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Interferon- γ Resets Muscle Cell Fate by Stimulating the Sequential Recruitment of JARID2 and PRC2 to Promoters To Repress Myogenesis

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Abstract

The inflammatory cytokine interferon- γ (IFN- γ) orchestrates a diverse array of fundamental physiological processes. IFN- γ and the class II transactivator (CIITA) play essential roles in inhibiting muscle development during the inflammatory response. Here, we describe the mechanism through which IFN- γ and CIITA inhibit myogenesis by repressing gene expression in muscle cells subjected to inflammation. In mice, the presence of increased amounts of circulating IFN- γ resulted in the increased abundance of polycomb repressive complex 2 (PRC2) in muscle fibers, a tissue in which PRC2 is not normally present in the adult. We showed that CIITA first interacted with the Jumonji family protein JARID2, a noncatalytic subunit of PRC2, which caused an RNA polymerase II (RNAPII), phosphorylated at serine 5, to pause at target promoters. Additional subunits of the PRC2 complex, including the catalytic subunit EZH2, were then recruited in a JARID2-dependent manner that was concurrent with the loss of RNAPII and the methylation of lysine 27 of histone H3 (H3K27), which is associated with gene repression. By inducing the formation and recruitment of the PRC2 complex, which is normally not present in differentiated muscle cells, CIITA and IFN- γ block myogenesis in culture. Together, these data indicate that increased amounts of IFN- γ reset myogenic cell fate through a multistep mechanism that culminates in the recruitment of PRC2 to silence muscle-specific genes.

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Supplementary Materials Fig. S1. CIITA in transfected C2C12 cells is similar in abundance to that in IFN- γ -stimulated untransfected cells.

Fig. S2. Muscle-specific genes are repressed in the presence of exogenous CIITA throughout cellular differentiation.

Fig. S3. CIITA interacts with pTEFB in muscle cells.

Fig. S4. H3K27 is methylated at *Hoxb7* in control and CIITA-expressing C2C12 cells on days 2 and 6 of differentiation.

Fig. S5. Trimethylation of H3K4 is inhibited in CIITA-expressing C2C12 cells.

Fig. S6. H3K9 is not methylated on CIITA-repressed genes.

Fig. S7. Phosphorylation of the CTD is inhibited in IFN- γ -stimulated cells.

Fig. S8. Genes encoding components of the PRC2 complex are increased in expression in *Mdx* mice.

Fig. S9. Detection of macrophages in the liver, but not muscle, of mice injected with IFN- γ -producing CHO cells.

Introduction

The differentiation of skeletal muscle is controlled by four highly related basic helix-loop-helix proteins known as the myogenic regulatory factors (MRFs), which include Myf5, MyoD, myogenin, and Myf6. In the combined absence of Myf5, Myf6 and MyoD, myoblasts are not specified, and no skeletal muscle forms, which results in lethality in embryos (1). Myogenin is the only MRF that singly is required for viability (2, 3). Mice deficient in *myogenin* have myoblasts, but very few muscle fibers, which suggests that myogenin is not required for the specification of skeletal muscle, but is required for the later stages of myofiber fusion.

Skeletal muscle cells are susceptible to injury and damage from direct trauma, neurological disorders, intense physical activities, and genetic defects. A remarkable property of skeletal muscle cells is their ability to regenerate in response to injury or damage. After injury, a sequence of finely tuned cellular and molecular responses is orchestrated that has many similarities to the process of muscle development during embryogenesis. The repair of skeletal muscle is divided into three phases: the inflammation phase, which involves the accumulation of macrophages, neutrophils, and lymphocytes; the repair and regeneration phase, during which quiescent myogenic precursor cells called satellite cells are activated; and the final remodeling phase, in which satellite cells proliferate, differentiate, and fuse to form multinucleated muscle fibers (4).

The inflammatory cytokine interferon- γ (IFN- γ) is produced by skeletal muscle cells after injury, and endogenous IFN- γ is required for efficient muscle regeneration and healing (5). IFN- γ regulates many physiological processes and exerts transcriptional control over a number of genes. It is mainly secreted by cytotoxic lymphocytes and natural killer (NK) cells (6), and its production is also controlled by cytokines that are produced by professional antigen-presenting cells (APCs), which serve as a bridge linking infection with IFN- γ production in innate immune responses (7, 8). IFN- γ serves many important functions, such as activation of phagocytes, stimulation of antigen presentation, and control of cell proliferation and apoptosis, and IFN- γ regulates the transport of specific immune cells to inflammatory sites (9).

Although transient administration of IFN- γ improves muscle healing (10), excess IFN- γ can lead to harmful effects. When IFN- γ is present in excess at the neuromuscular junction in transgenic mice, the mice demonstrate an age-dependent necrotizing myopathy (11). IFN- γ is both a strong inducer of the M1 phenotype of macrophages, a pro-inflammatory response that promotes injury, and a suppressor of the immune-modulatory and tissue-remodeling phenotype of M2 macrophages, which promote repair (12). Mouse models of muscular dystrophy show enhanced amounts of IFN- γ in the serum compared to wild-type mice (13), and ablation of IFN- γ in these animals improves muscle function (12).

The class II transactivator (CIITA) is the master regulator of the transcription of the gene encoding major histocompatibility complex II (MHC II). CIITA is a member of the nucleotide-binding domain and leucine-rich repeat (NLR)-containing gene family of proteins that regulate the immune response, and these proteins share key structural features

(14, 15). Mutations in CIITA result in bare lymphocyte syndrome (BLS), a genetic condition in which the expression of MHC class II genes is severely compromised (16-18). CIITA lacks DNA-binding activity, but is recruited to proximal promoters by interactions with sequence-specific DNA-binding factors (19-22). CIITA is also critical for IFN- γ -induced repression of target genes, including those encoding collagen, interleukin-4 (IL-4), IL-10, and cathepsin E. CIITA consists of 1130 amino acid residues, and it contains several functional domains (23). The recruitment of CIITA to the promoters of MHC II-encoding genes correlates with an enrichment for RNA polymerase II (RNAPII), enhanced acetylation of several residues of histones H3 and H4 (24, 25), and an accumulation of trimethylated Lys⁴ in histone H3 (H3K4) (25).

Polycomb repressor proteins are important in development, and they play essential roles in skeletal muscle specification. The two major polycomb complexes, polycomb repressive complex 1 (PRC1) and PRC2, are responsible for the ubiquitylation of histone H2A on lysine 119 (H2AK119) and the methylation of H3 lysine 27 (H3K27), respectively (26-28). Methylation of H3K27 is thought to recruit PRC1, which then catalyzes the ubiquitylation of H2AK119. The methylation of H3K27 by PRC2 requires the presence of one of the enhancer of zeste (EZH) proteins, EZH1 or EZH2. Di- and tri-methylation of H3K27 is primarily associated with facultative heterochromatin, whereas mono-methylation is associated with intergenic heterochromatin. Promoters and enhancers of many lineage-specific genes contain di- and trimethylated H3K27, and many of these elements lose their methylation profiles upon differentiation (29). EZH2 is co-expressed with Paired box 7 (PAX7), which is essential for the specification and maintenance of satellite cells in muscle (30), and EZH2 appears to be required for homeostasis of the satellite cell pool (31). Consistent with this role, EZH2 abundance is reduced upon the differentiation of myoblasts and the expression of muscle-specific genes (32). JARID2 is the catalytically inactive founding member of the Jumonji family of proteins, and it is a sub-stoichiometric component of PRC2, which appears to optimize the enzymatic activity of PRC2 and is thought to function in recruiting PRC2 to target genes (33). JARID2 is involved in targeting the PRC2 complex, but how JARID2 recognizes target sites is unclear. In embryonic stem (ES) cells, JARID2 is required to establish or maintain the phosphorylation of Ser⁵ of RNAPII, the initiating form of RNAPII that characterizes genes primed for mRNA synthesis before productive transcription occurs (34).

We previously demonstrated roles for CIITA and IFN- γ in modulating myogenesis and showed that IFN- γ acts as a reversible inhibitor of muscle differentiation (35). Here, we sought to gain insight into how CIITA inhibits the activity of myogenin. We demonstrated that CIITA repressed muscle genes in a stepwise manner that first involved inhibition of both the recruitment of RNAPII and the elongation of nascent transcripts, and, in a subsequent step, RNAPII was displaced and the PRC2 complex was recruited. CIITA resulted in a paused RNAPII by recruiting JARID2 independently of the additional components of PRC2. We showed that JARID2 was necessary to establish paused RNAPII and for the repression mediated by CIITA. Subsequently, additional members of the PRC2 complex, including the catalytic subunit EZH2, were recruited to the promoters of muscle-specific genes. We also showed that stimulation with IFN- γ or expression of CIITA maintained the presence of PRC2 components, which are normally silenced in differentiated

cells. Finally, we demonstrated that CIITA, EZH2, and suppressor of zeste 12 homolog (SUZ12), a core PRC2 subunit, were increased in abundance in muscle tissue in vivo when IFN- γ was exogenously expressed, during muscle damage, and in *Mdx* mice, a mouse model of muscular dystrophy that exhibits chronically increased amounts of IFN- γ in the serum. Thus, these results reveal the inhibitory mechanism mediated by CIITA and show that IFN- γ resets the differentiation program of skeletal muscle cells by maintaining them in an undifferentiated state. Together, these findings enhance our understanding of the molecular effects of chronic inflammation.

Results

Myogenin recruits CIITA to the promoters of muscle-specific genes

We previously showed that CIITA is recruited to muscle-specific genes in response to IFN- γ (35). To determine whether the recruitment of CIITA was dependent on myogenin, we depleted myogenin by transfecting C2C12 cells with a plasmid expressing a short hairpin RNA (shRNA) specific for myogenin. We first confirmed that the abundances of myogenin mRNA and protein were reduced in cells expressing the specific shRNA (Fig. 1A). To determine whether myogenin was required for the recruitment of CIITA to the promoters of muscle-specific genes, we performed chromatin immunoprecipitation (ChIP) analysis on C2C12 cells transfected with plasmid expressing either scrambled control shRNA or myogenin-specific shRNA. We found that in cells depleted of myogenin, CIITA was not recruited to the *Lmod2* and *Tnni2* promoters (Fig. 1B), suggesting that CIITA is recruited to muscle-specific gene promoters through a myogenin-dependent mechanism. Because we previously showed that exogenous CIITA represses the expression of *myogenin* and myogenin-dependent genes (35), we sought to determine whether the repression of muscle-specific gene expression in this case was a result of the loss of myogenin or the lack of CIITA recruitment. We first determined that myogenin was detectable by Western blotting after immunoprecipitation from C2C12 cells transfected with plasmid encoding CIITA, which suggests that myogenin is present at low abundance in these cells (Fig. 1C). To determine whether myogenin and CIITA were recruited to muscle-specific genes, we performed ChIP analysis on C2C12 cells transfected with either plasmid expressing exogenous CIITA or an empty plasmid control. Myogenin was detectable at these promoters, although at reduced abundance when compared to that in samples from cells transfected with the control plasmid (Fig. 1D). Exogenous CIITA was also present at muscle-specific gene promoters (Fig. 1E), which implies that the observed repression of gene expression in this cell line was mediated by the myogenin-dependent recruitment of CIITA.

CIITA represses muscle-specific gene promoters by pausing RNAPII

We previously showed that when CIITA is recruited to muscle-specific genes in response to IFN- γ , the abundance of RNAPII at these promoters is reduced, but that RNAPII is still detectable (35). To examine this further, we examined the status of RNAPII in a C2C12 cell line expressing exogenous CIITA compared with that in a control C2C12 cell line transfected with empty plasmid. These cell lines were chosen for our studies because exogenous CIITA was present at an abundance comparable to that in nontransfected cells

stimulated with IFN- γ (fig. S1). The C-terminal domain (CTD) of the largest subunit of RNAPII, RPB1, undergoes a cycle of phosphorylation events during a transcription cycle (36). RNAPII with an unphosphorylated CTD is recruited to promoters, serine 5 (Ser⁵) of the CTD is then phosphorylated as the polymerase initiates transcription, and Ser² is phosphorylated during the elongation phase of the cycle (36).

We first analyzed the status of the unphosphorylated form of the CTD of RPB1. We previously showed that muscle-specific gene expression is repressed when CIITA is exogenously expressed (35), and here, we confirmed the repression of muscle-specific gene expression for both time points used in this study, day 2 (D2) and day 6 (D6) of differentiation (fig. S2). In experiments with antibodies against the N-terminus of RPB1 (35), we observed that the unphosphorylated form of the CTD was recruited to the promoters of *Lmod2* and *Tnni2* during the differentiation of C2C12 cells, consistent with our previous data. As anticipated, we did not see the association of RNAPII with these promoters in proliferating cells in the presence or absence of CIITA, because these genes are not expressed in proliferating cells (Fig. 2A). In C2C12 cells, recruitment of RNAPII to these promoters was detectable after two days of differentiation (Fig. 2B) and was increased in extent after six days (Fig. 2C). In C2C12 cells expressing exogenous CIITA, we found that RNAPII was still recruited to the *Lmod2* and *Tnni2* promoters, albeit at reduced amounts compared to that in C2C12 cells (Fig. 2B); however, we also observed that this recruitment was lost after six days of differentiation (Fig. 2C).

We next looked for the presence of phosphorylated Ser⁵ (pSer⁵) in the CTD of RPB1 as an indicator of transcriptional initiation. We found that pSer⁵ was detectable at the promoters of both *Lmod2* and *Tnni2* in the presence or absence of CIITA (Fig. 2D); however, in C2C12 cells transfected with control plasmid, pSer⁵ was also detected in the coding regions of these genes, consistent with the presence of elongating RNAPII. However, in C2C12 cells expressing exogenous CIITA RNAPII-pSer⁵ in the coding regions of these genes was substantially reduced in abundance. We also found that the abundance of RNAPII-pSer⁵ greatly increased from day 2 to day 6 in C2C12 cells transfected with control plasmid (Fig. 2, D and E). In C2C12 cells expressing exogenous CIITA, the association of RNAPII-pSer⁵ with the promoters was lost after 6 days, consistent with the data from the analysis of unphosphorylated CTD in these cells (Fig. 2E).

Next, we assayed for the presence of pSer² as an indicator of the form of RNAPII associated with elongating transcripts. We detected RNAPII-pSer² at the promoters and coding regions of *Lmod2* and *Tnni2* in C2C12 cells transfected with control plasmid at days 2 and 6 (Fig. 2, F and G). However, we could not detect pSer² in C2C12 cells expressing exogenous CIITA (Fig. 2, F and G). Thus, RNAPII appeared to be blocked at the step of elongation in C2C12 cells expressing CIITA because of an inhibition in the phosphorylation of Ser².

We next sought to confirm the presence of arrested RNAPII-pSer⁵ at target promoters in experiments with cells stimulated with IFN- γ , which was added to C2C12 cells while they were proliferating, and then cells were allowed to differentiate for two days. We detected RNAPII-pSer⁵ in cells treated with IFN- γ , and, as observed earlier, RNAPII did not appear to move into the coding regions of the target genes (Fig. 2H). We then found that the

phosphorylation of Ser² was also inhibited in C2C12 cells stimulated by IFN- γ (Fig. 2I). Together, our data suggest that RNAPII is recruited to the promoters of muscle-specific genes when CIITA is present. Ser⁵ of RNAPII was phosphorylated, but the subsequent phosphorylation of Ser² was inhibited, thus halting the elongation process and preventing gene transcription.

pTEFB and NELF do not appear to contribute to pausing RNAPII at target promoters

The presence of a paused RNAPII on CIITA-repressed genes was unexpected, because CIITA interacts with the positive transcription elongation factor (pTEFB), which phosphorylates Ser² of RBP1 (37, 38). Because the interaction between CIITA and pTEFB has not been demonstrated in muscle cells, we first sought to understand how pTEFB abundance was regulated during muscle cell differentiation. We also examined the abundance of the negative elongation factor (NELF). We found that pTEFB, as determined by detection of cyclin-dependent kinase 9 (CDK9), one of the two subunits of pTEFB, and NELF were present in C2C12 cells in the presence and absence of CIITA throughout proliferation and differentiation (fig. S3A). Next, we found that CIITA physically interacted with pTEFB in differentiated C2C12 cells expressing exogenous CIITA (fig. S3B). To confirm that the interaction occurred under physiological conditions, we repeated the experiment, this time in C2C12 cells stimulated with IFN- γ , and we again observed the physical interaction between CIITA and pTEFB (fig. S3C). We then performed ChIP assays to determine whether the recruitment of these factors to muscle-specific genes was altered in the presence of CIITA. We also studied suppressor of Ty5 homolog (SPT5), an elongation factor that accompanies RNAPII and promotes productive transcript elongation (39, 40). We found that in C2C12 cells transfected with control plasmid, pTEFB, NELF, and SPT5 were recruited to the *Tnni2* promoter and traveled with the elongating RNAPII, as detected by their enrichment in an intronic region (fig. S3D). In C2C12 cells expressing exogenous CIITA, pTEFB, NELF, and SPT5 were recruited to the *Tnni2* promoter, but they were not detectable in intronic regions, consistent with the presence of paused RNAPII at the promoter (fig. S3D). However, the amounts of pTEFB and SPT5 at the promoter were greater in cells expressing exogenous CIITA than in control cells, which suggests that the lack of pTEFB or the enhanced recruitment of NELF were not responsible for the paused RNAPII. Indeed, the enhanced enrichment of pTEFB and SPT5 is consistent with a promoter that is poised for expression, but is inhibited by an alternative mechanism.

JARID2 and the PRC2 complex are recruited in a stepwise manner to CIITA-repressed genes

To identify the mechanism responsible for inducing the paused RNAPII, we next looked for the presence of JARID2, a noncatalytic subunit of the PRC2 complex that is required to establish or maintain RNAPII-pSer⁵ in ES cells (34). We examined the presence of JARID2 and additional members of the PRC2 complex in proliferating cells and in cells induced to differentiate for 2 or 6 days in the presence or absence of CIITA. We found that JARID2 bound to CIITA-repressed promoters on day 2, but we did not observe the association of other PRC2 subunits with these promoters (Fig. 3A). On day 6, we found that JARID2, EZH2, and SUZ12 were associated with the repressed genes. At this late time point, RNAPII was lost at these promoters (Fig. 2C). Because the recruitment of JARID2 to these

promoters was independent of the recruitment of EZH2, we also assayed for the presence of H3K27^{me3}, the epigenetic modification associated with EZH2. We found that, similar to the recruitment of EZH2 and SUZ12, the methylation of H3K27 was not observed until day 6 in C2C12 cells expressing exogenous CIITA (Fig. 3B). As a positive control for these experiments, we also assayed for the *Hoxb7* promoter, which is methylated at H3K27 (41), and confirmed the association of H3K27^{me3} with this promoter at both time points (fig. S4).

Because these experiments were performed in cells expressing exogenous CIITA, we repeated them in cells stimulated with IFN- γ . We found that cells differentiated for 2 days in the presence of IFN- γ also showed recruitment of JARID2 to *Tnni2* and *Lmod2*, with the subsequent recruitment of the additional constituents of PRC2 by day 6 (Fig. 3C). We could not detect H3K27^{me3} after 2 days of differentiation, but did observe it by 6 days of differentiation in cells stimulated with IFN- γ (Fig. 3D). Thus, the recruitment of JARID2 was not sufficient to induce the methylation of H3K27. We were also interested in the acetylation status of H3K27, because CIITA recruits the histone acetyltransferase CREB-binding protein (p300/CBP) to acetylate this residue. Indeed, we found that exogenous expression of CIITA enhanced the amount of H3K27^{ac} at target promoters on day 2 when compared to the enrichment present in cells deficient in CIITA; however, by day 6, no enrichment of H3K27^{ac} was observed in cells expressing exogenous CIITA, and instead, H3K27 was methylated (Fig. 3E). Thus, it appears that H3K27 was acetylated by day 2 through the recruitment of CIITA, but that it was methylated at day 6 through the recruitment of EZH2.

JARID2 is required for CIITA-mediated repression of muscle-specific genes

To determine whether JARID2 was required to induce the paused RNAPII, we used shRNA constructs to deplete JARID2 from C2C12 cells expressing exogenous CIITA. We first confirmed that the shRNA resulted in depletion of both JARID2 mRNA and protein (Fig. 4A). We repeated the ChIP experiments to pull down the Ser⁵- and Ser²-phosphorylated forms of the CTD, and found that JARID2 was required to induce the poised RNAPII (Fig. 4B). Depletion of JARID2 resulted in the detection of RNAPII-pSer⁵ throughout the coding region, and in the restoration of RNAPII-pSer², which was also detected throughout the coding region of *Tnni2* (Fig. 4B). We also examined the expression of CIITA-repressed genes in JARID2-deficient cells. We found that JARID2 was required for the ability of CIITA to repress the expression of a number of genes, including *Lmod2*, *Tnni2*, *Acta1*, and *Myog* (Fig. 4C). To determine how JARID2 was recruited to CIITA-bound promoters, we investigated the binding of CIITA to JARID2. We found that CIITA physically interacted with JARID2 in human embryonic kidney (HEK) 293 cells transfected with plasmid encoding CIITA (Fig. 4D). This interaction was also detected in C2C12 cells expressing exogenous CIITA at both day 2 and day 6 (Fig. 4E). To confirm the interaction and to determine whether it was mediated by DNA, we repeated the coimmunoprecipitation experiments in C2C12 cells stimulated with IFN- γ for 2 days in the presence or absence of ethidium bromide. We found that endogenous CIITA and JARID2 physically interacted in these cells and that the interaction observed was not mediated by DNA, because it was not affected by the addition of ethidium bromide to the immunoprecipitation reaction (Fig. 4F).

Thus, these data suggest that CIITA recruits JARID2 to repressed promoters to pause RNAPII.

The epigenetic mark on CIITA-repressed genes is not bivalent, because H3K4 is not methylated

The recruitment of CIITA to promoters correlates with the presence of H3K4^{me} (25), whereas the recruitment of JARID2 correlates with a bivalent domain that contains both transcriptionally repressive (H3K27^{me}) and transcriptionally permissive (H3K4^{me}) modifications in ES cells (34). Thus, we mapped the pattern of methylation on H3K4 to determine whether CIITA induced a bivalent state using antibodies specific to the mono-, di- and tri-methylation of H3K4. Trimethylation of H3K4 correlates with gene activation (42). In vertebrates, di-methylation of H3K4 largely co-localizes (is present in the same locus) with H3K4^{me3} (43, 44). As anticipated, we detected tri-methylation of H3K4 at the promoters of *Lmod2* and *Tnni2* during differentiation in C2C12 cells transfected with control plasmid (fig. S5A). The abundance of H3K4^{me3} was relatively low after 2 days of differentiation, but was markedly increased after 6 days of differentiation, which correlated with the peak of gene expression for each of these genes (45). However, in cells expressing exogenous CIITA, the abundance of H3K4^{me3} at promoters after 2 days of differentiation was low and it decreased even further after 6 days of differentiation (fig. S5A). H3K4^{me3} was also detected in the coding region of *Tnni2* in control C2C12 cells, but it was less abundant in C2C12 cells expressing exogenous CIITA (fig. S5A).

We detected low amounts of di-methylated H3K4 that co-localized with H3K4^{me3} in control C2C12 cells after two days of differentiation, and these were modestly reduced in abundance in C2C12 cells expressing exogenous CIITA (fig. S5B). The extent of di-methylation increased substantially in control C2C12 cells after 6 days of differentiation, whereas di-methylation decreased in C2C12 cells expressing exogenous CIITA (fig. S5B).

Mono-methylation was not greatly affected by CIITA, because increased amounts of monomethylated H3K4 were observed at the promoters of *Lmod2* and *Tnni2* after two days of differentiation in cells in either the absence or presence of CIITA (fig. S5C). However, we also detected increased amounts of mono-methylated H3K4 in the coding region of *Tnni2* in control C2C12 cells, and these were substantially reduced in C2C12 cells expressing exogenous CIITA. Thus, the methylation patterns of H3K4 in C2C12 cells expressing CIITA correlated with the lack of expression of these genes and suggest that the H3K27^{me3} modification at day 6 is not bivalent with H3K4^{me3}.

The methylation of H3K9 is not observed on CIITA-repressed genes

Because we had observed the methylation of H3K27 on CIITA-repressed genes on day 6 of differentiation, we were interested in whether H3K27 was the only repressive modification present on these promoters. To address this, we examined the presence of H3K9^{me3}, a well-characterized mark of repression (46). As anticipated, we observed low amounts of H3K9^{me3} in differentiating C2C12 cells transfected with control plasmid, and these amounts were reduced in C2C12 cells expressing exogenous CIITA on days 2 and 6 (fig. S6A). This result indicated that the methylation of H3K9 was not involved in the repression observed at

Lmod2 and *Tnni2*. As positive controls for these experiments, we also examined modifications on promoters known to be enriched for H3K9^{me3} (41), and we observed an enrichment for H3K9^{me3} on the promoters of *Tas2r124* and *Pcdhb18* (fig. S6B).

The pausing of RNAPII is reversible

We previously showed that the repression of myogenesis mediated by IFN- γ and CIITA is reversible, with gene expression being fully restored upon the withdrawal of IFN- γ (35). Thus, we sought to determine whether the paused RNAPII described here was also fully reversible upon withdrawal of IFN- γ . We compared C2C12 cells stimulated with IFN- γ while proliferating and then differentiated for 2 days in the presence of IFN- γ with C2C12 cells that were treated with IFN- γ while proliferating, differentiated for two days in the presence of IFN- γ , and then differentiated for an additional day in the absence of IFN- γ . Consistent with our earlier data with antibodies against RPB1 (35), we detected the unphosphorylated form of RNAPII on the promoters of *Lmod2* and *Tnni2* in response to IFN- γ (fig. S7A). Phosphorylation of Ser⁵ was detected at the promoter regions (fig. S7B), but Ser² phosphorylation was not observed (fig. S7C). None of the forms of RNAPII, either phosphorylated or unphosphorylated, were detected in the coding region of *Tnni2* in the presence of IFN- γ . We also assayed for the presence of H3K4^{me3} and did not detect this modification in cells stimulated with IFN- γ (fig. S7D). In the experiment in which IFN- γ was withdrawn after 2 days, we observed patterns consistent with actively transcribed genes. H3K4^{me3} was robustly detected at the promoters of *Tnni2* and *Lmod2*, and we observed enhanced amounts of the unphosphorylated CTD recruited to each of these promoters. We detected pSer⁵ at the promoters of *Lmod2* and *Tnni2* and at the first intron of *Tnni2*. We observed an inverse pattern for pSer², with low amounts detected at the promoters and intron 1, and increased amounts detected at downstream coding regions and intron 5. Thus, these data suggest that the pausing of RNAPII induced by CIITA is reversible in cells.

IFN- γ and CIITA maintain the abundance of PRC2, and CIITA recruits PRC2 to muscle-specific genes

Our results demonstrating the association of PRC2 with muscle-specific genes after 6 days of differentiation were unexpected, because PRC2 abundance is decreased upon differentiation (31). We examined the abundances of PRC2 components in untreated and IFN- γ -treated C2C12 cells, and found that both IFN- γ and CIITA maintained the abundance of PRC2 in these cells (Fig. 5A). We detected EZH2 in undifferentiated cells, but it became undetectable immediately upon differentiation. SUZ12 was detectable by immunoprecipitation and Western blotting analysis of cells stimulated with IFN- γ , but not of differentiated cells in the absence of IFN- γ (Fig. 5B). To confirm this finding, we repeated the EZH2 experiment in primary myoblasts and found that IFN- γ maintained the abundance of EZH2 in primary muscle cells (Fig. 5C).

To determine whether CIITA was required to maintain EZH2, we stimulated cell lines depleted of CIITA (35) with IFN- γ and induced them to differentiate. We found that CIITA was required for the presence of EZH2 (Fig. 5D). We showed that CIITA interacted with JARID2 (Fig. 4D), so we next asked whether CIITA could also interact with EZH2. Extracts from cells overexpressing CIITA were subjected to immunoprecipitation with antibodies

against EZH2 and JARID2, and samples were analyzed by Western blotting with antibody specific for CIITA. We found that CIITA physically interacted with EZH2 as well as with JARID2 (Fig. 5E). Next, we asked whether JARID2 was required for the interaction between CIITA and EZH2 by performing the co-immunoprecipitation experiments in JARID2-depleted cells, and we found that CIITA did not interact with EZH2 in the absence of JARID2 (Fig. 5F). Thus, it appears that CIITA recruits JARID2 to arrest RNAPII, and that JARID2 then subsequently recruits the additional components of the PRC2 complex, which leads to stable epigenetic silencing of target genes.

To confirm that IFN- γ was required for the increased abundance of PRC2, we used an antibody that recognizes the IFN- γ receptor (IFNGR) and blocks its stimulation with IFN- γ to neutralize IFN- γ . We treated C2C12 cells with IFN- γ in the presence or absence of the IFNGR-blocking antibody, and we found that the expression of *Ezh2*, *Suz12*, and *Ciita* was decreased in the presence of the IFNGR-blocking antibody (Fig. 5G). Blocking the stimulation by IFN- γ also restored the expression of *Myog*, *Tnni2*, and *Acta1* (Fig. 5G), which are inhibited upon stimulation with exogenous IFN- γ (Fig. 5G) (35). To confirm these findings, we repeated these experiments in primary myoblasts, and the results recapitulated what was observed for C2C12 cells (Fig. 5H).

IFN- γ increases the abundance of the PRC2 complex in vivo

To determine whether our results could be recapitulated in vivo, we examined the expression of genes encoding components of the PRC2 complex in mice subjected to large amounts of IFN- γ or to muscle damage. First, we injected CHO cells expressing IFN- γ into the hindlimbs of nude mice. We then measured the expression of four genes normally silenced in differentiated cells (*Ifngr*, *Ciita*, *Ezh2*, and *Suz12*) in the gastrocnemius muscle. We found that *Ifngr* and *Ciita* were increased in expression in mice treated with CHO-IFN- γ cells compared to mice treated with control CHO cells, consistent with the constitutive presence of IFN- γ (Fig. 6A). We also found that the expression of *Ezh2* and *Suz12* was increased in these animals (Fig. 6A). To investigate PRC2 abundance during muscle damage, we injected cardiotoxin (CTX) into the gastrocnemius muscles of mice. The early phase of CTX-induced muscle injury is characterized by muscle degeneration and necrosis, which is accompanied by the inflammatory response, including the secretion of IFN- γ . Inflammation in response to CTX is most active within 1 to 4 days of injection. After this, the regeneration phase activates muscle repair 5 to 6 days after injection, and by 10 days after injection, the overall architecture of the muscle is restored. Thus, we examined the expression of *Ciita* and of genes encoding PRC2 components 4 and 9 days after injection. We found that *Ciita*, *Ezh2*, and *Suz12* were increased in expression during the inflammatory phase (Fig. 6B). By day 9, the expression of each of these genes was decreased and was similar to that in undamaged muscle (Fig. 6B). To understand how chronic IFN- γ affected this gene expression profile, we performed the same experiments in animals injected with IFN- γ -producing CHO cells. As anticipated, we found that the expression of *Ciita*, *Ezh2*, and *Suz12* was not decreased 9 days after injection in the presence of IFN- γ (Fig. 6C).

***Mdx* mice exhibit increased amounts of PRC2**

To model these events in a case of chronic inflammation, we chose *Mdx* mice, the mouse model for muscular dystrophy. We chose *Mdx* mice for this study because these mice have chronically increased amounts of IFN- γ in the serum (13) because of the constant muscle damage that occurs in these animals, and ablation of IFN- γ in these mice improves muscle function (12). We analyzed gene expression in the gastrocnemius and quadriceps (Quad) muscles from 6-week-old *Mdx* mice and wild-type control mice. We found that *Ifngr*, *Ciita*, *Ezh2*, and *Suz12* were increased in expression in the gastrocnemius muscles (Fig. 6D) and the Quad muscles (fig. S8) of *Mdx* mice. We then demonstrated the increased abundance of EZH2 protein in the gastrocnemius muscle of *Mdx* mice by Western blotting analysis (Fig. 6E). To confirm that the increase in the abundance of the PRC2 complex occurred in the muscle fiber, we immunostained gastrocnemius muscles from *Mdx* mice and from nude mice injected with IFN- γ -secreting CHO cells with antibodies against SUZ12. The presence of myofibers was confirmed by staining with antibodies against myosin heavy chain (MyHC). We found that SUZ12 was present in the myofibers of *Mdx* mice and of mice injected with CHO cells secreting IFN- γ , but not in control animals (Fig. 7A). The sections were also stained with antibodies against macrophages to detect the inflammatory infiltrate, but we did not observe any positive staining (fig. S9A). As a positive control, the liver was immunostained with macrophage-specific antibodies, which confirmed the presence of macrophages in that tissue (fig. S9B). Thus, the increased abundances of CIITA and the PRC2 complex in muscle in response to IFN- γ signaling appeared to be specific to the muscle fibers. These data suggest that our results may help in understanding the pathology of inflammatory muscle diseases, such as muscular dystrophy.

Discussion

Here we showed that, upon stimulation of muscle cells with IFN- γ , CIITA was recruited to muscle-specific genes in a myogenin-dependent manner, where it recruited JARID2, thus preventing RNAPII-mediated elongation of transcripts. Subsequently, JARID2 then recruited the PRC2 complex, which methylated H3K27 (Fig. 7B). The recruitment of PRC2 is important, because we also showed that IFN- γ or CIITA maintained the abundance of this complex in cells induced to differentiate. Our data suggest that under sustained conditions of increased amounts of IFN- γ , RNAPII eventually dissociates from the promoter and PRC2 is recruited, leading to stable epigenetic repression. However, under normal conditions, transient stimulation with IFN- γ leads to a paused RNAPII on these genes, enabling the rapid reversal to normal gene expression once IFN- γ is withdrawn. It is likely that the repression is also reversible even after PRC2 is recruited, because PRC2 abundance would be reduced upon withdrawal of IFN- γ . It is intriguing that IFN- γ uses EZH2 in repressing gene expression, because the inflammatory cytokine tumor necrosis factor α (TNF- α) also uses EZH2 to repress *Notch* expression in myoblasts (47).

Promoter-proximal pausing is thought to be a major rate-limiting step in gene regulation (48). Transcriptionally engaged RNAPII paused at the 5' end of a silenced gene was first observed on adult β -globin-encoding genes (49). This finding was subsequently extended to stress-inducible genes such as *Drosophila melanogaster Hsp70*, where promoter-proximal

pausing was first characterized (50). Other work has revealed that promoter-proximal pausing may be much more widespread than was previously thought. In several genome-wide studies, RNAPII was localized at many inactive genes (51-53). RNAPII has been detected on many transcriptionally inert promoters, whereas it has not been detected in the coding regions of these genes. The stalling of RNAPII occurs on hundreds of genes that respond to specific stimuli or developmental cues (53). Another genome-wide study revealed that RNAPII is paused on individual genes within stimulus-responsive gene networks, especially those involved in immune responsive pathways (54). This study is consistent with our results that demonstrated that IFN- γ , a key regulator of the immune response, induced paused RNAPII through CIITA. However, in the earlier work (54), the paused RNAPII was dependent on NELF, whereas we found here that the paused RNAPII induced by IFN- γ was dependent on JARID2. JARID2 is essential for normal development, and the function of JARID2 has been best characterized in ES cells, in which it is thought to function with the PRC2 complex to facilitate transcriptional priming of developmental regulator genes (55). In the absence of JARID2, reduced binding of PRC2 and PRC1 to target gene loci is observed, indicating that JARID2 functions in targeting the polycomb complexes to these loci (34). How JARID2 is targeted is not currently understood. A study showed that *Jarid2*^{-/-} ES cells lack enrichment of RNAPII-pSer⁵, thus implicating JARID2 in establishing or maintaining paused RNAPII (34). Here, we showed that in committed muscle cells induced to differentiate, JARID2 was recruited independently to target genes through an interaction with CIITA, where it induced a paused RNAPII phosphorylated on Ser⁵. Although our work suggests that CIITA and JARID2 physically interact, we do not currently understand whether the interaction is direct or mediated through additional unidentified proteins. JARID2 subsequently recruits the other members of the PRC2 complex and H3K27 is methylated. The epigenetic mark is not bivalent, unlike what has been observed in ES cells. In cardiac cells, JARID2 functions as a repressor of *Notch1* through the recruitment of the histone methyltransferase SET domain, bifurcated (SETDB1), which catalyzes the methylation of H3K9 (56). Here, we did not detect the involvement of the methylation of H3K9 in JARID2-mediated repression.

Our finding that PRC2 is recruited to muscle-specific by CIITA both addresses how PRC2 is recruited to target genes and shows that CIITA maintains PRC2 abundance in cells induced to differentiate. PRC2 is required to maintain satellite cell self-renewal and maintenance (31), thus, the presence of PRC2 in cells induced to differentiate is likely to have widespread effects on gene expression. PRC2 has widespread roles in modulating cell fate during development (57, 58). A study of the cardiac system revealed that PRC2 controls the silencing of SIX homeobox 1 (SIX1), a transcriptional regulator that functions in cardiac progenitor cells (59), highlighting the central role of the PRC2 complex in guiding developmental fate. Thus, our finding that IFN- γ , through CIITA, maintains the abundance of PRC2 has potential implications for the influence of the inflammatory infiltrate resulting from injury or disease on gene regulation during muscle regeneration and repair..

Here, we defined a previously uncharacterized repressive mechanism for CIITA in inhibiting the expression of muscle-specific genes that culminates in the recruitment of PRC2. We showed that IFN- γ or overexpression of CIITA alone maintained the abundance

of an epigenetic modulator of cell fate, PRC2, in muscle cells induced to differentiate, which would be expected to have substantial effects on gene regulation. We demonstrated a role for JARID2 in a committed cell to identify genes targeted for repression through its interaction with the co-activator and co-repressor, CIITA. Our work also shows that JARID2 works by inhibiting the elongation of transcripts by RNAPII before subsequently recruiting the additional components of the PRC2 complex, which catalyze the methylation of H3K27. This work increases our understanding of the molecular mechanisms used by IFN- γ to modulate myogenesis, and shows that IFN- γ alters cell fate by repressing the myogenic program and inducing expression of the genes encoding components of the PRC2 complex. Given the established role of IFN- γ in inflammation and muscle repair, these data enhance our current knowledge of how cytokines modulate muscle homeostasis and regulate muscle regeneration and repair.

Materials and Methods

Cell culture

Proliferating C2C12 cells (ATCC) and HEK 293 cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone) in a humidified CO₂ incubator at 37°C, according to standard protocols (60). To induce differentiation into myotubes, cells were grown to 70% confluence, and the medium was exchanged for DMEM supplemented with 2% horse serum (HS, Hyclone). C2C12 cells were grown in differentiation medium for the number of days indicated in the figure legends. Primary myoblasts were isolated according to standard protocols (61). Stable C2C12 cell lines overexpressing exogenous CIITA were generated as previously described (35). Individual clones were isolated and propagated.

Stimulation of cells with IFN- γ

Cells were treated with recombinant murine IFN- γ (Peprotech). Except where noted, cells were stimulated with 100 U/ml IFN- γ (equivalent to 10 ng/ml). Cells were harvested for the analysis of RNA or protein at defined time points after stimulation with IFN- γ . IFN- γ was added to the medium and replenished every time the medium was changed. For differentiating cells, medium was changed every other day. IFN- γ function was blocked with an IFN γ -specific neutralizing antibody (GR20, ATCC).

Real-time reverse-transcriptase polymerase chain reaction

Isolation of RNA, preparation of complementary DNA (cDNA), and real-time reverse transcription polymerase chain reaction (RT-PCR) assays were performed as described previously (35). Real-time RT-PCR data were collected on an ABI 7300 (Applied Biosystems) with Power SYBR green master mix (Applied Biosystems) for detection. Real-time RT-PCR data were calculated with the comparative Ct method (Applied Biosystems). Standard deviations from the mean of the []Ct values were calculated from three independent RNA samples. Primers specific for the coding regions of *Lmod*, *Tnni2*, *Acta1*, *Myog*, and *Hprt1* were described previously (35). Where possible, we used intron-spanning primers. All real-time RT-PCR assays were performed in triplicate, and three independent RNA samples were assayed for each time point.

Western blotting analysis

Cell extracts were made by lysing cell pellets that had been washed in phosphate-buffered saline (PBS) in radio-immunoprecipitation assay buffer (RIPA) supplemented with protease inhibitors (Complete protease inhibitor, Roche Diagnostics). Protein concentrations were determined by the Bradford assay (Bio-Rad). For each sample, 30 µg of protein was loaded on each gel. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes with a tank blotter (Bio-Rad). The membranes were then blocked with 5% milk and 1× TBST (Tris-buffered saline containing 0.1% Tween 20) and incubated with primary antibody overnight at 4°C. Membranes were then washed with 1× TBST and incubated with the corresponding secondary antibody. Membranes were again washed with 1× TBST and then incubated with chemiluminescent substrate (SuperSignal, Pierce) according to the manufacturer's protocol and bands were visualized by autoradiography. The antibodies used included: anti-myogenin (F5D, DSHB and M225, Santa Cruz Biotechnologies), anti-CIITA (7-1H, Sigma), anti-myc (9E10, Roche), anti-CDK9 (#2316, Cell Signaling), anti-NELF (H-140, Santa Cruz Biotechnologies), anti-JARID2 (H00003720-A01, Novagen), anti-EZH2 (#5246, Cell Signaling), anti-SUZ12 (#3737, Cell Signaling), and anti-GAPDH (Millipore).

Chromatin immunoprecipitation assays

ChIP assays were performed and quantified as described previously (45) with the following modifications. 1×10^7 cells were used for each immunoprecipitation, and protein A agarose beads (Invitrogen) were used to immunoprecipitate the antibody:antigen complexes in all cases, except for the immunoglobulin M (IgM) antibodies specific for phosphorylated Ser² and Ser⁵; these complexes were immunoprecipitated with anti-IgM agarose beads (Sigma) (62). The following antibodies were used: anti-CIITA (7-1H, Sigma), anti-MyoG (F5D, DSHB), anti-CTD (8WG16, Covance), anti-serine 5 (H14, Covance), anti-serine 2 (H5, Covance), anti-NELF (H-140, Santa Cruz Biotechnologies), anti-CDK9 (#2316, Cell Signaling), anti-SPT5 (D-3, Santa Cruz Biotechnologies), anti-H3K4me3 (ab71998, Abcam), anti-H3K4me2 (ab32356, Abcam), anti-H3K4me1 (ab8895, Abcam), anti-H3K9me3 (#4473, Cell Signaling), anti-H3K27ac (#8173, Cell Signaling), anti-H3K27me3 (#9733, Cell Signaling), anti-EZH2 (#5246, Cell Signaling), anti-SUZ12 (#3737, Cell Signaling) and anti-JARID2 (H00003720-A01, Novagen). Rabbit IgG (Santa Cruz Biotechnology) was used as a nonspecific control. Primers spanning the promoters of *Lmod2* (*Lmod2* E1 Forward (F): 5' CACCCTCTCCACATTGTCAC 3' and *Lmod2* E1 Reverse (R): 5' AAAGAACCAGGCATTCAAGG 3'); *Tnni2* (*Tnni2* E12 F: 5' GCCAAAGGAGCAAGAGTTAAAAAT 3' and *Tnni2* E12 R: 5' AGGAGAAAGTGTTCCCAAAATGTC 3'); *Tas2r124* (*Tas2r124* P F: 5' CTCAGAACTCAAGACTGCATAACC 3' and *Tas2r124* P R: 5' CAGCAGATGATTTAGAAACAAAGG 3'); *Pcdhb18* (*Pcdhb18* P F: 5' CATAAAGATGTATGTGTGGGCAC 3' and *Pcdhb18* P R: 5' CAGACTCACTTTAATCTCCCTTGC 3'); and *Hoxb7* (*Hoxb7* F: 5' AAGAGGAAAAAGGGGGTCCT 3' and *Hoxb7* R: 5' TTTGTAGTCGGGGACGTTTT 3') were used to detect promoter enrichment. Primers spanning the coding region for *Tnni2* were *Tnni2 int1* (*Tnni2* int1 F: 5' GCAGAGGGTAAACTGAGGC 3' and *Tnni2* int1 R: 5' CCATGCCAGACTTCTCCTC 3') and *Tnni2 int5* (*Tnni2* int5 F: 5'

CTATCTCCCTGCCCTTTGTG 3' and Tnni2 int5 R: 5' CACCTCCTCACTGTCCCAG 3'). All data were normalized to an E box-containing gene desert region of the *IgH* locus (IgH F: 5' GCCGATCAGAACCAGAACACCTGC 3' and IgH R: 5' TGGTGGGGCTGGACAGAGTGTTC 3'). Real-time PCR analysis on an ABI 7300 (Applied Biosystems) with Power SYBR green master mix (Applied Biosystems) was performed on triplicate samples. Data were analyzed according to the Fold Enrichment method, which represents the ChIP signal as the fold-increase relative to the background signal. Values of $-Ct$ were calculated with the following formula based on the comparative Ct method: $Ct, \text{ template (antibody)} - Ct, \text{ template (IgG)} = -Ct$. Fold enrichments ($-Ct$) were determined with the formula: $2^{-Ct} (\text{experimental}) / 2^{-Ct} (\text{reference, IgH})$. Error bars reflect the standard deviation (SD), which was calculated from replicate $-Ct$ values derived from independent experiments. All ChIP assays shown represent at least three individual experiments.

shRNA-mediated knockdowns

Myogenin was depleted with specific shRNA constructs (pLOK.1, Sigma), as previously described (45). JARID2 knockdown cell lines were generated with shRNA constructs designed by the RNAi Consortium in the pLOK.1 plasmid (Open Biosystems). Five constructs targeting murine JARID2 and one scrambled control were linearized and were used to transfect C2C12 cells, which were selected in culture medium containing puromycin (2 $\mu\text{g/ml}$). Pooled clones were selected and propagated. CIITA knockdown cell lines were selected as described previously (35).

Coimmunoprecipitations

For immunoprecipitation of endogenous proteins, whole-cell extracts were prepared in RIPA buffer, and 300 μg of extract was used for each immunoprecipitation with 1 μg of antibody. To immunoprecipitate exogenous proteins, HEK 293 cells were transiently transfected with a plasmid expressing CIITA (J. Boss, Emory University), and whole-cell extracts were prepared 24 hours after transfection. The antibodies used included: anti-myogenin (F5D, DSHB), anti-CIITA (7-1H, Sigma), anti-myc (9E10, Roche), anti-JARID2 (H00003720-A01, Novagen), and anti-CDK9 (#2316, Cell Signaling). After an overnight incubation, antibody-antigen complexes were collected with protein A agarose beads (Invitrogen). The beads were washed with RIPA buffer and resuspended in protein loading dye. Immunoprecipitated samples with appropriate controls were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membranes for Western blotting analysis. For each immunoprecipitation, the blot was incubated with both antibody specific for the reciprocal factor, to test for the coimmunoprecipitation, and with the antibody used for the immunoprecipitation, to confirm that the immunoprecipitation reaction was successful. All immunoprecipitations were performed at least twice to confirm the results. To detect myogenin in the presence of exogenous CIITA, 600 μg of cell extract was subjected to immunoprecipitation with one anti-myogenin antibody raised in mouse (F5D, DSHB) and detected with another anti-myogenin antibody raised in rabbit (M225, SCBT).

Murine injections and cardiotoxin-induced muscle injury

Mice were injected with CHO cells engineered to secrete IFN- γ (a gift of D. Guttridge, Ohio State University) or with unmodified CHO cells. The CHO cells were harvested by trypsin treatment, counted, washed with PBS, and suspended at 10^{10} cells/100 μ l of PBS. 1×10^{10} cells were subcutaneously injected into the hind flanks of 12-week-old female, athymic nude mice (*Foxn1^{nu}/Foxn1^{nu}*, Jackson Laboratory). There were eight animals in each experimental group. Animals were injected with CHO-IFN- γ cells or with control CHO cells in the left flank. Two weeks after injection, when tumor growth was detectable, animals were either harvested for muscle after euthanasia or were injected with 100 μ l of 10 μ M cardiotoxin (CTX) in the gastrocnemius muscle. Mice were euthanized 4 or 9 days after injection. All animal experiments were conducted according to procedures approved by the Institutional Animal Care and Use Committee at Southern Illinois University.

Immunohistochemistry

5- μ m sections from paraffin-embedded muscle were used for immunohistochemistry. Sections were treated with citrate buffer for 20 min for antigen retrieval, and subsequently were treated with peroxide for 20 min. To detect SUZ12, sections were blocked with 2% normal goat serum, and further treated with streptavidin-biotin block (Vector labs) to reduce background. Sections were then incubated with primary antibodies against SUZ12 (1:100, #3737, Cell Signaling) and detected with a biotinylated secondary antibody (Vector labs) and a fluorochrome-conjugated streptavidin (Jackson ImmunoResearch). To detect myosin heavy chain (MyHC), sections were processed with the MOM kit (Vector labs), with primary antibodies against MyHC (1:100, MF20, Developmental Studies Hybridoma Bank) and Alexa Fluor 488-conjugated anti-mouse secondary antibody (1:500, Invitrogen). Macrophages were detected by blocking sections with 2% normal HS, incubating with primary antibodies against F4/80 (1:50, A-19, Santa Cruz Biotechnology) and detecting with Alexa Fluor 488-conjugated anti-goat secondary antibody (1:500, Invitrogen). Images were captured on a florescent microscope at a magnification of 400 $\times$. Cell nuclei were stained by incubating with DAPI (1 μ M, Invitrogen) for 5 min at room temperature.

Statistical analysis

Data are presented as means $\pm$ the standard deviation (SD). Statistical comparisons were performed by unpaired two-tailed Student's t tests, with a probability value of $P < 0.05$ taken to indicate statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Myogenin is required for the recruitment of CIITA to the promoters of muscle-specific genes

(A) A stable cell line of C2C12 cells depleted of myogenin was established by transfection with a myogenin-specific shRNA construct. Depletion of myogenin was confirmed by analysis of mRNA abundance by quantitative real-time RT-PCR (data are from 3 biological replicates; error bars show SD) and of protein by Western blotting analysis (data are representative of two biological replicates). (B) CIITA is not recruited to muscle-specific gene promoters in the absence of myogenin. Cells described in (A) were stimulated with IFN- γ while undergoing proliferation and differentiation for 6 days. ChIP assays were performed with antibodies against CIITA, and DNA was detected with primers specific for the promoters of *Lmod2* and *Tnni2*. Three biological replicates are represented in the graphs, and error bars represent SD. (C) Myogenin is present at low abundance in cells transfected with plasmid encoding CIITA. Extracts from C2C12 cells transfected with plasmid expressing exogenous CIITA were subjected to immunoprecipitation with antibody specific for myogenin and samples were analyzed by Western blotting to detect myogenin, and GAPDH as a loading control. Immunoprecipitations were performed in duplicate, and a single representative blot is shown. (D) Myogenin is recruited to *Lmod2* and *Tnni2* promoters in C2C12 cells expressing exogenous CIITA. ChIP assays were performed with control C2C12 cells (CIITA –) and C2C12 cells expressing exogenous CIITA (CIITA +) with antibodies against myogenin, and DNA was detected with primers specific for the promoters of *Lmod2* and *Tnni2*. Three biological replicates are represented in the graphs, and error bars represent SD. (E) CIITA is recruited to *Lmod2* and *Tnni2* promoters in C2C12 cells expressing exogenous CIITA. ChIP assays were performed as described in (D) with antibodies against CIITA. Three biological replicates are represented in the graphs, and error bars represent SD.

Fig. 2. CIITA inhibits the recruitment of RNAPII to muscle-specific genes and blocks its elongation of transcripts by inhibiting phosphorylation of RNAPII at Ser²

(A) RNAPII is not recruited to promoters in proliferating cells. Assays were performed on C2C12 cells transfected with control plasmid (CIITA –) or with a plasmid encoding CIITA (CIITA +). ChIP assays were performed on undifferentiated, proliferating cells (UD) with antibodies against the unphosphorylated CTD of RPB1, and DNA was detected with primers specific for the promoters of *Lmod2* and *Tnni2*. (B) RNAPII is recruited to repressed promoters in the presence of CIITA, but is not detected in the coding region. ChIP assays were performed as described in (A) in cells after 2 days of differentiation (D2) with primers specific for the promoter (prom.), the first intron (int.1), and the fifth intron (int.5) of *Tnni2*, as well as the promoter of *Lmod2* (prom.). (C) RNAPII is not detected after 6 days of differentiation. ChIP assays were performed as described in (A) on cells after 6 days of differentiation (D6). (D) Ser⁵ is phosphorylated at the promoters of repressed genes in the presence of CIITA after 2 days of differentiation, but is not detected in the coding region. ChIP assays were performed as described in (A) with antibodies against pSer⁵ and the primers described in (B). (E) pSer⁵ is not detected after 6 days of differentiation. ChIP assays were performed as described in (D). (F) Phosphorylation of Ser² is blocked at day 2 on repressed genes in the presence of CIITA. ChIP assays were performed as described in (A) with antibodies against pSer² and DNA was detected with the primers described in (B). (G) Phosphorylation of Ser² is blocked at day 6 on repressed genes in the presence of CIITA. ChIP assays were performed as described in (F). (H) IFN- γ stimulates the phosphorylation of pSer⁵. ChIP assays were performed as described in (D) with C2C12 cells differentiated for two days (D2) in the presence of IFN- γ . (I) IFN- γ blocks the phosphorylation of Ser². ChIP assays were performed as described in (F) with C2C12 cells differentiated for two days (D2) in the presence of IFN- γ . For (A) to (I), three biological replicates are represented in the graphs, and error bars represent SD.

Fig. 3. JARID2 is recruited to repressed genes and subsequently recruits EZH2 and SUZ12

(A) ChIP assays were performed on C2C12 cells transfected with control plasmid (C2C12 + vector) or with plasmid expressing exogenous CIITA (C2C12 + CIITA) with antibodies against JARID2, EZH2, and SUZ12, and primers against the *Tnni2* promoter. Three biological replicates are represented in the graph, and error bars represent SD. (B) Repressed genes exhibit H3K27^{me3} after 6 days of differentiation. ChIP assays were performed on C2C12 cells expressing exogenous CIITA while proliferating (UD) and after 2 (D2) and 6 (D6) days of differentiation with antibodies against H3K27^{me3}, and DNA was detected with primers specific for *Lmod2* and *Tnni2*. Three biological replicates are represented in the graphs, and error bars represent SD. (C) IFN- γ stimulates the recruitment of JARID2 and PRC2 to repressed genes. ChIP assays were performed on C2C12 cells treated with IFN- γ while proliferating and during differentiation for 2 days (D2) and 6 days (D6) and DNA was detected with primers as described in (B). Three biological replicates are represented in the graphs, and error bars represent SD. (D) IFN- γ stimulates trimethylation of H3K27 by day 6. ChIP assays were performed on C2C12 cells that were treated as described in (B). Three biological replicates are represented in the graphs, and error bars represent SD. (E) Repressed genes show acetylation of H3K27 after 2 days of differentiation and trimethylation of H3K27 after 6 days of differentiation. ChIP assays were performed on C2C12 cells expressing exogenous CIITA after 2 (D2) and 6 (D6) days of differentiation with antibodies against H3K27^{ac} or H3K27^{me3}, and DNA was detected with primers specific for the *Tnni2* promoter. Three biological replicates are represented in the graphs, and error bars represent SD.

Fig. 4. JARID2 is required to pause RNAPII

(A) Stable cell lines expressing shRNA constructs were used to deplete JARID2, which was confirmed by analysis of JARID2 mRNA and protein by real-time RT-PCR (data are from two biological replicates; error bars represent SD) and Western blotting analysis (data are from two biological replicates; error bars represent SD), respectively. (B) JARID2 is required for poised RNAPII. ChIP assays were performed on C2C12 cells expressing exogenous CIITA that were transfected with scrambled shRNA (scr) or JARID2-specific shRNA (JARID2 KD) constructs with antibodies against pSer⁵ or pSer² and primers spanning *Tnni2*. Three biological replicates are represented in the graphs, and error bars represent SD. (C) JARID2 is required for the repression mediated by CIITA. The cell lines used in (B) were analyzed by real-time RT-PCR for the expression of *Lmod2*, *Tnni2*, *Acta1*, and *Myog*. Four biological replicates are represented in the graphs, and error bars represent SD. (D) JARID2 interacts with CIITA. HEK 293 cells were transfected with plasmid encoding myc-tagged CIITA, and cell extracts were subjected to immunoprecipitation with antibody specific for JARID2 or with nonspecific IgG as a negative control. The Western blot was analyzed with antibodies against myc (to detect CIITA) and JARID2. Whole-cell extracts (EXT) were analyzed by Western blotting as a control. Transfections and immunoprecipitations were performed in duplicate, and a representative experiment is shown. (E) JARID2 interacts with CIITA in C2C12 cells. Whole-cell extracts of C2C12 cells expressing exogenous CIITA after the indicated number of days of differentiation were subjected to immunoprecipitation with antibodies against JARID2, and samples were analyzed by Western blotting with antibody against myc to detect exogenous CIITA. Immunoprecipitations were performed in triplicate, and a single representative experiment is shown. (F) Endogenous CIITA and JARID2 interact independently of DNA. The immunoprecipitation experiment described in (E) was performed on C2C12 cells stimulated with IFN- γ in the presence or absence of ethidium bromide (EtBr, 50 μ g/ml), and samples were analyzed by Western blotting with antibodies specific for JARID2 and CIITA. Immunoprecipitations were performed in duplicate, and a single representative experiment is shown.

Fig. 5. CIITA interacts with PRC2 and maintains the presence of the PRC2 complex in differentiated cells

(A) Both IFN- γ and CIITA maintain the presence of EZH2 in differentiated cells. Time-course analysis of cell extracts of control C2C12 cells or CXC12 cells expressing CIITA that were untreated or were treated with IFN- γ when the cells were not undergoing differentiation (UD) or when cells were undergoing differentiation for the indicated numbers of days (D1 to D8). Samples were analyzed by Western blotting with antibodies specific for the indicated proteins. Western blotting was performed in triplicate with independent extracts, and a representative blot is shown. (B) Stimulation with IFN- γ maintains the abundance of SUZ12 in differentiated cells. Cell extracts from C2C12 cells that were untreated or were stimulated with IFN- γ for 6 days were subjected to immunoprecipitation and Western blotting analysis with antibodies against SUZ12. Immunoprecipitations were performed in duplicate, and a single representative blot is shown. (C) IFN- γ maintains the abundance of EZH2 in differentiated primary myoblasts. Primary myoblasts were differentiated in the presence or absence of IFN- γ , as indicated, and were analyzed by Western blotting as described in (A). Western blotting was performed in duplicate with independent extracts, and a single representative blot is shown. (D) CIITA is required for the maintenance of EZH2. Cells depleted for CIITA (CIITA KD) were stimulated with IFN- γ and differentiated for 2 days (D2). Samples were then analyzed by Western blotting as described in (A). Western blotting was performed in duplicate with independent extracts and a representative blot is shown. (E) CIITA interacts with EZH2 and JARID2. C2C12 cells expressing exogenous CIITA were differentiated for 6 days and then subjected to immunoprecipitation with antibodies against EZH2 and JARID2 or with nonspecific IgG as a negative control. Samples were analyzed by Western blotting with antibodies against CIITA, EZH2, and JARID2. Immunoprecipitations were performed in duplicate, and a single representative blot is shown. (F) JARID2 is required for the interaction between CIITA and EZH2. Whole-cell extracts of C2C12 cells expressing exogenous CIITA and transfected with scrambled (scr) or JARID2-specific shRNA (JARID2 KD) were subjected to immunoprecipitation with antibodies against EZH2, and samples were analyzed by Western blotting with antibodies against myc and EZH2. Immunoprecipitations were performed in duplicate, and a representative blot is shown. (G) IFN- γ is required to induce expression of genes encoding components of the PRC2 complex and to repress myogenesis. C2C12 cells were left untreated or were treated with IFN- γ and differentiated for 2 days in the presence or absence of a neutralizing antibody against IFNGR (Neut. ab). Gene expression was assayed by real-time RT-PCR

and the fold-increases in expression of the indicated genes were determined relative to their expression in untreated cells. Three biological replicates are represented in the graphs, and error bars represent SD. (H) Primary myoblasts were treated and analyzed by real-time RT-PCR as described in (G).

Fig. 6. IFN- γ increases the amounts of CIITA and PRC2 in vivo

(A) Gene expression was analyzed by real-time RT-PCR in samples from the gastrocnemius (GA) muscle from mice injected with control CHO cells (CHO) or with CHO cells expressing IFN- γ (CHO+IFN- γ). Eight biological replicates are represented in the graphs, and error bars represent SD. (B) Mice were left untreated (UT) or were injected with cardiotoxin (CTX) and GA muscles were harvested 4 (4D) and 9 (9D) days later. The fold-change in the expression of the indicated genes in injured versus untreated mice was determined by real-time RT-PCR analysis. Four biological replicates are represented in the graph, and error bars represent SD. (C) Mice injected with CHO-IFN- γ cells were injected with cardiotoxin (CTX) and GA muscles were harvested 4 (4D) and 9 (9D) days after injury. The fold-increases in the expression of the indicated genes in treated versus untreated mice were determined by real-time RT-PCR analysis. Four biological replicates are represented in the graph, and error bars represent SD. (D) Expression of genes encoding PRC2 components is increased in *Mdx* mice. Gene expression analysis was performed in GA muscles isolated from 6-week-old wild-type (WT) and *Mdx* mice. Three biological replicates are represented in the graph, and error bars represent SD. (E) Extracts from GA muscles isolated from WT and *Mdx* mice were analyzed by Western blotting with antibodies specific for the indicated proteins. Western blotting was performed in triplicate with independent extracts, and a single representative experiment is shown.

Fig. 7. IFN- γ increases the abundances of CIITA and PRC2 in mouse myofibers

(A) Serial sections from age-matched *mdx* and WT nude mice injected with CHO-IFN- γ cells or with control CHO cells were immunostained with antibodies specific for SUZ12 and MyHC (to detect myofibers) and were stained with DAPI to detect nuclei. Images were taken at a magnification of 400 $\times$. (B) Model for repression of muscle-specific gene expression mediated by IFN- γ and CIITA. Left: During myogenic differentiation (represented by the fusion of green myoblasts to form myofibers), the recruitment of myogenin to promoters activates gene expression. RNAPII is recruited to promoters where it undergoes a phosphorylation cycle from pSer⁵ to pSer². Under normal conditions, the PRC2 complex is silenced upon differentiation. Right: Upon stimulation with IFN- γ , CIITA is induced and recruited to myogenin-dependent genes. CIITA recruits JARID2, which induces a paused RNAPII-pSer⁵ on the promoter. The PRC2 complex, which is increased in abundance in the presence of IFN- γ or CIITA, is then recruited to repressed genes where it methylates H3K27, leading to stable epigenetic silencing of muscle specific genes.