# Functional multi-omics reveals genetic and pharmacologic regulation of surface CD38 in multiple myeloma

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10    **Running Title:** CD38 regulation in myeloma

11    **Number of Figures:** 6

12    **Number of Tables:** 0

13    **Number of Supplementary Figures:** 6

14    **Number of Supplementary Tables/Datasets:** 5

15    **Abstract/Manuscript Main Text Word Count:** 197/4,127

1    **Abstract**

2    CD38 is a surface ectoenzyme expressed at high levels on myeloma plasma cells and is the target

3    for the monoclonal antibodies (mAbs) daratumumab and isatuximab. Pre-treatment CD38 density

4    on tumor cells is an important determinant of mAb efficacy. Several small molecules have been

5    found to increase tumor surface CD38, with the goal of boosting mAb efficacy in a co-treatment

6    strategy. Numerous other CD38-targeting therapeutics are currently in preclinical or clinical

7    development. Here we sought to extend our currently limited insight into CD38 surface expression

8    by using a multi-omics approach. Genome-wide CRISPR-interference screens integrated with

9    patient-centered epigenetic analysis confirmed known regulators of *CD38*, such as RARA, while

10   revealing XBP1 and SPI1 as other key transcription factors governing surface CD38 levels. *CD38*

11   knockdown followed by cell surface proteomics demonstrated no significant remodeling of the

12   myeloma "surfaceome" after genetically-induced loss of this antigen. Integrated transcriptome and

13   surface proteome data confirmed high specificity of all-trans retinoic acid in upregulating CD38 in

14   contrast to broader effects of azacytidine and panobinostat. Finally, unbiased phosphoproteomics

15   identified inhibition of MAP kinase pathway signaling in tumor cells after daratumumab treatment.

16   Our work provides a resource to design strategies to enhance efficacy of CD38-targeting

17   immunotherapies in myeloma.

18

19   **Keywords:** myeloma, CD38, daratumumab, CRISPR interference, proteomics

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**Introduction**

Harnessing the immune system to treat myeloma has rapidly become the most exciting therapeutic frontier in this disease. The first such immunotherapy agent to achieve United States Food and Drug Administration (FDA) approval was the monoclonal antibody (mAb) daratumumab[1]. Daratumumab targets CD38, a cell surface ectoenzyme highly expressed on myeloma plasma cells. Daratumumab is currently FDA-approved for use as either monotherapy or combination therapy in the relapsed/refractory setting, or front-line therapy in combination with other small molecule agents[1]. A second mAb targeting CD38, isatuximab, was also recently approved for relapsed/refractory myeloma; at least 15 additional CD38-targeting agents are in development[2]. Extensive and encouraging clinical data has already been obtained with daratumumab, though resistance appears to inevitably occur[3,4]. Biologically, this process appears to be quite complex, with determinants of resistance ranging from alteration of surface antigens on tumor cells[4-6] to dysfunction of the tumor immune microenvironment[7,8]. While it remains unclear whether CD38 downregulation on tumor cells after mAb treatment is a marker of resistance[5,9], or, instead, successful therapy[10], compelling preclinical and clinical data suggests that CD38 surface antigen density prior to treatment strongly correlates with mAb efficacy[5,11].

This latter observation has led to numerous efforts to identify small molecules which can increase tumor surface antigen density of CD38, representing potential co-treatments with CD38-targeting mAbs. The first such example of a CD38-boosting small molecule was all-trans retinoic acid (ATRA)[12]. Subsequent studies identified the pan-histone deacetylase (HDAC) inhibitor panobinostat[13], the thalidomide analog lenalidomide[14], the Janus kinase (JAK) inhibitor ruxolitinib[15], and the DNA methyltransferase inhibitor azacytidine[16] as agents that could lead to myeloma surface CD38 increase. A clinical trial combining ATRA with daratumumab has led to encouraging outcomes in patients previously refractory to daratumumab[17].

1       While these published strategies suggest ways to improve CD38 mAb outcomes, they also

2    leave many questions unanswered. Most notably, we do not yet have a broad global sense of the

3    transcriptional or post-transcriptional networks that most strongly impact CD38 expression. Bi- and

4    tri-specific antibodies[18] and chimeric antigen receptor (CAR) T cells[19] targeting CD38 are also in

5    clinical development. As seen for similar modalities against other targets[20], efficacy of these novel

6    agents, in addition to mAbs, is likely to also be impacted by CD38 antigen density on tumor cells.

7    Furthermore, prior studies showed that CD38 downregulation after daratumumab treatment was

8    accompanied by increases in the complement inhibitors CD55 and CD59 (ref.[5]). Are there other

9    features of myeloma surface remodeling driven by CD38 downregulation? And, for the small

10    molecules noted above, it is unknown how they more generally impact the makeup of the myeloma

11    cell surface proteome beyond CD38. The tumor cell surface not only harbors opportunities for

12    immunotherapeutic targeting but also is the interface for communication between tumor and

13    microenvironment, potentially leading to other alterations in myeloma biology after changes in

14    surface proteomic profile. To address these questions, here we have taken advantage of CRISPR

15    interference-based functional genomic screens, cell surface proteomics, epigenetic analyses, and

16    phosphoproteomics to provide a multi-omic perspective on CD38 regulation and tumor cell

17    consequences of targeting CD38 in myeloma.

18

**Methods**

*CRISPR interference screening and hit validation*

21    Genome-wide CRISPRi screening was performed as described previously[21]. Briefly, RPMI-8226

22    cells stably expressing dCas9-KRAB were transduced with a genome-wide library comprised 5

23    sgRNA/protein coding gene. After 14 days cells were stained for surface CD38 and flow-sorted to

24    enrich for populations of cells expressing low or high cell surface levels of CD38. Cell populations

1     were then processed for next-generation sequencing as previously described[22] and sequenced on a

2     HiSeq-4000 (Illumina). Reads were analyzed by using the MAGeCK pipeline as previously

3     described[23]. Further validation was performed by knockdown with individual sgRNA's extracted

4     from the genome-wide library with conformation by flow cytometry or Western blotting. Antibody-

5     dependent cytotoxicity assays were performed using NK92-CD16 cells as described previously[16].

6     Additional details in Supplementary Methods.

7

8     *Epigenetic analysis and machine learning for* CD38 *transcriptional regulation*

9     Publicly available ATAC-seq data from primary myeloma samples (ref.[24]) was analyzed with the

10     Homer tool findPeaks. Motif binding in the identified ATAC peak regions was called with PROMO

11     tool[25]. Newly-diagnosed patient tumor RNA-seq data in the Multiple Myeloma Research

12     Foundation CoMMpass trial (MMRF; research.themmrf.org) was used to correlate expression of

13     predicted transcription factors with *CD38* expression. To build a predictive model for *CD38*

14     expression as a function of transcription factor expression, we developed an XGBoost (Extreme

15     Gradient Boosting) model with randomized search with cross validation to find optimal parameters.

16     80% of CoMMpass data was used for training and the remainder for model testing. Additional

17     details in Supplementary Methods.

18

19     *Cell surface proteomics and phosphoproteomics*

20     Cell surface proteomics was performed using an adapted version of the *N*-glycoprotein Cell Surface

21     Capture[26] method, as we have described previously[27]. Unbiased phosphoproteomics was performed

22     using immobilized metal affinity (IMAC) chromatography using methods described previously[28].

23     All samples were analyzed on a Thermo Q-Exactive Plus mass spectrometer with data processing in

24     MaxQuant[29]. Additional details in Supplementary Methods.

1

**Results**

*A CRISPR interference-based screen reveals regulators of CD38 surface expression*

We first sought to use an unbiased approach to identify regulators of surface CD38 in myeloma

tumor cells. We specifically employed genome-wide screening with CRISPR interference

(CRISPRi), which leads to much higher specificity of knockdown than shRNA while avoiding

potential toxicity of double-strand breakage with CRISPR deletion[30]. We recently used this

approach to characterize regulators of surface B-cell Maturation Antigen (BCMA) in myeloma[21].

Here, we employed an RPMI-8226 cell line with the dCas9-KRAB machinery, required for

CRISPRi, as described previously[21]. We confirmed that this RPMI-8226 cell line robustly expressed

CD38 (**Supp. Fig. 1A**).

The genome-wide screen was performed as shown in **Fig. 1A**. Briefly, RPMI-8226 cells

were transduced with a pooled genome-wide sgRNA library. After 14 days the cells were then

stained with fluorescently-labeled anti-CD38 antibody and flow sorted into low- and high-CD38

populations. Frequencies of cells expressing each sgRNA was quantified using next generation

sequencing. As an important positive control, increasing confidence in the screen results, we first

noted that knockdown of *CD38* itself strongly decreased surface CD38 expression (**Fig. 1B**). On the

other hand, several dozen genes, when repressed, did indeed lead to increased surface CD38 (right

side of volcano plot in **Fig. 1B**; **Supp. Table 1**). As another positive control, one of these top hits

included *RARA*, whose degradation is catalyzed by ATRA treatment to drive CD38 increase[12].

To find pathways that may be useful for pharmacologic targeting, we first applied Gene

Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to the list of

genes that, when inhibited, significantly increased CD38 (**Fig. 1C**). We were intrigued to find that

many of the strongest effects appeared to be driven by transcriptional or other epigenetic factors.

7

1    These specifically included pathways such as "DNA replication", "mRNA processing", "DNA-

2    templated transcription", and "spliceosome".

3          We considered whether any hits associated with these pathways may be "druggable", with

4    the goal of expanding our repertoire of small molecules that enhance surface CD38 in myeloma.

5    *SS18*, a component of the BAF (BRG1/BRM associated factor) chromatin remodeling complex,

6    scored highly as a hit. However, treatment with the proposed BAF inhibitor caffeic acid phenol

7    ester (CAPE)[31] did not lead to consistent increases in surface CD38 (**Supp. Fig. 1B**). Similarly, the

8    lysine demethylase *KDM4A* was a prominent hit, but treatment with the inhibitory metabolite (*R*)-2-

9    hydroxyglutarate[32] also had no effect (**Supp. Fig. 1B**).

10         The strongest hits for genes whose knockdown increased surface CD38 were two

11   transcription factors, *HEXIM1* and *TLE3*. Validation studies using individual sgRNA knockdown

12   confirmed increased surface CD38 (**Fig. 1D**, **Fig. 2A** and **Supp. Fig. 2A**), as well as functional

13   impact in NK cell antibody-dependent cellular cytotoxicity (ADCC) assays with daratumumab (**Fig.**

14   **2B**). However, these proteins are known to be widespread negative regulators of transcription[33,34],

15   suggesting little scope for specific therapeutic targeting at the *CD38* locus.

16         We were surprised that other targets proposed to increase CD38 expression after

17   pharmacologic inhibition, such as HDACs[3], or catalyzed degradation, such as IKZF1/3 (ref.[25]), did

18   not appear as prominent hits (**Fig. 1B**). However, this result may reflect a limitation of functional

19   genomic screens. A pharmacologic agent may inhibit multiple members of a protein class to drive a

20   phenotype, whereas, with single gene knockdown, functional redundancy may prevent this

21   phenotype from appearing[35] (i.e., multiple HDACs may need to ablated at once, or both IKZF1 and

22   IKZF3 simultaneously, to drive increased CD38). We speculate this is the case with DNA

23   methyltransferases (DNMTs). We previously showed that treatment with the DNMT inhibitor

24   azacytidine, which promotes degradation of all cellular DNMTs[36], could robustly increase surface

1    CD38 (ref.[16]). Here, however, we found that knockdown of any individual DNMT only led to minor

2    CD38 increase (**Fig. 1B**).

3         Given these findings, we therefore shifted our focus to genes that, when knocked down, led

4    to CD38 decrease (left side of volcano plot in **Fig. 1B**). We reasoned this approach could still reveal

5    important biological inputs that regulate the surface expression of CD38. Examining specific genes,

6    we found that the transcription factor *SPI1* was the strongest hit besides *CD38* that, when knocked

7    down, repressed surface CD38 expression. We also noted that *NFKB1* and *NFKB2* knockdown

8    appeared to drive CD38 decrease. This finding was intriguing given the known importance of NF-

9    κB signaling in myeloma proliferation and survival[37]. KEGG and GO analysis of genes whose

10   knockdown significantly decreased CD38 showed enrichment for MAP kinase pathway and protein

11   phosphorylation more broadly (**Supp. Fig. 1C**), suggesting key roles for intracellular signaling in

12   regulating surface CD38.

13        Validation experiments with individual sgRNAs confirmed that *SPI1* knockdown strongly

14   decreased CD38 surface expression by flow cytometry, with a lesser decrease in surface CD38 with

15   *NFKB2* knockdown (**Fig. 1D, Fig. 2C, Supp. Fig. 2C-D**). These alterations also led to functional

16   impacts. RPMI-8226 cells with knockdown of these genes showed significantly decreased NK cell

17   lysis in ADCC assays (**Fig. 2D**). We further probed this dynamic *in vivo*, finding that RPMI-8226

18   cells with *SPI1* knockdown were relatively resistant to daratumumab in a murine model (**Fig. 2E**).

19   We note that we attempted to expand these results to additional cell lines. However, our four other

20   myeloma cell lines harboring the CRISPRi machinery[21] all express extremely low levels of *SPI1*

21   (**Supp. Fig. 2B**) and attempted knockdown in two of them (AMO1, KMS12PE) did not elicit any

22   phenotype (not shown). Therefore, this finding suggests that SPI1 may play an important role in

23   regulating CD38 expression in some myeloma tumors, but it is less likely to be a universal

24   regulator.

9

1

2      *Epigenetic analysis suggests XBP1 as a key determinant of CD38 in primary myeloma tumors*

3      Our CRISPRi results suggest that epigenetic and/or transcriptional regulation is a critical

4      driver of surface CD38 levels. However, we do not know whether these specific hits in a myeloma

5      cell line will extend to primary myeloma tumors. We therefore took a complementary approach to

6      find potential transcriptional regulators of *CD38*. Using ATAC-seq data from 24 primary myeloma

7      tumor samples[24], we extracted open chromatin motifs near the *CD38* promoter (**Supp. Fig. 3A**) to

8      identify a list of 46 transcription factors with potential binding sites at this locus (**Supp. Table 2**).

9      We then correlated expression (via Pearson *R*) of these transcription factors with *CD38* expression

10     across 664 primary patient tumors at diagnosis in the Multiple Myeloma Research Foundation

11     CoMMpass database (research.themmrf.org; release IA13). In this analysis, we found the

12     transcription factor most negatively correlated with *CD38* expression was *RARA* (**Fig. 3A**),

13     consistent with our CRISPRi screen data, and underscoring the promise of ATRA as a co-treatment

14     to increase *CD38*. Intriguingly, the transcription factor with strongest positive correlation was *XBP1*

15     (**Fig. 3A-B**), a central driver of plasma cell identity[38]. *XBP1* also showed strong positive

16     correlations with *CD38* in two other patient tumor gene expression datasets[39,40] (**Supp. Table 2**).

17     To further extend this analysis, we sought to build a predictive model which could estimate

18     *CD38* transcript level as a function of transcription factor expression. We used an XGBoost method

19     applied to CoMMpass mRNA-seq data to find weights of transcription factor expression that most-

20     influence *CD38* levels in patient tumors. We first tested this analysis on 80% of patient data as a

21     training set with 20% left out as test set. We found this model, solely based on transcription factor

22     expression, could predict about half of the variance (coefficient of variation $R^2 = 0.49$ using 5-fold

23     cross validation) in test set *CD38* levels (**Fig. 3C**). Using model weights and Shapley Additive

24     Explanations (SHAP) analysis (see Supplementary Methods) to determine transcription factors that

1    have the greatest impact, either positive or negative, on CD38 expression, we found that *XBP1*

2    played the strongest role overall. Other strong hits from both of our analyses included *IRF2*, *ATF1*,

3    and *STAT1*. *SPI1* also appeared in the top 10 most relevant transcriptional regulators (**Fig. 3D;**

4    **Supp. Fig. 3B**), consistent with our CRISPRi results, suggesting that SPI1 may play a key role in

5    regulating *CD38* in a subset of tumors.

6         We further evaluated XBP1 given its prominent role in these two complementary

7    bioinformatic analyses. In a prior dataset of shRNA knockdown of *XBP1* in myeloma plasma

8    cells[41], *CD38* mRNA was decreased ~3-fold after *XBP1* silencing (**Supp. Fig. 3C**). This finding

9    was consistent with results in our CRISPRi screen, where *XBP1* knockdown led to significant

10   surface CD38 decrease (**Fig. 1B**). We further validated this relationship by using a doxycycline-

11   inducible sgRNA construct for CRISPRi vs. *XBP1*, finding a clear dose-response between degree of

12   XBP1 knockdown and loss of surface CD38 by flow cytometry (**Supp. Fig. 3D-G**). Supporting

13   relevance of this link, a recent myeloma tumor whole genome sequencing study found that deletion

14   of *XBP1* was one of the strongest determinants of clinical response to daratumumab[42]. We

15   attempted to perform promoter activation assays to directly link XBP1 binding to *CD38* expression,

16   but were unable to successfully generate a reporter construct that reflected all 8 loci where XBP1

17   may bind at *CD38* regulatory elements (**Supp. Fig. 3H**). Taken together, these results nominate

18   XBP1 as a particularly strong determinant of surface CD38 in myeloma plasma cells, though future

19   investigation will be required to validate a direct or indirect relationship to *CD38* transcription.

20

21   *No consistent large-scale remodeling of the myeloma surface proteome after CD38 downregulation*

22         We next evaluated CD38 surface regulation from the perspective of monoclonal antibody

23   therapy. In clinical samples, CD38 loss after daratumumab was accompanied by increases in CD55

24   and CD59, which may inhibit complement-dependent cytotoxicity and contribute to daratumumab

1    resistance[5]. In preclinical studies, macrophage trogocytosis has been proposed as a mechanism

2    contributing to CD38 loss after mAb treatment, that also leads to alterations in other surface

3    antigens including CD138/SDC1 (ref.[7]). However, we hypothesized that given its enzymatic

4    activity and role as a cellular differentiation marker[43], loss of CD38 on its own may influence

5    surface expression of other myeloma antigens. Such alterations may reveal new biology or

6    (immuno)therapeutic vulnerabilities of CD38 mAb-treated disease.

7        To test this hypothesis, we employed a method we recently developed termed "antigen

8    escape profiling"[27]. We used CRISPRi to transcriptionally repress *CD38* in RPMI-8226, AMO-1,

9    and KMS12-PE myeloma cell lines, using this genetic approach to partially mimic loss of surface

10   antigen seen after mAb therapy (**Fig. 4A**). We then performed Cell Surface Capture proteomics[26,27]

11   to uncover surface proteome alterations in a relatively unbiased fashion. Across cell lines, analyzed

12   in biological triplicate with *CD38* knockdown vs. non-targeting sgRNA, we quantified 897 proteins

13   annotated as membrane-spanning in Uniprot (minimum of two peptides per protein) (**Supp. Table

14   3**). As a positive control, in all lines we found that the strongest signature was decrease of CD38

15   itself (**Supp. Fig. 4A-C**). However, when aggregating proteomic data, we found no significant

16   alterations in any surface antigens beyond CD38 itself (**Fig. 4B**). Integration with RNA-seq data

17   revealed only THY-1/CD90 as upregulated >3-fold at both the mRNA and surface proteomic level

18   after *CD38* knockdown (**Fig. 4C**). Intriguingly, CD90 is known as a marker of "stemness" in early

19   hematopoietic lineage cells that is lost when CD38 expression is increased[44]. CoMMpass analysis

20   also confirmed increased *THY1* expression in tumors with lower *CD38* (**Supp. Fig. 4D**). However,

21   further validation as to whether CD90 is truly altered after CD38 mAb will require pre- and post-

22   treatment clinical specimens, beyond the scope of our work here. Overall, we conclude that loss of

23   CD38 in isolation leads to limited remodeling of the myeloma surface proteome.

24

1    *Integrated surface proteomic and transcriptional analysis suggests ATRA is highly specific in CD38*

2    *upregulation*

3    Data from our group[16] and others[12-15] have suggested that several small molecules can

4    increase myeloma surface CD38. However, the broader impacts of these agents on membrane

5    antigens beyond CD38 have not been directly compared. We performed integrated cell surface

6    proteomics and transcriptional analysis of RPMI-8226 cells treated with 10 nM all-trans retinoic

7    acid (ATRA), 2 μM azaciditine (Aza), and 10 nM Panobinostat (Pano), all treated for 72 hr, in

8    comparison to DMSO (**Supp. Table 4**). These doses are chosen as they have been previously

9    published to significantly increase myeloma surface CD38 by flow cytometry[12,13,16]. In this

10   integrated analysis we found much broader impacts of azacytidine and panobinostat than ATRA on

11   the "surfaceome" of plasma cells, beyond increasing CD38 (**Fig. 5A**). These results suggest that, at

12   doses driving CD38 upregulation, for Aza or pano altering CD38 is just a small component of their

13   impact on myeloma tumor cells, whereas ATRA is much more specific in driving CD38

14   upregulation.

15   Toward understanding how CD38 is modulated after drug treatment, in our previous work[16]

16   we noted that the mechanism of *CD38* increase after Aza treatment was unclear. We thus further

17   investigated the global transcriptional response (i.e. not limited to membrane) after Aza (**Supp.**

18   **Table 4**). Prior studies have suggested that Aza anti-tumor effect is largely mediated by reactivation

19   of endogenous retroviruses stimulating a tumor-autonomous interferon response[45,46]. Consistent

20   with this work, we found a pronounced increase in interferon-responsive genes after Aza, but not

21   ATRA, including *IRF1*, *IFITM1*, *IFITM2*, and *IFITM3* (**Fig. 5B**). KEGG analysis also confirmed

22   this effect (**Fig. 5C**). Given evidence across multiple systems that interferon upregulates *CD38*

23   expression[47,48], our transcriptional profiling thus also supports an interferon-based mechanism

24   driving surface CD38 increase in plasma cells after Aza treatment.

1

2 *Plasma cell proliferative signaling pathways are inhibited by mAb binding to CD38*

3     In our final set of experiments related to targeting surface CD38, we were intrigued as to

4 whether binding of a therapeutic mAb leads to specific cellular phenotypes within myeloma plasma

5 cells. For example, isatuximab is known to directly lead to apoptosis of plasma cells[49], and

6 daratumumab can do so after crosslinking[50]. However, the mechanism underlying this transduction

7 of extracellular mAb binding to intracellular phenotype remains unclear. In addition, our CRISPRi

8 screen data (**Fig. 1B and Supp. Fig. 1C**) suggests that surface CD38 expression may be strongly

9 impacted by intracellular phospho-signaling pathways.

10     Therefore, we used unbiased phosphoproteomics by mass spectrometry to probe intracellular

11 signaling effects driven by CD38 mAb binding. In RPMI-8226 cells we compared 20 µM

12 daratumumab treatment vs. IgG1 isotype control. We chose a time point of 20 minutes of treatment

13 given known rapid alterations in signaling pathways in similar phosphoproteomic experiments[51]. In

14 total, across triplicate samples we quantified 5430 phosphopeptides (**Supp. Table 5; Supp. Fig.**

15 **5A**). Analyzing phosphopeptide changes by Kinase Substrate Enrichment Analysis (KSEA)[52], we

16 were intrigued to find downregulation of phosphorylation motifs consistent with both cyclin-

17 dependent kinases as well as several kinases of the MAP kinase pathway (**Fig. 6A**). Downregulation

18 of phosphorylation on several central nodes in the MAP kinase as well as AKT pathway was also

19 apparent via KEGG analysis (**Supp. Fig. 5B**). Across a time course we further confirmed effects on

20 MAP kinase pathway (reported by phosphorylation of MAPK (ERK1/2), a key node in this

21 response) and AKT signaling after daratumumab treatment via Western blotting in RPMI-8226 and

22 MM.1S cell lines, respectively (**Fig. 6B**). While the absolute value of changes in MAPK signaling

23 are modest, both by phosphoproteomics and Western blot, these results indicate that daratumumab

1    binding to CD38 can at least partially inhibit this central proliferative pathway within myeloma

2    tumor cells, and thus may form a component of daratumumab's anti-tumor effect.

3

4    **Discussion**

5    Our studies here present a "multi-omics" view of therapeutically targeting CD38 in multiple

6    myeloma. Our integrated functional genomics and epigenetic analysis point to the central role of

7    transcriptional regulators in governing CD38 surface expression. Using surface proteomics, we

8    further identify that loss of CD38 in isolation is unlikely to drive large changes in the "surfaceome",

9    while known pharmacologic strategies to increase CD38 have largely divergent impacts on other

10    surface antigens. Finally, unbiased phosphoproteomics reveals that binding of anti-CD38 mAb can

11    impair intracellular proliferative signaling within plasma cells.

12    Our CRISPRi screen illustrated the central role of numerous transcription factors, such as

13    SPI1, HEXIM1, and TLE3, in regulating surface CD38. This functional genomic study suggests that

14    regulation of surface CD38 largely occurs at the transcriptional, as opposed to protein trafficking,

15    level. This finding was in sharp contrast to our prior CRISPRi results with BCMA, where we found

16    that post-transcriptional mechanisms, such as proteolytic cleavage by γ-secretase and protein

17    trafficking via the SEC61 translocon, played the strongest roles in determining surface BCMA

18    levels[21].

19    Another recent study used genome-wide CRISPR deletion screening to find genes that,

20    when knocked out, could abrogate IL-6-mediated downregulation of surface CD38 (ref.[15]). The

21    strongest hits in this prior study included the transcription factors *STAT1* and *STAT3*, demonstrating

22    a role for JAK-STAT signaling in regulating tumor CD38 expression within the bone marrow

23    microenvironment[15]. In support of this notion, our integrated epigenetic and machine learning

24    analyses, extracted from bone marrow-derived patient tumor samples, also support a critical role for

1    STAT1 in governing surface CD38. However, in our CRISPRi screen in an *in vitro* monoculture

2    system, neither *STAT1* or *STAT3* affected CD38 surface expression (**Fig. 1B**). This result suggests

3    that JAK-STAT signaling may not play a major role in CD38 regulation in the absence of

4    exogenous tumor stimulation. This finding illustrates the complementary nature of our genome-

5    wide screen to that previously published under the context of IL-6 stimulation[15].

6         Toward the goal of finding key regulators of CD38 that were not previously known, our

7    epigenetic and machine learning approaches suggest that *XBP1* is a critical regulator of plasma cell

8    *CD38*. To our knowledge, there are not currently any known pharmacologic mechanisms to

9    potentiate XBP1 activity. Given the important role of *XBP1* splicing in myeloma plasma cells[53],

10   future work will investigate the role spliced vs. unspliced XBP1 in specifically regulating CD38, as

11   this strategy may provide new avenues for CD38 manipulation. Future work will also investigate

12   the role of *XBP1* deletion in determining clinical response to daratumumab[42].

13        Given that plasma cells demonstrate frequent loss of CD38 after daratumumab treatment[5], a

14   pressing question is whether CD38-low, daratumumab-resistant cells have novel

15   immunotherapeutic vulnerabilities. However, our recently-described strategy of "antigen escape

16   profiling"[27] – CRISPRi knockdown followed by unbiased cell surface proteomics (**Fig. 4**) –

17   suggests that other surface antigens on plasma cells do not exhibit consistent changes due to CD38

18   downregulation alone. This finding supports the notion that alterations in surface proteins found

19   after mAb treatment on patent tumors, such as increases in CD55 and CD59 (ref.[5]), are caused by

20   other therapy-induced selective pressure within the tumor microenvironment, not CD38 loss.

21        While *in vitro* assays have suggested a strong relationship between CD38 antigen density

22   and either NK-cell[54] or macrophage-mediated antibody-dependent cell killing[6], these experiments

23   cannot readily take into account the critical role of the immune microenvironment in determining

24   daratumamab response or resistance[8]. Furthermore, even with the higher specificity of ATRA, there

16

1    is the potential to alter CD38 expression on other hematopoietic cells, which may impact clinical

2    responses to daratumumab[17]. Notably, current clinical data is most consistent with pre-treatment

3    tumor CD38 antigen density positively correlating with daratumumab depth of response[5,11].

4    Analysis of transcriptional data in CoMMpass demonstrates that while increased *CD38* expression

5    was associated with improved outcomes in all patients, only those treated with daratumumab

6    showed a divergence in survival curves coinciding with the median time of initial daratumumab

7    treatment (**Supp. Fig. 6**). Pharmacologic manipulation of CD38 density on tumor cells may

8    ultimately be most fruitful pre-treatment rather than in the context of daratumumab resistance.

9    Similar strategies may also be most beneficial for other CD38-targeting immunotherapeutics[2].

10        Also directly related to mAb therapeutic effects, our unbiased phosphoproteomic results

11    suggest that daratumumab binding to CD38 can directly decrease signaling along the MAP kinase

12    and PI3K-AKT pathways. It remains to be investigated whether this inhibition of central

13    proliferative signaling pathways plays a role in the anti-tumor effect of daratumumab in patients.

14        In terms of limitations of our work, the most prominent is that the many of our studies are

15    derived from large-scale "omics" experiments in myeloma cell lines. There may be biological

16    differences between our findings *in vitro* and primary tumors growing within the bone marrow

17    microenvironment.

18        Taken together, our multi-omic studies comprise a resource that reveals new insight into the

19    genetic, epigenetic, and pharmacologic regulation of surface CD38 in myeloma plasma cells. We

20    anticipate these findings will have utility in deriving new strategies to enhance CD38-targeting

21    therapies in myeloma, including mAbs in current clinical practice as well as emerging antibody and

22    cellular therapies[2]. The technologies described here also comprise a blueprint to comprehensively

23    assess determinants of surface antigen regulation, and impacts of associated therapeutic

24    manipulation, that could be applied across targets in hematologic malignancies.

1

7

8    **Author Contributions:** P.C., P.R., M.K., and A.P.W. conceived and designed the study. P.C.,

9    C.K., B.P.E., A. Kang, A. Kishishita, S.R., J.C.P., O.G., L.S., Y-H.T.L., P.R., N.P., and M.M.

10    performed experiments. P.C., B.P.E., N.P., Y-H.T.L., B.J.B., and P.R. analyzed data. D.W., P.P., V.

11    Steri, and B.H. performed murine studies. H.G. analyzed patient epigenetic data. V. Sarin

12    performed machine learning analysis. P.C. and A.P.W. wrote the manuscript with input from all

13    authors.

14

15    **Disclosures:** P.C. is currently an employee and shareholder of Genentech/Roche, though during the

16    time of completing this project she was fully employed by the University of California, San

17    Francisco. P.R. is currently an employee and shareholder of Senti Biosciences, though during the

18    time of completing this project she was fully employed by the University of California, San

19    Francisco. A.P.W. is an equity holder and scientific advisory board member of Indapta

20    Therapeutics, LLC and Protocol Intelligence, LLC. M.K. has filed a patent application related to

21    CRISPRi screening (PCT/US15/40449); and serves on the Scientific Advisory Boards of Engine

22    Biosciences, Cajal Neuroscience and Casma Therapeutics. The other authors declare no relevant

23    conflicts of interest.

24

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1    **FIGURE LEGENDS**

2

3    **Figure 1. CRISPR interference (CRISPRi) screening reveals genetic determinants of surface**

4    **CD38 regulation. A.** Schematic of CRISPRi screen design. **B.** Results of CRISPRi screen

5    demonstrating genes that, when knocked down, regulate surface CD38 in RPMI-8226 cells. X-axis

6    indicates phenotype (epsilon) from MAGeCK[55] statistical analysis. Dashed line indicates cutoff for

7    significant change at False Discovery Rate (FDR) < 0.05. Genes of interest are specifically labeled.

8    4,000 negative control non-targeting sgRNAs are in grey. **C.** Gene Ontology (GO) Biological

9    Process and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of all genes that when

10   knocked down lead to significant CD38 upregulation. **D.** Follow-up flow cytometry validation of

11   CRISPRi screen hits using two individual sgRNAs per gene demonstrates *TLE3* knockdown drives

12   increased CD38, while *SPI1* knockdown leads to CD38 decrease.

13

14   **Figure 2. Validation of CRISPRi screen hits as functionally impacting daratumumab efficacy.**

15   **A.** Knockdown of HEXIM1 and TLE3 with two independent sgRNA's/gene (AMO1 myeloma

16   cells, *n* = 3) followed by flow cytometry shows significant surface CD38 increase with TLE3_i2

17   sgRNA and trend toward increased CD38 with HEXIM1_i1 sgRNA. Scri = non-targeting control

18   sgRNA. **B.** Results from ADCC assays with AMO1 cells stably expressing the noted sgRNA's and

19   incubated with the indicated concentration of daratumumab or isotype control antibody (1:20

20   myeloma:NK ratio, 20 hours, *n* = 2). The percent lysis by ADCC was calculated using the following

21   formula : % Lysis = (signal in presence of daratumumab – signal in presence of IgG1 control

22   antibody) x100 / signal in presence of IgG1 control antibody. At 10 µM daratumumab, both

23   HEXIM1 and TLE3 knockdown led to significant increase in ADCC. **C.** Similar to A., sgRNA

24   knockdown of *NFKB1*, *NFKB2*, and *SPI1* with fold-change in CD38 by flow cytometry (RPMI-

1    8226 cells, $n = 3$). **D.** Similar to B., knockdown with the most effective sgRNA for each gene show

2    significant decreases in NK-cell ADCC at 10 μM daratumumab in the RMPI-8266 cells ($n = 3$). **E.**

3    *In vivo* validation of *SPI1* knockdown driving daratumumab resistance. NOD *scid* gamma mice

4    were I.V. implanted with CRISPRi RPMI-8226 cells stably expressing both luciferase and noted

5    sgRNA, then treated with 200 μg daratumumab on the noted schedule. Bioluminescence imaging

6    measurement of tumor burden demonstrates significantly increased fold-change in tumor burden

7    (normalized to pre-daratumumab intensity) with either CD38 or SPI1 knockdown compared to

8    scramble sgRNA. For **A-E**, *$p < 0.05$, **$p < 0.01$ by two-tailed *t*-test.

9

10    **Figure 3. Patient-centered epigenetic analysis and machine learning predicts most potent**

11    **transcriptional regulators of *CD38*. A.** 46 transcription factors predicted to bind to the CD38

12    locus were derived from motif analysis of published ATAC-seq data (see Supplementary Figure 3).

13    Gene expression of each transcription factor (TF) was correlated with *CD38* expression in the

14    Multiple Myeloma Research Foundation (MMRF) CoMMpass database (release IA13), with RNA-

15    seq data from CD138+ enriched tumor cells at diagnosis ($n = 664$ patients). Top predicted positive

16    and negative regulators are shown based on Pearson correlation ($R$). **B.** CoMMpass RNA-seq data

17    illustrates strong positive correlation between *XBP1* and *CD38* expression. **C.** XGBoost machine

18    learning model was used to extract features of transcription factor gene expression that best-model

19    CD38 expression in CoMMpass tumors (shown in log2 TPM (Transcripts per Million)). 80% of

20    data was used as a test set with 20% left out as a training set. Coefficient of variation ($R^2$) for

21    predictive model $= 0.49$ after five-fold cross validation. **D.** Shapley Additive Explanations (SHAP)

22    analysis indicates transcription factors whose expression most strongly impacts CD38 expression

23    levels in CoMMpass tumors.

24

1 **Figure 4. Minimal alterations of the myeloma cell surface proteome after CD38 loss. A.**

2 Schematic of "antigen escape profiling" approach to reveal new cell-surface therapeutic

3 vulnerabilities in the context of CD38 downregulation. **B.** Cell surface capture proteomics

4 comparing *CD38* knockdown vs. non-targeting sgRNA control, with aggregated data across three

5 cell lines (CRISPRi-expressing RPMI-8226, AMO1, and KMS12-PE; $n = 3$ replicates per cell line

6 per sgRNA) reveals minimal changes in the cell surface proteome beyond CD38 knockdown at

7 significance cutoff of $p < 0.05$ and $\log_2$ fold-change $>|1.5|$. **C.** Integrated analysis of cell surface

8 proteomics and mRNA-seq ($n = 2$ per cell line per guide) across three cell lines reveals the only

9 consistent change at both protein and transcript level after *CD38* knockdown is *THY1*/CD90

10 upregulation. $\log_2$ fold-change cutoff = $|1.5|$.

11

12 **Figure 5. ATRA drives CD38 upregulation with limited additional cellular impact while Aza**

13 **leads to a broad interferon-mediated response. A.** Integrated mRNA-seq ($n = 2$ per drug

14 treatment) and cell surface proteomics ($n = 2$ per drug treatment) across RPMI-8226 treatment with

15 10 nM all-trans retinoic acid (ATRA), 2 μM azacytidine (Aza), and 10 nM panobinostat (Pano).

16 All plots are in comparison to control replicates treated with 0.1% DMSO. Doses chosen are based

17 on those previously published to lead to CD38 upregulation for each agent. Data points shown are

18 for proteins and genes corresponding to Uniprot-annotated membrane-spanning proteins. $\log_2$-fold

19 change cutoffs shown at $|0.5|$ for ATRA and $|2.0|$ for Aza and Pano to increase clarity of plots given

20 many fewer changed genes with ATRA treatment. **B.** RNA-seq for same samples with ATRA or

21 Aza treatment vs. DMSO but here showing all mapped genes, not just those annotated as

22 membrane-spanning. Significance cutoff at $p < 0.05$ with $\log_2$ fold-change cutoff set at $|0.8|$ to

23 illustrate prominent differences above this level in transcriptome alteration after either ATRA or

1    Aza treatment. **C.** KEGG analysis of genes from RNA-seq dataset meeting cutoff criteria of $p <$

2    0.05 and $\log_2$ fold-change $>0.8$ after Aza treatment.

3

4    **Figure 6. Unbiased phosphoproteomics reveals downregulation of proliferative signaling after**

5    **daratumumab treatment. A.** RPMI-8226 cells were treated with 20 μM daratumumab or IgG1

6    isotype control for 20 minutes ($n = 3$ each) and then harvested for unbiased phosphoproteomics with

7    immobilized metal affinity chromatography enrichment for phosphopeptide enrichment. Plot

8    displays results of Kinase Substrate Enrichment Analysis, indicating modest decrease in

9    phosphorylation of numerous predicted substrates of MAPK pathway kinases as well as cyclin-

10   dependent kinases (cutoffs of $p < 0.05$, $\log_2$ fold-change $>|0.5|$). **B.** Western blot in RPMI-8226 of

11   MAPK (ERK1/2) (Thr202/Tyr204) relative to total MAPK demonstrates modest decrease in MAPK

12   phosphorylation after 5, 10, or 15 min daratumumab (Dara) treatment; magnitude of change

13   normalized to IgG1 control at each time point (red) appears consistent with phosphoproteomic data.

14   **C.** Western blot of MM.1S cells treated with daratumumab and blotted for p-AKT (Ser473) and

15   total AKT, with quantification of p-AKT relative to total AKT and normalized to IgG1 at each time

16   point. All images representative of two independent Western blots.

# FIGURE 1

# FIGURE 2

# FIGURE 3



**A** TF Expression Correlation with *CD38* (CoMMpass IA13 mRNA-seq FPKM)

**B** CoMMpass data $R = 0.38$ $p < 2.2e^{-16}$

**C** Training Data (CoMMpass) [80%] / Testing Data (CoMMpass) [20%]

**D** TF Expression Impact on Model Prediction
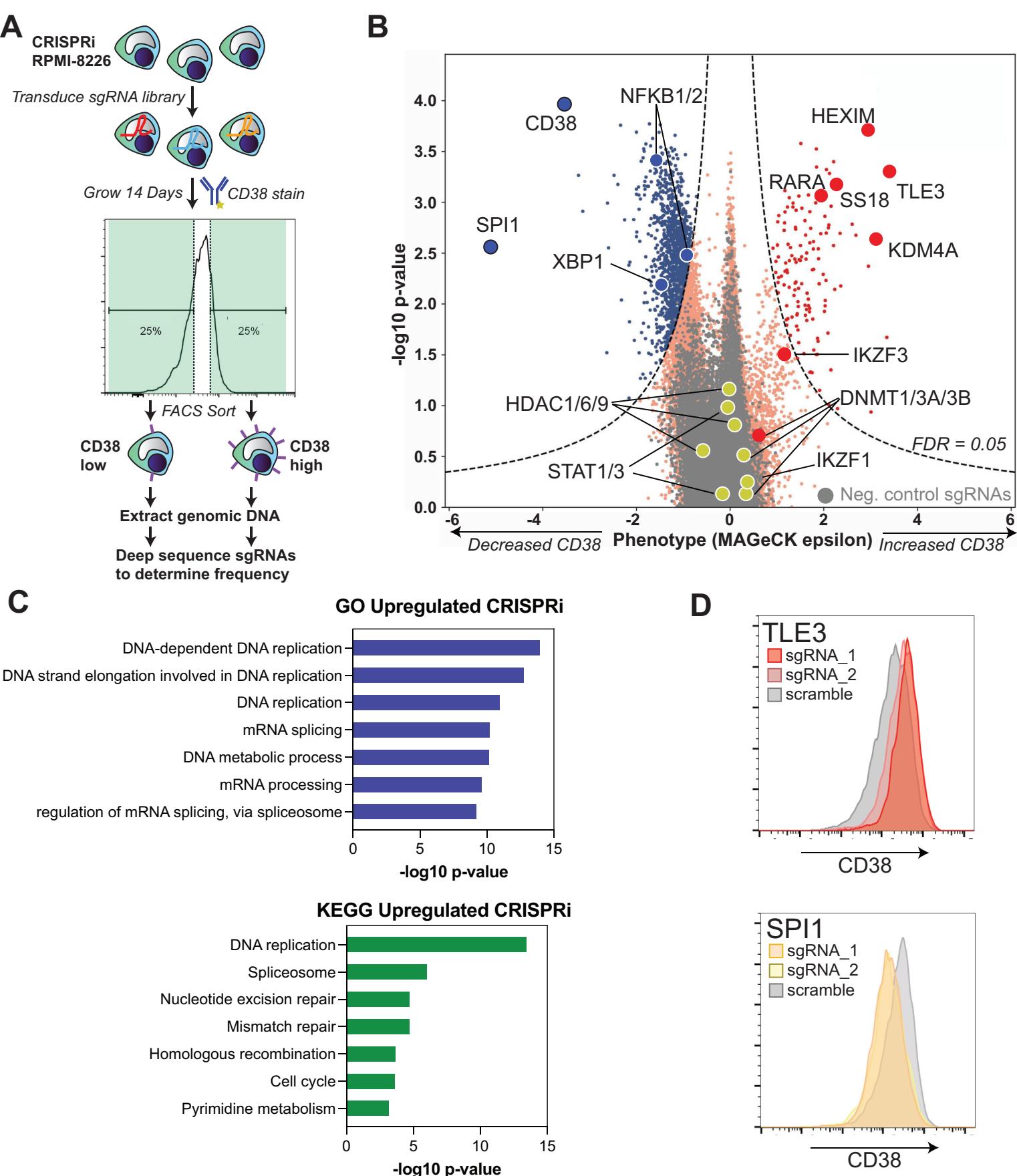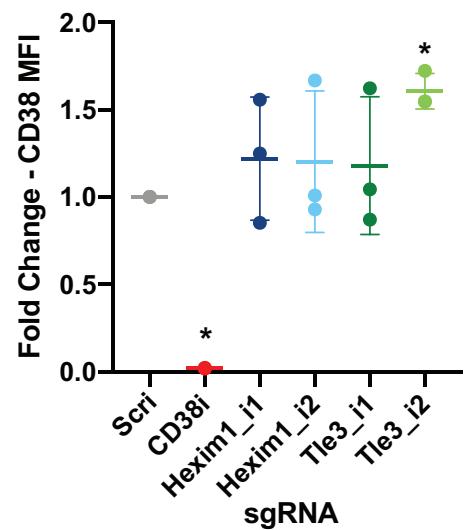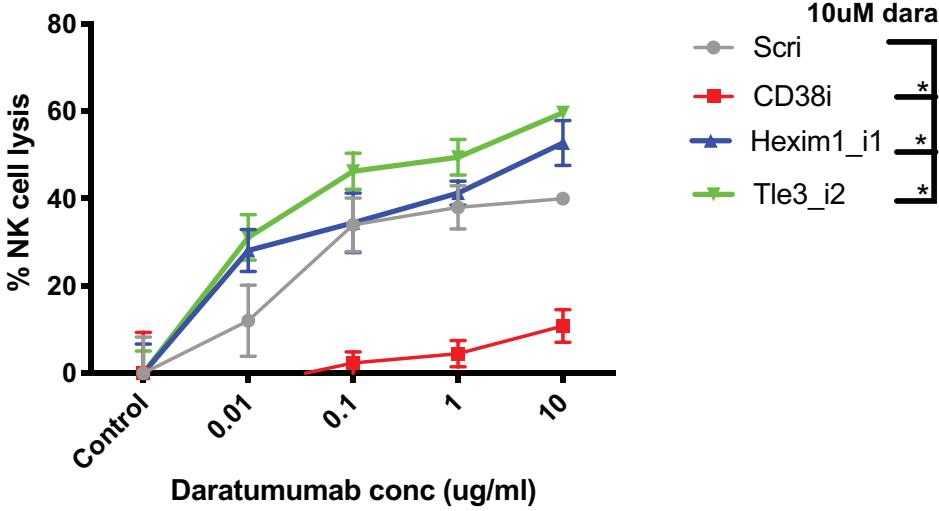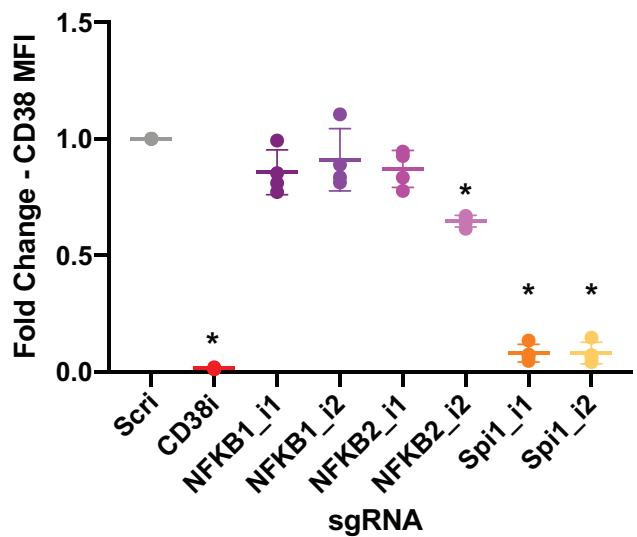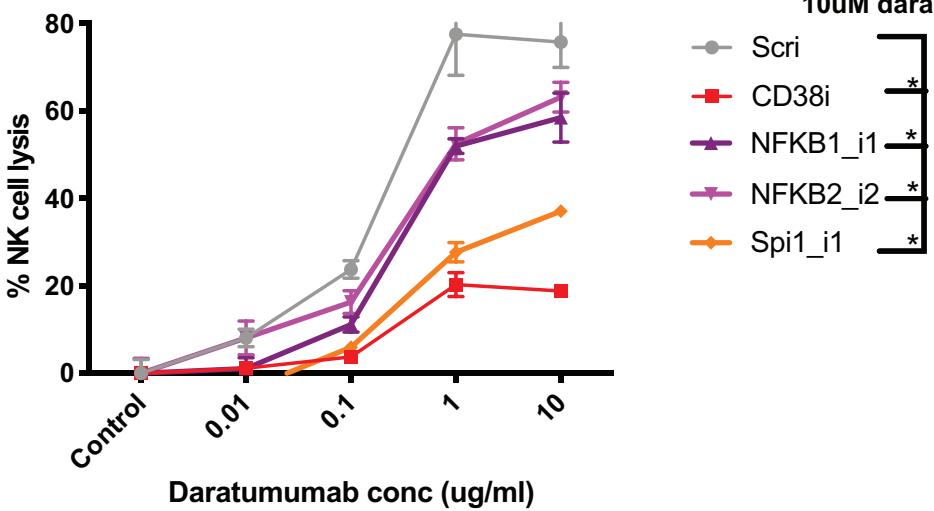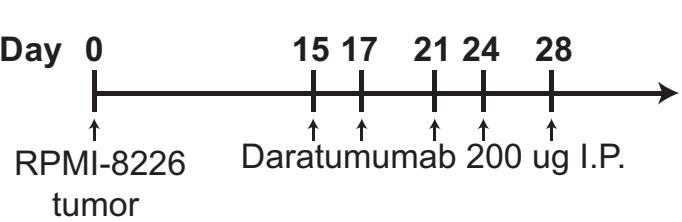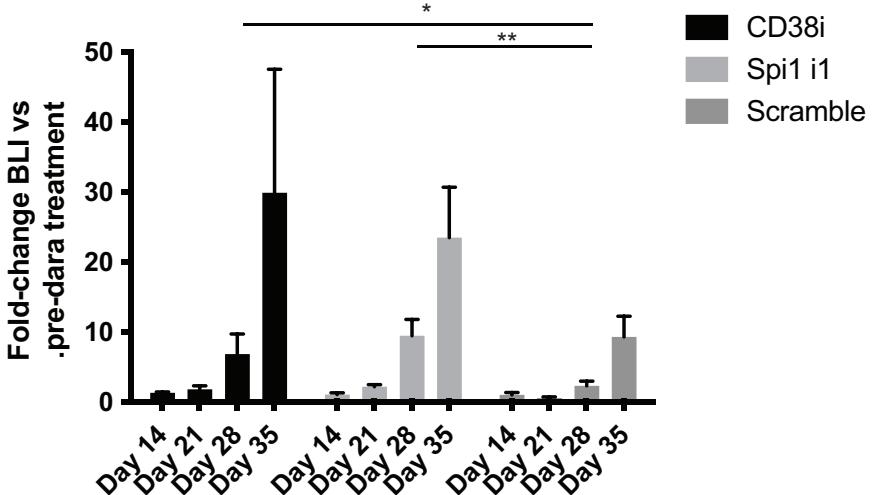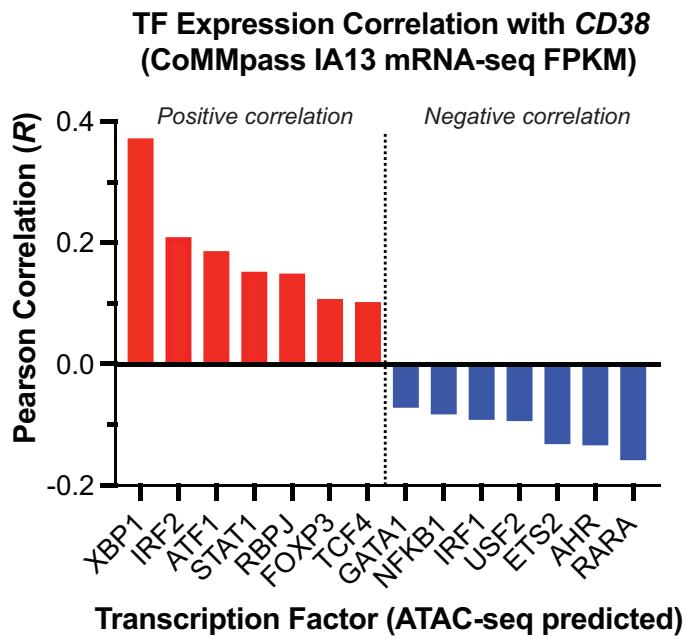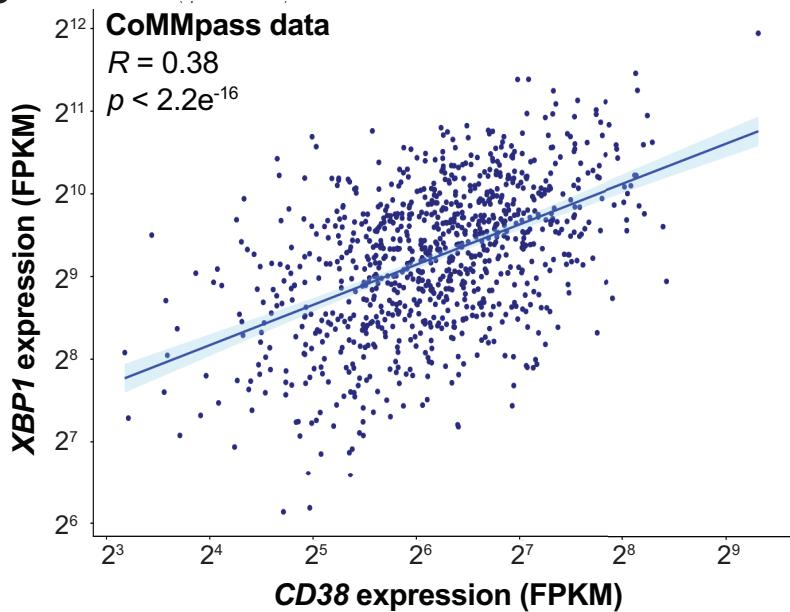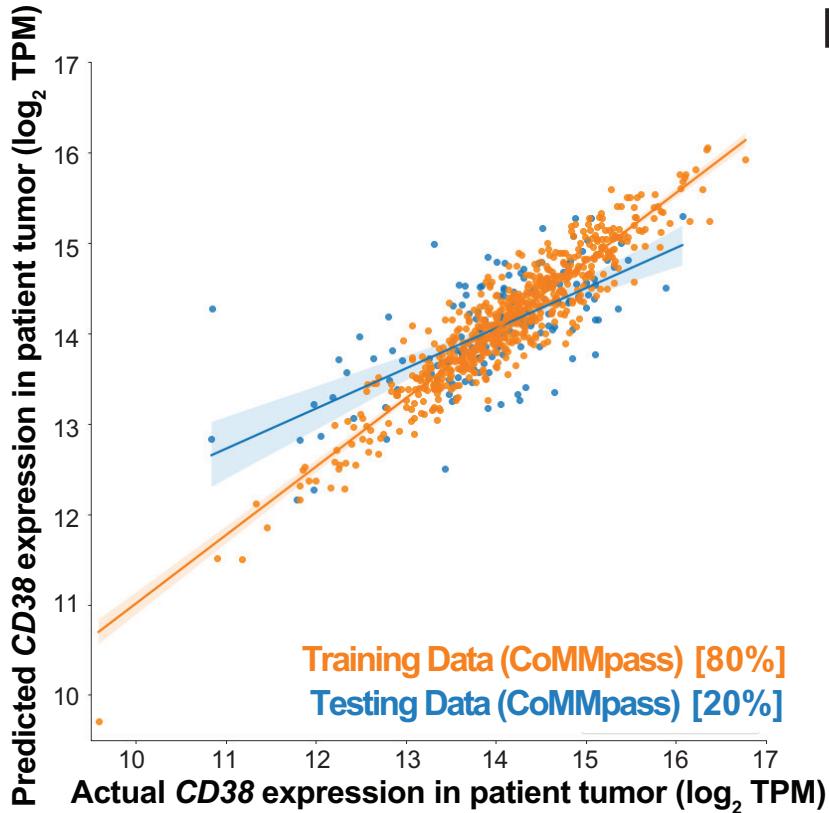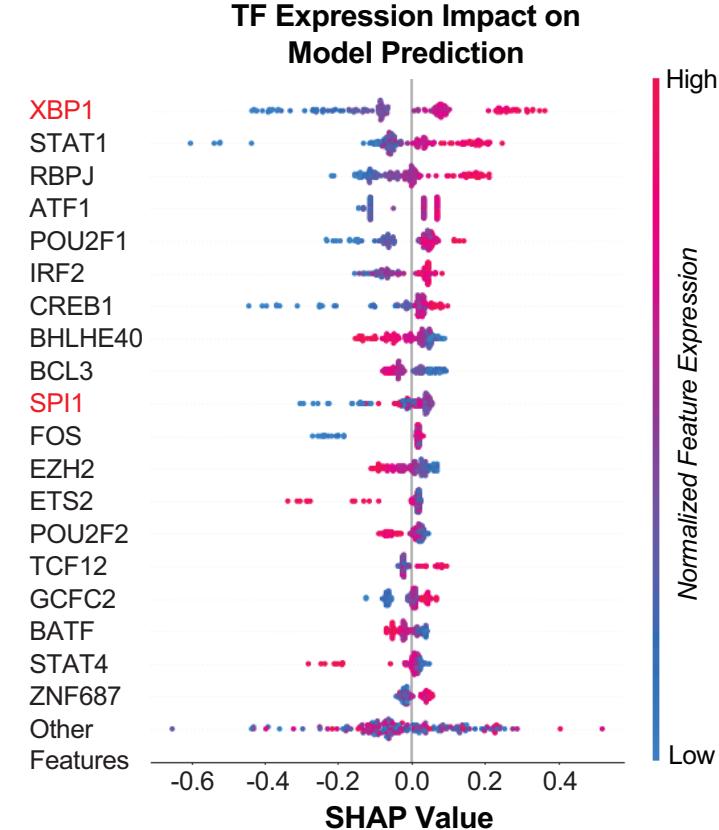
# FIGURE 4



**A** CRISPRi: RPMI-8226, AMO1, KMS12-PE — CD38 sgRNA / scramble sgRNA → surface proteomics + mRNA-seq → new (immuno) therapeutic vulnerabilities after CD38 loss?

**B** Volcano plot: Log2FC surface proteins (CD38 sgRNA/scramble) vs -log10 p-val; CD38 and THY1 labeled; p = 0.05

**C** Scatter plot: Log2FC mRNA vs Log2FC surface proteins (CD38 sgRNA/scramble); THY1 and CD38 labeled

**FIGURE 5**

# FIGURE 6

## A



## B