Detection of *CEBPA* Double Mutants in Acute Myeloid Leukemia Using a Custom Gene Expression Array

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Double (bi-allelic) mutations in the gene encoding the CCAAT/enhancer-binding protein- α (*CEBPA*) transcription factor have a favorable prognostic impact in acute myeloid leukemia (AML). Double mutations in *CEBPA* can be detected using various techniques, but it is a notoriously difficult gene to sequence due to its high GC-content. Here we developed a two-step gene expression classifier for accurate and standardized detection of *CEBPA* double mutations. The key feature of the two-step classifier is that it explicitly removes cases with low *CEBPA* expression, thereby excluding *CEBPA* hypermethylated cases that have similar gene expression profiles as a *CEBPA* double mutant, which would result in false-positive predictions. In the second step, we have developed a 55 gene signature to identity the true *CEBPA* double-mutation cases. This two-step classifier was tested on a cohort of 505 unselected AML cases, including 26 *CEBPA* double mutants, 12 *CEBPA* single mutants, and seven *CEBPA* promoter hypermethylated cases, on which its performance was estimated by a double-loop cross-validation protocol. The two-step classifier achieves a sensitivity of 96.2% (95% confidence interval [CI] 81.1 to 99.3) and specificity of 100.0% (95% CI 99.2 to 100.0). There are no false-positive detections. This two-step *CEBPA* double-mutation classifier has been incorporated on a microarray platform that can simultaneously detect other relevant molecular biomarkers, which allows for a standardized comprehensive diagnostic assay. In conclusion, gene expression profiling provides a reliable method for *CEBPA* double-mutation detection in patients with AML for clinical use.

Introduction

ONE OF THE hallmarks of acute myeloid leukemia (AML) is the accumulation of undifferentiated myeloid cells carrying specific genetic aberrations in the bone marrow (Löwenberg *et al.*, 1999). The CCAAT/enhancer-binding protein- α gene (*CEBPA*) encodes a transcription factor that has been found to play a critical role in granulopoiesis and is mutated in 5%–14% of patients with AML (Pabst *et al.*, 2001; Gombart *et al.*, 2002; Preudhomme *et al.*, 2002; Snaddon *et al.*, 2003; van Waalwijk van Doorn-Khosrovani *et al.*, 2003; Fröhling *et al.*, 2004; Nerlov, 2004; Bienz *et al.*, 2005). Two types of mutations in *CEBPA* are more frequently found in AML; first, out-of-frame mutations or mutations introducing a stop codon in the N-terminal part of the gene causing premature stops in translation (Zhang *et al.*, 2004), and second, in-frame mutations in the C-terminal basic leucine zipper (bZIP) region of the gene, which appear to impair DNA binding and/or homo- and heterodimerization (Nerlov, 2004). Most AML cases with mutant *CEBPA* have two mutations, most probably bi-allelic (referred to as *CEBPA* dm, double mutant,

here). The majority of these cases carry a combination of an N-terminal and C-terminal mutation (Nerlov, 2004; Leroy *et al.*, 2005; Pabst and Mueller, 2007). A minority of *CEBPA* mutant AML cases are mono-allelic, with a single mutation (referred to as *CEBPA* sm, single mutant) (Nerlov, 2004; Leroy *et al.*, 2005).

The WHO has defined AML with *CEBPA* mutations (single and double collectively) as a provisional favorable subtype of AML (Swerdlow *et al.*, 2008). AML cases with a *CEBPA* mutation are predominantly found among the cytogenetically intermediate risk group, mainly those with normal karyotypes (Grimwade *et al.*, 1998). However, AMLs carrying *CEBPA* double mutations rather than *CEBPA* single mutations represent a distinct prognostically favorable group of AML cases, and therefore, *CEBPA* double mutants provide a clinically useful marker for risk stratification of the leukemia (Pabst *et al.*, 2009; Renneville *et al.*, 2009; Wouters *et al.*, 2009; Green *et al.*, 2010; Taskesen *et al.*, 2011). Finally, AML cases have been identified that are *CEBPA* wt, yet are hypermethylated (Figuroa *et al.*, 2009). Not surprisingly, these AMLs display gene expression patterns that are very similar

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to those of *CEBPA* double mutants because a shared set of downstream transcripts are low either due to the bi-allelic mutations or the hypermethylated promoter status of *CEBPA* (Figuroa *et al.*, 2009; Taskesen *et al.*, 2011). However, *CEBPA* double-mutation AMLs and *CEBPA* hypermethylated AMLs are different as regard to the gene expression levels of *CEBPA* itself, which are generally higher in the double-mutant AML subtypes, but low to absent in the hypermethylated subtype.

Classification of *CEBPA* dm cases has been previously demonstrated using gene expression profiling (Wouters *et al.*, 2009); however, misclassified cases were the *CEBPA* hypermethylated AMLs (Figuroa *et al.*, 2009; Taskesen *et al.*, 2011). Herein we have developed a two-step classifier, which explicitly distinguishes the *CEBPA* double mutants from the *CEBPA* hypermethylated. Accuracy of this two-step classifier was established by means of a double-loop cross validation on a cohort of 505 primary AML cases. The two-step classifier was then incorporated on a custom chip, along with detection capabilities for *AML1-ETO*, *CBFB-MYH11*, *PML-RARA*, other mutations such as *NPM1* (Van Vliet *et al.*, Submitted), as well as overexpression of prognostic genes *EVII* and *BAALC* (Brand *et al.*, in preparation). Thus, this gene expression assay provides a standardized test for multiple molecular biomarkers relevant for AML at the time of diagnosis.

Materials and Methods

Custom in vitro diagnostic gene expression array

A customized Affymetrix (Santa Clara, CA) GeneChip microarray was designed (AMLprofiler™). This custom chip includes a subset of the probe sets present on the Affymetrix HG-U133 Plus2 platform and a subset of probes designed for specific purposes. Its resulting .CEL file is automatically routed through proprietary software that reports 0 or 1 for *CEBPA* wt and *CEBPA* dm, respectively.

Datasets

We employed the blood or bone marrow specimens from 505 AML patients, who had been enrolled in the Dutch–Belgian Hematology–Oncology Cooperative group protocols -04, -04A, -29, and -42 (HOVON-SAKK, the Erasmus University Medical Center, Rotterdam), (Valk *et al.*, 2004; Wouters *et al.*, 2009). The 505 cases employed here are a subset from the 524 cases (Wouters *et al.*, 2009) for which the hybridization cocktail was available. The 505 set contains all 26 *CEBPA* dm and 12 *CEBPA* sm cases from the Wouters *et al.* (2009) cohort (Table 1). Sample processing and quality control were performed as

previously described (Wouters *et al.*, 2009). Bi-allelic mutant, single mutant, and wild-type *CEBPA* annotations were confirmed in all cases by the entire *CEBPA* coding region investigation by denaturing high-performance liquid chromatography (dHPLC), analysis of selected regions by agarose gel analysis (van Waalwijk van Doorn-Khosrovani *et al.*, 2003), and/or nucleotide sequencing (Wouters *et al.*, 2009). *CEBPA* promoter hypermethylation was determined using the HELP assay (HpaII tiny fragment enrichment by ligation-mediated PCR), as previously described (Valk *et al.*, 2004). Table 1 shows an overview of the *CEBPA* mutation status of all AML cases in the cohort.

Hybridization cocktails were obtained for all cases (Verhaak *et al.*, 2009), and were hybridized onto the AMLprofiler platform. All .CEL files were preprocessed using the MAS5 algorithm (scaling to 1500), and probe set intensities below 30 truncated to 30. Subsequently, geometric mean centering was applied per probe set relative to a subset of 244 AML cases.

AML profiler CEL files are available at the Gene Expression Omnibus (National Center for Biotechnology Information) under accession number GSE42194.

All patients provided written informed consent in accordance with the Declaration of Helsinki.

Double-loop cross validation

To build diagnostic classifiers from high throughput data, we used the double-loop cross-validation framework (Wessels *et al.*, 2005). We adopted this methodology combined with forward filtering as the feature selector, the signal to noise ratio as a criterion to evaluate the individual genes, and ClaNC (Dabney, 2005) a simple classifier that is known to perform

FIG. 1. *CEBPA* hypermethylated cases are often FP in *CEBPA* double-mutant classifiers. The scatter plot showing the output of a one-step classifier trained on the 505 cases as determined by the double-loop cross-validation procedure. Numbers indicate how often a case was misclassified in the 100 repeats (samples always correctly classified do not have a number printed). Color images available online at www.liebertpub.com/gtmb

TABLE 1. OVERVIEW OF CASES IN THE COHORT, AND DISTRIBUTION OF *CEBPA* MUTATIONS

<i>CEBPA</i> status	Label	Training cohort		Above step 1 threshold	
		n	%	n	%
<i>CEBPA</i> WT	Neg	458	90.7%	48	60.8%
<i>CEBPA</i>	Neg	9	1.8%	0	0.0%
Hypermethylated					
<i>CEBPA</i> single mutant	Neg	12	2.4%	5	6.3%
<i>CEBPA</i> double mutant	Pos	26	5.1%	26	32.9%
Total		505	100.0%	79	100.0%

TABLE 2. DOUBLE-LOOP CROSS-VALIDATION PERFORMANCES OF THE CLASSIFIERS FOR PREDICTING *CEBPA* DOUBLE MUTATIONS

Classifier	n	TP	FN	TN	FP	Sens	95% CI		Spec	95% CI	
							LL	UL		LL	UL
Overall classifier	505	24.79	1.21	474.70	4.30	95.3	80.0	99.1	99.1	97.8	99.6
Two-Step classifier	505	25.00	1.00	479.00	0.00	96.2	81.1	99.3	100.0	99.2	100.0

Sens, sensitivity; spec, specificity; CI, confidence interval; LL, lower limit; UL, upper limit.

well on this type of data. The double-loop cross validation was executed with 100 repeats of 26-fold cross validation in the outer (validation) loop, and 10-fold cross validation in the inner loop. Learning curves were constructed for up to 100 genes, using the average of the sensitivity and specificity as criterion (reported as percentages, 50% is random classification, 100% is perfect classification) to be optimized. At all points, data splits were stratified with respect to the class prior probabilities. To optimize a classifier toward less FN or less FP cases, the prior probabilities were adjusted, such that the classifier boundary gets shifted.

Results

Development of a classifier for *CEBPA* dm

We set out to develop a classifier for detecting *CEBPA* dm AML cases in a cohort of 505 patients with AML (Table 1). For this, we consider the *CEBPA* dm as the positive group, and the remainder as negative (*CEBPA* wt, sm, or hypermethylated). This provides a six gene classifier with high performance, as shown in Figure 1 and Table 2. The misclassified cases are predominantly the hypermethylated cases (Fig. 1), which are consistent with their biological background, as the downstream gene expression profiles from a nonfunctional *CEBPA* protein due to the bi-allelic gene mutations may be equivalent to the absence of the protein due to the hypermethylated gene status. Although this classifier has a high accuracy, from a clinical utility point of view, false-positive cases are undesirable as they might be at risk of undertreatment given the favorable prognosis of *CEBPA* double mutants. Therefore, we investigated methods to exclude the hypermethylated cases before the application of a classifier.

Development of a two-step classifier for *CEBPA* dm

Since AML cases with hypermethylated *CEBPA* show reduced *CEBPA* gene expression levels, we propose a two-step classifier. In step 1, the *CEBPA* expression level is assessed in comparison with a threshold. In step 2, only the selected cases exceeding that threshold will subsequently be input into the classifier (Fig. 2).

The step 1 threshold was developed as follows. The expression levels for the different *CEBPA* mutation groups were compared (Fig. 3A). As expected, the hypermethylated *CEBPA* cases all have notably low *CEBPA* expression values. The *CEBPA* wt cases show a wide range of variable *CEBPA* expression levels, whereas the *CEBPA* sm cases have slightly elevated *CEBPA* expression. However, the *CEBPA* dm cases all share high *CEBPA* expression levels. The aim of the step 1 classifier is to classify all hypermethylated cases below the threshold and all *CEBPA* dm cases above the threshold. The threshold is determined by the intersection of the two fitted

normal distributions on the group of *CEBPA* dm cases and *CEBPA* hypermethylated cases. At that point, the groups have an equal chance of correct classification (i.e., where the overall chance of misclassification is minimal), indicated by the red line in Figure 3A. A total of 79 cases exceeds this threshold, including all 26 *CEBPA* dm cases, 5 *CEBPA* sm cases, and 48 *CEBPA* wt cases (see Table 1).

The step 2 classifier was developed using the 79 cases that exceed the threshold of the first step of the classifier. On these 79 cases, we trained a classifier without FPs and minimal FNs. This results in a 55-gene classifier with 0 FP and 1 FN (sensitivity 96.2%; specificity 100.0%; Fig. 3B). This classifier has a better sensitivity and specificity compared with the classifier trained on all 505 cases (see Table 2). The 55 probe sets used in the classifier are provided in Table 3.

Discussion

Given the favorable prognostic value of *CEBPA* double mutants, accurate detection of *CEBPA* mutations at the time of AML diagnosis is important. Current methods include dHPLC followed by Sanger sequencing, direct sequencing, melting curves, etc. However, *CEBPA* is a notoriously difficult gene to sequence due to its high GC content. In addition, due

FIG. 2. Overview of the two-step classifier for predicting *CEBPA* dm.

FIG. 3. (A) Step 1: Scatter plot indicating the distribution of the *CEBPA* expression level (*y*-axis) for all 505 cases split across the four groups of *CEBPA* status (*x*-axis). The red line indicates the threshold chosen for the first classifier. (B) Step 2: Scatter plot showing the classifier trained on the 79 cases that are above the threshold in (A), as determined by the double-loop cross-validation procedure. Numbers indicate how often a case was misclassified in the 100 repeats (samples always correctly classified do not have a number printed). Color images available online at www.liebertpub.com/gtmb

to short sequence reads, most next generation sequencing (NGS) techniques cannot confirm whether two co-occurring mutations inactivate both alleles or reside on the same allele. Alternatively, a gene expression classifier for *CEBPA* dm in AML has been built previously (Wouters *et al.*, 2009), but not

one that explicitly prevented *CEBPA* promoter hypermethylated AML cases as false-positive *CEBPA* double mutants (Figueroa *et al.*, 2009; Taskesen *et al.*, 2011).

Given the favorable prognosis of *CEBPA* double mutants, FP cases might be at risk of undertreatment. *CEBPA*

TABLE 3. 55 PROBE SETS USED IN THE CLASSIFIER

AffyID	Gene name	Ensembl gene	AffyID	Gene name	Ensembl gene
223095_at	MARVELD1	ENSG00000155254	204069_at	MEIS1	ENSG00000143995
200765_x_at	CTNNA1	ENSG00000044115	1559477_s_at	MEIS1	ENSG00000143995
200764_s_at	CTNNA1	ENSG00000044115	213844_at	HOXA5	ENSG00000106004
1555630_a_at	RAB34	ENSG00000109113	235521_at	HOXA3	ENSG00000105997
232227_at	LOC100505976	ENSG00000228401	205453_at	HOXB2	ENSG00000173917
1553183_at	UMODL1	ENSG00000177398	235753_at	HOXA7	ENSG00000122592
209191_at	TUBB6	ENSG00000176014	225368_at	HIPK2	ENSG00000064393
1556599_s_at	ARPP21	ENSG00000172995	204039_at	CEBPA	ENSG00000184771
210844_x_at	CTNNA1	ENSG00000044115	206940_s_at	POU4F1	ENSG00000152192
206622_at	TRH	ENSG00000170893	209686_at	S100B	ENSG00000160307
222423_at	NDFIP1	ENSG00000131507	204082_at	PBX3	ENSG00000167081
202252_at	RAB13	ENSG00000143545	209994_s_at	ABCB4	ENSG00000005471
215772_x_at	LOC283398	ENSG00000172340	225841_at	C1orf59	ENSG00000162639
217226_s_at	SFXN3	ENSG00000107819	224428_s_at	CDCA7	ENSG00000144354
205382_s_at	CFD	ENSG00000197766	211682_x_at	UGT2B10	ENSG00000109181
217853_at	TNS3	ENSG00000136205	206847_s_at	HOXA7	ENSG00000122592
241706_at	CPNE8	ENSG00000139117	228904_at	HOXB3	ENSG00000120093
213147_at	HOXA10	ENSG00000153807	216733_s_at	GATM	ENSG00000171766
209905_at	HOXA9	ENSG00000078399	202746_at	ITM2A	ENSG00000078596
210762_s_at	DLC1	ENSG00000164741	226065_at	PRICKLE1	ENSG00000139174
228365_at	CPNE8	ENSG00000139117	206772_at	PTH2R	ENSG00000144407
214651_s_at	HOXA9	ENSG00000078399	211341_at	POU4F1	ENSG00000152192
216667_at	ECRP/RNASE2	ENSG00000136315	206111_at	RNASE2	ENSG00000169385
225116_at	HIPK2	ENSG00000064393	203509_at	SORL1	ENSG00000137642
213150_at	HOXA10	ENSG00000153807	206589_at	GFI1	ENSG00000162676
224822_at	DLC1	ENSG00000164741	209360_s_at	RUNX1	ENSG00000159216
201841_s_at	HSPB1	ENSG00000106211	202718_at	IGFBP2	ENSG00000115457
217800_s_at	NDFIP1	ENSG00000131507			

hypermethylated cases have a gene expression pattern that is overall very similar to *CEBPA* double-mutant cases (Figueroa *et al.*, 2009; Taskesen *et al.*, 2011), and therefore, they are more likely than others to end up as FP. Therefore, we have built a two-step classifier that first eliminates *CEBPA* hypermethylated cases based on their low *CEBPA* expression level, which is the only difference that distinguishes them from *CEBPA* double-mutant cases. Next, a classifier with unequal prior probability is employed such that there were zero FPs. This two-step classifier provides an accurate method for predicting *CEBPA* dm status in AML.

Validation of a classifier on an independent cohort is a common way to estimate its performance. However, such gene expression data measured using the custom Affymetrix GeneChip platform is currently unavailable. As an alternative, we employed the double-loop cross-validation procedure, which estimates the classifier's performance using multiple re-samplings of the same data (generalization performance, Wesels *et al.*, 2005). Finally, the two-step classifier presented here will also be validated in an ongoing prospective clinical trial.

In conclusion, the two-step classifier provides an accurate means to detect *CEBPA* dm status. Moreover, the *CEBPA* dm classifier has been included on the AML profiler Affymetrix platform that also detects the presence of t(8;21), t(15;17), inv(16)/t(16;16), *NPM1* mutations, *EVII* overexpression, and *BAALC* overexpression, in a single standardized test. Together, this enables a more efficient analysis at diagnosis, with a single sample work up that is suitable for clinical use.

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Author Disclosure Statement

M.H.V.V. performed research, design of experiments, data analysis, data interpretation, and manuscript writing. P.B., L.D.Q., J.P.M.B., L.C.M.B., and H.V. performed research. B.L. and P.J.M.V. conceptualized the project, and were involved in research, design of the experiments, data interpretation, and manuscript writing. EHVB performed research, design of experiments, data interpretation, and manuscript writing. M.H.V.V., P.B., L.D.Q., J.P.M.B., L.C.M.B., H.V., B.L., P.J.M.V., and E.H.V.B. have declared ownership interests in Skyline Diagnostics.

References

- Bienz M, Ludwig M, Leibundgut EO, *et al.* (2005) Risk assessment in patients with acute myeloid leukemia and a normal karyotype. *Clin Cancer Res* 11:1416–1424.
- Dabney AR (2005) Classification of microarrays to nearest centroids. *Bioinformatics* 21:4148–4154.
- Figueroa ME, Wouters BJ, Skrabanek L, *et al.* (2009) Genome-wide epigenetic analysis delineates a biologically distinct immature acute leukemia with myeloid/T-lymphoid features. *Blood* 113:2795–2804.
- Fröhling S, Schlenk RF, Stolze I, *et al.* (2004) *CEBPA* mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. *J Clin Oncol* 22:624–633.
- Gombart AF, Hofmann, W-K, Kawano S, *et al.* (2002) Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias. *Blood* 99:1332–1340.
- Green CL, Koo KK, Hills RK, *et al.* (2010) Prognostic significance of *CEBPA* mutations in a large cohort of younger adult patients with acute myeloid leukemia: impact of double *CEBPA* mutations and the interaction with FLT3 and NPM1 mutations. *J Clin Oncol* 28:2739–2747.
- Grimwade D, Walker H, Oliver F, *et al.* (1998) The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. *Blood*, 92, 2322–2333.
- Leroy H, Roumier C, Huyghe P, *et al.* (2005) *CEBPA* point mutations in hematological malignancies. *Leukemia* 19:329–334.
- Löwenberg B, Downing JR, Burnett A (1999) Acute myeloid leukemia. *N Engl J Med* 341:1051–1062.
- Nerlov C (2004) C/EBPalpha mutations in acute myeloid leukemias. *Nat Rev Cancer* 4:394–400.
- Pabst T, Mueller BU, Zhang P, *et al.* (2001) Dominant-negative mutations of *CEBPA*, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet* 27:263–270.
- Pabst T, Mueller BU (2007) Transcriptional dysregulation during myeloid transformation in AML. *Oncogene* 26:6829–6837.
- Pabst T, Eyholzer M, Fos J, *et al.* (2009) Heterogeneity within AML with *CEBPA* mutations; only *CEBPA* double mutations, but not single *CEBPA* mutations are associated with favourable prognosis. *Br J Cancer* 100:1343–1346.
- Preudhomme C, Sagot C, Boissel N, *et al.* (2002) Favorable prognostic significance of *CEBPA* mutations in patients with *de novo* acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). *Blood* 100: 2717–2723.
- Renneville A, Boissel N, Gachard N, *et al.* (2009) The favorable impact of *CEBPA* mutations in patients with acute myeloid leukemia is only observed in the absence of associated cytogenetic abnormalities and FLT3 internal duplication. *Blood* 113:5090–5093.
- Snaddon J, Smith ML, Neat M, *et al.* (2003) Mutations of *CEBPA* in acute myeloid leukemia FAB types M1 and M2. *Genes Chromosomes Cancer* 37:72–78.
- Swerdlow SH, Campo E, Harris NL, *et al.* (2008) WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. IARC Press, Lyon.
- Taskesen E, Bullinger L, Corbacioglu A, *et al.* (2011) Prognostic impact, concurrent genetic mutations, and gene expression features of AML with *CEBPA* mutations in a cohort of 1182 cytogenetically normal AML patients: further evidence for *CEBPA* double mutant AML as a distinctive disease entity. *Blood* 117:2469–2475.
- Valk PJM, Verhaak RGW, Beijen MA, *et al.* (2004) Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* 350:1617–1628.
- van Waalwijk van Doorn-Khosrovani SB, Erpelinck C, Meijer J, *et al.* (2003) Biallelic mutations in the *CEBPA* gene and low *CEBPA* expression levels as prognostic markers in intermediate-risk AML. *Hematol J* 4:31–40.
- Verhaak RGW, Wouters BJ, Erpelinck CAJ, *et al.* (2009) Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica* 94:131–134.
- Wessels LFA, Reinders MJT, Hart AAM, *et al.* (2005) A protocol for building and evaluating predictors of disease state based on microarray data. *Bioinformatics* 21:3755–3762.

- Wouters BJ, Löwenberg B, Erpelinck-Verschueren CAJ, *et al.* (2009) Double *CEBPA* mutations, but not single *CEBPA* mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood* 113:3088–3091.
- Zhang P, Iwasaki-Arai J, Iwasaki H, *et al.* (2004) Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity* 21:853–863.

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