SUPPLEMENTAL MATERIALS & METHODS

Quantitative Real-Time PCR and Western Blot Analysis

Relative transcript expression levels were measured by quantitative real-time PCR using a SYBR Green-based method. The primer sequences are summarized in Table S4. Average fold changes were calculated by differences in threshold cycles (Ct) between pairs of samples. The β-Amyloid precursor gene was used for normalizing the cDNA concentration of each sample.

Protein lysates were prepared by resuspending cell pellets in Laemmli sample buffer, and proteins were separated by electrophoresis on 4% to 12% Tris-glycine gels and transferred onto a polyvinylidene difluoride membrane. After blocking, the membrane was incubated with anti-V5 antibody (Invitrogen) overnight, washed, incubated with horseradish peroxidase-conjugated secondary antibody, and detected with enhanced chemiluminescence solution (Thermo Scientific).

Luciferase Reporter Assay

DNA fragments spanning +1212 to –497 bp of the DLGAP5 promoter region were obtained by PCR using OVCAR3 genomic DNA as template, and were subcloned into pGL3-basic. PIN1 promoter reporter plasmid was a kind gift from Dr. Giannino Del Sal. Deletion mutation constructs were produced by two-step PCR amplification. All PCR products were subcloned into pGL3-basic plasmid (Promega) and validated by DNA sequencing. pRL-Renilla reporter plasmid (Promega) was co-transfected with the pGL3 plasmid to test for transfection efficiency.

DLGAP5 Expression Vector and Rescue Assay

The PCR product of DLGAP5 cDNA was cloned into the pLentiToV5HisPuro expression vector. For the rescue assay, OVCAR3 and MCF7 cells were first transfected with the pLenti-DLGAP5 vector (or empty pLentiToV5HisPuro vector as a negative control) by Lipofectamine 2000 (Invitrogen). The transfected cells were then treated with either MRK003 or DLGAP5 shRNAs. Cell numbers from each group were measured at different time points. The sequence for NOTCH3 shRNA4 is CCGGCTCGGTAGTAATGCTGGAGATCTCGAGATCTCCAGCAT TACTACCGAGTTTTT; the sequence for NOTCH3 shRNA5 is CCGGCCAGTTCACCTGTATCTGTATCTCGAGATACAGATACAGGTGAACTGGTTTTT.

Gene Knockdown and Growth Assay

shRNAs that targeted DLGAP5 were cloned into the pLKO.1 vector (Sigma). Their sequences were: DLGAP5 shRNA1 (CCGGGCACATTGGTCAAACAACTCGAGTT GTTTGACCAACTGCTGTGCTTTTTG); DLGAP5 shRNA3 (CCGGGCATTCCACACTA CATCTCGAGATGTAGTTTGTTGTGGAATGCTTTTTG). The packaging plasmid psPAX2 and envelop plasmid pMD2.G were co-transfected with each of the shRNA vectors into HEK293FT cells (Invitrogen). Viral supernatants were harvested two days after plasmid transfection. Filtered viral supernatants were used directly to transduce ovarian cancer cells, and transduced cells were harvested 24-72 hours post-transduction for quantitative real-time PCR analysis, cell growth assay, and apoptosis detection. For cell growth assays, cells were plated in 96-well plates at a density of 6,000 per well. Cell number was assessed by measuring the incorporation of SYBR Green I using a fluorescence microplate reader (FLUOstar, BMG). One million transduced OVCAR5 or A2780 cells were injected into subcutaneous tissue of nude mice. Animals were sacrificed for necropsy at 2, 3, and 5 weeks.

Cell Cycle and Apoptosis Analysis

DAPI (4',6-diamidino-2-phenylindole) (5 mg/ml) was used as a nuclear stain for cell cycle analysis. Annexin V-FITC apoptosis kit (Biovision) was used to detect early apoptotic cells. Forty-eight hours after DLGAP5 shRNA lentivirus infection, cells were stained with either DAPI or Annexin V-FITC/PI, and were quantified in a BD LSRII cytometer. Data were analyzed using FlowJo software version 8.7 (TreeStar, Ashland, OR).