**Supplementary Data**

**ROBO1 Promotes Homing, Dissemination, and Survival of Multiple Myeloma within the Bone Marrow Microenvironment**

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**Bioinformatic analysis of publicly available databases**

Expression of ROBO1 in normal plasma cells (NPC) versus monoclonal gammopathy of undermined significance (MGUS) versus smoldering MM (SMM) was analyzed based on previously deposited Affymetrix Human Genome Gene Expression Arrays.16,17 The IFM170 database was used for comparison of ROBO1 expression in MM, NPC and MM-BMSC. For RNA-sequencing analysis of the CoMMpass dataset, we used the publicly available TPM files (IA14 version). We transformed the raw transcripts counts in log2TPM for the analysis. All the clinical and biological information have been downloaded from the same CoMMpass version.

**Immunohistochemistry (IHC) of human tissues**

De-identified, formalin-fixed, and paraffin-embedded plasmacytoma or bone marrow biopsies were accessed from our pathology repository and processed for IHC to detect CD138 (# 12922, Cell Signaling, Danvers, MA) or ROBO1 (# ab7279, Abcam, Cambridge UK).

**Short hairpin-mediated ROBO1 KD**

ShRNA constructs for ROBO1 silencing were ordered from Dharmacon, Thermo Scientific (the RNAi Consortium (TRC) Lentiviral shRNA).

Mature antisense sequences are listed below. shRNA4 was selected for further experiments based on deepest silencing in 293T cells. shRNA5 was used for confirmatory experiments.

|  |  |  |
| --- | --- | --- |
| TRCN0000060413 | ShRNA 2 | ATAATGTGACTGTATTTCTGC |
| TRCN0000060414 | ShRNA 3 | AACACAAACATATTTGCCAGC |
| **TRCN0000060415** | **ShRNA 4** | **TTGCTTGTTCTCTTTGTCTGC** |
| TRCN0000060416 | ShRNA 5 | TTCTTCCATGAAATGGTGGGC |
| TRCN0000060417 | ShRNA 6 | AAATACATCCTTGCTGGTGGC |

**Generation of ROBO1 FL and mutant constructs**

ROBO1 cDNA was subjected to mutagenesis PCR followed by fusion PCR to delete an internal BamHI restriction site. The following primers were used:

Fwd Primer 1: cgggatccATGATTGCGGAGCCCGC;

Rev Primer 1: GCTGTTGTAGGAaCCTCCTGTGTTC;

Fwd Primer 2: GAACACAGGAGGtTCCTACAACAGC;

Rev Primer 2: ttgcggccgcGAACACAGGAGGtTCCTACAACAGC. Preparative PCR was digested with BamHI/NotI and cloned into pCDNA3.1(+) (Invitrogen).

This construct was used as template for the following mutants:

|  |  |  |  |
| --- | --- | --- | --- |
| Forward Primer | Reverse Primer | Backbone vector | Final construct |
| cgggatccATGATTGCGGAGCCCGC | cccaagcttGGCTTTCAGTTTCCTCTAATTCTT | pCDNA3.1-c3XFLAG | pDNA3.1-FLROBO1-c3XFLAG |
| cgggatccATGATTGCGGAGCCCGC | cccaagcttTCTCTTCTTGCGGTGTCG | pCDNA3.1-c3XFLAG | pCDNA3.1-DCytROBO1-c3XFLAG |
| cgggatccATGATTGCGGAGCCCGC | ttgcggccgctaCAGCTCTCTCTGGACCTG | pCDNA3.1 | pCDNA3.1-G674\*ROBO1 |
| cgggatccTATCGACACCGCAAGAAGA | ttgcggccgcCTATTTGTCATCGTCATCCTTATA | pCDNA3.1-c3XFLAG | pCDNA3.1-CytROBO1-c3XFLAG |

MyrPalmpCDNA3.1 was generated by annealing MyrPalm TOP (ctagcATGGGCTGCATCAAGAGCAAGCGCAAGGACAACCTGAACGACGACGGCGTGGACg

) and MyrPalm BOTTOM (gatccGTCCACGCCGTCGTCGTTCAGGTTGTCCTTGCGCTTGCTCTTGATGCAGCCCATg) oligos followed by ligation into pCDNA3.1 previously digested with NheI/BamHI. CytROBO1-c3XFLAG was subcloned into MyrPalmpCDNA3.1 via BamHI/NotI digest to generate pCDNA3.1-MyrPalm-CytROBO1-c3XFLAG. ROBO1 inserts were subcloned from pCDNA3.1 vector into pCDH-Ubc-MCS-PURO lentivirus via NheI/NotI digest. Sanger sequencing was used to confirm the identity of all vectors used in the study. Lentivirus transfection was performed as previously described.17 Monoclonal sorting via FACS was performed for FL-ROBO1 OPM2 cells and monoclones expanded. pCNDA3.1-SLIT2 was generated via cloning PCR of original Dharmacon vector with the following cloning primers: Forward Primer: cgggatccATGCGCGGCGTTGGCT; Reverse Primer: ttgcggccgCTAGGACACACACCTCGTA. Preparative PCR was digested with BamHI/NotI and subcloned in pCDNA3.1. pCDNA3.1-SLIT2N-cV5 was generated via NEB assembly for 5’ subcloning and by using the following reverse primer: ttgcggccgcCATGGGTGGAGAAAACTCA into a pCDNA3.1-cV5 backbone vector.

**Cell lysis, SDS-PAGE and western blotting**

RIPA buffer (Boston Bioproducts, Ashland, MA) was used for whole cell lysates unless otherwise specified. For cytosolic fractionation, cells were lysed in hypotonic lysis buffer (25mM HEPES pH 7.9, 1.5mM MgCl2, 10mM NaCl plus complete protease inhibitor and phosphatase inhibitors). Nuclei were lysed in 50mM HEPES pH 7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100 plus complete protease inhibitor and phosphatase inhibitors.

SDS–PAGE was performed on NuPage Bis-Tris gels (Thermofisher) by using either MOPS or MES running buffers. Gels were wet -transferred on 0.45µm nitrocellulose membranes (Amersham Hybond ECL, GE Healthcare) in Tris/Glycine, 0.1% SDS, 20% methanol transfer buffer. Following transfer, Ponceau S (Sigma Aldrich) staining was performed to assess for equal loading and adequate transfer and membranes were blocked in 5% BSA in T-BST, followed by incubation in primary antibody and secondary antibody in 5% BSA, T-BST. Detection of proteins was carried out with Amersham ECL (GE Healthcare). The following antibodies were used: ROBO1 C-terminus (# ab7279, Abcam, Cambridge UK), ROBO1 N-terminus (# 114103, Genetex, Irvine, CA), SLIT2 (# 118220, Genetex, Irvine, CA), Abl (# 2862, Cell Signaling, Danvers, MA), FLAG (# 1804, Millipore-Sigma, St. Louis, MO or # AHP1074, Bio-Rad, Hercules, California), MMSET (#65127, Cell Signaling, Danvers, MA), GFP (#2956, Cell Signaling, Danvers, MA) TP53 (#2527, Cell Signaling, Danvers, MA). GAPDH (# 5174), beta-tubulin (# 2128), beta-actin (# 3700) and HDAC1 (#34589), all from Cell Signaling, Danvers, MA, were used as loading controls.

**RNP CRISPR-Cas9 KO of TP53 and MMSET (NSD2)**

Each crRNA or tracrRNA was resuspended in Nuclease-Free Duplex Buffer (30 mM HEPES, pH 7.5, 100 mM potassium acetate) as a 200 μM stock solution. Equimolar crRNA and tracrRNA were diluted in Nuclease-Free Duplex Buffer for a final concentration of 43.2μM and annealed by incubating at 95°C for 5 minutes to generate the crRNA:tracrRNA complex, followed by cooling to room temperature (15–25 °C). For each reaction, 0.2 μL of Resuspension Buffer R was added to 0.3 μL of a 62 μM Cas9 protein stock solution (18 pmol), and then the solution was mixed with 0.5 μL of 43.2 μM crRNA:tracrRNA complex (0.22pmol). The mixed solution was incubated at room temperature for 10-20 minutes to form the RNP complex. Meanwhile, 5 x 105 cells MM cells per RNP reaction were harvested and washed once in collected in 1X PBS without Ca2+and Mg2+(Gibco). Cell pellets were resuspended in 10 μL of Resuspension Buffer R and added to the RNP complex. The electroporation of the mixture was carried out by the Neon™ Transfection System (Thermo Fisher Scientific) with the following program (1 pulse, 1600 Volts, 20ms pulse width) via a 10μL pipet tip (Neon 10μLkit Invitrogen) per reaction, according to manufacturer’s instructions. Cells were seeded immediately after electroporation in a 6-well polystyrene tissue culture plate with 2mL of pre-warmed RPMI supplemented with 10% FBS and without antibiotics. Volumes and cell numbers were scaled up accordingly for multiple reactions. For TP53 monolonal KO, cells were harvested 1-hour post electroporation and sorted based on DAPI negativity and RFP positivity as single cell clones in 96 well plates. For MMSET KO, cells were used as bulk and harvested 10 days post electroporation. Gene target KO was validated by immunoblots.

**Drugs and reagents**

SLIT2N was purchased from PeproTech (150-11) and resuspended at a concentration of 500 µg/mL per manufacturer instructions. Alternatively, conditioned media obtained from 293T cells transfected with pCDNA3.1-SLIT2N-cV5 or pCDNA3.1-V5 for 72 hours was subjected to centrifugation with Amicon Ultra-15, 100KDa cut off tubes (UFC910008, Millipore) and used fresh. Presence of SLIT2 in conditioned media was confirmed via western blotting against SLIT2 and/or V5 (data not shown).

**Flow cytometry apoptosis assay**

Cells were harvested at indicated times and washed once with PBS before being pelleted by centrifugation at 1400 rpm for 5 minutes. The cell pellet was re-suspended in 1X Annexin V binding buffer with 1 mg/ml of Annexin V (BD Biosciences, 556419). Cells were stained with Annexin V for 20 mins at room temperature. Prior to acquisition, 1mg/ml of propidium iodide (or DAPI) was added, and cells were acquired using a flow cytometer (BD LSRFortessa™) and analyzed using FlowJo (TreeStar). Cells that were propidium iodide (PI) negative and Annexin V negative were considered alive; PI negative and Annexin V positive cells were considered early apoptotic; double positive cells were considered late apoptotic.

**MM cell adhesion assay**

Primary bone marrow stromal cells (BMSCs) or human endothelial BMEC60 and TrHBMEC were plated at a density of 1 x 104 per well in a 96-well plate 1 day before the adhesion assay. Plates were pre-coated with gelatin for TrHBMEC adhesion. MM cells were pre-labeled by incubating in the presence of 1 mM calcein AM (Molecular Probes) at 37 ºC for 30 min in RPMI media with 1% serum. After washing with PBS with calcium and magnesium (PBS+) twice, labeled cells were resuspended in PBS+ at a concentration of 1 x 106/ml and seeded at a concentration of 1 x 105 cells/well in 9 replicates. Baseline fluorescent intensity was measured prior to incubation at 37ºC for 1 hour. At the end of the incubation, wells were washed three times with PBS, and ﬂuorescent intensity of adherent cells was then quantiﬁed using a ﬂuorescence plate reader (SpectraMax M3, Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. The percentage adhesion was determined by calculating the ratio of the ﬂuorescent intensity of the post-wash sample to that of the pre-wash sample.

**Confocal microscopy**

For immunofluorescent staining, 500,000 cells were adhered to poly-L-lysine coated coverslips. Cells were fixed in methanol free formaldehyde 16% solution (Piercenet 28906) in PBS for 10 minutes, washed in PBS, incubated with 0.1M glycine and then permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Non-specific signal was blocked with PBS 1% BSA, 0.04% Triton-X for 30 minutes. For phalloidin staining, each coverslip was incubated for 1 hour with 5 µL methanolic stock solution of phalloidin into 200 µL PBS+ 1% BSA, washed 3 times with PBS and nuclei counterstained with DAPI at a concentration of 1 µg/mL. For ROBO1 and FLAG IF, coverslips were incubated with primary antibody (ROBO1 # ab7279, Abcam, Cambridge UK and FLAG #AHP1074, Bio-Rad, Hercules, California) for 2 hours at RT, washed and then incubated with secondary antibody Alexafluor488 (Thermofisher) for 1 hour and then washed. Coverslips were mounted with Vectashield antifade mounting medium (Cole-Parmer), sealed with nail polish and examined with a Zeiff confocal fluorescent microscope. Images were analyzed with ImageJ.

**Genomic PCR primers to confirm ROBO1 KO**

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| --- | --- | --- | --- | --- | --- |
| |  | | --- | | hsRobo1\_Sg2\_gDNA\_Fp1 | | hsRobo1\_Sg2\_gDNA\_Rp1 | | |  | | --- | | TGGAGTCAATTCTCTTTAAGGGT | | TCAGCGAGGAAATTATTCCACAGA | |
| |  | | --- | | hsRobo1\_Sg2\_gDNA\_Fp2 | | hsRobo1\_Sg2\_gDNA\_Rp2 | | |  | | --- | | GCTTAGAAAGCAGTCTTCACTTAT | | TTATTCCACAGAGTCCCCTCA | |
| |  | | --- | | hsRobo1\_Sg2\_gDNA\_Fp3 | | hsRobo1\_Sg2\_gDNA\_Rp3 | | |  | | --- | | GCTTAGAAAGCAGTCTTCACTTATG | | TTCTGATTTCTTCTCTCCTCTTTCT | |
| |  | | --- | | hsRobo1\_Sg3\_gDNA\_Fp1 | | hsRobo1\_Sg3\_gDNA\_Rp1 | | |  | | --- | | TTTTAGGGGGTGGGTAGAAAGAG | | CCTAAGGCTCCCTGATTCGTG | |
| |  | | --- | | hsRobo1\_Sg3\_gDNA\_Fp2 | | hsRobo1\_Sg3\_gDNA\_Rp2 | | |  | | --- | | GGAGGACTAGGAGGAAGCACTA | | TCCTGCCTACTACCAAACCA | |
| |  | | --- | | hsRobo1\_Sg3\_gDNA\_Fp3 | | hsRobo1\_Sg3\_gDNA\_Rp3 | | |  | | --- | | GAGGAAGCACTAAACCTAGGGA | | CTGGCCCCTGATAGAGGATTC | |

**Gelatin methacrylate preparation for 3D proliferation studies**

To synthetize gelatin methacrylate(GelMA), 10 g gelatin was dissolved in 100 mL of PBS at 50°C. Eight mL of methacrylic anhydride (MA) was added into the gelatin solution dropwise to achieve methacrylation. The reaction was performed for 4 h under constant stirring at 200 rpm. 300 mL of DPBS was added into the mixture to stop the reaction. The resulting solution was dialyzed against deionized water using 12-14 kDa Mw cut-off membrane for seven days at 40°C. The solution was then frozen at -80°C and then lyophilized for a week. The final product was stored in -80°C until the three-dimensional (3D) encapsulation experiments. The polymer precursor that contained 5% (w/v) GelMA and 0.5% (w/v) Irgacure 2959 (photoinitiator) was prepared by dissolving them in DPBS at 70˚C for 3D cell encapsulation.

**3D Proliferation Assay**

Gelatin methacrylate was synthetized as described in supplemental methods. OPM2 or KMS11 cells were resuspended in the prepolymer solution at a 5x106 cells/mL density per condition. The cell-laden prepolymer solution was crosslinked using a 150 μm spacer upon exposure to UV light for 4s at 2.5 mW/cm2 power. The cell-loaded hydrogel was then rinsed with DPBS and cultured in a 24-well plate for up to 7 days. Four replicates were carried out for each experimental group. Cell proliferation was evaluated by an Alamar Blue assay on days 0, 1, 4, and 7 following the manufacturer’s instructions. The fluorescence of the Alamar Blue solution was determined using a microplate reader at 560/600 nm (Ex/Em). Proliferation at d7 was normalized against d0 reading.

**18F-FDG mouse PET imaging**

2-deoxy-2-[18F]fluoro-D-glucose (18F-FDG) PET imaging was carried out on a dedicated small animal scanner (Inveon Multimodality System, Siemens Medical Solutions USA, Inc., Hoffman Estates, IL). One hour prior to radiotracer administration, mice were warmed on Gaymar circulating warm water heating pads to reduce brown fat uptake, and anesthetized through sevoflurane/air inhalation. The mice were injected with a bolus intravenous injection (through the lateral tail vein) of approximately 7.4 MBq of 18F-FDG (PETNet Solutions, Woburn MA), and remained under anesthesia throughout the 60 min uptake period. The mice were subsequently placed into the scanner. Static PET emission scans were then acquired in list-mode format over 10 min, and corrected for decay and dead time. A low dose CT scans was then acquired (80 kVp, 0.5 mA) for anatomical reference and to provide guidance for the delineation of selected tissue volume of interest (VOI). The acquired data were then sorted into 0.5 mm sinogram bins and 1-time frame for image reconstruction using FORE/3D-OSEM-MAP image reconstruction. The reconstructed PET/CT images were analyzed with the Siemens Inveon Research Workplace software. The radioactivity retention within the selected VOI was obtained from mean voxel intensity values within the VOI and then converted to megabecquerels per milliliter using the calibration factor determined for the Inveon PET System. These values were then divided by the administered activity dose and body weight to obtain an image VOI-derived Standardized Uptake Value (SUV). We used the maximum SUV value (SUVmax) within a VOI as a quantitative imaging metric. The represented PET images are axial, coronal, and sagittal sections, with the mice placed in prone position.

**RNA sequencing analysis from primary mouse tumors**

Three tumors harvested from 3 distinct ROBO1 WT mice and three tumors harvested from 3 distinct ROBO1 KO mice (plasmacytoma model) were used to extract RNA and perform RNAseq. To avoid biases related to hypoxia, tumors were chosen of comparable volume. RNeasy (Qiagen) was used to extract RNA. After passing quality control, RNAseq was performed using polyA and stranded library prep and single end 75bp sequencing on next 500 platform pooling all 6 samples in one chip. RNA-seq data in fastq format were aligned to reference human genome using a STAR RNAseq aligner. We used the DeSeq2 method based on negative binomial generalized linear models (DOI: 10.1186/s13059-014-0550-8) to compare ROBO1 WT and KO tumors. Subsequently, a gene set enrichment analysis (DOI: 10.1073/pnas.0506580102), based on the DeSeq2 output, was implemented by the “fgsea” R package (DOI: 10.1101/060012). The GSEA analysis is able to detect biological common enriched pathways in a peculiar dataset. We ranked and plotted the enriched pathways based on the normalized enrichment score and their false discovery rate. The p-value was adjusted based on Benjamin-Hochberg test.

**Proteomic analysis**

For interactomic analysis of ROBO1 FL and MP-CYT pull down we used STRING (v 11.0) (https://string-db.org), a public database able to predict protein-protein interaction and to perform enrichment pathway analysis based on the protein expression input (DOI: 10.1093/nar/gky1131). The STRING database is able to predict direct and indirect protein-protein interactions. These predictive interactions are generated by the simultaneous interrogation of several transcriptomic and proteome datasets. Differential protein representaion analysis was performed with the DEP R package. This package provides a workflow of analysis for high-throughput mass spectrometry experiments. The workflow is composed by the setup of and experimental expression and design matrix, the filtering of missing values, normalization and statistical analysis for differential represented proteins between ROBO1 KO (2 distinct clones) and ROBO1 FL OPM2 clones (3 distinct clones), (false discovery rate < 0.1, log2fold change >1.5). The package provides also volcano plot visualization functions. Pathway analysis was performed by a multiple test correction according to the Benjamin-Hochberg test. We used the hierarchical “treemap” representation of interacting proteins based on the R package. The size of each square derived from the number of proteins that enriched that peculiar pathway, the color from the false discovery rate (FDR < 0.05).