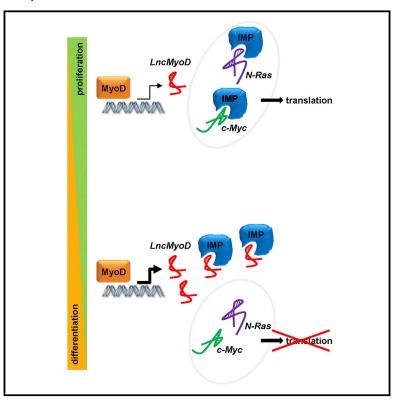
# **Developmental Cell**

### A Long Non-coding RNA, LncMyoD, Regulates **Skeletal Muscle Differentiation by Blocking IMP2-Mediated mRNA Translation**

### **Graphical Abstract**



#### **Authors**

Chenguang Gong, Zhizhong Li, Krishnan Ramanujan, ..., Yunyu Zhang, Sophie Lemire-Brachat, David J. Glass

#### Correspondence

david.glass@novartis.com

#### In Brief

Long non-coding RNAs are regulators of various biological functions. Gong and Li et al. show that LncMyoD is a LncRNA target of MyoD during myogenesis and is required for myoblast differentiation by affecting IMP2-mediated mRNA translation. *LncMyoD* is functionally conserved between mouse and human, despite limited sequence homology.

#### **Highlights**

- LncMyoD is directly activated by MyoD during myogenesis
- LncMyoD binds to IMPs and regulates mRNA translation
- Human and mouse LncMyoD are functionally conserved despite low sequence homology

#### **Accession Numbers**

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## A Long Non-coding RNA, *LncMyoD*, **Regulates Skeletal Muscle Differentiation** by Blocking IMP2-Mediated mRNA Translation

Chenguang Gong,<sup>1,3</sup> Zhizhong Li,<sup>1,3</sup> Krishnan Ramanujan,<sup>1</sup> Ieuan Clay,<sup>2</sup> Yunyu Zhang,<sup>1</sup> Sophie Lemire-Brachat,<sup>2</sup> and David J. Glass1,\*

#### **SUMMARY**

Increasing evidence suggests that long non-coding RNAs (LncRNAs) represent a new class of regulators of stem cells. However, the roles of LncRNAs in stem cell maintenance and myogenesis remain largely unexamined. For this study, hundreds of intergenic LncRNAs were identified that are expressed in myoblasts and regulated during differentiation. One of these LncRNAs, termed LncMyoD, is encoded next to the Myod gene and is directly activated by MyoD during myoblast differentiation. Knockdown of LncMyoD strongly inhibits terminal muscle differentiation, largely due to a failure to exit the cell cycle. LncMyoD directly binds to IGF2-mRNA-binding protein 2 (IMP2) and negatively regulates IMP2-mediated translation of proliferation genes such as N-Ras and c-Myc. While the RNA sequence of LncMyoD is not well conserved between human and mouse, its locus, gene structure, and function are preserved. The MyoD-LncMyoD-IMP2 pathway elucidates a mechanism as to how MyoD blocks proliferation to create a permissive state for differentiation.

#### **INTRODUCTION**

Long non-coding RNAs (LncRNAs) constitute a new class of genes recently identified in various tissues (Cabianca et al., 2012; Guttman et al., 2009, 2011; Huarte et al., 2010; Lee, 2012; Ørom et al., 2010; Rinn et al., 2007; Tsai et al., 2010). Thousands of LncRNAs have been annotated in various cells, but few have been functionally studied. LncRNAs play important roles in normal physiology as well as in many diseases, including embryonic stem cell maintenance, organ development, and cancer progression (Gupta et al., 2010; Guttman et al., 2011; Klattenhoff et al., 2013; Loewer et al., 2010; Nagano et al., 2008; Pasmant et al., 2007; Yildirim et al., 2013). However, the number of LncRNAs expressed in skeletal muscle stem cells and the determination as to whether they are

biologically important remains largely unknown (Cesana et al., 2011; Mousavi et al., 2013). In additional, few functionally conserved LncRNAs between mouse and human have been identified.

While many early studies have been focused on LncRNAs' roles in epigenetic modification, it is clear that LncRNAs are a large collection of functionally diverse genes that regulate biology through many additional mechanisms (Wang and Chang, 2011). In addition to their roles in epigenetic regulation, they may also participate in regulating RNA transcription, splicing and trafficking, RNA stability, microRNA regulation, translation, etc. (Wang and Chang, 2011). It is important to identify new protein-binding partners of LncRNAs and mechanisms of LncRNA biology to help categorize LncRNAs into functional

Myogenesis is a highly coordinated process that includes sequential steps of activation of muscle stem cells, proliferation and then differentiation of myoblasts, and cell fusion to form multinucleated myotubes (Berkes and Tapscott, 2005). Of all the factors that have been identified in regulating myogenesis, MyoD is one of the most critical transcriptional factors (Berkes and Tapscott, 2005). Overexpression of MyoD alone is sufficient to reprogram fibroblasts into muscle cells (Tapscott et al., 1988; Choi et al., 1990). MyoD is particularly important in myoblasts and controls early myogenesis (Berkes and Tapscott, 2005), which requires both myoblast cell-cycle arrest and initiation of a differentiation program. It is well established that MyoD can induce myoblast differentiation by activating downstream myogenic factors such as myogenin. However, how MyoD controls myoblast cell-cycle exit, a crucial step in myogenesis, is not entirely understood (Crescenzi et al., 1990).

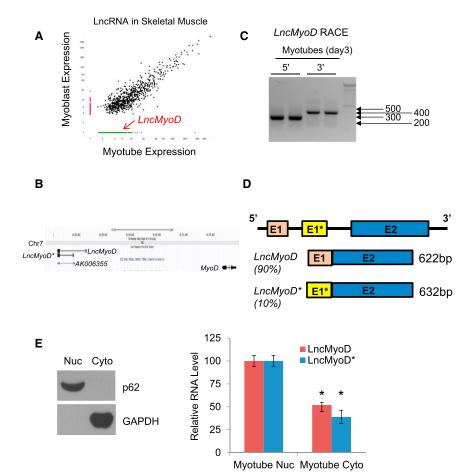
In the current study, we sought to identify novel and functionally important LncRNAs during myogenesis. We identified hundreds of LncRNAs that are actively regulated in myoblasts. Further, we determined through loss-of-function experiments that LncMyoD is a key downstream target of MyoD and an important regulator of myoblast cell-cycle exit and myogenesis. We also demonstrate that human LncMyoD and mouse LncMyoD are functionally conserved without strong sequence homology, supporting the argument that RNA sequence conservation is not indicative of LncRNA function.



<sup>&</sup>lt;sup>1</sup>Novartis Institutes for Biomedical Research, Cambridge, MA 02139, USA

<sup>&</sup>lt;sup>2</sup>Novartis Institutes for Biomedical Research, Basel 4002, Switzerland

<sup>\*</sup>Correspondence: david.glass@novartis.com http://dx.doi.org/10.1016/j.devcel.2015.05.009



	ID	C/NC	CODING POTENTIAL SCORE
	LncMyoD	noncoding	-0.772592
	LncMyoD*	noncoding	-0.71427
	MyoD	coding	0.691843
	HOTAIR	noncoding	-1.18564

#### **RESULTS**

F

## Identification of Skeletal Muscle LncRNAs, Including LncMyoD

To systematically identify LncRNAs in the skeletal muscle system, we analyzed deep RNA sequencing (RNA-seq) data from C2C12 myoblasts and early myotubes (3 days after differentiation) (Trapnell et al., 2010). After eliminating protein-coding genes, 1,183 intergenic LncRNAs and 27 microRNA precursors were found to be expressed in C2C12 cells (Figure 1A; Table S1). Most of the identified microRNAs have been previously shown to be expressed and to play important roles in muscle differentiation, such as *Mir1*, *Mir24*, *Mir27*, *Mir133*, *Mir205*, and *Mir296* (Figure S1A) (Chen et al., 2006; Ge and Chen, 2011).

Among the 1,183 LncRNAs identified, 738 were expressed at similar levels before and after myoblast differentiation (myoblasts versus myotubes) (Figure 1A; Table S1). Interestingly,

### Figure 1. Identification and Characterization of *LncMyoD*

(A) A total of 1,183 LncRNAs were identified from skeletal muscle as regions of contiguous coverage in mRNA-seq data from undifferentiated and differentiated C2C12 cells. Most of these were found to be expressed at similar levels in myoblasts and myotubes (black dots), while subsets were either enriched in undifferentiated myoblasts (pink dots) or differentiated multi-nuclear myotubes (green dots). *LncMyoD* was one of the top LncRNAs that was found to be highly enriched in myotubes.

(B) *LncMyoD* locates next to the *MyoD* gene on mouse chromosome 7. *LncMyoD* is encoded by the (-) DNA strand, while *MyoD* is coded by the (+) DNA strand.

(C) 5' RACE and 3' RACE of LncMyoD in myotubes.

(D) LncMyoD and LncMyoD\* both contain two exons and share the second exon. LncMyoD is the major isoform and accounts for 90% of the transcripts.

(E) LncMyoD was enriched in myotube nuclei, since about 70% of the spliced LncMyoD was located in the nucleus. LncMyoD mRNA levels were upregulated during myoblast differentiation. \*p < 0.05. Error bars depict mean ± SEM.

(F) LncMyoD and LncMyoD\* were predicted to be non-coding RNAs. The RNA sequences of LncMyoD, MyoD, and HOTAIR were put into the Coding Potential Calculator (CPC) program, and both LncMyoD and HOTAIR were predicted to be non-coding RNAs, while MyoD RNA was identified to code for protein.

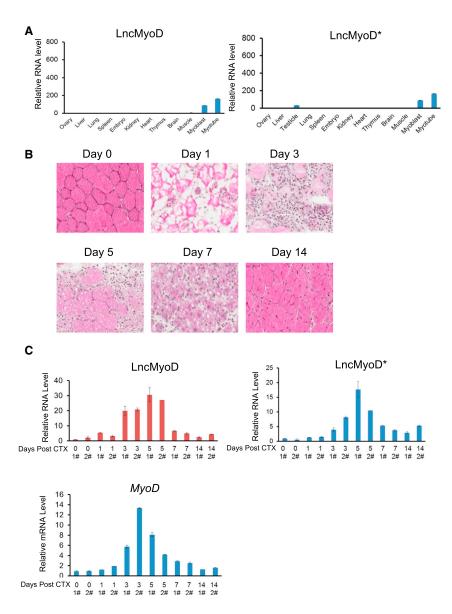
there were 51 LncRNAs that were enriched in myoblasts (downregulated during differentiation) and 394 LncRNAs that were enriched in myotubes (upregulated during differentiation) (Figure 1A; Table S1).

We first focused our study on those LncRNAs that were temporally regulated during myoblast differentiation because

they could serve as regulators of myogenesis. Since many LncRNAs have been shown to either positively or negatively regulate neighbor genes (Ørom et al., 2010; Rinn et al., 2007; Tsai et al., 2010), the genomic locations of these were further characterized. Furthermore, recently, it was pointed out that many regulatory RNAs seem to be expressed proximal to the muscle regulatory genes that regulate myogenesis: MyoD and myogenin. These "eRNAs" include long non-coding RNAs that act in cis and trans (Mousavi et al., 2013). We, therefore, decided to focus on one LncRNA in particular that is located about 30 kb upstream of the mouse Myod1 gene (Figure 1B) and named this LncRNA LncMyoD.

### Characterization of the *LncMyoD* Sequence and Expression Pattern

5' RACE (Rapid Amplification of cDNA Ends) and 3' RACE demonstrated that the length of *LncMyoD* was 622 base pairs



(bp) (Figures 1C and S1B). The gene contains two exons and one intron (Figure 1D), and, like many LncRNAs, it is poly(A) tailed (Figure S1C). We also identified a minor isoform (10% of the RACE product), termed *LncMyoD*\*, that shares the large Exon2 with LncMyoD but has a different Exon1 (Figures 1D and S1B). LncMyoD was not previously annotated, and LncMyoD\* significantly overlaps with a known transcript AK006355. Both LncMyoD and LncMyoD\* are strongly upregulated upon differentiation from myoblasts to myotubes (Figures 1A, S1A, and S1C). Cell fractionation followed by qRT-PCR demonstrates that about 70% of the spliced LncMyoD transcript resides in the nucleus (Figure 1E). Consistent with LncMyoD being a non-coding RNA, it harbors no open reading frames (ORFs) larger than 200 bp; the CPC (coding potential calculator) computational algorithm (Kong et al., 2007) predicts that LncMyoD has a very low coding potential, similar to HOTAIR, a well-known LncRNA (Figure 1F). We found no evidence of a protein product from *LncMyoD*, using an in vitro translation system (data not shown).

#### Figure 2. Expression Pattern of LncMyoD in Different Tissues and during Muscle Regeneration In Vivo

(A) LncMyoD expression was only detected in myoblasts and early myotubes, but not in any other tissues examined, including ovary, liver, lung, spleen, embryo, kidney, heart, thymus, brain and (notably) mature skeletal muscle. Error bars depict mean ± SEM.

(B) Skeletal muscles were injected with CTX, and samples were harvested at different time points from day 0 to day 14. Tissue samples were stained by H&E and the staining clearly demonstrated the steps of muscle regeneration from intact muscle (day 0). tissue damage (day 1), myoblast proliferation (day 3), muscle differentiation (days 5-7) and completed regeneration (day 14).

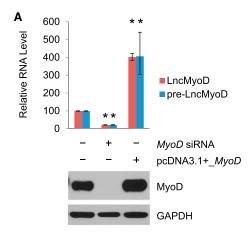
(C) LncMyoD was induced after muscle injury and mirrored the MyoD mRNA expression pattern during skeletal muscle regeneration. LncMyoD was strongly upregulated at day 3 and day 5 after CTX-induced muscle regeneration and began to decrease after day 7. Skeletal muscle mRNAs were extracted from two animals at each time point during muscle regeneration. Error bars depict mean  $\pm$  SEM.

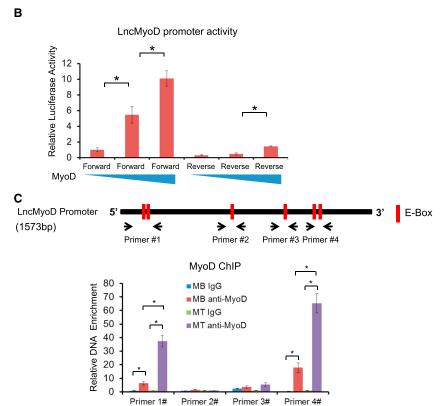
We next examined the expression profile of LncMyoD in multiple adult tissues. LncMyoD was not expressed in any adult tissues tested (Figure 2A). Interestingly, like MyoD, LncMyoD is not expressed in mature skeletal muscle (Figure 2A), suggesting that the gene is temporally upregulated during early differentiation of myoblasts to myotubes but eventually turned off as the muscle matures into post-differentiated muscle fibers. To test the correlation between LncMyoD levels and differentiation in vivo, the gene was examined during a period of induced muscle regeneration, using a cardiotoxin (CTX)-injury assay (Figure 2B). The LncMyoD level was low in unin-

jured muscle and strongly upregulated at 3-5 days after muscle injury. It began to be downregulated after day 5, when the muscle regeneration entered the late stage (Figure 2C). Notably, this expression pattern is almost identical to that of MyoD mRNA (Figure 2C). While the LncMyoD\* level was significantly lower in all experiments, it followed the same expression pattern as that of LncMyoD (Figures 2A and 2C).

#### LncMyoD Is a Direct Target of MyoD

To determine the upstream factor (or factors) regulating LncMyoD during myogenesis, we analyzed the 5' and intron sequences of the gene and found that there are six canonical MyoD binding sites (E-Boxes) in this region (Figures S2A and S2B), raising the possibility that LncMyoD is a direct target of MyoD. Such a finding would be consistent with the coincident expression profiles of LncMyoD and MyoD. Knocking down or overexpressing MyoD led to downregulating or upregulating *LncMyoD* transcription, respectively (Figure 3A).





We next cloned the LncMyoD regulatory element into a PGL3 luciferase reporter construct. When the reporter was cotransfected with a MyoD overexpression construct, it showed dose-dependent activation by MyoD (Figure 3B), suggesting that MyoD could directly promote LncMyoD transcription. Unlike enhancers, the activity of a promoter is usually orientation dependent. Consistent with this, the "reverse reporter" had much weaker baseline activity and could only be modestly activated by MyoD (Figure 3B). To directly determine which site(s) MyoD binds to within the LncMyoD promoter, chromatin immunoprecipitation (ChIP) was performed. MyoD was found to strongly bind to a 5' E-Box tandem and 3' E-Box tandem in the LncMyoD promoter (Figure 3C). Importantly, the binding of MyoD was significantly weaker in myoblasts than in myotubes,

#### Figure 3. MyoD Binds to the LncMyoD Promoter and Directly Activates LncMyoD Transcription

(A) MyoD regulates LncMyoD at the transcriptional level. Knocking down or overexpressing MyoD, downregulates or respectively. upregulates LncMyoD and pre-LncMyoD. \*p < 0.05. Error bars depict mean ± SEM.

(B) The LncMyoD promoter could be activated by MyoD. A LncMyoD-Pro-Luciferase construct demonstrated a dose-dependent activation by MyoD protein. When the promoter was reversed, its activity significantly decreased, and the response to MvoD was modest. \*p < 0.05. Error bars depict mean ± SEM.

(C) MvoD bound to E-boxes on the LncMvoD promoter. ChIP experiments indicated that MyoD bound to the first and last E-Boxes on the LncMyoD promoter regions in vivo. Notably, such binding was stronger in myotubes than in myoblast. \*p < 0.05. Error bars depict mean ± SEM.

consistent with low expression of LncMyoD in myoblasts (Figures 1A and 1E). Together, these data demonstrate that LncMyoD is a direct MyoD target.

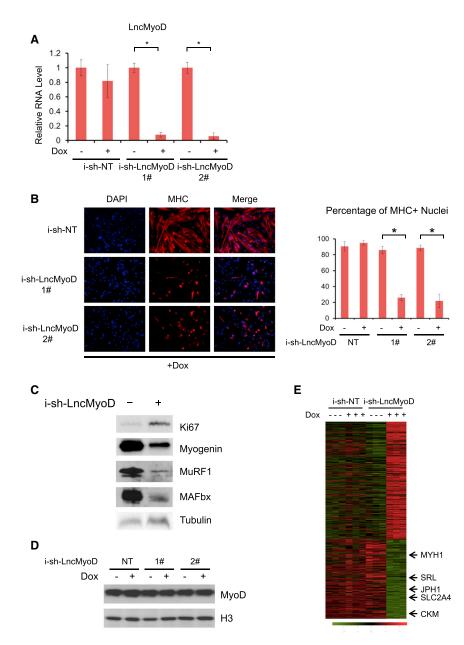
#### LncMyoD Is Required for Myoblast **Differentiation without Altering MyoD** mRNA or Protein Level

expression profile of LncMyoD prompted us to investigate its function during muscle differentiation. Two doxycycline (Dox)-inducible shRNAs successfully knocked down LncMyoD levels by more than 80% (Figure 4A). When myoblasts were induced to differentiate, knockdown of LncMyoD resulted in significant inhibition of terminal differentiation, as demonstrated by the reduced Myosin Heavy Chain (MHC) staining coincident with the knockdown (Figure 4B). After LncMyoD knockdown, a high percentage of myoblasts initially maintained an undifferentiated, round morphology (Figure S3A) and still expressed relatively high levels of Ki67 (a marker of proliferation),

even while in differentiation medium (Figure 4C), suggesting that LncMyoD is essential for cell-cycle withdrawal, a key step in myoblast differentiation.

Notably, while LncMyoD knockdown blocked myoblast differentiation, it did not change MyoD mRNA (Figure S3B) or protein levels (Figure 4D). This finding is consistent with the idea that MyoD is upstream of and regulates LncMyoD and that there is no feedback signaling of LncMyoD to MyoD, thus suggesting that LncMyoD is different from other MyoD neighbor LncRNAs (Mousavi et al., 2013) and leaving open the question of the downstream pathway controlled by this LncRNA.

In order to perform an unbiased search for downstream signaling pathways perturbed by LncMyodD downregulation,



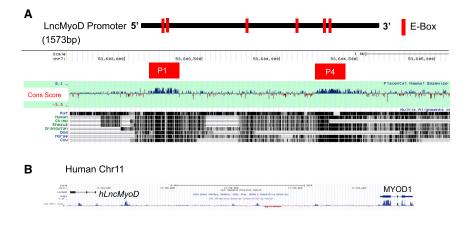
#### Figure 4. LncMyoD Plays Important Roles in **Myoblast Differentiation**

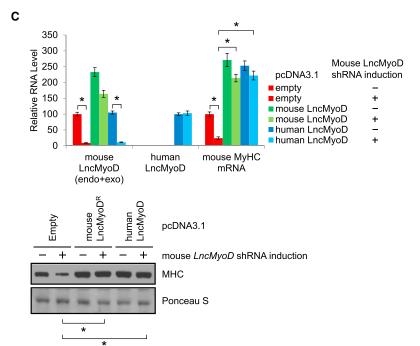
- (A) Knockdown of LncMvoD using inducible shRNAs. Two different Dox-inducible shRNAs targeting LncMyoD successfully knocked down LncMyoD levels by more than 80% after Dox treatment. NT shRNA was used as control. \*p < 0.05. Error bars depict mean ± SEM.
- (B) Knockdown of LncMyoD inhibited myoblast differentiation. Myoblasts stably infected with either LncMyoD or NT shRNAs were induced to differentiate, with or without Dox treatment. Three days after differentiation, cells were fixed and stained for MHC, a marker of terminal muscle differentiation. The percentage of nuclei that were associated with MHC-positive staining was significantly reduced by the LncMyoD shRNAs but not by the NT shRNA. \*p < 0.05. Error bars depict mean + SFM
- (C) LncMyoD knockdown leads to increased Ki67 expression and decreased myogenin activation during myoblast differentiation. Myoblasts stably infected by LncMyoD or NT shRNAs were induced to differentiate with or without Dox treatment. 48 hr after differentiation, protein samples were harvested, and Ki67, myogenin, MuRF1, and MAFbx proteins were detected by western blots. Tubulin was used as a loading
- (D) LncMyoD knockdown does not regulate MyoD protein levels. Western blots demonstrated that MyoD protein levels remained consistent before and after LncMyoD knockdown, suggesting that, unlike some LncRNAs, LncMyoD does not directly regulate the expression of its closest neighbor gene MyoD. Histone 3 (H3) was used as a loading control.
- (E) Knockdown of LncMyoD leads to a reduction of myogenic genes. A heatmap indicates that, after LncMyoD shRNA treatment, a large number of genes were perturbed. Notably, multiple genes involved in muscle functions, including Ckm, Slc2a4, Jph1, Srl, and Col6a1, were significantly downregulated, consistent with decreased myoblast differentiation.

microarrays were performed on myoblasts treated with control versus LncMyoD shRNAs. LncMyoD shRNAs altered the levels of hundreds of mRNAs (Figure 4E; Table S2). Muscle differentiation markers Creatine Kinase Muscle (CKM) and Myosin Heavy Chain 1 (MHC1) are among the most downregulated genes (Figure 4E). Further, we looked at myogenin, MuRF1, and MAFbx as additional markers of differentiation (Bodine et al., 2001) and found that these too were regulated by knockdown of LncMyoD (Figure 4C). When grouped by biological role, genes involved in skeletal muscle-specific functions, such as muscle contractility and myofibril-specific proteins, were among the most downregulated by LncMyoD knockdown (Table S3). These data further confirmed that LncMyoD is required for muscle differentiation, establishing differentiation-specific genes as markers of the knockdown.

#### **Identification of Functionally Conserved Human hLncMyoD**

LncRNAs are generally poorly conserved at the RNA sequence level, and no homolog of mouse LncMyoD was identified in human when we ran a BLAST analysis. However, a few position-conserved LncRNAs are also conserved in function (Ulitsky et al., 2011). In addition, during our study of LncMyoD regulation by MyoD, we noticed that MyoD binding sites are highly conserved across many mammalian species, including human (Figure 5A). This suggests that human MyoD might also regulate an important gene at that locus. By specifically analyzing human RNA-seq data surrounding the genome locus of MYOD1 in human chromosome 11, we identified a LncRNA (which we have termed hLncMYOD), located about 20 kb upstream of the human MYOD1 gene-very similar to mouse LncMyoD's





location relative to mouse *MyoD1*. RACE experiments showed that human *hLncMYOD* is a transcript of 600-bp length and containing three exons (Figures 5B and S4A).

To investigate whether *LncMyoD* functions are conserved between mouse and human, we overexpressed either mouse *LncMyoD* or human *hLncMYOD* exogenously in mouse *LncMyoD* knockdown myoblasts. Overexpression of LncMyoD alone has little effect on MHC expression, suggesting that it is required but not sufficient to drive differentiation (Figure S4B). Remarkably, both mouse *LncMyoD* and human *hLncMYOD* can restore the expression of MHC after endogenous mouse *LncMyoD*'s downregulation (Figures 5C and S4C). This suggests that *LncMyoD* function in myoblast differentiation is conserved between mouse and human.

#### LncMyoD Binds to IMPs and Regulates mRNA Translation

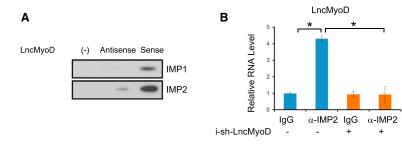
To further investigate the mechanism by which *LncMyoD* may regulate the cell cycle and myoblast differentiation, we attempt

### Figure 5. Identification of Human hLncMyoD

- (A) MyoD binding sites on LncMyoD promoter are conserved in human and other mammals. Cons score, conservation score.
- (B) Location and exon structure of human hLncMyoD.
- (C) Conserved function of *LncMyoD* between mouse and human. *LncMyoD* shRNA-treated myotubes were transfected with plasmid as indicated. Cells were harvested, qRT-PCRs were performed to measure each RNA level, and western blots were performed to detect MHC. Ponceau S staining was used as a loading control. endo+exo, endogenous plus exogenous.

ted to identify LncMyoD-interacting proteins, using a biotinylated-LncMyoD protein pull-down assay. Both non-biotinylated LncMyoD and biotinylated antisense RNA were used as controls. After screening a set of RNA-binding proteins, IGF2 mRNA-binding proteins (IMPs) were found to strongly bind to LncMyoD (Figure 6A). As a specificity control, the antisense strand was also used, and this did not pull down the IMPs (Figure 6A), demonstrating that the interaction is specific to the "sense" strand of the LncMyoD. The IMP family has three members: IMP1, IMP2, and IMP3. While we confirmed that LncMyoD at least binds to both IMP1 and IMP2, focused our efforts on IMP2, because this protein has been shown to be required for myogenesis as a result of its binding and upregulating the translation of proliferation-relevant target mRNAs (Boudoukha et al., 2010; Li et al., 2012).

It was important to determine whether IMP2 and LncMyoD interact in vivo; therefore, endogenous IMP2 protein was immunoprecipitated using an IMP2 antibody, and LncMyoD was indeed detected in the complex (Figure 6B). IMPs contain four signature K-homology-type (KH-type) RNA-binding domains and bind to CAUH (H = A, U, or C) RNA sequences (Hafner et al., 2010). Notably, LncMyoD contains multiple potential IMP-binding sequences (Figure S5A), and only full-length, but not truncated, variants of LncMyoD can rescue the downregulation of endogenous LncMyoD (Figure S6). The direct binding of LncMyoD to IMP2 raised the possibility that LncMyoD may regulate IMP2 protein levels and/or function. We first examined the protein level of IMP2 using western blot analysis and found that LncMyoD knockdown caused an increase in IMP2 protein levels (Figure 6C); this increase was not due to the upregulation of transcription or protein stability (Figure S7). Immunofluorescence demonstrated that IMP1 and IMP2 proteins were strongly enriched in the perinuclear space after LncMyoD knockdown (Figures S5A and S5B). Interestingly, IMP2 bound to its own mRNA, suggesting a positive



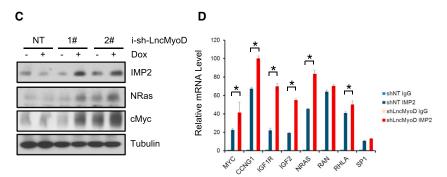


Figure 6. LncMyoD Regulates IMP Function

(A) LncMyoD bound to IMP1 and IMP2. Biotinylated LncMyoD was pulled down by streptavidin beads, and co-eluted proteins were detected by western blot. Non-biotinylated LncMyoD and biotinylated antisense-LncMyoD were used as controls.

(B) LncMyoD bound to IMP2. IMP2 immunoprecipitation was performed using antibodies against IMP2. RNAs interacting with IMP2 were eluted, reverse transcribed, and quantified by real-time PCR. \*p < 0.05. Error bars depict mean ± SEM.

(C) Knockdown of LncMyoD upregulated IMP2, NRAS, and MYC proteins. Dox-induced shRNAs were used to knock down LncMvoD. IMP2. NRAS, and MYC proteins were detected by western blot.  $\alpha$ -Tubulin was used as a loading control.

(D) LncMyoD knockdown caused a significant increase in the binding of IMP2 to many target mRNAs, including Myc, Ccng1, Igf1r, Igf2, Nras, and Rhla. IMP2 immunoprecipitation was performed using antibodies against IMP2. RNAs interacting with IMP2 were eluted, reverse transcribed, and quantified by real-time PCR. \*p < 0.05. Error bars depict mean ± SEM.

feedback loop where IMP2 protein can modulate the stability and/or translation of its own mRNA (Figure 7A). Knockdown of LncMyoD enhanced the interaction between IMP2 and Imp2 mRNA, which helps to explain the upregulation of IMP2 protein levels (Figures 6B and 6C). IMP1 protein showed a similar but modest upregulation after LncMyoD knockdown, suggesting that LncMyoD may regulate multiple IMPs (Figure S5C).

IMP2 regulates myoblast growth through binding to and enhancing the translation of mRNAs involved in proliferation, such as Nras and Myc (Li et al., 2012). LncMyoD knockdown caused a significant increase in the binding of many target mRNAs to IMP2, including Myc, Ccng1, Igf1r, Igf2, Nras, and Rhla (Figure 6D). Consequently, proteins like NRAS and MYC were maintained at high levels even upon differentiation stimuli (Figure 6C), which could contribute to the failure of terminal differentiation, given the undifferentiated phenotype of the LncMyoD knockdown myoblasts (Figure 4B). However, knocking down IMP2 by siRNA could not rescue the phenotype of LncMyoD knockdown (data not shown) which indicates that LncMyoD regulates more than just IMP2 during differentiation (for example, as noted, we also showed that it can regulate IMP1 as well).

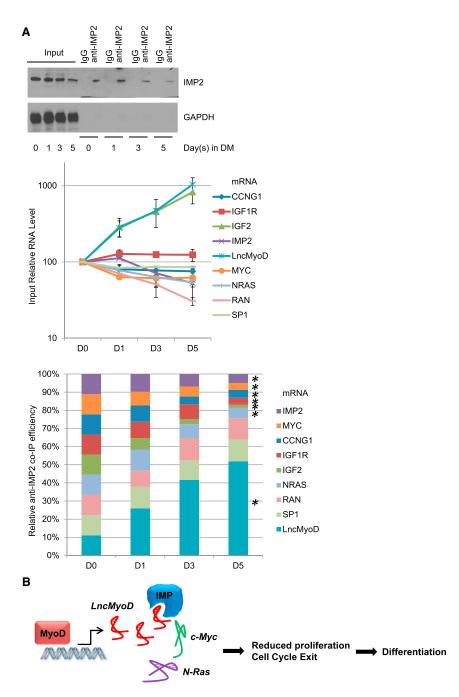
Finally, we pulled down the IMP2/RNP complex from myoblasts and myotubes at different time points. qRT-PCR showed that the increase of LncMyoD enlarged the amount of IMP2 bound, competing out other mRNAs including Imp2, Myc, Ccng1, Igf1r, Igf2, and Nras (Figure 7A). Taken together, our data suggest that induction of LncMyoD could perturb the positive feedback of IMP mRNA and IMP protein and, therefore, facilitate cell-cycle exit and promote terminal differentiation.

#### **DISCUSSION**

The number of LncRNAs identified in the human has been found to be comparable to the number of protein-coding genes (Volders et al., 2013), yet only a small fraction of the LncRNA genes have been functionally studied. In this paper, we report the identification of LncMyoD, a muscle-specific LncRNA that targets MyoD and functions as a regulator of myogenesis. We further use RNA pull-down experiments to identify IMPs as important binding partners of LncMyoD. To our knowledge, LncMyoD is the first long non-coding RNA that has been shown to act by blocking IMP-mediated shuttling of particular RNAs; it thus inhibits translation and thereby perturbs the many genes that require IMP for efficient translation.

One of the interesting findings in this study is that the human homolog of LncMyoD, hLncMyoD, could rescue the myogenesis deficiency in mouse myoblasts depleted of mouse LncMyoD. This demonstrates that LncMyoD gene function is conserved between human and mouse. hLncMyoD and LncMyoD genome locations are conserved, and the MyoD-binding sites on their promoters are highly conserved, strongly suggesting that LncMyoD is a functionally important target of MyoD. However, despite the location and functional conservation, the sequence homology between hLncMyoD and LncMyoD is low. This finding is consistent with previous reports that LncRNAs and mRNAs face distinct selection pressures during evolution and that sequence conservation is not a good filter for functionally important LncRNAs (Ulitsky et al., 2011). In fact, the primary RNA sequences of XIST and TERC, two of the best studied mammalian LncRNAs, are not conserved between mouse and human; however, their functions are the same and crucial in both species. It is thought that LncRNA conservation is at the secondary or tertiary level, and recent structure biology work on the telomerase RNA component-telomerase reverse transcriptase (TERC-TERT) complex supports this notion (Huang et al., 2014).

We have previously shown that the DNA hook protein HMGA2 mediates IMP2 activation in skeletal muscle (Li et al., 2012) and that this is required to maintain activated satellite cells in a



proliferation state. The finding of LncMyoD adds another regulatory node to translation of IMP2-chaperoned proteins, establishing an HMGA2/IMP2/LncMyoD pathway. Other MyoD-localized LncRNAs could also perturb muscle differentiation (Mousavi et al., 2013), by both cis and trans mechanisms; for example, it was also reported that LncRNA could regulate myogenesis by working as a microRNA sponge (Cesana et al., 2011). It will be of interest to see whether there is any crosstalk between these LncRNAs or whether the newly discovered IMP2 binding is a mechanism particular to the MyoD-LncMyoD-IMP pathway (Figure 7B). Since IMPs are involved in regulating cell proliferation and organ development in many tissues (Hansen et al., 2004;

#### Figure 7. LncMyoD Contributes to Myogenesis by Competing for IMP2 Binding

(A) I ncMvoD competes for IMP2 binding, IMP2 BNP complex were pulled down from myoblast or myotubes at different time points. qRT-PCRs were performed to analyze RNA from input and co-eluted with IMP2. DM, differentiation media. \*p < 0.05. Error bars depict mean ± SEM.

(B) Model of the MyoD-LncMyoD-IMP pathway in promoting muscle differentiation. LncMyoD is induced by MyoD and competes with mRNAs like c-Myc and N-Ras for IMP binding. This leads to reduced mRNA translation, cell-cycle exit, and myoblast differentiation.

Li et al., 2012, 2013), it will be of great interest to identify and study IMP-binding LncRNAs in other systems.

Disturbed myogenesis may be a component of the mechanism of diseases such as cachexia, sarcopenia, and especially muscular dystrophy (where a pattern of muscle breakdown and regeneration repeats itself until the muscle's regenerative capacity is depleted). The newly identified MyoD-LncMyoD-IMP pathway provides an additional mechanism that may be amenable to intervention in disease settings. We are currently examining the activity of this pathway in a variety of muscle diseases in order to determine whether targeting LncMyoD or LncMyoD-IMP2 interaction would be beneficial for patients.

#### **EXPERIMENTAL PROCEDURES**

#### **LncRNA** Identification

Confluent C2C12 myoblast differentation model mRNA sequencing (mRNA-seq) raw sequence read data, from Trapnell et al. (2010) (data series GSE20846), was downloaded from the Sequence Read Archive (SRA: http://www.ncbi.nlm.nih.gov/ sra); specifically, data relating to the data series SRX017794 ("-24 hours"; ref http://www.ncbi. nlm.nih.gov/sra?term=SRX017794; mouse skeletal muscle C2C12 cells, exponential growth phase in high serum medium, taken as a model of undifferentiated myoblasts) and data series SRX017795 ("+60

hours"; ref http://www.ncbi.nlm.nih.gov/sra?term=SRX017795; confluent mouse skeletal muscle C2C12 cells, 60 hr post-switch to low serum medium, initiating myogenic differentiation, taken as a model of differentiated myotubes). SRA data archives were converted to FASTQ format (ref), using the SRA toolkit, and aligned against the mouse genome (build mm9/ncbim37: ref http://may2012.archive.ensembl.org/Mus\_musculus/Info/Index) using bowtie (Langmead et al., 2009) and using the "-m 1 -best" flags. In order to detect transcribed, non-genic regions of the genomes (Mitchell et al., 2012), SeqMonk software (http://www.bioinformatics.babraham.ac.uk/projects/ seqmonk/) was used, and contiguous regions of genome coverage were identified. Using a 1-kbp sliding window allowing for 500 bp between reads, each non-genic region was annotated using the nearest gene feature, and reads per kilobase per million (RPKM) (Mortazavi et al., 2008) coverage statistics were

#### **Protein Extraction and Immunoblotting**

Cells were lysed with NP40 buffer (25 mM HEPES, 100 mM NaCl, 5 mM MgCl $_{2}$ , 10% glycerol, 0.2% NP-40, phosphatase inhibitor cocktail-1 and -2 (Sigma) and protease inhibitor cocktail (Roche) for total protein characterization. When nuclear extraction was required, cells were extracted using a CHEMI-CON Nuclear Extraction Kit (Millipore, #2900). Equal amounts of cell lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and detected using an enhanced chemiluminescence system (Pierce Biotechnology). Primary antibodies used were p62 (Abcam, #ab24609), GAPDH (Cell Signaling, #5174), Ki67 (Abcam, #ab15580), Myogenin (BD Pharmingen, #556358), MuRF1 (Abcam, #ab172479), MAFbx (Abcam, #ab168372), Tubulin (Abcam, #ab6160), MyoD (Santa Cruz Biotechnology, #sc760), H3 (Cell Signaling, #9517), MHC (Millipore, #05-833), IMP1 (Cell Signaling, #2852), IMP2 (MBL, #RN008P), NRas (Santa Cruz, #sc31), and cMyc (Cell Signaling, #5605).

#### **Muscle Regeneration**

Muscle degeneration/regeneration by cardiotoxin (CTX)-mediated injury was performed as previously described (Li et al., 2012). In brief, left TA muscles of anesthetized 8- to 12-week-old C57BL/6 WT or Hmga2 KO mice were intramuscularly injected with 100  $\mu$ l of 10  $\mu$ M Naja mossambica mossambica CTX. Right TA muscles of the same mice were injected with PBS as control. Muscle samples were harvested for immunohistochemistry at day 0, day 1, day 3, day 5, day 7, and day 14 after injection and stained with H&E and specific antibodies. Regeneration was clearly activated in the first 3 days and recovered by 14 days after injury.

#### Isolation of RNA and RT-PCR

Total RNAs were isolated with RNeasy Kits (QIAGEN), and cDNA was made using an iScript cDNA Synthesis Kit. SYBR Green dye-based quantitative real-time PCR was performed using the SYBR Green PCR Master Mix and 7900HT Fast Real-Time PCR System from Applied Biosystems. Individual gene primers were designed and synthesized by Integrated DNA Technologies.

#### **Immunofluorescence**

Staining of differentiating myoblasts was performed as previously described. Briefly, cells grown in chamber slides were fixed in 4% paraformaldehyde (PFA) for 15 min in room temperature and permeabilized by 0.5% Triton X-100. Samples were then stained with primary antibody for 2 hr, and with secondary antibody for 30 min at room temperature. Nuclei were labeled with DAPI. The primary antibody used was MHC (Millipore, #05-833). Anti-mouse secondary antibody used was from Invitrogen.

#### 3' BACE and 5' BACE

The RNA-ligase-mediated RACE (RLM-RACE) was carried out with total RNA extracted from primary myoblasts culture and was used to determine the transcription start points and the size of the LncMyoD transcripts. Rapid amplification of 5' or 3' cDNA ends was carried out using a FirstChoice RLM-RACE kit (Ambion), according to the manufacturer's instructions. Due to the low copy number of LncMyoD in cells, nested PCR was performed for each reaction. Primers used are as follows:

- 5' RACE External (CTA AAG AGA AGC CAG CAG CCA TG);
- 5' RACE Internal (CCC CAG TCA GGC CTT GAG ATG AG).
- 3' RACE External (AGC AGC AGC AGG GCT CTG AAG G); and
- 3' RACE Internal (GGG GAG AAG CCA CAC CCA TC).

#### **RNA-Binding Protein Immunoprecipitation**

RNA-binding protein immunoprecipitation (RIP) was performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, primary mouse myoblasts were harvested by adding RIP lysis buffer. Clear supernatant containing IMP2 protein, Protein G beads, and IMP2 antibody (or immunoglobulin G [IgG] control) was mixed to perform the immunoprecipitation. After washing, RNAs binding to IMP2 were eluted and quantified. RT-PCR and real-time PCR were performed to examine whether certain mRNAs were co-immunoprecipitated with the IMP2 antibody.

#### **Myoblasts Isolation and Culture**

The isolation procedure for mouse myoblasts and subsequent pre-plating methods have been described previously (Li et al., 2012). All procedures were performed in accordance with the standards of the U.S. Department of Health and Human Services and were approved by the Novartis Animal Care and Use Committee. Briefly, limbs of 21-day-old male C57BL/6 mice were isolated, and muscle was pulled from the bone and cartilage with sterile forceps. The muscle was treated for 1 hr with Collagenase (Sigma) and Dispase II (Roche) to degrade collagen and to disrupt cell contacts. The cells were plated for 2 hr to allow fibroblasts, which rapidly adhere, to attach to the Petri dish. The supernatant, containing a mixture of myoblasts and fibroblasts, was removed, and the cells were passaged again to another Petri dish. After 24 hr, the cells were again passaged and subsequently transferred to another culture dish. To grow primary myoblasts, cells were cultured in F10 (GIBCO) + 20% FBS (GIBCO) + 2.5 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen) + 1% penicillin/streptomycin. When induced for differentiation, myoblasts were switched to differentiation medium (DMEM supplemented with 5% horse serum).

#### **Luciferase Reporter Assay**

LncMyoD forward or reverse promoter regions were cloned into a PGL3 luciferase reporter vector (Promega). To overexpress MyoD protein, the mouse Myod1 coding region was cloned into the pcDNA3.1(+) vector. LncMyoD-Pro-Luciferase, Renilla, and MyoD plasmids were transfected into 293 cells seeded in 96-well plates, using the FUGENE 6 (Roche) transfection reagent, following the manufacturer's protocol. 48 hr later, cells were lysed, and luciferase assavs were performed using a Dual-Luciferase Reporter Assav System (Promega) on a luminometer. Transfection of each construct was performed in triplicate in each assay. Luciferase readings were taken as singlets. Ratios of Renilla luciferase readings to Firefly luciferase readings were taken for each experiment, and triplicates were averaged.

#### Inducible shRNA Knockdown

For knockdown of *LncMyoD*, ten shRNA sequences targeting *LncMyoD* were cloned into a Tet-pLKO-puro vector (Addgene #21915). Lentivirus was produced using a ViraPower Lentiviral Packaging Mix (Life Technologies) in 293T cells, filtered, and used to infect myoblast cells. The efficacy of shRNA constructs was screened after Dox treatment for 48 hr followed by RT-PCR. A non-targeting (NT) shRNA sequence was used as negative control (adapted from Sigma SHC002). Two shRNAs achieved more than 80% knockdown efficacy and were, therefore, selected for sequential experiments:

The target sequence for sh-LncMyoD 1# is AGCCTCAGTTTCTTGTC ATGT.

The target sequence for sh-LncMyoD 2# is GAGTTGTCACCCAAGGC AAGA.

#### **Microarray and Analysis**

Total RNA was extracted using the TRIzol reagent (Invitrogen) and purified with QIAGEN RNeasy separation columns (QIAGEN). For microarray analysis, firststrand cDNA was synthesized and hybridized to GeneChip Mouse Genome 430 2.0 Array (Affymetrix).

Statistical analysis of the microarray data was performed within the R statistical environment, using bioinformatics packages from Bioconductor. The raw signals from CEL files were normalized and summarized into probe-set level intensities using the PLIER (Probe Logarithm Intensity ERror) method. Affymetrix MAS5 present/absent calls were calculated, and probe sets with "present" calls in less than 50% of samples within all treatment groups were removed for further statistical analyses. The moderated F test implemented in the limma package (Smyth, 2004) was applied to determine the significance of the differential expression of each probe set between treatments. The p values were further adjusted using the Benjamini and Hochberg multiple testing procedures for false discovery rate (FDR) control. Pathway and upsteam regulator analyses were conducted using Ingenuity Pathway Analysis (Ingenuity Systems).

An Active Motif's ChIP-IT Express Kit was used for the ChIP experiment according to the manufacturer's instructions. Briefly, cells were crosslinked with 1% formaldehyde for 10 min at room temperature and lysed in SDS lysis buffer. Samples were then sonicated or enzymatically digested to obtain DNA fragments with an average length of 200–800 bp. Supernatant containing DNA-protein complexes was used for immunoprecipitations using an anti-MyoD antibody (Santa Cruz, sc-760) or a normal rabbit IgG control. Immunoprecipitated chromatin was collected using protein G magnet beads, and, after washing and elution, reverse crosslinking was carried out with 0.2 M NaCl at 65°C overnight. The chromatin was then digested by 20 µg of Proteinase K (Invitrogen) for 1 hr at 45°C and isolated by phenol chloroform extraction. PCR reactions were performed using the SYBR Green PCR Master Mix (Applied Biosystems) and primers against *LncMyoD* promoter regions. Data were normalized to the input signal and IgG values.

#### **Statistical Analyses**

Descriptive statistics were generated for all quantitative data with presentation of means and SEs. Results were assessed for statistical significance using Student's t test (Microsoft Excel) or ANOVA with the SAS Enterprise Guide 3.0 or SigmaPlot 11.0 software.

#### **ACCESSION NUMBERS**

The accession number for the microarray data presented in Table S1 of this paper is NCBI GEO: GSE68842.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015. 05.009.

#### **AUTHOR CONTRIBUTIONS**

C.G., Z.L., and K.R. contributed significantly to the project and experimental design, executed the majority of the experiments, and contributed to data interpretation, statistical analysis, and writing of the manuscript. D.G. contributed significantly to the experimental design, data interpretation, and manuscript writing. I.C., Y.Z., and S.L.-B. contributed significantly to the experimental design and bioinformatics analysis.

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