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Ldb1 regulates carbonic anhydrase 1 during erythroid differentiation

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Abstract

Carbonic anhydrase 1 (Car1), an early specific marker of the erythroid differentiation, has been used to distinguish fetal and adult erythroid cells since its production closely follows the γ- to β-globin transition, but the molecular mechanism underlying transcriptional regulation of Car1 is unclear. Here, we show that Car1 mRNA decreases significantly when erythroid differentiation is induced in MEL cells. The Ldb1 protein complex including GATA1/SCL/LMO2, binds to the Car1 promoter in uninduced cells and reduced enrichment of the complex during differentiation correlates with loss of Car1 expression. Knockdown of Ldb1 results in a reduction of Ser2 phosphorylated RNA Pol II and Cdk9 at the Car1 promoter region, suggesting that Ldb1 is required for recruitment of Pol II as well as the transcription regulator P-TEFb to enhance elongation of Car1 transcripts. Taken together, these data show that Ldb1 forms a regulatory complex to maintain Car1 expression in erythroid cells.

Keywords

Ldb1; Car1; Car2; erythroid differentiation

1. Introduction

Carbonic anhydrases (CAs) catalyze the reversible hydration/dehydration of carbon dioxide ($CO_2+H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO3^-$) and are therefore essential for normal carbon dioxide-bicarbonate exchange [1]. Erythrocytes possess both low activity and high activity CA isozymes (CA1 and CA2, respectively) that are major components of the membrane [1]. CA expression has been classically used to distinguish fetal and adult erythroid cells [2]. The synthesis of CAs is coordinately controlled with the key switch from fetal hemoglobin to adult hemoglobin production [3]. The CA switching event subsequently occurs as Car1 expression decreases and Car2 expression increases [2]. Car1 expression is also a

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differentiation marker of the colonic mucosa and loss of Car1 expression is associated with the disappearance of differentiated epithelial cells [4]. Moreover, aberrant production of CAs was observed in leukemia [3] and was correlated with biological aggressiveness of colorectal cancer [5], supporting the importance of Car gene expression for biological processes. However, the mechanism of developmental regulation of Car gene switching during erythroid development is unknown.

Ldb1, a widely expressed nuclear adaptor protein, is essential for the developmental progression of a number of tissues [6]. Ldb1 forms a complex with GATA1, LMO2, and SCL that is involved in multiple developmental pathways [7]. Ldb1 functions as a positive regulator of erythroid gene expression including α- and β-globin and the red cell membrane components protein 4.2 (Epb4.2) and glycophorin A (Gypa) [8–11]. Ldb1 is essential for erythroid development and is also necessary for transcriptional mechanisms that regulate the maintenance of hematopoietic stem cells [12, 13].

In this study, we examined participation of Ldb1 in transcriptional regulation of the Car1 and Car2 genes. Reduction of Ldb1 using shRNA resulted in decreased transcription of both genes in MEL cells suggesting that Ldb1 is important for their transcriptional regulation. We found that Ldb1 robustly occupied the Car1 promoter in uninduced MEL cells and occupancy decreased along with transcription during MEL cell differentiation. However, Ldb1 did not occupy the Car2 gene in either uninduced or induced MEL cells. Thus, Ldb1 is likely to regulate transcription of the Car1 and Car2 genes by direct and indirect means, respectively.

2. Materials and methods

2.1. Cell culture

MEL cells and stable clones for control or Ldb1-knockdown MEL cells were cultured and differentiated with DMSO as described previously [9].

2.2. Microarray analysis of Ldb1 reduction by RNAi

Affimetrix GeneChips Mouse Genome 430 2.0 arrays were used to profile mRNA expression in MEL cells that had been stably transformed with a control or Ldb1 shRNA before and after induction of differentiation [9]. Three biological replicates were used for each treatment. RNA expression values were calculated in R using the "affy" package and Benjamini-Hochberg adjusted p-values for each contrast were calculated using the "limma" package. For genes that had multiple probe sets, the probe set with the lowest adjusted p value was selected as representative for that gene. Genes in Table 1 were selected based on biological interest.

2.3. RT-PCR and Western blot analysis

Total RNAs were isolated and reverse transcribed as described [14]. Whole cell extracts were prepared, and western blot analysis was performed as previously described [9].

2.4. ChIP assay and Quantitative Real-Time PCR

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [15]. Quantitative real-time polymerase chain reaction (qPCR) using either TaqMan (Applied Biosystems) or SYBR Green (Bio-Rad) chemistry was performed to determine enriched DNA or cDNA using the ABI Prism 7900HT (Applied Biosystems) as described previously [16]. The enrichment of target DNA over input was calculated by the $\Delta\Delta C_t$ method with results presented as mean plus or minus SEM [16]. The PCR primers used for ChIP assay and qRT–PCR appear in Supplemental Table 1.

2.5. Antibodies

Antibodies used in this study include: Ldb1 (SC-11198), GATA1 (SC-1233), Pol II (SC-899), c-Myb (SC-517), and Cdk9 (SC-484) were from Santa Cruz Biotechnology, RNA polymerase II (phospho S2) was from Abcam, LMO2 was from R&D Systems and Car1 was from Rockland. SCL antibodies were kindly provided by Dr. Catherine Porcher [17].

3. Results and Discussion

3.1. Developmental changes in erythroid gene expression during MEL cell differentiation

To assess changes in gene expression during erythroid differentiation, several erythroid specific genes were selected for study in MEL cells that have been widely used as a model system for studying erythropoiesis and hemoglobin gene regulation [18]. Typical characteristics of erythroid maturation are observed in this system including loss of proliferative capacity, onset of abundant hemoglobin synthesis and morphological changes after induction. As described previously [8, 19], transcription of α-globin increased significantly upon DMSO-induced differentiation of MEL cells (Fig. 1A). Up-regulation of red cell membrane proteins, such as Gypa, Epb4.2, and Slc4a1, also occurred (Fig. 1B-D) [10, 20, 21]. In addition, the developmental shift in CA protein isoforms from CA1 to CA2 that occurs in normal erythroid maturation was recapitulated during DMSO induction of MEL cells as was the case during maturation of G1E cells induced by GATA1 [22]. Car1, a well-known early specific marker of erythroid differentiation, is expressed in uninduced MEL cells, and decreased during MEL cell maturation (Fig. 1E) [2, 22]. In contrast, Car2 was up-regulated after DMSO induction (Fig. 1F).

3.2. Reduction of Ldb1 dis-regulates Car1 and Car2 gene expression

We had previously observed that Ldb1 is crucial for induction of β-globin transcription in MEL cells [9, 13] and other data indicated that Ldb1 plays a role in regulation of α-globin and transcription of the red cell membrane proteins Epb4.2 and Gypa [10, 11]. However, it is unknown whether Ldb1 participates in regulation of the CAs. We profiled gene expression in MEL cells using Affymetrix GeneChips and mRNA from cells stably transformed with a control or Ldb1 lentiviral shRNA vector both before and after induction. The α- and β-globins were reduced upon Ldb1 reduction in induced MEL cells, as expected [8, 9, 19] (Table 1). The microarrays likely underestimated the magnitude of α- and β-globin RNA changes due to saturation of the detection system by these highly abundant transcripts [22]. Car1 was among the genes most strongly down-regulated after Ldb1 reduction both in uninduced and induced MEL cells. Car2 was modestly down-regulated in uninduced cells. In addition, the red cell membrane proteins Gypa, Epb4.2 and Slc4a1 were decreased by Ldb1 knockdown in induced cells. Neither uninduced nor DMSO-induced MEL cells expressed the Car3 gene that resides between Car1 and Car2 (data not shown). Thus, Car1 and Car2 potentially join a group of late erythroid genes that are positively regulated by Ldb1.

In Fig. 2, the fold change in expression for all probes on the microarray is plotted as a function of rank. For each gene highlighted in Table 1, the probe for that gene with the lowest adjusted p value is indicated. Induction contrasts the Car1 gene, which is expressed in uninduced cells and decreases, with the globin and red cell membrane genes that have the opposite expression pattern. The value for Car2 is included in this plot although the p value was below the level of significance (see Table 1). In uninduced MEL cells, reduction of Ldb1 most greatly affects Car1 which is the only one of the highlighted genes that is robustly expressed (p values for Gypa, Epb4.2 and Slc4a1 were not significant, see Table 1). In induced cells, the globin and red cell membrane genes, now expressed, are all

downregulated by Ldb1 reduction. As is evident from Table 1, Car2 was only modestly affected by Ldb1 knockdown.

3.3. Car1 but not Car2 is a target of the Ldb1/GATA1/LMO2/SCL complex

Recent ChIP-sequencing experiments in MEL cells and in mouse bone marrow indicated strong occupancy of Ldb1 at a set of peaks between Car2 and Car3 (Fig. S1A) [12, 21]. At the Car1 promoter there was a small peak of Ldb1 occupancy in uninduced MEL cells that diminished after induction. Ldb1 occupancy at Car2 was not noted. To investigate the connection between our microarray results and Ldb1 occupancy in the Car locus, we performed ChIP assays with an antibody to Ldb1 for uninduced and induced MEL cells. We observed enrichment of Ldb1 at the Car1 promoter and far upstream of Car2 (-9K and -8K in Fig. 3A, 3B) in uninduced MEL cells but Ldb1 was absent from the Car2 promoter, consistent with the ChIP-seq data (Fig. S1A). GATA1 co-occupied the Ldb1 sites, suggesting that the Ldb1 complex was present at these sites (Fig. 3C). Further ChIP assays with antibodies to LMO2 and SCL confirmed occupancy of the Car1 promoter in uninduced cells by the core Ldb1 complex (Fig. 3D and E). Ldb1 complex occupancy was strongly reduced at the Car1 promoter after MEL cell induction, correlating with Car1 down regulation but was unchanged at the sites upstream of Car2. These results suggest the Ldb1 complex occupies and directly regulates Car1 and loss of complex binding results in Car1 down regulation. In contrast, the comparatively modest effect of Ldb1 knockdown on Car2 expression may be an indirect effect since Ldb1 did not occupy the promoter. Car1 regulation by GATA1 in concert with c-Myb had been previously observed [23] and c-Myb occupancy of the Car1 promoter paralleled that of the Ldb1 complex (Fig. S1B).

3.4. Recruitment of transcription complex components to the Car1 promoter region during erythroid maturation of MEL cells

To begin to investigate how Ldb1 influences Car1 expression, we analyzed the binding of transcription components at the Car1 promoter in uninduced and induced MEL cells using the ChIP assay. Car1 has two alternative transcription initiation sites separated by about 30 kb (Fig. 4A) [24]. The proximal promoter (near Exon 2) directs high levels of Car1 expression in adult colon epithelia [4]. However, the distal promoter (Car1 pro/Ex1 in Fig. 4A) is erythroid cell-specific [23]. ChIP analysis revealed robust recruitment of Pol II at the Car1 erythroid-specific core promoter in uninduced MEL cells with a strong decrease after induction commensurate with Car1 down regulation (Fig. 4B). Pol II increased at the promoter of the Car2 gene during differentiation, as expected, but this was not associated with Ldb1 complex occupancy (Fig. S2 and Fig. 3).

Next, we examined the recruitment to Car1 of the fully elongation competent form of Pol II, which is phosphorylated at Ser2 of the C-terminal domain (CTD) [25] in uninduced and induced MEL cells. We also performed ChIP assays with antibodies to Cdk9, a subunit of the positive elongation factor b (P-TEFb) complex that catalyzes Pol II Ser2 phosphorylation [25]. Ser2P Pol II was detected across the Car1 gene in undifferentiated MEL cells (Fig. 4C). Surprisingly, in contrast to previous studies [25, 26], elongation competent Pol II peaked at the transcriptional start sites of Car1 and lower levels were detected at the 3’ end of the gene in uninduced MEL cells. On the other hand, Cdk9 was recruited only to the Car1 promoter (Fig. 4D). Both Ser2P Pol II and Cdk9 were very strongly reduced after MEL cell differentiation (Fig. 4C and D). Together, these results suggest that decreased Car1 expression during MEL cell differentiation is associated with reduced recruitment of transcription machinery components.

3.5. Importance of Ldb1/GATA1/LMO2/SCL complex for Car1 expression

To investigate the specific role of the Ldb1 complex in Car1 transcription in MEL cells, we used MEL cells with Ldb1 reduced by RNAi [9]. Western blotting confirmed that Ldb1 was reduced substantially in 2 Ldb1-shRNA expressing MEL cell clones (Fig. 5A). The steady-state level of Car1 protein was also markedly decreased upon knockdown of Ldb1, while expression of GATA1 remained largely unaffected [9]. Transcription of Car1 was reduced after Ldb1-knockdown, consistent with our microarray results and suggesting the importance of the Ldb1 complex for Car1 expression (Fig. 5B and Table 1).

Next, we assessed the effect of Ldb1 reduction on Car1 promoter occupancy. ChIP assays with MEL cells expressing Ldb1-shRNA, showed that enrichment of Ldb1 was reduced fivefold on average compared to MEL cells expressing control-shRNA (Fig. 5C). Although repression of Ldb1 had no effect on GATA1 expression (Fig. 5A), decreased Ldb1 expression was associated with reduced binding of GATA1 at the Car1 promoter (Fig. 5D). Under these conditions, enrichment of the other Ldb1 complex members, LMO2 and SCL, was also reduced (Fig. S3A and B). These results strongly support the idea that Ldb1 plays a role in stabilizing the entire Ldb1 complex at the Car1 promoter to regulate gene expression [9, 15]. Pol II was also reduced at Car1 after Ldb1 reduction and, interestingly, knockdown of Ldb1 led to depletion of c-Myb at the Car1 promoter, suggesting that the Ldb1 complex may act to maintain c-Myb on the Car1 gene in uninduced MEL cells (Fig. S3C and D) [23]. c-Myb expression itself has been reported to be positively regulated by the Ldb1 complex [27]. However, we observed no effect of Ldb1 reduction on transcription of the c-Myb gene (Fig. S4) suggesting that c-Myb occupancy at Car1 in MEL cells is influenced by the Ldb1complex.

Ldb1 can directly interact with Cdk9, the kinase component of P-TEFb [28]. Moreover, Ldb1 is required for efficient recruitment of P-TEFb to stimulate transcription elongation through phosphorylating Ser2 of Pol II at the β-globin locus [15]. To test whether Ldb1 is required for recruiting elongation-competent Pol II complexes to the Car1 promoter, ChIP experiments were performed with Ldb1-shRNA expressing MEL cell clones. Upon Ldb1 depletion, the levels of both Ser2P Pol II and its kinase Cdk9 were reduced at the Car1 promoter and, for Pol II Ser2P, within the gene (Fig. 5E and F). Taken together, and in agreement with our previous results with β-globin gene transcription [15], Ldb1 is necessary for P-TEFb enrichment and thus for Ser2 phosphorylation of Pol II to promote Car1 expression.

In summary, Ldb1 is required to stabilize the Ldb1/GATA1/TAL1/LMO2 complex at the promoter of the Car1 gene. Ldb1 also contributes to efficient recruitment of Cdk9 to the Car1 promoter, which is coincident with active transcription of the gene in uninduced MEL cells. Down regulation of the Ldb1 complex at the promoter of Car1 gene subsequently results in the loss of Car1 transcription in these cells. Previously, we have shown that Ldb1 is required for induction of β-globin gene expression during MEL cell differentiation through the establishment of a chromatin loop between the gene and the distant β-globin LCR where Ldb1 is also localized [9]. We considered whether the Ldb1 sites upstream of Car2 might serve as such a distant enhancer for Car1 but we were unable to obtain data supporting chromatin looping between these regions (data not shown). This may be explained by the presence of multiple binding sites for CTCF, the insulator protein, between Car1 and these distant sites. Thus, it remains unclear for Car1, as for Gypa and Epb 4.2, whether the transcriptional regulatory role of Ldb1 is exerted solely through the promoter or includes as yet unidentified long range enhancer sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CA	carbonic anhydrase
Epb4.2	erythrocyte membrane protein band 4.2
Gypa	glycophorin A
DMSO	Dimethyl sulfoxide
qPCR	quantitative real-time polymerase chain reaction

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Highlights

- Ldb1 is essential for embryonic and definitive erythropoiesis.
- The Ldb1/GATA1/SCL/LMO2 complex directly regulates transcription of Car1.
- Ldb1 is required to recruit the Ldb1 complex to the promoter of the Car1 gene.

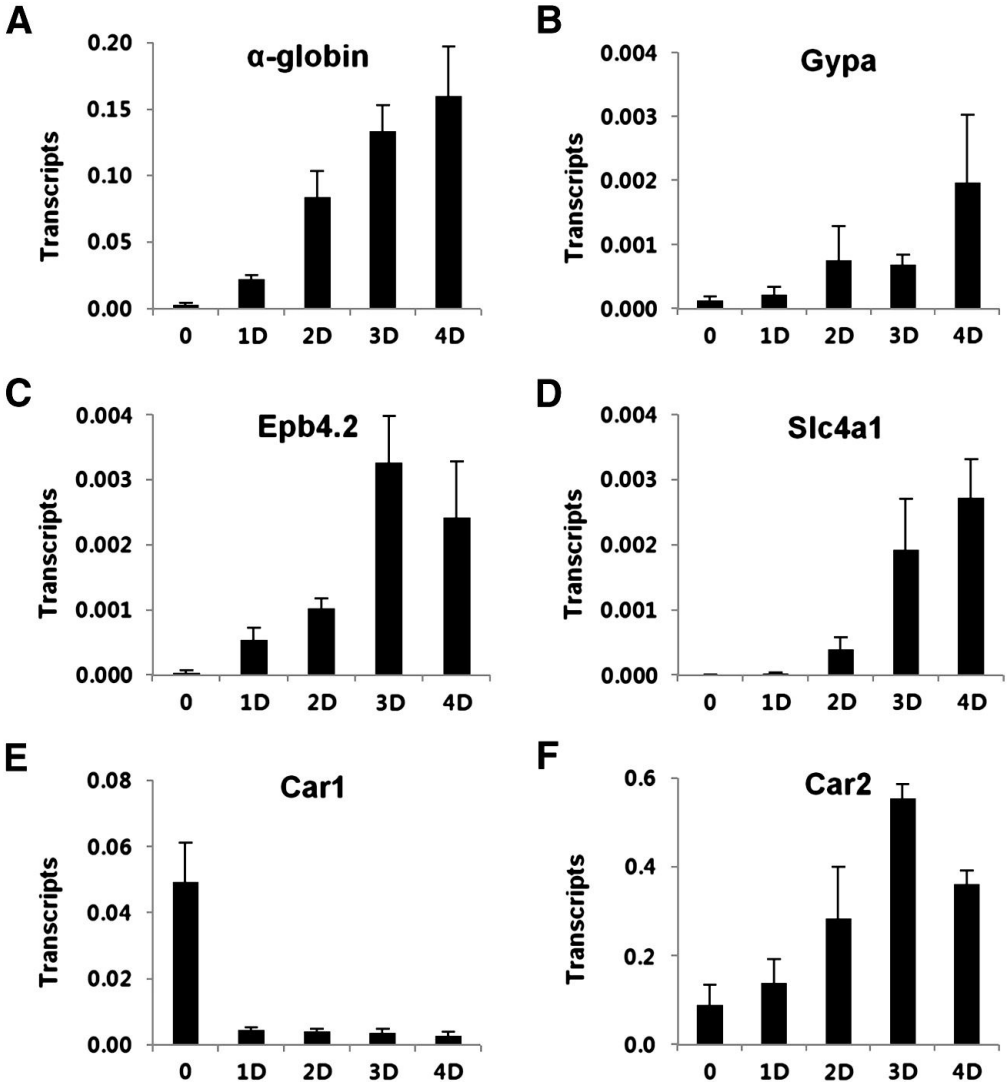

Fig. 1. Changes in erythroid-specific gene transcription during differentiation of MEL cells

MEL cells were treated with 2% DMSO, and then total RNA was prepared at the indicated times (days). (A) α-globin, (B) Gypa, (C) Epb4.2, (D) Slc4a1, (E) Car1, and (F) Car2 mRNA expression was analyzed by qRT-PCR. Each value was normalized with 18S ribosomal RNA. Error bars represent the SEM for three independent RNA preparations.

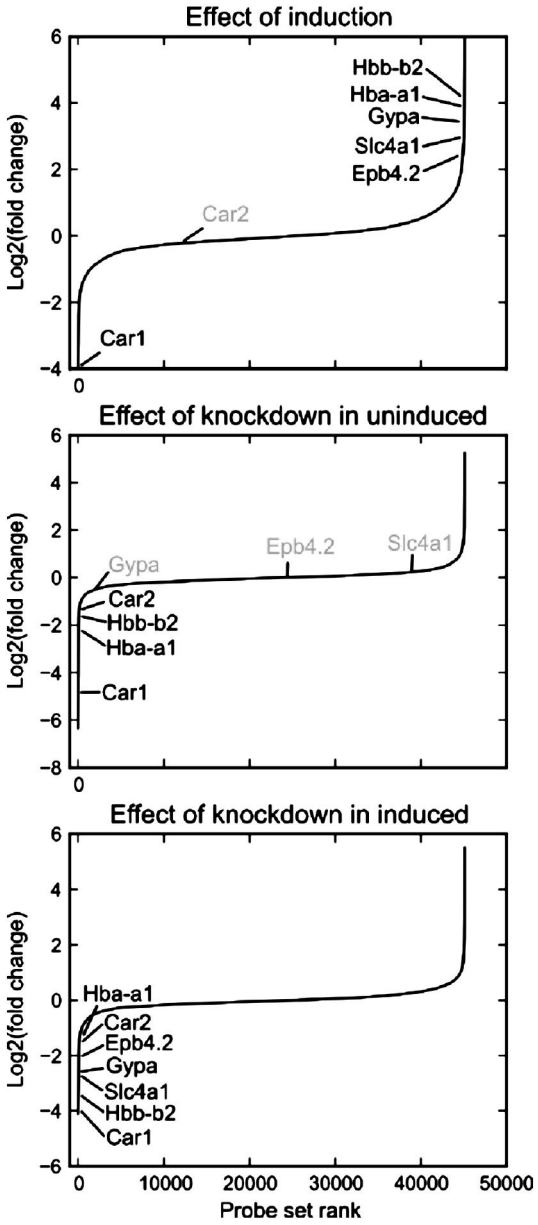

Fig. 2. Rank plots of induction or Ldb1 reduction in MEL cells

For each contrast in the microarray experiment (effect of induction in WT cells, effect of knockdown in uninduced cells and effect of knockdown in induced cells), probe sets in the array were sorted by fold change. For each gene highlighted in Table 1, the probe set with the best adjusted p-value is indicated. Those probes with an adjusted p-value > 0.01 are indicated in gray.

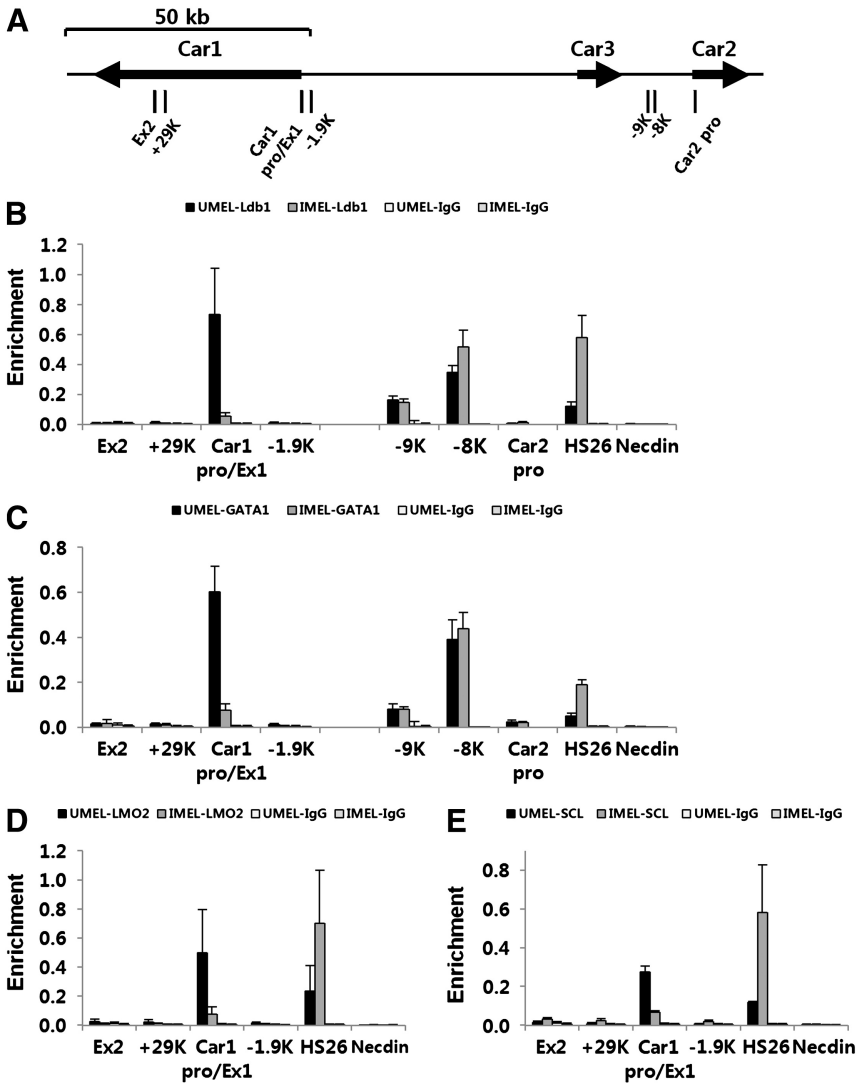

Fig. 3. Enrichment of the Ldb1/GATA1/LMO2/SCL complex at the Car1 promoter during MEL cell differentiation

(A) The Car1-3 locus is illustrated to scale showing the location of primer pairs used for qPCR with names below. ChIP was carried out using MEL cells before (UMEL) and after 4 days of 2% DMSO induction (IMEL) using antibodies to (B) Ldb1, (C) GATA1, (D) LMO2, and (E) SCL. HS26 (hypersensitive site 26) of mouse α-globin locus served as a positive control and necdin as a negative control for Ldb1 complex binding [19]. Error bars represent the SEM among four independent chromatin preparations.

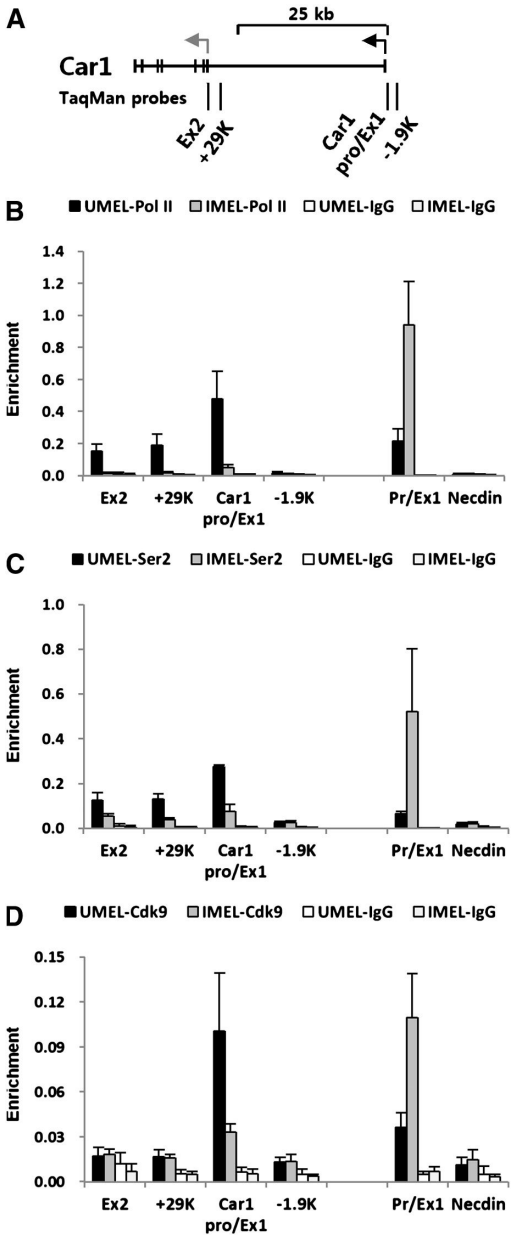

Fig. 4. Dissociation of transcription complex components from the Car1 promoter during MEL cell differentiation

(A) The mouse Car1 gene is illustrated to scale. Exons are indicated by vertical lines. The positions of amplicons used for qPCR are indicated below and are named. Car1 pro/Ex1 amplifies an E box/GATA motif at position −203 to −198 of the erythoid-specific distal promoter/exon1 region [24]. Ex2 denotes an E box/GATA motif over the intron1/exon2 region (proximal promoter for adult colon epithelia). −1.9K and +29K are putative E box/GATA motifs upstream and downstream of the erythoid-specific promoter, respectively. Pr/Ex1 denotes a positive control sequence at the promoter of the mouse α-globin gene [19]. Inactive necdin served as a negative control [9]. Chromatin was prepared from MEL cells before (UMEL) and after 4 days of 2% DMSO treatment (IMEL). ChIP was performed with antibodies to (B) RNA Pol II, (C) Ser2P Pol II, or (D) Cdk9. The results of four independent experiments ± SEM are graphed.

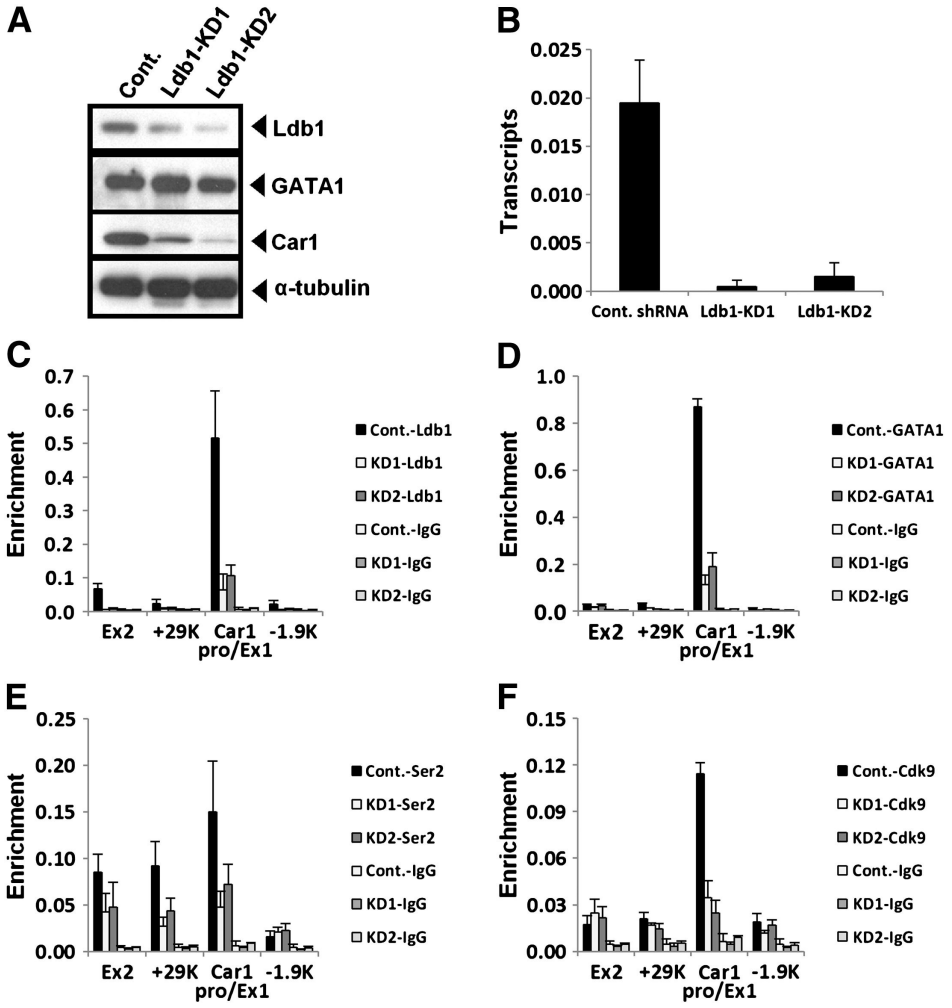

Fig. 5. Knockdown of Ldb1 by shRNA results in failure of Ldb1/GATA1/LMO2/SCL complex formation at the Car1 promoter

MEL cells were stably transfected with control or Ldb1-shRNA [9]. Ldb1-KD1 and Ldb1-KD2 represent two stable Ldb1- shRNA expressing MEL cell clones. (A) Western blot analysis was performed with the indicated antibodies. α-tubulin served as an internal control. (B) Car1 mRNA expression was analyzed by qRT-PCR from control or stable Ldb1-shRNA clones. Each value was normalized with 18S rRNA. Error bars represent the SEM of three independent experiments. (C-F) Chromatin was prepared from control or stable Ldb1-shRNA clones. ChIP assay was carried out with antibodies to (C) Ldb1, (D) GATA1, (E) Ser2P Pol II, and (F) Cdk9. Data are mean ± SEM of four independent experiments.

Table 1

Effect of stable Ldb1 knockdown (KD) in induced and uninduced MEL cells, as well as effect of induction, on select erythroid genes

	KD, Uninduced MEL		KD, Induced MEL		Induction, WT	
	Fold change	**adj pval**	**Fold change**	**adj pval**	**Fold change**	**adj pval**
Car1	−30.83	2.64E-08	−17.3	1.36E-08	−15.55	2.20E-08
Car2	−2.69	6.29E-05	−2.98	2.40E-06	NS	-
β-globin	−3.29	1.93E-02	−11.65	1.67E-05	17.57	3.60E-06
α-globin	−5.02	3.30E-04	-2.51	1.75E-03	13.99	4.71E-07
Slc4a1	NS	-	−7.12	9.30E-05	7.62	5.05E-05
Gypa	NS	-	−6.27	7.60E-06	12.19	5.21E-07
Epb4.2 (NS, not significant)	NS	-	−4.24	8.85E-07	4.73	2.01E-07