The hydroxymethylome of multiple myeloma identifies FAM72D as a 1q21 marker linked to proliferation

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SUPPLEMENTAL DATA

SUPPLEMENTAL METHODS

Human Myeloma Cell Lines (HMCLs)

XG human myeloma cell lines were obtained as previously described.[1] 1 HMCLs were cultured in the presence of recombinant IL-6. HMCLs were authenticated according to their short tandem repeat profiling and their gene expression profiling using Affymetrix U133 plus 2.0 microarrays deposited in the ArrayExpress public database under accession numbers E-TABM-937 and E-TABM-1088.[1]

Construction of HMCLs overexpressing *FAM72D*

FAM72D cDNA was cloned in the pLenti4-mGFP-Tagged cloning vector (Origene). HMCLs were transduced with *FAM72D* lentiviruses (MOI = 2) and stable transduced cells were obtained after selection using cell sorter. HMCLs transduced with control lentiviruses were used as control.[2,3]

Cell growth assay

HMCLs were cultured for 4 days in RPMI 1640 medium, 10% FCS, and 2 ng/ml IL-6 (control medium) in the presence or absence of graded concentrations of FDI-6 (Sigma). HMCLs overexpressing or not FAM72D were then IL-6- and serum-starved for 2 hours and cultured for 4 days in 96-well flat-bottom microtiter plates in serum-free culture medium without cytokine (control) or with graded concentrations of IL-6 as described.[4,5] Cell growth was evaluated by quantifying intracellular ATP amount with a Cell Titer Glow Luminescent Assay (Promega, Madison, WI, USA) using a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Sensitivity of primary myeloma cells to HDACi/DNMTi combination

Primary myeloma cells of 17 patients were cultured with or without 2 µM 5azacitidine and 300 nM SAHA (Sigma). MMC cytotoxicity was evaluated using anti- CD138-PE monoclonal antibody (Immunotech, Marseille, France) as described.[6]

Identification of genes deregulated by the HDACi/DNMTi combination

HMCLs were treated with 0.5 µmol/L decitabine (Sigma, St Louis, MO) for 7 days in RPMI 1640, 10% fetal bovine serum supplemented with IL-6 for IL-6-dependent HMCLs. During the last 24 h, 0.33 µmol/L trichostatin A (TSA; Sigma) was added as described.[6] Whole-genome GEP was assayed with Affymetrix U133 2.0 plus microarrays (Affymetrix).

Knock-down of FAM72D in MCF-7 cells and mitotic defect analysis

For siRNA experiments, MCF-7 cells were plated in 6-well plates (250,000 cells/well) in DMEM supplemented with 10% fetal calf serum and antibiotics. After 24 hours, cells were switch to opti-MEM medium and transfected for 6 hours with 10 nM of control or FAM72D siRNAs (Cohesion Biosciences, ref CRJ8695) diluted in opti-MEM and oligofectamine (Thermo Fischer Scientific). After transfection, cells were further cultured for 48 hours in DMEM supplemented with 10% serum before processing either for RT-qPCR or mitotic defect analysis. Total RNAs were extracted with TRIzol (Invitrogen) and reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) and Pd(N)6 random hexamers (Amersham Pharmacia Biosciences). Analysis of the efficiency of the siRNAs to knock-down FAM72D was assessed by qPCR using SYBR green master mix (Biorad) and the following oligonucleotides: FAM72D forward: 5'-tgtgattgttccatgtagttcct-3', FAM72D reverse: 5'-actctcttctatctctggcaagt-3',

FOXM1 forward: 5'-gccatcaacagcactgagag-3', FOXM1 reverse: 5'-tggggtgaatggtccagaag-3', NUF2 forward: 5'-gccgggtgaatgactttgag-3', NUF2 reverse: 5'-tttcacggcatgcttctctg-3'. For phenotypic analysis, cells grown on coverslips were fixed with 4% paraformaldehyde for 10 minutes and permeabilized for 15 minutes in 0.2% Triton X-100 in PBS. Cells were next mounted in Vectashield medium (VECTOR Laboratories) containing DAPI. All experiments were run in triplicates and repeated 3 times. A total of 180 anaphases were monitored for lagging chromatids, 180 metaphases for misaligned chromosomes, and 1,800 cells for micronuclei in both control and FAM72D siRNA conditions.

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SUPPLEMENTAL FIGURES S1 to S10 (pages 5 to 14)

A

oxBS-450K		SCL-exo		SCL-seq	
GO Biological Process	**raw *P*-value**	**GO Biological Process**	**raw *P*-value**	**GO Biological Process**	**raw *P*-value**
antigen processing and presentation of exogenous peptide antigen via MHC class I	3.6860e-36	apoptotic signaling pathway	0.0000	cytokine-mediated signaling pathway	1.1114e-306
		response to endoplasmic reticulum stress	0.0000	immune response-activating cell surface receptor signaling pathway	2.0178e-212
antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	6.0225e-36	immune response-activating cell surface receptor signaling pathway	5.7229e-308	intrinsic apoptotic signaling pathway	7.1023e-199
		Notch signaling pathway	1.7355e-284	response to endoplasmic reticulum stress	1.1773e-172
Mouse Phenotypes	**raw *P*-value**	**Mouse phenotypes**	**raw *P*-value**	**Mouse phenotypes**	**raw *P*-value**
No terms		abnormal B cell differentiation	0.0000	abnormal B cell differentiation	0.0000
		abnormal B cell proliferation	0.0000	abnormal B cell proliferation	0.0000
		abnormal mature B cell morphology	0.0000	abnormal mature B cell morphology	0.0000
		increased hemolymphoid system tumor incidence	0.0000	abnormal hematopoietic system physiology	0.0000
				abnormal lymph node size	0.0000
Disease Ontology	**raw *P*-value**	**Disease Ontology**	**raw *P*-value**	**Disease Ontology**	**raw *P*-value**
No terms		chronic leukemia	0.0000	herpes simplex	4.9466e-115
		dsDNA virus infectious disease	0.0000	Hodgkin's lymphoma	1.3254e-84
		chronic lymphocytic leukemia	0.0000	progressive multifocal leukoencephalopathy	2.2362e-76
		head and neck squamous cell carcinoma	0.0000		
MSigDB Pathway	**raw *P*-value**	**MSigDB Pathway**	**raw *P*-value**	**MSigDB Pathway**	**raw *P*-value**
No terms		Genes involved in Signaling by NOTCH	0.0000	Genes involved in Cytokine Signaling in Immune system	1.2445e-262
		C-MYB transcription factor network	0.0000		
		Cell cycle	0.0000	Genes involved in Signaling by NOTCH	3.2818e-181
		IL2-mediated signaling events	0.0000	Genes involved in Unfolded Protein Response	3.3832e-153
		Regulation of nuclear SMAD2/3 signaling	1.4831e-275	p38 MAPK signaling pathway	1.7645e-138
		Genes involved in Signaling by SCF-KIT	3.0805e-274	Regulation of nuclear SMAD2/3 signaling	7.1719e-127
		Caspase cascade in apoptosis	4.4654e-267	Genes involved in Signaling by SCF-KIT	2.5653e-126
		Chronic myeloid leukemia	4.2969e-265	IFN-gamma pathway	1.6899e-118
		MAPKinase Signaling Pathway	2.1543e-252	Validated targets of C-MYC transcriptional repression	3.2832e-118
		Regulation of Telomerase	1.6354e-237		
MSigDB Perturbation	**raw *P*-value**	**MSigDB Perturbation**	**raw *P*-value**	**MSigDB Perturbation**	**raw *P*-value**
Genes within amplicon 16q24 identified in a copy number alterations study of 191 breast tumor samples.	5.0686e-320	Genes down-regulated in multiple myeloma (MM) cell lines treated with both decitabine [PubChem=451668] TSA [PubChem=5562].	0.0000	Genes down-regulated in multiple myeloma (MM) cell lines treated with both decitabine [PubChem=451668] TSA [PubChem=5562].	0.0000
Genes within amplicon 7p22 identified in a copy number alterations study of 191 breast tumor samples.	2.1938e-173	Genes down-regulated in the MM1S cells (multiple myeloma) after treatment with aplidin [PubChem=44152164], a marine-derived compound with potential anti-cancer properties.	0.0000	Up-regulated genes in B-CLL (B-cell chronic leukemia) patients expressing high levels of ZAP70 and CD38 [GeneID=7535;952], which are associated with poor survival.	0.0000
Genes within amplicon 17q21-q25 identified in a copy number alterations study of 191 breast tumor samples.	6.2204e-97	IRF4 [GeneID=3662] target genes up-regulated in primary myeloma vs. mature B lymphocytes.	0.0000	IRF4 [GeneID=3662] target genes up-regulated in primary myeloma vs. mature B lymphocytes.	0.0000
		Genes up-regulated in plasma cells compared with B lymphocytes.	0.0000	Genes up-regulated in plasma cells compared with B lymphocytes.	0.0000
		The 'MLL signature 1': genes up-regulated in pediatric AML (acute myeloid leukemia) with rearranged MLL [GeneID=4297] compared to all AML cases with the intact gene.	0.0000	The 'MLL signature 1': genes up-regulated in pediatric AML (acute myeloid leukemia) with rearranged MLL [GeneID=4297] compared to all AML cases with the intact gene.	0.0000

B

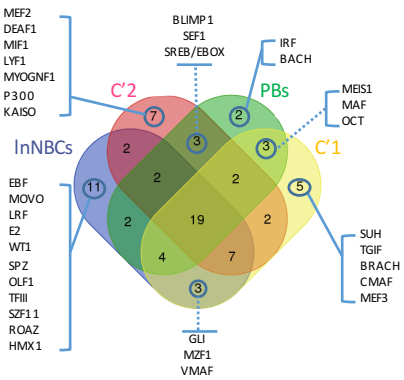


Figure S1: SCL-exo and SCL identify active genomic regions in MM patients. (**A**) Aggregated 5hmC positive regions (40,586 CpGs for oxBS-450K; 86,591 CpGs for SCL-exo; 64,424 regions for SCL-seq) were annotated using GREAT with default settings. (**B**) C'1 and C'2 subgroup-specific sets of 5hmCpGs obtained through heatmap clustering were analyzed for the presence of transcription factor binding motifs in their vicinity. Sets of significantly enriched (p<0.05) motifs were compared to motifs enriched in lymph node NBCs and PBs through a Venn diagram.

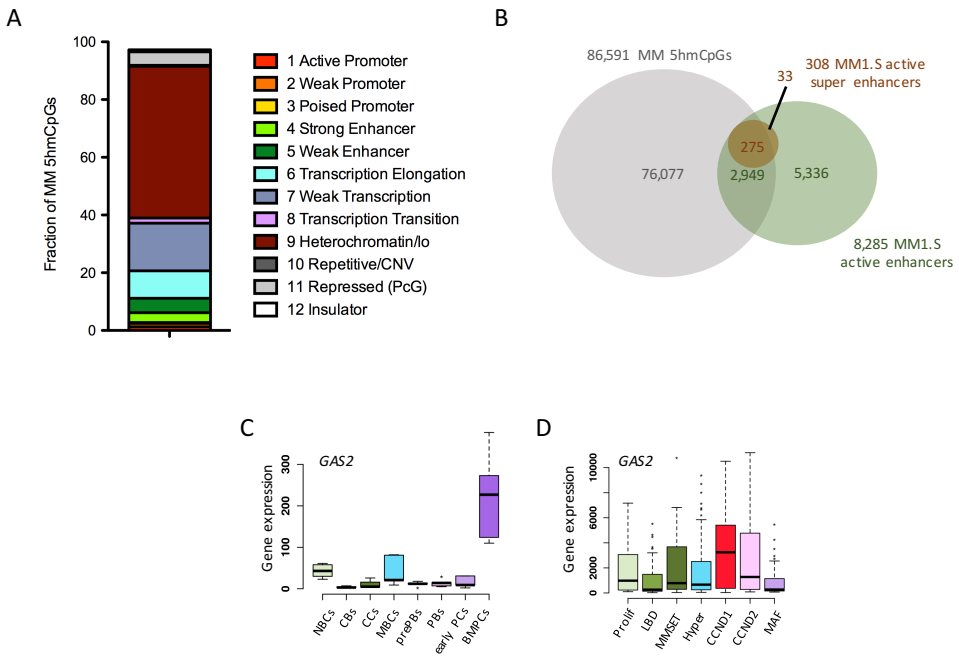


Figure S2: SCL-exo and SCL identify active genomic regions in MM patients. (**A**) Association of the MM 5hmCpGs with chromatin states (ChromHMM) from the GM12878 lymphoblastoid cell line. (**B**) Venn diagram analysis of the distribution of MM 5hmCpGs in genomic regions identified as active enhancers and active super enhancers (SEs) in MM1.S cells. (**C**) *GAS2* expression levels in normal cells: naive B cells (NBCs), centroblasts (CBs), centrocytes (CCs), memory B cells (MBCs), pre-plasmablasts (prePBs), plasmablasts (PBs), early plasma cells (early PCs), and bone marrow plasma cells (BMPCs). Graphs were generated by GenomicScape (http://genomicscape.com/). (**D**) *GAS2* expression levels in MMPCs from patients of the Arkansas cohort (n=414) classified in the following molecular groups: proliferation (Prolif), low bone disease (LBD), MMSET, hyperdiploid (Hyper), CCND1, CCND2, and MAF.

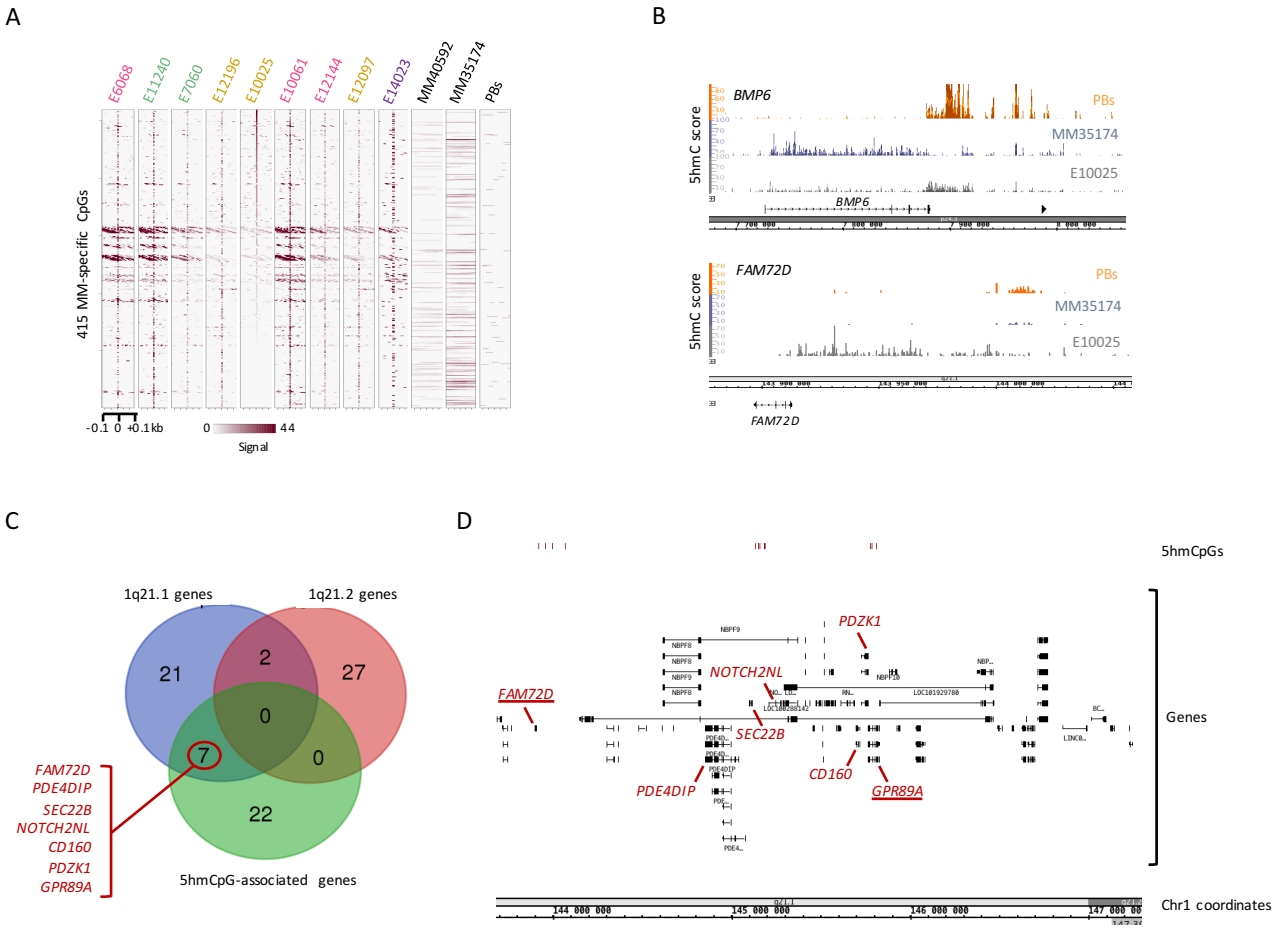


Figure S3: The 1q21.1 region is enriched in 5hmCpG-associated genes. (**A**) Heatmap of the 5hmC signal at 415 MM-specific 5hmCpGs. (**B**) Integrated genome browser (IGB) view of the 5hmC signal at the *BMP6* and *FAM72D* loci in PB (orange), MM35174 (dark blue) and E10025 (grey) samples. (**C**) Venn diagram comparing the list of genes associated with 5hmC and those located at 1q21.1 and 1q21.2. (**D**) Genomic organization of the 1q21.1 cytoband. Genes associated with 5hmC are indicated in red font.

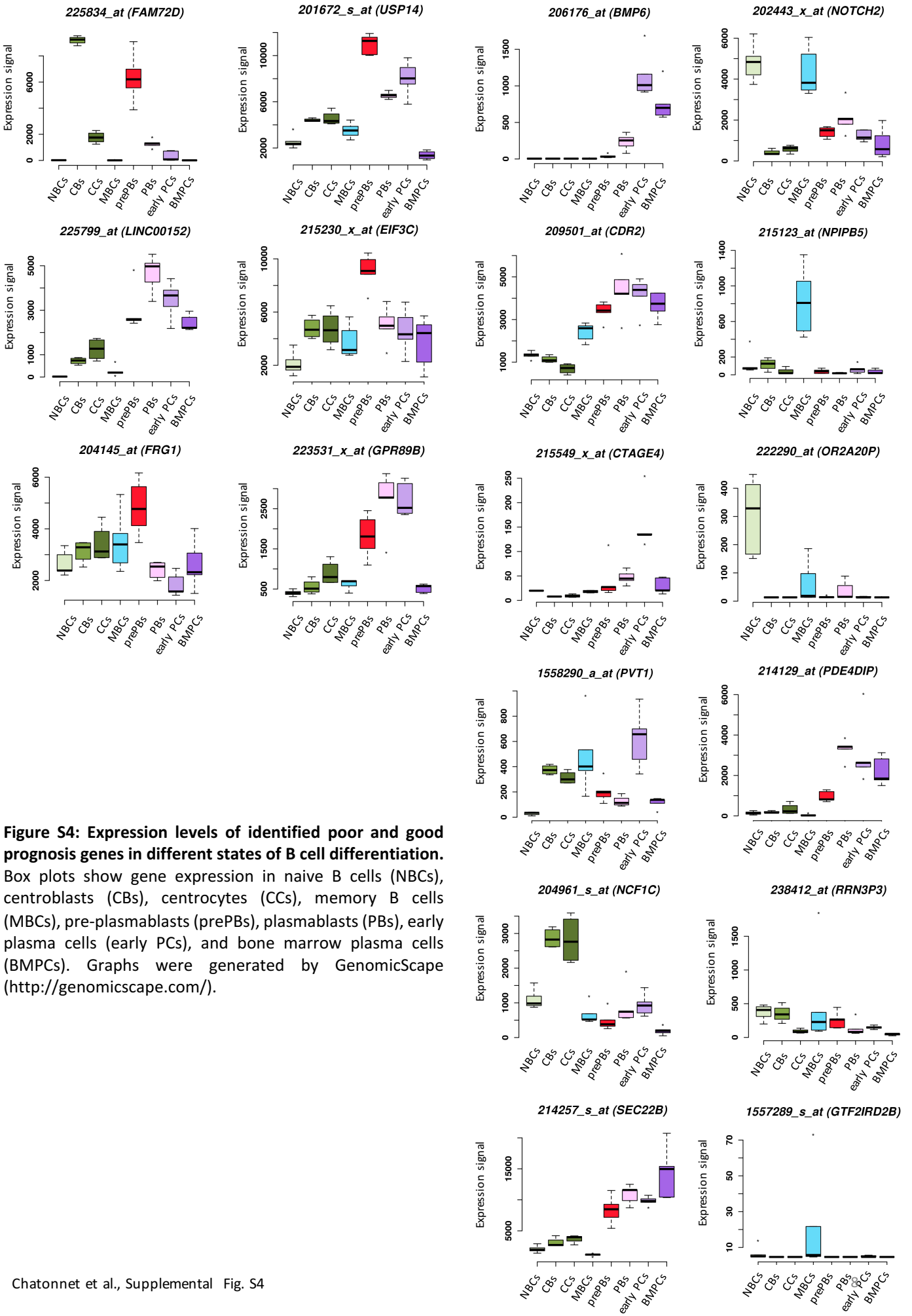


Figure S4: Expression levels of identified poor and good prognosis genes in different states of B cell differentiation. Box plots show gene expression in naive B cells (NBCs), centroblasts (CBs), centrocytes (CCs), memory B cells (MBCs), pre-plasmablasts (prePBs), plasmablasts (PBs), early plasma cells (early PCs), and bone marrow plasma cells (BMPCs). Graphs were generated by GenomicScape (http://genomicscape.com/).

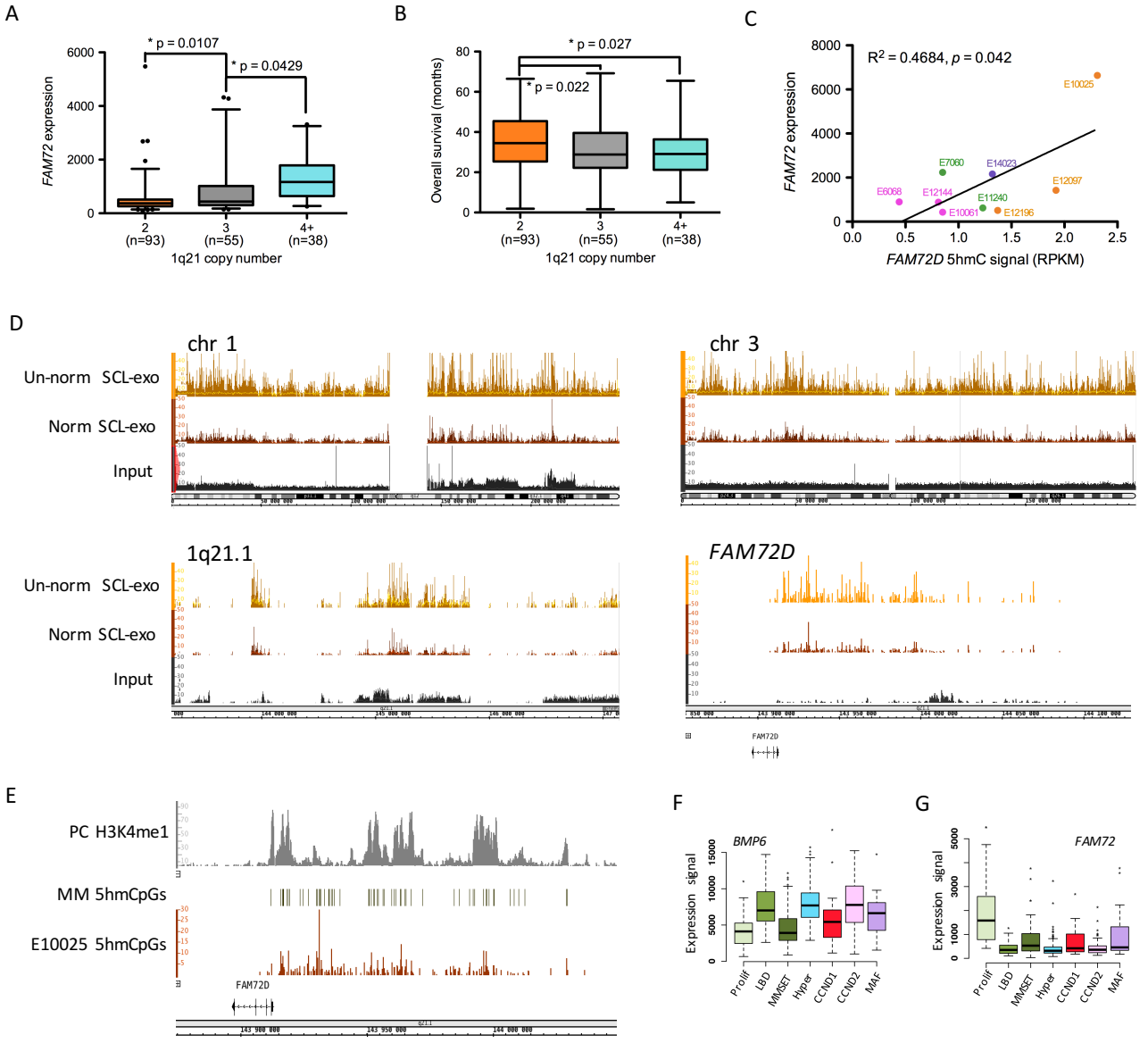
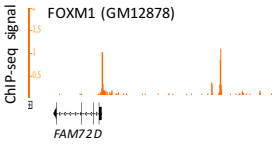


Figure S5: (**A**) *FAM72* expression in patients from the Arkansas cohort with 2, 3 or 4 and more (4+) copies of 1q21. (**B**) Overall survival of patients from the Arkansas cohort with 2, 3 or 4 and more (4+) copies of 1q21. (**C**) Correlation analysis of *FAM72D* hydroxymethylation (RPKM: reads per kb per million reads) and *FAM72* expression levels in MM patients. (**D**) Integrated genome browser (IGB) snapshots showing input-seq, un-normalized and normalized SCL-exo signals, along the full chromosome 1 (chr1) and 3 (chr3), as well as close up views of 1q21.1 and the *FAM72D* locus. (**E**) IGB snapshot showing H3K4me1 signal form normal plasma cells (PC H3K4me1), the presence of highly significant MM 5hmCpGs, and the SCL-exo signal from E10025 at the *FAM72D* locus. (**F**) *BMP6* expression levels in the different molecular subgroups from the Arkansas cohort. (**G**) *FAM72* expression levels in the different molecular subgroups from the Arkansas cohort.

A



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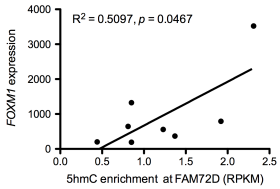
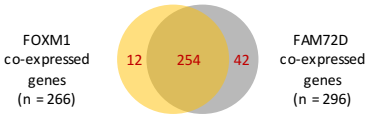


C



D

GO term CC	Benjamini
Kinesin complex	2e-8
Midbody	5e-7
Spindle microtubule	9e-5
Kinetochore	1e-2
Chromosome, centromeric region	1e-2

E

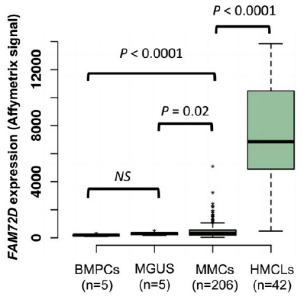


Figure S6: *FAM72* is part of *FOXM1* network. (**A**) IGB view of FOXM1 ChIP-seq signal at the *FAM72D* locus in GM12878 lymphoblastoid cells. (**B**) Correlation analysis of *FAM72D* hydroxymethylation (RPKM: reads per kb per million reads) and *FOXM1* expression levels in MM patients. (**C**) Venn diagram showing the overlap between genes coregulated (correlation coefficient above 0.5) with *FOXM1* or with *FAM72* in patients from the Proliferation group. (**D**) Functional annotation of the top-50 genes coregulated with *FAM72* in the proliferation group. Benjamini indicates the corrected *p*-values of these annotations. (**E**) Expression levels of *FAM72* in bone marrow plasma cells (BMPCs), monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma cells (MMCs) and human myeloma cell lines (HMCLs).

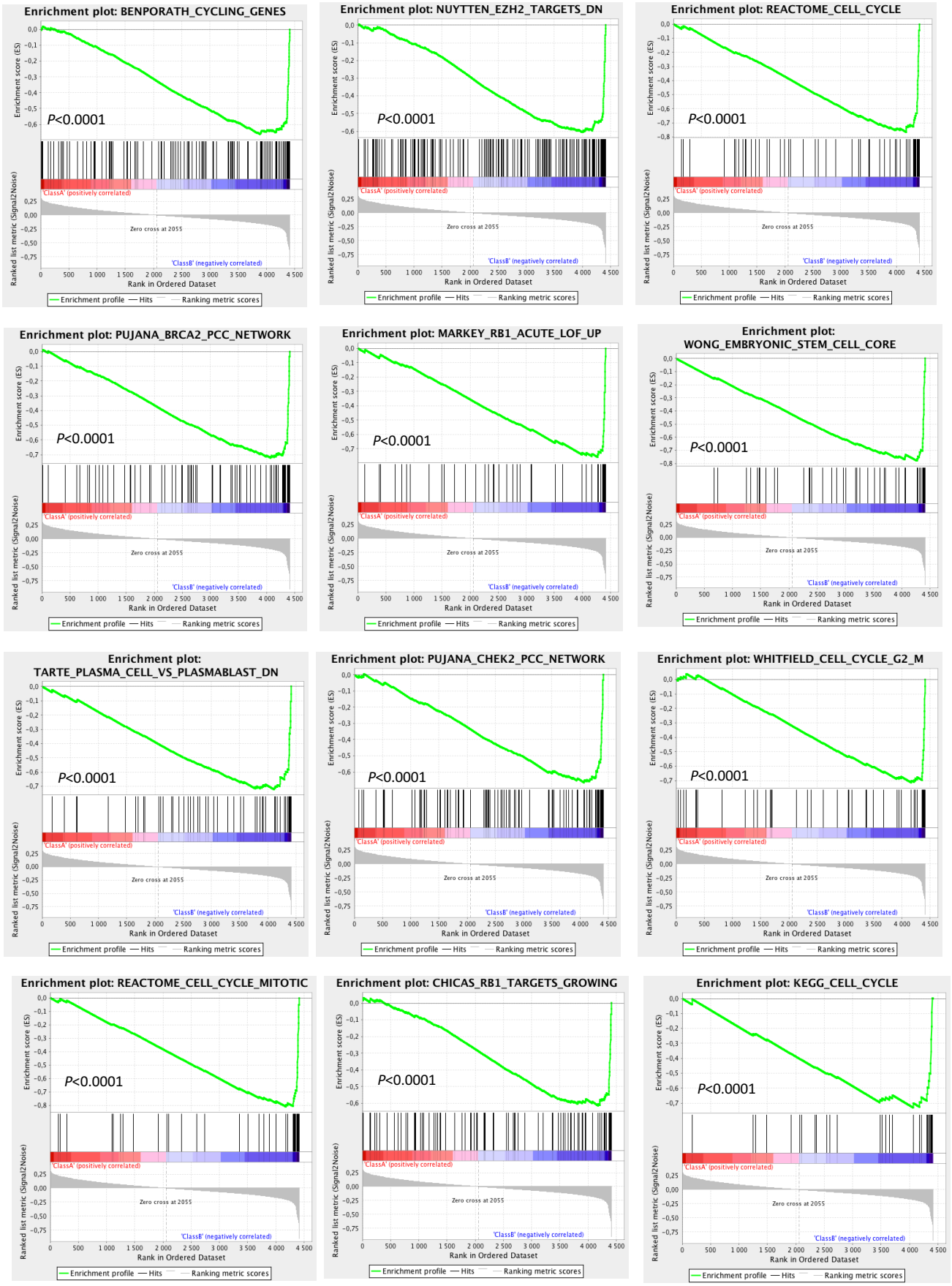


Figure S7: Top gene sets significantly associated with high *FAM72* expression in MM (TT2 cohort). GSEA enrichment plots with the absolute enrichment *P* value and the normalized enrichment score of the gene set.

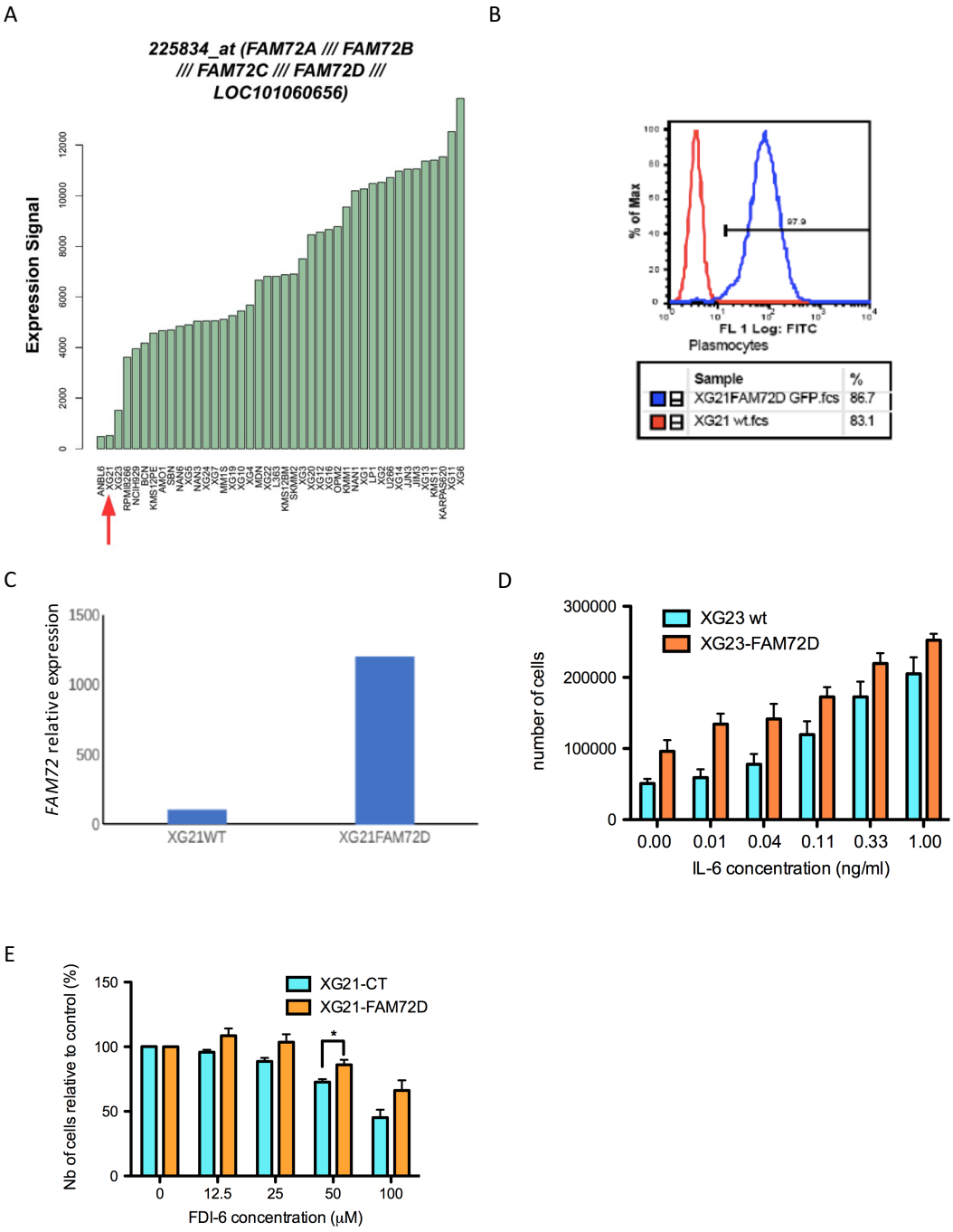


Figure S8: Overexpression of FAM72D::GFP in the multiple myeloma cell line XG21. (**A**) Expression levels of *FAM72* in human myeloma cell lines (HMCLs). Graphs in (A) and (B) were generated by GenomicScape (http://genomicscape.com/). (**B**) Fluorescence acquired cell sorting of XG21 cells expressing FAM72D::GFP. (**C**) RT-qPCR analysis of FAM72D expression in XG21 and XG21-FAM72D cells. (**D**) Cell proliferation assay with control and FAM72D-overexpressing XG23 cells in the presence of increasing concentrations of IL-6. (**E**) Impact of increasing concentration of FDI-6 on XG21 and XG21-FAM72D HMCLs.

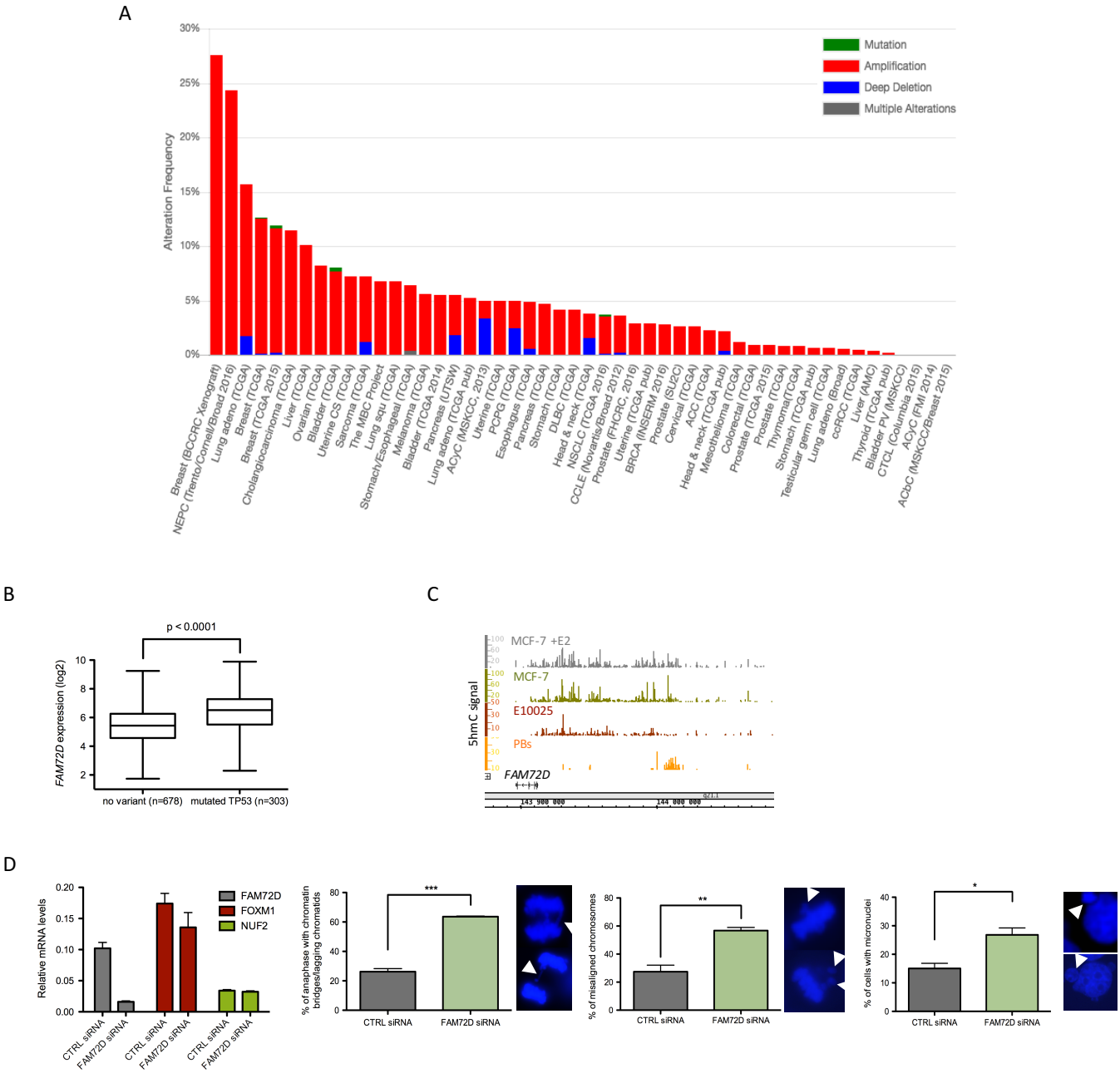


Figure S9: Alterations of FAM72D levels in breast cancer. (**A**) FAM72D alterations in various cancer samples (http://www.cbioportal.org/). (**B**) Box plot analysis of *FAM72D* gene expression as a function of TP53 mutations in 981 breast cancer patients from the TCGA database (breast invasive carcinoma cohort, The Cancer Genome Atlas). (**C**) IGB view of the 5hmC signal at the *FAM72D* locus in PBs, MM E10025, and MCF-7 samples. (**D**) Inactivation of FAM72D in MCF-7 cells. From left to right: RT-qPCR analysis of *FAM72D*, *FOXM1* and *NUF2* expression in MCF-7 cells transfected with control or FAM72D siRNAs; quantification of chromatin bridges and lagging chromatids in anaphases of control and *FAM72D* siRNA transfected MCF-7 cells; quantification of misaligned chromosomes in metaphases of control and *FAM72D* siRNA transfected MCF-7 cells; quantification of cells with micronuclei in control and *FAM72D* siRNA transfected MCF-7 cells. Images exemplify mitotic defects which are indicated by white arrowheads.

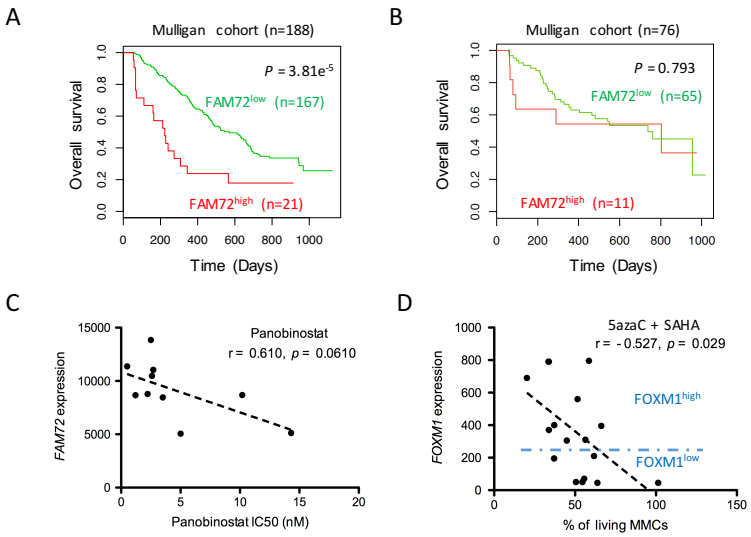


Figure S10: MM cells with high *FAM72* expression show distinct sensitivity to drugs. (**A**) High *FAM72* expression is associated with a shorter overall survival (OS) in a cohort of 188 patients at relapse treated with bortezomib monotherapy (Mulligan cohort). (**B**) OS in a cohort of 76 patients at relapse treated with dexamethasone monotherapy (Mulligan cohort). Cut-points for *FAM72* expression were similar in (A) and (B). (**C**) 10 HMCLs were cultured with graded concentrations of Panobinostat for 4 days and IC50 were calculated with mean values of five experiments determined on sextuplet culture wells. A trend between high *FAM72* expression (Affymetrix microarrays) and a higher sensitivity to Panobinostat was identified. (**D**) *FOXM1* expression predicts 5azacitidine/SAHA combination sensitivity of primary myeloma cells of patients. Mononuclear cells from tumor samples of 17 patients with MM were cultured for 4 days in the presence of IL-6 (2 ng/ml) with or without 2 µM 5azacitidine and 300 nM SAHA. At day 4 of culture, the count of viable MMCs was determined using CD138 staining by flow cytometry.