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Latent Enhancers Activated by Stimulation in Differentiated Cells

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SUMMARY

According to current models, once the cell has reached terminal differentiation, the enhancer repertoire is completely established and maintained by cooperatively acting lineage-specific transcription factors (TFs). TFs activated by extracellular stimuli operate within this predetermined repertoire, landing close to where master regulators are constitutively bound. Here, we describe latent enhancers, defined as regions of the genome that in terminally differentiated cells are unbound by TFs and lack the histone marks characteristic of enhancers but acquire these features in response to stimulation. Macrophage stimulation caused sequential binding of stimulus-activated and lineage-determining TFs to these regions, enabling deposition of enhancer marks. Once unveiled, many of these enhancers did not return to a latent state when stimulation ceased; instead, they persisted and mediated a faster and stronger response upon restimulation. We suggest that stimulus-specific expansion of the *cis*-regulatory repertoire provides an epigenomic memory of the exposure to environmental agents.

INTRODUCTION

Mammals contain about 200 specialized cell types whose distinct transcriptional outputs reflect the selection during development of unique combinations of coding and regulatory elements. Indeed, the repertoire of *cis*-regulatory elements active in each distinct lineage, as identified by chromatin marks or occupancy by transcriptional regulators (Barski et al., 2007; Heintzman et al., 2007; Wang et al., 2008), is only a small fraction of all possible genomic regulatory elements, with minimal overlap between different cell types (Ernst et al., 2011; Heintzman et al., 2009; Visel et al., 2009).

Cell-type-specific usage of the *cis*-regulatory information reflects the combinatorial activity of sequence-specific transcription factors (TFs) involved in lineage determination and maintenance of cell identity (Natoli, 2010). Enhancers active in a given tissue are typically co-occupied by multiple TFs enforcing cell-type-specific gene expression programs (Chen et al., 2008; He et al., 2011; Junion et al., 2012; Siersbæk et al., 2011; Song et al., 2011; Tijssen et al., 2011). Shaping this genomic *cis*-regulatory landscape entails multiple TF activities, including recruitment of chromatin modifiers, nucleosome remodelers, and histone chaperons (Hu et al., 2011; Ram et al., 2011), eventually resulting in the demarcation of short DNA stretches that contain accessible TF binding sites (TFBS) bracketed by positioned nucleosomes (He et al., 2010; Schones et al., 2008). Enhancers are marked by monomethylated K4 on the histone H3 N-terminal tail (H3K4me1) (Heintzman et al., 2007, 2009) and have been classified into poised and active enhancers based on the absence or presence of histone acetylation, respectively (Creyghton et al., 2010; Rada-Iglesias et al., 2011).

Each cell-type-specific enhancer repertoire provides a unique *cis*-regulatory context in which TFs activated by environmental stimuli operate to modify gene expression. For instance, STAT1 was recruited to preexisting H3K4me1-marked enhancers in HeLa cells within minutes after interferon gamma (IFNγ) stimulation (Heintzman et al., 2009). In mammary epithelial cells estrogen receptor alpha (ERα) was recruited to accessible chromatin sites established by the forkhead TF FoxA1 (Hurtado et al., 2011). FoxA1 was also found to organize the enhancer repertoire of prostate cancer cells, thus directing androgen receptor (AR) recruitment to predefined genomic sites (Wang et al., 2011). Transforming growth factor beta (TGFβ) stimulation of different cell types resulted in the recruitment of Smad2/3 to distinct sets of regulatory elements determined by cell-type-specific TFs (Mullen et al., 2011). Finally, recruitment of stimulus-induced TFs such as NF-κB and Lxr in activated macrophages occurs at cell-type-specific enhancers controlled by the master regulator Pu.1 (Ghisletti et al., 2010; Heinz et al., 2010; Natoli et al., 2011).

So far, the prevailing concepts are that the enhancer repertoire represents a predetermined landscape generated and enforced by the same TFs that control cell identity, and that all transcriptional regulatory events in the differentiated cell occur within this landscape. This model implies that external stimuli that activate a transient response cannot change the repertoire of genomic regulatory elements but solely act on the one that is already available. Clearly, this represents a homeostatic mechanism that guarantees robustness in maintenance of cell identity in spite of a changing environment.

Nevertheless, the behavior of some cell types can be persistently affected by transient stimuli to the point that the resulting

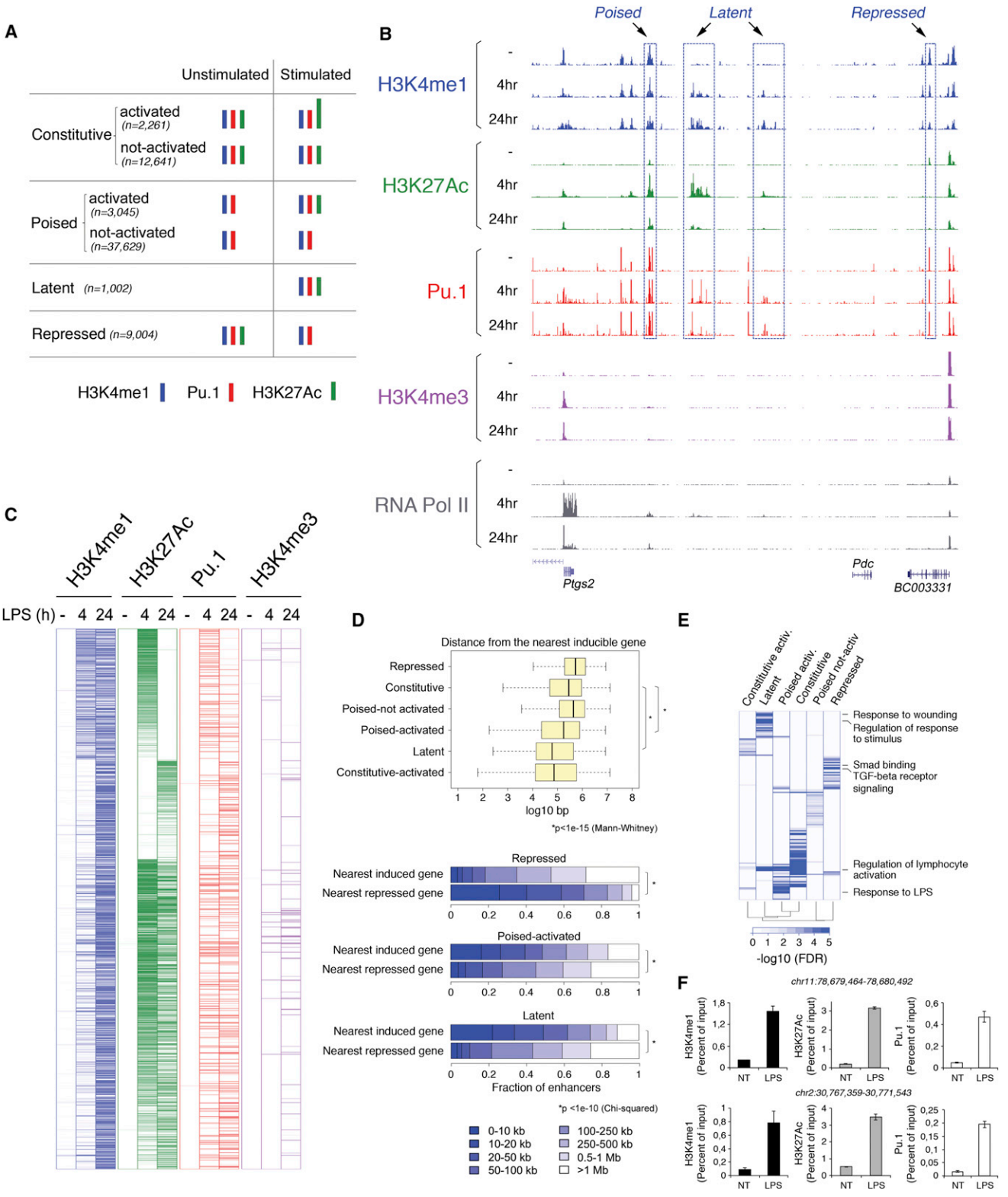

Figure 1. Latent Enhancers Unveiled by LPS Stimulation of Macrophages

(A) Classification of macrophage enhancers based on their response to LPS stimulation. Numbers refer to the 24 hr time point.

(B) A representative genomic region showing latent enhancers induced by LPS stimulation.

(C) Heatmap showing the dynamic behavior of histone marks and Pu.1 at latent enhancers induced by a 4 hr or 24 hr LPS treatment.

(legend continued on next page)

cell, while retaining its identity, acquires new functional properties and a corresponding transcriptional output, which together can define a new cellular subtype. This phenomenon, often indicated as plasticity, is well known in cells of the myeloid lineage such as macrophages, neutrophils, and dendritic cells, whose exposure to different stimuli results in subtypes with specific properties (Biswas and Mantovani, 2010; Fridlender et al., 2009; Lawrence and Natoli, 2011).

In this study, we addressed the hypothesis that stimulation-induced plastic changes in cell behavior may be associated with a partial reprogramming of the available *cis*-regulatory information. To this aim we carried out a systematic analysis of the impact of stimuli affecting macrophage properties and transcriptional outputs (Mosser and Edwards, 2008) on their enhancer repertoire. We classified macrophage enhancers in several categories based on their response to distinct stimuli, thus determining general principles regulating the dynamic usage of the genomic regulatory information. As expected, a large number of pre-existing, poised enhancers were specifically activated in response to distinct stimuli, and in a reciprocal fashion many active elements were repressed upon stimulation. Each stimulus also allowed the surfacing of a distinct subset of latent enhancers, namely genomic regulatory elements that were unbound by TFs and unmarked in unstimulated cells. These enhancers recruited the macrophage master regulator Pu.1 only in response to stimulation and conditionally to the recruitment of stimulus-activated TFs such as STAT1 and STAT6, thus indicating a mechanisms of enhancer induction and activity that is radically different from that of classical enhancers established during differentiation by cooperative binding of lineage-determining TFs. Washout of the stimulus resulted in the rapid loss of acetylation and Pu.1 occupancy, whereas residual H3K4me1 was sustained and associated with a faster and stronger induction upon restimulation, thus providing an epigenomic memory of the initial stimulation.

RESULTS

Latent Enhancers Are a Distinct Class of *cis*-Regulatory Elements

TFs activated by macrophage stimulation with a canonical inflammatory agent (lipopolysaccharide [LPS]), such as NF-κB and IRFs, land at regulatory elements predefined by Pu.1 and constitutively marked by H3K4me1 (Barish et al., 2010; Escoubet-Lozach et al., 2011; Ghisletti et al., 2010; Heinz et al., 2010). Although this is the most common occurrence, these data do not rule out the possibility that stimulation may also modify the pre-existing regulatory landscape. We therefore generated a panel of ChIP-seq data sets exploring the regulatory repertoire of mouse bone marrow-derived macrophages stimulated with LPS for 4 and 24 hr.

Data obtained with each antibody at different time points showed higher correlation (e.g., R^2 = 0.936 considering H3K4me1 in untreated and 4 hr LPS-treated cells) compared to data obtained with a different antibody (e.g., $R^2 \sim 0.1$ when comparing H3K4me1 with H3K4me3 in untreated cells), indicating high reproducibility. Overall, response to stimulation was highly dynamic, allowing the classification of ~65,000 regulatory elements in multiple classes (Figure 1A; Table S1 available online). Constitutive enhancers were associated with both H3K4me1 and H3K27Ac in unstimulated cells. Of the 14,902 constitutive enhancers we detected, 2,261 showed increased acetylation after a 24 hr LPS treatment (constitutive-activated enhancers; Table S1). Poised enhancers showed basal H3K4me1 without H3K27Ac. Most of them (37,629) were unaffected by LPS stimulation at either time point, whereas a comparatively small subset (poised-activated enhancers) acquired H3K27Ac (3,904 at 4 hr and 3,045 at 24 hr). Poised enhancers unaffected by stimulation likely represent a heterogeneous set that may also include constitutively repressed elements (Bonn et al., 2012). Repressed enhancers (11,150 at 4 hr and 9,004 at 24 hr) displayed constitutive H3K27Ac that was either lost or greatly reduced upon stimulation (in most of these cases H3K4me1 was unaffected).

The most unexpected behavior was observed at genomic regions that were unmarked in untreated macrophages and acquired enhancer features upon LPS stimulation (Figures 1A–1C; Table S1). We named these putative regulatory elements, whose dynamic behavior differed from all previously identified enhancer classes, latent enhancers to highlight the notion that they are inactive, unbound, and unmarked in the basal state, being selectively unveiled by stimulation.

Latent enhancers were identified on the basis of two restrictive criteria: (1) the lack of H3K4me1, H3K27Ac, and Pu.1 in unstimulated cells even when using relaxed statistical thresholds (see Extended Experimental Procedures); and (2) the presence of an LPS-induced H3K4me1 peak using a restrictive threshold ($p < 10^{-10}$ using MACS). Although the induction of H3K27Ac was not included among the criteria (because acetylation is not invariably detected at enhancers), in the overwhelming majority of cases acquisition of H3K4me1 was accompanied by the appearance of H3K27Ac (Figures 1B and 1C). Frequently, histone acetylation was only transiently increased at 4 hr and then returned to low-to-undetectable levels at 24 hr (see Figure 1B and the upper area of Figure 1C). At the same regions where this behavior was detected, in most cases H3K4me1 remained steadily elevated at 24 hr, indicating an uncoupling of these two marks at later time points. Transient H3K4me1 was detected at a comparatively small number of regions.

Using these criteria, 514 latent enhancers were identified at 4 hr and 1,002 at 24 hr post-LPS stimulation (Table S1). Although LPS-induced latent enhancers represented a relatively small fraction of the total enhancer repertoire, they made up 15.8% of the total pool of enhancers activated by LPS in this system (1,002/6,308).

(D) Proximity of latent enhancers to LPS-inducible genes.
(E) GO categories of genes assigned to LPS-activated latent enhancers (as inferred from a GREAT analysis) (McLean et al., 2010).
(F) In vivo induction of two latent enhancers in LPS-induced peritoneal macrophages. Data are expressed as mean ± SEM.
See also Figure S1 and Tables S1, S2, and S3.

We also considered additional features known to be associated with enhancers, including increased accessibility of genomic DNA (Gross and Garrard, 1988), binding of histone acetyltransferases (HATs) (Heintzman et al., 2007), RNA Pol II, and noncoding transcription (De Santa et al., 2010; Kim et al., 2010). None of these marks could be used to identify latent enhancers in untreated macrophages, as virtually no overlap could be observed between latent enhancers and regions of basal genomic DNA accessibility (measured by formaldehyde-assisted isolation of regulatory elements [FAIRE]; 0.79%) (Giresi et al., 2007), basal p300 (0.37%) (Ghisletti et al., 2010), RNA Pol II (0.6%), and noncoding chromatin-associated transcripts (Bhatt et al., 2012) (Figures S1A and S1B). Although we cannot rule out the existence of additional, as yet unknown features that may be associated with latent enhancers prior to stimulation, these data support the definition of latent enhancers as a class of regulatory elements that cannot be identified in unstimulated cells using currently available markers and appear in terminally differentiated cells conditionally to stimulation.

To evaluate the contribution of latent enhancers to the LPS-induced gene expression program, we generated ChIP-seq data sets for RNA Pol II in naive and LPS-stimulated macrophages and analyzed the genomic distribution of latent enhancers relative to the transcription start site (TSS) of LPS-regulated genes. We assigned enhancers from different classes to the nearest gene induced in response to stimulation (Table S2). Proximity to LPS-responsive genes was different among enhancer classes, latent enhancers being generally associated with LPS-inducible genes ($p < 1 \times 10^{-15}$ in a chi-square test, Figure 1D). Latent enhancers were located closer to the assigned genes than both poised-activated and constitutive enhancers ($p = 1.5 \times 10^{-8}$ and $p = 2.8 \times 10^{-25}$) (Figure S1C). We then scored latent enhancers for the associated functional categories of the nearby genes using an independent approach (McLean et al., 2010). Strong functional enrichments were observed (Figure 1E), suggesting that latent enhancers contribute to the activation of specific components of the LPS response.

Genes are activated by LPS with very different kinetics. Therefore, we analyzed the association of latent enhancers with genes activated at different times after LPS stimulation. Considering the seven recently reported kinetic classes of LPS-induced genes (Bhatt et al., 2012), we found that the classes of genes with faster activation kinetics were underrepresented among the inducible genes associated with latent enhancers ($p = 8.9 \times 10^{-6}$ in a chi-squared test). Therefore, latent enhancers may be mainly dedicated to the activation of slowly induced genes. Conversely, no obvious bias was observed for genes subjected or not to LPS tolerance (i.e., genes resistant to a second LPS stimulation) (Foster et al., 2007).

Additional features that support the functionality of latent enhancers include: (1) their sequence conservation (Figure S1D), (2) the enrichment for specific TFBS (Table S3), and (3) their ability to promote expression of a reporter gene driven by a minimal promoter (14/24, 58.8% of tested enhancers) (Figure S1E). Finally, latent enhancer unveiling could also be observed in vivo in LPS-elicited peritoneal macrophages (Figure 1F), thus further supporting their potential biological relevance.

A Complex Repertoire of Latent Enhancers Unveiled by Distinct Stimuli

These data suggest that the emergence of latent enhancers may represent a general feature of the response of differentiated cells to external stimuli. Macrophages are equipped with a plethora of receptors allowing them to sense, and react to, a broad panel of agonists (Medzhitov and Horng, 2009). Thus, we treated macrophages with different stimuli with varying degrees of functional overlap, and analyzed alterations of the enhancer repertoire and transcriptional outputs. Stimuli included (1) two Toll-like receptor (TLR) agonists specific for TLR2 (macrophage-activating lipopeptide-2 [MALP-2]) and TLR9 (unmethylated CpG-containing oligonucleotide) (Kawai and Akira, 2011); these stimuli differ from LPS (acting on TLR4) mainly because of their inability to induce IRF3-dependent transcription of the interferon beta (IFNβ) gene, which generates a secondary wave of inducible transcription in this system (Doyle et al., 2002); (2) interleukin 4 (IL-4) and IFNγ, two cytokines that induce macrophage polarization toward an anti-inflammatory (M2) or pro-inflammatory (M1) phenotype, respectively (Mosser and Edwards, 2008); (3) tumor necrosis factor-alpha (TNFα) and interleukin 1-beta (IL-1β), two inflammatory cytokines; and (4) the immunoregulatory cytokine TGFβ.

Each stimulus induced histone acetylation at a discrete subset of poised enhancers ranging from 2,142 (TGFβ) to 3,371 (MALP-2) elements (Figure 2A; Table S4). An even higher number of constitutively acetylated enhancers that ranged between 4,174 (IL-1β) and 8,046 (IFNγ), displayed loss or reduction of acetylation upon stimulation. All stimuli were also able to unveil distinct and comparatively small sets of latent enhancers (Figures 2A and 2B; Table S4), indicating that the unveiling of selected regulatory elements is a hallmark of the cellular reaction to changing environments. As seen with LPS, induction of H3K4me1 usually occurred concurrently with histone acetylation and often with recruitment of Pu.1 (Table S4).

The data sets described above allowed us to identify 2,140 latent enhancers that emerged in response to at least one stimulus. Because we used a limited panel of agonists, representative of just a fraction of the environmental complexity macrophages can be exposed to, and given that for all stimuli except LPS just a single time point was analyzed, it appears evident that the hidden repertoire of regulatory elements that may emerge in response to stimulation is much larger.

We next interrogated our ChIP-seq data sets to determine to what extent stimuli induced shared rather than unique sets of latent enhancers. The overlap between different stimuli closely reflected their functional similarity. TLR agonists activated largely overlapping groups of latent enhancers, with each stimulus also retaining the capacity to unveil distinct and specific regulatory regions (Figure 2B). A significant fraction of the latent enhancers common to TLR agonists was also activated by proinflammatory cytokines (TNFα and IL-1β). The immunoregulatory cytokine TGFβ, apart from inducing a unique repertoire of latent enhancers, also activated enhancers responsive to inflammatory agents, suggesting a possible integration of multiple signals at these regions. IFNγ and IL-4 were able to induce two large sets of unique and nonoverlapping latent enhancers (Figures 2B and S2), consistent with their opposing functions as

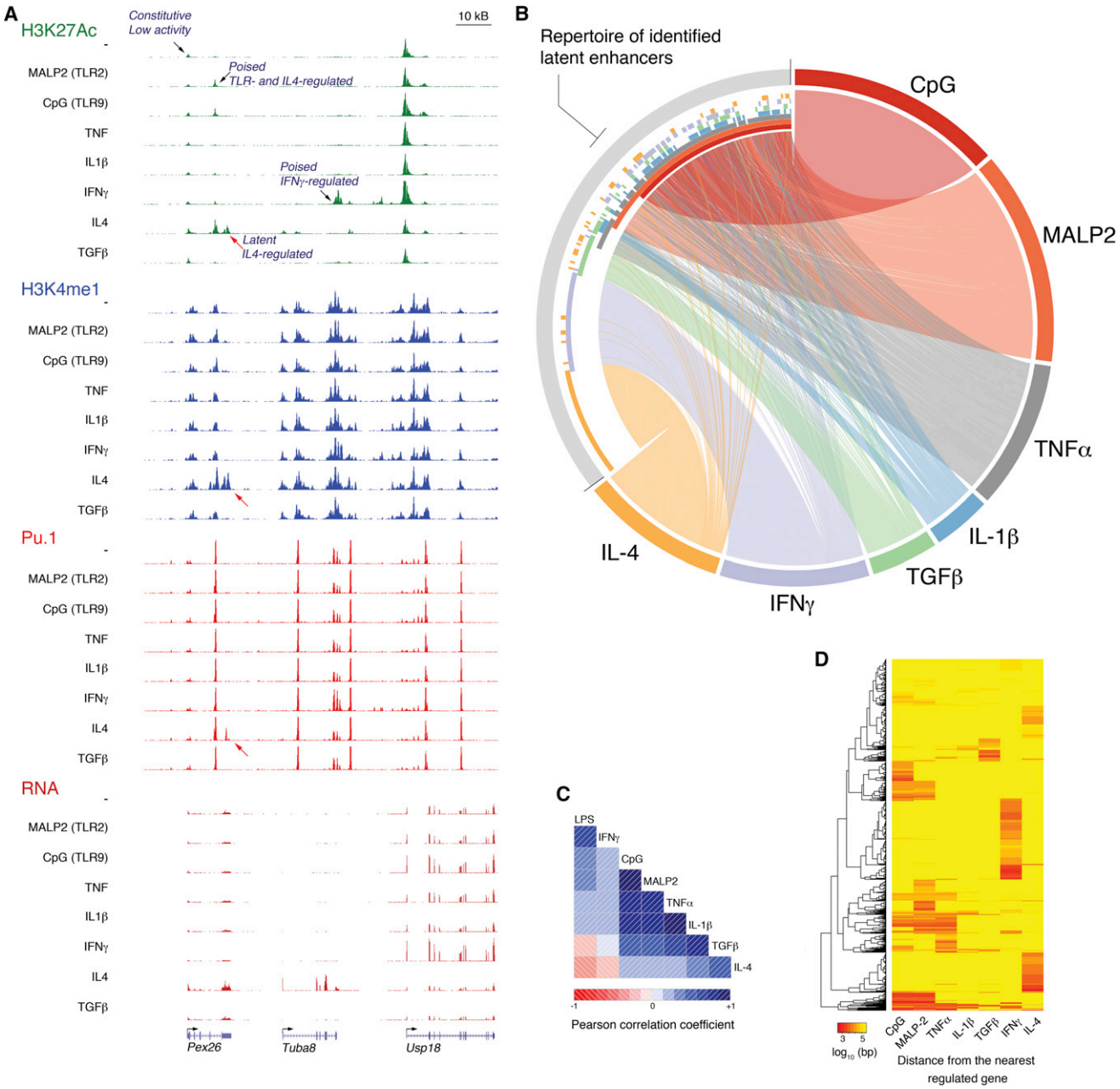

Figure 2. The Complex Repertoire of Latent Enhancers Induced by Distinct Macrophage Activators

(A) Genomic snapshot showing poised-activated and latent enhancers induced in response to a panel of macrophage activators.

(B) Circos visualization of latent enhancers induced by multiple stimuli.

(C) Correlation matrix of the number of reads ($\log_2$) for latent enhancers activated by each stimulus. Pearson correlation values are shown by color and intensity of shading. Variables were ordered by PCA (principal component analysis).

(D) Heat-map showing the distance between each latent enhancer (represented by an individual line) and the closest gene regulated by the same stimulus. See also Figure S2 and Tables S4, S5, and S6.

M1 and M2 macrophage polarizers, respectively. Overall, CpG DNA, MALP-2, and TNFα were highly correlated, whereas LPS and IFNγ were anticorrelated to IL-4 (Figure 2C). Latent enhancers induced by a given stimulus were frequently at a short distance from a gene regulated by the same stimulus (Figure 2D) and, as shown above for LPS, they were significantly closer to candidate target genes than poised-activated ($p = 2.2 \times 10^{-16}$) or constitutive ($p = 1.04 \times 10^{-13}$) enhancers. Finally, different functional categories were enriched in sets induced by different stimuli (Table S5).

To gain insight into the process of unveiling of latent enhancers, we identified TFBS overrepresented in the sets

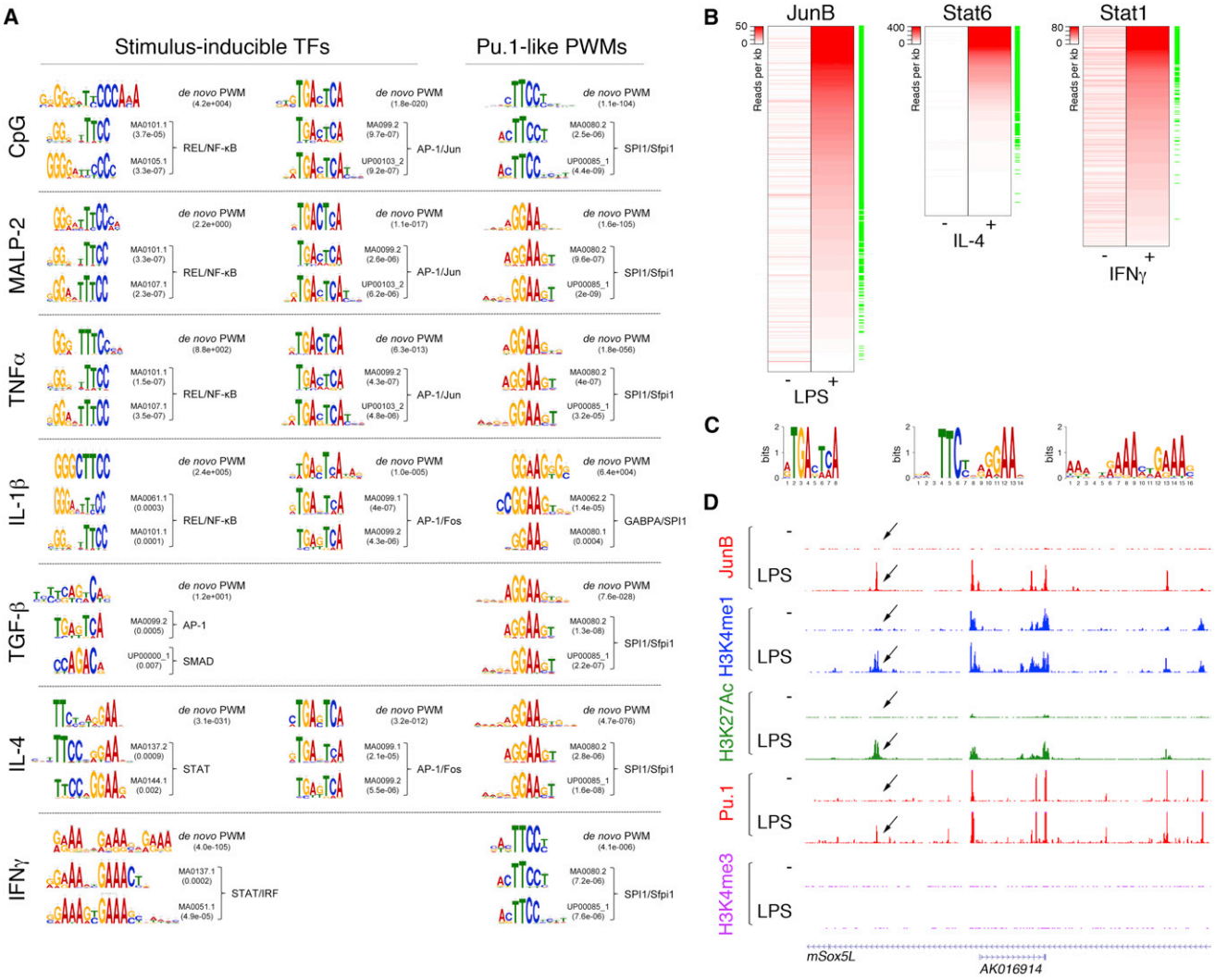

Figure 3. Sequence-Specific TFs Recruitment to Latent Enhancers

(A) TFBS overrepresented in latent enhancers unveiled by different stimuli. For all agonists, PWMs identified by de novo motif discovery and relative *E* values (obtained using MEME) are shown at the top line. Below each motif the two top-ranking Jaspar matrices are shown.

(B and C) ChIP-seq analysis of JunB, Stat6, and Stat1 recruitment to latent enhancers induced in response to LPS, IL-4, and IFNγ, respectively. Heatmaps show the normalized number of reads in the genomic area of each latent enhancer before and after stimulation. Regions are sorted based on tag density after stimulation. The green flags on the right highlight the presence of a statistically significant peak at 4 hr (MACS, $p \leq 1 \times 10^{-5}$). The PWMs obtained by de novo motif discovery on the peaks of each TF at the respective latent enhancer set are shown in (C).

(D) A representative snapshot showing JunB recruitment to an LPS-induced latent enhancer.

See also Figure S3.

induced by different stimuli (Figure 3A; Table S6). Latent enhancers induced by TLR agonists were mainly enriched in AP-1 sites, which may relate to the reported ability of AP-1 family TFs to promote chromatin opening (Biddie et al., 2011). Consistently, the AP-1 protein JunB was inducibly recruited to the majority (79.6%) of LPS-dependent latent enhancers (Figure 3B). De novo motif discovery on sequences bound by JunB in latent enhancers retrieved a canonical AP-1 matrix (Figure 3C). A representative snapshot is shown in Figure 3D. Enhancers induced by TGFβ were associated with a SMAD-type binding site and recruited Smad3 (Figure S3), whereas different types of STAT sites were overrepresented in the IL-4 and IFNγ sets (Figure 3A). IL-4-induced latent enhancers were associated with an inverted repeat 5′-TTCNNNNGAA-3′ that matches the STAT6 binding motif identified in ChIP-seq experiments (Elo et al., 2010). Consistently, analysis of STAT6 genomic distribution in IL-4-treated (4 hr) macrophages revealed an extensive association with IL-4-induced latent enhancers (Figure 3B), and de novo motif discovery on these sequences retrieved the same inverted repeat (Figure 3C). IFNγ-induced latent enhancers were associated with an IRF-type STAT site. Homodimeric STAT1 binds canonical STAT binding sites (5′-TTCCNGGAA-3′)

(Robertson et al., 2007); however, within a trimeric complex with STAT2 and IRF9, which can be activated by IFNγ (Matsumoto et al., 1999) binding occurs at IRF-type binding sites formed by direct repeats of 5′-GAAA-3′ units (Taniguchi et al., 2001). A Stat1 ChIP-seq confirmed its broad association with latent enhancers activated by IFNγ (Figure 3B), and the underlying binding site identified by de novo motif discovery was indeed an IRF-type site (Figure 3C), thus confirming that the STAT1 isoform involved in the formation of latent enhancers in response to IFNγ is probably a complex with IRFs.

In addition, binding sites similar but not perfectly identical to those for the macrophage master regulator Pu.1 were overrepresented in all latent enhancer sets.

Mechanistic Aspects of Latent Enhancers

We focused on IL-4 and IFNγ because they both operate through an experimentally tractable two-step pathway that includes a receptor-associated JAK family tyrosine kinase and a STAT family TF whose phosphorylation triggers nuclear translocation and transcriptional activation (Darnell et al., 1994).

We first analyzed the kinetics of increase of H3K4me1, H3K27Ac, and Pu.1 at representative enhancers. With both IL-4 and IFNγ, signals increased slowly and in a progressive manner and their kinetics were similar (Figures 4A and 4B). Consistently, FAIRE indicated that basal accessibility of latent enhancers was low and identical in macrophages and in Hepa1-6 hepatocytes, an unrelated cell line used for comparison (Figures 4A and 4B). In response to stimulation, FAIRE signals increased in a progressive fashion that was indicative of an ongoing nucleosomal reorganization, eventually leading to an increased central region of high accessibility bracketed on both sides by modified nucleosomes (Figure 4C).

STAT6 and STAT1 recruitment to latent enhancers showed peculiar kinetic properties (Figures 4A and S4A). The slow increase in STAT1 and STAT6 occupancy, which resembled the slow changes in accessibility and the deposition of histone modifications, was dissociated from bulk changes in TF phosphorylation, as this was already maximal at 10 min poststimulation (Figure 4D). However, consistent with its rapid activation and nuclear translocation, STAT6 was recruited with fast kinetics to other regulatory regions, such as the promoter of the IL-4-inducible *Ccl12* gene (Figure S4B). Therefore, although STAT6 is fully competent for DNA binding at early time points, it could not be quickly recruited to latent enhancers.

To determine the kinetic properties of latent enhancers at genome scale, we generated ChIP-seq data sets at multiple time points after IL-4 (Figure 4E) or IFNγ treatment (Figure S4D). Genomic data confirmed that the activation of latent enhancers is a relatively slow process, particularly if compared to the kinetics of TF activation (Figure 4D) and to the kinetics of TF recruitment to poised enhancers, which was often maximal at 15 min poststimulation (enhancer marked by asterisks in Figure 4E). Moreover, kinetics of STAT TF recruitment often preceded the deposition of H3K4me1 and in some cases also Pu.1 recruitment (Figures 4E and S4D).

Mechanistically, the kinetic discrepancy between STAT1 or STAT6 activation and their recruitment to latent enhancers, together with the slow increase in accessibility and histone mark deposition described above, indicate that the STAT TF binding sites in latent enhancers are not immediately available for binding. Clearly, the time lag between stimulation and maximal occupancy must be interpreted in the context of a population analysis, and be taken as evidence of a progressively increasing fraction of cells in which STAT binding sites in latent enhancers are exposed. TFBS at latent enhancers were not covered by positioned nucleosomes in unstimulated macrophages, as indicated by homogeneous H3 signals (Figure S4C). Upon stimulation, nucleosomal occupancy was increased on both sides of a centrally located, H3-depleted area of the enhancer (Figures S4C and 4C), likely because of the barrier effect exerted by newly recruited TFs (Jiang and Pugh, 2009; Segal and Widom, 2009; Valouev et al., 2011). Lack of a well-positioned nucleosome in the basal state implies that nucleosomes at latent enhancers are allowed to undergo lateral movements leading to stochastic fluctuations in their positions. In turn, such movements may create windows of opportunity for TF binding. Once the STAT TF are bound they may recruit Pu.1 and together stabilize the open and accessible state by recruiting chromatin-remodelers (Cui et al., 2004; Huang et al., 2002; Liu et al., 2002), as demonstrated at the promoters of inflammatory genes (Ramirez-Carrozzi et al., 2009).

To determine the role of STAT TFs in latent enhancer unveiling, we first used a Jak kinase inhibitor (Jak-i) that blocked STAT TF phosphorylation without affecting other pathways activated by these cytokine receptors (such as the ERK/MAPK pathway) (Figure 5A). Jak-i blocked the induction of H3K4me1 and H3K27Ac in response to IL-4 and IFNγ (Figures 5B and 5C). Pu.1 recruitment was also entirely dependent on Jak-Stat signaling.

We next analyzed at genome scale the impact of *Stat6* and *Stat1* gene loss on IL-4 and IFNγ induction of latent enhancers. De novo histone mark deposition and Pu.1 recruitment to latent enhancers were entirely dependent on STAT TFs activation, as virtually no latent enhancer could be detected in their absence (Figures 5D and 5E). A representative snapshot is shown in Figure 5F.

We noticed that new Pu.1 peaks were in some cases located within inducible genes (Figure S5A) and therefore we considered the possibility that in those specific cases RNA-Pol II-dependent nucleosome displacement and chromatin opening may increase accessibility of latent sites. Blockade of RNA Pol II elongation by the Cdk9 inhibitor DRB blocked Pu.1 recruitment to one such enhancer located within the *Nos2* gene (Figure S5B). Therefore, in some cases RNA Pol II may play an active role in promoting the surfacing of latent enhancers contained within partially inaccessible chromatin. Conversely, the protein synthesis inhibitor puromycin only marginally impaired H3K4me1 induction (Figure S5C).

A most intriguing observation was the lack of constitutive Pu.1 binding at latent enhancers in spite of the presence of Pu.1 sites. Indeed, Pu.1 is considered a pioneer factor able to invade nucleosomal chromatin and to promote both the formation of nucleosome-free regions and the deposition of H3K4me1 at adjacent nucleosomes (Ghisletti et al., 2010; Heinz et al., 2010). Lack of basal Pu.1 binding at latent enhancers may be explained by different mechanisms, including: (1) an actively enforced repressive chromatin state that renders the underlying binding sites

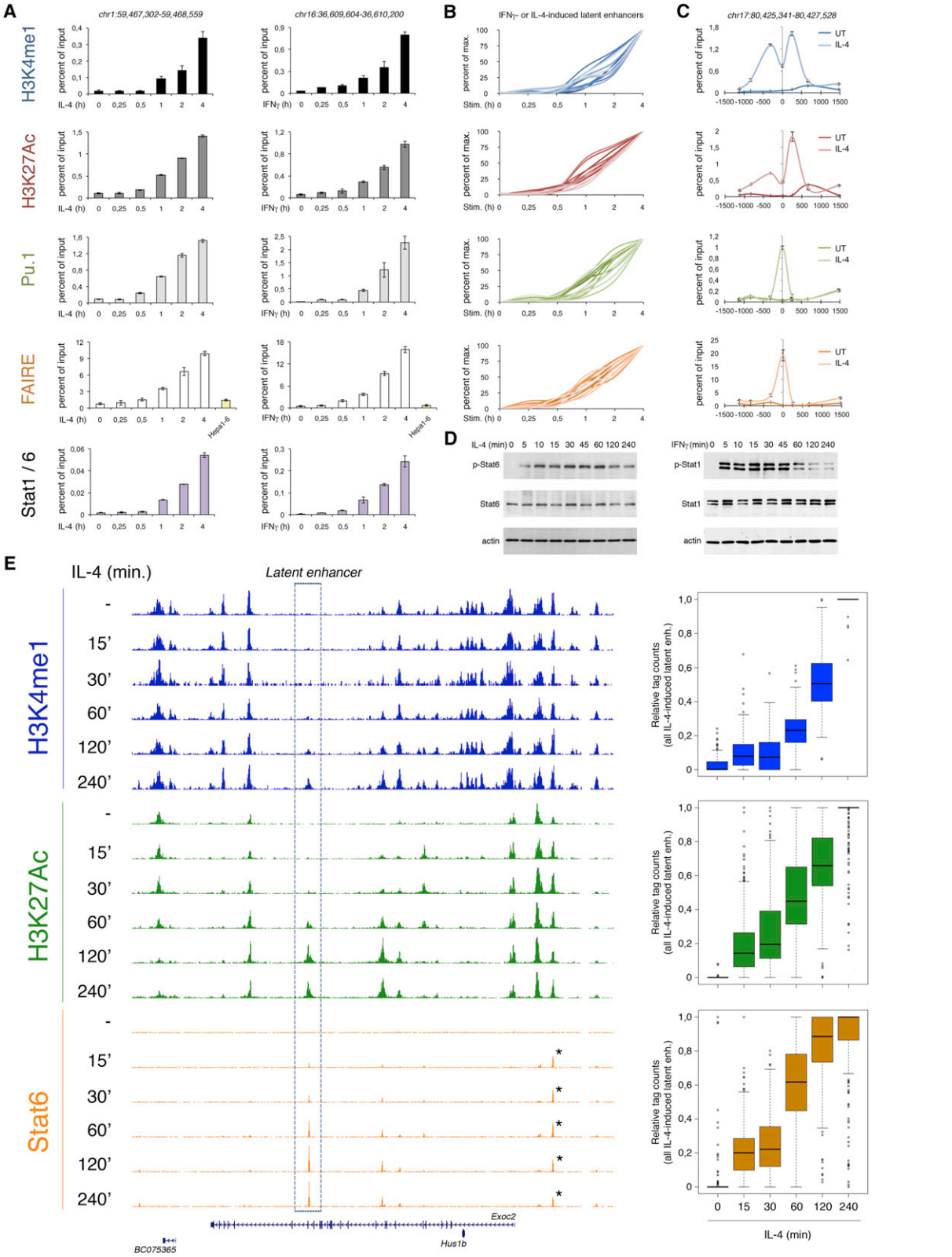

Figure 4. Kinetic Analysis of Latent Enhancers

(A) Kinetics of H3K4me1 and H3K27Ac deposition, Pu.1 and Stat1 (or Stat6) recruitment, DNA accessibility (FAIRE) at representative IL-4- (left) and IFNγ-induced (right) latent enhancers. Hepa1-6, mouse hepatocyte cell line. Data are expressed as mean ± SEM.

(legend continued on next page)

unavailable for binding, and (2) the presence of low affinity binding sites that cannot support recruitment of Pu.1 without cooperation provided by a partner TF activated by stimulation. An additional possibility is that Pu.1's pioneering function may be dependent on cooperative binding or synergy with constitutively expressed TFs that bind adjacent sites, which may be absent at the latent enhancers. Regarding the first mechanism, we investigated by ChIP the presence of negatively acting histone marks. Levels of H3K27me3, H3K27me2, and H3K9me3 were undetectable or very low at all latent enhancers tested (Figures 6A and S6A), indicating that these marks were not involved in maintenance of basal repression.

Consistent with the second mechanism, Pu.1 binding sites within latent enhancers displayed lower affinities as compared to those in constitutively bound enhancers. First, using in vitro determined binding affinities (Wei et al., 2010), we computed the Pu.1 binding affinities for the consensus sites found in the different classes of enhancers. The sites in latent enhancers showed much lower affinities for Pu.1 relative to constitutive enhancers (Wilcoxon test p value 7.06×10^{-54}) and poised-activated enhancers (p value 1.01×10^{-65}). Instead poised-activated and constitutive enhancers showed similar binding affinities (p value 0.02) (Figure 6B). Second, we computed the distributions of best matches for the Pu.1 position weight matrix (PWM) derived from ChIP-seq data (Heinz et al., 2010) for each class of enhancers. The cumulative distribution of PWM scores for constitutive, steady and poised enhancers showed nearly overlapping distribution frequencies (Wilcoxon test p value 0.017) (Figure 6C), whereas latent enhancers showed a significant divergence in sequence composition (p values 6.931×10^{-117} and 1.113×10^{-95} relative to poised and constitutive enhancers, respectively) (Figure 6C). Overall, these data indicate that the composition of Pu.1 binding sites in latent enhancers diverges from canonical high affinity sites enough to prevent Pu.1 binding in basal conditions; however, these low affinity sites allow Pu.1 recruitment in the presence of cooperating stimulus-activated TFs.

Latent Enhancers and Short-Term Transcriptional Memory

We therefore investigated whether newly activated latent enhancers could be maintained in the absence of a sustained stimulation. Cells were treated with IL-4 or IFNγ for either 4 hr or 24 hr, washed and incubated in fresh medium for additional 48 hr before harvesting (Figure 6D). Complete loss of STAT1 or STAT6 phosphorylation after washout confirmed efficient signal termination (Figure 6D). Histone acetylation, accessibility and Pu.1 occupancy all returned to prestimulation levels upon cytokine removal (Figure 6E). STAT1 and STAT6 binding to latent enhancers were also lost after washout (Figure 6E). These observations indicate that following the initial recruitment, sustained Pu.1 occupancy required the constant presence of cytokine-activated STAT1 or STAT6, thus suggesting a strong functional cooperativity. As a consequence of the removal of STAT1/Pu.1 or STAT6/Pu.1 from DNA, histone acetylation and accessibility of latent enhancers were completely lost. Instead, H3K4me1 persisted for days after the washout (Figures 6E and S6B), indicating an autonomous sustainment of this mark. We generated H3K4me1 ChIP-seq data sets to determine the fraction of latent enhancers that were retained after extensive washout. We found that of the 1,002 newly formed H3K4me1 peaks that were detected at 24 hr of IFNγ treatment and persisted after 72 hr of continuous stimulation, 307 (30.6%) were also detected after a 48 hr washout (Figure 6F). These data show that a large fraction of latent enhancers remains stably marked in the absence of continuous stimulation. Therefore, by uncovering a specific and previously hidden repertoire of enhancers, each stimulus generates an epigenomic signature of its exposure.

Residual marking by H3K4me1 was indicative of a poised state of these enhancers after cytokine washout. When primed cells were restimulated with the same agonist used in primary stimulation, faster acetylation kinetics were observed (Figure 7A). Moreover, final levels of acetylation were consistently higher than those achieved in response to primary stimulation. These data are consistent with the notion that the H3K4me1 mark primes enhancers for recruitment of HATs (Jeong et al., 2011). Finally, genes adjacent to latent enhancers were induced faster, and often to higher levels in response to secondary stimulation (Figure 7B). These enhancing effects were not due to a stronger induction of the Jak/Stat signal transduction pathway in restimulated cells, because levels of phospho-STATs were comparable to those found in unprimed macrophages (Figure 7C).

As shown above (Figure 2), stimuli differed in their ability to cause the emergence of latent enhancers. However, the presence of complex combinations of TFBS may in principle enable cross-reactivation once the latent enhancer has surfaced. To test this hypothesis, we primed macrophages with IL-4 and after cytokine washout, we restimulated them with a panel of different agonists. In some cases, latent enhancers were promiscuously induced by multiple stimuli and retained increased responsiveness to the same agonists upon restimulation (Figure 7D, left). At other enhancers induced selectively by IL-4, responsiveness to secondary stimulation was exquisitely restricted to IL-4 (Figure 7D, middle). Finally, in other cases, reactivation of a surfaced IL-4-induced latent enhancer was highly promiscuous (Figure 7D, right) indicating that although the primary exposure of these elements was highly selective, the very same enhancers could be subsequently acted upon by TFs activated by multiple stimuli.

(B) Data from multiple latent enhancers activated by either IL-4 or IFNγ are shown. Each line represents a different enhancer.
(C) A representative IL-4-induced latent enhancer was analyzed using multiple primers spanning a ~3 kb region. The central position (0) corresponds to the summit of the inducible Pu.1 peak. Data are expressed as mean ± SEM.
(D) Western blots with phospho-specific STAT6 and STAT1 antibodies showing their activation kinetics.
(E) ChIP-seq analysis of IL-4-dependent latent enhancer induction kinetics. A representative genomic region is shown on the left. The asterisks (bottom right) indicate a poised enhancer where Stat6 recruitment was already maximal at 15′, as opposed to the latent enhancer shown on its left, where recruitment was progressive. The box plots indicate relative tag counts at all mapped IL-4 latent enhancers.
See also Figure S4.

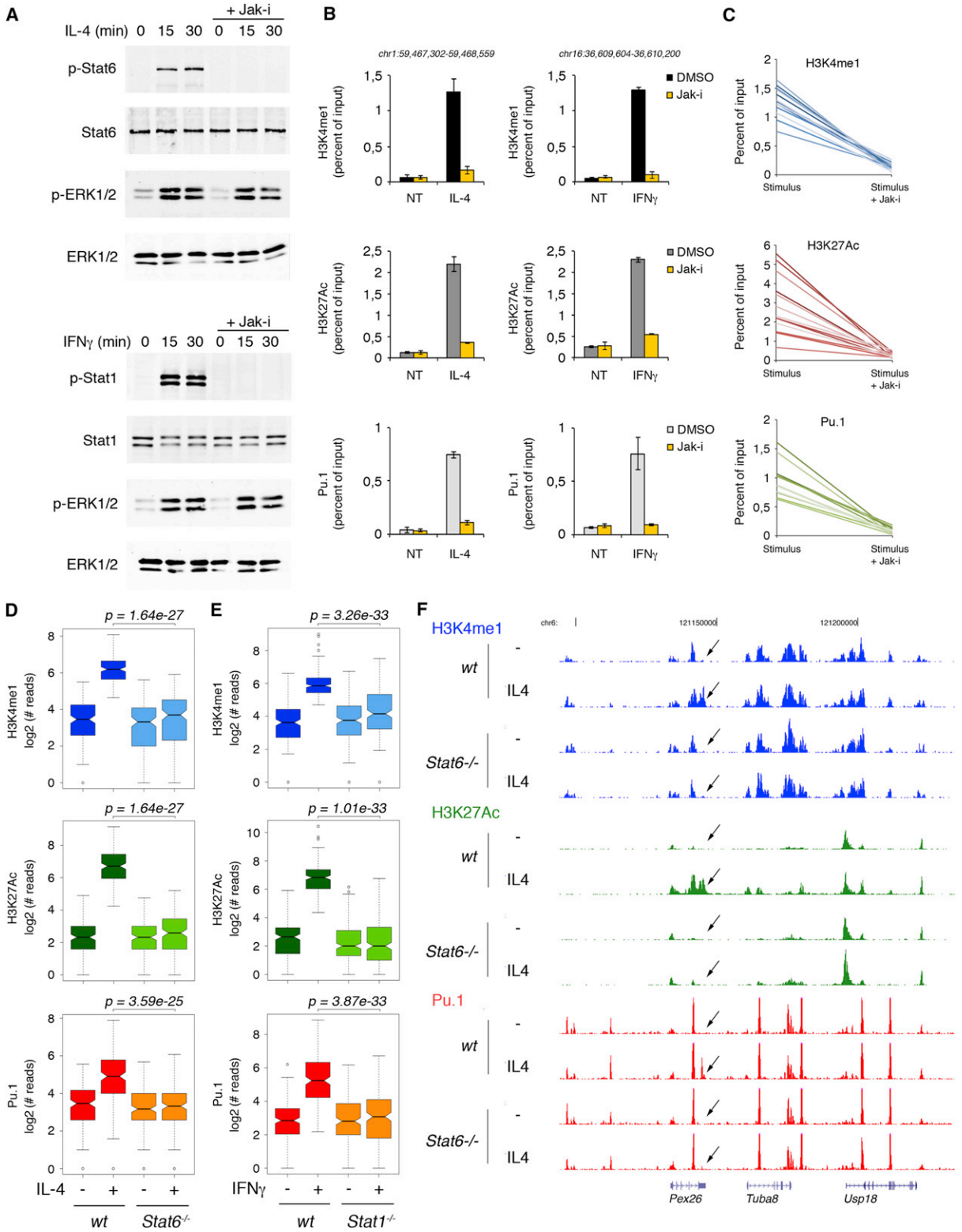

Figure 5. STAT TFs Requirement for the Unveiling of Latent Enhancers in Response to IL-4 and IFNγ Stimulation

(A) Inhibition of STAT tyrosine phosphorylation but not MAPK ERK phosphorylation by a selective Jak kinase inhibitor (Jak-i).

(B and C) Inhibition of histone marks deposition and Pu.1 recruitment by Jak-i at individual enhancers (B) or at a panel of IL-4- or IFNγ-induced latent enhancers (C). Data in (B) are expressed as mean ± SEM.

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DISCUSSION

In this study, we report a distinct class of transcriptional enhancers whose main distinguishing features are: (1) lack of basal marking and TF binding in fully differentiated cells, (2) acquisition of enhancer marks and accessibility upon stimulation, (3) dependence on both stimulus-activated TFs and lineage determining TFs, (4) selective retention of H3K4me1 upon signal termination, and (5) faster and/or augmented response upon secondary stimulation. Although the repertoire of available *cis*-regulatory elements in a given cell type appears to be a specific property of its differentiated state and to be directly controlled by the enhancer-organizing activity of lineage-determining TFs (Natoli, 2010), the repertoire of surfaced latent enhancers is determined by the functional cooperation between stimulus-activated TFs and lineage-restricted TFs, thus integrating cell-type-specific and stimulus-specific inputs.

We suggest that the unveiling, and the subsequent retention, of an agonist-specific repertoire of latent enhancers can be considered an epigenomic footprint (or scar) of the stimuli the cell has previously been exposed to. The surfacing of latent enhancers also implies an expansion of the repertoire of accessible regulatory elements, which effectively changes the way cells respond to a subsequent stimulation. It has been previously reported that in macrophages that underwent a prolonged exposure to LPS, although some genes cannot be reactivated upon washout and secondary LPS stimulation, many others were hyperinduced (Foster et al., 2007), a phenomenon that may relate to the expansion of the enhancer repertoire we described here. In this context, it is interesting to note that once unveiled, some latent enhancers become responsive to stimuli that were unable to induce their exposure in naive cells. Therefore, not only does the surfacing of latent enhancers change the way cells respond to repeated challenges with the same stimulus, but also the way a preconditioned cell will respond to stimuli it never encountered before.

Mechanistically, latent enhancers can be distinguished from those poised enhancers whose basal inactivity is associated with both H3K4me1 and H3K27me3 (Rada-Iglesias et al., 2011). Our data suggest that lack of marking of latent enhancers in the basal state reflects the fact that these enhancers are ignored because of a combination of two main factors: nucleosome occlusion and a comparatively low affinity for Pu.1, which would otherwise be able to invade nucleosomal DNA and induce enhancer formation (Ghisletti et al., 2010; Heinz et al., 2010). Upon stimulation, Pu.1 can gain access to such sites (and promote the organization of an active enhancer) because of the functional cooperation provided by stimulus-activated TFs. However, the slow kinetics of surfacing of latent enhancers is not compatible with a simple model of cooperative binding, because in this case Pu.1 recruitment and enhancer organization would occur immediately after the nuclear translocation of inducible TFs such as STAT1 or STAT6. Conversely, the time lag between activation of the STAT TFs and their recruitment (together with Pu.1) to latent enhancers suggests the existence of an additional regulatory layer that may be due to nucleosomal interference with DNA binding. Reorganization of the nucleosomal spacing at latent enhancers may reflect either an active process mediated by chromatin remodelers or random fluctuations in the position of nonfixed nucleosomes. In the first case, TFs such as the STAT TF and AP-1 endowed with the ability to recruit chromatin-remodeling complexes (Cui et al., 2004; Huang et al., 2002; Liu et al., 2002; Biddie et al., 2011) would initially make short contacts with partially occluded binding sites within latent enhancers, thus promoting (in combination with Pu.1) their opening via an iterative process involving the repetition of transient binding events followed by progressively increased site exposure. The second possibility is that either nucleosome turnover or spontaneous lateral movements of nucleosomes that are not constrained because of the lack of either adjacent barriers or positioning signals (Valouev et al., 2011) would create windows of opportunity for TF binding, thus eventually leading to the stabilization of the open state. Upon stimulus termination and release of the bound TFs, loss of the barrier activity they exerted would allow spontaneous nucleosome repositioning and loss of accessibility. Nevertheless, H3K4me1 marking will persist and promote a faster reinduction at restimulation, which may reflect a direct reading of this mark by HATs (Jeong et al., 2011).

Our experimental system does not allow determining the potential for transmission of the unveiled repertoire of latent enhancers across DNA synthesis. Therefore, retention of the H3K4me1 mark—and the associated functional consequences—after stimulus termination can only be discussed here in the context of short-term transcriptional memory mechanisms. In any case, such short-term epigenomic memory may have great functional implications in postmitotic cells such as neurons. Indeed, it will be interesting to determine if brain memory circuits, in which epigenetic phenomena have been suggested to play an important role (Levenson and Sweatt, 2005), also involve latent enhancers.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, Cell Culture Procedures, Cloning, Transfections, and RNA Analyses

For information on antibodies, reagents, cell culture procedures, cloning, transfections, and RNA analyses, please refer to the Extended Experimental Procedures.

Chromatin Immunoprecipitation and Sequencing

ChIP was carried out using either a previously described protocol (Ghisletti et al., 2010) or a high-throughput protocol (Garber et al., 2012). Briefly, 5–15 × 10^6 (ChIP-seq for histone marks and Pu.1) or 100 × 10^6 (ChIP-seq for other TFs and RNA Pol II) fixed macrophages were lysed with 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. For ChIP-qPCR experiments, 10%–20% of cells indicated above were used, and IP was performed using 1–3 μg of antibody. After immunoprecipitation, beads were recovered using a 96-well magnet, washed,

(D–F) STAT6 (D) and STAT1 (E) requirement for IL-4- or IFNγ-mediated induction of latent enhancers. Box plots show the consequence of STAT TF absence on de novo deposition of histone marks and Pu.1 recruitment at latent enhancers, as measured by ChIP-seq (p values calculated with paired-samples Wilcoxon signed-rank test). A representative snapshot from *Stat6*$^{-/-}$ macrophages is shown in (F).
See also Figure S5.

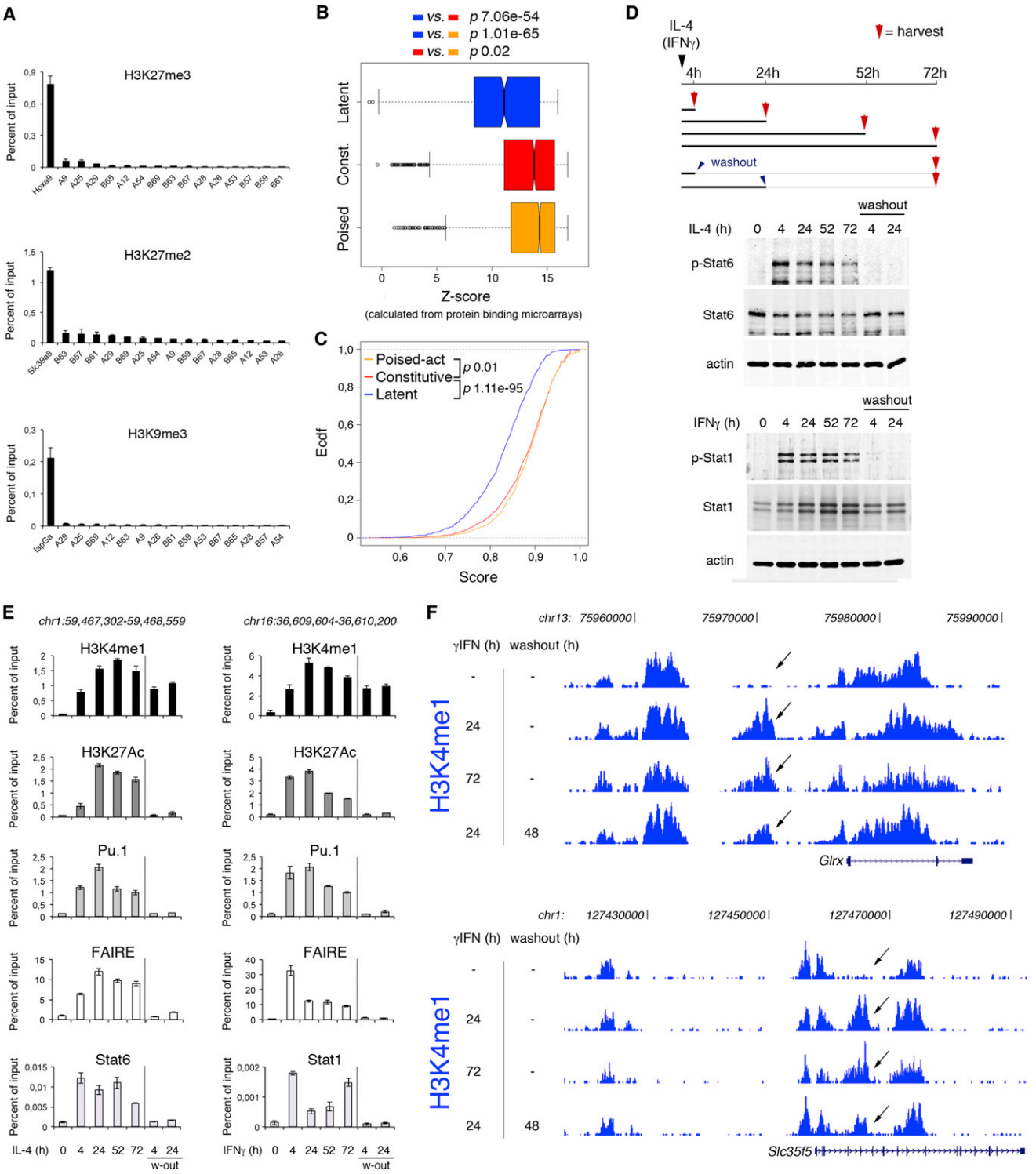

Figure 6. Mechanistic Dissection of Latent Enhancer Formation

(A) Repressive histone marks at representative latent enhancers. The first column on the left is a positive control for each modification. For each modification, the enhancers were ranked based on decreasing signal intensity. Data are expressed as mean ± SEM.

(B) Analysis of Pu.1 binding sites at latent enhancers as compared to constitutively bound Pu.1 sites (in constitutive and poised-activated enhancers). The *Z* score was calculated using Pu.1 affinity data obtained from protein binding microarrays (Wei et al., 2010). The p values shown were obtained using a Wilcoxon test.

(C) Cumulative distribution for PWM scores in latent enhancers and two control groups. The reference PWM used was generated from in vivo binding data (Heinz et al., 2010). The p values were generated by a Wilcoxon test. Ecdf, empirical cumulative distribution function.

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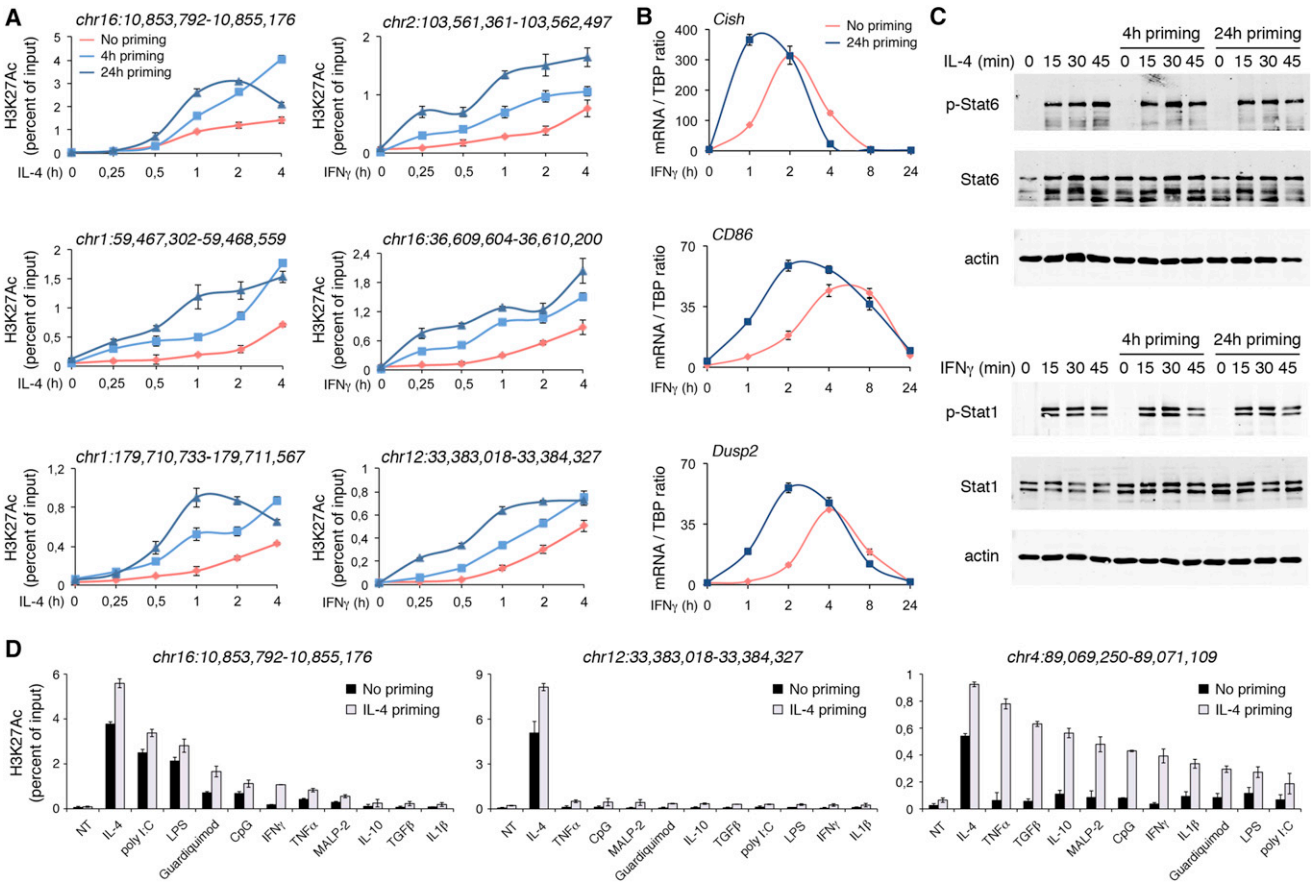

Figure 7. A Short-Term Memory Associated with Latent Enhancers

(A) Acetylation kinetics at latent enhancers upon restimulation.

(B) Induction of genes adjacent to latent enhancers upon restimulation.

(C) STAT TF activation upon secondary stimulation.

(D) Selective versus promiscuous activation of latent enhancers by alternative stimuli. Three different representative behaviors are shown. The enhancer on the left was activated in a promiscuous manner, the one in the middle was selectively activated by IL4 both at primary and secondary stimulation, and the one on the right was selectively activated by IL4 at primary stimulation but promiscuously at restimulation. ChIP data in (D) refer to a priming of 24 hr and a (re)stimulation of 4 hr. Data in (A), (B), and (D) are expressed as mean ± SEM.

and DNA was eluted and decrosslinked overnight at 65°C. DNA was then purified with Solid-phase reversible immobilization (SPRI) beads (Agencourt AMPure XP, Beckman Coulter) and quantified with PicoGreen (Invitrogen). For ChIP-QPCR experiments, 0.4 μl of purified DNA was used for amplification on an ABI 7500 machine. Primers used for ChIP-QPCR are in Table S7. ChIP DNA was prepared for Solexa GAII or HiSeq2000 sequencing following standard protocols. Basic data processing was carried out using the Fish-The-ChIP pipeline (Barozzi et al., 2011). A detailed description of the computational analyses is provided in the Extended Experimental Procedures.

ACCESSION NUMBERS

Raw data sets have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) and are available under the accession GSE38379. R was used to compute statistics and generate plots.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.12.018.

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(D) Cytokine washout results in signal termination and loss of STAT TF phosphorylation.

(E) Pu.1 binding to latent enhancers, acetylation and increased accessibility, but not H3K4me1, require continuous cytokine signaling. Data are expressed as mean ± SEM.

(F) Two representative ChIP-seq snapshots showing H3K4me1 behavior at two IFNγ-induced latent enhancers upon cytokine washout.

See also Figure S6.

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