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Genome-wide Kinase-Chromatin Interactions Reveal the Regulatory Network of ERK Signaling in Human Embryonic Stem Cells

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SUMMARY

The extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase signal-transduction cascade is one of the key pathways regulating proliferation and differentiation in development and disease. ERK signaling is required for human embryonic stem cells' (hESCs') self-renewing property. Here, we studied the convergence of the ERK signaling cascade at the DNA by mapping genome-wide kinase-chromatin interactions for ERK2 in hESCs. We observed that ERK2 binding occurs near noncoding genes and histone, cell-cycle, metabolism, and pluripotency-associated genes. We find that the transcription factor ELK1 is essential in hESCs and that ERK2 co-occupies promoters bound by ELK1. Strikingly, promoters bound by ELK1 without ERK2 are occupied by Polycomb group proteins that repress genes involved in lineage commitment. In summary, we propose a model wherein extracellular-signaling-stimulated proliferation and intrinsic repression of differentiation are integrated to maintain the identity of hESCs.

INTRODUCTION

During development, cells divide, grow, and differentiate to form the embryo. Growth factors act as signaling molecules between cells to ensure that embryonic development proceeds in a highly coordinated fashion (Böttcher and Niehrs, 2005). These growth factors are recognized by cell-surface receptors, which initiate a signal-transduction cascade that results in cell proliferation or specification of cell fate. One of the pivotal transmitters of growth-factor signaling is the extracellular signal-regulated kinase (ERK) pathway (Treisman, 1996). The ERK pathway belongs to the family of mitogen-activated protein kinase (MAPK) cascades. Actuation of the ERK/MAPK pathway by growth factors activates a Ras-like GTPase, which subsequently induces a three-step kinase cascade involving phosphorylation of family members of Raf, MEK, and ERK. Activated ERK translocates to the nucleus, where it phosphorylates its interaction partners and induces changes in gene expression (Bonni et al., 1999; Hu et al., 2009; Khokhlatchev et al., 1998; Yang et al., 2003). ERK signaling has been shown to be involved in cell-cycle progression (Yamamoto et al., 2006), differentiation (Lai et al., 2001), and proliferation (Zhang and Liu, 2002), and deregulation of ERK can lead to severe diseases such as cancer (Keld et al., 2011; Suojun et al., 2012).

A particular interest has focused on the role of fibroblast growth factor 2 (FGF2)/ERK signaling in embryonic stem cells (ESCs) (Greber et al., 2011; Kim et al., 2012). ESCs are derived from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman, 1981; Thomson et al., 1998). Whereas the cells of the inner cell mass undergo differentiation, the pluripotent capacity of ESCs can be preserved in vitro through indefinite self-renewal. This property holds great potential for regenerative medicine and cell-based therapy in which functional cells might be used to replace the loss of tissue function in certain human diseases. Human ESCs (hESCs) are grown in the presence of FGF2, which activates the ERK signaling cascade (Lanner and Rossant, 2010). This is in stark contrast to mouse ESCs (mESCs), which require leukemia inhibitory factor (LIF) for self-renewal in serum containing media, whereas ERK signaling is dispensable for their proliferation (Kunath et al., 2007; Ng and Surani, 2011). Hence, there is a clear difference in the signaling networks for the maintenance of pluripotency between mESCs and hESCs. It is also of interest to note that pluripotent mouse epiblast stem cells derived from postimplantation embryos are also dependent on FGF2. This similarity with hESCs suggests that signaling networks may be conserved between the pluripotent stem cells from the two species (Nichols and Smith, 2009).

In mESCs, external signaling is integrated into a network of transcription factors and chromatin regulators that bind to regulatory regions in the genome (Chen et al., 2008; Göke et al., 2011; Kim et al., 2008; Marson et al., 2008; Ng and Surani, 2011; Orkin

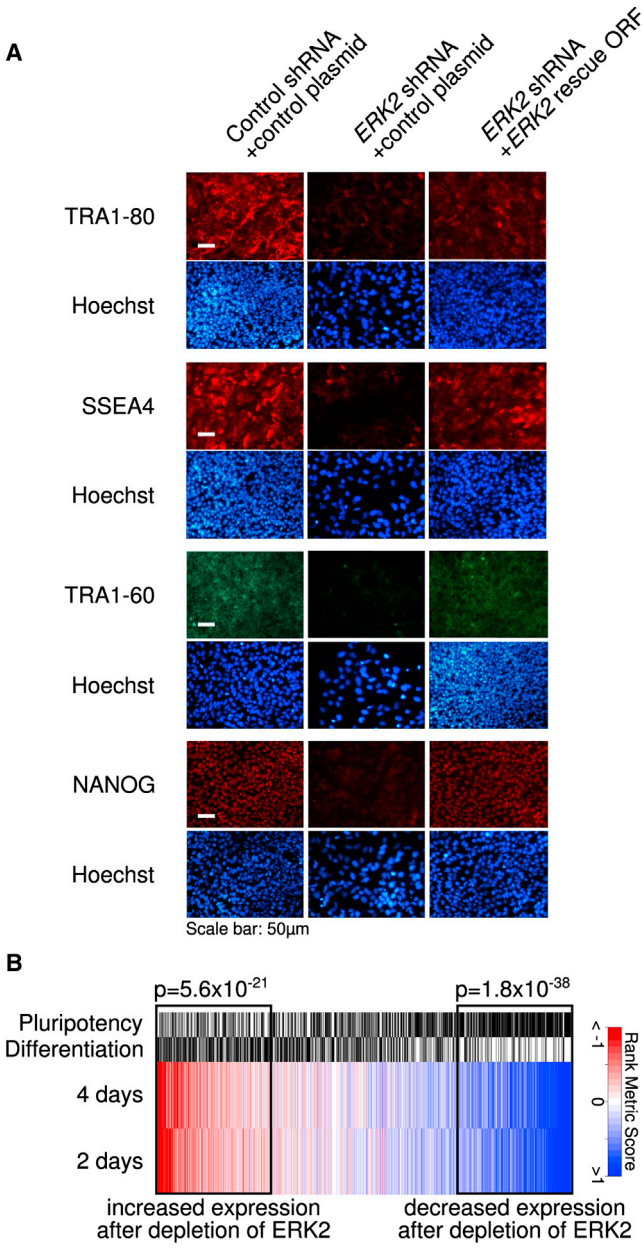

Figure 1. *ERK2* RNAi Knockdown Leads to Loss of Pluripotency

(A) hESCs were transfected with shRNA constructs targeting the *Luciferase* transcript or *ERK2*. Constructs expressing the wild-type ERK2 or the RNAi-immune ERK2 were cotransfected with the ERK2 shRNA constructs. Shown are representative immunostainings for pluripotency marker after *ERK2* knockdown and rescue. Nuclei are stained with DAPI (blue fluorescence). ORF, open reading frame.

(B) Expression changes (normalized signal-to-noise ratio) after depletion of ERK2 for hESC-specific genes and differentiation genes; significance was calculated using Fisher's test. See also Figure S1.

and Hochedlinger, 2011). It has previously been shown that downstream effectors (Stat3 and Smad1) of LIF and BMP signaling converge at enhanceosomes, which play a role in activating pluripotency associated genes in mESCs (Chen et al., 2008). The interplay of these transcriptional regulators activates or represses transcription, and accordingly, the underlying regulatory elements can be in an active or repressive epigenetic state. Active regulatory regions are marked by histone modifications associated with active transcription, such as H3K27ac or H3K4me3, and mark genes associated with pluripotency. Repressed regulatory regions are marked by H3K27me3, which is catalyzed by the Polycomb group proteins (Bernstein et al., 2006; Cao et al., 2002). Regulatory regions which are marked by active and repressive histone modifications are termed bivalent domains. These bivalent domains occur largely near developmental genes to prevent differentiation and maintain the pluripotent capacity (Lee et al., 2006).

Although the importance of FGF2 signaling in human pluripotent cells is generally acknowledged (Greber et al., 2010, 2011; Nichols and Smith, 2009), little is known about the downstream transcriptional regulatory network that is formed by the convergence of ERK signaling at the DNA. In this study, we investigate the function and mechanism of the ERK-mediated transcriptional response by mapping genome-wide chromatin interaction sites of ERK2 in hESCs. We find that ERK2 binds at active promoters of genes involved in metabolic pathways and cell-cycle progression, many of which are essential for survival, proliferation, and pluripotency of hESCs. We observe that binding of ERK2 correlates with the occurrence of specific DNA sequence motifs, such as binding sites for the transcription factor ELK1. By integrating genome-wide binding data with epigenetic profiles and loss-of-function gene-expression experiments, we provide a description of the transcriptional network controlled by ERK2 and ELK1. Our study identifies many targets of ERK2 and highlights how kinase-chromatin interactions integrate extracellular signaling into the regulatory network that promotes self-renewal and prevents differentiation for maintenance of the pluripotent state of hESCs.

RESULTS

ERK2 Is Required for Maintaining the hESC Identity

To probe the function of ERK2 in hESCs, we depleted ERK2 expression using RNAi. Consistent with the importance of FGF signaling in hESCs, ERK2 knockdown results in the loss of pluripotency markers (Figure 1A) and a concomitant loss of the typical hESC morphology. To ensure that the phenotype is not the consequence of off-target effects of the small hairpin RNA (shRNA), we constructed an RNAi-immune complementary DNA (cDNA) encoding ERK2 by introducing silent mutations into the target sequence. Coexpression of this RNAi-immune cDNA substantially restored the expression of pluripotency markers in ERK2-depleted hESCs (Figure 1A), thus confirming that ERK2 is indeed required for the identity of hESCs. To further characterize the phenotype of ERK2 depletion in hESCs, we measured genome-wide expression 2 and 4 days after knockdown of ERK2 and calculated differential expression for pluripotency and differentiation genes (Assou et al., 2007) (Figure 1B; Figures S1A and S1B available online). Genes that are downregulated after loss of ERK2 are highly enriched in pluripotency genes (e.g., *SOX2*, *PRDM14*, *DNMT3B*, and *TERF1*; $p = 1.8 \times 10^{-38}$). In contrast, upregulated genes are enriched in

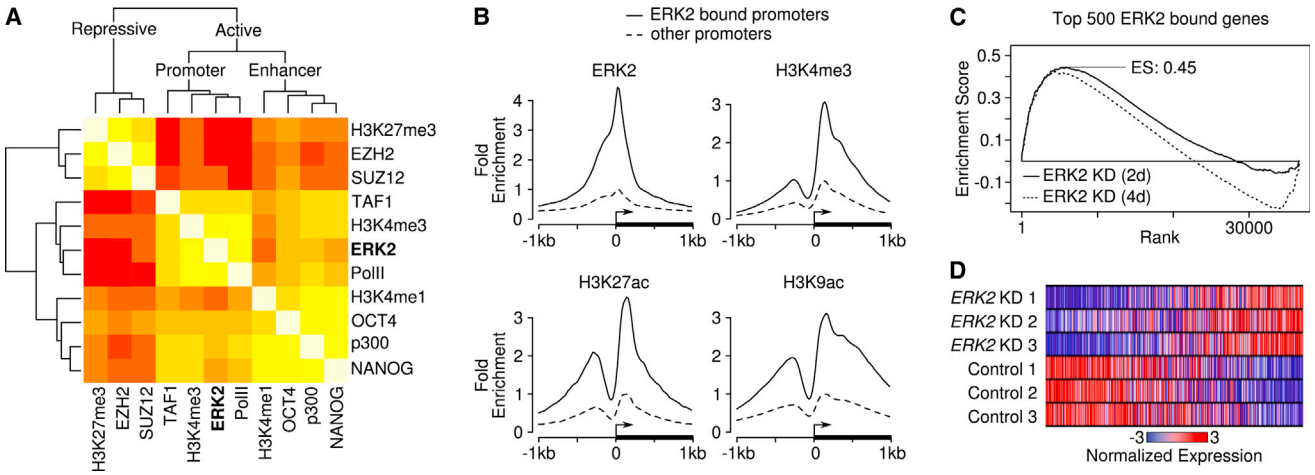

Figure 2. ERK2 Binds at Active Promoters in hESCs

(A) Hierarchical clustering of genome-wide binding data sets with ERK2. The color indicates the similarity based on the Pearson correlation coefficients of the log-transformed number of ChIP-seq reads. ERK2 binds preferentially to active promoters as indicated by H3K4me3, PolII, and TAF1.

(B) Profile of ERK2, H3K4me3, H3K27ac, and H3K9ac at promoters bound by ERK2 as a fold enrichment over promoters without ERK2 binding.

(C) GSEA of genes bound by ERK2, 2 and 4 days after knockdown of ERK2. Genes are ranked according to the signal-to-noise ratio (control/knockdown). A positive enrichment score (ES) indicates enrichment in the set of genes which show reduced expression after the knockdown.

(D) Heatmap showing the expression of genes with nearby ERK2 binding, 4 days after knockdown (KD) of ERK2. See also Figure S2 and Table S1.

differentiation genes ($p = 5.6 \times 10^{-21}$). This further confirms the loss of the hESC identity after depletion of ERK2, possibly involving both direct and indirect regulation of pluripotency genes.

Genome-wide Kinase-Chromatin Interactions Identify Transcriptional Targets of ERK2 Signaling

In order to identify target genes of ERK2 signaling, we mapped the genome-wide profile of ERK2-chromatin interactions using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). We obtained 12,070 loci wherein ERK2 binds to chromatin (henceforth referred to as ERK2 binding sites, Table S1), 64% of which lie within 1,000 base pairs (bp) of a transcription start site (TSS). To further characterize ERK2 binding sites, we calculated the correlation of ERK2 binding intensity with markers for repressive promoters (H3K27me3 and Polycomb group proteins EZH2 and SUZ12), active promoters (H3K4me3, PolII, and TAF1), enhancers (H3K4me1 and p300), and transcription factor binding sites (OCT4 and NANOG). ERK2 forms one cluster with PolII, TAF1, and H3K4me3, suggesting that ERK2 interacts with chromatin at active promoters (Figures 2A and S2). Indeed, promoters bound by ERK2 show significantly higher levels of histone modification associated with active transcription (H3K4me3, H3K27ac, and H3K9ac) compared to promoters in general (p value $< 10^{-16}$, Figure 2B).

The observation that ERK2-chromatin interactions occur mainly at active promoters raises the question of whether ERK2 binding itself regulates active transcription. To estimate the impact of ERK2 binding on gene expression, we tested whether ERK2-bound genes are among the differentially expressed genes after ERK2 depletion using the gene set enrichment analysis (GSEA) software (Subramanian et al., 2005) (Figures 2C and 2D). Indeed, genes wherein the promoter is bound by ERK2 show a significant enrichment in the set of genes with reduced expression after knockdown (p value < 0.001). This supports the hypothesis that ERK2-chromatin interactions identify transcriptional targets of ERK2 signaling and that ERK2 binding is associated with active transcriptional activity.

ERK2 Targets Noncoding Genes and Genes Involved in Metabolism, Cell Cycle, and Transcription

To assess the biological response triggered by ERK2 signaling, we calculated the enrichment of gene ontology (GO) terms for genes with nearby binding of ERK2 (Figures 3A, S3A, and S3B). ERK2-bound genes show significant enrichment in GO terms associated with cellular metabolism and translation (e.g., *RPS6*, Figure 3B), cell cycle (e.g., *FOXN3*, Figure S3C), chromatin organization (e.g., histone cluster 1, Figure 3D), and transcription (e.g., *POLR2A*, *SOX2*, and *HMGA1*; Figures 3C, 3E, 3F, and S3D). ERK2 signaling has been reported to affect metabolism and cell cycle through its kinase activity (Chambard et al., 2007; Yamamoto et al., 2006). The finding that these properties are reflected in ERK2 binding data indicates that besides the kinase activity, kinase-chromatin interactions are involved in mediating the transcriptional response.

Interestingly, the genes with the highest ERK2 binding intensity are noncoding genes, including small nuclear RNAs (snRNAs) such as U1-U2-U5 spliceosomal RNAs (Figure 3G), small nucleolar RNAs, and noncoding snRNA host genes, some of which have been shown to be involved in hESC-specific regulation of gene expression (O'Reilly et al., 2013). Furthermore, among the noncoding genes with nearby binding of ERK2 are multiple instances of the retrovirus *HERVH*, which is exclusively expressed in hESCs (Figures 3H and S3E).

ERK signaling is active in various cell types, and accordingly, the functions associated with binding of ERK2, such as cell cycle

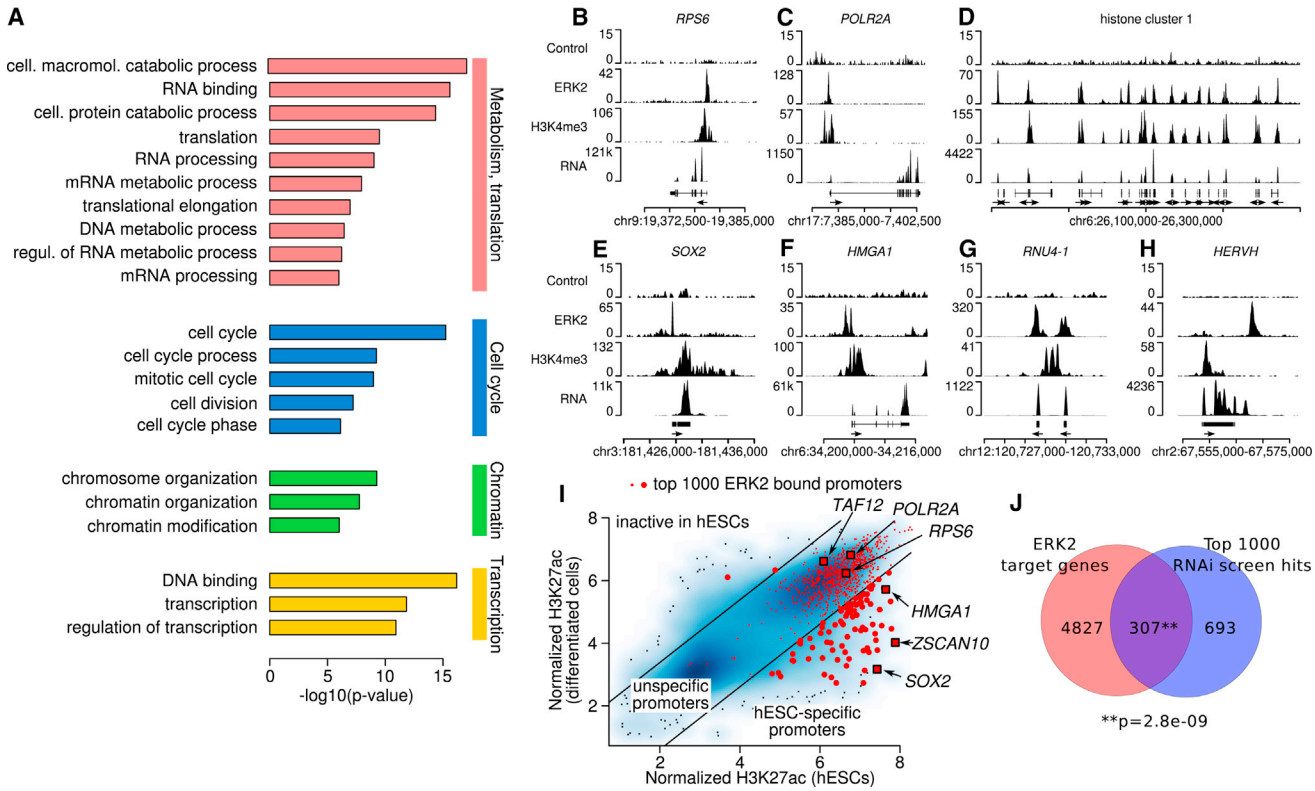

Figure 3. ERK2 Targets Noncoding Genes and Genes Involved in Metabolism, Cell Cycle, and Transcription

(A) GO enrichment analysis of genes what are bound by ERK2 at the promoter (within 1 kb of the TSS).

(B–H) Binding of ERK2 at the promoter of (B) *RPS6*, (C) *POLR2A*, (D) histone cluster 1, (E) *SOX2*, (F) *HMGA1*, (G) *RNU4-1*, and (H) *HERVH*.

(I) Average normalized H3K27ac levels at promoters in two pluripotent cells (H1 and H9 hESCs) and in 20 differentiated cells (log-scale). Promoters were classified as hESC-specific if the average normalized H3K27ac was at least 4-fold higher than the average normalized H3K27ac level in differentiated cells (as indicated by the black line).

(J) Overlap of genes bound by ERK2 at the promoter, with the top 1,000 genes from a genome-wide RNAi screen where the RNAi knockdown led to the strongest decrease in the number of nuclei. Significance was tested using Fisher's exact test. See also Figure S3.

and metabolism, are of broad importance for cell survival. However, depletion of ERK2 induces loss of pluripotency, indicating that ERK signaling in hESCs contains a hESC-specific component. To systematically dissect the cell-type-independent component from the hESC-specific component of ERK signaling, we investigated binding of ERK2 at hESC-specific and cell-type-unspecific promoters (Figure 3I). Indeed, ERK2 binding occurs at promoters that are active in multiple cell types (e.g., *POLR2A*, *TAF12*, and *RPS6*) and at hESC-specific promoters (e.g., *SOX2*, *HMGA1*, and *ZSCAN10*). The binding of ERK2 near hESC-specific genes such as *SOX2* or *HERVH* potentially represents key events that mediate the pluripotency-specific response of ERK signaling. Yet many genes from the cell-type-independent component of ERK signaling are similarly required for the maintenance of pluripotency, as evident from the overlap with a genome-wide RNAi screen in hESCs (Figures 3J and S3F–S3H) (Chia et al., 2010). Among the top 1,000 genes with the most severe effects of the RNAi on survival and proliferation of hESCs, 31% are bound by ERK2, many of which are not cell-type specific. Precise regulation of metabolic processes, RNA processing, translation, and cell-cycle progression is crucial for self-renewing pluripotent cells (Dejosez et al., 2010; Medina et al., 2012). Thus, the loss of pluripotency in response to ERK2 depletion is most likely a result of both the loss of direct regulation of pluripotency genes and disruption of these fundamental self-renewal pathways by ERK2.

Motif Finding Identifies Candidate Interaction Partners of ERK2

Regulation of gene expression by ERK2 is achieved through phosphorylation of transcription factors that can bind to the DNA (Carlson et al., 2011). Therefore, binding of ERK2 at genomic DNA is likely to reflect binding of sequence-specific transcription factors that interact with ERK2. To identify potential interaction partners of ERK2 acting at the chromatin, we searched for overrepresented DNA motifs in the ERK2-bound loci (Figure 4A). Several of the identified motifs resemble DNA binding motifs from known substrates of MAPK signaling, such as KLF4 (Kim et al., 2012), SP1 (Milanini-Mongiat et al., 2002), CREB (Arthur and Cohen, 2000), E2F (Wang et al., 1999), and ELK1 (Hipskind et al., 1994; Sharrocks, 1995; Treisman, 1996).

To address which of these candidates are relevant downstream effectors for ERK2 in hESCs, we depleted the expression

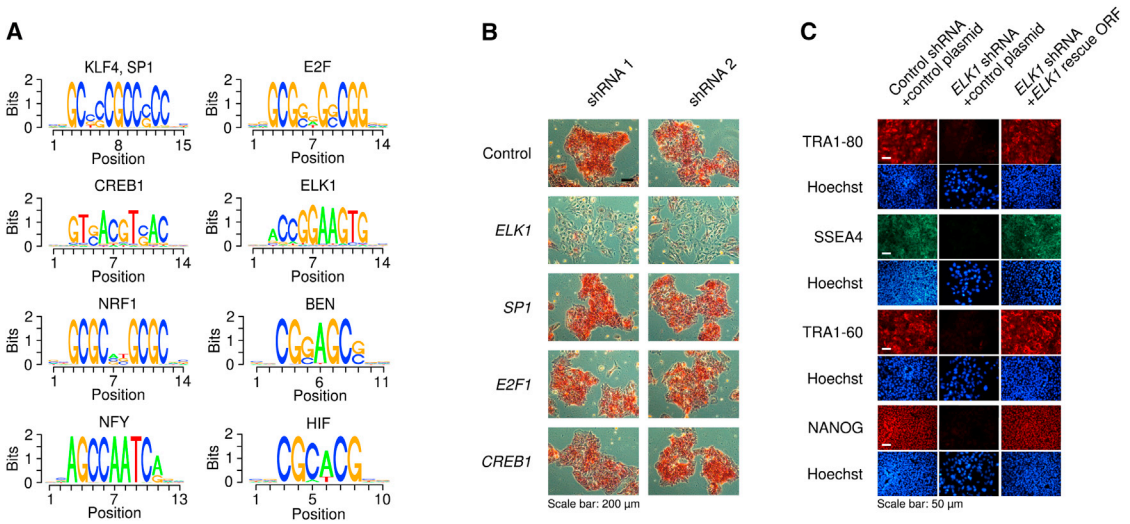

Figure 4. Motif Finding identifies ELK1 as a Candidate Interaction Partner of ERK2 in hESCs

(A) De novo motif finding in ERK2 binding sites identifies known ERK phosphorylation targets and additional candidates.

(B) Alkaline phosphatase staining of hESCs. Whereas knockdown of *CREB1*, *E2F1*, and *SP1* had no visible effect, depletion of *ELK1* significantly decreased alkaline phosphatase activity.

(C) ELK1 RNAi knockdown reduces pluripotency marker expression. The phenotype can be rescued with RNAi immune ELK1 expression. hESCs were transfected with shRNA constructs targeting the Luciferase transcript or *ELK1*. Constructs expressing the wild-type ELK1 or the RNAi-immune ELK1 were co-transfected with the ELK1 shRNA constructs. Shown are representative immunostains for pluripotency marker after *ELK1* knockdown and rescue. Nuclei are stained with DAPI (blue fluorescence). See also Figure S4.

of *ELK1*, *CREB1*, *E2F1*, and *SP1* using two independent shRNAs for each transcript (Figure S4A). Knockdown of *CREB1*, *E2F1*, and *SP1* had no effect, whereas depletion of *ELK1* induced a fibroblast-like morphology with significantly decreased alkaline phosphatase activity, suggesting a role of ELK1 in the maintenance of pluripotency (Figure 4B). ELK1 depletion also decreased the expression of pluripotency markers, confirming the loss of hESCs' undifferentiated state (Figure 4C). This effect is specific for ELK1, as the ectopic expression of an RNAi-immune ELK1 construct is able to rescue the phenotype. Genome-wide expression data further confirm the severe effect of ELK1 depletion in hESCs. Genes that are upregulated after knockdown of ELK1 are significantly enriched in differentiation genes ($p = 3.4 \times 10^{43}$), wheres genes that are downregulated are enriched in hESC-specific genes ($p = 7.2 \times 10^{42}$, Figure S4B). These results show that ELK1 is required for the maintenance of the undifferentiated state of hESCs; therefore, we decided to investigate the role of ELK1 as a downstream target of ERK2.

ERK2 Co-occupies Promoters Bound by the Transcription Factor ELK1 in hESCs

To test whether ELK1 and ERK2 co-occupy genomic loci in hESCs, we generated the genome-wide binding profile for ELK1. We detected 7,679 binding sites (Table S2), 2,966 of which were also bound by ERK2. Inhibition of the phosphorylation of ERK2 by MEK inhibitors leads to reduced binding of ERK2 and ELK1 at these co-occupied loci (Figures S5A–S5F), suggesting that binding is phosphorylation dependent. Interestingly, ERK2 colocalization with ELK1 almost exclusively (96%) occurs within 1 kb of a coding transcript, which is significantly higher than what is observed for ERK2 (64%, $p < 10^{-16}$) or ELK1 (46%, $p < 10^{-16}$) in general (Figure S5G). The same CCGGAAG motif that we detected in the ERK2 data is highly enriched at the TSS of genes that are co-occupied by ERK2 and ELK1 (Figures 5A, 5B, and S5H). Interestingly, ERK2 binding occurs on average 60 bp downstream of ELK1 toward the TSS, indicating a spatial constraint (Figure 5B). In line with our initial observations for ERK2, promoters bound by ERK2 and ELK1 show increased levels of active histone modifications (Figure 5C). Similar to ERK2, ELK1-bound genes are significantly enriched in GO terms associated with metabolic processes (Figure S5I), supporting that ERK2 and ELK1 synergistically target genes involved in metabolic processes in hESCs.

To further investigate whether ELK1 is required for the activation of gene expression downstream of ERK2 signaling, we calculated whether genes that are bound by ELK1 and ERK2 show differential expression after knockdown (Figure 5D). Indeed, knockdown of ELK1 leads to reduced expression of genes which are co-occupied by ERK2 ($p < 0.001$). Importantly, knockdown of ERK2 similarly leads to decreased expression ($p < 0.001$). Together, these results support that ELK1 binds to promoters of genes which are targeted by ERK2, thereby linking this kinase with sequence-specific regulation of gene expression in hESCs.

ERK2 Colocalization with ELK1 Discriminates between Activation and Repression

The above data indicates that ELK1 is a transcriptional activator downstream of ERK2. However, gene-expression data shows that knockdown of ELK1 leads to both reduced and elevated

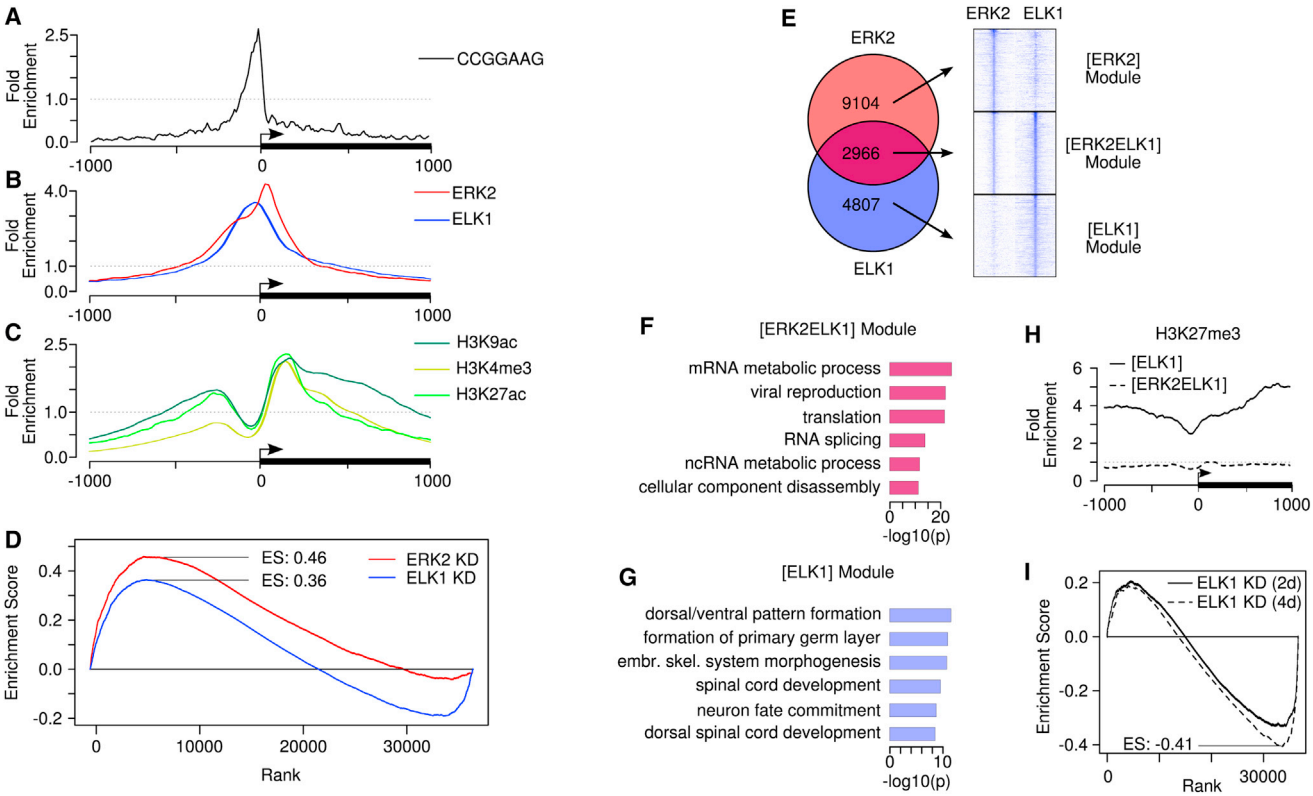

Figure 5. Colocalization of ERK2 and ELK1 Identifies Functionally Distinct Modules

(A) Number of occurrences of the ELK1 consensus motif in promoters bound by ERK2 and ELK1 as a fold enrichment over the number of occurrences observed in all other promoters.

(B) Average binding profile of ERK2 and ELK1 at promoters which are co-occupied. Shown are the mean number of ChIP-seq reads as a fold enrichment over the average binding profile in promoters without ERK2 and ELK1 colocalization.

(C) Average number of ChIP-seq reads for H3K9ac, H3K4me3, and H3K27ac at promoters which are cooccupied by ERK2 and ELK1 as a fold enrichment over the average ChIP-seq profile in promoters without ERK2 and ELK1 colocalization.

(D) GSEA of genes which are co-occupied by ERK2 and ELK1, 2 days after knockdown of ERK2 and ELK1. Genes are ranked according to the signal-to-noise ratio (control/knockdown). A positive enrichment score (ES) indicates enrichment in the set of genes which show reduced expression after the knockdown.

(E) Overlap of genome-wide binding data for ERK2 and ELK1 in hESCs. Based on colocalization, three binding modules can be defined: the [ERK2] module consists of promoters bound by ERK2 without ELK1, the [ERK2ELK1] module consists of promoters bound by ERK2 and ELK1, and the [ELK1] module consists of promoters bound by ELK1 without ERK2.

(F) GO enrichment analysis of genes which are co-occupied by ERK2 and ELK1 ([ERK2ELK1] module).

(G) GO enrichment analysis of genes which are bound by ELK1 without ERK2 ([ELK1] module).

(H) Average ChIP-seq profile for H3K27me3 at promoters bound by ELK1 without ERK2 ([ELK1] module), compared to promoters bound by ELK1 with ERK2 ([ERK2ELK1] module).

(I) GSEA of genes from the [ELK1] module 2 and 4 days after knockdown of ELK1. A negative enrichment score (ES) indicates enrichment in the set of genes which show stronger expression after knockdown of ELK1. See also Figure S5 and Table S2.

gene expression (Figure S5J), indicating that ELK1 might act as a repressor as well. Surprisingly, and in stark contrast to ERK2, ELK1 binding is highly enriched near genes involved in developmental processes (Figure S5I), further suggesting that ELK1 has a partially distinct function from ERK2. In order to understand the discrepancy between ERK2 and ELK1, we investigated their genome-wide colocalization pattern. ERK2 colocalization with ELK1 at promoters defines three distinct sets of genomic loci, henceforth referred to as binding modules (Figure 5E). Module 1 ([ERK2] module) are promoters bound by ERK2 without ELK1; module 2 ([ERK2ELK1] module) are promoters bound by ERK2 and ELK1, and module 3 ([ELK1] module) are promoters bound by ELK1 without ERK2. Strikingly, ERK2 colocalization alone is sufficient to discriminate ELK1 binding sites near developmental genes ([ELK1] module) from ELK1 binding sites near metabolism related genes ([ERK2ELK1] module, Figures 5F and 5G). These differences are also reflected in an antagonistic epigenetic state. Without colocalization of ERK2, ELK1-bound loci show significantly higher levels of the repressive histone modification H3K27me3 (Figures 5H and S5K). Gene-expression data shows that ELK1 indeed acts as a repressor of transcription at loci without ERK2 colocalization (Figure 5I), confirming that ERK2 colocalization discriminates between activation and repression of ELK1 target genes.

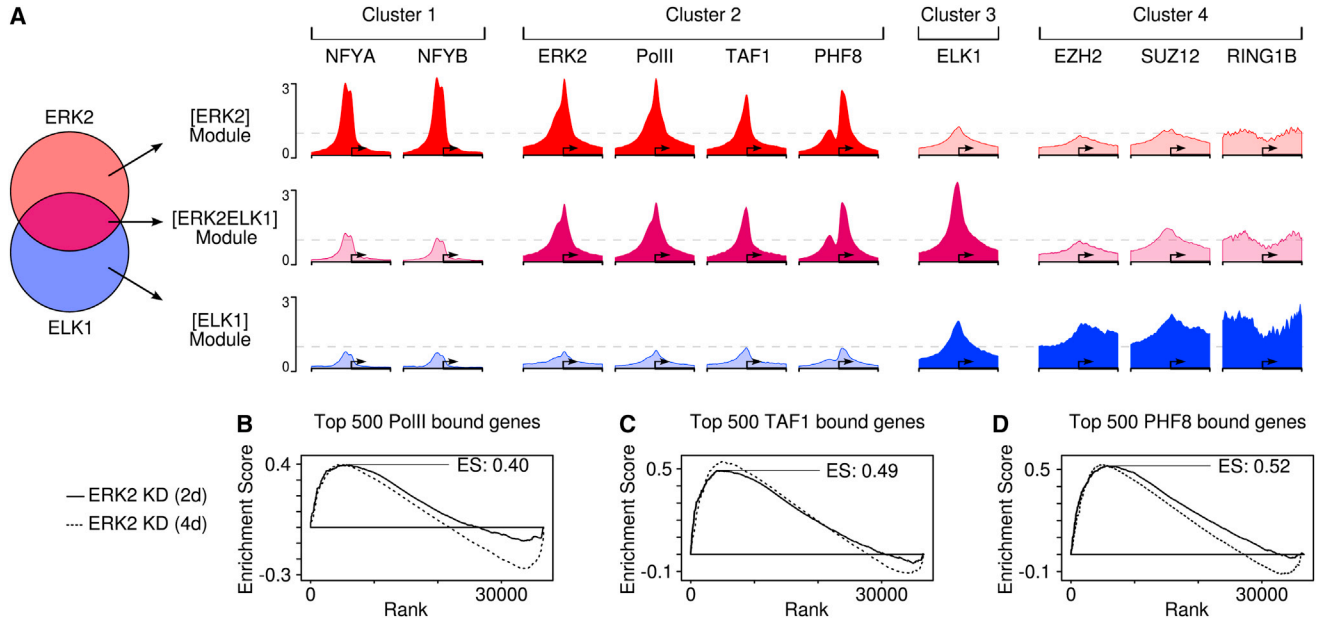

Figure 6. The [ERK2], [ERK2ELK1], and [ELK1] Modules Are Bound by Distinct Combinations of Transcription Factors and Chromatin Regulators

(A) Average binding profiles at promoters bound by ERK2, ERK2, and ELK1, and ELK1 for proteins that colocalize with ERK2 or ELK1. Profiles that have at least a 2-fold enrichment are highlighted in dark. Cluster 1 (NFYA and NFYB) shows high binding intensity in the [ERK2] module; cluster 2 (ERK2, PolII, TAF1, and PHF8) shows high binding intensity when ERK2 binds, independently of ELK1 ([ERK2] or [ERK2ELK1] modules); cluster 3 (ELK1) is the only cluster that connects activating and repressing protein complexes; cluster 4 (EZH2, SUZ12, and RING1B) shows high binding intensity in the [ELK1] module. All data is from hESCs, except NFYA/NFYB (K562).

(B–E) GSEA for the 500 genes with the highest binding intensities of (B) PolII, (C) TAF1, (D) PHF8, 2 and 4 days after knockdown of ERK2. Genes are ranked according to the signal-to-noise ratio (control/knockdown). A positive enrichment score (ES) indicates reduced expression after knockdown of ERK2. See also Figure S6.

The [ERK2], [ERK2ELK1], and [ELK1] Modules Are Bound by Distinct Combinations of Transcription Factors and Chromatin Regulators

The modular structure of the ERK2 and ELK1 binding data raises the questions of how ERK2 is able to co-occupy only a very specific, functionally coherent subset of ELK1 target genes ([ERK2ELK1] module) and how ERK2 establishes chromatin interactions without ELK1 ([ERK2] module). Given that the DNA sequence and the epigenetic state seem to be important for ERK2-chromatin interactions, we investigated whether specific combinations of transcription factors and chromatin regulators can be associated with the individual modules. We screened 36 genome-wide binding data sets for increased binding at promoters in one of the three modules compared to all other promoters in the genome (Bernstein et al., 2010; Chan et al., 2012; Costessi et al., 2011; Dunham et al., 2012; Ku et al., 2008; Kunarso et al., 2010; Ram et al., 2011). Hierarchical clustering reveals that eight proteins that occur in four different combinations (clusters) can be associated specifically with binding of ERK2 and ELK1 in hESCs (Figures 6A and S6A).

Cluster 1 is formed by proteins which are enriched in the [ERK2] module. This cluster consists of NFYA and NFYB, which bind the CAAT box motif that is enriched in the ERK2 binding data (Figure 4A). Similar to NFYA and NFYB, the CAAT box motif occurs significantly more often in the [ERK2] module compared to the other modules or promoters in general (Figures S6B and S6C). Transcription factors that recognize the NFY-CAAT motif could therefore be involved in establishing ERK2-chromatin interactions without ELK1 in hESCs.

Cluster 2 (PolII, TAF1, and PHF8) are proteins which co-occur with ERK2 irrespectively of ELK1 colocalization ([ERK2] and [ERK2ELK1] modules). PolII, TAF1, and PHF8 are part of protein complexes involved in transcriptional activation. PHF8 and TAF1 have further been shown to be involved in cell-cycle progression and could be potential targets of the ERK2 signaling pathway (Liu et al., 2010; Martin et al., 1999). Interestingly, the binding of ERK2 is highly similar to that of PolII (Figures S6D–S6F), suggesting that the PolII position might be coupled to the position of ERK2 at promoters. To estimate whether ERK2 colocalization with PolII, TAF1, or PHF8 has an impact on transcription, we investigated the effect of depletion of ERK2 on genes bound by each of them (Figures 6B–6D). Genes bound by PolII, TAF1, and PHF8 show reduced expression after loss of ERK2 ($p < 0.001$ for each set), suggesting that colocalization with ERK2 indeed may reflect a direct or indirect, functional interaction. Because promoters bound by ERK2 show active histone modifications, colocalization with TAF1, PHF8, or PolII might be important for establishing this epigenetic state and initiating transcription in hESCs.

Cluster 3 consists of ELK1 alone, as this is the only factor associated with both activation and repression. Interestingly, the ELK1 motif occurs much more frequently in the subset of

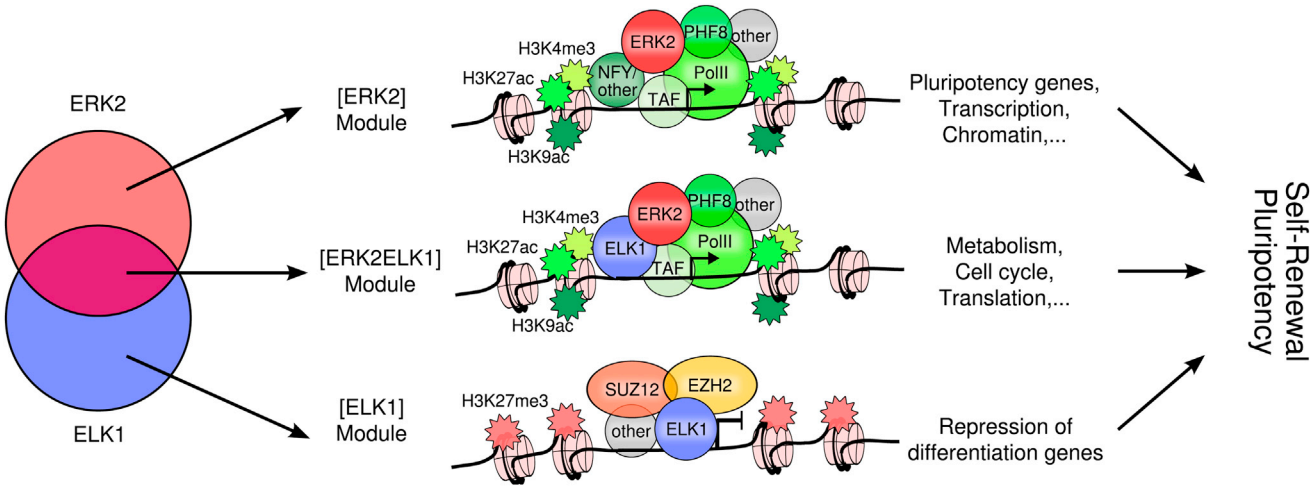

Figure 7. Model of the Transcriptional Regulatory Network of ERK2 Signaling in hESCs

Transcription factors such as ELK1 link ERK2 to sequence-specific regulation of gene expression. ERK2 and ELK1 colocalization defines three distinct modules that target different sets of genes. In this model, combinatorial binding of ERK2 and ELK1 with transcription factors, chromatin regulators, and the basal transcriptional machinery integrates external signaling into the cell-type-specific regulatory network. In hESCs, ERK2 and ELK1 participate in the regulation of pluripotency and self-renewal pathways, whereas differentiation genes are repressed.

ELK1 binding sites which are co-occupied by ERK2 ([ERK2ELK1] module, Figures S6G and S6H). This indicates that sequence-specific binding of ELK1 at the CCGGAA motif is involved in the establishment of ERK2-chromatin interactions and activation of gene expression.

Cluster 4 consists of proteins that are enriched at ELK1 binding sites where ERK2 does not colocalize ([ELK1] module). Strikingly, all proteins in this cluster (EZH2, SUZ12, and RING1B) are part of the Polycomb repressive complexes, PRC1 and PRC2, which catalyze the repressive H3K27me3 histone modification (Cao et al., 2002; Lee et al., 2006). The repressive role of ELK1 might therefore be closely linked to Polycomb-mediated gene silencing (Figures S6I–S6K and S6M). This is indeed confirmed by the gene-expression data. Genes bound by EZH2 or SUZ12 are expressed at a significantly higher level after knockdown of ELK1 ($p < 0.001$, Figure S6L). A comparison of ELK1 binding with three differentiated cell lines (Dunham et al., 2012) shows that a large fraction of these repressed loci are bound by ELK1 solely in hESCs (Figure S6N). Together, these results support a hESC-specific role of ELK1 in Polycomb-mediated gene silencing in the absence of ERK2 at the DNA.

DISCUSSION

The MAPK/ERK signaling cascade is one of the key signaling pathways in embryonic development (Corson et al., 2003). ERK signaling contributes to cell-cycle control, proliferation, and differentiation (Grassian et al., 2011; Yang et al., 2003). Due to its prevalent role in disease, ERK signaling is targeted by a number of drugs (English and Cobb, 2002; Suojun et al., 2012), highlighting the clinical relevance of understanding this pathway. Several kinase substrates are known for ERK2 (Carlson et al., 2011), yet the convergence of ERK2 signaling at regulatory regions in the DNA has not been studied in the context of pluripotency and development. Here, we mapped genome-wide chromatin interaction sites for ERK2 in hESCs, leading to the elucidation of the ERK signaling pathway. We observe that the functions which have been described previously for ERK2 are reflected in the genes which are bound by ERK2, indicating that ERK2 chromatin interaction is central for ERK signaling.

The genome-wide binding profile for ERK2 provides a large number of regulatory regions involved in ERK signaling. By applying de novo motif finding to these regions, we are able to identify sequence-specific features of ERK2 in a completely unbiased way. It has been reported that ERK2 itself can bind to the DNA (Hu et al., 2009), but we did not find this motif in our analysis. However, we found many motifs which are recognized by transcription factors that are known ERK2 substrates, such as ELK1, highlighting the validity of this approach. Importantly, the ERK2 binding data not only identifies ERK2 interaction partners, but also transcription factors required for pluripotency. By combining knockdown experiments with genome-wide mapping of binding sites in hESCs we were able to assign a previously unrecognized function in pluripotency to ELK1.

Surprisingly, the same transcription factor that activates expression of metabolism-related genes (Boros et al., 2009) together with ERK2 acts as a repressor of developmental genes without colocalization of ERK2 (Figure 7). Even though a repressive potential of ELK1 has been described (Witty et al., 2010; Yang and Sharrocks, 2004), it is not evident how the separation into two functionally distinct binding modules is achieved. Because ELK1-mediated activation and repression occurs at two distinct sets of promoters, we speculate that the promoter sequences might be partially responsible. In support of this, promoters bound by both ERK2 and ELK1 frequently contain the ELK1 binding motif, whereas promoters bound by ELK1 without ERK2 do not have this motif (Figure S6H). The repressive mode might therefore require a different binding motif or interaction

with additional transcription factors and chromatin regulators (Odrowaz and Sharrocks, 2012). The increased level of EZH2, SUZ12, and RING1B at ELK1-bound loci indicates that the repressive mode is linked to Polycomb-mediated gene silencing, which is a key process in maintaining pluripotency (Lee et al., 2006). Although additional experiments are required for understanding how ELK1 can have parallel active and repressive functions, it is very likely that this dual role is of importance in hESCs. The integration of ERK2 signaling and Polycomb-mediated gene silencing by a single transcription factor reflects the balance between self-renewal and differentiation frequently described as one of the key properties of pluripotent cells (Bernstein et al., 2006; Boyer et al., 2005; Loh et al., 2006; Wang et al., 2006).

By analyzing the ERK2 and ELK1 binding data we were able to identify the CAAT box binding transcription factors NFYA and NFYB as additional candidate interaction partners of ERK2 at the DNA. The CAAT motif is specifically enriched in promoters bound by ERK2, but not ELK1. Therefore, NFY transcription factors might be involved in establishing binding of ERK2 in the absence of ELK1 ([ERK2] module). NFYA and NFYB have been shown to be important in ESCs (Dolfini et al., 2012; Grskovic et al., 2007), and the CAAT motif has been detected previously in ERK responsive genes (Jürchott et al., 2010). Even though the CAAT motif occurs with lower frequency in ERK2-bound regions than the ELK1 motif, it would be of interest to further investigate this putative interaction and its relevance for stem cell biology and ERK2 signaling in general. We additionally identified PHF8, TAF1, and PolII as potential interaction partners of ERK2. Although colocalization is insufficient to support direct phosphorylation, these results demonstrate that large-scale data of kinase-chromatin interactions generates many new hypotheses for future investigation.

ESCs can differentiate into all cell types of the three germ layers, yet they proliferate without differentiation. This unique pluripotent property distinguishes hESCs from differentiated cells. ERK signaling is required for pluripotency in hESCs; however, it is active in a variety of differentiated cell types as well. In line with this, we find that the ERK signaling pathway consists of a hESC-specific and a cell-type-independent component. We hypothesize that interaction of ERK2 with stem cell-specific transcriptional regulators and the stem cell-specific epigenetic landscape facilitates the integration of this essential signaling pathway into the regulatory network of hESCs (Figure 7). Importantly, even though the hESC-specific component targets hESC-specific genes, the cell-type-unspecific component appears to be similarly required for the maintenance of pluripotency. In line with this, many potential ERK2 target genes found in this study, such as the histone genes *TAFs*, *SOX2*, or *HERVH*, are indispensible for the maintenance of the human stem cell identity (Chia et al., 2010; Medina et al., 2012; Pijnappel et al., 2013). For hESCs, we propose a model wherein ERK2 and ELK1 integrate external signaling with sequence-specific DNA binding and epigenetic control to form a regulatory network that supports proliferation and prevents differentiation, thereby maintaining the pluripotent state (Figure 7).

Signaling pathways have been studied for many years and the ERK2 cascade is central in development and pluripotency. By deciphering the kinase-chromatin interactions in a genome-wide manner we identified many targets of ERK2, such as noncoding genes, histone genes, and many more with a function in RNA processing, translation, cell-cycle progression, or transcriptional control. Additional studies in other organisms, conditions, and cell types will ultimately help to understand the plasticity and dynamics of ERK signaling in development and disease. Here, we provide a first step toward this goal by outlining the role of ERK2 and its interaction partners at the DNA for the maintenance of pluripotency in hESCs.

EXPERIMENTAL PROCEDURES

Cell Culture

The cells (hESC line H1, WiCell) were maintained as feeder-free culture on Matrigel (BD Bioscience). The medium, containing 20% KnockOut serum replacement, 1 mM L-glutamine, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (Invitrogen) in Dulbecco's modified Eagle's medium/F12 (Invitrogen), was conditioned with mitotically inactivated mouse embryonic fibroblast for 24 hr. An additional 8 ng/ml of basic fibroblast growth factor (Invitrogen) was supplemented to conditioned medium before usage. Medium was changed daily. The hESCs were passaged with 1 mg/ml collagenase IV (GIBCO) upon confluency.

ChIP and RNA Expression Analysis

ChIP was performed with ERK2 antibody (D-2; Santa Cruz Biotechnology) and ELK1 antibody (I-20; Santa Cruz). For generating the ChIP-seq library, 5–15 ng of ChIP-enriched DNA was modified with the ChIP-seq DNA Sample Prep Kit (IP-102-1001, Illumina). The DNA end was repaired with exonucleases and an adaptor was ligated to the end, followed by PCR amplification for 15 rounds. The amplified DNA was gel purified, and the 200–300 bp fragments were selected for sequencing (Illumina). Total RNA extraction, reverse transcription, and quantitative real-time PCR were performed as described previously (Chew et al., 2005). For expression profiling, messenger RNAs (mRNAs) derived from ERK2, ELK1, and luciferase shRNA-treated H1 hESCs were reverse transcribed, labeled, and analyzed on the Illumina microarray platform (HumanHT-12 v.3.0). Arrays were processed according to manufacturer's instructions. For all conditions, biological replicate microarray data were generated. Rank invariant normalization was used to normalize the microarrays.

shRNA Knockdown

shRNA constructs for each gene were designed to target 19 bp gene-specific regions. Oligonucleotides were cloned into pSuper-puro (BglII and HindIII sites; Oligoengine). H1 cells were trypsinized (0.25% trypsin EDTA, GIBCO) and seeded in small clumps (~10 cells) 16–24 hr before transfection. Cells were transfected with 1.5 μg of knockdown constructs. For rescue experiments, silent mutations on the shRNA target sequences were introduced into the cDNA of ERK2 and ELK1 in the PCAG plasmid for generation of the RNAi-immune constructs. 200 ng of the RNAi-immune constructs were co-transfected with the knockdown constructs for the rescue experiments. See Supplemental Information for shRNA and primer sequences.

Computational Analysis

Reads were mapped against the hg19 reference genome using Bowtie (0.12.5) (Langmead et al., 2009); peak calling was done with MACS 1.4.0) (Zhang et al., 2008). The GO enrichment analysis was done using DAVID (Huang et al., 2009) and GREAT (McLean et al., 2010). For motif finding, we used RSAT on repeat masked sequences 500 bp around the summit of the peaks (Thomas-Chollier et al., 2012). In order to calculate the fold enrichment of ChIP-seq reads at specific sets of loci, we obtained a set of TSSs for all coding genes that are currently annotated (Ensembl v.69, total number: 19,978). In the case of multiple TSSs, we selected only the TSS with the maximum number of PolII ChIP-seq reads within 4 kb around the TSS, assuming that this represents the primary TSS for the respective genes in hESCs. This set was used to calculate the average ChIP-seq profile ±2 kb around the TSSs in nonoverlapping windows of 20 bp in size ("reference profile"). For the subsets of interest, we

calculated the average profile similarly. The fold enrichment was then defined as the average read count in every window divided by the maximum read count of the reference profile. The enrichment of DNA words was calculated similarly, using the number of word occurrences instead of the number of reads. Significance was estimated using the two-sample Wilcoxon test on the sum of reads within 4 kb. See Supplemental Experimental Procedures for accession numbers of public data and parameters for data processing.

ACCESSION NUMBERS

All data is accessible at the ArrayExpress archive under accession numbers E-MTAB-1565 (ERK2 and ELK1 ChIP-seq) and E-MTAB-1583 (ERK2 and ELK1 knockdown microarrays).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.04.030.

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