Abstract

**Background:** Core Binding Factor or CBF is a transcription factor composed of two subunits, Runx1/AML-1 and CBF beta or CBFβ. CBF was originally described as a regulator of hematopoiesis.

**Methodology/Principal Findings:** Here we show that CBF is involved in the control of skeletal muscle terminal differentiation. Indeed, downregulation of either Runx1 or CBFβ protein level accelerates cell cycle exit and muscle terminal differentiation. Conversely, overexpression of CBFβ in myoblasts slows terminal differentiation. CBF interacts directly with the master myogenic transcription factor MyoD, preferentially in proliferating myoblasts. In addition, we show a preferential recruitment of Runx1 protein to MyoD target genes in proliferating myoblasts. The MyoD/CFB complex contains several chromatin modifying enzymes that inhibits MyoD activity, such as HDACs, Suv39h1 and HP1β. When overexpressed, CBFβ induced an inhibition of activating histone modification marks concomitant with an increase in repressive modifications at MyoD target promoters.

**Conclusions/Significance:** Taken together, our data show a new role for Runx1/CFBβ in the control of the proliferation/differentiation in skeletal myoblasts.

Introduction

Runx1 (for Runt-related transcription factor 1, also known as AML1 for Acute Myeloid Leukemia 1, CBFA2 or PEBP2zb) belongs to a family of highly homologous heterodimeric transcription factors named Core Binding Factors or CBF (reviewed in [1]). In addition to the Runx1 subunit which binds DNA directly, CBF is composed of a non-DNA-binding subunit named CBFbeta (CBFβ) [2]. Runx1 binds better DNA in the presence of CBFβ. Runx1 was originally identified at a breakpoint on human chromosome 21 in the t(8;21) translocation, known as the most common target of chromosomal translocations in human leukemia [3,4]. Genetic studies showed that Runx1 is essential in the developing murine embryo for definitive hematopoiesis of all lineages [5,6].

There is now strong evidence that Runx proteins are also important for differentiation of multiple cell types, including osteoblasts [7], neurons [8,9], hematopoietic cells of all lineages [5,6,10] and skin epidermis and hair follicle stem cells [11,12]. Runx1 is also involved in promoting senescence in primary mouse fibroblasts [13], and in cell cycle regulation [14–16].

Runx proteins have the potential to either activate or repress transcription in a context dependent manner. Runx1 seems to promote proliferation in progenitor cells, whereas in differentiating cells it cooperates with tissue-specific transcription factors to regulate tissue-specific gene expression. For example, Runx1 cooperates with C/EBPα and C/EBPβ to regulate hematopoiesis and osteogenesis, respectively [17,18]. The dual role of Runx1 in regulating proliferation and differentiation could depend on differential interactions with protein partners, specific for each stage of cell development. The molecular mechanisms underlying such a switch in Runx1 function remain however to be deciphered.

Runx1 and CBFβ have also been linked to skeletal muscle differentiation [19–21], and prevention of muscle wasting [20]. In skeletal muscle, proliferation and differentiation are mutually exclusive. Indeed, skeletal muscle terminal differentiation begins with an irreversible withdrawal from the cell cycle, followed by muscle-specific marker expression [22]. Irreversible cell cycle exit involves a definitive silencing of proliferation-associated genes (reviewed in [23] and references therein). Terminal muscle differentiation is orchestrated by myogenic bHLH transcription factors, such as MyoD and Myf5, two master myogenic determination factors. MyoD is expressed in proliferating myoblasts, but is unable to activate its target genes even when...
spectrometry in the MyoD complex corresponding to CBF
proliferating (prolif.) or differentiating myoblasts (48 h, indicated as
recognizing the native proteins (Figure S8).

We then performed a complementary experiment by transfect-
ing HA-tagged MyoD and/or HA-tagged Runx1 into HeLa cells
stably expressing Flag-HA-CBFβ (ectopic, eCBFβ). We showed
that, indeed, MyoD co-precipitated with eCBFβ (Figure 1C, lane
b). We showed

Here we show that CBF associates with MyoD preferentially in
proliferating myoblasts, and knockdown of Runx1 or CBFβ
accelerates cell cycle exit and terminal differentiation. Conversely,
overexpression of CBF slows cell cycle exit and delays muscle
differentiation. In proliferating myoblasts, the MyoD/CFB complex
contains several chromatin modifying enzymes such as
HDACs. In agreement with this, when overexpressed, CBFβ
maintains histone H3 hypoacetylated, hypomethylated on lysine 4
and hypermethylated on lysine 9, on MyoD target promoters,
along with HDAC1 recruitment, even in differentiation condi-
tions. Finally, Runx1 is recruited to MyoD target genes
preferentially in proliferating myoblasts, when these genes are
repressed. Altogether, our data suggest that CBF transcription
factor plays a pivotal role as a negative regulator of skeletal muscle
terminal differentiation.

Results

CBF Subunits, Runx1 and CBFβ, Interact with MyoD in
Proliferating Myoblasts

In an attempt to characterize MyoD protein partners, we
carried out double-affinity purification of HA-Flag MyoD stably
expressed in HeLa cells (see purification scheme on Figure S1).
MyoD protein complex composition was then analyzed by mass
spectrometry (MS) and western blot (WB). MS analysis of the
purified protein complex revealed some already known partners of
MyoD (Figure S2), such as Id, Pbx1, PC4 and E12/E47, and
partners that had never been described to interact with MyoD.

Figure 1. Runx1 and CBFβ interact with MyoD in vitro and in
proliferating myoblasts. A. Peptide sequences identified by mass
spectrometry in the MyoD complex corresponding to CBFβ protein.
B. Western blot analysis of double-purified Flag-HA-MyoD (MyoD), or
eeluuate from HeLa control cells (Mock) with the indicated antibodies (WB
Ab). S: soluble; C: chromatin associated. C. HeLa cells stably expressing
Flag-HA-CBFβ (eCBFβ) or HeLa control cells stably transfected with the
empty vector (Ctr) were transiently transfected with expressing vectors
for HA-tagged MyoD and/or HA-tagged Runx1. 24 hours post-
transfection, cells were harvested and lysates were used for immuno-
precipitation (IP) with Flag resin to precipitate Flag-HA-CBFβ.
Precipitates were then subjected to western blot using HA Ab (WB
α-HA) to simultaneously detect HA-Runx1, HA-MyoD, and Flag-HA-CBFβ
(discriminated on the gel by their molecular weight). However, we have
checked the identity of each HA-revealed band by using antibodies
recognizing the native proteins (Figure S8). D. Nuclear extracts from
proliferating (prolif.) or differentiating myoblasts (48 h, indicated as
CBFβ and Runx1 are expressed in the C2C12 myoblasts and their protein level do not vary significantly during differentiation (Figure S3). We found that MyoD, and the other myogenic determination factor Myf5, co-precipitated with CBFβ preferentially in proliferating compared to differentiating C2C12 myoblasts (Figure 1D).

To assay whether MyoD has the ability to interact directly with CBFβ, we performed GST pull-down experiments, which showed that GST-Myc-MyoD strongly interacts with Runx1 (Figure 1E, lane 4), but not with CBFβ (Figure 1E, lane 5). The interaction of MyoD with Runx1 was specific; we did not detect any Runx1 signal in the presence of GST protein alone (Figure 1E, lane 8) nor any luciferase signal with GST-MyoD (Figure 1E, lane 7). Interestingly, GST-MyoD interacts with CBFβ only in the presence of Runx1 (Figure 1E, compare lanes 5 and 6), in agreement with our previous findings (Figure 1C). These results show that MyoD interacts directly with Runx1 subunit.

The bHLH and the Transactivating Domains of MyoD, and the Transcription Regulation Domain of Runx1 Are Required for Their Interaction

In an attempt to delimit the domain of MyoD responsible for interaction with Runx1, we used HA-tagged mutants of MyoD transfected into HEK 293 cells. Anti-HA immunoprecipitation revealed an interaction of Runx1 and CBFβ with wild-type MyoD as expected, and only with a MyoD 82-318 mutant, which retains the bHLH and the C-terminal transactivating domains (Figure 2A). Runx1 and CBFβ failed to interact with truncated MyoD versions lacking either the bHLH, i.e., mutants Nter and Cter, or the C-terminal domain, i.e., mutants ΔCter, Nter and bHLH (Figure 2A). These experiments clearly show that the bHLH and the C-terminal transactivating domains are required for interaction with Runx1. Notably, MyoD deletion mutants that do not interact with Runx1 do not interact with CBFβ (Figure 2A). This result, combined with the results presented on Figure 1E, indicate that Runx1 is most likely the subunit that directly interact with MyoD.

To delimit the Runx1 domain involved in the interaction with MyoD, we performed a GST-pull down experiment (Figure 2B). Our results show that the transcription regulation domain located in the C-terminal part of Runx1 is required for the interaction with MyoD (Figure 2B). The interaction of MyoD with Runx1 was specific; we did not detect any Runx1 signal in the presence of GST protein alone nor any luciferase signal with GST-MyoD (Figure 2B).

CBF Negatively Regulates Cell Cycle Exit and Terminal Differentiation in Skeletal Myoblasts

We used siRNAs to decrease Runx1 level in order to investigate its role in differentiating myoblasts (Figure 3A, see Runx1 quantification). Downregulation of Runx1 resulted in a more efficient differentiation (Figure 3A); both the expression of muscle markers and the proportion of multinucleated cells were higher in Runx1-depleted cells (Figure 3A and Figure S4A). In particular, myogenin and MCK (Muscle Creatine Kinase) were expressed at higher levels in Runx1-depleted cells (Figure 3A). Interestingly, cyclin D1 level decreased more rapidly when Runx1 is downregulated (Figure 3A). Similarly, CBFβ downregulation induced an accelerated differentiation (Figure 3B, top panel) and a more rapid decrease in cyclin D1 (Figure 3B, lower panel). Indeed, we could detect MCK expression in CBFβ-depleted myoblasts as soon as 24 h after the induction of differentiation (Figure 3B, top panel). These cells moreover exhibited larger myotubes (Figure S4B). We confirmed these results in primary myoblasts (Figure 3C).

Figure 2. The domains of MyoD and Runx1 involved in their interaction. A. The bHLH domain and the C-terminal transactivating domain of MyoD are involved in the interaction with Runx1 and CBFβ. Top panel, Schematic diagram of MyoD functional domains, TAD: transactivation domain, bHLH: basic Helix Loop Helix. Lower panel, expression vectors for wild-type MyoD and its deletion mutants were transfected in HEK 293 cells using Calcium phosphate precipitation as described in Material and Methods section. 48 h post-transfection, cells were lysed and lysates were subjected to anti-HA immunoprecipitation. Precipitates were then analyzed by western blotting using with the indicated antibodies (WB Ab). *: specific bands. B. The transcription regulation domain located in C-terminal part of Runx1 is required for the interaction with MyoD. Top panel, Schematic diagram of Runx1 protein. The Runx, transcription activation (TA), and transcription inhibition (ID) domains are indicated. Lower panel, Runx1 and its deletion mutants, or luciferase (Luc) were in vitro translated in the presence of 35S-Methionine (inputs shown on the right panel) and incubated with equivalent amounts of GST-MyoD or GST agarose beads (normalization is shown in the lower panel). GST pull-down was then conducted as described in the Material and Methods section, and the radiolabeled proteins were detected by autoradiography.

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In agreement with our previous results, we have found that Runx1 or CBFβ downregulation led to a significant decrease of S-phase cells proportion concomitant with an increase in G1-phase cells (Figure 3D). This suggests that CBF positively regulates myoblasts proliferation.

As for the specificity of the siRNAs, we obtained the same phenotype with three different siRNAs that target CBF: the two targeting CBFβ subunit and the one targeting Runx1 subunit isoforms. Thus, the observed effects are unlikely due to any off-target effect.

To complete our analysis, we studied the effect of CBFβ overexpression on terminal differentiation. Overexpression of CBFβ in C2C12 myoblasts (C2C12-CBFβ) was well tolerated and did not lead to morphological abnormality (Figure S5). However, in differentiation conditions, C2C12-CBFβ cells showed a delay in cell cycle exit, as measured by early-G1 phase cyclin D1 level that decreased with a 24 to 48 h delay compared to control cells (Figure 4A), but not that of late-G1 cyclins A and E (Figure 4C). Note that proliferating C2C12-CBFβ cells contain more cyclin D1 protein then the control cells (Figure 4A), but not cyclins A2 and E (Figure 4C). The delayed cell cycle exit correlated with delayed expression of muscle markers such as myogenin (48 h delay), MCK (24 h delay), and MHC (Myosin Heavy Chain, not detected at 120 h) (Figure 4A). In contrast to control cells, C2C12-CBFβ cells exhibited smaller and mainly mononucleated myotubes (Figure 4B and Figure S5) with low expression of MCK and MHC (Figure 4B) in differentiation conditions. This suggests that differentiation kinetics was not completely impaired but greatly delayed when CBFβ was overexpressed. Further analysis of cell cycle regulators expression showed that, in addition to a delayed decrease in cyclin D1 (Figure 4A and C), cyclin D3 and p21 expression is delayed in C2C12-CBFβ cells compared to C2C12 control cells (Figure 4C).

Altogether, these results suggest that CBF plays a dual role during skeletal muscle differentiation by regulating cell cycle withdrawal and expression of muscle markers.

**CBF Transcription Factor Is Located to MyoD Early Target Genes and Regulates Negatively Their Expression**

The fact that CBF is a transcription factor which interacts with MyoD preferentially in proliferation conditions, led us to investigate whether it would be targeted to MyoD target genes to repress their transcription. Using an in silico approach, we first found that on early target gene promoters of MyoD, Runx1 and CBF were adjacent (Figure S6A). In order to test these observations, we performed ChIP experiments. Our results showed a preferential enrichment in Runx1 on early target genes to regulate negatively their expression in proliferating C2C12 myoblasts. Our ChIP assays showed that Runx1 was not located on late target genes of MyoD, such as Desmin, MHC and MCK (data not shown), while it was on early muscle differentiation genes myogenin, p21 and cyclin D3. In addition, we did not find Runx1 binding sites adjacent to E-boxes on late MyoD target genes' promoters. These findings suggest that Runx1 would essentially regulate early events of skeletal muscle terminal differentiation. Taken together, our results strongly suggest that Runx1 could be recruited onto MyoD early target genes to regulate negatively their expression in proliferating myoblasts.

To gain insights into the mechanism of action of CBF on MyoD target genes, we purified CBFβ protein complex from proliferating C2C12-CBFβ cells via its Flag tag. As expected, CBFβ co-purified......
deacetylases HDACs 1, 2 and 3 (Figure 5B). These interactions
are mediated by Runx1. Indeed, these proteins are already
known partners of Runx1 on the one hand [30], and repressors
of MyoD activity on the other hand [31–33].

Given the association of CBFβ with chromatin-modifying
enzymes, we studied the chromatin status of three target gene
promoters of MyoD in differentiating C2C12-CBFβ using
chromatin immunoprecipitation (ChIP). Our results showed that,
in contrast to control cells in which histone H3 acetylation (a mark
associated with transcription activation) on myogenin, cyclin D3
and p21 promoters increased in differentiation compared with
proliferation conditions, histone H3 acetylation levels at these
promoters did not vary in C2C12-CBFβ cells (Figure 5C). More
generally, in differentiation conditions, we found that activating
marks (histone H3 acetylation, histone H3 lysine 4 tri-methylation)
are abnormally lower on myogenin and cyclin D3 promoters in
C2C12-CBFβ cells compared to control cells (Figure 5D).
Concomitantly, repressive marks (histone H3 lysine 9 tri-
methylation, presence of HDAC1) are higher (Figure 5D).

These results are in agreement with our findings showing that
CBF associates with chromatin modifying enzymes, such as
HDACs, Suv39h1 and HP1β (Figure 5B), which are known to
repress MyoD activity in proliferating myoblasts. Thus, CBF has
an effect on the chromatin structure of three early target genes of
MyoD and contributes to maintain a repressive chromatin state.

Runx1 Represses MyoD Transcriptional Activity

To investigate the effects of Runx1 on MyoD transcriptional
activity, we used a luciferase reporter gene under the control of the
myogenin promoter, which is a direct target promoter of MyoD that
harbors a Runx-binding site adjacent to MyoD-binding site
(Figure S6A). Co-transfection experiments were performed in
HeLa cells line that does not express MyoD endogenously. We
observed that the co-transfection of Runx1-expressing plasmid
with MyoD expression vector resulted in the inhibition of
myogenin promoter activity in a dose-dependent manner (Figure 6).
The inhibitory effect of Runx1 is specific and is MyoD-dependent.
Indeed, it was not seen with Renilla-luciferase expression under a
CMV promoter (used as a normalization control for transfection
efficiency). Thus, Runx1 inhibits MyoD activity.

Discussion

Here we show that CBF transcription factor, composed of
Runx1 and CBFβ subunits, is expressed in proliferating myoblasts
where it interacts with MyoD. Modulating the expression levels of
either Runx1 or CBFβ impaired cell cycle exit and terminal
myogenic differentiation.

Figure 4. Overexpression of CBFβ delays cell cycle exit and
muscle terminal differentiation. A–C. C2C12 cells stably overex-
pressing Flag-HA-CBFβ (C2C12-CBFβ) or control cells (C2C12-Ctr) were
differentiated at the indicated times (in A and C, in hours), and analyzed
by western blotting with the indicated antibodies (WB Ab) (A, C) or by
immunofluorescence (IF) (63× magnification) (B). The kinetic studies were
carried out in the same 10 cm diameter cell culture dish (A, C). IF
experiments using anti-MCK or anti-MHC antibodies were performed 48 h
and 72 h respectively after induction of differentiation. Cells were DAPI-
stained prior to fluorescent microscopy analysis (63× magnification).

with the myogenic factor MyoD and with its dimerization partner
Runx1 (Figure 5B). The other partners that co-purified specifically
with CBFβ are proteins known to be involved in transcriptional repression: the histone 3 lysine 9 (H3K9) methyltransferase
Suv39h1, the heterochromatin protein HP1β, and the histone
decacetylasymes HDACs 1, 2 and 3 (Figure 5B). These interactions

Figure 5. Runx1/CBFβ interact with MyoD

Mass spectrometry analysis revealed that CBFβ is part of MyoD
complex. Further western blot analyses of the same MyoD
complex revealed the presence of Runx1 subunit. We have
narrowed our study to Runx1 because of all the three Runx
proteins, to our knowledge, Runx1 is the only one linked to
skeletal muscle [20]. In addition, Runx1 is one of the MyoD target
genes in myoblasts [34]. Among the other Runx members, Runx2
is mainly involved in osteogenesis, and it has already been shown
that Runx2 expression is not detectable in myoblasts [35].
Moreover, ectopic expression of Runx2 in myoblasts triggers
osteogenic transdifferentiation [35–37]. Finally, concerning
Runx3, it has been shown that it is not expressed in skeletal
muscle and is mainly linked to neurogenesis [38–42]. Thus, we
explored the role of Runx1/CBFβ during muscle skeletal terminal
differentiation. However, Runx1 has been reported to play an

Discussion

Here we show that CBF transcription factor, composed of
Runx1 and CBFβ subunits, is expressed in proliferating myoblasts
where it interacts with MyoD. Modulating the expression levels of
either Runx1 or CBFβ impaired cell cycle exit and terminal
myogenic differentiation.
important role in protecting denervated, fully differentiated, myofibers from atrophy and autophagy [20]. In this paper [20], Runx1 has been assigned a role of a growth-promoting factor of muscle cells to limit muscle wasting. In this very elegant study, the authors generated knock-in homozygous mice carrying inactive Runx1 in skeletal muscle cells. However, Runx1 inactivation was conducted under the indirect control of MCK promoter, which is active only at late stages of terminal differentiation. Thus, with this system, we cannot see the effect of Runx1 inactivation on early stages of terminal differentiation, where MCK promoter is not yet active.

Runx1 and CBFβ Regulate Cell Cycle Exit and Terminal Differentiation

Runx1 or CBFβ downregulation in myoblasts induced an accelerated cell cycle exit. Indeed, cyclin D1 protein disappears are the mean of 3 independent experiments. B. Western blot analysis with the indicated antibodies (WB Ab) of Flag-purified Flag-HA-CBFβ stably expressed in C2C12 cells (CBFβ), or from C2C12 control cells (Ctr). Both inputs are probed in lanes 1–2 and eluates are shown in lanes 3–4. C. Chromatin immunoprecipitation (ChIP) experiments using anti-acetyl H3 antibody were performed from either proliferating (grey bars) or differentiating (black bars) C2C12 control (left) and C2C12-CBFβ (right) cells. We quantified copy numbers of the myogenin (myog.), cyclin D3 and p21 promoter regions harboring the MyoD target sequences. Results are the mean of three measurements. D. ChIP experiments using antibodies against acetyl histone H3 (AceH3), trimethylated histone 3 lysine 4 (me3H3K4), trimethylated histone 3 lysine 9 (me3H3K9), Histone Deacetylase 1 (HDAC1) were performed from differentiating C2C12 control (grey bars) or C2C12-CBFβ (black bars) cells (48 h differentiation time). We quantified copy numbers of the myogenin and cyclin D3 promoter regions harboring the MyoD target sequences. Results with the transcription activating marks (AceH3 and me3H3K4) were normalized using the expressed housekeeping gene 36B4, while the repressive marks (me3H3K9 and HDAC1) were normalized using the repressed major satellite repeats. Results are the mean of three measurements.

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Figure 5. CBF in Muscle Differentiation

A. Western blot analysis with the indicated antibodies (WB Ab) of Flag-purified Flag-HA-CBFβ stably expressed in C2C12 cells (CBFβ), or from C2C12 control cells (Ctr). Both inputs are probed in lanes 1–2 and eluates are shown in lanes 3–4. C. Chromatin immunoprecipitation (ChIP) experiments using anti-acetyl H3 antibody were performed from either proliferating (grey bars) or differentiating (black bars) C2C12 control (left) and C2C12-CBFβ (right) cells. We quantified copy numbers of the myogenin (myog.), cyclin D3 and p21 promoter regions harboring the MyoD target sequences. Results are the mean of three measurements. D. ChIP experiments using antibodies against acetyl histone H3 (AceH3), trimethylated histone 3 lysine 4 (me3H3K4), trimethylated histone 3 lysine 9 (me3H3K9), Histone Deacetylase 1 (HDAC1) were performed from differentiating C2C12 control (grey bars) or C2C12-CBFβ (black bars) cells (48 h differentiation time). We quantified copy numbers of the myogenin and cyclin D3 promoter regions harboring the MyoD target sequences. Results with the transcription activating marks (AceH3 and me3H3K4) were normalized using the expressed housekeeping gene 36B4, while the repressive marks (me3H3K9 and HDAC1) were normalized using the repressed major satellite repeats. Results are the mean of three measurements.

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Figure 6. Runx1 represses MyoD-mediated transcription. Co-transfection into HeLa cells with a myogenin promoter-driven Firefly luciferase reporter plasmid (kind gift of V. Sartorelli, NIH) without or with a fixed amount of MyoD expression vector (800 ng), and increasing amounts of vector expressing Runx1. The quantities of Runx1 expression vector used were: 0, 30, 60, 150, and 300 ng. The total amount of plasmid was normalized when necessary to 300 ng with the empty vector. The inhibitory effect of Runx1 was specific, indeed, it was not seen with a Renilla luciferase expression under a CMV promoter (used as a normalization control for the transfection). Results are the mean of 2 independent experiments performed each in triplicates.

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Figure 6. Runx1 represses MyoD-mediated transcription. Co-transfection into HeLa cells with a myogenin promoter-driven Firefly luciferase reporter plasmid (kind gift of V. Sartorelli, NIH) without or with a fixed amount of MyoD expression vector (800 ng), and increasing amounts of vector expressing Runx1. The quantities of Runx1 expression vector used were: 0, 30, 60, 150, and 300 ng. The total amount of plasmid was normalized when necessary to 300 ng with the empty vector. The inhibitory effect of Runx1 was specific, indeed, it was not seen with a Renilla luciferase expression under a CMV promoter (used as a normalization control for the transfection). Results are the mean of 2 independent experiments performed each in triplicates.

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24 h earlier in differentiation conditions. In addition, Runx1 or CBFβ downregulation led to an increase in G1-phase cells, suggesting that CBF promotes myoblasts proliferation. Conversely, CBFβ overexpression delayed the early-G1 phase cyclin D1 disappearance in differentiation conditions (cyclin D1 is still detectable 72 hours after the induction of differentiation), but not that of late-G1 and early-S phase cyclins A2 and E1. Note that proliferating C2C12-CBFβ cells contain more cyclin D1 protein, but not cyclins A2 and E, than the control cells. Together, these results suggest that CBFβ overexpression impacts on early G1 markers. In addition, expression of the cell cycle exit regulators p21 and cyclin D3 [43,44] is delayed in differentiating CBFβ-overexpressing cells, suggesting a delayed or impaired cell cycle exit and terminal differentiation entry. In agreement with this, our ChIP results showed that in proliferating myoblasts, CBF (via Runx1) is recruited to repressed p21 and cyclin D3, which encode cell cycle exit regulators [43,44]. Together, these results suggest that CBF regulates positively proliferation, and negatively terminal differentiation, of skeletal myoblasts. Thus, CBF impacts on the proliferation/differentiation switch in myoblasts (see model on Figure S7).

In differentiation conditions, C2C12-CBFβ cells showed delayed molecular differentiation (expression of muscle markers), and delayed appearance of myotubes that are abnormally small and mainly mono-nucleated. One alternative explanation is that these cells managed to differentiate correctly, although in a delayed manner, but exhibited a specific block in fusion and multinucleation. Indeed, CBFβ subunit is retained in the cytoplasm by cortical filamins [45]. In muscle cells notably, structural proteins and cell adhesion proteins are required for the reorganization of the cell cytoskeleton during cell fusion to form myotubes [46]. Some studies do suspect a cytoplasmic role for CBFβ [47]. Thus, CBFβ overexpression in myoblasts, especially in the cytoplasm, could have a role in impairing the correct cytoskeleton reorganization during fusion. This could explain the phenomenon we observe in differentiating CBFβ-overexpressing myoblasts. Moreover, we observed a very low expression of myogenin transcription factor in myoblasts overexpressing CBFβ and since that myogenin is involved in cell fusion, this is an alternative explanation of the observed the mono- or di-nucleated myotubes.

**CBF Regulates Muscle Differentiation via a Direct Interaction between MyoD and the Runx1 Subunit**

Our results revealed that the role of CBF in myoblasts is likely to be partly mediated through direct interaction with MyoD. GST pull-down experiments showed that MyoD interacts directly with Runx1 subunit, but not with CBFβ. This is confirmed by the use of MyoD deletion mutants in living cells. Indeed, MyoD deletion mutants that fail to interact with Runx1 do not interact neither with CBFβ. MyoD/Runx1 interaction implicates the bHLH and the C-terminal transactivating domains of MyoD, and the transcription regulation domain of Runx1.

The preferential association of MyoD and CBF in proliferating myoblasts could mean that CBF might be acting as a negative co-factor of MyoD. Indeed, we provide evidence that CBF is recruited to early MyoD target genes, via Runx1, in proliferating myoblasts, where MyoD is mainly associated with transcriptional repressors [24,26,31]. This suggests that CBF may serve for assembly of a transcription repression complex at early MyoD target genes such as myogenin, p21 and cyclin D3 (see our model on Figure S7). As for example, such a mechanism could be involved in the repression of the skeletal muscle acetylcholine receptor gene, which contains a repressive E-box that mediates its repression in proliferating myoblasts [48]. In agreement with this, we found that in proliferating myoblasts, CBF associates with many chromatin modifying enzymes, such as Histone Deacetylases (HDACs 1, 2 and 3), the histone H3 lysine 9 (H3K9) methylase Suv39h1, and Heterochromatin Protein beta (HP1β), which are known to repress MyoD activity in proliferating myoblasts [24,31,49,50]; and already known to interact with Runx1 [30,51,52]. In agreement with this, in myoblasts overexpressing CBFβ, histone H3 acetylation and its trimethylation on lysine 4 (marks of active transcription) are delayed on early MyoD target genes in differentiation conditions. Concomitantly, these genes remain abnormally marked by histone repressive marks (meH3K9, HDAC1).

Interestingly, although MyoD is expressed both in proliferating and differentiating cells, we found that the interaction between MyoD and CBF was lost in differentiating cells. In addition, we showed that overexpression of CBFβ in myoblasts led to stabilization of Runx1 subunit that could more efficiently repress MyoD transactivating activity, which induces a delay in terminal differentiation. In agreement with this, we have shown that Runx1 represses MyoD activity in a gene reporter assay.

We did not succeed to show the concomitant presence of Runx1 and MyoD on MyoD target genes, given that it has previously been demonstrated that in proliferating conditions, only a small fraction of MyoD contributes to the repressive remodeling of its target genes, prior differentiation. Alternatively, Runx1 could prevent the proper binding of MyoD and the recruitment of the transcriptional machinery. Notably, the displacement of Runx1 in differentiating conditions is concomitant with a strong binding of MyoD to its target promoters (data not shown).

MyoD and Runx1 are both subject to post-translational modifications. Notably, MyoD is phosphorylated during the cell cycle while it becomes acetylated during differentiation. Runx1 can also be phosphorylated, acetylated or methylated, while these modifications still need to be characterized in muscle cells and during muscle differentiation. We propose that these modifications could favor or impair MyoD interaction with Runx1, respectively.

**Conclusion**

Our findings concerning the role of CBF in the regulation of the proliferation/differentiation balance are in agreement with several reports. CBF was indeed implicated in skin epidermis and hair follicle differentiation [11,12], as well as in neuronal differentiation [8,9]. These data support an emerging role for Runx proteins in cell fate regulation in many cell lineages. Furthermore, MyoD was also shown to regulate osteogenic differentiation [53]. In addition, it has been shown that muscle satellite cells can differentiate into osteocytes or adipocytes under some conditions [54,55]. Thus, our results point to a model in which CBF and myogenic bHLH protein families could act in concert to induce cell-lineage-specific gene expression, dependent on the extra-cellular stimuli.

In summary, we propose that CBF transcription factor might participate in recruiting chromatin modifying enzymes to repress MyoD early target genes by locally inducing a repressive chromatin structure. Our data reveal a new critical role of CBF in the regulation of the balance between proliferation and differentiation in skeletal muscle cells. They also demonstrate a new mechanism of repression of differentiation genes in proliferating myoblasts.

**Methods**

**Cell Culture**

C2C12, HEK 293 and HeLa-S3 cells were cultured under standard conditions. C2C12 cells and mouse primary myoblasts were cultured and differentiated as described in: [56].
Stable Cell Lines Establishment and Plasmid Construction

A HeLa cell line stably expressing MyoD was established with a transgene encoding for full-length MyoD; and HeLa and C2C12 cell lines expressing CBFβ were established with a transgene encoding for full-length CBFβ. The transgenes were tagged with double-HA (Haemagglutinin) and double-FLAG epitopes at the N-terminus as described in [57].

Control cell lines transduced with the empty vector were also established. Murine CBFβ cDNA (a kind gift from Dr Nancy A. Speck) was amplified by PCR with specific primers with protruding restriction sites (fw-Pspx1: CCGGCTCGAGGCACGCGC-CCGGTCGTCGCCGGG; rev-Not1: ATTTCTATATCGGC-CCGCTAAAGAAGTTGACATCG), and sub-cloned into the Xhol-Not1 sites in the pREV retroviral vector after Pspxl and Not1 digestion (Pspxl is compatible with Xho cloning). Cells were transduced via lentiviral transduction with the empty pREV vector has been established. We carried out double-affinity purification of Flag-HA-CBFβ from HeLa cells (Figure S1), using either nuclear soluble or chromatin fractions. For this, cells were resuspended in a hypotonic buffer (10 mM Tris-HCl pH 7.65; 1.5 mM MgCl₂; 10 mM KCl) and disrupted with 20 strokes of a tight-fitting Dounce homogenizer. The cytosolic fraction was separated from nuclei by 7 min centrifugation at 32000 rpm for 1 h at 4°C. Nuclear soluble and chromatin fractions were then ultracentrifugated at 32000 rpm for 1 h at 4°C. Tagged-MyoD complex were then purified using anti-FLAG antibody immobilized on agarose beads (Sigma). After elution with the FLAG peptide (Ansynth, The Netherlands), the bound complexes containing nucleosomes were further affinity-purified on anti-HA antibody-conjugated agarose (Sigma) and eluted with the HA peptide (Ansynth, The Netherlands). Double-immunopurified complexes were resolved on 4–12% SDS-PAGE bis-Tris acrylamide gradient gel in MOPS buffer (Invitrogen), and stained using either the SilverQuest kit (Invitrogen, Cergy-Pontoise, France) [31], or with Colloidal blue (Invitrogen) for mass spectrometry (MS) analyses. In the latest, bands corresponding to proteins were cut from the gel, trypsin-digested using 0.4 µg of sequencing-grade trypsin (Promega, Charbonnières, France), and identified by MS analysis.

To purify CBFβ complex from C2C12 cells, 3 grams of C2C12-CBFβ cell pellet were used to purify tagged CBFβ using a simple-affinity purification method using Flag resin.

Preparation of Nuclear Extracts

Cells were scraped in a minimal volume of PBS and centrifuged 2 min at 400 g. The pellet was resuspended in 5 volumes of: 20 mM HEPES pH 7, 0.15 mM EDTA, 0.15 mM EGTA, 10 mM KCl, then lysed by addition of NP-40 up to 4.5%. Nuclei were immediately neutralized with addition sucrose buffer (50 mM HEPES pH 7, 0.25 mM EDTA, 10 mM KCl, 70% (m/v) sucrose). After centrifugation (5 min, 2000 g), nuclei were suspended in glycerol buffer (10 mM HEPES pH 8, 0.1 mM EDTA, 100 mM NaCl, 25% glycerol) to remove any trace of cytosolic components and centrifuged again. The nuclei were then resuspended in sucrose buffer n2 (20 mM Tris pH 7.65; 60 mM NaCl; 15 mM KCl; 0.34 M Sucrose) then lysed in a final concentration of 250 mM NaCl using High Salt Buffer (20 mM Tris pH 7.65; 0.2 mM EDTA; 25% glycerol; 900 mM NaCl; 1.5 mM MgCl₂). The lysates were sonicated 5 times for 15 s with a BioRuptor (Diagenode, Liège, Belgium) on “High”, then centrifuged 10 min at 13900 rpm to harvest the total nuclear extracts (supernatant). Protein concentration for each sample was estimated with BCA kit (Perbio, Brebières, France).

Transient Transfections, Flag-Affinity Precipitation of Flag-HA-CBFβ, HA-MyoD Precipitation

For plasmid transfection, 25 µg of pRcCMV-HA-Runx1 (kind gift of Dr I. Kitabayashi, Japan), pCMV-HA-MyoD or pRC-CMV backbone were transfected into HeLa-CBFβ cells, using calcium phosphate pH 7.12, and Flag IPs was performed 24 h post-transfection (results presented on Figure 1C). Each IP was performed with 1.5 mg of total nuclear extracts and with 25 µL stock of ssDNA and BSA-pre-blocked Agarose Flag M2 resin from Sigma. IP was performed on wheel overnight at 4°C. Resin was then washed 5 times with TEGN buffer (20 mM Tris pH 7.65; 0.1 mM EDTA; 10% glycerol; 150 mM NaCl; 0.5% NP-40) and eluted by competition with high-purity Flag peptide at a final concentration of 0.2 mg/ml. The resin-free eluate was retrieved using Clean-up Post reaction columns (Sigma).

For interaction experiments using MyoD deletion mutants, HEK 293 cells were transiently transfected using calcium phosphate at pH 7.12. We used for a 10-cm dish 5 µg of a pCMV-3HA-MyoD or its deletion mutants (Cter 241-318, Nter 1-82-172, bHLH 82-172, ΔNter 82-318), or the empty vector, along with 5 µg of pcDNA3-Runx1 and 5 µg of pcDNA3-CBFβ vectors. 48 h post-transfection, cells were lysed in lysis buffer (300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.4% NP-40, 10 mM MgCl₂) to extract proteins. Anti-HA immunoprecipitation was then performed as described above.

siRNA Transfection

siRNAs were purchased from Sigma (Saint-Quentin Fallavier, France) and were transfected using Hi-Perfect reagent (Qiagen, Courtaboeuf, France) according to the manufacturer recommendations. We usually transfect 0.2 µmol of siRNA per 100 mm cell culture dish. CBFβ targeting siRNA sequences used are: C1: CCGGGAUAUGUGCGACUAU, and C2: UAAUCUUGGGCGGG-GUGAU; Runx1 siRNA: CUGUGAAGUCUUCGAUUU; and the scrambled siRNA: ACUUAACCGGCAUCGCCCTT.

Immunoprecipitation of Endogenous Proteins

For IP, we usually use 2 µg of antibodies, 10 µL protein A/G Sepharose beads from Perbio and 1.2 mg of nuclear extracts from C2C12 cells, or 0.5 mg from primary myoblasts. Elution was performed with 40 µL of 0.1 M glycine pH 2.5, 15 min at 25°C, the eluate was recovered using Spin cleaning-up post-reaction column (Sigma). Acidity was neutralized with Tris pH 8.0 before adding loading buffer.

Western Blotting

For western blotting, protein samples were resolved on pre-cast NuPage 4–12% bis-Tris acrylamide gradient SDS-PAGE gel (Invitrogen, Cergy-Pontoise, France). Proteins were then trans-
ferred onto nitrocellulose membrane during 1 h at 400 mA in transfer buffer (25 mM Tris, 150 mM Glycine, 0.1% SDS and 20% methanol). Membranes are blocked 1 hour in PBS-0.2% Tween, 10% skimmed milk and incubated overnight at 4 °C with primary antibodies. Membranes were incubated with the appropriate secondary antibodies coupled to HRP and revealed using West Dura from Pierce (Perbio, Brevières, France) and ChemiS-mart 5000 system (Vilber Lourmat, Marne-La-Vallée, France).

**Plasmids, GST Fusions and GST Pull-Down**

GST and GST-MyoD plasmid constructs were expressed in *Escherichia coli* strain BL21 and purified using glutathione-sepharose beads according to the manufacturer (Sigma, Saint Quentin-Fallavier, France). Purified proteins were quantified by coomassie staining after SDS-PAGE separation. In vitro transcription and translation (TNT) of pcDNA3-Rux1 and its deletion mutants aa 1-189 and 1-242, pcDNA3-CBFβ and luciferase were performed with Riboprobe in vitro transcription systems (Promega, Charbonnières, France) in the presence of [35S]-labelled methionine.

Agarose beads coated with equal amounts of GST or GST-MyoD (1 µg) were incubated with 10 µL of radioactive TNT reaction in reaction buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.1% Triton) during 2 h at 4 °C. Beads were washed 5 times with wash buffer (50 mM Tris pH 7.6, 300 mM NaCl, 0.5% Triton 100), resuspended and proteins resolved by SDS-PAGE gel and revealed by autoradiography.

**Immunofluorescence**

Cells were cultured in Labtecks permanox (Falcon) and fixed briefly with 4% formaldehyde in PBS. Residual formaldehyde was neutralized with 0.1 M Glycine pH 8.0, and washed with PBS. Cells were permeabilized and blocked using 1% BSA, 1% goat serum, 0.3% Triton-X100 in PBS. Primary and secondary antibodies were diluted in the permeabilizing/blocking solution neutralized with 0.1 M Glycine pH 8.0, and washed with PBS. Cells are then centrifuged and resuspended in PBS. Nuclei are stained with DAPI and the glass lid is fixed using an anti-fading mounting media.

**Antibodies**

The anti-MyoD (C-20), anti-myogenin (M-225), anti-Myf5 (C-20), anti-CBFβ (FL-182), anti-cyclin A2 (C19, sc-996), anti-cyclin D1 (72-13G, sc-450), anti-cyclin D3 (C-16, sc-182), anti-cyclin E (sc-23030), anti-p21 (C-19, sc-397) and normal rabbit IgG antibodies were all purchased from Santa Cruz (Santa Cruz, CA, USA). Rabbit polyclonal anti-Suv39h1 (07-550), anti-trimethyl histone 3 lysine 9 (07-442) and rabbit anti-acetyl histone H3 (06-599) antibodies were obtained from Upstate Biotech (Lake Placid, NY, USA). Anti-HP1β (M0D1A9AS) was from Euro-medex (SouffleWeysehheim, France). Anti-HDAC1 (pAB-653-050) was from Diagenode (Liège, Belgium). Anti-trimethyl H3K4 was from Abcam (Paris, France). Rabbit polyclonal anti-MCK antibody was developed by Dr H. Ito [59]. Anti-Flag and anti-α-tubulin antibodies were purchased from Sigma (Saint-Quentin Fallavier, France). Rat anti-HA antibody was purchased from Roche (Meylan, France). Mouse anti-Runx1 antibody (MAB10062) was purchased from Millipore (Saint Quentin en Yvelines, France) and mouse anti-HDAC 1-3 antibody (611125) from BD Biosciences (Le Pont de Claiix, France). Goat anti-rat IgG Alexa-488-conjugated, anti-rabbit IgG Alexa-488 were from Invitrogen (Cergy-Pontoise, France) and anti-mouse IgG TRITC (T7657) were from Sigma (Saint-Quentin Fallavier, France).

**FACS Analysis**

C2C12 were transfected with the siRNAs as indicated in the Material and Method section. 48 hours post-transfection, cells were washed with PBS, then scraped in 500 µL of PBS. Cells were kept on ice while 4.5 ml of ethanol 70% were added. Then cells are kept at least overnight at −20°C. Propidium iodide (PI) staining proceeds as follows: cells are centrifuged and the pellet is washed with PBS. Cells are then centrifuged and resuspended in 2.0 µL PI solution (PI 25 ng/ml, RNase 200 ng/ml, Triton 0,1%) 30 minutes and kept in the dark. Cells are homogenized by vortexing before analysis. We worked on a Beckman and Coulter FACSc apparatus and we counted at least 3000 events for each condition.

**Chromatin Immunoprecipitation (ChIP)**

ChIP protocol and primers have been described in: [56]. The yet unpublished primers used are: Myogenin f/w: TTATCGAATCTGCTCAGGA, rev: ACGCCAAGCTGCTGCAAGCCA. Cyclin D3 f/w: CTGCTGCTCGCTGCCTCTCA, rev: GACCCATGTCAGATGCCTC. 36B4 f/w: ATTCGACGCTGATGAAAGC.TG; rev: CTGTGATGTCGACACCTGAG.

**Gene Reporter Assays**

HeLa cells at 60% confluence were co-transfected by Calcium Phosphate co-precipitation with a myogen promoter-driven Firefly luciferase reporter plasmid (kind gift of V. Sartorelli, NIH) without or with a fixed amount of MyoD expression vector (800 ng) and increasing amounts of vector expressing Runx1. The quantities of Runx1 expression vector used were: 0, 30, 60, 150, and 300 ng. The total amount of plasmid was normalized when necessary to 300 ng with the empty vector. A Renilla luciferase expression under a CMV (cytomegalovirus) promoter was used as a normalization control for the transfection. 24 h post-transfection, cells were lysed in a reporter lysis buffer (Promega, Charbonnières, France). Luciferase activity was determined using Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was then normalized to the level of Renilla luciferase and to the total protein amount.

**Supporting Information**

**Figure S1** Schematic representation of the purification protocol used to purify the MyoD complex from HeLa cells.  
Found at: doi:10.1371/journal.pone.0009425.s001 (0.38 MB EPS)

**Figure S2** MyoD known partners identified by mass spectrometry in the MyoD complex.  
Found at: doi:10.1371/journal.pone.0009425.s002 (0.05 MB PDF)

**Figure S3** Expression of Runx1 and CBFβ proteins during muscle terminal differentiation. Cellular extracts from proliferating or differentiating C2C12 myoblasts (left panel) or from mouse primary myoblasts (right panel) were subjected to western blot analyses for the expression of CBFβ, Runx1, MyoD, myogenin (Myog) and Muscle Creatine Kinase (MCK). α-tubulin is detected as a loading control. Differentiation times are indicated in hours on the top of each panel. Lanes 1 and 7 correspond to proliferating cells. Rat anti-HA antibody was purchased from Roche (Meylan, France). Mouse anti-Runx1 antibody (MAB10062) was purchased from Millipore (Saint Quentin en Yvelines, France) and mouse anti-HDAC 1-3 antibody (611125) from BD Biosciences (Le Pont de Claiix, France). Goat anti-rat IgG Alexa-488-conjugated, anti-rabbit IgG Alexa-488 were from Invitrogen (Cergy-Pontoise, France) and anti-mouse IgG TRITC (T7657) were from Sigma (Saint-Quentin Fallavier, France).

**Figure S4** Downregulation of CBF subunits expression accelerates muscle terminal differentiation entry. A. C2C12 myoblasts were transfected with control siRNA (Scrambled, Scr) or with Runx1 siRNA as indicated. 48 hours post-transfection (Proliferation) cells were placed in differentiation medium (Differentiation)
for 86 hours. Cells were then analyzed by microscopy (10× magnification). B. As in A, except that we used CBFβ siRNA.

**Figure S5** C2C12-CBFβ cells characterization. A. Expression level of CBFβ in C2C12 control (ctr) and in C2C12-CBFβ, in proliferating myoblasts (0 h) or at the indicate differentiation times (in hours), as measured by western blot using anti-CBFβ antibody. Ex.: exogenous; end: endogenous. B. C2C12 cells stably overexpressing Flag-HA-CBFβ (C2C12-CBFβ) or control cells (C2C12-Ctr) were differentiated for 72 hours and analyzed by light microscopy (10× magnification).

**Figure S6** A. Runx1 and MyoD binding sites found in silico on MyoD target genes myogenin and p21. B. Western blot analysis (with the indicated antibodies) of cell extracts used for the ChIP experiment presented on Fig. 5A. α-tubulin (α-tub.) is used as a loading control.

**Figure S7** Proposed model of skeletal muscle terminal differentiation regulation by CBF and MyoD. Note that in muscle system, proliferation inhibition or cell cycle exit is a pre-requisite to terminal differentiation. Thus, cell cycle exit regulators, such as p21 and cyclod3, are activated early during terminal differentiation. Muscle specific early markers, such as myogenin, are also activated before late muscle markers. In proliferating myoblasts, Runx1/CBFβ (CBF dimer) proteins repress MyoD target genes, possibly via a direct interaction with MyoD. Thus, in proliferating myoblasts, CBF binds early MyoD target genes via Runx1 subunit and recruits chromatin modifying enzymes such as HDAC1, Suv39h1 and HP1. Upon triggering of terminal differentiation, CBF dissociates from the promoters and MyoD recruits activating chromatin modifying enzymes, such as HATs. HDAC1: Histone Deacetylase 1; HAT: Histone acetyltransferase.

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**Author Contributions**

Conceived and designed the experiments: SASA. Performed the experiments: OP VJ OAM PR LF SASA. Analyzed the data: OP VJ OAM PR LF SASA. Contributed reagents/materials/analysis tools: SASA. Wrote the paper: SASA.

**References**

1. Mikail FM, Sinha KK, Samartharajah Y, Nucifora G (2006) Normal and transforming functions of RUNX1: a perspective. J Cell Physiol 207: 562–585.
2. Wang Q, Stacy T, Miller JD, Lewis AF, Gu TL, et al. (1996) The CBFβ subunit is essential for CBFA2alpha (AML2) function in vivo. Cell 87: 697–708.
3. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, et al. (1991) (9;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc Natl Acad Sci U S A 88: 10431–10434.
4. Golob TR, Barker GF, Bohlander SK, Hiebert SW, Ward DC, et al. (1995) Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 92: 4917–4921.
5. Wang Q, Stacy T, Binder M, Marin-Patilla M, Sharpe AH, et al. (1996) Disruption of the Cbfα2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci U S A 93: 3444–3449.
6. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR (1996) The target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 84: 321–330.
7. Komori T, Kishimoto T (1996) Cbfα in bone development. Curr Opin Genet Dev 6: 494–499.
8. Chen CL, Brooun DC, Liu Y, de Nooij JC, Li Z, et al. (2006) Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. Neuron 49: 363–377.
9. Theriault FM, Notall HN, Dong Z, Lo R, Barnabe-Heider F, et al. (2005) Role for Runx1 in the proliferation and neuronal differentiation of selected progenitor cells in the mammalian nervous system. J Neurosci 25: 2050–2061.
10. Ichikawa M, Asai T, Saito T, Seo S, Yamazaki I, et al. (2004) AML-1 is required for megakaryocytopoiesis maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat Med 10: 299–304.
11. Ravet E, Cohen S, Levanon D, Negreanu V, Groner Y, et al. (2006) Dynamic expression of Runx1 in skin affects hair structure. Dev Biol 293: 842–850.
12. Osorio KM, Lee SE, McDermitt DJ, Waghmare SK, Zhang YV, et al. (2006) Runx1 modulates developmental, but not injury-driven, hair follicle stem cell activation. Development 133: 1059–1068.
13. Wotton SF, Blyth K, Kilbey A, Jenkins A, Terry A, et al. (2004) RUNX1 transformation of primary embryonic fibroblasts is revealed in the absence of p53. Oncogene 23: 5476–5486.
14. Britos-Bray M, Ramirez M, Cao W, Wang X, Liu PP, et al. (1999) CBFBeta-SMMHC, expressed in M4eo acute myeloid leukemia, reduces p53 induction and slows apoptosis in hematopoietic cells exposed to DNA-damaging agents. Blood 92: 4544–4552.
30. Reed-Inderbitzin E, Moreno-Miralles I, Vanden-Eynden SK, Xie J, Lutterbach B, et al. (2006) RUNX1 associates with histone deacetylases and SUV39H1 to repress transcription. Oncogene 25: 5777–5786.
31. Yahi H, Fritsch L, Philipot O, Guascioni V, Soudi M, et al. (2006) Differential cooperation between heterochromatin protein HP1 isoforms and MyoD in myoblasts. J Biol Chem 281: 23692–23700.
32. Andreu N, Garcia-Rodriguez M, Volpini V, Frecha C, Molina IJ, et al. (2006) A novel Wiskott-Aldrich syndrome protein (WASP) complex mutation identified in a WAS patient results in an aberrant product at the C-terminus from two transcripts with unusual polyA signals. J Hum Genet 51: 92–97.
33. Ayyanathan K, Lechner MS, Bell P, Mail G, Schultz DC, et al. (2003) Regulation of HP1 in a euchromatin gene induces mitotic instability, ephemeral gene silencing: a mammalian cell culture model of gene variation. Genes Dev 17: 1855–1869.
34. Ishibashi J, Perry RL, Asakura A, Rudnicki MA (2005) BMP2 regulates the transcriptional activity of the t(8;21) and t(12;21) fusion proteins in acute myeloid leukemia. Oncogene 24: 175–185.
35. Inoue K, Ozaki S, Shiga T, Ito K, Masuda T, et al. (2002) Runx3 controls the expression of AML2/CBFA3 in hematopoietic cells through the retinoic acid receptor alpha-dependent signaling pathway. J Biol Chem 274: 21651–21658.
36. Gersbach CA, Byers BA, Pavlath GK, Garcia AJ (2004) Runx2/Cbfa1 and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20: 8783–8792.
37. Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, et al. (2000) Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20: 8783–8792.
38. Ait-Si-Ali S, Guascioni V, Fritsch L, Yahi H, Sekhri R, et al. (2004) A Suv39h1-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. Embo J 23: 4343–4354.
39. Mal AK (2006) Histone methyltransferase Snur39h1 represses MyoD-stimulated myogenic differentiation. Embry J 23: 3323–3334.
40. Ishibashi J, Perry RL, Asakura A, Rudnicki MA, Sodek J, Cheifetz S (2004) MyoD promotes osteoblast differentiation by stimulation of the osteonectin promoter. Endocrinology 149: 3698–3707.
41. Levanon D, Negreanu V, Bernstein Y, Bar-Am I, Avivi L, et al. (1994) AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. Genomics 23: 425–432.
42. Levanon D, Negreanu V, Bernstein Y, Bar-Am I, Avivi L, et al. (1994) A large variety of alternatively spliced and differentially expressed mRNAs are encoded by the human acute myeloid leukemia gene AML1. DNA Cell Biol 13: 175–185.
43. Meyers S, Lenny N, Sun W, Hiebert SW (1996) AML-2 is a potential target for transcriptional regulation by the c(8;21) and t(12;21) fusion proteins in acute leukemia. Oncogene 13: 303–312.
44. Le XF, Groner Y, Kornblau SM, Gu Y, Hittelman WN, et al. (1999) Regulation of AML2/CBFA3 in hematopoietic cells through the retinoid acid receptor alpha-dependent signaling pathway. J Biol Chem 274: 21651–21658.
45. Halevy O, Novitch BG, Spacer DB, Skapek SX, Rhee J, et al. (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science 267: 1018–1021.
46. Cenciarelli C, De Santa F, Puri PL, Mattei E, Ricci L, et al. (1999) Critical role played by cyclin D3 in the MyoD-mediated arrest of cell cycle during myoblast differentiation. Mol Cell Biol 19: 5203–5217.
47. Yoshida N, Ogata T, Tanabe K, Li S, Nakazato M, et al. (2005) Filamin A interacts with PEBP2beta/CBFbeta and is retained in the cytoplasm and prevented from functioning as a partner of the Runx1 transcription factor. Mol Cell Biol 25: 1003–1012.
48. Buckingham M (1992) Making muscle in mammals. Trends Genet 8: 144–148.
49. Chiba N, Watanabe T, Nomura S, Tanaka Y, Shimizu T, et al. (1997) The histone variant mH2A1.1 interferes with transcription by down-regulating the acetylcholine receptor delta-subunit gene during myogenesis. Mol Cell Biol 17: 4661–4670.
50. Mal AK (2006) Histone methyltransferase Suv39h1 represses MyoD-stimulated myogenic differentiation. Embry J 23: 3323–3334.
51. Chakraborty S, Sinha KK, Senyuk V, Nacifora G (2003) SUV39H1 interacts with AML1 and abrogates AML1 transactivity. AML1 is methylated in vitro. Oncogene 22: 5229–5237.
52. Taniuchi I, Littman DR (2004) Epigenetic gene silencing by Runx proteins. Oncogene 23: 4343–4345.
53. Hewitt J, Lu X, Gilbert L, Nanes MS (2008) The muscle transcription factor MyoD promotes osteoblast differentiation through cooperation of its NH2- and COOH-terminal regions. Genes Dev 22: 3608–3617.
54. Komaki M, Asakura A, Rodnicki MA, Sodek J, Cheifetz S (2004) MyoD enhances BMP7-induced osteogenic differentiation of myogenic cell cultures. J Cell Sci 117: 1457–1468.
55. Asakura A, Komaki M, Rodnicki MA (2003) Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation 68: 245–253.
56. Ait-Si-Ali S, Guascioni V, Fritsch L, Yahi H, Sekhri R, et al. (2004) A Suv39h1-dependent mechanism for silencing Sphase genes in differentiating but not in cycling cells. Embo J 23: 605–615.
57. Robin P, Fritsch L, Philipot O, Swinarchuk F, Ait-Si-Ali S (2007) Post-translational modifications of histones H3 and H4 associated with the histone methyltransferases Snur39h1 and G9a. Genome Biol 8: R270.
58. Ouazarhui K, Hadj-Slimane R, Ait-Si-Ali S, Robin P, Mietton F, et al. (2006) The histone variant mH2A1.1 interferes with transcription by down-regulating PARP-1 enzymatic activity. Genes Dev 20: 3324–3336.
59. Ito H, Kamei K, Inaguma Y, Kato K (2001) Regulation of the levels of small heat-shock proteins during differentiation of C2C12 cells. Exp Cell Res 266: 213–221.