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A General Approach for Controlling Transcription and Probing Epigenetic Mechanisms: Application to the Cd4 Locus

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Synthetic regulatory proteins such as tetracycline (tet)-controlled transcription factors are potentially useful for repression as well as ectopic activation of endogenous genes and also for probing their regulatory mechanisms, which would offer a versatile genetic tool advantageous over conventional gene targeting methods. In this study, we provide evidence supporting this concept using Cd4 as a model. Cd4 is expressed in double-positive and Cd4 cells but irreversibly silenced in Cd8 cells. The silencing is mediated by heterochromatin established during Cd8 lineage development via transient action of the Cd4 silencer; once established, the heterochromatin becomes self-perpetuating independently of the Cd4 silencer. Using a tet-sensitive Cd4 allele harboring a removable Cd4 silencer, we found that a tet-controlled repressor recapitulated the phenotype of Cd4-deficient mice, inhibited Cd4 expression in a reversible and dose-dependent manner, and could surprisingly replace the Cd4 silencer to induce irreversible Cd4 silencing in Cd8 cells, thus suggesting the Cd4 silencer is not the (only) determinant of heterochromatin formation. In contrast, a tet-controlled activator reversibly disrupted Cd4 silencing in Cd8 cells. The Cd4 silencer impeded this disruption but was not essential for its reversal, which revealed a continuous role of the silencer in mature Cd8 cells while exposing a remarkable intrinsic self-regenerative ability of heterochromatin after forced disruption. These data demonstrate an effective approach for gene manipulation and provide insights into the epigenetic Cd4 regulatory mechanisms that are otherwise difficult to obtain. The Journal of Immunology, 2013, 190: 737–747.

Several methods have been devised to achieve reversible gene regulation. In one method, endogenous genes are modified so that their expression is now driven by regulatable synthetic transcription activators expressed from the endogenous regulatory elements, thus allowing for reversible gene regulation, but it is difficult to recapitulate the expression levels of the endogenous genes with the synthetic activators (7–10). Regulated expression of short hairpin RNA is also useful for reversible gene repression, but the repression is usually incomplete (2). Finally, removable transcription stop cassettes can be inserted into target genes, leading to constitutive KO that can be conditionally rescued (1, 3). This method, however, is not fit for inducing gene repression. Furthermore, none of these methods are fit for achieving ectopic expression or probing the molecular mechanisms of gene regulation.

A potential method for overcoming all three limitations in the conventional gene KO strategies would be to manipulate the endogenous genes using conditional regulatory proteins, the most popular of which are tetracycline (tet)-sensitive transcription factors such as tet-regulated Transcription Silencer (tTS) and tet-regulated Transcription Activator (tTA) (11, 12). tTS and tTA consist of the same bacterial tet-sensitive DNA binding domain fused to the mammalian KRAB repressor domain or the viral VP16 activation domain, respectively, and both proteins bind the cognate Tet-Response Element (TRE) only in the absence of Tet or its derivative doxycycline (Dox). In addition, a modified version of tTA, reverse tTA (rtTA), binds the TRE only in the presence of Tet or Dox (13). Surprisingly, although the Tet system is routinely used for reversible regulation of simple target/reporter genes bearing the TRE and a minimal promoter (11, 12), little is known about whether the system can directly control endogenous genes via perturbation of their natural regulatory mechanisms and whether the perturbation, if achieved, can be exploited to probe the regulatory mechanisms. Indeed, two decades after the intro-

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T.C. conceived the project and wrote the manuscript. M.W. and S.L. created the Cd4 mice. All authors participated in the analysis of the mice.

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; DN, double-negative; Dox, doxycycline; ISP, immature single-positive; KO, knockout; LN, lymph node; MFI, mean fluorescence intensity; rTA, reverse tet-regulated Transcription Activator; SP, single-positive; TAM, tamoxifen; tet, tetracycline; TRE, Tet-Response Element; tTA, tet-regulated Transcription Activator; tTS, tet-regulated Transcription Silencer.

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duction of the Tet system, it has been applied to only three endogenous genes (Hoxa2, Htr1a, and Mlc1) and in a rather preliminary manner (14–18). Specifically, iTS is shown to repress all three genes, with the reversibility of repression demonstrated only for Hoxa2 and Htr1a. Furthermore, ectopic expression was shown only for Mlc1, and mechanistic studies performed for none. A detailed characterization of the Tet system at these genes is hampered by a technical difficulty: the expression of these genes must be measured in embryos (for Hoxa2) or adult brains (for Htr1a and Mlc1). Additional studies using genes whose expression is readily quantifiable are therefore warranted to characterize better the system.

Two major subsets of T lymphocytes in the adaptive immune system are the CD4 helper and CD8 cytotoxic T cells that express the Ag coreceptor CD4 or CD8, respectively. CD4 acts to strengthen TCRβ–MHC class II interaction and to help trigger downstream signaling cascade after TCRβ ligation, thus playing essential roles in T cell activation and function. CD4 is also essential for CD4 lineage development. Intrathymic T cell development starts with double-negative (DN) cells lacking CD4 or CD8 expression (Fig. 1A) (19, 20). DN cells develop through four successive stages, DN1–4. DN4 cells then upregulate CD8 to become immature single-positive (ISP) cells before they express CD4 to become double-positive (DP) cells. DP cells express an entire repertoire of TCRβ, but only those cells expressing TCRβ with moderate affinity/avidity for self-MHC are signaled to survive. These cells then develop into CD4 and CD8 single-positive (SP) thymocytes based on the specificity of TCRβ, with the MHC class II– and MHC class I–restricted TCRβ coupled to the CD4 and CD8 lineages, respectively (21, 22). The survival and further maturation of DP cells are both controlled by TCR signaling and hence dependent on the Ag coreceptors, with CD4 selectively required for signaling by MHC class II–restricted TCRβ and hence for CD4 lineage development.

Given the biological importance of Cd4, much effort has been devoted to studying its mechanisms of regulation (23, 24). Cd4 transcription is driven by the Cd4 promoter and enhancer but repressed by the Cd4 silencer (Fig. 1B). The silencer is inactive in DP and CD4 cells, allowing CD4 expression in these cells, but is active in DN and CD8 cells where it inhibits the Cd4 promoter/ enhancer; in the absence of the silencer, both DN and CD8 cells express CD4. Notably, although the silencer is required to establish and maintain Cd4 repression in DN cells, it seems only required to establish repression in CD8 cells during CD8 lineage development, because the silencer can be conditionally deleted in mature CD8 cells without causing CD4 derepression under physiological conditions (24–27). Thus, the Cd4 silencer induces two types of repression in T cells: reversible repression in DN cells and irreversible repression or epigenetic silencing in CD8 cells. Epigenetic Cd4 silencing is thought to be mediated by a certain self-perpetuating repressive heterochromatin structure at the Cd4 locus, although little is known about the molecular feature of this structure (24–28). The Cd4 silencer is perhaps the only silencer in mammalian cells known to be capable of epigenetic silencing. It is unclear how the Cd4 silencer establishes epigenetic silencing in the CD8 lineage, whether the Cd4 silencer has any potential role in maintaining Cd4 repression in CD8 cells under special, more challenging conditions, or how stable the heterochromatin is. Such questions are difficult to address using conventional genetic or molecular approaches.

In this study, we characterized the behavior of the Tet system at the Cd4 gene in detail and used the system to address the mechanistic questions mentioned earlier. Our work is made possible in part by the ability to quantify CD4 expression at the single-cell level in a nonlethal manner. Our data illustrate the power of the Tet system for controlling the expression of endogenous genes and probing their physiological regulatory mechanisms.

Materials and Methods

Mice

The construct for producing the Cd4* allele was generated using standard molecular biology techniques on the pEZ TET plasmid backbone (a gift of K. Rajewsky), where the left and right arms flanking the Cd4 silencer were PCR-amplified from C57Bl6 mice. The construct including the PCR-generated DNA was verified by sequencing. ES cells (from 129Sv strain) were selected on G418 and screened by PCR. Founder mice carrying the targeted allele were mated with a line ubiquitously expressing Fpe (a gift from Dr. Flavell) to delete the neomycin resistance cassette, thus generating the Cd4* allele. To repress Cd4*, the mice were mated with a transgenic line widely expressing iTS from the β-actin promoter (a gift from M. Mallo) (15). To activate Cd4*, the Cd4* mice were mated with a transgenic line ubiquitously expressing rtTA from the Rosa26 locus [a gift from R. Jaenisch (29)]. As the Rosa26-rtTA and the Cd4 loci are both situated on chromosome 6 (but separated by ~1 million bp or 1 cm), to obtain Cd4*; rtTA+/*–interbreeding, we obtained two Cd4*; rtTA+/* founders, which were used to produce the Cd4*; rtTA+/* offspring. No difference in Cd4 regulation was detected between the two lineages, and so one of the lineages was used for all subsequent experiments. Mice expressing ER-Cre (from the Rosa26 promoter) were provided by Dr. Thomas Ludwig (Columbia University) (30–32). All mice analyzed were on the C57Bl6 × 129Sv background.

FACS analysis of lymphocytes

Adult (4–8 wk old) mice were used. In general, cells were stained with anti-Cd4–allophycocyanin, anti-Cd8–PE–Cy7, anti-B220–PE, and anti-TCR–FITC or anti-Cd3–Pacific blue, and anti-TCR–FITC or anti-Cd3–Pacific blue. However, to determine Cd4 expression throughout T cell development (Figs. 3A, 4A), thymocytes were stained with anti-Cd4–allophycocyanin, anti-Cd8–PE–Cy7, anti-Cd25–PE, anti-Cd4–FITC, and anti-Cd3–Pacific blue, and the data, collected on the LSRII flow cytometers, were analyzed as described (33–35). The FACS plots shown in this article are representative of a total of at least three mice per condition; for the most part, these mice were analyzed in several independent experiments.

Adoptive transfer

CD8 cells were magnetically purified from Cd4*; rtTA+/* mice using Dynabeads FlowComp mouse CD8 purification kit (Invitrogen). The purity was ~95%. One million cells (in 100 μl PBS) per mouse were injected retro-orbitally into Rag2 KO mice and Dox exposure (1 mg/ml) initiated 1 mo thereafter.

RT-PCR

Lymph node (LN) Cd4 and Cd8 cells were purified to near homogeneity using FlowComp Dynabeads (Invitrogen). RNA was isolated with RNAeasy plus (Qiagen). iTS and CD4 mRNA were quantified using Quant-iT TEB SYBR Green RT-PCR Kits (Qiagen). PCR was performed in triplicate and the values normalized to β-actin. PCR primers are as follows: ITS, 5′-CTC-GCCCAGAAGCTAGGTGTAAGC-3′ and 5′-CTGTAATAGTCGGGCGTACCTAGAC-3′; Cd4, 5′-AAGTCTCGAGCCCTCATATACACA-3′ and 5′-CTCGGCCACATGTTGTCTGCCAGG-3′; 5′-CTCGGCCACATGTTGTCTGCCAGG-3′; β-actin, 5′-GCACCGACAGACAGCTTCT-3′ and 5′-CTTTCGCACTAGGCGCAAG-3′.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed essentially as described (28), but with important modifications that significantly reduced nonspecifically precipitated signals. Briefly, LN cells were fixed with 1% formaldehyde at room temperature for 5 min, and cells resuspended in RIPA (0.8 M NaCl) before chromatin was sheared to ~300 bp with a Bioruptor sonicator. Chromatin from 1 million cells was diluted with equal volume of RIPA (0.8 M NaCl) before the addition of histone Abs (0.5 μg; Biolegend), salmon sperm DNA (final 1 μg/ml), and BSA (final 1 μg/ml). The samples were rocked at 4°C overnight and precipitate removed by centrifugation. Dynal protein G magnetic beads (10 μl) preblocked with salmon DNA and BSA were added to the supernatant and the
samples rotated for 3 h. The beads were washed sequentially in RIPA (0.3 M NaCl), RIPA (0 M NaCl), and TE. Then, 10 μl DirectPCR DNA Extraction Buffer (Viagen Biotech) containing 1 μg/ml proteinase K was added, and the samples incubated at 60°C overnight. The samples were diluted with 40 μl H2O and used directly for real-time multiplex PCR, which quantified the Cd4 promoter and silencer together with the internal control β-actin. Input DNA was quantified in parallel, which yielded % input for both target sequences and β-actin. The % input of the ChIP DNA was then plotted relative to the % input of β-actin as “fold over actin.” Of note, using the magnetic beads, DNA nonspecifically precipitated by rabbit IgG-beads was negligible and hard to quantify and thus ignored. Anti-H3K9ac and H3K4me2 were obtained from SABiosciences (no. GAM-1209 and no. GAM-3203, respectively). The primer pairs and probes (Biosearch Technologies) are as follows: Cd4 silencer primers, 5′-GACGCTACTGAACACCAAG-3′ and 5′-CTCTTTTCCTACCTCCTGAT-3′; Cd4 silencer probe, CAL Fluor Red 610 5′-GCTCCGTAGGCCCAGATGT-3′; Cd4 promoter primers, 5′-TGGAGGCTGTCTCAGCT-3′ and 5′-TTTGCCCTCACAGATGTCTGTA-3′; Cd4 promoter probe, Quasar 670 5′-CTGTCGACAGGACCTACGAC-3′; β-actin primers, 5′-CAGGGCTTATGAACCCAGTGG-3′ and 5′-GCTCTGACAGGGCAGATGT-3′; β-actin probe, FAM 5′-TGCCTCAACAAGGCAGGGT-3′.

Conditional Cd4 silencer deletion
Tamoxifen solution (20 mg/ml) was prepared by dissolving 200 mg tamoxifen (free base, Sigma-Aldrich T5648) in 0.5 ml 10% ethanol before adding 9.5 ml autoclaved peanut oil. The solution was sonicated and stored at −20°C.

To delete the Cd4 silencer, 100 μl of the solution was injected i.p. into adult Cd4−/−: ER-Cre−/− mice once a day for 3 consecutive days. To quantify the deletion efficiency, 50 μl of peripheral blood was drawn. RBC was lysed and CD8 cells purified to near homogeneity using FlowCyt Dynabeads (Invitrogen). The cells were resuspended in 10 μl DirectPCR Lysis buffer (Viagen). Quantitect SYBR Green PCR Kits (Qiagen) were used to quantify the Cd4 silencer and enhancer, the latter serving as an internal control. The primers have been described before (28).

DNA methylation of the Cd4 promoter
Genomic DNA (1 μg) was bisulfite-converted using Epitexit Bisulfite kit (Qiagen). The resulting DNA was amplified using PyroMark PCR Kit (Qiagen) with a primer pair targeting the Cd4 promoter. The primer sequences are 5′-GTGGGAGGAGGAGTTT-3′ and 5′-CCAATCCCTCGCTCCTCCTCT-3′. An internal primer (5′-GGAGGATTGGTGGG-3′) was then used to sequence the amplicon in the PyroMark Q24 system (Qiagen).

Results
The tet-sensitive Cd4 allele bearing a removable Cd4 silencer
To use the Tet system to manipulate Cd4 expression and explore its regulatory mechanisms, we created a bifunctional Cd4 allele (Cd4tTS) bearing the TRE and a floxed Cd4 silencer (Fig. 1), which allows for Dox-mediated transcription regulation as well as Cre-mediated silencer deletion (Fig. 2A). The TRE was inserted immediately upstream of the Cd4 silencer, which could presumably avoid nonspecific disruption of Cd4 regulation by the TRE insertion while allowing TRE-bound factors to impact the Cd4 promoter/enhancer via long-range interactions between the regulatory elements (36).

tTS reproduces the phenotype in Cd4 KO mice and causes reversible and graded Cd4 repression in DP cells
We bred mice homozygous for the Cd4+ allele and also carrying a transgene that widely expresses tTS from the human β-actin promoter (Cd4tTS+/+; tTS+/−) (15). We found these mice recapitulated the phenotype of the Cd4 KO mice, namely Cd4 lineage elimination due to loss of Cd4 expression in DP cells (37), as indicated by the lack of TCRβ CD4+ cells (Fig. 3A, columns 2–3, second row) and conversion of the normal DP (CD4+CD8−/−) into CD4+CD8−/− cells (Fig. 3A, column 1, second row). Furthermore, as in the case of Cd4 KO mice, a TCRβ CD4+CD8−/− population with unknown function was present in the thymus and periphery (Fig. 3A, columns 2–3). Cd4 repression was reversible upon tTS inactivation by Dox: Cd4 expression was restored on 10% of DP cells within 24 h after Dox administration (100 μg/ml) and in most DP cells by day 4, with concomitant emergence of Cd4 SP thymocytes (Fig. 3A, columns 1–2). By day 20, the thymus was indistinguishable from control mice, and substantial numbers of peripheral Cd4 cells had accumulated (Fig. 3A, column 3). Of note, on day 3, the “Cd4 SP” thymocytes weakly expressed Cd8 (Fig. 3A, circle, row 5, column 2), suggesting they were the “intermediate cells,” namely, positively selected DP cells that have recently terminated CD8 transcription (22, 38).

Having shown that tTS can fully repress Cd4 in DP cells, we examined the feasibility of graded Cd4 repression by treating adult Cd4tTS+/+; tTS+/− mice with decreasing concentrations of Dox (from 100 to 0.1 μg/ml) for 3 d. Dox as low as 10 μg/ml was able fully to inactivate tTS and restore Cd4 expression in >95% of DP cells, and Dox at 1 μg/ml successfully produced a partial effect, in that Cd4 was expressed only in 69% of DP cells and only to a low level [mean fluorescence intensity (MFI) was 24 U compared with 78 U induced by Dox at 10 μg/ml] (Fig. 3B, 3C). Curiously, whereas high doses of Dox produced the TCRβCD4+CD8−/− “intermediate cells” as previously described, such cells were absent in mice exposed to Dox at 1 μg/ml (Fig. 3B, circle, row 3, column 2) even after prolonged (3 wk) exposure (Fig. 3D). As CD4 transcription in the intermediate cells is weaker than in DP cells (38) and thus perhaps more vulnerable to tTS, the “intermediate cells” might have completely lost Cd4 expression and merged into the Cd4 CD8−/− TCRβ compartment that normally exist in Cd4−/−; tTS+/− mice. Indeed, as shown in Fig. 3B, cells in this compartment did seem to accumulate concomitantly with the loss of intermediate cells, reaching 35% in mice with 10 and 15% in mice with 100 and 10 μg/ml Dox exposures, respectively.

These data indicate that tTS can prevent Cd4 expression if acting constitutively throughout development. To determine whether tTS can also terminate ongoing Cd4 transcription in preexisting Cd4−/− cells, Cd4tTS+/−; tTS+/− newborns were raised to adulthood in the presence of Dox (5 μg/ml in drinking water to the mothers), which inactivated tTS and allowed for normal T cell development (Fig. 3E, column 1, day 0). Dox was then removed and mice analyzed at various times thereafter. Cd4 expression on DP cells began to

FIGURE 1. Regulation of Cd4 expression during T cell development. (A) The expression of Cd4 (small dots), in conjunction with that of Cd8 (data not shown), marks the four major stages in T cell development; namely, DN, ISP, DP, and mature CD4/CD8 cells. Cd4 is expressed in DP and CD4 cells but repressed in the rest of the T cell types by the Cd4 silencer. (B) The regulatory elements in the Cd4 locus. The Cd4 promoter (P) and enhancer (E) are constitutively active, but its activity in CD8 cells is suppressed by the Cd4 silencer (S) located 1.8 kb downstream of the transcription start site. The sizes of the regulatory elements are indicated below the DNA.
To determine whether rtTA can induce ectopic CD4 expression, we used a transgene (rtTA) that ubiquitously expresses rtTA from the Rosa26 promoter (29). In adult Cd4*; rtTA+/− mice, after 15 d of Dox exposure [1 mg/ml, which is around saturating dose (39)], CD4 was highly induced in all CD4+ thymocyte subsets except the earliest ones; that is, in DN4, ISP, and CD8 SP but not DN1−3 cells (Fig. 4A, columns 1–5, and data not shown). In contrast to the thymocytes, peripheral CD8 cells were rather resistant to Dox, and Cd4 induction occurred in a stochastic, all-or-none rather than uniform manner, with ∼30% of the cells fully expressing CD4 and the rest fully repressing CD4 (Fig. 4, column 6), consistent with the pattern of gene de-repression resulting from probabilistic heterochromatin disruption. Finally, B cells and macrophages were completely resistant to Dox (data not shown).

We next sought to increase the efficiency of ectopic Cd4 induction. Because these mice described earlier were heterozygous for the Cd4* allele and the rtTA transgene, we wondered whether homozygosity could potentiate the effect of rtTA (by doubling its cellular concentrations). We therefore generated mice homozygous for both the Cd4* allele and the rtTA transgene (Cd4+; rtTA+/+). As the Rosa26-rtTA and the Cd4 loci are both located on chromosome 6, we used meiotic recombination to create the Cd4+; rtTA+/+ mice (see Materials and Methods). We then administered a saturating amount of Dox (1 mg/ml) to adults and monitored Cd4 induction in peripheral blood. Dox induced Cd4 in CD8 cells in a slow and all-or-none manner, Cd4+ CD8 cells becoming detectable within 2 d and gradually reaching 47 ± 12% on day 17 with MFI being ∼320 (Fig. 4B, filled circles; data not shown), which is indeed more efficient than the heterozygous Cd4+; rtTA+/− mice. However, as Dox also induced Cd4 in CD8 SP thymocytes, which were continuously exported into the periphery, the peripheral CD8 cell pool was contaminated with the recent thymic emigrants, thus confounding data interpretation. To address this issue, CD8 cells were isolated from Cd4+; rtTA+/− mice and adoptively transferred into Rag2 KO mice. The mice were challenged with Dox 1 mo thereafter, when CD8 cells had populated the peripheral blood (Supplemental Fig. 2). Notably, Dox induced Cd4 on the transferred cells more efficiently than in Cd4+; rtTA+/− mice, with the Cd4+ expressing cells reaching 63 ± 6% on day 17 (Fig. 4B, open circles). The higher induction frequency might reflect the fact that the adoptively transferred CD8 cells had undergone and perhaps were still undergoing homeostatic proliferation in the recipients, which might facilitate gene expression by, for example, decompacting chromatin. To explore this further, peripheral blood cells were stimulated overnight with anti-CD3/28 Ab in vitro in the presence of Dox. Anti-CD3/28, known to activate TCR signaling and globally decondense chromatin in T cells, indeed greatly facilitated Cd4 induction, leading to CD4 expression in >40% of CD8 cells compared with ∼6% in the absence of stimulation (Fig. 4C, left). As expected, ionomycin and PMA, which can also activate TCR signaling, similarly enhanced Cd4 inducibility (Fig. 4C, right).

We conclude that Dox can rather effectively induce ectopic Cd4 expression in peripheral CD8 cells when adult Cd4+; rtTA+/− mice were exposed to Dox for 17 d, and the induction is further enhanced by TCR signaling. However, neither the 17-d Dox treatment of Cd4+; rtTA+/− mice in vivo nor ionomycin plus PMA stimulation in vitro could effectively induce Cd4 in B cells or macrophages (data not shown). In contrast, when mice were
exposed to Dox for 1 mo starting at birth, namely from days 20 to 50 postconception, CD4 was successfully induced in 49% of B cells, although the expression level was low (MFI = 47); under this condition, 89% of CD8 cells expressed CD4 to very high levels (Fig. 4D).

CD4 induction was most robust if Dox exposure was initiated prenatally: in mice exposed from conception until 1 mo after birth (days 0–50), CD4 was expressed in all CD8 cells and 90% of B cells to higher levels (MFI = 495 and 95 U for CD8 and B cells, respectively; Fig. 4E, left and middle, top histograms), and furthermore, CD4+ macrophages/monocytes also emerged (Fig. 4E, top right). Notably, upon Dox withdrawal, whereas CD4 induction was rapidly (within 4 d) reversed in macrophages/monocytes (Fig. 4E, right), in B and CD8 cells, the reversal proceeded in two distinct phases: within the first 4 d after Dox withdrawal, CD4 expression was rapidly and rather uniformly reduced by 3- to 6-fold (MFI from 495 to 90 in CD8 and from 95 to 28 in B cells), which was followed by the second phase (days 4–15) where the CD4 expression level no longer declined but the frequencies of CD4-expressing cells dwindled, the kinetics of which was slow especially in B cells where CD4 expression still lingered on in 33% of B cells as late as 15 d after Dox withdrawal (Fig. 4E, left and middle). The mechanisms of the reversal in the two phases may differ (see Discussion).

Finally, we examined histone modifications in CD8+CD4+ cells produced after 1 wk of Dox exposure. In unexposed CD8 cells, H3K9a and H3K4me2 were depleted at the Cd4 promoter and silencer, whereas both markers were increased after Dox stimulation (Fig. 4F). Thus, the effect of rtTA (in CD8 cells) is opposite
to the effects of tTS (in thymocytes; Fig. 3G), consistent with their respective roles in Cd4 regulation.

We conclude that rtTA can reversibly induce ectopic CD4 expression in multiple cell types, but the efficiencies are cell type specific and also dependent on functional states of the cells.

tTS triggers epigenetic Cd4 silencing in CD8 and early T cells

We next used the Tet system to address biological questions regarding Cd4 silencing. One of the most interesting features of Cd4 regulation during T cell development is that the Cd4 silencer can induce irreversible Cd4 repression (i.e., epigenetic silencing) in CD8 cells. It has been proposed that the silencer achieves this feat by recruiting special repressors to establish heritable heterochromatin at the Cd4 locus; the heterochromatin, once formed, can then maintain Cd4 repression independently of the Cd4 silencer (24–26). However, it remains possible that the Cd4 locus in CD8 cells is uniquely prone to the formation of heterochromatin. In this scenario, even a repressor such as tTS typically only capable of reversible repression might cause epigenetic Cd4 silencing in CD8 cells.

To test this idea, we created the Cd4* allele lacking the Cd4 silencer (Cd4pDs, Fig. 2A) by deleting the Cd4 silencer in the germline using a transgenic line that constitutively expresses Cre (gift from Dr. R. Flavell). Silencer deletion de-repressed Cd4 in all CD4+ T cells, including the early (DN3, DN4, ISP) and DP cells, where the DN4 and ISP cells are rare proliferating blasts that are clearly detectable only by gating on the large cells within the post-DN3 compartment (column 3). Shown is a representative of three mice from a single experiment. (B) Cd4 induction in CD8 cells in the peripheral blood in adult Cd4p/ mice (left) or in Rag2 KO mice adoptively transferred with CD8 cells isolated from Cd4p/ rtTA+ mice (right). Mice were exposed to Dox (1 mg/ml) for 17 d. Blood was drawn from the tail vein at various times and the cells stained for CD4, CD8, and TCR before analysis. Data are pooled from two independent experiments. Each symbol represents a mouse. (C) TCR activation facilitated rtTA-mediated Cd4 induction in CD8 cells. Peripheral blood cells from Cd4p/; rtTA+ mice were stimulated overnight in vitro with plate-bound anti-CD3/28 (left) or 1 μM ionomycin plus 10 ng/ml PMA (right) in the presence of Dox (100 ng/ml). Cd4 expression in CD8 cells was analyzed. (D) Cd4p/; rtTA+ mice were exposed to Dox from birth to adulthood before analysis of CD4 expression in the peripheral blood. (E) Cd4p/; rtTA+ mice were exposed to Dox from conception to adulthood. Dox was then discontinued and Cd4 expression in peripheral blood cells monitored, where macrophages/monocytes were identified by their large size. (F) rtTA induced epigenetic changes at the Cd4 promoter and silencer in peripheral CD8 cells. Cd4p/; rtTA+ mice were exposed to Dox for 1 wk (+), and the peripheral CD8 cells expressing CD4 were isolated and analyzed as described in Fig. 3G. CD8 cells from the mice lacking Dox exposure (−) were used as a control. *p < 0.05 (statistically significant effects of Dox).
mice were hemizygous for the tTS transgene as opposed to the homozygous mice in Fig. 3A). To determine whether the tTS-mediated repression was reversible, we treated the mice with Dox (100 ng/ml) for 3 d. Dox restored CD4 expression in DP but, importantly, not in the other T cells including CD8 cells, demonstrating that tTS had epigenetically silenced Cd4 in these cells (circles, row d). Physiological Cd4 silencing induced by the Cd4 silencer is known to be mitotically stable, surviving multiple rounds of cell division (27). To determine the mitotic stability of tTS-induced silencing, LN cells from Cd4<sup>ΔsΔs</sup>; tTS mice were stimulated in vitro with plate-bound anti-CD3/CD28 and cells cultured for 5 d in the presence of Dox (100 ng/ml) and IL-2. Cells from Cd4<sup>ΔsΔs</sup>; rTATA mice were used as a control for the effect of Dox. In these mice, Dox induced Cd4 in all CD8 cells, as expected (Fig. 5B, left). In sharp contrast, in CD8 cells from Cd4<sup>ΔsΔs</sup>; tTS mice, Cd4 remained completely silenced under this condition, demonstrating tTS-triggered silencing was mitotically stable as in the case of the physiological silencing.

To address the mechanism of action of tTS, we first compared the effects of tTS versus the Cd4 silencer on H3K9a and H3K4me2 at the Cd4 promoter in CD8 cells. The markers were absent in normal CD8 cells (as described in Fig. 4F) and enriched by silencer deletion, but this enrichment was abolished by tTS (Fig. 5C). Thus, tTS can replace the Cd4 silencer to remove the two markers. To gain further mechanistic insights, we examined CpG methylation at the Cd4 promoter. It was reported that the Cd4 promoter is highly methylated in CD8 cells (27), and so we wondered whether tTS could similarly induce Cd4 promoter methylation. Unexpectedly, we found little methylation in CD8 cells in the control mice with an intact Cd4 silencer (Fig. 5D, group b), the level (∼10%) being comparable to that in CD4 cells (group a) and total thymocytes (group e). Also, deleting the Cd4 silencer did not further reduce the methylation (group c), indicating the silencer was not responsible for this baseline-level methylation. In contrast, in the presence of tTS, we observed near maximal (∼87%) Cd4 promoter methylation in CD8 cells (group d) and thymocytes (group g). This result suggests that tTS induced Cd4 promoter de novo methylation. Alternatively, under the physiological condition, the Cd4 promoter may be “naturally” methylated in the DN cells but becomes de-methylated in DP cells and remains so in CD8 cells. In this scenario, tTS acts not by inducing de novo methylation but by preventing de-methylation, and future studies are needed to distinguish between the two possibilities. Despite this uncertainty, the data strongly suggest that the mode of action of tTS is different from that of the Cd4 silencer, even though tTS and the silencer can both trigger irreversible epigenetic silencing, which has mechanistic implications (see Discussion). Of note, CpG methylation, if necessary, was not sufficient for tTS-mediated epigenetic silencing, given that tTS also methylated the Cd4 promoter in DP cells and yet the repression in DP cells was reversible.

We conclude that tTS can replace the Cd4 silencer to trigger epigenetic Cd4 silencing in CD8 cells even though tTS is typically a reversible repressor and seems to repress Cd4 via a mechanism distinct from that used by the Cd4 silencer.
Roles of the Cd4 silencer versus heterochromatin in maintaining epigenetic Cd4 silencing in CD8 cells

We sought to expose the role, if any, of the Cd4 silencer in maintaining Cd4 repression in CD8 cells. To this end, we used rtTA to challenge the Cd4 locus before and after conditional deletion of the Cd4 silencer in CD8 cells. Conditional deletion was achieved with a transgene ubiquitously expressing ER-Cre, a fusion protein between Cre and modified estrogen receptor that is normally trapped in the cytoplasm until stimulation by the estrogen analogue tamoxifen (TAM) (30–32). In mice carrying the Cd4+ allele and expressing both rtTA and ER-Cre (Cd4+; rtTA+/−; ER-Cre+/−), three consecutive TAM injections induced Cd4 silencer deletion in virtually all peripheral CD8 cells (Fig. 6A). Twenty-four hours after the last injection, we challenged the mice with Dox (1 mg/ml) for various times and monitored CD4 expression in peripheral blood CD8 cells. In the control mice without TAM treatment, the frequencies of CD4+CD8 cells remained at the baseline level 1 d after Dox administration and reached ∼19 ± 3% on day 4 (Fig. 6B, left column, rows a–b; Fig. 6D, group a). In contrast, in the mice with prior TAM treatment and hence silencer deletion, the frequency of CD4+CD8 cells already reached ∼13 ± 2% on day 1 and was further increased to ∼33 ± 3% on day 4 (Fig. 6B, row b, middle and right columns; Fig. 6D, groups b–c). Thus, rtTA-mediated Cd4 induction was much faster in the absence of the silencer, revealing an important role of the silencer in protecting against rtTA-mediated disruption of Cd4 repression in CD8 cells. In contrast to CD8 cells, silencer deletion did not facilitate Dox-induced Cd4 derepression in B cells, consistent with the fact that the silencer is inactive in B cells (data not shown).

We next determined whether the silencer is also important for restoring Cd4 repression after forced disruption by rtTA. In the control mice, Cd4 expression was fully reversed by day 10 after Dox withdrawal (Fig. 6B, left column, row f; Fig. 6D, group d). In fact, the reversal seemed complete within 4 d: although on day 4 after Dox withdrawal, Cd4 expression persisted in ∼10% of CD8 cells (Fig. 6B, left column, row e, circle), the expression level was higher than on day 1 or 2, suggesting these CD4+CD8 cells were not preexisting CD8 cells with lingering Cd4 expression but rather were recently generated CD8 cells that were unable to establish...
Cd4 silencing because of rtTA interference during development. Importantly, in TAM-treated mice with a brief (1-d) Dox exposure, Cd4 expression was reversed in a manner hardly distinguishable from the control mice (Fig. 6B, middle column), except for the presence of a Cd4*Cd8* population (comprising 7% of CD8 cells) on day 10 after Dox withdrawal (Fig. 6B, circle, middle column, row e). These cells, however, were not derived from preexisting CD8 cells, but perhaps were recently exported CD8 cells that aberrantly expressed CD4 due to TAM-mediated premature Cd4 silencer deletion occurring before the establishment of Cd4 silencing, as indicated by the following data: in mice treated with TAM alone, the CD4*CD8* cells slowly accumulated in the periphery, reaching ~6% of the CD8 population on day 10 (Fig. 6C). Correcting for these recent thymic emigrants, we found that in mature CD8 cells where the silencer had been conditionally deleted, Cd4 expression induced by a brief (1-d) Dox exposure was readily reversible in the absence of the silencer (Fig. 6D, group e). Thus, the silencer was dispensable for effective restoration of Cd4 expression if the repression was disrupted by a brief Dox exposure. However, the silencer became important if Dox exposures were prolonged: in mice challenged with Dox for 4 d, Cd4 expression persisted in 25% of CD8 cells on day 10 after Dox withdrawal, which amounted to 15% of mature CD8 cells after correcting for the recent thymic emigrants (Fig. 6B, right column, row f). Considering that immediately after the 4-d Dox exposure, Cd4 was expressed on 35% of CD8 cells (Fig. 6B, right column, row b), we estimate that ~40% of the silencer-deleted Cd4* alleles whose Cd4 repression was disrupted by the 4-d Dox challenge failed to restore Cd4 repression within 10 d after Dox withdrawal. Thus, the Cd4 silencer is important for efficient restoration of Cd4 repression after the prolonged, 4-d Dox challenge. However, under this condition, ~60% of the silencer-deleted Cd4* allele managed to restore the repression (Fig. 6D, group f; averaged value being 67 ± 5%).

These data expose a role of the Cd4 silencer in stabilizing Cd4 repression in CD8 cells, but also reveal a remarkable potential of the heterochromatin to regenerate itself and restore Cd4 repression after forced disruption.

Discussion
To our knowledge, this study represents the first in-depth characterization of the Tet system in the context of an endogenous gene and the first use of this system to probe its regulatory mechanism. Generally speaking, our study demonstrates the feasibility of controlling endogenous genes and probing their regulatory mechanisms via recruitment of synthetic regulatory proteins. This recruitment-based approach should have diverse applications. For example, one can recruit a histone-modifying enzyme to explore the role of a relevant histone mark, a nuclease to study DNA repair, or GFP for real-time imaging. Of note, because the Tet system can deliver any effector protein, it is adaptable for diverse purposes. In the following we will discuss the characteristics of the Tet system when applied to Cd4 and the mechanistic insights this system generated regarding Cd4 regulation; such insights are difficult to obtain with any other tool.

Tet system as a tool for regulating endogenous genes: advantages, limitations, and potential solutions
We show that the Tet system can reproduce the phenotype in Cd4 KO mice and is additionally capable of reversible Cd4 repression and reversible ectopic Cd4 activation, thus confirming its advantages over conventional gene KO methods. The limitation of the Tet system, as revealed in our study, is that the efficacy of gene regulation is variable among different cell types. However, this variability may not be intrinsic to the Tet system (see further). Of note, efficiency of Cre-mediated deletion can also be cell type specific reflecting perhaps from variable accessibility of the LoxP sites (see, e.g., Refs. 3, 40), and just as in the case of the Cre-Lox system, the limitation in the Tet system is overwhelmed by the advantages; indeed, the Tet system is indispensable for certain applications and an attractive alternative to the conventional gene targeting methods. Below we will discuss in detail the cell type--specific variation in the efficiency of the Tet system.

First, tTS could repress Cd4 in DP but unexpectedly not in CD4 cells. Although this may suggest that tTS function is cell type specific, which would be an intrinsic weakness of the Tet system, we found that tTS expression in CD4 cells was 10- to 20-fold lower than that in thymocytes, pointing to a different but readily manageable culprit. tTS transcription in the transgene used in our study is directed by the ubiquitous human β-actin insulator. Despite this, in situ hybridization assays reveal that although tTS is widely expressed in the mouse embryos, the expression levels vary significantly in different regions of the embryos (Fig. 1E in Ref. 15), consistent with variable tTS expression in thymocytes versus peripheral T cells. Different mouse lines are therefore needed to direct uniform, high-level tTS expression.

Second, rtTA could readily induce ectopic Cd4 expression in some cell types (DN4, ISP, and CD8) but not others (DN1–3 thymocytes, B cells, and macrophages). This might reflect variation in rtTA expression, but the following data suggest a more significant possibility. Specifically, the ability of rtTA to induce Cd4 in various T cell subsets paralleled the effects of Cd4 silencer KO: in mice with germline silencer deletion, Cd4 was highly expressed in DN4, ISP, and CD8 cells but not DN1–3 cells, presumably because the latter do not possess strong endogenous activators of Cd4 transcription (Fig. 5A). Because endogenous activators can facilitate rtTA function, their variation among different cell types can contribute to the variable rtTA sensitivity. Furthermore, endogenous repressors of Cd4 transcription, which can antagonize rtTA, may also be variable among different cell types, which might further diversify rtTA sensitivity. We should stress that despite this variability in the efficiency, rtTA successfully induced Cd4 in the diverse cell types examined.

Finally, rtTA-induced Cd4 expression was reversed very slowly in B cells compared with CD8 or macrophages. Notably, in both B and CD8 cells, the reversal proceeded in two phases: a uniform drop in Cd4 expression in all cells to a stable level, followed by a shutoff of the remaining expression in a stochastic, all-or-none manner. The reversal in the first phase was rapid in both B and CD8 cells, and the rapid kinetics resembled that at simple tetrathionate-responsive transgenes (41–43), suggesting the reversal resulted from the departure of rtTA. But how was Cd4 expression sustained thereafter, how was it stochastically terminated, and why was this termination so slow in B cells? We imagine that both B and CD8 cells contain endogenous activators of Cd4 transcription. These activators are presumably excluded from the Cd4 locus by the heterochromatin, but rtTA can disrupt heterochromatin (44–46) and open up the Cd4 locus, which would allow the activators to bind Cd4 and sustain its expression after rtTA’s departure. However, the damaged heterochromatin tends to regenerate itself to restore Cd4 repression (see further), which is expected to occur in the observed stochastic manner as in the case of other heterochromatin-mediated phenomena such as position effect variegation. This heterochromatin-mediated reversal is facilitated by the Cd4 silencer in CD8 cells but not in B cells where the silencer is inactive, thus explaining the slow reversal kinetics in B cells.
In summary, the Tet system is not equally effective in regulating Cd4 in different cell types, which might reflect tissue-specific TTS/rtTA expression and/or the extremely complex cell type–specific Cd4 regulatory mechanisms. The former problem is extrinsic to the Tet system, and the latter may be insignificant for genes with less complex regulatory mechanisms. Indeed, to date, the Tet system has been applied to three other endogenous genes in mice beside Cd4 (Hoxa2, Htr1a, and Mlci1), and whenever tested, rTTS and rtTA can effectively alter target gene expression (see Introduction). These data, together with ours, strongly suggest general applicability of the Tet system, which will be greatly facilitated by transgenic lines with various rTTS and tTTS expression patterns, many of the lines already available (11). Furthermore, the efficiency of the Tet system may be enhanced by, for example, moving the TRE closer to the promoter, increasing the copy number of the cognate DNA binding sites for tet-controlled factors (there are five copies in our TRE), or altering the physiological state of the target cells (Fig. 4C). In addition, we found that a few days of exposures of Cd4<sup>-/-</sup>; rtTA+/+ mice to Dox during fetal development suffices to greatly facilitate subsequent rtTA-mediated Cd4 induction in adult mice (data not shown), suggesting another method for improving the efficiency of ectopic induction.

The Cd4 promoter/enhancer in CD8 cells is predisposed to epigenetic silencing: the silencer is not the key? rTTS induced epigenetic Cd4 silencing in CD8 and early (DN and ISP) T cells. Paradoxically, rTTS-mediated repression of simple transgenes in adult mice and tumor lines is reversible, as is its repression of Hoxa2 in early mouse embryos (15, 47–49) and Cd4 in DP cells. Although rTTS can irreversibly repress the ubiquitin and β-actin promoters in transgenic mice if it acts during the first few days of embryogenesis (50), the epigenetic Cd4 silencing did not require fetal rTTS action (data not shown). These data raise the possibility that the Cd4 locus in CD8 and early T cells is predisposed to epigenetic silencing so that even a reversible repressor such as rTTS can induce epigenetic silencing. The fact that rTTS but not the Cd4 silencer affects CpG methylation in CD8 cells reinforces the notion that epigenetic Cd4 silencing can be induced via different mechanisms. We thus propose that the Cd4 silencer, just like rTTS, is not the (only) determinant of epigenetic Cd4 silencing, which is testable by asking whether the silencer can trigger epigenetic silencing at heterologous genes in CD8 cells. It is of great interest to understand the putative silencer-independent mechanisms that predispose the Cd4 locus to epigenetic silencing.

Cd4 silencer, heterochromatin resilience, and maintenance of Cd4 repression in CD8 cells

In CD8 cells, Cd4 silencer deletion facilitated rtTA-mediated disruption of Cd4 repression and could hamper its restoration, indicating that the Cd4 silencer continuously exerts repressive effects on Cd4 transcription. Although the heterochromatin at the Cd4 locus is sufficient for Cd4 repression under normal conditions, the Cd4 silencer may become additionally required under pathological conditions such as defective heterochromatin formation or aberrant expression of Cd4 transcriptional activators.

Interestingly, although the Cd4 silencer could facilitate restoration of Cd4 repression after Dox challenges, the heterochromatin at the Cd4 locus was at least partially sufficient for the restoration, particularly when the Dox exposures were brief. Because Cd4 induction necessitates the disruption of the heterochromatin structure, the data reveal remarkable resilience of this structure that allows it to regenerate or “heal” itself after rtTA-inflicted damage. To our knowledge, this is the first demonstration of such resilience of heterochromatin. rTTS and the VP16 activation domain it carries has been used to disrupt heterochromatin at randomly integrated reporter templates in tumor cell lines (44–46), but the reversibility of the disruption has not been explored, let alone the contribution of heterochromatin resilience to this reversibility. Indeed, these systems are not fit for addressing heterochromatin resilience because the mechanisms that establish heterochromatin at the reporter genes persist after the establishment, analogous to the situation at the wild-type Cd4 locus that carries the Cd4 silencer, which would obscure the intrinsic property of heterochromatin. Of note, for the same reason, the ability of B cells to restore Cd4 repression after rtTA-mediated disruption does not prove the resilience of heterochromatin (or other forms of repressive chromatin) because the repressive chromatin structure is not established by the Cd4 silencer but rather by an unknown, presumably persistent mechanism. The molecular basis of heterochromatin resilience is a mystery, and the Cd4 locus in CD8 cells provides an excellent model for addressing this fundamental question, which has implications for the mechanisms of epigenetic memory and plasticity.

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References


