**Supplementary Materials and Methods**

**DNA extraction**

We also evaluated DNA and RNA from lung primary tumors and blood (or normal lung tissues) from 108 patients diagnosed with LSCCs. For genomic DNA extraction, freshly frozen tissue from tumors was meticulously dissected to ensure enriched material comprising at least 40% tumor cells. Approximately 10-20-μm sections were collected from normal and tumor samples and placed in 1% SDS/proteinase K (10 mg/ml) at 58ºC overnight. Digested tissue was then subjected to phenol–chloroform extraction and ethanol precipitation, following standard protocols. Available slides were reviewed and classified according to WHO criteria and informed consent was obtained from each patient.

***PARD3* direct sequencing and loss of heterozygosity analysis**

We analyzed loss of heterozygosity (LOH) using three microsatellite markers (D10S1666, D10S1780 and D10S1791) spanning 4 Mb of the short arm of chromosome 10. PCRs were performed using fluorescent-labeled primers and according to the previously described protocol (1). The normal matching genomic DNA from each tumor was used for comparison. Ratios greater than or equal to 1.35 or less than or equal to 0.67 were considered to be unambiguously indicative of LOH. Homozygous cases were considered to be not informative (NI) for LOH.

The sequence of primers used for the cloning and the mutation screening of the *PARD3* gene are provided in Supplementary Table S2. The primers were not designed to amplify the short variants of the *PARD3* gene. PCR products were cycle-sequenced using Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA) with an ABI PRISM 3700 DNA Analyzer (Perkin Elmer LifeSiences, Inc., Boston, MA). All of the variants identified in the study were confirmed by the resequencing of independent PCR products.

**Methylation specific and Multiplex ligation-dependent probe amplification analysis (MS-MLPA) of PARD3**

After the MS-MLPA reaction, fragments were separated on an ABI 3130XL apparatus (Applied Biosystems). MLPA fragment analysis and comparative analysis were performed using Coffalyser.Net software. The MS-MLPA probemix contains four probes with an HhaI recognition site for detecting the methylation status of the promoter region of the *PARD3* gene and 15 probes for detecting the PARD3 copy number status. In addition, 11 reference probes were included that were located at genomic regions known to be relatively silent in lung cancer. In short, for both patient and reference samples, 100 ng of genomic DNA were denatured and SALSA MLPA buffer and MS-MLPA probemix were added. Probes were hybridized overnight

(~16 h) at 60°C. The mixture was then diluted at RT with H2O and ligase buffer A, and divided equally between two tubes. A mixture containing ligase-65 (MRC-Holland), HhaI (Promega) and ligase buffer B was added to one tube. For the second tube, the HhaI enzyme was replaced with H2O. Simultaneous ligation and digestion were then performed by incubating for 30 min at 48°C, followed by 5 min heat inactivation of the enzymes at 98°C. PCR was performed following the manufacturer’s instructions (MRC-Holland). MLPA fragment analysis and comparative analysis were performed using Coffalyser (2-3).Net software, which is available at <http://www.intechopen.com/books/modern-approaches-to-quality-control/analysis-of-mlpa-data-using-novel-software-coffalyser-net-by-mrc-holland>.

**Bisulfite treatment and bisulfite genomic sequencing**

To validate the MS-MLPA data we performed bisulfite genomic sequencing of the promoter region of *PARD3*. Genomic DNA was converted using the EZ DNA Methylation Gold kit (Zymo Research, Orange, CA). The modified DNA was used as a template for bisulfite genomic PCR. The PCR products were cloned in the pGEM-T Easy Vector System (Promega, Madison, WI). A minimum of eight single clones was interrogated for each sample and the methylation frequency was calculated in each case. The sequence of primers spanning the CpG island of the *PARD3* gene promoter, used for bisulfite genomic sequencing, is available upon request.

**Expression vectors, transfections and infections**

To determine the frequency of the various *PARD3* variants we amplified the entire coding sequence of *PARD3* by RT-PCR in two separate cDNA pools, each consisting of a mixture of four cDNA products from normal RNAs. The amplified products were cloned in a TOPO-TA vector, following the manufacturer’s instructions. Positive colonies were picked, grown and processed using the NucleoSpin Multi-96 Plus plasmid purification kit (Macherey-Nagel, Bethlehem, PA) and automatically sequenced. More than 80 clones were individually sequenced. We determined that the transcript lacking 9 bp between exon 15 and exon 16 NM\_001184785.1 was the most abundant in normal lung, accounting for almost 30% of all transcripts of *PARD3.*

For ectopic expression of PAR3 proteins, full-length human *PARD3* wild type (NM\_001184785) and HA-tag were cloned into the HindIII and NotI restriction sites of the vector pcDNA4TO (Invitrogen, Carlsbad, CA). The different mutants were obtained by directed mutagenesis in the same vector. All constructs were verified by automatic sequencing. For transient transfections we used Lipofectamine 2000 (Invitrogen), following the manufacturer’s protocol. The stable and conditional PAR3tr cells, which carry the tet-repressor plasmid, were generated using the Retro-X Tet in an advanced inducible expression system (Clontech, Mountain View, CA). The H157 and the glioblastoma-derived T98G cells were infected with the tetracycline (tet) transactivator expression construct and the tet-controlled expression vector (tet-on) alone (H157tr and T98Gtr) or containing the entire wild type (H157tr-wtPAR3 and T98Gtr-wtPAR3) or various PAR3 mutant coding sequences (H157tr-41\_74del, H157tr-R345H, H157tr-T861S, H157tr-I1043M and T98Gtr-41\_74del, T98Gtr-R345H, T98Gtr-T861S, T98Gtr-I1043M). Individual colonies were selected and analyzed for inducible expression of PAR3 by western blot, immunofluorescence and real-time quantitative PCR. To avoid clone-related bias that might affect the analysis, three clones of each wild type and mutant clone were pooled to obtain the different H157 and T98G- derived cells.

To deplete PAR3 expression we used shRNAs, which were purchased from SIGMA-MISSION (LentiExpress™ Technology, Sigma-Aldrich, St. Louis, MO) as a glycerol stock of 5 pLKO plasmids carrying *PARD3-*specific shRNA sequences. To obtain stable expression of the shRNAs different cell populations were selected with puromycin for 2-3 weeks.

**Colony formation, MTT and cell migration assays**

For colony formation, assays with the indicated mammalian expression vectors cells were seeded at a density of 1,500,000 cells/100 mm diameter plate in RPMI media containing 10% (v/v) fetal bovine serum. After 24 h, cells were transfected and stained 14-15 days later with Cristal Violet (Merck Millipore, Darmstadt, Germany). Each assay was performed in triplicate. The information regarding the empty vector as a control was not included in results. We found a larger number of colonies in the empty vector control (data not shown), as compared to the wtPAR3 and the to the various PAR3 mutant proteins. However this could not be attributable to a higher capability to form colonies, but rather to an increased in the transfection efficiency, because of the differences in size between the control vector, pcDNA4TO, and the pcDNA4TO-PARD3 (either wild type or mutants), that exceed the 2500–bp.

For the MTT assays, cells were seeded at a density of 5,000 cells/well on 96-well plates. After induction of PAR3 expression with doxocyclin, the number of viable cells was measured by the 3-(4,5- dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 10 μL of a solution of 5 mg/mL MTT (Sigma Chemical, Zwijndrecht, The Netherlands) was added. After incubation for 3 hr at 37ºC, the medium was discarded, the formed formazan crystals were dissolved in 100 ml DMSO and absorbance was determined at 560 nm by means of a microplate reader (Bio-Rad, Hercules, CA).

Cell motility was examined by a real-time migration assay through the xCELLigence system (Roche Applied Science). The upper chamber of the CIM-plates was coated with 0,22 mg/ml of collagen I or with 2 μg/μL of fibronectin. 40000 cells/well were seeded onto the upper chamber in serum free media. Fresh RPMI 10% FBS was added to each well of the lower chamber. CIM plates were placed onto the Real-Time Cell Analyzer (RTCA) station (xCELLigence System, Roche, Mannheim, Germany). Cell migration was continuously monitored by measuring changes in the electrical impedance at the electrode/cell interface, as a population of cells migrated from the top to the bottom chamber. Continuous values are represented as cell index (CI), a dimensionless parameter that reflects a relative change in measured electrical impedance, and quantified as a slope (h-1) between two different 4 h intervals (n=3). The rate of migration

was determined by calculating the slope of the line between two given time points.

**Quantification of protrusions and PAR3 staining in the membrane**

To evaluate the localization of PAR3 in cells, transiently transfected cells were fixed and immunostained against HA. Random fields from each digital image were counted. The number of cells, expressed as a percentage of the total number of cells examined, showing stronger PAR3 staining at the membrane than of the cytoplasm were judged to have predominant PAR3 at the membrane. 100-120 cells were randomly selected and counted for each condition.

**RAC1 activation assay and calcium switch assays**

 To determine the levels of RAC1 bound to GTP (activated RAC1) we used the Rac1/Cdc42 Activation Assay Kit (Millipore, Temecula, CA). Equal amounts of cell lysates were prepared and incubated with 10 μg of PAK-1 PBD agarose to bind activated RAC1 (RAC1-GTP). The proteins bound to PAK-1 PBD were separated by SDS-PAGE, transferred to PVDF and probed with anti-RAC1 mouse monoclonal antibody. To ensure equal protein loading, we also subjected the same amounts of cell lysates to Western blot analysis using antibodies against RAC1.

To disrupt cell-cell junctions we used calcium switch experiments. Briefly, cells were grown up to confluence and then were incubated with 4 mM EGTA for 30-60 min at 37 °C. After that, the cells were cultured in regular media at the indicated hours, to re-establish the cell-cell contacts.

**Antibodies, western blot, immunofluorescence, immunoprecipitation and immunostaining**

Anti-PAR3 (07-330, Millipore, Temecula, CA), anti-ZO1 (339194, Invitrogen), anti-HA (H6908, Sigma-Aldrich, Saint Louis, MO), anti-TUBULIN (21058, Abcam, Cambridge, MA), anti-PHALLOIDIN (A12380, Invitrogen), anti-aPKC (17837, Santa Cruz Biotechnologies, Santa Cruz, CA), anti-phospho-aPKC (76129, Abcam), anti-BACTIN (1616, Santa Cruz Biotechnologies), anti-STAT3 (9132, Cell Signaling, Danvers, MA), anti-phosphoSTAT3 (4113, Cell Signaling) and anti RAC1 (05-389, Millipore) antibodies were used for western blot, immunoprecipitation and/or immunofluorescence. For western blots, cells were scraped from the dishes into lysis buffer and 50 μg of total protein was separated by SDS-PAGE, transferred to a PVDF and western blotted according to the antibody manufacturer’s suggestions. The secondary antibodies anti-mouse HRP and anti-rabbit HRP (DAKO, Glostrup, Denmark) were used at a final dilution of 1:5000. For immunofluorescence, cells growing on coverslips were treated with doxycycline or infected transiently with the various PAR3 constructs. After 24 h the coverslips were fixed in 4% formaldehyde (Electron Microscopy Sciences, Hatfield, UK) for 20 min. The preparation was washed in PBS and blocked with 2% BSA/0.1%Triton X-100 in PBS, then incubated for 1 h with the corresponding antibody. Excess antibody was removed by washing three times in 0.1% tween in PBS. Labeling was revealed with anti-mouse IgG-Alexa488 and IgG-Alexa594 (Molecular Probes, Eugene, OR) at 1:200 and incubated for 1 h. Cell nuclei were revealed by incubation with 1 µM DAPI (48,6-diamidino-2-phenylindole) for 5 min. All preparations were mounted with Mowiol mounting medium (Sigma-Aldrich). Fluorescence was analyzed using the Leica TCS SP5 inverted confocal spectral fluorescence microscope and the Zeiss Axio Observer Z1 Apotome.

For immunoprecipitations, H157 cells were transfected with the indicated plasmids. After 24 h, cells were lysed in 10 mM Tris-HCl, pH 8.5 mM EDTA, 100 mM NaCl, 10 mM NaF and 0.05% Triton X-100 supplemented with protease and phosphatase inhibitors, followed by clearance of lysates by microcentrifugation. The soluble supernatants were precleared with protein A-Agarose beads (Millipore) for 1 h and incubated with the specific antibody ON at 4ºC. The immunocomplexes were precipitated from solution using the same beads, washed three times with lysis buffer, and eluted by boiling in SDS-PAGE sample buffer, and then subjected to immunoblot analysis with the indicated antibodies.

Formalin-fixed paraffin-embedded tissue blocks from lung primary tumors and HNSCCs were used for tissue microarrays, following a previously described protocol (4). We performed immunohistochemical staining with PAR3 antibody (1:1000 dilution) and pY-STAT3 antibody (1:150 dilution). Three-micrometer-thick sections from the tumors distributed in tissue microarrays were transferred to silanized glass slides. After deparaffinization and quenching the endogenous peroxidase, the slides were boiled in citrate buffer for 30 min. After antibody incubation, immunodetection was performed with the Super SensitiveTM Link-Label IHC Detection System Label (Biogenex, The Hague, The Netherlands) and with diaminobenzidine chromogen as the substrate (Invitrogen). Sections were counterstained with hematoxylin and evaluated under a Leica DM1000 microscope. Four investigators, including two pathologists (EC and EBr), evaluated the sections using uniform criteria. PAR3 immunostaining was scored as follows: no staining, (-); low-intensity staining, (+); moderate-intensity staining, (++); and strong-intensity staining (+++). In the case of HNCCs, all the slides were stained simultaneously in an automated horizontal slide-processing system (Dako Autostainer Plus). Negative controls with either omitting the primary antibody or with a normal mouse IgG (Santa Cruz Biotechnology, Inc.) in the primary incubation were also included. pY-STAT3 immunostaining was scored as follows: no staining or less than 10% of cells with low-intensity staining, (-); low-intensity staining or less than 10% of cells with strong-intensity staining (+); strong-intensity staining (++).

**Microarray global gene expression analysis and quantitative RT-PCRs**

For scanning we used a G2505B DNA microarray scanner. Images were quantified using Agilent Feature Extraction Software (version 9.5). Fluorescence intensity of each array element was subtracted from the local background. Data were normalized as previously described (5). To determine the mRNA levels we used real-time RT-quantitative PCRs. DNase-treated RNA was reverse-transcribed. cDNA was amplified using an ABI Prism 7900 Sequence detector (Applied Biosystems), and levels of genes were measured by SYBR green real-time PCR. Reactions were carried out in triplicate. As controls we used human ACTB and PP1A to correct for inter-individual/tumor variation.

We selected transcripts that fulfilled the following criteria: i) at least 1.5-fold induction or repression in the wild type compared with mock cells; ii) adjusted p<0.05. The differentially expressed genes were functionally classified according to the gene ontology (GO) database and exhaustive searches of PubMed. Gene Set Enrichment Analysis (GSEA) was applied to the ranked list using the *PARD3* gene expression signature as a gene set.

***In vivo* experiments**

For the *in vivo* experiments we used athymic mice male nu/nu, aged 4-5 weeks (Harlan-Laboratories, Inc., Indianapolis, IN), maintained in a sterile environment. The animals were maintained under pathogen-free conditions. The experimental designs were approved by the Institutional Animal Care and Use Committee of the IDIBELL. All the animals drank 2% sucrose plus 2 mg/ml doxycycline in water *ad libitum* to induce PAR3 ectopic expression.

For the spleen metastasis model, 1x106 cells were injected into the spleen of 13 mice (6 and 7 mice for the H157tr and H157tr-PAR3wt cells, respectively). Briefly, cells were intrasplenically injected with a tuberculin syringe into the middle of the mouse’s spleen. To minimize local tumor growth, mice were anesthetized and the spleen surgically resected 48 h after injection. Four days after cell injection, PAR3 expression was induced in mice that drank water supplied with 2% sucrose plus 2 mg/ml doxycycline *ad libitum*. All the mice were killed 5 weeks later and the hepatic and lung metastases were examined. Hepatic and lung metastases were examined macroscopically and microscopically following hematoxylin and eosin tissue staining.

To generate the orthotopic model, athymic male *nu/nu* mice aged 4-5 weeks were maintained in a sterile environment. First, the H157tr and H157tr-PAR3wt cells (1.5x106 cells) were grafted subcutaneously in anesthetized nude mice (n=3, for each cell type). Once the tumor had grown to 600-800 mm3, mice were sacrificed, tumors harvested, cut into equally sized small fragments of 3x3 mm3 and maintained in DMEM supplied medium with 10% fetal bovine serum and penicillin/streptomycin. Solid fragments with absent or minimal presence of necrotic areas were selected for orthotopic implantation into the lung. Mice were anesthetized with a continuous flow of 1% to 3% isoflurane/oxygen mixture (2 l/min) and subjected to right thoracotomy. Mice were situated in the left lateral decubitus position and a small transverse skin incision (around 5-8 mm) was made in the right chest wall. Chest muscles were separated by a sharp dissection and costal and intercostal muscles were exposed. An intercostal incision of 2-4 mm was made on the third or fourth rib on the chest-wall and a small tumor piece of 2-4 mm3 was introduced into the chest cavity. The tumor specimen was anchored to the lung surface (in the second or third lung lobule) with Prolene 7.0. Next, the chest wall incision was closed with surgical staples, and the chest muscles and skin were closed (6-7). To induce ectopic PAR3 expression ten days postimplantation the engrafted animals drank water supplied with 1% sucrose plus 2 mg/ml doxycycline *ad libitum*. Mice were inspected daily, and monitored for evidence of breathing problems, and sacrificed when they displayed serious respiratory difficulty, which was subsequently confirmed to be associated with lung tumor burden. For histological analysis of lung and liver tumors, lungs and livers were fixed and embedded in paraffin. Five-micrometer sections were stained with H&E, using standard protocols, and examined by light microscopy in a blinded fashion.

**Statistical and bioinformatic analysis**

Analysis consisted of chi-square, two-tailed unpaired Student’s t, Mann-Whitney and ANOVA tests. Results of the Student’s t tests are presented as means and standard deviations. We considered significant any test with a value of p<0.05. To identify genes that are important for distinguishing the *PARD3* gene expression signature we carried out an ANOVA.

**References**

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