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Overabundant FANCD2, alone and combined with NQO1, is a sensitive marker of adverse prognosis in breast cancer

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Background: Defective DNA repair is central to the progression and treatment of breast cancer. Immunohistochemically detected DNA repair markers may be good candidates for novel prognostic and predictive factors that could guide the selection of individualized treatment strategies.

Patients and methods: We have analyzed nuclear immunohistochemical staining of BRCA1, FANCD2, RAD51, XPF, and PAR in relation to clinicopathological and survival data among 1240 paraffin-embedded breast tumors, and additional gene expression microarray data from 76 tumors. The antioxidant enzyme NQO1 was analyzed as a potential modifier of prognostic DNA repair markers.

Results: RAD51 [hazard ratio (HR) 0.81, 95% confidence interval (CI) 0.70–0.94, $P = 0.0050$] and FANCD2 expression (HR 1.50, 95% CI 1.28–1.76, $P = 1.50 \times 10^{-7}$) were associated with breast cancer survival. High FANCD2 expression correlated with markers of adverse prognosis but remained independently prognostic in multivariate analysis (HR 1.27, 95% CI 1.08–1.49, $P = 0.0043$). The FANCD2-associated survival effect was most pronounced in hormone receptor positive, HER2-negative tumors, and in tumors with above-median NQO1 expression. In the NQO1-high subset, patients belonging to the highest quartile of FANCD2 immunohistochemical scores had a threefold increased risk of metastasis or death (HR 3.10, 95% CI 1.96–4.92). Global gene expression analysis indicated that FANCD protein overabundance is associated with the upregulation of proliferation-related genes and a downregulated nucleotide excision repair pathway.

Conclusion: FANCD2 immunohistochemistry is a sensitive, independent prognostic factor in breast cancer, particularly when standard markers indicate relatively favorable prognosis. Taken together, our results suggest that the prognostic effect is linked to proliferation, DNA damage, and oxidative stress; simultaneous detection of FANCD2 and NQO1 provides additional prognostic value.

Key words: DNA repair, breast cancer, prognosis, FANCD2, NQO1

introduction

Deficient DNA repair is a common feature of cancer. The two major breast cancer susceptibility genes, *BRCA1* and *BRCA2*, are involved in the homologous recombination repair of DNA double strand breaks and, in the case of *BRCA2*, the Fanconi Anemia (FA) pathway of interstrand cross-link repair [1–3]. Other genes in the FA pathway have also been implicated in breast cancer, notably *PALB2* [4].

Different DNA repair pathways overlap and interact in complex ways. While the accumulation of DNA damage is a driving force of tumorigenesis, deficient DNA repair mechanisms represent a weakness of tumor cells that can be exploited by oncologists in cancer treatment [5]. Defects in DNA repair pathways can render tumor cells hypersensitive to genotoxic agents at concentrations that are relatively safe for healthy tissues, enabling various effective forms of chemotherapy [6, 7]. The interactions between different DNA repair pathways have raised considerable interest in this context, and simultaneous suppression of complementary pathways has been proposed as a treatment strategy [8].

The redox environment of the cell exerts profound functional effects on cell cycle control and DNA repair. Crucially, oxidative

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stress is a direct cause of various types of DNA damage [9, 10]. The key tumor suppressor p53 itself plays a central role in sensing and modulating ROS levels [11, 12]. Additionally, the FA pathway has been reported to contribute to cellular antioxidant defense through functional interaction between FANCD2 and FOXO3a, a finding that broadens the potential role of the FA pathway beyond DNA repair mechanisms [13].

The multifunctional antioxidant enzyme NAD(P)H:quinone dehydrogenase 1 (NQO1) is involved in several cellular processes that are highly relevant in cancer. In addition to its role in the management of ROS, it stabilizes key stress response proteins such as p53 and p73 and modulates the NFκB pathway [14–16]. It also localizes to the mitotic spindle in dividing human cells [17], which may suggest a presently undiscovered role in the maintenance of chromosomal integrity. This makes NQO1 an attractive marker to study in combination with DNA repair markers, particularly FANCD2.

Immunohistochemical (IHC) staining of DNA repair markers may facilitate the discovery of novel prognostic and predictive factors that could guide the selection of individualized treatment strategies. Here, we have analyzed five such markers in relation to the clinicopathological, prognostic, and predictive associations of 1240 breast tumors, using automated image analysis and a scoring method with minimal a priori assumptions. The panel of markers included the homologous recombination pathway proteins BRCA1 and RAD51, the central FA pathway protein FANCD2, and the nucleotide excision repair (NER) protein XPF. Activation of PARP-1, involved in the repair of single-strand breaks, was determined by detecting poly-ADP-ribose (PAR), the product of PARP-1 activity. We have additionally investigated NQO1 protein expression as a potential modifier of these markers.

materials and methods

patients and clinicopathological data

In total, 1240 paraffin-embedded invasive tumor specimens were available for this study. Of these tumors, 603 originated from a prospective series of 884 unselected, consecutive Finnish female breast cancer cases ascertained for primary breast tumors, while 637 tumor specimens were obtained from additional familial, BRCA1/2 mutation negative cases. All cases were ascertained at the departments of Oncology and Clinical Genetics, Helsinki University Central Hospital; see supplementary Methods, available at *Annals of Oncology* online, for in-depth details on the collection of clinicopathological data. The treatment and follow-up statistics of the cases, and the flow of samples through the various stages of the study, have been summarized in supplementary Table S1, available at *Annals of Oncology* online and supplementary Figure S1, available at *Annals of Oncology* online.

This study was carried out with patients' informed consent and permissions from the Ethics Committee of the Helsinki University Central Hospital and the Ministry of Social Affairs and Health in Finland.

immunohistochemistry

Four 0.6-mm cores were taken from the most representative area of each formalin-fixed, paraffin-embedded tumor sample, and assembled into tumor tissue microarrays (TMAs) as previously described [18]. The TMAs were stained using the following antibodies: mouse monoclonal anti-FANCD2 clone FI17; mouse monoclonal anti-XPF clone SPM228; mouse monoclonal anti-poly ADP-ribose clone 10H; rabbit polyclonal anti-RAD51 (AB3756; Merck Millipore) and mouse monoclonal anti-BRCA1 clone MS110. Microscope slide images were scanned into a digital pathology platform (Aperio Technologies, Inc., Vista, CA) and scored as the percentage of positive tumor nuclei (averaged over four cores), and the average staining intensity of the positive cells. The oxidoreductase NQO1, for which standard immunohistochemistry data have been published previously [19], was re-stained and re-scored using the above methodology. For technical details, see supplementary Methods, available at *Annals of Oncology* online.

gene expression microarrays

Gene expression microarray data (Geo Dataset GSE24450) was available for 76 tumors that were also represented on the TMAs. The platform used was Illumina HumanHT-12 v3 Expression BeadChip; the data were processed as previously described [19]. The relationship between FANCD2 protein abundance and gene expression was analyzed with a Spearman correlation test and corrected for false discovery rate using the Benjamini–Hochberg method. The resulting gene list was analyzed for enrichment of GO biological processes against the Illumina HumanHT-12 v3 background using *DAVID* and *GO Trimming 2.0* software [20, 21].

statistical analysis

Differences in staining between various phenotypic subgroups were investigated using the Kruskal–Wallis test. Staining intensity was analyzed only for markers staining positive for >80% positive cells at median. Benjamini–Hochberg correction was used to adjust the *P*-values against multiple testing, and the threshold of statistical significance was set at $P < 0.01$.

In an effort to achieve a balance between resolution and statistical power, quartile scoring of the markers was used in survival analysis. The end points of all survival analyses were distant metastasis or death from breast cancer (BDDM). In univariate Kaplan–Meier analyses, no formal correction for multiple testing was done, but a *P*-value of 0.005 was chosen as the threshold of statistical significance. Multivariate Cox models were employed to evaluate prognostic effects detected in univariate analyses in the presence of clinically relevant covariates. In subgroup analyses, heterogeneity between mutually exclusive subgroups was determined by two-sample *z*-tests. See supplementary Methods, available at *Annals of Oncology* online, for a more detailed description of the statistical analyses. All statistical analyses were done in the R 2.13.0 statistical computing environment (http://www.r-project.org/).

results

immunohistochemistry of DNA repair markers in breast tumors

After the exclusion of missing, damaged and unrepresentative (no tumor cells) cores, an average of 87.7% (86.5%–89.9%) of all tumor samples were successfully scored for FANCD2, XPF, RAD51, BRCA1, and PAR nuclear staining. Representative images of staining for the five markers are displayed in supplementary Figure S2, available at *Annals of Oncology* online. The median proportions of positively stained cells were as follows: BRCA1 83.5%, FANCD2 12.5%, PAR 89.4%, RAD51 24.2%, and XPF 99.9%.

Associations between the DNA repair markers and tumor clinicopathological characteristics are described in detail in supplementary Table S2, available at *Annals of Oncology* online and supplementary Figure S3, available at *Annals of Oncology* online. Briefly, RAD51 and FANCD2 followed opposite patterns of association with most of the clinicopathological markers. RAD51 was more abundant in hormone receptor positive, HER2-negative, low-grade cancer with normal p53 expression, small tumors, and a low Ki67 index. In contrast, high FANCD2 expression associated with high-grade, highly proliferating (high Ki67), HER2-positive, hormone receptor negative cancer and p53 overexpression. The proportion of FANCD2-positive cells was also higher in tumors with γH2AX-positive cells compared with tumors with negative γH2AX staining (17.5% versus 10.9%, respectively). High PAR and BRCA1 levels were associated with markers of favorable prognosis: tumors that are hormone receptor positive, low-grade, and with low Ki67 score. XPF abundance associated only with positive ER status.

FANCD2 immunohistochemistry is an independent prognostic marker in breast cancer

The quartile thresholds and complete results of the univariate Kaplan–Meier analysis of 5-year BDDM survival for all DNA repair proteins are displayed in supplementary Table S3, available at *Annals of Oncology* online. Among these markers, RAD51 and FANCD2 emerged as statistically significant. High FANCD2 abundance was associated with poor survival ($P = 1.50 \times 10^{-7}$, hazard ratio (HR) 1.50, 95% confidence interval (CI) 1.29–1.76; Figure 1A). In contrast, abundant RAD51 was associated with better prognosis ($P = 0.0050$, HR 0.81, 95% CI 0.70–0.94; supplementary Figure S4, available at *Annals of Oncology* online). Only FANCD2 was independently prognostic in a multivariate analysis when adjusted for hormone receptor status, tumor size, lymph node metastasis, and grade ($P = 0.0043$, HR 1.27, 95% CI 1.08–1.49), and remained independent even after further adjustment for additional FANCD2-correlated prognostic markers: p53, Ki67 and HER2 ($P = 0.0084$, HR 1.26, 95% CI 1.06–1.49; Table 1). Corresponding multivariate models for RAD51 are presented in supplementary Table S4, available at *Annals of Oncology* online.

the FANCD2-associated prognostic effect is modified by NQO1 protein expression

Next, we investigated the prognostic value of FANCD2 within a number of phenotype- and treatment-based subgroups (Figure 1). The prognostic value of FANCD2 immunohistochemistry varied by HER2 status ($P_{(het)} = 0.0040$), NQO1 protein level ($P_{(het)} = 0.0005$) and, to a lesser degree, Ki67 ($P_{(het)} = 0.0450$) and hormone receptor status ($P_{(het)} = 0.0294$ and 0.0409 for ER and PgR, respectively). NQO1 remained a statistically significant modifier even after strict Bonferroni correction (adjusted $P = 0.006$). FANCD2 was associated with high hazard in the NQO1-high subgroup (HR 3.10; 95% CI 1.96–4.92 for the highest quartile; $P = 1.40 \times 10^{-6}$; Figure 1B and C). Kaplan–Meier survival curves illustrating the nominally significant subgroup results are displayed in supplementary Figure S4, available at *Annals of Oncology* online.

In light of the strong correlation between FANCD2 protein expression and the proliferation marker Ki67 (supplementary Table S2, available at *Annals of Oncology* online), we further tested whether the modifier effect seen for NQO1 is specific to FANCD2 and not confounded by cell proliferation. NQO1 expression did not modify the prognostic value of Ki67 itself (HR 1.48 versus 1.29 in the NQO1 high and low groups, respectively, $P_{(het)} = 0.4035$). The immunohistochemical scores for FANCD2 and NQO1 were not correlated ($r = 0.116$).

Table 1. Multivariate survival analysis of quartile-scored FANCD2 protein expression

Covariate[a]	HR	(95% CI)	*P* (Wald)
(a) Multivariate model with FANCD2 and basic prognostic covariates (*N* = 921, 144 events)			
FANCD2	1.27	(1.08–1.49)	0.0043
ER	0.77	(0.47–1.28)	0.3118
PgR	0.76	(0.48–1.20)	0.2354
T	1.83	(1.52–2.20)	1.10×10^{-10}
N	2.89	(1.96–4.27)	8.33×10^{-8}
Grade	1.46	(1.09–1.94)	0.0104
(b) Multivariate model with additional FANCD2-correlated covariates (*N* = 846, 135 events)			
FANCD2	1.26	(1.06–1.49)	0.0084
ER	0.95	(0.56–1.62)	0.8447
PgR	0.71	(0.44–1.12)	0.1397
T	1.80	(1.49–2.18)	1.70×10^{-9}
N	2.89	(1.93–4.34)	2.75×10^{-7}
Grade	1.55	(1.13–2.13)	0.0070
P53	1.38	(0.91–2.07)	0.1252
Ki67	0.86	(0.70–1.07)	0.1800
HER2	1.32	(0.87–2.00)	0.1849

[a]Statistics for FANCD2 are for linear trend across quartiles. For ER, PgR, p53, and HER2, the statistics are for positive status versus negative, and for N, any lymph node metastases (N1+) versus none. For T (1-4), Ki67 (0-3), and Grade (1-3), the statistics represent linear trend across categories.

genes of the NER pathway are downregulated in tumors with abundant FANCD2

FANCD2 protein expression correlated moderately with the mRNA transcript level of the *FANCD2* gene itself ($r = 0.329$). Global correlation analysis in relation to FANCD2 protein expression yielded 329 negatively and 241 positively correlated genes ($P < 0.05$ after Benjamini–Hochberg adjustment). Three Gene Ontology biological processes were specifically enriched in the negatively correlated group: GO:0044265 (cellular macromolecule catabolic process; $P = 0.0021$), GO:0000718 (nucleotide-excision repair, DNA damage removal; $P = 0.0050$), and GO:0019941 (modification-dependent protein catabolic process, $P = 0.0068$).

Among the positively correlated genes, 11 GO biological processes were significantly enriched. Nine of these pathways involved the cell cycle and DNA replication, most notably GO:0007049 (cell cycle; $P = 5.66 \times 10^{-7}$). Additional pathways detected in the positively correlated set were GO:0050728 (negative regulation of inflammatory response; $P = 0.0008$) and GO:0006974 (response to DNA damage stimulus; $P = 0.0048$). The complete results from the DAVID pathway enrichment analysis can be viewed in supplementary Table S5, available at *Annals of Oncology* online.

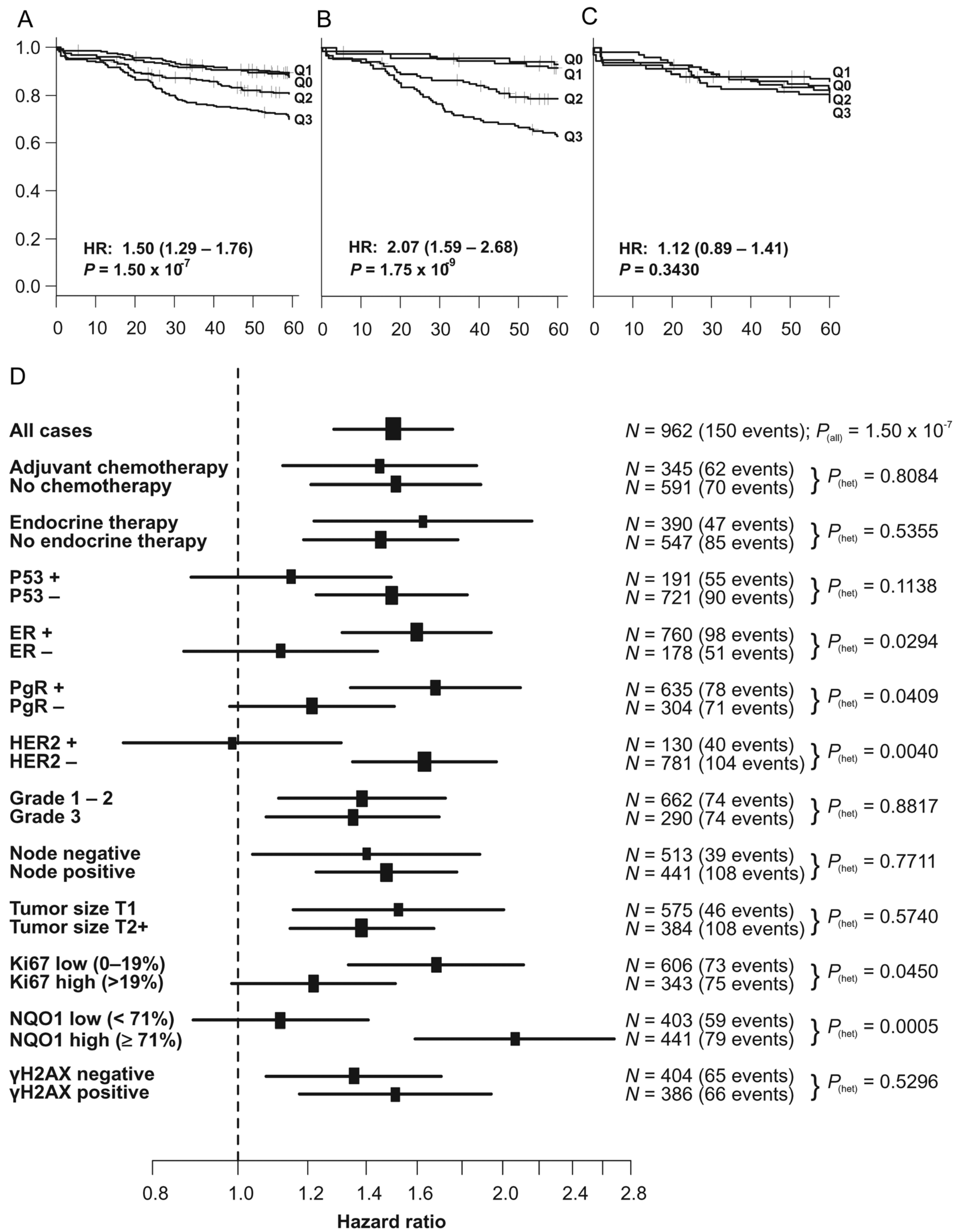

Figure 1. Kaplan–Meier and hazard ratio plots illustrating the prognostic effect associated with FANCD2 protein abundance alone and within clinicopathological subgroups. The end points used in the analysis were distant metastasis or death from breast cancer. (A) Survival by FANCD2 quartile in the entire sample set. The lines represent the quartile-scored immunohistochemistry score (% positive cells). The *P*-value is for linear trend across quartiles. (B) Survival by FANCD2 quartile in tumors with above-median NQO1 protein expression (>70% cells positive). (C) Survival by FANCD2 quartile in tumors with below-median NQO1 protein expression (≤70% cells positive). (D) Forest plot of the hazard ratios and their confidence intervals for FANCD2 in the entire sample set and within subgroups. For subgroup analyses, the *P*-values from two-sample *z*-tests of heterogeneity between subgroup pairs are displayed ($P_{(\mathrm{het})}$).

discussion

In contrast to the other markers examined, which tended to associate with markers of favorable prognosis, FANCD2 expression correlated with features of aggressive cancer: hormone receptor negativity, HER2 amplification, elevated p53 expression, high grade, and proliferation. An increasing FANCD2 score was also associated with worse prognosis of breast cancer patients. These findings are consistent with a study by van der Groep et al., who reported that high FANCD2 expression associated with increased proliferation, hormone receptor negativity, and worse overall survival among 122 breast tumors [22]. FANCD2 appears to be a highly sensitive marker, however, as it remained independently prognostic in multivariate analyses, and FANCD2 overabundance was a predictor of poor survival especially in subgroups of generally quite favorable prognosis: tumors that are hormone receptor positive, HER2-negative and/or have low Ki67 expression.

The association between FANCD2 and breast cancer survival was strongest when the tumors expressed above-median levels of the NQO1 protein, and indeed appeared to be restricted to this subgroup, with a threefold increased risk of metastasis or death among patients with highest FANCD2 and NQO1 expression levels. This finding suggests a clinically relevant link between FANCD2 protein levels and oxidative stress in breast tumors, although the specifics of the underlying biological mechanisms can only be speculated on at this point.

The mechanism behind a high FANCD2 expression, and its association with tumor progression and survival, cannot be fully elucidated based on these data alone. Given that the correlation between FANCD2 immunohistochemistry and mRNA expression wasn't very strong, it is plausible that aberrant turnover of the FANCD2 protein could be involved. For example defective deubiquitination of FANCD2 can lead to hyperaccumulation of monoubiquitinated FANCD2 [23]. Unfortunately, we are only able to observe the overall protein level on TMAs, as the FI17 monoclonal antibody is specific to both monoubiquitinated and non-monoubiquitinated forms of FANCD2 [24]. Another possible explanation is that FANCD2 is a sensitive marker of proliferation, an attractive idea given that (i) FA-mediated DNA repair is coupled to replication [25], (ii) FANCD2 is expressed in many rapidly proliferating normal tissues concomitantly with Ki67 [26], and (iii) FANCD2 and Ki67 IHC scores were strongly correlated in this study. If this were the case, then FANCD2 would appear to be a better marker of proliferation than Ki67 in our sample material, given that the FANCD2-associated HR is higher in Ki67-low tumors, and FANCD2 remained statistically significant in multivariate survival analysis whereas Ki67 did not. Furthermore, NQO1 modulates the prognostic value of FANCD2 alone, in our dataset, not Ki67. It would therefore appear that FANCD2 immunohistochemistry provides additional prognostic information beyond the proliferation state of the tumor.

It could also be speculated that FANCD2 overabundance is indicative of defects in one or more DNA repair pathways. Indeed, we found the proportion of FANCD2-positive cells to be higher in tumors positive for γH2AX, a sensitive marker of DNA damage [27]. Our gene expression data provide some support for this hypothesis: genes in the nucleotide excision repair (NER) pathway were downregulated in FANCD2-high tumors. This is an intriguing finding, as a functional NER pathway is required for complete FA-mediated DNA repair of interstrand cross-links [28], and one of the downregulated genes, *XPC*, is required for the recruitment of the core FA complex to sites of DNA damage [29]. Increased FANCD2 expression may therefore be an indicator of downregulated or defective NER in breast cancer.

In conclusion, our results provide evidence that FANCD2 immunohistochemistry is an independent prognostic factor in breast cancer. As this effect is most pronounced in tumor subgroups of otherwise relatively favorable prognosis, it can be relevant to the selection of an optimal treatment strategy: tumors likely to develop a metastasizing phenotype may benefit from more aggressive treatment, e.g. with adjuvant chemotherapy. The prognostic effect of FANCD2 appears to be linked to oxidative stress, proliferation, and/or the regulation of the NER pathway, a complex framework that warrants further study. Finally, simultaneous detection of FANCD2 and NQO1 protein expression may be a particularly effective method of identifying a subset of breast carcinomas with poor prognosis.

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disclosure

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