Cell Stem Cell
Article

Pax3/7BP Is a Pax7- and Pax3-Binding Protein that Regulates the Proliferation of Muscle Precursor Cells by an Epigenetic Mechanism

Yarui Diao,[1,6] Xing Guo,[1] Yanfeng Li,[1] Kun Sun,[2,4,5] Leina Lu,[3,4,5] Lei Jiang,[1] Xinrong Fu,[1] Han Zhu,[1] Hao Sun,[2,4,5] Huating Wang,[3,4,5] and Zhenguo Wu[1,6,*]
[1]Division of Life Science, State Key Laboratory of Molecular Neuroscience, The Hong Kong University of Science and Technology, Clearwater Bay, Kowloon, Hong Kong, China
[2]Department of Chemical Pathology
[3]Department of Obstetrics and Gynaecology
[4]Li Ka-Shing Institute of Health Sciences
The Chinese University of Hong Kong, China
[5]Prince of Wales Hospital, Shatin, Hong Kong, China
[6]Biomedical Research Institute, Shenzhen-PKU-HKUST Medical Center, Shenzhen, Guangdong 518036, China
*Correspondence: bczgwu@ust.hk
http://dx.doi.org/10.1016/j.stem.2012.05.022

SUMMARY

In mouse skeletal muscles, Pax7 uniquely marks muscle satellite cells and plays some important yet unknown functions at the perinatal stage. To elucidate its in vivo functions, we initiated a yeast two-hybrid screening to look for Pax7-interacting proteins and identified a previously uncharacterized Pax7- and Pax3-binding protein (Pax3/7BP). Pax3/7BP is a ubiquitously expressed nuclear protein, enriched in Pax7+ muscle precursor cells (MPCs), and serves as an indispensable adaptor for Pax7 to recruit the histone 3 lysine 4 (H3K4) methyltransferase (HMT) complex by bridging Pax7 and Wdr5. Knockdown of *Pax3/7BP* abolished the Pax3/7-associated H3K4 HMT activity and inhibited the proliferation of Pax7+ MPCs from young mice both in culture and in vivo. *Id3* and *Cdc20* were direct target genes of Pax7 and Pax3/7BP involved in the proliferation of Pax7+ MPCs. Collectively, our work establishes Pax3/7BP as an essential adaptor linking Pax3/7 with the H3K4 HMT to regulate the proliferation of MPCs.

INTRODUCTION

Two similar but distinct waves of myogenesis exist in vertebrates: embryonic myogenesis and adult myogenesis. In mice, the former starts with muscle precursor cells (MPCs) in the myotome around embryonic day 8 with the expression of Myf5, one of the four myogenic regulatory factors (MRFs) (Puri and Sartorelli, 2000; Sabourin and Rudnicki, 2000; Tapscott, 2005), while the latter is executed by muscle stem cells, also called muscle satellite cells (MSCs), which originate from the central region of the dermomyotome with the expression of Pax7 and Pax3 (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). In adult muscles, MSCs are quiescent, located between basal lamina and the sarcolemma of myofibers, and are mainly responsible for postnatal muscle growth and muscle regeneration in response to either injury or exercise (Biressi and Rando, 2010; Buckingham and Relaix, 2007; Le Grand and Rudnicki, 2007; Wagers and Conboy, 2005; Zammit, 2008).

In the past decade, intensive efforts have been made to characterize MSCs at the molecular level. A major breakthrough in this regard came with the discovery of Pax7 as a specific marker for all MSCs (Seale et al., 2000). Pax3, a close paralog of Pax7, is also coexpressed in MSCs of a subset of muscles including the diaphragm (Relaix et al., 2005, 2006). Both Pax7 and Pax3 are transcription factors of the Pax family proteins and contain a characteristic set of domains, including a paired domain (PD), an octapeptide motif, and a homeodomain (HD) (Buckingham and Relaix, 2007; Lang et al., 2007) (Figure 1A). While the PD and HD domains in Pax proteins are mainly involved in DNA binding, both of them and the octapeptide are also capable of interacting with other proteins (Buckingham and Relaix, 2007; Lang et al., 2007). Although Pax7 is also known to be expressed in other nonmuscle tissues, Pax7 only uniquely marks MSCs in skeletal muscles (Seale et al., 2000). In addition to the quiescent MSCs (i.e., Pax7+/MyoD−), Pax7 is also expressed in activated and proliferating myoblasts (i.e., Pax7+/MyoD+) that are also called MPCs. However, upon differentiation, Pax7 is rapidly downregulated (Olguin and Olwin, 2004; Zammit et al., 2004). The number of MSCs/MPCs was found to be comparable in newborn mice with or without Pax7 (Oustanina et al., 2004; Relaix et al., 2006). Intriguingly, the Pax7 null MSCs quickly disappeared in adult mice (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006). While one report showed that Pax7 null MSCs were prone to apoptosis (Relaix et al., 2006), another suggested that Pax7 null MSCs failed to establish quiescence and underwent precocious differentiation (Lepper et al., 2009). The importance of Pax7 in MSCs is further underscored by the findings that in a few Pax7 null mice that survived to adulthood, there was a major defect in injury-induced muscle regeneration (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006).

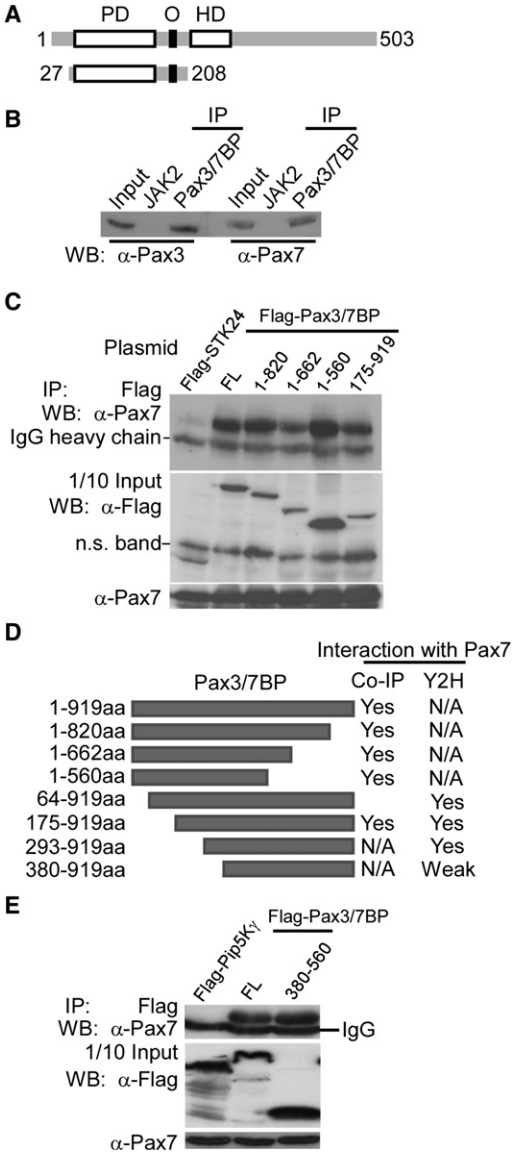

Figure 1. Pax3/7BP Is a Pax7-Interacting Protein

(A) A schematic diagram of the full-length and the truncated mouse Pax7 used in the yeast two-hybrid (Y2H) screening. PD, paired domain; O, Octapeptide; HD, homeodomain.

(B) Five-hundred micrograms of whole cell extract (WCE) prepared from trunk muscles of P5 mice were immunoprecipitated (IP) with polyclonal antibodies against either Pax3/7BP or JAK2 and the coprecipitated Pax7 and Pax3 was revealed by western blot (WB) analysis. Fifty micrograms of WCE was used as the input control.

(C) C2C12 cells were cotransfected with a Pax7 construct together with various Pax3/7BP constructs as indicated. Flag-tagged Pax3/7BP proteins were immunoprecipitated from an equal amount (300 μg) of WCE, and the coprecipitated Pax7 was revealed by WB analysis. Thirty micrograms of WCE was used as the input. n.s., nonspecific.

(D) A schematic summary of Pax3/7BP fragments in different Pax7-binding assays. Co-IP, coimmunoprecipitation assays; Y2H, yeast two-hybrid assays; N/A, not assayed.

(E) 293T cells were transfected with Pax7 together with PIP5Kγ (control) or different Pax3/7BP constructs. Flag-tagged proteins were immunoprecipitated from WCE and the coprecipitated Pax7 was detected by WB analysis. FL, full-length. See also Figure S1.

Recently, an unexpected finding revealed that Pax7 is completely dispensable for the maintenance or survival of MSCs and for injury-induced muscle regeneration in adult mice (Lepper et al., 2009). However, Pax7 was found to play a critical role in MSCs/MPCs in young mice (i.e., those younger than 3 weeks old) (Lepper et al., 2009). It remains unclear what role Pax7 plays in MSCs or MPCs in young mice.

To elucidate the function of Pax7 in vivo, it would be very helpful to identify Pax7-interacting proteins. Using C2C12 myoblasts stably expressing Pax7 and combining immunoprecipitation with mass spectrometry, McKinnel et al. showed that Pax7 could recruit the mixed-lineage leukemia (MLL)-containing histone methyltransfearse (HMT) complex that specifically catalyzes dimethylation and trimethylation of lysine 4 in histone 3 (H3K4) (McKinnell et al., 2008). The MLL-containing HMT is frequently recruited to the regulatory regions of the transcription-active genes (Ansari and Mandal, 2010; Cosgrove and Patel, 2010; Malik and Bhaumik, 2010; Shilatifard, 2008). The H3K4 HMT complex consists of a set of core components including a catalytic protein of the MLL family, Wdr5, Rbbp5, and Ash2L (Dou et al., 2006). It is unclear whether Pax7 directly interacts with any of the core components of the MLL complex. To look for direct Pax7-binding proteins, we conducted a yeast two-hybrid screening using Pax7 as bait, and we identified a previously uncharacterized Pax7- and Pax3-binding protein (Pax3/7BP). We provide evidence here to show that Pax3/7BP bridges Pax7 and Wdr5 to facilitate the recruitment of the H3K4 HMT complex to the regulatory regions of Pax7 target genes. Both Pax7 and Pax3/7BP play key roles in regulating the proliferation of Pax7+ MPCs. *Cdc20* and *Id3* are direct target genes of Pax7 and Pax3/7BP involved in regulating the proliferation of MPCs. Our work suggests that Pax3/7BP could serve as an adaptor protein linking the H3K4 HMT complex to selected transcription factors and participate in epigenetic regulation of their target genes.

RESULTS

Pax3/7BP Is a Pax7- and Pax3-Interacting Protein

To better understand how Pax7 exerts its functions in MSCs/MPCs, we decided to search for Pax7-interacting proteins using yeast two-hybrid screening. For reasons unknown, among several Pax7 bait constructs we generated, including the full-length Pax7, only Pax7 (aa 27–208) that spans the entire PD and the octapeptide motif (O) was expressed in yeast (Figure 1A). We then used this fragment as bait to screen a mouse 17-day embryo cDNA library. Among 31 positive clones identified in our screening, most of them encoded members of the fibulin family (our unpublished data), which are all extracellular proteins. Because Pax7 is a nuclear transcription factor, we reasoned that its interacting protein should also be a nuclear protein. By name, one positive clone from our screen fit the criterion: it encodes GC-rich sequence DNA binding factor 1 (GCFC1, NP_080386.3), a protein of 919 amino acids that is conserved in metazoa and has not been functionally characterized in any previous publications. Because the sequence in the Pax7 fragment used as bait is highly conserved in Pax3 (Figure S1A,available online), we also tested whether the corresponding fragment in Pax3 interacted with the GCFC1 clone

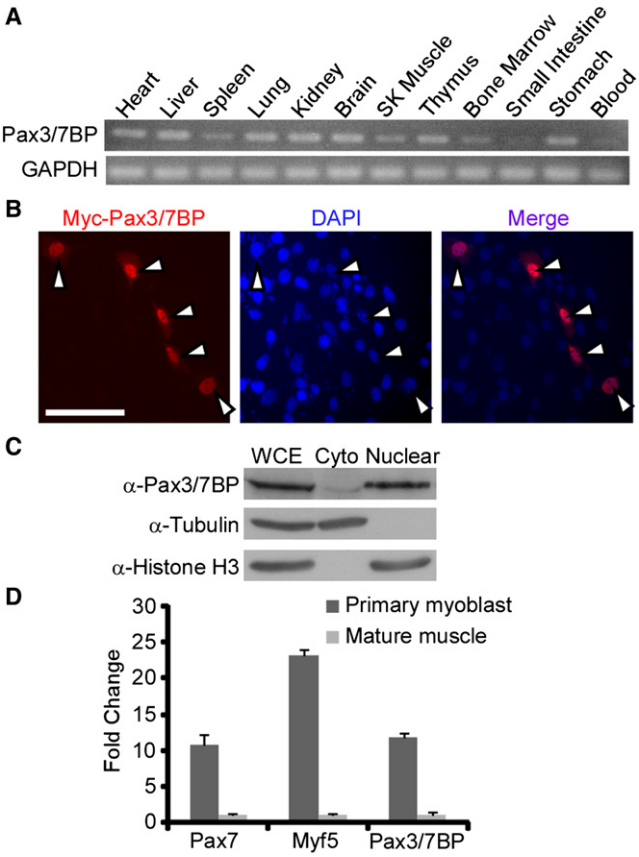

Figure 2. Pax3/7BP Is a Nuclear Protein Enriched in Primary Myoblasts

(A) Total RNA was isolated from different mouse tissues and subjected to semiquantitative RT-PCR analysis to detect *Pax3/7BP* mRNA. *GAPDH*: the glyceraldehyde 3-phosphate dehydrogenase gene.

(B) C2C12 cells were transfected with a plasmid encoding Myc-Pax3/7BP. Twenty-four hours after transfection, the cells were fixed and subjected to immunostaining using an anti-Myc antibody. The arrowheads indicated the nuclei that were double-positive for DAPI and Myc-Pax3/7BP. Scale bar: 100 μm.

(C) WCE and the cytoplasmic (Cyto) and the nuclear fractions (Nuclear) were prepared from C2C12 myoblasts and separated on SDS-PAGE, followed by WB analysis with the indicated antibodies.

(D) Total RNA isolated from either primary myoblasts or mature tibialis anterior muscles was subjected to RT-qPCR analysis to reveal the relative mRNA levels of *Pax7*, *Myf5*, and *Pax3/7BP* with *GAPDH* as the internal control. The fold change was calculated as the ratio of the relative amount of an mRNA in primary myoblasts over that in mature muscles. Data were presented as mean ± SD.

we pulled out from the library. As shown in Figure S1B, Pax3 also specifically interacted with GCFC1 in yeast two-hybrid assays. Because there is no direct evidence showing that GCFC1 is a direct DNA binding protein, the current name in the database of the National Center for Biotechnology Information (NCBI) could be confusing. Therefore, we renamed this protein Pax3/7-binding protein or Pax3/7BP. To facilitate our work, we generated two polyclonal antibodies against Pax3/7BP: one was found to be good for immunoblotting, while the other was suitable for immunoprecipitation (Figures S1C and S1D). To confirm whether Pax3/7BP interacts with Pax7 or Pax3 under physiological conditions, we performed coimmunoprecipitation assays using lysates from trunk muscles of 5-day-old (P5) mice in which Pax7, Pax3, and Pax3/7BP are all known to be expressed (Relaix et al., 2005, 2006; Seale et al., 2000). We found that both the endogenous Pax7 and Pax3 preferentially coprecipitated with Pax3/7BP but not JAK2 (a negative control) (Figure 1B). We next tried to map the Pax7-interacting domain in Pax3/7BP. We generated a series of deletion constructs of Pax3/7BP. By combining both coimmunoprecipitation and yeast two-hybrid assays, we found that a region of Pax3/7BP that spans aa 380–560 was both necessary and sufficient to interact with Pax7 (Figures 1C–1E).

Pax3/7BP Is a Nuclear Protein Enriched in MPCs

To functionally characterize Pax3/7BP, we first checked its tissue expression profile. By semiquantitative RT-PCR, we found that the *Pax3/7BP* mRNA was ubiquitously expressed in all tissues examined including skeletal muscles (Figure 2A). To examine the subcellular localization of Pax3/7BP, we performed immunostaining assays. In C2C12 cells transfected with a Myc-Pax3/7BP-expressing construct, we found that Pax3/7BP was exclusively localized in the nuclei of C2C12 cells (Figure 2B). To further verify this result, we separated the soluble whole cell extract (WCE) of C2C12 myoblasts into the cytoplasmic and nuclear fractions. Consistently, we found that the endogenous Pax3/7BP was predominantly localized in the nuclear fraction (Figure 2C). To examine whether Pax3/7BP is expressed in Pax7+ MPCs, we isolated total RNA from both tibialis anterior (TA) muscles of adult mice and freshly isolated Pax7+ MPCs and performed quantitative RT-PCR. After normalizing the mRNA levels of *Pax3/7BP* to those of *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), we found that the *Pax3/7BP* mRNA was highly enriched in MPCs instead of mature muscles, the latter consisting of a large quantity of differentiated myofibers and a small fraction of quiescent MSCs/MPCs (Figure 2D). This differential expression pattern of *Pax3/7BP* in MPCs and mature muscles was similar to that of *Pax7* and *Myf5* (Figure 2D), both of which are known to be enriched in MSCs/MPCs (Beauchamp et al., 2000; Seale et al., 2000).

Pax3/7BP Is Associated with the H3K4 HMT Complex through Wdr5

To understand the physiological relevance of the interaction between Pax7 and Pax3/7BP, we turned to a recent finding that Pax7 could recruit the H3K4 HMT in myoblasts (McKinnell et al., 2008). Interestingly, we noticed that the domain of Pax7 involved in recruiting HMT overlapped with what was used in our yeast two-hybrid screening. Thus, we tested if Pax3/7BP was also associated with the HMT complex. The WCE prepared from muscles of P5 young mice were subjected to immunoprecipitation with various antibodies against key components of the H3K4 HMT complex. As shown in Figure 3A, we found that Pax3/7BP specifically coprecipitated with Wdr5, Rbbp5, Ash2L, and different catalytic subunits of the H3K4 HMT complex: Set1, Set2, and MLL1–4, but not with a control antibody. Furthermore, by direct H3 methylation assays, we demonstrated that the full-length Pax3/7BP immunoprecipitated from C2C12 myoblasts displayed HMT activity toward H3 (Figures 3B and S2A). The Pax3/7BP-associated HMT activity was

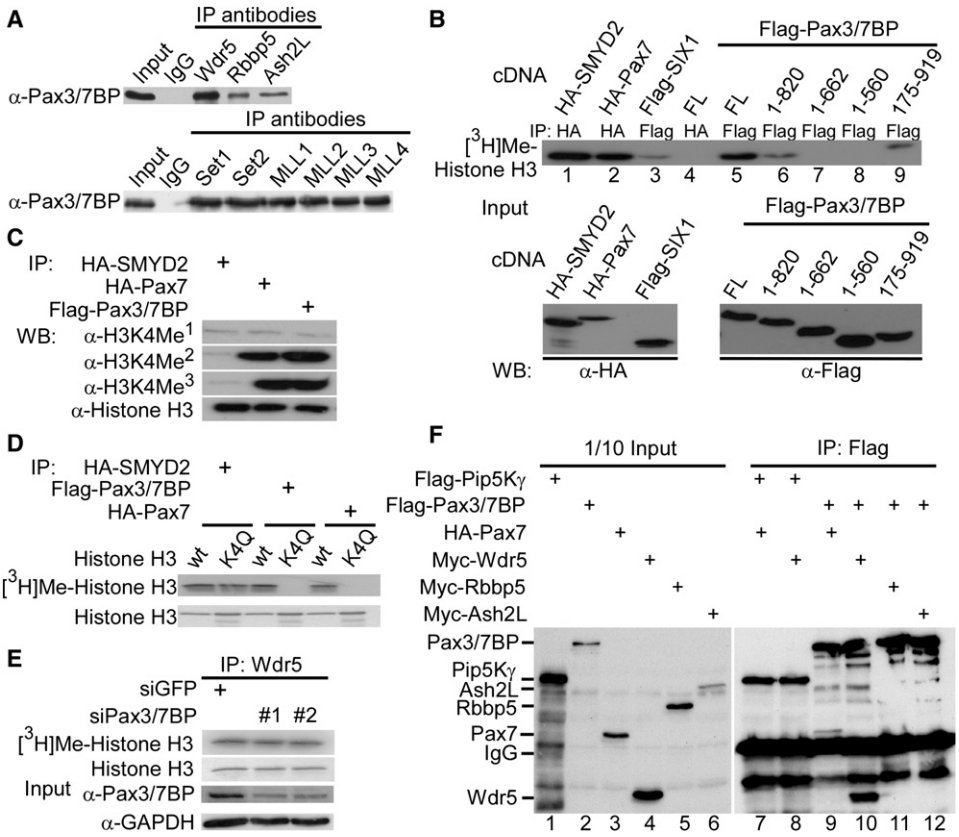

Figure 3. Pax3/7BP Interacts with the H3K4 HMT Complex through Wdr5

(A) One milligram of WCE from muscles of P5 mice was subjected to immunoprecipitation using various antibodies as indicated. The coprecipitated Pax3/7BP was revealed by WB analysis.

(B–D) C2C12 cells were transfected with various plasmids as indicated. An equal amount of WCE (500 μg) was subjected to immunoprecipitation using either the anti-HA or anti-Flag antibody. The immunoprecipitates were subjected to in vitro HMT assays with the recombinant histone H3 as a substrate. The reaction mixtures were separated on SDS-PAGE followed by gel drying and fluorography (B and D) or WB analysis (C). K4Q, a mutant H3 with K4 replaced by Q; FL, full-length. Me^1, Me^2, and Me^3 denote the monomethylated, dimethylated, or trimethylated H3K4, respectively.

(E) WCE was prepared from C2C12 cells transfected with various siRNAs. Wdr5 was immunoprecipitated from 500 μg of WCE and the immunoprecipitates were subjected to in vitro HMT assays toward H3. In (D and E), after fluorography (top panel), the dried gel was rehydrated and stained with Coomassie blue to reveal the amount of H3 used in each reaction (second panel). Fifty micrograms of input WCE was subjected to WB analysis with indicated antibodies.

(F) Pairs of in vitro translated proteins (left panel) were mixed as indicated, followed by immunoprecipitation with the anti-Flag antibody. The coprecipitated protein was revealed by WB analysis (right panel). See also Figure S2.

comparable to that associated with Pax7, Pax3, or SMYD2, another HMT specific for H3K36 (Shilatifard, 2008). Small N- or C-terminal deletion of Pax3/7BP [i.e., Pax3/7BP (aa 175–919) and Pax3/7BP (aa 1–820)] reduced, but not completely eliminated, its associated HMT activity. However, a larger C-terminal truncation in Pax3/7BP [i.e., Pax3/7BP (aa 1–662) and Pax3/7BP (aa 1–560)] completely abolished its associated HMT activity (lanes 7 and 8). Because Pax3/7BP is ubiquitously expressed, we showed that Flag-Pax3/7BP that was immunoprecipitated from several nonmuscle cell lines, like COS7, 293T, or NIH 3T3, also displayed HMT activity (Figure S2B). Furthermore, using antibodies specifically recognizing monomethylated, dimethylated or trimethylated H3, we showed that Pax3/7BP, just like Pax7, mainly associated with the HMT that catalyzes dimethylation or trimethylation of H3 (Figure 3C). Using an H3K4Q mutant protein, we also further confirmed that the HMT that was associated with either Pax3/7BP or Pax7 was specific for H3K4 (Figure 3D). In contrast, SMYD2, an HMT specific for H3K36, methylated both the wild-type H3 and the H3K4Q mutant. We then tested whether Pax3/7BP is a key component of the H3K4 HMT complex. We transfected C2C12 myoblasts with either a control siRNA against the *green fluorescence protein* (*GFP*) gene or two different siRNAs against *Pax3/7BP*. We then immunoprecipitated Wdr5, a core component of the H3K4 HMT complex (Dou et al., 2006; Wysocka et al., 2005), from these C2C12 myoblasts and measured its associated HMT activity. As shown in Figure 3E, knockdown of *Pax3/7BP* did not affect the HMT activity associated with Wdr5, suggesting that Pax3/7BP is not a key component in the H3K4 HMT complex. To find out which component of the H3K4 HMT complex interacts with Pax3/7BP, we expressed individual proteins in rabbit reticulocyte lysates using the coupled in vitro transcription/translation system and mixed different proteins together as indicated in Figure 3F. We then immunoprecipitated

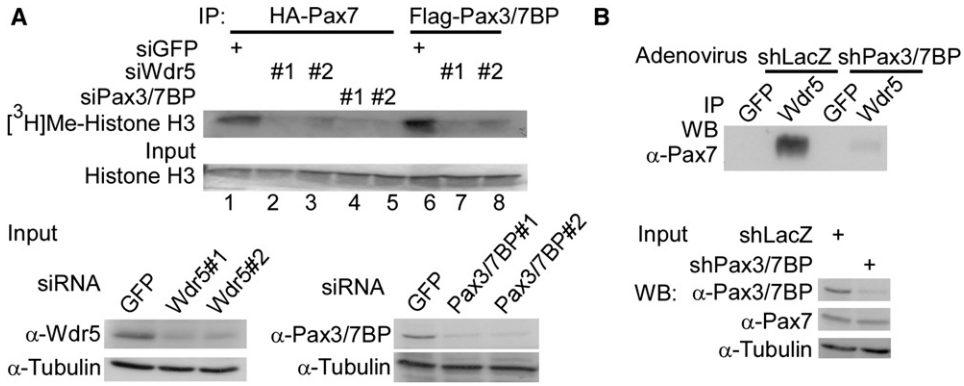

Figure 4. Pax3/7BP Bridges Pax7 and the H3K4 HMT Complex

(A) WCE was prepared from C2C12 myoblasts cotransfected with various plasmids and siRNAs as indicated. HA-Pax7 and Flag-Pax3/7BP were immunoprecipitated from 500 μg of WCE and the immunoprecipitates were subjected to in vitro HMT assays toward H3. The dried gel was first subjected to fluorography (top) followed by rehydration and Coomassie blue staining (middle). Fifty micrograms of input WCE was subjected to WB analysis with the indicated antibodies (bottom).

(B) Primary myoblasts were infected with adenoviruses expressing shRNAs targeting *LacZ* or *Pax3/7BP*. One milligram of WCE was subjected to immunoprecipitation with either an anti-GFP antibody or the anti-Wdr5 antibody. The coprecipitated Pax7 was revealed by WB analysis (top panel). Fifty micrograms of input WCE was analyzed by WB analysis (bottom panel). See also Figures S3 and S4.

Pax3/7BP and looked for its coprecipitated partners. As a positive control, we showed that Pax7 coprecipitated with Pax3/7BP (lane 9). Importantly, we found that Wdr5 (lane 10), but not Rbbp5 or Ash2L (lanes 11 and 12), also coprecipitated with Pax3/7BP. As a negative control, Pax7 or Wdr5 did not coprecipitate with PIP5Kγ (lanes 7 and 8). Our results showed that Pax3/7BP was associated with the H3K4 HMT complex through Wdr5.

Pax7-Associated HMT Activity Depends on Pax3/7BP

Because both Pax7 and Pax3/7BP associate with the H3K4 HMT complex, we next asked whether Pax3/7BP serves as an essential adaptor to link the H3K4 HMT with Pax7. In C2C12 myoblasts, as a positive control, we first showed that both the Pax7- and Pax3/7BP-associated HMT activity was nearly abolished when *Wdr5* was knocked down (Figure 4A, top panel: lanes 2–3 and 7–8), consistent with the fact that both Pax7 and Pax3/7BP interact with the H3K4 HMT through Wdr5. Importantly, we found that the Pax7-associated HMT activity was also greatly reduced when *Pax3/7BP* was knocked down (lanes 4 and 5). Our control experiments showed that siRNAs for *Wdr5* and *Pax3/7BP* worked well (Figure 4A, bottom two panels). Similarly, we found that the Pax3-associated HMT activity was also dependent on Pax3/7BP (Figure S3). To assess whether Pax3/7BP was required for the recruitment of the HMT complex by other transcription factors, we examined MEF2, which is known to associate with the H3K4 complex in differentiating myocytes (Rampalli et al., 2007). Unlike Pax7, the MEF2-associated H3K4 HMT activity was not affected by *Pax3/7BP* knockdown (Figure S4). Our results suggested that Pax3/7BP specifically links Wdr5 with Pax7 or Pax3. To directly prove this, we infected primary myoblasts with adenoviruses expressing small-hairpin RNAs (shRNA) specifically targeting either *lacZ* (control) or *Pax3/7BP*, and we showed that Pax3/7BP-shRNA was effective in knocking down *Pax3/7BP* (Figure 4B, bottom panel). When we immunoprecipitated Wdr5 from myoblast lysates, we found that the endogenous Pax7 only coprecipitated with Wdr5 in myoblasts infected with the lacZ-shRNA-expressing adenovirus (Figure 4B, top panel). In contrast, in myoblasts infected with the Pax3/7BP-shRNA-expressing adenovirus, Wdr5 failed to coprecipitate Pax7. This result indicated that the physical binding between Wdr5 and Pax7 was dependent on Pax3/7BP.

Pax3/7BP Is Involved in Myoblast Proliferation in Cell Culture

Existing evidence suggested that Pax7 plays a role in myoblast proliferation (Chen et al., 2010; Oustanina et al., 2004; Palacios et al., 2010; Relaix et al., 2006). We therefore tested whether Pax3/7BP is also involved in this process. To address this issue, we isolated Pax7+ MPCs from P5 mice and expanded them in vitro. We then transfected the same number of Pax7+ MPCs with individual siRNAs specifically targeting *GFP*, *Pax7*, or *Pax3/7BP*. Myoblasts were subjected to two different cell proliferation assays: in the BrdU incorporation assays, after 24 hr of growth, cells were labeled with BrdU for 30 min before harvest. Consistently, we found that knockdown of *Pax7* indeed reduced the proliferation of MPCs (Figures 5A and 5B). Interestingly, knockdown of *Pax3/7BP* also inhibited myoblast proliferation to a similar extent. Using the WST-1 assays that directly measure the activity of the mitochondrial succinate-tetrazolium reductase, which is proportional to the number of live cells, we confirmed that the growth of Pax7+ MPCs was inhibited when either *Pax7* or *Pax3/7BP* was knocked down (Figure 5C). Thus, our results above showed that Pax3/7BP was required for the proliferation of Pax7+ MPCs in cell culture.

Pax3/7BP Is Required for the Proliferation of MPCs In Vivo

Mouse Pax7 was shown to have some essential yet unknown functions in the first 3 weeks after birth, which is a period when Pax7+ MPCs undergo extensive proliferation (Lepper et al., 2009). To find out whether Pax3/7BP also plays a role at this

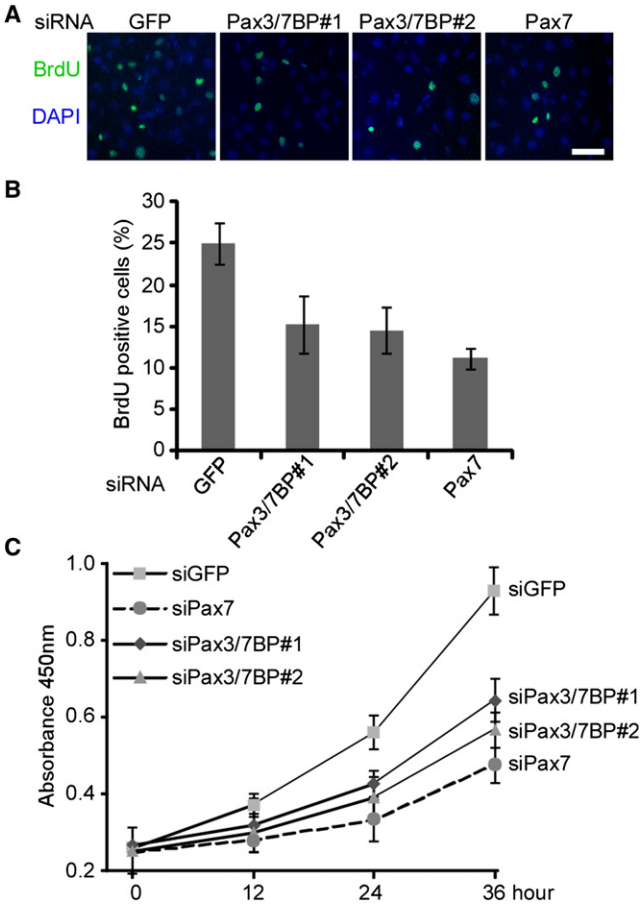

Figure 5. Pax3/7BP Is Required for the Proliferation of Primary Myoblasts in Culture

(A–C) Primary myoblasts were transfected with various siRNAs as indicated and cultured in the growth medium. (A and B) Twenty-four hours after transfection, cells were labeled with 10 μM of BrdU for 30 min, then fixated and immunostained (A). The nuclei were counterstained with DAPI. Scale bar: 100 μm. The percentage of BrdU+ cells was calculated as the ratio of the number of BrdU+ cells over that of DAPI+ cells and the data were presented as mean ± SD in (B). (C) Twenty-four hours after transfection, cells were trypsinized, and an equal number of cells (3,000 cells/well) was replated into 96-well plates. Six duplicates were used for each sample at each time point. At various time points, the number of live cells in each well was indirectly quantified by the WST-1 assays. The experiment was done three times with similar results and the data from one representative experiment were presented as mean ± SD.

stage, we directly injected Pax3/7BP siRNA mixed with RNAiMAX (Invitrogen) into the TA muscles of young mice at postnatal days 6, 8, and 10. A GFP siRNA was injected into the contralateral TA muscle to serve as a control. The TA muscles were isolated for further analysis at postnatal day 12. To make sure that an injected siRNA could knock down its intended target gene in MPCs, we employed Pax7 siRNA as a positive control. As shown in Figure 6A (left panel), the *Pax7* mRNA was indeed knocked down in the TA muscles after Pax7 siRNA injection. Because most of the Pax7+ cells were MPCs at this stage, this result suggested that siRNA could be effectively delivered into these MPCs. Similarly, the *Pax3/7BP* mRNA was also effectively knocked down in TA muscles by this approach (Figure 6A, right panel). Consistent with a key role of Pax7 in postnatal muscle growth (Kuang et al., 2006), we found that knockdown of *Pax7* reduced the total mass of TA muscles in young mice by about 35% (Figure 6B). Importantly, knockdown of *Pax3/7BP* also reduced the weight of the TA muscles to a similar extent. This result implied that knockdown of *Pax3/7BP* may reduce the size of myofibers in young mice. To directly test this, we stained for laminin on the cross-sections of TA muscles of young mice to visualize individual myofibers and measured their sizes (i.e., cross-sectional area or CSA). As shown in Figures 6C and 6D, knockdown of *Pax3/7BP* reduced the percentage of large myofibers (i.e., those with CSA $\geq$ 1,000 μm^2) with a concomitant increase in the percentage of smaller myofibers (i.e., those with CSA $\leq$ 999 μm^2). Moreover, we found that the number of Pax7+ MPCs also decreased when *Pax3/7BP* was knocked down (Figures 6C and 6E). As a control, we also examined whether knockdown of *Pax3/7BP* affected the proliferation of Pax7+ MPCs in adult mice. Four-week-old dystrophin null (i.e., *mdx*) mice were used because their trunk and limb muscles are known to contain a large number of proliferating Pax7+ MPCs due to cycles of spontaneous myofiber degeneration and regeneration (Durbeej and Campbell, 2002; Watchko et al., 2002). We separately electroporated GFP siRNA and Pax3/7BP siRNA into TA muscles of *mdx* mice on each side, and we showed that the endogenous *Pax3/7BP* could be effectively knocked down (Figure S5A). However, the number of Pax7+ MPCs was similar between TA muscles treated with GFP siRNA or Pax3/7BP siRNA (Figures S5B, S5C), suggesting that Pax3/7BP is dispensable for the proliferation of MPCs in adult muscles. Collectively, our data suggested that Pax3/7BP was mainly required for the proliferation of MPCs in vivo in young mice.

Pax7 and Pax3/7BP Regulate the Expression of *Id3* and *Cdc20* in MPCs

We showed above that both Pax7 and Pax3/7BP were required for the proliferation of MPCs. However, the underlying molecular mechanisms remained unclear. A recent report showed that *Id3* is a direct target of Pax7 (Kumar et al., 2009). Using primary myoblasts from P5 mice combined with siRNA knockdown and chromatin immunoprecipitation (ChIP) assays, we confirmed that Pax7 was indeed recruited to the *Id3* promoter and that knockdown of *Pax7* reduced the expression of *Id3* (Figures 7A and 7B). Knockdown of *Pax3/7BP* also reduced the expression of *Id3* to a similar extent, suggesting that Pax3/7BP facilitates Pax7 to regulate *Id3* (Figure 7B). To directly test whether knockdown of *Pax3/7BP* affected the recruitment of Pax7 or the H3K4 HMT to the *Id3* promoter, we infected primary myoblasts with adenoviruses expressing shRNAs against either *lacZ* or *Pax3/7BP*. By ChIP assays, we found that the recruitment of the H3K4 HMT, but not Pax7, to the *Id3* promoter was clearly reduced when *Pax3/7BP* was knocked down (Figure 7C). Consistently, we also found that there was much less trimethylated H3K4 at the *Id3* promoter when *Pax3/7BP* was knocked down (Figure 7D). In contrast, the levels of trimethylated H3K4 at the *myogenin* promoter were not affected by knockdown of *Pax3/7BP* (Figure 7D) (Rampalli et al., 2007), suggesting that Pax3/7BP is only required for the recruitment of the H3K4 HMT

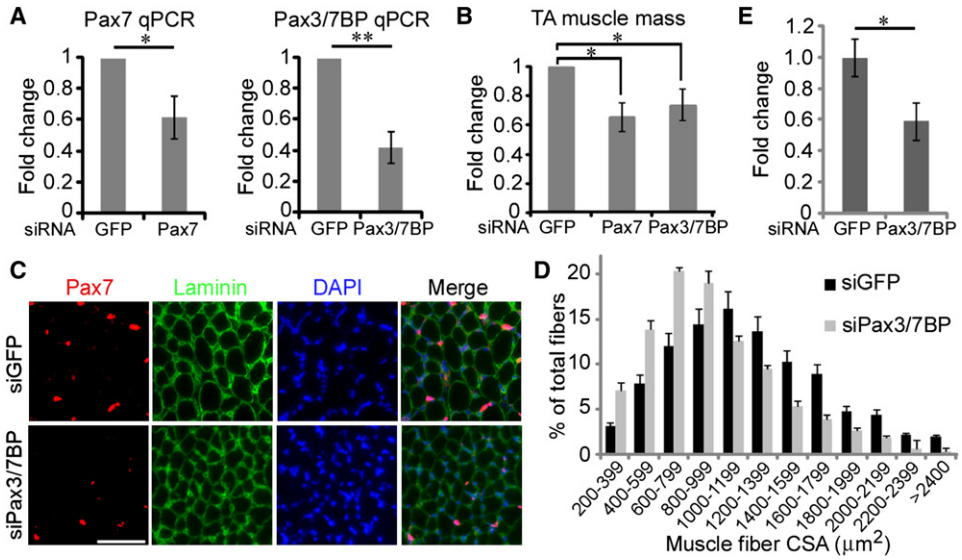

Figure 6. Pax3/7BP Is Required for the Proliferation of Pax7+ MPCs In Vivo

(A–E) Different siRNAs were repeatedly injected into TA muscles of young mice at P6, P8, and P10. For each mouse, one leg was injected with Pax7 or Pax3/7BP siRNA, while the contralateral leg was injected with GFP siRNA. The TA muscles were isolated at P12 for further analysis.

(A) Total RNA was harvested from TA muscles and the mRNA levels of *Pax7* or *Pax3/7BP* were analyzed by RT-qPCR.

(B) The TA muscles were isolated and weighed and the fold change was calculated as the ratio of the weight of muscles injected with Pax7 siRNA or Pax3/7BP siRNA over that with GFP siRNA.

(C) TA muscle sections were analyzed by immunostaining. The nuclei were counterstained with DAPI. Scale bar: 100 μm.

(D) The cross-sectional area (CSA) of individual myofibers was measured using PhotoShop software. Myofibers with similar CSA were grouped and their percentages were calculated and presented. About 2,000 myofibers were examined.

(E) The number of Pax7+ cells was counted in each microscopic field under a 20× objective lens. For each mouse, one microscopic field from each of the five consecutive slides was analyzed. The fold change was calculated as the ratio of the number of Pax7+ cells from TA muscles treated with Pax3/7BP siRNA over that with GFP siRNA. In (A and B), 12 mice were treated with the Pax7 siRNA, while 11 were treated with the Pax3/7BP siRNA. In (C)–(E), 18 mice were used. The data were presented as mean ± SD. *$p < 0.05$; **$p < 0.01$. See also Figure S5.

complex to a subset of active promoters/enhancers. Although the effect of *Pax3/7BP* knockdown on *Id3* gene expression paralleled that of *Pax7* knockdown, it did not necessarily mean that they work together. To further prove that Pax7 regulates *Id3* through Pax3/7BP, we carried out a Pax7-dependent reporter assay in C2C12 myoblasts with a luciferase reporter gene under the control of a 934 bp proximal *Id3* promoter (Kumar et al., 2009). As shown in Figure 7E, the *Id3* reporter was indeed activated by cotransfected Pax7 in a Pax3/7BP-dependent manner. To directly prove that Id3 was involved in the proliferation of MPCs, we knocked down *Id3* in Pax7+ MPCs (Figure 7G, right panel) and examined BrdU incorporation. As shown in Figures 7F and 7G (left panel), we found that knockdown of *Id3* reduced the incorporation of BrdU, which was indicative of decreased cell proliferation.

To look for additional target(s) of Pax7 and Pax3/7BP that are potentially involved in regulating the proliferation of MPCs, we performed two sets of genome-wide ChIP-sequencing (ChIP-seq) using antibodies against Pax7 and the trimethylated H3K4 and chromatin isolated from TA muscles of P5 mice. Among a list of promoter/enhancer fragments that were associated with both Pax7 and the trimethylated H3K4, we noticed that one fragment about 9.3 kb upstream of the transcription start site of *Cdc20* contains two closely linked Pax7 binding sites (Figure S6). Because Cdc20 is a key molecule involved in the mitotic checkpoint control and cell proliferation (Kim and Yu, 2011; Pesin and Orr-Weaver, 2008), we decided to further examine this gene. Using ChIP-qPCR, we confirmed that both Pax7 and the trimethylated H3K4 specifically associated with this region of *Cdc20* (Figure 7H). Using primary myoblasts of young mice, we confirmed that knockdown of either *Pax7* or *Pax3/7BP* decreased the expression of Cdc20 (Figure 7I). Finally, using ChIP assays and chromatin prepared from primary myoblasts infected with GFP-expressing or Flag-Pax3/7BP-expressing adenoviruses, we directly proved that Pax3/7BP was indeed recruited to the regulatory sites in *Id3* and *Cdc20* that were bound by Pax7 and the trimethylated H3K4 (Figure 7J).

DISCUSSION

Pax3/7BP Facilitates the Recruitment of HMT by Pax7 and Pax3

To understand the functions of Pax7 in MSCs/MPCs, it is important to identify its interacting proteins. Unfortunately, very little is known about Pax7-interacting proteins. A recent breakthrough in this area came when Pax7 was shown to recruit the H3K4 HMT complex via Wdr5 (McKinnell et al., 2008). However, it is unclear how Pax7 recruits Wdr5. As an important epigenetic regulator, the MLL/Set complex itself does not recognize specific sequences in the promoter/enhancer regions of its target genes. Instead, it is recruited to these regions via sequence-specific transcription factors (Ruthenburg et al.,

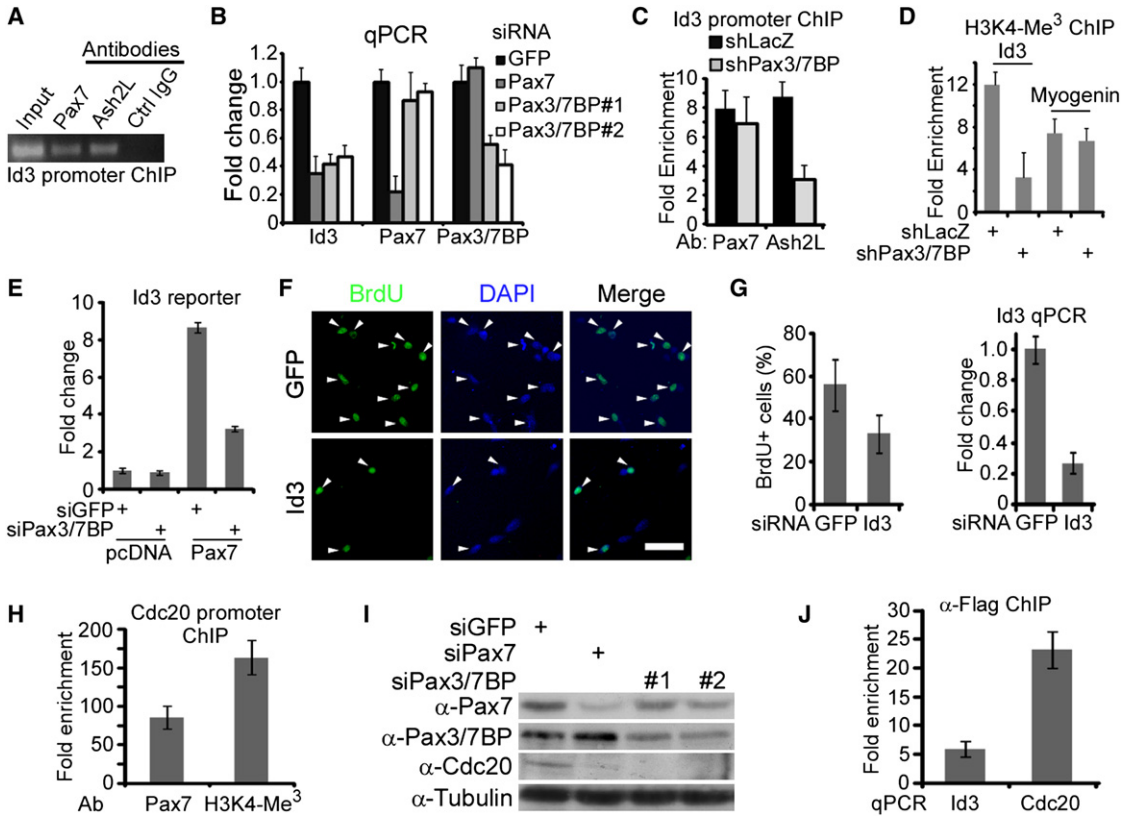

Figure 7. Pax3/7BP Facilitates Pax7 to Directly Regulate *Id3* and *Cdc20*

(A) Primary myoblasts freshly isolated from P5 mice were subjected to ChIP analysis. The input genomic DNA and the immunoprecipitated DNA fragments were subjected to PCR to amplify the Pax7 binding site in the *Id3* promoter.

(B) Total RNA was isolated from primary myoblasts transfected with various siRNAs and subjected to quantitative (q) PCR analysis. The mRNA levels of *Id3*, *Pax7*, and *Pax3/7BP* in cells treated with Pax7 siRNA or Pax3/7BP siRNA over that treated with GFP siRNA was calculated and presented.

(C and D) Primary myoblasts from P5 mice were infected with adenoviruses expressing shRNAs targeting *LacZ* or *Pax3/7BP*. Thirty-six hours after infection, the cells were harvested and analyzed by ChIP assays. The Pax7 binding site (C) and the trimethylated H3K4 binding sites (D) in the *Id3* and *myogenin* promoters (D) were amplified by qPCR.

(E) C2C12 myoblasts were cotransfected with an *Id3-luc* reporter together with various plasmids and siRNAs as indicated. Twenty-four hours after transfection, the WCE was subjected to luciferase assays. The fold change was calculated as the ratio of luciferase activities in experimental samples over that in control cells transfected with an empty vector and GFP siRNA.

(F and G) Primary myoblasts were transfected with siRNAs against *Id3* or *GFP*. Twenty-four hours after transfection, one batch of cells was labeled by BrdU and immunostained with an anti-BrdU antibody (F), while the other was subjected to RT-qPCR to measure the relative mRNA levels for *Id3* (G, right panel). The percent of BrdU+ cells over DAPI+ cells was calculated and is presented in (G) (left panel). Scale bar: 100 μm.

(H) ChIP-qPCR was performed on chromatin from P5 muscles to confirm the ChIP-seq result.

(I) Primary myoblasts were transfected with various siRNAs and cultured for another 30 hr. The WCE was prepared and subjected to WB analysis.

(J) Primary myoblasts were infected with GFP-expressing or Flag-Pax3/7BP-expressing adenoviruses. Thirty-six hours after infection, chromatin was isolated and subjected to ChIP-qPCR using the anti-Flag antibody. The Pax7 binding sites in the *Id3* and *Cdc20* promoters were amplified by qPCR. The fold enrichment was calculated with the amount of DNA obtained either with a specific antibody over that with a control IgG (C, D, and H) or with the anti-Flag antibody from Flag-Pax3/7BP-expressing cells over that from GFP-expressing cells (J). The data in (B)–(E), (G), (H), and (J) were presented as mean ± SD. Ab, antibody. See also Figure S6.

2007). Some transcription factors directly recruit this complex by interacting with specific components of this complex. For example, Oct4 was recently shown to recruit H3K4 HMT via its specific interaction with Wdr5 (Ang et al., 2011), while both AP2δ and MEF2D recruit this complex by specifically interacting with Ash2L (Rampalli et al., 2007; Tan et al., 2008). Other transcription factors recruit the H3K4 HMT via unique adaptor proteins. For example, Pax2, another member of the Pax family proteins, recruits the H3K4 HMT via PTIP to regulate its target gene expression (Patel et al., 2007). Interestingly, like Pax3/7BP, PTIP is also a ubiquitously expressed nuclear protein and facilitates the recruitment of the HMT complex to target gene promoters by interacting with specific transcription factors (Muñoz and Rouse, 2009; Patel et al., 2007). It remains unclear which component of the H3K4 HMT complex directly associates with PTIP. Our work here shows that Pax7 does not directly recruit the H3K4 HMT. Instead, Pax3/7BP serves as a critical adaptor to link Wdr5 with Pax7. Even though we found that Pax3/7BP directly interacts with Wdr5 using in vitro translated proteins, it remains possible that Pax3/7BP does so via another

bridging molecule. More detailed study is needed to understand the structural basis underlying the interaction between Pax7 and Pax3/7BP as well as that between Pax3/7BP and Wdr5.

A More General Role of Pax3/7BP in Metazoa

Unlike Pax7 or Pax3, Pax3/7BP is ubiquitously expressed and well conserved in metazoa, suggesting that Pax3/7BP has other Pax3/7-independent functions. We found that the Pax3/7BP-associated H3K4 HMT activity is readily detectable not only in myoblasts but also in nonmuscle cell lines (Figure S2B). Therefore, it is likely that Pax3/7BP can link the H3K4 HMT with transcription factors other than Pax7 in nonmuscle cells. A search for Pax3/7BP-interacting proteins may identify such transcription factors. Furthermore, it remains to be established whether Pax3/7BP has additional functions other than transcriptional regulation.

The Role of Pax7 and Pax3/7BP in the Proliferation of MPCs

An unexpected recent finding revealed that mouse Pax7 only plays a crucial role during the first 3 weeks after birth (Lepper et al., 2009), a period when Pax7+ MPCs are known to undergo extensive proliferation (Buckingham and Relaix, 2007; Chargé and Rudnicki, 2004; Le Grand and Rudnicki, 2007). However, the exact functions of mouse Pax7 in the perinatal period remain unclear. Here, we showed that Pax7 regulates the proliferation of MPCs at this stage, which was supported by data from other groups (Chen et al., 2010; Oustanina et al., 2004; Palacios et al., 2010; Relaix et al., 2006). Importantly, Pax3/7BP also functions in MPCs from young mice, because knockdown of *Pax3/7BP* inhibits the proliferation of Pax7+ MPCs from young mice both in vitro and in vivo. In contrast, knockdown of *Pax3/7BP* has no effect on the proliferation of MPCs in adult muscles. This is consistent with the fact that Pax7 becomes dispensable in MSCs/MPCs of adult muscles (Lepper et al., 2009). Presumably, other mechanisms have compensated for the roles of Pax7 and Pax3/7BP in adult MSCs/MPCs. In addition to Pax7, we showed that Pax3/7BP also interacts with Pax3, a close paralog of Pax7 with overlapping functions in a subset of MSCs/MPCs (Relaix et al., 2005, 2006). Like Pax7, Pax3 also depends on Pax3/7BP to recruit the H3K4 HMT. This suggests that, in addition to regulating the proliferation of MPCs, Pax3/7BP may also participate in processes uniquely controlled by Pax3, such as migration of MPCs from the hypaxial dermomyotome to limbs during embryonic myogenesis (Buckingham and Relaix, 2007).

As to the molecular mechanisms underlying the Pax7 and Pax3/7BP-mediated MPC proliferation, we showed that both *Id3* and *Cdc20* are direct target genes of Pax7 and that Pax3/7BP facilitates Pax7 to recruit the H3K4 HMT to the regulatory regions in these genes. *Id3* is expressed in both the quiescent MSC and proliferating MPC population and its overexpression in C2C12 cells enhances myoblast proliferation but inhibits differentiation (Atherton et al., 1996; Kumar et al., 2009). Moreover, we directly demonstrated that knockdown of *Id3* decreases the proliferation of Pax7+ MPCs (Figures 7F and 7G). As for Cdc20, a key regulator of the mitotic checkpoint, its role in cell proliferation is well established (Kim and Yu, 2011; Pesin and Orr-Weaver, 2008). In agreement with our findings, *Cdc20* was recently found to be expressed in quiescent MSCs and was further induced in proliferating MPCs (Pallafacchina et al., 2010). With more detailed analysis of our ChIP-seq data, we expect to identify additional direct targets of Pax7 and Pax3/7BP that could participate in the proliferation of MPCs in perinatal mice.

EXPERIMENTAL PROCEDURES

In Vivo siRNA Delivery

To deliver siRNA into the TA muscles of young mice, 6 μl of siRNA (50 μM) was first mixed with 24 μl of Opti-MEM medium, while 6 μl of lipofectamine RNAiMAX was diluted with 24 μl of Opti-MEM for 5 min. Then, the two parts were mixed for another 20 min. The siRNA/RNAiMAX mixtures were repeatedly injected into the TA muscles of young mice. For each mouse, one leg was injected with an experimental siRNA, while the contralateral leg was injected with a control GFP siRNA. At postnatal days 6, 8, and 10, the injection volume for each TA muscle was 10 μl, 10 μl, and 15 μl, respectively. The TA muscles were isolated for further analysis at P12. To deliver siRNA into adult *mdx* mice, the TA muscles of 4-week-old *mdx* mice were pretreated with 30 μl of hyaluronidase (0.4 U/μl) (Sigma) 2 hr before siRNA injection. For each TA muscle, 6 μl of siRNA (50 μM) was diluted with 0.9% NaCl to a final volume of 30 μl, and injected into the TA muscle and followed with electroporation using a BTX ECM 830 generator (mode: LV; field strength: 175V/cm; pulse length: 20 ms; number of pulses: 8) and a pair of 7 mm Tweezertrodes (BTX) with one electrode attached to the TA muscle and the other to the gastrocnemius muscle. The siRNA injection/electroporation was repeated every 24 hr for 5 days.

In Vitro HMT Assays

In a final reaction volume of 30 μl, the immune complex, which was pulled down with specific antibodies and Protein A/G beads, was incubated at 30°C for 2 hr in a reaction buffer containing 50 mM Tris-HCl [pH 8.5], 100 mM NaCl, 10 mM DTT, 1 μM ^{3}H-labeled S-adenosyl-L-methionine (^{3}H-SAM, PerkinElmer, Boston, MA), and 2 μg of recombinant histone H3.1 (NEB, Ipswich, MA). The reaction was stopped by the addition of SDS sample loading buffer, and the HMT reaction products were then separated on 15% SDS-PAGE. The gel was incubated with the Amplify Fluorographic Reagent (GE Healthcare), dried, and subjected to autofluorography. To visualize the recombinant histone protein used in HMT reactions, the dried gel was rehydrated and stained with Coomassie blue.

ChIP Assays and ChIP-Seq

ChIP assays were performed using a standard protocol. Briefly, in primary myoblasts or muscle tissues from P5 mice, after crosslinking of proteins to DNA with 1% formaldehyde followed by cell lysis and sonication to generate chromatin fragments with an average size of 200–300 bp, immunoprecipitation was performed with 5 μg of the anti-Pax7 or the anti-trimethylated H3K4 antibody bound to Protein A/G-Sepharose beads. After extensive washing and reversal of crosslinking, proteinase K and RNase A digestion, chromatin fragments were purified by phenol chloroform extraction, and ethanol precipitation was performed. For regular ChIP assays, the purified DNA was amplified by qPCR. For ChIP-seq, we used a protocol from Illumina Inc. Briefly, the purified DNA (10 ng) was end-repaired, and A-nucleotide overhangs were added by incubation with the Taq Klenow fragment lacking exonuclease activity. After the attachment of anchor sequences, fragments were PCR amplified using Illumina-supplied primers. The purified DNA library products were evaluated using Bioanalyzer (Agilent) and SYBR qPCR and diluted to 10 nM for cluster generation in situ on the GAIIx paired-end flow cell using the CBot automated cluster generation system, along with massively parallel sequencing (2 × 50 bp) on GAIIx. After sequencing, we employed a data analysis pipeline CASAVA 1.8 (Illumina) to perform the initial bioinformatic analysis including base calling. The raw sequence reads were aligned against the unmasked mouse genome (mm9) using SOAP2 with two mismatches allowed. MACS (v1.4.1) (Zhang et al., 2008) was subsequently used for genome-wide identification of Pax7 binding sites or DNA sequences

associated with the trimethylated H3K4, with the IgG pulldown as negative control and a False Discovery Rate (FDR) below 0.05.

Statistical Analysis

To evaluate the statistical significance, the Student's t test was employed to analyze the experimental data sets.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.05.022.

ACKNOWLEDGMENTS

We thank Drs. K. Ge (NIH) and W. Herr (U of Lausanne) for the anti-MLL3/MLL4 and anti-Wdr5 antibodies, respectively; Drs. M. Rudnicki (University of Ottawa) and A. Lassar (Harvard Medical School) for Flag-Pax3 and the *Id3-Luc* reporter constructs, respectively; Dr. G. Zhu (HKUST) for HA-SMYD2; and Dr. M. Zhang (HKUST) for the 6xHis-3C-pET32M vector and the Rosetta *E. Coli* strain. We also thank Ms. C. Wong and the Shenzhen Biomedical Research Support Platform for technical help. The work was supported by grants from the Hong Kong Research Grant Council (663609), Shenzhen Science and Technology Project (JC200903180682A), and the State Key Laboratory of Molecular Neuroscience at HKUST.

Received: January 4, 2012
Revised: April 16, 2012
Accepted: May 11, 2012
Published: August 2, 2012

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