ORIGINAL ARTICLE

Waldenström’s macroglobulinemia harbors a unique proteome where Ku70 is severely underexpressed as compared with other B-lymphoproliferative disorders

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Waldenström’s macroglobulinemia (WM) is a clonal B-cell lymphoproliferative disorder (LPD) of post-germinale center nature. Despite the fact that the precise molecular pathway(s) leading to WM remain(s) to be elucidated, a hallmark of the disease is the absence of the immunoglobulin heavy chain class switch recombination. Using two-dimensional gel electrophoresis, we compared proteomic profiles of WM cells with that of other LPDs. We were able to demonstrate that WM constitutes a unique proteomic entity as compared with chronic lymphocytic leukemia and marginal zone lymphoma. Statistical comparisons of protein expression levels revealed that a few proteins are distinctly expressed in WM in comparison with other LPDs. In particular we observed a major downregulation of the double strand repair protein Ku70 (XRCC6); confirmed at both the protein and RNA levels in an independent cohort of patients. Hence, we define a distinctive proteomic profile for WM where the downregulation of Ku70—a component of the non homologous end-joining pathway—might be relevant in disease pathophysiology.

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INTRODUCTION

Waldenström’s macroglobulinemia (WM)—alternatively designated as lymphoplasmacytic lymphoma—is an uncommon B-cell lymphoproliferative disorder (LPD) characterized primarily by a lymphoplasmacytic infiltration of the bone marrow and by an immunoglobulin M monoclonal gammapathy.1,2

Although the precise molecular path(s) leading to WM remain largely unknown, there is clear evidence for the post-germinal center nature of the WM clonal cells, as the IGH (immunoglobulin heavy chain gene) is almost always subjected to somatic hypermutation.3,4 although without the presence of any intraclonal variation.5 Moreover and in contrast to immunoglobulin M multiple myeloma (MM) and chronic lymphocytic leukemia (CLL), post-switch clonotypic immunoglobulins are undetectable in WM B cells, suggesting therefore that absence of isotype switching;5,6 although the latter could be achieved in vitro in response to appropriate stimuli for example, CD40-ligand and IL-4.7 Immunoglobulin class switching requires a functional activation-induced cytidine deaminase8 and uses the robust non homologous end-joining (NHEJ) pathway.9 The Ku (Ku70/Ku80) heterodimer is a key factor in this pathway, acting as a scaffold for the recruitment of NHEJ core or such processing factors as the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the XRCC4/ligase IV complex.10,11

To progress in the understanding of molecular pathway(s) underlying the advent of the disease, gene-expression profiling of WM cells has been previously performed; revealing a homogeneous expression profile, more similar to that of CLL than that of MM.12 A small set of genes was thereafter identified to be distinctly expressed in WM. They include interleukin-6 (IL6) and genes of the mitogen-activated protein kinase pathway. Upregulation of IL6 in WM was confirmed by an independent study.13 Aiming to compare WM cells with B-cell morphology and those with plasma cell morphology, this work concluded that B cells and plasma cells from WM patients exhibit distinct patterns of gene expression as compared with B cells and plasma cells from patients with CLL and MM.13

Few proteomic studies have been performed in WM. These include a proteomic analysis of signaling pathways performed in WM and MM samples, before and after treatment with a proteasome inhibitor.14 Clustering analysis allowed to identify proteins that were expressed by either of these disorders but not both, indicating differences in cellular responses to proteasome inhibition.14 Hatjiharissi et al.15 on the other hand compared—using an antibody-based protein microarray method—the patterns of protein expression between untreated WM cells and normal bone marrow controls. These analyses identified upregulation of proteins of the Ras and Rho family, as well as of...
Proliferation, cytostatic activity, and the incidence of apoptosis were assessed by both flow cytometry and conventional microscopy. Flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA) and the CellQuest Pro software (BD Biosciences). Cells were stained with annexin V (BD Biosciences) and propidium iodide (Sigma-Aldrich) to monitor early and late apoptosis, respectively. The percentage of apoptotic cells was calculated using the CellQuest Pro software. The results were expressed as mean percentages of apoptosis ± standard deviation (SD). All experiments were performed at least in triplicate.

Data analysis was performed using GraphPad Prism software (version 7.0). Differences between groups were analyzed using the Student's t-test for unpaired samples. A p-value < 0.05 was considered statistically significant.

Results

Flow cytometry analysis revealed a significant increase in the percentage of apoptotic cells in the treatment group compared to the control group (p < 0.05). The annexin V and propidium iodide staining results showed a higher proportion of early and late apoptotic cells in the treated group, indicating a more effective induction of apoptosis.

Conventional microscopy images showed a marked morphological change in the cells, with increased shrinkage and nuclear condensation, indicative of apoptosis.

Discussion

The results of this study suggest that the tested compound has a potent effect on inducing apoptosis in the selected lymphoma cell line. The combination of flow cytometry and conventional microscopy provided a comprehensive assessment of the apoptotic process, allowing for a more accurate interpretation of the results.

Future directions

Further experiments are needed to investigate the potential mechanisms of action and the long-term effects of the compound on lymphoma cell survival. Additionally, in vivo studies are warranted to evaluate the therapeutic potential of this compound in a lymphoma model.
Proteomics of WM vs other LPDs
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Western-blot analysis
Cell lysates were prepared by incubating purified B-cell pellets on ice for 30 min in 200 μl of a lysis buffer containing 150 mM NaCl, 50 mM Tris pH 8, 1% NP40, 10% glycerol, 0.5 mM EDTA and protease inhibitor Complete 1X (Roche Diagnostics, Mannheim, Germany). The samples were then centrifuged in a vacuum centrifuge and then resuspended in 0.1% TFA 50% acetonitrile in an ultrasonic bath during 10 min to extract residual peptides. The supernatants were collected and subjected to overnight trypsin digestion. The protein concentration of the supernatant was determined using a bicinchoninic acid (BCA) assay. Peptides were then dried completely in a vacuum centrifuge and re-dissolved in 10% acetonitrile, 0.5% Triton X100, 0.5% SB3-10, 40 mM DTT and 0.5% TFA with 7 M urea, 2 M thiourea, 1% CHAPS, 10% isopropanol, 10% isobutanol, 0.5% Triton X100, 0.5% SB3-10, 40 mM DTT and 0.5% IPG buffer 4–7 for a total volume of 460 μl. Rehydration of a 24 cm Immobiline pH 4–7 DryStrip (GE Healthcare) was achieved in the dark during 16 h. Iso-electric focusing was then performed at 20 °C for a total of 85 000 Vh using the Ettan II IPGphor system (GE Healthcare). After migration, the strips were equilibrated in SDS containing buffer (reduction and alkylation) before being loaded onto SDS polyacrylamide gels for separation according to molecular weight using an Ettan DALT Six Electrophoresis System (GE Healthcare). After migration, 2D-E gels were scanned using an Ettan Dige Imager (GE Healthcare) according to the manufacturer’s instructions. Image analysis and statistical calculations were performed using the Progenesis SameSpots software (NonLinear Dynamics, Newcastle, UK) and the ‘Multiple stains per gel without internal standards’ comparison method. All sample gel images were first aligned. Spots were then automatically detected and filtered to eliminate non-protein spots. Statistical analyses (analysis of variance and principal component analyses) were performed on normalized spots data. For multigroup analysis of variance test, a q-value (a FDR corrected P-value) of <0.05 was considered significant. The threshold chosen to appreciate variations was a 2.5-fold change when comparing WM versus CLL groups.

Protein identification
Analytical gels were stained with SYPRO Ruby (Invitrogen Corporation, Carlsbad, CA, USA) and used for robotized spot picking (EXQuest spot cutter, Bio-Rad, Marnes-la-Coquette, France). Gel plugs were washed twice with 25 mM ammonium bicarbonate in 50% acetonitrile then dehydrated in 100% acetonitrile, before being subjected to overnight in-gel trypsin digestion. Briefly, each spot was incubated in a solution containing 50 mM ammonium bicarbonate, 10% acetonitrile and 20 μg/ml trypsin (G-Biosciences) on ice during 1 h, then overnight at 37 °C. The supernatants were collected and gel plugs were incubated in 0.1% TFA 50% acetonitrile in an ultrasonic bath during 10 min to extract residual peptides. The supernatants were newly pooled with the former. Peptides were dried completely in a vacuum centrifuge then resuspended in 0.1% TFA 50% acetonitrile, to be analyzed by mass spectrometry (MS or MS/MS). Peptides were spotted onto a MALDI plate with matrix solution (50% acetonitrile, 0.1% TFA, HCCA at saturation) diluted four-fold in 50% acetonitrile, 0.1% TFA and analyzed by MS using an Autoflex MALDI-TOF (Bruker Daltonics, Bremen, Germany). This instrument was operated in positive ion mode and externally calibrated in the peptide mass range of 700–3200 m/z. MS/MS analysis was performed using the Ultimate 3000 nanoLC system (Dionex, Voisin le Bretonneux, France) coupled to an Esquire HCTUltra nESI-IT-MS (Bruker Daltonics). A search for protein identity was carried out with MASCOT (http://www.matrixscience.com). Confident matches were defined by the MASCOT score and statistical significance (P<0.05), the number of matching peptides and the percentage of total amino acid sequences covered by matching peptides.

RESULTS
Here we present a first comprehensive 2D-E analysis of WM versus other LPDs.

WM is a unique proteomic entity
Quantitative proteomic analysis by 2D-E was performed for 10 protein extracts obtained from B cells isolated from peripheral blood and/or bone marrow of 2 WM (3 samples), 2 MZL (3 samples) and 3 CLL (4 samples) patients. The inclusion of blood and bone marrow cells from the same patients (WM1 and MZL2) was aimed to foresee the extent to which cell origin/developmental stage might have any influence in proteomic profile in a same individual.

Multivariate analysis of protein expression was first performed using a principal component analysis. Used as an explorative tool to investigate the clustering of all WM, MZL and CLL samples, principal component analysis analysis demonstrated that WM clustered distinctly from MZL and CLL (Figure 1). Hence, the protein content of WM cells is significantly different from those of MZL or CLL. Of note, one WM and one MZL patient segregated quite differently in principal component analysis, although without overlapping with other LPDs, and the ‘stray’ WM patient still was nearest to the other two (Figure 1).

Several proteins are differentially expressed in WM as compared with MZL and CLL
Among the 1051 polypeptide spots analyzed within different samples, 356 showed a differential expression. Most differentially expressed polypeptide spots (with a 2.5-fold change) between WM and CLL samples, as identified by mass spectrometry, are presented in Table 2. The first highlight from this analysis is that the detected differentially expressed proteins are not—for the vast majority—among the candidate genes/proteins, which would have expected to be critical in WM—that is, in direct connection with leukomogenesis,
immunoglobulin class switching—the candidate protein retained for validation on a larger cohort of patients. First, western blot experiments performed on 9 untreated patients (whose characteristics are summarized in Table 1) clearly confirmed a much lower expression that is, quasi absence of Ku70 in WM as compared with MZL and CLL cells. Several components of the cytoskeleton were also differentially expressed in WM cells, namely moesin, gelsolin and lamin-B1. Ku70 proteome profile was examined more thoroughly: 2D and 3D representations together with statistical expression data are shown in Figure 2. No variation of the proteomic content between blood and bone marrow cells, neither for WM nor MZL was noted for this protein.

Lower expression of Ku70 (XRCC6) protein and gene expression in WM cells by comparison with other LPDs

Ku70 was—for obvious reasons of its direct implication in immunoglobulin class switching—the candidate protein retained for validation on a larger cohort of patients. First, western blot experiments performed on 9 untreated patients (whose characteristics are summarized in Table 1) clearly confirmed a much lower expression that is, quasi absence of Ku70 in WM as compared with MZL and CLL (Figure 3). Second, in order to investigate whether the quantitative defect of Ku70 protein was related to a lack of XRCC6 RNA transcript or to a post-translational process, gene expression of XRCC6 was assessed using quantitative RT-PCR (using GUSB as reference gene) in 14 patients with WM or other lymphoid malignancies. XRCC6 expression was found to be significantly lower in MW cells as compared with other LPDs including MZL, CLL and MCL (Figure 4); thus suggesting that the observed protein down-regulation was due to a transcriptional shut down.

**DISCUSSION**

WM, a rare, distinct, B LPD, is defined by lymphoplasmacytic infiltration of the bone marrow and a monoclonal immunoglobulin M paraproteinaemia, respectively, ascertained by bone marrow biopsy and serum protein immunoelectrophoresis/immunofixation. However, before performing the latter tests in order to reach diagnostic certitude, there is/are at present no unique non invasive diagnostic biomarker(s) available helping to guide the clinician in an in many diversity of nonspecific early symptoms for example, fatigue, anemia, thrombocytopenia, hepatitisplenomegaly and lymphadenopathy.

Array technology, especially transcriptome analysis, has been widely used in clonal hematological malignancies.18,19 WM has not escaped this trend, as mentioned earlier. Proteome analysis—more cumbersome presently—has been less widely used and in case of WM has been performed in only two reports with self imposed limitations/biases. Here, we present a first comprehensive 2D-E analysis of WM, as compared with other LPDs. This study yielded more significant differences for 17 proteins (Table 2). Besides, the possibly critical downregulation of Ku70, a few other modifications of housekeeping/structural proteins were observed. These include molecules involved in glucose metabolism, redox balance, cell communication, protein metabolism, regulation of translation or nucleic acid binding. Phosphoglucotuse-2 appears to be selectively overexpressed in WM compared with CLL and MZL.20,21 Inversely, glutaredoxin-3 (GLRX3), lactoylglutathione lyase (GLO1) and peroxiredoxin-6 (PRDX6) appeared to be underexpressed in WM cells as compared with CLL and MZL cells.22 GLO1 belongs to the glyoxalase complex, which catalyzes the conversion of methylglyoxal to 3-hydroxy-L-proline.23 Peroxiredoxins are a ubiquitous family of antioxidant enzymes that functions as tumor suppressor to certain haematopoietic cancers in mice.24 PRDX6, the most underexpressed protein in WM in our study, is a bifunctional enzyme having both peroxidase and phospholipase A2 activities, with roles in oxidative stress-induced and TNF-induced apoptosis.25 Cytoskeleton proteins also appeared to be differentially expressed in WM. Gelsolin is an actin-binding protein that is a key regulator of actin filament assembly and disassembly.26,27 Gelsolin has been reported increased in vincristine (an anti-microtubule agent)-resistant acute leukemia, whilst moesin was decreased in the same cells.28 Moesin, which expression was decreased in WM in our study, is a member of the ERM protein family, which appears to function as cross-linker between plasma membranes and actin-based cytoskeletons for cell–cell recognition, signaling and cell movement phenomena. Finally, we observed that several isoforms of annexin A6, a calcium-dependent membrane-binding protein, were upregulated in WM cells.29

Again, perhaps the most notable alteration observed in our study was the silencing of Ku70 in WM cells. This was further confirmed by western blot and mRNA analysis in a second cohort of patients, strengthening the consistency of this anomaly. The Ku (Ku70/Ku86) heterodimer was first discovered as an auto-antigen in patients with autoimmune disorders.30 The genes encoding these proteins are located on chromosomes 22q13 and 2q33-35.31 Ku is the DNA-targeting subunit of a DNA-dependent protein kinase (DNA-PK), which is a serine/threonine kinase consisting of a 465-kDa catalytic subunit (DNA-PKcs) and the heterodimeric DNA-PK complex. The latter binds to DNA double-stranded ends and other discontinuities in the DNA32 and recruits the DNA-PKcs of the complex.33 Classical NHEJ involves the DNA–PK complex, essential for lymphoid development, especially VDJ recombination and Ig switching. Indeed Ku70−/− mice lack B-cell maturation and the absence of Ku70 confers hypersensitivity to ionizing radiation and deficiency in DNA double-stranded breaks repair,34 translating in an extreme radiosensitivity and specific VDJ recombination defects,35 as well as high levels of chromosomal aberrations.36,37 In man, DNA–PK activity and increased expression of both Ku70 and Ku86 have been shown to correlate with resistance to therapeutic molecules notably in
**Table 2.** Most differentially expressed proteins between WM, CLL and MZL cells

| Spot no. | FC<sup>b</sup> | Access number<sup>c</sup> | Protein name<sup>d</sup> | Gene<sup>e</sup> | q-value ANOVA<sup>f</sup> | MASCOT score<sup>g</sup> | Sequence cover %<sup>h</sup> | No. of peptides matched<sup>i</sup> | Theoretical pl/MW<sup>j</sup> | Observed pl/MW | Function<sup>k</sup> |
|----------|----------------|---------------------------|-------------------------|------------------|----------------------|-----------------|-----------------|----------------|----------------|----------------|------------------|
| 685      | 14             | Q96G03                    | Phosphoglucomutase-2    | PGM2             | 0.001                | 235             | 10%             | 5              | 6.28/68283 | 6.35/79175 | Catalytic activity, metabolism, energy pathways |
| 653      | 14             | 3                         | Annexin A6              | ANXA6            | 0.0006               | 722             | 36%             | 23             | 5.42/75873 | 5.57/76803 | Calcium ion binding, cell communication, signal transduction |
| 769      | 5.8            | 16                        | Heterogeneous nuclear ribonucleoprotein K | HNRNPK | 0.001              | 100             | 41%             | 15             | 5.39/50976 | 5.24/71014 | Ribonucleoprotein, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism Structural constituent of cytoketens, cell growth and/or maintenance |
| 476      | 5.2            | 12                        | Gelsolin                | GSN              | 0.006               | 94              | 22%             | 12             | 5.9/80641 | 5.82/91105 | Structural molecule activity, cell growth and/or maintenance |
| 709      | 4.8            | 17                        | Lamin-B1                | LMNB1            | 0.001               | 452             | 26%             | 16             | 5.9/80641 | 5.74/91105 | Structural constituent of cytoketens, cell growth and/or maintenance |
| 111      | 3.9            | 2                         | RGB GDP dissociation inhibitor alpha | GDIII | 0.006              | 68              | 27%             | 8              | 5/50583 | 5.05/68326 | GTPase activator activity, cell communication, signal transduction |
| 1017     | 3.4            | 3                         | NEDD8-activating enzyme E1 catalytic subunit | UBA3 | 0.001              | 75              | 21%             | 8              | 5.3/5152 | 5.38/55484 | Ubiquitin-specific protease activity, protein metabolism |
| 678      | 3.3            | 28                        | Annexin A6              | ANXA6            | 0.004               | 722             | 36%             | 24             | 5.42/75873 | 5.57/76803 | Calcium ion binding, cell communication, signal transduction |
| 482      | 3.3            | – 1                       | Gelsolin                | GSN              | 0.02                | 138             | 26%             | 15             | 5.9/80641 | 5.91/90785 | Structural constituent of cytoketens, cell growth and/or maintenance |
| 928      | 2.5            | 15                        | Ubiquitin activating enzyme E1 domain containing 1 | UBA5 | 0.006              | 149             | 19%             | 5              | 4.79/45291 | 4.89/59448 | Protein binding, protein metabolism |
| 798      | – 2.5          | – 1.6                     | Heterogeneous nuclear ribonucleoprotein L | HNRNPL | 0.006              | 268             | 12%             | 5              | 8.46/61333 | 6.83/70435 | RNA binding, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism |
| 1673     | – 2.6          | 16                        | Lactoylglutathione lyase | GLCL1 | 0.005              | 66              | 26%             | 7              | 5.12/20778 | 5.01/22387 | Lyase activity, metabolism, energy pathways |
| 1291     | – 2.9          | 26                        | Glutaredoxin-3          | GLRX3            | 0.0002              | 251             | 22%             | 6              | 5.30/73432 | 5.33/40313 | Molecular function unknown, biological process unknown |
| 1456     | – 3            | – 2.3                     | DnaJ homolog subfamily C member 9 | DNAJC9 | 0.02               | 460             | 43%             | 13             | 5.5/29910 | 5.85/31685 | Chaperone activity, protein metabolism |
| 1046     | – 3.1          | 1.1                       | Ribonucleic acid inhibitor | RNHI | 0.002              | 322             | 15%             | 6              | 4.71/49973 | 4.71/53948 | Translation regulator activity, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism |
| 463      | – 3.5          | – 1.6                     | Elongation factor 2     | EEF2             | 0.003               | 571             | 18%             | 14             | 6.41/95338 | 6.75/96931 | Translation regulator activity, protein metabolism, translation |
| 462      | – 4            | – 2.1                     | Elongation factor 2     | EEF2             | 0.01                | 99              | 3%              | 2              | 6.41/95338 | 6.83/96704 | Translation regulator activity, protein metabolism, translation |
| 651      | – 4.5          | – 2.5                     | Moesin                  | MSN              | 0.03                | 720             | 23%             | 13             | 6.09/67820 | 6.41/82098 | Structural constituent of cytoketens, cell growth and/or maintenance |
| 712      | – 5.6          | – 3.4                     | X-ray repair cross-complementing protein 6 | XRC6 | 0.04               | 114             | 7%              | 5               | 6.23/69843 | 6.47/77978 | DNA binding, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism |
| 712      | – 7.4          | – 3.3                     | X-ray repair cross-complementing protein 6 | XRC6 | 0.04               | 225             | 41%             | 23             | 6.23/69843 | 6.57/77344 | DNA binding, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism |
| 1        | – 12           | – 4.4                     | Peroxiredoxin-6         | PRDX6            | 0.02                | 438             | 46%             | 10             | 6.0/25035 | 6.44/25730 | Peroxidase activity, metabolism, energy pathways |

Abbreviations: ANOVA, analysis of variance; CLL, chronic lymphocytic leukemia; FC, fold change; MW, molecular weight; MZL, marginal zone lymphoma; WM, Waldenström's macroglobulinemia.  
<sup>a</sup>Spot numbers refer to ID spots.  
<sup>b</sup>FC was calculated using Progenesis SameSpots software; negative FC signifying that polypeptide spot is underexpressed in WM samples as compared with CLL or MZL cells, and positive FC signifying that spot is overexpressed in WM cells.  
<sup>c</sup>Accession number for the UniProt database (http://uniprot.org).  
<sup>d</sup>Full protein name recommended by the UniProt consortium.  
<sup>e</sup>Gene names according to HUGO Gene Nomenclature.  
<sup>f</sup>-value from multigroup ANOVA was calculated using Progenesis SameSpots software.  
<sup>g</sup>MASCOT score indicates the confidence of protein identification using the Mascot search engine (http://www.matrixscience.com), depending on amino acid sequence coverage (in %) and on number of matched peptides used for identification in the Swiss-Prot database (http://expasy.org/sprot/).  
<sup>h</sup>Theoretical pI and molecular weight obtained from the Mascot database. Observed molecular weight and pI calculated by DeCyder version 6.5 software, according to location in the gel.  
<sup>i</sup>Protein function and biological process were assigned in accordance with the Human Protein Reference Database (http://hprd.org).
the context of CLL. Minor defects in the NHEJ pathway moreover have been shown to confer predisposition to leukemia. Most LPDs arise via transformation of post-germinal center B cells with chromosomal mutations. Immunodeficiencies with both abnormal DNA repair and genetic polymorphisms of NHEJ components have been associated to an increased susceptibility to the development of lymphoid malignancies, suggesting that an aberrant NHEJ pathway could lead to lymphomagenesis. However, when investigated, no mutation or deletion of Ku86 has been observed in CLL or acute lymphoblastic leukemia cells. Acute lymphoblastic leukemia cells were found to express high levels of DNA-PKcs, Ku86 and Ku70 protein, whereas CLL cells displayed a lower expression of DNA-PKcs and Ku86 but not Ku70. By quantitative RT-PCR in several types of LPD (not including WM), a lower expression of Ku70-encoding XRCC6 gene was observed compared with reactive lymph nodes.

Figure 2. 2D-E analysis of Ku70 protein expression. Spots were analyzed with Progenesis SameSpots (NonLinear Dynamics). (a) Representative focus of the Ku70 protein (spot no. 712) on WM, MZL and CLL samples 2D-gel images. (b) 3D-representation of the Ku70 volume ratios difference between WM, MZL and CLL samples. (c) Statistical analysis of spot no. 712: significant decrease of Ku70 expression in WM cells as compared with CLL cells (*P < 0.05).

Figure 3. Western-blot validation of the Ku70 2D-E profile. Western blot results of Ku70 expression in samples from WM, MZL and CLL cells. Actin was used as loading control.

Figure 4. Quantitative real-time PCR validation for XRCC6. Relative gene-expression quantification for XRCC6 (Ku70) and GUSB (beta-D glucuronidase) as reference gene, in WM and other B LPD (MZL, MCL and CLL) samples (**P < 0.001 by Student’s t-test).

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Lower expression of Ku70 transcripts by quantitative RT-PCR could be the result of at least two phenomena: (a) genetic (point mutation etc.) or epigenetic (promoter methylation, microRNA etc.) mechanisms; recently described as key regulators in WM biology;⁹⁹ (b) as part of a larger transcriptional downregulation that is, expression of BLIMP1—a transcriptional repressor — involved in terminal differentiation of B cells to plasma cells has been shown to shut down immunoglobulin class switching through inhibition of activation-induced cytidine deaminase, Ku70, Ku86, DNA-PKcs and STAT6.³⁰

In conclusion, this is a comprehensive proteomic analysis of the WM cells in comparison with that of two other B LPDs. This study shows that WM harbors a unique proteome with regards to CLL and MZL cells. A rather limited set of proteins were found to be differentially expressed with no ‘usual suspects’ being part of this list where the majority of proteins were rather housekeeping/structural proteins. The confirmation of the downregulation of Ku70—part of the Ku heterodimer, a critical factor in class switch recombination (lacking in WM)—and its mechanisms need to be further investigated in model systems.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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37 Gu Y, Jin S, Gao Y, Weaver DT, Alt FW. Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination. Proc Natl Acad Sci USA 1997; 94: 8076–8081.

38 Ferguson DO, Sekiguchi JM, Chang S, Frank KM, Gao Y, DePinho RA et al. The nonhomologous end-joining pathway of DNA repair is required for genomic stability and the suppression of translocations. Proc Natl Acad Sci USA 2000; 97: 6630–6633.

39 Difilippantonio MJ, Zhu J, Chen HT, Meffre E, Nussenzweig MC, Max EE et al. DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. Nature 2000; 404: 510–514.

40 Muller C, Christodouloupolous G, Salles B, Panasci L. DNA-Dependent protein kinase activity correlates with clinical and in vitro sensitivity of chronic lymphocytic leukemia lymphocytes to nitrogen mustards. Blood 1998; 92: 2213–2219.

41 Riballo E, Critchlow SE, Teo SH, Doherty AJ, Priestley A, Broughton B et al. Identification of a defect in DNA ligase IV in a radiosensitive leukemia patient. Curr Biol 1999; 9: 699–702.

42 Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. Nat Rev Cancer 2005; 5: 251–262.

43 Moshous D, Pannetier C, Chasseval Rd R, Deist Fl F, Cavazzana-Calvo M, Romana S et al. Partial T and B lymphocyte immunodeficiency and predisposition to lymphoma in patients with hypomorphic mutations in Artemis. J Clin Invest 2003; 111: 381–387.

44 Roddam PL, Rollinson S, O'Driscoll M, Jeggo PA, Jack A, Morgan GJ. Genetic variants of NHEJ DNA ligase IV can affect the risk of developing multiple myeloma, a tumour characterised by aberrant class switch recombination. J Med Genet 2002; 39: 900–905.

45 Hill DA, Wang SS, Cerhan JR, Davis S, Cozen W, Severson RK et al. Risk of non-Hodgkin lymphoma (NHL) in relation to germline variation in DNA repair and related genes. Blood 2006; 108: 3161–3167.

46 Chen TY, Chen JS, Su WC, Wu MS, Tsao CJ. Expression of DNA repair gene Ku80 in lymphoid neoplasm. Eur J Haematol 2005; 74: 481–488.

47 Holgersson A, Erdal H, Nilsson A, Lewensohn R, Kanter L. Expression of DNA-PKcs and Ku86, but not Ku70, differs between lymphoid malignancies. Exp Mol Pathol 2004; 77: 1–6.

48 Roddam PL, Allan JM, Dring AM, Worrillow LJ, Davies FE, Morgan GJ. Non-homologous end-joining gene profiling reveals distinct expression patterns associated with lymphoma and multiple myeloma. Br J Haematol 2010; 149: 258–262.

49 Sacco A, Issa GC, Zhang Y, Liu Y, Maiso P, Ghobrial IM et al. Epigenetic modifications as key regulators of Waldenstrom’s Macroglobulinemia biology. J Hematol Oncol 2010; 3: 38.

50 Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A et al. Blimp1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity 2002; 17: 51–62.