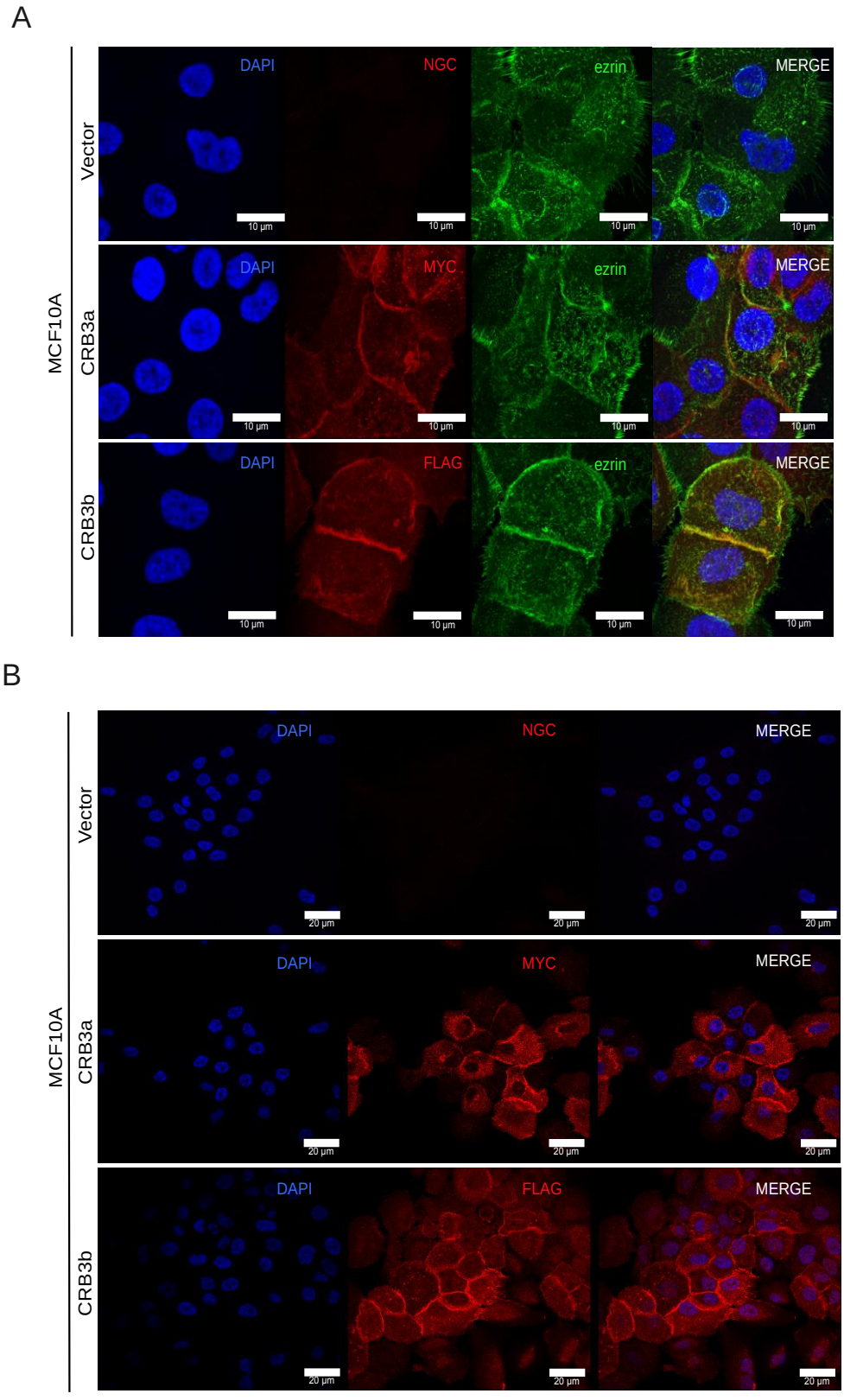
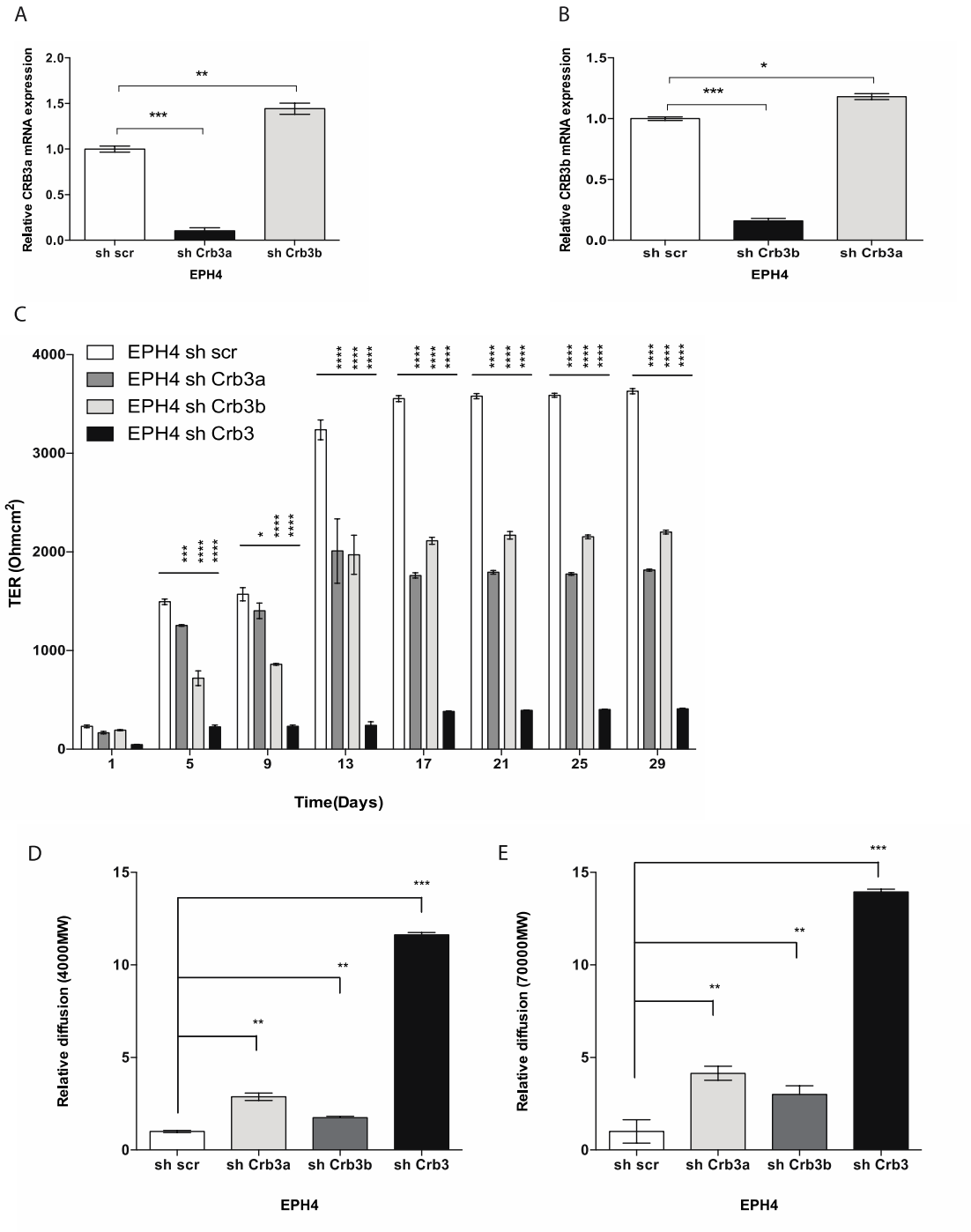
Supplementary Figures



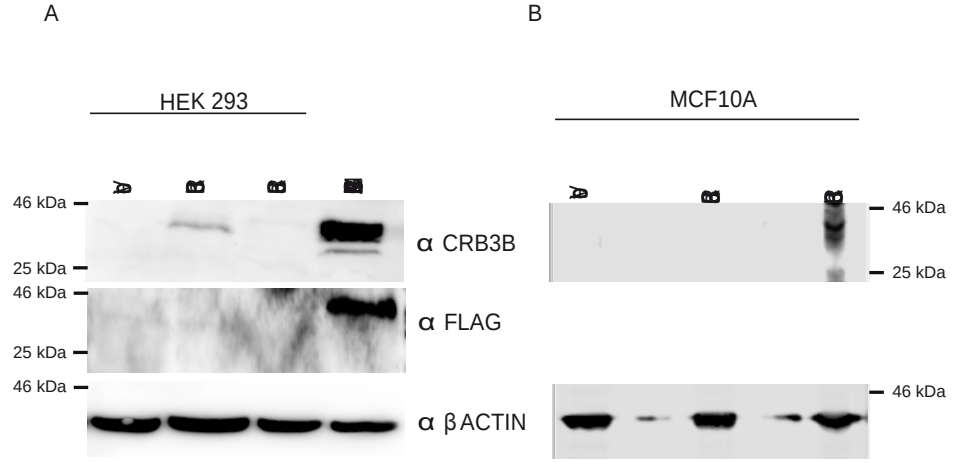
Supplementary Figure S1. (A) Immunofluorescent confocal images of MCF10A-Vector, MCF10A-CRB3a, and MCF10A-CRB3b cells. Monolayers were stained with anti-Myc to detect CRB3a, anti-FLAG to detect CRB3b and anti-Ezrin. Both CRB3a and CRB3b localize to the regions of cell-to-cell contacts along with ezrin, which is

also present at the cell junctions. Images were taken using a Leica SP8 confocal microscope at 63X magnification. Scale bar= 10 µm. (B) Immunofluorescent confocal images of MCF10A-Vector, MCF10A-CRB3a, and MCF10A-CRB3b cells. Monolayers were stained with anti-Myc to detect CRB3a; anti-FLAG to detect CRB3b. CRB3a localizes in the cytoplasm and cell junctions and is excluded from the nucleus. CRB3b localizes in the cytoplasm, cell junctions and in the nucleus. Images were taken using a Leica SP8 confocal microscope at 40X magnification. Scale bar= 20 µm.

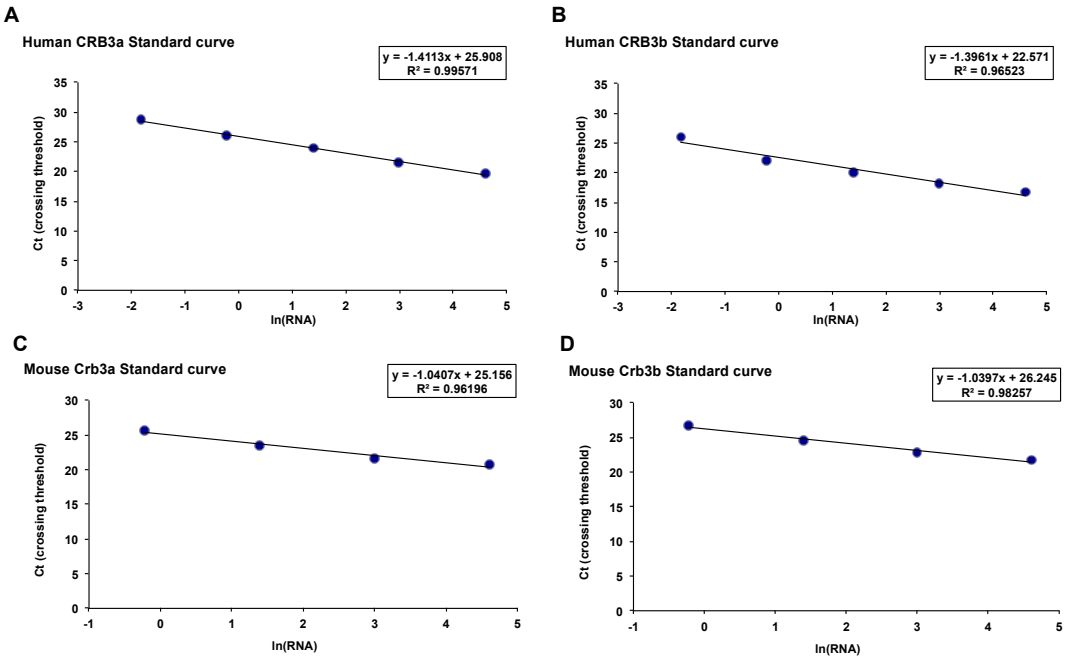


Supplementary Figure S2. Quantitative real-time PCR analysis of the levels of the

two isoforms of Crb3 in EPH4 cells. β-Actin was used to normalize for variations in input cDNA. Error bars represent ±s.d (n=9). For all experiments, a student's test was used. *=p<0.05, ***=p<0.001. (A) The relative levels of Crb3a in EPH4-ShSCR, EPH4-ShCrb3a and EPH4-ShCrb3b. (B) The relative levels of Crb3b in EPH4-ShSCR, EPH4-ShCrb3a and EPH4-ShCrb3b. (C) EPH4 sh scr, EPH4 sh Crb3a, EPH4 sh Crb3b cells and EPH4 sh Crb3 cells were grown on 0.4 μm PET membranes for 29 days, and TER was recorded at 4 day intervals. Error bars represent ±s.d (n=6). A two-way ANOVA was used with Bonferroni post-tests to compare against EPH4 sh scr, *=P<0.05, *** =P<0.001, **** =P<0.0001. (D) Size-selective assessment of TJ paracellular flux on EPH4 sh scr, EPH4 sh Crb3a, EPH4 sh Crb3b cells and EPH4 sh Crb3 cells using fluorescently labelled dextrans. Cells were grown for 5 days on 0.4 μm PET membranes prior to addition of fluorescent 4 kDa-dextran–FITC. Error bars represent ±s.d (n=6). A two-way ANOVA was used with Bonferroni post-tests to compare against EPH4 sh scr, **=P<0.01, *** =P<0.001. (E) Size-selective assessment of TJ paracellular flux on EPH4 sh scr, EPH4 sh Crb3a, EPH4 sh Crb3b cells and EPH4 sh Crb3 cells using fluorescently labelled dextrans. Cells were grown for 5 days on 0.4 μm PET membranes prior to addition of fluorescent 70 kDa-dextran–Rhodamine. Error bars represent ±s.d (n=6). A two-way ANOVA was used with Bonferroni correction to compare against EPH4 sh scr, **=P<0.01, *** =P<0.001.



Supplementary Figure S3. To show the specificity of the CRB3 antibody used for the detection of CRB3b in normal and tumour tissue. (A) Using HEK293 cells that have been transiently transfected with the empty pBabe Vector, **untagged** CRB3a or **untagged** CRB3b. MCF10A stably expressing CRB3b.FLAG was used as a positive control and the blot was probed for β actin as a loading control. (B) MCF10A stably expressing the empty pBabe Vector, **untagged** CRB3a or **untagged** CRB3b was probed using a antibody for CRB3b. The MCF10a expressing CRB3b isoform was detected indicating the specificity of the antibody for CRB3b.



E

Organism	Isoform	Slope	Efficiency	R^2
Human	CRB3a	-3.2495	103.11	0.996
	CRB3b	-3.2147	104.68	0.965
Mouse	Crb3a	-3.3797	97.64	0.962
	Crb3b	-3.2410	103.49	0.983

Supplementary Figure S4. The standard curves plotted with the Ct values against the log quantity of the amount of RNA used for the absolute quantification of human (A) CRB3a and (B) CRB3b and mouse (C) Crb3a and (D) Crb3b isoforms. The standard curves were performed along side unknown samples for every qPCR experiment. (E) The primer efficiency percentage for each primer pair was calculated based on the slope of the standard curve where efficiency =($(10^{-1/slope})$-1).

Supplementary Materials and methods

Immunoprecipitation

MCF10A cells expressing either the empty vector, CRB3b or the CRB3bΔFBM were grown on 10cm^2 plates. The cells were lysed in lysis buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% TritonX-100). Immunoprecipitation was carried out using anti-FLAG M2 Magnetic Beads (Sigma) according to manufacturer's instructions. The end product was run on NuPAGE® Novex® 4-12% Bis-Tris Gels (Life technologies, UK) using NuPAGE® MES SDS Running Buffer (Life Technologies, UK). The lanes were cut into gel chunks before analysis on the TripleTOF 5600.

Analysis on the TripleTOF 5600

The gel chunk was excised and cut into 1 mm cubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Genomic Solutions, Ann Arbor, MI) using standard protocols. Briefly the gel cubes were destained by washing with acetonitrile and subjected to reduction and alkylation before digestion with trypsin at 37℃[1]. The peptides were extracted with 10% formic acid and concentrated down to 20 μL using a SpeedVac (ThermoSavant). The peptides were then separated on an Acclaim PepMap 100 C18 trap and an Acclaim PepMap RSLC C18 column (ThermoFisher Scientific), using a nanoLC Ultra 2D plus loading pump and nanoLC as-2 autosampler (Eskigent). The peptides were eluted with a gradient of increasing acetonitrile, containing 0.1 % formic acid (5-50% acetonitrile in 30 min, 50-95% in a further 1 min, followed by 95% acetonitrile to clean the column, before reequilibration to 5% acetonitrile). The MS/MS data file generated via the 'Create mgf file' script in PeakView (ABSciex) was analysed using the Mascot algorithm (Matrix Science), initially against the NCBInr database Aug 2013 with no species restriction, trypsin as the cleavage enzyme and carbamidomethyl as a fixed modification of cysteines and methionine oxidation and deamidation of glutamines and asparagines as a variable modifications, to confirm protein identification.

Western blot analysis

Cells were lysed in 10 mM Tris (pH 8.0), 150 mM NaCl, 1% Na deoxycholate, 1% Nonidet P-40, 1% sodium dodecyl sulphate, 1 mM ethylenediaminetetracetic acid and

protease inhibitor. Proteins were separated by SDS-PAGE gel electrophoresis gel and the lanes were equally loaded with total protein. The proteins were then transferred onto PVDF membrane (GE Healthcare, Amersham, UK), protein expression was detected by specific antibodies. Primary antibodies used were: ezrin 3C12 (Neomarkers, USA), Importin β1 [#8673] (Cell Signalling, USA), Merlin [ab88457] (Abcam, UK), FRMD6/Willin [EPR12261] (Abcam, UK), FLAG and β-actin (Sigma-Aldrich). Secondary antibodies used were anti-mouse horseradish peroxidase and anti-rabbit horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., USA). Protein expressions on immunoblots were analyzed using ImageJ analysis and a Student's unpaired t-test was performed to test significance.

Immunohistochemistry

Tissue samples were obtained from patients undergoing treatment for histologically confirmed HNSCC at Montefiore Medical Center in Bronx, NY. All patients provided written consent for participation in this study under a protocol approved by the Institutional Review Board at Montefiore Medical Center. Slides were dewaxed in Xylene 3 times, for 5 mins each, followed by sequential rehydration twice in 100% ethanol, 80% ethanol, 50% ethanol for 2 mins after which they were washed in running water for 2 mins. Slides were pre-treated with 0.3% H_2O_2 for 10 min and washed in PBS with 1%Tween 20 (PBS/T). Antigen retrieval was performed using pH 6.0 10 mM sodium citrate buffer in a steamer for 20 min, then cooled for 30 min at RT. Slides were blocked in protein block (DAKO X0909) for 1 h at RT before incubating with the following antibodies: ezrin (1/100, Neomarkers, 3C12), CRB3b (1/100, Proteintech, 12315-1-AP) overnight at 4C diluted in antibody dilutent (DAKO, S0809). Slides were washed 4 times, 5 min each with PBS/T before using Peroxidase labelled polymer conjugated to goat anti-mouse (DAKO, K4000) or Peroxidase labelled polymer conjugated to goat anti-rabbit (DAKO, K4002) at 1/500 for 1 h at RT. Slides were washed 3 times, for 10 mins each with PBS/T. Slides were then threated with freshly prepared DAB (DAKO, K3467) applied for 3.5 min (CRB3b) and 1.5 min (ezrin). The slides were washed with running water for 2 mins and stained with haematoxylin and washed under running water for 2 mins. The slides were dehydrated in 50% ethanol, 80% ethanol, for 30sec each, twice in 100% ethanol,

for 2 mins each and in Xylene 3 times for 5 mins each. The slide were mounted and imaged for analysis.

In vitro wound healing assay

Stable pools of infected cells were seeded at $8x10^5$ per well (6-well plates) and allowed to grow until they reached confluency. Monolayers of confluent cultures were gently scratched with a sterile micropipette tip and migration towards the wound was monitored at different time points. Phase-contrast images were captured after the scratch for each one of the time points. The percentage of scratch covered was measured by quantifying the total distance the cells moved from the edge of the scratch to complete closure the scratch, using Image J software and MiToBo algorithms. Data is presented as the mean percentage of scratch covered in nine independent experiments.

Migration Assay using Boyden Chamber

Serum-free cell suspension (4×10^5 EPH4 cells) were added to the top chamber of 24-well chambers with 8.0 μm pores (BD Biosciences) and complete media supplemented with 20% serum, was added to the lower chamber. MCF10A assay media cell suspension (5×10^5 MCF10A cells,) were added to the top chamber of 24-well chambers with 8.0 μm pores (BD Biosciences) and assay media supplemented with 20% serum, was added to the lower chamber. Cells were incubated for 24h at 37℃ in a humidified atmosphere of 5% (v/v) CO_2. After 24h, the top of the insert membrane was scrubbed free of cells by using a cotton swab and three PBS washes. The bottom side was stained with 0.3% crystal violet (Sigma-Aldrich) in 80% PBS/ 20% ethanol solution previously filtered through a sterile 0.22 μm syringe driven filter (Elkay). The number of cells on the lower surface of each chamber was counted using a Zeiss Axiovert 40CFL microscope and representative images of migration Boyden chambers taken when indicated.

Quantification of tight junction formation

To allow for the quantification of TJs, we defined a TJ structure as a completely enclosed ring of smooth, contiguous, apical ZO-1 staining. Cells were immunostained for ZO-1 and examined with a microscope. For each cell sample, the number of tight junction structures were counted in six different fields under 20 × magnification using

a Leica DM5500b microscope and the mean number of tight junction structures per field was determined. Experiments were performed three times and the mean number of tight junction structures per field from three independent experiments was calculated. Error bars represent s.e.m.

Immunostaining and Imaging

MCF10A and EPH4 cells were grown on sterile coverslips prior to fixing in 4% paraformaldehyde for 15 min at room temperature followed by a 2x5 minute washes in PBS. Permeabilization was carried out in 1% NP-40/PBS for 10 minutes. Coverslips were washed in PBS before blocking in 3% BSA/PBS for 1 hour at room temperature. Samples were incubated with mouse anti-ZO1 (339100, Invitrogen) and rabbit anti-occludin (Sigma Aldrich) overnight at 4℃. Coverslips were washed with PBS before probing with secondary anti-mouse antibodies conjugated to Dylight488 and Dylight594 fluorochromes (Jackson ImmunoResearch Laboratories, Inc., USA) ProLong Gold Antifade containing DAPI (Invitrogen) was used to visualize nuclei. Images were acquired on either a Leica TCS SP8 or on a N-SIM confocal microscope (NIKON, Japan).

Global RNA expression

RNA from tumours was extracted using the same methodology as previously described (17).

Statistical analysis

Statistical differences in cancer survival probabilities were estimated for each CRB3 probe using Kaplan-Meier analyses and Log-rank tests comparing HNSCC stratified by CRB3 alone, defined using median cut-off levels in RNA expression in the tumor, as well as by cytoplasmic ezrin status defined assuming a +2 cut-off in protein expression level (based on a scale of 0 to 3). Survival was assessed from date of diagnosis to death due to HNSCC cancer. Patients who did not die of HNSCC were censored at date of death from other causes, or the last recorded follow-up visit they were known to be alive.

QPCR PRIMERS

hCRB3a FW	GGAAGAGCGGCTCATCTGAA
hCRB3a RV	GGTGTACAAGCCAGCAACCC
hCRB3b FW	CCAGTAGCGAGGAGCAGTTC
hCRB3b RV	GGAGAGGGGACCTAGATGGG
hACTIN FW	CAGGAAGGAAGGCTGGAAGA
hACTIN RV	GCTGTGCTATCCCTGTACGC
mCRB3a FW	GCTCATGCGAAAACTTCGGG
mCRB3a RV	CCAGGCGTTGGTAGTGATGA
mCRB3b FW	CCCAGGTGATGAGTCCAACG
mCRB3b RV	CGTGGGAAAACTGCTCCTCA
mACTIN FW	GGCTGTATTCCCCTCCATCG
mACTIN RV	CCAGTTGGTAACAATGCCATGT

Table Sç: This table shows the sequences of primers used to detect the levels of endogenous CRB3 isoforms in human and mouse cell lines. Primers for actin which was used as a reference gene is listed.