



Journal of The Ferrata Storti Foundation

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

by Michela Colombo, Silvia Garavelli, Mara Mazzola, Natalia Platonova, Domenica Giannandrea, Raffaella Colella, Luana Apicella, Marialuigia Lancellotti, Elena Lesma, Silvia Ancona, Maria Teresa Palano, Marzia Barbieri, Elisa Taiana, Elisa Lazzari, Andrea Basile, Mauro Turrini, Anna Pistocchi, Antonino Neri, and Raffaella Chiaramonte

Haematologica 2019 [Epub ahead of print]

Citation: Michela Colombo, Silvia Garavelli, Mara Mazzola, Natalia Platonova, Domenica Giannandrea, Raffaella Colella, Luana Apicella, Marialuigia Lancellotti, Elena Lesma, Silvia Ancona, Maria Teresa Palano, Marzia Barbieri, Elisa Taiana, Elisa Lazzari, Andrea Basile, Mauro Turrini, Anna Pistocchi, Antonino Neri, and Raffaella Chiaramonte. Multiple myeloma exploits Jagged1 and Jagged2 to promote intrinsic and bone marrow-dependent drug resistance.

Haematologica. 2019; 104:xxx

doi:10.3324/haematol.2019.221077

Publisher's Disclaimer.

E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.

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

Michela Colombo^{1§}, Silvia Garavelli¹, Mara Mazzola², Natalia Platonova¹, Domenica Giannandrea¹, Raffaella Colella¹, Luana Apicella¹, Marialuigia Lancellotti¹, Elena Lesma¹, Silvia Ancona¹, Maria Teresa Palano¹, Marzia Barbieri^{3,4}, Elisa Taiana^{3,4}, Elisa Lazzari¹, Andrea Basile³, Mauro Turrini⁵, Anna Pistocchi², Antonino Neri^{3,4}, Raffaella Chiaramonte^{1§}.

¹ Department of Health Sciences, Università degli Studi di Milano, via di Rudinì, 8 – 20142 Milano, Italy

² Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Via Vanvitelli 32 - 20133 Milano, Italy

³ Department of Oncology and Hemato-oncology, Università degli Studi di Milano, Via Festa del Perdono 7 – 20122 Milano, Italy

⁴ Hematology, Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico, Via della Commenda, 10, 20122 Milano, Italy.

⁵ Department of Hematology, Division of Medicine, Valduce Hospital, via Dante Alighieri, 11 - 22100 Como, Italy

§ Correspondence to:

Raffaella Chiaramonte

Department of Health Sciences, Università degli Studi di Milano, via di Rudinì, 8 – 20142 Milano, Italy

e-mail: raffaella.chiaramonte@unimi.it

Michela Colombo

Current address: Haematopoietic Stem Cell Biology Laboratory,

MRC Weatherall Institute of Molecular Medicine, University of Oxford, OX3 9DS Oxford, United Kingdom.

e-mail: michela.colombo@ndcls.ox.ac.uk

Jagged1/2 and drug resistance in multiple myeloma

Keywords: Multiple myeloma, bone marrow, tumor microenvironment, Notch, CXCR4, BCL2, Survivin, ABCC1

Abstract

Multiple myeloma is still incurable due to an intrinsic aggressiveness or, more frequently, to the interactions of malignant plasma cells with bone marrow microenvironment. Myeloma cells educate bone marrow cells to support neoplastic cell growth, survival, acquisition of drug resistance resulting in disease relapse.

Myeloma microenvironment is characterized by Notch signaling hyperactivation due to the increased expression of Notch1 and 2 and the ligands Jagged1 and 2 in tumor cells. Notch activation influences myeloma cell biology and promotes the reprogramming of bone marrow stromal cells.

In this work we demonstrate, by *in vitro*, *ex vivo* and using a zebrafish multiple myeloma model, that Jagged inhibition causes a decrease in both myeloma-intrinsic and stromal cell-induced resistance to currently used drugs, i.e. bortezomib, lenalidomide and melphalan.

The molecular mechanism of drug resistance involves the chemokine system CXCR4/SDF1 α . Myeloma cell-derived Jagged ligands trigger Notch activity in bone marrow stromal cells. These, in turn, secrete higher levels of SDF1 α in the bone marrow microenvironment increasing CXCR4 activation in myeloma cells, which is further potentiated by the concomitant increased expression of this receptor induced by Notch activation. Consistently with the augmented pharmacological resistance, SDF1 α boosts the expression of BCL2, Survivin and ABCC1.

These results indicate that a Jagged-tailored approach may contribute to disrupting the pharmacological resistance due to intrinsic myeloma cell features or to the pathological interplay with bone marrow stromal cells and, conceivably, improve patients' response to standard-of-care therapies.

1. Introduction

Multiple myeloma (MM) is the second most common hematological malignancy, still incurable, with a median overall survival that has not been substantially extended since the introduction of anti-myeloma agents such as melphalan, lenalidomide, and bortezomib (1). The typical clinical course of MM displays a remission-relapse pattern due to the appearance of drug-resistant malignant cells, reducing the numbers of effective salvage regimens (2). Therefore, a more stable response requires the development of a therapeutic approach that prevents drug resistance.

MM cells accumulate in the bone marrow (BM), where they establish anomalous signaling loops with BM-residing non-tumor cells, resulting in the exchange of anti-apoptotic factors which critically induce drug resistance (3).

The Notch pathway includes four transmembrane receptors (Notch1-4) activated by the interaction with five ligands (Jagged1-2 and Dll1-3-4) on adjacent cells (4-6). Notch receptors and ligands have been found to be aberrantly expressed in MM cells (7-10). We recently demonstrated that Jagged1 and the Notch transcriptional target HES5 are increasingly expressed in MM and in primary plasma cell leukemia (11). Moreover, Jagged1 and Notch1 are overexpressed during progression from the benign monoclonal gammopathy of uncertain significance (MGUS) to MM (12), while Jagged2 overexpression is detected already at the MGUS stage (13) and can be ascribed to aberrant acetylation of its promoter (14) or to altered post-translational processing due to aberrant expression of the ubiquitin ligase Skeletrophin (15). Finally, Notch2 hyperexpression is associated with the high-risk translocations t(14;16)(q32;q23) and t(14;20)(q32;q11) (16).

Recently, we and other groups pointed out the importance of Jagged ligands in providing MM cells with the ability to shape the surrounding microenvironment, interacting with osteoclast progenitors (17), and promoting a release of BM stromal cell (BMSC) key factors, including IL6, IGF1 and VEGF (11, 13).

Aberrant levels of Notch signaling are associated with pharmacological resistance in different tumor settings (6) and correlate with the expression of anti-apoptotic genes, such as *BCL2* (18) and *Survivin/BIRC5* (19), or regulates the expression of *ABCC1* (46), which contributes to multidrug resistance in MM (20).

Thereby, we hypothesized that the aberrant expression of Notch receptors and ligands in MM cells may portend the development of drug resistance by inducing autonomous activation of Notch in MM cells, and by triggering Notch signaling in the surrounding BMSCs and boosting their ability to support MM cell drug resistance (21, 22).

Previous works investigated how BMSCs support the development of drug resistance in MM cells by activating Notch signaling (23-25). *Viceversa*, here we show that also the overexpression of MM cells-derived Jagged ligands triggers Notch signaling dysregulation in the BM niche and promotes MM cell intrinsic pharmacological resistance as well as BMSC-dependent drug resistance.

2. Methods

2.1 Cell lines and primary cells

The human MM cell lines (HMCLs), OPM2 (ACC-50) and U266 (ATCC® TIB-196) were purchased from the DSMZ and ATCC, respectively. Primary cells were isolated from patient BM aspirates and MM cells were purified using the Human Whole Blood CD138⁺ Selection Kit EasySep (StemCell Technologies). Detailed information is in Table S1. Primary BMSCs were isolated as previously reported (11). The Ethical Committee of Milano University approved this study (approval n.8/15). All cell treatments have been detailed in Supplementary information.

2.2 Luciferase reporter assay

HS5 cells were transiently transfected with a Notch reporter plasmid pNL2.1 carrying a 6xCSL Notch responsive element (26) and with the vector expressing constitutively the firefly luciferase upon the thymidine kinase promoter (pGL4.54[luc2/TK]). After 24 h HS5 cells were cultured alone or placed in co-culture with scrambled (Scr) or Jagged1 and Jagged2 knockdown (J1/2KD) HMCL and incubated for 24 h. Luciferase activity was measured using Nano-Glo® Dual-Luciferase® Reporter assay kit (Promega) on the Glowmax instrument (Promega).

2.3 *In vivo* experiments on xenografted Zebrafish embryos

Zebrafish AB strains obtained from the Wilson lab, University College London, were maintained according to the national guidelines (Italian decree 4/03/2014 2014, n.26). All experiments have been conducted within 5 days hpf.

Dechorionated Zebrafish embryos were injected with Scr or J1/2KD U266 cells stained with the CM-Dil dye into the yolk (200 cells in 10 nl, 5-20 nl injection volume/embryo) with a manual microinjector (Eppendorf, Germany) using glass microinjection needles.

Xenograft-positive embryos divided randomly into the following groups: Scr-injected embryos treated with DMSO, Scr-injected embryos treated with 10 nM bortezomib, J1/2KD-injected embryos treated with DMSO and J1/2KD-injected embryos treated with 10 nM bortezomib. Tumor growth was evaluated 48 hpi by fluorescence microscopy. Further details are in Supplementary Information.

Further details and information concerning cell cultures, RNA isolation and quantitative Real Time PCR (qRT-PCR), RNAi assay, apoptosis assays, flow cytometry, ELISA, western blot and statistical analysis can be found in Supplemental experimental procedures.

3. Results

3.1 Jagged1/2 inhibition improves MM cell response to standard-of-care drugs by increasing the anti-apoptotic background

To assess if Jagged1 and Jagged2 contribute to MM intrinsic drug resistance, we took advantage of an established knockdown (KD) approach using specific siRNAs for Jagged ligands (11, 17) and analyzed MM cell response to three standard-of-care drugs: bortezomib (Bor), melphalan (Melph) and lenalidomide (Len). As reported in experimental timeline in Figure 1A, two HMCLs, OPM2 and U266 cells, were transfected with Jagged1 and Jagged2 (J1/2KD) or the scrambled control (Scr) siRNAs and then were treated with 6 nM Bor or 30 μ M Melph or with 15 or 30 μ M Len (respectively for U266 and OPM2 cells). The efficacy of J1/2KD was assessed by evaluating the expression of Jagged ligands and the active forms of the two Notch receptors expressed in MM cells, Notch intracellular domains 1 and 2 (NICD1 and NICD2), by Western blot (Fig.1B).

The apoptosis rate of J1/2KD HMCLs treated or not with the mentioned drugs was analyzed by flow cytometry. The graphs in Figure 1C show the effect of Bor, Melph and Len on HMCLs normalized on DMSO-treated cells compared to J1/2KD HMCLs treated with the drugs and normalized on untreated J1/2KD HMCLs. As shown, in the graph of Figure 1C, J1/2KD induced an appreciable increase in HMCLs sensitivity to standard-of-care drugs, with statistical significance reached in all cases, with the exception of U266 cells treated with Bor and Melph ($p=0.06$), that anyway keep the trend. The basal apoptotic effect of J1/2KD is shown in Fig. S1. Concerning Len treatment, it is worth mentioning that, although Scr HMCLs are resistant to this drug, J1/2KD cells acquire drug sensitivity. The selective inhibition of Jagged1 or Jagged2 is clearly less effective in comparison with the simultaneous J1/2KD, that maximizes the biological outcome (Fig. S2).

These results indicate that the expression of Jagged1 and 2 stimulates autonomous Notch activity in MM cells that, consequently, may be inhibited by Jagged silencing. This evidence prompted us to verify if the increased pharmacological sensitivity of MM cells induced by J1/2KD was associated to variations in the expression of recognized anti-apoptotic Notch targets, such as BCL2 (18) and *Survivin/BIRC5* (19), or with the levels of *ABCC1* reported to have a significant impact in MM (19, 20, 27). J1/2KD, validated by the decrease in Jagged1, 2 and HES1 and 6 gene expression, significantly inhibited the expression of the studied anti-apoptotic genes analyzed by qRT-PCR (Fig. 2A-B). Fig. S1 confirms that the effect of J1/2KD on gene expression was not due to increased apoptosis rate in HMCLs (approximately 15%). J1/2KD effect on anti-apoptotic effectors was assessed at protein levels by flow cytometry (Fig. 2C-D and S3) and Western blot (Fig. S4). By contrast, the selective inhibition of Jagged1 or Jagged2 was not sufficient to significantly downregulate the expression of these genes (Fig. S5).

3.2 Jagged1 and Jagged2 silencing contributes to MM cells ability to promote BMSC-mediated drug resistance

MM cells localize within the BM and interact with several cell types, hijacking their functions to promote tumor progression. BMSCs are a crucial target in this process, that sustains malignant cell proliferation and survival (22). Since Jagged-mediated activation of Notch pathway is involved in cell-cell communication (6), we hypothesized that MM cell-derived Jagged ligands could activate Notch in BMSCs, possibly determining BMSC-mediated drug resistance.

To explore this hypothesis, we first verified that HMCL-derived Jagged1 and Jagged2 were able to trigger the activation of Notch signaling in a BMSC line, HS5, using a Notch reporter assay. Results in Fig. 3A show that scr HMCLs are able to activate Notch signaling in co-cultured HS5 cells, while this ability is lost by J1/2KD HMCLs, indicating that MM-derived Jagged may activate Notch signaling in BMSCs.

To verify if Jagged-mediated activation of Notch in BMSCs affected the ability of these cells to promote drug resistance in MM cells, we analyzed by flow cytometry the apoptotic rate of Scr or J1/2KD HMCLs cultured alone or co-cultured with HS5 cells after treatment with standard-of-care drugs. As expected, in Fig. 3 B-C, HS5 cells show a clear trend of protection of HMCLs from apoptosis induced by Bor (15% in OPM2 and 26% in U266), Melph (20% in OPM2 and 11% in U266) and Len (14% in OPM2), although the statistical significance was reached only in the case of OPM2 treated with Bor. Conversely and more importantly, J1/2KD induced a statistically significant increase of apoptosis, re-establishing HMCLs drug sensitivity by hampering BMSC-mediated protection (HS5 cells do not display any significant increase of apoptosis - data not shown). Notably, although U266 cells were resistant to Len treatment in culture alone or in the presence of HS5 cells, apoptotic rate increased up to approximately 20% upon J1/2KD. The basal apoptotic effect of J1/2 KD on MM cells cultured with HS5 cells is reported in Fig. S6. As before, the selective Jagged1 or Jagged2 silencing was less effective respect the simultaneous J1/2KD (Fig. S7).

Since HS5 cells could act as a source of paracrine/autocrine Jagged ligands, we wondered why they cannot rescue J1/2KD in MM cells. Western blot analysis in Fig. S8 indicates that the expression levels of Jagged1 and Jagged2 in HS5 cells are significantly lower than those expressed by OPM2 and U266 cells. This can reasonably explain why, in our co-culture system, Notch signaling activated in HMCLs by BMSCs, is not sufficient to rescue the loss of Jagged1 and Jagged2 in MM cells.

We further explored if Jagged-mediated Notch activation in BMSCs could promote the pharmacological resistance of MM cells by upregulating the anti-apoptotic effectors previously analyzed, Survivin, BCL2, and ABCC1. To evaluate gene expression changes, we took advantage of a co-culture system including OPM2 or U266 cells with a non-human mimic model of BMSCs, the murine cell line of NIH3T3 fibroblasts. This approach enabled us to precisely assess the

expression levels of human (HMCL-derived) anti-apoptotic genes in co-culture by using species-specific primers. Results showed that BMSCs were able to promote the expression of the anti-apoptotic effectors *Survivin*, *BCL2*, and *ABCC1* in Scr HMCLs, while BMSCs co-cultured with J1/2KD HMCLs lost this ability (Fig. 4A-B). Importantly, using an entirely human co-culture system, we observed the same effects measuring by flow cytometry the protein expression of *Survivin*, *BCL2*, and *ABCC1* in Scr or J1/2KD HMCLs co-cultured with human GFP⁺ HS5 (Fig. 4C-E and Fig. S9).

3.3 The CXCR4/SDF1 α axis is a mediator of Notch pathway ability to determine drug resistance in multiple myeloma

To further study the molecular mechanisms underlying BMSC-induced drug resistance generated by Notch activation in MM microenvironment, we explored the possible involvement of the chemokine system CXCR4/SDF1 α , a key player in MM development and progression, and a downstream regulator of Notch signaling (28, 29). We hypothesized that Notch ability to promote pharmacologic resistance in MM cells might be mediated by SDF1 α . We reasoned that the main source of SDF1 α in the BM was the stromal cell population, therefore we explored if Jagged ligands, expressed by MM cells, could trigger the BMSC-mediated production of SDF1 α and if J1/2KD might inhibit this effect.

The analysis was performed by taking advantage of co-culture systems of Scr or J1/2KD HMCLs grown on a layer murine (NIH3T3) or human (HS5) stromal cells to measure the variations of SDF1 α gene or protein expression. Results obtained by qRT-PCR with murine-specific primers shown in Fig. 5A indicate that HMCLs promoted the activation of Notch signaling (HES5) and SDF1 α gene expression in NIH3T3 cells, which could be reverted by J1/2KD.

Similar results were observed at protein level as assessed by flow cytometry analysis (Fig. 5B and S10) on co-cultures composed by HMCLs and the human GFP⁺ HS5 cells. Of note, the selective inhibition of Jagged1 or Jagged2 is clearly less effective if compared with the simultaneous J1/2KD, that maximizes the outcome on SDF1 α inhibition (Fig. S11). Flow cytometric results were validated by ELISA on conditioned media (Fig. 5C) indicating that MM cell-derived Jagged can increase SDF1 α production by BMSCs. We further confirmed that the variation of SDF1 α expression was the consequence of Jagged-activated Notch signaling in BMSCs by assessing that the stimulation with Jagged1 and/or Jagged2 peptides can increase HS5 cell-mediated secretion of SDF1 α , measured by ELISA (Fig. 5D). Additionally, we knocked down Notch1 expression in HS5 cells (N1KD HS5) by using a specific siRNA as previously reported (11) and observed that SDF1 α expression significantly decreased in comparison to control HS5 cells (Fig. 5E). Since Notch1 silencing does not significantly affect HS5 cell viability (Fig. S12), we could exclude that reduction of SDF1 α expression might be due to HS5 cell apoptosis.

On the other side, we verified that J1/2KD was associated to a reduced CXCR4 expression in HMCLs used in co-culture experiments. As shown in Fig. 5F and S13, J1/2KD HMCLs significantly decreased CXCR4 expression in comparison to Scr HMCLs.

We assessed the outcome of SDF1 α stimulation on the anti-apoptotic background of HMCLs, by analyzing the levels of Survivin, BCL2, and ABCC1 in U266 cells treated with 500 ng/ml SDF1 α for 48 h. We observed an increase in *Survivin*, *BCL2*, and *ABCC1* gene expression by qRT-PCR analysis (Fig. 5G) confirmed at protein level by Western blot (Fig. 5H). These results suggest that SDF1 α can promote MM cell ability to survive to drug administration, at least in part, by stimulating tumor cell anti-apoptotic defenses (Survivin, BCL2) and detoxification ability (ABCC1). Consistently, the treatment of U266-HS5 co-culture system with 50 μ M AMD3100 (an antagonist of SDF1 binding to CXCR4), abrogated BMSC-induced resistance to the analyzed drugs (Fig. 5I).

3.4 Translational potential of approaches inhibiting Jagged-mediated Notch activation in multiple myeloma microenvironment

We further verified whether the ability of MM cells to promote BMSC-induced drug resistance was dependent on Jagged1 and Jagged2 expression by using primary co-culture systems of highly purified CD138⁺MM cells and BMSCs isolated from BM aspirates of patients at MM onset (Table S1 in Supplementary information).

Primary CD138⁺ cells were transduced with the lentiviral vector pLL3.7 carrying Jagged1/2 shRNAs or Scr shRNAs and the efficiency was assessed by flow cytometry (Fig. S14). In order to maintain CD138⁺ cells viability during *ex vivo* drug administration, after lentiviral transduction, they were co-cultured with primary BMSCs stained with PKH26. Co-cultures were maintained for 72 h and treated for the last 24 h with 6 nM Bor (8 patients) or 30 μ M Melph (10 patients) or for the last 48 h with 15 μ M Len (9 patients) or the corresponding vehicle. The apoptotic rate of MM cells (expressing the GFP codified by the pLL3.7 vector) was detected by flow cytometry analyzing the GFP⁺/Annexin-V-APC⁺ subpopulation (Fig. 6A). Results showed that J1/2KD significantly increased apoptosis of primary MM cells treated with all the analyzed drugs, in agreement with the obtained *in vitro* findings.

To verify if the inhibitory approach based on J1/2KD had a translational potential, we recapitulated the experiments of MM-BMSC interplay by using IGOR1, a novel small molecule recently developed in our laboratory (30) to uncouple Notch-Jagged interaction. Results in Fig. 6B and C show that IGOR1 is able to inhibit Notch activation in OPM2 cells and significantly increases the efficacy of the administered drugs, with a higher efficiency for Mel and Len.

3.5 Jagged1 and Jagged2 blockade promotes sensitivity to bortezomib in a zebrafish xenograft myeloma model

Bortezomib is one of the most commonly used drugs for the treatment of newly-diagnosed and refractory MM patients (31). In the recent years several studies supported the hypothesis that the development of resistance to such treatment is strongly dependent upon the BM microenvironment, with a significant contribution of the CXCR4/SDF1 α axis (32-34). Due to the results obtained *in vitro* concerning the role of this chemokine axis in the development of pharmacological resistance to Bor, we validated the effect of J1/2KD on MM cell resistance to Bor by taking advantage of a novel zebrafish xenograft MM model.

Zebrafish embryos were recently validated as a complementary *in vivo* model for MM that allows the rapid screening of MM cells response to chemotherapeutic drugs (35). Moreover, this model fully recapitulates the cytokine milieu present in the human BM, since zebrafish-secreted growth factors, such as IL6 and SDF1 α , support MM cells growth *in vivo* (33, 35). To validate our *in vitro* and *ex vivo* findings, Scr or J1/2KD U266 cells vitally labelled with the fluorescent dye CM-Dil were injected in the yolk area of 48 hpf zebrafish embryos. Xenotransplanted embryos were visualized by fluorescent microscopy to verify the presence of MM cells at the injection site at 2 hpi (Fig. 7A-B-C-D), treated or not with 10 nM Bor and, visualized at 48 hpi for tumor cell growth (Fig. 7A'-B'-C'-D'). Representative images of whole embryos are shown in Fig. S15.

As shown, the addition of 10 nM Bor to the embryo medium inhibited tumor growth of about 57% compared to controls (Fig. 7A'-B'), without affecting fish viability. A similar effect was induced by J1/2KD (Fig. 7A'-C'), while the combination of J1/2KD and Bor significantly reduced tumor growth in comparison to all other experimental groups (-82% in comparison to the control) (Fig. 7A'-B'-C'-D').

4. Discussion

MM progression is characterized by development of drug resistance causing patients' relapse and contributing to the fatal outcome of this disease. The close interaction of MM cells with BM healthy cells represents an important source of factors able to promote malignant cell growth and survival.

Notch is a pathway able to mediate the cell-cell communication. Current evidence provided by different groups including ours, highlighted the importance of Jagged ligands in the pathological communication between tumor and healthy cells within the myeloma BM. MM-derived Jagged ligands activates Notch receptors in the nearby BM cells inducing osteoclastogenesis, osteolysis (17), angiogenesis (36) and BMSC-mediated release of key cytokines including IL6, IGF1 and VEGF (11, 13). Moreover, the activation of Notch signaling in MM cells, induced by tumor cell- (37, 38) or BMSC-derived Jagged1 (25), stimulates MM cells proliferation (38), resistance to apoptosis (37) and decrease of drug sensitivity (25).

This work is specifically focused on the pathological communication of MM cells and BMSCs mediated by Notch signaling and on its outcome on MM drug resistance. Notably, the Notch pathway is known to be a key player in BM-induced drug resistance in other haematologic

malignancies. Indeed, Krampera's group provided several evidences of how the BM-driven activation of Notch3 and Notch4 in B-ALL (39-41) and Notch1, Notch2 and Notch4 in chronic lymphocytic leukemia (42), results in chemoresistance, while Notch1-Jagged1 crosstalk supports BM-induced drug resistance in AML (43). Concerning MM, although the recent advances in this field, it is still missing a complete picture of the bidirectional crosstalk between BMSCs and MM cells, that is predictable on the basis of the expression of Notch receptors and ligands on both cell types (11, 12, 16, 23, 25, 44). This work aims to fill some gaps by providing novel information about the effects of the aberrant expression of MM-derived Jagged ligands on the intrinsic tumor cell drug resistance and investigating a key aspect never explored before, the outcome of MM-derived Jagged ligands on BMSC-induced drug resistance.

To address these issues, we interfered with the mRNA expression of MM-derived Jagged ligands and investigated J1/2KD outcomes in tumor cells and in surrounding BMSCs. We observed *in vitro* that MM cell-derived Jagged ligands could trigger Notch signaling in the nearby MM cells by homotypic interaction. Notch activation resulted in the increased expression of anti-apoptotic effectors including BCL2, Survivin and the multidrug resistance transporter ABCC1, along with the increase of MM cell survival to standard-of-care drugs, such as bortezomib, melphalan and lenalidomide. Notably, besides observing homotypic activation of Notch signaling among MM cells, we found out that HMCLs can trigger Notch signaling in the neighboring BMSCs and, in turn, Notch activation boosts BMSCs ability to increase the pharmacological resistance of MM cells. This effect was clearly dependent on MM-derived Jagged ligands, since J1/2KD completely abrogated BMSCs support. At least in part, the pro-tumor effect of Notch-“educated” BMSCs was due to their ability to increase SDF1 α level in the BM microenvironment. Indeed, soluble or MM cell-derived Jagged ligands may induce a Notch-dependent increase of SDF1 α secretion by BMSCs; on the contrary, J1/2KD HMCLs lose this ability and N1KD interferes with BMSCs to release SDF1 α . The Notch-dependent activation of SDF1 α secretion by BMSCs is potentially more relevant than the previously observed secretion induced by Notch activation in MM cells (29), since BMSCs are the most effective producers of this cytokine in the BM.

To complete the picture of Notch signaling effect on the SDF1 α /CXCR4 axis in myeloma BM, we also demonstrated that MM cell-derived Jagged ligands may further enhance the anti-apoptotic signaling of SDF1 α by stimulating the expression of its receptor CXCR4 on MM cell surface.

The contribution of SDF1 α /CXCR4 axis to MM pharmacological resistance was confirmed by the ability of the antagonist molecule AMD3100 to abrogate U266 cell resistance to bortezomib, melphalan and lenalidomide induced by BMSCs consistently with findings of Azab *et al.* (32).

Although the downstream molecular mechanisms of Notch-associated drug resistance in MM still need to be fully elucidated, we showed that the secreted SDF1 α can stimulate general mechanisms including tumor cell antiapoptotic background, by up-regulating BCL2 and Survivin, or drugs extrusion mediated by ABCC1. These antiapoptotic proteins are particularly relevant.

Indeed, BCL2 and Survivin are overexpressed in MM cells, where they play an important role in cell survival, and significantly correlate with disease stage (20, 27, 45); on the other side, xenobiotic transporters, such as ABCC1, are well known mediators of MM multidrug resistance (20), modulated by Notch in different cancer settings (46).

The general validity of these novel findings stems from the observed improvement in drug-response promoted by J1/2KD in *ex-vivo* co-culture systems of CD138+ MM cells and BMSCs from BM aspirates of newly-diagnosed MM patients.

Additionally, *in vivo* validation of these findings in a zebrafish xenograft MM model engrafted with U266 cells, confirmed that J1/2KD promoted an increased response to Bor *in vivo*, showing a wider decrease of tumor burden compared to the control.

The present results provide novel and relevant information to improve the current picture on the effect of Notch mediated communication in myeloma BM. Indeed, since both BMSCs and MM cells carry Notch receptors and ligands, it is necessary to consider their bidirectional crosstalk. We sought to fill the information gap on the role of MM cells as Notch signaling sending cells in the BM and, here, we discuss our findings according to the previous literature data in order to summarize the overall picture (Fig. 8). Previous work reported the consequences of Notch activity in MM cells (mainly using γ -secretase inhibitors), identifying the following molecular mechanisms: i) up-regulation of p21 inducing MM cells growth inhibition and increased survival (23; ii) Notch/HES1 mediated down-regulation of the pro-apoptotic protein Noxa (24); iii) Notch up-regulated expression of integrin $\alpha\beta 5$ resulting in increased adhesion to vitronectin and consequent protection from pro-apoptotic drugs (47); iv) upregulation of the enzyme cytochrome P450 (44), implicated in drug metabolism and in the onset of several malignancies (48). Concerning the contribution of Notch in BMSC-dependent drug resistance, previous investigations were focused on the autonomous contribution of BMSC-derived Notch ligands in MM cell behavior (23, 25, 44) (Fig. 8).

In this work we found that the alteration induced in the BM by the presence of MM cells aberrantly expressing Jagged ligands is a key step in “educating” the tumor microenvironment to a pro-tumor behavior. Indeed, MM cell-derived Jagged1 and 2 may switch on Notch signaling in tumor and non-tumor BMSCs by triggering Notch signaling, activating MM cell anti-apoptotic background, increasing SDF1 α level in the BM, and finally resulting in support to MM cell resistance to standard-of-care drugs (Fig. 8).

Overall, our findings provide the proof-of-principle that selective targeting of Jagged ligands in MM cells can restore tumor cell sensitivity to therapy, laying the foundation for the development of combined low-toxic therapeutic options to restore drug sensitivity and overcome fatal drug resistance of relapsing MM patients. Recently, inhibitory small molecules (30) or neutralizing antibodies (49) directed to inhibit the activation of Notch signaling mediated by Jagged ligands have been developed. This prompted us to confirm the translational potential of our results by

testing the anti-tumor effect of an inhibitory small molecule developed in our laboratory, IGOR1, which was directed to uncouple Notch-Jagged interaction. *In vitro* results showed that IGOR1 had the ability to increase MM cell pharmacological response, with higher efficacy if combined with Melphalan and Lenalidomide.

The importance of our results stems from the evidence that a Jagged-tailored therapy might represent a more suitable clinical approach to achieve the inhibition of Notch signaling in the BM of MM patients. Indeed, it lacks potential adverse effects of pan-Notch blockade obtained with γ -secretase inhibitors (GSIs), that provided promising results in *in vivo* MM model by increasing the chemotherapeutic effect of doxorubicin and melphalan (24), but are associated with severe gastrointestinal toxicity due to intestine metaplasia (50, 51).

5. Acknowledgments

This study was supported by grants from Associazione Italiana Ricerca sul Cancro, AIRC Investigator Grant to RC (20614) and AN (16722), My First Grant to AP (18741); Fondazione Italiana per la Ricerca sul Cancro to MC (post-doctoral fellowship 18013) and ET (post-doctoral fellowship 19370); Università degli Studi di Milano to RC (Linea 2B-2017 - Dept. Health Sciences), to NP (post-doctoral fellowship type A), SG, MTP and EL (PhD fellowship in Molecular and Translational Medicine), DG (PhD fellowship in Experimental Medicine).

Conflict of interest statement

The authors declare no conflicts of interest.

Supplementary Information is available at journal website.

References

1. Kumar SK, Rajkumar V, Kyle RA, et al. Multiple myeloma. *Nat Rev Dis Primers*. 2017;3:17046.
2. Nijhof IS, van de Donk N, Zweegman S, Lokhorst HM. Current and New Therapeutic Strategies for Relapsed and Refractory Multiple Myeloma: An Update. *Drugs*. 2018;78(1):19-37.
3. Manier S, Sacco A, Leleu X, Ghobrial IM, Roccaro AM. Bone marrow microenvironment in multiple myeloma progression. *J Biomed Biotechnol*. 2012;2012:157496.
4. Chiaramonte R, Basile A, Tassi E, et al. A wide role for NOTCH1 signaling in acute leukemia. *Cancer Lett*. 2005;219(1):113-120.
5. Colombo M, Mirandola L, Chiriva-Internati M, et al. Cancer Cells Exploit Notch Signaling to Redefine a Supportive Cytokine Milieu. *Front Immunol*. 2018;9(1823).
6. Platonova N, Lesma E, Basile A, et al. Targeting Notch as a Therapeutic Approach for Human Malignancies. *Curr Pharm Des*. 2017;23(1):108-134.
7. Colombo M, Mirandola L, Platonova N, et al. Notch-directed microenvironment reprogramming in myeloma: a single path to multiple outcomes. *Leukemia*. 2013;27(5):1009-1018.
8. Mirandola L, Comi P, Cobos E, Kast WM, Chiriva-Internati M, Chiaramonte R. Notch-ing from T-cell to B-cell lymphoid malignancies. *Cancer Lett*. 2011;308(1):1-13.
9. Platonova N, Manzo T, Mirandola L, et al. PI3K/AKT signaling inhibits NOTCH1 lysosome-mediated degradation. *Genes Chromosomes Cancer*. 2015;54(8):516-526.
10. Colombo M, Galletti S, Garavelli S, et al. Notch signaling deregulation in multiple myeloma: A rational molecular target. *Oncotarget*. 2015;6(29):26826-26840.
11. Colombo M, Galletti S, Bulfamante G, et al. Multiple myeloma-derived Jagged ligands increases autocrine and paracrine interleukin-6 expression in bone marrow niche. *Oncotarget*. 2016;7(35):56013-56029.
12. Skrtic A, Korac P, Kristo DR, Ajdukovic Stojisavljevic R, Ivankovic D, Dominis M. Immunohistochemical analysis of NOTCH1 and JAGGED1 expression in multiple myeloma and monoclonal gammopathy of undetermined significance. *Hum Pathol*. 2010;41(12):1702-1710.
13. Houde C, Li Y, Song L, et al. Overexpression of the NOTCH ligand JAG2 in malignant plasma cells from multiple myeloma patients and cell lines. *Blood*. 2004;104(12):3697-3704.

14. Ghoshal P, Nganga AJ, Moran-Giuati J, et al. Loss of the SMRT/NCOR2 corepressor correlates with JAG2 overexpression in multiple myeloma. *Cancer Res.* 2009;69(10):4380-4387.
15. Takeuchi T, Adachi Y, Ohtsuki Y. Skeletrophin, a novel ubiquitin ligase to the intracellular region of Jagged-2, is aberrantly expressed in multiple myeloma. *Am J Pathol.* 2005;166(6):1817-1826.
16. van Stralen E, van de Wetering M, Agnelli L, Neri A, Clevers HC, Bast BJ. Identification of primary MAFB target genes in multiple myeloma. *Exp Hematol.* 2009;37(1):78-86.
17. Colombo M, Thummler K, Mirandola L, et al. Notch signaling drives multiple myeloma induced osteoclastogenesis. *Oncotarget.* 2014;5(21):10393-10406.
18. Ye QF, Zhang YC, Peng XQ, Long Z, Ming YZ, He LY. Silencing Notch-1 induces apoptosis and increases the chemosensitivity of prostate cancer cells to docetaxel through Bcl-2 and Bax. *Oncol Lett.* 2012;3(4):879-884.
19. Ju JH, Yang W, Oh S, et al. HER2 stabilizes survivin while concomitantly down-regulating survivin gene transcription by suppressing Notch cleavage. *Biochem J.* 2013;451(1):123-134.
20. Buda G, Ricci D, Huang CC, et al. Polymorphisms in the multiple drug resistance protein 1 and in P-glycoprotein 1 are associated with time to event outcomes in patients with advanced multiple myeloma treated with bortezomib and pegylated liposomal doxorubicin. *Ann Hematol.* 2010;89(11):1133-1140.
21. Kawano Y, Moschetta M, Manier S, et al. Targeting the bone marrow microenvironment in multiple myeloma. *Immunol Rev.* 2015;263(1):160-172.
22. Colombo M, Platonova N, Giannandrea D, Palano MT, Basile A, Chiaramonte R. Re-establishing Apoptosis Competence in Bone Associated Cancers via Communicative Reprogramming Induced Through Notch Signaling Inhibition. *Front Pharmacol.* 2019;10(145).
23. Nefedova Y, Cheng P, Alsina M, Dalton WS, Gabilovich DI. Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. *Blood.* 2004;103(9):3503-3510.
24. Nefedova Y, Sullivan DM, Bolick SC, Dalton WS, Gabilovich DI. Inhibition of Notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy. *Blood.* 2008;111(4):2220-2229.
25. Muguruma Y, Yahata T, Warita T, et al. Jagged1-induced Notch activation contributes to the acquisition of bortezomib resistance in myeloma cells. *Blood Cancer J.* 2017;7(12):650.
26. Kato H, Taniguchi Y, Kurooka H, et al. Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. *Development.* 1997;124(20):4133-4141.
27. Khan N, Kahl B. Targeting BCL-2 in Hematologic Malignancies. *Target Oncol.* 2018;13(3):257-267.
28. Chiaramonte R, Colombo M, Bulfamante G, et al. Notch pathway promotes ovarian cancer growth and migration via CXCR4/SDF1alpha chemokine system. *Int J Biochem Cell Biol.* 2015;66:134-140.
29. Mirandola L, Apicella L, Colombo M, et al. Anti-Notch treatment prevents multiple myeloma cells localization to the bone marrow via the chemokine system CXCR4/SDF-1. *Leukemia.* 2013;27(7):1558-1566.
30. Platonova N, Parravicini C, Sensi C, et al. Identification of small molecules uncoupling the Notch::Jagged interaction through an integrated high-throughput screening. *PLoS One.* 2017;12(11):e0182640.
31. Richardson PG, Sonneveld P, Schuster M, et al. Extended follow-up of a phase 3 trial in relapsed multiple myeloma: final time-to-event results of the APEX trial. *Blood.* 2007;110(10):3557-3560.
32. Azab AK, Runnels JM, Pitsillides C, et al. CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. *Blood.* 2009;113(18):4341-4351.
33. Sacco A, Roccaro AM, Ma D, et al. Cancer Cell Dissemination and Homing to the Bone Marrow in a Zebrafish Model. *Cancer Res.* 2016;76(2):463-471.

34. Waldschmidt JM, Simon A, Wider D, et al. CXCL12 and CXCR7 are relevant targets to reverse cell adhesion-mediated drug resistance in multiple myeloma. *Br J Haematol.* 2017;179(1):36-49.
35. Lin J, Zhang W, Zhao J-J, et al. A clinically relevant in vivo zebrafish model of human multiple myeloma (MM) to study preclinical therapeutic efficacy. *Blood.* 2016;128(2):249-252.
36. Saltarella I, Frassanito MA, Lamanuzzi A, et al. Homotypic and Heterotypic Activation of the Notch Pathway in Multiple Myeloma–Enhanced Angiogenesis: A Novel Therapeutic Target? *Neoplasia.* 2019;21(1):93-105.
37. Jia CM, Tian YY, Quan LN, Jiang L, Liu AC. miR-26b-5p suppresses proliferation and promotes apoptosis in multiple myeloma cells by targeting JAG1. *Pathol Res Pract.* 2018;214(9):1388-1394.
38. Jundt F, Probsting KS, Anagnostopoulos I, et al. Jagged1-induced Notch signaling drives proliferation of multiple myeloma cells. *Blood.* 2004;103(9):3511-3515.
39. Nwabo Kamdje AH, Krampera M. Notch signaling in acute lymphoblastic leukemia: any role for stromal microenvironment? *Blood.* 2011;118(25):6506-6514.
40. Nwabo Kamdje AH, Mosna F, Bifari F, et al. Notch-3 and Notch-4 signaling rescue from apoptosis human B-ALL cells in contact with human bone marrow-derived mesenchymal stromal cells. *Blood.* 2011;118(2):380-389.
41. Takam Kamga P, Dal Collo G, Midolo M, Adamo A, Delfino P. Inhibition of Notch Signaling Enhances Chemosensitivity in B-cell Precursor Acute Lymphoblastic Leukemia. *Cancer Res.* 2019;79(3):639-649.
42. Nwabo Kamdje AH, Bassi G, Pacelli L, et al. Role of stromal cell-mediated Notch signaling in CLL resistance to chemotherapy. *Blood Cancer J.* 2012;2(5):e73-e73.
43. Takam Kamga P, Bassi G, Cassaro A, et al. Notch signalling drives bone marrow stromal cell-mediated chemoresistance in acute myeloid leukemia. *Oncotarget.* 2016;7(16):21713-21727.
44. Xu D, Hu J, De Bruyne E, et al. Dll1/Notch activation contributes to bortezomib resistance by upregulating CYP1A1 in multiple myeloma. *Biochem Biophys Res Commun.* 2012;428(4):518-524.
45. Romagnoli M, Trichet V, David C, et al. Significant impact of survivin on myeloma cell growth. *Leukemia.* 2007;21(5):1070-1078.
46. Cho S, Lu M, He X, et al. Notch1 regulates the expression of the multidrug resistance gene ABCC1/MRP1 in cultured cancer cells. *Proc Natl Acad Sci U S A.* 2011;108(51):20778-20783.
47. Ding Y, Shen Y. Notch increased vitronectin adhesion protects myeloma cells from drug induced apoptosis. *Biochem Biophys Res Commun.* 2015;467(4):717-722.
48. Go R-E, Hwang K-A, Choi K-C. Cytochrome P450 1 family and cancers. *J Steroid Biochem Mol Biol.* 2015;147:24-30.
49. Li D, Masiero M, Banham AH, Harris AL. The Notch Ligand Jagged1 as a Target for Anti-Tumor Therapy. *Front Oncol.* 2014;4:254.
50. Milano J, McKay J, Dagenais C, et al. Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol Sci.* 2004;82(1):341-358.
51. Wong GT, Manfra D, Poulet FM, et al. Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. *J Biol Chem.* 2004;279(13):12876-12882.

Figure legends

Figure 1. J1/2 silencing increases drug response in MM cells. (A) Timeline of the experiment to study J1/2KD effect on HMCL drug. (B) Representative western blots showing the expression of Jagged1, Jagged2, NICD1 and NICD2 in OPM2 and U266 cells following single and combined Jagged1 and/or Jagged2 silencing. β -actin was used as loading control. (C) The effect of J1/2KD on OPM2 (left) and U266 (right) cell response to Bor, Melph and Len was evaluated on culture by Annexin V staining. MM cells were transfected with two specific siRNAs targeting Jagged1 and Jagged2 (J1/2KD) or the corresponding scrambled control (Scr) and treated with Bor, Melph or Len. Values of apoptosis of Scr HMCLs were normalized to the corresponding DMSO treated controls and values of J1/2KD HMCLs treated with drugs were normalized to DMSO treated J1/2KD HMCLs. Results are shown as the mean values \pm SEM from at least 3 independent experiments and statistical analysis was performed using Mann-Whitney test (*= $p < 0.05$; **= $p < 0.01$).

Figure 2. J1/2 withdrawal affects MM cells anti-apoptotic background. We analyzed how J1/2KD affects Notch activation and the expression of anti-apoptotic genes in HMCLs. (A-B) Confirmation of J1/2KD efficacy in OPM2 (A) and U266 (B) cells was obtained by qPCR assay assessing the relative gene expression variation of Jagged1 and Jagged2 and Notch target genes HES1 and HES6 (normalized to GAPDH) in cells transfected with J1/2KD siRNA compared to cells transfected with Scr siRNA, calculated by the $2^{-\Delta\Delta Ct}$ formula. The expression levels of the anti-apoptotic effectors BCL2, Survivin and ABCC1 were also analyzed. Data are expressed as mean value \pm SD of at least three independent experiments. Two-tailed t-test confirmed statistically significant downregulation of the tested genes; (C-D) Histograms display the levels of BCL2, Survivin and ABCC1 protein (black lines) analyzed by flow cytometry in J1/2KD OPM2 or Scr OPM2 (C) and J1/2KD U266 or Scr U266 (D) and an isotype-matched control (gray line). Histograms are representative of at least 3 independent experiments.

Figure 3. Effect of J1/2 inhibition on MM cells ability to promote BM-induced drug resistance. (A) A Notch-responsive dual luciferase assay was carried out in HS5 cells cultured alone or in the presence of Scr or J1/2KD HMCLs for 24 h. Data were normalized on luciferase activity in HS5 cells cultured alone (=100). Mean values \pm SD of three independent experiments are shown. Statistical analysis was performed using one-way ANOVA and Tukey post-test (**= $p < 0.01$). (B-C) Co-cultures of J1/2KD or Scr HMCLs with the BMSC line HS5 were established to evaluate the effect of J1/2KD on BMSC-induced drug resistance. The experimental timeline is reported. Graphs display the percentage of apoptotic OPM2 (B) or U266 (C) cells (Annexin V⁺/GFP⁻). Values of apoptosis of each type of culture (Scr alone, Scr + HS5 and J1/2KD + HS5)

treated with drugs are normalized to the corresponding controls treated with DMSO. Results are shown as the mean \pm SEM from at least 3 independent experiments. Statistical analysis was performed using Kruskal-Wallis and Dunn post-test (*= $p < 0.05$; **= $p < 0.01$).

Figure 4. J1/2KD is crucial to determine BM-induced drug resistance. We investigated how J1/2KD affects the molecular outcome of MM cell crosstalk with BMSCs.

qRT-PCR for BCL2, Survivin and ABCC1 gene expression in Scr or J1/2KD OPM2 cells (A) and Scr or J1/2KD U266 cells (B) cultured in the presence of the NIH3T3 cell line. Graphs show the relative expression levels normalized to GAPDH and compared with Scr cells cultured alone (=1), calculated by the $2^{-\Delta\Delta Ct}$ formula. Mean values \pm SD are shown. Statistical analysis was performed using two-tailed t-test (* = $p < 0.05$; ** = $p < 0.01$).

Histograms display the levels of intracellular BCL2 (C), Survivin (D) and ABCC1 (E) (black lines) analyzed by flow cytometry in Scr or J1/2KD OPM2 cells (left panels) and Scr or J1/2KD (right panels) in single culture or co-cultured with GFP⁺ HS5 cells. The isotype-matched control is shown in gray. Histograms are representative of at least 3 independent experiments.

Figure 5. MM cell-derived Jagged ligands promote resistance to apoptosis through the modulation of the CXCR4/SDF1 α axis in the BM niche. We evaluated the effect of J1/2KD in HMCLs on the CXCR4/SDF1 α axis in the myeloma BM and the consequence on the pharmacological resistance.

(A) qRT-PCR for SDF1 α and HES5 gene expression in NIH3T3 cells co-cultured with J1/2KD or Scr OPM2 cells (left panel) or J1/2KD or Scr U266 cells (right panel) compared to NIH3T3 cultured alone (=1), calculated by the $2^{-\Delta\Delta Ct}$ formula. HES5 cells were used as a control for Notch pathway activity. The mean values \pm SD of 4 experiments are shown. Statistical analysis was performed using two-tailed t-test (* = $p < 0.05$; *** = $p < 0.001$). (B) Intracellular SDF1 α level in HS5 cells co-cultured with J1/2KD HMCLs. Histograms display the levels of intracellular SDF1 α (black lines) analyzed by flow cytometry in GFP⁺ HS5 cells cultured alone or co-cultured with J1/2KD or Scr OPM2 cells (left panel) and J1/2KD or Scr U266 cells (right panel), and the isotype-matched control (dotted line). Histograms are representative of at least 3 independent experiments. Due to a high percentage of SDF1 α expressing HS5 cells cultured with OPM2 we show also Δ GeoMFI. The apparent discrepancy between the two different basal levels of SDF1 α produced by HS5 cells used as control in the co-culture systems with OPM2 or U266 cells is due to the effect of the different HS5 cell concentrations (see methods in supplementary information). (C) SDF1 α levels in conditioned media of Scr or J1/2KD HMCLs, HS5 cells or co-culture systems have been assessed by ELISA. Statistical analysis was performed using one-way ANOVA and Tukey post-test (* = $p < 0.05$; ** = $p < 0.01$). (D) The effect of stimulation with Jagged1 and Jagged2 peptides on the secretion of SDF1 α by HS5 cells. Statistical analysis was performed using one-way ANOVA and Tukey post-test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). (E) Contribution

of the Notch pathway to the ability of stromal cells to produce SDF1 α . SDF1 α levels were measured in Scr or N1KD HS5 cells. Flow cytometry histograms (left) and graphs (right) display the levels of intracellular SDF1 α (Δ GeoMFI) analyzed in HS5 Scr (green) or HS5 N1KD cells (blue) and an isotype-matched control (gray); the graph shows the mean values \pm SEM of SDF1 α expression levels. Statistical analysis has been performed by t-test (*= p<0.05). (F) Status of CXCR4 expression in Scr or J1/2KD HMCLs used in co-culture experiments with HS5 cells. Values in the graph represents the mean values \pm SEM of CXCR4 expression levels (Δ GeoMFI) measured by flow cytometry. Statistical analysis has been performed by t-test (*= p<0.05, **=p<0.01). (G) To evaluate if SDF1 α contributes to BCL2, Survivin and ABCC1 expression, U266 cells were cultured in the presence of 500 ng/ml recombinant SDF1 α for 48 h and analyzed by qRT-PCR. Graphs show the relative expression levels of the indicated genes compared with the corresponding values in BSA-treated cells (= 1), calculated by the $2^{-\Delta\Delta C_t}$ formula. Mean values \pm SD of three independent experiments are shown. Statistical analysis was performed using two-tailed t-test(* = p < 0.05). (H) Results were further confirmed by Western blot analysis. Images were acquired using the UV-tech Alliance system and are representative of 3 independent experiments. (I) To assess if the SDF1 α /CXCR4 axis affects MM cells drug resistance, U266 cells cultured alone or with GFP $^+$ HS5 cells were treated with 6 nM Bor, 30 μ M Melph, 15 μ M Len or DMSO in the presence or absence of 50 μ M AMD3100. Apoptotic MM cells were measured by flow cytometry as Annexin-V $^+$ /GFP $^-$ cells. Graph shows the mean values \pm SEM of at least 3 independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's post-test: *= p<0.05; **= p<0.01; ****= p<0.0001.

Figure 6. Translational potential of Jagged1/2 inhibition: outcome on ex vivo cultures of MM patients' cells and treatment with small molecule affecting Notch-Jagged interaction

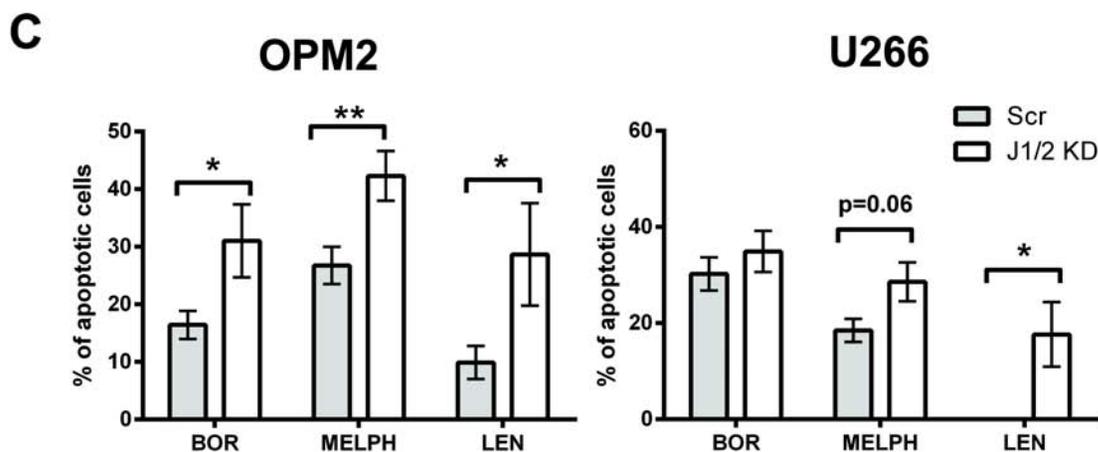
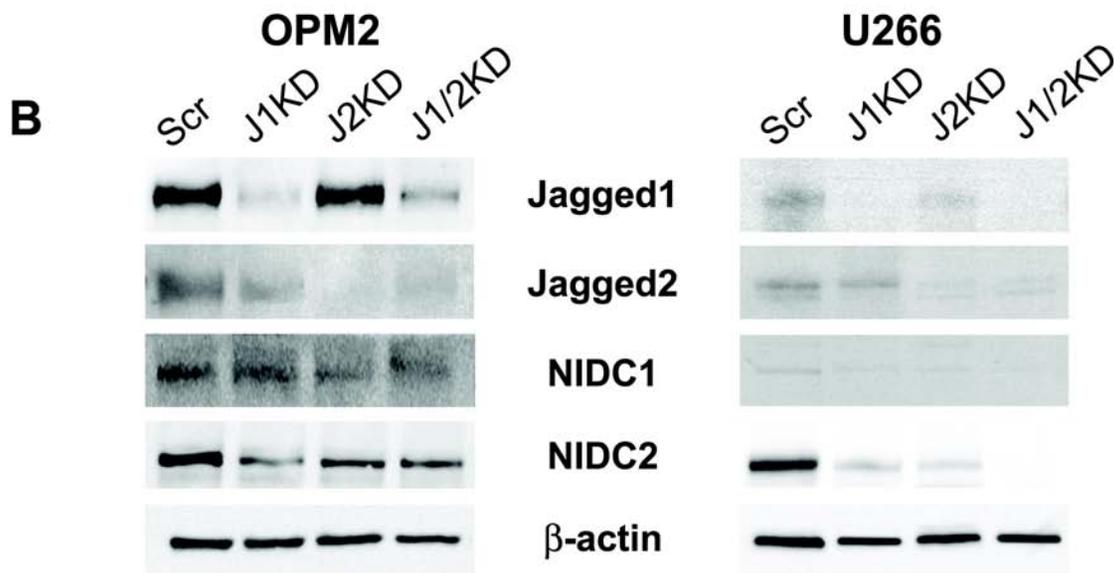
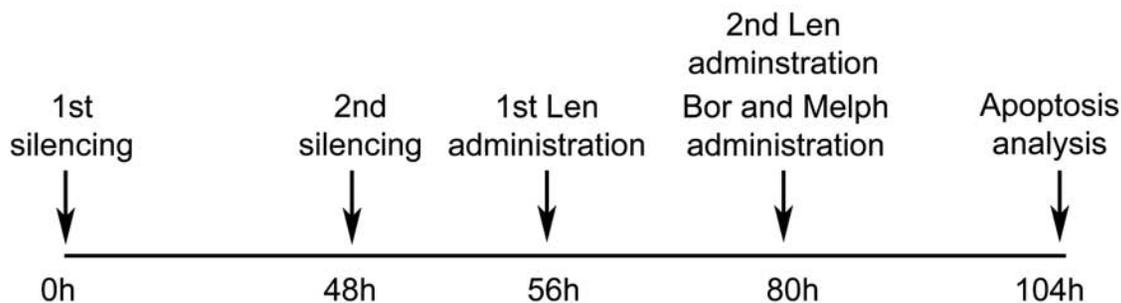
(A) Outcome of J1/2KD on primary CD138 $^+$ MM cells response to standard-of-care drugs in a primary co-culture system with BMSCs. Levels of apoptosis were analyzed by flow cytometry on primary MM cells transduced with the lentiviral vector pLL3.7 codifying for the Jagged1 and 2 shRNAs (J1/2) or the corresponding scrambled control (Ctrl), and then co-cultured with BMSCs from MM patients. Co-cultures were maintained for 72 h and treated for the last 24 h with 6 nM Bor (left panel; 8 patients) or 30 μ M Melph (central panel; 10 patients) and for 48 h with 15 μ M Len (right panel; 9 patients) or DMSO. The percentage of infected MM cells that underwent apoptosis (GFP $^+$ /AnnexinV $^+$) was detected by flow cytometry. Statistical analysis was performed using one-way ANOVA and Tukey post-test (*=p<0.05; **=p<0.01; ***=p<0.001). (B-C) Effect of the inhibitory small molecule, IGOR1, on MM drug resistance. OPM2 cells treated with 30 μ M IGOR1 were cultured on a monolayer of HS5 GFP $^+$ cells in the presence or the absence of different drugs as described in Methods. (B) qPCR assay shows that IGOR1 inhibit Notch pathway in OPM2 cells as demonstrated by the downregulation of Notch target genes, HES1 and HES6. Relative gene

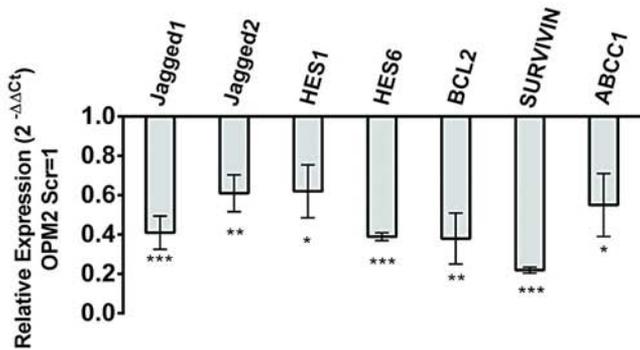
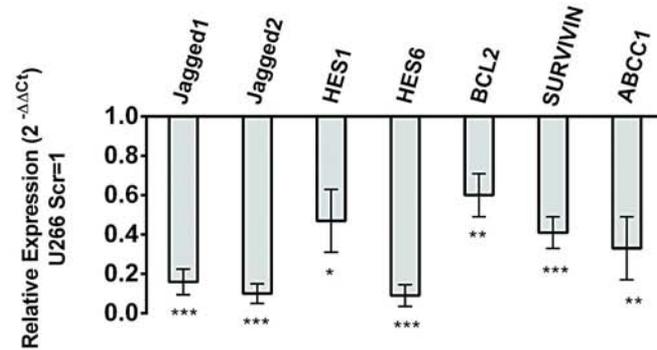
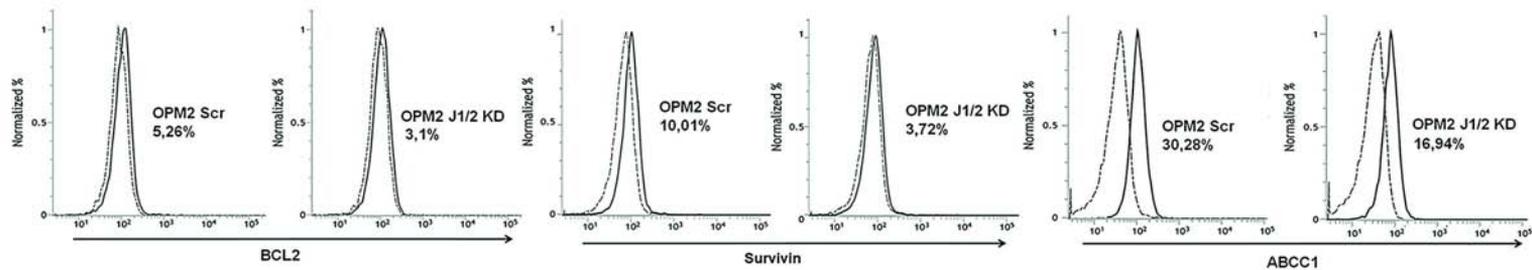
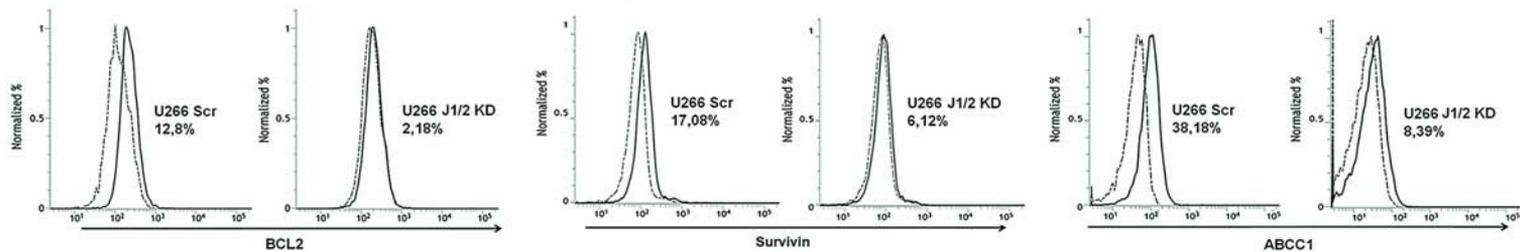
expression variation was normalized to GAPDH and calculated by the $2^{-\Delta\Delta C_t}$ formula. The mean values \pm SD of 3 experiments are shown. Statistical analysis was performed using two-tailed t-test (* = $p < 0.05$). (C) The levels of apoptosis of OPM2 cells treated with IGOR1 and the indicated drugs were measured by staining with Annexin-V-APC (C). Graph shows the mean values \pm SEM of at least 3 independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's post test (* = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$).

Figure 7. Evaluation of tumor growth inhibition of myeloma cells xenotransplanted in zebrafish embryos. Fluorescent microscopy images of CM-Dil stained MM xenografts at 2 hpi (A-D) and 48 hpi (A'-D') into the yolk of zebrafish embryos. (A'-D') Tumor growth analyses indicates that MM xenografts are responsive to Bor treatment (compare A' and B'). Xenotransplanted J1/2KD cells also show reduced tumor growth (compare A' and C'). These effects are increased combining the injection of J1/2KD cells with Bor treatment (compare A', B', C' and D'). (E) Dot-plot shows the increase in tumor burden at 48 hpi, normalized to tumor area at 2 hpi (Scr+DMSO= 20 embryos; Scr+ Bor= 26 embryos; J1/2KD+ DMSO= 35 embryos; J1/2KD+ Bor= 31 embryos). Statistical analysis was performed using one-way ANOVA and Tukey post-test (***= $p < 0.001$; ****= $p < 0.0001$).

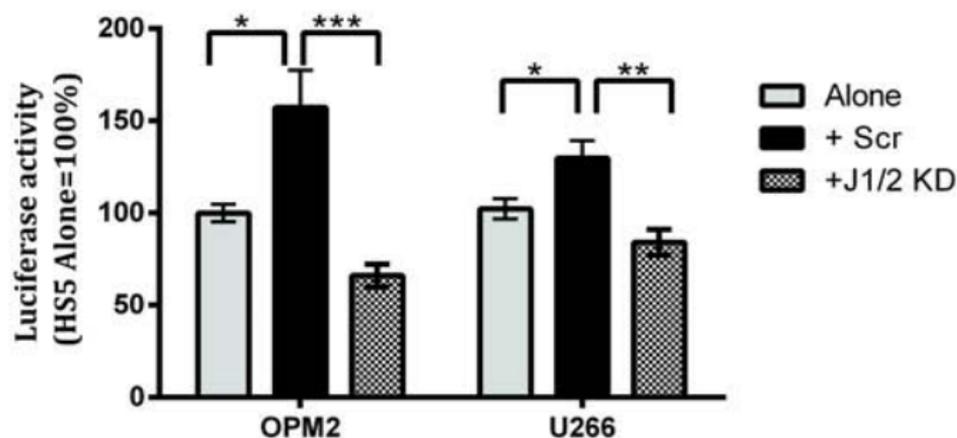
Figure 8. Mechanism underlying Notch ability to promote drug resistance in MM microenvironment. Jagged1/2 overexpression in MM cells causes hyperactivation of Notch signaling in the BM milieu, which, in turn, promotes drug resistance by modifying both MM cell and BMSC behavior. Indeed, (1) Notch activation in MM cell triggered by Jagged1/2 through homotypic interactions sustains resistance to drug-induced apoptosis in different ways. Notch can (2) promote the expression of the pro-survival factors BCL2, Survivin, and ABCC1 and the chemokine receptor CXCR4; (3) upregulates Hes1, which in turn inhibits the expression of the pro-apoptotic protein Noxa; (4) promotes the expression of integrin $\alpha\beta 5$, thus enhancing MM cell adhesion to vitronectin. (5) MM-derived Jagged1/2 may also activate Notch in BMSCs, (6) boosting its ability to produce SDF1 α , which in turn, by activating CXCR4 signaling in MM cell, promotes the expression of the anti-apoptotic factors BCL2, Survivin, and ABCC1, improving MM cell pharmacological resistance. On the other hand, (7) BMSCs through their basal expression of Jagged1 and Dll4 activate the Notch pathway in MM cells, (8) promoting the expression of cytochrome P450 and p21 thereby supporting MM cells resistance to therapy.

A Experiment timeline

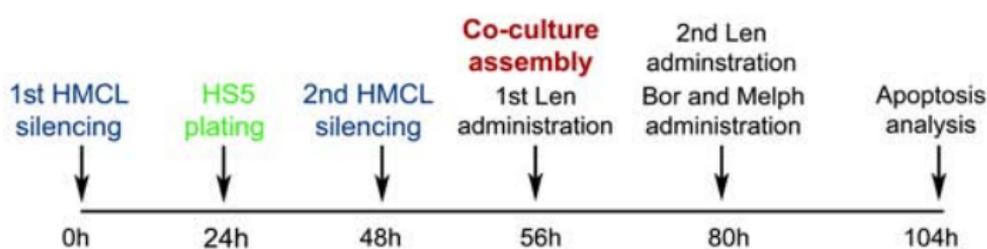


A**J1/2KD vs Scr OPM2****B****J1/2KD vs Scr U266****C****Effect of J1/2KD on the expression of anti-apoptotic effectors in HMCLs****D**

A Notch reporter assay

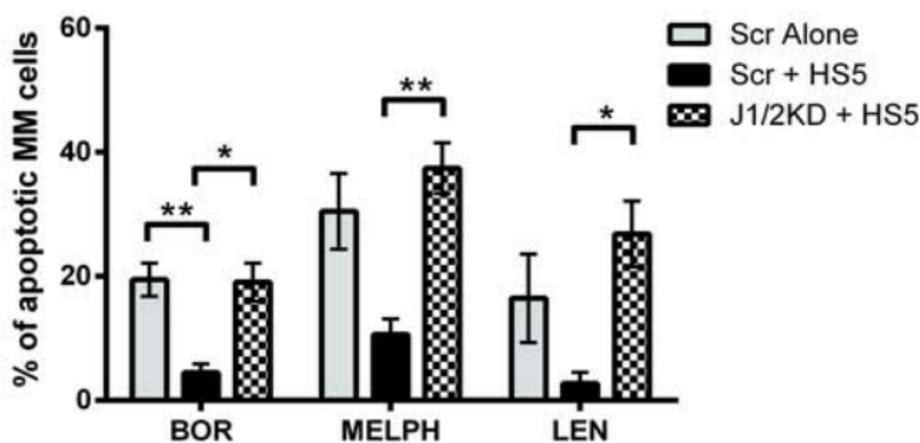


Experiment timeline



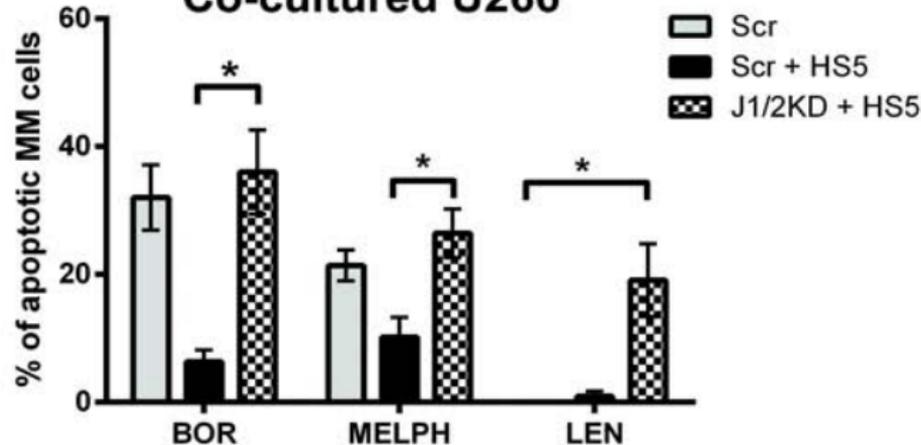
B

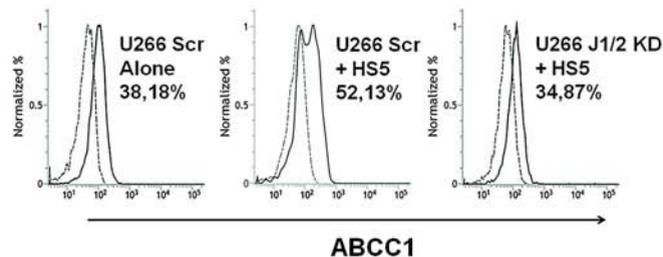
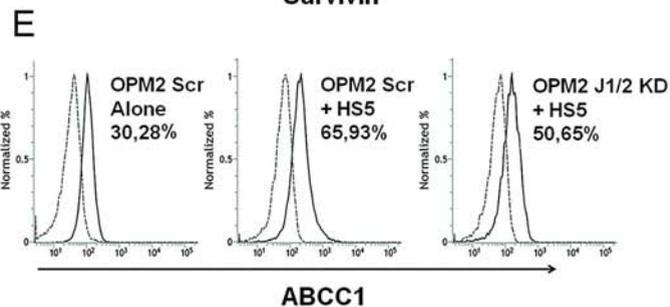
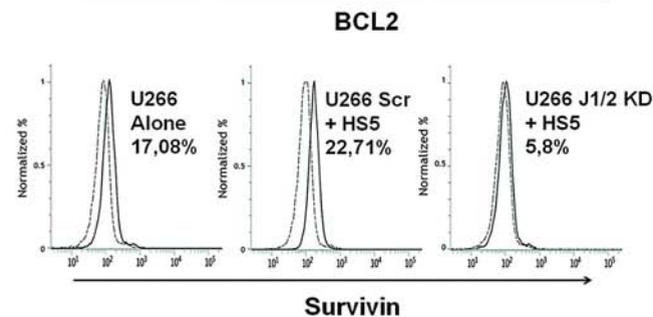
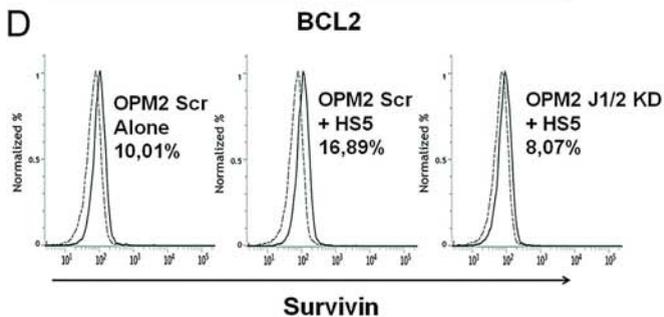
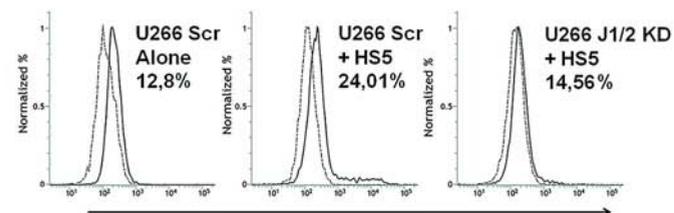
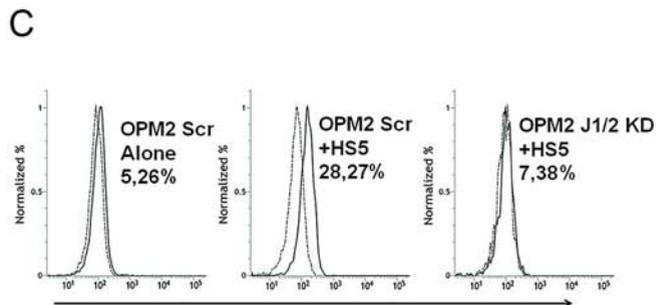
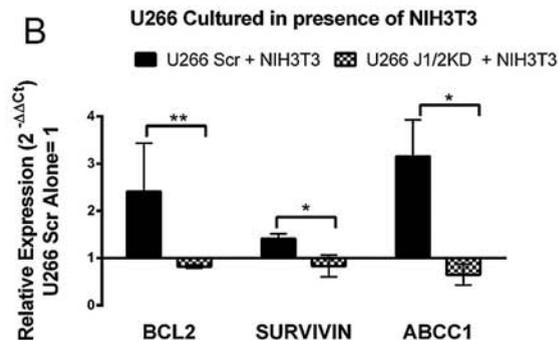
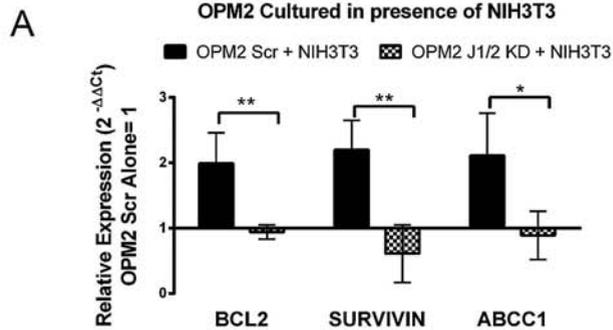
Co-cultured OPM2



C

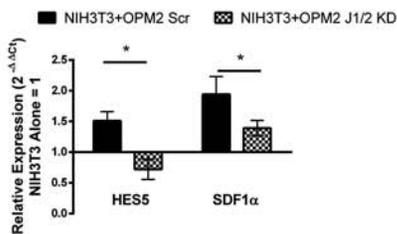
Co-cultured U266



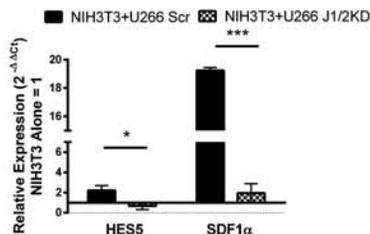


A

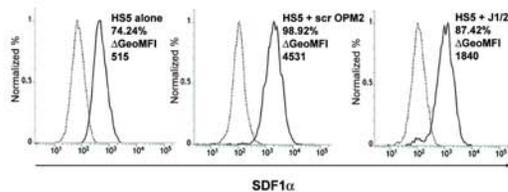
NIH3T3 cultured in the presence of OPM2



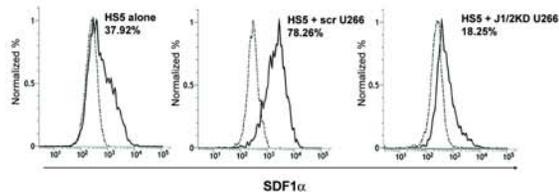
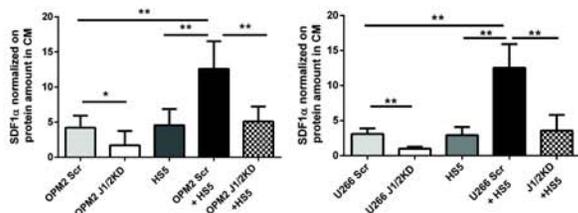
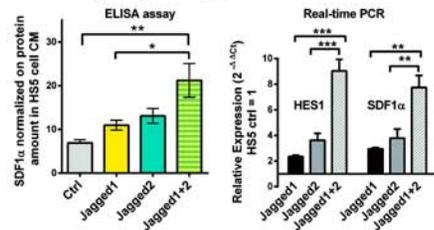
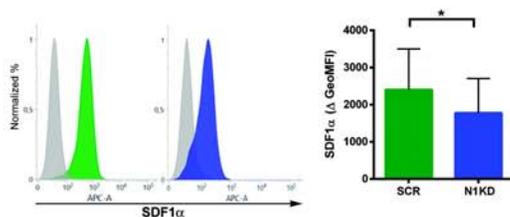
NIH3T3 cultured in the presence of U266

**B**Effect of J1/2KD on the expression of SDF1 α in HS5 cells

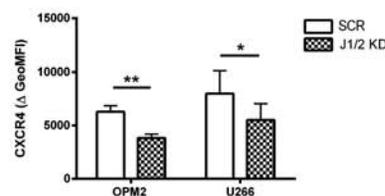
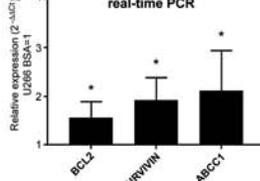
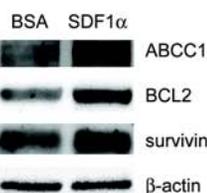
HS5 cultured in the presence of OPM2



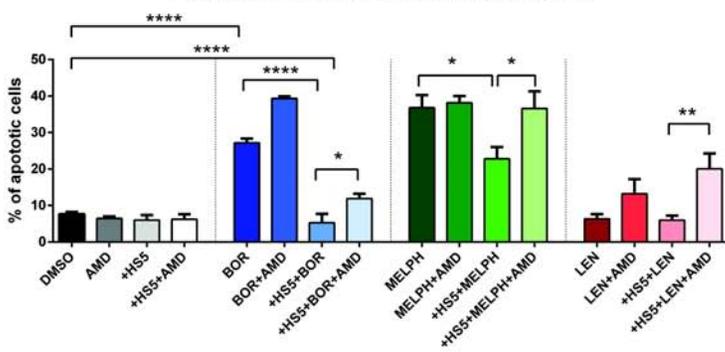
HS5 cultured in the presence of U266

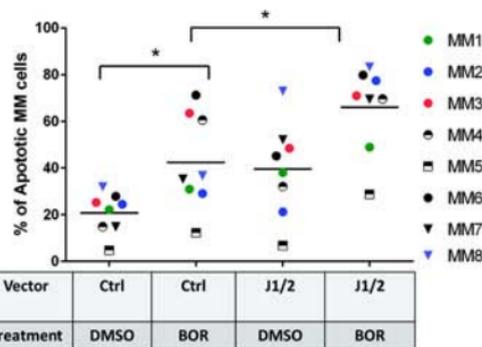
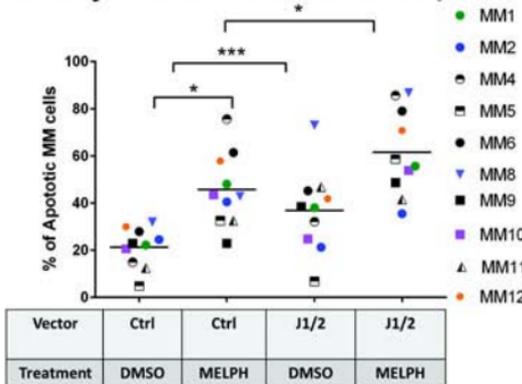
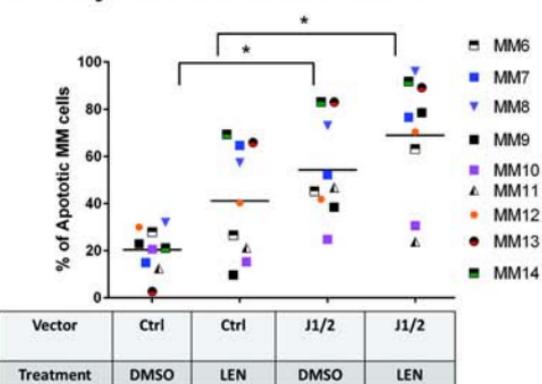
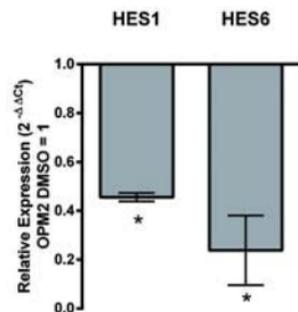
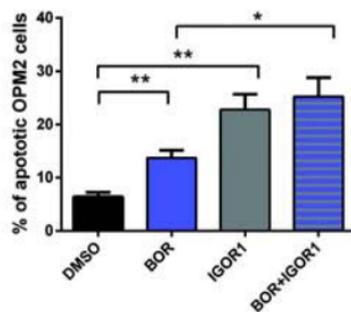
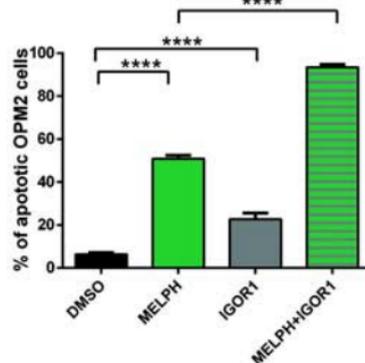
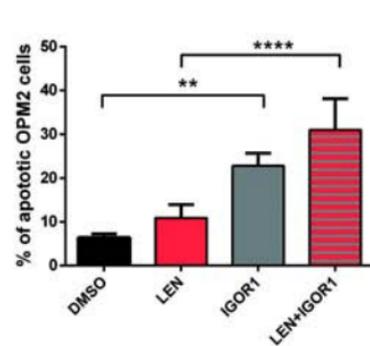
**C**SDF1 α released by HMCLs and HS5 cultured alone or co-cultured**D**Jagged ligands trigger HS5 secretion of SDF1 α **E**SDF1 α expression in N1KD HS5 cells**F**

CXCR4 expression in J1/2KD OPM2 and U266

**G**U266 treated with SDF1 α real-time PCR**H****I**

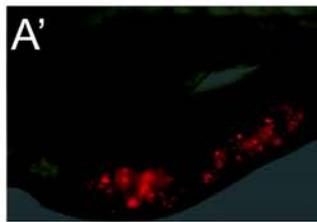
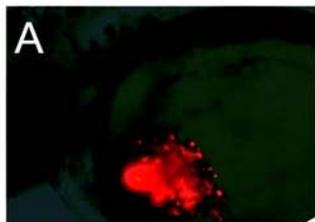
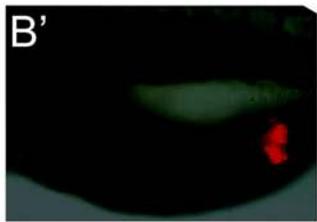
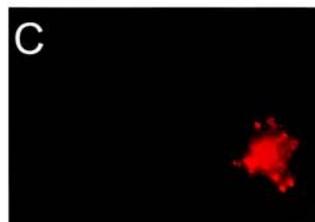
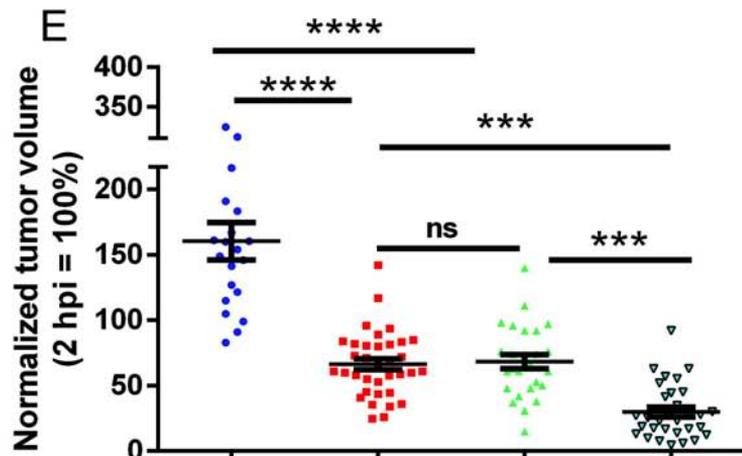
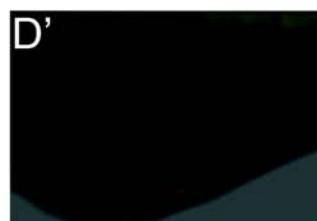
Effect of AMD3100 on U266 cell drug resistance



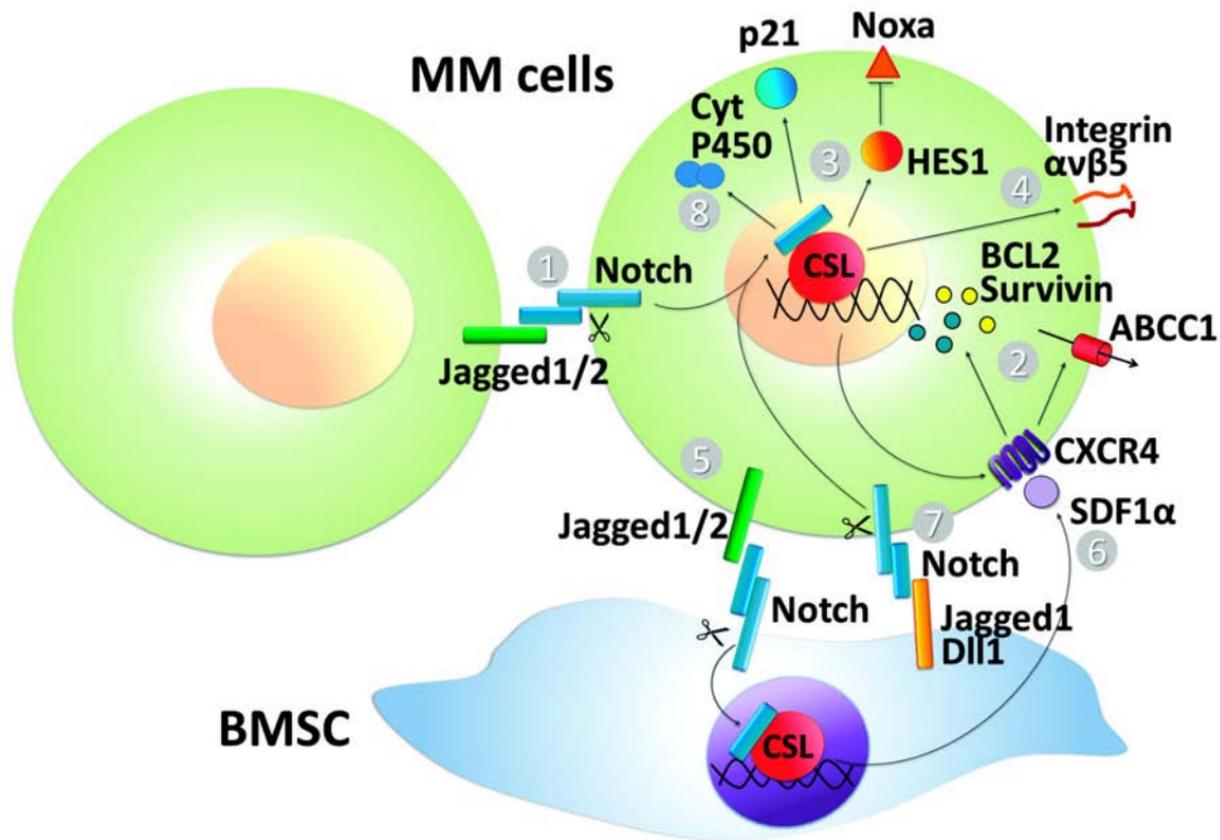
A**Primary co-culture treated with Bor****Primary co-culture treated with Melph****Primary co-culture treated with Len****B****OPM2 treated with IGOR1****C****OPM2/HS5 co-culture treated with Bor and IGOR1****OPM2/HS5 co-culture treated with Melph and IGOR1****OPM2/HS5 co-culture treated with Len and IGOR1**

2 hpi

48 hpi

Scr
+DMSOScr
+BorJ1/2 KD
+DMSOJ1/2 KD
+Bor

Injected Cells	U266 Scr	U266 Scr	U266 J1/2 KD	U266 J1/2 KD
Treatment	DMSO	BOR	DMSO	BOR



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

Michela Colombo, Silvia Garavelli, Mara Mazzola, Natalia Platonova, Domenica Giannandrea, Raffaella Colella, Luana Apicella, Marialuigia Lancellotti, Elena Lesma, Silvia Ancona, Maria Teresa Palano, Marzia Barbieri, Elisa Taiana, Elisa Lazzari, Andrea Basile, Mauro Turrini, Anna Pistocchi, Antonino Neri, Raffaella Chiaramonte.

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.

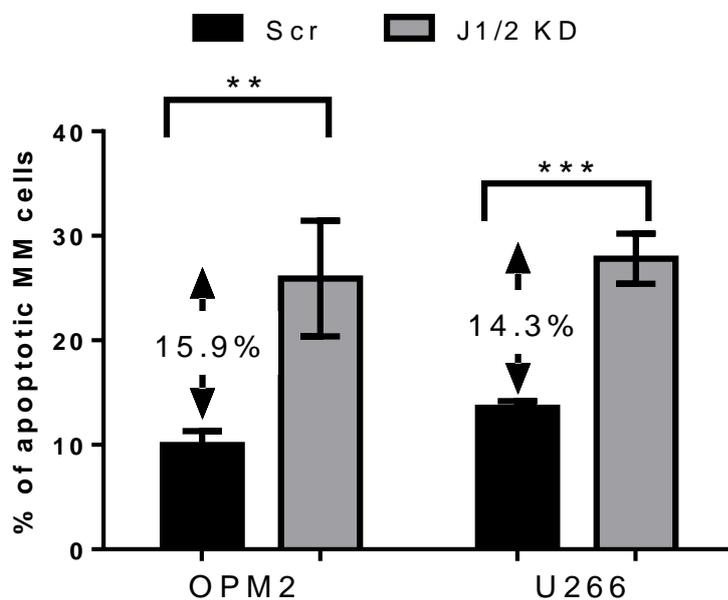


Fig. S1. Effect of J1/2KD on MM cell apoptosis. Results are shown as the mean values \pm SEM. Statistical analysis was performed using one-tailed t-test (**=p<0.01; ***=p<0.001).

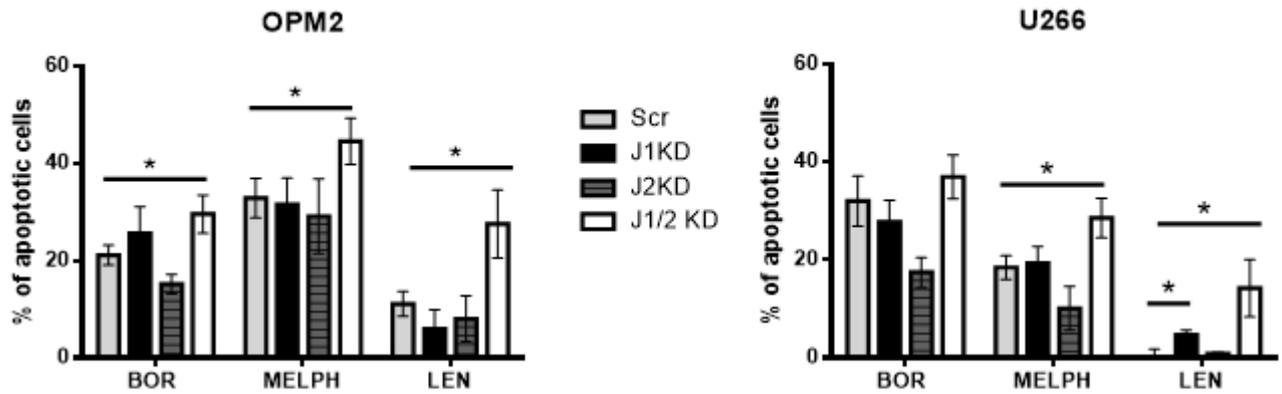


Fig. S2. Effect of the selective silencing of Jagged1 or Jagged2 on MM cells response to standard-of-care drugs. Values of apoptosis of Scr HMCLs are normalized on the corresponding DMSO treated controls and values of KD HMCLs treated with drugs are normalized on the corresponding KD cells treated with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

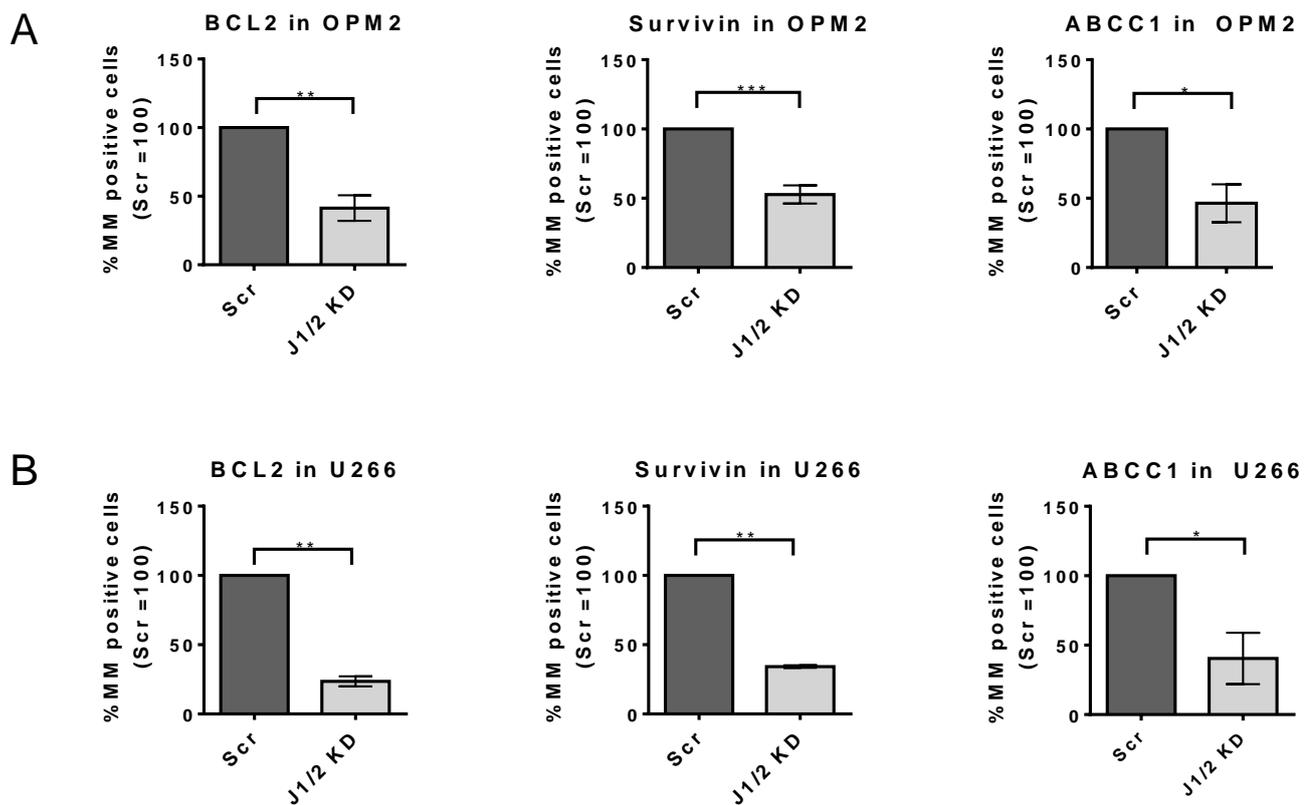


Fig. S3. Effect of J1/2KD on the expression of anti-apoptotic factors. The graph shows changes in the expression levels of BCL2, Survivin and ABCC1 obtained from the flow cytometric analysis on OPM2 (A) or U266 (B) cells transfected or not with siRNAs targeting Jagged1 and Jagged2 (Scr cells cultured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

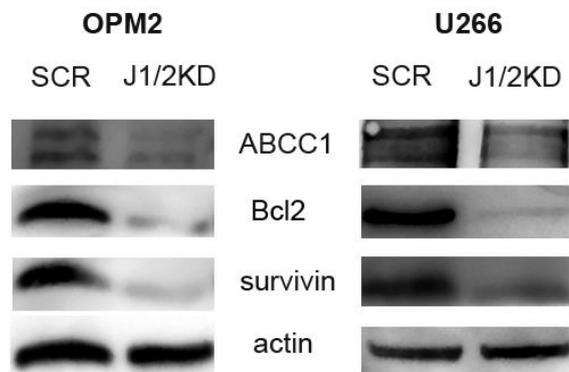


Fig. S4. Effect of J1/2KD on MM cell anti-apoptotic background. Western blot analysis of the effect of J1/2KD on the expression of Bcl2, Survivin and ABCC1. Images were acquired using the UV-tech Alliance system and are representative of 3 independent experiments with similar results.

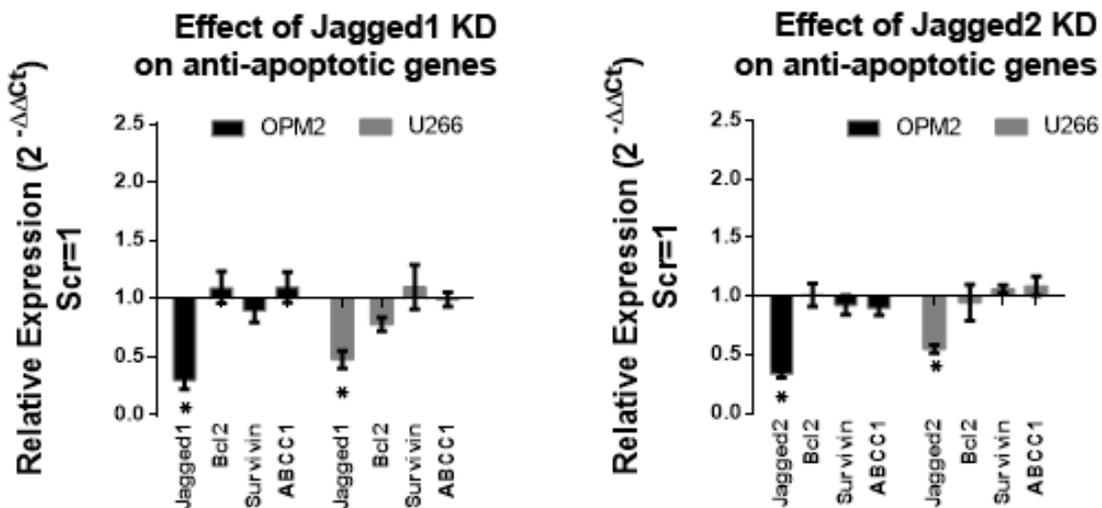


Fig. S5. Effect of the selective silencing of Jagged1 or Jagged2 on the expression of anti-apoptotic genes in MM cells. Results are shown as the mean \pm SEM from 3 independent experiments (Scr cells treated with DMSO=1). Statistical analysis was performed using two-tailed t-test (*= $p < 0.05$).

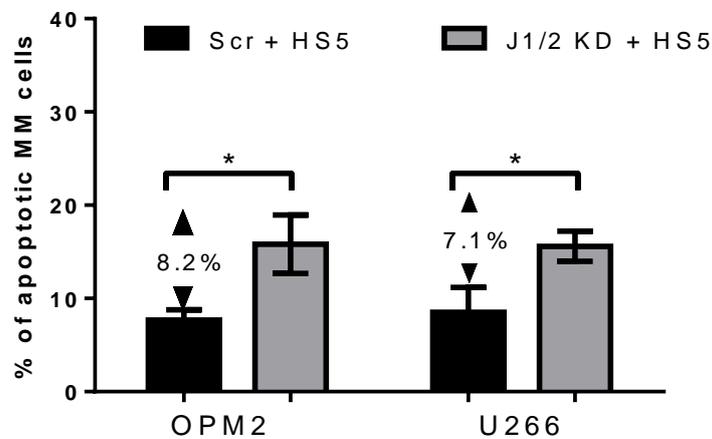


Fig. S6. Pro-apoptotic effect of J1/2KD on MM cell cultured in the presence of BMSCs. Results are shown as mean values \pm SEM. Statistical analysis was performed by one-tailed t-test (*= $p < 0.05$).

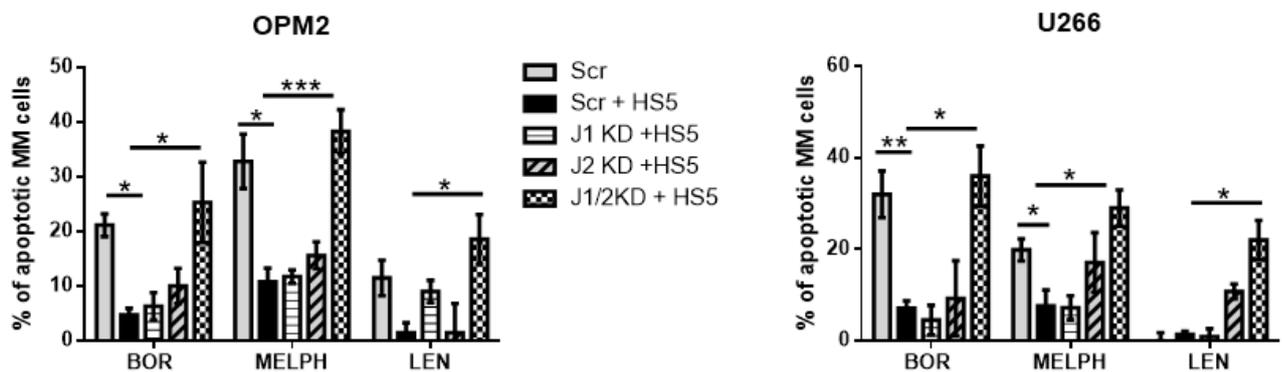


Fig. S7. Effect of the selective silencing of Jagged1 or Jagged2 on the response to standard-of-care drugs of MM cells cultured with BMSCs. Values of apoptosis of each type of culture (Scr alone, Scr+ HS5, J1KD+ HS5, J2KD+ HS5 and J1/2KD + HS5) treated with the reported drugs are normalized on the corresponding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

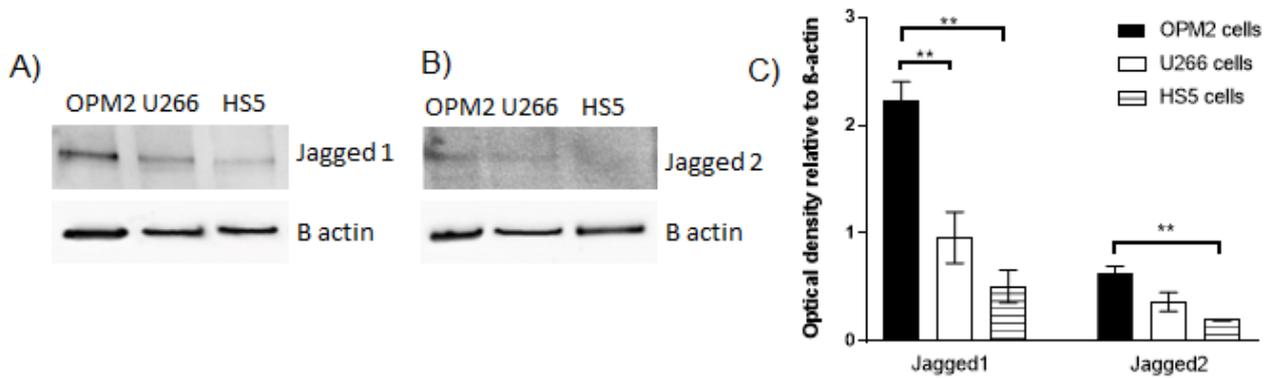


Fig. S8. Differences among Jagged 1 and 2 expression in OPM2, U266 and HS5 cells. Representative western blots show Jagged 1 (A) and Jagged 2 (B) expression in OPM2, U266 and HS5 cells. β-actin was used as loading control. Relative intensity obtained by densitometric analysis was evaluated relatively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

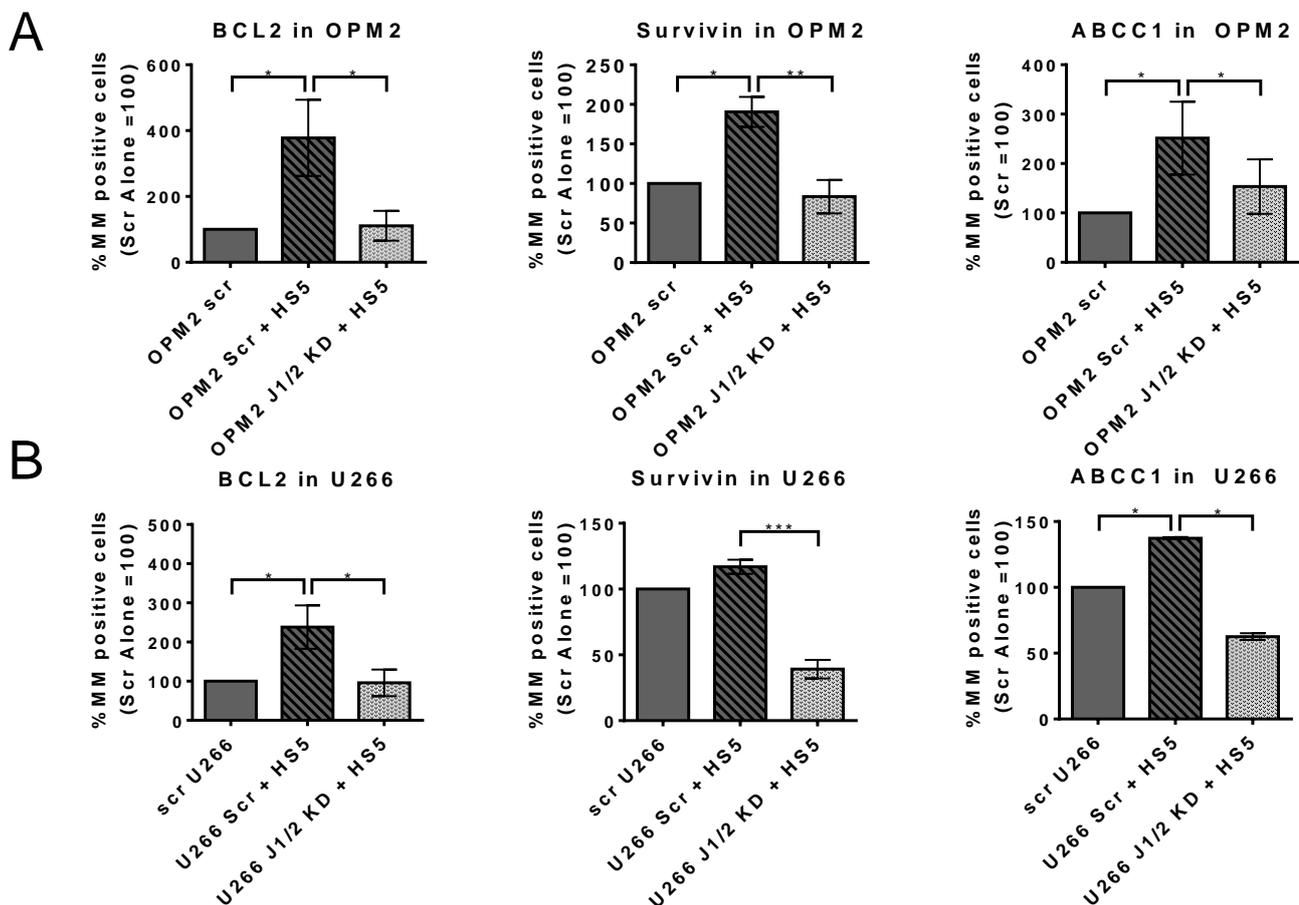


Fig. S9. Effect of J1/2KD on the expression of anti-apoptotic factors in HMCLs cultured with HS5 cells. The graphs show the changes in the expression levels of BCL2, Survivin and ABCC1 obtained by flow cytometric analyses on OPM2 (A) or U266 (B) cells transfected or not with siRNAs targeting Jagged1 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 (*= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$).

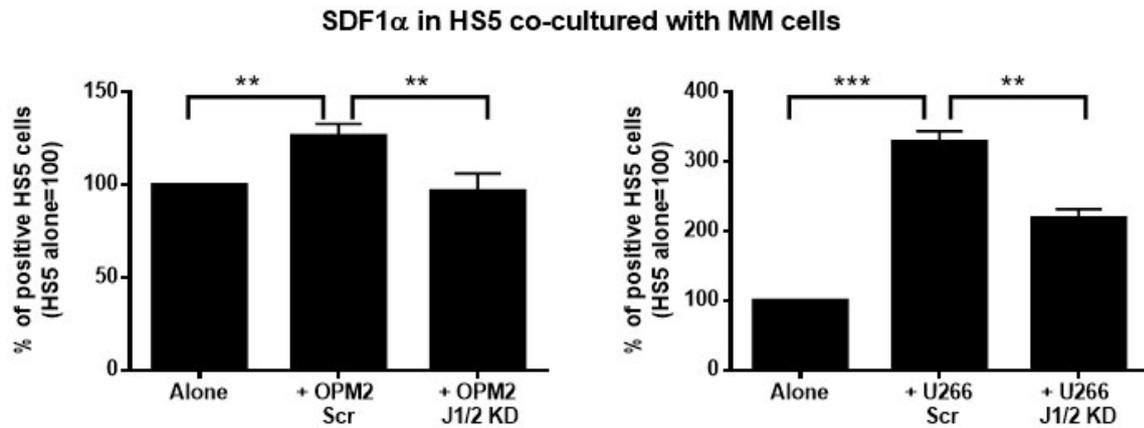


Fig. S10. Changes in SDF1 α expression by HS5 cells cultured with HMCLs. The graph shows changes in the expression levels of SDF1 α in HS5 cells cultured alone or co-cultured with Scr HMCLs or J1/2KD HMCLs (HS5 cells cultured alone=100%). The bars represent the mean values \pm SD. Statistical analysis was performed using one-way ANOVA and Tukey post-test (**=p<0.01; ***=p<0.001).

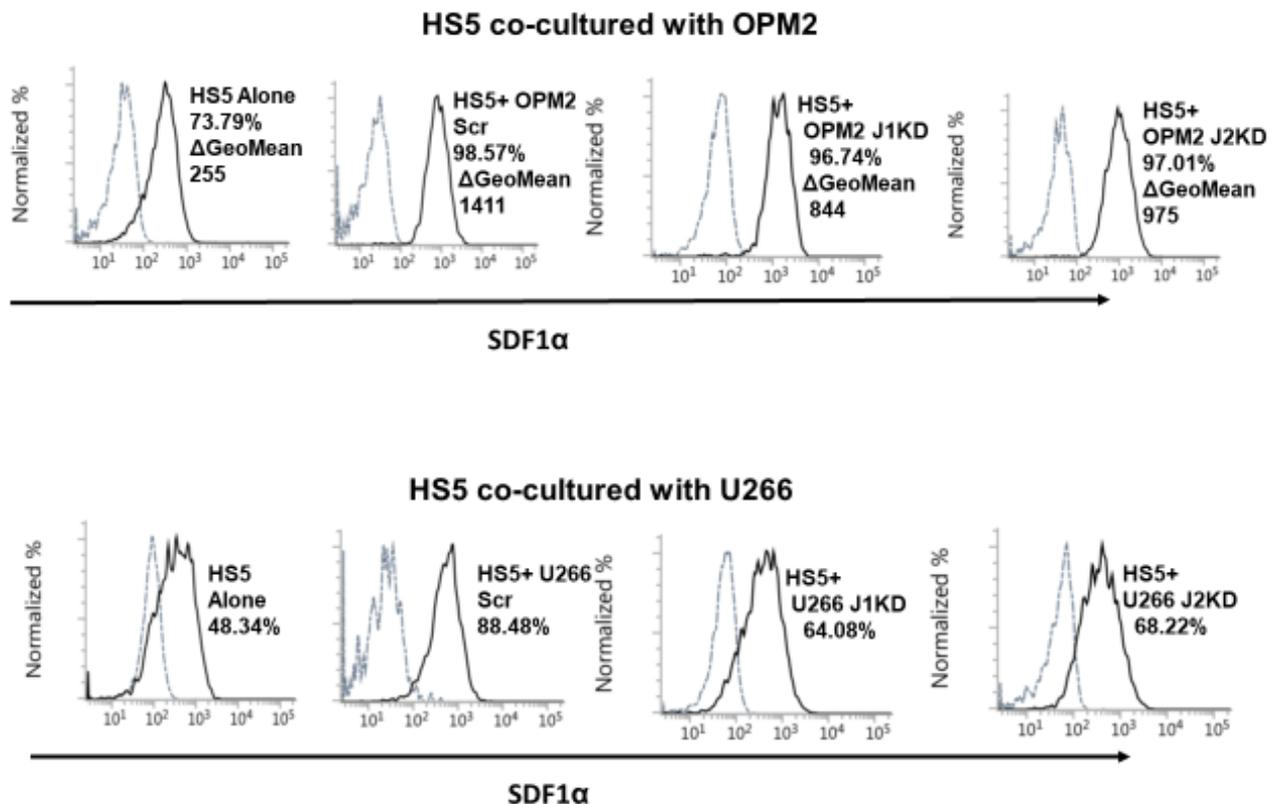


Fig. S11. Effect of the selective silencing of Jagged1 and Jagged2 on the ability of MM cells to boost SDF1 α in HS5 cells. Histograms display the levels of intracellular SDF1 α (black lines) analyzed by flow cytometry in GFP⁺ HS5 cells cultured alone or co-culture with Scr OPM2, Jagged1 KD OPM2 or Jagged2 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 SDF1 α in HS5 cultured with OPM2, Δ GeoMFI is reported, too.

Effect of Notch1 silencing on the apoptosis of HS5

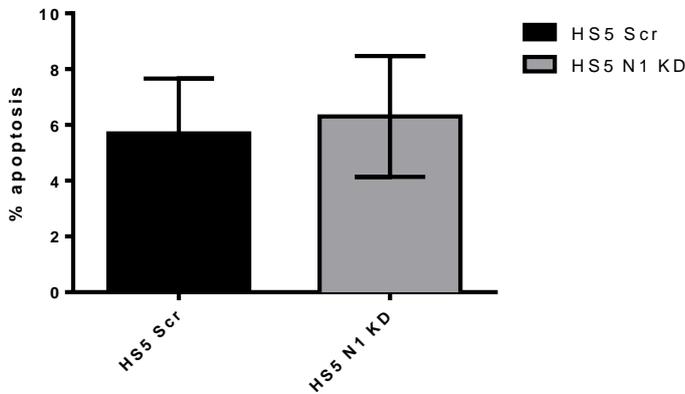


Fig. S12. Notch1 KD does not induce apoptosis in HS5 cells.

The graph shows the levels of apoptosis in Scr or N1KD HS5 cells. The bars represent the mean values \pm 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

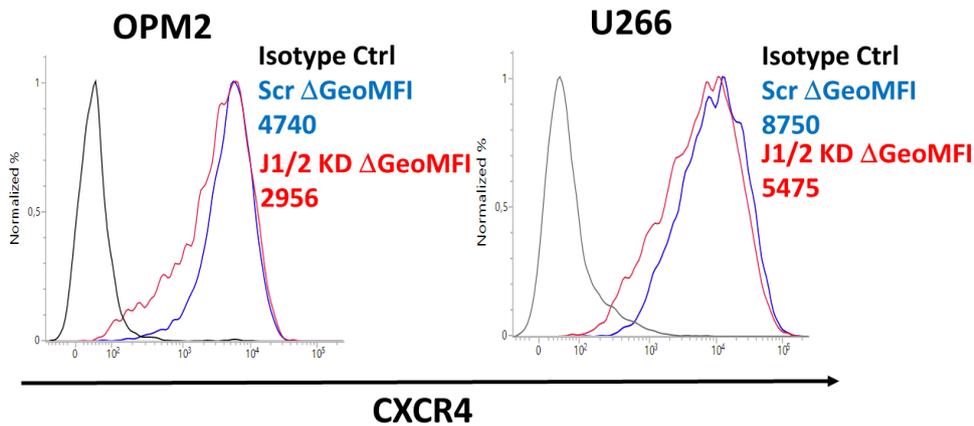


Fig. S13. CXCR4 expression in scr and J1/2KD OPM2 and U266 cell lines. Histograms representative of the expression levels (ΔGeoMFI) of CXCR4 in Scr (blue) and J1/2KD HMCLs (red) used in co-culture experiments with HS5 cells analyzed by flow cytometry. The gray lines represent the isotype-matched control.

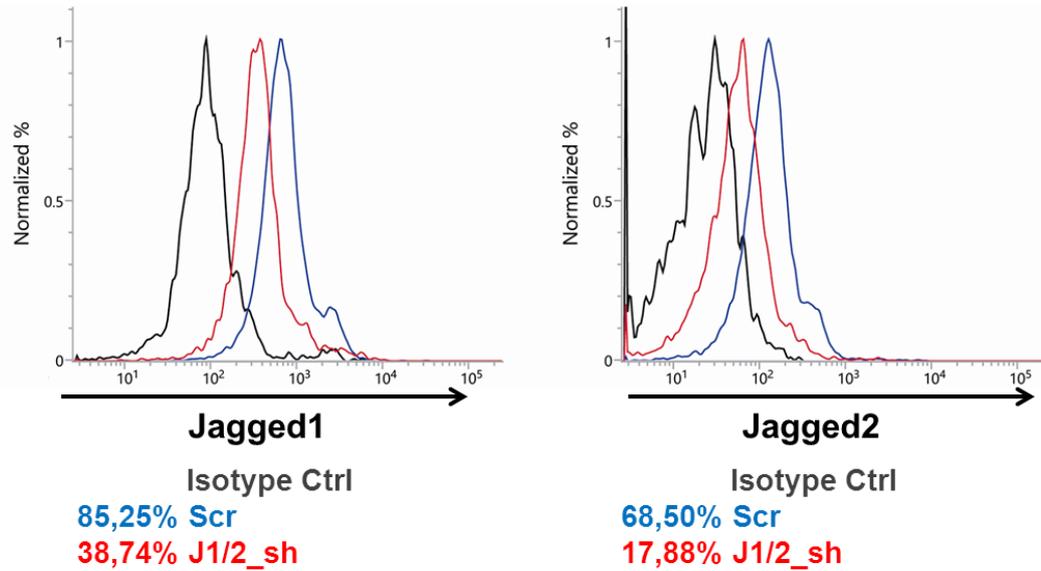


Fig. S14. Infection with pLL3.7 carrying Jagged1/2 shRNAs causes the downregulation of Jagged1/2 in MM cells. OPM2 cells were transduced with the lentiviral vector pLL3.7, carrying Jagged1/2 shRNAs (J1/2_sh, in red) or Scr shRNAs (Scr, in blue). The efficiency of Jagged1/2 silencing was evaluated on 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.

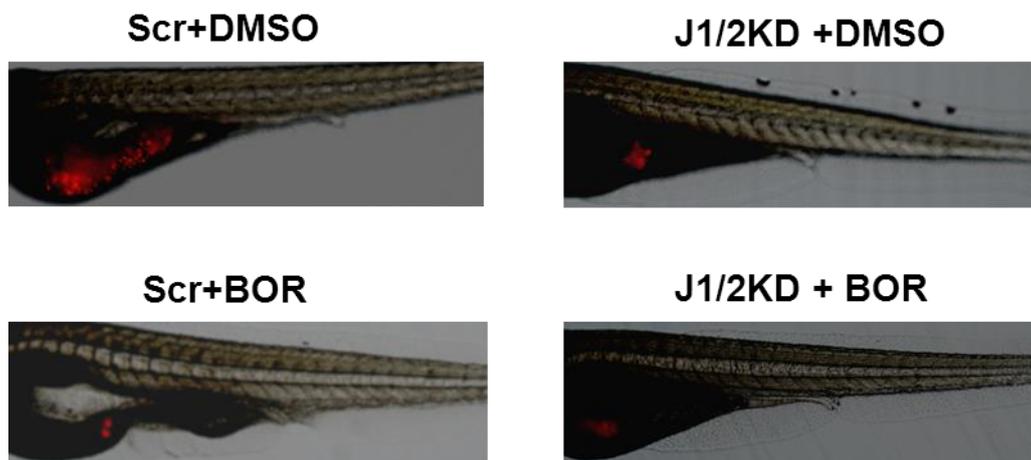


Fig. S15. Fluorescent microscopy (magnification 4X) images of myeloma-stained xenografts at 48 hpi into the yolk of 96hpf (48hpi) zebrafish embryos.

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. Treatment 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 μ M 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 (3×10^5 cells/ml), and were treated with the appropriate drugs as reported above.

For treatment with inhibitory small molecule, OPM2 cells were cultured with 30 μ M 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 μ M) were performed 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 μ g/ml polybrene (Sigma Aldrich), 20 ng/ml IL6 (Peprotech, USA) and 20ng/ml IGF1 (Peprotech, USA). Samples showing $\geq 30\%$ 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 μ M melphalan, or for the last 48 h with 15 μ M lenalidomide or the corresponding amount 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 manufactures' 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	TCGTGCCACATGCACCCAC
mSDF1 α	CAGCTCTGCAGCCTCCGGC	AAGAACCGGCAGGGGCATCG
hGAPDH	ACAGTCAGCCGCATCTTCTT	AATGGAGGGGTCATTGATGG
hHES1	GATGCTCTGAAGAAAGATAGC	GTGCGCACCTCGGTATTAAC
hHES6	ATGAGGACGGCTGGGAGA	ACCGTCAGCTCCAGCACTT
hJAG1	TTCGCCTGGCCGAGGTCCTAT	GCCCGTGTTCTGCTTCAGCGT
hJAG2	CCGGCCCCGCAACGACTTTT	CCTCCCTTGCCAGCCGTAGC
hCXCR4	AGCAAAGTGACGCCGAGGGC	CCCTGAGCCATTTCTCGGT
hSurvivin	AGCCAGATGACGACCCCAT	CTTGGCTCTTTCTCTGTCCAGT
hBcl2	GTCATGTGTGTGGAGAGCGT	GCCGTACAGTTCCACAAAGG
hABCC1	TAATCCCTGCCAGAGTCCA	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 - CaCl₂ 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 SDF1 α expression was assessed using anti-CXCR4 antibody (clone 555976, BD Biosciences) and anti-SDF1 α 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 SDF1 α 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 μ g) 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

1. Platonova N, Parravicini C, Sensi C, et al. Identification of small molecules uncoupling the Notch::Jagged interaction through an integrated high-throughput screening. *PloS one*. 2017;12(11):e0182640.
2. Colombo M, Galletti S, Bulfamante G, et al. Multiple myeloma-derived Jagged ligands increases autocrine and paracrine interleukin-6 expression in bone marrow niche. *Oncotarget*. 2016;7(35):56013-29.
3. Colombo M, Thummler K, Mirandola L, et al. Notch signaling drives multiple myeloma induced osteoclastogenesis. *Oncotarget*. 2014;5(21):10393-406.
4. Rubinson DA, Dillon CP, Kwiatkowski AV, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet*. 2003;33:401.
5. Chiamonte R, Colombo M, Bulfamante G, et al. Notch pathway promotes ovarian cancer growth and migration via CXCR4/SDF1alpha chemokine system. *Int J Biochem Cell Biol*. 2015;66:134-40.
6. Mirandola L, Apicella L, Colombo M, et al. Anti-Notch treatment prevents multiple myeloma cells localization to the bone marrow via the chemokine system CXCR4/SDF-1. *Leukemia*. 2013;27(7):1558-66.
7. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Develop dynamics*. 1995;203(3):253-310.
8. Lin J, Zhang W, Zhao J-J, et al. A clinically relevant in vivo zebrafish model of human multiple myeloma (MM) to study preclinical therapeutic efficacy. *Blood*. 2016.
9. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods*. 2007;39(2):175-91.
10. Motulsky HJ, Brown RE. Detecting outliers when fitting data with nonlinear regression – a new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics*. 2006;7(1):123.