

# Metabolic, Anti-apoptotic and Immune Evasion Strategies of Primary Human Myeloma Cells Indicate Adaptations to Hypoxia

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## In Brief

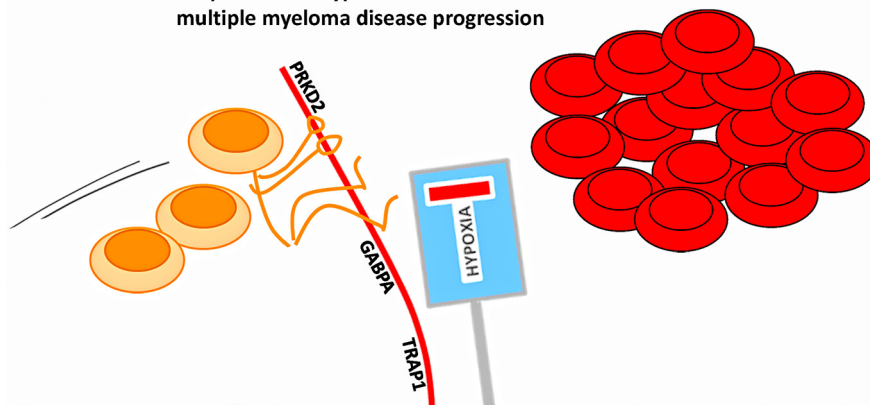
In-depth proteome profiling of primary human CD138-positive plasma cells, derived from bone marrow biopsies from patients with different stages of multiple myeloma, have been performed on a Q Exactive orbitrap. Analysis of the 6218 identified proteins using label-free quantification with the MaxQuant software revealed strategies adopted by myeloma cells to overcome limitations imposed by hypoxic conditions in the bone marrow microenvironment, including specific immune evasion mechanisms and metabolic adaptations.

## Highlights

- In-depth proteome profiling of primary human myeloma cells
- Characteristics of myeloma cells are related to hypoxic bone marrow conditions
- Myeloma cells show specific immune evasion strategies
- Metabolic adaptations involve tumor and stroma cells

## Graphical Abstract

Adaptations to hypoxia associated with multiple myeloma disease progression





# Metabolic, Anti-apoptotic and Immune Evasion Strategies of Primary Human Myeloma Cells Indicate Adaptations to Hypoxia\*<sup>§</sup>

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Multiple Myeloma (MM) is an incurable plasma cell malignancy primarily localized within the bone marrow (BM). It develops from a premalignant stage, monoclonal gammopathy of undetermined significance (MGUS), often via an intermediate stage, smoldering MM (SMM). The mechanisms of MM progression have not yet been fully understood, all the more because patients with MGUS and SMM already carry similar initial mutations as found in MM cells. Over the last years, increased importance has been attributed to the tumor microenvironment and its role in the pathophysiology of the disease. Adaptations of MM cells to hypoxic conditions in the BM have been shown to contribute significantly to MM progression, independently from the genetic predispositions of the tumor cells. Searching for consequences of hypoxia-induced adaptations in primary human MM cells, CD138-positive plasma cells freshly isolated from BM of patients with different disease stages, comprising MGUS, SMM, and MM, were analyzed by proteome profiling, which resulted in the identification of 6218 proteins. Results have been made fully accessible via ProteomeXchange with identifier PXD010600. Data previously obtained from normal primary B cells were included for comparative purposes. A principle component analysis revealed three clusters, differentiating B cells as well as MM cells corresponding to less and more advanced disease stages. Comparing these three clusters pointed to the alteration of pathways indicating adaptations to hypoxic stress in MM cells on disease progression. Protein regulations indicating immune evasion strategies of MM cells were determined, supported by immunohistochemical staining, as well as transcription factors involved in MM development and progression. Protein regulatory networks related to metabolic adaptations of the cells became apparent. Results were strengthened by targeted analyses of a selected panel of metabolites in MM cells and MM-associated fibroblasts. Based on our data, new opportunities may arise for developing therapeutic strategies targeting myeloma disease

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Multiple myeloma (MM)<sup>1</sup> is a hematological tumor localized primarily in the bone marrow (BM), characterized by the proliferation of malignant antibody-secreting plasma cells. The disease is accompanied by severe health problems such as bone lesions, hypercalcemia, renal failure, anemia and immunodeficiency (1). Even though several new therapeutic strategies extending the survival time of patients have been developed over the last years, MM remains an incurable disease (2). Further, genetic alterations in MM cells are heterogeneous and it is difficult to predict disease course or therapeutic response for individual patients (3). Even though first-line treatments may be successful, relapses are to be expected and tumors then typically become more aggressive and more difficult to treat. MM develops from a premalignant stage, monoclonal gammopathy of undetermined significance (MGUS), and typically progresses through an intermediary stage, smoldering MM (SMM). It is still not completely understood what drives this progression, as patients with MGUS and SMM already carry similar mutations as found in MM cells, suggesting that these mutations are necessary but not enough for tumor development (4–6).

Over the last years, the tumor microenvironment has been recognized to play an important role in MM progression, as well as in immune evasion and drug resistance of MM cells (7, 8). New therapeutic approaches have thus been developed, targeting not only the tumor itself but also tumor-supporting stromal cells such as fibroblasts and endothelial cells, as well as cells of the immune system (9, 10). Further, significance has been attributed to hypoxic conditions in the BM and the role of hypoxia in MM biology, offering novel possibilities for treatment strategies (11–13). MM cells encountering hypoxic

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environments in the BM apparently enhance pathways that allow them to survive and proliferate under these conditions. Consequently, processes such as angiogenesis and anti-apoptotic survival and proliferation strategies may be induced, and alternative metabolic pathways may be activated in the tumor cells (14–16). As a result, hypoxia-surviving MM cells may become more aggressive and more resistant to therapies (17).

Recent advances in proteomics technologies have given us the opportunity to better understand cellular processes involved in development and progression of diseases (18–22). Proteomics has already been used by others to assess specific aspects of MM such as development of drug resistance (23–26). In line with a recent work on chronic lymphocytic leukemia (CLL) B cells (22), in the present study we have investigated primary human multiple myeloma (MM) cells. The aim was to apply proteome profiling to get deeper insights into pathways MM cells follow in response to hypoxic conditions in the BM microenvironment. Hypoxia-related proteome signatures of MM cells, associated with survival, proliferation and mechanisms to escape apoptosis and immune response were investigated, as well as new targets for therapeutic interventions. To this end, we analyzed primary human CD138-positive plasma cells freshly isolated from the BM of patients with different disease stages, comprising MGUS, SMM, and MM. Another aim was to find out if, like chronic lymphocytic leukemia cells (22), MM cells of individual donors, despite MM being associated with a rather heterogeneous genotype, would show consistent protein patterns indicating pathways commonly adapted by MM cells. The study design should also allow investigating whether there were differences in the proteome of cells from premalignant and earlier stages compared with more advanced stages of the disease, which could provide insights into the pathogenesis and progression of multiple myeloma.

#### EXPERIMENTAL PROCEDURES

**Study Cohort and Bone Marrow Sampling**—For proteome profiling experiments, bone marrow (BM) samples from thirteen patients with different stages of multiple myeloma (MM), including monoclonal gammopathy of undetermined significance (MGUS), smoldering MM (SMM) and diagnosed MM, were obtained from routinely taken BM aspirates at the Vienna General Hospital. Written informed consent was obtained from all patients, as well as approval of the Ethics Committee of the Medical University of Vienna (application nr. 1181/2013). In the same way another five BM aspirates were taken from patients with diagnosed MM for targeted analyses of a selected panel

<sup>1</sup> The abbreviations used are: MM, multiple myeloma; BM, bone marrow; ER, endoplasmic reticulum; FASP, filter aided sample preparation; GO, gene ontology; HCD, high-energy collisional dissociation; IR, infiltration rate; LDL, low density lipoprotein; LFQ, label-free quantification; MACS, magnetic activated cell sorting; MGUS, monoclonal gammopathy of undetermined significance; MHC, major histocompatibility complex; MWCO, molecular weight cut off; PCA, principle component analysis; SMM, smoldering multiple myeloma; TF, transcription factor.

of metabolites (described below). From three of these samples, plasma cells were isolated. From the two other samples, as well as from two biopsies also used for proteomics experiments (indicated in Table I), BM fibroblasts were prepared as described below. Both, BM plasma cells and BM fibroblasts were subjected to the analysis of selected metabolites.

For immunohistochemical staining, BM biopsies from eight patients with different stages of MM were used. These biopsies were obtained for routine diagnostics at the University Hospital of Regensburg. No additional biopsies for research purposes were taken. After routine diagnostics, remaining material was used for immunohistochemistry (IHC) with approval of the Ethics Committee of the University of Regensburg (application nr. 051097). Clinical parameters, as well as if patients have obtained MM-specific treatments or not, are indicated in Table I for all patients included in the present study.

**Plasma Cell Isolation**—Bone marrow aspirates were filtered through 40  $\mu$ m filter (40  $\mu$ m Nylon Cell Stainer, BD Falcon), diluted 1:2 with PBS, overlaid on Ficoll Paque (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden), and centrifuged at  $720 \times g$  for 20 min at room temperature with minimal acceleration and deceleration settings. Mononuclear cells were collected from the resulting interface, were washed with PBS, then resuspended in PBS. Plasma cells were isolated using magnetic activated cell sorting (MACS). To this end, mononuclear cells were incubated at 4 °C for 15 min, after adding magnetic bead-coupled anti-CD138 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). After another PBS washing step, cells were resuspended in MACS buffer ( $1 \times$  PBS, 0.5% FBS, 2 mM EDTA) and pipetted onto a preconditioned MACS LS column mounted on a magnetic holder (both Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were washed with MACS buffer and CD138-positive plasma cells eluted by removing the MACS LS column from the magnet and pressing 4 ml of MACS buffer through the column. The plasma cell-containing eluate was diluted to 10 ml and cell number as well as viability was determined using the MOXI Z Mini Automated Cell Counter (ORFLO Technologies, Ketchum, ID). Cells were then pelleted by centrifugation at  $590 \times g$  for 5 min at 4 °C.

**Cell Lysis and Subcellular Fractionation of Primary Human Bone Marrow Plasma Cells**—Cell lysis and subcellular fractionation were performed applying a previously established protocol (27). In short, CD138-positive cells were resuspended in lysis buffer supplemented with protease inhibitors at 4 °C to achieve cell lysis. After centrifugation, the cytoplasmic fraction was collected in the supernatant. The pellet was dissolved in 500 mM NaCl solution and subsequently diluted in NP40-buffer; after centrifugation, nuclear protein extracts were collected in the supernatant. Cytoplasmic and nuclear proteins were precipitated in ice-cold ethanol overnight and solubilized in sample buffer (7.5 M urea, 1.5 M thiourea, 4% CHAPS, 0.05% SDS, 100 mM DTT). Protein concentrations were assessed by applying a Bradford assay (Bio-Rad-Laboratories, Vienna, Austria).

**Proteolytic Digestion and Sample Clean-up for LC-MS/MS Analysis**—Protein fractions were subjected to a filter-assisted proteolytic digestion with a modified version of the FASP protocol (28, 29). In short, 20  $\mu$ g of proteins were loaded onto a prewetted MWCO filter (Pall Austria Filter GmbH, Vienna, Austria) with a pore size of 3 kDa, followed by reduction of disulfide bonds with dithiothreitol (DTT), alkylation with iodoacetamide (IAA) and washing steps with 50 mM ammonium bicarbonate buffer. Digestion of proteins was achieved by applying two times Trypsin/Lys-C with Mass Spec Grade quality (Promega, Mannheim, Germany), at first overnight, and in a second step for 4 h. Resulting peptides were eluted through the filter by centrifugation, and clean-up was performed using C-18 spin columns (Pierce, Thermo Fisher Scientific, Austria).

**LC-MS/MS Analysis**—For LC-MS/MS analyses, samples were reconstituted in 5  $\mu$ l 30% formic acid (FA), supplemented with four

synthetic peptide standards for internal quality control, and diluted with 40  $\mu$ l mobile phase A (97.9% H<sub>2</sub>O, 2% ACN, 0.1% FA). Of this solution 10  $\mu$ l were injected into a Dionex Ultimate 3000 nano LC-system coupled to a Q Exactive orbitrap mass spectrometer equipped with a nanospray ion source (Thermo Fisher Scientific, Austria). All samples were analyzed as technical replicates. As a preconcentration step, peptides were loaded on a 2 cm  $\times$  75  $\mu$ m C18 Pepmap100 pre-column (Thermo Fisher Scientific, Austria) at a flow rate of 10  $\mu$ l/min using mobile phase A. Elution from the precolumn to a 50 cm  $\times$  75  $\mu$ m Pepmap100 analytical column (Thermo Fisher Scientific, Austria) and subsequent separation was achieved at a flow rate of 300 nL/min using a gradient of 8% to 40% mobile phase B (79.9% ACN, 2% H<sub>2</sub>O, 0.1% FA) over 235 min with a total chromatographic run time of 280 min. For mass spectrometric detection, MS scans were performed in the range from  $m/z$  400–1400 at a resolution of 70000 (at  $m/z$  = 200). MS/MS scans of the eight most abundant ions were achieved through HCD fragmentation at 30% normalized collision energy and analyzed in the orbitrap at a resolution of 17,500 (at  $m/z$  = 200).

**Data Analysis**—The MaxQuant software (version 1.6.0.1), including the Andromeda search engine, was used for data analysis (30). For positive protein identification, as a minimum two peptides, at least one of them being unique, had to be detected. Trypsin/P was specified in the digestion mode. Peptide mass tolerance was set to 50 and 25 ppm for the first and the main search, respectively. The false discovery rate (FDR) was set to 0.01 both on peptide and protein level. The database applied for the search was the human Uniprot database (version 06/2017, with 20100 reviewed entries and 22088 isoforms). Carbamidomethylation was set as fixed modification, methionine oxidation and N-terminal acetylation as variable modifications. Each peptide could have a maximum of two missed cleavages and two modifications. “Match between runs” was enabled and the alignment and match time window set to 25 and 1 min, respectively.

In parallel, to allow data submission to the ProteomeXchange Consortium via the PRIDE partner repository (31), raw files were analyzed using Proteome Discoverer 1.4 (Thermo Fisher Scientific, Austria) using Mascot 2.5 (Matrix Science, UK). Protein identification was performed by searching raw files against the SwissProt Database (version 11/2015 with 20 193 entries), applying a mass tolerance of 50 ppm at MS1 level and 100 mmu at MS2 level, and allowing for up to two missed cleavages per peptide. Fixed and variable modifications were set in the same way as for the MaxQuant search described above. Resulting data were submitted to the ProteomeXchange Consortium via the PRIDE partner repository and can be accessed via [www.proteomeexchange.org](http://www.proteomeexchange.org) with the identifier PXD010600.

**Experimental Design and Statistical Rationale**—Plasma cells isolated from bone marrow of ten patients with multiple myeloma (MM) and three patients with premalignant stages of MM (MGUS or SMM), as indicated in Table I, were used for proteome profiling experiments. Only patients diagnosed according to the guidelines provided by the International Myeloma Working Group (32) were included. Patients suffering from severe comorbidities were excluded. In addition to the MM cell data set, data previously obtained from six biological replicates of peripheral B cells (22), isolated from six healthy donors by applying magnetic activated cell sorting with anti-CD19 antibodies, were included for comparative purposes. B cells were used as reference cell system, representing precursors of plasma cells which have hardly experienced hypoxia. All biological samples were measured as technical replicates with LC-MS/MS, which resulted in 19 independent data sets and a total of 38 measurements. The MaxQuant software (version 1.6.0.1), including the Andromeda search engine, was used for data analysis (30). For statistical data evaluation, the Perseus software (version 1.6.0.2) was used (30, 33). Reverse sequences, potential contaminants as well as proteins identified only by site were

removed. Additionally, the normal distribution of data points was manually checked via the histogram function implemented in the Perseus software. Label-free quantification (LFQ) values were logarithmized to base 2, and technical replicates were averaged. Protein groups were filtered for valid values, keeping only those identified in at least 70% of B cell or MM cell samples. Missing values were then replaced from a normal distribution with a down shift of 1.8 and a width of 0.3 in order to enable t-testing and volcano plots. A principle component analysis (PCA), based on label-free quantification of cytoplasmic proteins, was performed. To determine protein groups significantly regulated between resulting clusters, two-sided t-tests employing multiparameter correction with a q-value  $\leq$  0.01 (permutation-based FDR correction with  $s_0$  = 0.5) were applied. For selected proteins, heat maps representing LFQ values determined in each sample were generated by a custom R (<https://www.r-project.org>) script. Protein groups found to be significantly regulated were further submitted to the oPOSSUM software (version 3.0) (34), which allowed the detection of over-represented conserved transcription factor binding sites in the corresponding sets of genes.

**Targeted Analyses of a Selected Panel of Metabolites**—For targeted analyses of a selected panel of metabolites, one million of CD138-positive plasma cells isolated from BM biopsy material of three patients with diagnosed MM (Table I) was suspended in lysis buffer (10 mM phosphate buffer in 85% ethanol) and lysed by three freeze-thaw cycles. The obtained cell lysates were analyzed with the AbsoluteIDQ p180 kit (Biocrates Life Sciences AG, Innsbruck, Austria), as described previously (20). In short, LC-MS and flow injection (FIA)-MS analyses were carried out on a 4000 QTRAP MS system (AB Sciex, Framingham, MA) coupled to a 1200 RR HPLC system (Agilent, Palo Alto, CA), using the Analyst 1.6.2 software (also AB Sciex). Data evaluation was performed with the software supplied with the kit (MetIDQ, version 5–4–8-DB100-Boron-2607, Biocrates Life Sciences). Results were again compared with data obtained previously from the analysis of B cells (22).

In parallel, from BM samples of four MM patients (Table I), fibroblasts were prepared as previously described (8). In short, BM samples were filtered through a 40  $\mu$ m mesh (40  $\mu$ m Nylon Cell Stainer, BD Falcon), the residue in the filter was transferred into a culture flask with fibroblast basal medium (FBM, Lonza Clonetics, # CC-3131) supplemented with one FGM BulletKit (Lonza Clonetics, # CC-3130) and 10% FCS. The culture flask was placed in an incubator at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>, refreshing the medium after 24 h. For comparative purposes, three biological replicates of human mesenchymal stem cells (hMSC, passage 4 to 5; Lonza), cultured in mesenchymal stem cell growth medium (Lonza) supplemented with the associated Bulletkit and 100 U/ml penicillin/streptomycin (ATCC/LGC Standards, London, UK), were used. After reaching 75% confluence, fibroblasts and hMSC were detached by trypsin-EDTA (Sigma-Aldrich) treatment. Cells were washed twice with PBS and one million of each of these cells was processed and used for targeted analyses of a selected panel of metabolites as described above for CD138-positive plasma cells.

**Immunohistochemistry**—Immunohistochemical stainings were performed on 3  $\mu$ m thick formalin-fixed, paraffin-embedded tissue sections. Antigen retrieval was performed with Ventana cell conditioning solution 1 (Tris-EDTA buffer pH 8 at 100 °C 32 min) (Ventana Medical Systems, Tucson, AZ). Subsequently, the slides were incubated with primary antibodies against SDC1 (CD138; mouse monoclonal, dilution 1:100, Dako, Denmark), SIGIRR (rabbit polyclonal, dilution 1:500, abcam, United Kingdom), CD46 (rabbit monoclonal, dilution 1:750, abcam, United Kingdom), SLAMF7 (rabbit polyclonal, dilution 1:150, Sigma Aldrich, Germany) for 32 min at 36 °C. The immunoreactivity was visualized with the Optiview DAB IHC Detection Kit (Ventana Medical Systems). Slides were counterstained with hematoxylin.



## RESULTS

*In-depth Proteome Profiling of Primary Human Myeloma Cells*—Primary human CD138-positive plasma cells were isolated from freshly isolated bone marrow (BM) biopsy material of ten patients with diagnosed MM, one patient with monoclonal gammopathy of undetermined significance (MGUS), the premalignant stage of MM, and two patients with smoldering multiple myeloma (SMM), an intermediary stage of MM (Table I). Cells were fractionated into cytoplasm and nuclear extracts and analyzed by in-depth proteome profiling using a Q Exactive orbitrap. In total, 6038 and 3415 proteins were identified in cytoplasmic and nuclear fractions of the cells, respectively, (supplemental Tables S1 and S2) by means of the MaxQuant Andromeda search engine (30). A principle component analysis (PCA), based on label-free quantification of cytoplasmic proteins, distinguished three groups: (1) B cells; (2) premalignant stages of MM, and diagnosed MM with BM plasma cell infiltration rates up to 20%, termed MM<sub>low</sub>; (3) diagnosed and more advanced stages of MM which match to BM plasma cell infiltration rates of at least 40%, termed MM<sub>high</sub> (Fig. 1A). Remarkably, in both groups, MM<sub>high</sub> and MM<sub>low</sub>, patients who had obtained MM-specific therapies and patients who had not been specifically treated before were included (indicated in Table I). Therefore, it was particularly relevant to observe that proteome profiles were that homogeneous in each of these groups, and displayed more commonalities than differences, indicating that protein expression profiles of MM cells were more dependent on the disease stage and hardly on patient treatments. In total, 637 and 605 cytoplasmic proteins were found significantly regulated between MM cells and B cells, and between MM<sub>high</sub> and MM<sub>low</sub> cells, respectively, illustrated by volcano plots in Fig. 1B and listed in supplemental Table S1. In the nuclear extracts, 414 proteins were found significantly regulated between MM cells and B cells, and 158 proteins between MM<sub>high</sub> and MM<sub>low</sub> cells (supplemental Table S2). MGUS, SMM, and MM cells, when compared all together to B cells, were referred to as “MM cells.” Several well-known differentiation markers of plasma cells, such as CD19, MS4A1 (CD20) and CD38, as well as marker proteins characteristic for myeloma cells, such as CD37, PTPRC (CD45), and SDC1 (CD138) (35, 36), were found regulated as expected in MM versus B cells (Fig. 1C).

*Proteins Involved in Apoptosis Regulated in MM Cells*—To determine biological processes regulated in MM cells and possibly related to the hallmarks of cancer (37), we used different resources such as DAVID functional annotation tool (38, 39), UniProt information about proteins (40), and performed thorough research of scientific literature. In this way, first, we determined pathways that point to suppression of apoptosis in MM cells, including upregulation of anti-apoptotic proteins and downregulation of pro-apoptotic proteins (Fig. 1D). Some of these proteins were found regulated in all MM cells relative to B cells, exemplified by apoptosis regula-

tor BAX, whereas others were only regulated at advanced stages of the disease, such as, for example, CASP10 (Fig. 1E).

*Proteins Involved in ER Activities Regulated in MM Cells*—Further, several proteins related to biological activities in the endoplasmic reticulum (ER) such as folding and modification of newly synthesized proteins, as well as degradation of incorrectly folded proteins, and transport processes from the ER to the Golgi apparatus, were found at significantly elevated levels in MM cells (Fig. 2A). Most of these proteins were upregulated in a progressive way between B and MM<sub>low</sub> cells, and further between MM<sub>low</sub> and MM<sub>high</sub> cells (Fig. 2B and 2C), consistent with the increasing amounts of immunoglobulins produced by malignant plasma cells on disease progression. This was further accompanied by a significant upregulation of proteins involved in translational processes, according to GO terms (highlighted in Fig. 1B). Similarly, upregulation of proteins involved in control of redox stress caused by disulfide bond formation, such as PRDX4 and TXNDC11 (41, 42), was observed (Figs. 2A–2C). In parallel, pathways that limit ER stress-induced apoptosis seemed to be enhanced especially in MM<sub>high</sub> cells, involving downregulation of BAX, TRAF2, ITPR1, and PRAF2 (43–45) and upregulation of SDF2L1, MYDGF, SDF4, RCN1, and TXNDC5 (46–49) (Figs. 1D, 1E, 2A and 2C).

*Regulation of Proteins Related to Respiratory Activities and to Hypoxia*—Numerous mitochondrial proteins were found deregulated in MM cells. Although reduced levels of proteins of the respiratory chain, such as COX6C, MT-CO<sub>2</sub>, or ATP5L, were determined in all MM cells compared with B cells (supplemental Table S1), several other proteins of the respiratory chain, especially chaperones necessary for the assembly of respiratory complexes, were found at significant higher levels in MM<sub>high</sub> than in B cells and MM<sub>low</sub> cells (Fig. 3A). These findings may point to a downshift of respiratory activities in MM<sub>low</sub> cells and a reactivation in MM<sub>high</sub> cells. In line with this, proteins involved in mitochondrial translation, according to GO terms, appeared to be significantly upregulated in MM<sub>high</sub> versus MM<sub>low</sub> cells (highlighted in Fig. 3B). Additionally, several proteins related to hypoxia were found regulated. HIF1AN and VHL (Fig. 3C), known to prevent activation of hypoxia-inducible factor (14, 50–53), were determined at reduced levels in MM cells, similarly to TP53I11 and PTPN1, described to be targets of hypoxia-induced micro-RNA-210 (54). Remarkably, DIMT1, described to be downregulated by micro-RNA-210 likewise (16), was found at lower levels only in MM<sub>low</sub> cells, but was upregulated again in MM<sub>high</sub> cells. Similarly, higher levels of FYN and SRC, two tyrosine-protein kinases of the same family, were determined at higher levels only in MM<sub>low</sub> cells; hypoxia has been shown to activate FYN either by phosphorylation or by upregulation of protein expression (55, 56). Also consistent with this, GTF2F2, reported to be downregulated under hypoxic conditions (57), was found at lower levels in MM<sub>low</sub> than in MM<sub>high</sub> cells. These findings indicate that MM cells activate mechanisms in re-

## Adaptations to Hypoxia Associated With Myeloma Progression

TABLE I  
Clinical parameters for patients included in the present study and type of experiment performed with the respective biopsy sample

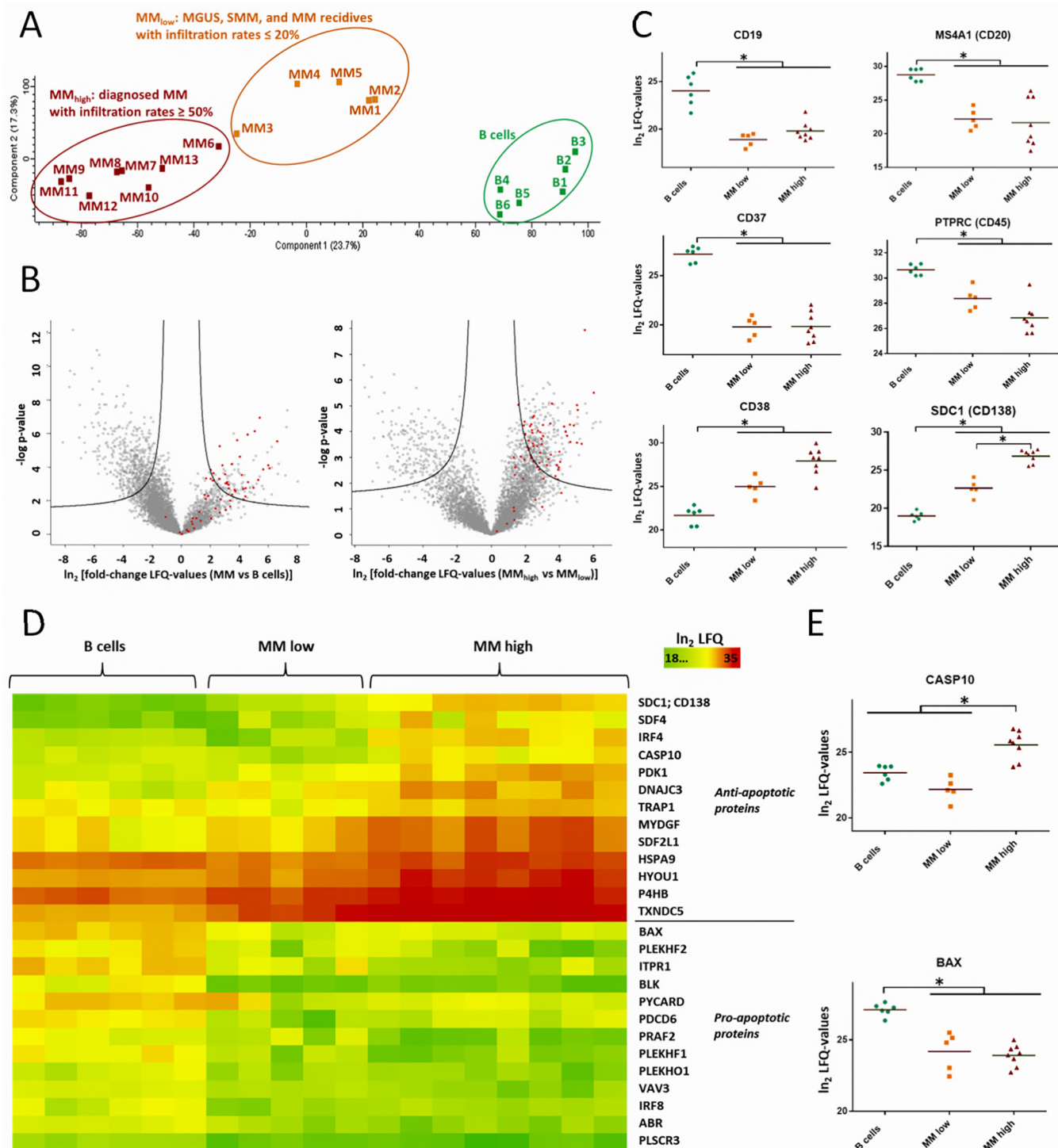
Patient #	Diagnosis	MM-specific treatment	Experiments			
			Proteomics	PC-Metabolites	Fib-Metabolites	IHC
MM1	cyclin D1-neg. MGUS; IR 5%	no	x			
MM2	κ-pos., cyclin D1-neg. IgA-SMM; IR 50%	no	x			
MM3	λ-pos., cyclin D1-neg., IgH-MAF/t(14,16)-pos., monosomy 14 & 16-pos. IgA-SMM; IR 40%	no	x			
MM4	λ-pos., cyclin D1-neg. plasma cell myeloma relapse; IR 20%	yes	x			
MM5	λ-pos., cyclin D1-neg. plasma cell myeloma relapse; IR 10–15%	yes	x			
MM6	λ-pos., cyclin D1-pos. secondary plasma cell myeloma after ASCT; IR 70%	yes	x			
MM7	κ-pos., cyclin D1-neg., low-secretory plasma cell myeloma relapse after ASCT; IR 60%	yes	x			
MM8	λ-pos., cyclin D1-neg. newly diagnosed plasma cell myeloma; IR 70%	no	x			
MM9	λ-pos., IgA-positive, newly diagnosed plasma cell myeloma; IR 70%	no	x			
MM10	κ-pos., cyclin D1-neg., IgH-MAF/t(14,16)-pos., TP53 Deletion-pos., polysomy 4, 14 & 16-pos., trisomy 17-pos. newly diagnosed light-chain plasma cell myeloma; IR 85%	no	x			x
MM11	λ-pos., cyclin D1-neg., polysomy 14-pos., trisomy 17-pos., newly diagnosed light-chain plasma cell myeloma; IR >90%	no	x			x
MM12	κ-pos., bcl1-pos., low-secretory plasma cell myeloma; IR 80%	yes	x			
MM13	λ-pos., heterozygous IgH and p53 deletion-pos., ATM amplification-pos., newly diagnosed plasma cell myeloma; IR 50%	no	x			
MM14	λ-pos., cyclin D1-neg. newly diagnosed plasma cell myeloma; IR 70%	no		x		
MM15	IgG-κ-pos. plasma cell myeloma; IR 40%	yes		x		
MM16	IgG-λ-pos., bcl1-pos. plasma cell myeloma; advanced stage; IR N/A	yes		x		
MM17	λ-pos., cyclin D1-neg., plasma cell myeloma; IR 50%	yes				x
MM18	IgD-λ-pos., cyclin D1-neg., newly diagnosed plasma cell myeloma; IR 50%	no				x
MM19	κ-pos, cyclin-D1-neg., newly diagnosed IgG-myeloma; IR:10%	no				x
MM20	κ-pos, cyclin-D1-neg.,13q14 deletion, newly diagnosed IgA-myeloma; IR:15%	no				x
MM21	λ-pos, cyclin-D1-neg., newly diagnosed IgA-myeloma; IR:20%	no				x
MM22	λ-pos, cyclin-D1-neg., newly diagnosed IgA-myeloma; IR:40%	no				x
MM23	κ-pos, cyclin-D1-neg., 13q14 deletion, trisomy 3,9,11, newly diagnosed IgA-myeloma; IR:60%	no				x
MM24	λ-pos, cyclin-D1-neg., newly diagnosed light chain-myeloma; IR:70%	no				x
MM25	κ-pos, cyclin-D1-neg., 13q14-deletion, TP53-deletion, newly diagnosed IgA-myeloma; IR:70%	no				x
MM26	λ-pos, cyclin-D1-neg., newly diagnosed IgG-myeloma; IR:80%	no				x
MM27	κ-pos, cyclin-D1-neg.,13q14-deletion, 17p-deletion, newly diagnosed IgG-myeloma; IR:80%	no				x

PC-Metabolites, targeted analyses of amino acids performed with BM plasma cells (PCs); Fib- Metabolites, targeted analyses of amino acids performed with MM-associated BM fibroblasts; IHC, immunohistochemistry; IR, BM infiltration rate with PCs; ASCT, autologous stem cell transplantation.

sponse to hypoxic conditions in the BM and that adaptation processes take place in MM<sub>high</sub> cells.

**Regulation of Proteins Involved in Metabolic Processes**—In addition, mitochondrial and cytoplasmic proteins involved in

several metabolic processes appeared to be deregulated in myeloma cells. Fig. 4A shows metabolic pathways in which proteins were found significantly up- or downregulated between B cells, MM<sub>low</sub> cells and MM<sub>high</sub> cells, the most impor-

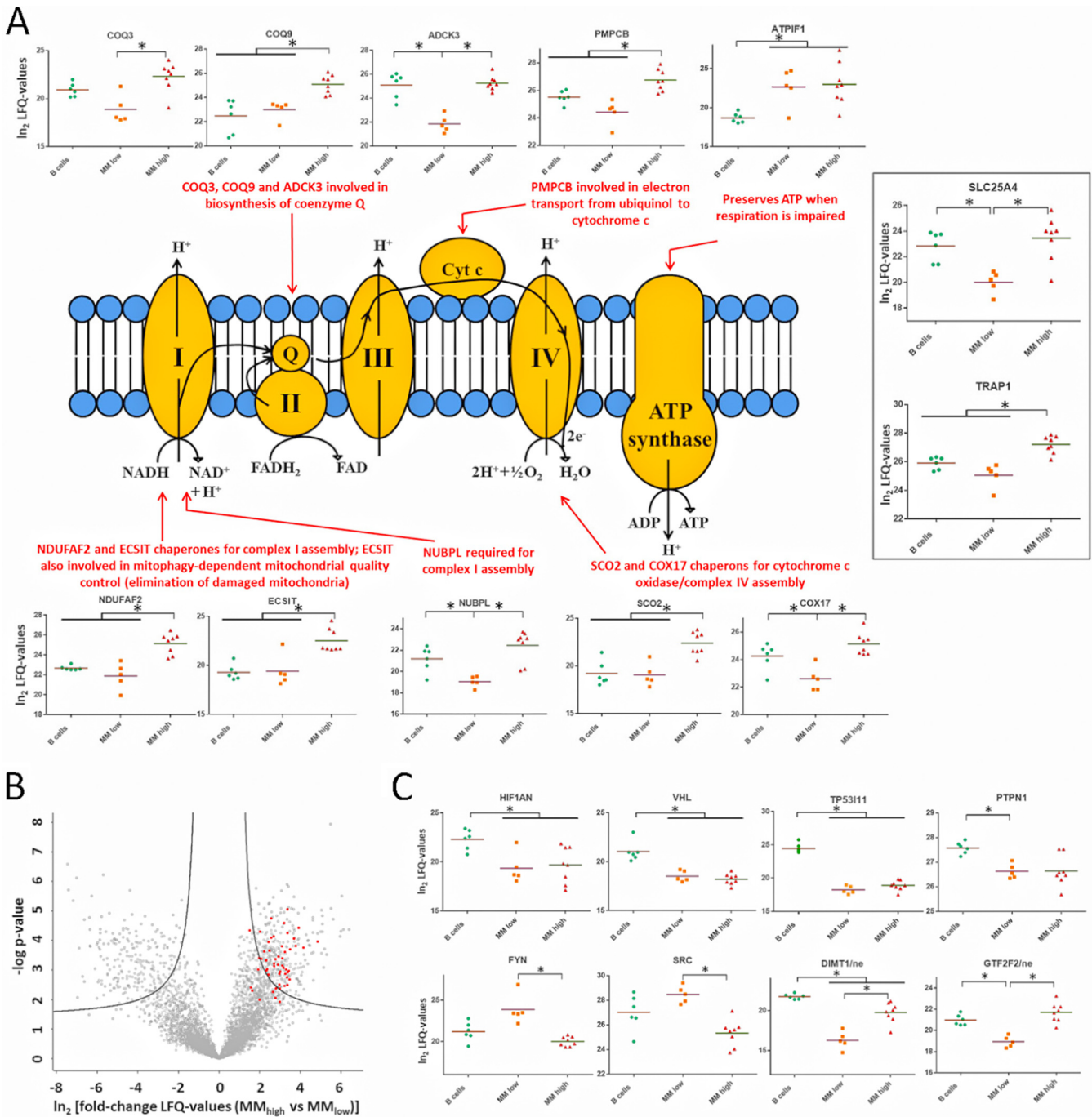


**FIG. 1. Proteome profiling of primary human MM cells and comparison to B cells.** The Perseus software (30, 33) was used to perform statistical data analyses. **A**, PCA based on label-free quantification (LFQ) of 6038 cytoplasmic proteins detected in B cells and MM cells, showing three groups: B cells; MM<sub>low</sub> cells from patients with MGUS or SMM, as well as from patients with MM with BM infiltration rates with MM cells ≤ 20%; and MM<sub>high</sub> cells from patients with diagnosed MM and BM infiltration rates ≥ 40%. **B**, Volcano plots comparing the abundance levels of the same proteins in MM *versus* B cells (left panel) and in MM<sub>high</sub> *versus* MM<sub>low</sub> cells (right panel). Proteins, which were found significantly up- or downregulated, are delineated in each plot in the right and in the left area above the black curves, respectively. Proteins involved in translational processes, according to GO terms, are highlighted in red. **C**, Levels of typical markers for B cell differentiation and/or for MM cells. **D**, Heat maps representing LFQ values of proteins involved in pro- or anti-apoptotic processes. **E**, Levels of two proteins involved in apoptosis. Significant regulations are indicated by asterisks.







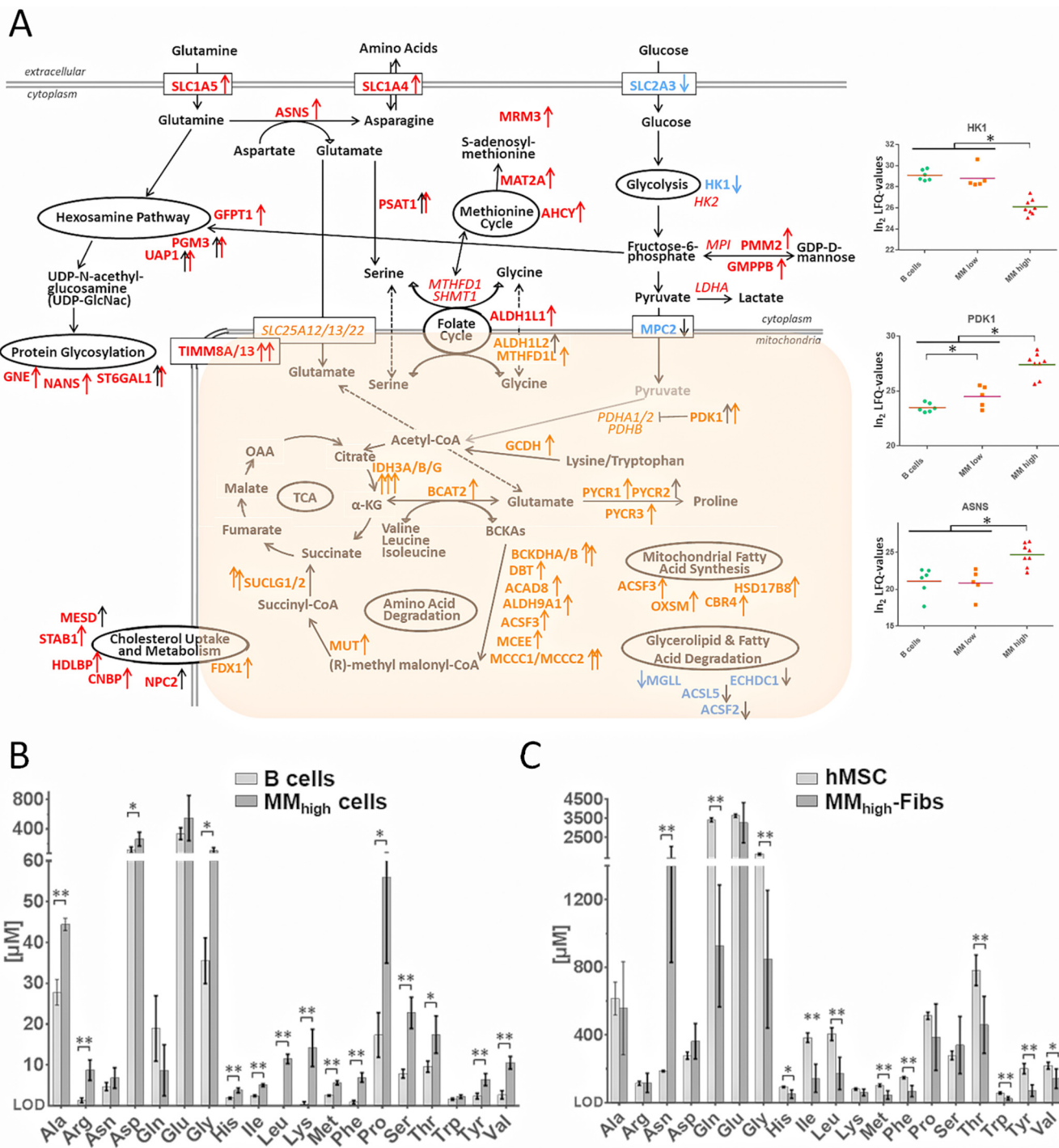


**FIG. 3. Regulation of proteins related to respiration and protein regulation by hypoxia.** **A**, Levels of selected proteins related to the respiratory chain are represented, and biological functions are indicated for some proteins. **B**, Volcano plot comparing the protein levels in  $MM_{high}$  versus  $MM_{low}$  cells. Proteins involved in mitochondrial translation processes, according to GO terms, are highlighted in red. **C**, Levels of selected proteins regulated by hypoxia, detected in the nuclear (ne) or in the cytoplasmic fraction (not specified). Significant regulations are indicated by asterisks.

tant regulatory events occurring between  $MM_{low}$  and  $MM_{high}$  cells. Most strikingly, the hexosamine pathway, amino acid interconversion reactions and degradation processes, the folate and the methionine cycle, cholesterol uptake and metabolism, and mitochondrial fatty acid synthesis were found up-regulated in myeloma cells of advanced disease stages.

These pathways seem thus to play an important role in the progression of multiple myeloma.

To support these results, we also performed targeted analyses of amino acids with both, plasma cells and MM-associated fibroblasts isolated from the BM of patients with advanced disease stages, using B cells and human mesen-



**FIG. 4. Regulation of proteins related to metabolic processes taking place in MM cells and results of metabolomics experiments.** A, Metabolic pathways for which enzymes were found significantly up- or downregulated in MM versus B cells (black arrows) and in MM<sub>high</sub> versus MM<sub>low</sub> cells (red and orange arrows for proteins upregulated in the cytoplasm and in mitochondria, respectively, blue arrows for downregulated proteins). Some proteins found at high levels but not regulated are indicated in italics. For three selected proteins, determined LFQ values are represented as dot plots. B, Levels of amino acids in B and MM<sub>high</sub> cells. C, Levels of amino acids in MM-associated BM fibroblasts (MM-Fibs) and in mesenchymal cells of the BM (hMSC) that were used as reference system. Significant regulations are indicated by asterisks. For metabolomics experiments: \*\* p-values < 0.01; \* p-values < 0.05.

chymal stem cells of the BM (hMSCs) as reference cell system, respectively. In this way, higher amounts of several amino acids were determined in MM<sub>high</sub> cells compared with

B cells (Fig. 4B), strengthening our proteomics data, which suggest that MM cells may use amino acids not primarily for the synthesis of immunoglobulins, but possibly also for other

## DISCUSSION

purposes such as catabolism. In parallel, MM-associated fibroblasts, essential supporters of MM cells in the BM microenvironment (8), proved to have rather low levels of amino acids, except for asparagine, which was present at extremely high levels in these cells when compared with hMSCs (Fig. 4C). Fibroblasts seem thus to be deregulated in a concerted fashion to MM cells.

**Protein Regulation Associated with Immune Evasion Strategies**—Several pathways leading to immune evasion are known for MM cells. Our data independently confirmed upregulation of SLAMF7 and CD46 (10, 58), and downregulation of MHC-IIs such as HLA-DQB1 (35), together with positive expression of MHC-I molecules such as HLA-C in MM cells (Fig. 5A). Moreover, we were able to determine new candidates possibly involved in immune escape mechanisms of MM cells likewise. Regulation of such candidates, including STAB1, SIGIRR, DAPP1 as well as VSIR (Fig. 5B), have been described to suppress immune responses and to be involved in the patho-mechanisms of different diseases (59–63). Immunohistochemistry independently verified the relative expression levels of the proteins SLAM7, CD46, and SIGIRR together with SDC1 (CD138), which depend on the disease stage (Fig. 5C).

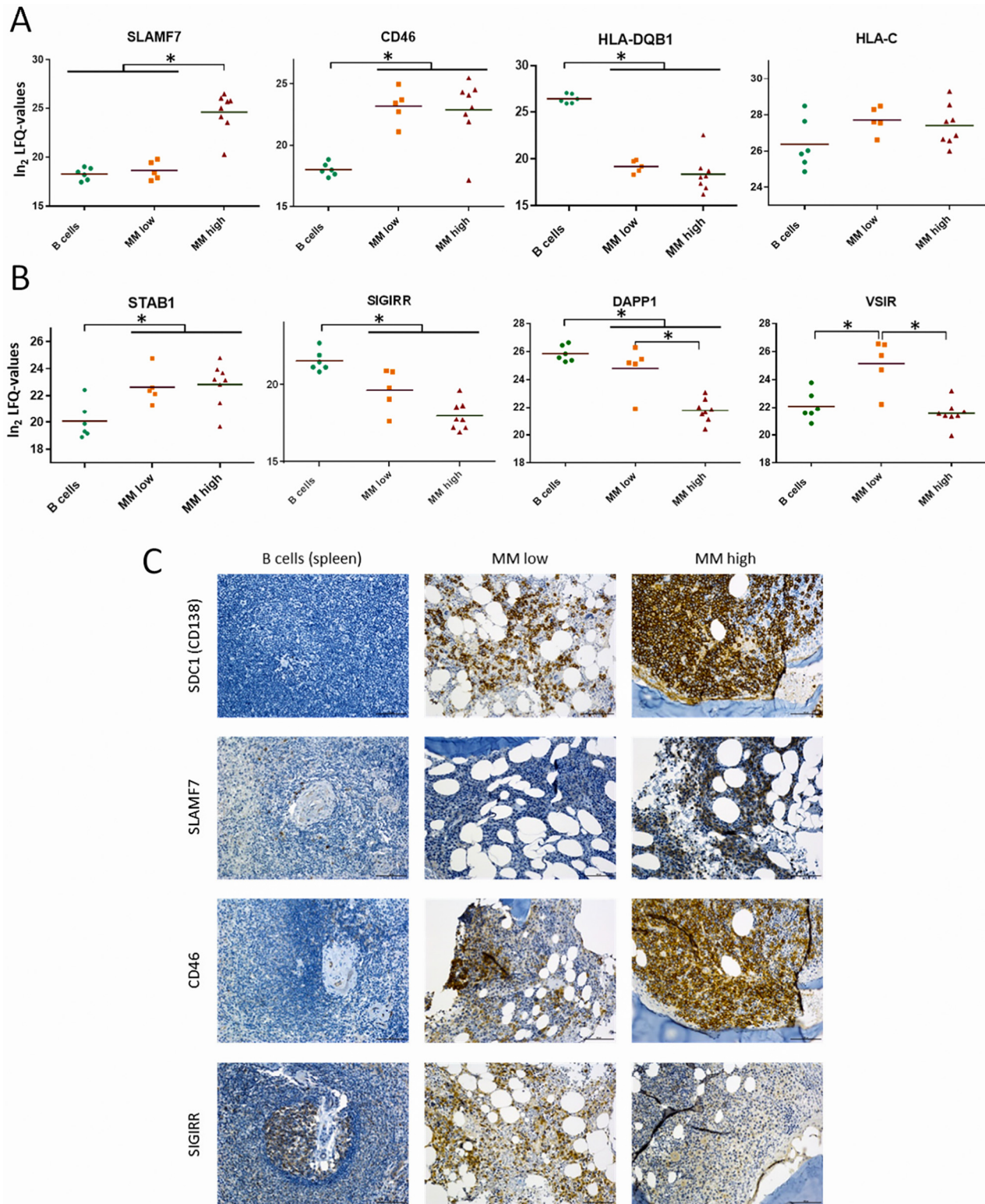
**Transcription Factors Involved in Regulating Proteins in Primary Human Myeloma Cells**—Finally, in order to determine over-represented conserved transcription factor binding sites and, thus, the main transcription factors responsible for the observed proteome alterations in MM cells, the corresponding sets of genes were submitted to the oPOSSUM software version 3.0 (34). This allowed us to find out transcription factors apparently playing an important role in the development and/or progression of multiple myeloma. Besides transcription factors known to be activated in MM, such as MYC, HIF1A, and ARNT (13, 64, 65), or to be suppressed in MM, such as SPI1 (PU.1) and KLF4 (66, 67), we were able to determine also NFYA, GABPA, ELK4, and PAX4 as transcription factors that may play an important role in the pathogenesis of MM (Fig. 6A). Levels of GABPA and NFYA determined in the nuclear fractions of MM cells are represented in Fig. 6B, together with the nuclear levels of two other transcription factors, PAX5 and IRF4, known to be involved in MM likewise. Downregulation of PAX5 is commonly associated with plasma cell differentiation and was related to multiple myeloma cell survival (68, 69). IRF4, originally identified as the product of a proto-oncogene involved in chromosomal translocations in multiple myeloma (70), has been described to be transcribed by MYC, whereas SPI1 seems to suppress the expression of IRF4 (64, 66). Finally, levels of two target proteins, PRKD2 and BLK are also shown in Fig. 6B. PRKD2 expression depends on the activity of transcription factor GABPA (71). BLK has been shown to be suppressed by MYC via downregulation of PAX5 (72).

**Myeloma Cells are Adapted to Hypoxic Conditions in the Bone Marrow Microenvironment**—It is generally recognized that the bone marrow (BM) is rather hypoxic compared with peripheral blood (13, 73). Silva and Gatenby have demonstrated using computer simulation that adaptation to hypoxic conditions in the BM microenvironment is essential for myeloma cells to progress to an aggressive stage (74). HIF1A and ARNT have been described to be upregulated by tumor cells including myeloma cells under low oxygen conditions (13, 65). Both transcription factors seem to be responsible for the induction of relevant proteins in MM cells (Fig. 6A). The observed downregulation of HIF1AN and VHL (Fig. 3C) also points to hypoxic conditions in the BM microenvironment of the cells (11, 14). Remarkably, even though expression of HIF1AN and VHL may also be affected by typical anti-myeloma treatments such as Bortezomid or Dexamethasone (52, 75), levels of these proteins were rather similar in all MM cells and apparently independent on patient treatments. Further, reduced levels of proteins of the respiratory chain, such as COX6C, MT-CO<sub>2</sub> or ATP5L (supplemental Table S1) indicate a limited oxygen consumption rate in MM cells. In line with this, ATPase inhibitory factor ATPIF1 was found highly induced in MM cells (Fig. 3A); this protein is necessary to preserve ATP at the expense of the mitochondrial membrane potential when respiration is impaired, and protects cells from ATP depletion in response to hypoxia and glucose deprivation (76, 77).

However, a trend of increased synthesis of proteins of the respiratory complexes in MM<sub>high</sub> relative to MM<sub>low</sub> cells was also observed, together with upregulation of the ADP/ATP translocase SLC25A4 (Fig. 3A). Further, proteins involved in mitochondrial translation, as well as TRAP1, an important mitochondrial chaperon that directly interacts with respiratory complexes and contributes to their stability and activity (78) were found at significantly elevated levels in MM<sub>high</sub> cells (Figs. 3A and 3B). Although its role in carcinogenesis is controversial, TRAP1 has been found highly expressed in several cancers, as reviewed by Matassa *et al.* (79), providing tumor cells the ability to use spare respiratory capacity when oxygen and glucose are limited (80). Additionally, mitochondrial enzymes of the folate cycle, highly relevant for mitochondrial translation and described to be strongly upregulated in proliferating lymphocytes (81), were found upregulated in MM<sub>high</sub> cells as well (Fig. 4A). Thus, it seems that, at advanced stages of the disease, myeloma cells get access to more oxygen, consistent with the “angiogenic switch” necessary for MM progression, as claimed by Silva and Gatenby (74), and with the pro-angiogenic phenotype associated with MM-related endothelial cells (15).

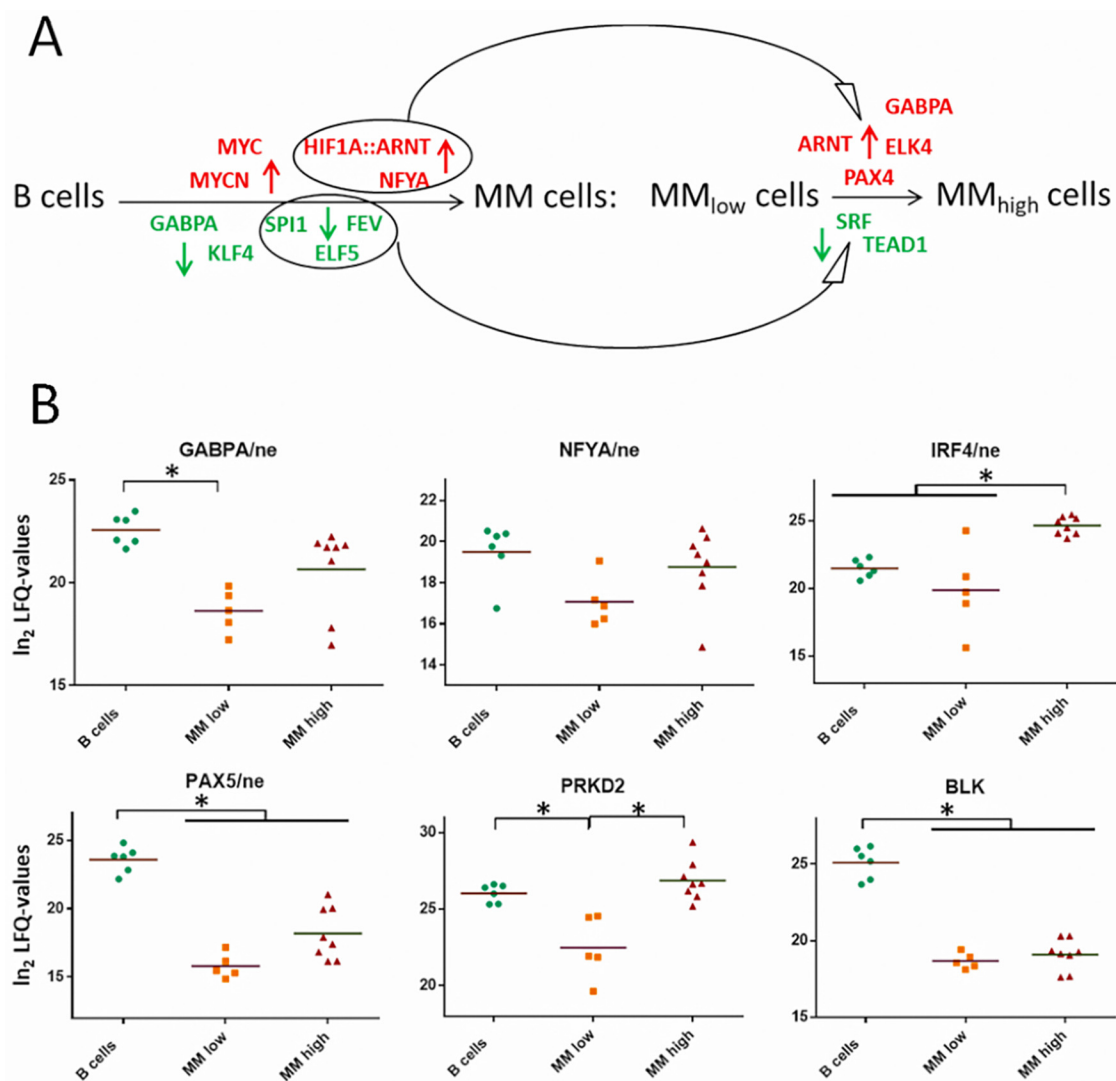
**Metabolic Adaptations in MM Cells as Response to Hypoxic Conditions in the Bone Marrow**—Myeloma cells have to adapt their metabolic pathways to be able to survive hypoxia. PDK1





**FIG. 5. Regulation of proteins related to immune evasion strategies of MM cells.** *A*, Levels of selected proteins known to be involved in immune evasion strategies of MM cells. *B*, Levels of selected proteins potentially contributing to immune evasion strategies of MM cells likewise. *C*, Selected proteins were verified by immunohistochemistry (IHC). Stainings were performed on BM biopsies of patient MM19–27, MM19–21 representing MM<sub>low</sub> and MM22–27 MM<sub>high</sub> cases. Infiltration rates (IR) were assessed by evaluation of the entire tissue section. Because of focal infiltrates, the IR might vary within one section. Depicted image sections resemble representative areas. B cells in germinal centers of the spleen were used as reference system.





**FIG. 6. Transcription factors (TFs) mainly responsible for the observed up- and downregulations of proteins in MM cells.** *A*, Well-known and newly determined TFs that may play an important role in MM development and/or progression. Red, TFs involved in upregulation of proteins; green, TFs involved in downregulation of proteins. HIF1A, ARNT, FYA, SPI1, FEV and ELF5 may be responsible for both, regulation of proteins in MM relative to B cells and in MM<sub>high</sub> relative to MM<sub>low</sub> cells. *B*, Levels of TFs detected in nuclear fractions (ne), and of two known TF targets, PRKD2 and BLK, detected in cytoplasmic fractions. Significant regulations are indicated by asterisks.

is one of the key enzymes regulating metabolic adaptations in response to hypoxia, allowing cell proliferation under these conditions (82). We have found this enzyme significantly up-regulated in MM *versus* B cells, and further in MM<sub>high</sub> *versus* MM<sub>low</sub> cells (Fig. 4A). This kinase prevents the formation of acetyl-coenzyme A from pyruvate by inhibiting the activity of pyruvate dehydrogenase, thus disconnecting glycolysis and citric acid cycle. This strategy is well-known as Warburg-effect, a strategy commonly used by tumor cells, as reviewed by Nissim Hay (83). Inhibition of PDK1 has been proposed as target for MM therapy (84). Further, we found mitochondrial pyruvate carrier MPC2 significantly downregulated in MM cells (Fig. 4A); loss of MPC has been associated with carcinogenicity as well as with a stem cells phenotype (85, 86). Consistent with this, hypoxia has been shown to induce a

stem cell-like phenotype in MM cells (87), and GABPA and its target PRKD2 (Figs. 6A and 6B) have been described to control proliferation of hematopoietic stem cells as well as development of leukemia (71).

Moreover, glucose import and the glycolytic enzyme HK1 appeared to be significantly downregulated in MM<sub>high</sub> cells (Fig. 4A), pointing to a reduced glycolysis rate in MM cells of advanced stages of the disease. This is in accordance with the already discussed upregulation of ATP1F1 and TRAP1 in MM<sub>high</sub> cells. On the other hand, uptake of glutamine seems to be highly increased in MM<sub>high</sub> cells (Fig. 4A), consistent with the observed glutamine dependence of MM cells (88). Glutamine is required for the hexosamine pathway, which is highly upregulated in MM<sub>high</sub> cells (Fig. 4A). This pathway leads to protein glycosylation as part of the completion proc-

ess of newly synthesized proteins. Multiple myeloma is characterized by aberrantly glycosylated immunoglobulins (89), which are synthesized in high amounts by MM cells. Most importantly, glutamine can also be interconverted into asparagine, which might be used for the import of other amino acids via reciprocal exchange (90); levels of glutamine-hydrolyzing asparagine synthetase ASNS and transporter proteins involved in these processes, SLC1A4 and SLC1A5, were found significantly increased in MM<sub>high</sub> cells (Fig. 4A). Results of the targeted analyses of amino acids further point to the likelihood that BM fibroblasts may supply MM cells with several of the necessary amino acids, probably in exchange for asparagine (Figs. 4B and 4C). This view is supported by the fact that BM stromal cells such as fibroblasts are widely used as feeder layers for MM cells in cell culture experiments (91). Remarkably, asparagine synthesis has been associated with survival of glutamine-dependent tumor cells, and expression of ASNS was correlated with poor prognosis in several tumors (92). Studies have indeed suggested good anti-myeloma activity of asparaginase (93).

Glutamate arising from the interconversion of glutamine to asparagine may be used for the synthesis of serine, which feeds into the folate cycle (Fig. 4A). Interfering with the serine metabolism has been proposed to improve anti-myeloma therapies (94). Alternatively, glutamate may be transported into mitochondria to be channeled into the citric acid cycle, directly or via interconversion into other amino acids (Fig. 4A). As depicted above, it appears that myeloma cells of advanced stages of the disease are capable of increasing respiration again. Our data indicate that fuels for oxidative phosphorylation in MM<sub>high</sub> cells may arise from degradation of amino acids (Fig. 4A). Remarkably, we also observed increased mitochondrial fatty acid synthesis and upregulation of proteins involved in the methionine cycle (Fig. 4A). These two pathways give rise to lipoic acid, a cofactor of the enzyme BCKDH, which is necessary for branched chain amino acid degradation (95) and was found upregulated in MM<sub>high</sub> cells (Fig. 4A). There is, furthermore, evidence that mitochondrial fatty acid synthesis is essential for respiration and mitochondrial biogenesis (96).

Two proteins involved in mitochondrial fusion, CLPP and CLPX (97), were also found significantly upregulated in MM<sub>high</sub> cells, whereas OMA1, which prevents fusion (98) and SLC25A46, which is involved in mitochondrial fission (99) were found significantly downregulated in MM cells (supplemental Table S1). Mitochondria, which are metabolically challenged when cells switch to use amino acids or fatty acids for ATP production, have been described to undergo active fusion to avoid oxidative stress and mitochondrial damage (100). Enlarged mitochondria in multiple myeloma cells have already been described in the 1980s by Ghadially *et al.* (101). Further, we also found upregulation of ECSIT in MM<sub>high</sub> cells relative to MM<sub>low</sub> and B cells (Fig. 3A); this protein, besides being involved in the assembly of mitochondrial complex I, also plays an essential role in mitophagy-dependent mito-

chondrial quality control, preventing accumulation of damaged mitochondria (102).

In addition, cholesterol uptake as well as cholesterol metabolic processes were found upregulated in MM cells and especially in MM<sub>high</sub> cells (Fig. 4A). This is in accordance with observations about hypocholesterolemia in the plasma of MM patients, associated with increased LDL clearance and use of cholesterol by MM cells, which seem to be necessary for MM cell survival (103, 104).

Responsible for deregulation of metabolic processes in MM<sub>high</sub> versus MM<sub>low</sub> cells may be the activation of transcription factor NFYA, which appeared to be involved in protein regulation in MM cells and showed a trend of upregulation in MM<sub>high</sub> cells (Figs. 6A and 6B). This transcription factor promotes the expression of proteins fueling metabolic pathways commonly altered in cancer cells, such as the serine, the one carbon, the glycine, the glutamine pathways and the cholesterol pathway (105). Further, MYC is involved in regulating glutamine metabolism as well. Very recently, Gonsalves *et al.* have demonstrated that higher levels of MYC in smoldering myeloma cells correlate with an increased use of glutamine for the citric acid cycle and a shorter time to progression to MM (106). This is consistent with our data indicating an increased uptake of glutamine in MM<sub>high</sub> cells, which apparently channel this amino acid into the citric acid cycle (Fig. 4A).

*Myeloma Cells Adapt ER Activities to Hypoxic Conditions in the Bone Marrow*—Stress proteins of the endoplasmic reticulum (ER) such as glucose-regulated and heat-shock proteins have been shown to be involved in resistance against environmental stress such as that caused by hypoxia, in avoidance of stress-induced apoptosis, and in cancer development (107, 108). It has also been demonstrated that protein-disulfide isomerases are upregulated in response to hypoxia and are critical for cell viability under these conditions (109). In line with this, we found massive upregulation of HSP90B1 (GRP94), HSPA5 (GRP78), HYOU1 (GRP170), and HSPA13 in MM versus B cells, and moreover in MM<sub>high</sub> versus MM<sub>low</sub> cells; the same trend was observed for protein-disulfide isomerases such as P4HB, PDIA4, PDIA5, and PDIA6 (Figs. 2A–2C). Azoitei *et al.* have shown that signals from hypoxia and HSP90 pathways are interconnected via PRKD2 (Figs. 6A and 6B), which is apparently necessary to prevent apoptosis and promote tumor angiogenesis and tumor growth (110). Activation of transcription factor PAX4 (Fig. 6A), proposed as candidate oncogene in hematologic malignancies (111), has also shown to contribute to protect against ER stress-induced apoptosis (112).

*Myeloma Cells Have Strategies to Prevent Apoptosis Under Hypoxic and Normoxic Conditions*—Ikeda *et al.* have demonstrated that myeloma cells are compatible with both hypoxic and normoxic conditions (16). Under hypoxic conditions, HIF apparently activates micro-RNA-210 that blocks the DIMT1-IRF4 axis and leads to glycolysis and to quiescence of the cells. Under normoxic conditions, active DIMT1 and IRF4

induce myeloma cell maturation and proliferation. Remarkably, we found IRF4 and DIMT1 to be present at significantly higher levels in MM<sub>high</sub> than in MM<sub>low</sub> cells (Figs. 3C and 6B). This fits to our model in which MM<sub>low</sub> cells are confronted with more hypoxic conditions than MM<sub>high</sub> cells, and that glycolysis is rather downregulated in MM<sub>high</sub> versus MM<sub>low</sub> cells. Most importantly, myeloma cells are obviously capable of preventing apoptosis under both conditions (16). In line with this, we were able to determine several proteins that may support cell death escaping mechanisms in MM<sub>low</sub> and MM<sub>high</sub> cells (Fig. 1D). In MM<sub>low</sub> cells hypoxia-induced anti-apoptotic factors such as SRC and FYN (55, 113) were found upregulated (Fig. 3C). This was accompanied by downregulation of pro-apoptotic proteins such as hypoxia-regulated proteins BAX (114), TP53I11 (115), and BLK (116) (Figs. 1E, 3C, and 6B). In MM<sub>high</sub> cells, elevated levels of anti-apoptotic proteins such as HYOU1 (117), SDF4 (47, 118), TRAP1 (119), and the ADP/ATP translocase SLC25A4 (120) (Figs. 1D, 2C, and 3A) were detected, together with IRF4 (121), which may also act indirectly in an anti-apoptotic way via induction of CASP10 (122) (Figs. 1E and 6B).

**Myeloma Cells Develop Hypoxia-driven Strategies to Escape Immune Response**—Besides evading intrinsic apoptosis, MM cells have pathways that limit extrinsic apoptosis induced by the immune system, involving for example upregulation of CD38, CD46, SLAMF7 (CS1), and MHC-I molecules (Figs. 1C, 5A, and 5C) (10, 58). In case of CD38 and SLAM7 therapeutic antibodies are already tested in clinical trials for combination therapies (123). Remarkably, SLAM7, showed different expression levels depending on the disease stage (Figs. 5A and 5C), and may thus be applied as stratification marker of use in antibody therapy. Reduced levels of MHC-II molecules, as well as MHC-II-associated proteins such as CD37 (Figs. 1C and 5A), may also contribute to immune evasion by impeding the antigen presentation capacity of MM cells and their interactions with T cells (124, 125). Upregulation of MYC (Fig. 6A) has been shown to disrupt MHC-II-mediated immune recognition of human B cell tumors (126).

The presently applied untargeted method also revealed novel candidates potentially involved in immune escape mechanisms of MM cells, such as DAPP1 and STAB1 (Fig. 5B) (59, 62). Further, VSIR (Fig. 5B) may negatively regulate T cell functions, at least at earlier stages of the disease. This protein has been found highly expressed in tumor microenvironments where it seems to suppress the proliferation of T cells without affecting B cells, as described by Lines *et al.* (63). Reduced levels of SIGIRR (Figs. 5B and 5C) may also be involved in immune escape mechanisms of MM cells. This protein has also been found downregulated in chronic lymphocytic leukemia (61). It is however worth mentioning that, when comparing immune evasion strategies of MM cells with those of CLL cells described in a previous study of our group (22), more differences than similarities became evident.

Remarkably, hypoxic conditions in the BM microenvironment may induce mechanisms leading to immune evasion of MM cells. As mentioned before, Ikeda *et al.* have shown that under hypoxic conditions micro-RNA-210 is activated in MM cells (16). Noman *et al.* have demonstrated that, in lung cancer and melanoma, high levels of micro-RNA-210 dramatically decrease tumor cell susceptibility to cytotoxic T cell-mediated lysis via coordinate silencing of PTPN1, HOXA1 and TP53I11 (54). Our data indicate that PTPN1 is significantly reduced in MM<sub>low</sub> relative to B cells and a similar trend was observed for MM<sub>high</sub> versus B cells (Fig. 3C). HOXA1 was not detected in any sample investigated in the present study. TP53I11 was found significantly downregulated in both MM<sub>low</sub> and MM<sub>high</sub> cells (Fig. 3C), and the higher oxygen availability of MM<sub>high</sub> cells seems not to be sufficient to restore high levels of this protein.

**Conclusion and Outlook**—The present proteome profiling data strongly support that adaptation of MM cells to hypoxia accompanies myeloma disease progression. Our results demonstrate that in-depth proteome profiling is very well suited to give deep insights into processes that are contributing to tumor development and progression. The data clearly reproduced established knowledge on myeloma cells, but also present novel findings and causal relations of relevant pathways taking place in these tumor cells. Importantly, it seems that strategies exploited by myeloma cells to allow survival and proliferation, including immune evasion mechanisms and metabolic adaptations, are more dependent on the disease state than on the genetic background. This study may thus support the development of improved stratification and anti-myeloma treatment strategies.

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#### DATA AVAILABILITY

Proteomics data were submitted to the ProteomeXchange Consortium via the PRIDE partner repository. Results are fully accessible via [www.proteomeexchange.org](http://www.proteomeexchange.org) with the identifier PXD010600.

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The authors declare no competing financial interests.

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