Multiple myeloma exploits Jagged1 and Jagged2 to promote intrinsic and bone marrow-dependent drug resistance

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SUPPLEMENTARY INFORMATION

INVENTORY

Supplemental Information contains the Supplemental Data (15 figures and 2 tables), Supplemental experimental Procedures and Supplemental References.

Supplemental Data:

Table S1: Patients' information.

Figure S1-2 are related to Fig.1.

Figure S1, S3-5 are related to Fig.2.

Figure S6-S8 are related to Fig.3.

Figure S9 is related to Fig.4.

Figure S10-13 is related to Fig.5

Figure S14 is related to Fig.6.

Figure S15 is related to Fig.7.

Table S2: Sequence of RT-qPCR Primers.

Patients informations

	Gender	del17	t(4;14)	t(14;16)	1p loss/1q gain
MM1	**M**	**-**	**-**	**-**	**ND**
MM2	**M**	**-**	**-**	**-**	**ND**
MM3	**M**	**-**	**+**	**-**	**- 1p loss/+ 1q gain**
MM4	**M**	**-**	**-**	**-**	**ND**
MM5	**M**	**-**	**-**	**-**	**ND**
MM6	**M**	**ND**	**ND**	**ND**	**ND**
MM7	**M**	**-**	**-**	**-**	**- 1p loss/+ 1q gain**
MM8	**M**	**-**	**+**	**+**	**- 1p loss/+ 1q gain**
MM9	**F**	**-**	**-**	**-**	**- 1p loss/- 1q gain**
MM10	**F**	**ND**	**ND**	**ND**	**ND**
MM11	**M**	**-**	**-**	**-**	**- 1p loss/- 1q gain**
MM12	**M**	**ND**	**ND**	**ND**	**ND**
MM13	**F**	**-**	**-**	**-**	**- 1p loss/+ 1q gain**
MM14	**M**	**+**	**+**	**-**	**- 1p loss/+ 1q gain**

Table S1. Patients' information. All the analyzed MM patients were newly diagnosed patients and did not received any therapeutic treatment at the time of the study. Gender and key chromosomal aberrations have been reported. M: male; F: female; ND: not determined.

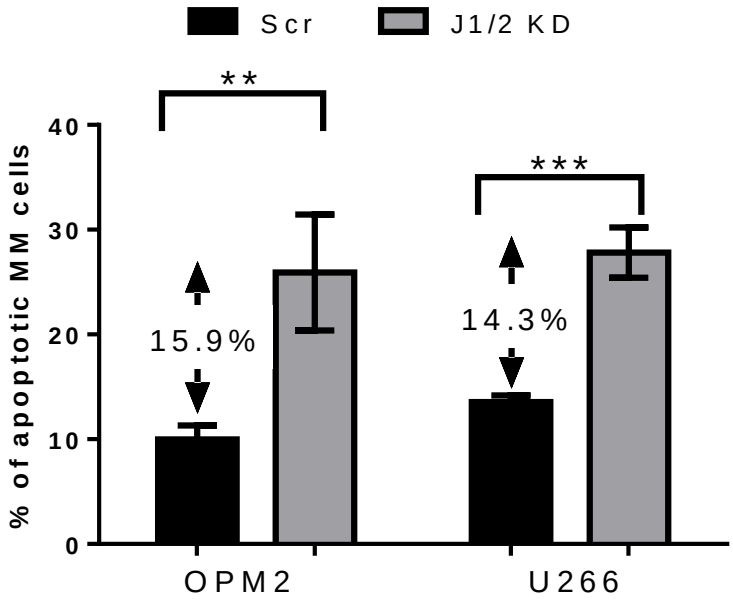

Fig. S1. Effect of J1/2KD on MM cell apoptosis. Results are shown as the mean values ± SEM. Statistical analysis was performed using one-tailed t-test (**=p<0.01; ***=p<0.001).

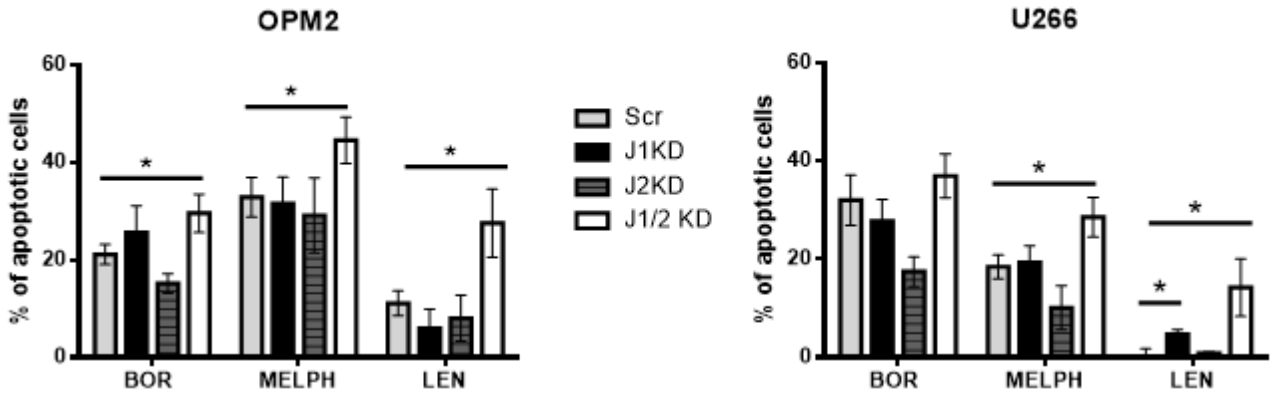

Fig. S2. Effect of the selective silencing of Jagged1 or Jagged2 on MM cells response to standard-of-care drugs. Values of apoptosis of Scr HMCLs are normalized on the corresponding DMSO treated controls and values of KD HMCLs treated with drugs are normalized on the corresponding KD cells treated with the vehicle. Results are shown as the mean ± SEM from 3 independent experiments. Statistical analysis was performed using one way ANOVA and Tukey post test (*=p<0.05).

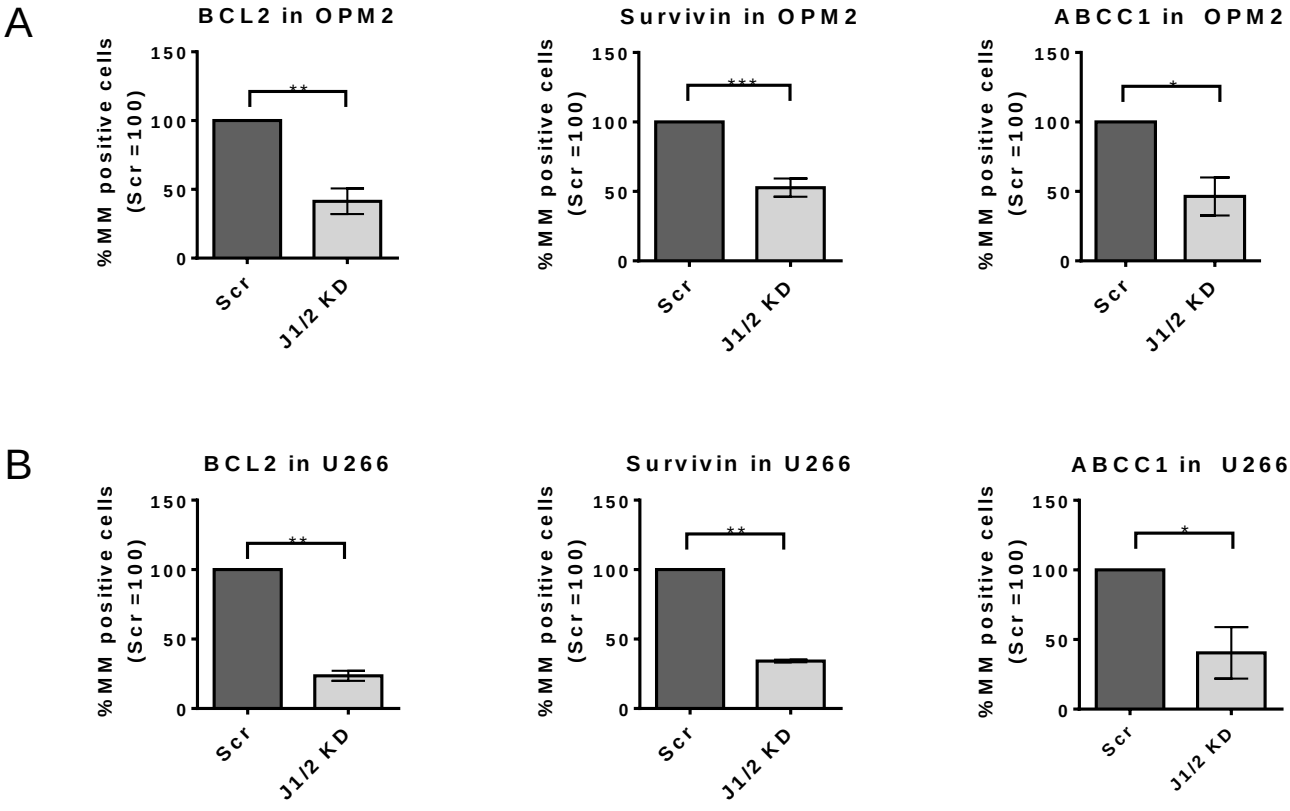

Fig. S3. Effect of J1/2KD on the expression of anti-apoptotic factors. The graph shows changes in the expression levels of BCL2, Survivin and ABCC1 obtained from the flow cytometric analysis on OPM2 (A) or U266 (B) cells transfected or not with siRNAs targeting Jagged1 and Jagged2 (Scr cells cultured alone=100%). The bars are the mean values ± SEM. Statistical analysis was performed using one-tailed t-test (*=p<0.05; **=p<0.01; ***=p<0.001).

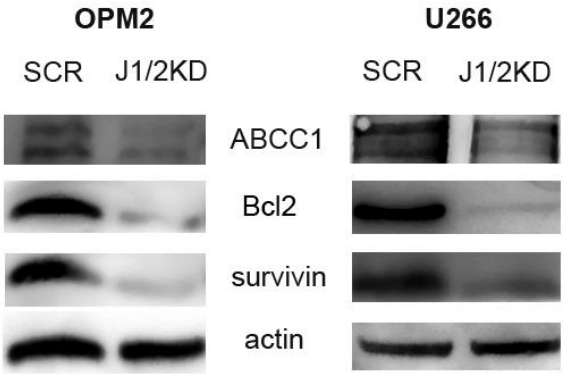

Fig. S4. Effect of J1/2KD on MM cell anti-apoptotic background. Western blot analysis of the effect of J1/2KD on the expression of Bcl2, Survivin and ABCC1. Images were acquired using the UV-tech Alliance system and are representative of 3 independent experiments with similar results.

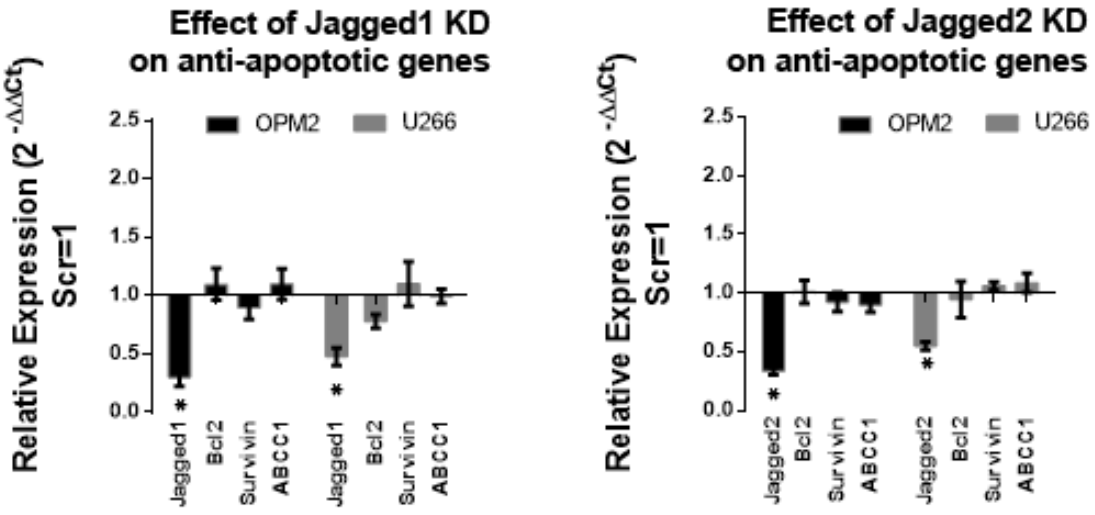

Fig. S5. Effect of the selective silencing of Jagged1 or Jagged2 on the expression of anti-apoptotic genes in MM cells. Results are shown as the mean ± SEM from 3 independent experiments (Scr cells treated with DMSO=1). Statistical analysis was performed using two-tailed t-test (*= $p<0.05$).

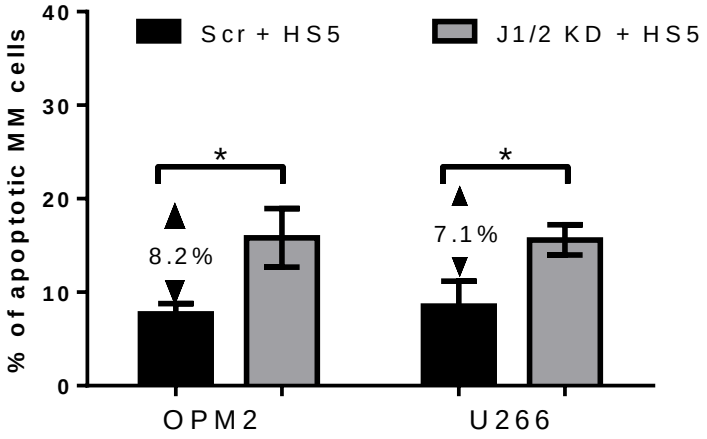

Fig. S6. Pro-apoptotic effect of J1/2KD on MM cell cultured in the presence of BMSCs. Results are shown as mean values ± SEM. Statistical analysis was performed by one-tailed t-test (*=p<0.05).

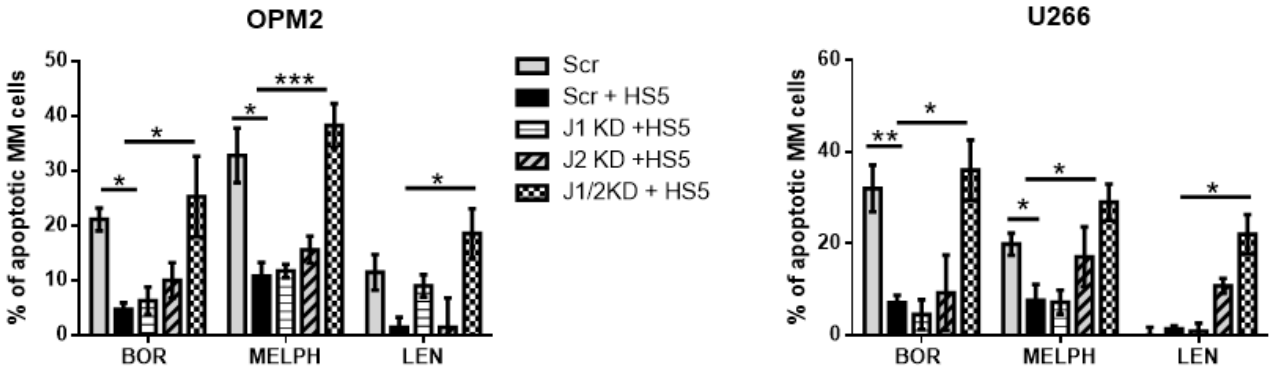

Fig. S7. Effect of the selective silencing of Jagged1 or Jagged2 on the response to standard-of-care drugs of MM cells cultured with BMSCs. Values of apoptosis of each type of culture (Scr alone, Scr+ HS5, J1KD+ HS5, J2KD+ HS5 and J1/2KD + HS5) treated with the reported drugs are normalized on the corresponding controls treated with DMSO. Results are shown as mean ± SEM from at least 3 independent experiments and statistical analysis was performed using one-way ANOVA and Tukey post test (*=p<0.05;**= p<0.01; ***=p<0.001).

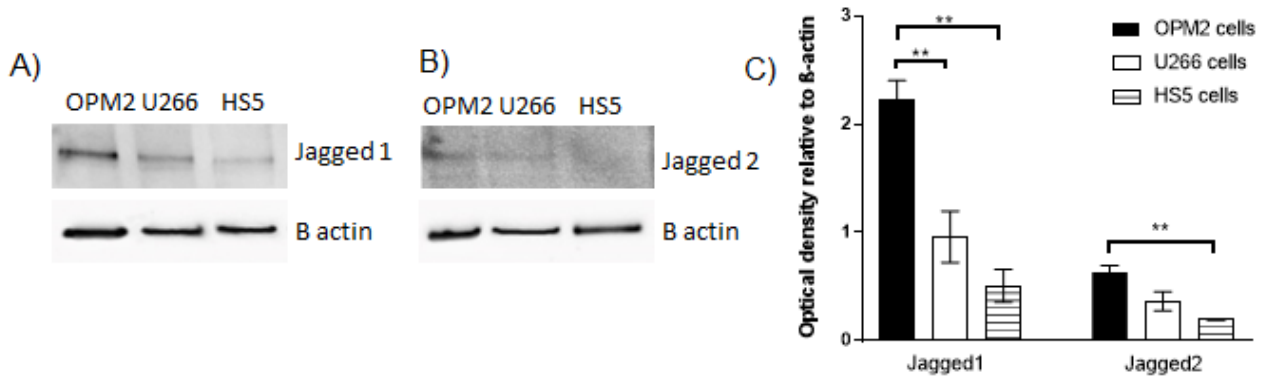

Fig. S8. Differences among Jagged 1 and 2 expression in OPM2, U266 and HS5 cells. Representative western blots show Jagged 1 (A) and Jagged 2 (B) expression in OPM2, U266 and HS5 cells. β-actin was used as loading control. Relative intensity obtained by densitometric analysis was evaluated relatively to β-actin levels (C). Error bars represent SEM of three experiments. Statistical analysis was performed using ANOVA and Tukey post-test (** =p<0.01).

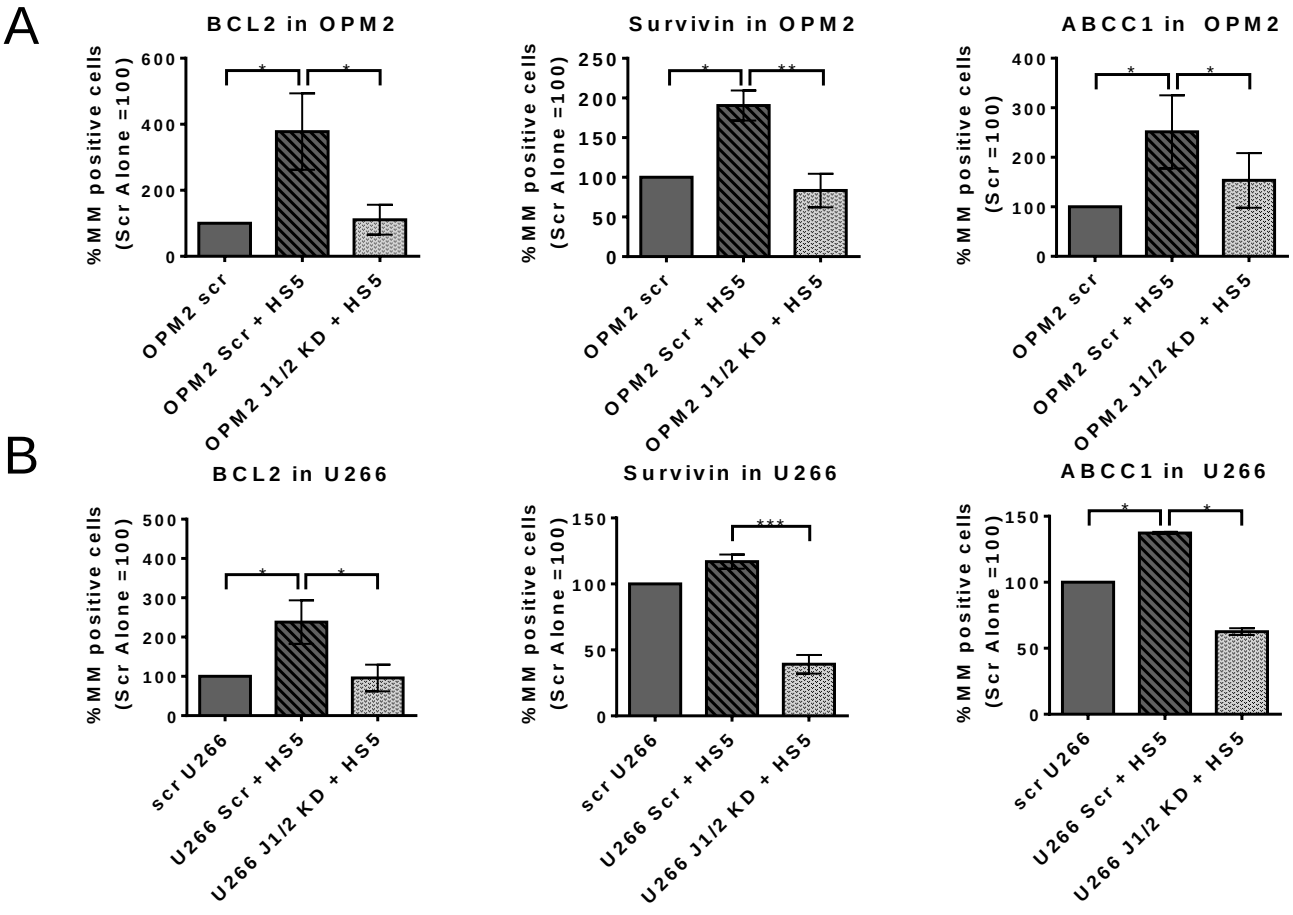

Fig. S9. Effect of J1/2KD on the expression of anti-apoptotic factors in HMCLs cultured with HS5 cells. The graphs show the changes in the expression levels of BCL2, Survivin and ABCC1 obtained by flow cytometric analyses on OPM2 (A) or U266 (B) cells transfected or not with siRNAs targeting Jagged1 and Jagged2 (Scr cells cultured alone=100%) and co-cultured or not with HS5 cells. The bars represent the mean values ± SEM. Statistical analysis was performed using one-way ANOVA and Tukey post-test (*=p<0.05; **=p<0.01; ***=p<0.001).

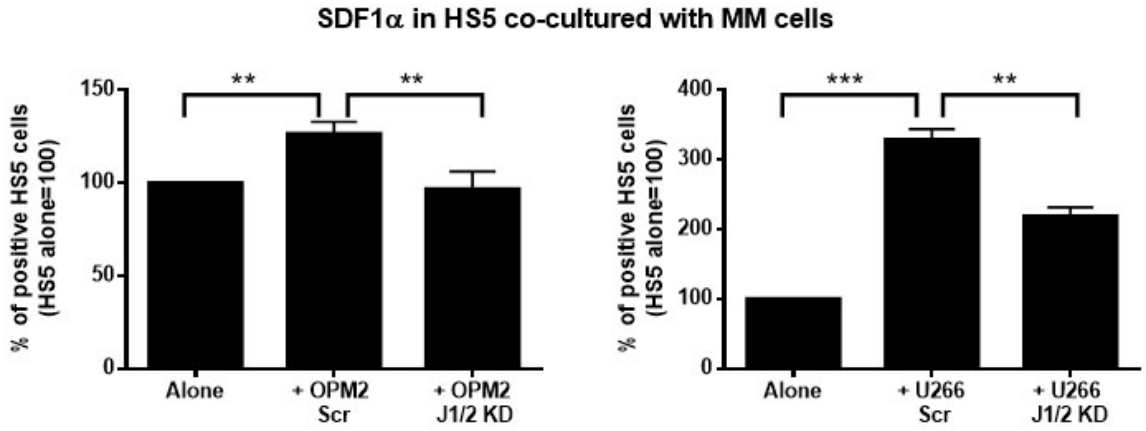

Fig. S10. Changes in SDF1α expression by HS5 cells cultured with HMCLs. The graph shows changes in the expression levels of SDF1α in HS5 cells cultured alone or co-cultured with Scr HMCLs or J1/2KD HMCLs (HS5 cells cultured alone=100%). The bars represent the mean values ± SD. Statistical analysis was performed using one-way ANOVA and Tukey post-test (**=p<0.01; ***=p<0.001).

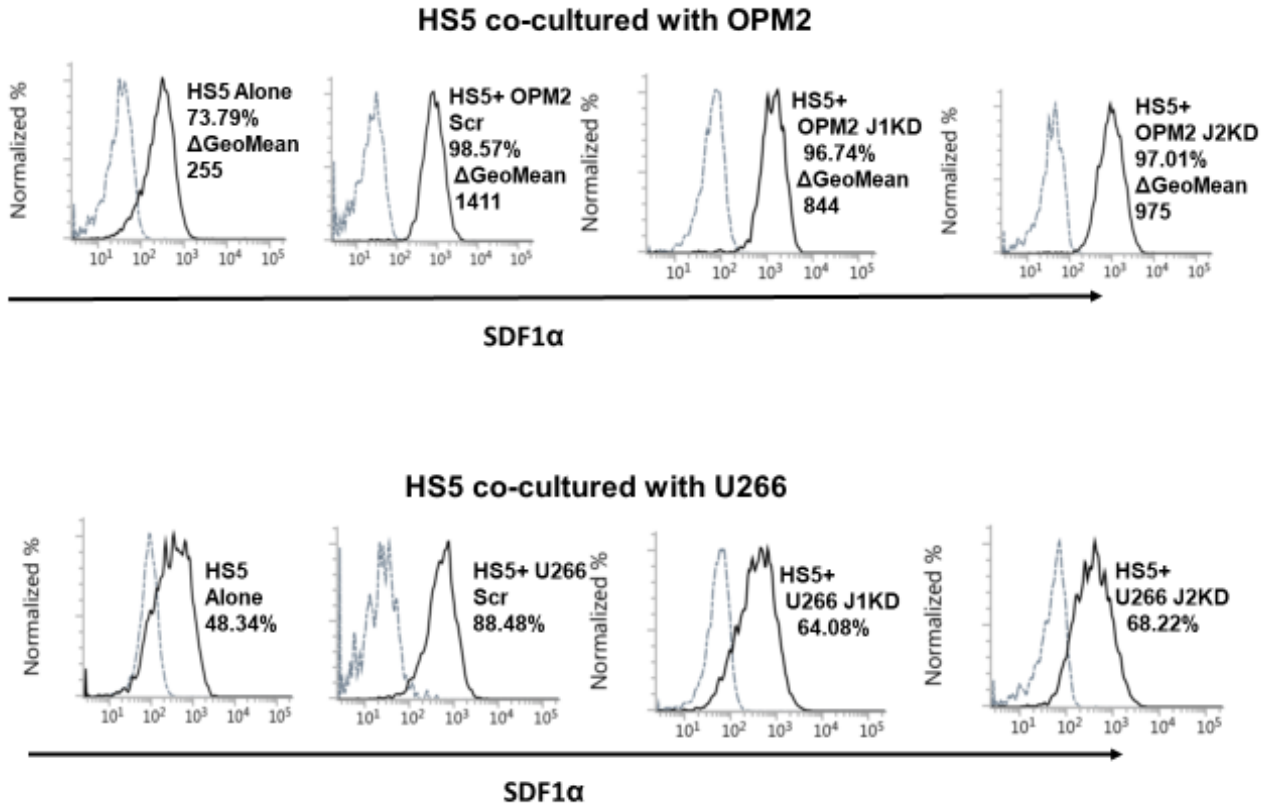

Fig. S11. Effect of the selective silencing of Jagged1 and Jagged2 on the ability of MM cells to boost SDF1α in HS5 cells. Histograms display the levels of intracellular SDF1α (black lines) analyzed by flow cytometry in GFP$^+$ HS5 cells cultured alone or co-culture with Scr OPM2, Jagged1 KD OPM2 or Jagged2 KD OPM2 cells (upper panel) and Scr U266 or Jagged1 KD U266 or Jagged2 KD U266 (lower panel) cells, and the isotype-matched control (gray line). Histograms are representative of at least 3 independent experiments. Due to the high expression of SDF1α in HS5 cultured with OPM2, ΔGeoMFI is reported, too.

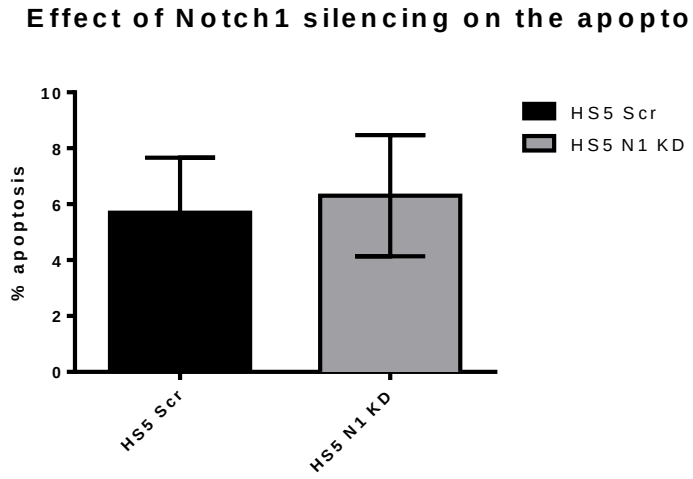

Fig. S12. Notch1 KD does not induce apoptosis in HS5 cells.
The graph shows the levels of apoptosis in Scr or N1KD HS5 cells. The bars represent the mean values ± SD. Statistical analysis performed using one-tailed t-test indicates no significant variation in the apoptotic rates.

Effect of J1/2KD on CXCR4 expression in MM cells

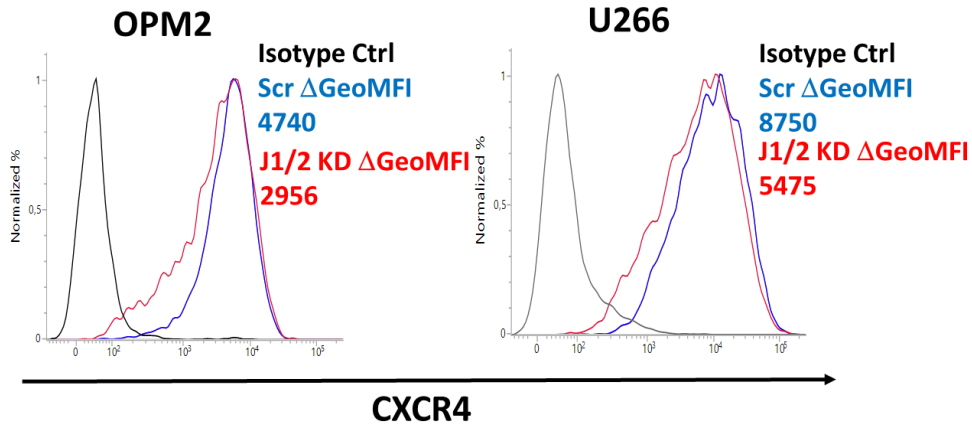

Fig. S13. CXCR4 expression in scr and J1/2KD OPM2 and U266 cell lines. Histograms representative of the expression levels (ΔGeoMFI) of CXCR4 in Scr (blue) and J1/2KD HMCLs (red) used in co-culture experiments with HS5 cells analyzed by flow cytometry. The gray lines represent the isotype-matched controsl.

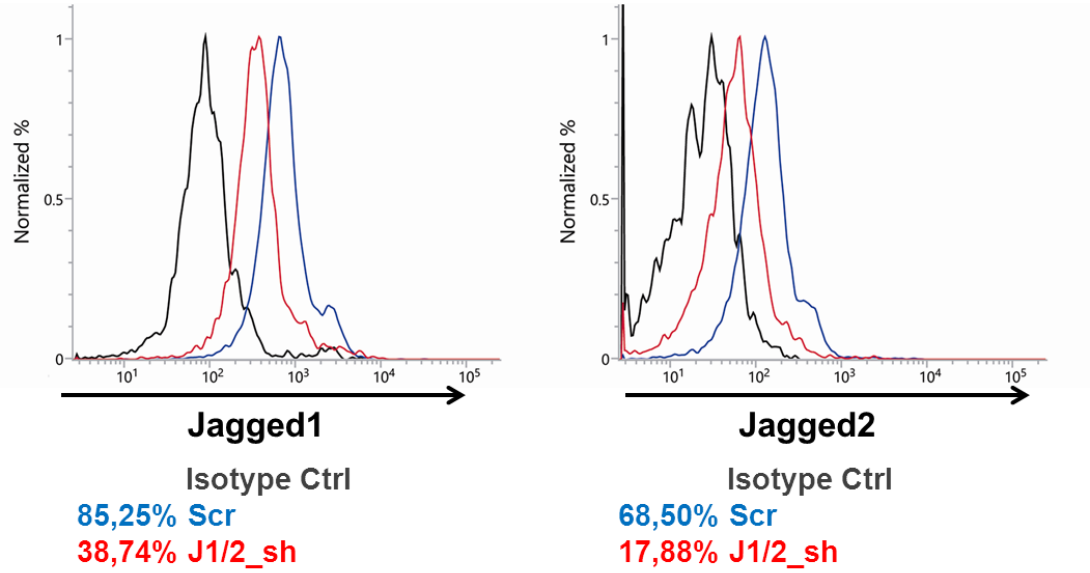

Fig. S14. Infection with pLL3.7 carrying Jagged1/2 shRNAs causes the downregulation of Jagged1/2 in MM cells. OPM2 cells were transduced with the lentiviral vector pLL3.7, carrying Jagged1/2 shRNAs (J1/2_sh, in red) or Scr shRNAs (Scr, in blue). The efficiency of Jagged1/2 silencing was evaluated on transduced GFP+ cells 72h post-infection by flow cytometry using primary antibody anti-Jagged1-APC (R&D Systems, clone #188331) and anti-Jagged2-PE-Vio770 (Miltenyi Biotec, clone #MHJ2-523) or the appropriate isotype controls.

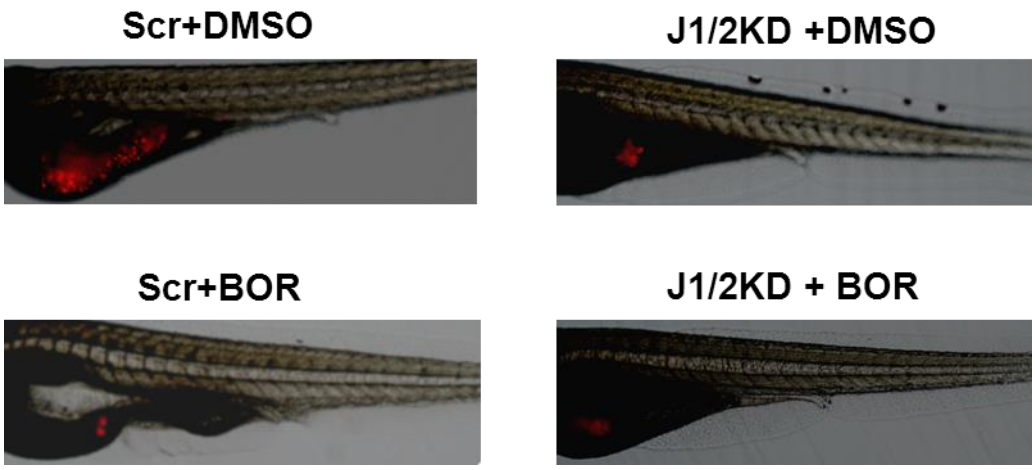

Fig. S15. Fluorescent microscopy (magnification 4X) images of myeloma–stained xenografts at 48 hpi into the yolk of 96hpf (48hpi) zebrafish embryos.

Supplemental experimental procedures

Cells and reagents

MM cells were cultured in RPMI1640 (Lonza, Italy) supplemented with 10% FBS (Euroclone, Italy). NIH3T3 (ATCC® CRL-1658™) and human bone marrow stromal cell line HS5 (ATCC® CRL-11882™) were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Italy) with 10% FBS (Euroclone, Italy). All cell lines were regularly tested to avoid mycoplasma contamination. Primary cells were isolated from patient BM aspirates and MM cells were purified using the Human Whole Blood CD138+ Selection Kit EasySep (StemCell Technologies). Bortezomib (Selleckchem) was used at 6 nM concentration, Melphalan at 30 μM (Santa Cruz Biotechnology) Lenalidomide (Sigma Aldrich) at 15 μM or 30μM, for respectively U266 and OPM2 cells, AMD3100 (Sigma-Aldrich) at 50 μM and IGOR1 (1) (BAS 00693376, Asinex) at 30 μM. Recombinant human SDF1α/CXCL12 (Peprotech) was used at 500 ng/ml for 48 h. Treatement with recombinant human Jagged ligands was performed for 72 h, using Jagged1 (#188-204 – AnaSpec) at 10μg/ml and Jagged2 (R&D Systems Inc., Minneapolis, MN, USA) at 40μg/ml, according to manufacturers' instructions.

RNAi Assays

The siRNAs targeting Notch1, Jagged1 and Jagged2 were previously reported (2, 3). Results of RNA interference were compared with those obtained with the scrambled siRNAs (Scr).Transfections were performed using lipofectamine RNAiMAX (Invitrogen) adding twice 50 nM siRNAs every 48 h (see experimental timelines in the figures).

To knock down Jagged1 and Jagged2, we cloned the lentiviral vector pLL3.7_J1/2Sh. This carries EGFP and two shRNAs targeting the Jagged1 coding sequence (817-837 nt - Gene Bank Sequence ID: AF028593.1) and the Jagged2 coding sequence (2764-2784 nt - Gene Bank Sequence ID: NM_002226.5) separated by a spacer sequence and cloned in the pLL3.7 HpaI and XhoI restriction sites.

Cell culture treatments

For treatment with siRNAs and the used drugs, single cultures OPM2 and U266 cells were transfected with Jagged1 and Jagged2 siRNAs or with the Scr control siRNA, after 48 h transfection was repeated for 8 h and then cells were treated twice every 24 h with

lenalidomide for a total of 48 h or only in the last 24 h with 6 nM bortezomib or 30 μM melphalan; control cells were treated with the corresponding amount of DMSO. Co-cultures were settled taking into account the different growth rate of the two HMCLs. In consideration of the lower growth rate of U266 cells respect OPM2 cells, to obtain the protective effect of stromal cells, 1.5×10^5 cells/ml HS5 cells or NIH3T3 cells were seeded in a 48-well plate for co-cultures involving OPM2 cells and 0.75×10^5 cells/ml for co-cultures with U266 cells. The day after, co-cultures were set up for 48h by adding HCMLs ($3x10^5$cells/ml), and were treated with the appropriate drugs as reported above.

For treatment with inhibitory small molecule, OPM2 cells were cultured with 30 μM IGOR1 for 48 h and then co-cultured with HS5 cells in the presence of the same amount of IGOR1 and the appropriate concentration of drugs as described above; control cells were treated with the corresponding amount of the vehicle. Experiments with ADM3100 (50 μM) were perfomed in the same way. To distinguish apoptotic HMCLs from BMSCs in co-culture systems analysed by flow cytometry, we used HS5 cells constitutively expressing GFP (GFP^+ HS5) (2).

For primary cell co-cultures, BMSCs were stained with PKH26 (Sigma-Aldrich) and allowed to adhere overnight. Primary $CD138^+$ cells were infected with pLL3.7_J1/2Sh, or with the corresponding Scr control (4). Infections were carried out in the presence of 10 μg/ml polybrene (Sigma Aldrich), 20 ng/ml IL6 (Peprotech, USA) and 20ng/ml IGF1 (Peprotech, USA). Samples showing ≥ 30% GFP^+ cells were included in the study. After 24 h, cells were seeded on a monolayer of PKH+ BMSCs (at 50-60% confluence) and cultured for 72 h, adding for the last 24 h 6 nM bortezomib or 30 μM melphalan, or for the last 48 h with15μM lenalidomide or the corresponding amunt of DMSO.

RNA isolation and quantitative Real Time PCR

Total RNA was isolated using the Trizol reagent (Sigma Aldrich) following the manufacturer protocol and cDNA was prepared using RevertAid M-MuLV Reverse Transcriptase (Thermo Fisher Scientific) according to manifactures' instructions. Quantitative real time PCR (qRT-PCR) was performed as previously described (5). Primer sequences are reported in Table S2.

Sequence of RT-qPCR Primers

RT-qPCR primers	Forward Primer 5'-3'	Reverse Primer 5'-3'
mGAPDH	TTGGCCGTATTGGGCGCCTG	CACCCTTCAAGTGGGCCCCG
mHES5	GGCTCACCCCAGCCCGTAGA	TCGTGCCCACATGCACCCAC
mSDF1α	CAGCTCTGCAGCCTCCGGC	AAGAACCGGCAGGGGCATCG
hGAPDH	ACAGTCAGCCGCATCTTCTT	AATGGAGGGGTCATTGATGG
hHES1	GATGCTCTGAAGAAAGATAGC	GTGCGCACCTCGGTATTAAC
hHES6	ATGAGGACGGCTGGGAGA	ACCGTCAGCTCCAGCACTT
hJAG1	TTCGCCTGGCCGAGGTCCTAT	GCCCGTGTTCTGCTTCAGCGT
hJAG2	CCGGCCCCGCAACGACTTTT	CCTCCCTTGCCAGCCGTAGC
hCXCR4	AGCAAAGTGACGCCGAGGGC	CCCTGAGCCCATTTCCTCGGT
hSurvivin	AGCCAGATGACGACCCCAT	CTTGGCTCTTTCTCTGTCCAGT
hBcl2	GTCATGTGTGTGGAGAGCGT	GCCGTACAGTTCCACAAAGG
hABCC1	TAATCCCTGCCCAGAGTCCA	ACTTGTTCCGACGTGTCCTC

Table S2. Primer sequences. Sequences of all the primer sets used for RT-qPCR analysis are reported.

Apoptosis assays

Cells were harvested, washed once with ice-cold PBS, resuspended in binding buffer 1X (HEPES 0,1 M - NaCl 1,4 M - $CaCl_2$ 0,025 M) and stained with Annexin V-APC (ImmunoTools GmbH, Germany) for 10 minutes in the dark and at room temperature. Cells were analyzed using the BD FACSVerse™ System (BD Biosciences). In experiments involving co-culture systems apoptosis of HMCLs was measured as AnnexinV+ cells gated on the GFP-negative population, while in experiments with primary co-cultures, we measured AnnexinV+ cells in the gated GFP^+ population (pLL3.7-infected $CD138^+$).

Flow cytometry analyses

CXCR4 and SDF1α expression was assessed using anti-CXCR4 antibody (clone 555976, BD Biosciences) and anti-SDF1α antibody (clone IC350A, R&D Systems, Inc, US) as previously reported (6). Survivin, BCL2 and ABCC1 expression was determined in GFP-negative MM cells. Briefly, cells were fixed with 4% formaldehyde, permeabilized with 0.5% saponin and stained with primary antibodies anti-human Survivin (ab469, Abcam Plc, UK), anti-human BCL2 (sc-7382, Santa Cruz Biotechnology, CA, USA) or anti-human ABCC1 (IC19291P, R&D Systems, Inc, USA) or the appropriate isotype matched controls. After 1 h

incubation at 4°C in the dark, cells were washed, and the PE-conjugated secondary antibodies were added (sc-3750 anti-rabbit antibody, Santa Cruz Biotechnology, CA, USA; 22549914 anti-mouse antibody ImmunoTools GmbH, Germany). Samples were incubated for 30 minutes in the dark at 4°C, washed and acquired using the BD FACSVerse™ System (BD Biosciences). Data were normalized on the appropriate isotype control.

ELISA

The amount of SDF1α secreted in conditioned media of Scr or J1/2KD HMCLs, HS5 cells or co-culture systems was determined using Human CXCL12/SDF-1 DuoSet ELISA Kit (R&D system) and normalized on total amount of proteins measured within the harvested conditioned medium.

Western Blot assay

Whole cell extracts were prepared in RIPA lysis buffer with proteases and phosphatases inhibitors cocktail (Sigma Aldrich). Protein samples (50–70 µg) were run on Bolt™ 4-12% Bis-Tris Plus Gels (Invitrogen), transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Bioscience, Italy), and blocked with 5% nonfat milk in TBS-T (20mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). Membranes were incubated overnight at 4°C with human primary antibodies anti-BCL2 (1:500, sc-7382, Santa Cruz Biotechnology, CA, USA), anti-Survivin (1:1000, ab469, Abcam, UK), anti-ABCC1 (1:1000, ab233383 Abcam, UK), anti-ICN1 (1:500, sc-4147, Cell Signaling Technology), anti-ICN2 (1:500, SAB4502022, Sigma Aldrich), anti-Jagged1 (1:500, sc-70109, Cell Signaling Technology), anti-Jagged2 (1:500, sc-2205, Cell Signaling Technology) or anti-β-actin (1:10000, ab197277 Abcam, UK) antibodies, and then with the appropriated HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc). Detection was performed by ECL (Promega, Milano, Italy) according to the manufacturer's instructions.

Zebrafish housing and microinjections

Zebrafish AB strains obtained from the Wilson lab, University College London, London, United Kingdom were maintained according to the national guidelines (Italian decree 4th March 2014, n.26) at 28°C on a 14 h light/10 h dark cycle in Petri dishes with fish water (Instant Ocean, 0,1% Methylene Blue). Embryos were collected by natural spawning, staged according to Kimmel and colleagues (7) and raised at 28°C in Petri dishes with fish water (Instant Ocean, 0,1% Methylene Blue), according to established techniques. Embryonic

ages are expressed as hours post fertilization (hpf). At 24 hpf, to prevent pigmentation, 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich, Saint Louis, Missouri, USA) was added to the fish water. All experiments were conducted within 5 days hpf. Zebrafish embryos were dechorionated for 5 to 10 minutes with 1 mg/ml pronase 48 hpf and anaesthetized with 0.016% tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich). Scr or J1/2KD U266 cells were stained with the vital fluorescent dye CM-Dil, resuspended in PBS with 3% polyvinyl pyrrolidone (PVP) and injected into the yolk (200 cells in 10 nl, 5-20 nl injection volume/embryo) with a manual microinjector (Eppendorf, Germany) using glass microinjection needles. Following injections, embryos were kept at 28 °C for 30 minutes and at 32 °C for the duration of the experiments.

Xenotransplant engraftment analysis and drug administration

The efficiency of tumor xenografts was evaluated 2 hours post injection (hpi) by fluorescence microscopy using Leica DM 5500B microscope equipped with DC480 camera. Xenograft-positive embryos were placed into 24-well plates (1 embryo per well) and divided randomly into the following experimental groups: Scr-injected embryos treated with DMSO, Scr-injected embryos treated with bortezomib, J1/2KD-injected embryos treated with DMSO and J1/2KD-injected embryos treated with bortezomib. Bortezomib was added to embryos in 24-well plates at the final concentration of 10nM as previously reported (8). Tumor growth was evaluated 48 hpi by fluorescence microscopy. We measured the area of fluorescence on photomicrographs using ImageJ software and estimated tumor xenograft volume in control and drug-treated animals at 48 hpi normalizing it to the signal obtained at 2 hpi. Images were processed using the Adobe Photoshop program.

Statistical analysis

Statistical analyses were performed using Student's t-test or Mann-Whitney for single comparison and analysis of variance was performed by one-way ANOVA with Tukey post-test or Kruskal-Wallis and Dunn post-test for multiple comparison.

The sample minimum size for each *in vivo* experiment was determined based on *a priori* power analysis for a one-way ANOVA with an alpha level of 0.05 aimed to have power of 0.95, performed on data from a pilot study with 5 embryos for each condition (G-power 3.2 software) (9). Each *in vivo* experiment involved at least 16 embryos divided in 4 group. The final analysis was performed by one-way ANOVA with Tukey post-test on data from 4

independent experiments, excluding outliers identified through the ROUT method (Q=1%) (10).

Supplemental References

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