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Promoter hypermethylation as a reversible mechanism of resistance to GPRC5D-directed therapy in multiple myeloma

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Title:

Promoter hypermethylation as a reversible mechanism of resistance to GPRC5D-directed therapy in multiple myeloma

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Contributions:

S.H., U.M., J.M.W., and K.M.K. conceptualized and designed the study. Experimental work and data acquisition were conducted by S.H., M.T., E.G., L.P., A.H., S.K., S.N., C.V., E.B., N.R., J.L., and M.K. Data processing and statistical analyses were performed by S.H., U.M., M.T., E.G., F.S., Y.T., X.Z., T.S., C.H., A.R., J.M.W., and K.M.K. The manuscript was drafted by S.H., M.T., J.M.W., and K.M.K., and revised and approved by all authors.

Running head:

Promoter methylation drives anti-GPRC5D resistance

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Data sharing statement:

De-identified methylation data from whole-genome bisulfite sequencing (WGBS) are available under access number 10.5281/zenodo.17709806. Adherence to the Datenschutz-Grundverordnung (https://dsgvo-gesetz.de/ and https://data.consilium.europa.eu/doc/document/ST-5419-2016-REV[1]1/en/pdf) is mandatory for sharing genome-wide sequencing data. Thus, the raw genome-wide sequencing data of a single patient cannot be shared according to European law. Processed data from which the identification of a patient is not possible can be made available upon reasonable request.

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To the Editor:

GPRC5D-directed immunotherapies, such as the GPRC5D×CD3 bispecific antibody talquetamab (TAL) and the anti-GPRC5D CAR T-cell products MCARH109 and Arlocabtagene autoleucel (Arlo-cel), have shown significant clinical activity in heavily pretreated multiple myeloma (MM) patients[1-3]. Most patients however eventually develop resistance, and while biallelic loss of *GPRC5D* on chromosome 12p13.1 is now established as a resistance mechanism in GPRC5D-exposed patients[4-6], Derrien and colleagues have recently described long-range chromatin closure and loss of promoter accessibility at the *GPRC5D* locus as a novel form of epigenetic resistance in talquetamab-resistant patients[7]. Similarly, Ma and colleagues demonstrated that altered *GPRC5D* promoter methylation results in antigen loss in patients relapsing after anti-GPRC5D CAR-T cell therapy[8]. Although both studies offered novel mechanistic insights, direct functional implications of *GPRC5D* promoter hypermethylation as a reversible and putatively druggable mechanism of target evasion have yet to be demonstrated.

Building on our previously published findings[9], we first analyzed an expanded whole-genome sequencing (WGS) dataset comprising 402 TAL-naïve patients. All patients provided informed consent in accordance with the Declaration of Helsinki (Ethics Vote 8/21, University of Wuerzburg). Monoallelic *GPRC5D* alterations (including deletions, frameshift or missense mutations) were detected in 12.9% of cases, whereas the frequency of *GPRC5D* loss, defined as either deletion or copy-neutral loss of heterozygosity (CN-LOH), was substantially enriched at 61.5% (8/13) in a second cohort of TAL-exposed patients with more extensive pretreatment (**Figure 1A**). Of these TAL-exposed patients, 4/8 patients exhibited clonal biallelic *GPRC5D* alterations (biallelic deletions or CN-LOH with a clonal mutation), whereas the remaining four patients carried isolated monoallelic deletions or monoallelic loss combined with subclonal events.

From the subset of patients with monoallelic *GPRC5D* alterations, we here report on a 58-year-old patient (MM1) with penta-refractory high-risk MM, including del17p and high-risk gene expression profiling by Sky-92 testing[10]. In his tenth line of therapy, the patient was treated with TAL and daratumumab as part of the phase Ib TRIMM-2 clinical trial (*NCT04108195*) and achieved a stringent complete remission (sCR) according to IMWG criteria lasting for a total of 15 months. At relapse, WGS of bone marrow-derived $CD138^+$ plasma cells revealed an acquired monoallelic 12p deletion spanning 23.3 Mb, which included the *GPRC5D* gene, along with three subclones carrying *GPRC5D* mutations (p.Tyr12*, VAF 6%; p.Trp126*, VAF 9%; p.Trp199*, VAF 5%) (**Figure 1B**). Immunohistochemistry (IHC) analysis demonstrated complete loss at protein level (**Figure 1C**) and a 94% reduction of *GPRC5D* mRNA expression ($p < 0.0001$) by quantitative PCR (qPCR) (**Figure 1D**). Given the subclonal nature of the single-nucleotide variants (SNVs)

which affected the remaining allele, we concluded that these additional genomic events were unlikely to fully account for the near-complete loss of *GPRC5D* expression, but rather support a scenario in which a second epigenetic hit, e.g. by promoter methylation, may have contributed to inactivation of the residual allele[11].

To further explore this hypothesis, we assessed promoter methylation in MM1 using whole-genome bisulfite sequencing (WGBS). This analysis revealed a 26.5% increase in *GPRC5D* promoter methylation in the TAL relapse sample of MM1 compared to baseline (**Figure 2A**). In further validation of this observation, we generated CRISPR-Cas9 OPM2 MM cell line models with either monoallelic or biallelic *GPRC5D* knockouts (KO). Biallelic KO models showed an expected 91.5% reduction in *GPRC5D* mRNA levels, whereas monoallelic alterations reduced *GPRC5D* expression to 57.2% of WT (42.8% reduction) (**Figure 2B**), confirming that monoallelic gene loss was indeed not sufficient to fully abrogate *GPRC5D* expression. We next investigated if altered promoter methylation may account for the loss of *GPRC5D* expression in MM1. To this end, regulatory promoter activity was studied by Firefly/Renilla dual-luciferase assay[12] using a CpG-free vector (CpGL) containing the *GPRC5D* promoter and the differentially methylated segment for chr12 (12,952,081-12,952,935). *In vitro* methylation reduced the reporter activity by ~278-fold as compared to the unmethylated construct (**Figure 2C)**, providing direct evidence that hypermethylation of this regulatory element suppresses the transcriptional activity of *GPRC5D*. Next, AMO1 MM cells, intrinsically characterized by a hypermethylated *GPRC5D* promoter, were treated with the pre-clinical non-covalent DNA methyltransferase 1 inhibitor (DNMTi) GSK-3484862. DNMT1 inhibition led to a 2.2-fold increase in *GPRC5D* expression by day 3 and a 5.9-fold increase at day 7 after treatment (**Figure 2D)** along with a significant decrease of promoter methylation (mean methylation difference: -8.6%, $p < 0.001$, **Figure 2E)**.

Taken together, the functional and clinical data presented in this report support an epigenetic second-hit model, in which promoter hypermethylation contributes to *GPRC5D* downregulation under the selective pressure of GPRC5D-targeted therapy.

Over the past years, cellular plasticity and epigenetic adaptation, either through permissive chromatin accessibility or enhancer rewiring, have emerged as key mechanisms of resistance in cancer which enable rapid cellular adaptation without the need for genetic alterations[13, 14]. In an elegant study, Derrien and colleagues previously combined WGS with single-nucleus RNA- and ATAC-sequencing to describe loss of promoter accessibility together with long-range chromatin closure (~1.15 Mb) around the *GPRC5D* locus in TAL-resistant patients. These observations indicate that epigenetic repression of the promoter may contribute to genetic silencing and subsequent antigen loss[7]. Beyond chromatin accessibility, Ma and colleagues provided further single-base-resolved DNA methylation data to describe multiple hypermethylated CpGs across *GPRC5D* regulatory regions in post-CAR-

T relapses lacking biallelic genetic lesions[8]. The methylation levels of these regions showed an inverse correlation with GPRC5D mRNA expression across MM cell lines. However, the specific regulatory elements demethylated by treatment, as well as a direct causal relationship at the promoter site, were not identified. Our study adds to this gap in knowledge by demonstrating a direct link between epigenetic alterations at the *GPRC5D* promoter and reduced *GPRC5D* expression. Using CRISPR gene edited mono- versus biallelic *GPRC5D* knockout models, we further delineate that monoallelic *GPRC5D* loss does not fully abolish expression but rather implicates the need for a second epigenetic hit for full transcriptional loss. These findings propose a model in which MM patients with baseline monoallelic GPRC5D deletions may be particularly susceptible to secondary promoter hypermethylation (**Figure 2F**). Moreover, our data provide first evidence that promoter hypermethylation can be overcome by targeted inhibition of DNA methyltransferases. This co-treatment strategy, which aims at preventing target evasion, may have broader implications for overcoming epigenetic resistance to immunotherapies across other targets, including the BCMA-coding gene *TNFRSF17*[15], as well as for other malignancies beyond MM.

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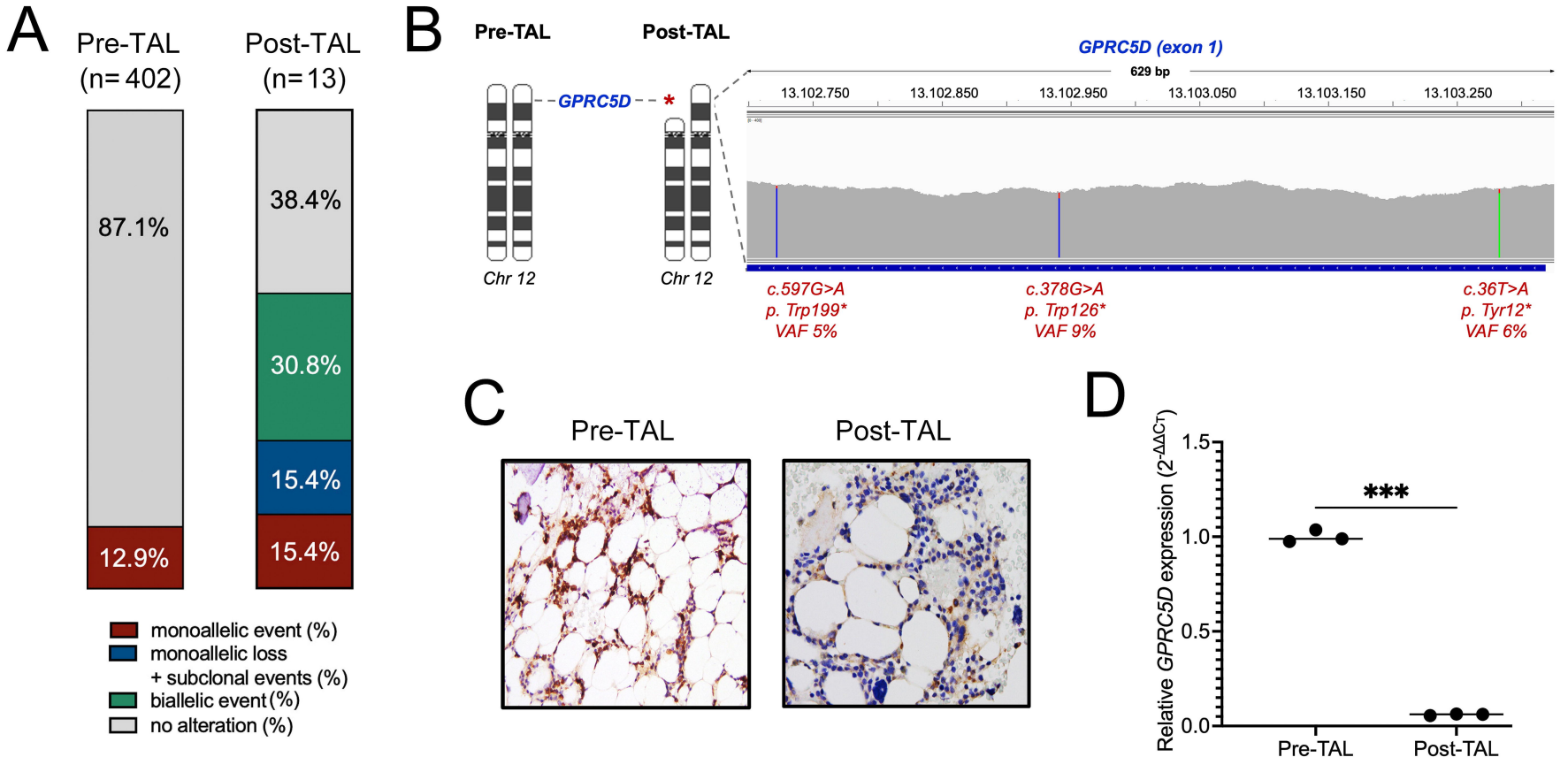
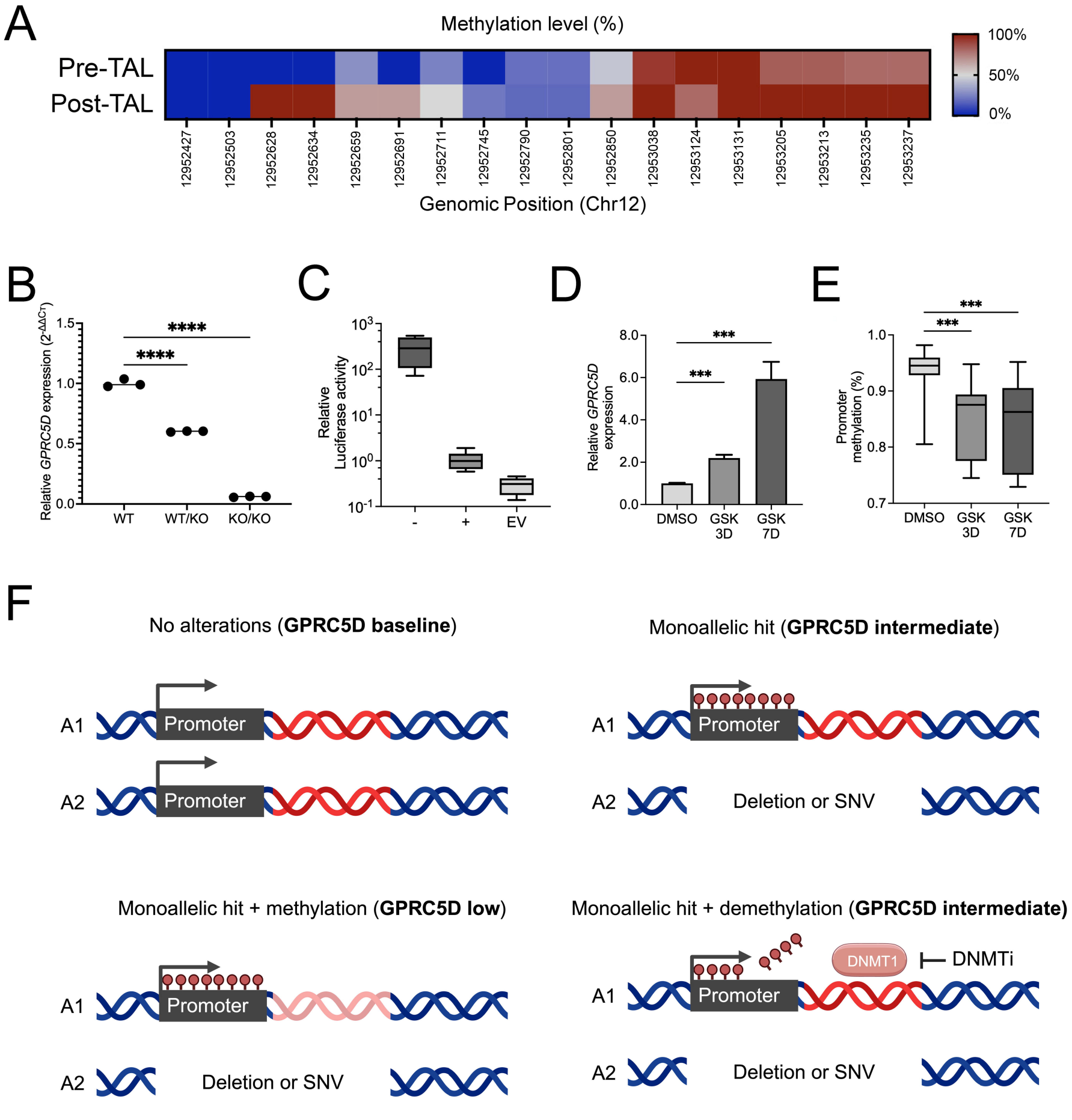
Figure Legends:

Figure 1. Clinical and functional evidence of monoallelic *GPRC5D* loss. (A) Bar chart demonstrating frequency of *GPRC5D* alterations in TAL-naïve (n = 402) vs. TAL-exposed MM patients (n = 13). **(B)** 23.3 Mb monoallelic deletion on chromosome 12p including the *GPRC5D* gene and three subclonal *GRPC5D* mutations detected in MM1 by whole-genome sequencing. **(C)** Immunohistochemistry showing complete loss of GPRC5D at relapse. **(D)** qPCR analysis showing a 94% reduction of *GPRC5D* mRNA expression at TAL relapse vs. baseline. *Abbreviations: TAL= talquetamab, GPRC5D= G-protein coupled receptor class C group 5 member D*

Figure 2. Functional validation of promoter hypermethylation and therapeutic intervention *in vitro*. (A) Whole-genome bisulfite sequencing (WGBS) in MM1 at relapse vs. baseline visualizes increase in *GPRC5D* promoter methylation. **(B)** qPCR analysis comparing *GPRC5D* mRNA expression in OPM2 with wildtype vs. monoallelic vs. biallelic knockout status.
(C) Dual-luciferase reporter assay using CpG-free constructs containing the unmethylated (-) *GPRC5D* promoter region vs. methylated construct (+) vs. empty vector (EV). **(D)** qPCR analysis for *GPRC5D* mRNA expression in AMO1 cells treated with the DNMTi GSK-3484862 after 3 and 7 days of treatment. **(E)** Promoter methylation in AMO1 cells treated with GSK-3484862 after 3 and 7 days of treatment. **(F)** Proposed mechanism of *GPRC5D* regulation in patients with pre-existing monoallelic alterations, suggesting promoter hypermethylation as a second hit and potential target for DNMTi. *Abbreviations: TAL= talquetamab, GPRC5D= G-protein coupled receptor class C group 5 member D, DNMTi= DNA methyltransferase 1 inhibitor, GSK= GSK-3484862, 3D/7D= after 3/7 days, DMSO= dimethyl sulfoxide. Figure 2F created in BioRender under publication license https://BioRender.com/hv0ij0d.*


A
Pre-TAL
(n= 402)
Post-TAL
(n= 13)
87.1%
12.9%
38.4%
30.8%
15.4%
15.4%
monoallelic event (%)
monoallelic loss
+ subclonal events (%)
biallelic event (%)
no alteration (%)
B
Pre-TAL
Post-TAL
GPRC5D
Chr 12
Chr 12
GPRC5D (exon 1)
629 bp
13.102.750
13.102.850
13.102.950
13.103.050
13.103.150
13.103.250
c.597G>A
p. Trp199*
VAF 5%
c.378G>A
p. Trp126*
VAF 9%
c.36T>A
p. Tyr12*
VAF 6%
C
Pre-TAL
Post-TAL
D
Relative GPRC5D expression (2-ΔΔCT)
1.5
1.0
0.5
0.0
***
Pre-TAL
Post-TAL


A
Methylation level (%)
Pre-TAL
Post-TAL
100%
50%
0%
12952427
12952503
12952628
12952634
12952659
12952691
12952711
12952745
12952790
12952801
12952850
12953038
12953124
12953131
12953205
12953213
12953235
12953237
Genomic Position (Chr12)
B
Relative GPRC5D expression (2^-ΔΔCT)
****
****
WT
WT/KO
KO/KO
C
Relative Luciferase activity
-
+
EV
D
Relative GPRC5D expression
***
***
DMSO
GSK 3D
GSK 7D
E
Promoter methylation (%)
***
***
DMSO
GSK 3D
GSK 7D
F
No alterations (GPRC5D baseline)
A1
Promoter
A2
Promoter
Monoallelic hit (GPRC5D intermediate)
A1
Promoter
A2
Deletion or SNV
Monoallelic hit + methylation (GPRC5D low)
A1
Promoter
A2
Deletion or SNV
Monoallelic hit + demethylation (GPRC5D intermediate)
DNMT1
DNMTi
A1
Promoter
A2
Deletion or SNV