PIAS1 confers DNA-binding specificity on the Msx1 homeoprotein

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The mechanisms by which homeoproteins bind selectively to target genes *in vivo* have long remained unresolved. Here we report that PIAS1 confers DNA-binding specificity on the Msx1 homeoprotein by regulating its subnuclear localization and proximity to target genes. We demonstrate that the interaction of Msx1 with PIAS1, but not its sumoylation, is required for Msx1 to function as an inhibitor of myoblast differentiation through repression of myogenic regulatory genes, such as *MyoD*. We find that PIAS1 enables Msx1 to bind selectively to a key regulatory element in *MyoD*, the CER, in myoblast cells and to distinguish the CER from other nonregulatory TAAT-containing sequences. We show that PIAS1 is required for the appropriate localization and retention of Msx1 at the nuclear periphery in myoblast cells. Furthermore, we demonstrate that myogenic regulatory genes that are repressed by Msx1, namely *MyoD* and *Myf5*, are located at the nuclear periphery in myoblast cells. We propose that a key regulatory event for DNA-binding specificity by homeoproteins *in vivo* is their appropriate targeting to subnuclear compartments where their target genes are located, which can be achieved by cofactors such as PIAS1.

[**Keywords:** Homeobox genes; protein–protein interactions; myoblast differentiation; *PIAS* genes; DNA-binding specificity; sumoylation]

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Homeobox genes encode an extensive family of transcriptional regulators that control a myriad of developmental processes (Manley and Levine 1985; Gehring 1987; Levine and Hoey 1988; Krumlauf 1994). Although homeoproteins display exquisite specificities *in vivo*, presumably reflecting their selective regulation of target genes, they display broad DNA-binding specificities *in vitro* (Hayashi and Scott 1990). While it is widely believed that target gene specificity *in vivo* is achieved through selective protein interactions (Mann and Af-folter 1998), unraveling the mechanistic details has proven to be a daunting task.

The vertebrate *Msx* homeobox genes play key roles in regulating cellular differentiation during development. This family consists of three members, of which *Msx1* and *Msx2* are closely related with respect to sequence (Supplementary Fig. 1A), expression patterns, and biochemical and biological functions (Davidson 1995; Bendall and Abate-Shen 2000). Despite their expression in

tissues of unrelated embryological origin, a unifying feature of *Msx* expression is its restriction to progenitor populations that ultimately give rise to terminally differentiated cells (Bendall and Abate-Shen 2000). This is exemplified in the muscle lineage, where *Msx1* is transiently expressed in myoblast precursor cells but down-regulated upon terminal differentiation (Bendall et al. 1999; Houzelstein et al. 1999). In accordance with its inverse correlation of expression and cellular differentiation, forced expression of *Msx1* prevents precursor cells from undergoing terminal differentiation (Song et al. 1992; Woloshin et al. 1995; Bendall et al. 1999; Odelberg et al. 2000; Hu et al. 2001), while mutations of *MSX1* and *MSX2* are responsible for several human genetic disorders that are manifested as abnormalities of differentiation (Jabs et al. 1993; Vastardis et al. 1996; van den Boogaard et al. 2000; Wilkie et al. 2000; Jumlongras et al. 2001).

Msx homeoproteins function as regulators of differentiation through their activities as transcriptional repressors. In particular, inhibition of myoblast differentiation by *Msx1* reflects, in part, its ability to repress the expression of myogenic regulatory genes, such as *MyoD* (Woloshin et al. 1995; Bendall et al. 1999), by binding to

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a key regulatory element in the *MyoD* promoter called the core enhancer region (CER) (Goldhamer et al. 1992; Kucharczuk et al. 1999; Lee et al. 2004). Binding of Msx1 to the CER, as well as transcriptional repression and inhibition of differentiation, requires the selective interaction of Msx1 with a linker histone, mouse H1b (human H1e) (Lee et al. 2004). In contrast to its selective interaction with the CER in myoblast cells, Msx1 can interact in vitro with many DNA sites containing a TAAT core motif (Catron et al. 1993), which is typical of other homeoproteins (Scott et al. 1989). This contrast emphasizes the important distinction between the apparent DNA-binding properties of Msx1 in vitro and its actual function in myoblast cells.

In pursuit of Msx1-interacting proteins that contribute to DNA-binding specificity in a cellular context, we have now isolated *PIAS1* by yeast two-hybrid screening. *PIAS1* is a member of a family of at least five genes and/or spliced variants (*PIAS1*, *PIAS3*, *PIASy*, *PIASx α* , *PIAS x β*), one of which (*PIASx β*) was isolated as a protein partner for Msx2 (and called Miz-1, for Msx-2-interacting protein 1) (Wu et al. 1997). PIAS proteins were initially described as inhibitors of STAT signaling, hence their name protein inhibitor of activated STATs (Liu et al. 1998). Subsequent studies revealed that PIAS proteins have numerous partners, many of which are transcription factors (Schmidt and Muller 2003). The consequences of these interactions are often manifested as transcriptional repression (Schmidt and Muller 2003) and PIAS proteins have themselves been shown to interact with DNA via an N-terminal region called the SAP domain (Fig. 1A; Sachdev et al. 2001). Further insights regarding their mechanisms of repression were provided by the discovery that PIAS proteins can function as E3 ligases for sumoylation mediated by a ring-finger domain in the C-terminal region (Fig. 1A; Kahyo et al. 2001; Sachdev et al. 2001; Kotaja et al. 2002; Schmidt and Muller 2002). We now report that Msx1 interacts with *PIAS1*, which results in modification of Msx1 by sumoylation. We find that the interaction of Msx1 with *PIAS1*, but not its sumoylation, is required for transcriptional repression and inhibition of differentiation. *PIAS1* facilitates the selective interaction of Msx1 with a key regulatory element on the *MyoD* promoter and results in the coincident localization of Msx1 with the *MyoD* gene, as well as the *Myf5* gene, at the nuclear periphery. Thus, our findings highlight a new role for *PIAS1* proteins as regulators of homeoprotein DNA-binding specificity through their ability to control the localization of homeoproteins within the nucleus and their proximity to target genes.

Results

Msx1 interacts specifically with PIAS1

Among the genes that we found to interact with Msx1 by yeast two-hybrid screening, we focused on *PIAS1* because (1) we had also isolated *Ubc9*, which encodes an E2 enzyme in the sumoylation pathway (Seeler and Dejean 2003; Hay 2005); and (2) Msx2 had been previously

shown to interact with another member of the PIAS family, *PIASx β* (Miz-1) (Wu et al. 1997). We verified the interaction of Msx1 with endogenous *PIAS1*, as well as with the exogenous protein, by coimmunoprecipitation assays (Fig. 1B). By analyses of truncated Msx1 proteins, we found that the C-terminal region of Msx1 was necessary for interaction with *PIAS1* (Fig. 1A,C); in fact, this interaction was disrupted by deletion of the last five amino acids of Msx1 (Supplementary Fig. 2A). Conversely, analyses of truncated *PIAS1* proteins in vitro revealed that an N-terminal region (amino acids 1–200), including the SAP domain, was necessary for interaction with Msx1 (Fig. 1D; Supplementary Fig. 2B).

The interaction of Msx1 with *PIAS1* was highly specific since no such interaction was observed with several other homeoproteins, including its closest homolog Msx2 (Fig. 1E). This prompted us to further verify that Msx1, but not Msx2, interacted with *PIAS1* (Supplementary Fig. 1B). Conversely, we found that Msx1 did not interact with *PIASx β* , which was shown previously to interact with Msx2 (Fig. 1F; Wu et al. 1997). Taken together, these findings demonstrate that the interaction between Msx1 and *PIAS1* is highly selective, even among members of their respective protein families. To our knowledge this is the first biochemical activity that discriminates Msx1 and Msx2, which are otherwise virtually indistinguishable (Catron et al. 1996; Bendall and Abate-Shen 2000).

The reported expression patterns of *Msx1* and *PIAS* genes, including *PIAS1*, are largely overlapping during mouse development (Bendall and Abate-Shen 2000; Sturm et al. 2000) and *Msx1* and *PIAS1* are coexpressed in undifferentiated C2C12 myoblast cells (Supplementary Fig. 3). Moreover, both *Msx1* and *PIAS1* are down-regulated during terminal differentiation of these cells, as is evident from the appearance of myotubes and by expression of *myogenin*, a marker of muscle cell differentiation (Supplementary Fig. 3; Olson and Klein 1994). Therefore, Msx1 and *PIAS1* are coexpressed in biologically relevant contexts.

The interaction of Msx1 with PIAS1, but not its modification by sumoylation, is required for Msx1 activity

Considering that PIAS proteins function as E3 SUMO ligases (Kahyo et al. 2001; Sachdev et al. 2001; Kotaja et al. 2002; Schmidt and Muller 2002), we next investigated whether the interaction of Msx1 with *PIAS1* resulted in its modification by sumoylation. We found that Msx1 was indeed sumoylated in 293T cells (Fig. 1G). This modification was stimulated by exogenous *PIAS1* (Supplementary Fig. 4A), while an Msx1 protein lacking the *PIAS1*-interacting region [Msx1(1–271)] was not sumoylated (Fig. 1G). Detection of exogenous Myc-SUMO revealed two modified forms of Msx1 (indicated by asterisks in Fig. 1G), the upper one being predominant, as well as additional slower-mobility forms [indicated by SUMO(n) in Fig. 1G], which likely represent multimeric SUMO-modified Msx1 proteins (Fig. 1G). Accordingly,

Figure 1. Msx1 interacts specifically with PIAS1. (A) Schematic diagrams of Msx1 and PIAS1. Functional domains of Msx1 (shaded boxes) include the homeodomain and the histone H1b and PIAS1 interaction regions. Functional domains of PIAS1 include the SAP domain, which interacts with DNA, and the Ring finger, which is required for E3 SUMO ligase activity. The amino acids corresponding to the truncated Msx1 and PIAS1 proteins (as shown in C,D; Supplementary Fig. 2) are indicated. (B) Msx1 interacts with PIAS1. 293T cells were transfected with expression plasmids encoding Flag-Msx1 alone (Endogenous) or with HA-PIAS1 (Exogenous) followed by immunoprecipitation with α -Flag and immunoblotting with α -PIAS1 (top) or α -HA (bottom) to detect the endogenous or exogenous PIAS proteins, respectively. Tubulin is shown as a control. In all experiments, lanes labeled "Input" contain 10% of the total cell extract. (C) The C-terminal region of Msx1 interacts with PIAS1. 293T cells were cotransfected with HA-PIAS1 along with Flag-Msx1 or the indicated truncated Flag-tagged Msx1 proteins followed by immunoprecipitation with α -Flag and immunoblotting with α -HA or α -Flag. The asterisk indicates lower-mobility Msx1 proteins, which correspond in size to potential SUMO-modified forms. In all experiments, lanes labeled "Vector" contain a control plasmid. (D) The N-terminal region of PIAS1 interacts with Msx1 in vitro. PIAS1 or the indicated truncated or mutated derivatives were produced by in vitro transcription/translation and interaction assays were performed using MBP-Msx1. Bound proteins were resolved by SDS-PAGE and visualized by autoradiographic detection of the radio-labeled PIAS1 proteins. (E) Msx1, but not other homeoproteins, interacts with PIAS1. 293T cells were cotransfected with plasmids encoding HA-PIAS1 along with Flag-Msx1 or the indicated Flag-tagged homeoproteins, immunoprecipitated with α -Flag, and probed with α -HA or α -Flag. (F) Msx1 interacts preferentially with PIAS1. 293T cells were cotransfected with Myc-Msx1 alone or with the indicated Flag-PIAS proteins, immunoprecipitated with α -Flag, and immunoblotted with α -Myc or α -Flag. (G) Msx1 is sumoylated at Lys 15 and Lys 133. 293T cells were transfected with Myc-SUMO along with Flag-Msx1 or the indicated Flag-Msx1 proteins. To dissociate Msx1 from other sumoylated proteins, cell lysates were denatured with 1% SDS in the presence of 50 mM of N-ethylmaleimide (NEM). Denatured lysates were diluted with 10 volumes of RIPA buffer followed by immunoprecipitation with α -Flag and probed with α -Myc or α -Flag. A description of all proteins is provided in Supplementary Table 2.

Msx1 has two lysine residues in the N-terminal region (K15 and K133) that are putative consensus sites for sumoylation (ψ KXE) (Supplementary Fig. 1A; Hochstrasser 2001; Seeler and Dejean 2003; Hay 2005). Substitution of these, but not other, lysine residues with arginine [Msx1(K15R); Msx1(K133R); and Msx1(K15R,K133R)] rendered Msx1 proteins that were defective for sumoylation (Fig. 1G; Supplementary Fig. 4A,B).

However, these sumoylation-defective Msx1 proteins [Msx1(K15R), Msx1(K133R), and Msx1(K15R,K133R)]

were capable of interacting with PIAS1 (Fig. 2A; Supplementary Fig. 4C). Conversely, PIAS1 proteins with defective E3 SUMO ligase activity [HA-PIAS1(W372A) and HA-PIAS1(C350S) (Kahyo et al. 2001; Kotaja et al. 2002)] interacted with Msx1 (Fig. 1D). Thus, while PIAS1 sumoylates Msx1, this modification is not required for its interaction with Msx1.

The major sumoylation site (K133) is located within an N-terminal region that is required for several key functions of Msx1, namely interaction with histone

Figure 2. The interaction of Msx1 with PIAS1 is necessary for its activity. (A) Summary of the relative activities of Msx1 and truncated or mutated derivatives. The asterisks indicate the positions of amino acid substitutions. A description of the proteins is provided in Supplementary Table 2. (B) Cells were transfected with an expression plasmid expressing Msx1 or the indicated Msx1 truncated or mutated derivatives (100 ng) along with a *MyoD* luciferase reporter plasmid (100 ng). Data are expressed as fold repression relative to the vector control. (C) Cells were infected with a retrovirus expressing Msx1 or the indicated Msx1 derivatives and grown in conditions that promote differentiation to myotubes (Supplementary Fig. 5B). Expression of *myogenin*, relative to *GAPDH*, was determined by real-time PCR. For comparison, control cells were grown in conditions that maintain their undifferentiated state (Vector, UD). (D) Cells were infected with a retrovirus expressing Msx1 or the indicated Msx1 derivatives and grown in conditions that maintain their undifferentiated state. Binding to the CER of the endogenous *MyoD* gene was examined by ChIP analyses. Data were normalized to *Actin* as a control. Insets in B–D are Western blots showing the expression levels of the exogenous Msx1 and its derivatives. Data in B–D represent the averages of at least three independent experiments done in triplicate; error bars indicate standard deviation.

H1b, transcriptional repression, inhibition of differentiation, and binding to the CER regulatory element in the *MyoD* promoter (Supplementary Fig. 1A; Lee et al. 2004). However, we found that sumoylation of Msx1 was not necessary for any of these activities (Fig. 2A–D; Supplementary Fig. 5A,B). In fact, rather than being decreased, each of these activities of the sumoylation-defective Msx1(K15R,K133R) were modestly but reproducibly increased relative to that of wild-type Msx1. In striking contrast, an Msx1 protein lacking the PIAS1 interaction region [Msx1(1–271)] was significantly attenuated in its ability to repress transcription, inhibit myoblast differentiation, and bind to the CER (Fig. 2A–D). These findings suggest that the interaction of Msx1 with PIAS1, but not its sumoylation, is required for Msx1 to inhibit differentiation and repress *MyoD* expression.

We directly investigated the requirement of PIAS for these activities of Msx1 by performing knock-down experiments using short hairpin RNA interference (shRNAi) (Fig. 3). We found that depletion of endogenous PIAS1, but not PIASxβ, attenuated the activities of Msx1 in transcriptional repression and inhibition of myoblast

differentiation (Fig. 3A,B; Supplementary Fig. 5C; data not shown). Notably, the consequences of PIAS1 depletion were analogous to removal of the PIAS1 interaction region [Msx1(1–271)] (Fig. 2A–D). Therefore, the activities of Msx1 in transcriptional repression and inhibition of differentiation are dependent on PIAS1.

PIAS1 confers DNA-binding specificity on Msx1 in myoblast cells

Considering that an Msx1 protein lacking the PIAS1 interaction region [Msx1(1–271)] was impaired in its ability to interact with the CER (Fig. 2D), we next asked whether PIAS1 affected DNA-binding specificity of Msx1 in myoblast cells. Inspection of sequences up to 25 kb upstream of the *MyoD* promoter revealed numerous (>80) DNA sequences containing one or more TAAT motifs (Fig. 3C), which are potential Msx1-binding sites (Catron et al. 1993). While Msx1 bound to most of these TAAT sites in vitro (V.A. Swiss and C. Abate-Shen, unpubl.), it bound exclusively to the CER (fragment A) of endogenous *MyoD* in the C2C12 myoblast cells (Fig. 3C;

Figure 3. PIAS1 is required for target site recognition by Msx1 in myoblast cells. (A,B) PIAS1 depletion attenuates the functions of Msx1. (A) Transcriptional repression analyses were done following infection of C2C12 cells with retroviruses expressing two different PIAS1 shRNAi, or a control (empty vector), and transfection with the indicated *Msx1* plasmids (400 ng) and a *MyoD* luciferase reporter (200 ng). Data are expressed as fold repression relative to the control (vector); error bars indicate standard deviation. The *p* values were calculated for *Msx1*-containing samples with the control RNAi versus PIAS1 RNAi (1) or RNAi (2). (Inset) Western blot showing knock-down of endogenous PIAS1 protein with PIAS1 shRNAi (1). Unless otherwise indicated shRNAi (1) was used for all experiments. (B) Inhibition of differentiation was assessed following infection of C2C12 cells with retroviruses expressing PIAS1, PIASxβ, or a control (empty vector) shRNAi along with retroviruses expressing *Msx1* (or Vector) and grown in conditions that promote differentiation (Supplementary Fig. 5C). Expression of *myogenin* and *GAPDH* were determined by real-time PCR. Error bars indicate standard deviation; the *p* value was calculated for *Msx1*-expressing samples with the control versus PIAS1 RNAi.

(C) Diagram of the upstream region of the *MyoD* gene. TAAT sequences are shown by dark boxes; the lighter box (H, DRR) has no TAAT sequences. The positions of *MyoD* regulatory regions, CER and DRR, are indicated. Summaries of the DNA-binding activity of Msx1 in vitro (V.A. Swiss and C. Abate-Shen, unpubl.) and in vivo (Supplementary Fig. 5D) are shown. (D) PIAS1 binds selectively to the CER in C2C12 myoblast cells. ChIP assays were done in C2C12 cells infected with retroviruses expressing Flag-PIAS1, with or without Myc-Msx1. Anti-Flag was used to isolate fragments A, G, or H as indicated. Shown are representative data from two experiments done in triplicate; error bars indicate standard deviation. (E) Depletion of PIAS1 decreases the DNA-binding specificity of Msx1 in myoblast cells. ChIP assays were done in C2C12 cells following infection with retroviruses containing a control RNAi or two different PIAS1 shRNAi, or a PIASxβ shRNAi and a retrovirus expressing Flag-Msx1. Data show the average of three independent experiments done in triplicate; error bars indicate standard deviation. The *p* values compare binding of Msx1 in cells expressing the control versus PIAS1 shRNAi (1) for sites A or G as indicated. Relative binding was determined using *Actin* as a control. (Inset) Western blot showing that the levels of Msx1 are not altered by the PIAS1 shRNAi.

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Supplementary Fig. 5D). These observations extend our previous findings (Lee et al. 2004) and demonstrate the ability of Msx1 to distinguish between a potential binding site in vitro and an authentic target site in vivo.

Importantly, we found that PIAS1 also interacts specifically and preferentially with the CER of the endogenous *MyoD* gene in myoblast cells (Fig. 3D). Furthermore, using shRNAi to knock-down endogenous PIAS1, we found that depletion of PIAS1, but not PIASxβ, resulted in decreased binding of Msx1 to the CER in C2C12 cells (from fourfold to 1.8-fold, $p < 0.0001$) (Fig. 3E). On the contrary, depletion of PIAS1 increased binding to another TAAT-containing site (fragment G) that is not bound by Msx1 in vivo (from 1.1- to 1.8-fold, $p < 0.01$), although it did not promote binding of Msx1 to DNA sites lacking any TAAT sequences (fragment H;

Figure 3C,E). These findings suggest that PIAS1 permits Msx1 to distinguish its authentic target sites, such as the CER, from other irrelevant TAAT-containing sequences in myoblast cells.

PIAS1 is required for localization of Msx1 at the nuclear periphery

To further investigate the significance of the interaction between Msx1 and PIAS1, we examined their subnuclear localization in myoblast cells. In the absence of exogenous Msx1, exogenous PIAS1 displayed a punctate distribution within the nucleus (Fig. 4F,K), as has been reported in other cell types (Sachdev et al. 2001; Kotaja et al. 2002). However, when coexpressed with Msx1, PIAS1 exhibited a striking redistribution to the nuclear periph-

Figure 4. Colocalization of Msx1 and PIAS1 at the nuclear periphery. (A–O) C2C12 cells were transfected with plasmids expressing HA-PIAS1 and Flag-Msx1 or the indicated Flag-Msx1 proteins (or a control vector) and visualized by immunofluorescence with α -Flag (Msx1, green) or α -HA (PIAS1, red). Images were collected using a Leica confocal microscope. Merged images in K–O were generated using Adobe Photoshop. (P–S) Endogenous Msx is associated with the nuclear periphery in the embryonic limb. Msx protein expression was visualized by immunofluorescence in cryosections of mouse embryos (10.5 dpc). (P) Shown is the forelimb, where Msx expression is localized along the perimeter in the mesenchyme and epithelium (arrows). Higher-power views (boxed area, P) show Msx expression in the vicinity of the nuclear periphery (arrows, Q,R). (S) Topro shows the staining of the nuclei. (T–V) C2C12 cells expressing Flag-Msx1 were infected with a retrovirus expressing a control, PIAS, or PIASx β shRNAi. Flag-Msx1 was detected by immunofluorescence with α -Flag (Msx1, green). Note that PIAS1 depletion results in diffuse staining of Msx1 throughout the nucleus rather than concentrated at the periphery.

ery where it was colocalized with Msx1 (Fig. 4B,G,L). Importantly, the distribution patterns of these proteins, either individually or together, were not dependent on fixation conditions or other experimental parameters (see Materials and Methods).

We extended these studies by examining the localization of endogenous Msx1 in mouse embryos, focusing on the forelimb where both *Msx1* and *PIAS1* are known to be coexpressed (Bendall and Abate-Shen 2000; Sturm et al. 2000). Using a monoclonal antibody that recognizes both Msx1 and Msx2 (hence, we refer to Msx expression) (Liem et al. 1995), we observed that endogenous Msx proteins were also located at the nuclear periphery in the embryonic forelimb (Fig. 4P–S). Therefore, localization of Msx1 at the nuclear periphery is not likely to be a consequence of its exogenous expression in cell culture.

Colocalization of Msx1 and PIAS1 at the nuclear periphery required their interaction, since proteins lacking their respective interaction regions were not colocalized at the periphery (Fig. 4D,I,N; Supplementary Fig. 6). In contrast, a sumoylation-defective Msx1 protein [Msx1(K15R,K133R)]

and, conversely, E3 SUMO-ligase-defective PIAS1 proteins [PIAS1(W372A) and PIAS1(C350)] were colocalized with PIAS1 or Msx1, respectively, at the periphery (Fig. 4C,H,M; Supplementary Fig. 6C',D',G',H',K',L'). Notably, Msx1-A, which is not active in transcriptional repression but interacts with PIAS1 (Figs. 1C, 2A,B; Zhang et al. 1996), was not colocalized with PIAS1 at the nuclear periphery (Fig. 4E,J,O). Taken together, these findings indicate that, regardless of its sumoylation status, Msx1 can colocalize with PIAS1 at the nuclear periphery, provided that it is able to interact with PIAS1 and function as a transcriptional repressor.

We investigated whether localization of Msx1 at the nuclear periphery was dependent on endogenous PIAS1 using shRNAi knock-down. We found that depletion of PIAS1, but not PIASx β , rendered Msx1 no longer concentrated at the periphery but instead diffusely localized throughout the nucleus (Fig. 4T–V). Notably, this expression pattern was similar to that of an Msx1 protein lacking the PIAS1 interaction region [Msx1(1–271)] (Fig. 4D,I,N). Therefore, PIAS1 is necessary for localization of Msx1 to the nuclear periphery.

Figure 5. PIAS1 restricts the mobility of Msx1 within the nucleus. C2C12 cells expressing CFP-Msx1 were infected with retroviruses expressing a control (empty vector), PIAS1, or PIASxβ shRNAi. (Top) Representative images from an individual cell taken prior or subsequent to photobleaching for the indicated time points; the red box shows a representative photobleached area. (Bottom) Scatterplot of the percent fluorescence recovery versus time, with $t_{1/2}$ indicated. Data were collected from 10–20 cells in three independent experiments; the scatterplot shows the average of these experiments. The p value compares the $t_{1/2}$ for the PIAS1 shRNAi versus the control.

PIAS1 restricts the mobility of Msx1 within the nucleus

We extended these observations by investigating whether PIAS1 affects the kinetics of movement of Msx1 in living cells. We performed fluorescence recovery after photobleaching (FRAP) using fluorescently tagged derivatives of Msx1 [CFP-Msx1, CFP-Msx1(1–271) and CFP-Msx1(K15R,K133R)] expressed in C2C12 cells. We observed that CFP-Msx1 displayed a time for half-recovery ($t_{1/2}$) in the range of 20–22 sec (Fig. 5; Supplementary Fig. 7), which is in the order of reported values for other transcriptional regulatory proteins (Carrero et al. 2003). However, following depletion of PIAS1, but not PIASxβ, the mobility of CFP-Msx1 within the nucleus was significantly increased ($t_{1/2} = 13$ sec; $p = 0.01$). Similarly, an Msx1 protein lacking the PIAS1 interaction region [CFP-Msx1(1–271)] displayed rapid kinetics of movement within the nucleus (Supplementary Fig. 7). Although the resolution of this live-cell imaging experiment does not permit effective visualization of Msx1 at the nuclear periphery, we observed that CFP-Msx1 displayed slower kinetics of movement near the periphery relative to

more internal regions of the nucleus ($t_{1/2} = 24$ vs. 20 sec, respectively) (Supplementary Fig. 7). These findings suggest that PIAS1 is not only required for the localization of Msx1 at the nuclear periphery, it also prevents its dispersion by restricting the mobility of Msx1 within the nucleus.

Msx1 target genes are located in the nuclear periphery

Considering the involvement of PIAS1 in the localization and retention of Msx1 at the nuclear periphery, we next performed DNA FISH to ask where Msx1 target genes are located in myoblast cells. Strikingly, we found that *MyoD* was localized to the nuclear periphery in >98% (116 of 118) of C2C12 cells but seldom (24 of 102) in nonmyoblast cells, such as HC11 (Fig. 6A,E). This localization of *MyoD* to the nuclear periphery was not dependent on expression of Msx1 or PIAS1 (Fig. 6H; data not shown); however, it was dependent on the differentiation status of the cells, since *MyoD* was not localized to the periphery in differentiated C2C12 cells (Fig. 6D).

In addition to *MyoD*, *Myf5*, another myogenic regulatory gene that is repressed by Msx1 (Lee et al. 2004) (Y.

Figure 6. Target genes that are repressed by Msx1 in myoblast cells are localized to the nuclear periphery. DNA FISH analyses showing the localization (white arrowheads) of the *MyoD*, *Myf5*, or *mCAT2* genes in undifferentiated myoblast cells (C2C12, A–C) and nonmyoblast cells (HC11, E–G). Also shown is the localization of the *MyoD* gene in differentiated C2C12 cells (D) and in undifferentiated C2C12 cells expressing a PIAS1 RNAi (H).

Chen and C. Abate-Shen, in prep.), was also localized to the nuclear periphery in >90% (58 of 63) of C2C12 cells but not in HC11 cells (29 of 69) (Fig. 6B,F). In contrast, *mCAT2* (MacLeod 1996), a gene that is expressed in muscle cells but not repressed by Msx1 (Y. Chen and C. Abate-Shen, unpubl.), displayed no preference for localization to the nuclear periphery in either C2C12 or HC11 cells (41 of 89) (Fig. 6C,G). Cumulatively, our findings suggest that PIAS1 regulates the localization Msx1, the consequences of which are to bring Msx1 to the proximity of genes that are targets for its repressor function (Fig. 7).

Discussion

Understanding how homeoproteins achieve target recognition in vivo remains a critical problem in developmental biology. Indeed, bona fide target genes for homeoproteins, as well as the regulatory sequences to which they bind in vivo, remain poorly defined. To a large extent this reflects the striking contrast between the promiscuous DNA-binding specificities of homeoproteins in vitro and their selective recognition of target sites in vivo, which makes it virtually impossible to extrapolate from an in vitro binding site to a bona fide target gene. So, the challenge is to investigate the biochemical properties of homeoproteins within biologically relevant contexts.

In our analyses of the mechanisms underlying DNA-binding specificity of the Msx1 homeoprotein in vivo, we have now found that PIAS1 is a critical determinant

of target site recognition by Msx1 in myoblast cells. Our current studies complement our previous ones in which we demonstrated that the functions of Msx1 as a transcriptional repressor and regulator of differentiation require its interaction with histone H1b (Lee et al. 2004). Notably, the interaction of Msx1 with PIAS1 occurs through a distinct domain from that required for its interaction with H1b and, unlike the interaction with H1b, the interaction of Msx1 with PIAS1 is not conserved with Msx2, which itself interacts with a different member of the PIAS family (Wu et al. 1997). Thus, we envision that target site recognition by Msx1 (and extrapolating more generally to other homeoproteins) represents the culmination of several distinct protein interactions, some of which are shared with Msx2 (or other homeoproteins) while others are exclusive for Msx1.

Interestingly, the E3 SUMO ligase activity of PIAS1 does not appear to be required for its ability to confer DNA-binding specificity on Msx1 in vivo or for inhibition of differentiation or transcriptional repression. Although PIAS proteins sumoylate many protein partners, the consequences of this modification differ dramatically depending on the particular partner (Hochstrasser 2001; Gill 2003; Seeler and Dejean 2003; Hay 2005). In some cases, sumoylation has profound effects, as exemplified by sumoylation of Sp3, which renders it transcriptionally inactive and also affects its subnuclear localization (Ross et al. 2002; Sapetschnig et al. 2002). However, for other proteins, such as Lef1, the consequences of sumoylation are not readily apparent (Sachdev et al. 2001). In the case of Msx1, the lack of a requirement for sumoylation seems counterintuitive, since the primary sumoylation site is located within the minimal conserved region required for inhibition of differentiation and transcriptional repression (Lee et al. 2004). Indeed, rather than being less active, an Msx1 protein that is defective for sumoylation is slightly *more active*, which leads us to speculate that sumoylation of Msx1 may be inhibitory for these activities. One possibility is that sumoylation provides a clearance mechanism for rendering Msx1 inactive and thereby “fine-tuning” the extent and/or timing of its repressive capabilities.

While colocalization of PIAS proteins with their partners is common, colocalization to the nuclear periphery is unusual; the interaction of PIAS proteins with part-

Figure 7. Model showing how PIAS1 confers DNA-binding specificity on Msx1 by regulating its proximity to target genes. In the absence of PIAS1, Msx1 is free to diffuse within the nucleus and access irrelevant TAAT sites, whereas with PIAS1, Msx1 is retained at the nuclear periphery where it binds specific targets, such as the CER of *MyoD*.

ners generally results in sequestration to nuclear bodies, as exemplified by the association of PIASy with the activator, Lef1 (Sachdev et al. 2001). Considering that Lef1 and Msx1 have complementary biological functions in at least one biological context (Chen et al. 1996; Kratochwil et al. 1996), it is tempting to speculate that their actions are regulated, at least in part, by the ability of PIAS proteins to shuttle Lef1 and Msx1 to distinct subnuclear compartments, potentially toward or away from distinct sets of target genes.

It has long been recognized that the organization of chromosomes within the nucleus is an active process, in which chromosomes partition into distinct compartments depending on the cell type, differentiation status, and other biological parameters (Marshall et al. 1997; Vazquez et al. 2002; Harmon and Sedat 2005). Therefore, although not previously appreciated, it is perhaps not surprising that the shuttling of a homeoprotein protein to the nuclear compartment where its target genes reside plays a critical role in target recognition. Notably, the colocalization of Msx1 and PIAS1 at the nuclear periphery requires that Msx1 be competent for repression, since a transcriptionally inert form of Msx1 (Msx1-A) does not colocalize with PIAS1 at the periphery. Therefore, although localization of Msx1 to the periphery requires PIAS1, Msx1 is clearly not an innocent bystander but rather an active participant in the dynamic localization of its proximity to its targets. Notably, the nuclear periphery is now recognized as a critical compartment not only for gene silencing but also for regulation of transcriptional repression (Gasser 2001; Kosak and Groudine 2004; Misteli 2004). Our findings, which implicate the nuclear periphery as the site for repression of *MyoD* by an Msx1–PIAS1 complex in undifferentiated myoblast cells, provide further support for the idea that this nuclear compartment plays a key regulatory role in transcriptional repression.

In conclusion, we have uncovered a hitherto unappreciated function for PIAS1 as a critical determinant of homeoprotein DNA-binding specificity through its ability to regulate the localization of Msx1 within the nucleus and its proximity to target genes (Fig. 7). We propose that PIAS1 retains Msx1 at the nuclear periphery where it binds to the CER regulatory element of the *MyoD* gene and presumably regulatory elements of other target genes, such as *Myf5*. Therefore, our findings support the idea that, in addition to primary sequence determinants, recognition of bona fide target sequences by Msx1 requires a dynamic interplay with the nuclear architecture, which culminates in its localization to target genes. More broadly, this model can readily be extended to explain how specific target recognition occurs for other homeoproteins, regardless of whether they use PIAS proteins or analogous regulatory partners.

Materials and methods

Plasmids

Plasmids encoding Flag- or Myc-tagged Msx1, Msx1-A [Msx1(K174A,R176A,F179A)], the truncated Flag-Msx1 deriva-

tives, other Flag-tagged homeoproteins, and MBP-Msx1 were described in Lee et al. (2004) and references therein. Sequences encoding Msx1 derivatives with substitutions of lysine residues and those encoding the CFP-tagged Msx1 proteins were generated by PCR mutagenesis. Sequences corresponding to the coding region of *PIAS1* were generated by PCR from image clone #1296138 (Research Genetics/Invitrogen) using oligonucleotides that introduced the Flag or HA epitope tags at the N terminus. Sequences encoding truncated PIAS1 proteins and PIAS1 proteins with substitutions of residues 372 and 350 were made by PCR. Other PIAS cDNAs were described in Liu et al. (1998). Sequences corresponding to full-length *SUMO* were isolated by PCR from image clones #1547915 (SUMO2) or #849895 (SUMO1). All data shown were obtained using SUMO2; similar results were obtained with SUMO1. All DNA sequences generated by PCR were sequence confirmed. Expression plasmids were as follows: (1) for production of MBP-tagged proteins in *Escherichia coli*, pMAL-c2E (New England Biolabs); (2) for transient transfection, pcDNA3 (Invitrogen); (3) for retroviral gene transfer, pLZRS-IRES-GFP (Lee et al. 2004); and (4) for retroviral delivery of shRNAi, pMSCV-HpaI-Gateway (Hemann et al. 2003). Sequences of primers used in this study are provided in Supplementary Table 1; a description of all proteins used is provided in Supplementary Table 2.

Yeast two-hybrid screen

Yeast two-hybrid screening was performed using the DupLEX-A yeast two-hybrid system (OriGene Technologies, Inc.) with the full-length Msx1 coding region fused to LexA as the bait. The library was made from mammary glands of day 12.5 pregnant mice (OriGene Technologies, Inc.), where *Msx1* is known to be expressed and functionally relevant (Bendall and Abate-Shen 2000). A total of 2.7×10^6 transformants were screened for potential interactors. Following four independent verifications, 29 positive interactors were obtained, which corresponded to 12 independent genes. Among these were one positive clone that corresponded to *PIAS1* and one that corresponded to *Ubc9*.

Cell culture, immunoprecipitation, and chromatography in immunoprecipitation (ChIP) assays

Cell culture studies were done using human 293T cells or mouse C2C12 myoblast cells. Transcriptional repression was measured by transfection of *Msx1* (or other) expression plasmids along with a *MyoD* reporter plasmid (pGL2-F3/-2.5 *MyoD*-promoter-luciferase) as in Hu et al. (2001) and Lee et al. (2004). For myoblast differentiation assays, undifferentiated C2C12 cells were grown in media containing 10% serum and differentiation was induced by shifting cells to media containing 0.1% serum (Hu et al. 2001; Lee et al. 2004).

For immunoprecipitation assays, cells were lysed in RIPA buffer and proteins were immunoprecipitated with anti-Flag M2 agarose (Sigma) (Lee et al. 2004). Immunoprecipitated proteins were analyzed by immunoblotting using ECL plus Western Blotting Detection (Amersham). For the detection of endogenous PIAS1, membranes were blocked with 10% normal donkey serum (Jackson Immunological) and incubated with anti-PIAS1 antibody (C-20, Santa Cruz). Other antibodies used were anti-Flag (M2, Sigma), anti-Myc (Hu et al. 2001), anti-HA (HA-7, Sigma), and anti-V5 (V5-10, Sigma).

Primers (Supplementary Table 1) were used to PCR amplify 11 fragments (~300 base pairs each) from the upstream region of the *MyoD* gene (as per Fig. 3C) and ChIP was done as described (Lee et al. 2004). Samples were quantified by real-time PCR

using an Mx4000 Multiplex Quantitative PCR System (Stratagene).

Immunofluorescence in cells and mouse embryos

Immunofluorescence assays were done in C2C12 cells expressing *Msx1* and/or *PIAS1* and/or the indicated *PIAS* shRNAi. Cells were fixed in 4% paraformaldehyde, blocked with 10% goat serum, and incubated with the following antibodies: Cy3-conjugated anti-Myc (Sigma), FITC-conjugated anti-FLAG (Sigma), and anti-HA (Sigma) visualized with Texas Red (Molecular Probes). Immunofluorescence was visualized with a Leica DMIRE2/TCS SP2 inverted confocal microscope equipped with argon/krypton and helium/neon lasers using excitation wavelengths 488, 543, and 594 nm. For Cy3 and Texas Red, the collection range of emission spectra was narrowed to eliminate the possibility of bleed-through from these two fluors. Similar results to those shown in Figure 4 and Supplementary Figure 6 were obtained in independent experiments performed in cells fixed with 10% formalin or 95% methanol/5% acetic acid as well as different antibody combinations.

Immunofluorescence assays were done on cryosections of mouse embryos from post-coital day 10.5 (10.5 dpc) that had been fixed in 4% paraformaldehyde. *Msx* proteins were visualized using the 4G1 antibody (Developmental Studies Hybridoma Bank), which recognizes both *Msx1* and *Msx2* (Liem et al. 1995).

FRAP assays

C2C12 cells expressing the CFP-*Msx1* with or without the indicated *PIAS* shRNAi were plated on 35-mm glass-bottom microwell dishes (MatTek). FRAP was performed using a Leica confocal microscope (as above) fitted with an INC-2000 chamber/incubator (20/20 Technology, Inc.) at a constant temperature of 37°C and with constant levels of CO₂ (5%). Photobleaching was performed using a 488-nm argon/krypton laser at full laser power (100% for 0.828 sec). Three regions of interest were chosen for each cell analyzed: one in the center of the nucleus, a second near the nuclear periphery, and a third in the cytoplasm (used for calculation of background). Fluorescence recovery was monitored for a period of 300 sec using a 63× oil objective by scanning at low laser power (5%) at 0.828-sec increments. Recovery curves were generated using Leica Confocal Software.

DNA FISH

C2C12 cells were pre-extracted in cytoskeletal buffer (CSK: 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, and 10 mM PIPES at pH 6.8) containing 0.5% Triton-X 100 for 5 min on ice and fixed with 3.7% freshly prepared formaldehyde for 15 min at room temperature. The cells were washed in 1× PBS (pH 7.2) and heat denatured in 70% formamide and 2× SSC at 72°C for 5 min, followed by hybridization with the nick-translated BAC probes in 2× SSC, 50% formamide, 10% dextran sulfate, yeast tRNA, and Cot-1 DNA overnight at 37°C as previously described (Xing et al. 1995). BAC clones were nick-translated using spectrum red-conjugated dUTP as a substitute nucleotide according to the manufacturer's instructions (VYSIS, Inc.). The BAC clones used were as follows: *MyoD*, RP24-358H24; *Myf5*, RP23-57E8; *mCAT2*, RPCI-23-94B23 (all from CHORI).

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