Supplemental Methods

Tissue specimens and immunohistochemistry

Primary antibodies for immunohistochemistry were used as follows: mouse anti E-cadherin, 1:100 (clone 36, Becton Dickinson, San Jose, CA); mouse anti keratin 18, 1:800 (clone CY-90, Sigma, Deisenhofen, Germany); goat anti ZEB1-N-term, 1:100 (E-20, S.Cruz, Biotech, S. Cruz, CA); rabbit anti Lgl2 C-term (1:200, own production). For double stainings, the samples were first incubated with anti-ZEB-1 antibody for 1 hour at room temperature, washed with PBS three times, and incubated for 1 hour at room temperature with anti-goat IgG-biotin (Vector Laboratories, Inc., Burlingame, CA). Samples were then incubated for 30 minutes at room temperature with avidin-horseradish peroxidase (Vectastain Elite ABC reagent, Vector Laboratories), washed with PBS, and treated with diaminobenzidine (DAB kit, Vector laboratories). Samples were washed three times with PBS and incubated overnight at 4°C either with Lgl2 antiserum. After extensive rinsing with PBS, samples were incubated for 1 hour at room temperature with appropriate secondary antibodies coupled to biotin. Sections were rinsed with PBS, processed as for the first ZEB1 antibody, washed with PBS then incubated with Vector VIP. The color reaction was stopped by rinsing with water. The slides were rinsed, counterstained with hematoxylin, dehydrated and mounted.

DNA constructs

For sequence comparison to detect homologies and conserved repressor binding sites in the mouse and human gene we used the ClustalW algorithm (PubMed). For construction of the human Lgl2 promoter reporter plasmid, nucleotides -244 to +135 (relative to Lgl2 transcriptional start site) were amplified from human genomic DNA and cloned into pGL3basic (Promega, Mannheim, Germany).

Cell culture and various assays

All experiments were done at least three times.

For immunoblot of Lgl2 we used a rabbit antiserum diluted 1:1000 raised against the 27 C-terminal amino acids of human Lgl2. For quantitative RT-PCR a microscope-aided, gross microdissection of normal and tumor tissue using a scalpel was performed.

For EMSAs the following sequences were used as double-stranded DNA-oligonucleotides for probes and for competition:

lgl2,E2/3: ctgcgtccaggtgcgcgcaggtgaggccgg;
lgl2,E2M/3: ctgcgtctaggcgcgcgcaggtgaggccgg;
lgl2,E2/3M: ctgcgtccaggtgcgcgtaggcgaggccgg;
lgl2,E2M/3M: ctgcgtctaggcgcgcgtaggcgaggccgg;
IL2 NRE: tgtcagacaggtaaagtctttg.

For ChIP analyis the ChIP IT kit (Active Motif, Carlsbad, CA) was applied according to manual instructions. 2 µg of control rabbit antiserum or antiserum against ZEB1 (E20, S.Cruz, Biotech, S. Cruz, CA) were used for IP. The following primers were used for PCR analysis:
lgl2 1. s: cggggttccaggtgagat; as: cagagaagtttcccgcagaa;
lgl2 2. s: gggaactatttccgcttgcg; as: ctgcgctcctgccaatc;
Crumbs3 1. s: atacataccccgtgcgtctc; as: cgtgacccggaaagtttagt;
Crumbs3 2. s: ccggccatctgttttctgta; as: gaaaattagccagatgtgatgg;
INADL s: ttgatcttgggtctcgctct; as: aaggagggaggacggttg
Gapdh s: tactagcggttttacgggcg; as: tcgaacaggaggagcagagagcga

For real time RT-PCR, the following primer pairs were used:
Lgl2 Ex3/4s: 5'-tttaacaagacggtggagca-3'; as: 5'-gagcttgatggctccagaac-3';
snail s: 5'-gctgcaggactctaatccaga-3'; as: 5'-atctccggaggtgggatg-3';
slug s: 5'-tggttgcttcaaggacacat-3'; as:5'-gttgcagtgagggcaagaa-3';
twist s: 5'-tccgcgtcccactagca-3'; as: 5'-ttctctggaaacaatgacatctaggt;
ZEB1 s: 5'-AAGAATTCACAGTGGAGAGAAGCCA; as: 5'-CGTTTCTTGCAGTTTGGGCATT
E-cadherin s: 5'-GTCCTGGGCAGACTGAATTT; as: 5'-GACCAAGAAATGGATCTGTGG
Vimentin s: 5'-CGAGGAGAGCAGGATTTCTC; as: 5'-GGTATCAACCAGAGGGAGTGA

For transient knock down the following siRNAs were used (all genes were knocked down by at least two different specific siRNAs):
Lgl2 1.: 5´-ACCAGAUCCUGAUCGGCUAdTT-3'
Lgl2 2.: 5´-GCCAGCAACUGGAGAACAUdTT-3`
ZEB1 1.: 5´-AGAUGAUGAAUGCGAGUCGdTT-3'
ZEB1 2.: 5´-UGAUCAGCCUCAAUCUGCAdTT-3´
Control si 1.: 5´-GCUACCUGUUCCAUGGCCAdTT-3'
Control si 2.: 5´-GGCAGCAAUUCAUUGAGUAUdTT-3´

Matrigel 3D cultures were performed as described (Debnath et al., Methods, Vol. 30, 256-268, 2003). For collagen 3D cultures $2.5x10^4$ cells per 24-well were seeded in 1ml of a mixture containing 250µl CollagenG (Serva, Heidelberg, Germany), 300µl CollagenR (Biochrom, Berlin, Germany), 100µl PBS, 100µl 10xDMEM (Biochrom, Berlin, Germany), 100µl FCS, 22mg NaHCO3, pH 7,5. The solidified collagen gel was overlaid with 1ml DMEM containing 10% FCS. Medium was changed twice a weak. After 10d cultures were fixed in 5% formalin embedded in paraffin and subjected to immunofluorescence analysis. The following antibodies were used for staining: rabbit anti Lgl2 (1:200, own production), mouse anti E-cadherin (clone 36, 1:100 (Becton Dickinson, San Jose, CA), rabbit anti beta-catenin 1:1000 (Sigma, Taufkirchen, Germany), rabbit anti cleaved caspase 1:50 (Cell Signaling, Danvers, MA).

Migration assay:

For the monolayer wounding cell migration assays, $1x10^6$ cells were seeded in 6-well culture plates, a scratch was made with a pipette tip and the groove was documented using a photo microscope. Increased proliferation can mimick an increased cell migration in the wounding assay. However in our experiments ZEB1 knockdown not only resulted in decreased migration, but also in a slightly increase in proliferation. Thus slightly increased proliferation may have even weakened the effect of reduced migration after ZEB1 knockdown in the wounding assay.

Collagen invasion assay:

Rat tail type I collagen R (4 mg/ml) (Biochrom, Berlin, Germany) and calf skin type I collagen G (2 mg/ml) (Serva, Heidelberg, Germany) were mixed at a ratio of 1:1, 0.1 vol of sodium bicarbonate (22 mg/ml) and 0.1 vol of 10 x DMEM was added, and the solution was neutralized with sodium hydroxide. Aliquots of 1.2 ml/well were allowed to gel in 6-well culture dishes at 37°C. 1×10^5 tumor cells were added per well. After 48 h at 37°C assays were fixed with 3% paraformaldehyde. Cells invading the collagen gel were detected by focusing down into the matrix, and quantified by counting 30 optical fields per well