High constitutive activity of a broad panel of housekeeping and tissue-specific cis-regulatory elements depends on a subset of ETS proteins

Alessia Curina,1,5 Alberto Termanini,2,5 Iros Barozzi,1,4 Elena Prosperini,1 Marta Simonatto,1 Sara Polletti,1 Alessio Silvola,1 Monica Soldi,1 Liv Austenaa,1 Tiziana Bonaldi,1 Serena Ghisletti,2,6 and Gioacchino Natoli1,3,6

1Department of Experimental Oncology, European Institute of Oncology (IEO), 20139 Milan, Italy; 2Humanitas Clinical and Research Center, 20089 Rozzano-Milan, Italy; 3Humanitas University, 20089 Rozzano-Milan, Italy

Enhancers and promoters that control the transcriptional output of terminally differentiated cells include cell type-specific and broadly active housekeeping elements. Whether the high constitutive activity of these two groups of cis-regulatory elements relies on entirely distinct or instead also on shared regulators is unknown. By dissecting the cis-regulatory repertoire of macrophages, we found that the ELF subfamily of ETS proteins selectively bound within 60 base pairs (bp) from the transcription start sites of highly active housekeeping genes. ELFs also bound constitutively active, but not poised, macrophage-specific enhancers and promoters. The role of ELFs in promoting high-level constitutive transcription was suggested by multiple evidence: ELF sites enabled robust transcriptional activation by endogenous and minimal synthetic promoters, ELF recruitment was stabilized by the transcriptional machinery, and ELF proteins mediated recruitment of transcriptional and chromatin regulators to core promoters. These data suggest that the co-option of a limited number of highly active transcription factors represents a broadly adopted strategy to equip both cell type-specific and housekeeping cis-regulatory elements with the ability to efficiently promote transcription.

[Keywords: ETS; enhancers; housekeeping genes; macrophages; transcription]

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ETS proteins is IBP39, a protein that, in the primitive line with this notion, the first identifiable ancestor of contribution to the control of core promoter activity. In close proximity of ETS motifs to TSSs suggests their direct giogenesis (for review, see Hollenhorst et al. 2011). The of biological processes, including hematopoiesis and an-
lectively associated with the constitutively acetylated expressed genes. Unexpectedly, ELF proteins were also se-
regions immediately adjacent to the TSSs of highly ex-
ence (with an ACA motif preceding the central core),
ference. The PU.1 motif is the most divergent one and
same class as GABPA, showed a distinct binding prefer-
a CC dinucleotide. FLI1, even if previously assigned to the
motif in which the central 5′ subset of ETS proteins is associated with the TSSs of housekeeping genes
A subset of ETS proteins is associated with the TSSs
To identify the TFs that control constitutive gene expression in bone marrow-derived macrophages [BMDM], we determined the TF consensus DNA-binding motifs that are statistically overrepresented in the TSSs of all genes highly expressed in basal conditions [Supplemental Material; Supplemental Table S1]. In addition to some GC-rich motifs that likely depend on the abundance of CpG island-containing promoters in this set of genes, this analysis retrieved (|P| ≤ 3.6 × 10^{-246}) an ETS motif different from the one recognized by the myeloid-specific ETS protein PU.1 (Ghisletti et al. 2010; Heinz et al. 2010). Whereas all ETS proteins recognize a core GGAAT motif via a shared 85-amino-acid ETS domain [Gallant and Gilkeson 2006], divergences in amino acid residues involved in DNA recognition determine different affinities for nucleo-
tides 5′ and 3′ from such cores and define four classes [I–IV] [Wei et al. 2010].
Therefore, we used ChIP-seq to comparatively explore the genomic distribution of ETS proteins that we found to be highly expressed in macrophages and that, based on current annotations [Wei et al. 2010], display distinct DNA-binding specificities; namely, FLI1 and GABPA [class I], ELF1 and ELF4 [class II], and PU.1 [class III; the only class IV family member, SPDEF, is not expressed in mouse macrophages]. Some obvious trends were apparent irrespective of the threshold used. Specifically, ELF1, ELF4, and GABPA showed a significant preference for TSS-proximal regions [TSS ± 2.5 kb], which was increasingly more evident when considering peaks with a progressively higher score (Fig. 1A, fold enrichment bins 1–4). Conversely, PU.1 showed a strong preference for TSS-distal [intergenic and intragenic] regions, while FLI1 displayed an intermediate behavior, with ~50% of the binding events called at intermediate and high stringency being associated with gene promoters [Fig. 1A]. The genomic distribution of the ETS proteins analyzed in genomic windows of ±10 kb surrounding the TSS is shown in Figure 1B.
A de novo motif discovery analysis [Fig. 1C] showed that the TSS-associated ETS proteins [the class I GABPA and the class II ELF1 and ELF4] recognize a virtually identical motif in which the central 5′-GGAA-3′ core is preceded by a CC dinucleotide. FLI1, even if previously assigned to the same class as GABPA, showed a distinct binding preference (with an ACA motif preceding the central core), which likely accounts for its different genomic distribution. The PU.1 motif is the most divergent one and is characterized by an extended purine-rich 5′ end. Overall, some ETS proteins (GAPBA, ELF1, and ELF4) have a
significant binding preference for TSS-proximal regions that correlated with distinct DNA-binding specificities and were only partially recapitulated by previous in vitro studies. Among the promoter-biased ETS proteins, GABPA and ELF1 (which bound a similar number of regions and were thus amenable to direct comparison) showed substantial overlap at promoters (4299 peaks) but also bound distinct sites (3278 GABPA-specific and
1031 ELF1-specific peaks (Supplemental Fig. S1). These data suggest that differences in the composition of the underlying DNA sequence may contribute to the generation of functional specificity between ETS proteins with the ability to recognize an identical motif. Indeed, when directly compared with each other, GABPA-specific peaks showed an overrepresentation of CpG-rich motifs such as E2F and SP1 motifs, while ELF1-specific peaks showed an overrepresentation of the canonical ELF motifs and PU.1-like motifs (Supplemental Table S2). Additional layers of specificity may arise from the differential transcription activation ability of proteins recruited to the same site.

To determine whether differences in genomic distributions reflect distinct functional specificities of the ETS proteins analyzed, we used GREAT (genomic regions enrichment of annotations tool) (McLean et al. 2010). GREAT links sets of genomic regions to putative biological functions based on the functional annotations of the nearby genes with a score that is based on the region–gene distance and therefore the probability of correct assignment. When considering the genomic regions bound by ELF1, ELF4, and GABPA, GREAT retrieved ontology terms related to housekeeping cellular functions (such as DNA repair, RNA metabolism, ribosome biogenesis, and protein folding), while ontology terms associated with PU.1 and FLI1 clustered separately and were related mainly to myeloid cell differentiation and functions (Fig. 1D; Suplementar Table S3). These results are in keeping with the known role of PU.1 and FLI1 in macrophage development and indicate that ELF proteins and GABPA are likely involved in the regulation of housekeeping gene promoters.

Since genes with constitutive expression in macrophages include cell type-specific genes and broadly expressed genes with housekeeping functions, we tested whether the genes bound by ELF1s are expressed across different tissues. To this aim, the expression of ELF4-positive genes was evaluated in almost 100 different tissues using publicly available data sets (Tippmann et al. 2012). In all tissues considered (Fig. 1E, top panel) and even more so in hematopoietic cells (Fig. 1E, bottom panel), ELF-associated genes were expressed at significantly higher levels than the ELF-negative ones, indicating that, irrespective of the cell type, ELF binding correlates with high and constitutive gene expression.

Finally, we used a restrictive window of 250 bp upstream of the TSS to determine the subset of ELF4-bound genes expressed at a basal state in macrophages (Austenaa et al. 2015). Eighty-one percent (2524 out of 3117) of ELF4 peaks were associated with the promoters of expressed genes. Representative snapshots are shown in Figure 1F.

Overall, these results indicate that a subset of ETS proteins is associated with the TSSs of highly expressed genes, including genes that are broadly expressed across tissues. Association of ELF proteins with the macrophage cis-regulatory repertoire

We next characterized the features of the TSS-proximal regions associated with the promoter-biased ETS proteins (ELFs and GABPA). RefSeq genes were divided into two groups based on their association with ELF4 (ELF4 genomic distribution extensively overlapping that of ELF1 is shown in Supplemental Fig. S2; specificity of the ELF1 and ELF4 antibodies is shown in Supplemental Fig. S3) and then analyzed for the occurrence of the TATA-box and INR motifs, the presence of a CpG island, and RNA Pol II binding (Fig. 2A). Consistent with previous data (FitzGerald et al. 2004, 2006), ETS sites occurred mainly near TSSs, mapping within 50–60 nucleotides [nt] from annotated TSSs. The TATA-box motif was depleted (but not completely excluded) from the TSSs of ELF-bound genes, while the INR motif showed the opposite trend, being almost absent at ELF-negative TSSs. The ELF-positive group was enriched for CpG islands and, consistent with the gene expression data shown above, was characterized by much higher constitutive levels of RNA Pol II.

An additional and most obvious difference between the two groups became evident when analyzing the organization of nucleosomes around the TSSs (Barozzi et al. 2014). ELF binding was almost invariably associated with intense nucleosome depletion upstream of the TSS and strong nucleosome phasing downstream from it, with a particularly prominent +1 nucleosome (Fig. 2B). Differences in nucleosome organization correlate with the different prevalence of CpG islands in the two groups, since a very high G + C content disfavors nucleosome assembly (Fenouil et al. 2012, Barozzi et al. 2014). Overall, ELF proteins showed a preference for GC-rich and INR-positive promoters characterized by well-defined nucleosome-depleted areas upstream of the TSS. Finally, when considering newly synthesized nascent transcripts (which reflect the actual transcriptional activity), ELF-associated genes were expressed at higher levels than the ELF-negative ones, whose activity was below the detection threshold in many cases (Fig. 2C). ELF-negative genes were also relatively enriched for tissue-specific genes. Using a mouse tissue-specific list of genes (from Pattern Gene Database version 10), we found that 4597 genes out of 10,610 (43.3%) of the ELF4-negative subsets and 1407 genes out of 6292 (22.4%) of the ELF4-positive subsets were tissue specific \( P < 2.2 \times 10^{-16} \) using a two-sample test for equality of proportions with continuity correction.

To expand the analysis of the relationship between ELF proteins and the activity of cis-regulatory elements, we focused on the genomic regions associated with PU.1, the master regulator of the myeloid lineage, which binds and regulates most macrophage-specific enhancers and a fraction of the macrophage-specific promoters (Ghisletti et al. 2010; Heinz et al. 2010; Mancino et al. 2015). We first used PU.1 binding to map genome-wide the macrophage-specific cis-regulatory repertoire and then divided TSS-distal and TSS-proximal PU.1-bound regions based on their association with ELF1s. At TSS-distal regions, ELF binding was associated with significantly higher levels of H3K27ac, H3K4me1, RNA Pol II, and even PU.1 than those observed at ELF-negative cis-regulatory elements (Fig. 3A, top panels). GREAT analysis of TSS-distal
PU.1-positive regions indicated that both the ELF-positive and the ELF-4-negative groups were associated with ontology terms related to myeloid cell differentiation and function [Supplemental Table S4], suggesting that these putative enhancers are enriched for macrophage-specific cis-regulatory regions.

The correlation between ELF4 binding and marks of high activity was similarly detected at PU.1-bound TSS-proximal regions (Fig. 3A, bottom panels), where H3K4me3, H3K27ac, and RNA Pol II showed higher signals at ELF4-positive than at ELF4-negative promoters. The box plots in Figure 3B provide a quantitative description of the same data and show the strong correlation between ELF binding and indicators of transcriptional activity.

To gain further mechanistic insight into the relationship between ELF binding and underlying sequence features, we scanned the regions in the four groups of regulatory elements shown above for the presence of ELF and PU.1 motifs. ELF and PU.1 sites tended to co-occur in the PU.1-positive/ELF4-positive regions, while the ELF motif was either absent or low-scoring in the ELF4-negative regions [Fig. 3C], indicating that the regions bound by both TFs have a distinct motif composition that correlated with a high activity of the underlying cis-regulatory element.

Release of ELF proteins from promoters correlates with transcriptional shutdown

Stimulation of macrophages with inflammatory agonists such as lipopolysaccharide (LPS) results in the transcriptional activation of hundreds of genes as well as widespread gene repression [Glass and Natoli 2016]. Therefore, we investigated whether a relationship exists between LPS-induced transcriptional changes and ELF binding at gene promoters. LPS stimulation for 4 h resulted in a general reduction in ELF4 occupancy [Fig. 4A,B,
data at TSS; Supplemental Fig. S4, global data), with only a small fraction of regions showing increased binding at either the TSS (Fig. 4B) or elsewhere in the genome (Supplemental Fig. S4). While the induced ELF4 peaks in Figure 4B were significantly enriched for gene ontology (GO) categories related to immune cell activation, the repressed ones were associated with GO categories related to housekeeping functions such as DNA repair and RNA metabolism (Supplemental Table S5). To determine whether dynamic variations in ELF4 occupancy correlated with gene expression changes, we generated data sets of chromatin-associated nascent transcripts from unstimulated and LPS-stimulated (4 h) macrophages. In Figure 4C (left panel), genes whose transcription significantly changed in response to LPS stimulation (false discovery rate [FDR] ≤ 0.01) were ordered, with the most repressed genes on the left side and the most induced ones on the right [Supplemental Table S6]. The smoothed scatter plot in the right panel of Figure 4C shows ELF4 binding at the corresponding TSS. The overall trend of the plot and the polynomial regression fit [Fig. 4C, red line] indicate a significant, albeit imperfect, correlation between changes in transcriptional activity and ELF4 occupancy, with ELF4 release correlating with transcriptional repression and de novo ELF4 binding correlating with gene activation [Supplemental Table S6]. However, there was also a substantial fraction of genes (corresponding to the central area of the plot in Fig. 4C) in which partial ELF release correlated with induction. The promoters of these genes were associated, among others, with motifs recognized by stimulus-inducible TFs such as SRF, IRFs, STATs, and NF-kB, albeit their level of enrichment was lower than that found at the promoters of genes at the right side of the plot (namely, genes with a strong induction and increased ELF recruitment) [Supplemental Table S7]. We also correlated genome-wide changes in histone acetylation (H3K27ac) induced by LPS with changes in ELF4 occupancy. Again, we detected an imperfect correlation between loss of histone acetylation and ELF4 release on the one hand and gain in both acetylation and ELF4 occupancy on the other (Fig. 4D). Figure 4E includes two representative genomic snapshots showing ELF4 release from the TSS of a repressed gene (Cep55) and ELF4 recruitment to the TSS and an upstream transcribed enhancer of an LPS-activated gene (Ccl5). Taken together, these results indicate that recruitment of ELF TFs tightly correlates with the recruitment of the transcriptional machinery in a broad panel of cis-regulatory elements in both basal and stimulated conditions.

Functional activity of ETS sites in minimal promoters

The strong correlation between ELF binding and constitutive transcriptional activity of TSS-proximal and TSS-distal cis-regulatory elements as well as the vicinity of ETS sites to mapped TSSs prompted us to explore a direct role of ETS proteins in transcriptional activation. Attempts to simultaneously deplete all three ELF proteins expressed in macrophages (ELF1, ELF2, and ELF4) were not successful, and we had to resort to alternative strategies. We initially tested a large panel of endogenous core promoters consisting of short sequences of fixed length; namely, 60 bp from mapped TSSs. The promoters tested were selected based on the following criteria: (1) the presence of the canonical class II ETS site within 60 nt of the mapped TSS, (2) the absence of a TATA box [see Fig. 2], and (3) binding by ELF4 as determined by ChIP-seq. In the first set of experiments, all sequences were cloned upstream of a common sequence, including a single SP1 site and an INR sequence (Weis and Reinberg 1997) in a luciferase vector [pGL3-basic] devoid of either promoter or...
enhancer sequences and thus with very low to undetectable basal activity [Fig. 5A, Supplemental Table S8]. Importantly, the SP1–INR combination alone was ineffective at stimulating luciferase expression [Supplemental Fig. S5], while a synthetic promoter in which the SP1, the INR, or both motifs were eliminated retained ETS-dependent (albeit lower) transcriptional activity. Therefore, the ETS site can efficiently promote transcription in the absence of other TF DNA-binding sites or canonical core promoter elements (Supplemental Fig. S5). Upon transfection in a macrophage cell line (Raw264.7), all of the promoters tested stimulated the expression of the reporter gene [Fig. 5A]. In 13 out of 15 cases, the transcriptional activity of the promoter was almost completely dependent on the ETS site, since a mutation destroying 2 nt in its core abrogated luciferase expression (Fig. 5A, white bars). The same core promoters not only were able to activate transcription in two other nonhematopoietic cell types (hepatocytes and fibroblasts) [Fig. 5B] but also showed comparable relative strength in the three different cell types. Altogether, these data indicate that binding of ETS proteins close to the TSSs imparts constitutive activity to minimal promoters. Importantly, core promoter activity strictly required an ELF-type ETS site because its replacement with a PU.1 site (Fig. 5C) in nearly all cases (eight out of 10) abrogated transcriptional activity. Since ELFs also bound to active enhancers via their specific motifs (Fig. 3), we investigated whether ELF site-containing enhancers were similarly capable of activating transcription. Sixty-base-pair-long DNA fragments corresponding to ELF4-positive enhancers were cloned [Supplemental Table S8] and tested as described above: All enhancers tested were able to activate transcription of the reporter gene [Fig. 5D], and, in most cases, this activity was reduced when the ETS site was mutated [Supplemental Fig. S6].

Given the data above, it was critical to determine whether ELF sites in endogenous promoters in their own native genomic context are similarly required to activate gene transcription. Therefore, we resorted to CRISPR/
Cas9-mediated genome editing, taking advantage of the fact that the core ETS site (NGGAA) contains the PAM sequence of SpCas9 (NGG). We selected five genes containing a TSS-proximal ETS site and generated individual clones that were sequenced to identify small and mechanistically informative mutations. For two genes (Cep55 and Fuz), we obtained several clones in which both alleles were properly mutated. In all cases, mutations affecting the ETS site (even if removing a single nucleotide) almost completely abrogated the expression of the adjacent gene (Fig. 5E).

Overall, a subset of ETS sites bound by ELF proteins was necessary and sufficient to impart high and constitutive transcriptional activity to a broad panel of cis-regulatory elements.

Mutual interactions between ELF proteins and the transcriptional machinery

Since the ETS sites recognized by ELFs and GABPA are commonly found within 50 nt (and even more frequently within 20 nt) of mapped TSSs (FitzGerald et al. 2004, 2006), we determined the impact on transcriptional activation of the distance between the ETS site and the INR sequence. First, ELF motif-containing promoters (n = 51) were divided into five groups based on the distance between the ETS site and the INR sequence.
between the ETS site and the TSS (listed in Supplemental Table S8). Sixty-base-pair fragments were then cloned upstream of a common SP1–INR motif as above [Fig. 5A] and tested for luciferase activity. The median transcriptional activity of these fragments correlated with the distance of the ETS site from the TSS, with a shorter distance being generally associated with a higher transcriptional activity [Fig. 6A]. To analyze the effects of the distance between the ETS site and the INR in an identical sequence context, we created a synthetic 60-nt-long core promoter based on a transcriptionally inert backbone from a bacterial plasmid in which we inserted an ELF-type ETS site at variable

Figure 6. The relationship between TSS-proximal ELF4 binding and the transcriptional machinery. (A) Endogenous ELF site-positive core promoters (n = 51) [listed in Supplemental Table S8] as described in Figure 5A were divided into five groups depending on the distance from TSSs and tested for luciferase activity in macrophages. Each dot of the bee swarm plot represents one biological replicate out of three independent experiments. The central red bars indicate the median, and the gray bars indicate the first and third quartile. Data are shown as fold enrichment over promoterless vector (pGL3-basic). (B) Synthetic sequences containing an ELF site (red) or a PU.1 site (blue) at different distances from a common INR sequence (green) were tested for luciferase activity in macrophages. Data are shown as fold enrichment over promoterless vector (pGL3-basic). Error bars represent ±SD of at least three independent biological replicates. (C) Scatter plot indicating genomic regions (±1000 bp from TSSs) bound by ELF4 (left panel), GABPA (middle panel), and PU.1 (right panel), as determined by ChIP-seq in macrophages treated with 10 μg/mL α-amanitin for 5 h relative to untreated cells (UT). Blue dots indicate regions where the ELF4 signal is reduced upon α-amanitin treatment. (D) A representative genomic region showing ELF4 binding loss upon α-amanitin treatment, while GABPA and PU.1 binding were not affected.
distances from the INR. The combination of an ETS site and the INR was sufficient to activate luciferase expression in this minimal promoter context [Fig. 6B]. Consistent with the previous experiment, the synthetic promoter also showed a clear [albeit imperfect] trend in which a shorter distance of the ETS site from the INR was associated with higher activity [Fig. 6B], and the replacement of the ELF/GABPA site with a PU.1 site caused a loss of activity.

The distance constraints for optimal transcriptional activity revealed by these experiments hint at the occurrence of close interactions between ETS site-bound proteins and the transcriptional machinery. We therefore tested whether the association of ETS proteins with their DNA-biding site is influenced by the recruitment of the transcriptional machinery. To this aim, we depleted the large RNA Pol II subunit (Rbp1) with an extended (5-h) treatment with α-amanitin [Supplemental Fig. S7A] and generated ELF4, GABPA, and PU.1 ChIP-seq data sets [Fig. 6C]. While RNA Pol II depletion did not greatly impact PU.1 and GABPA association with TSS-proximal regions, it nearly completely [7745 out of 7902 peaks; 98%] abrogated ELF4 binding [Fig. 6C] without affecting its abundance [Supplemental Fig. S7A]. The effects of α-amanitin on ELF4 binding were not associated with differences in promoter accessibility, as indicated by an ATAC-seq [assay for transposable-accessible chromatin [ATAC] with high-throughput sequencing] analysis [Supplemental Fig. S7B,C].

These data show that binding of ELF4 [but not PU.1 or GABPA, which contains two domains absent in ELFs] to active TSS-proximal regions is stabilized by RNA Pol II, which is likely indirect evidence of a close physical interaction between components of the transcriptional machinery and DNA-bound ELF proteins. A representative snapshot is shown in Figure 6D.

**ETS-dependent recruitment of transcriptional and chromatin regulators to core promoters**

To obtain insight into the mechanism of action of ELF proteins in transcriptional activation, we used a DNA affinity purification approach coupled to mass spectrometry analysis. Briefly, we terminally labeled with biotin a 200-bp DNA fragment [−150/+50 relative to the annotated TSS] corresponding to the *Scamp2* gene promoter, which contains a canonical ELF site and is efficiently bound in vivo [Fig. 7A]. As a control, we generated a probe with a point mutation in the ELF site. Triplicate experiments were set up in which wild-type and mutant probes [Fig. 7B] were separately incubated with a nuclear lysate in the presence of an excess of nonspecific competitor. Biotinylated DNA fragments were retrieved with streptavidin paramagnetic beads and extensively washed before analyzing bound proteins by label-free mass spectrometry. We identified 1353 proteins, the majority of which [1149 out of 1353; 84.9%] was pulled down equally with the wild-type and ETS site mutant probes. The 204 proteins specifically enriched in the pull-down with the wild-type probe were considered putative ELF interactors [Supplemental Material; Supplemental Fig. S8; Supplemental Table S9]. In addition to the retrieval of ETS proteins [ELFs and FLI1] within this group, we identified a number of proteins involved in different aspects of transcriptional regulation [Fig. 7C; Supplemental Fig. S8], including chromatin remodelers [BAF57 and CHD1] and proteins affecting RNA Pol II processivity [NELFB, the Integrator complex subunit INTS10, PRMT5, and CSTF1/2]. NELFB and Integrator complex subunits interact to control RNA Pol II release from promoter-proximal pausing [Gardini et al. 2014; Stadalmeyer et al. 2014] and enable productive transcriptional elongation. CHD1 was shown previously to bind the TSSs of highly active genes and remove the nucleosomal barrier downstream from the TSSs, thus enabling RNA Pol II promoter escape [Skene et al. 2014] and maintaining high-level transcription [Guzman-Ayala et al. 2015]. We first validated some of the affinity-isolated proteins by Western blot in independent experiments. ELF4 was used as a specificity control and in fact was selectively pulled down with the wild-type probe [Fig. 7D]. Both NELFB and INTS10 were more enriched in affinity purifications with the wild-type probe compared with those with the mutant probe [Fig. 7D], with ratios that, overall, were consistent with those observed in the mass spectrometry analysis. In particular, recruitment of INTS10 was very selectively dependent on an intact ELF site.

The ELF-dependent association of proteins controlling promoter escape of RNA Pol II [CHD1] and transcriptional elongation [NELFB and Integrator] prompted us to further characterize the functional consequences of ETS site editing at the *Cep55* gene [Fig. 5E]. Since we found that nascent RNAs were down-regulated all over the gene body [Fig. 7E], we performed an RNA Pol II ChIP-seq to discriminate between elongation and initiation defects. This analysis revealed a similar occupancy of the TSS-proximal region by the paused RNA Pol II regardless of ETS site mutation but a 2.9-fold reduction in the number of intragenic reads [Fig. 7F], indicating a defective entry of RNA Pol II inside the coding region. Remarkably, histone acetylation downstream from the TSS was almost completely abrogated in ETS site mutant cells [Fig. 7G].

**Discussion**

The functional specificity of the thousands of cis-regulatory elements that control the transcriptional output of higher eukaryotic cells is determined by the characteristic combination and arrangement of TF motifs that eventually establish the unique profile of activity of each of them [Farley et al. 2015]. The focus of this study was the repertoire of constitutively active enhancers and promoters, with the objective of identifying the possible existence of shared molecular determinants of their high activity.

The data that we obtained suggest that, irrespective of their tissue-restricted or broad activity across cell types, a large panel of constitutively active promoters and enhancers may rely on a more limited number of TFs (such as the ELF proteins) than could be expected, in principle, based on their functional divergence.
ETS proteins may have originally appeared in primitive eukaryotes to bridge core promoters and the transcriptional machinery (Schumacher et al. 2003). The data reported in this study suggest that while some ETS proteins (such as PU.1 and FLI1) acquired in mammal tissue-specific functions related mainly to enhancer specification (Ghisletti et al. 2010; Heinz et al. 2010; Lichtinger et al. 2012), some others (such as ELFs and GABPA) may have retained ancestral functions related to core promoter regulation and specifically the maintenance of a subset of transcriptionally competent enhancers and promoters. Such functional differences among ETS family members are reflected by the fine specificity of the DNA motifs recognized by each of them, which results in completely different genomic distribution profiles, with enhancer-biased and promoter-biased ETS proteins at the opposite ends of the spectrum.

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The tight link between ELF proteins and efficient transcription is underscored by the fact that the relatively small fraction of bound enhancers was also the one with by far the strongest activity (as inferred by both histone acetylation and RNA Pol II levels). Both the close proximity of ELF-specific ETS sites to the TSSs and the negative impact of RNA Pol II depletion on ELF occupancy are consistent with the occurrence of a tight interaction between DNA-bound ELFs and the transcriptional machinery. However, the precise steps at which promoter-associated ETS proteins act to control transcription will require additional investigation. Our proteomic and genomic data hint at a role of ELFs in the control of transcriptional elongation rather than initiation. Specifically, RNA Pol II density in the body of a gene with a mutated TSS-proximal ETS site was strongly reduced, while loading at the TSS was only marginally affected. The identification of NELF and an Integrator complex subunit as ELF interactors is consistent with a direct impact of TSS-proximal ELFs on promoter escape by RNA Pol II. Moreover, recruitment of the chromatin remodeler CHD1, which moves promoter-proximal nucleosomes (Skene et al. 2014), may critically contribute to transcriptional elongation at genes (such as those bound by ELFs) (Fig. 2) with a prominent +1 nucleosome. However, additional mechanisms linking ELFs to transcriptional control and specifically to RNA Pol II recruitment and initiation cannot be ruled out, particularly considering that unstable and weak interactions may have been overlooked by the affinity purification strategy that we used. In fact, the sufficiency at activating transcription of a minimal synthetic promoter containing only an ELF site and no strong core promoter elements points to direct interactions with the transcriptional machinery that may be sufficient by themselves to recruit RNA Pol II and initiate transcription.

The general principle that can be drawn from these data is that the co-optation of a limited number of TFs (such as the ELFs) capable of strongly facilitating transcription may represent a transversal strategy broadly adopted across cell types to equip cis-regulatory elements with disparate functional roles and specificity with the ability to efficiently promote transcription. Conversely, the absence of the same motifs in a cis-regulatory region may represent a prerequisite for its tighter regulation in response to specific microenvironmental or developmental cues. Overall, when considering cell type–specific cis-regulatory elements, the emerging paradigm is that they are generated by a combination of motifs recognized by tissue-restricted TFs (commonly endowed with the ability to displace nucleosomes and generate accessible chromatin) (Zaret and Carroll 2011; Glass and Natoli 2016) and motifs for TFs that impart specific functional properties such as inducible (e.g., NF-kB and AP-1) or constitutive (such as ELFs) activity to that specific element. Finally, the data shown in our study contribute to provide a mechanistic framework for previous observations linking ubiquitously or broadly expressed TFs to critical tissue-restricted functions (Gilmour et al. 2014).

Materials and methods

Cell culture and reagents

Macrophages were derived from the BM of C57/BL6 mice (Harlan) as described (Austenaa et al. 2015). The project was approved by the Italian Ministry of Health and performed under the supervision of the Institutional Committee for Animal Welfare. RAW264.7 (mouse macrophages), Hepa1-6 (mouse hepatoma cells), and L-Wnt-3A (mouse fibroblasts) were purchased from American Type Culture Collection (ATCC). α-Amanitin (Sigma) was used at 10 μg/mL. For Western blots, the following antibodies were used: Rpb1 (Santa Cruz Biotechnology, sc-899), vinculin (Sigma), ELF4 (homemade rabbit polyclonal antibody raised against residues 555–655 of the mouse protein), ELF1 (Santa Cruz Biotechnology, sc-631x), INTS10 (Origene, TA337360), and NELFB (Proteintech, 16418-1-AP).

ChIP-seq

Fixed macrophages [8 × 10^7 for FLI1 and ELF1, 4 × 10^7 for GABPA and ELF4, or 5 × 10^5 for PU.1] and RAW264.7 cells [8 × 10^5 for RNA Pol II and 2 × 10^7 for H3K27ac] were lysed in RIPA buffer and, after chromatin shearing by sonication, incubated overnight at 4°C with protein G Dynabeads (Invitrogen) that were previously coupled with 3–10 μg of antibody (Austenaa et al. 2015). The following antibodies were used: ELF1 (Santa Cruz Biotechnology, sc-631x), FLI1 (Santa Cruz Biotechnology, sc-356x), GABPA (Santa Cruz Biotechnology, sc-22810), RNA Pol II (Santa Cruz Biotechnology, sc-899x), and H3K27ac (Abcam, ab4729). The rabbit polyclonal antibodies for PU.1 (Mancino et al. 2015) and ELF4 were generated in-house. ChIP-seq libraries were generated as described (Austenaa et al. 2015) and sequenced on an Illumina HiSeq 2000 (Supplemental Material).

Cloning, transfections, and luciferase assays

The genomic regions of interest [Supplemental Table S8] were placed upstream of a characterized SP1–INR sequence [S′-CCCGGCTATCCTTG-3′; the −1/+1 Py/Pu dinucleotide is underlined] (Weis and Reinberg 1997) and cloned by KpnI and NheI digestion in the pGL3-basic luciferase reporter vector [Promega]. The loss-of-function mutants of the ETS site (GGAA > CCAA), ELF-to-PU.1 mutant site (CCCGGAGGT > AGAGGAAAGT), and synthetic core promoter sequences [cloned by NheI and NcoI] are listed in Supplemental Table S8. Transient transfections were performed using Lipofectamine 2000 [Invitrogen] according to the manufacturer’s protocol. RAW264.7 cells were transiently transfected in a 24-well format [150 × 10^3 cells per well] with 150 ng of empty vector or vectors containing the specified genomic region together with 50 ng of pRL-TK-renilla vector [Promega]. Hepa1-6 and L-Wnt-3A cells were transiently transfected in a 48-well format (30 × 10^3 cells per well) with 50 ng of promoter vector and 25 ng of the pRL-TK-renilla vector. The luciferase assay [Dual-Glo Lumaciferase assay system, Promega] was performed 24 h after transfection. Values were normalized on the Renilla signal and expressed as fold change relative to the empty vector.

ATAC-seq

The original ATAC-seq protocol [Buenrostro et al. 2013] was modified according to Lara-Astiaso et al. (2014). A detailed description of the ATAC-seq experiment is in the Supplemental Material.
CRISPR/Cas9 genome editing

Single-guide sequences specific to the ETS site of Cep55 and Fuz core promoters [listed in Supplemental Table S8] were designed using the CRISPR design tool [http://tools.genome-engineering.org] and cloned into lentivirus vectors [Sanjana et al. 2014]. After RAW264.7 infection, single cells were seeded in 96-well plates by dilution and expanded. Clones were first screened evaluating gene expression by qPCR (expression primers listed in Supplemental Table S8). Positive clones were subjected to Sanger sequencing.

DNA affinity purification and mass spectrometry-based proteomics analysis

Biotin-conjugated DNA baits [240 bp] corresponding to the SCAMP2 gene promoter (mm9 coordinates chromosome 9: 57408589–57408808) and its ETS site mutant were generated by PCR using the primers listed in Supplemental Table S8. A detailed description of the pull-down experiment and the subsequent mass spectrometry analysis and label-free protein quantitation to identify specific interactors is in the Supplemental Material.

Computational methods

Short reads obtained from Illumina HiSeq 2000 runs were analyzed as described [Austenaa et al. 2015]. Detailed computational methods are described in the Supplemental Material.

Accession numbers

Raw data sets are available in the Gene Expression Omnibus (GEO) database [http://www.ncbi.nlm.nih.gov/geo] under accession number GSE88702, which comprises ChIP-seq data [GSE88699, ATAC-seq data [GSE88698], and expression data [GSE88700].

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