL), or anti–caspase-9 (Santa Cruz Biotechnology; 0.8 μg/mL) antibodies and protein-A (Sigma) or protein-G Sepharose (GE Healthcare). Washes were done in 50 mM Hepes pH 7.6, 150 mM NaCl, 5 mM EDTA (four washes). For Western blot and coimmunoprecipitation performed to detect ubiquitinated caspase-9, HEK293T cells were harvested 48 h after transfection. For Western blot, cells were lysed in 30 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol and 1% SDS, or in 0.1M NaH_2PO_4, 10 mM Tris-HCl, 500 mM NaCl, 0.1% Triton-X100, 10 mM β-mercaptoethanol, and 8 M Urea in the presence of protease inhibitor mixture (Roche). For coimmunoprecipitation, cells were heated in SDS 1% at 95 °C for 5 min and then diluted in 30 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol. Lysates were further incubated with anti–caspase-9 (Santa Cruz Biotechnology; 0.8 μg/mL) or anti-HA (Sigma; 2.4 μg/mL) antibodies and protein-A Sepharose (Sigma). Washes were performed in 30 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol (four washes). Immunoblots were performed as previously described (1) using anti-Myc (Sigma; 1/1,000), anti-FlagM2 (Sigma; 1/5,000), anti-HA (Sigma; 1/10,000), anti–Ptc-1 (Santa Cruz Biotechnology; 1/1,000), anti–caspase-9 (Cell Signaling; 1/2,000), anti–Caspase-9 (Immunotech; 1/2,000), anti–ß-actin (Millipore; 1/5,000), anti-polyUbiquitin (EnzoLife; 1/2,000), anti-Ubiquitin, Lys63-specific (Millipore; 1/2,000), anti-Ubiquitin, Lys48-specific (Millipore; 1/2,000), and anti-p21 (Dako; 1/1,000) antibodies.

Cell Death Analysis and Caspase Assays. Cell death was analyzed 24 h after transfection using Trypan blue staining procedures. Caspase-3 activity assay was performed 24 h after transfection using the caspase-3 fluorometric assay kit (BioVision), as described previously (1). Caspase-9 activity was measured 18 h after transfection using the luminescent Caspase-Glo 9 Assay according to the manufacturer's instructions (Promega).

Quantitative RT-PCR. To assay Nedd4 mRNA expression in HEK293T cells transfected with siRNA against Nedd4, total RNA was extracted from cells with the Nucleospin RNAII kit (Macherey-Nagel) and 1 μg of RNA was reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad). Real-time quantitative RT-PCR was performed on a LightCycler 2.0 apparatus using the LightCycler TaqMan Master kit (Roche Applied Science) according to the manufacturer's instructions. The ubiquitously expressed β-actin gene was used as internal control. Primers and probe were given by Universal Probe Library Assay Design Center Web site (Roche Applied Science). Sequences are shown in Table S1.

Statistics. The statistical significance of differences between groups was evaluated by the Mann–Whitney *U* test. Mean values for all outcome variables are presented with SEMs. Data presented are representative of at least four independent experiments. All statistical tests were two-sided, and *P* values less than 0.05 were considered to be statistically significant.

1. Mille F, et al. (2009) The Patched dependence receptor triggers apoptosis through a DRAL-caspase-9 complex. *Nat Cell Biol* 11:739–746.

2. Forcet C, et al. (2001) The dependence receptor DCC (deleted in colorectal cancer) defines an alternative mechanism for caspase activation. *Proc Natl Acad Sci USA* 98:3416–3421.

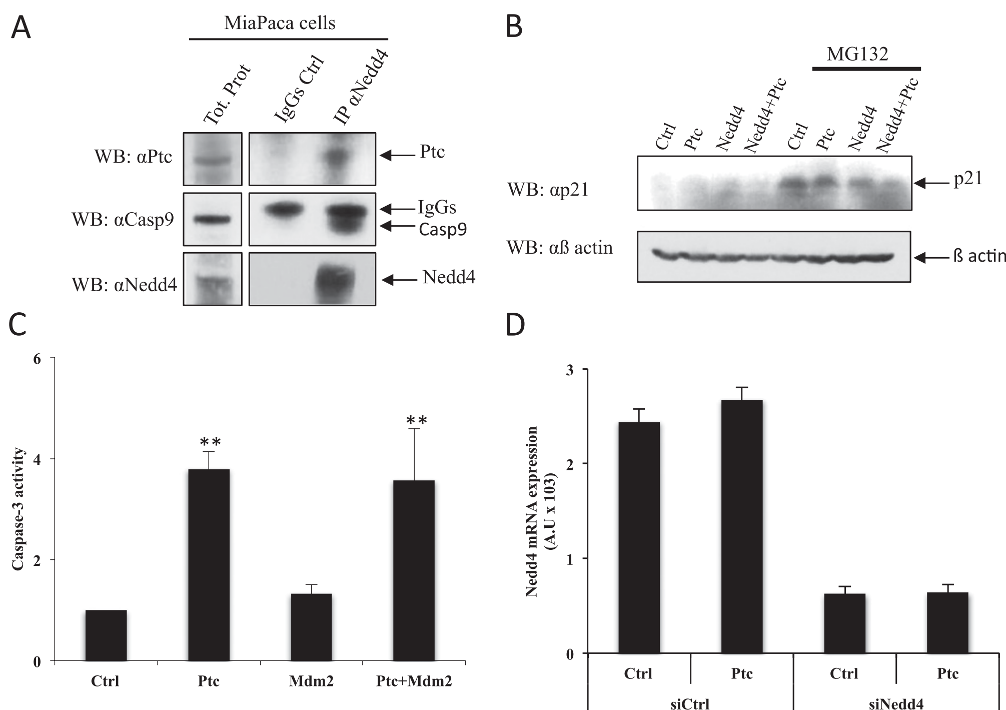

Fig. S1. Controls for Nedd4 specificity. (*A*) Coimmunoprecipitation experiments were performed on Mia-Paca-2 cells. Pull-down with anti-Nedd4 antibody was used to immunoprecipitate Nedd4 followed by Western blot using anti-Ptc (αPtc), anti–caspase-9 (αCasp9), or anti-Nedd4 (αNedd4), as a control of immunoprecipitation efficiency. (*B*) Immunoblot using p21 antibody on HEK293T cells transfected with an empty vector (Ctrl) or with Ptc-encoding vector (Ptc), Nedd4-encoding vector (Nedd4), or Ptc- and Nedd4-encoding vectors (Ptc+Nedd4) in the absence or in the presence of MG132 (0.5 μg/mL). (*C*) Caspase-3 activity assay was performed on HEK293T cells 24 h after transient transfection with an empty vector (Ctrl) or with Ptc-encoding vector (Ptc), Mdm2-encoding vector (Mdm2), or Ptc- and Mdm2-encoding vectors (P+Mdm2). Folds over control are represented and error bars are SEM. $^{**}P < 0.01$. (*D*) Nedd4 mRNA relative expression assessed by real-time quantitative RT-PCR in HEK293T cells transfected with siRNA control (siCtrl) or siRNA Nedd4 (siNedd4) in the absence (Ctrl) or in the presence of Ptc (Ptc).

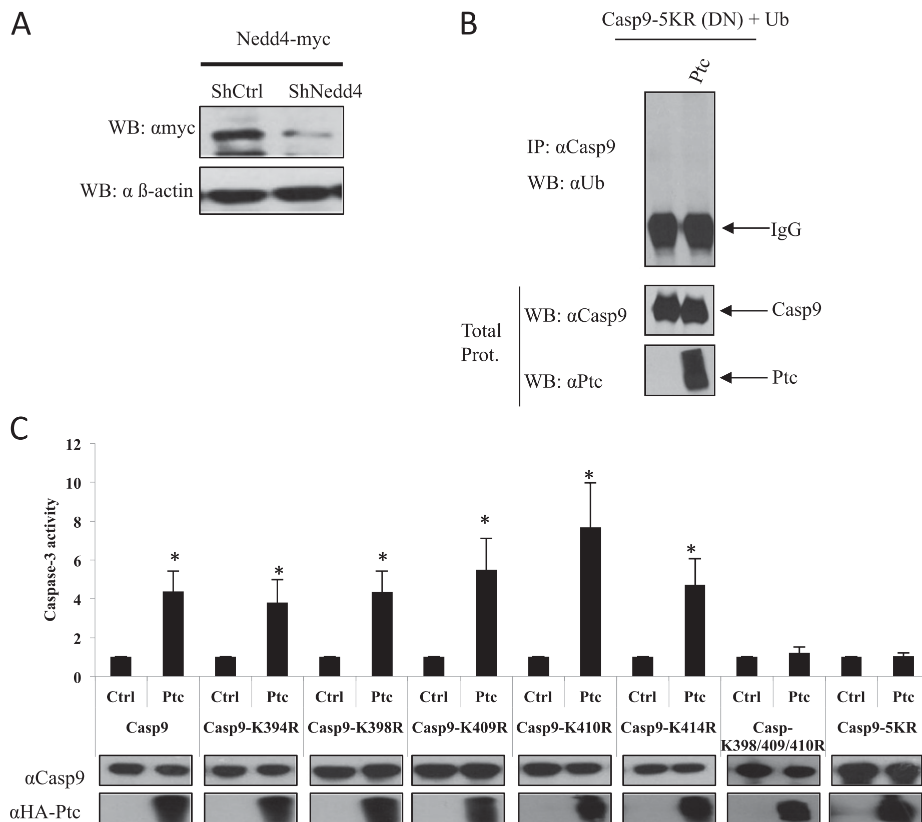

Fig. S2. Controls for caspase-9 lysines involved in Ptc-induced apoptosis. (*A*) Immunoblot performed 48 h after transfection on HEK293T cells overexpressing Nedd4-myc in the presence of shRNA control (shCtrl) or shRNA against Nedd4 (shNedd4) using myc and β-actin antibodies. (*B*) Immunoprecipitation of caspase-9 (IP: αCasp9) was performed on SDS-lysed HEK293T cells expressing catalytically dead caspase-9 5KR [Casp9-5KR (DN)] and Ubiquitin proteins (Ub) in the absence or in the presence of Ptc (Ptc-HA). Western blot using an anti-ubiquitin (αUb) shows the absence of ubiquitination of 5KR mutated caspase-9. (*C*) Caspase-3 activity assay was performed in HEK293T cells 24 h after transfection with wild-type caspase-9 (Casp9), caspase-9 mutated on each single lysines (Casp9-K394R, Casp9-K398R, Casp9-K409R, Casp9-K410R, and Casp9-K414R), caspase-9 mutated on three lysines (Casp9 K398/409/410R), or caspase-9 mutated on five lysines (Casp9-5KR) together with either an empty vector (Ctrl) or a Ptc-encoding vector (Ptc). Anti-HA (αHA-Ptc) and anti–caspase-9 (αCasp9) immunoblot are shown as a control of loading. Folds over control are represented and error bars are SEM. *$P < 0.05$.

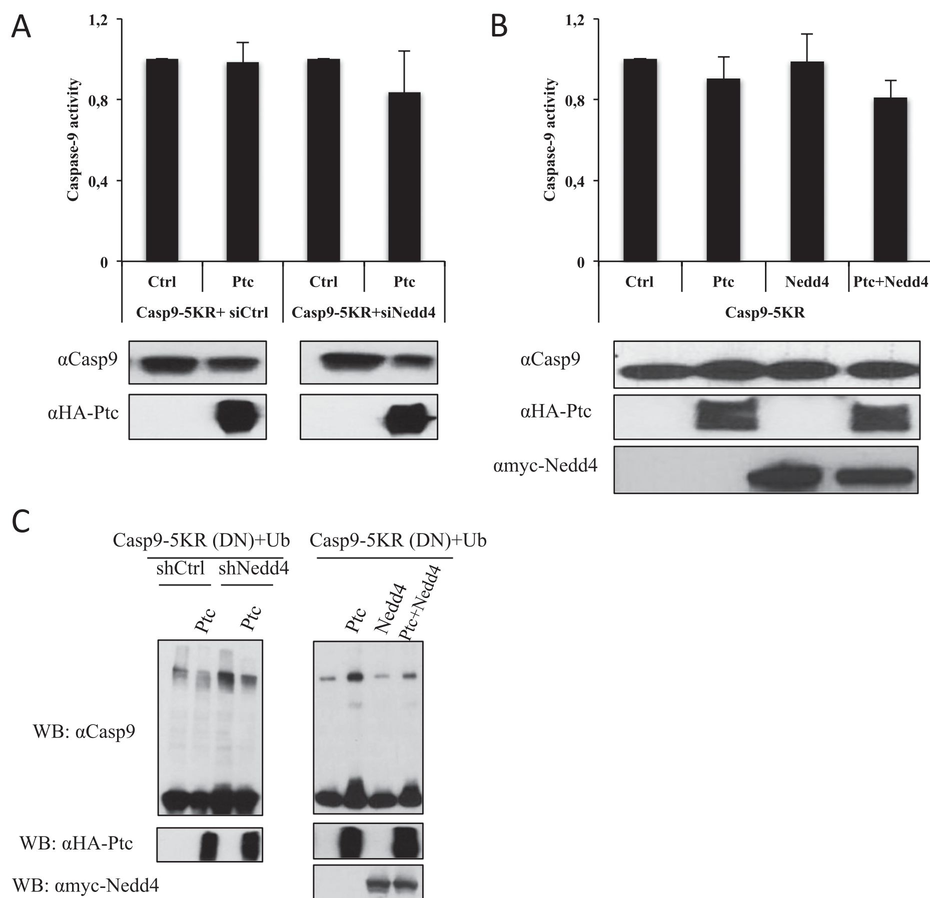

Fig. S3. Controls for caspase-9 5KR activation and ubiquitination. (*A* and *B*) Caspase-9 activity assays were performed using a proluminogenic caspase-9 substrate 18 h after transfection of HEK293T cells with caspase-9 5KR together (*A*) with siRNA control (Casp9-5KR+siCtrl) or siRNA Nedd4 (Casp9-5KR+siNedd4), in the absence (Ctrl) or in the presence of Ptc (Ptc) (*B*) with empty vector (Ctrl), Ptc-encoding vector (Ptc), Nedd4-expressing construct (Nedd4), or Ptc- and Nedd4-encoding vectors (Ptc+Nedd4). Anti-HA (αHA-Ptc), anti–caspase-9 (αCasp9), and anti-myc (αmyc-Nedd4) immunoblots are shown as a control of specificity and loading. Folds over control are represented and error bars are SEM. (*C*) Western blot using caspase-9 antibody was performed on HEK293T cells lysed in 8 M urea lysis buffer, 48 h after transient transfection of a Casp9-5KR (DN) and ubiquitin proteins (Ub) together with shRNA control (shCtrl) or a shRNA against Nedd4 (shNedd4) in the presence of an empty vector or Ptc-encoding vector (Ptc-HA) or together with an empty vector, with Ptc-encoding vector (Ptc-HA), with Nedd4-encoding vector (Nedd4-myc), or with Nedd4- and Ptc-encoding vectors (Ptc-HA+ Nedd4-myc). Anti-HA (αHA-Ptc) and anti-myc (αmyc-Nedd4) immunoblot are shown as a control of loading.

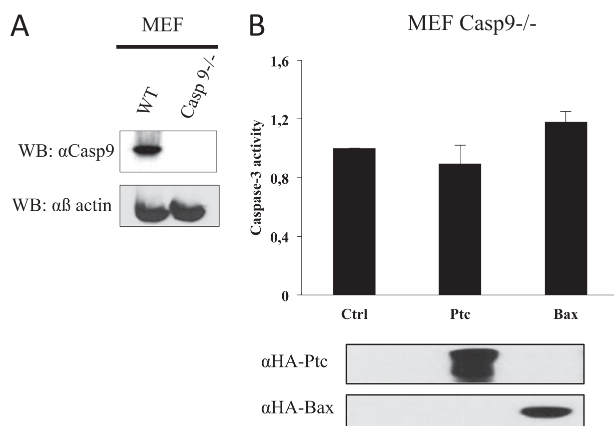

Fig. S4. Controls in caspase-9–deficient MEF cells. (*A*) Immunoblot performed on wild-type (WT) and caspase-9$^{-/-}$ MEF cells (*Casp9*$^{-/-}$) using caspase-9 and β-actin antibodies. β-Actin was used as control of loading. (*B*) Caspase-3 activity assay was performed in MEFs deficient for caspase-9 (MEF *Casp9*$^{-/-}$) 24 h after transfection with either an empty vector (Ctrl), a Ptc-encoding vector (Ptc), or a Bax-encoding vector (Bax). Anti-HA (αHA-Ptc and αHA-Bax) and anti–caspase-9 (αCasp9) immunoblots are shown as controls of specificity and loading. Folds over control are represented and error bars are SEM.

Table S1. Oligonucleotides used for direct mutagenesis and real-time quantitative RT-PCR

Primers and probes	Sequences
Nedd4WW-F	5′-CTCCTCTACCTCCAGGGTGGGAAGAGA-3′
Nedd4WW-R	5′-CACCCTCGAGCAATCTTGGATCTTCCCAGGTGGTGGT-3′
Nedd4C1286S -F	5′-AGCTGCCAAGAGCTCATACCTCATTTAATCGCCTGGACTTGCC-3′
Nedd4C1286S-R	5′-GGCAAGTCCAGGCGATTAAATGAGGTATGAGCTCTTGGCAGCT-3′
NCC9-D315A -F	5′-CTGGCAGTAACCCCGAGCCAGCTGCCACCCCGTTCCAGGAAGGTTT-3′
NCC9-D315A -R	5′-AAACCTTCCTGGAACGGGGTGGCAGCTGGCTCGGGGTTACTGCCAG-3′
NCC9-D330A -F	5′-GTTTGAGGACCTTCGACCAGCTGGCCGCCATATCTAGTTTGCCCACACCCA-3′
NCC9-D330A -R	5′-TGGGTGTGGGCAAACTAGATATGGCGGCCAGCTGGTCGAAGGTCCTCAAAC-3′
C9-K394R-F	5′-GCTAATGCTGTTTCGGTGCGAGGGATTTATAAACAGATG-3′
C9-K394R-R	5′-CATCTGTTTATAAATCCCTCGCACCGAAACAGCATTAGC-3′
C9-K398R-F	5′-CGGTGAAAGGGATTTATCGACAGATGCCTGGTTGCTT-3′
C9-K398R-R	5′-AAGCAACCAGGCATCTGTCGATAAATCCCTTTCACCG-3′
C9-K409R-F	5′-GCTTTAATTTCCTCCGGCGAAAACTTTTCTTTAAAAC-3′
C9-K409R-R	5′-GTTTTAAAGAAAAGTTTTCGCCGGAGGAAATTAAAGC-3′
C9-K410R-F	5′-TTAATTTCCTCCGGAAACGACTTTTCTTTAAAACATC-3′
C9-K410R-R	5′-GATGTTTTAAAGAAAAGTCGTTTCCGGAGGAAATTAA-3′
C9-K414R-F	5′-CTCCGGAAAAAACTTTTCTTTCGAACATCAGACTACAAGG-3′
C9-K414R-R	5′-CCTTGTAGTCTGATGTTCGAAAGAAAAGTTTTTTCCGGAG-3′
TaqNedd4-F	5′-GGCTCAGAAGATGATAATGCAGA-3′
TaqNedd4-R	5′-GCATCTGGTTGGTCCAAAAC-3′
Taqßactin-F	5′-ATTGGCAATGAGCGGTTC-3′
Taqßactin-R	5′-GGATGCCACAGGACTCCAT-3′
Probe TaqNedd4	5′-GGCTGAGG-3′
Probe Taqßactin	5′-CTTCCAGC-3′