MLL5, a trithorax homolog, indirectly regulates H3K4 methylation, represses cyclin A2 expression, and promotes myogenic differentiation

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Most cells in adult tissues are nondividing. In skeletal muscle, differentiated myofibers have exited the cell cycle permanently, whereas satellite stem cells withdraw transiently, returning to active proliferation to repair damaged myofibers. We have examined the epigenetic mechanisms operating in conditional quiescence by analyzing the function of a predicted chromatin regulator mixed lineage leukemia 5 (MLL5) in a culture model of reversible arrest. MLL5 is induced in quiescent myoblasts and regulates both the cell cycle and differentiation via a hierarchy of chromatin and transcriptional regulators. Knocking down MLL5 delays entry of quiescent myoblasts into S phase, but hastens S-phase completion. Cyclin A2 (CycA) mRNA is no longer restricted to S phase, but is induced throughout G_0/G_1, with activation of the cell cycle regulated element (CCRE) in the CycA promoter. Overexpressed MLL5 physically associates with the CCRE and impairs its activity. MLL5 also regulates CycA indirectly: Cux, an activator of CycA promoter and S phase is induced in RNAi cells, and Brm/Brg1, CCRE-binding repressors that promote differentiation are repressed. In knockdown cells, H3K4 methylation at the CCRE is reduced, reflecting quantitative global changes in methylation. MLL5 appears to lack intrinsic histone methyl transferase activity, but regulates expression of histone-modifying enzymes LSD1 and SET7/9, suggesting an indirect mechanism. Finally, expression of muscle regulators Pax7, Myf5, and myogenin is impaired in MLL5 knockdown cells, which are profoundly differentiation defective. Collectively, our results suggest that MLL5 plays an integral role in novel chromatin regulatory mechanisms that suppress inappropriate expression of S-phase-promoting genes and maintain expression of determination genes in quiescent cells.

Brm | CCRE | Pax7 | quiescence | reversible arrest

In adult skeletal muscle, terminally differentiated myofibers and satellite stem cells exist in distinct states of cell cycle exit. Myofibers develop by fusion of committed myoblasts, which permanently withdraw from the cell cycle and express tissue-specific genes under the control of transcription networks orchestrated by the MyoD family (1). By contrast, mononucleated satellite myoblasts idle in a reversibly arrested state and do not express muscle genes; in response to tissue damage, they exit G_0, reactivate MyoD expression and proliferate (2). Restoration of the quiescent stem cell state is accompanied by repression of MyoD (3, 4).

The cell cycle and differentiation are coordinated to ensure the correct balance of stem cells and differentiated cells in regenerating tissue. The decision between proliferation and differentiation is regulated in G_1 (5–7), before the retinoblastoma (Rb)-regulated restriction point (8). In cycling cells, S-phase genes (such as replication proteins and cell cycle regulators) are repressed by Rb, whose inactivation triggers the G_1/S transition. During differentiation, permanent arrest triggered by cdk inhibitor p21 couples Rb function to stable activation of tissue-specific genes by basic helix–loop–helix muscle regulatory factors (MRFs) (9), and S-phase genes are permanently repressed (10). Little is known about control of reversible quiescence where neither p21, Rb, nor the MRFs are expressed (6).

Silencing of S-phase genes in differentiating cells also involves chromatin modulation by enzymes such as Suv39h, a histone methyltransferase (HMT) (11). Histone modifications create instructive chromatin configurations, which control stable activation or repression of gene expression during differentiation (12). Epigenetic regulatory mechanisms in reversible arrest are largely unexplored.

C2C12 myoblasts are an established culture model of satellite cell growth control and differentiation (13). Mitogen deprivation of adherent C2C12 cultures triggers permanent arrest, fusion, and differentiation into multinucleated myotubes and is not reversed by mitogen addition. However, suspension cultures of myoblasts in mitogen-rich methyl-cellulose medium arrest in an undifferentiated state that is reversed when surface contacts are restored, accompanied by appropriate regulation of genes expressed by satellite cells in vivo (6, 14).

To investigate the program of reversible quiescence, we identified genes that are induced in G_0 in undifferentiated C2C12 myoblasts (15). Here, we analyze the role of a quiescence-induced gene, mixed lineage leukemia 5 (MLL5). MLL5 is a putative tumor suppressor encoding a SET and PHD domain protein homologous to *Drosophila trithorax* (16–18) and yeast SET3 (19). Recent studies establish a role for MLL5 in hematopoietic stem cells (20–22), but its molecular targets are unknown. We dissected the role of this predicted chromatin modulator in myoblasts by using RNAi and overexpression. Our results suggest a model in which MLL5 regulates a mechanism to suppress inappropriate expression of S-phase-promoting genes and maintain expression of muscle determination genes in quiescent cells.

Results

Delayed S-Phase Entry but Rapid S-Phase Progression in MLL5 Knockdown Myoblasts. We identified the *Trithorax* homolog MLL5 in a gene trap screen for quiescence-induced genes in synchronized C2C12 mouse myoblasts (15). MLL5 forms nuclear foci in G_0, with reduced expression in S phase (Fig. 1*A* and Fig. S1). To study the

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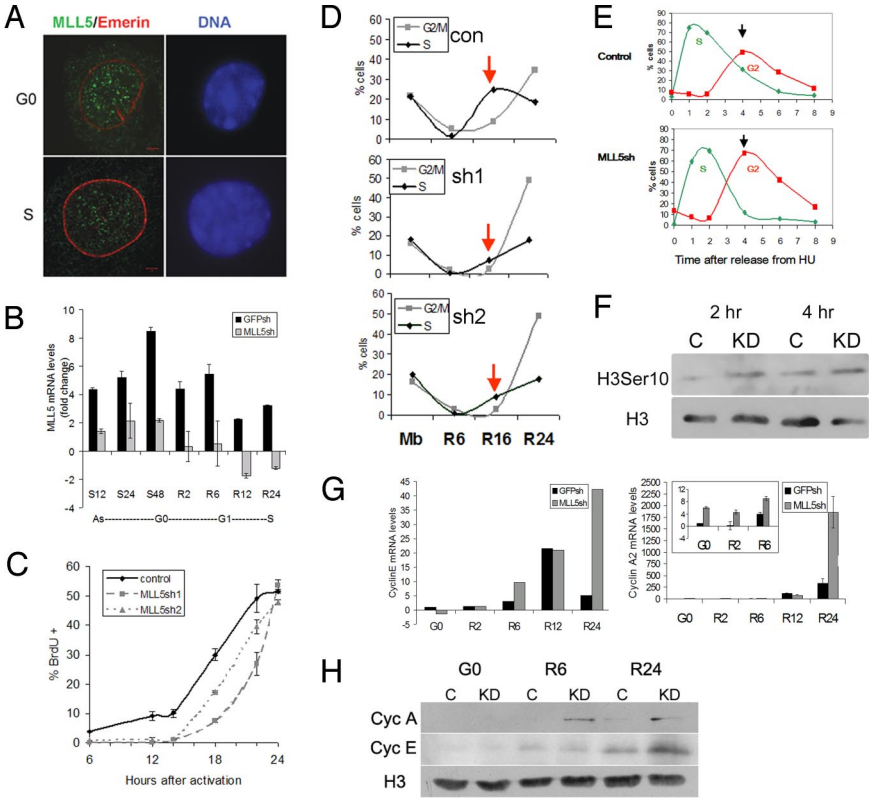

Fig. 1. Delayed G_1/S transition but rapid completion of S phase in MLL5 RNAi myoblasts. (*A*) MLL5 foci in G_0 nuclei; expression declines in S phase. Emerin (nuclear rim) is induced in S. (Magnification: 400×.) (*B*) MLL5 mRNA is induced in control myoblasts (GFPsh2) during arrest in suspension for 12–48 h (S12-S48) and declines during cell cycle reactivation for 2–24 h (R2-R24). Quantitative RT-PCR shows knockdown of MLL5 RNA throughout the cell cycle (As, asynchronous). Values are normalized fold differences relative to levels in growing myoblasts (mean ± SEM, $n = 3$). (*C*) BrdU labeling during release from G_0 (6–24 h) shows delayed S-phase entry of both MLLsh1 and sh2 cells, but by 24 h RNAi cells catch up with controls (mean ± SEM, $n = 2$). (*D*) FACS analysis of DNA content. Mb, asynchronous; R6-24, G_0-synchronized followed by reactivation for 6–24 h. For clarity, only cells in S (black line) and G_2/M (gray line) are shown. RNAi cells arrest like control, but lag in S-phase entry (red arrow). At 24 h, more RNAi cells have transited to G_2, indicating faster traverse of S. (*E*) MLL5 controls duration of S phase. FACS analysis of S-phase progression in double-synchronized cells is shown: percentage cells in S (green) and G_2/M (red). At 4 h (arrow), controls show an S/G_2/M ratio of 30:50 (*Upper*), whereas in the MLLsh1 pool (*Lower*) the ratio is 10:70, indicating faster S traverse. Two independent experiments show similar results. (*F*) H3Ser10 phosphorylation is induced earlier in RNAi cells, consistent with more rapid onset of M. (*G*) Quantitative RT-PCR analysis of S-phase regulatory cyclins. In MLL5sh1 cells, cyclin E and CycA2 are strongly induced. (*Inset*) CycA2 is activated even in G_0/G_1 in RNAi (mean ± SEM, $n = 2$). (*H*) Western blot analysis confirms induction of cyclin E and CycA proteins.

role of MLL5 in muscle cells we used RNAi. MLL5sh1 was the most efficient of 5 different shRNAs tested (Fig. S2). In control cells (GFP shRNA), MLL5 expression waned from G_0 to S, suggesting incompatibility with progression. The MLL5sh1 stable pool showed reduced immunoreactivity (Fig. S1) and lower MLL5 mRNA through out the cell cycle (Fig. 1*B*).

To determine the effect of MLL5 knockdown on cell cycle progression, we used cumulative labeling with BrdU (Fig. 1*C*), scoring all cells reentering S phase from G_0. RNAi cells arrested efficiently, suggesting that MLL5 is not essential for G_0 arrest per se. However, 2 pools derived by using different shRNAs (sh1 and sh2) showed delayed S-phase entry, indicating dependence of the G_1–S transition on MLL5. FACS analysis confirmed delayed progression (Fig. 1*D* and Fig. S3). At 16 h after release, few knockdown cells had entered S, but by 24 h, more knockdown cells than control had reached G_2/M, indicating faster progress overall. Thus, despite delayed entry from G_1 into S phase, G_2 cells appear more rapidly, suggesting that RNAi cells "catch up" with controls during S-phase transit.

To directly estimate the duration of S phase, control and knockdown cells were synchronized at the G_1/S border by using hydroxyurea (HU), released for different periods and S-phase transit monitored by FACS (Fig. 1*E* and Fig. S4). MLL5sh1 cells completed S and progressed to G_2/M faster. At 4 h after release from HU, when control cells showed a ratio of 30% S/50% G_2/M, knockdown cells showed a ratio of 10% S/70% G_2/M, indicating that the MLL5sh1 population as a whole had moved more rapidly through S. During the release from HU, phospho-histone 3, a marker of mitosis appeared earlier in MLLsh1 cells (Fig. 1*F*), consistent with faster entry into M phase. Thus, reducing MLL5 expression leads to complex deregulation at one or more points in the cell cycle.

MLL5 Knockdown Induces Expression of S-Phase Regulators. G_1 regulators cyclin D1 and c-myc were mildly repressed in MLL5sh1 cells, consistent with slower G_1–S progression (Fig. S5). Notably, cyclin E and cyclin A (CycA) (which control initiation and completion of S phase, respectively) were strongly induced in MLL5sh cells (Fig. 1 *G* and *H*). CycA expression, normally restricted to S phase (23), was seen in G_0 and throughout G_1/S reentry in sh1 cells. Thus, MLL5, a candidate tumor suppressor, negatively regulates CycA, a key regulator of S phase.

MLL5 Represses the CycA Promoter. CycA expression is restricted to S phase by a negative cell cycle regulatory element (CCRE) in the proximal promoter, mutation of which causes elevated promoter activity and deregulated timing (24). We confirmed induction of mutant CCRE-driven luciferase activity in control cells. Notably, in MLL5sh cells, WT CCRE activity was derepressed 2.5-fold (Fig. 2*A*). Overexpressed human MLL5 (17), which is RNAi-resistant because of sequence divergence from mouse MLL5, caused dose-dependent repression of CCRE activity in both control and knockdown cells (Fig. 2*B*), also suppressing endogenous CycA protein, DNA synthesis, and MyoD expression (Fig. 2 *C–E*), indicating arrest in G_0. Collectively, these results demonstrate that in myoblasts elevated MLL5 leads to quiescence and that MLL5 represses CycA2 via the CCRE.

Transcriptional Regulators of Cyc A and S Phase Are Altered by MLL5 RNAi. Microarray analysis of S-phase enriched cultures (Table S1) revealed 2 strong candidates as cell cycle effectors of MLL5. Cux, a homeodomain factor known to induce expression of CycA and cyclin E (25) was up-regulated, and Snf2h, an ISWI chromatin protein required for replication machinery function in late G_1/S (26), was down-regulated in MLL5sh1 cells (Fig. 2 *F* and *G*). Thus, transcriptional regulators of CycA/S-phase downstream of MLL5 may contribute to the altered cell cycle.

CCRE-Binding SWI/SNF Repressors Are Suppressed in MLL5 Knockdown Cells. Repression of CycA CCRE activity in G_0/G_1 involves binding of SWI–SNF protein Brm/Snf2a, an ATP-dependent chromatin remodeling helicase (24). Expression of Brm and closely related Brg1/Snf2b was reduced >2-fold in MLL5 RNAi cells (Fig. 2 *F–H*).

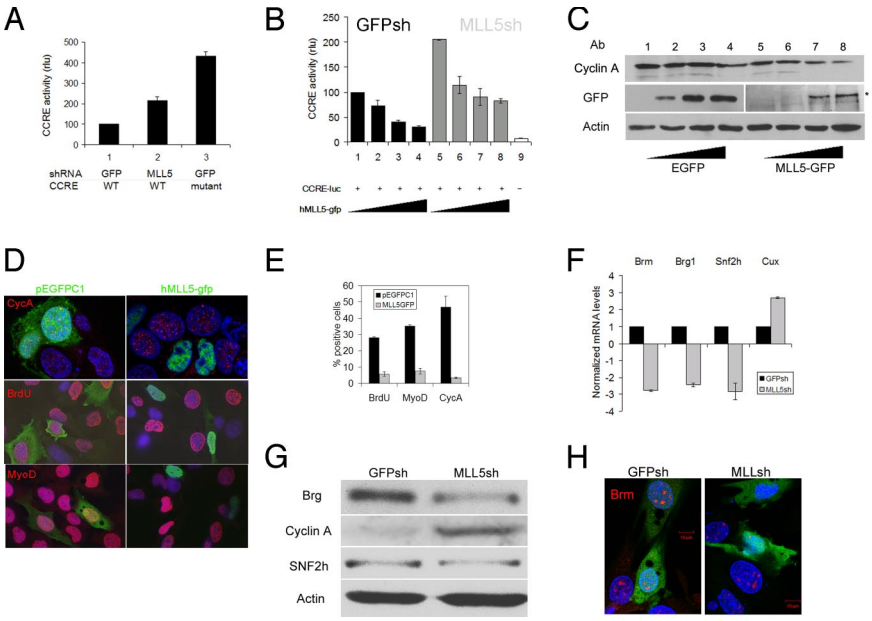

Fig. 2. MLL5 negatively regulates the CycA2 promoter and SWI/SNF chromatin remodeling factors. (*A*) WT CycA2 promoter CCRE-luc activity in GFPsh cells (lane 1) and derepressed in MLLsh myoblasts (lane 2). Mutant CCRE (lane 3) shows elevated promoter activity in control (GFPsh) myoblasts, confirming a negative role. (*B*) RNAi-resistant hMLL5-gfp suppresses deregulated WT CCRE activity in MLL5sh1 cells. CCRE-luc was transfected into GFPsh (lanes 1–4) or MLL5sh1 cells (lanes 5–8) with increasing amounts of hMLL5-gfp plasmid. Luciferase activity (percentage of control, mean ± SEM, $n = 3$). (*C*) Endogenous CycA protein levels repressed by hMLL5-gfp (transfection as in *B*). * denotes 200 kDa hMLL5-gfp fusion protein. (*D*) hMLL5-gfp forms dense nuclear foci and suppresses BrdU incorporation and CycA and MyoD expression. (Magnification: CycA, 150×; BrdU, MyoD, 70×.) (*E*) Quantification of staining shown in *D* (mean ± SEM, $n = 2$). (*F*) MLL5 RNAi reduces expression of CycA regulators. Qunatitative RT-PCR analysis of Brm and Brg1 (mean ± SEM, $n = 2$) and 2 S-phase regulators revealed by microarray analysis. Cux is up-regulated in MLL5 RNAi, whereas Snf2h is down-regulated (mean ± SEM, $n = 3$). (*G*) Western blot analysis confirms reduced Brg and Snf2h levels. (*H*) Immunostaining confirms reduced Brm levels. (Magnification: 100×.)

Importantly, in MLL5 knockdown myoblasts, ectopic Brm could repress the elevated CCRE activity (Fig. 3*A*), and Brg's association with the CCRE was reduced (Fig. 3*B*), consistent with a role as effectors of MLL5. Thus, chromatin remodeling at the CCRE may mediate the effects of MLL5 on CycA transcription, and along with Cux and Snf2h, may account for cyclin deregulation.

Histone Methylation Status Is Altered in MLL5 Knockdown Myoblasts. Because of its divergent SET domain (conserved histone lysine methyl transferase motif), classification of MLL5 as an H3K4 methyl transferase has been debated (27, 28). We asked whether reduced MLL5 expression affects histone methylation. Immunoblot analysis (Fig. 3*C*) showed quantitative changes: Whereas global

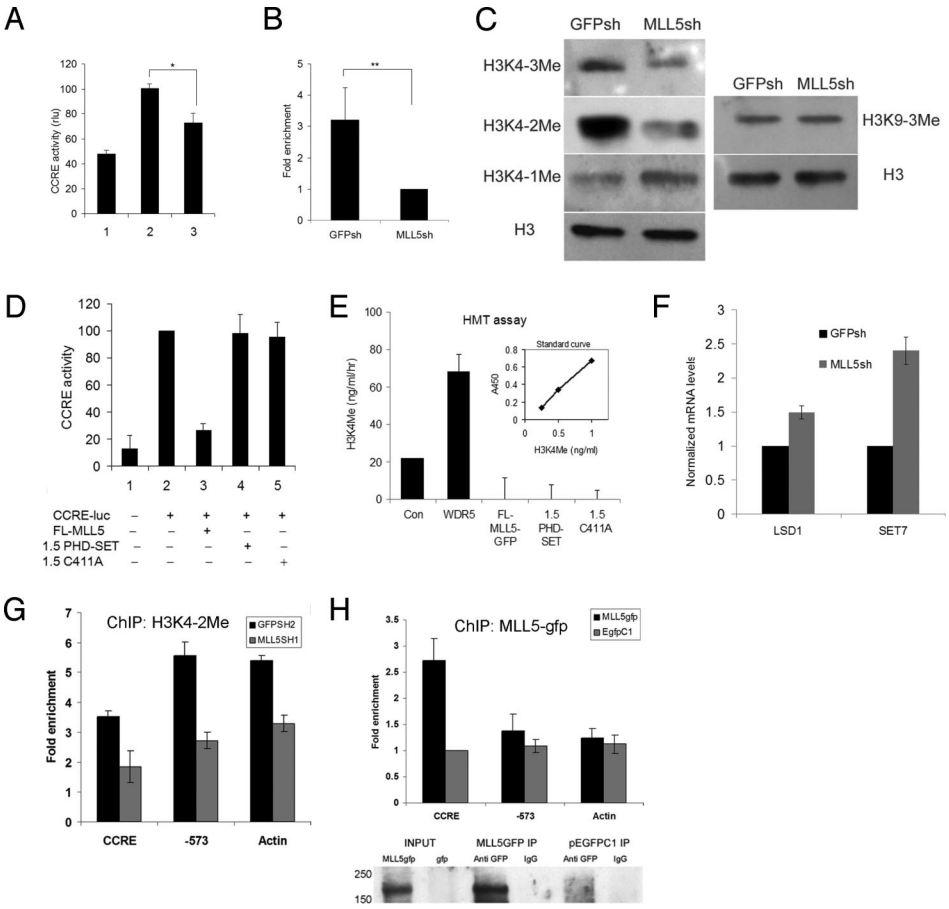

Fig. 3. MLL5 indirectly regulates H3K4 methylation and physically associates with CycA2 CCRE. (*A*) Elevated WT CCRE-luc activity in MLL5sh cells (lane 2 vs. lane 1, GFPsh), is repressed by ectopic Brm (lane 3) (mean ± SEM, $n = 3$; *, $P = 0.05$). (*B*) ChIP analysis shows reduced Brg association with the CCRE in MLLsh1 cells (mean ± SEM, $n = 4$; **, $P = 0.008$). (*C*) Quantitative changes in global H3K4 methylation (immuno-blot). Relative to total histone H3, H3K4–3Me and H3K4–2Me levels are reduced in MLL5 RNAi, H3K4–1Me is increased; H3K9 is unchanged (representative of 3 independent experiments). (*D*) CCRE activity (lane 1, untransfected; lane 2, transfected); full-length hMLL5 GFP represses CCRE activity (lane 3); WT 1.5-kb truncated MLL5 (PHD + SET domain), and SET domain point mutant C411A do not (lanes 4 and 5). (*E*) HMT assay of purified control enzyme (Epigentek) and immunoprecipitates of transiently transfected WDR5, WT FL-hMLL5gfp, WT 1.5 PHD + SET fragment, and C411A mutant (mean + SEM, $n = 3$). Only WDR5 immune complexes show HMT activity. (*Inset*) H3K4-Me standard curve. (*F*) Expression of LSD1 and SET7/9 is elevated in MLL5 shRNA cells ($n = 2$). (*G*) Locus-specific reduction of methylation in MLL5 sh1 cells. ChIP analysis of CycA2 (CCRE and −573 element) and β-actin promoter shows reduced H3K4–2Me at all 3 regions; values are normalized to β-globin (mean ± SEM, $n = 3$). (*H*) hMLL5-GFP physically associates with CycA2 CCRE in chromatin. ChIP analysis of C2C12 cells transiently transfected with hMLL5-gfp or control EGFPC1 by using anti-GFP antibody. (*Inset*) Specific pull-down of ≈200-kDa hMLL5-GFP. hMLL5-GFP specifically binds the CCRE but not CycA2 −573 or β-actin elements.

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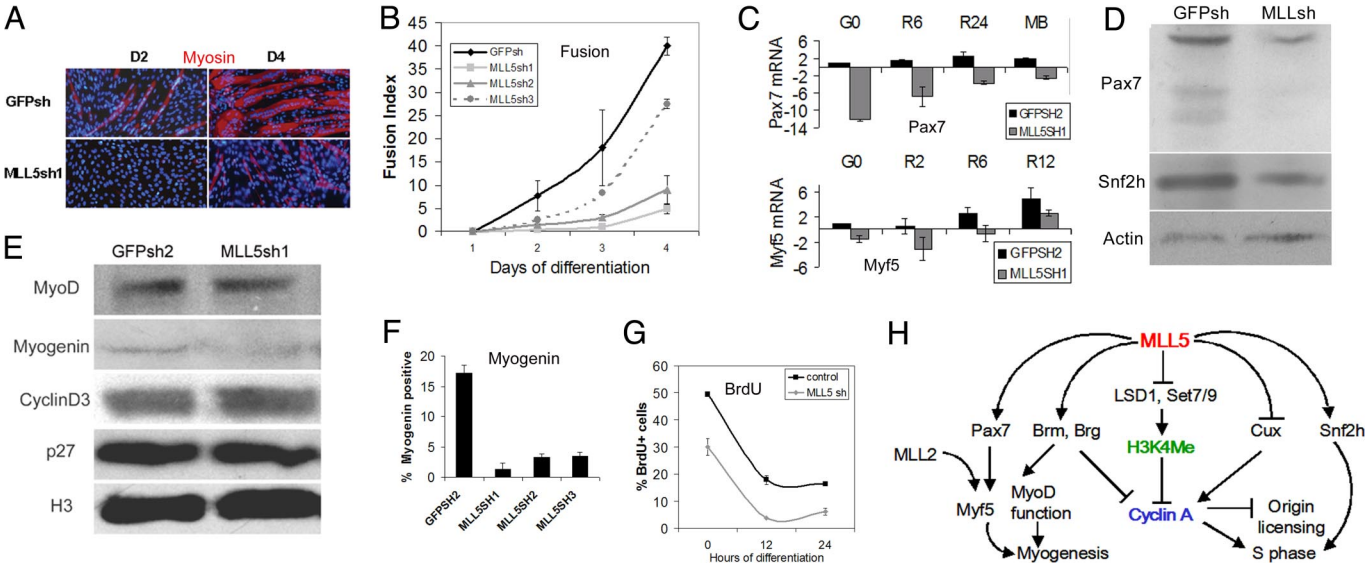

Fig. 4. MLL5 regulates myogenic differentiation. (*A*) Fusion is delayed and reduced-myosin heavy chain, a late marker of myogenesis at days 2 and 4 in fusion medium. (Magnification: 20×.) (*B*) Estimation of fusion index at 1–4 days of differentiation in control and RNAi pools generated with 3 distinct shRNAs (MLL5sh1–3) (mean ± SEM, $n = 2$). (*C*) Pax7 is suppressed throughout cell cycle in MLL5sh1, most strongly during G_0 (mean + SEM, $n = 2$). Myf5, a direct target of Pax7, is also inhibited. (*D*). Pax7 (3 isoforms) and Snf2h protein levels are reduced in MLL5 sh1 myoblasts. (*E*). Immunoblot analysis at 24 h in fusion medium (FM). MyoD targets are differentially affected by MLL5. Myogenin is suppressed in MLLsh1 cells. Cyclin D3 is unaffected. p27 levels are normal, consistent with ability of MLL5 RNAi cells to arrest. (*F*) Reduced myogenin was confirmed by staining of 3 different knockdown pools for 24 h in FM (mean ± SEM, $n = 2$). (*G*) MLL5 RNAi cells arrest in FM and BrdU incorporation at 12 and 24 h after medium shift is shown (mean and SEM, $n = 2$). (*H*) Model of MLL5-regulated network. MLL5 presides over a hierarchy of chromatin and transcriptional regulators to control the cell cycle and myogenesis (see *Discussion*).

levels of H3K4–2Me and H3K4–3Me were reduced in MLL5 RNAi cells, intriguingly, H3K4–1Me levels were increased. H3K9 levels were unchanged, confirming specificity for H3K4. Confocal microscopy confirmed this analysis (Fig. S6). Another report (21) suggests that recombinant MLL5 does not possess intrinsic HMT activity. We assessed activity of overexpressed full-length hMLL5, a 1.5-kb PHD + SET fragment and a SET domain point mutant C411A, after antibody pull-down. Unlike FL-MLL5-GFP, neither WT-truncated MLL5 nor C411A mutant could repress CCRE activity (Fig. 3*D*), and none of the MLL5 proteins catalyzed H3K4 methylation under conditions where WDR5, a known component of MLL1 H3K4 transferase complex was active (Fig. 3*E*). Our results support the evidence (21) that MLL5 may not possess HMT activity.

Increased H3K4–1Me and decreased H3K4–2Me levels might be achieved indirectly, by elevated expression of lysine demethylase LSD1 (29) or SET7/9 monomethyltransferase (30). Indeed, both genes were induced in MLL5 knockdown cells (Fig. 3*F*), supporting an indirect mechanism for the altered global H3K4 methylation.

Altered H3K4 Status at CycA CCRE Correlates with MLL5 Binding. H3K4 dimethylation and trimethylation usually correlate with increased transcription when present at the 5′ ends of genes, but monomethylation of Lys-4 correlates with repressive euchromatin (31). ChIP analysis of the CCRE and an upstream CycA promoter site (−573) revealed a ≈2-fold decrease in H3K4–2Me levels in MLL5 knockdown cells (Fig. 3*G*). No change was detected in H3K4–3Me or H3K9–3Me status (Fig. S6*C*). Thus, global reduction of H3K4–2Me was reflected at a locus-specific level on the CycA promoter, correlating with its derepression.

To investigate whether MLL5 directly binds to chromatin at the CCRE, we used ChIP analysis of transfected hMLL5-GFP (ChIP-grade anti-MLL5 antibodies being unavailable). Pull-down of full-length hMLL5-GFP protein was confirmed by immunoblot (Fig. 3*H Lower*). Indeed, MLL5-GFP is enriched at the CCRE but not at −573 or the β-actin promoter (Fig. 3*H*).

Taken together, our observations show that MLL5 physically associates with chromatin at the CCRE but may affect H3K4 methylation indirectly, perhaps via regulation of other histone-modifying enzymes, such as LSD1 and SET7/9.

Reduced Expression of Myogenic Regulators and Defective Differentiation in MLL5 Knockdown Myoblasts. Chromatin modulation by SWI/SNF regulators plays a key role in myogenic differentiation (32). Repression of 3 such factors (Brm, Brg1, and Snf2h) in MLL5sh1 myoblasts suggested the likelihood of altered myogenesis. Although some differentiation and expression of the late muscle marker Myosin eventually occurred (Fig. 4*A*), 3 different MLL5sh pools generated thin myotubes with few nuclei (Fig. 4*B*).

Regulators of myogenic commitment/determination (Pax7, Myf5), and early differentiation (myogenin) were suppressed (Fig. 4 *C–F*). Pax7, an upstream regulator of Myf5 (33, 34), was reduced throughout the cell cycle in MLL5sh1 cells, and Myf5 expression was suppressed in G_0, with delayed G_1 induction (Fig. 4 *C* and *D*). MyoD levels were unchanged but expression of MyoD's targets was differentially affected: CycD3 was unaltered, but myogenin was strongly suppressed in 3 independent knockdown pools (Fig. 4 *E* and *F* and Fig. S7). Knockdown cells showed normal levels of the CDKI p27 (not a target of MyoD). Differential expression of MyoD's target genes is consistent with reduced levels of Brm/Brg1 in MLL5 RNAi cells, because MyoD recruits these factors to remodel chromatin at the myogenin promoter (32), but not at the CycD3 promoter. Thus, defective differentiation in MLL5 knockdown myoblasts correlates with altered expression of regulators of commitment (Pax7, Myf5), altered function of MyoD, and inefficient activation of myogenin (a likely effect of reduced SWI/SNF-dependent chromatin remodeling).

MLL5sh1 cells exited the cell cycle efficiently after mitogen withdrawal (Fig. 4*G*). Thus, the differentiation deficit is not caused by an inability of MLL5 RNAi cells to arrest in low serum.

Collectively, the results indicate that MLL5 controls CycA directly by chromatin association at the CCRE and indirectly via reciprocal regulation of repressors and activators of CycA transcription. MLL5 also indirectly regulates histone methylation glo-

bally and at the CCRE, possibly by repression/recruitment of histone-modifying enzymes. MLL5 controls muscle differentiation via altered expression of Pax7 and Myf5 and SWI-SNF factors that impact MyoD's transcriptional function. Thus, MLL5 coordinates the cell cycle and myogenic differentiation by presiding over a hierarchy of chromatin remodeling and transcriptional regulators (Fig. 4*H*).

Discussion

In this study, we have investigated the function of MLL5, a TrxG homolog induced in G_0 myoblasts (15). We establish that MLL5 is a negative regulator of the cell cycle whose molecular targets include SWI/SNF chromatin-remodeling enzymes and the S-phase regulator CycA. We demonstrate that MLL5 physically associates with the CycA promoter whose activity it represses. We provide evidence that MLL5 regulates histone methylation and the expression of histone-modifying enzymes, but that this divergent MLL may not possess intrinsic methyl transferase activity. We show that MLL5 positively regulates the promyogenic genes Pax7, Myf5, and myogenin. Our findings suggest a model where MLL5 may coordinate direct and indirect mechanisms to prevent inappropriate expression of proliferation genes and preserve expression competence of myogenic genes in quiescent myoblasts.

MLL5 Is Not Required for Arrest but Controls Gene Expression During Quiescence and Activation. In myoblasts, MLL5 expression wanes from G_0 to S, suggesting incompatibility with progression, forced expression causes arrest in G_0 (ref. 17 and Fig. 2), and knockdown accelerates S phase. MLL5 knockout mice show defects in bone marrow stem cell self-renewal (20–22). These findings implicate MLL5 in cell cycle inhibition, consistent with potential tumor suppressor function (16). By contrast, in human tumor lines, MLL5 siRNA arrests cells in both G_1 and G_2 (18), suggesting a role in progression. This finding may reflect cell type differences or stronger inhibition by siRNA generating an extreme phenotype, precluding analysis of S phase. In myoblasts, MLL5 function is not essential for arrest per se, but for the normal transcriptional program of G_0: altered CycA, Pax7, and Myf5 expression in G_0 suggests control of aspects of the quiescent state other than cell cycle exit. These genes regulate later events (S phase and differentiation), and MLL5 might affect these programs via a cascade initiated in G_0/G_1.

MLL5 Represses CycA Transcription by Direct and Indirect Mechanisms. Several lines of evidence show that MLL5 negatively regulates CycA expression at a transcriptional level. CycA promoter activity is elevated in MLL5 RNAi myoblasts and repressed by overexpression of hMLL5 that binds chromatin at the CCRE, strongly suggesting that CycA is a direct target. Reduced MLL5 levels led to decreased expression of repressors of CycA (Brm/Brg) and induction of a transcriptional activator (Cux), which may cooperate in derepression of CycA2 throughout the cell cycle.

CycA as an Effector of MLL5 Action on the Cell Cycle. CycA has 2 functions in DNA replication: activate replication complexes preloaded onto origins in G_1 and inhibit origin licensing (new replication complex assembly). This dual mechanism prevents reinitiation to ensure precise doubling of the genome (35). Our results suggest that MLL5 induction in G_0/G_1 may permit origin licensing by repressing CycA. Declining MLL5 expression in mid-late G_1 would derepress CycA, curtail new licensing, and activate firing of prelicensed origins. In MLL5 knockdown cells, CycA expression is elevated throughout the cell cycle. Consequently, premature inhibition of licensing in G_1 would delay S-phase onset, which is signaled when replication complex assembly is complete. Origin licensing requires chromatin remodeling by Snf2h complexes that are recruited to chromatin by H3K4 methylation (36). In MLL5 RNAi, delayed S-phase entry may result from reduced Snf2h expression and reduced H3K4 methylation, which would reduce the number of docking sites for Snf2h complexes.

CycA phosphorylates different targets at the beginning and end of S phase (37). At the start of S, CycA/cdk2 can phosphorylate Rb, releasing E2F/DP1 to activate transcription. Levels of CycA rise during S, and CycA/cdk2 targets DP1, which dissociates from E2F, inhibiting S-phase gene transcription. CycA/cdk1 activity also contributes to the S/G2 transition by targeting proteins such as Cux (38). Thus, increased CycA in MLL5 RNAi cells may hasten the end of S by rapidly achieving levels required for the S/G_2 transition.

Similar Cell Cycle Regulation by MLL5 and Brm, a Direct Repressor of CycA. Complex alterations of cell cycle kinetics in which 2 consecutive phases are differentially regulated are rare, but strikingly, have been reported for Brm null fibroblasts (23, 24). MLL5 RNAi cells resemble $Brm^{-/-}$ cells not only in the delayed G_1/S transition coupled with a reduced S phase, but also in deregulated expression of cyclin E and CycA. Brm specifically represses CycA in quiescent cells (23) by remodeling chromatin at the CCRE. Notably, in MLL5 knockdown cells, Brm expression is reduced and ectopic Brm can repress elevated CCRE activity, albeit mildly, because other CCRE-activating conditions (high Cux, low Snf2h) persist. Finally, Brg's association with the CCRE is reduced in MLL5 sh1 cells. Taken together, our data support a model where MLL5 acts via Brm/Brg to repress CycA expression.

MLL5 May Regulate Differentiation via Control of Pax7 and Brm in G_0/G_1. In the myogenic program, Pax7 participates in specification and survival, whereas commitment requires cell cycle-dependent activation of Myf5 and MyoD. Pax7 expression is sustained in G_0, when MRFs are repressed (4). Loss of Pax7 expression in RNAi cells points to the existence of an MLL5-dependent memory mechanism in quiescence. The Pax7 deficit in MLL5sh cells would contribute to the differentiation defect, because Pax7 recruits the related MLL2 HMT to the Myf5 promoter to activate its expression and myogenic commitment (34). Overt differentiation is activated by extrinsic signals that unmask the transcriptional functions of MyoD and is restricted to a window in G_1 when MyoD induces expression of its target genes, of which myogenin, cyclin D3, and p21 are critical early players. MyoD's target promoters show a differential requirement for SWI–SNF factors: activation of myogenin by MyoD requires Brm/Brg, whereas activation of cyclin D3 does not (32). Because Brm/Brg enhance MyoD function by remodeling repressive chromatin specifically at the myogenin promoter, lower SWI/SNF expression in MLL5 RNAi cells would impact myogenesis.

MLL5 Regulates Histone H3K4 Methylation Indirectly. Our study supports the recent report (21) that MLL5 lacks HMT activity, which is consistent with the sequence divergence of the MLL5 SET domain from MLL family H3K4 methyl transferases (27, 28). We provide evidence for a role in chromatin regulation, showing quantitative changes in H3K4Me status when MLL5 is suppressed. Global reduction of H3K4 dimethylation and trimethylation with concomitant increases in monomethylation correlate with elevated expression of genes known to affect H3K4 methylation, the demethylase LSD1 and the H3K4–1Me transferase Set7/9. At the CCRE, H4K4–2Me is reduced, whereas H3K4–3Me is unaffected. Interestingly, LSD1 can demethylate H3K4–2Me but not H3K4–3Me (29). Together, these indirect mechanisms could account for altered H3K4 methylation.

Histone methylation is associated with epigenetic inheritance of permissive or repressive chromatin states (12). In general, H3K4–2Me and H3K4–3Me at transcriptional start sites increase accessibility and correlate with activation, whereas monomethylation correlates with repression (31). However, in MLL5 knockdown cells, elevated CCRE activity correlates with decreased H3K4–2Me but may result from reduced recruitment of repressors,

Brm/Brg. Ectopic MLL5 is located at the CCRE and represses its activity. Taken together, these findings strongly implicate MLL5 in direct targeting of the CycA negative element, perhaps by assembly of regulatory complexes in an H3K4 methylation-sensitive mechanism for active gene repression (39).

The founding member of the family, MLL1, participates in a large SET1 complex that acts to maintain transcriptional activation states (40). However, MLL5 is suggested to interact with NCoR and HDAC, components of the SET3 repressor complex (19). Further, microarray analysis showed that ≈90% of genes affected by MLL5 RNAi were up-regulated (Table S1). Taken together with its repression of Cux and CycA, these findings raise the possibility that MLL5 plays a predominantly repressive role.

In summary, this study provides evidence that MLL5 presides over a chromatin regulatory network that prevents inappropriate expression of S-phase genes in quiescent muscle cells. Other SET-domain proteins such as Suv39 (an H3K9 methyl transferase) suppress S-phase gene activity by distinct mechanisms in proliferating and differentiating muscle cells (11). We propose that MLL5 may regulate a transcriptional memory mechanism preventing silencing of promyogenic genes in reversibly arrested cells. Unraveling the mechanisms downstream of MLL5 will contribute to understanding how reversible and irreversible cell cycle exits are distinguished. Chromatin compartmentalization can also affect the replication timing of specific loci, an interesting future avenue for understanding MLL5 function.

Materials and Methods

Additional details are in *SI Text*. For antibodies used in this study see Table S2. For primer sequences used see Table S3.

RNAi. RNA hairpins targeting MLL5 and GFP control were cloned into mU6 vector, and stable pools of shRNA-expressing cells were derived (sequences in *SI Text*).

Double Synchronization for Estimation of S Phase. Cells were suspension-arrested in G_0, replated in GM with HU (1 mM) for 16 h to synchronize at G1/S border, fed with fresh GM, and used at 0–8 h after HU removal for FACS.

Immunofluoresence. Immunofluorescence was performed by standard methods (see *SI Text*) and recorded on a Zeiss 510 Meta laser scanning confocal microscope (63×, Plan Apochromat Zeiss objective, 1.4 N.A.; LSM5 software).

Quantitative Real-Time RT-PCR. Quantitative real-time RT-PCR analysis was performed on an ABI 7900HT cycler (Applied Biosystems).

ChIP. Chromatin was cross-linked, sonicated, and precipicated by using rabbit anti-H3K4–3Me or anti-H3K4–2Me or H3K9–3Me (Upstate), anti-H3 (Abcam), or anti-Brg (Upstate) (see *SI Text* for details). DNA was purified and analyzed by quantitative PCR targeting CycA2 CCRE (+9 to +175), upstream CycA element (−573 to −449), or β-actin promoter (−818 to −992). Signals were normalized against β-globin.

Histone Methyl Transferase Activity. Histone methyl transferase activity was measured with an ELISA kit (Epigentek) after immunoprecipitation of transiently transfected proteins from HEK nuclear lysates.

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