

# Cell cycle gene-specific control of transcription has a critical role in proliferation of primordial germ cells

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**Transcription elongation is stimulated by positive transcription elongation factor b (P-TEFb), for which activity is repressed in the 7SK small nuclear ribonucleoprotein (7SK snRNP) complex. We show here a critical role of 7SK snRNP in growth control of primordial germ cells (PGCs). The expression of *p15<sup>INK4b</sup>*, a cyclin-dependent kinase inhibitor (CDKI) gene, in PGCs is selectively activated by P-TEFb and its recruiting molecule, Brd4, when the amount of active P-TEFb is increased due to reduction of the 7SK snRNP, and PGCs consequently undergo growth arrest. These results indicate that CDKI gene-specific control of transcription by 7SK snRNP plays a pivotal role in the maintenance of PGC proliferation.**

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Transcription is a highly regulated multistep process, and transcription elongation by RNA polymerase II (RNAPII) is a key step in the production of mature mRNA. Transcription elongation is stimulated by the positive transcription elongation factor b (P-TEFb), and the 7SK small nuclear ribonucleoprotein (7SK snRNP) forms a complex

with P-TEFb to suppress P-TEFb activity (Diribarne and Bensaude 2009). Once the amount of any component of the 7SK snRNP (including Larp7) is reduced, the inhibitory complex becomes destabilized, and P-TEFb is released in an active form (Diribarne and Bensaude 2009). Recruiting molecules, such as Brd4, then recruit activated P-TEFb to the transcription start site (TSS) of target genes and phosphorylate Ser2 in the C-terminal domain (CTD) of RNAPII to stimulate transcription elongation (Jang et al. 2005; Yang et al. 2005). The 7SK snRNP is therefore considered as reservoir machinery to generally control transcription elongation.

Primordial germ cells (PGCs) are precursors of eggs and sperm and emerge as clusters of small numbers of cells in early embryos (Matsui and Okamura 2005; Hayashi et al. 2007; Saitou 2009). PGCs subsequently proliferate rapidly and increase in number but stop proliferation in later gestational stages in mammalian embryos (Western 2009). Male germ cells subsequently arrest in G1 phase until birth, while female germ cells immediately enter meiotic prophase. PGC proliferation is therefore dynamically regulated, but mechanisms underlying cell cycle progression of PGCs are not fully understood.

Eukaryotic cell cycle progression is tightly controlled by a number of molecular pathways. In particular, cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs) play pivotal roles in cell cycle control (Sherr and Roberts 1999). CDKIs belonging to the INK4 family, including *p15<sup>INK4b</sup>*, inhibit cyclinD-CDK4/6 and induce cell cycle arrest in G1 phase. Transcriptional regulation of CDKI expression has an important role in establishing G1 arrest under various biological conditions (Massague 2004). Previous studies indicate that expression of CDKI genes is up-regulated in PGCs that have stopped proliferating (Western et al. 2008), suggesting their critical roles in regulation of PGC proliferation. In this study, we found that 7SK snRNP-mediated selective repression of *p15<sup>INK4b</sup>* is necessary for PGCs to actively proliferate in mouse embryos.

## Results and Discussion

We isolated *Larp7*, a gene encoding a La-related protein, and a component of the 7SK snRNP using differential cDNA screening because it was preferentially expressed in mouse PGCs (Supplemental Fig. S1A–F). We generated knockout mice lacking functional *Larp7* to investigate *Larp7* functions in PGCs (Supplemental Fig. S1G–I). Mice heterozygous for the *Larp7* mutation were healthy and fertile. Homozygous mutant embryos were histologically normal (Supplemental Fig. S2C–S) but tended to be slightly smaller than their heterozygous mutant and wild-type littermates at embryonic day 16.5 (E16.5) (Supplemental Fig. S2A,B), and all homozygous mutants were, for unknown reasons, dead between E17.5 and the time of birth (Supplemental Fig. S3A). Because a few homozygous embryos were obtained at E19, it is likely that any pups that were actually born were eaten by their parents just after birth. A few *Larp7* homozygous mutant embryos with a higher contribution of 129Sv genetic background presented with a failure of neural tube closure (Supplemental Fig. S3B), but this abnormality was not observed in *Larp7* homozygous mutant embryos derived from backcrosses with C57BL/6 mice.

[**Keywords:** primordial germ cell; CDK inhibitor; transcription; P-TEFb; 7SK snRNP; Larp7]

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We focused on PGC abnormalities in *Larp7* homozygous mutant embryos. We first examined PGCs in mutant and wild-type embryos at E10.5 and found that the position and number of migrating PGCs in the homozygous mutants were indistinguishable from those in wild-type littermates (Supplemental Fig. S4A–E). We next examined PGCs in the embryonic gonads at E13.5; there were significantly fewer PGCs in male and female homozygous mutants than in wild-type embryos (Fig. 1A–E). These results indicate that *Larp7* is necessary for PGCs to increase in number after E10.5.

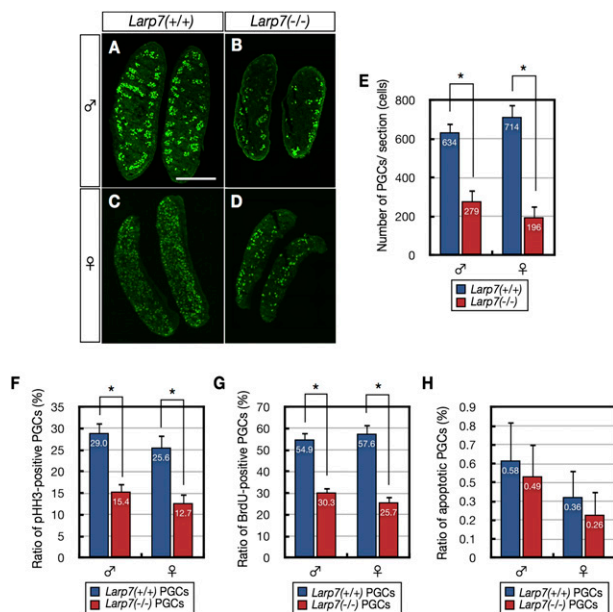
We next assessed whether *Larp7* functioned in the proliferation or survival of PGCs. Ratios of PGCs expressing phosphohistone H3 (pHH3), a marker for cells in G2–M phase of the cell cycle, and ratios of PGCs incorporating bromodeoxyuridine (BrdU), a marker of passage through S phase, were lower in mutant embryos than in wild-type embryos (Fig. 1F,G; Supplemental Fig. S5A–X,a–h), but the ratios of active caspase 3-positive apoptotic PGCs in mutant embryos were not significantly different from those in wild-type embryos at E12.5 (Fig. 1H; Supplemental Fig. S5i–x). The number of germ cells in both males and females was still lower in homozygous mutant embryos

than in wild-type embryos at E19 (Supplemental Fig. S4F–M). Taken together, these findings indicate that *Larp7* functions in the proliferation and cell cycle progression of PGCs, most likely at the G1–S transition, but is dispensable for their survival.

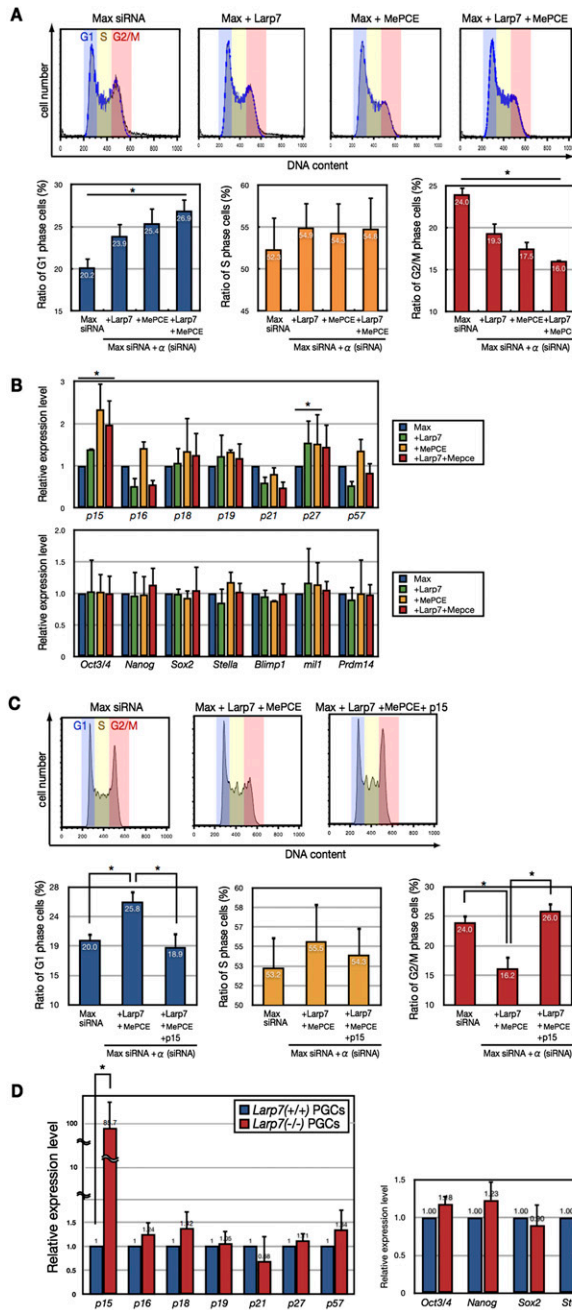
We also examined differentiation of germ cells in the homozygous mutant embryos. TDRD1 (Chuma et al. 2003) and Dnmt3L (Sakai et al. 2004) are specifically expressed in male germ cells after E15.5 and E14.5, respectively, and the expression and localization of those proteins were normal in the homozygous mutant testis at E17.5 (Supplemental Fig. S6A–P). *Scp3* is a component of the synaptonemal complex in meiotic germ cells and is specifically expressed and localized in the axial core in female germ cells after E14.5 (Yuan et al. 2000). Localization of *Scp3* was normal in mutant germ cells in embryonic ovaries at E17.5 (Supplemental Fig. S6Q–X). Taken together, these findings demonstrate that differentiation of both male and female germ cells is not affected in the *Larp7*-deficient embryos, indicating that *Larp7*-mediated transcription control specifically regulates the cell cycle of PGCs. Although *Larp7*-deficient PGCs prematurely undergo growth arrest in genital ridges (Fig. 1), the expression of TDRD1 in male PGCs and of *Scp3* in female PGCs is normally repressed until the correct time of their expression (Supplemental Fig. S6a–p), suggesting that differentiation of PGCs, including the initiation of meiosis in female germ cells, and their growth arrest are independently controlled.

We examined the functions of *Larp7* in cell cycle control in PGCs in more detail using embryonic stem (ES) cell-derived PGC-like cells in culture. We recently found that ES cells subjected to siRNA-mediated knockdown of the Max transcription factor (Hurlin and Huang 2006) are efficiently converted into PGC-like cells that express several germ cell-specific genes. We identified the PGC-like cells by monitoring expression of the *Vasa-Venus* transgene using flow cytometry; *Vasa-Venus* is specifically expressed in germ cells (Imamura et al. 2010). We introduced siRNAs targeting *Larp7*, 7SK methylphosphate capping enzyme (*MePCE*; another component of 7SK snRNP) (Jeronimo et al. 2007), or both, along with siRNA targeting *Max* into ES cells. The siRNAs for those and other related genes efficiently reduced their expression in ES cells and in Max knockdown cells (Supplemental Fig. S7). We analyzed the cell cycle of the PGC-like cells using flow cytometry (Fig. 2A). The number of cells in G1 phase was significantly increased, while the number in G2/M phase was apparently decreased, by knockdown of *Larp7* or *MePCE*; simultaneous knockdown of *Larp7* and *MePCE* resulted in additive effects (Fig. 2A; Xue et al. 2009). These results are consistent with the finding that the cell cycle in *Larp7*-deficient PGCs in embryos is abnormal (Fig. 1F,G). Moreover, these results indicate that 7SK snRNP positively controls the G1–S transition in PGCs. *Larp7/MePCE* knockdown in ES cells without Max knockdown resulted in an effect on the cell cycle (Supplemental Fig. S8) similar to that in Max knockdown ES cells (Fig. 2A), but the cell cycle profile of ES cells was apparently different from that of Max knockdown ES cells; the ratio of cells in G2 phase in Max knockdown cells (Fig. 2C) was substantially higher than that in ES cells, which resembled the cell cycle profile of PGCs (Seki et al. 2007), supporting the idea that the Max knockdown cells are a valid model to study cell cycle regulation in PGCs.

Because 7SK snRNP negatively regulates transcription, it is likely that 7SK snRNP suppressed transcription of



**Figure 1.** Reduced number of gonadal PGCs caused by the proliferation defect in *Larp7* mutant embryos. (A–D) PGC number in the E13.5 genital ridges of *Larp7* mutant embryos (B,D) and of wild-type littermates (A,C) was estimated by detecting Stella-positive PGCs using immunostaining. (E) The average number of Stella-positive PGCs in the E13.5 genital ridge per section. PGCs in five sections from the middle part of individual genital ridges were counted, and the number of PGCs in three embryos of each genotype was determined. (F–H) Loss of *Larp7* function induced cell cycle arrest of PGCs in G1 phase but had no influence on apoptotic cell death of PGCs. The ratios of pHH3-positive PGCs in G2/M phase (F) and of PGCs incorporating BrdU during S phase (G) were determined using immunostaining of a frozen section of genital ridges from E12.5 embryos of each genotype. (H) Apoptotic cells were detected by immunostaining for active caspase 3. PGCs were identified by the expression of Oct3/4 (F,H) and by the expression of Stella (G). (F–H) A total of 1500–2500 PGCs in eight sections of both genital ridges of an embryo were counted, and ratios of the pHH3-positive, BrdU-positive, and active caspase 3-positive PGCs were determined for three embryos of each genotype; the average ratios are presented. (E–G) (\*)  $P < 0.01$ . Bar, A–D, 0.3 mm.

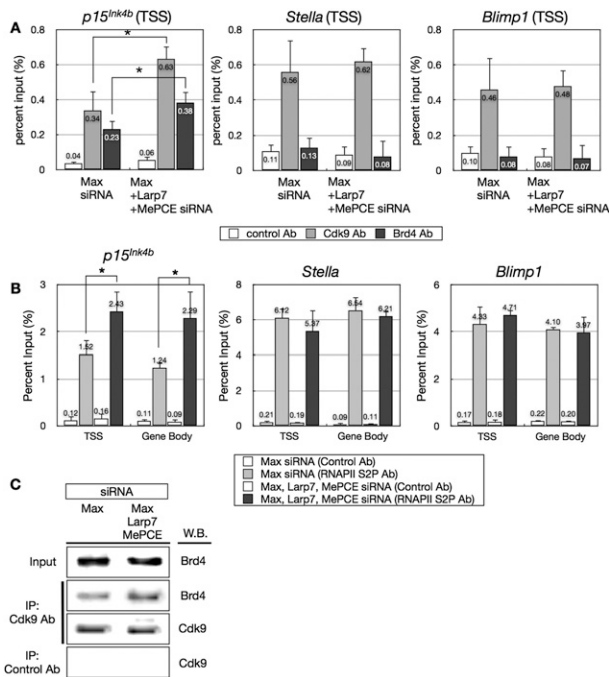


**Figure 2.** Induction of G1 arrest and CDKI gene expression in PGCs lacking Larp7, MePCE, or both. (A) The PGC-like cells were induced from the ES cells harboring the Vasa-Venus transgene by transfecting siRNA for *Max*; in some ES cultures, siRNA for *Larp7*, *MePCE*, or *Larp7* and *MePCE* were transfected along with *Max* siRNA. The cells were stained by propidium iodide (PI), and the cell cycle of the Vasa-Venus-positive PGC-like cells was analyzed using flow cytometry. (B) Quantitative RT-PCR was used to assess expression of the CDKI and germ cell-related genes in purified Vasa-Venus-positive PGC-like cells with or without knockdown of Larp7, MePCE, or both. The average values from three independent experiments are presented. (C) Simultaneous *p15<sup>INK4b</sup>* knockdown in Larp7 and MePCE knockdown PGC-like cells rescued G1 arrest. (A, C) In each experiment, 30,000 Vasa-Venus-positive cells from each sample were scored ( $n = 3$ ). (D) Quantitative RT-PCR was used to assess expression of the CDKI and the germ cell-related genes in purified GFP-positive PGCs from *Larp7* homozygous mutant and wild-type E12.5 embryos harboring the *mil1*-GFP transgene. PGCs of three independent embryos of each genotype were used to estimate expression levels. (\*)  $P < 0.05$  [A-C]; (\*)  $P < 0.01$  [D]. [A, B] Statistically significant differences between *Max*-only knockdown and other knockdown conditions are indicated.

CDKI genes that inhibited the G1-S transition in growing PGCs. We tested this hypothesis using the ES-derived PGC-like cells and found that expression of *p15<sup>INK4b</sup>* and *p27<sup>Kip1</sup>*, as estimated by quantitative RT-PCR, was up-regulated by knockdown of Larp7, MePCE, and a combination of both (Fig. 2B). In contrast, expression of other CDKI genes as well as of a number of PGC-specific genes was not affected by knockdown of either gene or a combination of both, showing that 7SK snRNP selectively repressed expression of only some CDKI genes. In ES cells without *Max* knockdown, Larp7/MePCE knockdown also induced expression of *p15<sup>INK4b</sup>*, but instead of *p27<sup>Kip1</sup>*, *p21<sup>Cip1</sup>* was additionally induced (Supplemental Fig. S9). To confirm the importance of increased *p15<sup>INK4b</sup>* expression to inhibition of the G1-S transition in PGC-like cells, we determined whether the G1 arrest caused by Larp7/MePCE knockdown could be rescued by additionally introducing siRNA targeting *p15<sup>INK4b</sup>*; the proportions of PGC-like cells in G1 phase or G2/M phase were similar in cells treated simultaneously with four siRNAs (*p15<sup>INK4b</sup>*, *Larp7*, *MePCE*, and *Max*) and those treated with only *Max* siRNA (Fig. 2C). We further investigated whether Larp7 deficiency and *Larp7* siRNA caused the same abnormal gene expression in embryonic PGCs and PGC-like cells in culture, respectively. For this experiment, we purified *mil1*-GFP-positive PGCs (Tanaka et al. 2004) from *Larp7* homozygous mutant or wild-type embryos at E12.5. The results reveal that expression of *p15<sup>INK4b</sup>* is markedly higher in PGCs from homozygous embryos than in PGCs from wild-type embryos (Fig. 2D). Together, these results indicate that 7SK snRNP selectively represses expression of *p15<sup>INK4b</sup>* in growing PGCs, and when the function of 7SK snRNP is impaired by reduced expression of Larp7 or MePCE, more active P-TEFb may be preferentially recruited to the *p15<sup>INK4b</sup>* locus to activate transcription.

Our previous studies demonstrated that the bromodomain protein Brd4 associates with P-TEFb to form an active complex, and this complex is recruited to TSSs of genes. We hypothesized that Brd4 was involved in the selective recruitment of active P-TEFb to *p15<sup>INK4b</sup>* in PGC-like cells lacking Larp7, MePCE, or both, and we assessed this possibility using a chromatin immunoprecipitation (ChIP) assay. We investigated binding of Cdk9, a subunit of P-TEFb (Peterlin and Price 2006), and of Brd4 to the TSS of *p15<sup>INK4b</sup>* in ES-derived PGC-like cells. Binding of Cdk9 and Brd4 to *p15<sup>INK4b</sup>* was significantly increased after simultaneous knockdown of Larp7 and MePCE (Fig. 3A). In contrast, binding of Cdk9 to the TSS of *Stella* (*Dppa3*) or of *Blimp1*(*Prdm1*), representative PGC-specific genes (Saitou et al. 2002; Sato et al. 2002; Ohinata et al. 2005), was not changed in cells treated with *Larp7* and *MePCE* siRNA. Moreover, Brd4 was not detected on the TSS of *Stella*(*Dppa3*) or of *Blimp1*(*Prdm1*) (Fig. 3A).

Because P-TEFb phosphorylates Ser2 in the CTD of the RNAPII large subunit, we examined changes in binding of the Ser2-phosphorylated



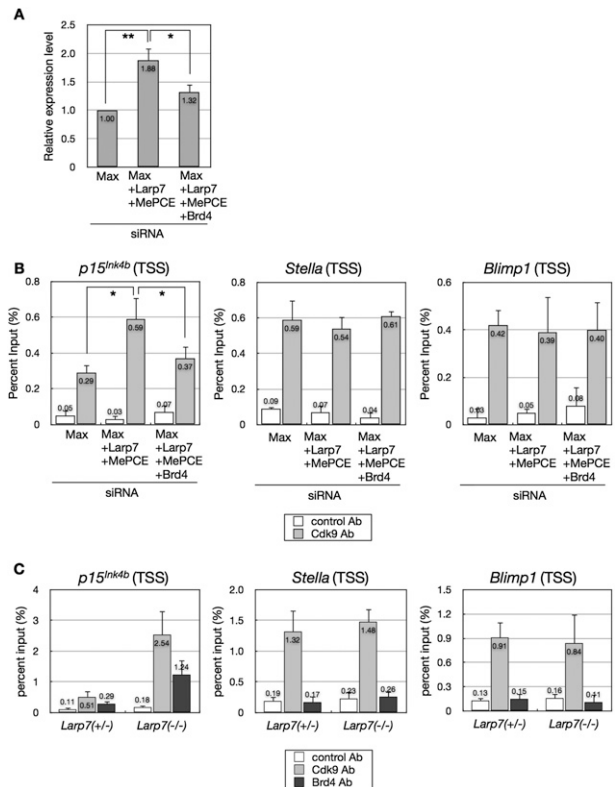
**Figure 3.** Selective increase of Cdk9, Brd4, and activated RNAPII binding at the TSS of *p15<sup>INK4b</sup>* in PGCs lacking Larp7, MePCE, or both. (A) ChIP analysis of Cdk9 and Brd4 binding at the *p15<sup>INK4b</sup>*, *Stella*, and *Blimp1* TSSs in PGC-like cells and those lacking Larp7 and MePCE; Max siRNA induced the PGC-like state. The results of three independent experiments are presented. (\*)  $P < 0.05$ . (B) ChIP analysis of S2P-RNAPII (phosphorylation of Ser2 in the CTD of the largest subunit of the RNAPII enzyme) association at TSS and the gene body of *p15<sup>INK4b</sup>* (left), *Stella* (middle), and *Blimp1* (right) in PGC-like cells and those lacking Larp7 and MePCE; Max siRNA induced the PGC-like state in ES cells. The results of three independent experiments are presented. (\*)  $P < 0.05$ . (C) Increased interaction of Cdk9 with Brd4 by knockdown of Larp7 and MePCE in PGC-like cells. Extracts of PGC-like cells with or without knockdown of Larp7 and MePCE were subjected to immunoprecipitation with control or anti-Cdk9 antibody and analyzed by immunoblot with the antibodies shown on the right. Essentially the same results were obtained by two independent experiments.

(S2P)-RNAPII to the TSSs as well as to the gene bodies of the genes by knockdown of Larp7 and MePCE by ChIP assay (Fig. 3B). Binding of S2P-RNAPII to the TSSs and the gene bodies of *p15<sup>INK4b</sup>* was significantly increased after simultaneous knockdown of Larp7 and MePCE in PGC-like cells, but its binding to *Stella* and *Blimp1* was not affected by knockdown (Fig. 3B). The results suggest that knockdown of Larp7 and MePCE results in the enhancement of phosphorylation of RNAPII and the subsequent stimulation of transcription elongation in *p15<sup>INK4b</sup>*. We confirmed that the amount of Cdk9 associated with Brd4, which should be the active form, in PGC-like cells was increased by simultaneous treatment with *Larp7* and *MePCE* siRNAs (Fig. 3C; Yang et al. 2008). Therefore, Brd4 most likely selectively recruited P-TEFb to *p15<sup>INK4b</sup>* in PGCs when the amount of active P-TEFb was increased due to repression of Larp7, MePCE, or both, and the recruited P-TEFb stimulated transcription elongation via phosphorylation of RNAPII. We confirmed the involvement of Brd4 in the activation of *p15<sup>INK4b</sup>* expression by additional knockdown of Brd4 in the Larp7/MePCE

knockdown PGC-like cells and observed decreased expression of *p15<sup>INK4b</sup>* and decreased binding of Cdk9 to the TSS of *p15<sup>INK4b</sup>* by knockdown (Fig. 4A,B).

We finally indicated that more Cdk9 and Brd4 bound to *p15<sup>INK4b</sup>* in PGCs from Larp7-deficient embryos than those from heterozygous mutant embryos (Fig. 4C). Therefore, cell cycle progression in PGCs after E10.5 was ensured by 7SK snRNP-mediated repression of *p15<sup>INK4b</sup>*.

As shown above, Brd4 selectively recruited active P-TEFb to *p15<sup>INK4b</sup>* when the amount of active P-TEFb was increased due to the reduction of Larp7 in mutant PGCs (Fig. 4C). Brd4 associates with acetylated histone (Dey et al. 2003), but its DNA sequence-specific binding is not known. It is therefore unclear how P-TEFb and Brd4 are preferentially recruited to *p15<sup>INK4b</sup>* in PGCs. To answer this question, we searched promoter regions of *p15<sup>INK4b</sup>*, *Stella*, and *Blimp1* and found that a conserved binding sequence of a transcription factor, IRF-1 (Miyamoto et al. 1988), existed only within the promoter region of *p15<sup>INK4b</sup>*, and ChIP analysis indeed indicated specific binding of IRF-1 to *p15<sup>INK4b</sup>* in genital ridge cells (Supplemental Fig.



**Figure 4.** Involvement of Brd4 in the activation of *p15<sup>INK4b</sup>* (A,B) and the selective increase of Cdk9 and Brd4 binding at the TSS of *p15<sup>INK4b</sup>* (C) in Larp7-deficient PGCs in vivo. (A) Brd4 knockdown attenuated expression of *p15<sup>INK4b</sup>* induced by Larp7 and MePCE knockdown in PGC-like cells; Max siRNA induced the PGC-like state in ES cells. ES cells transfected with the indicated siRNA were applied for quantitative RT-PCR. The results of three independent experiments are presented. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ . (B) ChIP analysis of Cdk9 binding at the *p15<sup>INK4b</sup>* (left), *Stella* (middle), and *Blimp1* (right) TSSs in PGC-like cells and those lacking Larp7 and MePCE. The results of three independent experiments are presented. (\*)  $P < 0.05$ . (C) ChIP analysis of Cdk9 and Brd4 binding at the *p15<sup>INK4b</sup>*, *Stella*, or *Blimp1* TSSs in the genital ridge cells of E12.5 Larp7 homozygous mutant and heterozygous embryos. The results of two independent experiments are presented.

S10A). IRF-1 was originally identified as a regulator of virus-inducible enhancer-like elements of the human INF- $\beta$  gene and binds to a conserved sequence termed IRF-E (Tanaka et al. 1993). We then examined the association of Cdk9 and Brd4 with IRF-1 by a pull-down assay, and the result indicates that IRF-1 forms a complex with both Cdk9 and Brd4 (Supplemental Fig. S10B). We also found that additional knockdown of IRF-1 in *Larp7*/MepCE knockdown PGC-like cells resulted in decreased expression of *p15<sup>INK4b</sup>* (Supplemental Fig. S10C). Together, these results suggest that the association of Brd4 and P-TEFb with IRF-1 results in preferential binding of P-TEFb to *p15<sup>INK4b</sup>*. We also found that IRF-1 is preferentially expressed in PGCs in embryos (Supplemental Fig. S10D,E).

7SK snRNP and Brd4 control transcription elongation in different kinds of cells (Wu and Chiang 2007; Barboric et al. 2009), but the phenotype of *Larp7* mutant embryos revealed cell type-specific functions of 7SK snRNP in embryos. We investigated the expression of components of 7SK snRNP, Brd4, and IRF-1 in various embryonic tissues at E12.5. Although the expression of *Hexim1*, *Hexim2*, *MePCE*, and 7SK snRNA did not show apparent embryonic tissue specificity (Supplemental Fig. S11C–F), *Larp7*, *Brd4*, and IRF-1 were preferentially expressed in PGCs (Supplemental Figs. S11A,B, S10E), suggesting that selective expression of *Larp7*, *Brd4*, and IRF-1 contributed to the PGC-specific function of 7SK snRNP in embryos.

Our results reveal that the 7SK snRNP, in combination with Brd4, exerts gene-specific transcriptional regulation in PGCs via quantitative control of active P-TEFb (Supplemental Fig. S12), and this transcriptional regulation is critical for PGCs to actively proliferate. Previous studies indicate that *Larp7* or the 7SK snRNP had anti-proliferative and tumor-suppressing functions in some kinds of cells, including hematopoietic cell lineage in *Drosophila* (Remillieux-Leschelle et al. 2002), cardiac myocytes in adult mice (Sano et al. 2002), and cultured cells (e.g., HeLa cells) (He et al. 2008). In addition, it was reported that Brd4 promotes cell cycle progression (Yang et al. 2008) and that Brd4 deficiency causes reduced proliferation of embryonic liver and lung cells (Houzelstein et al. 2002). In contrast, our results demonstrate that *Larp7* has positive regulatory function in the cell cycle progression of PGCs (Figs. 1, 2) and that Brd4 positively controls *p15<sup>INK4b</sup>* expression (Figs. 3, 4). The discrepancy indicates that 7SK snRNP may show cell type-specific or developmental stage-dependent functions or both in transcriptional regulation, and these context-dependent functions may be controlled, at least in part, by sequence-specific transcription factors such as IRF-1 that may control preferential recruitment of P-TEFb to specific target genes, as discussed above (Supplemental Figs. S10, S12).

Our results indicate that the functions of *Larp7* on cell cycle regulation are restricted in gonadal PGCs because migrating PGCs in *Larp7*-deficient embryos are apparently normal (Supplemental Fig. S4), and the expression of *Larp7* (Supplemental Fig. S1) and of other components of 7SK snRNP (data not shown) is up-regulated at E14 when PGCs undergo mitotic arrest. We speculate that additional *p15<sup>INK4b</sup>*-specific transcription repressors and activators may ensure the active proliferation of early PGCs and the growth arrest of PGCs at E14. In summary, we demonstrated that the 7SK snRNP-Brd4-mediated regulation of P-TEFb activity exerts cell type-specific and gene-specific control of transcription activation in

mouse embryos and that this regulation plays a pivotal role in cell cycle progression in actively proliferating PGCs.

## Materials and methods

### *Mouse strains and staging of embryos*

Embryos were obtained from female mice of the outbred strain ICR that were mated with male BDF1 (B6D2F1) mice or with *mil1*-GFP transgenic male mice (Tanaka et al. 2004), and *Larp7* mutant embryos were obtained by intercrosses between *Larp7* heterozygotes harboring the *mil1*-GFP transgene.

### *Targeted disruption of the Larp7 locus*

The *Larp7* targeting vector (Supplemental Fig. S1G) was subjected to electroporation into the E14.1 ES cell line (129/Ola), which was injected into C57BL/6 blastocysts. We backcrossed the chimera mice and heterozygous mutant mice to C57BL/6.

### *Real-time PCR*

Real-time PCR was performed using gene-specific primer sets (Supplemental Table 1) or Power SYBR Green PCR master mix according to the manufacturer's instructions (Applied Biosystems).

### *Assay for PGC proliferation*

PGC proliferation was assayed by pulse-labeling with BrdU as described (Seki et al. 2007) with minor modifications.

### *Immunohistochemistry*

Immunohistochemical analyses with cryosections (10  $\mu$ m) were performed as described previously (Yamaguchi et al. 2005).

### *RNAi*

VV3 ES cells harboring the Vasa-Venus transgene were transfected with siRNA oligonucleotides (Millipore) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

### *Cell cycle analysis by fluorescence-activated cell sorting (FACS)*

Dissociated ES cells were fixed in 1% paraformaldehyde, treated overnight in 70% EtOH, and then incubated with DNase-free RNase (Roche) and propidium iodide (Sigma).

### *ChIP*

ChIP was performed using ES cells cultured for 72 h after siRNA transfection or dissociated genital ridge cells at E12.5 by the method described in the online protocol provided by Nippon Gene Co., Ltd. ([http://www.nippongene.com/pdf/manual/epigenetics/man\\_chip\\_reagents\\_090514.pdf](http://www.nippongene.com/pdf/manual/epigenetics/man_chip_reagents_090514.pdf)).

### *Statistical analysis*

Statistical differences were calculated using the Student's *t*-test.  $P < 0.05$  and  $P < 0.01$  were considered statistically significant.

The details of all experimental procedures are described in the Supplemental Material.

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