

The enhancer HS2 critically regulates GATA-3-mediated *Il4* transcription in T_H2 cells

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GATA-3 is a master regulator of T helper type 2 (T_H2) differentiation. However, the molecular basis of GATA-3-mediated T_H2 lineage commitment is poorly understood. Here we identify the DNase I-hypersensitive site 2 (HS2) element located in the second intron of the interleukin 4 locus (*Il4*) as a critical enhancer strictly controlled by GATA-3 binding. Mice lacking HS2 showed substantial impairment in their asthmatic responses and their production of IL-4 but not of other T_H2 cytokines. Overexpression of *Gata3* in HS2-deficient T cells failed to restore *Il4* expression. HS2 deletion impaired the trimethylation of histone H3 at Lys4 and acetylation of histone H3 at Lys9 and Lys14 in the *Il4* locus. Our results indicate that HS2 is the target of GATA-3 in regulating chromosomal modification of the *Il4* locus and is independent of the *Il5* and *Il13* loci.

Naive CD4⁺ T cells differentiate into two main subsets of helper T cells, T helper type 1 (T_H1) and T_H2, defined on the basis of their nonoverlapping cytokine profiles and immunoregulatory functions. T_H1 cells secrete the proinflammatory cytokines interferon- γ (IFN- γ) and tumor necrosis factor and are responsible for cell-mediated immunity and protection against intracellular pathogens, whereas T_H2 cells produce interleukin 4 (IL-4), IL-5, IL-6, IL-10 and IL-13 and mediate humoral immunity and protection against helminthic infection^{1–3}. A third subset of helper T cells, T_H17 cells, has been identified as IL-17-producing CD4⁺ T cells responsible mainly for adaptive immunity against certain bacteria and fungi; the development of this subset is tightly associated with the pathogenesis of certain autoimmune disorders^{4,5}.

The mouse T_H2 cytokine locus contains *Il4*, *Il5* and *Il13* and the constitutively expressed *Rad50* and *Kif3a* genes, and the locus composition is well conserved in mammals⁶. T_H2 cytokine expression is heritably regulated by transcriptionally permissive and repressive chromatin structure at *cis*-acting regulatory elements, which have been experimentally identified by the detection of DNase I-hypersensitive (HS) sites and histone modifications^{7–9}. In the mouse T_H2 locus, *Il4* transcription is regulated by elements that map to conserved sequence 1 (HSS1 and HSS2), located between *Il13* and *Il4*; HS0, located in the 5' region of *Il4*; HS1, located in the promoter region; HS2 and HS3 in the second exon; and HS5a and HS5, located 3' of *Il4* (refs. 10–13). A T_H2 cytokine locus-control region has been characterized at several HS sites (*Rad50* HS4 (RHS4), RHS5, RHS6 and RHS7) located between exon 22 and exon 25 of *Rad50* (refs. 14–17). The *Il13* locus contains two promoter regions, HS1 and HS2 (refs. 11,18). HS1 is the conserved GATA-3-response element (CGRE) upstream of the

proximal promoter HS2 (refs. 19,20). This CGRE has been shown to control widespread chromosomal modification of the *Il13* and *Il4* loci²⁰. HS5a is a T_H2-specific stimulation-dependent site involved in commitment to the T_H2 cell fate¹². In contrast, HSS3 and HS4 appear in all naive cells as well as T_H1 and T_H2 cells^{10,11}.

Studies of *Il4*-regulatory elements in transgenic reporter mice have indicated that the coordination of multiple elements is necessary for proper lineage-specific *Il4* expression²¹. T_H2 cytokine production is appreciably lower in mice with a germline deletion of conserved non-coding sequence 1 (CNS-1) or of HS5a and HS5 (refs. 22,23). However, those observations conflict with results obtained in the transgenic reporter experiments showing that the coordination of several regions is required for maximum production of T_H2 cytokines²¹. Therefore, these deletion experiments still leave open the question of whether each element regulates *Il4* transcription or T_H2 differentiation. A solution may have been provided with the finding that RHS7, located 3' of *Rad50*, satisfies most of the criteria for being an locus-control region and that disruption of RHS7 leads to considerable impairment in T_H2 cytokine production¹⁷.

GATA-3 is a master regulator of T_H2 differentiation^{24–26} and activates genes encoding T_H2 cytokines mainly through epigenetic modifications^{21,27–29}. For example, ectopic expression of GATA-3 can induce T_H2 differentiation even under T_H1-skewing conditions in which STAT6, a transducer of IL-4 signaling, is absent^{27,30,31}. Furthermore, GATA-3 can autoactivate its own transcription³¹. An *in vivo* requirement for GATA-3 in the initiation of *Il4* transcription during T_H2 differentiation and for direct regulation of the *Il5* and *Il13* promoters has been demonstrated by analyses of mice with conditional knockout of *Gata3* (ref. 25). This direct regulation is consistent with

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Figure 1 Histone modification of the *Il13* and *Il4* loci and regulation of IL-4 by HS sites.

(a) ChIP analysis of chromatin from C57BL/6 CD4⁺ T cells (left unstimulated (Naive) or stimulated under T_H1- or T_H2-polarizing conditions) immunoprecipitated with antibody to H3K9ac and H3K14ac, anti-H3K4me3, anti-H3K9me3 or anti-H3K27me3, then analyzed by real-time PCR of DNA from immunoprecipitated chromatin components or an equivalent mass of input DNA (purified from chromatin fractions before immunoprecipitation). Results are presented as the ratio of 2^{-CT(IP)} to 2^{-CT(input)}, where 'IP' is immunoprecipitated chromatin and 'CT' indicates the cycling threshold. (b) ICS analysis of IL-4 and IFN- γ (top) and ELISA of IL-4 production (bottom) in naive CD4⁺ T cells isolated from mice with deletion (KO) of CGRE, CNS-1, HS2, 3' UTR, HS4 or CNS-2 (above plots) and their wild-type littermates (WT), then stimulated for 7 d under T_H2-polarizing conditions and then restimulated with monoclonal antibody (mAb) to TCR β . Numbers in or below quadrants indicate percent cells in each. **P* < 0.05 (Student's *t*-test). Data are representative of three independent experiments with similar results (error bars (b), s.d.).

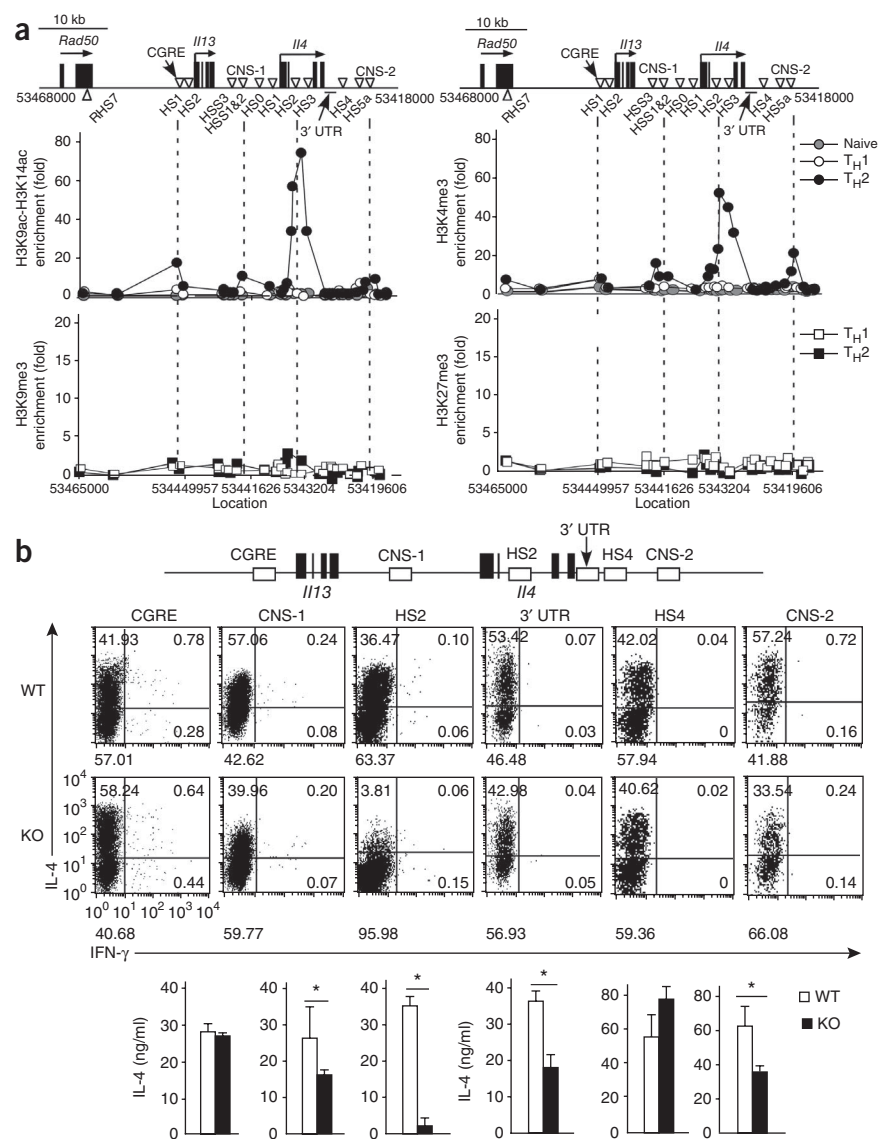
the idea that the binding of GATA-3 activates the *Il5* and *Il13* promoters^{18,32–34}. GATA-3 also binds to the first intron of *Il4*, HS5a, HS5, RHS7 and CGRE^{12,17,20,35,36}. However, the precise mechanisms by which GATA-3 regulates epigenetic modification of the *Il4* locus during T_H2 lineage commitment and identification of the critical target elements remain unclear.

Here we demonstrate that HS2 in the *Il4* locus is a critical GATA-3-binding site that regulated lineage-specific *Il4* expression. Deletion of HS2 markedly impaired the maintenance of acetylation of histone H3 at Lys9 (H3K9ac) and Lys14 (H3K14ac) and the onset of methylation of histone H3 at Lys4 (H3K4me) on the *Il4* locus, both of which are required for transcriptional permissibility, which led to the lack of *Il4* transcription. GATA-3 overexpression failed to reconstitute those defects. We found that HS2, as a specific target of the GATA-3-mediated epigenetic modification on the *Il4* locus, was an indispensable enhancer. Therefore, our work proposes a distinct role for GATA-3 in the regulation of genes encoding T_H2 cytokines.

RESULTS

H3 modifications in canonical T_H1 and T_H2 cells

Rapid acquisition of differences in the acetylation and methylation of histone H3 in T_H1 and T_H2 cells indicates a developmentally early role for these modifications in cytokine gene expression during the process of helper T cell fate determination^{28,37}. For histone H3, modifications can be classified as those associated with transcriptionally repressed chromatin (di- and trimethylation at Lys9 or Lys27 (H3K9me2, H3K9me3, H3K27me2 and H3K27me3) or transcriptionally permissive chromatin (H3K9ac and H3K14ac, and trimethylation at Lys4 (H3K4me3))³⁸. We prepared canonical T_H1 and T_H2 cells from naive CD4⁺ T cells from C57BL/6 mice and analyzed their *Il13*–*Il4* loci by chromatin immunoprecipitation (ChIP) with antibodies specific



for H3K9ac and H3K14ac, as well as H3K4me3, H3K9me3 and H3K27me3, plus the appropriate primers (Supplementary Table 1). Naive T cells had no detectable signal with any of the antibodies (Fig. 1a). In contrast, we observed a consistent signal for H3K9ac and H3K14ac and for H3K4me3 in T_H2 cells at the CGRE (*Il13* distal promoter), CNS-1 enhancer, *Il4* intronic enhancer (HS2 and HS3) and CNS-2 enhancer, with the most prominent signal in the intronic enhancer (Fig. 1a). However, these stimulated T_H2 cells showed no enrichment for the transcriptionally repressed signatures H3K9me3 and H3K27me3 (Fig. 1a), despite having a strong signal for the control locus (Supplementary Fig. 1). Therefore, permissive chromatin modifications specifically occur at several HS sites in T_H2 cells, with HS2 and HS3 showing the most prominent H3K9ac-H3K14ac and H3K4me3 modifications. In contrast to T_H2 cells, in T_H1 cells there was no sign of repressive or permissive chromatin modifications of the *Il4* locus.

Abrogation of *Il4* expression in HS2-deficient mice

We established a series of mutant mice lacking each HS site in the *Il4*–*Il13* locus to answer the question of whether the lineage-specific regulation of T_H2 cytokines is determined by a single element, such

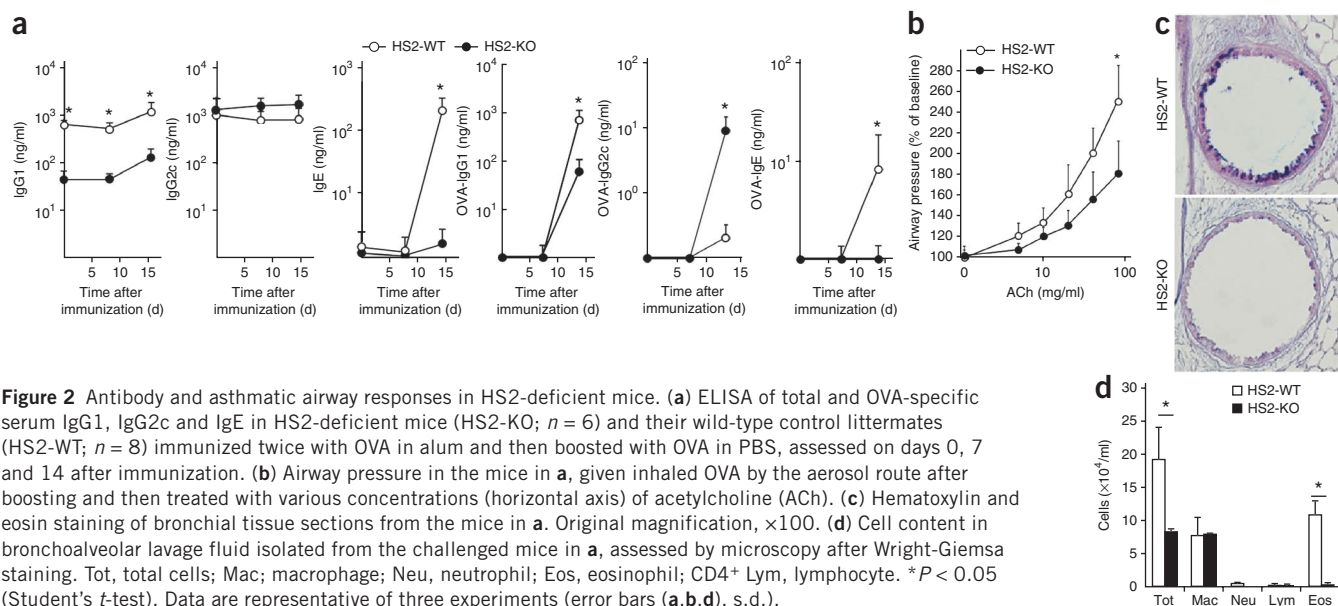


Figure 2 Antibody and asthmatic airway responses in HS2-deficient mice. **(a)** ELISA of total and OVA-specific serum IgG1, IgG2c and IgE in HS2-deficient mice (HS2-KO; $n = 6$) and their wild-type control littermates (HS2-WT; $n = 8$) immunized twice with OVA in alum and then boosted with OVA in PBS, assessed on days 0, 7 and 14 after immunization. **(b)** Airway pressure in the mice in **a**, given inhaled OVA by the aerosol route after boosting and then treated with various concentrations (horizontal axis) of acetylcholine (ACh). **(c)** Hematoxylin and eosin staining of bronchial tissue sections from the mice in **a**. Original magnification, $\times 100$. **(d)** Cell content in bronchoalveolar lavage fluid isolated from the challenged mice in **a**, assessed by microscopy after Wright-Giemsa staining. Tot, total cells; Mac, macrophage; Neu, neutrophil; Eos, eosinophil; CD4⁺ Lym, lymphocyte. * $P < 0.05$ (Student's t -test). Data are representative of three experiments (error bars (**a,b,d**), s.d.).

as the locus-control region, or by the coordinated activity of multiple elements. To study the enhancer activity of each HS site, we generated mice with a series of HS deletions—deletion of the CGRE, HS2, the 3' untranslated region (UTR), HS4 (targeting strategy for the construction of these mutants, **Supplementary Fig. 2**) or CNS-2 (ref. 39)—by homologous recombination and replacement with a *loxP*-flanked neomycin-resistance cassette in embryonic stem cells derived from C57BL/6 mice (for CGRE deletion) and 129 $\times$ C57BL/6 hybrid mice (for deletion of HS2, 3' UTR, HS4 or CNS-2; **Fig. 1b**). After germline deletion of these elements by crossing of those mice with mice that express Cre recombinase, we backcrossed the offspring with C57BL/6 mice for at least four generations and then intercrossed those offspring to obtain mice with homozygous gene deletion. None of the deletion lines showed any developmental defects or growth abnormalities in T cells. We also examined previously established CNS-1-deficient mice (on the BALB/c background)²².

To investigate the function of each HS site in the expression of T_H2 cytokines, we isolated naive CD62L^{hi}CD44^{lo} CD4⁺ T cells from wild-type littermate mice and mice with deletion of CGRE, CNS-1, HS2, 3' UTR, HS4 or CNS-2, stimulated them with antibody to the T cell antigen receptor (anti-TCR) and anti-CD28 under T_H2-polarizing conditions and measured expression of IL-4 and IFN- γ . IL-4 production by T_H2 cells lacking CGRE or HS4 was similar to that of wild-type T cells, and T_H2 cells lacking CNS-1, 3' UTR or CNS-2 had slightly less IL-4 production (**Fig. 1b**). However, the greatest effect was in T_H2 cells derived from HS2-deficient mice, which had much less IL-4 production (**Fig. 1b**). These results indicate that HS2 is a potent enhancer for the regulation of *Il4* expression during early T_H2 priming.

Abrogation of the allergic response in HS2-deficient mice

We next examined the function of the HS2 enhancer in the induction of an *in vivo* allergic response regulated mainly by T_H2 cytokines. We first examined the concentration of immunoglobulin E (IgE) and IgG1 in serum, as switching to these isotypes is regulated by IL-4 (ref. 40). The basal serum concentration of IgG1 in unprimed HS2-deficient mice was about 10% as much as that in control mice (**Fig. 2a**). After immunization with aluminum hydroxide (alum)-conjugated ovalbumin (OVA), HS2-deficient mice had about 10%

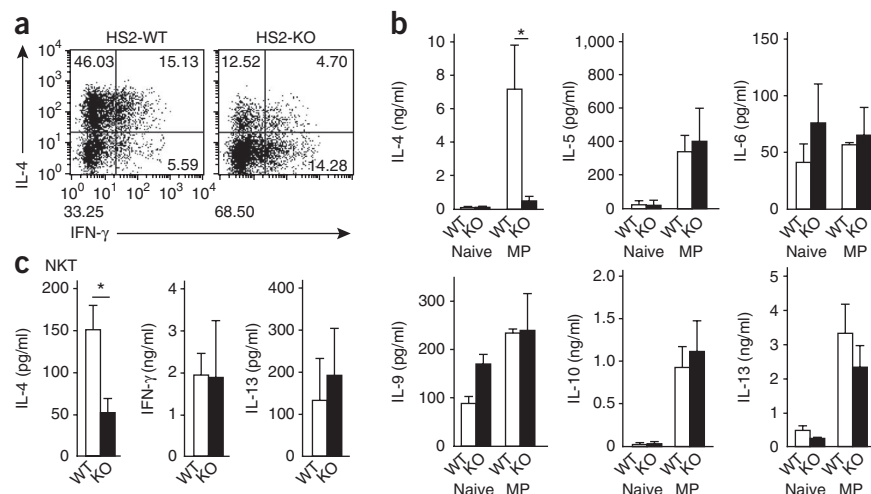
as much OVA-specific IgG1 and IgE as their control littermates had (**Fig. 2a**, bottom row). In contrast, HS2-deficient mice had ~50-fold more antigen-specific IgG2c (a T_H1-associated antibody) than did control mice. These results indicate that HS2 is a critical element in the regulation of IL-4-mediated antibody responses.

We further investigated the role of the HS2 enhancer in a model of OVA-induced bronchial asthma. After treating mice by systemic sensitization to OVA and aerosolized OVA challenge, we induced airway responsiveness by administering aerosolized acetylcholine. Control littermate mice demonstrated airway hyper-responsiveness to acetylcholine and airway pathogenesis, whereas HS2-deficient mice had much lower responses (**Fig. 2b,c**). Sensitization and challenge with OVA resulted in more eosinophils in bronchoalveolar lavage fluid from control mice, but there was no eosinophil infiltrate in the HS2-deficient mice; also, their total number of infiltrating cells of all types was 25–33% that of control mice (**Fig. 2d**), and we observed no significant difference in the number of macrophages or lymphocytes. These data suggest that the HS2 enhancer contributes to the onset and development of IgE production and eosinophil-mediated asthmatic disease.

HS2 regulates initial IL-4 production by T cells and NKT cells

As HS2-deficient mice showed a defect in IL-4 production by T_H2 cells and T_H2-mediated allergic *in vivo* responses, we next sought to determine whether the HS2 enhancer influences the initial IL-4 production from memory phenotype (MP) T cells and NKT cells, which are the main source of IL-4 during primary T cell activation⁴¹. Indeed, T_H0-induction cultures of bulk CD4⁺ T cells also had a lower frequency of IL-4⁺ cells but no effect on IFN- γ ⁺ cells (**Fig. 3a**). Therefore, we speculated that the HS2 defect was manifested in MP T cells and NKT cells. We separated CD4⁺ T cells into CD62L^{hi}CD44^{lo} (naive) and CD62L^{lo}CD44^{hi} (MP) fractions. We stimulated sorted cells for 48 h with anti-TCR and anti-CD28 and assessed cytokine production by enzyme-linked immunosorbent assay (ELISA). CD44^{lo} naive CD4⁺ T cells produced no detectable T_H2 cytokines except IL-6 and IL-9, whereas CD44^{hi} MP CD4⁺ T cells had readily detectable expression of several T_H2 cytokines, including IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (**Fig. 3b**). However, MP T cells lacking HS2 produced very small amounts of IL-4, even though their production of other T_H2 cytokines

Figure 3 HS2 is critical for the primary production of IL-4 by MP T cells and NKT cells. **(a)** ICS analysis of IFN- γ and IL-4-producing cells among wild-type and HS2-deficient CD4⁺ T cells stimulated for 7 d under T_H0 conditions, then restimulated for 6 h with mAb to TCR β . Numbers in or below quadrants indicate percent cells in each. **(b)** ELISA of cytokine production by CD44^{hi}CD62L^{lo} (MP) and CD44^{lo}CD62L^{hi} (Naive) wild-type and HS2-deficient T cells stimulated for 48 h with mAb to TCR β and mAb to CD28. **(c)** ELISA of IL-4, IL-13 and IFN- γ in splenic wild-type and HS2-deficient NKT cells stimulated for 48 h with α -galactosylceramide (10 ng/ml). * P < 0.05 (Student's t -test). Data are representative of three independent experiments (error bars (b,c), s.d.).



was similar to that of control MP cells (Fig. 3b). We further examined whether NKT cells had a similar defect. We stimulated NKT cells for 48 h with α -galactosylceramide and assessed cytokine production by ELISA. IL-4 production by NKT cells was greatly impaired in the absence of HS2, whereas the production of IFN- γ and IL-13 was unaffected (Fig. 3c). These data indicate that the HS2 enhancer is an essential regulatory element for initial IL-4 production by MP T cells and NKT cells.

HS2 regulates IL-4 production in T_H2 cells

We next sought to determine whether HS2 is essential for the T_H2-commitment process or for IL-4 expression by T_H2 cells. For this, we isolated naive CD4⁺ T cells by sorting and then stimulated the cells with anti-TCR and anti-CD28 under neutral and T_H2-skewing conditions. After 7 d, we restimulated cells with anti-TCR and measured

cytokine production by ELISA and intracellular cytokine staining (ICS). Under T_H0 conditions, few control or HS2-deficient T cells produced IL-4 or IFN- γ (Fig. 4a). In contrast, naive T cells from control mice generated substantial numbers of IL-4⁺ cells under T_H2 conditions, and most of these cells coexpressed IL-13. However, the frequency of HS2-deficient T cells that produced IL-4 was much lower, although there was no effect on the number of IL-13-producing cells (Fig. 4a). The profile of secreted cytokines was similar to the results obtained by ICS: there was much less IL-4 production by HS2-deficient T_H2 cells, but the amount of other T_H2 cytokines (IL-5, IL-9, IL-10 and IL-13) was nearly identical to that in control T cell cultures (Fig. 4b). These results indicate that the HS2 enhancer is indispensable for IL-4 expression by T_H2 cells.

We extended our analysis to the CGRE, which corresponds to the distal *Il13* promoter and CNS-2 elements, to determine whether

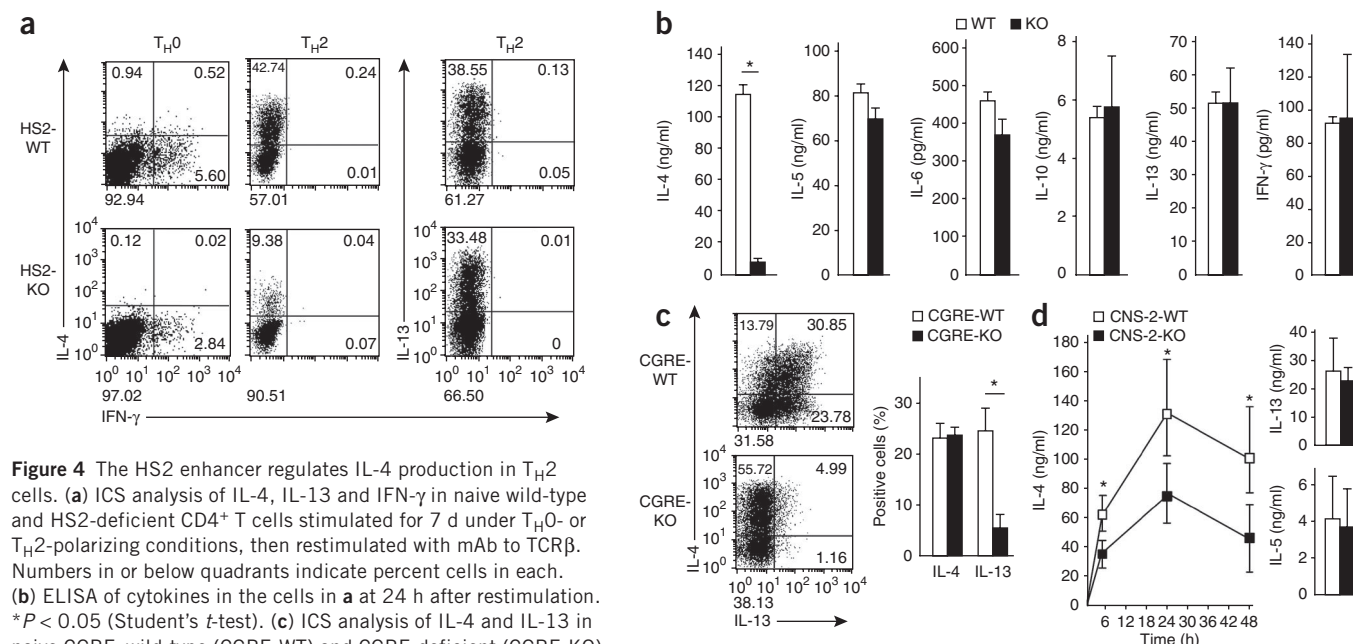


Figure 4 The HS2 enhancer regulates IL-4 production in T_H2 cells. **(a)** ICS analysis of IL-4, IL-13 and IFN- γ in naive wild-type and HS2-deficient CD4⁺ T cells stimulated for 7 d under T_H0- or T_H2-polarizing conditions, then restimulated with mAb to TCR β . Numbers in or below quadrants indicate percent cells in each. **(b)** ELISA of cytokines in the cells in **a** at 24 h after restimulation. * P < 0.05 (Student's t -test). **(c)** ICS analysis of IL-4 and IL-13 in naive CGRE-wild-type (CGRE-WT) and CGRE-deficient (CGRE-KO) CD4⁺ T cells stimulated for 7 d under T_H2-polarizing conditions, then restimulated with mAb to TCR β . Numbers in or below quadrants (left) indicate percent cells in each. * P < 0.05 (Student's t -test). **(d)** ELISA of IL-4, IL-13 and IL-5 in naive CNS-2-deficient (CNS-2-KO) and control C57BL/6 (CNS-2-WT) CD4⁺ T cells stimulated for 7 d under T_H2-polarizing conditions, then restimulated with mAb to TCR β . * P < 0.05 (Student's t -test). Data are representative of three independent experiments with similar results (**a**), three independent experiments (**b**) or four experiments with four mice per group (**c,d**; error bars (**b-d**), s.d.).

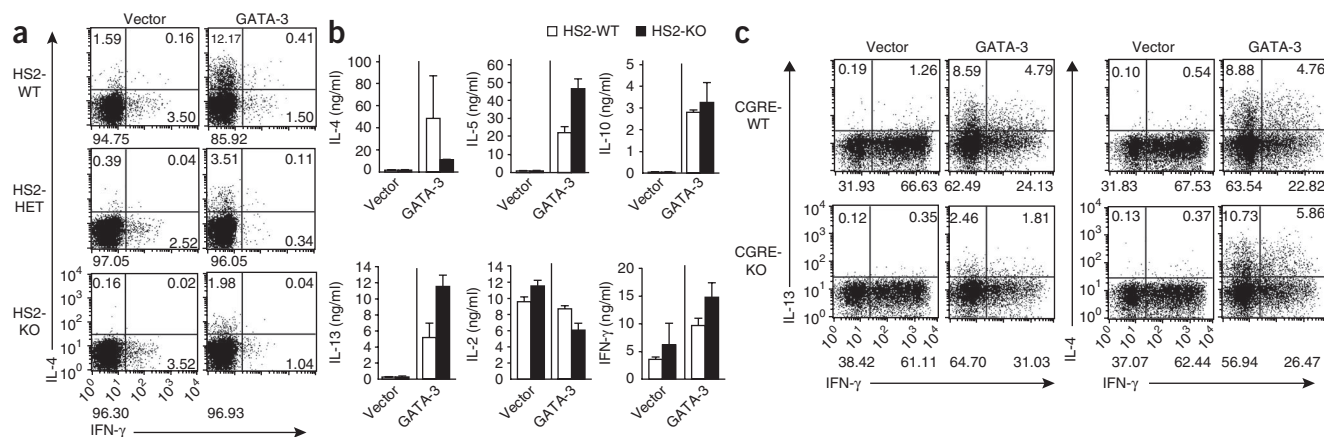


Figure 5 The HS2 enhancer is required for GATA-3-mediated production of IL-4. **(a)** Production of IL-4 and IFN- γ in green fluorescent protein-positive (GFP⁺) cells among CD4⁺ T cells derived from wild-type mice (HS2-WT) and mice with heterozygous (HS2-HET) or homozygous (HS2-KO) deletion of HS2, then transduced with retroviral vector encoding GFP alone or GFP and GATA-3, cultured for 7 d under T_H1-polarizing conditions and restimulated with mAb to TCR β . **(b)** ELISA of cytokine production by cells transduced and restimulated as in **a**. **(c)** Production of IL-4, IL-13 and IFN- γ in GFP⁺ cells among CD4⁺ T cells derived from wild-type and CGRE-deficient mice, then transduced for 7 d with retrovirus as in **a** under T_H1-polarizing conditions and then restimulated with mAb to TCR β . Numbers in or below quadrants (**a,c**) indicate percent cells in each. Data are representative of three independent experiments with similar results (**a,c**) or are from three independent experiments (**b**; error bars, s.d.).

each element has an important role in regulating IL-4 and IL-13. Naive T cells lacking CGRE generated substantial numbers of IL-4⁺ cells under T_H2 conditions, but most of these cells failed to express IL-13 (Fig. 4c), which indicated that the CGRE regulates IL-13 expression. T_H2 cells lacking CNS-2 had a phenotype similar to that of HS2-deficient T_H2 cells, although effect on IL-4 expression was much milder than that in HS2-deficient T_H2 cells (Figs. 1b and 4d). These results indicate that CGRE is a potent enhancer in the regulation of *Il13* transcription; however, the enhancer function of CNS-2 is qualitatively similar to but much weaker than that of HS2.

HS2 required for GATA-3-mediated IL-4 production

We next examined the question of whether GATA-3 protein is directly linked to the function of the HS2 enhancer. If the interaction were direct, overexpression of GATA-3 protein would be expected to fail to reconstitute IL-4 expression in HS2-deficient T cells. Therefore, we overexpressed *Gata3* cDNA via retrovirus in T_H1-skewed cells from wild-type mice and mice with heterozygous or homozygous deletion of HS2. As expected, overexpression of *Gata3* in wild-type T_H1 cells resulted in many more IL-4-producing cells and much more secretion of IL-4 (Fig. 5a,b). In contrast, overexpression of *Gata3* in HS2-deficient T_H1 cells failed to induce IL-4 production, whereas the production of IL-5 and IL-13 was greater than that of wild-type T cells (Fig. 5a,b). This enhancement might have been due to an additive effect with the presence of excess GATA-3. However, CGRE-deficient T_H1 cells failed to undergo induction of IL-13 production but not of IL-4 production (Fig. 5c). These results demonstrate that HS2 is a critical enhancer of GATA-3-mediated *Il4* regulation and may be a specific target of GATA-3 for this regulation.

Prominent binding of GATA-3 to HS2

GATA-3-binding sites have been reported at some HS sites in the T_H2 locus, such as RHS7, CGRE, intron 1, CNS-1 and CNS-2 (refs. 12,17,20,35,36). ChIP analysis with a GATA-3-specific antibody showed no enrichment for GATA-3 in naive T cells, but in T_H2 cells there was enrichment of more than tenfold for GATA-3 at CGRE and CNS-2 (Fig. 6a). There was also weak binding at RHS7,

intron 1 and CNS-1, but the greatest enrichment (50- to 60-fold) was at HS2 (Fig. 6a). We next examined spatiotemporal binding of GATA-3 at the *Il13* and *Il4* loci under T_H2-polarizing conditions. There was enrichment of more than tenfold for GATA-3 at HS2 after 24 h and at CGRE, intron 1 and CNS-2 after 48 h. With time, HS2 showed a further increase in GATA-3 binding, reaching enrichment of over 90-fold after 72 h (Fig. 6b). Therefore, GATA-3 bound to CGRE, HS2 and CNS-2 during T_H2 differentiation, and HS2 showed rapid and strong binding of GATA-3.

H3-modification kinetics during T_H2 differentiation

Next we examined how spatiotemporal changes in the acetylation of H3K9 and H3K14 and methylation of H3K4 at each regulatory element correlated with GATA-3 binding. Naive T cells showed no signal for H3K9ac-H3K14ac or H3K4me (Fig. 1a), but after 24 h in T_H2-polarizing conditions, there was modest but significant enrichment of H3K9ac and H3K14ac (all less than sixfold) at the HS1-HS3 and CNS-1 and CNS-2 regions. The acetylation of H3K9 and H3K14 become more prominent after 48 h in T_H2 cells than in T_H1 cells (Fig. 6c, left). Published studies have reported modest acetylation of H3K9 and H3K14 across the *Il4-Il13* locus in T_H1 and T_H2 cells primed for 24–48 h (refs. 20,28,37,42,43). That observation is consistent with the idea that the *Il4-Il13* locus is transcriptionally permissive at the early stage of both T_H1 priming and T_H2 priming. Acetylation of H3K9 and H3K14 during T_H1 priming was much lower at 72 h, whereas T_H2 priming further enhanced the acetylation of H3K9 and H3K14 at HS2-HS3 until it was more than eightfold above background, and HS2 showed the greatest enrichment (Fig. 6c, left).

In contrast to the results reported above, another permissive marker, H3K4me, was completely absent at 24 h except at the *Il13* promoter, including the CGRE (Fig. 6c, right). The H3K4 methylation signal at the CGRE gradually increased in a time-dependent manner. The *Il13* locus had already acquired transcriptional permissivity at 24 h, and this occurred under both T_H1 conditions and T_H2 conditions. We detected enrichment of H3K4me after 48 h under T_H2 conditions but not under T_H1 conditions. These signals, which we observed mainly in the HS1-HS3 and CNS-1 and CNS-2 regions,

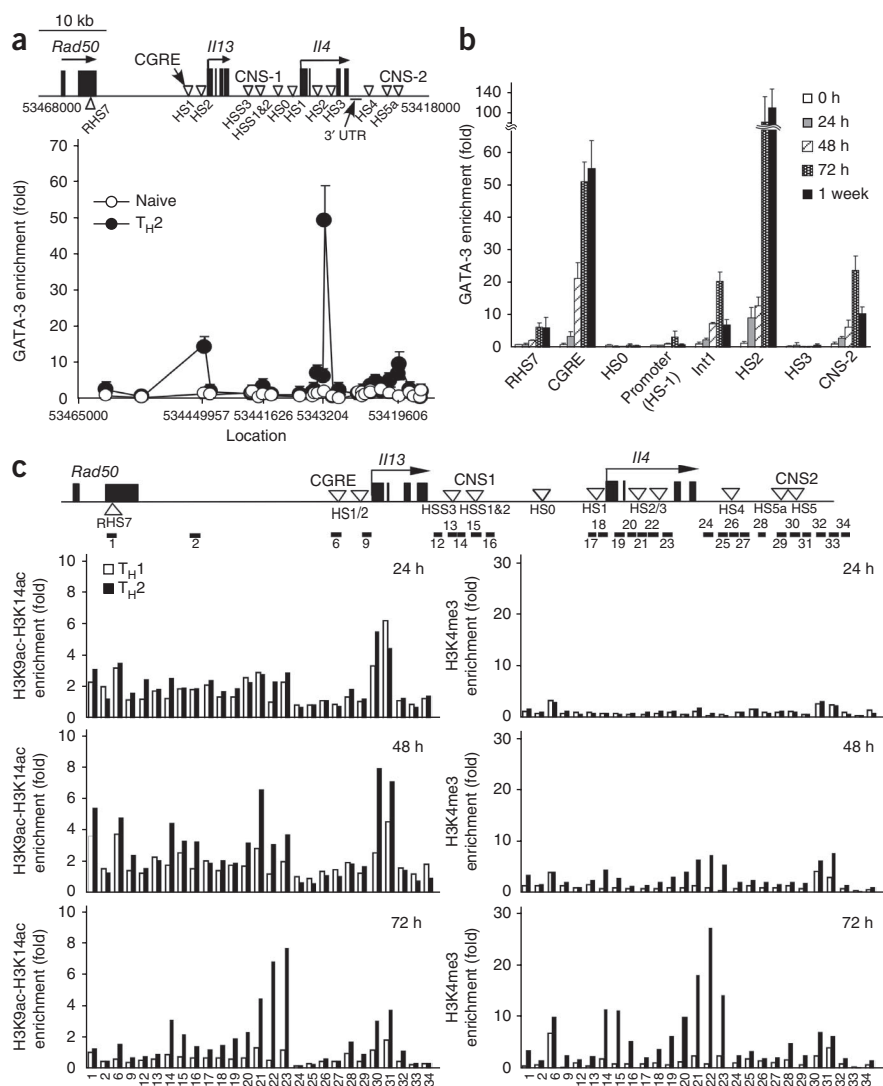


Figure 6 Histone H3 modification and binding of GATA-3 to the *Il13* and *Il4* loci. **(a)** ChIP analysis of chromatin extracts obtained from C57BL/6 naive T cells and TH2 cells, immunoprecipitated with anti-GATA-3-conjugated Sepharose beads and analyzed by real-time PCR as in **Figure 1a**. **(b)** ChIP analysis of C57BL/6 naive T cells stimulated for 0 h, 24 h, 48 h, 72 h and 1 week under TH2 conditions, then collected for immunoprecipitation with anti-GATA-3. **(c)** ChIP analysis of C57BL/6 naive T cells stimulated for 24, 48 or 72 h under TH1- or TH2-polarizing conditions, followed by detection of H3K9ac-H3K14ac and H3K4me3 with the primers at top (*Il13* and *Il4* loci). Data are representative of three (**a,b**) or two (**c**) independent experiments (error bars (**a,b**), s.d.).

We further examined the effect on HS2 deletion on spatiotemporal changes in the acetylation of H3K9 and H3K14 and methylation of H3K4. After 24 h, T cells isolated from both wild-type and HS2-deficient mice showed slight enrichment of H3K9ac and H3K14ac (all less than sixfold) at most of the HS sites examined, although HS2-deficient T cells had no enrichment at the HS2 region (**Fig. 7b**, left). By 72 h and later, control wild-type TH2 cells had much more enrichment at intron 1, HS2, HS3 and CNS-2; however, there was a lack of such enrichment in HS2-deficient T cells. Control mice had more enrichment of H3K4me after 48 h. Although enrichment at CNS-1 and CGRE was similar in control and HS2-deficient T cells, in the latter cells there was no detectable enrichment at the HS site spanning the promoter and CNS-2 region (**Fig. 7b**, right). These results indicate that the HS2 enhancer has a critical role in the acetylation of H3K9

generated a very sharp peak (6- to 30-fold) at 72 h. After 72 h, HS2 again showed the greatest enrichment (**Fig. 6c**, right). These results indicated that the two permissive histone marks, H3K9ac-H3K14ac and H3K4me, were regulated differently in a spatiotemporal manner and that the timing of H3K4me-mediated transcriptional permissivity correlated well with the spatiotemporal change in the binding of GATA-3 to the HS2 enhancer.

HS2 regulates histone acetylation and methylation

A published report has indicated that GATA-3 directly regulates transcriptional permissivity of the *Il4* locus^{21,27–29,44}. If HS2 were a specific target of GATA-3 in the initiation of transcriptional permissivity, then deletion of HS2 would cause impairment of the binding of GATA-3 only to the *Il4* locus, eventually leading to impairment in the permissive histone modification. Therefore, we examined acetylation of H3K9 and H3K14 and binding of GATA-3 in wild-type and HS2-deficient T cells under TH2-skewing conditions by comprehensive ChIP plus microarray analysis spanning chromosome 11 (52,205,742–54,365,252), including the *Il4* and *Il13* loci, and chromosome 2 (8,801,255–10,826,256), including the *Gata3* locus. HS2-deficient TH2 cells showed considerable impairment in the enrichment of H3K9ac-H3K14ac and GATA-3 only at the *Il4* locus but not at other loci (**Fig. 7a** and **Supplementary Fig. 3**).

and H3K14 and the increase in methylation of H3K4 at the *Il4* locus without affecting the *Il13* locus. In contrast, the CGRE-deficient T cells showed a lack of methylation of H3K4 at the *Il13* locus but not at the *Il4* locus (**Fig. 7c**). Therefore, the HS2 and CGRE enhancers regulate the transcription of *Il4* and *Il13*, respectively.

DISCUSSION

Using targeted deletion of a series of HS sites located in the *Il4*-*Il13* locus in mice, we have identified a critical function for HS2 in the regulation of *Il4* expression and in the chromatin-remodeling events underlying the TH2 lineage-commitment process. We found that CD4⁺ T cells from HS2-deficient mice had much lower IL-4 expression and TH2 responses after antigenic stimulation both *in vivo* and *in vitro*. The deletion of HS2 largely impaired *Il4* transcription even in the presence of large amounts of GATA-3, which indicates that GATA-3 regulates *Il4* transcription via its binding to HS2. We also found that HS2 was an essential enhancer for the formation of an active chromatin structure; it was required for the acetylation and dimethylation of histone H3 on the *Il4* locus. Our study suggests, therefore, that HS2 is indispensable for GATA-3-mediated *Il4* expression by formation of active chromatin at the *Il4* locus.

In a kinetic analysis of histone modification, we found modest acetylation occurred equally across the *Il4*-*Il13* locus during the

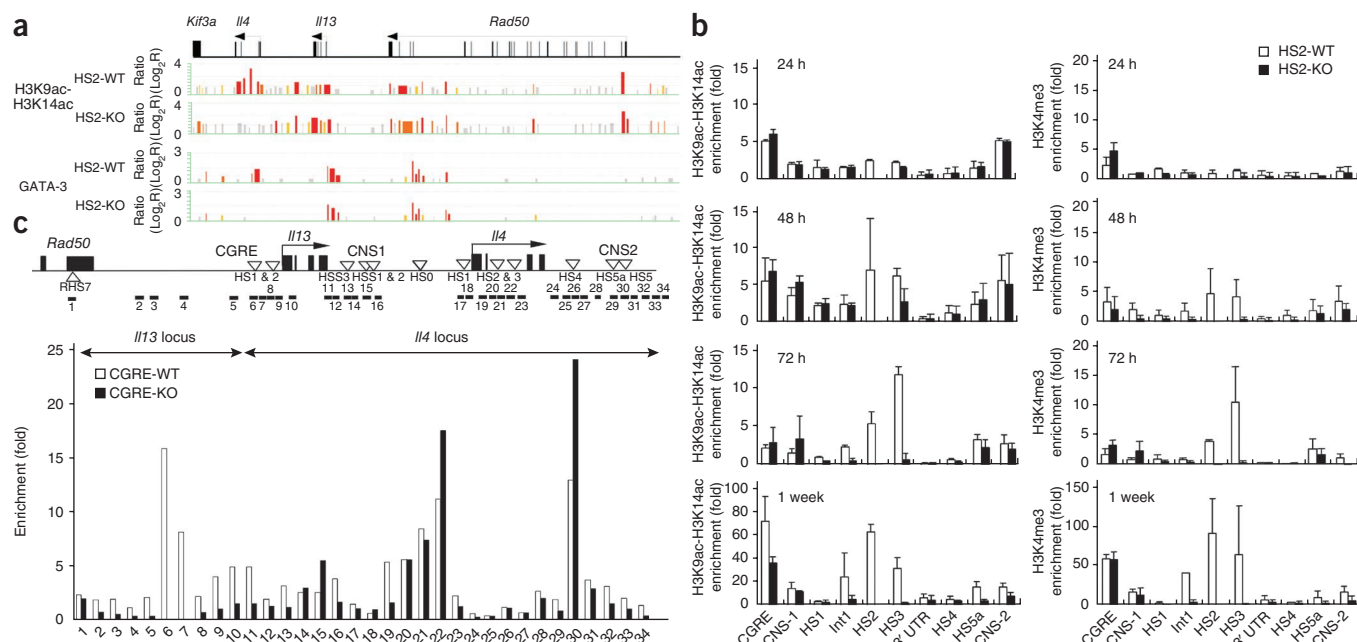


Figure 7 HS2-deficient T cells have a defect in histone acetylation and methylation. (a) ChIP plus microarray analysis of the binding of H3K9ac-H3K14ac and GATA-3 to regions in chromosome 11 (53238000–53359000) including the *Il4* and *Il13* loci, in chromatin extracts obtained from wild-type and HS2-deficient T_H2 cells, presented as the logarithmic ratio of the hybridization intensity of H3K9ac-H3K14ac or GATA-3 to that of the control, with colors assigned on the basis of the false-discovery rate score: red, ≤ 0.05 ; orange, $0.05 \leq 0.1$; yellow, $0.1 \leq 0.2$; gray, > 0.2 . Data are representative of genome-wide ChIP-plus-microarray analysis. (b) ChIP analysis of wild-type and HS2-deficient naive T cells stimulated for 24 h, 48 h, 72 h or 1 week under T_H2 conditions, followed by detection of H3K9ac-H3K14ac and H3K4me3 with various primer sets (horizontal axis). Data are representative of three independent experiments. (c) ChIP analysis of H3K4me3 in wild-type and CGRE-deficient T_H2 cells, detected with the primers above (in the *Il13* and *Il4* loci). Data are representative of three experiments.

priming of both T_H1 and T_H2 cells. However, this acetylation was maintained only in T_H2 cells and subsequently led to transcriptionally permissive chromatin. These acetylation kinetics are consistent with the published observation that small amounts of acetylation are present at all regions of the *Il4-Il13* locus even under T_H1 culture conditions at early time points^{20,37} but that later the amount of histone acetylation increases substantially only under T_H2-skewed conditions^{20,28,37,43}. Our data indicated that methylation of H3K4 occurred with a timing similar to that of the increase in acetylation and GATA-3 binding to the HS2 region. Therefore, we speculate that T cell activation initiates permissive modification of the *Il4-Il13* locus and that these processes allow GATA-3 to interact with its potential binding motif. HS2 is a critical binding site in the direct regulation of chromatin modification at later stages of T_H2 differentiation, and binding of GATA-3 to HS2 directly controls a permissive chromatin structure marked by trimethylation of H3K4.

HS2 was originally identified as a mast cell-specific enhancer^{45,46}, and analysis of reporter mice with transgenic expression of green fluorescent protein under the control of HS2 has confirmed mast cell-specific HS2 expression³⁹. Here, deletion of HS2 caused less production of IL-4 but not of other T_H2 cytokines (IL-5, IL-10 and IL-13) under T_H2-skewed conditions, which indicates that HS2 is a specific enhancer component for *Il4*. GATA-3 facilitates the conversion of the *Il4* locus to an open conformation through its binding to HS2. However, GATA-3 seems to regulate *Il5* and *Il13* independently, because GATA-3 expression in T_H2-skewed HS2-deficient T cells promoted normal amounts of IL-5 and IL-13. Our data suggest that GATA-3 regulates three T_H2 cytokines, IL-4, IL-5 and IL-13, in a distinct way. That conclusion is consistent with the observation that conditional deletion, after lineage commitment, of *loxP*-flanked

Gata3 alleles in mice expressing Cre recombinase under control of the promoter of the gene encoding the T cell–costimulatory molecule OX40 results in the abrogation of IL-5 and IL-13 but not of IL-4, although GATA-3 is required for the differentiation and proliferation of T_H2 cells²⁵. Functional GATA-3-binding sites have been reported in both the *Il5* and *Il13* promoters but not in the *Il4* promoter^{12,18,32–34}. Furthermore, histone modification of the distal promoter of *Il13*, CGRE, seemed to be constitutively permissive, and deletion of CGRE attenuated *Il13* expression without affecting IL-4 production. Finally, forced expression of *Gata3* induces *Il5* promoter activity^{32,33}. These results together suggest two distinct GATA-3 activities in T_H2 cells: it directly activates the *Il5* and *Il13* promoters, whereas its binding to HS2 regulates chromatin modification on the *Il4* locus.

The mechanisms for coordinated regulation of T_H2 cytokines have remained an inconclusive and controversial area for a long time¹⁹. The finding of a T_H2 locus-control region, RHS7, in an intronic interval of the constitutively expressed gene *Rad50* has provided the new insight that genes encoding T_H2 cytokines are located in close spatial proximity because of long-range intrachromosomal interactions^{16,47}. Our results indicate that HS2 regulates T_H2-specific chromatin configuration through binding of GATA-3. Therefore, GATA-3 should be a key participant in the control of long-range intrachromosomal interactions and, in this case, GATA-3 may function as a hub to achieve the appropriate chromosomal interactions among several distinct loci. However, our data raise the following alternative possibility: that GATA-3 independently regulates multiple T_H2 cytokine genes without intrachromosomal interactions. That hypothesis is consistent with our finding that forced expression of *Gata3* induced enhanced expression of *Il5* and *Il13* in HS2-deficient T cells, even though *Il4* expression remained impaired. If coordinated regulation of

T_H2 cytokines were controlled by long-range intrachromosomal interactions through GATA-3 as a T_H2-specific hub protein, HS2 would be dispensable. Therefore, we speculate that T_H2-specific expression of GATA-3 causes synchronized expression of multiple genes encoding T_H2 cytokines by its individual control of each regulatory element in these genes.

The data obtained with our series of deletion mice also indicate that HS2 is not the only element that regulates *Il4* transcription during initial T_H2 priming, although the effect of each element is different. Indeed, the deletion of CNS-1, 3' UTR or CNS-2 also impaired IL-4 expression, albeit to a much lesser extent. These data are consistent with published observations of CNS-1- and CNS-2-deficient mice^{22,23} and with studies of transgenic *Il4* promoter reporter mice indicating that the coordination of multiple regulatory elements is necessary for proper lineage-specific *Il4* expression²¹. Individual HS sites in the *Il4* locus (CNS-1; HS2 and HS3; HS4; or HS5a and HS5) do not enhance *Il4* promoter activity in T_H2 cells^{21,41}. Our data support the coordination model even though HS2 is a potent enhancer that determines T_H2-specific epigenetic modification on *Il4*. However, it still remains a possibility that individual cells use distinct elements to initiate *Il4* expression. Future studies will need better technology to allow investigation of the activity of each enhancer element in single cells. In conclusion, we have shown here that HS2 is essential for a normal T_H2 response and *Il4* transcription and for the proper chromatin modification of this locus. Our results also shed new light on how GATA-3 functions during cell lineage commitment.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

S.T. built the initial constructs, generated mouse lines and confirmed mouse lines *in vivo*; Y.M. did ChIP analysis; Y.S. screened mouse lines; R.Y. built the initial constructs; H.I. did airway hyper-responsiveness experiments; S.M. distributed materials; and M.K. designed experiments, supervised the project and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice and gene targeting. The targeting vector was constructed in a phosphoglycerate kinase–neomycin-resistance (neo) cassette flanked by *loxP* sites and a gene encoding diphtheria toxin A. CGRE, HS2, 3' UTR and HS4 were replaced by neo flanked by two *loxP* sites, which was excised from the mutated allele by Cre (**Supplementary Fig. 2**) as follows: mice were crossed with mice transgenic for Cre expression (under the control of the chicken β -actin promoter coupled with the cytomegalovirus immediate-early enhancer)⁴⁸, followed by homologous recombination and replacement with a *loxP*-flanked cassette in embryonic stem cells (B6JN/1 (C57BL/6) for CGRE and M-1 (129 $\times$ C57BL/6 hybrid) for HS2, 3' UTR, HS4 and CNS-2); mice with deletion of HS2, 3' UTR and HS4 were backcrossed onto the C57BL/6 strain for at least four generations. Microsatellite analysis indicated that offspring of the four-generation backcross were genetically more than 98% identical to C57BL/6 mice. CNS-1 deficient mice on a BALB/c background were provided by R. Locksley^{22,49}; CNS-2-deficient mice have been described³⁹. All mice were maintained under specific pathogen-free conditions and animal care was in accordance with guidelines of the RIKEN Yokohama Institute.

Antibodies. ELISA used mAb to IL-4 (biotin-conjugated BVD4-1D11 and BVD6-24G2, BD Biosciences), mAb to IL-13 (biotin-conjugated BAF413 and MAB413; R&D Systems), mAb to IgE (R35-72 and R35-118; BD Biosciences), and polyclonal anti-IgG1 (A90-105P) and anti-IgG2c (A90-136A; both from Bethyl Laboratory). ELISA of OVA-specific IgE used an ELISA kit system (Dai-Nippon Seiyaku). The PV-1 mAb to CD28 was a gift from R. Abe. Anti-NK1.1 (PK136), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD62L (MEL-14), mAb to IFN- γ (XMG1.2) and mAb to IL-4 (11B11; all from BD Biosciences) were used for flow cytometry. ChIP analysis used antibody to H3K9ac and H3K14ac (06-599; Upstate), anti-H3K4me3 (ab8580; Abcam), anti-H3K9me3 (ab8898; Abcam), anti-H3K27me3 (ab6002; Abcam) and mAb to GATA-3 (HG3-31AC; Santa Cruz).

Preparation and culture of CD4⁺ T cells. CD4⁺ T cells were isolated from spleen with the MACS bead system (Miltenyi). Naive (CD25[−]CD44^{lo}CD62L^{hi}) and MP (CD25[−]NK1.1[−]CD44^{hi}CD62L^{lo}CD4⁺) T cells were isolated by cell sorting with a FACS Vantage (BD Biosciences). Cells were stimulated with plate-bound mAb to TCR β (10 μ g/ml; H57-597) and soluble mAb to CD28 (PV-1; both from BD Biosciences). For T_H2 induction, cells were stimulated in the presence of mouse IL-4 (100 U/ml; Peprotech) and mAb to IFN- γ (R46A2). For T_H1 polarization, cells were cultured in the presence of mouse IL-12 (Peprotech) and mAb to IL-4 (11B11).

ICS analysis and cytokine production. Cells were restimulated for 6 h with mAb to TCR β in the presence of 2 μ M monensin (Sigma), then were fixed with 4% (vol/vol) paraformaldehyde and made permeable with 0.5% (vol/vol) Triton X-100. After blockade with 3% (wt/vol) BSA in PBS, mouse cells were stained with the following antibodies: fluorescein isothiocyanate-conjugated mAb to IFN- γ (XMG1.2; BD Biosciences), phycoerythrin-conjugated mAb to IL-4 (11B11; BD Biosciences) and Alexa Fluor 647-labeled mAb to IL-13 (eBio13A; eBiosciences). A FACSCalibur and CellQuest software (BD Biosciences) were used for flow cytometry. Cytokine production was measured by ELISA³⁹ with the Bio-Plex suspension array system (Luminex 200; Bio-Rad Laboratories).

ChIP assay. Nuclear fractions were extracted as chromatin from sonicated cells. After preclearance with protein G agarose (Amersham), chromatin

fractions were incubated with the appropriate antibody. Input samples were DNA extracts prepared from untreated chromatin. DNA was extracted from immunoprecipitated chromatin, and concentrations were quantified with a PicoGreen fluorescence assay (Molecular Probes). Equivalent masses of immunoprecipitated and input DNA were analyzed by real-time PCR (primers, **Supplementary Table 1**), with results presented as the ratio of the cycling threshold value of immunoprecipitated DNA to that of input DNA. ($2^{-CT(IP)}$ to $2^{-CT(IP)}$, where 'IP' is immunoprecipitated chromatin and 'CT' indicates the cycling threshold.)

ChIP plus microarray analysis. After standard ChIP, DNA was purified with a QIAquick PCR purification kit (Qiagen). NimbleGen Systems analyzed the oligonucleotide arrays as a part of a Chromatin Immunoprecipitation Array Service (Hokkaido System Science). Custom synthetic tiling microarrays containing 2.5 Mb of sequence around the T_H2 cytokine loci and the *Gata3* locus were used. Each DNA spot (50 nucleotides; forward and reverse strands) was designed at an interval of every 100 bases and was printed at two random locations on the array. The logarithmic ($\log_2$) ratio of the hybridization intensity of ChIP DNA to that of control DNA was calculated using the 'subtraction values' (sample ChIP – ChIP with control immunoglobulin) for each probe. NimbleScan software detects peaks by searching for four or more probes with signals above a specified cutoff value (mean + 6 s.d.). The ratio data were then randomized 20 times and each peak was then assigned a false-discovery rate score on the basis of the randomization. Possible binding regions were identified by Signal Map software. Results were assigned colors according to the false-discovery rate as follows: red, ≤ 0.05 ; orange, $0.05 \leq 0.1$; yellow, $0.1 \leq 0.2$; gray, > 0.2 .

Antibody formation and airway hyper-responsiveness. Mice were immunized on day 1 and day 10 by intraperitoneal injection of 100 μ g OVA (grade VI; Sigma Aldrich) emulsified in alum (Pierce), followed by boosting on day 23 with OVA in PBS. Serum was obtained on days 0, 7 and 14 after initial immunization, and total and OVA-specific IgG1, IgG2c and IgE were measured by ELISA. OVA-immunized mice were challenged with OVA by inhalation after boosting at day 23 and acetylcholine-dependent airway hyper-responsiveness was measured⁵⁰. Cellular content of the bronchoalveolar lavage fluid was assessed by Wright-Giemsa staining and cell counting.

Retroviral transduction. The retroviral vector pMX-IRES-GFP or pMX-Gata3-IRES-GFP was transfected into Platinum-E packaging cells (T. Kitamura), and supernatants of these cultures were used as the source of viral particles. Samples were enriched for naive CD4⁺ T cells via sorting with a FACS Vantage, followed by stimulation for 24 h with mAb to TCR β and mAb to CD28, then activated CD4⁺ T cells were transduced with viral particles.

Statistical analysis. A two-tailed Student's *t*-test was used for statistical analysis. Differences with a *P* value of less than 0.05 were considered significant.

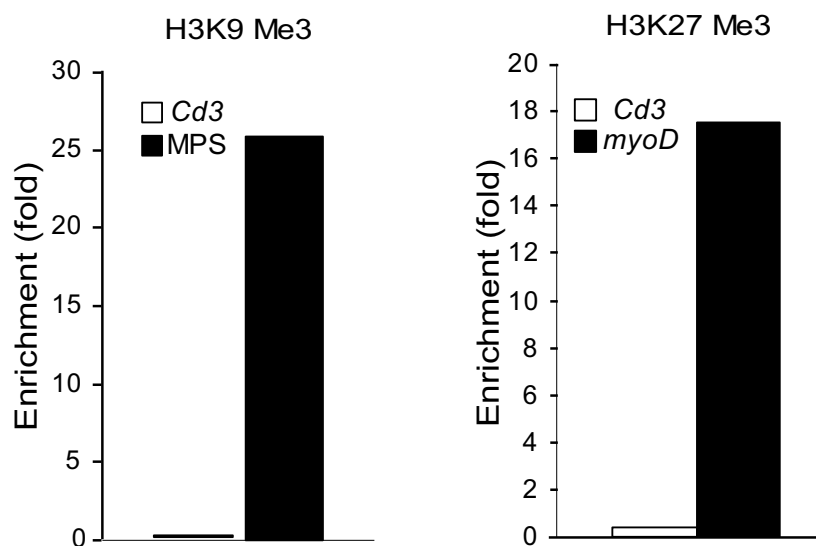
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The enhancer HS2 critically regulates GATA-3-mediated IL4 transcription in TH2 cells

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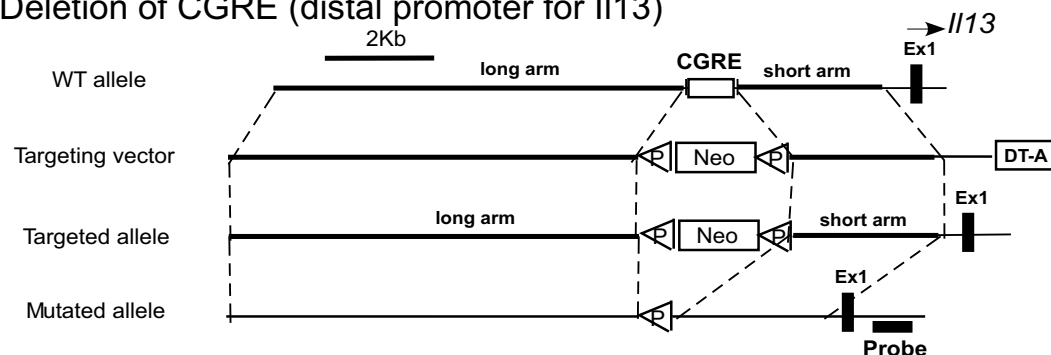


Supplementary Figure 1

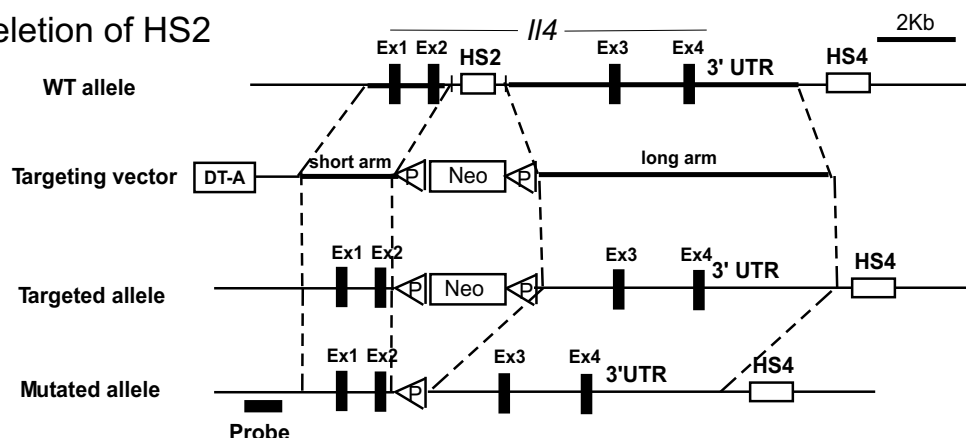
Chromatin fractions were extracted from developing TH2 cells from C57BL/6 mice and immunoprecipitated with antibodies against H3K9 Me3 and H3K27 Me3. Primers for major pericentric satellite (MPS) and myoD are positive controls for H3K9 Me3 and H3K27 Me3, respectively, and primers for the Cd3 promoter are a negative control. Data are representative of two independent experiments.

Maj; 5-gacgacttgaaaaatgacgaaatc-3' 5-catattccaggctcctcagtggtgc-3' .
 MyoD; 5-cgccctactacactcctattg -3' 5-aaggttctgtgggttgaatg-3' .
 CD3; 5-ggccccgatttctcagtta-3' 5-gccatagtaggatgaaggag-3' .

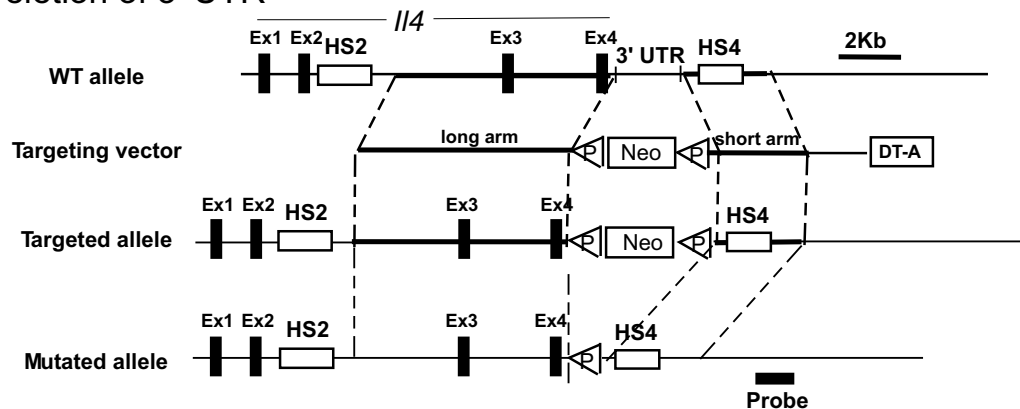
a Deletion of CGRE (distal promoter for *Il13*)



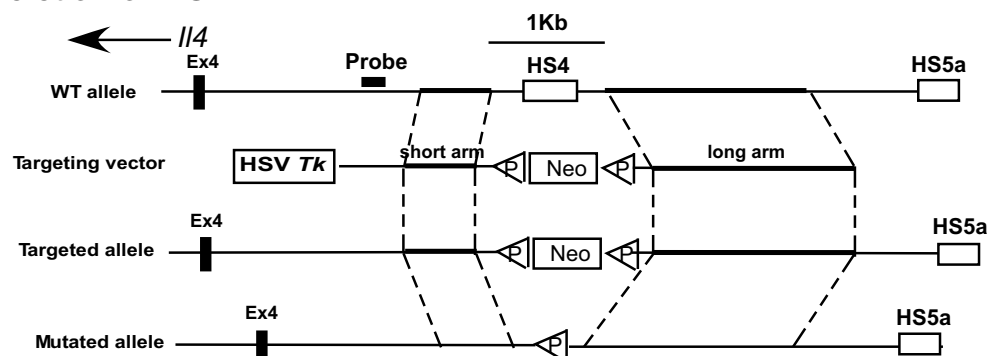
b Deletion of HS2



c Deletion of 3' UTR

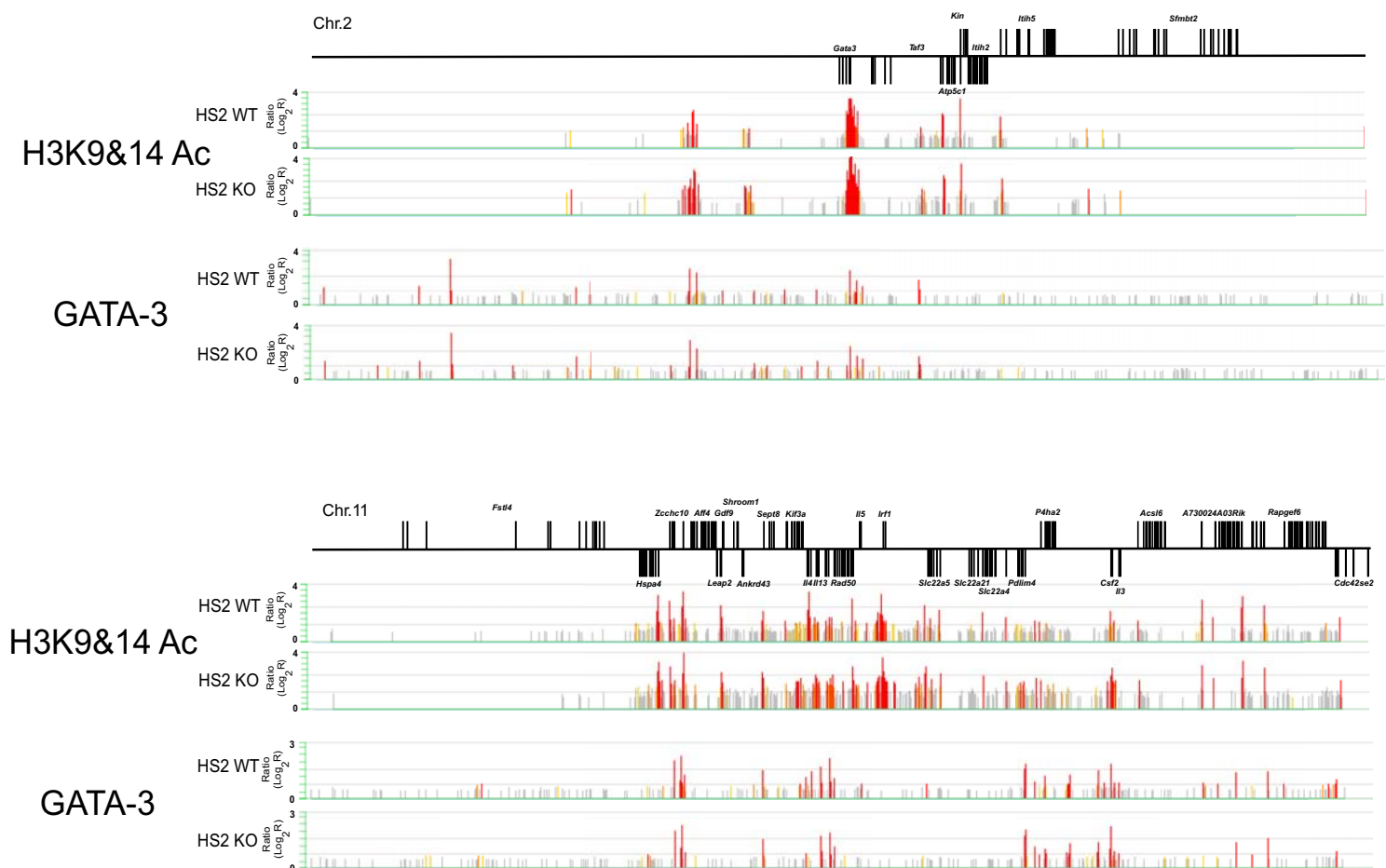


d Deletion of HS4



Supplementary Figure 2

Schematic representation of wild-type (WT) and mutated *Il4* alleles together with the targeting vector containing the diphtheria toxin A gene (DT-A), loxP (P) flanked neomycin resistance gene (Neo), and homologous arms. The targeting vector was constructed in a PGK neo cassette flanked by LoxP sites and a diphtheria toxin A gene with short (-875 to +756) and long homology arms (+2250 to +8140) for HS2 deletion (b), short (+8315 to +10968) and long arms (+2250 to +6220) for 3' UTR deletion (c), short (+9354 to +10967) and long arms (+2250 to +8140) for HS4 deletion (d) flanking the *Il4* locus (the translation start codon was defined as 0). For the generation of CGRE deletion mice (a), fragments of -1425 to +97 and -6818 to -1818 for pIL-13 flanking the *Il13* locus (the translation start codon was defined as 0) were used as homology arms. The mutant alleles were generated by replacement of CGRE, HS2, 3' UTR or HS4 with the Neo gene



Supplementary Figure 3

(a) The representative views of the results from H3K9 & K14Ac and GATA-3 ChIP-on-chip analysis. Chromatin extracts were obtained from HS2 WT and KO TH2 cells. The logarithmic ratio (log₂R) of hybridization intensities between H3 K9&14Ac or GATA-3 ChIP DNA and Control Ig ChIP) was used. The subtracted ratio values (H3 K9&14Ac H3K9 & K14Ac or GATA-3 ChIP-Control Ig ChIP) of each DNA spot (50 nucleotides) in the chromosome2 (8,801,255-10,826,256) (upper), the chromosome11 (52,205,742-54,365,252) (lower) are shown. Each peak represented as color-coded based on the assignment of a FDR score. The red bars (≤ 0.05); orange (0.05 ≤ 0.1); yellow (0.1 ≤ 0.2); gray (> 0.2).

Supplementary Table 1 The primers list for ChIP analysis

Gene	HS site	No.	Forward	Reverse
II13	RHS7	1	tcctgtgaatcctcatgtcaagc	aagtgtcatcatcgctctg gaggc
	-	2	caatctgttcccccag tag	aagatggaaatcatgccagt
	-	3	ggaccctcagagctacatcctcag	gagtggtttggatgcccagc
	-	4	actggatgcactccaagag	ggcatcagatcccatgaac
	-	5	ccagaagaagaggg catcaa	cga ctgctctccaaaggtc
	CGRE	6	gtcctcttatcgaccccatc	aaaggcttggggaaacac
	HS1-2	7	gcatgccttctgcttg tctga	gaactggaaatctgcctcctgc
	HS2-1	8	gtctatatccctcccactcgt	ggg caggtgagatcagctcta
	HS2-2	9	gtctatatccctcccactcgt	ggg caggtgagatcagctcta
	Introl1	10	tctatgctacccgaggatg	aggaagcaaagagaccgtga
	3' UTR	11	caccctaggaggg cacataa	gggattaaaggcgtgtgcta
	-	12	tcaagcctgatgacctatgc	agggctccagaagatagcc
	-	13	gaatgccagtgctctacagt	catgccagttgagattgt
	HSS3	14	gcaaaagaaactctcagcaaggcc	ccgtcctctcgcctcagt
	HSS1&2	15	cgaggcctcattatctcat	ggagatgacgagg ttgcta
	-	16	tgcattgactttggtagggtc	agagtggcactgagacttag
II4	HS1	17	caaagagagacccggtctcc	ggaggttctgggctagggtg
	HS1	18	aggttaag accccagagtcagcttc	tcagcgtagggttgccactg
	Intron1	19	cacttgagagagatcatcgg	ccacctctctagcaactcag
	HS2	20	gccttgcttgatacggatct	gcctgtagggaccatacga
	HS2	21	aggcctctttctggtcacctaaca	gtcccctcccactacaaatgga
	HS3	22	cccagcgttacatgagc	gggtgtgaataagccatattg
	-	23	gcatttagcctaccgtggc	aagctgctatgtgagtgccg
	3' UTR	24	tggcactacacctaccttccat	atgcgtatgtggaggtcag aacac
	-	25	cccaggctattcccagtggtg	cagtctccttgccctgtctta
	HS4-1	26	catctgacaccatttctga	catagagcatctctgacgg
	HS4-2	27	ctgtgagtacttcgtgcag	agagtccaagtcactg ttac
	-	28	gtgcgcacgttgaagtatgtga	accaaccgccagcttattgac
	HS5a	29	ggcagctactgcaggaactg	cgctaagcacaattccatt
	CNS2	30	tcagagagatgcagccttgactaa	acagcctgttctctcagcatt
	-	31	gtggtgcacacagtgtaggc	tgctgttaccgaccaggaaaca
	-	32	ctgccattccttaattcac	agccagaacatcagggttag
	-	33	tcacatgagccttgaagaca	ggctgcggatgactgatcag
	-	34	caggccacactcacaatagaagatg	tgagacttccacgtgaggataaa