Supplementary Tables I and II, related to Figure 3

Table I

Selected list of proteins analyzed using RPPA data available for the RATHER cohorts. Protein expression was compared in PDLIM2-negative versus positive tumors and include comparison with PDLIM2 expression from Immunohistochemical staining weighted scores (IHC score1-3), as described in main text. p-values were determined using Wilcoxon test (Rank Sum) and significant differences are highlighted in bold.

Table IIa and b

Selected list of genes analyzed using Microarray data available for the RATHER cohorts. mRNA expression was compared in PDLIM2-negative versus positive tumors and include comparison with PDLIM2 expression from Immunohistochemical staining weighted scores (IHC score1-3), as described in main text. p-values were determined using Wilcoxon test (Rank Sum) and significant differences are highlighted in bold. Included is sub-table IIb highlighting p-values from mRNA expression analysis of proteins shown to be differentially expressed from RPPA data and cell model experiments.

Supplementary Methods

Table 1: Details of growth media used for culturing Breast cell lines used in this study, as previously described [1-3]

Cell Line	Basal Medium*	Supplements
MDA-MB-231-LUC2	EMEM	10% Fetal bovine serum
MCF-10A	50:50 Ham's F12:DMEM	5% Horse serum, 1 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone
MDA-MB-231, MDA-MB-157, HS578T, HEK293T	DMEM	10% Fetal bovine serum
HCC70, HCC1806	RPMI	10% Fetal bovine serum, 1% Glucose, 1 mM Sodium Pyruvate

MDA-MB-436	50:50 Leibovitz (L15) media:RPMI	10% Fetal bovine serum
CAL-51	50:50 Ham's F12:DMEM	10% Fetal bovine serum
BT549	RPMI	10% Fetal bovine serum, 1µg/ml (0.023IU) Insulin

*All media supplemented with 1% penicillin/streptomycin (except MCF10A) and 2mM L-Glutamine. Cell culture reagents were purchased from Sigma (Dublin, Ireland).

Table 2: Details of primers used for qPCR

Target	**5'-3' Primer sequences**
PDLIM2 variant 2 (Mystique 2; NM_021630)	*Forward:* cagcccgcagggtacttt *Reverse:* gatgggcgtgtggaaatc
PDLIM2 variant 3 (Mystique 3; NM_198042)	*Forward:* gagagaggaaccaggctgtg *Reverse:* taagagcaactgcccaccat
Beta 2 Microglobulin (housekeeping gene)	*Forward:* ccagcagagaatggaaagtc; *Reverse:* cctccatgatgctgcttaca

Primers were synthesized by Integrated DNA Technologies (IDT Europe, Leuven, Belgium). 5'-3' Primer sequences are listed. Quantitative PCR was carried out using the LightCycler instrument (Roche Molecular Biochemicals, East Sussex, UK) using FastStart Essential DNA Green Master kit (Roche Diagnostics, West Sussex, UK, Cat#6402712001). The $2^{-\Delta\Delta CT}$ method was used to analyze data and determine relative mRNA expression levels normalized to the housekeeping gene as previously described [4].

Reagents, Cell Lines and Antibodies:

Table 3: Cell line Sources

Cell line	Company, Cat#, RRID
MDA-MB-231-LUC2	Caliper Life Sciences, #124319, RRID:CVCL_D582
MCF-10A	ATCC Cat# CRL-10317, RRID:CVCL_0598
MDA-MB-231	ATCC Cat# CRM-HTB-26, RRID:CVCL_0062
HCC70	ATCC Cat# CRL-2315, RRID:CVCL_1270
MDA-MB-157	ATCC Cat# CRL-7721, RRID:CVCL_0618
HS578T	ATCC Cat# HTB-126, RRID:CVCL_0332
HCC1806	ATCC Cat# CRL-2335, RRID:CVCL_1258
MDA-MB-436	ATCC Cat# HTB-130, RRID:CVCL_0623
CAL-51	DSMZ Cat# ACC-302, RRID:CVCL_1110
BT549	ATCC Cat# HTB-122, RRID:CVCL_1092

Table 4: Antibodies

Antibody Target	Company, Cat#, RRID
PDLIM2	(Abcam Cat# ab119243, RRID:AB_10903715) or
	(Abcam Cat# ab68220, RRID:AB_2268037)
PARP	(Cell Signaling Technology Cat# 9542, RRID:AB_2160739)
PHOSPHO-BETA CATENIN Ser675	(Cell Signaling Technology Cat# 4176S, RRID:AB_1903925)
BETA CATENIN NON-PHOSPHO Ser45	(Cell Signaling Technology Cat# 19807, RRID:AB_2650576)
PHOSPHO-IGF-1R Tyr1135/1136	(Cell Signaling Technology Cat# 3024, RRID:AB_331253)
PHOSPHO-EGFR Tyr1068	(Cell Signaling Technology Cat# 3777, RRID:AB_2096270)
PHOSPHO-FAK Tyr397	(Cell Signaling Technology Cat# 8556S, RRID:AB_10891442)
PHOSPHO-Met Tyr1349	(Cell Signaling Technology Cat# 3133, RRID:AB_2181539)
PHOSPHO-AKT Ser473	(Cell Signaling Technology Cat# 9271, RRID:AB_329825)
E-CADHERIN	(BD Biosciences Cat# 610181, RRID:AB_397580)
ACTIVE β1-INTEGRIN (CD29)	(Millipore Cat# MAB2247, RRID:AB_94606)
PHOSPHO-SMAD2 Ser465/467	(Millipore Cat# AB3849, RRID:AB_11213091)
β1-INTEGRIN	(Millipore Cat# AB1952P, RRID:AB_571025)
VINCULIN	(Santa Cruz Biotechnology Cat# sc-25336, RRID:AB_628438)
VIMENTIN	(Santa Cruz Biotechnology Cat# sc-32322, RRID:AB_628436)
ALPHA-TUBULIN	(Santa Cruz Biotechnology Cat# sc-23948, RRID:AB_628410)
BETA-CATENIN	(Santa Cruz Biotechnology Cat# sc-7963, RRID:AB_626807)

Reagents

TRITC-Phalloidin was purchased from Sigma (Dublin, Ireland, Cat#P1951). A custom anti-PDLIM2 polyclonal Rabbit antibody has been described previously [1, 4]. Alexa 488- and Cy3-conjugated secondary antibodies were purchased from Jackson ImmunoResearch

Laboratories (Cambridgeshire, UK, Cat#711-545-152 and 715-165-150). IRDye® 680 and Cat# 926-68070 and 925-32211). TGF-β1 was from Millipore (Cork, Ireland, Cat#GF111), IGF-1 was from PeproTech EC Ltd (London, UK, Cat#100-11), and Y27632 was purchased from ChemDea (Ridgewood, NJ, USA, Cat#CD0141). Lipofectamine 2000 and oligofectamine were from Invitrogen, (Paisley, UK, Cat#11668-019) and G418 from Calbiochem, (Millipore, Cork, Ireland, Cat#345810). PDLIM2 and control siRNAs were purchased from OriGene Technologies GmbH, (Herford, Germany, cat#SR312004). 96-well OptiPlate microplates (Cat#6005290) were from PerkinElmer, Dublin, Ireland. *Renilla reniformis* luciferase construct (pRL-TK; Cat#E2241) and the dual luciferase Assay System kit (Cat#E1910) were from Promega (MyBio LTD, Kilkenny, Ireland). All other reagents were from Sigma (Dublin, Ireland), unless otherwise noted.

In vivo studies

Animals were weighed at regular intervals (twice each week) for the duration of the experiment and all animals were healthy weights. Animals were administered 200µl D-luciferin solution (3mg/ml; Caliper Life Sciences #119222), 3 minutes prior to anaesthesia with a Ketamine/Xylazine mix, dosed according to weight. When fully anaesthetised (total time 10 minutes following luciferin injection), animals were imaged. Images were taken of groups of 3-4 animals and following background signal removal, the Bioluminescent signal (luminescence counts) per animal was measured. The color scale depicts the photon flux (photons per second) emitted from animals.

Cell imaging

Brightfield images (wound healing and 3D on top assays): images were captured using a Nikon Eclipse TE300 Microscope with a Hamamatsu C11440 ORCA-flash 4.0LT Digital Camera attachment. The widths of the wound tracks were analyzed using Nikon NIS-Elements imaging software AR version 4.50 (Aquilant Scientific, Dublin, Ireland). A minimum of 6 measurements were taken per cell type, per time-point.

For fluorescence imaging, cells were photographed using a Nikon Eclipse E600 microscope, equipped with a SPOT digital camera and SPOT software version 4.6 (Diagnostic Instruments Inc, Sterling Heights, MI, USA).

Immunohistochemical (IHC) staining:

Northern Ireland Biobank (NIB) cohort: IHC staining using PDLIM2 antibody (Abcam, 1:200 dilution) was performed using an automated system (Almac Diagnostics, Craigavon, Northern Ireland). Staining was scored based on negative (0), low (1), moderate (2) or high (3) expression scoring system, blinded to patient clinicopathological and outcome data and consensus scores from tissue sample triplicate cores were determined.

RATHER TNBC cohorts: Tissues were stained with PDLIM2 antibody (Abcam; 1:50-150) using DAKO Real^{TM} EnVision^{TM} Detection System (Agilent Technologies Ireland Ltd, Cork, Ireland, DAKO Cat#K4065), according to manufacturer's instructions, and counterstained with Mayer's Hematoxylin (Sigma, Dublin, Ireland, Cat#MHS-16).

Heidelberg samples cohort: 0.5-1 μm–thick sections of the paraffin-embedded triple negative breast cancer tissues were prepared (n=15). Immunoperoxidase-based immunohistochemistry was used to stain the sections: Heat induced epitope retrieval (HIER) was performed at 100°C for 20 min, using pH9 target retrieval solution, (Leica Biosystems, Dublin, Ireland, Cat#RE7119) (DAKO, Agilent, Santa Clara, CA, USA). PDLIM2 antibody was used at 1:150 dilution. Detection was performed by using the immunoperoxidase/DAB-based DAKO REAL detection system and counterstained as above. The intensity of the PDLIM2 staining was analyzed by a pathologist (negative=0, weak staining =1; moderate staining =2 and strong staining =3).

All IHC staining was scored independently three times and consensus scores used for subsequent analysis. Data were analyzed for statistical significance using a Student's *t*-test to compare samples where appropriate.

RATHER cohorts RPPA, gene expression and subtype survival analysis

Data from the quantification of raw fluorescence intensities are presented as units of 'Q-N Log2 Intensity' which refers to quantile-normalized log2 transformed intensity for mRNA expression and 'Norm. RFU' which is normalized RPPA fluorescence units. All analysis of RPPA and gene expression data was conducted in R [5] with plots produced using "ggplot2" package [6]. TNBC patients were identified and clinical data for this cohort was retrieved; genes and proteins of interest were subset from the microarray (n=219 probes, n=139 genes) and RPPA (n=65 targets) expression data respectively. Absolute PDLIM2 status of TNBC patients is presented as either NEG(ative) or POS(itive), or consensus status as NEG, POS _1, POS _2 or POS _3, where annotated NEG versus POS, and NEG versus each POS level of

consensus, was tested using the two-sided Wilcoxon Rank Sum test. Results are visualized as boxplots with Tukey style whiskers. Similarly, RPPA data were used to test protein expression against absolute and consensus PDLIM2 status.

To assess the distribution, differential expression and survival of Burstein *et al.* molecular subtypes [7] in RATHER TNBC data, we used their 80-gene signature in the "genefu" package [8]. Subtypes are described as MES: Mesenchymal, BLIA: Basal-like Immune-Activated, BLIS: Basal-like ImmunoSuppressed, LAR: Luminal Androgen Receptor. Subtype results were plotted with the "pheatmap" package [9]. Differential expression was conducted using "limma" package [10], adjusting for false discoveries using Benjamini-Hochberg correction.

Survival analysis was conducted using the "survival" package [11]. Samples without a conclusive single subtype, or in the BLIA subtype which had very low occupancy, were censored (n=11; majority were due to LAR/BLIS overlap). The log-rank test was used to determine significance of the three subtypes MES, LAR and BLIS based on *PDLIM2* expression.

RATHER tumor samples analyses: The entire cohort contained 138 samples, of these, 128 were successfully scored for PDLIM2 staining by IHC. The number of samples used in further analyses of the cohorts was determined by the availability of additional information such as clinicopathiological parameters, survival details and gene expression data, including for the Burstein gene signatures of TNBC subtype [7]. These numbers (n=) are indicated in figures and/or legends.

Generation of stable clones of cells with PDLIM2 overexpressed or suppressed

Stable clones of MDA-MB-231-LUC2 cell lines expressing shRNAs or BT549 expressing HA-Empty Vector or HA-PDLIM2 were generated after selection in 1-1.2mg/ml G418 (Calbiochem, La Jolla, CA, Cat#345810) and screened by western blotting, as previously described [4]. pSUPER vectors encoding shRNA targeting PDLIM2 with the following sequences ACATAATCGTGGCCATCAA; shPDLIM2(1) or AAGATCCGCCAGAGCCCCT; shPDLIM2 (2) or a control shRNA (shScramble, TGACATGATAATACTCTCT, 2 clones used in experiments).

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