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Received: October 10, 2023.
Accepted: February 13, 2024.


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A NOTCH3-CXCL12-driven myeloma-tumor niche signaling axis promotes chemoresistance in multiple myeloma

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Author contributions

the data analysis and interpretation. J.D.C and H.M.S. wrote the manuscript. All authors reviewed the manuscript.

**Running head:** NOTCH3 and chemoresistance in multiple myeloma

**Keywords:** multiple myeloma, Notch, Bortezomib, bone, chemoresistance

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**Data sharing statement.** The IA15 datasets used for the analyses described in this work were downloaded from the Multiple Myeloma Research Foundation CoMMpass (MMRF CoMMpass [SM] (Relating Clinical Outcomes in MM to Personal Assessment of Genetic Profile) study (www.themmrfrf.org)) researcher gateway. Other non-public datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

**Abstract word count:** 200

**Main text count:** 4000

**Number of figures and tables:** 7 main figures and 13 supplementary files

**References:** 39
Acknowledgments: The authors would like to acknowledge the services provided by the TBAPS of the UAMS Winthrop P. Rockefeller Cancer Institute and the MMRF for providing the CoMMpass IA15 dataset. These data were generated as part of the Multiple Myeloma Research Foundation Personalized Medicine Initiatives (https://research.themmrf.org and www.themmrf.org). The authors thank Dr. Onal for her assistance with CRISPR NOTCH3 activation and sgRNA design.

Funding: This work was supported by the National Institutes of Health (NIH) R37CA251763, R01CA209882, R01CA241677 to J.D.C., P20GM125503 to J.D.C and I.N., and F31CA284655 to H.M.S., the UAMS Musculoskeletal Hub Award to J.D.C. and I.N., the UAMS Translational Research Institute (TRI), grant KL2 TR003108 through the National Center for Advancing Translational Sciences of the NIH to C.A., and the UAMS Winthrop P. Rockefeller Cancer Institute Seeds of Science Award and Voucher Program awarded to J.D.C.

Disclosure of Conflicts of Interest: The authors have declared that no conflict of interest exists.
Abstract

Multiple myeloma (MM) remains incurable due to disease relapse and drug resistance. Notch signals from the tumor microenvironment (TME) confer chemoresistance, but the cellular and molecular mechanisms are not entirely understood. Using clinical and transcriptomic datasets, we found that NOTCH3 is upregulated in CD138^+ cells from newly diagnosed MM (NDMM) patients compared to healthy individuals and increased in progression/relapsed MM (PRMM) patients. Further, NDMM patients with high NOTCH3 expression exhibited worse responses to Bortezomib (BOR)-based therapies. Cells of the TME, including osteocytes, upregulated NOTCH3 in MM cells and protected them from apoptosis induced by BOR. NOTCH3 activation (NOTCH3OE) in MM cells decreased BOR anti-MM efficacy and its ability to improve survival in in vivo myeloma models. Molecular analyses revealed that NDMM and PRMM patients with high NOTCH3 exhibit CXCL12 upregulation. TME cells upregulated CXCL12 and activated the CXCR4 pathway in MM cells in a NOTCH3-dependent manner. Moreover, genetic or pharmacologic inhibition of CXCL12 in NOTCH3OE MM cells restored sensitivity to BOR regimes in vitro and in human bones bearing NOTCH3OE MM tumors cultured ex vivo. Our clinical and preclinical data unravel a novel NOTCH3-CXCL12 pro-survival signaling axis in the TME and suggest that osteocytes transmit chemoresistance signals to MM cells.
Introduction

Multiple myeloma (MM) is a hematological cancer characterized by the accumulation of malignant plasma cells in the bone marrow and the overproduction of monoclonal proteins (M-proteins). First-line therapy in MM includes proteasome inhibitors, such as Bortezomib (BOR), administered alone or in combination regimens.\(^1\) Despite high response rates to BOR-based therapies, MM remains incurable due to the development of chemoresistance and disease recurrence after transient remissions.

Although MM cells exhibit genetic and molecular heterogeneity, they depend highly on the bone marrow niche. MM cells localize in specialized niches in the marrow where tumor microenvironment (TME) cells promote their proliferation and allow them to escape anti-MM therapies by promoting \textit{de novo} chemoresistance.\(^2\)-\(^4\) Notch signaling activation downstream of the four NOTCH (1-4) receptors in MM cells plays a critical role in transforming the bone marrow into a permissive niche for MM progression and chemoresistance.\(^5\), \(^6\) Pharmacologic pan-inhibition of Notch in the TME induces apoptosis in MM cells and enhances sensitivity to chemotherapy.\(^6\), \(^7\) Notch signals from stromal cells contribute to \textit{de novo} drug resistance to proteasome inhibitors in MM cells.\(^8\) Yet, the role of other TME cells and how MM cells integrate and execute TME Notch signals are not completely understood.

Prior studies have focused on NOTCH1 or 2, as they are expressed at relatively higher levels than NOTCH3 or 4 in NDMM patients.\(^9\) However, we recently reported that 30\% of NDMM patients exhibit NOTCH3 expression levels comparable to NOTCH1 or NOTCH2.\(^9\) Moreover, we showed that osteocytes, the most abundant bone cells,\(^10\) rapidly upregulate NOTCH3 expression in MM cells,\(^9\) emphasizing the need to understand the role of NOTCH3 in MM further. In this study, we describe a novel NOTCH3-CXCL12 signaling axis of TME-mediated
chemoresistance and identify the osteocyte as a new TME cell capable of influencing MM therapeutic responses to BOR-based regimes.

**Methods**

**Study population.** The mRNA expression of NOTCH receptors was studied in CD138⁺ plasma cells from a previously described institutional cohort of newly diagnosed MM (NDMM) patients (n=52; t(4;14)=8; t(11;14)=10; t(14;16)=9; t(14;20)=6; D1=12; D2=7) and age-matched healthy donors (n=4). To study the impact of NOTCH3 on the transcriptome and clinical outcomes of MM patients, we obtained clinical and gene expression data from NDMM patients from the Multiple Myeloma Research Foundation (MMRF) CoMMpass registry (NCT01454297, version IA15).

**Bioinformatic analyses.** Gene expression and mutation analyses are described in Supplementary Methods.

**Cell culture.** MM-osteocyte (5:1) or MM-stroma (5:1) co-cultures were established as described before. Co-cultures were treated with Plerixafor (25uM), BOR (3nM), VRd (BOR: 2nM, Lenalidomide: 1uM, Dexamethasone: 10nM) and refreshed every 24h. Cell characteristics, reagents, and methods for apoptosis/proliferation assays are described in Supplementary Methods.

**Gene expression.** Methods to quantify mRNA (qPCR) and protein expression (Western Blot and ELISA) are described in Supplementary Methods.

**Genetic inhibition/activation in MM cells.** Methods used to manipulate NOTCH3/CXCL12 expression are described in Supplementary Methods.
**Ex vivo organ cultures.** Ex vivo MM-murine bone organ cultures were established as described before. Ex vivo MM-human bone organ cultures were established with human cancellous bone fragments similar in size obtained from femoral heads discarded after hip arthroplasty (see **Supplementary Methods** for details).

**Animal studies.** 7-week-old immunodeficient littermate NSG female and male mice were injected intravenously with $5 \times 10^5$ OPM2-Scr MM cells, OPM2-Notch$^{3OE}$ MM cells, or saline. Equal numbers of female and male mice were used per group. After one week, mice were randomized based on tumor burden and bone disease to two groups (1) vehicle (saline) or (2) 0.1mg/kg BOR (i.p.) 5x/wk for 3 weeks. To assess survival, the health of mice was monitored daily, and mice were euthanized at first sign of back leg paralysis. The sample size was calculated based on previous studies. MicroCT analyses were performed as shown before.

**Statistics.** Data were analyzed using GraphPad (GraphPad Software Inc, San Diego, CA, USA). Differences in means were analyzed using a combination of unpaired t-test, One-Way, or Two-Way ANOVA tests, followed by pairwise multiple comparisons (Tukey post hoc test). Values were reported as means ± SD. P values ≤ 0.05 were considered statistically significant. Data analysis was performed in a blinded fashion.

**Study Approvals.** All procedures involving animals were performed in accordance with guidelines issued by the University of Arkansas for Medical Sciences IACUC (protocol #202220000489). Collection and de-identification of human bone samples was coordinated by the UAMS Winthrop P. Rockefeller Cancer Institute TBAPS and approved by the UAMS IRB (protocol # 262940). All participants provided written, informed consent before study procedures occurred, with continuous consent ensured throughout participation. NDMM patients and healthy
Results

**NOTCH3 expression is increased in newly diagnosed patients (NDMM) by a non-mutational, TME cell-dependent mechanism.** To investigate the integration of Notch signals by MM cells, we first compared the expression of the NOTCH receptors in CD138+ plasma cells using an institutional cohort of NDMM patients of major molecular MM subgroups, including primary translocations t(4;14), t(11;14), t(14;16) and t(14:20), hyperdiploid subgroups D1 and D2, and age-matched healthy donors (Figure 1A). We found no differences in the expression of NOTCH1 or NOTCH2 in NDMM patients compared to healthy donors, except for a NOTCH2 upregulation detected in the t(14;16) MM subgroup. NOTCH4 was decreased in all the MM subgroups, except the t(4;14) MM subgroup. In contrast, NOTCH3 expression was elevated in all NDMM subgroups, although it did not reach statistical significance in the t(14;16) subgroup. To understand the mechanisms behind NOTCH3 upregulation in NDMM, we next mined the MMRF CoMMpass cohort dataset to investigate whether NOTCH3 expression levels in CD138+ plasma cells from NDMM patients correlated with gain- or loss-of-function mutations in the NOTCH3 gene. We identified several mutations in the NOTCH3 gene (Supplementary Table 1) but did not find an association with NOTCH3 expression (Figure 1B). In addition, we investigated the presence of NOTCH3 mutations in a panel of 69 human MM cell lines and identified only one cell line, FLAM76 (t11;14), with a frameshift mutation (AGGGG/AGGG). Next, we investigated if constituents of the TME upregulate NOTCH3 in MM cells. As shown before, co-culture with osteocytes upregulated NOTCH3 and increased the expression of the
Notch target gene *HES1* in several murine and human MM cell lines (*Supplementary Figure 1A-C*). In contrast, no changes were detected in *NOTCH1*, 2, or 4. Like osteocytes, co-culture with stromal cells, another important cellular component of the MM-TME, selectively upregulated *NOTCH3* expression and activated Notch signaling in MM cells (*Supplementary Figure 1D-F*). Together, these data support that non-mutational, TME cell-dependent NOTCH3 activation occurs in specialized niches in the bone marrow of NDMM patients.

**NOTCH3 expression correlates with worse responses to Bortezomib (BOR)-based therapies in NDMM patients.** Further bioinformatic mining of the CoMMpass cohort revealed that NDMM patients with high NOTCH3 expression exhibited upregulation and enrichment in genes associated with processes involved in chemoresistance, including *Responses to drugs* (*Figure 2A*) and *Cell-adhesion pathways* (*Figure 2B*). Moreover, gene set enrichment analysis (GSEA) revealed that the transcriptome of high NOTCH3 NDMM patients is enriched in genes associated with poor responses to BOR therapy (*Figure 2C*).15 Poised by these observations, we next investigated if high expression levels of NOTCH3 are associated with poor prognosis in NDMM patients. No significant correlations were observed between the expression of NOTCH3 and OS or PFS (*Supplementary Figure 2A-B*). However, after stratification by therapy (combined graph is shown in *Supplementary Figure 2C*), we observed that NDMM patients with high NOTCH3 had significantly worse PFS when receiving BOR-based therapies vs. other therapies not including BOR (*Figure 2D*). In contrast, NDMM patients with low NOTCH3 levels exhibited similar PFS regardless of the therapy received. Next, we rationalized that if CD138+ MM cells expressing high NOTCH3 are chemoresistant, the expression of NOTCH3 should increase in PRMM patients. Consistent with this notion, we found an increase in NOTCH3 expression in PRMM patients using paired diagnosis-relapse samples of MM patients included in
the CoMMpass cohort (Figure 2E). These results suggest that NOTCH3-regulated transcriptional reprogramming of MM cells promotes drug resistance to BOR-based therapies and associates with poor clinical outcomes.

**NOTCH3 signaling mediates TME-mediated BOR chemoresistance in MM cells.** To investigate the impact of NOTCH3 signaling on drug resistance to BOR-based therapies, we selected murine 5TGM1 and human U266 MM cells, which exhibit higher levels of NOTCH3 expression/activation, and human OPM2 MM cells, with lower NOTCH3 levels (Supplementary Figure 3A), and determined that BOR therapy does not affect NOTCH expression in these cell lines (Supplementary Figure 3B). Then, we knocked down NOTCH3 (NOTCH3KD) in 5TGM1 and U266 MM cells (Supplementary Figure 3C) and established co-cultures with cells of the TME (Figure 3A). Control (Scr) and NOTCH3KD MM cells cultured alone exhibited similar apoptotic responses to BOR and the triple regime VRd (BOR+dexamethasone+lenalidomide), frequently used in induction therapy for NDMM. Co-culture with osteocytes decreased by ~50% the apoptosis induced by BOR or VRd in control MM cells, while this protection was lost in Notch3KD MM cells (Figure 3A-B). Second, we used CRISPR-mediated transcriptional activation from the endogenous NOTCH3 loci to promote a more physiological activation of NOTCH3 in OPM2 MM cells (NOTCH3OE cells) while permitting further NOTCH3 regulation by the TME (Supplementary Figure 3D). NOTCH3 activation did not alter the anti-MM efficacy of these therapies in MM cells cultured alone but enhanced the pro-survival effects of osteocytes in in vitro co-cultures exposed to BOR or VRd (Figure 3C). Similar responses to NOTCH3 inhibition/activation in MM cells exposed to BOR or VRd were also observed in co-cultures with stromal cells (Supplementary Figures 4A-C).
Co-culture with osteocytes or genetic manipulation of NOTCH3 did not affect the baseline levels of apoptosis in MM cells cultured in the absence of BOR (Supplementary Figures 4D-E).

Next, we injected Scr and NOTCH3OE OPM2 MM cells in immunodeficient mice and treated them with BOR after the tumors engrafted. BOR decreased tumor progression, reduced tumor burden (~60%), and improved survival in mice injected with control MM cells (Figure 4A-C). In contrast, BOR only reduced tumor burden by 23% in mice bearing NOTCH3OE MM cells and had no impact on survival. Lastly, to assess the effects of NOTCH3 inhibition on responses to BOR-based therapies, we established ex vivo MM-bone organ cultures (Figure 4D), a system that recapitulates the spatial dimension, cellular diversity, and molecular networks of the TME in a controlled setting. Scr or NOTCH3KD MM cells were allowed to colonize calvarial bones, treated with BOR, and MM-secreted paraprotein levels were quantified in the media to assess tumor growth (Figure 4E-F and Supplementary Figures 5A and C). BOR exhibited higher anti-MM efficacy in bones bearing 5TGM1 or U266 NOTCH3KD MM cells compared to Scr MM cells (Figure 4E-F). Similarly, treatment of bones bearing 5TGM1 NOTCH3KD MM cells with VRd resulted in better tumor reduction compared to bones bearing control 5TGM1 tumors (Supplementary Figure 5B). Together, this set of experiments supports that NOTCH3 integrates TME-mediated Notch signals in MM cells and confers chemoresistance/sensitivity to BOR-based therapies.

Because we previously reported that NDMM patients with NOTCH3 have a gene signature consistent with increased osteoclastogenic potential, we examined the impact of NOTCH3 activation on MM-induced bone disease. We found that NOTCH3OE MM tumors led to greater reductions in cancellous bone mass and higher levels of the bone resorption biomarker CTX compared to control tumors (Supplementary Figure 6A-C), but no differences were detected in
the levels of the bone formation marker P1NP (Supplementary Figure 6D). BOR therapy improved cancellous bone mass and P1NP and reduced CTX in mice bearing control MM cells, but had no effect on NOTCH3OE MM-bearing mice. Consistent with increased MM osteoclastogenic potential, NOTCH3OE MM cells expressed higher mRNA levels of RANKL, which were further enhanced by co-culture with osteocytes or stromal cells (Supplementary Figure 6E).

NOTCH3 transcriptional reprogramming increases the expression of CXC chemokines in MM cells. To determine the molecular mechanism(s) by which TME-mediated NOTCH3 signaling dictates responses to BOR-based therapies in MM cells, we compared the transcriptome of NDMM patients with high vs. low NOTCH3 expression (Supplementary Figure 7). We found enrichment in GO terms related to Chemokine signaling, CXCR chemokine signaling, Chemotaxis, and Cell adhesion (Supplementary Figure 7A-C), and upregulation of Cytokine-cytokine receptor interaction, Chemokine signaling pathways, and Cell Adhesion Molecules pathways in NDMM patients with high NOTCH3 expression (Figure 5A). Moreover, several members of the CXC chemokine family were upregulated in NDMM patients with high NOTCH3 (Supplementary Figure 7D). We focused on CXCL12 because it has been previously linked to cell adhesion-mediated drug resistance in MM.17, 18 We found a strong positive correlation between CXCL12 and NOTCH3 expression in NDMM and PRMM patients (Figure 5B). Similar to NOTCH3, the expression of CXCL12 also increased in PRMM patients compared to levels at diagnosis (Figure 5C). Additionally, we mined a previously published single-cell RNAseq data set of CD138+ plasma cells from PRMM patients19 and found colocalization of NOTCH3 and CXCL12 expression in a subset of CD138+ plasma cells from patients with primary refractory MM (Supplementary Figure 8). Based on this clinical data, we hypothesized
that TME-mediated NOTCH3 signaling increases CXCL12 expression in MM cells. Osteocytes increased the expression of CXCL12 in murine and human MM cells. This increase was prevented in NOTCH3KD cells and further increased in NOTCH3OE MM cells (Figure 5D-F). A similar regulation of CXCL12 by NOTCH3 was seen in co-cultures with stromal cells (Supplementary Figure 9). Together, these clinical and cellular data demonstrate that NOTCH3 signaling regulates CXCL12 expression in MM cells.

Autocrine CXCL12-CXCR4 signaling mediates NOTCH3-induced chemoresistance in MM cells. Next, we investigated the contribution of CXCL12 to the NOTCH3-mediated acquired chemoresistance triggered by the TME. We found that TME osteocytes activated NOTCH3 signaling by cleaving NICD3, but not NICD1 or 2 (Supplementary Figure 10) and increased the phosphorylation of the CXCL12 receptor CXCR4 and the downstream targets ERK 1/2 and AKT in MM cells. These effects were fully prevented in NOTCH3KD and enhanced in NOTCH3OE MM cells (Figure 6A-B and Supplementary Figure 11), indicating that CXCL12-CXCR4 signaling in MM cells depends on NOTCH3 signals. To further explore the role of CXCL12-CXCR4 signaling on responses to BOR-based therapies, we first employed Plerixafor, a selective inhibitor of CXCR4. Plerixafor fully restored sensitivity to BOR and VRd in NOTCH3OE MM cells co-cultured with osteocytes or stromal cells (Figure 6C-D and Supplementary Figure 12A). Because cells of the TME are thought to be an abundant source of CXCL12 in the MM-TME,20, 21 we examined the specific contribution of MM-derived CXCL12 by silencing CXCL12 in NOTCH3OE MM cells (Supplementary Figure 12B). As seen with Plerixafor, genetic inhibition of CXCL12 in MM cells restored the anti-MM efficacy of BOR and VRd to control levels (Figure 6E and Supplementary Figure 12C). These data identify the
existence of a novel autocrine NOTCH3-CXCL12-CXCR4 signaling axis promoted by the TME in MM cells.

**Pharmacological inhibition of CXCL12-CXCR4 or Notch signaling increases BOR sensitivity in high NOTCH3 MM cells.** Prompted by our *in vitro* studies with Plerixafor, we explored further the use of this agent in combination with BOR-based therapies using MM *ex vivo* 3D organ cultures established with murine and human bone. As seen *in vivo* with BOR, VRd’s anti-MM efficacy was significantly reduced in murine bones bearing *NOTCH3*<sup>OE</sup> vs. control MM cells (Figure 7A-B). Co-administration of Plerixafor increased the sensitivity of *NOTCH3*<sup>OE</sup> MM cells to VRd. We also tested the effects of VRd + Plerixafor in a novel human MM-human bone *ex vivo* system, which allowed us to study MM cell responses to chemotherapy in a TME closer to the one in patients (Figure 7C). MM cells engrafted human bones, and tumor growth was evident after four days (Figure 7C). *NOTCH3*<sup>OE</sup> MM cells exhibited resistance to VRd therapy compared to control MM cells, and co-administration of Plerixafor restored VRd’s anti-MM efficacy to control levels (Figure 7D). Lastly, we investigated if bone-targeted pan inhibition of Notch signals with a novel compound recently developed by our laboratory (BT-GSI)<sup>13</sup> overcomes the chemoresistance conferred by NOTCH3 activation in MM cells. Using *ex vivo* cultures established with murine bone and *Notch3*<sup>OE</sup> MM cells, we found that co-administration of BT-GSI doubled the anti-MM efficacy of BOR (Figure 7E-F). Together, these studies highlight the potential of combining BOR-based therapies with CXCL12-CXCR4 or bone-targeted Notch inhibitors to overcome TME-mediated drug resistance.

**Discussion**
Chemotherapy resistance is the leading cause of relapsed/refractory disease, decreased survival, and a major obstacle to more successful clinical outcomes in MM. In this study, we demonstrate that a signaling pathway involving NOTCH3 activation by the extrinsic TME in MM cells promotes resistance to BOR therapeutic regimes. Our data highlight that this pathway is present in NDMM patients, upregulated in PRMM patients, and predicts worse clinical responses to BOR-based chemotherapy. Further, genetic activation of NOTCH3 in MM cells is sufficient to promote resistance to BOR therapies. Conversely, we show that genetic or pharmacologic interruption of NOTCH3 signals in MM cells increases sensitivity to BOR and decreases tumor burden. Our clinical and preclinical data position NOTCH3 inhibition as a rational target to improve clinical responses to first-line regimes based on BOR in MM patients.

Osteocytes are best known for their role in bone remodeling, where they function as paracrine and endocrine cells controlling the activity of bone cells in the bone marrow and distant organs. Work from our group and others uncovered that osteocytes are also important components of the TME, capable of directly interacting with tumor cells, and have a pivotal role in tumor growth and cancer-induced bone disease. Yet, the role of osteocytes in chemoresistance has not been explored until now. Our studies extend beyond previous work on stromal cell-mediated mechanisms of resistance and identify the osteocyte as a new cell type of the TME contributing to resistance to chemotherapy via Notch communication. Along the same lines, another recent study reported that osteocytes can confer MM resistance to chemotherapy via exosomes. Because osteocytes are 95% of the cells in bone and, as stromal cells, can live for decades, these two cell types represent a major and long-lasting source of pro-survival signals for MM cells in MM. Future studies are needed to characterize further the contextual microenvironments and disease stages where these cell types preferentially operate.
It has been long appreciated that Notch signaling mediates communication between MM cells and other cells of the TME, supports tumor growth and bone destruction, and contributes to drug resistance and survival.\textsuperscript{5, 26} Furthermore, functional studies have suggested that inhibiting Notch activation downstream all NOTCH receptors with gamma-secretase inhibitors (GSI) decreases tumor burden, bone disease, and improves sensitivity to chemotherapeutic agents.\textsuperscript{6, 27} Although the evidence for the influence of NOTCH signals in MM progression is strong, the specific contribution of individual NOTCH components is less clear. Notably, our paper uncovers that the basal expression of NOTCH3 is dynamic and selectively upregulated by TME cells and describes a previously unknown role for NOTCH3 in MM chemoresistance. Previous \textit{in vitro} studies suggested that NOTCH1 and 2 are the main mediators of stroma-MM communication.\textsuperscript{28, 29} In contrast, our prior work showed that osteocytes preferentially employ NOTCH3 to communicate with MM cells.\textsuperscript{9} Although we cannot exclude the contribution of other NOTCH receptors to TME-mediated chemoresistance, this study suggests that NOTCH3 activation is a common molecular mechanism that TME cells utilize to communicate with MM cells and plays a pivotal role in promoting drug resistance. We reported before that homotypic NOTCH3 signaling mediates MM cell proliferation but does not affect MM cell viability.\textsuperscript{9} Consistent with this observation, homotypic NOTCH3 signaling (between MM cells) did not affect MM cell apoptotic responses to BOR or VRd, underlining their dependence on TME-derived Notch ligands for chemoprotection. Further studies beyond the scope of the current manuscript are granted to identify the TME Notch ligand(s) responsible for the activation of NOTCH3 and chemoresistance in MM cells.

We noted fascinating differences in the transcriptome of NDMM that are mechanistically dependent on NOTCH3 and lead to a gene signature predictive of poor clinical outcomes. Our
data show that NOTCH3 integrates signals from cells of the TME to increase CXCL12 expression in MM cells and provide evidence of NOTCH3-CXCL12 co-expression in CD138+ plasma cells from patients. Previous *in vitro* observations showed that inhibition of all NOTCH receptors with GSI decreases CXCL12 production in MM cells. Our findings are consistent with this study and support that NOTCH3 is a major molecular regulator of CXCL12. Although stroma-derived CXCL12/CXCR4 is a well-established symbiotic bridge linking MM cells and their stromal neighbors in oncogenic communication/drug resistance, this report is one of the first indications suggesting the existence of an active autocrine CXCL12-CXCR4 signaling axis in MM cells promoted by the TME. We show that interruption of NOTCH3 signals by inhibiting NOTCH3 cleavage at the membrane level (BT-GSI) or suppressing CXCL12 expression (siRNA) or signaling through CXCR4 (Plerixafor) led to comparable prevention of TME-induced BOR resistance in MM cells. Remarkably, we validated these observations in human *ex vivo* models, which showed that bones infiltrated with NOTCH3-activated MM cells have worse responses to VRd and, importantly, a robust reduction in tumor burden after co-administration of VRd and Plerixafor. Therefore, human (and murine) *ex vivo* organ cultures represent a powerful tool to model responses to chemotherapy in a physiologically relevant environment. Collectively, these findings support that NOTCH3 is activated in MM cells by the TME in specialized niches, resulting in a transcriptional response downstream of CXCL12 binding to CXCR4, which leads to chemoresistance.

In addition to its role in drug resistance, we confirmed that NOTCH3 signaling in MM cells exerts bone catabolic actions. We showed before that inhibition of NOTCH3 in MM cells reduces MM-induced bone disease. Conversely, we found that mice-bearing MM cells with activated NOTCH3 exhibited worse bone destruction in this study. Two potential mechanisms,
not mutually exclusive, may account for this observation. One, NOTCH3 activation by TME cells increases in MM cells the expression of RANKL, a pro-osteoclastogenic cytokine with a key role in the development of bone disease in MM patients. Second, TME-derived signals integrated by NOTCH3 stimulate the proliferation of MM cells and lead to greater tumors and, therefore, more bone disease. Our studies did not address the contribution of each potential mechanism, as both occur simultaneously in our model.

These findings have important clinical implications. Our results suggest the potential added value of NOTCH3 expression in MM treatment decision-making for NDMM and PRMM patients. Further validation of the impact of NOTCH3 expression in other cohorts is needed to strengthen this argument, which we acknowledge as a limitation of our study. In addition, these findings raise the possibility that NOTCH3 might be a useful target for MM patients. Anti-NOTCH3-antibodies have shown efficacy against solid tumors but have not yet been evaluated in MM models. Further, our data suggest the potential of a therapy targeting NOTCH3 to simultaneously decrease tumor growth, improve responses to BOR regimes, and stop bone destruction, as pharmacological inhibition of NOTCH3 in vivo is sufficient to decrease bone resorption in naïve mice. Of note, NOTCH3-antibodies do not exhibit dose-limiting side effects seen with pan-inhibition of Notch signaling in humans and mice. Similarly, our novel bone-targeted GSI inhibitor shows great potential to overcome drug resistance mediated by NOTCH3, and other NOTCH receptors, while circumventing toxicities. Lastly, our work provides a cellular and molecular rationale to combine BOR regimes with Plerixafor as a chemosensitization strategy in MM patients, a strategy proven successful recently in a small clinical trial (NCT00903968).
A better understanding of the cellular and molecular events leading to disease progression/relapse in MM is needed to bypass drug resistance. This study unravels a crucial role of NOTCH3 as a mediator of TME-mediated chemoresistance in MM. Complementary clinical and preclinical data and pharmacologic and genetic approaches in human and mouse systems support this conclusion. Further, we identified a previously unknown function of osteocytes as providers of Notch signals in the TME conducive to resistance to chemotherapy. Lastly, we demonstrate the beneficial effects of targeting NOTCH3 and its downstream signals to restore sensitivity to BOR-based therapies in MM cells. In summary, our findings support using existing and novel pharmacologic tools to interfere with NOTCH3 signals to overcome drug resistance and improve bone health in MM.
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Figure legends

**Figure 1.** *NOTCH3* expression is increased in newly diagnosed MM (NDMM) patients and regulated by the cells of the tumor niche. (A) Gene expression of *NOTCH* 1-4 receptors in CD138<sup>+</sup> cells from NDMM or healthy donors. N=52 patients. *p<0.05* vs. healthy donors by One-way ANOVA, followed by a Tukey post hoc test. Boxes show the data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean. (B) *NOTCH3* gene expression in CD138<sup>+</sup> cells from NDMM patients with or without point mutations in the *NOTCH3* gene. N=725 (12 with mutations) NDMM patients.

**Figure 2.** *NOTCH3* expression is increased in relapsed MM patients and correlates with poor responses to BOR-based therapies. Network plot of selected upregulated functional enrichment analysis of GO terms related to (A) Responses to Drugs or (B) Cell-Adhesion in CD138<sup>+</sup> cells from newly diagnosed (NDMM) patients with high vs. low *NOTCH3* expression. The size of the circles represents the number of genes in the individual GO terms. The thickness of the lines represents the number of overlapped genes between the individual GO terms. (C) Gene Set Enrichment Analysis (GSEA) shows NDMM patients with high *NOTCH3* have enrichment in genes involved in poor responses to Bortezomib (BOR) therapy in MM patients compared to NDMM patients with low *NOTCH3* expression. Data were analyzed using a weighted Kolmogorov-Smirnov-like statistical test. (D) Kaplan-Meier plot of the progression-free survival (PFS) of NDMM patients with high (top) vs. low (bottom) *NOTCH3* expression receiving BOR-based (blue line) vs. other therapies (other) not including BOR (red line). N=708 patients. Data were analyzed using a Log-rank (Mantel-Cox) test. (E) Gene expression of *NOTCH* 1-4 receptors in CD138<sup>+</sup> cells from paired diagnosis (NDMM) and progression/relapsed MM (PRMM) patients. N=70/group. *p<0.05* vs. diagnosis by Student’s *t*-test. Boxes show the
data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean.

**Figure 3. NOTCH3 integrates TME-mediated signals dictating MM cell responses to BOR-based therapies.** (A) MM cells were co-cultured with osteocytes (Ots) and treated with Bortezomib (BOR; 48h) or Dexamethasone+BOR+Lenalidomide (VRd; 24h). Percent apoptosis in scramble (Scr) or \( NOTCH3 \) knockdown (\( NOTCH3^{KD} \)) 5TGM1 or U266 (B) MM cells, and in Scr or \( NOTCH3 \) activated (\( NOTCH3^{OE} \)) OPM2 (C) MM cells co-cultured in the absence/presence of Ots and treated with/without BOR or VRd. N=4/group. *p<0.05 by Two-Way ANOVA, followed by a Tukey post hoc test. The dotted line represents the percent apoptosis in vehicle-treated Scr MM cells cultured alone. ns: non-significant. Data are shown as mean ± SD; each dot represents an independent sample. Representative experiments out of two are shown. DiD: cell label dye; PI: propidium iodide.

**Figure 4. NOTCH3 activation in MM cells promotes chemoresistance to BOR therapy.** (A) Experimental design. Tumor progression and Bortezomib (BOR)-induced tumor reduction (B) and probability of survival (C) in mice injected with Scramble (Scr) or \( NOTCH3 \) activated (\( NOTCH3^{OE} \)) OPM2 MM cells treated with/without BOR. N=6/11 mice/group. A Two-way ANOVA test was used for (B, endpoint), followed by a Tukey post hoc test. Tumor reduction by BOR therapy by Student’s \( t \)-test, *p<0.05 vs. mice bearing Scr tumors treated with BOR. For (C), a Log-rank (Mantel-Cox) test was performed. (D) Tumor reduction by BOR in \textit{ex vivo} organ cultures of calvarial disc bones from KaLwRijHsd bearing murine 5TGM1 (E) or NSG mice bearing human U266 (F) Scr/\( NOTCH3 \) knockdown (\( NOTCH3^{KD} \)) MM cells. N=4-8/group. *p<0.05 vs. Scr MM cells treated with BOR by Student’s \( t \)-test for each time point. ns: non-
significant. wk: week. Boxes show the data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean. (D, E, F).

**Figure 5. Activation of NOTCH3 transcriptional reprogramming by the TME increases CXCL12 expression in MM cells.** Top 20 most significantly upregulated pathways (A) in newly diagnosed (NDMM) patients with high vs. low NOTCH3 expression. N=768 patients. (B) CXCL12 and NOTCH3 expression correlation in CD138+ cells from NDMM and progression/relapsed (PRMM) patients. N=70/group. For (B), Pearson’s correlation tests were performed. (C) Gene expression of CXCL12 in CD138+ cells from paired diagnosis and relapsed MM patients. N=70/group. *p<0.05 vs. diagnosis by Student’s t-test. Boxes show the data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean. CXCL12 gene expression in Scramble (Scr)/NOTCH3 knockdown (NOTCH3KD) 5TGM1 (D) or U266 (E) MM cells and Scr/NOTCH3 activated (NOTCH3OE) OPM2 (F) MM cells cultured in the absence/presence of osteocytes (Ots). N=4/group. *p<0.05 by Two-way ANOVA, followed by a Tukey post hoc test. ns: non-significant. Data are shown as mean ± SD; each dot represents an independent sample; representative experiments out of two are shown (F).

**Figure 6. NOTCH3-CXCL12-CXCR4 signaling mediates TME-induced chemoresistance in MM cells.** Effects of osteocytes (Ots) and manipulation of NOTCH3 signaling in MM cells on protein levels of activated NOTCH3 receptor (NICD3), phosphorylated (p) CXCR4, pERK, pAKT in (A) Scramble (Scr)/NOTCH3 knockdown (NOTCH3KD) 5TGM1 MM cells and (B) Scr/NOTCH3 activated (NOTCH3OE) OPM2 MM cells. Representative images from 3 independent experiments are shown (see Supplementary Figure 11). (C) Experimental design. (D) Percent apoptosis of OPM2 NOTCH3OE MM cells treated with/without Plerixafor,
Bortezomib (BOR), or dexamethasone+BOR+Lenalidomide (VRd) in the absence/presence of Ots. N=4/group; BOR=48h, VRd=24h. (E) Percent apoptosis of OPM2 NOTCH3OE MM cells with/without CXCL12 silencing and treated with BOR or VRd in the absence/presence of Ots. *p<0.05 by Two-way ANOVA, followed by a Tukey post hoc test. The dotted line represents the percent apoptosis in vehicle-treated OPM2 NOTCH3OE MM cells cultured alone. ns: non-significant. DiD: cell label dye. siRNA: small interference RNA. Data are shown as mean ± SD; each dot represents an independent sample; representative experiments out of two are shown.

Figure 7. Pharmacological inhibition of CXCL12-CXCR4 or Notch signaling enhances therapeutic responses to BOR-based therapy in NOTCH3-activated MM cells. (A) Ex vivo bone-MM organ cultures established with Scramble (Scr)/ NOTCH3 activated (NOTCH3OE) OPM2 human MM cells and calvarial disc bones from NSG mice. (B) Percent tumor reduction by co-administration of dexamethasone+bortezomib (BOR)+lenalidomide (VRd) and Plerixafor. N=6/group. *p<0.05 vs. bones bearing Scr MM cells treated with VRd alone for 11 days by One-way ANOVA, followed by a Tukey post hoc test. (C) Ex vivo bone-MM organ cultures established with Scr/NOTCH3OE OPM2 human MM cells and femoral head bone fragments from healthy human donors. Representative bioluminescence images of human bones showing engraftment and growth of human MM cells through the length of the experiment. (D) Percent tumor reduction by co-administration of VRd and Plerixafor. N=6/group. *p<0.05 by Two-way ANOVA, followed by a Tukey post hoc test. (E) Ex vivo bone-MM organ cultures established with NOTCH3OE OPM2 human MM cells and calvarial disc bones from NSG mice. (F) Percent tumor reduction by co-administration of BOR and bone-targeted gamma-secretase inhibitor (BT-GSI) after 4 and 11 days. N=6/group. *p<0.05 vs. bones bearing NOTCH3OE MM cells treated with BOR alone by One-way ANOVA, followed by a Tukey post hoc test. ns: non-significant.
Boxes show the interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean. d: days.
Figure 1

A  NDMM patients

\[ \text{Log}_2 \text{ expression} \]

\[ \text{NOTCH1} \]

\[ \text{Log}_2 \text{ expression} \]

\[ \text{NOTCH2} \]

\[ \text{Log}_2 \text{ expression} \]

\[ \text{NOTCH3} \]

\[ \text{Log}_2 \text{ expression} \]

\[ \text{NOTCH4} \]

\[ \text{Healthy} \quad \text{t(4;14)} \quad \text{t(11;14)} \quad \text{t(14;16)} \quad \text{t(14;20)} \quad \text{D1} \quad \text{D2} \]

B  NDMM patients

\[ \text{Log}_2 \text{ expression} \]

\[ \text{NOTCH3} \]

Non-mutated

Mutated
Figure 2

A NDMM patients NOTCH3\textsuperscript{hi} vs NOTCH3\textsuperscript{low}

**Response to drug network**

Enrichment P value

- 0.01
- 0.0087
- 0.0007
- 0.0052
- 0.0035
- 0.0015
- 0.0017
- 0.0017

- GO:2001038 regulation of cellular response to drug (2)
- GO:0042493 response to drug (293)

B NDMM patients NOTCH3\textsuperscript{hi} vs NOTCH3\textsuperscript{low}

**Cell-adhesion network**

- GO:0060355 positive regulation of cell adhesion molecule production (4)
- GO:0051041 Positive regulation of calcium-independent-cell-adhesion (1)
- GO:0034116 positive regulation of heterotypic cell adhesion (14)
- GO:1904996 positive regulation of leukocyte adhesion to vascular endothelial cell (12)
- GO:1900026 positive regulation of substrate adhesion-dependent cell spreading (40)
- GO:1904906 positive regulation of endothelial cell-matrix adhesion via fibronectin (5)
- GO:0051894 positive regulation of focal adhesion assembly (27)
- GO:0010811 Positive regulation of cell-substrate adhesion (34)

C NDMM patients NOTCH3\textsuperscript{hi} vs. Notch3\textsuperscript{low}

**GSEA BOR response gene set classifier**

Enrichment score

- 0
- 0.2
- 0.4
- 0.6
- 0.8

Rank 0 1000 2000

p=0.001
FDR=0.002
NES=-2.06

D NDMM patients with NOTCH3\textsuperscript{hi}

- BOR-based
- Other

Probability PFS

p=0.043

Time (days)
0 1000 2000

E NDMM patients with NOTCH3\textsuperscript{low}

- BOR-based
- Other

Probability PFS

p=0.48

Time (days)
0 1000 2000

F MM patients

- NDMM
- PRMM

Log\textsubscript{2} expression

*NOTCH 1 2 3 4

0 5 10 15 20

*
Figure 3

A. DiD-MM cells or VRd

B. U266 cells

C. OPM2 cells

5TGM1 cells

BOR or VRd

Ot-like cells

BOR therapy

Alone + BOR Co-culture + BOR

% Apoptosis

NOTCH3KD

Scr

Ots - + - +

Scr NOTCH3KD

Ots - + - +

Scr NOTCH3KD

Ots - + - +

Scr NOTCH3OE

Ots - + - +
Figure 4

A

OPM2

BOR (0.1 mg/kg; 5x/wk)

wk0  wk1  wk2  wk3  wk4

B

Tumor burden

Days post tumor inoculation

Tumor reduction by BOR

Percent

C

Probability of survival

Days post tumor inoculation

D

Ex vivo bone organ culture

Mouse calvaria

MM cells

E

5TGM1 cells

BOR therapy

% Tumor reduction (IgG2b)

day 4 11

F

U266 cells

BOR therapy

% Tumor reduction (L-L-chain)

day 4 11
Figure 5

A. NDMM patients \( NOTCH3^{hi} \) vs \( NOTCH3^{low} \)
Top KEGG_pathways upregulated

- Viral protein interaction with cytokine and cytokine
- Receptor Hematopoietic cell lineage
- Staphylococcus aureus infection
- Protein digestion and absorption
- Rheumatoid arthritis
- Complement coagulation cascades
- amoebiasis
- Cytokine-cytokine receptor interaction
- Chemokine signaling pathway
- Cell adhesion molecules
- Osteoclast differentiation
- phagosome
- Neuroactive ligand-receptor interaction
- Transcriptional missregulation in cancer
- Tuberculosis
- PI3K-Akt signaling pathway
- Focal adhesion
- cAMP signaling pathway
- Rap1 signaling pathway
- Calcium signaling pathway

B. NDMM patients

\[ R=0.76 \quad P<2.2\times10^{-16} \]

C. MM patients

- Diagnosis
- Relapse

D. 5TGM1 cells

\[ \text{Scr} \quad \text{NOTCH3}^{KD} \]

E. U266 cells

\[ \text{Scr} \quad \text{NOTCH3}^{KD} \]

F. OPM2 cells

\[ \text{Scr} \quad \text{NOTCH3}^{OE} \]
Supplementary Table 1. Point mutations in the *NOTCH3* gene detected in newly diagnosed MM patients from the MMRF. N=725 patients. Chr: chromosome; Ref: reference nucleotide; Alt: alteration.

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Supplementary Figure 1. Cells of the TME increase \textit{NOTCH3} expression in MM cell lines. Gene expression of NOTCH receptors (\textit{NR}) 1-4 and the Notch target gene \textit{HES1} in co-cultures of osteocytes (Ots) and (A) murine 5TGM1, (B) human U266, or (C) human OPM2 MM cells after 48h. mRNA expression of (D) murine 5TGM1, (E) human U266, and (F) human OPM2 MM cells cultured in the absence and presence of stromal cells (ST2: murine stromal cells; HS-5: human stromal cells). N=4/group. *\(p<0.05\) vs. MM cells cultured alone by Student’s \(t\)-test. n.d.: not detected. Data are shown as mean ± SD; each dot represents an independent sample. Representative experiments out of two are shown.
Supplementary Figure 2. Correlation between NOTCH1-4 expression and survival in newly diagnosed MM patients. Kaplan-Meier plots of the overall survival (OS, A) and progression-free survival (PFS, B) of newly diagnosed MM (NDMM) patients with high (blue) vs. low (red) NOTCH 1-4 expression. (C) Kaplan-Meier plot of the PFS of NDMM patients with high NOTCH3 expression receiving Bortezomib (BOR)-based therapy (purple line), high NOTCH3 expression receiving other therapies not including BOR (other; sky blue line), low NOTCH3 expression receiving BOR-based therapy (green line), low NOTCH3 expression receiving other therapies not including BOR (other; red line). N=708 patients. Data were analyzed using a Log-rank (Mantel-Cox) test.
Supplementary Figure 3. Generation of NOTCH3 knockdown and activated MM cells. (A) NOTCH1-3 receptor expression and cleaved intracellular domain (NICD) protein levels in human U266 and OPM2 MM cells. N=4/group; 48h culture. *p<0.05 vs OPM2 cells by Student’s t-test. (B) NOTCH 1-3 gene expression in scramble (Scr) or genetically modified 5TGM1, U266, and OPM2 MM cells cultured alone and treated with Bortezomib (BOR; 3nM) for 24h. N=4/group; *p<0.05 vs. scramble MM cells by Two-Way ANOVA, followed by a Tukey post hoc test. NOTCH 1-3 and HES1 gene expression and cleaved NICD protein levels in (C) U266 Scr and NOTCH3 knockdown (NOTCH3KD) MM cells and (D) OPM2 Scr and NOTCH3 activated (NOTCH3OE) MM cells. N=4/group; 48h culture. *p<0.05 vs. Scr MM cells by Student’s t-test (C) or One-Way ANOVA, followed by a Tukey post hoc test (D). Data are shown as mean ± SD; each dot represents an independent sample. Representative experiments out of two are shown. sg: guide RNA.
Supplementary Figure 4. NOTCH3 acts as a signaling hub integrating stroma-mediated drug resistance signals. Percent apoptosis in (A) 5TGM1 scramble (Scr)/NOTCH3 knockdown (NOTCH3KD) MM cells, (B) U266 Scr/NOTCH3KD MM cells, and (C) OPM2 Scr/NOTCH3 activated (NOTCH3OE) MM cells cultured in the absence/presence of stromal cells treated with Bortezomib (BOR). Percent apoptosis in (D) 5TGM1 Scr/NOTCH3KD MM cells, (E) U266 Scr/NOTCH3KD MM cells, and (F) OPM2 Scr/NOTCH3OE MM cells cultured in the absence/presence of osteocytes cultured without BOR. N=4/group; BOR=48h. *p<0.05 by Two-way ANOVA, followed by a Tukey post hoc test. OtS: osteocyte-like cells; ST2: murine stromal cells; HS-5: human stromal cells. Dotted line represents the percent apoptosis in vehicle-treated Scr MM cells cultured alone. ns: non-significant. Data are shown as mean ± SD; each dot represents an independent sample. Representative experiments out of two are shown.
Supplementary Figure 5. Inhibition of NOTCH3 sensitizes MM cells to Bortezomib-based treatment. Ex vivo bone-MM organ cultures were established with murine scramble (Scr) and NOTCH3 knockdown (NOTCH3KD) 5TGM1 MM cells and calvarial disc bones from C57BL/KaLwRijHsd mice (A, B) or human Scr and NOTCH3KD U266 MM cells and calvarial disc bones from NSG mice (C). Cultures were treated with Bortezomib (BOR) (A, C) or dexamethasone+BOR+Lenalidomide (VRd) (B) for 4 and 11 days. N=4-8/group. *p<0.05 by Two-way ANOVA, followed by a Tukey post hoc test (A, C) or Student’s t-test (B). Data are shown as mean ± SD (A, C); each dot represents an independent sample. Boxes show the data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean (B).
Supplementary Figure 6. *NOTCH3* activation in MM cells impedes the beneficial effects of BOR on bone. (A) Representative microCT 3D reconstruction images of tibiae, (B) tibiae cancellous bone volume/tissue volume (BV/TV) quantification, serum CTX (C), and P1NP (D) in mice injected with Scramble (Scr) or *NOTCH3* activated (*NOTCH3OE*) MM cells and treated with/without Bortezomib (BOR). N=6-11 mice/group. *p<0.05 vs. saline by One-way ANOVA, followed by a Tukey post hoc test. Boxes show the data interquartile range, the middle line in the box represents the median, and whiskers the 95% confidence interval of the mean. (B, C, D). (E) *RANKL* gene expression in Scr or *NOTCH3OE* MM cells co-cultured with osteocytes (Ots) or stromal cells (HS5). N=4/group. *p<0.05 by Two-way ANOVA, followed by a Tukey post hoc test. Representative experiments out of two are shown (E). Data are shown as mean ± SD; each dot represents an independent sample (E).
Supplementary Figure 7. Gene expression and GO term bioinformatics differential analysis in newly diagnosed patients with high vs. low NOTCH3 expression. Gene ontology (GO) analysis of upregulated genes in NOTCH3 high vs. low newly diagnosed MM (NDMM) patients. Bar charts showing the top 20 GO terms for Molecular Function (A) and Biological Process (B) ranked by p-value. N=768 patients. (C) Network plot of selected upregulated functional enrichment analysis of GO terms related to Chemotaxis in NDMM patients with high vs. low NOTCH3 expression. The size of the circles represents the number of genes in the individual GO terms. N=768 patients. The thickness of the lines represents the number of overlapped genes between the individual GO terms. (D) Differentially expressed CXC-related genes and the corresponding fold changes and adjusted p values in NDMM patients with high vs. low NOTCH3 expression by Student’s t-test. N=768 patients.
Supplementary Figure 8. Identification of a population of CD138⁺ cells co-expressing NOTCH3 and CXCL12 in RRMM patients. Single-cell RNA-seq analysis, UMAP-based visualization of major classes of CD138⁺ cells based on a published single-cell RNA sequencing dataset and colored by patient (A) or cluster (B). Gene expression plot of NOTCH3 (C) and CXCL12 (D) of individual CD138⁺ cells on UMAP coordinates. (E) Top 20 most upregulated genes in cluster 2 containing NOTCH3⁺-CXCL12⁺ MM cells. (F) Patient and scRNAseq data characteristics.
Supplementary Figure 9. Stromal cell-mediated NOTCH3 signaling regulates CXCL12 expression in MM cells. mRNA expression of CXCL12 in (A) 5TGM1 Scramble (Scr) and NOTCH3 knockdown (NOTCH3KD), (B) U266 Scr and NOTCH3KD, and (C) OPM2 Scr and NOTCH3 activated (NOTCH3OE) MM cells cultured for 48h in the absence and presence of stromal cells. ST2: murine stromal cells; HS-5: human stromal cells. N=4/group. *p<0.05 by Two-way ANOVA, followed by a Tukey post hoc test. ns: non-significant. Data are shown as mean ± SD; each dot represents an independent sample. Representative experiments out of two are shown.
Supplementary Figure 10. Osteocytes do not activate NOTCH1 or NOTCH2 in MM cells. Cleaved NOTCH1 and 2 intracellular domain (NICD) protein levels in murine Scramble (Scr) or NOTCH3 knockdown (NOTCH3KD) 5TGM1 MM cells culture alone or with osteocytes (Ots).
Supplementary Figure 11. Osteocyte-mediated NOTCH3 signals activate CXCR4 signaling via AKT and ERK phosphorylation. Quantification of three independent western blots from experiments displayed in Figure 6 A-B using (A) Scramble (Scr) and NOTCH3 knockdown (NOTCH3KD) 5TGM1 and (B) Scr and NOTCH3 activated (NOTCH3OE) OPM2 MM cells co-cultured in the absence/presence of osteocytes (Ots). N=3/group. *p<0.05 Two-Way ANOVA, followed by a Tukey post hoc test. Data are shown as mean ± SD; each dot represents an independent sample.
Supplementary Figure 12. Genetic or pharmacologic inhibition of CXCL12 signaling prevents stroma-mediated drug resistance to Bortezomib-based therapies. (A) Percent apoptosis in NOTCH3 activated (NOTCH3OE) OPM2 MM cells treated with Bortezomib (BOR) in the presence/absence of Plerixafor and cultured with/without osteocytes (Ots). N=4/group; BOR=48h. *p<0.05 by Two-Way ANOVA, followed by a Tukey post hoc test. (B) CXCL12 gene expression in NOTCH3OE OPM2 MM cells treated with/without siRNAs against CXCL12 and cultured alone or with Ots. N=4; *p<0.05 by Two-way ANOVA, followed by a Tukey post hoc test. (C) Percent apoptosis in NOTCH3OE OPM2 MM cells treated with BOR in the presence/absence of siRNA-CXCL12 and cultured with/without Ots. N=4/group; BOR=48h. *p<0.05 by Two-Way ANOVA, followed by a Tukey post hoc test. Data are shown as mean ± SD; each dot represents an independent sample. Dotted line represents the percent apoptosis in vehicle-treated OPM2 MM cells cultured alone. siRNA: small interference RNA. Representative experiments out of two are shown.
Supplementary methods.

Reagents. RPMI 1640 media, Minimum Essential Media (MEM) α, fetal bovine serum, bovine calf serum, Normocin, antibiotics (penicillin/streptomycin), TriZol, and DiI/DiD cell trackers were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). Trypan Blue was purchased from Sigma Aldrich (St. Louis, MO, USA). γ-secretase inhibitor XX (GSI-XX) was purchased from Calbiochem (San Diego, CA, USA). Anti-GAPDH (Cat#2118S, RRID: AB_561053), anti-NOTCH1 (Cat #4380S, RRID: AB_10691684), anti-NOTCH2 (Cat #5732S, RRID: AB_10693319), anti-p-ERK (Cat#9101, RRID: AB_331646), anti-ERK (Cat#9102, RRID: AB_330744), anti-pAKT (Cat# 4060S), and anti-AKT (Cat# 9272S) antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA); anti-NOTCH3 (ab23426, RRID: AB_776841), anti-NOTCH4 (ab166605), and anti-pCXCR4 (ab74012, RRID: AB_1523429) antibodies were purchased from Abcam (Cambridge, MA, USA). Plerixafor (Cat#S8030), Bortezomib (Cat#S1013), and Lenalidomide (Cat#S1029) were purchased from Selleckchem (Houston, TX, USA); Dexamethasone (Cat#D4902) was purchased from Sigma (St. Louis, MO, USA); and CXCL12 siRNA (Cat#SR304303) was purchased from Origene Technology (Rockville, MD, USA). Puromycin (Cat#58-58-2) and Blasticidin (Cat#ant-bl) were purchased from InvivoGen (San Diego, CA, USA).

Cell culture. 5TGM1 murine MM cells (RRID: CVCL_VI66) were obtained from Dr. B. Oyajobi (University of Texas at San Antonio, TX, USA), and MLO-A5 murine osteocyte-like cells (RRID: CVCL_0P24) were purchased from Kerafast (Boston, MA, USA). OPM2 (RRID: CVCL_1625) and U266 (RRID: CVCL_0566) human MM cells were obtained from Dr. G. D. Roodman (Indiana University, IN, USA). ST2 murine stromal cells (RRID: CVCL_2205) were obtained from Dr. Jin-Ran Chen (University of Arkansas for Medical Sciences, AR, USA), and HS-5 human stromal
cells (RRID: CVCL_3720) were purchased from ATCC (Manassas, VA, USA). Cell lines were checked for mycoplasma weekly and routinely examined for proper morphology, population doubling, and paraprotein production. MM cell lines (5TGM1, OPM2, U266) were cultured in RPMI with 10% FCS and 1% P/S. Osteocyte-like cells (MLOA5) were cultured on calf skin collagen type I-coated plates in αMEM media with 2.5% FBS, 2.5% BCS, and 1% P/S. Stromal cells (ST2, HS5) were cultured in DMEM with 10% FBS and 1% P/S.

**Gene Expression.** Total RNA was isolated from MM cells and bone tissues using Trizol and converted to cDNA (Invitrogen Life Technologies), following the manufacturer's directions. EDTA incubations were used to separate MM cells from the osteocytes/stroma, as previously described.\(^1\)\(^,\)\(^2\) Gene expression was quantified by quantitative real-time PCR (qPCR) using TaqMan assays from Applied Biosystems (Foster City, CA, USA), following the manufacturer's directions. Gene expression levels were calculated using the comparative threshold (CT) method and were normalized to the housekeeping gene GAPDH.\(^1\)\(^,\)\(^2\)

**Western Blot.** Cell lysates (50μg) were boiled in the presence of SDS sample buffer (NuPAGE LDS sample buffer; Invitrogen) for 10 minutes and subjected to electrophoresis on 10% SDS-PAGE (Bio-Rad Laboratories). Proteins were transferred to PVDF membranes using a semidry blotter (Bio-Rad) and incubated in a blocking solution (5% nonfat dry milk in TBS containing 0.1% Tween-20) for 1 hour to reduce nonspecific binding. Immunoblots were performed using anti-GAPDH, pCXCL12, AKT, pAKT, ERK, pERK, NOTCH1, NOTCH2, and NOTCH3 antibodies (1:1000) followed by goat anti-rabbit secondary antibodies, conjugated to horseradish peroxidase (1:2000) in 5% milk (Santa Cruz Biotechnology). Western blots were developed using an enhanced chemiluminescence detection assay following the manufacturer's directions (Bio-Rad). Protein bands were quantified using ImageJ.
Enzyme-linked immunoassays (ELISA). The levels of the tumor biomarkers human lambda (Bethyl Laboratories; Cat#E88-116) or IgG2B (Invitrogen; Cat#88-50430-88) paraproteins produced by OPM2/U266 and 5TGM1 cells, respectively, were used to determine tumor growth/burden in vivo (serum) and ex vivo (conditioned media). The bone resorption biomarker C-telopeptide of type 1 collagen (CTX) (Immunodiagnostic systems; Cat#AC-06F1) and bone formation marker propeptide of type 1 collagen (P1NP) (Immunodiagnostic systems; Cat#AC-33F1) were analyzed in serum (in vivo). Tumor growth/burden, bone resorption, and bone formation were determined using commercially available specific ELISA and following the manufacturer's recommendations.

Apoptosis and proliferation assays. Apoptosis in MM cells was assessed by flow cytometry using the FITC (OPM2 or U266) or PerCP-Cyanine5.5 (5TGM1) Annexin V apoptosis Detection kit (BD Biosciences) following the manufacturer's recommendations. For analysis of apoptosis in co-cultures, OPM2 cells were selected by using mCherry fluorescence, and 5TGM1 and U266 cells were stained with the fluorescent cell-tracker DiD before plating cells (emission 665 nm; excitation 645 nm) following the manufacturer's recommendations. Samples were analyzed in a BD FACSCalibur (UAMS Core Facility for Flow cytometry) within 1h. At least 10,000 cells were used for each group, and the data were analyzed by FlowJo software to detect different cell populations. Co-cultures were treated with Plerixafor (25uM), BOR (3nM), VRd (BOR: 2nM, Lenalidomide: 1uM, Dexamethasone: 10nM) were refreshed every 24h. OPM2 cells were stained with a fluorescent cell tracker DiI before plating cells following the manufacturer's recommendations for proliferation analysis. After 48h of culture, cells were collected, re-suspended in PBS, and DiI fluorescence was read at 520 to 565 nM on a plate reader.
Genetic inhibition/activation in MM cells. Control and NOTCH3 knockdown 5TGMI MM cells were generated as described before. To generate stable control and NOTCH3-activated OPM2 MM cells, we first transduced cells with CRISPRa dCas9-VP64 lentiviral particles (MOI=10; Addgene, Watertown, MA, USA) and treated them with Blasticidin (5ug/mL) for selection. OPM2 MM cells stably expressing CRISPRa dCas9-VP64 were transduced with lentiviral particles carrying scramble or NOTCH3 directed sgRNAs (TCCGCGCGTCCCAGGCTGTG) and mCherry (MOI=10; Genecopoeia, Rockville, MD. USA) and treated with Puromycin (1ug/mL) for selection. To generate control and NOTCH3 knockdown U266 MM cells, cells were transduced with a plasmid containing a sgRNA sequence against NOTCH3 cloned into a guide RNA Cas9 vector (a gift from Dr. Helena Karlström, Karolinska Institutet, Sweden) and treated with Puromycin (3ug/mL) for selection. 5TGM1 transduced MM cells were maintained in complete media supplemented with 3ug/mL of Puromycin, OPM2 cells were maintained in complete media supplemented with 0.5ug/mL Puromycin and 2.5 μg/mL Blasticidin, and U266 cells were maintained in complete media supplemented with 1.5ug/mL Puromycin. To transiently inhibit CXCL12 in MM cells, we treated OPM2 MM cells with 1.0 nM of siRNA against CXCL12 for 24h before establishing MM-osteocyte/stroma co-cultures.

Ex vivo organ cultures. Ex vivo MM-murine bone organ cultures were established with calvarial bones from NOD.Cg-Prkdscid Il2rgtm1Wjl/SzJ (NSG; Jackson's Lab, strain: 005557) for human U266 or OPM2 MM cells or with calvarial bones from C57BL/KaLwRijHsd mice of murine 5TGM1 MM cells, as described before. For these ex vivo cultures, 5x10^4 MM cells were plated on calvarial bones, and 2x10^5 MM cells were plated on human bones. The human bone samples were obtained from 2 males, with no pathologies or medications that could affect bone mass or architecture. Treatments (Plerixafor 50uM; BT-GSI 10 uM; BOR 3nM; VRd: BOR 2nM,
Lenilidomide 1uM, Dexamethasone 10nM) were refreshed every 3 days. 50% of conditioned media was collected and replaced after 4 days of culture. All conditioned media was collected after 11 days of culture. Tumor growth was quantified by luciferase activity or ELISA.

**Bioinformatic analyses.** We segregated high vs. low NOTCH3 patients using quartile measures and performed comparative gene expression analysis as described before. The gene list for the GSEA on responses to BOR was constructed using data from Mulligan et al. Samples were classified as progressive/relapsed if they met two criteria: i) the patient must have a baseline sample, and ii) the patient must have a sample taken after either a complete or partial response. The scRNASeq analysis was conducted on data previously published by Cohen et al. (GSE161195). Salmon gene count data were imported into and normalized using the R package DESeq2. Differential expression analysis of NOTCH3 high versus low samples was performed using DESeq2, with an adjusted p-value cutoff of 0.05. Gene set enrichment analysis was then conducted using the R package fgsea. Survival analyses were performed and visualized in R using the survival and survminer packages. Comparison of gene expression between NDMM (n=768) and paired NDMM vs. progression/relapsed (PRMM; n=70) samples was performed and visualized using the R package ggpubr. Correlation analysis between CXCL12 and NOTCH3 was performed in both the NDMM and progressive/relapsed samples using the R package ggpubr. Mutation data for NOTCH3 was obtained from the MMRF Researcher Gateway for corresponding whole-exome sequencing samples (n=725). All single-cell analyses were performed using the R package Seurat as described before. The PIANO package was used to perform the gene-set enrichment analysis of Gene Ontology (GO) and gene networks using adjusted p-value and log2 fold changes as the input to calculate the enrichment p-value of the GO terms. For human cell line mutation analysis, the (HMCL69_Preliminary_Mutations_Samtools.xlsx) file was utilized to
determine which cell lines were mutated for NOTCH3, which was downloaded from the Keats Lab repository (https://www.keatslab.org).

References


Figure 6A

Scr NOTCH3KD

5TGM1 cells

NICD3 (Abcam ab23426)

pCXCR4 (Abcam ab74012)

GAPDH (Cell signaling 14C10)

pERK (Cell signaling 9101)

ERK (Cell signaling 9102)

pAKT (Cell signaling 9272S)

MW (kDa)

-75 -50 -25

-150 -100 -75

-100 -75 -50 -37 -25

-50 -37 -25

-75 -50 -37 -25
**Figure 6B**

- **NICD3** (Abcam ab23426)
- **pCXCR4** (Abcam ab74012)
- **GAPDH** (Cell signaling 14C10)
- **ERK** (Cell signaling 9102)
- **AKT** (Cell signaling 9272S)
Supp. Fig 3a

Notch1
(Cell signaling D6F11)

Notch2
(Cell signaling D76A6)

Notch3
(Abcam ab23426)

Gapdh
(Cell signaling 14C10)
Supp. Fig 3b

Notch1 (Cell signaling D6F11)
Notch2 (Cell signaling D76A6)
Notch3 (Abcam ab23426)
Gapdh (Cell signaling 14C10)
Supp. Fig 3c

**Notch1**
(Cell signaling D6F11)

**Notch2**
(Cell signaling D76A6)

**Notch3**
(Abcam ab23426)

**Gapdh**
(Cell signaling 14C10)
Supp. Fig 10

- **Gapdh**
  (Cell signaling 14C10)

- **Notch1**
  (Cell signaling D6F11)

- **Notch2**
  (Cell signaling D76A6)

**5TGM1 cells**

- **Scr**
  -
  +

- **NOTCH3KD**
  -
  +

**MW (kDa)**

- 150
- 100
- 75
- 100
- 75