
Multimodal antigenic escape to GPRC5D-targeted T cell engagers in multiple myeloma

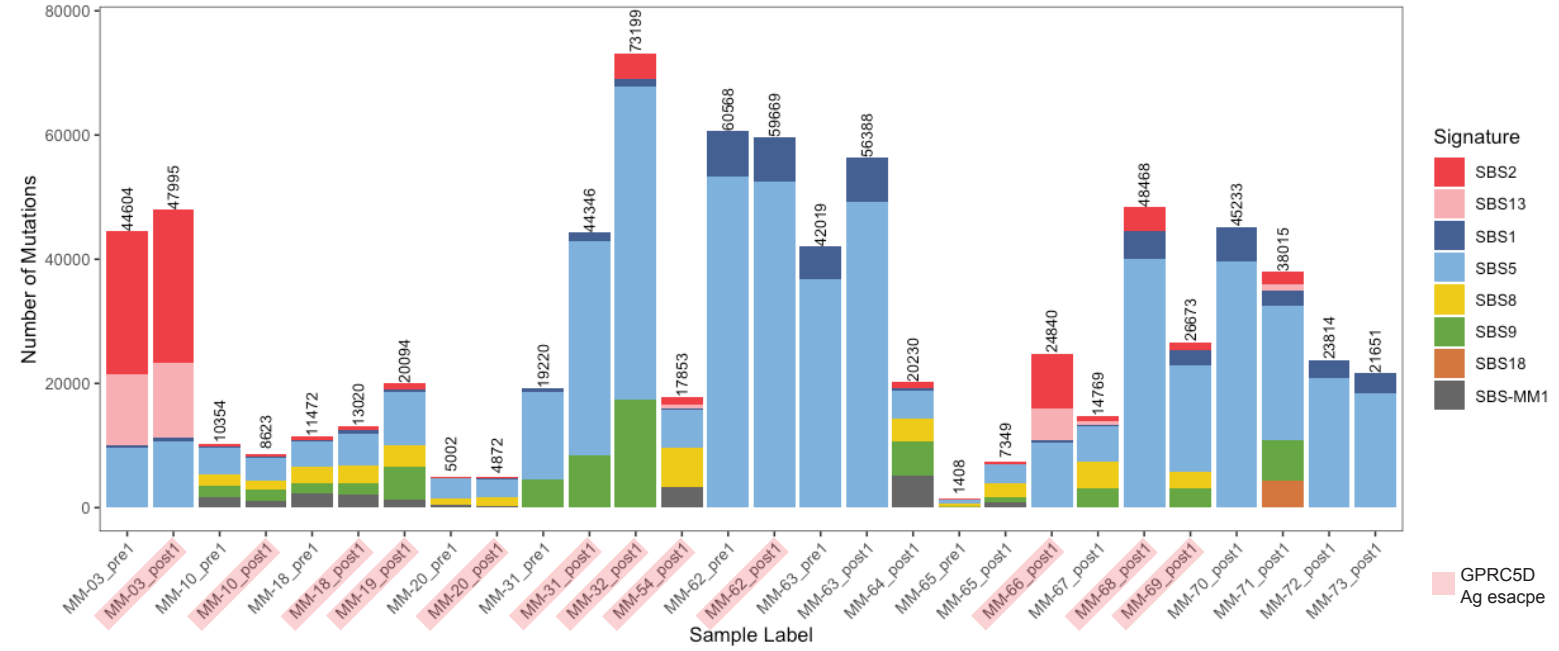
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Supplementary Figure Legend

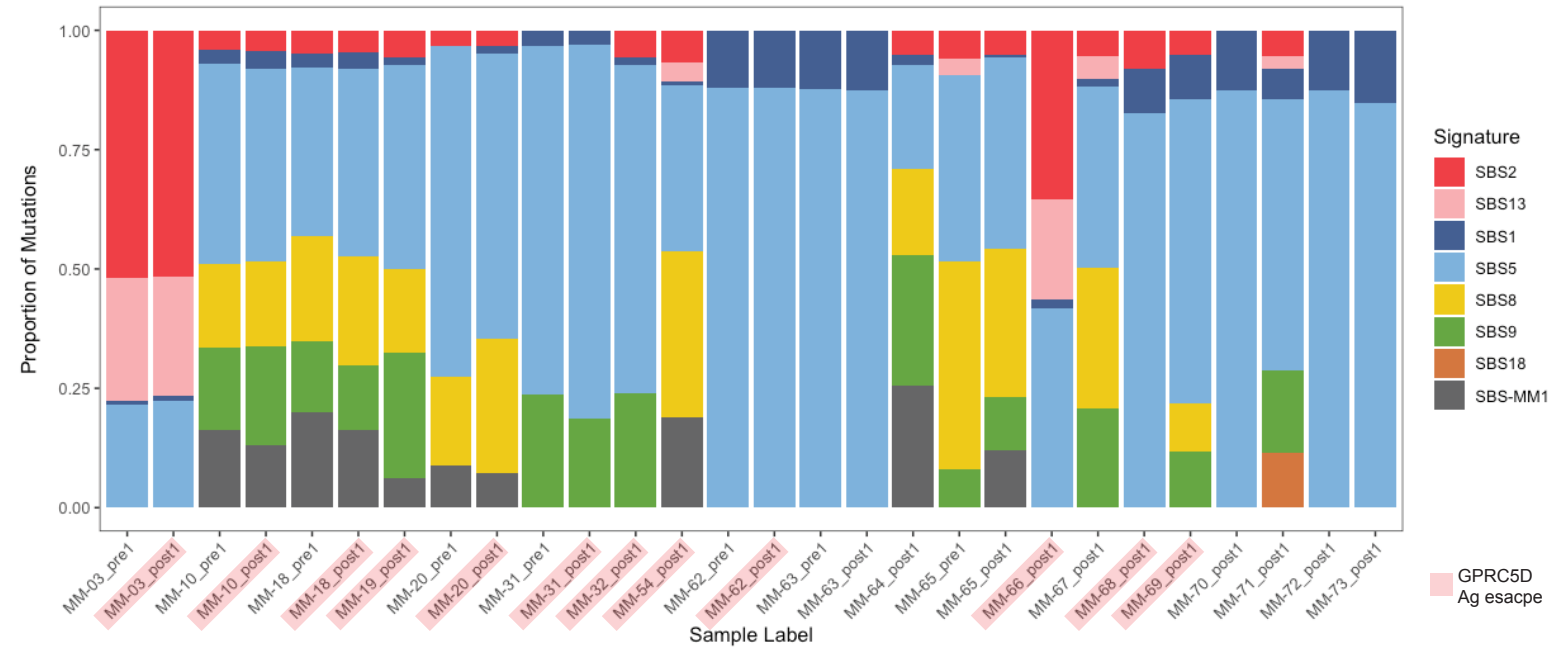
Supplementary Figure 1. Mutational burden and SBS COSMIC signature proportions per sample. **A.** Bar plot showing the total mutational burden per sample, with relative contributions of COSMIC single base substitution (SBS) signatures. **B.** Proportions of mutations attributed to each SBS signature as inferred by mmsig. Red highlighted samples on the X axis denote samples harboring *GPRC5D* alterations, including single-nucleotide variants, indels, copy number losses, or epigenetic changes.

Supplementary Figure 1

A. Mutational Burden per Sample



B. Mutational Signature Proportions per Sample

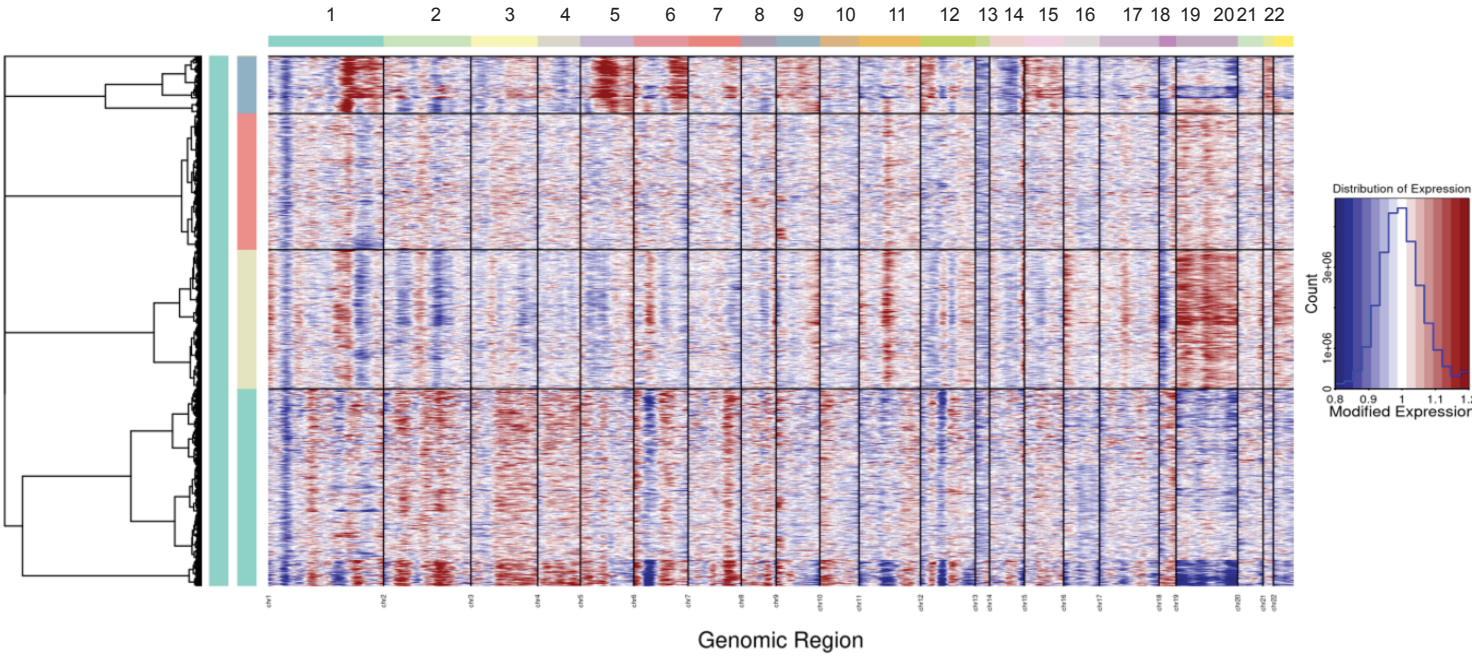


Supplementary Figure 2. Heatmap of inferred copy number changes in case MM-20 from scRNA-seq data (InferCNV), comparing pre- versus post-relapse CD138+ tumor cells. Bars on the left indicate variable clustering based on copy number variation. Red arrow indicates monoallelic copy number loss (inferCNV state 2 = one copy number loss) on chromosome 12p (chr12: 2,876,258-21,518,408)) overlapping the genomic coordinates of *GPRC5D* at progression post talquetamab (P4585_Post). Of note the focal biallelic deletion (104 kb) overlapping *GPRC5D* detected by WGS (100x) (Main **Figure 3B**) was not inferred by inferCNV with its resolution limited by the depth of scRNA-seq resolution.

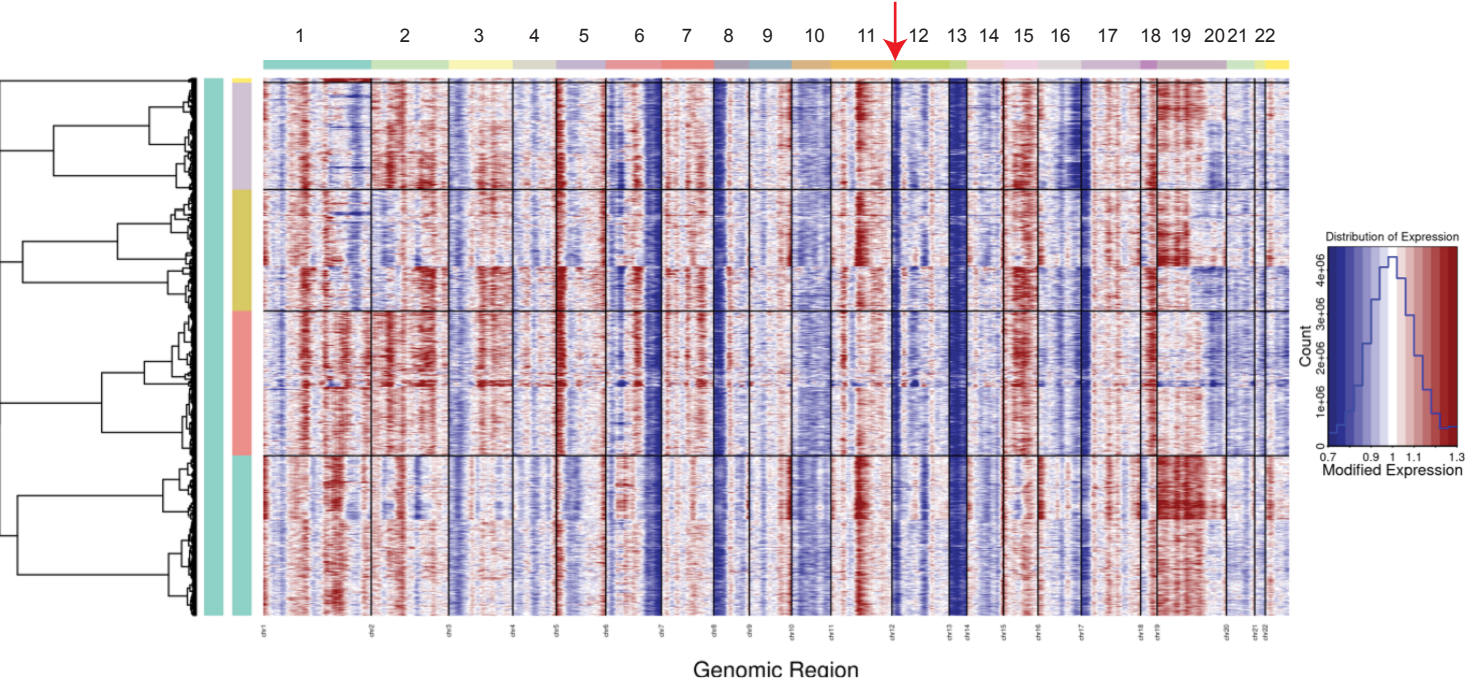
Supplementary Figure 2

MM-20

P1858_Pre

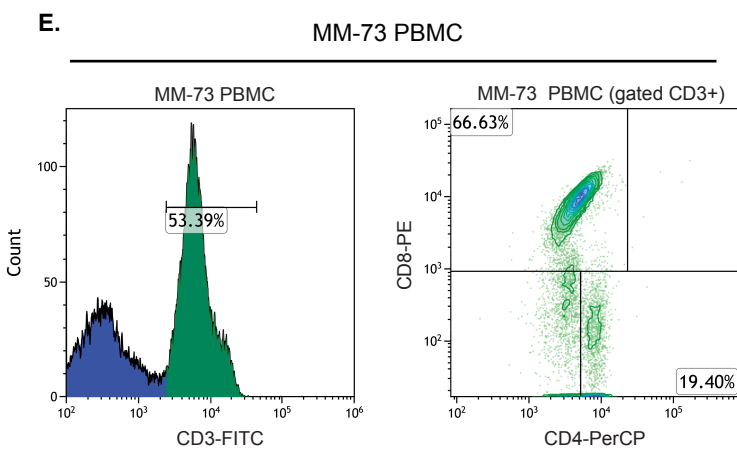
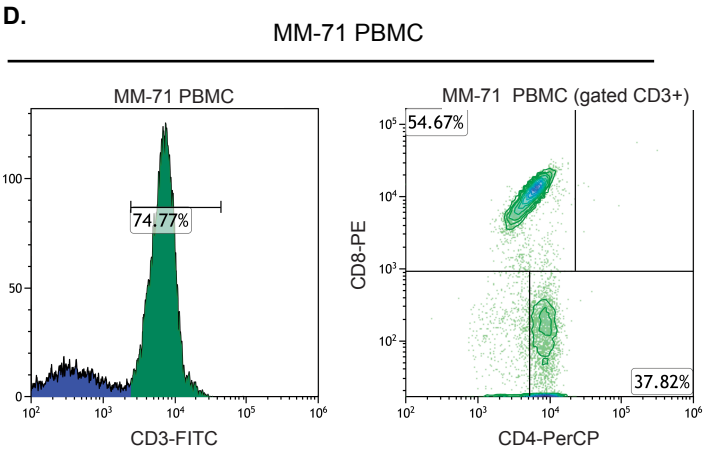
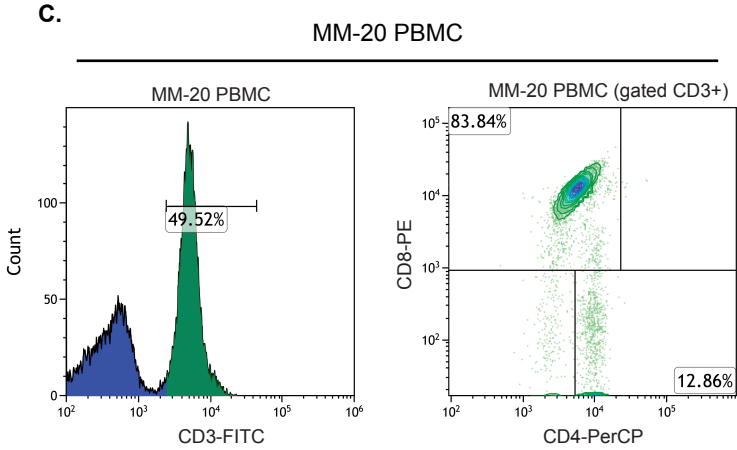
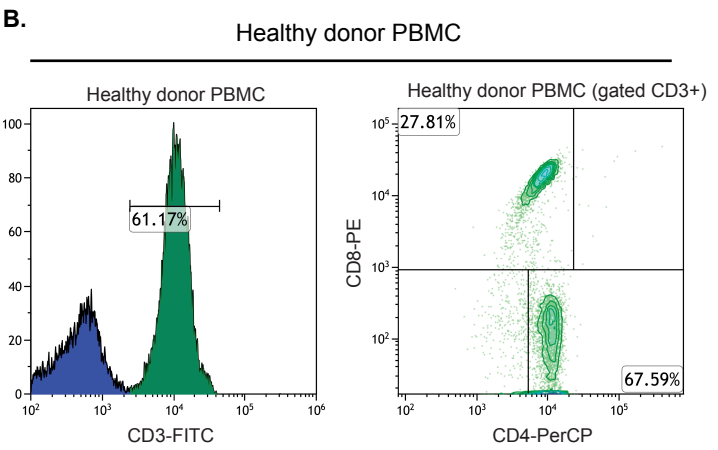
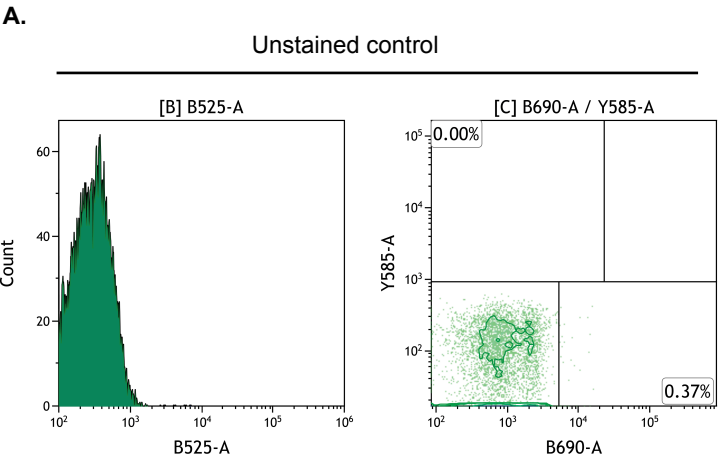


P4585_Post



Supplementary Figure 3. Proportion of CD3⁺ T cells and the CD4:CD8 ratio in peripheral blood mononuclear cells (PBMCs) by flow cytometry. A. Unstained control, B. Healthy donor, C. MM-20, D. MM-71, and E. MM-73.

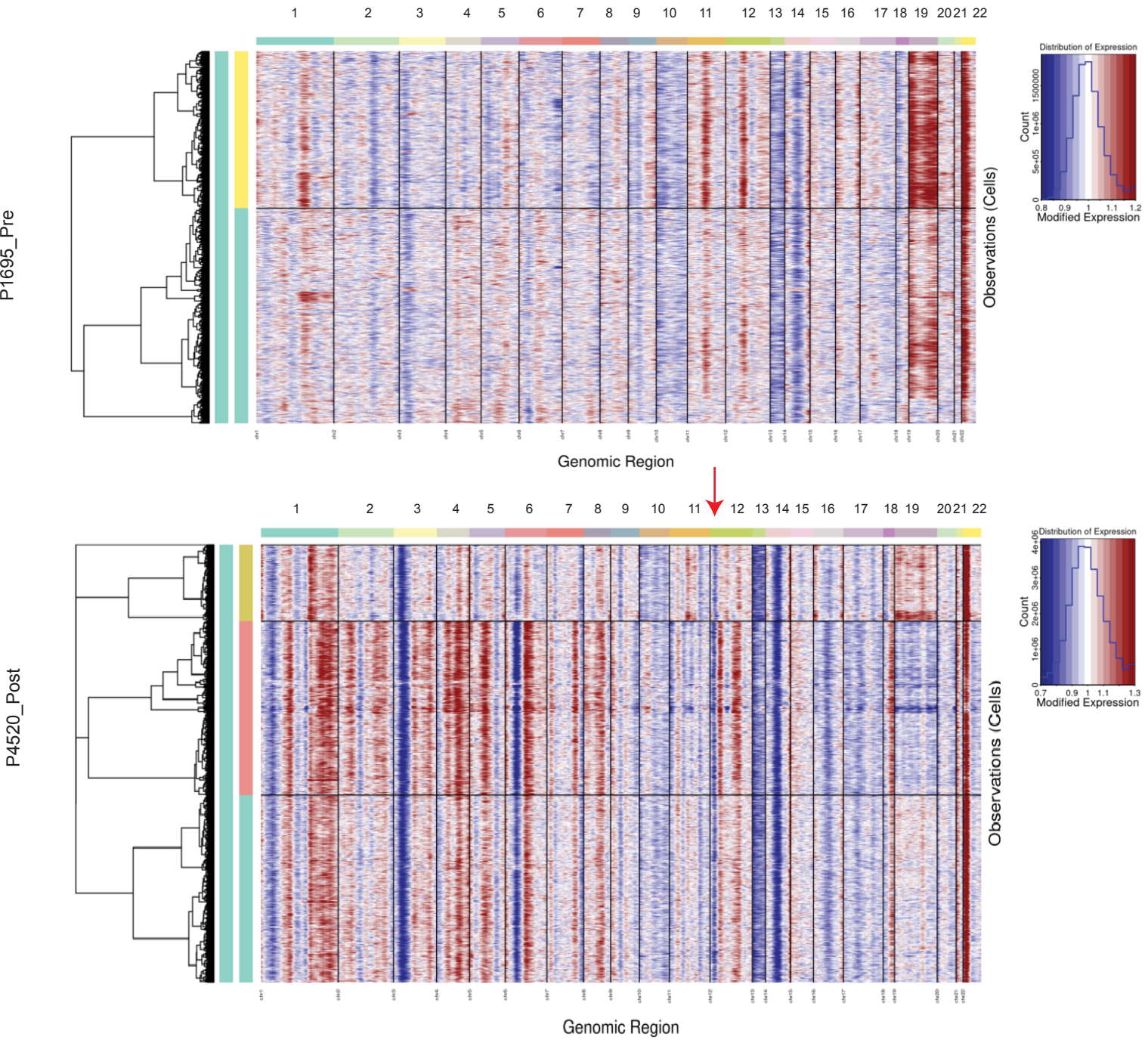
Supplementary Figure 3



Supplementary Figure 4. Heatmap of inferred copy number changes in case MM-10 from scRNA-seq data (InferCNV), comparing pre- versus post-relapse CD138+ tumor cells. Bars on the left indicate variable clustering based on copy number variation. Red arrow indicates monoallelic copy number loss (inferCNV state 2 = one copy number loss) on chromosome 12p (chr12: 6,786,858-14,961,728) overlapping the genomic coordinates of *GPRC5D* at progression post talquetamab (P4520_Post).

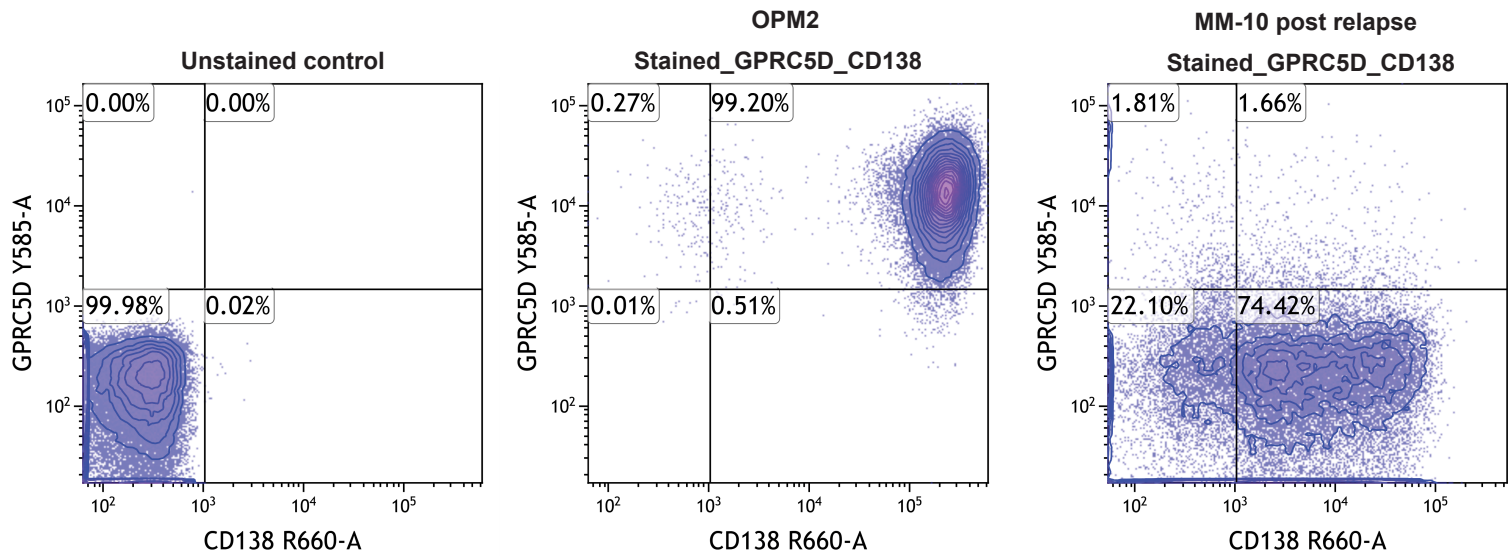
Supplementary Figure 4

MM-10



Supplementary Figure 5. Post-relapse CD138+ flow cytometry for case MM-10.
CD138+ sorted cells were stained with anti-GPRC5D and anti-CD138 flow antibodies.
OPM2 cells were used as positive control.

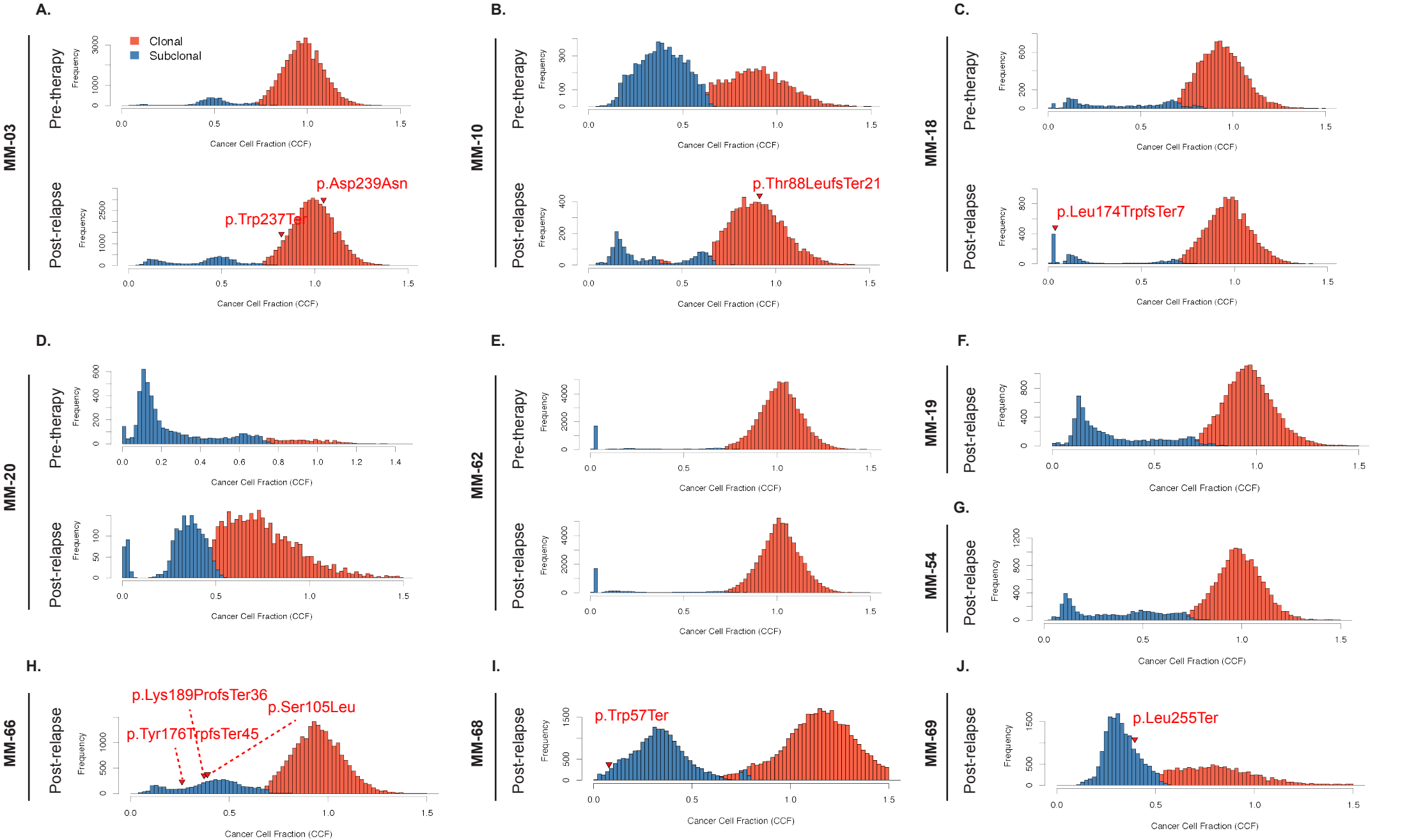
Supplementary Figure 5



Supplementary Figure 6. Pre- and post-relapse CD138⁺ scATAC-seq analysis in case MM-10. **A.** UMAP of pre-therapy and post-relapse bone marrow cells clustered by chromatin accessibility. **B.** Malignant plasma cells were defined by open chromatin accessibility at the canonical MM genes, *TNFRSF17* (shown), *CD38*, and *SDC1*. **C.** Tumor populations were subset based on open chromatin accessibility at the canonical MM gene loci (pre-relapse, red, n = 3,515; post-relapse, blue, n = 5,408). **D.** Chromatin accessibility at *TNFRSF17*, *CCND1*, *GPRC5D*, and *EMP1* in tumor populations. Dotted boxes highlight markedly reduced accessibility at *GPRC5D* and the distal *GPRC5D* enhancer near *EMP1* post-relapse. This is to be contrasted with the retained chromatin accessibility at *TNFRSF17* and *CCND1* pre and post relapse.

Supplementary Figure 7. Distribution of cancer cell fraction (CCF) of clonal and subclonal mutations for all samples with antigen escape calculated by Palimpsest. Orange = clonal, blue = subclonal, red arrows over the bars = *GPRC5D* SNV/ indels.

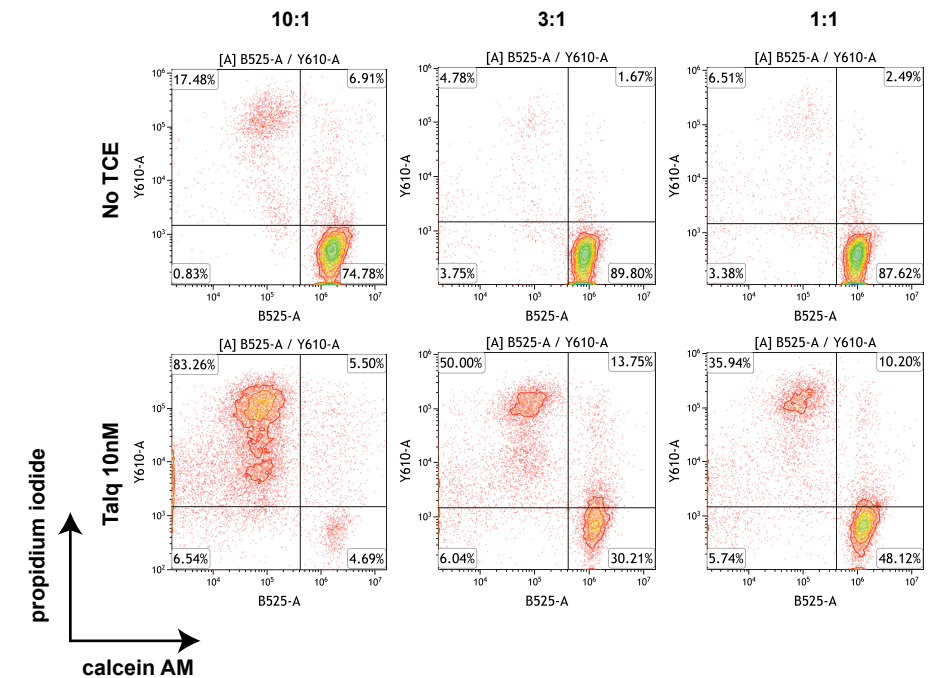
Supplementary Figure 7



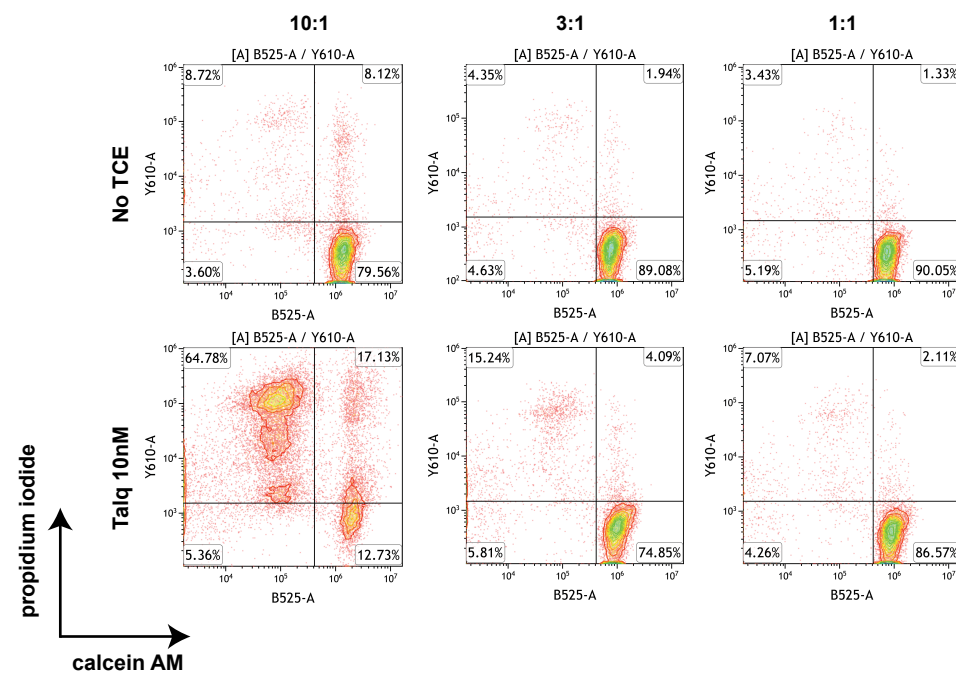
Supplementary Figure 8. Cytotoxicity assays were performed using patient peripheral blood mononuclear cells (PBMCs) obtained at relapse, co-cultured with OPM2 cells at effector:target (E:T) ratios ranging from 10:1 to 1:1 for 72 hours, in the presence or absence of 10 nM talquetamab. OPM2 MM cell viability was assessed by calcein AM⁺/propidium iodide⁻ staining. Healthy donor PBMCs served as a positive control. **A.** Healthy donor PBMC co-culture; **B.** MM-71 PBMC co-culture; **C.** MM-73 PBMC co-culture. Missing condition in panel C is due to limited availability of patient PBMCs.

Supplementary Figure 8

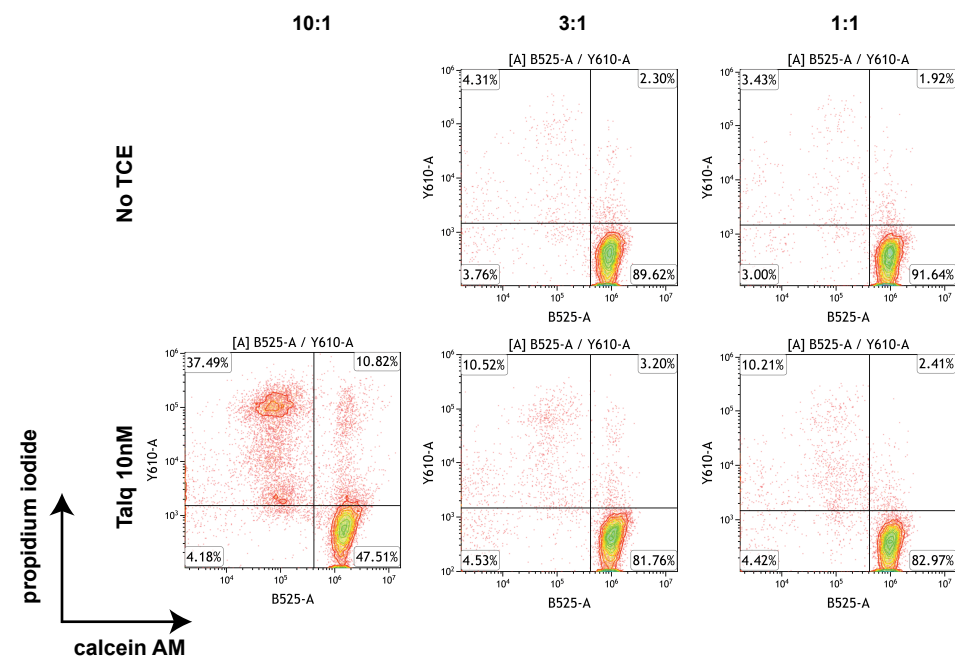
Healthy donor PBMC + OPM2 (E:T)



MM-71 PBMC + OPM2 (E:T)



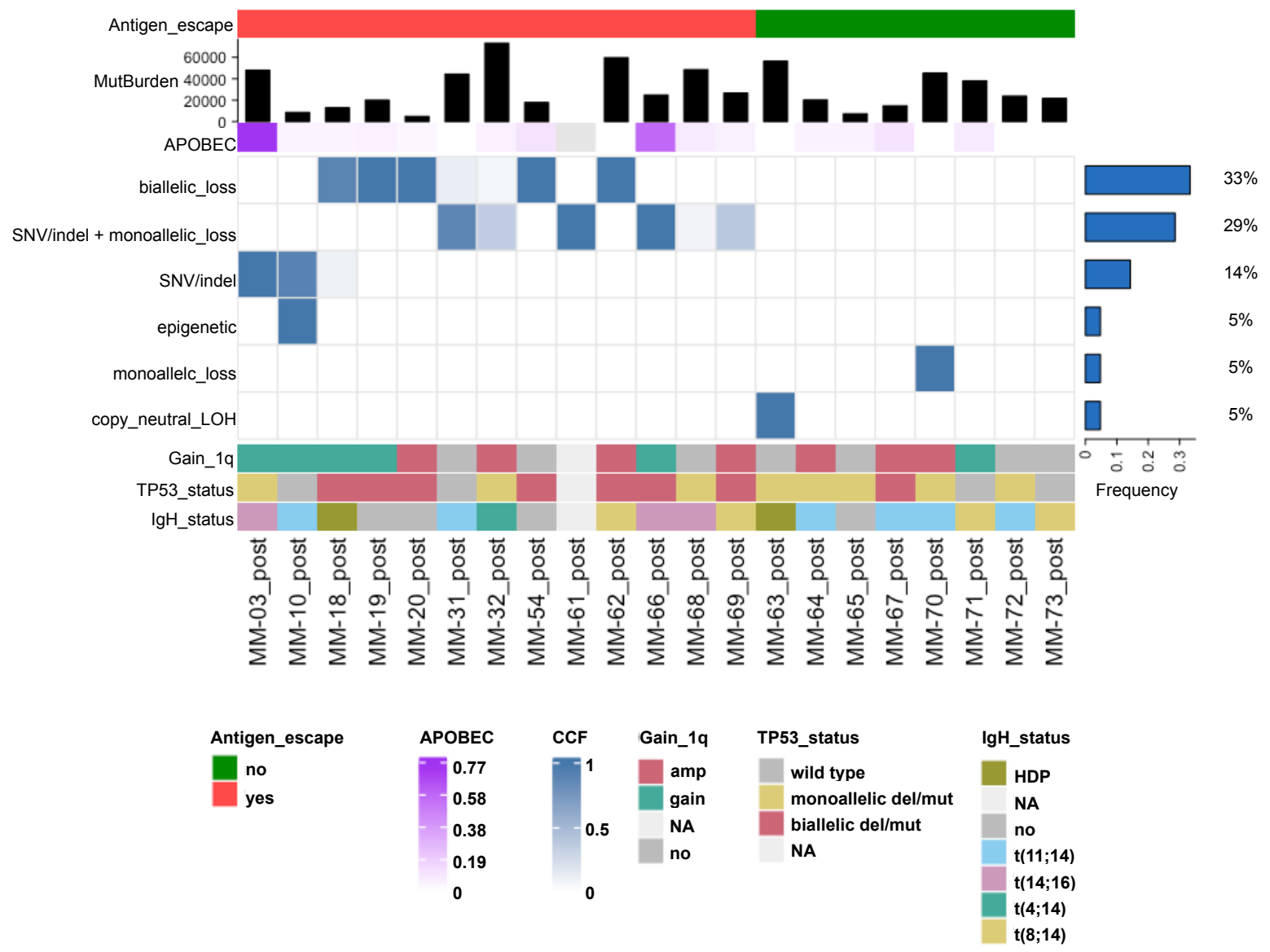
MM-73 PBMC + OPM2 (E:T)



Supplementary Figure 9. Oncoplot of post-relapse samples, highlighting GPRC5D antigen escape frequencies, mutational burden, and APOBEC activity.

Each column represents a post-relapse patient sample, grouped by GPRC5D antigen escape status. **Top annotations** show GPRC5D antigen escape (yes/no), mutational burden (total somatic mutations per sample, displayed as a bar plot), and APOBEC signature activity (% contribution of SBS2 + SBS13). **Bottom annotations** display additional genomic features, including 1q copy number (gain/amp), TP53 status, and IgH translocations or hyperdiploidy (HDP). The heatmap itself shows cancer cell fraction (CCF) of recurrent events across samples, with higher values indicating greater clonality. Row-wise barplots on the right indicate the frequency of each event across all post-relapse samples. CCF, cancer cell fraction; HDP, hyperdiploid; MutBurden, total somatic mutations. NA = not assessed.

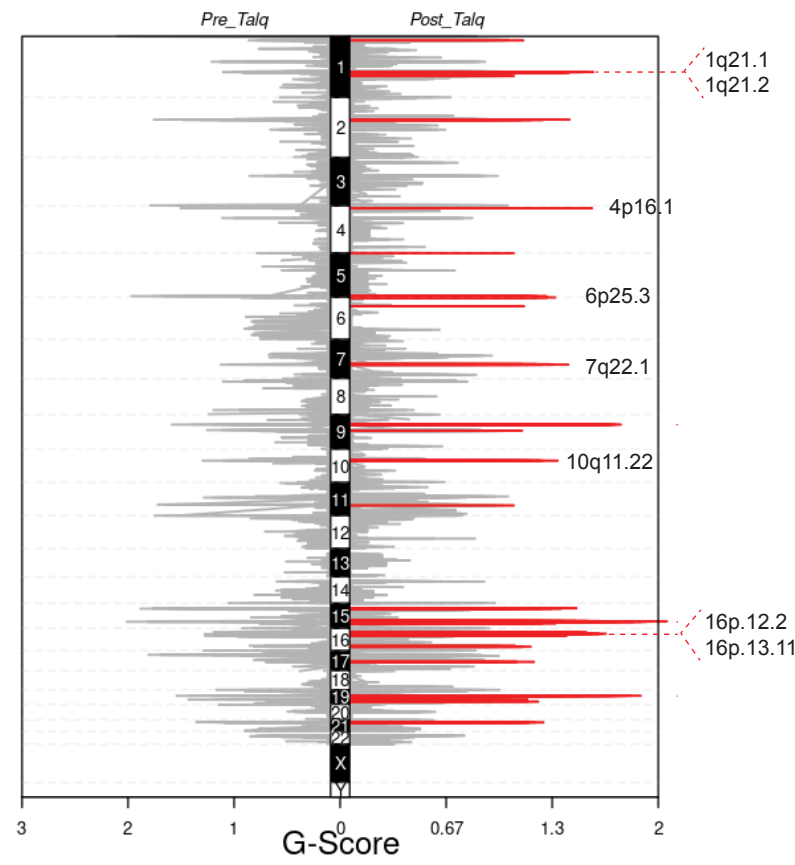
Supplementary Figure 9



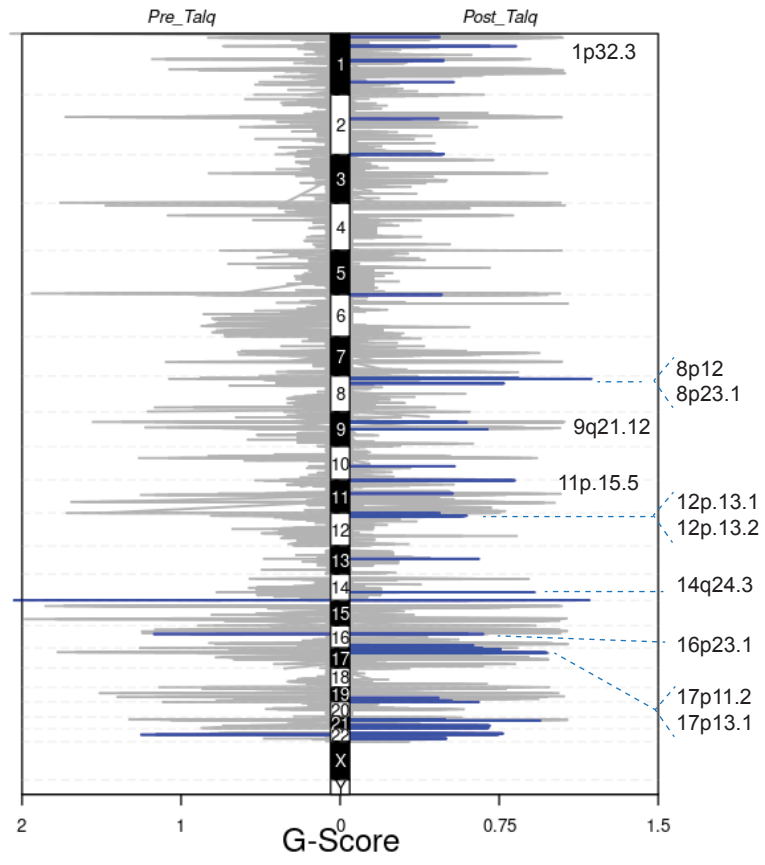
Supplementary Figure 10. GISTIC analysis of genomic copy number profiles in pre-therapy versus relapse samples. The x-axis represents GISTIC G-scores, and chromosomes are arranged along the central axis. Peaks to the left correspond to significant events in pre-therapy samples, and peaks to the right correspond to relapse samples. **A.** Red peaks denote regions of significant copy number gain. **B.** Blue peaks denote regions of significant copy number loss.

Supplementary Figure 10

A.

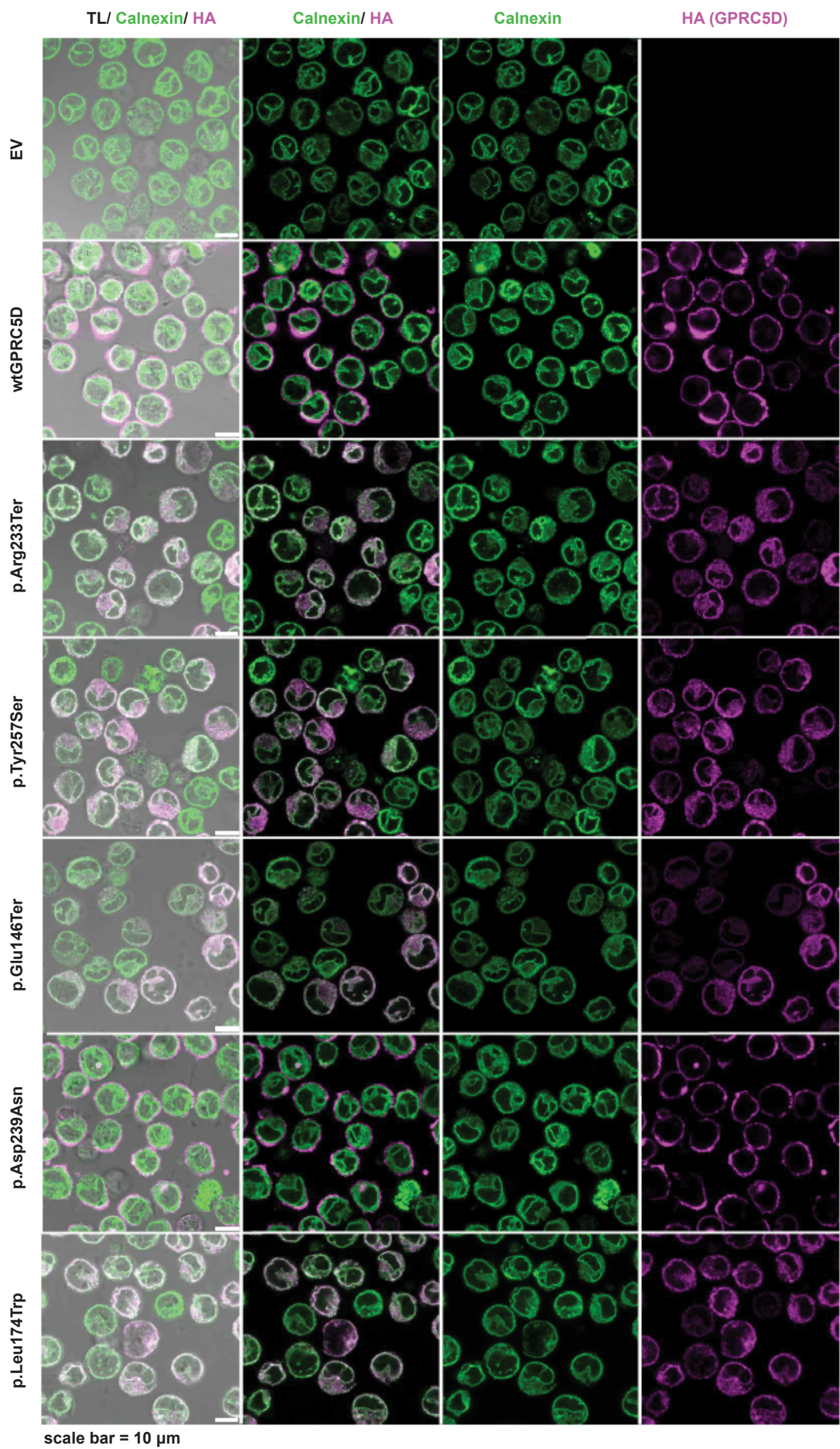


B.



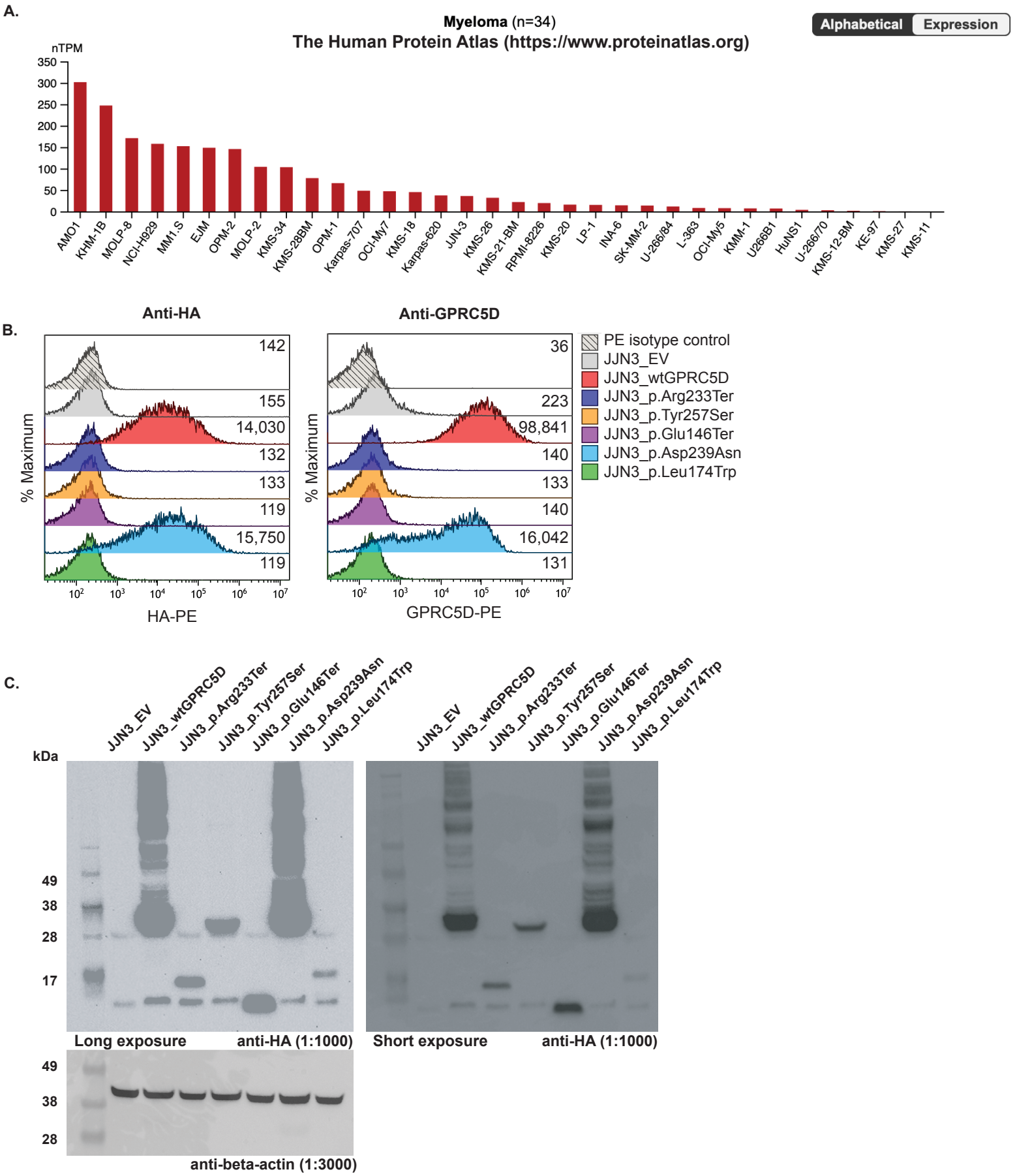
Supplementary Figure 11. Confocal imaging of K562 cells expressing wild-type (wt) or mutant GPRC5D (p.Arg233Ter, p.Tyr257Ser, p.Glu146Ter, p.Asp239Asb, p.Leu174Trp), stained with anti-calnexin (endoplasmic reticulum ER marker) and anti-HA (GPRC5D) antibodies. EV = empty vector K562 cells, TL = transmitted light. Shown is a representative experiment of 2 biologically independent replicates.

Supplementary Figure 11



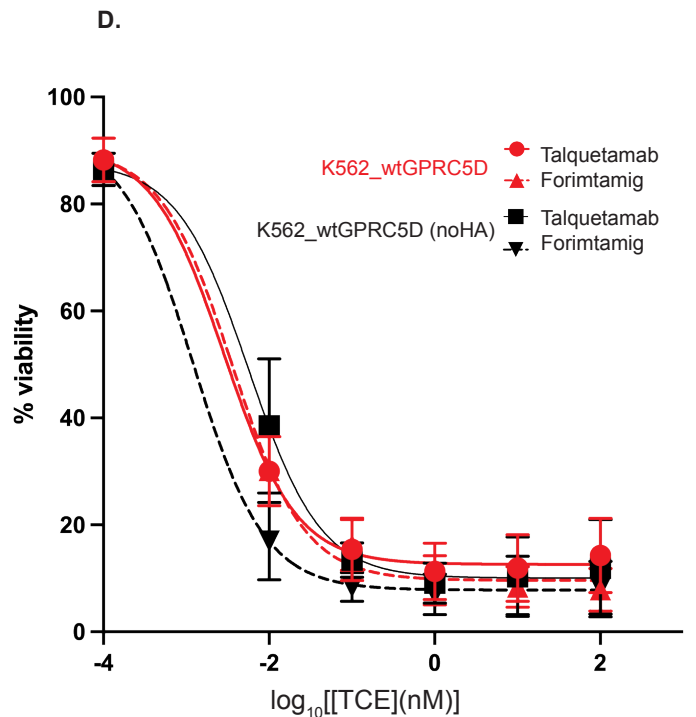
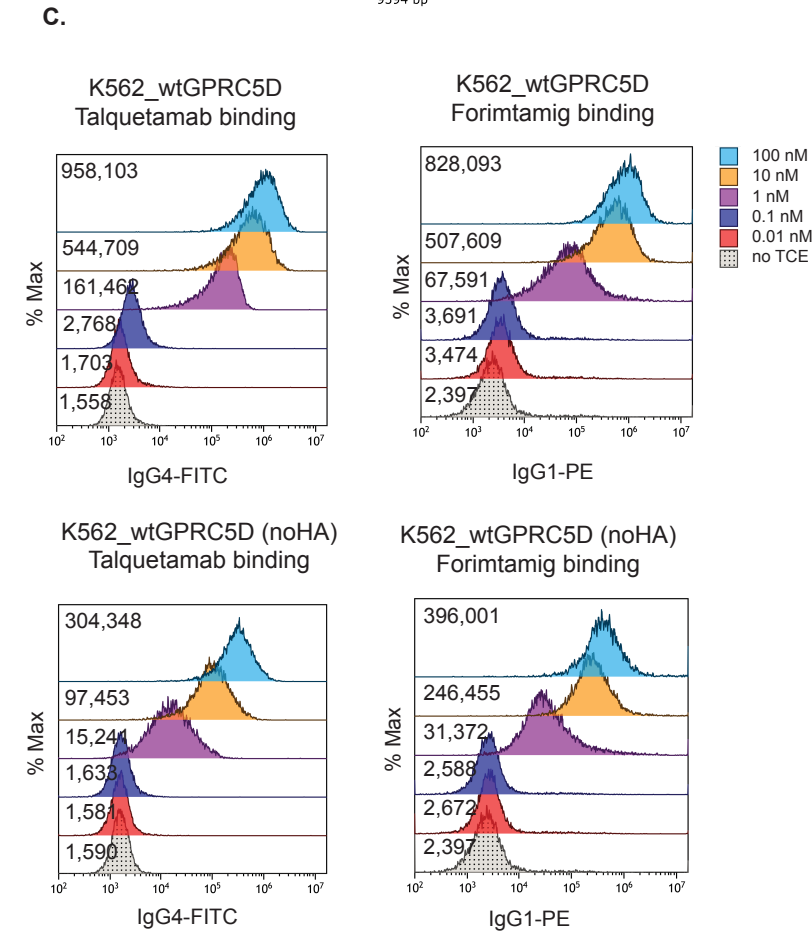
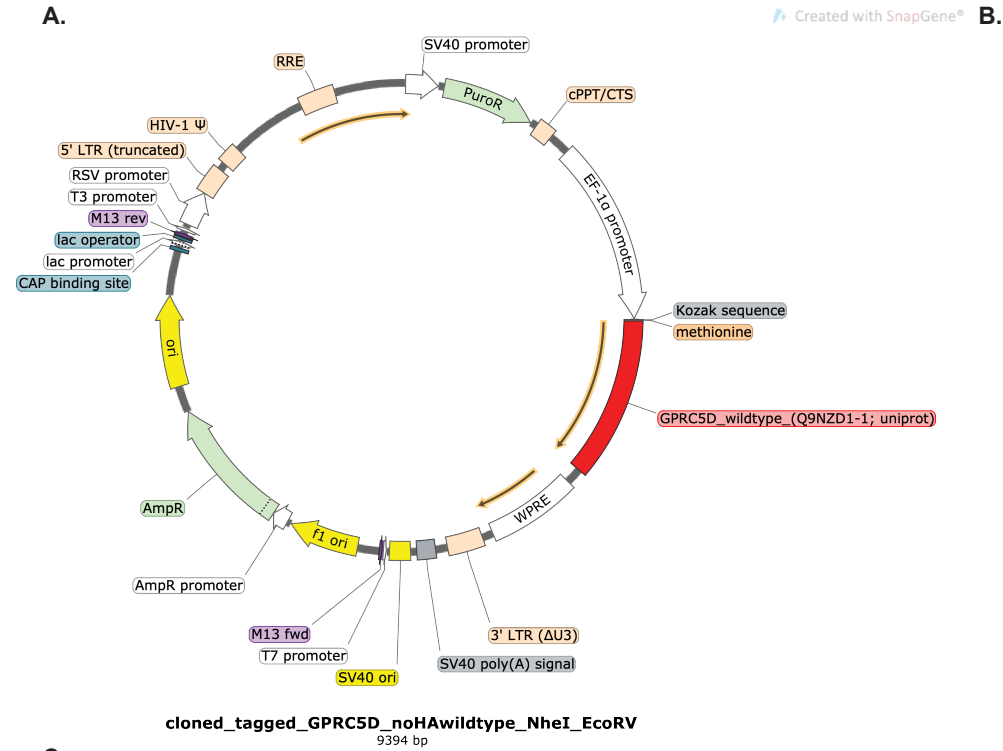
Supplementary Figure 12. Wild-type or mutant GPRC5D expression in JJN3 MM cell line. **A.** *GPRC5D* transcript expression levels (TPM, transcript per million) in multiple myeloma cell lines. Screenshot from The Human Protein Atlas, proteomics.proteinatlas.org¹. **B.** Flow cytometry histograms of transduced JJN3 cells stained with anti-HA or anti-GPRC5D antibodies; numbers indicate median fluorescence intensity (MFI). **C.** Western blot of GPRC5D in JJN3 and empty vector control (JJN3_EV); anti-HA blot shown with short and long exposures. Shown is a representative experiment of 2 biologically independent replicates.

Supplementary Figure 12



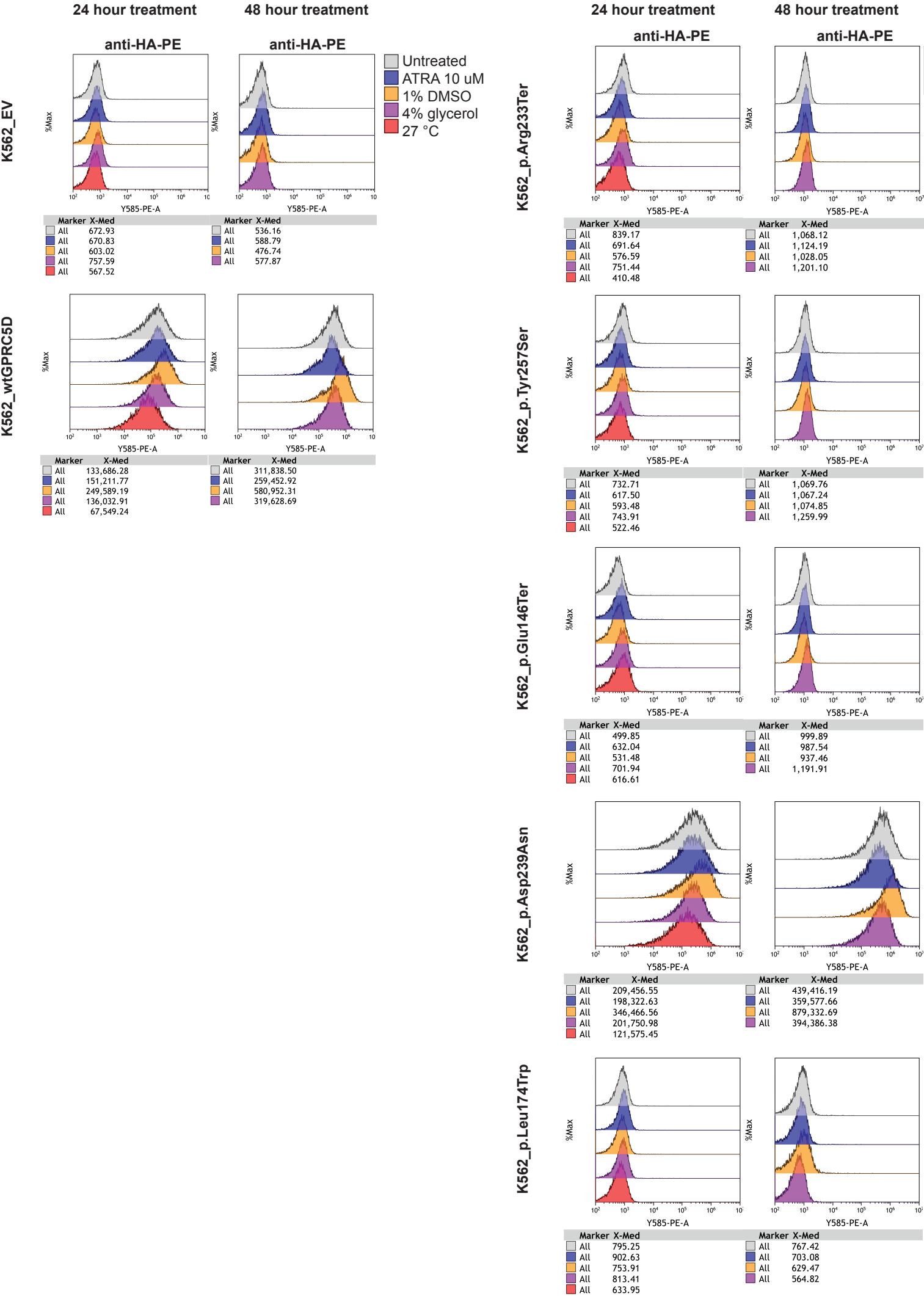
Supplementary Figure 13. GPRC5D N-terminal tagging with hemagglutinin (HA) does not interfere with trafficking, surface expression, antibody binding, or TCE cytolytic activity. **A.** Lentiviral transfer vector (pLEX_307, Addgene #41392) used to stably express wild-type GPRC5D without N-terminus HA tagging. Figure generated with SnapGene. **B.** Flow cytometry of K562 cells expressing empty vector, wild-type GPRC5D with HA tag, and wild-type GPRC5D without HA tag stained with anti-HA and anti-GPRC5D antibodies; MFIs indicated. **C.** Talquetamab and forimtamig binding assays on K562 cells expressing wtGPRC5D with or without HA tag, showing no difference in binding with or without GPRC5D HA-tagging. **D.** Dose-response cytotoxicity assay: K562 cells co-cultured with healthy donor PBMCs (E:T 10:1) $\pm$ TCEs for 48 hours; target cell viability (%) plotted versus $\log_{10}$ [TCE] (nM). Data are presented as mean $\pm$ standard deviation. Raw data and p values from triplicate biologically independent experiments (n=3 for each cell line) are available in **Supplementary Table 5.**

Supplementary Figure 13



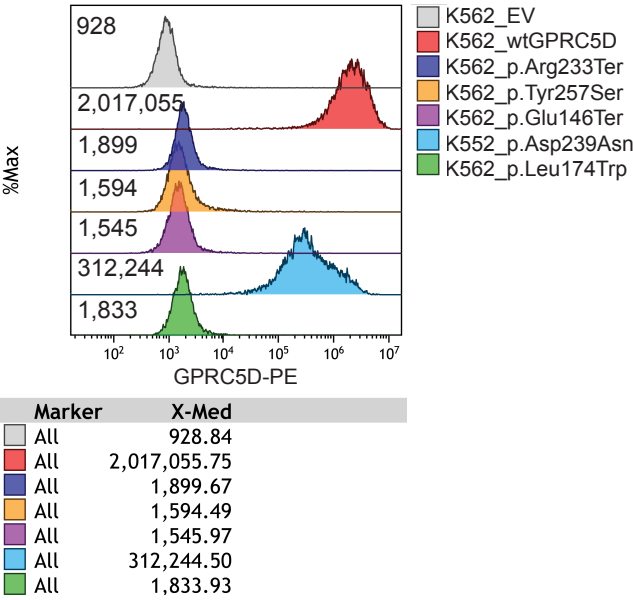
Supplementary Figure 14. Chemical chaperones or cold temperature treatment does not translocate mutant GPRC5D from ER to cell surface. Flow cytometry histograms of K562 cells transduced with GPRC5D treated with 10 μ M ATRA, 1% DMSO, 4% glycerol (24 and 48 hours) or cultured at 27°C (24 hours); stained with anti-HA antibody. Numbers under X-axis indicate median fluorescence intensity. Shown is a representative experiment of 2 biologically independent replicates.

Supplementary Figure 14

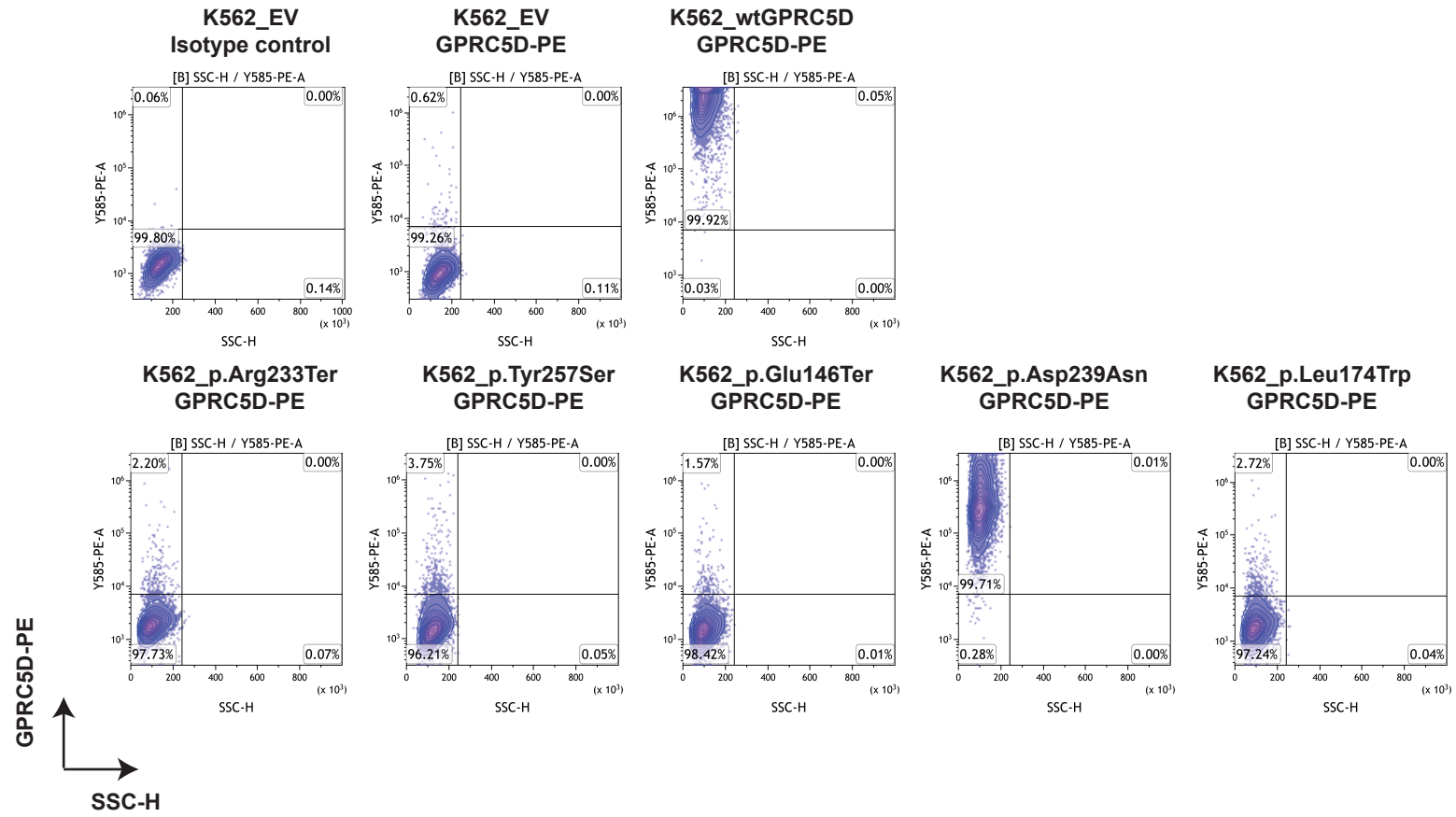


Supplementary Figure 15. Overnight incubation with anti-GPRC5D antibody does not detect mutant GPRC5D membrane expression. A. Flow cytometry histograms of GPRC5D-transduced K562 cells incubated with anti-GPRC5D-PE antibodies for 18 hours at 37°C. MFIs indicated. **B.** Dot plot of corresponding flow cytometry data. Shown is a representative experiment of 2 biologically independent replicates.

A. Overnight staining with GPRC5D-PE antibody



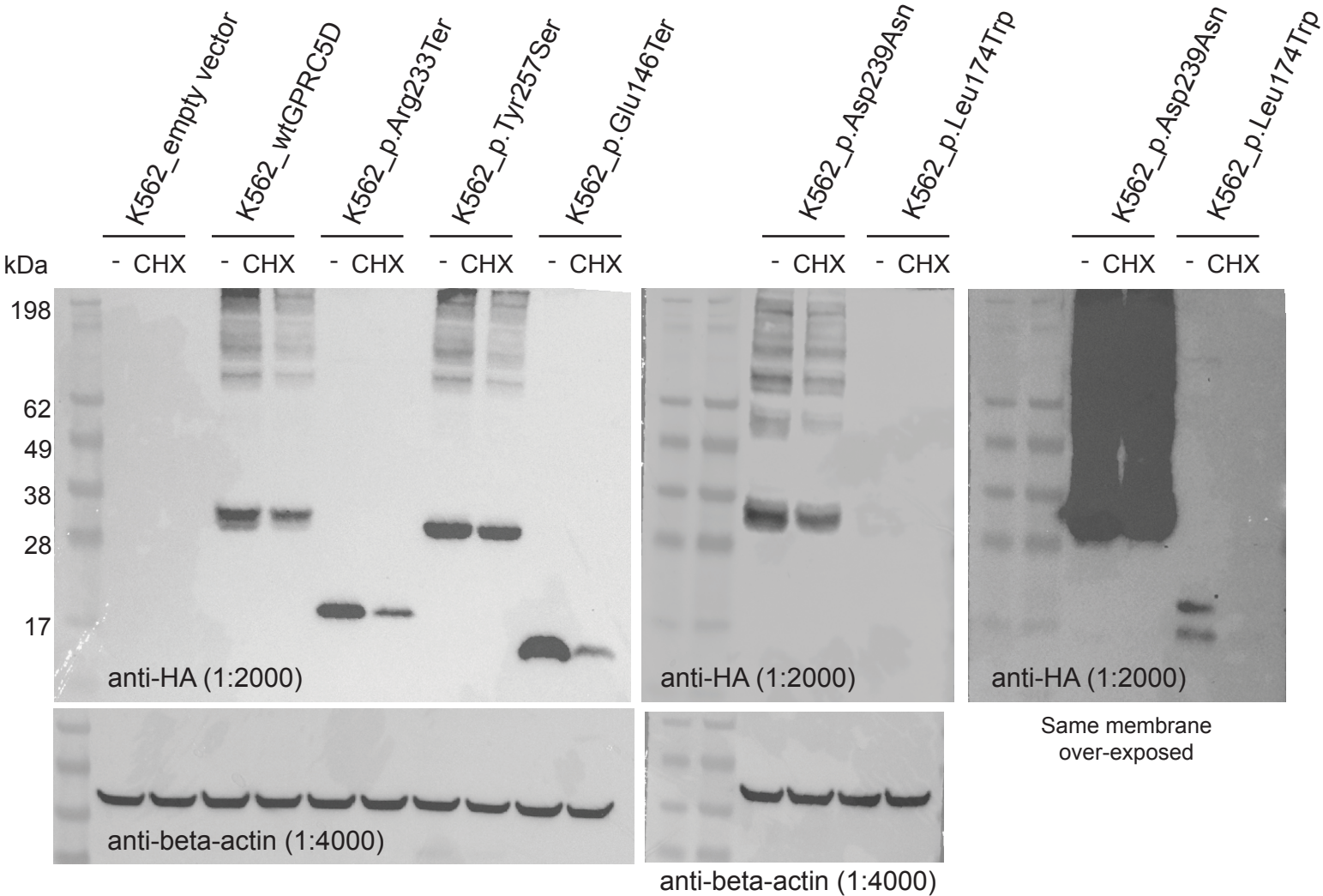
B.



Supplementary Figure 16. Cycloheximide (CHX) treatment does not demonstrate significant differences in wild type versus mutant GPRC5D protein stability.

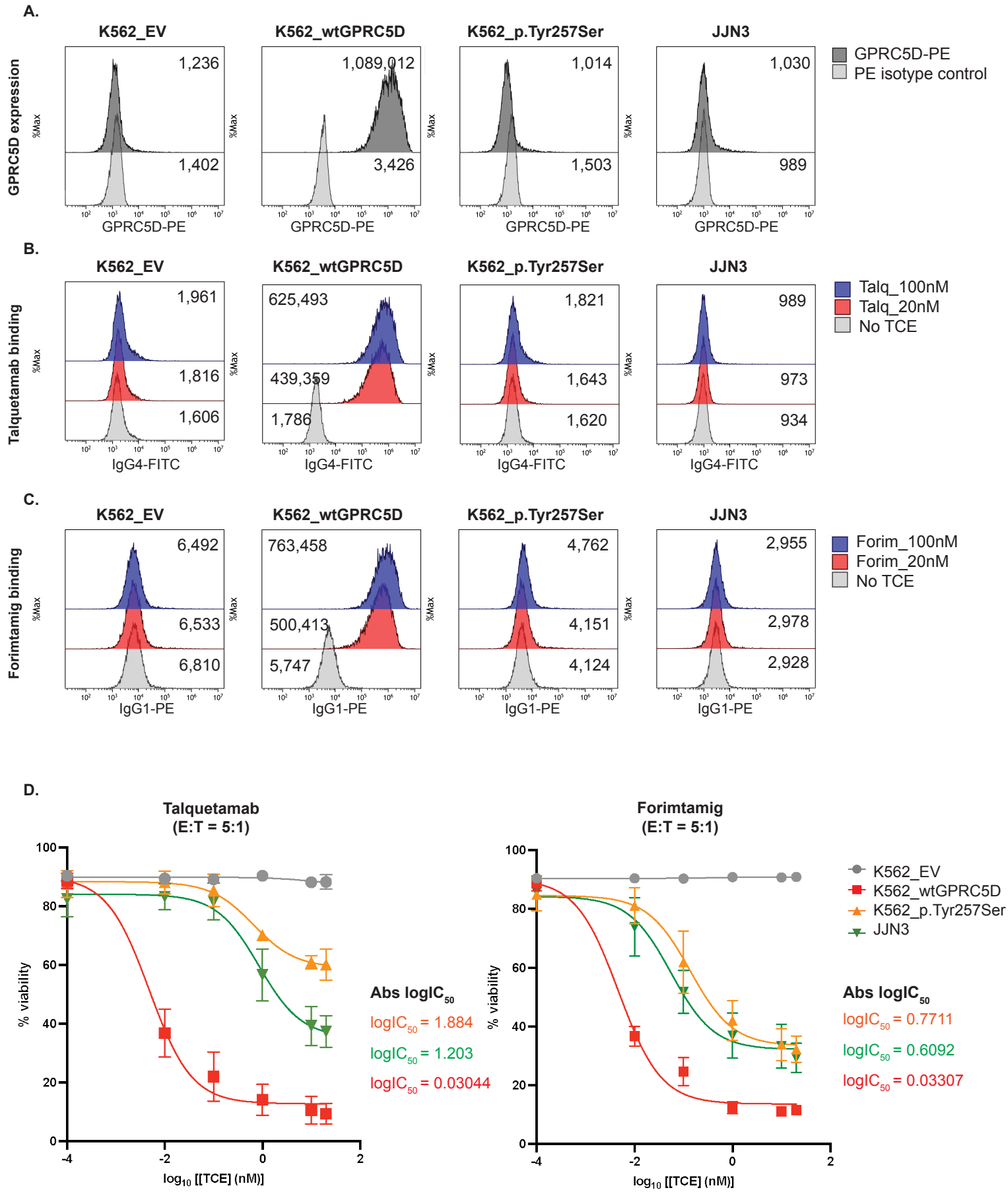
Western blotting of cell lysates from wild type or mutant GPRC5D expressing K562 cells treated $\pm$ cycloheximide (10 μ g/mL, 6 hours). The third panel shows an overexposed image of the membrane blot in the second panel. Shown is a representative experiment of 2 biologically independent replicates.

Supplementary Figure 16



Supplementary Figure 17. Anti-GPRC5D TCE cytolytic activity is retained against GPRC5D-low expressing cells. **A.** Flow cytometry histograms of membrane GPRC5D expression in K562 EV, K562 wtGPRC5D, K562 p.Tyr257Ser, and JJN3 MM cells; MFIs indicated. **B and C.** Talquetamab and forimtamig at 20 nM (red) and 100 nM (blue) binding assays by flow cytometry. **D.** Dose-response cytotoxicity assays: healthy donor PBMCs co-cultured with targets (E:T 5:1) $\pm$ TCEs; target viability (%) versus $\log_{10}$ [TCE] concentration shown. Data are presented as mean $\pm$ standard deviation. Raw data and p values from triplicate biologically independent experiments (n=3 for each cell lines) are available in **Supplementary Table 8**.

Supplementary Figure 17



Supplementary Figure 18. High-throughput Chromosome Conformation Capture (Hi-C) analysis of the GPRC5D-HEBP1 locus in GM12878 lymphoblastoid cell line². Analysis of Hi-C data previously published by Rao et al² in GM12878 cell line depicting topologically associating domain (TAD) spanning *GPRC5D* and *HEBP1*. Off-diagonal contacts indicate long-range chromatin looping between enhancer region near *EMP1* gene and *GPRC5D*. Figure generated using HiGlass³.

Supplementary Figure 18

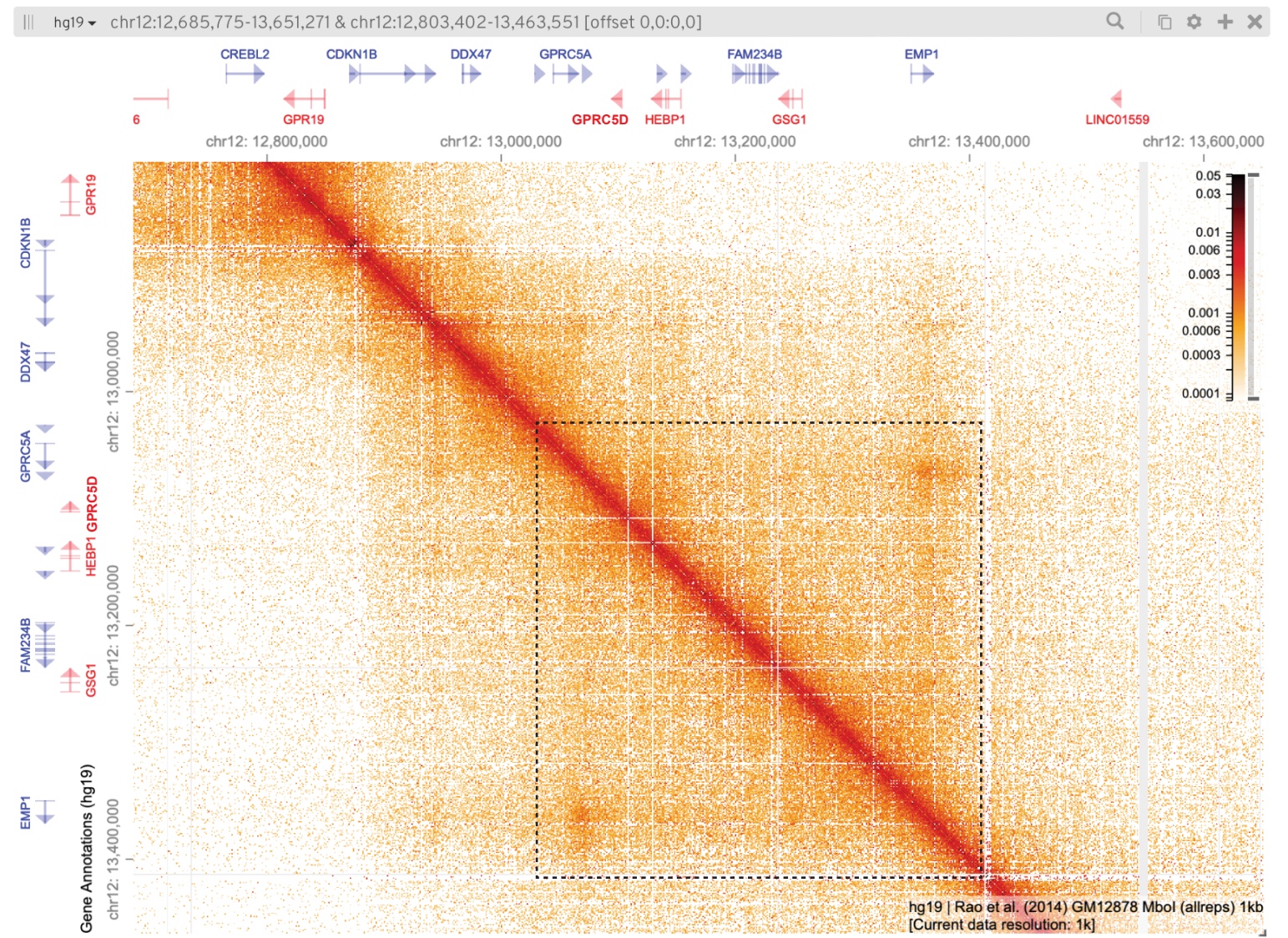
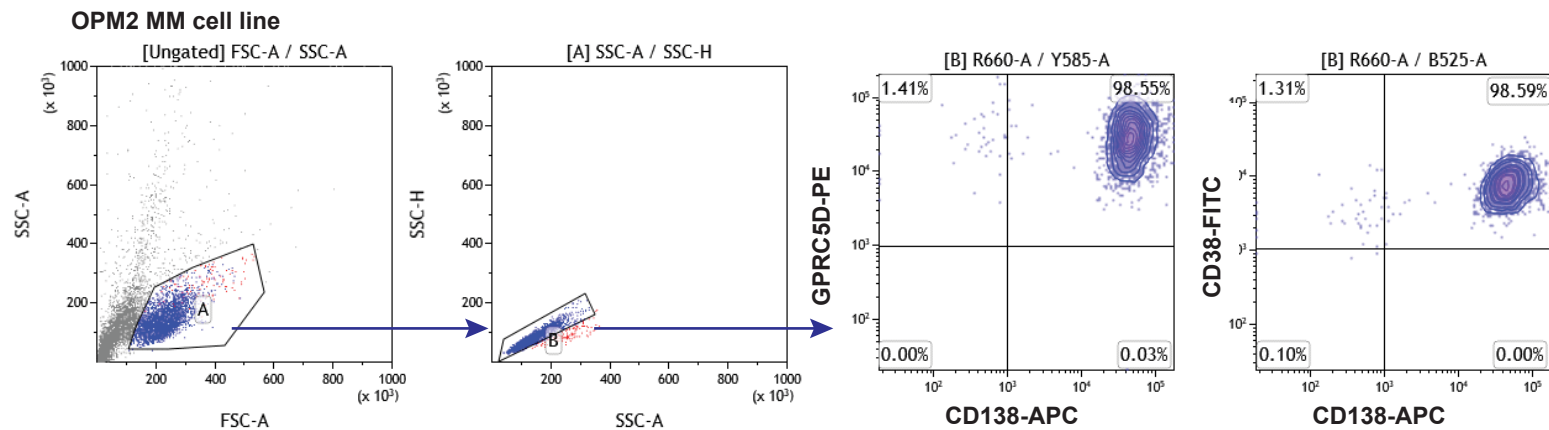


Figure generated using HiGlass (Kerpedjiev et al. HiGlass: Web-based visual comparison and exploration of genome interaction maps. Genome Biology, 19:125.2018)

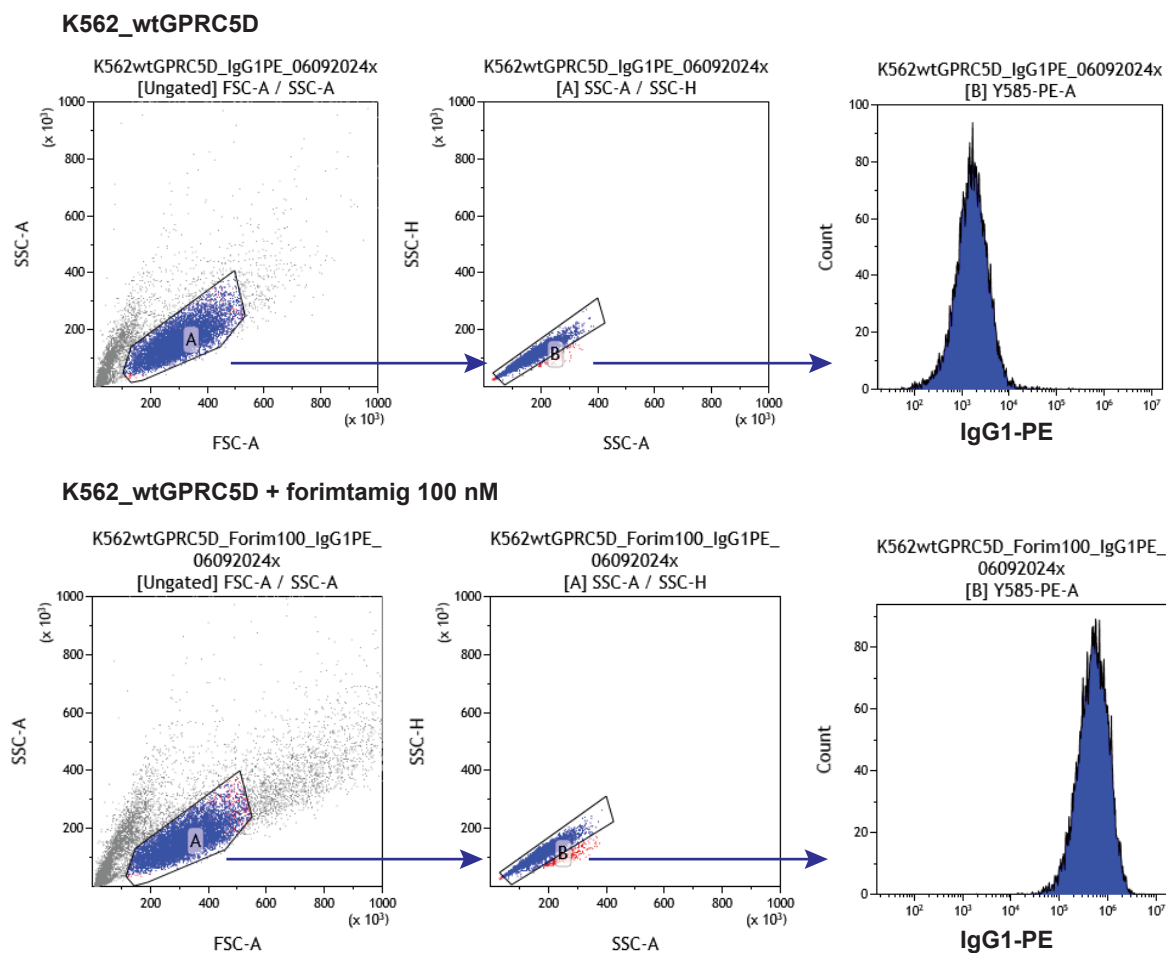
Supplementary Figure 19: Flow cytometry gating strategy. **A.** Live cells were selected from the forward scatter/side scatter plot, and doublets were excluded based on side scatter height versus area plot. CD138 (APC), CD38 (FITC), and GPRC5D (PE) antibody staining was performed and displayed as contour density plots in dot plots as shown. Dot plot gating was based on unstained controls. **B.** TCE binding assay: Live cells were incubated with or without 100 nM TCE (forimtamig) for 15 minutes, then washed and stained with anti-IgG1-PE antibody, followed by washing prior to flow cytometry analysis. Live cells were selected from the forward scatter/side scatter plot, and doublets were excluded based on side scatter height versus area plot. IgG1 binding was determined by median fluorescence intensity on single-parameter histograms (PE channel for anti-IgG1 antibody). **C.** Target cell viability measurement in cytotoxicity assay: CellTrace Violet positive cells were gated using the V450-PB-A channel (CellTrace Violet/BV421). The selected population was then plotted on a density plot of mcherry versus FITC to identify propidium iodide positive versus calcein AM positive cells. Viability was defined as the percentage of calcein AM positive propidium iodide negative cells.

Supplementary Figure 19

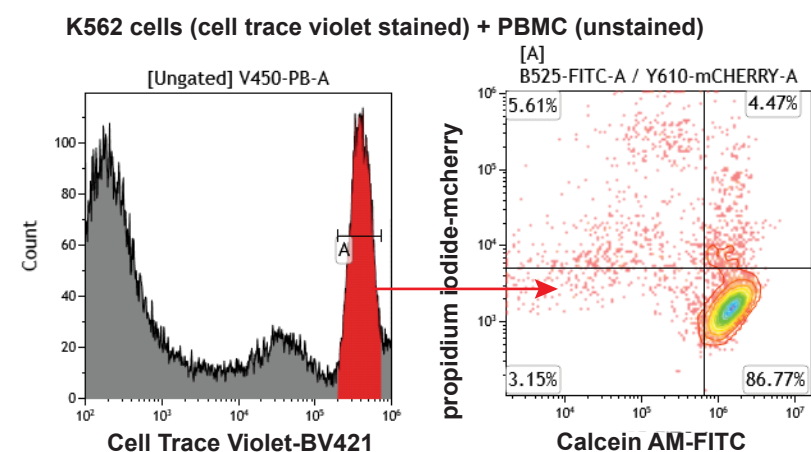
A. Gating strategy for CD138(APC), GPRC5D (PE), CD38 (FITC)



B. Gating strategy for anti-GPRC5D TCE binding assay



C. Gating strategy for cytotoxicity assays



Supplementary Table Legends

Supplementary Table 1. Clinical characteristics of 21 patients who progressed on anti-GPRC5D TCE are shown, along with the sample numbers used for whole genome sequencing (WGS), scATAC-seq, and scRNA-seq. Best responses were assessed by IMWG criteria: sCR, stringent complete remission; CR, complete remission; VGPR, very good partial remission; PR, partial response; SD, stable disease; PD, progressive disease. PFS, progression-free survival in months; OS, overall survival in months. R-ISS, revised multiple myeloma international staging system; FISH, fluorescence in situ hybridization; Ig_caller, IgH translocations called by IgCaller R tool⁴. # Prior therapy indicates number of prior lines of therapy; prior anti-BCMA indicates prior BCMA-directed therapies including T cell engagers (TCE), CAR T cells, or antibody-drug conjugates; Triple-drug indicates refractory or exposure to proteasome inhibitors (PI), immunomodulatory drugs (IMiD), and anti-CD38 antibodies. Penta drug refractory represents patient who had prior progression to 2 PI, 2 IMiDs, and anti-CD38 antibodies. Case MM-61 underwent targeted sequencing. Cases MM-65 and MM-67 exhibited primary refractory disease.

Supplementary Table 2. WGS sample purity, ploidy, and cancer cell fraction.

*Patient MM-61 underwent targeted sequencing only. ** cancer cell fraction (CCF) for copy number alterations using FACETS⁵. *** cancer cell fraction (CCF) for SNV or indels as per Palimpsest⁶. **** variant allelic fraction (VAF) values were not purity corrected. # CCF_CNV could not be accurately computed as the sample was subjected to targeted panel sequencing only. ## CCF_CNV could not be accurately computed as patient received prior allogenic stem cell transplantation with chimerism in germline reference.

Supplementary Tables 3. Results from dNdScv analysis of nonsynonymous mutation burden across genes in post-relapse samples (n = 20). Shown are counts of synonymous (n_syn), missense (n_mis), nonsense (n_non), splice site (n_spl), and indel (n_ind) mutations. The maximum-likelihood estimates of the dN/dS ratios are provided for missense (wmis_cv), nonsense (wnon_cv), splice site (wspl_cv), and indel (wind_cv) mutations. Corresponding p-values (pmis_cv, ptrunc_cv, pallsubs_cv, pind_cv) and q-values (qmis_cv, qtrunc_cv, qallsubs_cv, qind_cv) represent statistical significance. Global p- and q-values (pglobal_cv, qglobal_cv) indicate overall evidence of positive selection for each gene.

Supplementary Tables 4. Results from dNdScv analysis of nonsynonymous mutation burden across genes in pre-therapy samples (n=8). Shown are counts of synonymous (n_syn), missense (n_mis), nonsense (n_non), splice site (n_spl), and indel (n_ind) mutations. The maximum-likelihood estimates of the dN/dS ratios are provided for missense (wmis_cv), nonsense (wnon_cv), splice site (wspl_cv), and indel (wind_cv) mutations. Corresponding p-values (pmis_cv, ptrunc_cv, pallsubs_cv, pind_cv) and q-values (qmis_cv, qtrunc_cv, qallsubs_cv, qind_cv) represent statistical significance.

Supplementary Table 5. Raw data and statistical analysis for the dose-response cytotoxicity assay shown in Supplementary Figure 13D. Target cell viability (%) from triplicate biologically independent experiments (n=3 per cell line) is presented. Comparisons were made using ordinary two-way ANOVA followed by Tukey's multiple comparisons test with a single pooled variance. Exact two sided, multiplicity adjusted P values, mean differences, and 95% confidence intervals are reported.

Supplementary Table 6. Raw data and statistical analysis for the dose-response cytotoxicity assay shown in main Figure 6C. Target cell viability (%) from triplicate biologically independent experiments (n=3 per cell line) is presented. Comparisons of K562_p.Asp239Asn versus K562_wtGPRC5D cells treated with talquetamab and forimtamig were compared using ordinary two-way ANOVA followed by Tukey's multiple comparisons test with a single pooled variance. Exact two sided, multiplicity adjusted P values, mean differences, and 95% confidence intervals are reported.

Supplementary Table 7. Raw data and statistical analysis for the dose-response cytotoxicity assay shown in main Figure 6D. Target cell viability (%) from triplicate biologically independent experiments (n=3 per cell line) is presented. Comparisons of wild type and mutant GPRC5D cells treated with talquetamab or forimtamig were compared using ordinary two-way ANOVA followed by Tukey's multiple comparisons test with a single pooled variance. Exact two sided, multiplicity adjusted P values, mean differences, and 95% confidence intervals are reported.

Supplementary Table 8. Raw data and statistical analysis for the dose-response cytotoxicity assay shown in Supplementary Figure 17D. Target cell viability (%) from triplicate biologically independent experiments (n=3 per cell line) is presented. Comparisons were made using ordinary two-way ANOVA followed by Tukey's multiple comparisons test with a single pooled variance. Exact two sided, multiplicity adjusted P values, mean differences, and 95% confidence intervals are reported.

Supplementary Table 9. GPRC5D genome accessibility peak-to-gene links identified by ArchR⁷. Genomic positions and false discovery rates (FDR) included. Gene body positions are highlighted in blue. Regulatory regions are highlighted in red.

Supplementary Table 10. A. List of antibodies, reagents, and **B.** cell lines used in this study.

Supplementary Table 11. Wild type and mutant GPRC5D DNA sequences used for cloning into PLX307 constructs.

Supplementary Table 12. CLASTR short tandem repeat (STR) profiling results for OPM-2 multiple myeloma cell line. STR genotyping of the OPM2 cell line was analyzed using the CLASTR database to confirm cell line authenticity.

Supplementary Table 13. CLASTR short tandem repeat (STR) profiling results for JJN3 multiple myeloma cell line. STR genotyping of the JJN3 cell line was analyzed using the CLASTR database to confirm cell line authenticity.

References

1. Uhlen, M., *et al.* A pathology atlas of the human cancer transcriptome. *Science* **357**, eaan2507 (2017).
2. Rao, S.S., *et al.* A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. *Cell* **159**, 1665–1680 (2014).
3. Kerpedjiev, P., *et al.* HiGlass: web-based visual exploration and analysis of genome interaction maps. *Genome Biology* **19**, 125 (2018).
4. Nadeu, F., *et al.* IgCaller for reconstructing immunoglobulin gene rearrangements and oncogenic translocations from whole-genome sequencing in lymphoid neoplasms. *Nature Communications* **11**, 3390 (2020).
5. Shen, R. & Seshan, V.E. FACETS: allele-specific copy number and clonal heterogeneity analysis tool for high-throughput DNA sequencing. *Nucleic Acids Res* **44**, e131 (2016).
6. Shinde, J., *et al.* Palimpsest: an R package for studying mutational and structural variant signatures along clonal evolution in cancer. *Bioinformatics* **34**, 3380–3381 (2018).
7. Granja, J.M., *et al.* ArchR is a scalable software package for integrative single-cell chromatin accessibility analysis. *Nature Genetics* **53**, 403–411 (2021).