Purpose Ewing sarcoma (ES) is a primitive sarcoma defined by EWSR1-ETS fusions as the primary driver alteration. To better define the landscape of cooperating secondary genetic alterations in ES, we analyzed clinical genomic profiling data of 113 patients with ES, a cohort including more adult patients (> 18 years) and more patients with advanced stage at presentation than previous genomic cohorts.

Methods The data set consisted of patients with ES prospectively tested with the US Food and Drug Administration–cleared Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets large panel, hybrid capture-based next-generation sequencing assay. To assess the functional significance of ERF loss, we generated ES cell lines with increased expression of ERF and lines with knockdown of ERF. We assessed cell viability, clonogenic growth, and motility in these ES lines and performed transcriptomic and epigenetic analyses. Finally, we validated our findings in vivo using cell line xenografts.

Results Novel subsets were defined by recurrent secondary alterations in ERF, which encodes an ETS domain transcriptional repressor, in 7% of patients (five truncating mutations, one deep deletion, and two missense mutations) and in FGFR1 in another 2.7% (one amplification and two known activating mutations). ERF alterations were nonoverlapping with STAG2 alterations. In vitro, increased expression of ERF decreased tumor cell growth, colony formation, and motility in two ES cell lines, whereas ERF loss induced cellular proliferation and clonogenic growth. Transcriptomic analysis of cell lines with ERF loss revealed an increased expression of genes and pathways associated with aggressive tumor biology, and epigenetic, chromatin-based studies revealed that ERF competes with EWSR1-FLI1 at ETS-binding sites.

Conclusion Our findings open avenues to new insights into ES pathobiology and to novel therapeutic approaches in a subset of patients with ES.

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Background Ewing sarcoma (ES) is the second most common primary malignant bone neoplasm in children and young adults.\(^3\) Metastatic ES is often refractory to standard chemotherapy, resulting in a 5-year survival rate of approximately 30%.\(^3\)

In roughly 85% of ES, the primary driver alteration is the EWSR1-FLI1 fusion, generated by the classic t(11;22) (q24;q11.2).\(^4,5\) In the remaining 15% of ES cases, EWSR1 is fused to other genes from the ETS family of transcription factors, such as ERG, ETV1, E1AF, or FEV.\(^6,8\)

Although the oncogenic and transcriptional effects of the EWSR1-FLI1 fusion have been extensively studied, the role of cooperating secondary genetic alterations remains to be fully defined. Huang et al\(^9\) reported the strong and independent negative prognostic impact of TP53 mutations and p16/p14ARF deletions in patients with ES. Solomon et al\(^10\) identified mutations in STAG2—a gene encoding a member of the mitotic cohesin complex—in ES in 2011, and Tirode et al\(^11\) showed that STAG2 was mutated in 17% of ES and was associated with negative prognosis. The negative prognostic impact of STAG2 was further compounded when concurrent with TP53 mutations. These data confirmed the importance of STAG2 mutations in the molecular pathogenesis of ES,\(^12,13\) and recently published functional data support an effect of STAG2 loss on higher-order chromatin conformation and...
Ewing sarcoma (ES) is a primitive, highly aggressive sarcoma. Despite important insights afforded into the molecular pathogenesis of ES by the analysis of EWSR1-FLI1 and related fusions, the role of secondary genetic alterations and their functional significance remain underexplored. To expand our understanding of the genetic landscape of ES, we conducted a comprehensive analysis of clinical genomic profiling data on tumors from 113 pediatric and adult patients with ES.

**Knowledge Generated**

Clinical genomic profiling of ES reveals novel subsets of patients with inactivating mutations of the ERF ETS domain transcriptional repressor or activating FGFR1 mutations. Our functional analyses demonstrate that ERF loss results in increased tumorigenic and metastatic properties in vitro and in vivo.

**Relevance**

ERF loss-of-function mutations may be another pathway to enhance the oncogenicity of EWSR1-FLI1. Moreover, we report the presence of recurrent FGFR1 activating mutations in ES, the first potentially targetable recurrent alteration in this sarcoma.

**METHODS**

Cohort Selection and Statistical Analysis

All 113 patients with ES who underwent MSK-IMPACT testing were included in the analysis. The majority of patients were treated with the standard Memorial Sloan Kettering Cancer Center regimen of cyclophosphamide/doxorubicin/vincristine and ifosfamide/etoposide. Overall survival (OS) was defined as the time from diagnosis to death from all causes. Survival rates were estimated using a Kaplan-Meier estimator, and survival curves were compared using a log-rank test. Multivariable analyses were performed using the Cox regression model to determine hazard ratios and 95% CIs for OS while adjusting for other clinicopathologic features. Only patients with information available for all variables were included in the multivariable analysis. All tests were two-sided, and statistical significance was set at a P < .05. Statistical analyses were conducted using R v3.6.0 software and GraphPad Prism Software.

**MSK-IMPACT Analysis**

Clinical sequencing using the MSK-IMPACT panel was performed on matched tumor and blood samples from 113 patients (n = 118 samples). Somatic mutations, copy number alterations, and structural variants were assayed over a total of 468 genes (version 3), 410 genes (version 2), or 341 genes (version 1) as previously described. Of the genes discussed, ERF was the only one not covered on all versions. To address this, samples that had originally been analyzed by using version 1 or 2 were resequenced on version 3 to assess ERF status.

Detailed methods for ERF overexpression and knockdown functional analyses, animal experiments, gene expression analyses, and epigenomic analyses are given in the Data Supplement.

**RESULTS**

Validation of Prognostic Relevance of Alterations in TP53, STAG2, and CDKN2A in a Prospective, Clinical Genomic Profiling Cohort of ES

We analyzed genomic data of 113 consecutive cases of ES that underwent MSK-IMPACT testing at our institution. Clinical and pathologic features of these 113 patients are shown in Table 1.

The somatic genetic alterations detected are shown in Figure 1A. As expected, all cases harbored a fusion of EWSR1 (n = 111) or FUS (n = 2) with an ETS transcription factor gene. As previously described, TP53 mutation was the most common secondary genetic alteration (Fig 1A). STAG2 alterations were the next most common, occurring in 12 patients. Eleven patients were found to have CDKN2A...
Table 1. Baseline Clinical Characteristics

| Characteristic           | N = 113 No. (%) |
|--------------------------|-----------------|
| Age, years, median (IQR) | 20 (14–34)      |
| Age category, years      |                 |
| < 40                     | 93 (82.0)       |
| ≥ 40                     | 20 (18.0)       |
| Sex                      |                 |
| Female                   | 43 (38.0)       |
| Male                     | 70 (62.0)       |
| Fusion type              |                 |
| EWSR1-FLI1               | 94 (83.0)       |
| EWSR1-ERG               | 15 (13.0)       |
| FUS-FEV                  | 2 (1.8)         |
| EWSR1-ETV4              | 1 (0.9)         |
| EWSR1-FEV               | 1 (0.9)         |
| STAG2 alteration         | 12 (11.0)       |
| TP53 alteration          | 13 (12.0)       |
| ERF alteration           | 7 (6.2)         |
| CDKN2A alteration        | 11 (9.7)        |
| Stage                    |                 |
| Localized                | 67 (60.0)       |
| Metastatic               | 44 (39.0)       |
| Unknown                  | 2 (1.8)         |
| Primary tumor site       |                 |
| Axial                    | 66 (58.0)       |
| Peripheral               | 42 (37.0)       |
| Unknown                  | 5 (4.4)         |
| Disease status           |                 |
| AWD                      | 9 (8.0)         |
| DOD                      | 38 (34.0)       |
| NED                      | 49 (43.0)       |
| Unknown                  | 17 (15.0)       |

Abbreviations: AWD, alive with disease; DOD, dead of disease; IQR, interquartile range; NED, no evidence of disease.

alterations, with eight deep deletions, and in line with previous observations, the CDKN2A alterations were rarely concurrent with STAG2 alterations.

To assess whether our cohort of ES was representative of prior genomic cohorts of ES, we examined the prognostic impact of previously analyzed genomic alterations. As only patients who were alive were eligible for clinical MSK-IMPACT testing, to adjust potential left-truncation bias, we excluded from the survival analyses patients with MSK-IMPACT profiling performed more than 2 years after initial diagnosis. STAG2 mutation status was associated with a 48% OS at 5 years compared with 63% in the STAG2 wild-type group (P = .1; see the Data Supplement for the clinical profile of STAG2-mutant patients). TP53 mutations were associated with significantly worse OS at 5 years (P < .0001; see the Data Supplement for the clinical profile of TP53-mutant patients). Patients with neither TP53 nor STAG2 mutations showed a probability of survival at 5 years of 66%, whereas those who harbored both alterations all died within 18 months (Data Supplement). CDKN2A alterations were also strongly associated with worse OS (P < .03), consistent with previous reports (see the Data Supplement for the clinical profile of CDKN2A-mutant patients). Thus, the prognostically unfavorable impact of alterations in these three genes in our cohort was in line with most previous studies.

Although the above-referenced previous studies found that CDKN2A and TP53 alterations were associated with poor outcomes, it should also be noted that, in a secondary subset analysis of molecular data from a clinical trial for patients with localized ES, TP53 mutations (found in 8 of 93) and CDKN2A deletions (found in 12 of 107) were not associated with event-free survival.

ES Harbors Additional Secondary Genetic Alterations of Biologic or Clinical Interest, Including Recurrent Alterations in FGFR1 and ERF

Other recurrent secondary alterations included EZH2 mutations in 4% (all being known hotspot activating missense mutations: K515R, Y646H/N, A682G, and A692V), CREBBP mutations in 5%, BCOR mutations in 4%, SMARCA4 mutations in 2%, TERT promoter mutations in 4%, and KRAS hotspot mutations in 2% (G12D and Q61R; Fig 1A). The rare occurrence of RAS mutations in ES has been previously reported.

Notably, 3% of patients had activating FGFR1 alterations (one amplification and two hotspot activating kinase domain mutations: N577K and K687E). Although there is a previous report of a single ES case with an activating FGFR1 mutation (N546K) in one of 50 ES, our findings now establish activating FGFR1 mutations as a recurrent alteration in ES. Aside from the KRAS and FGFR1 mutations, there were no other known activating mutations in any other major genes in the MAPK pathway in this cohort (EGFR, ERBB2, MET, RET, IGF1R, NRAS, HRAS, BRAF, ARAF, MAP2K1, FGFR2, FGFR3, FGFR4).

As a previous study had reported a frequent high-level expression of FGFR1 in ES, we sought to confirm this using two independent mRNA-based data sets. First, from mining data generated using a targeted RNAseq assay that includes FGFR1 on the basis of the Archer Anchored Multiplex polymerase chain reaction technology. FGFR1 was highly expressed in the ES cohort (n = 42). Notably, the two patients with activating FGFR1 mutations in the ES cohort had a relatively high expression of FGFR1 (Data Supplement). In a separate legacy Affymetrix data set, FGFR1 was overexpressed compared with NTRK3 in ES (n = 28), but not in the desmoplastic small round tumor cohort (n = 28)—a sarcoma subtype that, conversely, is known to have a high expression of NTRK3. Moreover,
relative expression of FGFR1 was higher in ES than in desmoplastic small round tumor (Data Supplement). Notably, although numbers are small, all three known patients with activating FGFR1 mutations have presented with metastatic disease (Data Supplement). Together, these data confirm that FGFR1 expression is a common feature of ES, including in cases with canonical, targetable FGFR1-activating mutations.

The second notable finding was that tumors from eight patients (7%) harbored inactivating alterations in ERF, which encodes an ETS domain transcriptional repressor. These included five truncating mutations, one deep deletion, and two missense mutations (R86C and R70P).

Although rare ERF mutations had been noted in a previous genomic study of ES, their potential role in ES has not been studied. In our cohort, ERF mutations were nonoverlapping with STAG2 mutations. Because of the small number of patients with ERF alterations, our analyses lacked statistical power to detect associations with OS at 5 years in either univariable (Data Supplement) or multivariable analyses (Data Supplement; characteristics of the eight ERF-mutant patients, along with three previously identified cases, are presented in the Data Supplement). ERF status was not associated with advanced stage or primary site of diagnosis (P = .7 and P = .9, respectively, analysis not shown).
were only seen in cases with the \textit{EWSR1-FLI1} fusion, but this relationship was not statistically significant ($P = .21$, analysis not shown). Significantly, although truncating mutations were scattered across the \textit{ERF} gene, missense mutations clustered in the ETS DNA–binding domain (Fig 1B), similar to the pattern observed in prostate cancers sequenced with the MSK-IMPACT platform and in a previously published analysis examining...

\textbf{FIG 2.} Increased expression of ERF decreases growth, colony formation, and motility of ES cell lines in vitro. (A) Proliferation assay in the A673 ES cell line (A673 pcW) transfected with ERF (A673-ERF) versus empty vector control (A673-EV) and (B) TC71 ES cell line (TC71 pcW) transfected with ERF (TC71-ERF) versus empty vector control (TC71-EV). Cell viability was measured using the resazurin (alamarBlue) fluorescence assay. The mean and standard deviation of six replicates per condition were used for analysis. Growth in low attachment assay in (C) A673 pcW and (D) TC71 pcW; cell viability was measured using the resazurin (alamarBlue) fluorescence assay. The mean and standard deviation of six replicates per condition were used for analysis. (E) Representative photograph of anchorage-independent growth by the colony formation assay of A673 and TC71 cells with overexpression of ERF (pCW-ERF) compared with empty vector (pCW-EV) cells. Cells were incubated and grown for 10 days until they formed colonies. Colonies containing more than 50 cells were counted and normalized to the control. Quantification of the number of colonies for (F) A673 and (G) TC71 cells. (H) Representative photograph of cell invasion assay of A673 and TC71 cell lines for pCW ERF versus pCW-EV. Invading cells per view quantification of (I) A673 pcW and (J) TC71 pcW cells on the lower surface of the filter membrane were counted in five random squares (magnification, $\times200$). *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$. ES, Ewing sarcoma.
FIG 3. ERF knockdown increases cellular proliferation and cell invasion ability in ES cells in vitro. (A) Proliferation assay performed with the A673 ES cell line transfected with ERF shRNA (A673-sh1, A673-sh2) versus nontarget (A673-shNT). (B) Proliferation assay in the TC71 ES cell line transfected with ERF shRNA (TC71-sh1, TC71-sh2) versus nontarget (TC71-shNT). Cell viability was measured using the resazurin (alamarBlue) fluorescence assay. The mean and standard deviation of six replicates per condition were used for analysis. Growth in low attachment assay for ERF knockdown in (C) A673 shRNA and (D) TC71 shRNA; cell viability was measured (continued on following page)
the role of ERF in prostate cancer oncogenesis. As the functional significance of FGFR1 in ES had been previously studied, we focused our functional studies on the role of ERF status in ES.

**Increased Expression of ERF Decreases Growth, Colony Formation, and Motility of ES Cell Lines In Vitro**

Given the known role of inactivating ERF mutations in driving oncogenesis in prostate cancer, we sought to explore the functional significance of ERF in ES. To define the functional significance of ERF expression in vitro, we transfected an ERF expression plasmid into the ES cell lines, A673 and TC71, and confirmed inducible ERF expression (Data Supplement). Increased expression of ERF reduced cell viability in A673-pCW (Fig 2A and Data Supplement) and TC71-pCW (Fig 2B and Data Supplement) cells. Similar results were obtained in a growth in low attachment assay for both cell lines (Figs 2C and 2D). To explore the impact of ERF status on anchorage-independent growth, clonogenic growth assays were performed. ERF overexpression reduced cellular proliferation in pCW-ERF cells on the basis of visual assessment of the number of colonies (Fig 2E) and their quantification in A673 (Fig 2F) and TC71 cultures (Fig 2G). In a transwell assay, the number of invasive cells in pCW-Flag-ERF-DOX + ES cells was significantly decreased (Fig 2H) for both A673 (Fig 2I) and TC71 (Fig 2J). Taken together, these results demonstrate that increased expression of ERF decreases growth, colony formation, and motility of ES cell lines in vitro.

**ERF Loss Induces Cellular Proliferation and Clonogenic Growth in ES Cells In Vitro**

On the basis of the inverse association between increased ERF expression and cellular proliferation, we next asked whether ERF loss could be associated with tumorigenesis. On knockdown of ERF via short-hairpin RNA (shRNA; Data Supplement), we observed increased cell viability of A673 (Fig 3A and Data Supplement) and TC71 cells (Fig 3B and Data Supplement) and validated this finding in growth in low attachment assays in both A673-sh1 and TC71-sh2 cells (Figs 3C and 3D). To further validate the significance of ERF deficiency in ES, we investigated the effect of ERF knockdown on ES cell invasion. We used a Matrigel invasion assay comparing in vitro invasiveness of ERF wild-type and knockdown cells using a Transwell system. After 72 hours of incubation, the cells that invaded through the membrane were stained and representative fields were photographed (Fig 3E). The invasiveness of ERF knockdown cells was increased compared with that of ERF wild-type cells (Figs 3F and 3G). These results suggest that ERF loss contributes to cellular proliferation and growth.

**Overexpression of ERF Induces Cytotoxic Effects, Whereas ERF Loss Induces Tumor Growth in ES Cells In Vivo**

To validate our hypothesis that ERF acts as a tumor suppressor in ES, we generated cell line xenografts by mixing 10 million TC71 cells with Matrigel (1:1) and injecting them subcutaneously into the flank of female

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**FIG 3.** (continued) Using the resazurin (alamarBlue) fluorescence assay. The mean and standard deviation of six replicates per condition were used for analysis. (E) Representative photograph of cell invasion assay examining A673 and TC71 cells upon ERF knockdown. Invading cells per view quantification of (F) A673 shRNA and (G) TC71 shRNA cells on the lower surface of the filter membrane were counted in five random squares (magnification, ×200). *P < .05, **P < .01, ***P < .001. ES, Ewing sarcoma; NS, not significant; NT, nontargeting; shRNA, short-hairpin RNA.

**FIG 4.** Increased expression of ERF blocks tumor growth, whereas ERF loss induces tumor growth in Ewing sarcoma cells in vivo. Xenografts were generated from TC71 cells injected subcutaneously into a single flank of female NOD/SCID gamma mice. (A) TC71 pCW DOX–inducible ERF compared with controls TC71 pCW EV plus doxycycline. (B) Knockdown of ERF in TC71-sh1 and TC71-sh2 compared with nontarget control (TC71-shNT). shNT; nontarget. Tumor volumes at the last measurement were compared using Student’s t test. P = .160 for TC71 shERF2 versus TC71 shNT. **P < .01, ***P < .001. NT, nontargeting.
NOD/SCID gamma mice. When tumors reached a volume of 100 mm³, tumor size and body weight were measured twice weekly and tumor volume was calculated. All mice were sacrificed when tumors reached 1,500 mm³, before tumor-associated death. In line with the data observed in vitro, increased ERF expression was associated with decreased tumor growth (Fig 4A), whereas knockdown of ERF (both TC71-sh1 and TC71-sh2) was associated with enhanced tumor growth in this model (Fig 4B).

Transcriptomic and Epigenetic Studies of ERF Loss

We performed RNA sequencing analysis in TC71 cells in which ERF was knocked down versus nontargeting control to define a transcriptomic profile associated with ERF loss. Transcriptomic analysis revealed that ERF loss was highly associated with NOS1 overexpression (log₂fold change 3.5, \textit{P} < .0001; Fig 5A; a full list of genes is provided in the Data Supplement). NOS1 belongs to the family of nitric oxide synthases, which synthesize nitric oxide from L-arginine. NOS1 is ubiquitously expressed, with highest levels of expression in skeletal muscle. NOS1 overexpression has been associated with increased tumor proliferation, metastasis, and invasion in multiple cancer types. Pathway enrichment analyses revealed enrichment of a cancer progression pathway (\textit{P} < .001; Fig 5B) and a nitric oxide metabolic pathway (\textit{P} < .01; Fig 5C).

We then performed CUT&RUN to determine the relationship between the binding sites of ERF compared with EWSR1-FLI1. The ERF/EWSR1-FLI1 intersection analysis from the CUT&RUN peak calls revealed that in A673, about 50% of all EWSR1-FLI1 sites were also bound by ERF (Data Supplement), suggesting that ERF is competing with the

\[\text{\textit{Log}_2 \text{FC}}\]

\[\text{\textit{P}}\]

\[\text{\textit{NS}}\]

\[\text{\textit{Total}} = 564 \text{ variables} \]
fusion peaks at ETS family binding sites. We then grouped the peaks into three types (ERF only, EWSR1-FLI1 only, and cobound) and ran motif signatures on those peak sets from the cell lines included in our analysis. For A673 FLI1, the top two hits in the genome were exactly the two motif types expected for the fusion (Fig 5D). We then annotated the peaks for each motif class to find associated genes. Both SOX2 and NKX2-2 had distal peaks associated with them, and in both cases, and within all peaks, the motif signature was GGAA (Fig 5D). SOX2 expression is known to be modulated by EWSR1-FLI1,31 and high SOX2 is associated with poor outcomes in ES.32 NKX2-2 is a target of EWSR1-FLI1, is known to be upregulated in ES33 on the basis of array-based gene expression analysis, and the protein serves as an immunohistochemical marker for ES.34

**DISCUSSION**

In this study of clinical genomic data of 113 consecutive patients with skeletal or extraskeletal ES seen at an adult and pediatric cancer center, we found that 7% of patients harbored ERF alterations, a notably higher prevalence than previous studies that may be related to cohort differences. The median age at diagnosis for our cohort was 20 years, significantly older than in the ES genomic profiling cohorts published by Brohl et al,12 Tirode et al,11 and Crompton et al13 that had median ages of 12, 14, and 11 years, respectively. Higher proportions of older patients and of patients with advanced disease at presentation in our cohort could be relevant to the finding of more frequent ERF alterations as these were associated with more aggressive tumor biology in our in vitro and in vivo studies.

ERF, or ETS2 repressor factor, is a member of the ETS transcription factor family that functions as a strong transcriptional repressor.35 In a whole-exome sequencing series of 102 prostate cancers, Huang et al29 identified loss-of-function mutations in ERF in 3% of cases. ERF loss was associated with unfavorable prostate cancer clinicopathologic prognostic features. Knockdown of ERF conferred increased anchorage-independent growth and was also associated with a prominent expression signature of ETS-targeted activation and androgen signaling, whereas increased expression of ERF induced tumor control, pointing to a role for ERF as a tumor suppressor in prostate cancer. Proposing a reciprocal relationship between ERF and ERG, Bose et al28 showed that loss-of-function ERF mutations occurred only in prostate tumors without TMPRSS2-ERG and that, in prostatic organoid models, ERF loss nephocopied ERG gain of function.

Given the oncogenic role of ERF loss in a TMPRSS2-ERG–negative subset of prostate cancers,28 as described above, we examined the role of ERF in ES. We found that ERF overexpression decreases growth, colony formation, and motility of ES cell lines, whereas ERF loss was associated with enhanced tumor growth and cell invasion in vitro, and these findings were validated in vivo. Finally, transcriptome profiling revealed increased gene expression and pathway enrichment associated with tumor aggressivity, and our epigenome analysis suggested that ERF is competing with EWSR1-FLI1 at GGAA sites.

Aside from these novel observations regarding ERF loss of function in a subset of ES, our study also found that nearly 3% of patients in our cohort harbored activating FGFR1 mutations, a targetable alteration with numerous clinical trials currently evaluating FGFR1 inhibitors in other cancers (ClinicalTrials.gov identifier: NCT03210714). Although an FGFR1 activating mutation had previously been described in only a single case of ES,23 our findings now establish FGFR1 activating mutations as a recurrent secondary mutation in ES. In the previous study, whole-exome sequencing was performed in 51 patients with ES, identifying one patient with a FGFR1 N546K mutation in the tyrosine kinase domain.23 The investigators then transduced the FGFR1 N546K mutant retrovirally into ES cells, leading to enhanced proliferation, whereas knockdown of FGFR1 using shRNA inhibited growth of ES cells, and these findings were validated in vivo in mice. Together with these published functional data, our findings now nominate mutated FGFR1 as a recurrent targetable alteration in a small but potentially clinically important subset of patients with ES.

In summary, our study genetically profiling the tumors of 113 patients with ES revealed a previously unexplored role of ERF loss of function in this sarcoma. Our functional analyses of how ERF modulates EWSR1-FLI1 oncogenicity may open a new window into the pathobiology of ES. Moreover, our data suggest that close to 3% of patients with ES harbor activating FGFR1 mutations, the first recurrent, targetable kinase alteration in this sarcoma.

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