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MAPK/ERK2 phosphorylates ERG at serine 283 in leukemic cells and promotes stem cell signatures and cell proliferation

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Abstract

Aberrant *ERG* (v-ets avian erythroblastosis virus E26 oncogene homolog) expression drives leukemic transformation in mice and high expression is associated with poor patient outcomes in acute myeloid leukemia (AML) and T-acute lymphoblastic leukemia (T-ALL). Protein phosphorylation regulates the activity of many *ETS* factors but little is known about ERG in leukemic cells. To characterize ERG phosphorylation in leukemic cells, we applied liquid chromatography coupled tandem mass spectrometry and identified five phosphorylated serines on endogenous ERG in T-ALL and AML cells. S283 was distinct as it was abundantly phosphorylated in leukemic cells but not in healthy hematopoietic stem and progenitor cells (HSPCs). Overexpression of a phosphoactive mutant (S283D) increased expansion and clonogenicity of primary HSPCs over and above wild-type ERG. Using a custom antibody, we screened a panel of primary leukemic xenografts and showed that ERG S283 phosphorylation was mediated by mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling and in turn regulated expression of components of this pathway. S283 phosphorylation

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

YH, JAIT, MLT, KK, DB, VC, ENG and AB performed experiments and analyzed data. JO, SRT, KLM, JWHW and JEP analyzed data. SS, RBL, KLM and JHB provided essential reagents. YH, JAIT, JWHW and JEP wrote the paper.

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facilitates ERG enrichment and transactivation at the *ERG*+85 HSPC enhancer that is active in AML and T-ALL with poor prognosis. Taken together, we have identified a specific post-translational modification in leukemic cells that promotes progenitor proliferation and is a potential target to modulate ERG-driven transcriptional programs in leukemia.

INTRODUCTION

ERG (v-ets avian erythroblastosis virus E26 oncogene homolog) is an *ETS* family transcription factor with known roles in vasculogenesis, angiogenesis, hematopoiesis and bone development.^{1–4} In relation to the hematopoietic system, ERG is required for definitive hematopoiesis, normal hematopoietic stem cell (HSC) function and maintenance of normal peripheral blood platelet numbers.^{4–6} Conditional loss of ERG in hematopoietic cells has recently been shown to accelerate differentiation, resulting in depletion of functional HSCs.⁷ *ERG* is highly expressed in healthy human hematopoietic stem and progenitor cells (HSPCs) and high expression in subtypes of T-acute lymphoblastic leukemia (T-ALL) and cytogenetically normal acute myeloid leukemia (AML) has been reported to be associated with poor prognosis.^{8–10} Although ERG appears to be dispensable for the initiation and maintenance of *NOTCH-ICD* or *Nras*^{G12D} transformed murine T-ALL or MLL-ENL transformed murine AML, germline overexpression of ERG in hematopoietic cells have shown *ERG* to be a potent oncogene, promoting the development of both T-ALL and AML.^{6,11,12} ERG overexpression in human CD34+ HSPCs promotes clonal expansion and gene expression signatures that are evident in high ERG human leukemias.¹³

Protein phosphorylation is a major post-translational modification that modifies the activity of many *ETS* factors. For example, ELK1 translocates into the nucleus only when phosphorylated at T417, ETS1 phosphorylation auto-inhibits its DNA binding^{14,15} and FLI1 phosphorylation inhibits its interaction with RUNX1 and blocks megakaryocytic differentiation.¹⁶ However, although ERG has previously been recognized as a nuclear phosphoprotein,¹⁷ the specific residues of ERG that are targets of phosphorylation are largely obscure and vary depending on tissue and cell type.^{18–21} A recent global phosphoproteomic survey identified several serine residues of ERG that were phosphorylated in healthy CD34+ HSPCs.²⁰ However, whether leukemic cells have similar or distinct sites of ERG phosphorylation and the functional consequences of these differences are currently unknown.

In this study, we combined immunoprecipitation with phosphoenrichment and mass spectrometry to identify phosphorylation sites on endogenous ERG in leukemic cells and report that phosphorylation of serine 283 (pS283) on ERG3, the dominant ERG isoform in hematopoietic cells, is particularly abundant in leukemic cells compared with healthy HSPCs. This residue is phosphorylated by mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling and overexpression of a constitutively active pS283 ERG mutant results in activation of genes associated with MAPK/ERK activity, enrichment of the mutant protein at the *ERG*+85 stem cell enhancer (SCE) and expansion of CD34+ HSPCs.

MATERIALS AND METHODS

Immunoprecipitation

Whole-cell extracts from $>3 \times 10^7$ MOLT4 cells were prepared using lysis buffer with the following contents: 20 mM HEPES-NaOH (pH 7.8), 300 mM NaCl, 1 mM EDTA, 20% glycerol, 0.5% NP-40, protease inhibitor cocktails (Roche, Basel, Switzerland), 10 mM NaF and 1 mM Na_3VO_4 (1 ml lysis buffer per 1×10^7 cells). Cleared lysate was incubated overnight with anti-ERG antibody (sc-354x, Santa Cruz, Dallas, TX, USA) at 4 °C (1 μl antibody per 1×10^7 cells) and adsorbed onto protein G Dynabeads (Invitrogen, Carlsbad, CA, USA) for 3 h at 4 °C (20 μl Dynabead suspension per 1×10^7 cells) the next day. The beads were washed with low detergent lysis buffer (20 mM HEPES-NaOH (pH 7.8), 300 mM NaCl, 1 mM EDTA, 20% glycerol and 0.1% NP-40) and the precipitated proteins were subjected to gel electrophoresis or in-solution digestion.

Enzymatic digestion and phosphoenrichment

Immunoprecipitated ERG was digested with chymotrypsin (Roche)/trypsin (Promega, Madison, WI, USA) and enriched for phosphopeptides for phosphosite identification (see Supplementary Methods).

Mass spectrometry

Peptide mixtures were separated and analyzed using the Fourier Transform mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Mascot software (version 2.3.2; Matrix Science, London, UK) with the Swissprot database search algorithm was used for protein identification. Search parameters included three missed cleavages from trypsin proteolysis. Modifications selected were set as fixed carbamidomethylation of cysteins, variable oxidation of methionine and phosphorylation of serine, threonine and tyrosine residues. All peptides with high-quality tandem mass spectra were manually interpreted and confirmed. The sequences and phosphorylation sites of all identified phosphopeptides were carefully assigned accordingly.

Generation of ERG-pS283 specific antiserum

Mouse monoclonal phospho-specific antibody for ERG pS283 was produced by ProMab Biotechnologies, Inc. (Richmond, CA, USA) using the artificially synthesized phosphopeptide PTPQSKAAQP(p)*S*PSTVPKTEDQ, S283 highlighted by asterisk. The specificity of this antiserum for phosphorylated ERG is shown in Supplementary Figure S1.

Viral vectors and transduction

The open reading frame of human *ERG3* (NM_182918.3, corresponding to transcript variant 1, mRNA) was subcloned into the *pMIG+* retroviral vector that encodes the green fluorescence protein (GFP) downstream of an internal ribosomal entry site.²² Phoenix A cells were transiently transfected to generate virus-containing medium. Five rounds of retroviral transduction were performed over 3 days as previously described.¹³ See Supplementary Methods for details.

Site-directed mutagenesis

Various *ERG* constructs with point mutations were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The *pMIG⁺ ERG* plasmid was used as a backbone vector to generate mutant *ERG* constructs with alanine or aspartate mutations at S283. The mutation was subcloned into *pMIG⁺ HA-ERG* for tagged constructs. The mutagenesis primer sets are listed in Supplementary Table S1.

Cytokine-driven expansion culture

Transduced cells were expanded in cytokine-driven culture by plating 4×10^4 viable cells per ml in Iscove's modified Dulbecco's medium supplemented with 10% low endotoxin fetal bovine serum (16000-044, Thermo Fisher Scientific), 2 mM L-glutamine, 50 µg/ml gentamycin, human stem cell factor (50 ng/ml), thrombopoietin (10 ng/ml), Flt3 ligand (50 ng/ml) and interleukin-6 (10 ng/ml). Cells were passaged weekly, and either returned to cytokine-driven culture or used for colony-forming unit assays. See Supplementary Methods for CD34⁺ cell enrichment and transduction.

Colony-forming unit assay

Clonogenic progenitors were quantified in triplicate cultures of 1% methylcellulose supplemented with 20 ng/ml stem cell factor, 20 ng/ml interleukin-3, 20 ng/ml interleukin-6, 6 µg/ml erythropoietin and 20 ng/ml granulocyte-colony stimulating factor.²³ The optimal seeding density of HSPCs was determined empirically to yield 20–200 colonies per 35 mm plate. KG1 and ME1 were plated at 3000 cells/plate and 25 000 cells per plate, respectively. Cultures were incubated at 37 °C, 5% CO₂ for 14–16 days and then total and GFP⁺ colonies were counted.

RNA sequencing

Single-stranded TruSeq complementary DNA libraries were generated and sequenced using the Illumina HiSeq2000 analyzer (BGI, Hong Kong). The sequencing reads were aligned to the human genome (hg19) using the software package TopHat.²⁴ Transcript expression was quantified with the software suite HOMER²⁵ using standard parameters and DeSeq²⁶ to identify differentially expressed genes. Data have been deposited in Gene Expression Omnibus as GSE53665.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (v6.05, La Jolla, CA, USA), SAS software (v9.4, Cary, NC, USA) and R software (v3.0.2, R Foundation for Statistical Computing, Vienna, Austria). A *P*-value of <0.05 was considered significant. See Supplementary Methods for details.

Additional methods

For detailed descriptions of the protein degradation assay, immunostaining, fluorescence polarization assay, chromatin immunoprecipitation, quantitative real-time PCR, flow

cytometry and fluorescence-activated cell sorting and transactivation assay, see the previous studies^{6,13,27–30} and the Supplementary Methods.

RESULTS

ERG S283 is phosphorylated in human leukemic cells

To evaluate the role of phosphorylation on ERG activity, we first set out to identify sites of phosphorylation in endogenous ERG in MOLT4 T-ALL cells. To this end, we applied liquid chromatography coupled tandem mass spectrometry on immunoprecipitated ERG and identified five phosphopeptides corresponding to five unique phosphorylation sites: S55, S88, S103, S222 and S283 (Figure 1a and Supplementary Figure S2). The identity of ERG was confirmed by liquid chromatography coupled tandem mass spectrometry with over 60% sequence coverage from the in-gel trypsin digested sample and 20% sequence coverage from the in-gel chymotrypsin digested sample (Supplementary Figure S3). Apart from the detection of S55 that required chymotrypsin digestion, the other four serine residues were identified by trypsin digestion. Tandem mass spectra were manually curated to confirm phosphorylation (Figure 1b and Supplementary Figure S4).

To directly compare ERG phosphorylation in different leukemic cell types, we used a SILAC (stable isotope labeling of amino acids in cell culture)-based approach.³¹ MOLT4 cells cultured in media containing L-Arginine.HCl (¹³C₆, 99%) and L-Lysine.2HCl (¹³C₆, 99%) for six consecutive passages were ‘heavy’ labeled and their lysates were combined with lysates from KG1 AML cells cultured in normal media (‘light’). Phosphoproteomic analysis was also performed using primary mobilized CD34+ human HSPCs. ERG S88 was robustly phosphorylated in all three cell types, but ERG S283 phosphorylation (pS283), although abundant in T-ALL and AML cells, was minimal or undetectable in primary HSPCs (Figure 1c and Supplementary Figure S5). ERG phosphorylation at S103 and S222 was at or below the threshold for detection in KG1 and primary HSPCs.

We also extracted phosphoproteomic data from publicly available data sets to evaluate ERG phosphorylation in normal tissues (Figure 1d).^{18–20} Interrogation of phosphorylation at S55 requires chymotrypsin digestion and was not always performed in these studies. Following the curation of these data sets and comparison with ours, a striking feature was the abundance of ERG pS283 in leukemic cells in contrast with either low or undetectable phosphorylation in normal human HSPCs and normal mouse tissues or in prostate cancer cells expressing the TMPRSS2-ERG fusion protein (Figure 1d).^{18–20}

To investigate whether ERG pS283 was also present in primary leukemic cells, we generated a monoclonal antibody that specifically recognized this modification but not the unmodified protein (see Methods; Supplementary Figure S1) and screened a panel of AML and T-ALL patient-derived xenografts.^{32–35} Most T-ALL and AMLs had robust ERG S283 phosphorylation (Figure 1e). The ETP (early T-cell precursor) ALLs in particular had high ERG S283 phosphorylation that was minimal in healthy CD34+ HSPCs despite high total ERG expression (Figure 1e).

Overexpression of an ERG S283 phosphomimetic promotes maintenance and expansion of CD34+ HSPCs in cytokine-driven cultures

Following the identification of ERG pS283 and its relative abundance in leukemic cells, we set out to identify the biological impact of this modification on hematopoiesis. *ERG* overexpression in CD34+ cord blood (CB) cells results in the expansion of the progenitor pool, and a gene expression signature that shares features with normal HSCs, high ERG AMLs, ETP ALLs and leukemic stem cell signatures associated with poor clinical outcome.¹³ Given the frequency and relative abundance of pS283 in leukemic cell lines and in primary leukemic cells in contrast with CD34+ HSPCs, we investigated the impact of overexpressing phosphomimetic and phosphodeficient S283 mutants of ERG in CD34+ CB cells in cytokine-driven cultures (Figure 2 and Table 1). CB cells were transduced with retroviral vectors expressing either the empty vector (*pMIG+*), *wild-type* (*WT*) *ERG*, *ERG S283A* (phosphodeficient mutant) or *ERG S283D* (phosphomimetic mutant). Transduced cells were cultured in media supplemented with stem cell factor, thrombopoietin, Flt3 ligand and interleukin-6 with weekly passaging and harvesting for clonogenic assays, flow cytometry and molecular analysis (Supplementary Figure S6). Quantitative real-time PCR analysis of CB cells from three independent transductions confirmed that *ERG* expression levels in all three high ERG conditions were substantially elevated in comparison with the *pMIG+* cells at each time point (Supplementary Figure S7). Transduced cells were identified by the expression of GFP. The proportion of CB cells transduced with *ERG WT* and mutant *ERG* progressively increased in culture in contrast with those transduced with the empty vector (*pMIG+*) that progressively decreased (Figure 2a). These data were consistent with previous observations that *ERG* overexpression conferred HSPCs with a competitive advantage in cytokine-driven cultures.¹³

Overexpression of the *ERG S283D* mutant was associated with a significant increase in the expansion of the transduced primitive population (GFP+ CD34+) (Figure 2b and Table 1). Although there were inherent differences between CB samples, the cumulative expansion of CD34+ progenitors in *ERG S283D*-transduced cells was greater than the expansion of CB cells transduced with *ERG WT* in three independent experiments ($P < 0.001$, Table 1). As expected, transduced ERG WT was not phosphorylated at S283 in GFP+CD34+ cells (Supplementary Figure S8). Along with the expansion of CD34+ cells, overexpression of *ERG S283D* in CB cells also resulted in a cumulative increase in the numbers of CD34+ cells consistent with the absence of a gross defect in progenitor differentiation (Figure 2c and Table 1, $P < 0.0001$). Clonogenic assays are indicative of functional hematopoietic progenitors. Consistent with the increased numbers of CD34+ progenitors, there was an increase in the numbers of colony-forming units generated by cells transduced with *ERG S283D* compared with those transduced by *ERG WT* and *ERG S283A* that in turn was significantly greater than cells transduced with the *pMIG+* vector (Figure 2d and Table 1, $P < 0.01$).

To characterize the impact of high *ERG S283D* expression on the transcriptome of CD34+ HSPCs, we performed RNA sequencing on cell fractions sorted at week 0 following five rounds of transduction over 3 days. RNA sequencing was performed using two independent CB transductions (experiments 2 and 3). Gene set enrichment analysis showed that genes

that were high in HSCs (relative to granulocyte–macrophage progenitors) were enriched in *ERG S283D*-transduced CB cells (Figure 2ei). The *ERG +85* SCE is active in leukemic cells and we have previously shown that a signature derived from the activity of this enhancer is an independent risk factor for poor overall survival in AML.²⁷ *ERG S283D*-transduced CB cells were also enriched for genes in the +85 SCE (Figure 2eii) as well as gene sets associated with oncogenic processes (Supplementary Figure S9). Another notable observation was the enrichment of genes associated with RAS (Figure 2fi) and MEK (Figure 2fii) signaling in *ERG S283D*-transduced cells compared with *ERG WT*-transduced cells.

ERG S283 phosphorylation is mediated by the MAPK/ERK2 pathway

Given the abundant phosphorylation of ERG S283 in leukemic cells and the observation that ERG S283 phosphomimetic promotes a stem cell-like gene expression signature and expansion of CD34+ progenitors, we set out to identify the upstream pathways that mediate this modification in leukemic cells.

Human ERG3, the major hematopoietic isoform, contains a putative MAPK docking site (FxFP) encoded by exon 12 between the *PNT* and *ETS* domains (Figure 3a).^{21,36} This motif is also known as a *DEF* domain and facilitates ERK1/2 docking in preference to c-Jun N-terminal kinase or p38.³⁷ Given this and the observation that MEK/ERK activity is reinforced by ERG S283 phosphomimetic in CD34+ CB cells, we considered the possibility of a recursive loop and evaluated the ability of ERK1 and ERK2 to phosphorylate ERG S283 in *in vitro* kinase reactions using purified WT ERG followed by mass spectrometry (Supplementary Figure S10). ERK2 but not ERK1 successfully phosphorylated ERG S283. These reactions were repeated using purified WT ERG or an exon 12 deletion mutant of ERG (ERG- Ex12). Consistent with loss of its binding domain, the percent phosphorylation on S283 was significantly reduced in ERG- Ex12 (Figure 3b). Myelin basic protein (1.5 nmol per reaction) was included in each reaction to serve as an internal indicator of kinase efficiency (Supplementary Figure S11). ERK2 did not phosphorylate ERG S88, suggesting that the abundant phosphorylation of this residue in hematopoietic cells is likely mediated by another kinase (Figure 3b). S222, on the other hand, can be phosphorylated by ERK2 even in the absence of the docking domain on exon 12 (Figure 3b).

Phorbol 12-myristate 13-acetate (PMA), a direct protein kinase C activator, also activates MEK/ERK and increases phosphorylation on ERK substrates.³⁸ PMA also mediates phosphorylation of non-ERK substrates, but unlike the phosphorylation of ERK substrates, the former cannot be attenuated by pretreatment with ERK inhibitors. To investigate the *in vivo* role of ERK on ERG S283 phosphorylation, serum-starved MOLT4 cells were treated with an MEK/ERK inhibitor (U0126) and/or PMA (Figure 3c). The experiment was performed using a SILAC-based method, where cells grown in normal media were treated with U0126 ± PMA and phosphorylation levels analyzed by mass spectrometry simultaneously with lysates of vehicle control-treated cells that were grown in heavy media. MEK/ERK inhibition (U0126) reduced S283 phosphorylation in MOLT4 cells (Figure 3c). PMA treatment increased S283 phosphorylation but pretreatment with U0126 attenuated this increase. These data were consistent with pS283 in MOLT4 cells being predominantly an ERK-mediated modification. On the other hand, S222 phosphorylation was not exclusively

an ERK-mediated modification in that although U0126 reduced the degree of phosphorylation at this residue, it did not attenuate PMA-mediated phosphorylation (Figure 3c). Consistent with the *in vitro* assays in Figure 3b, S88 phosphorylation was largely independent of ERK (Figure 3c).

To evaluate whether ERG pS283 in primary leukemic cells was also mediated by MEK/ERK, we used two primary ETP ALL xenografts with high baseline pS283. Variations in ERG pS283 levels were determined using the custom anti-ERG pS283 antibody (Figure 3d). As with MOLT4 cells, U0126 reduced baseline pS283 levels in ETP ALL cells and the PMA-mediated increase in phosphorylation was attenuated by U0126 (Figure 3d). The variations in pERK levels with U0126 ± PMA serve as internal controls for target kinase inhibition and activation (Figure 3d). Taken together, these data were supportive of ERG pS283 in primary leukemic cells also being mediated by MEK/ERK.

To evaluate the relationship between pERK2 levels and ERG pS283, we screened a panel of patient-derived ETP ALL, AML xenografts and healthy CD34+ HSPCs (Figure 3e and Supplementary Figure S12). With some exceptions (for example, ETP1), the levels of pERK2 and ERG pS283 in individual leukemic samples were concordant and consistent across a second set of spleens from these xenografts (Supplementary Figure S13). Healthy CD34+ HSPCs, which have minimal level of pS283, had no discernible pERK2 (Figure 3e). However, MEK/ERK inhibition *per se* was not cytotoxic to these ETP ALL xenografts with high ERG pS283 (Supplementary Figure S14). Given the enhanced proliferation of CD34+ CB cells transduced with *ERG S283D*, we hypothesized that inhibiting MAPK/ERK signaling would have a greater impact on the clonogenicity of leukemic cells with high ERG pS283. To this end, we performed clonogenic assays using KG1 and ME1 AML cells in the presence of a MEK inhibitor, U0126. Although MEK inhibition affected colony-forming unit formation in both healthy CD34+ and leukemic cells, the relative impact was significantly higher on leukemic cells (see Materials and methods; Figure 3f).

The ERG S283 phosphomimetic is enriched on leukemia-associated enhancers

To assess how pS283 affected ERG activity, we evaluated protein stability, nuclear localization and DNA-binding properties of ERG S283 phosphomutants and that of WT ERG. Protein stability was measured by transfecting HEK293T cells with WT and mutant ERG and performing western blots on cell lysates collected at various intervals following the inhibition of *de novo* protein synthesis with cycloheximide. The protein half-lives were comparable and calculated at 38.5, 38.5 and 36.5 h for ERG WT, ERG S283A and ERG S283D, respectively (Figure 4a and Supplementary Figure S15).

ERG is a transcription factor and to explore whether pS283 of ERG alters its nuclear localization, we transduced MOLT4 T-ALL cells with WT ERG or the S283 mutants carrying an hemagglutinin tag to discriminate recombinant ERG from endogenous protein. Immunofluorescence images of WT and variant ERG showed little difference with all forms able to translocate into the nuclei of MOLT4 cells (Figure 4bi). This was further supported by immunoblots of the nuclear and the cytoplasmic fractions of the transduced cells (Figure 4bii).

Given the efficient nuclear localization of all forms, we evaluated possible differences in *in vitro* and *in vivo* DNA binding of ERG WT and S283D mutant. Glutathione *S*-transferase-tagged full-length ERG was bound *in vitro* to a DNA segment (5'-AGGACCGGAAGTAACT-3') containing the consensus *ETS* motif (5'-GGAA/T-3') in a fluorescence polarization assay. Comparable binding affinity of ERG WT ($K_D = 23$ nM) and S283D ($K_D = 22$ nM) was observed (Figure 4c, $n = 2$). *In vivo* DNA binding was assessed by performing chromatin immunoprecipitation assays in MOLT4 cells transduced with hemagglutinin-tagged WT and S283 mutant ERG (Figure 4di). An *ETS* deletion mutant of ERG (ERG- *ETS*), which is incapable of binding DNA, was used as a control. Chromatin immunoprecipitation was performed with an anti-hemagglutinin antibody and enrichment of transduced ERG forms was evaluated by quantitative real-time PCR at regions of the *ERG* +85 and *HHEX* +1 SCEs at which we have previously shown ERG binding in primary ALL.^{6,39} The activity of the +85 SCE is linked to both higher endogenous *ERG* expression and poor prognosis.^{6,27} There was greater enrichment of the phosphomimetic ERG S283D mutant at these enhancers *in vivo* compared with ERG WT or the phosphodeficient S283A mutant (Figure 4di). To investigate the impact of differential *in vivo* binding of these transduced ERG forms at the *ERG* +85 enhancer on endogenous *ERG* expression, we measured endogenous transcripts using allele specific quantitative PCR (see Materials and methods). Occupancy of the *ERG* + 85 enhancer by S283D resulted in significantly higher levels of endogenous ERG transcripts than either WT ERG or S283A (Figure 4dii). A similar trend was observed in relation to binding of transduced ERG forms at *HHEX* +1 and levels of endogenous *HHEX* transcripts.

To evaluate the impact of MEK inhibition on ERG enrichment at stem cell enhancers in leukemic cells,²⁸ we treated KG-1 AML cells with either U0126 or dimethyl sulfoxide for 30 min, which was sufficient to ablate pERK2 and reduce ERG pS283 levels (Supplementary Figure S16), and performed quantitative real-time PCR–chromatin immunoprecipitation. There was significant reduction of endogenous ERG at the *ERG* +85, *HHEX* +1 and *LMO2-23* stem cell enhancers (Figure 4ei) and concomitant reductions in endogenous gene expression (Figure 4eii).

Taken together, our results suggest a model in which cooperative interaction between RAS/ MAPK signaling and pS283 ERG contributes to the malignant phenotype of high ERG leukemias (Figure 5).

DISCUSSION

Targeting aberrantly regulated post-translational modifications has significant advantages over targeting the transcription factor itself to avoid deleterious effects to normal physiological function. This is the first study to our knowledge that evaluates ERG phosphorylation and its functional impact in leukemia. A notable aspect of this work was that ERG was purified directly from healthy human hematopoietic and leukemic cells without exogenous overexpression to map physiologically relevant phosphosites.

Baseline phosphorylation of ERG S283 in pooled healthy CD34+ HSPCs was minimal, although the level of this post-translational modification may vary in functionally distinct

CD34+ subfractions and possibly contribute to their proliferative vigor or indeed their susceptibility to leukemic transformation. The RAS/MAPK signaling cascade, which mediates ERG S283 phosphorylation, has well-described roles in promoting oncogenesis,⁴⁰ but the mechanism by which MAPK signaling promotes leukemia is poorly understood. Our data show that MAPK/ERK-mediated phosphorylation of ERG enhances stem cell and leukemic gene expression signatures, promotes HSPC proliferation and contributes to the malignant phenotype of high ERG leukemias. However, generic inhibition of the RAS/MAPK signaling pathway is not a viable therapeutic option given the broad range of physiological processes that it mediates. In contrast, given its relative enrichment in leukemic cells, specific inhibition of S283 phosphorylation has promise. One potential avenue would be to leverage the requirement for exon 12 or the *DEF* domain that we have shown is required for ERK-mediated phosphorylation of ERG S283. *ERG3-exon12* is a naturally occurring *ERG* isoform⁴¹ and loss of exon 12 is likely to be more tolerable than loss of total ERG3. Alternate approaches that interfere with specific enzyme–substrate interactions will require further clarity with regard to the structural relationship between ERK and ERG.

Our data show that instead of impaired protein degradation or increased nuclear transfer, enhanced cell proliferation by the ERG S283 phosphomimetic is likely to be mediated by consequence of increased *in vivo* binding of this variant to gene regulatory elements. Binding of transduced ERG WT to enhancers in MOLT4 leukemic cells *in vivo* was intermediate to the low binding of the S283A mutant and high binding of the S283D mutant (Figure 4d). MEK/ERK activity is high in MOLT4 cells (Figure 3c) and a proportion of the transduced WT ERG will gain S283 phosphorylation, although it is unlikely given that these proteins are being overexpressed that every molecule of WT ERG would carry this modification vis-a-vis overexpressed S283D ERG. Transcription factor regulation of downstream gene expression is complex and target gene activation relies on combinatorial interactions between multiple transactivating proteins and chromatin modifiers.^{42–44} Combinatorial transcription factor binding is a well-recognized phenomenon in the regulation of transcriptional programs in CD34+ HSPCs.^{27,28,45} An analogy in embryonic stem cell maintenance is instructive. Human OCT4, a transcription factor fundamental to maintaining cell pluripotency and self-renewal, has two critical phosphorylation sites at T234 and S235. Coimmunoprecipitation assays have identified protein partners that are enriched for ERK motifs, suggesting a shared upstream pathway regulating the activity of OCT4-associated transcriptional complexes.⁴⁶ With relevance to the hematopoietic system, we have previously shown that over 300 chromatin sites are bound by seven transcription factors constituting a ‘heptad’ of factors that are associated with stem cell signatures in HSPCs and leukemic cells.^{27,28} The heptad includes FLI1, the *ETS* factor that is most closely related to ERG in protein phylogeny, as well as ERG itself and RUNX1.^{47,48} FLI1 and RUNX1 show the highest binding overlap of any heptad transcription factor binding combination in healthy HSPCs. Phosphorylation of FLI1 affects its ability to complex with RUNX1 and impairs megakaryocytic differentiation.¹⁶ However, the identity of proteins that complex with ERG at hematopoietic enhancers in leukemic cells and how these are disrupted by S283 phosphorylation is currently not known and requires further investigation.

ERG is probably modified in many ways in addition to phosphorylation. A recent study reported that ERG acetylation by p300 contributed to its interaction with and recruitment of the chromatin reader protein BRD4 (bromodomain-containing protein 4) to specific promoter and enhancer regions.⁴⁹ Targeted recruitment of BRD4, a member of the BET (bromodomain and extraterminal) family of chromatin reader proteins, to hematopoietic enhancers by ERG is particularly interesting given the hypersensitivity of leukemic cells to BET inhibitors.^{50–52} In addition, different modifications on the same protein, especially at nearby locations, may interfere with each other.^{53,54} For example, S59 phosphorylation of SP1 is inversely related to its N-terminal sumoylation and leads to enhanced proliferation and cancer development.⁵⁵ Expanding the knowledge base of distinct post-translational modifications that are associated with specific subtypes of leukemia is a major undertaking but may be required in order to design targeted therapies with minimal toxicity to healthy cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ERG S283 is phosphorylated in human leukemic cells. **(a)** A schematic of hERG3 scaled to size showing sites of serine phosphorylation in MOLT4 cells relative to its two functional domains. The numbers correspond to the amino acid positions. ETS, E-twenty six; PNT, Pointed. **(b)** A representative tandem mass spectrum (MS) showing phosphorylated ERG tryptic peptide containing S283. The b- and y-ions are annotated and labeled on the spectrum with the MS1 accurate spectrum shown in the inset. Note that for clarity, some regions of the extracted ion chromatogram have been amplified 10-fold. **(c)** ERG S283 phosphorylation (pS283) level in MOLT4, KG1 and healthy CD34+ human HSPCs was quantified using MS and expressed as a ratio to the level of pS88 on a log-10 scale. **(d)** A table summarizing the presence (Y) or absence (N) of ERG phosphorylation. Detection of S55 requires chymotrypsin digestion and was not available for all studies. **(e)** Lysates of 24

primary leukemic xenografts including 5 ETP ALL, 14 non-ETP T-ALL and 5 primary AML xenografts were probed for pS283 in ERG using a custom anti-pS283 ERG monoclonal antibody. A lysate of healthy CD34+ HSPCs is shown for comparison. Numbers above blots are densitometry reading for pS283 ERG normalized to β -actin. Given variations in exposure time, these numerical values are for within blot interpretation and not for interpretation across individual blots. Expression levels of total ERG and β -actin are also shown. The patient characteristics relating to the xenografts are detailed in previous studies.
32–35

Figure 2.

Overexpression of ERG S283 phosphomimetic promotes expansion of CD34⁺ HSPCs in cytokine-driven culture. Human CB cells were transduced with empty vector (*pMIG+*), *WT* *ERG* or mutant *ERG* (S283A or S283D) and cultured with stem cell factor (SCF), thrombopoietin (TPO), Flt3 ligand (Flt3L) and interleukin-6 (IL-6) supplements until replicative exhaustion. Representative plots (experiment 2 (Exp 2)) showing (a) percent transduced (GFP⁺) cells determined by flow cytometry; (b) cumulative fold expansion of GFP⁺ CD34⁺ cells calculated relative to cell numbers at week 0 for each condition; (c) cumulative fold expansion of GFP⁺ CD34⁻ cells calculated relative to cell numbers at week

0 for each condition; **(d)** cumulative GFP+ CFUs (colony-forming units) derived from 10 000 GFP+ cells at week 0. **(e, f)** Gene set enrichment analysis (GSEA) of genes that were upregulated in *ERG S283D* cells compared with *ERG WT* cells (Exp 2 and 3). **(e)** GSEA plots showing enrichment of genes that were upregulated in data sets from gene expression analysis of (i) HSC versus GMP (granulocyte–macrophage progenitor) and (ii) the *ERG +85* SCE signature. **(f)** GSEA plots showing genes associated with (i) KRAS and (ii) MEK signaling.

Figure 3.

ERG S283 phosphorylation is mediated by the MAPK/ERK2 pathway. (a) A schematic showing the putative MAPK docking motif, *DEF* domain, located within ERG exon 12. The amino acid numbers and peptide sequence are shown. (b) A bar graph showing percent phosphorylation of serine residues in WT ERG and in an exon 12 deletion mutant of ERG (ERG-ΔEx12) measured using an *in vitro* ERK2 kinase assay. Peptide signal intensities of the phosphorylated and unphosphorylated versions were quantified by mass spectrometry (MS). Error bars represent s.d. for all peptide spectral matches quantified in this experiment ($n = 3$). **** $P < 0.0001$. (c) A bar graph showing changes in phosphorylation in MOLT4 cells in response to treatment with U0126 (10 μM) and/or PMA (100 nM). The levels of phosphorylation were quantified by MS and plotted as a ratio of treated/vehicle on a log-10 scale. (d) Western blots showing the responses of two ETP ALL xenografts with high-level ERG pS283 following treatment with U0126 (10 μM) and/or PMA (100 nM). The levels of ERG pS283, total ERG, active ERK1/2 (pERK1/2), total ERK1/2 and β-actin are shown. Numbers above blots are densitometry readings for pS283 ERG normalized to β-actin. Given variations in exposure time, these numerical values are for within blot interpretation and not for interpretation across individual blots. The upper band in the ERK1/2 panel represents ERK1 and the lower band represents ERK2. (e) Western blots showing levels of endogenous ERG pS283, total ERG and active ERK1/2 (pERK1/2) in a panel of ETP ALL and AML patient-derived xenografts. Levels of each in healthy CD34+ HSPCs are also

shown for comparison. Numbers above blots are densitometry reading for pS283 ERG normalized to β -actin. Given variations in exposure time, these numerical values are for within blot interpretation and not for interpretation across individual blots. (f) CFUs (colony-forming units) derived from vehicle-treated (0.1% dimethyl sulfoxide (DMSO)) or U0126-treated (10 μ M) KG1, ME1 and CD34+ HSPCs. Error bars represent s.d. from triplicate measurements, * P <0.05 for the relevant comparisons.

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Figure 4.

ERG S283 phosphomimetic shows enhanced *in vivo* DNA binding without change in stability or nuclear localization. **(a)** Western blots from a protein degradation assay to assess the stability of WT and mutant ERG. HEK293T cells expressing *ERG* (2.5 μg/well) were treated with 20 ng/ml cycloheximide 48 h post transfection and harvested at indicated time points post treatment. Protein levels were measured by immunoblotting and quantified by densitometry. The result shown is a representative from three independent experiments. **(b)** Confocal microscopy and immunoblotting to show nuclear localization of WT and mutant ERG. **(i)** Confocal images of MOLT4 cells expressing hemagglutinin (HA)-tagged WT and mutant *ERG*. The exposure was adjusted using negative controls (Mock, GFP negative; *pMIG*+, HA negative). The regions shown were representative of 10 randomly picked visual fields. **(ii)** The cytoplasmic (C) and nuclear (N) fractions of transduced MOLT4 cells expressing *HA-ERG* were blotted for total ERG, DNA TOP1 (nuclear protein) and β-actin. **(c)** Representative fluorescence polarization (FP) isotherms of ERG binding to DNA. Steady-state fluorescence anisotropy of DNA (5′-AGGACCGGAAGTAAGT-3′) extrinsically labeled with a Texas Red fluorophore on the 5′ end bound by various concentrations of full-length **(i)** WT and **(ii)** S283D glutathione *S*-transferase (GST)-ERG.

The sigmoidal curve is the best-fit binding isotherm with K_D of 22–24 nM. The experiment was performed in duplicate (representative plots shown). (di) Enrichment of WT and mutant HA-ERG in transduced MOLT4 cells at the *ERG* +85 SCE and the *HHEX*+1 enhancer measured by quantitative real-time PCR (qRT-PCR). The ERG *ETS* deletion mutant (ERG-ETS) served as an *ERG*-expressing but non-DNA binding control. Enrichment was normalized to corresponding IgG controls. Error bars represent s.d. from duplicate qRT-PCR measurements, * P <0.05 (t -test). (dii) Expression of endogenous ERG and HHEX in MOLT4 cells expressing WT or mutant HA-ERG. Error bars represent s.d. from duplicate qRT-PCR measurements, * P <0.05 (t -test). (ei) Enrichment of ERG in KG1 cells treated with U0126 or vehicle (30 min) at known ERG binding sites²⁸ (*ERG* +85 SCE, *HHEX* +1 enhancer, *LMO2*-23 enhancer) measured by qRT-PCR. Error bars represent s.d. from duplicate qRT-PCR measurements, * P <0.05 (t -test). (eii) Expression of ERG, HHEX and LMO2 in KG1 cells treated with U0126 or vehicle (24 h). Error bars represent s.d. from duplicate qRT-PCR measurements, * P <0.05 (t -test).

Figure 5. MAPK/ERK and ERG cooperate in leukemic cells. A diagram summarizing the cooperative interaction between MAPK/ERK signaling and ERG S283 phosphorylation that enhances stem cell and oncogenic gene expression signatures in leukemic cells.

Table 1

Expression of an ERG S283 phosphomimetic results in increased cumulative expansion in cytokine-driven culture

	GFP+ CD34+	GFP +CD34 –	GFP+CFU
<i>Exp 1^a</i>			
<i>pMIG+</i>	3.00×10^0	2.21×10^2	5.58×10^6
<i>ERG WT</i>	1.95×10^2	2.12×10^3	2.49×10^7
<i>ERG S283A</i>	6.94×10^2	1.96×10^3	8.11×10^7
<i>ERG S283D</i>	9.93×10^2	1.93×10^3	3.96×10^7
Fold change	5.07	0.91	1.59
<i>Exp 2</i>			
<i>pMIG+</i>	2.41×10^2	9.31×10^3	6.87×10^3
<i>ERG WT</i>	5.12×10^3	1.80×10^4	5.40×10^6
<i>ERG S283A</i>	2.86×10^3	4.53×10^4	1.36×10^6
<i>ERG S283D</i>	3.99×10^6	8.45×10^6	4.57×10^9
Fold change	779	469	845
<i>Exp 3^b</i>			
<i>pMIG+</i>	4.75×10^1	4.05×10^3	8.74×10^3
<i>ERG WT</i>	3.57×10^3	5.42×10^4	2.31×10^6
<i>ERG S283D</i>	5.27×10^3	9.36×10^4	5.05×10^6
Fold change	1.47	1.73	2.19
<i>P</i> -value for effect of <i>ERG S283D</i> over time	<i>P</i> <0.001	<i>P</i> <0.0001	<i>P</i> <0.01

Abbreviations: CFU, colony-forming unit; ERG, v-ets avian erythroblastosis virus E26 oncogene homolog; GFP, green fluorescence protein; WT wild type. Data show area under the curve calculation of cumulative expansion for each independent cord blood replicate. The *P*-value derives from a segmented regression approach using linear mixed model to separately estimate changes in cumulative expansion over time for ERG WT and ERG S283D with individual cord blood replicates as a random effect. Fold change calculated as ERG S283D÷ERG WT.

^aExperiment 1 (Exp 1) data calculated from week 0 to week 6.

^bExperiment 3 (Exp 3) does not have ERG S283A data.