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Véronique Gelsi-Boyer,1,2,3,* Nathalie Cervera,1,* François Bertucci,1,3 Mandy Brecqueville,1 Pascal Finetti,1 Anne Murati,1,2 Christine Arnoulet,7 Marie-Joelle Mozziconacci,1,2 Ken I. Mills,4 Nicholas C. P. Cross,5 Norbert Vey,1,6 and Daniel Birnbaum4

1Centre de Recherche en Cancérologie de Marseille; Laboratoire d’Oncologie Moléculaire; UMR1068 Inserm; Institut Paoli-Calmettes; Marseille, France; 2Département de BioPathologie, Institut Paoli-Calmettes, Marseille, France; 3Faculté de Médecine, Aix-Marseille Université, Marseille, France; 4Centre for Cancer Research and Cell Biology, Queens University Belfast, UK; 5Faculty of Medicine, University of Southampton, UK and Wessex Regional Genetics Laboratory, Salisbury, UK; and 6Département d’HématoLogie, Institut Paoli-Calmettes, Marseille, France

ABSTRACT

Chronic myelomonocytic leukemia is similar to but a separate entity from both myeloproliferative neoplasms and myelodysplastic syndromes, and shows either myeloproliferative or myelodysplastic features. We ask whether this distinction may have a molecular basis. We established the gene expression profiles of 39 samples of chronic myelomonocytic leukemia (including 12 CD34-positive) and 52 CD34-positive samples of myelodysplastic syndromes by using Affymetrix microarrays, and studied the status of 18 genes by Sanger sequencing and array-comparative genomic hybridization in 53 samples. Analysis of 12 mRNAs from chronic myelomonocytic leukemia established a gene expression signature of 122 probe sets differentially expressed between proliferative and dysplastic cases of chronic myelomonocytic leukemia. As compared to proliferative cases, dysplastic cases over-expressed genes involved in red blood cell biology. When applied to 32 myelodysplastic syndromes, this gene expression signature was able to discriminate refractory anemias with ring sideroblasts from refractory anemias with excess of blasts. By comparing mRNAs from these two forms of myelodysplastic syndromes we derived a second gene expression signature. This signature separated the myelodysplastic and myeloproliferative forms of chronic myelomonocytic leukemias. These results were validated using two independent gene expression data sets. We found that myelodysplastic chronic myelomonocytic leukemias are characterized by mutations in transcription/epigenetic regulators (ASXL1, RUNX1, TET2) and splicing genes (SRSF2) and the absence of mutations in signaling genes. Myelodysplastic chronic myelomonocytic leukemias and refractory anemias with ring sideroblasts share a common expression program suggesting they are part of a continuum, which is not totally explained by their similar but not, however, identical mutation spectrum.

Introduction

Chronic myelomonocytic leukemia (CMMML) is a malignant hematologic disease of the elderly characterized by peripheral blood monocytosis, overproduction of bone marrow monocytes with dysplasia of one or more lineages, and less than 20% of blasts in the bone marrow. Its prognosis is poor with a median survival of 12-18 months and a 15-20% risk of transformation into acute myeloid leukemia (AML).1,2 CMMML is classified by the World Health Organization (WHO) into the myelodysplastic/myeloproliferative neoplasms and, based on the number of blasts, subclassified into CMMML1 and CMMML2 (5-9% and 10-19%, respectively).3 Like myelodysplastic syndromes (MDS), CMMML shows dysplastic features that reflect ineffective hematopoiesis; however, dysplasia is associated with bone marrow proliferation.4-5 Because of this duality, CMMML had been separated into a myeloproliferative form (MP-CMMML) and a myelodysplastic form (MD-CMMML) based on a semi-arbitrary threshold of 15 x 10^9/L for peripheral white blood cell (WBC).6 However, due to its lack of impact on outcome, this separation is not included in the WHO classification.5 Yet, the prognosis of MD-CMMML but not MP-CMMML may be evaluated by the international prognostic scoring system, underlining a similarity of MD-CMMML with MDS. Moreover, even if, given the limited treatments currently available, MD and MP-CMMMLs have similar outcome, this situation may change with the advent of new therapies, in which case they would each need to be recognized separately.

Because CMMML has both dysplastic and proliferative features it is likely that the disease is heterogeneous. We wanted to determine whether these MD and MP features may have any relevant molecular basis that may help classify and understand CMMML. To this aim, we established the gene expression profiles and the mutational status of CMMML and compared them to those of MDS.

Design and Methods

Patients and samples

We selected 53 CMMML and 32 MDS bone marrow (BM) samples previously studied by array-comparative genome hybridization...
(aCGH) and sequencing of candidate genes. According to the French-American-British (FAB) and WHO classifications, the CMML series was made up of 31 MP and 22 MD cases (Online Supplementary Table S1) and the MDS panel 8 refractory anemia (RA) with ring sideroblasts (RAS), 15 RA with excess of blasts type 1 (RAEB1) and 11 RAEB type 2 (RAEB2). CMML and MDS cases selected for gene expression profiling were collected at the time of diagnosis or in therapeutic abstention; none had been treated. All patients signed an informed consent for research and the study was approved by our institutional review board ("Comité d’Orientation Scientifique" of the Institut Paoli-Calmettes).

CD34 enrichment

Samples were enriched in CD34-positive (CD34+) cells for 12 CMML and 32 MDS cases. Leukocytes were obtained after bone marrow red cell lysis and washing with PBS, and labeled with magnetic bead-conjugated anti-CD34 monoclonal antibody (AC34 MicroBead; Miltenyi Biotec, Auburn, CA, USA). CD34+ hematopoietic stem cell populations were then purified through a miniMACS magnetic cell separation column (Miltenyi Biotec).

RNA/DNA extraction

RNAs and DNAs were extracted from whole BM CMML samples. After BM aspiration, a red cell lysis was carried out, followed by rinses with PBS. Leukocytes were processed immediately or cryopreserved at −80°C at the sample bank of the Institute and processed later. DNA and RNA were extracted using Nucleobond RNA/DNA kit from Macherey-Nagel (Düren, Germany) as recommended by the supplier. RNA from CD34+ cells were similarly extracted using Nucleobond RNA/DNA kit from Macherey-Nagel.

Sequencing of 18 candidate genes

Mutations in ASXL1 (exon 12), CBL (exons 8, 9), DNMT3A (exons 15-23), EZH2 (all exons), FLT3 (exons 14, 15, 20), IDH1/2 (exons 4), JAK2 (exon 14), NFI (exons 1-50), NKRAS (exons 1, 2), PTPN11 (exons 3, 11), RUNX1 (exons 1-8), SF3B1 (exons 12-16), SUZ12 (exons 14-16), SRSF2 (exon 2), TET2 (exons 3-11), U2AF35/U2AF1 (exons 2, 6), and ZRSR2 (exons 1-11) were analyzed using BM DNA as previously described. Gene expression profiling

Gene expression profiling of 39 CMML (out of the 53) and 32 MDS (all from CD34+ cells) mRNAs were established. Among the 39 CMML cases, 37 were studied as BM (10 of these were also studied as CD34+) and 2 as CD34+ RNAs. In other words, 10 CMML samples were studied as both CD34+ and whole BM RNAs, and 2 as CD34+ only (12 CD34+ in total).

RNA quality and purity were assessed with Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Preparation of cRNA, hybridizations onto Affymetrix U133 Plus 2.0 human oligonucleotide microarrays, washes and detection were carried out as recommended by the supplier and as previously described. Data were analyzed by the Robust Multichip Average (RMA) method in R using Bioconductor and associated package, as previously described. Before analysis, a first filtering process removed from the data set the probe sets with low and poorly measured expression as defined by an expression value inferior to 100 units, thus retaining 19,780 probe sets in the 12 CMML CD34+ data set and 23,515 probe sets in the 32 MDS CD34+ data set.

Before hierarchical clustering, a second filter, based on the intensity of standard deviation (SD >0.5), was applied and retained 9,179 probe sets in the 37 CMML from the whole BM data set, 12,660 probe sets in the 12 CMML CD34+ data set, and 11,623 probe sets in the 32 MDS CD34+ data set. Filtered data were then log2-transformed and submitted to the Cluster program using data median-centered on genes, Pearson’s correlation as similarity metrics and centroid linkage clustering. Results were shown using the TreeView program.

Supervised analyses identified and ranked genes that discriminate two groups of samples. A discriminating score (DS) was calculated for each of the 19,730 probe sets for the 12 CMML and of the 23,515 probe sets for the 32 MDS. A ‘leave-one-out’ (LOO) cross-validation procedure was applied to estimate the accuracy of prediction of the signature and the validity of the supervised analysis. Functional processes and pathways were identified using Ingenuity software (Ingenuity Systems, Redwood City, CA, USA).

To test the performance of our signature on independent panels, we analyzed publicly-available external data sets collected from NCBIGenBank GEO database (series entry GSE4619 and entry GSE15061). Gene set enrichment analysis (GSEA) was carried out as reported. Fisher’s exact test was used when appropriate. All statistical tests were two-sided at the 5% level of significance. All statistical analyses were carried out in R (2.8.0) and its associated packages.

Results

Gene expression analysis separates MD- from MP-CMML cases

We first determined the gene expression profiles of 37 BM CMML samples. Unsupervised analysis identified two clusters (S1 and S2) including 17 and 20 cases, respectively (Online Supplementary Figure S1). S1 and S2 cases did not correlate with clinical or hematologic data and were not separated according to MD/MP features. We next determined the gene expression profiles of 12 available RNAs from CD34+ CMML samples (5 MD and 7 MP). Hierarchical clustering separated the 12 samples into two clusters (Online Supplementary Figure S2). The two clusters differed (Fisher’s exact test, P=0.04) in terms of leukocytosis and overlapped the MP/MD definition: the left cluster contained 4 of 5 MD-CMML cases whereas the right cluster comprised 6 of 7 MP-CMML cases (black boxes). This showed that the MD/MP distinction has a molecular basis at the transcriptional level on a whole-genome scale.

MD-CMML over-expresses genes involved in red blood cell biology as compared to MP-CMML

To understand this MD/MP difference, we compared the gene expression profiles from the 5 MD-CMML samples to those of the 7 MP-CMML samples in a supervised analysis. A total of 122 probe sets (corresponding to 96 unique genes and 6 ESTs; Online Supplementary Table S2) were differentially expressed between the two forms. The accuracy of prediction and validity of our procedure was cross validated by LOO with overall accuracy of 92% (Fisher’s exact test, P=0.015) with high sensitivity and specificity (86% and 100%; only one MP-CMML was misplaced) and with a theoretical number of false positive of 30.

Among the 122 probe sets, 61 were up-regulated and 61 were down-regulated in the MP samples (the top 20 up-regulated genes are listed in the Online Supplementary Table S3). Inspection of the list (hereafter called MD/MP CMML gene expression signature or CMML GES) showed that up-regulated genes in MD-CMML belonged to pathways and cell processes found in red blood cells: they encoded...
enzymes involved in heme synthesis (ALAS2, HMBS, FECH), glycoporins (GYP A, GYP B), globins (HBA1, HBB, HBM), and proteins associated with blood groups (RHD, RHCE) and erythrocyte differentiation (TRIM10). Ingenuity analysis of this GES confirmed that the most relevant over-expressed genes in MD-CMMML cases were involved in erythropoiesis (data not shown). Down-regulated genes in MD-CMMML included ZCCHC11/TUT4, PHC1 and BMI1.

We applied this CMMML GES to our 12 CD34+ CMMML RNAs. As expected, the MD and MP samples were separated (Fisher’s exact test, $P=1\times10^{-3}$) (Figure 1A). We applied this GES to the 37 BM CMMML RNAs (including 10 of the 10 CD34+ samples and 27 additional samples). Two clusters were observed: in the left cluster, 16 of the 19 samples were MP-CMMML, whereas in the right cluster, 13 of the 18 samples were MD-CMMML (Figure 1B), supporting the validity of our MD/MP CMMML GES (Fisher’s exact test; $P=1\times10^{-3}$).
Using GSEA, we confronted the gene expression profiles of our 37 BM CMML mRNAs to the 122 gene signature. We found a significant enrichment in the red cell genes of this signature in the MD-CMML samples (Enrichment Score=0.76; Normalized Enrichment Score=2.08; FDR q-value<0.01).

**CMML gene expression signature classifies MDS samples**

Overexpression of genes involved in red cell biology has been observed in previous gene expression analyses of RARS samples.\(^1\)\(^7\) When applied to our 32 CD34\(^+\) MDS samples, the CMML GES perfectly separated RARS from RAEB samples (Fisher’s exact test, \(P=1\times10^{-4}\); Figure 1C). When the CMML GES was applied to the pool of 12 CMML and 32 MDS CD34\(^+\) samples the MD-CMMLs clustered with the RARS samples and the MP-CMMLs with the RAEB samples (Figure 1D) (Fisher’s exact test, \(P=1.5\times10^{-5}\)). These results showed that MD-CMML and RARS share gene similar expression programs.

**MDS gene expression signature classifies CMML samples**

Then we derived an MDS GES by comparing the gene expression profiles of our 8 RARS to those of our 24 RAEB samples. A total of 428 probe sets (295 unique genes and 25 ESTs; Online Supplementary Table S4) were differentially expressed between RARS and RAEB (hereafter called MDS GES). The accuracy of prediction and validity of our procedure was cross-validated by LOO with overall accuracy of 78\% (Fisher’s exact test, \(P=6\times10^{-10}\)) with high sensitivity and specificity (72\% and 100\%, respectively) and with a theoretical number of false positive of 5.

A total of 304 probe sets were up-regulated and 124 were down-regulated in the RARS cases (the top 20 up-regulated genes are listed in Online Supplementary Table S3). Up-regulated genes in RARS belonged to the pathway and cell processes found in red blood cells (Online Supplementary Figure S3): they encoded proteins involved in heme synthesis (ALAS2, ALAD, HBBS, UROD, CPOX, PPOX, FECH), iron-sulfur cluster biogenesis (SLC25A57, GLRX5), mitochondrial biology (TRAK2), antioxidant defense (HAGH), glycoporphins (GYPA, GYPB, GYPE), globins (HBA1, HBA2, HBB, HBD, HBG1, HBM, HBO1), proteins associated with blood groups (RHCE, RHDS, DARC, KEL), red cell structure (ANK1, EPB42, EPB49, ERMAF, SPTA1, SLC4A1), differentiation (TRIM10) and regulation (GATA1, KLF1, TAL1, EFOR). Ingenuity analysis of this GES confirmed that the most relevant over-expressed genes in RARS were involved in erythropoiesis (data not shown). When applied to our 32 CD34\(^+\) MDS RNAs, the MDS GES perfectly separated the RARS from the RAEB samples (Figure 2A), as expected. More surprisingly, when applied to the 12 CD34\(^+\) (Figure 2B) or BM CMML mRNAs (Fisher’s exact test, \(P=8.4\times10^{-4}\); data not shown) it perfectly separated the MD-CMML from the MP-CMML samples.

Thus, MD-CMML and RARS share expression of genes involved in erythropoiesis and red blood cell biology, with a total of 38 probe sets corresponding to 25 unique genes common to the MD/MP CMML and MDS GESs (Online Supplementary Table S3).

**External validation on two independent data sets**

To validate these unexpected results, we applied our two GESs (CMML GES and MDS GES) to two external published data sets (a CMML and an MDS data set)\(^4\)\(^5\) and a GES obtained from one of these external data sets to our CMML and MDS samples.

First, we studied the external data sets with our two GESs. The first data set corresponded to the expression profiles of 25 CMML BM samples profiled using Affymetrix microarrays.\(^8\) Unfortunately, information on MD/MP forms was not associated with the data. Our CMML GES separated these external CMML cases into two groups, one of which over-expressed red blood cell genes (Online Supplementary Figure S4A, stripe 1). All but one of the 25 CMML samples was similarly sorted with our MDS GES (Online Supplementary Figure S4B, stripe 2). We studied a second external data set, consisting in the expression profiles of 66 CD34\(^+\) MDS samples studied by Affymetrix microarrays.\(^4\) Our MDS GES separated the RARS from the other MDS classes (Figure 3A), as did our CMML GES (Figure 3B).

Second, we derived a third GES (hereafter called MDS-ext GES) by comparing RARS and RAEB samples from the MDS external data set.\(^7\) This MDS-ext GES contained 738
probe sets (597 unique genes and 28 ESTs). The genes found over-expressed in RARS are involved in red blood cell biology. This GES was able to separate our RARS from RAEB samples (Online Supplementary Figure S4C) and our MD-CMML from MP-CMML CD34-positive samples (Figure 3C). When applied to the CMML external data set, it separated the 25 samples (stripe 3) in the same way as did our CMML and MDS GESs (Online Supplementary Figure S4D).

Thus, GESs obtained from comparison from either CMML or MDS were similarly able to distinguish CMML and MDS classes, showing that MD-CMML and RARS share common molecular features. The CMML GES, MDS GES and MDS-ext GES had 26 probe sets in common (corresponding to 16 genes and 1 EST) (Figure 3D), all overexpressed in MD-CMML and RARS and involved in red cell biology (Online Supplementary Table S3). This small core GES separated MD-CMML from MP-CMML (Figure 3E) and RARS from RAEB (Online Supplementary Figure S5) as efficiently as the three larger GESs.

Analysis of mutated genes in CMML and MDS
These results showed a molecular similarity between MD-CMML and RARS. Could this similarity be the result of gene mutations common and specific to the two diseases? We and others have previously studied several leukemogenic genes in CMML and RARS. However, several of those (e.g. ASXL1, RUNX1, TET2) are neither specific of MD-CMML nor of RARS and, therefore, can account neither for the similarity between the two diseases nor for the differences from the other myeloid malignancies.

Figure 3. Independent validation of the gene expression signatures. (A) Hierarchical clustering of 66 Pellagatti’s CD34+ MDS external samples using our 428-gene MDS GES. Legend similar to Figure 1A. MDS classes are indicated by colored boxes in the stripe under the dendrogram. The GES significantly separates the RARS from the other MDS classes. (B) Similar to (A), but using our 122-gene MD/MP CMML. The GES significantly separates the RARS from the other MDS classes. (C) Hierarchical clustering of our 12 CD34-positive CMML samples using the MDS-ext GES. MD/MP forms are indicated by black and white boxes in the stripe under the dendrogram. The GES significantly separates the MD-CMML samples from the MP-CMML samples. (D) Venn diagram showing the overlap between the three GES: 26 genes overlap the three GES. (E) Hierarchical clustering of the 12 mRNAs from CD34+ CMML samples using these 26 overlapping genes. This 26-gene GES separates the MD-CMML from the MP-CMML samples.
We studied the sequence of 18 genes involved in the regulation of transcription (ASXL1, DNMT3A, EZH2, IDH1, IDH2, RUNX1, SUZ12, TET2), splicing (SF3B1, SRSF2, U2AF1, ZRSR2) and signaling (CBL, FLT3, JAK2, KRAS, NRAS, PTPN11) in our series of 53 CMMLs. Results are shown in Figure 4, Online Supplementary Table S5 (see also Online Supplementary Figure S1). The samples had been studied by aCGH, and deletions of these genes were taken into account when appropriate. In CMML, splicing mutations have been recently described, but how mutations in 4 splicing genes combine with alterations in the other 14 genes has not yet been reported. Only seven samples (3 MD and 4 MP) did not show any mutation in the genes studied (15%). Among transcription/epigenetic regulators, ASXL1, RUNX1 and TET2 were the most frequently mutated genes.

Mutations in these genes were found in both MP and MD cases. DNMT3A mutations were only found in MD-CMML and EZH2 mutations in MP-CMML; however, the number of these events was low, confirming findings of previous studies. MD-CMML showed only one CBL mutation and one NFI deletion, whereas MP-CMML showed 14 mutations in signaling genes and one NFI deletion. Thus, mutations in these selected signaling genes account for a first difference between MD-CMML and MP-CMML.

Except in one case, mutations in splicing genes were mutually exclusive, as previously described and recently reviewed. We found one mutation in SF3B1, 3 in U2AF1, 2 in ZRSR2 and 24 in SRSF2. In total, 15 of 22 (68%) MD and 15 of 31 (45%) MP cases were mutated in the splicing genes studied. They were not differentially distributed between the two forms (P=0.17): the 22 MD cases showed no SF3B1 and 13 SRSF2 mutations (59%) whereas the 31 MP cases showed one SF3B1 and 11 SRSF2 mutations (38.7%). In addition, U2AF1 and ZRSR2 were mutated in one and one MD-CMML cases and in 2 and one MP-CMML cases, respectively.

To evaluate the possible relationships between gene mutations and gene expression we looked at the expression of the mutated genes (Online Supplementary Figure S2B). As expected, since they were not found in the GES, there was no difference in expression of the 18 genes between MD and MP-CMML. Gene mutations in our series of MDS cases have been described in previous studies, and will not be detailed here again for the sake of comparison with CMML. We found 6 mutations in SF3B1 and 3 in SRSF2 in our series of 32 MDs (Online Supplementary Table S6). They were differentially distributed between RARS and RAEB cases: the 8 RARS showed 5 SF3B1 and one SRSF2 mutations whereas the 24 RAEB cases showed one SF3B1 and 2 SRSF2 mutations. Three RAEB cases were also mutated in U2AF1 and ZRSR2.

Thus, both MD-CMML and RARS display frequent mutations in genes encoding components of the RNA splicing machinery. However, MP-CMML cases also show alterations of this process. RARS and MD-CMML also show more mutations in DNMT3A.

**Discussion**

We studied CMML by gene expression profiling and by sequencing analyses of 18 candidate genes. Unsupervised analysis of mRNAs from CD34-positive cells separated CMML into two molecular subtypes that overlapped with the MD and MP forms initially distinguished by the FAB classification. Supervised analysis established an MD/MP CMML GES characterized by the overexpression in MD-CMML of genes involved in red blood cells. The comparison of RARS and RAEB samples allowed the establishment of an MDS GES that was also characterized by the overexpression in RARS of the same genes and functions, as observed in previous studies. The CMML GES recognized the RARS and also the MDS GES recognized the MD-CMML samples. Thus, MD-CMML and RARS share

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**Figure 4.** Gene mutations in CMML. Profiles of concomitant gene mutations of 18 genes in 53 CMML cases. Mutations in RARS (Online Supplementary Table S6) are shown for comparison. The number of gene mutations per case is presented below. Missing data are indicated by gray squares.
a similar transcriptional program involving red blood cell homeostasis. These results were validated by using two external published data sets and a third GES (MDs-ext GES) suggesting the robustness of the results. In MD-CMML and RARS, several red blood cell functions were affected, including heme biosynthesis, iron-sulfur cluster biogenesis, calcium uptake, antioxidant defense, and transcription regulation, suggesting that MD-CMML and RARS share a common differentiation pathway characterized by an erythroidic program.

In MD-CMML and RARS these results could reflect a mere abundance of erythroid progenitors, an overexpression of the program to compensate for abnormal red blood cell maturation, or a true molecular defect with pathological consequences. This defect in MD-CMML and RARS may be different or similar. In the latter case at least two possibilities should be considered. First, genes present in the signatures and/or involved in the identified functions could be directly affected by mutations. Many of these functions take place in the mitochondrion and it is possible that the defect affects primarily this organelle; the role of the iron transporter gene ABCB7 had been suspected, but this gene had not mutated.19 Actually, mutations in mitochondrial genes are associated with congenital rather than acquired anemias. A second hypothesis is perhaps the most likely. Mutations in splicing factors may indirectly affect red cell processes by leading to abnormal processing of mRNAs, including RNAs involved in red cell biology.20 These frequently show alternative splicing41 which may make them hypersensitive to splicing mutations. SRSF2 mutations are prominent in MDS with ring sideroblasts (RS) (a hallmark of iron overload and dyserythropoiesis) such as RARS, and SRSF1 haploinsufficiency or inhibition leads to the formation of RS.42 In contrast, SRSF2 mutations are frequent in CMLL.31 Splicing defects in SRSF2 in CMLL and in SRSF1 in RARS may affect genes involved in erythropoiesis such as ABCB7, FLT3, GATA1, or HAMP for example, or a master transcription factor of hematopoietic cell lineages such as TIF1γ, which controls erythroid cell fate and acts as a tumor suppressor in CMLL.64,46 However, SRSF2 mutations are also found in MP-CMML which does not show a red cell program. MP-CMML is characterized by mutations in signaling genes, and this could modify the effect of SRSF2. Overexpression of components of the pathway may also compensate for the defect (e.g. DICER1, CUGBP2/CELF2, ZCCHC14/TUT4 and SYNC RIP which are involved in miRNA and mRNA editing).

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