

# Repression of interferon- $\gamma$ expression in T cells by Prospero-related Homeobox protein

Linfang Wang<sup>1,3</sup>, Jianmei Zhu<sup>1</sup>, Shifang Shan<sup>1,3</sup>, Yi Qin<sup>1</sup>, Yuying Kong<sup>1</sup>, Jing Liu<sup>2,4</sup>, Yuan Wang<sup>1</sup>, Youhua Xie<sup>1,2,4</sup>

<sup>1</sup>State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China; <sup>2</sup>Key Laboratory of Medical Molecular Virology, Institute of Medical Microbiology, Shanghai Medical College, Fudan University, Shanghai 200032, China; <sup>3</sup>Graduate School of Chinese Academy of Sciences, China; <sup>4</sup>Institute of Biomedical Sciences, Fudan University, Shanghai 200032, China

**Interferon-gamma (IFN- $\gamma$ ) is a major proinflammatory effector and regulatory cytokine produced by activated T cells and NK cells. IFN- $\gamma$  has been shown to play pivotal roles in fundamental immunological processes such as inflammatory reactions, cell-mediated immunity and autoimmunity. A variety of human disorders have now been linked to irregular IFN- $\gamma$  expression. In order to achieve proper IFN- $\gamma$ -mediated immunological effects, IFN- $\gamma$  expression in T cells is subject to both positive and negative regulation. In this study, we report for the first time the negative regulation of IFN- $\gamma$  expression by Prospero-related Homeobox (Prox1). In Jurkat T cells and primary human CD4+ T cells, Prox1 expression decreases quickly upon T cell activation, concurrent with a dramatic increase in IFN- $\gamma$  expression. Reporter analysis and chromatin immunoprecipitation (ChIP) revealed that Prox1 associates with and inhibits the transcription activity of IFN- $\gamma$  promoter in activated Jurkat T cells. Co-immunoprecipitation and GST pull-down assay demonstrated a direct binding between Prox1 and the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), which is also an IFN- $\gamma$  repressor in T cells. By introducing deletions and mutations into Prox1, we show that the repression of IFN- $\gamma$  promoter by Prox1 is largely dependent upon the physical interaction between Prox1 and PPAR $\gamma$ . Furthermore, PPAR $\gamma$  antagonist treatment removes Prox1 from IFN- $\gamma$  promoter and attenuates repression of IFN- $\gamma$  expression by Prox1. These findings establish Prox1 as a new negative regulator of IFN- $\gamma$  expression in T cells and will aid in the understanding of IFN- $\gamma$  transcription regulation mechanisms.**

**Keywords:** Prox1, IFN- $\gamma$ , T cell activation, gene regulation, repression, PPAR $\gamma$

*Cell Research* (2008) 18:911-920. doi: 10.1038/cr.2008.275; published online 12 August 2008

## Introduction

Interferon-gamma (IFN- $\gamma$ ) is a cytokine predominantly produced by activated T cells and large granular lymphocytes such as NK cells. More recently, IFN- $\gamma$  has also been found to be expressed in murine peritoneal macrophages and mast cells, human eosinophils, keratinocytes and primary B cells [1-5]. *In vivo*, IFN- $\gamma$  functions as both a proinflammatory effector and a regulator and plays a central part in a diverse array of immunological processes such as inflammatory reactions, cell-mediated immunity and autoimmunity. Irregularities in IFN- $\gamma$  expression have been implicated

in the pathogenesis of a variety of human disorders [6]. Agents capable of modulating IFN- $\gamma$  expression are being considered as potential therapeutics for such diseases.

To maintain a proper IFN- $\gamma$ -expression profile, IFN- $\gamma$  expression in T cells is subject to both positive and negative regulation [7]. Regulation of IFN- $\gamma$  expression involves mechanisms at pre-transcription, transcription and post-transcription levels. Transcription factors known to affect IFN- $\gamma$  expression include T-bet, NF-AT, NF- $\kappa$ B, YY1, AP-1 and STAT4 [8-15]. Compared to IFN- $\gamma$  transcription activators, repressors of IFN- $\gamma$  transcription have been less widely reported. Recent findings have unveiled an inhibitory role of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) in regulating the expression of IFN- $\gamma$  [16, 17]. However, the detailed molecular mechanisms have yet to be fully revealed.

Prospero-related Homeobox (Prox1) has been reported to be predominantly expressed in lens, heart, liver, kidney,

Correspondence: Youhua Xie

Tel/Fax: +86-21-54237973

E-mail: yhxie@sibs.ac.cn

Received 5 March 2008; revised 26 March 2008; accepted 31 March 2008;

published online 12 August 2008

spleen, skeletal muscle, pancreas and central nervous system [18]. Previous studies using mainly knockout mice have shown that Prox1 plays an essential role in lymphatic vasculature development [19] and is also involved in the development of lens, kidney and liver [20-22]. Although Prox1 is a key regulator in development, very few target genes whose expression is directly affected by Prox1 have been reported. Work conducted by our lab and another group showed that, in adult hepatocytes, Prox1 acts as a corepressor of the nuclear receptor liver receptor homologue 1 (LRH-1) to regulate cholesterol catabolism [23, 24].

The expression or function of Prox1 in T cells has not been reported previously. In this study, we report for the first time the expression of Prox1 in primary human CD4<sup>+</sup> T cells and cultured Jurkat T cells. In activated Jurkat T cells, Prox1 down-regulates IFN- $\gamma$  expression by repressing the activity of the IFN- $\gamma$  promoter. Our results also suggest

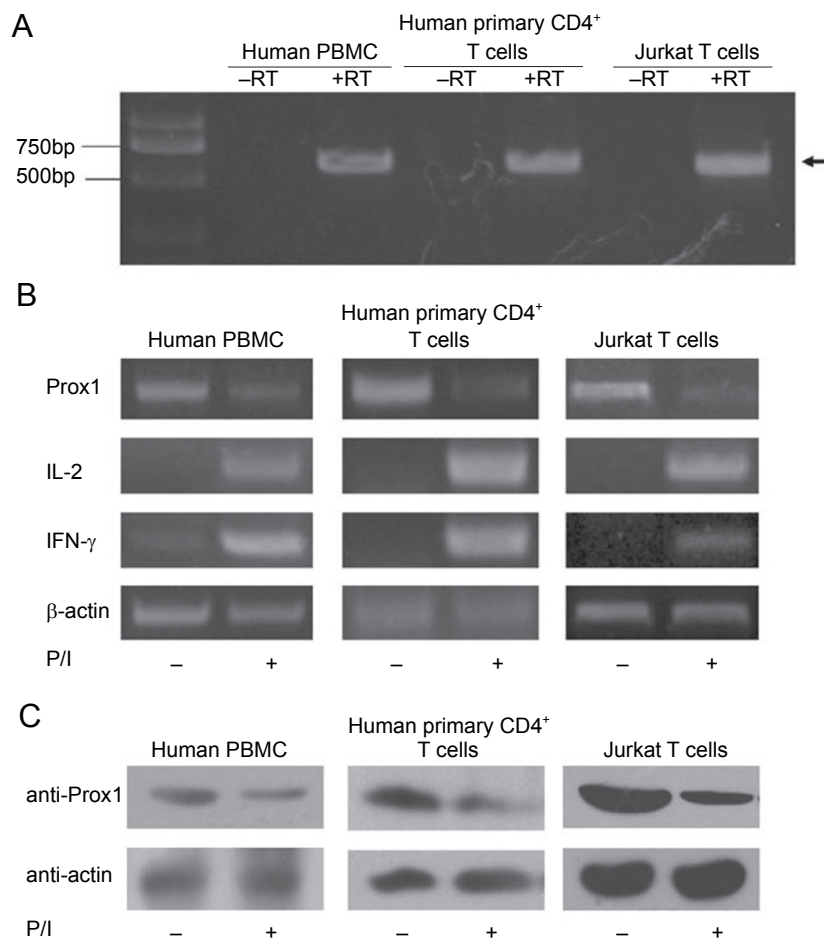
that repression of IFN- $\gamma$  expression by Prox1 is largely dependent upon its interaction with PPAR $\gamma$ . Implications of these findings for Prox1 *in vivo* functions and IFN- $\gamma$  expression regulation are discussed.

## Results

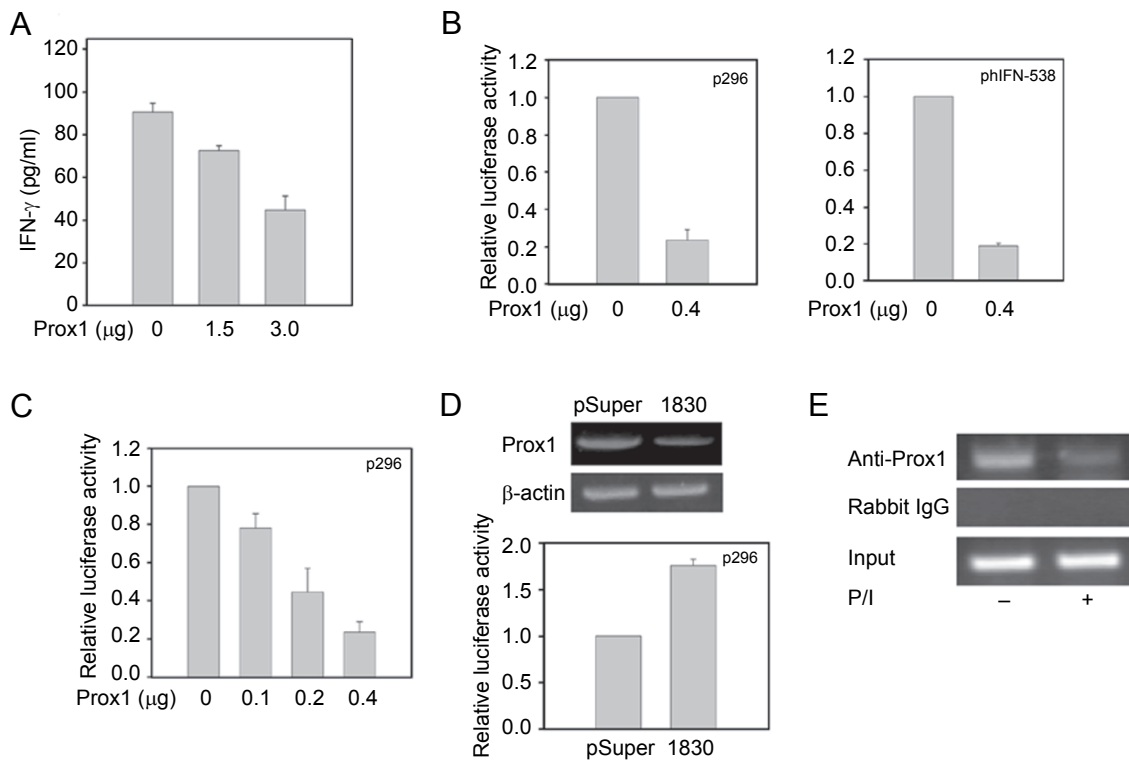
### *Prox1 expression in T cells correlates negatively with the expression of cytokines related to T cell activation*

In our study of Prox1 functions, Prox1 was found to be expressed in T lymphocytes, including human PBMCs, primary CD4<sup>+</sup> T cells and cultured Jurkat T cells using RT-PCR (Figure 1A). Prox1 expression in these cells has not been reported previously.

To identify possible roles played by Prox1 in T cell functions, a combination of PMA and ionomycin treatment was used to induce T cell activation and cytokine



**Figure 1** Prox1 expression in T cells decreases upon activation. **(A)** Expression of Prox1 in human PBMCs, primary CD4<sup>+</sup> T cells and Jurkat T cells as detected by RT-PCR. **(B)** Changes in expression of Prox1, IFN- $\gamma$  and IL-2 upon T cell activation as determined by RT-PCR. **(C)** Changes in expression level of Prox1 upon T cell activation as determined by western blot. P/I: treatment with PMA (25 ng/ml) and ionomycin (1  $\mu$ M) for 16 h.  $\beta$ -actin expression was used as internal control for both RT-PCR and western blot.



**Figure 2** Prox1 inhibits the activity of IFN- $\gamma$  promoter. **(A)** Prox1 overexpression decreases IFN- $\gamma$  expression induced by T cell activation. Jurkat T cells transfected with indicated amounts of Prox1 expression plasmid were stimulated with PMA (25 ng/ml) and ionomycin (1  $\mu$ M). IFN- $\gamma$  in the medium was measured by ELISA and the means s.d. of results from three tests are presented. **(B)** Prox1 inhibits both mouse (left) and human IFN- $\gamma$  promoter (right) activities. Jurkat T cells transfected with Prox1 expression plasmid along with indicated IFN- $\gamma$  promoter reporter constructs were stimulated with PMA (25 ng/ml) and ionomycin (1  $\mu$ M) and relative luciferase activities were measured. **(C)** Dose-dependent repression of IFN- $\gamma$  promoter (p296) by Prox1. Jurkat T cells transfected with p296 luciferase reporter (0.5  $\mu$ g) and indicated amounts of Prox1 expression plasmid were stimulated with PMA (25 ng/ml) and ionomycin (1  $\mu$ M) and relative luciferase activities were measured. **(D)** Knockdown of endogenous Prox1 expression enhances the activity of IFN- $\gamma$  promoter. Jurkat T cells were transfected with either pSuper vector or pSuper-Prox1-1830 along with p296 luciferase reporter (0.5  $\mu$ g). (top) Knockdown of Prox1 expression as determined by RT-PCR. (bottom) Relative luciferase activities were measured following stimulation with PMA (25 ng/ml) and ionomycin (1  $\mu$ M). **(E)** Association of Prox1 with IFN- $\gamma$  promoter as determined by ChIP assay. Chromatin was extracted from Jurkat T cells unstimulated or stimulated with PMA (25 ng/ml) and ionomycin (1  $\mu$ M) and precipitated with anti-Prox1 antibody or rabbit IgG as described in Materials and Methods.

production [25]. Expression of Prox1 as well as cytokines expressed by activated T cells including IFN- $\gamma$  and IL-2 was measured by RT-PCR in treated and untreated T cells (Figure 1B). In all three T cell preparations tested, Prox1 expression dropped significantly upon PMA/ionomycin-induced activation, whereas expression of IFN- $\gamma$  and IL-2 was markedly elevated, indicating proper T cell activation. The decrease in Prox1 expression was further confirmed by using anti-Prox1 antibody in Western blot (Figure 1C). These results demonstrate that in activated T cells Prox1 expression correlates negatively with the expression of IFN- $\gamma$  and IL-2, and suggest Prox1 as a possible repressor of the expression of these cytokines.

*Prox1 is a repressor of IFN- $\gamma$  expression in Jurkat T cells*

IFN- $\gamma$  is one of the most important cytokines produced by activated T cells and plays a pivotal role in inflammation and many fundamental biological processes. Since Prox1 expression in T cells correlates negatively with IFN- $\gamma$  expression, the possibility of Prox1 serving as a repressor of IFN- $\gamma$  expression was investigated.

First, Prox1 was overexpressed in Jurkat T cells by transfecting with increasing amounts of Prox1 expression plasmid. Upon PMA and ionomycin treatment, secreted IFN- $\gamma$  was measured by ELISA. As shown in Figure 2A, overexpression of Prox1 inhibited the production of IFN- $\gamma$  in activated T cells in a dose-dependent manner.

In order to test whether Prox1 represses IFN- $\gamma$  expres-

sion through acting on its promoter, mouse (-296/+111, p296) and human (-538/+64, phIFN-538) IFN- $\gamma$  promoter sequences were placed upstream of luciferase reporter gene and cotransfected with Prox1 expression plasmid into Jurkat T cells. As shown in Figure 2B, the activities of both mouse and human IFN- $\gamma$  promoters were repressed by Prox1 overexpression. Because IFN- $\gamma$  promoters are highly conserved among mammals [26, 27], mouse IFN- $\gamma$  promoter p296 was selected for more detailed studies. Similar to results obtained with endogenous IFN- $\gamma$  expression (Figure 2A), reporter analysis showed that IFN- $\gamma$  promoter activity in activated Jurkat T cells was repressed by Prox1 in a dose-dependent manner (Figure 2C). In order to further prove the inhibition of IFN- $\gamma$  promoter by Prox1, reporter assay was performed in which endogenous Prox1 expression in Jurkat T cells was knocked down by overexpression of a Prox1-specific siRNA. As shown in Figure 2D, knockdown of endogenous Prox1 resulted in an almost 2-fold increase in IFN- $\gamma$  promoter activity in stimulated Jurkat T cells.

Taken together, these results demonstrate that Prox1 functions as a repressor of IFN- $\gamma$  expression in T cells.

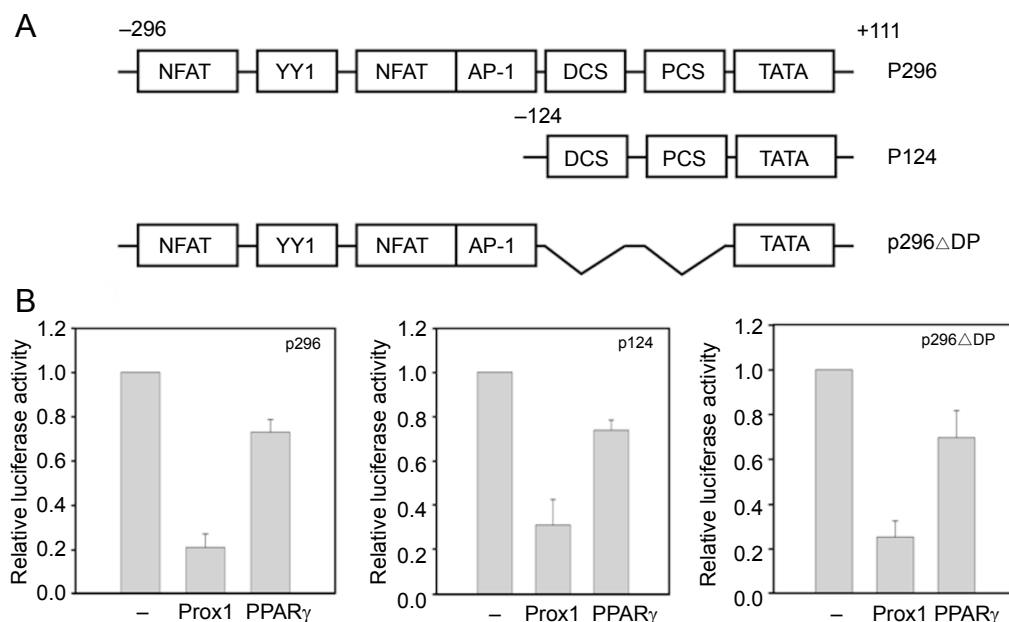
#### *Prox1 is associated with IFN- $\gamma$ promoter in Jurkat T cells*

In order to probe the mechanism of Prox1 repression of IFN- $\gamma$  promoter, Prox1 association with IFN- $\gamma$  promoter is tested. Although Prox1 contains a conserved homeo-

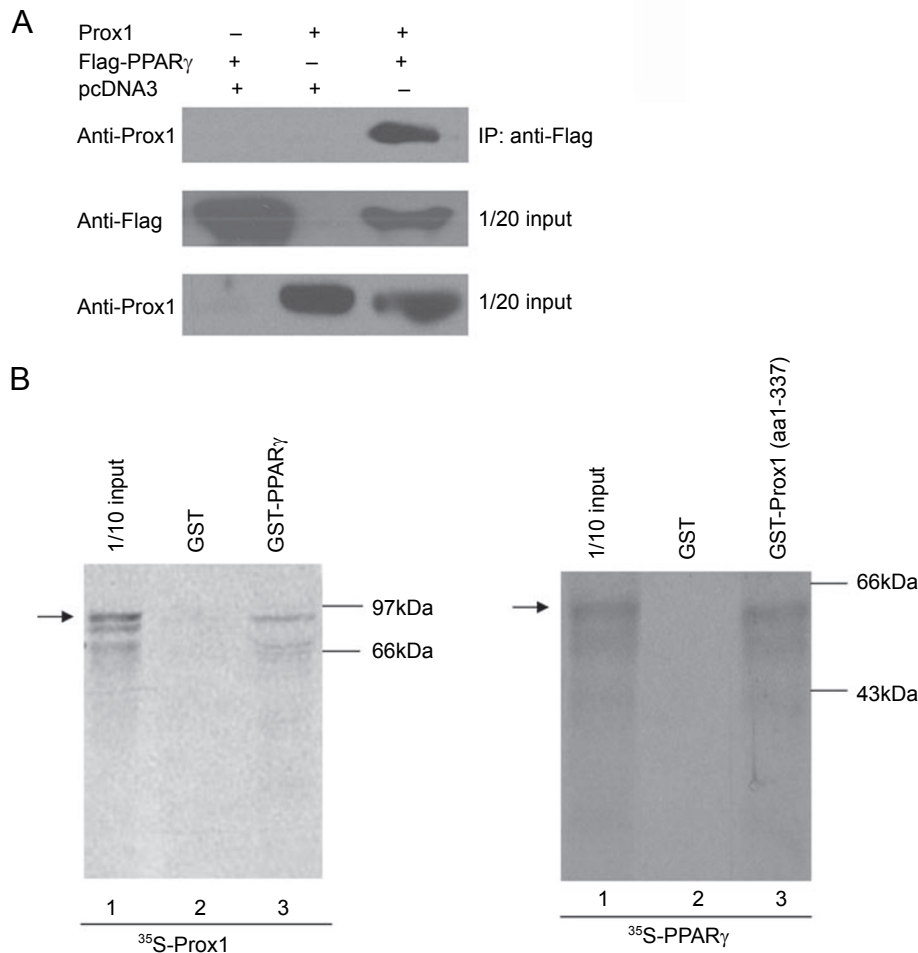
prospero domain at its C-terminus, which is a putative DNA-binding domain (DBD), it has not been shown convincingly to bind directly to DNA of target genes. We also failed to observe a direct binding between Prox1 and IFN- $\gamma$  promoter sequences (data not shown). However, chromatin immunoprecipitation (ChIP) assay using anti-Prox1 antibody not only proved the presence of Prox1 on IFN- $\gamma$  promoter in Jurkat T cells, but also showed that such presence was significantly reduced upon T cell activation (Figure 2E). These results suggested that Prox1 associates indirectly with IFN- $\gamma$  promoter through other proteins to exert its repressive effects.

#### *Similar regions in the IFN- $\gamma$ promoter mediate repression by Prox1 and PPAR $\gamma$*

Binding sites or responsive regions for many transcription factors and co-regulators have been characterized in the IFN- $\gamma$  promoter. In order to identify proteins that might be involved in Prox1's association with and repression of IFN- $\gamma$  promoter, we attempted to map Prox1 responsive regions on IFN- $\gamma$  promoter using deletion mutants (Figure 3A). As shown in Figure 3B, the minimal IFN- $\gamma$  promoter p124 [26], which lacks binding sites for most reported upstream regulators, was repressed by Prox1 to the same extent as p296 (compare the middle bars in Figure 3B left panel and middle panel). Located within the minimal IFN- $\gamma$  promoter are two conserved regions called distal conserved



**Figure 3** Mapping of responsive regions for Prox1 and PPAR $\gamma$  on IFN- $\gamma$  promoter. **(A)** Schematic diagram depicting IFN- $\gamma$  promoter. **(B)** Mapping of responsive regions for Prox1 and PPAR $\gamma$ . Prox1 expression plasmid was cotransfected with luciferase reporter controlled by indicated IFN- $\gamma$  promoter constructs into Jurkat T cells. Relative luciferase activities were measured following stimulation with PMA (25 ng/ml) and ionomycin (1  $\mu$ M).



**Figure 4** Prox1 directly interacts with PPAR $\gamma$ . **(A)** Prox1 co-immunoprecipitates with PPAR $\gamma$ . HEK293T cells were transfected with indicated expression plasmids or empty vector. Flag-PPAR $\gamma$ -associated proteins were immunoprecipitated using anti-Flag mAb. Co-immunoprecipitated Prox1 was detected using anti-Prox1 antibody (up). Expression of Flag-PPAR $\gamma$  and Prox1 was confirmed and normalized by using 1/20 of input protein extracts and detecting using corresponding antibodies (middle and bottom). **(B)** Prox1 binds to PPAR $\gamma$  in GST pull-down assay. In vitro translated Prox1 (left) and PPAR $\gamma$  (right) were pulled down using GST-PPAR $\gamma$  and GST-Prox1(aa1-337), respectively. Prox1(aa1-337) was used because full-length Prox1 GST fusion proteins were poorly expressed. Lane 1, 1/10 of input labeled protein; lane 2, purified GST as bait; lane 3, purified GST-PPAR $\gamma$  (left) or GST-Prox1(aa 1-337) (right) as bait. Arrows indicate positions of full-length target protein.

sequence (DCS) and proximal conserved sequence (PCS), respectively (Figure 3A). Because deletion of DCS and PCS from p124 renders the minimal promoter irresponsive to PMA and ionomycin treatment [16], DCS and PCS were deleted from p296 to create p296 $\Delta$ DP. Prox1 repressed p296 $\Delta$ DP activity to a similar degree as it did on p296 or p124 (compare the middle bars in Figure 3B).

PPAR $\gamma$  is a nuclear receptor that has been identified as a trans-repressor of IFN- $\gamma$  expression in Jurkat T cells [16]. Previous work by others and us has shown that Prox1 acts as a corepressor for another nuclear receptor LRH-1 by binding directly to LRH-1 to repress target gene transcription [23, 24]. This led us to speculate whether similar

mechanisms exist between Prox1 and PPAR $\gamma$  in the repression of IFN- $\gamma$  expression. To test this speculation, PPAR $\gamma$  repression of IFN- $\gamma$  promoter deletion mutants in Jurkat T cells was tested. As shown in Figure 3B, PPAR $\gamma$  represses p296, p124 and p296 $\Delta$ DP to a comparable degree (compare the right bars), which is similar to results obtained with Prox1. These data indicate that regions in the IFN- $\gamma$  promoter important for both Prox1- and PPAR $\gamma$ -dependent repression localize to the minimal promoter and probably do not require the presence of DCS or PCS.

#### *Prox1 interacts directly with PPAR $\gamma$*

Since similar regions of the IFN- $\gamma$  promoter mediate

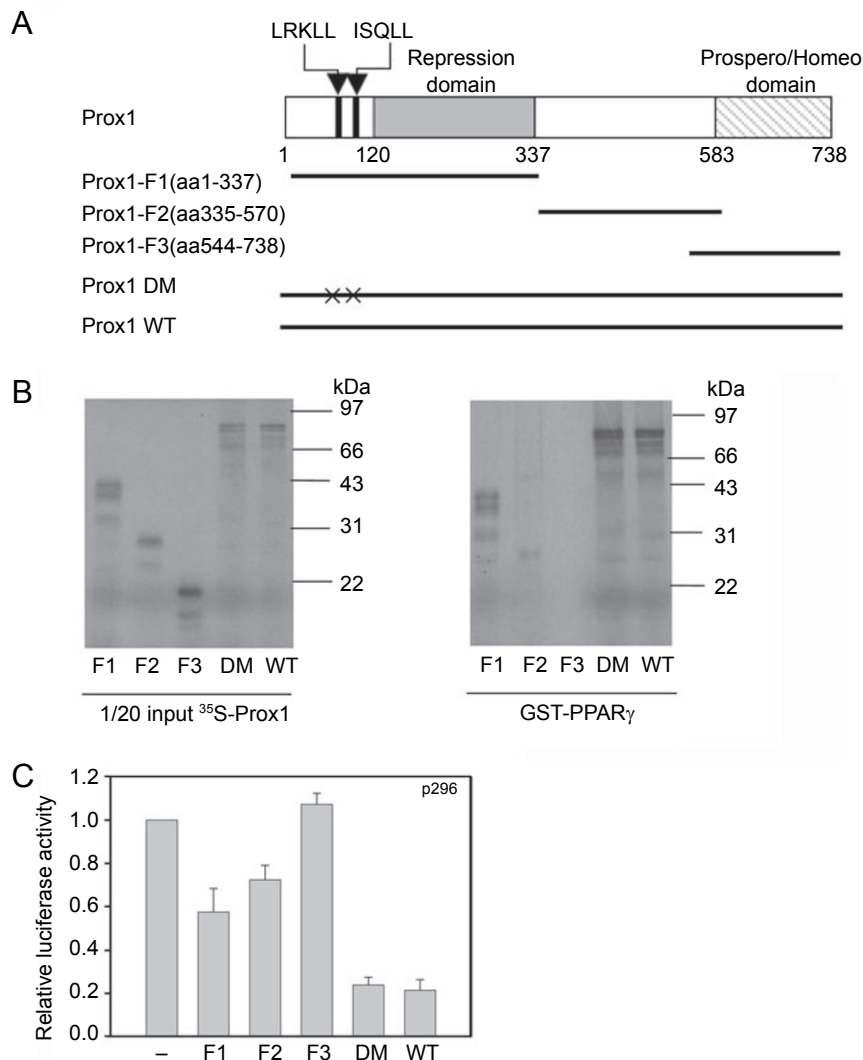


Prox1- and PPAR $\gamma$ -dependent repression, the possibility of the two repressors interacting with each other was then investigated. Co-immunoprecipitation and GST pull-down assays demonstrate a direct binding between Prox1 and PPAR $\gamma$  in cultured cells as well as *in vitro* (Figure 4).

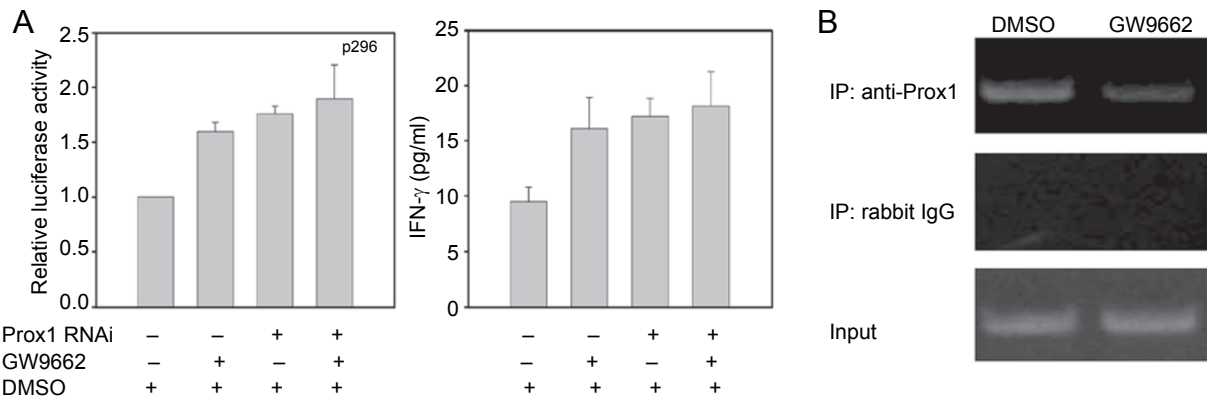
*Prox1-PPAR $\gamma$  interaction correlates with Prox1-mediated repression of IFN- $\gamma$  expression*

Deletions and mutations were introduced into Prox1 to map regions responsible for interacting with PPAR $\gamma$  (Figure 5A). As shown in Figure 5B, compared with full-

length wild-type Prox1, the N-terminal region of Prox1 (F1, aa 1-337) displayed weaker binding of PPAR $\gamma$  in GST pull-down assay, whereas the middle part of Prox1 (F2, aa 335-570) displayed only barely detectable binding. The C-terminal homeo-prospero domain of Prox1 (F3, aa 544-738) showed no binding of PPAR $\gamma$  at all. Mutation of two LXXLL-like motifs (aa70-74, aa93-97) in the N-terminal part of Prox1, which are required for Prox1 interaction with LRH-1, had no effect on its interaction with PPAR $\gamma$ . These results suggest that Prox1 interacts with PPAR $\gamma$  mainly using its N-terminal region but the two LXXLL-like motifs



**Figure 5** Prox1 interaction with PPAR $\gamma$  correlates with Prox1-mediated repression of IFN- $\gamma$  promoter. **(A)** Schematic representation of Prox1 domain organization and the truncated or mutated Prox1 proteins used in following experiments. Prox1 DM has both LXXLL-like motifs mutated. **(B)** Ability of different Prox1 proteins to bind PPAR $\gamma$  as determined by GST pull-down assay. (left) A fraction (1/20) of input Prox1 proteins were analyzed. (right) GST-PPAR $\gamma$  was used as bait. **(C)** Ability of different Prox1 proteins to repress the activity of IFN- $\gamma$  promoter in stimulated Jurkat T cells. Expression plasmids for indicated Prox1 proteins were cotransfected with p296 luciferase reporter. Relative luciferase activities were measured following stimulation with PMA (25 ng/ml) and Ionomycin (1  $\mu$ M).



**Figure 6** Effects of PPAR $\gamma$  antagonist GW9662 treatment on Prox1 repression of IFN- $\gamma$ . **(A)** Effects of GW9662 treatment and Prox1 knockdown on the activity of IFN- $\gamma$  promoter and expression. (left) Jurkat T cells were transfected with p296 luciferase reporter along with pSuper vector or Prox1 RNAi plasmid pSuper-Prox1-1830, stimulated with PMA (25 ng/ml) and ionomycin (1  $\mu$ M), and treated with GW9662 (10  $\mu$ M) in DMSO or DMSO alone. Relative luciferase activities were measured and the means $\pm$ s.d. of the results of three independent assays are presented. (right) Jurkat T cells were transfected with pSuper vector or pSuper-Prox1-1830 and transfected cells were isolated by FACS based on GFP expression from pSuper vector or pSuper-Prox1-1830. GFP-expressing cells were then stimulated with PMA (25 ng/ml) and ionomycin (1  $\mu$ M) and treated with GW9662 (10  $\mu$ M) in DMSO or DMSO alone. IFN- $\gamma$  in the medium was then measured by ELISA. **(B)** GW9662 treatment attenuates association between Prox1 and IFN- $\gamma$  promoter. Chromatin was extracted from Jurkat T cells stimulated with PMA (25 ng/ml) and ionomycin (1  $\mu$ M) and treated with GW9662. ChIP assay was performed using anti-Prox1 antibody or rabbit IgG as described in Materials and Methods.

are not involved.

To test for a possible link between Prox1-PPAR $\gamma$  interaction and Prox1 repression of IFN- $\gamma$  promoter, the various Prox1 mutant proteins were then overexpressed in Jurkat T cells. Reporter analysis demonstrated a clear correlation between Prox1-PPAR $\gamma$  interaction and Prox1 inhibition of IFN- $\gamma$  promoter transcription. As shown in Figure 5C, Prox1 WT and DM, which displayed strongest binding for PPAR $\gamma$  (Figure 5B), are the most effective repressors. The weak PPAR $\gamma$ -binders, Prox1 F1 and F2, showed weaker repression corresponding to their respective affinity with PPAR $\gamma$ . Prox1 F3, which does not interact with PPAR $\gamma$ , showed no repression of IFN- $\gamma$  promoter activity. Thus, Prox1 repression of IFN- $\gamma$  promoter transcription is likely dependent upon Prox1-PPAR $\gamma$  interaction.

*Prox1 repression of IFN- $\gamma$  expression is attenuated by PPAR $\gamma$  antagonist*

To further demonstrate PPAR $\gamma$ 's involvement in Prox1-mediated repression of IFN- $\gamma$  promoter, PPAR $\gamma$  antagonist GW9662 was used to treat Jurkat T cells prior to PMA/ionomycin stimulation. Data shown in Figure 6A indicate that inhibition of PPAR $\gamma$  activity by GW9662 resulted in a significant de-repression of IFN- $\gamma$  promoter activity (left) and the endogenous IFN- $\gamma$  expression (right). The scale of de-repression was comparable to that obtained by knocking down Prox1 using siRNA. More importantly, combination of GW9662 treatment and Prox1-specific RNA interference

failed to achieve significantly higher IFN- $\gamma$  expression, compared to using either of them alone. In addition, ChIP assay showed that Prox1 association with endogenous IFN- $\gamma$  promoter in stimulated Jurkat T cells was nearly abolished in the presence of GW9662 (Figure 6B). These results further demonstrate that PPAR $\gamma$  is actively involved in Prox1-dependent repression of IFN- $\gamma$  promoter.

**Discussion**

Due to its key functions in innate and adaptive immune responses, IFN- $\gamma$  expression is regulated by a vast array of protein factors functioning at pre-transcription, transcription and post-transcription levels. Studies of negative regulation of IFN- $\gamma$  expression have mainly focused on epigenetic mechanisms such as chromatin remodeling and DNA methylation. In this study, we show that Prox1 is a novel negative regulator of IFN- $\gamma$  expression in Jurkat T cells. Firstly, upon T cell activation, Prox1 expression quickly decreased while IFN- $\gamma$  and IL-2 expression markedly elevated (Figure 1). A similar pattern of reversely correlated Prox1 and IFN- $\gamma$  expression levels was also observed in T cells from experimental autoimmune encephalomyelitis mice (data not shown), an animal model of human multiple sclerosis. Secondly, reporter analysis demonstrated that Prox1 represses IFN- $\gamma$  promoter activity in a dose-dependent manner (Figure 2A-2D). Finally, ChIP assay confirmed the association of Prox1 with IFN- $\gamma$

promoter, which was significantly reduced upon T cell activation (Figure 2E).

Prox1 was originally discovered as a mammalian homologue of the *Drosophila melanogaster* prospero. Despite its vital functions in the development of many organs and tissues and the fact that it possesses a putative homeo-prospero DBD at the C-terminus, Prox1 has not been shown convincingly to bind directly to target gene DNA. Instead, previous work by others and our group has shown that Prox1 functions as a corepressor of nuclear receptor LRH-1 in the regulation of lipid catabolism-related genes [23, 24]. In such a regulation mechanism, Prox1 associates with target gene promoter through binding to LRH-1, which in turn directly binds to specific binding sites on the promoter. Since Prox1 also seems to associate with IFN- $\gamma$  promoter indirectly and repress its transcription, we wondered whether similar mechanisms are involved.

PPAR $\gamma$  is another nuclear receptor that has been found to repress IFN- $\gamma$  promoter [16]. Therefore, the possibility of PPAR $\gamma$  being involved in Prox1-dependent repression was investigated. Mapping of responsive regions for Prox1 and PPAR $\gamma$  on IFN- $\gamma$  promoter showed that both Prox1- and PPAR $\gamma$ -dependent repression mainly localize to the minimal promoter (-124/+111), while the conserved DCS and PCS sequences probably are not involved (Figure 3). We then went on to probe a possible physical interaction between Prox1 and PPAR $\gamma$ . Co-immunoprecipitation and GST-pull-down assays confirmed a direct binding between Prox1 and PPAR $\gamma$  (Figure 4). By introducing deletions or mutations into Prox1, we were able to demonstrate a strong correlation between Prox1-PPAR $\gamma$  interaction and Prox1-dependent repression of IFN- $\gamma$  promoter (Figure 5). Furthermore, PPAR $\gamma$  antagonist GW9662 attenuated repression of IFN- $\gamma$  promoter in activated Jurkat T cells. The extent of de-repression was not significantly different from that achieved by using Prox1-specific siRNA with or without GW9662 (Figure 6A). Correspondingly, ChIP assay also showed that GW9662 treatment resulted in an almost total loss of Prox1 association with the IFN- $\gamma$  promoter (Figure 6B). Taken together, these results indicate that repression of IFN- $\gamma$  promoter in Jurkat T cells by Prox1 involves PPAR $\gamma$  and is largely dependent upon a direct interaction between PPAR $\gamma$  and Prox1.

Detailed mechanisms of repression of the IFN- $\gamma$  promoter by PPAR $\gamma$  and Prox1 remain to be fully revealed. As neither Prox1 nor PPAR $\gamma$  seems to bind directly to the IFN- $\gamma$  promoter, their repression effect is therefore most likely achieved through other factors that directly bind to the promoter. Using artificial multimerized promoter sequences, Cunard *et al.* [16] reported that PPAR $\gamma$  repressed IFN- $\gamma$  promoter through inhibiting the effects of transcription activator c-Jun, which was found to bind to DCS in

the promoter. However, our results showed that repression by PPAR $\gamma$  is not significantly affected when DCS and PCS are removed from the promoter (Figure 3), indicating that c-Jun-related mechanisms may not play a major part in PPAR $\gamma$ -dependent repression. On the Prox1 side, its corepressor activity on other target genes has been attributed to the repression domain (Figure 5A), which has been reported to recruit histone deacetylase 3 (HDAC3) [24]. HDAC3 activity is related to negative epigenetic regulation of gene transcription. It is possible that HDAC3 might be involved in some way in the repression of IFN- $\gamma$  promoter by Prox1/PPAR $\gamma$ . Such a possibility warrants detailed study and will be addressed in our future work.

*In vivo* functions of Prox1 have mainly been deduced from studies of knockout mice. Homozygous Prox1 knockout mice die prematurely in the womb due to many developmental defects [18, 19]. Interestingly, heterozygous Prox1 knockout mice also die soon after birth, suggesting that normal Prox1 function heavily depends upon dosage [20]. Only by crossing with NMRI mice were viable heterozygous Prox1 mice obtained at a one out of thirty ratio. In those few viable hybrid heterozygous mice, an increased number of inflammatory cells were observed in the mesothelial membrane [28]. As Prox1 is a master regulator in lymphatic vasculature development, such an observation has been explained as the result of developmental defects in lymphatic vasculature and leakage of immune cells. Based on our findings presented in this study, however, lowered expression of Prox1 in these heterozygous knockout mice might constitute another contributing factor by causing abnormal IFN- $\gamma$  overexpression and IFN- $\gamma$ -mediated pro-inflammatory effects. It would be interesting to examine the IFN- $\gamma$  expression and reaction profile in such a mouse model and the results could shed new light on the *in vivo* functions of Prox1.

How Prox1 expression is down-regulated upon T cell activation to allow efficient IFN- $\gamma$  production is another important question that remains to be addressed. T cell activation signals and/or their downstream messenger molecules might directly or indirectly alter Prox1 promoter activity through certain pathways. Future studies of Prox1 promoter and its transcription regulators will no doubt result in a better understanding of the molecular details of T cell activation.

## Materials and Methods

### Plasmids and reagents

Luciferase reporter under the control of mouse IFN- $\gamma$  promoter (-296/+111, p296) is a generous gift from Dr Cunard. The minimal promoter (-124/+111, p124) [16] was then subcloned from p296 into pGL3-basic (Promega). p296 $\Delta$ DP was obtained by introducing internal deletions of DCS (-114/-98) and PCS (-76/-51) into p296.



The human IFN- $\gamma$  promoter (-538/+64, ph538) was kindly provided by Dr Wilson. For RNA interference of Prox1, coding sequences for Prox1-1830 siRNA (5'-AGT TCA ACA GAT GCA TTA C-3') were inserted in hairpin format into pSuper (OligoEngine). Prox1 expression plasmids have been described previously [23]. The cDNA for PPAR $\gamma$  was obtained by RT-PCR and inserted into pcDNA3 (Invitrogen). For GST pull-down assays, PPAR $\gamma$  and Prox1 fragment (aa 1-337) were subcloned into pGEX-3X (GE Healthcare) for the production of GST fusion proteins.

PMA, ionomycin and GW9662 were purchased from Sigma, Calbiochem, and Cayman Chemicals, respectively.

#### Transfections and reporter assays

Jurkat T cells (ATCC) were cultured in RPMI-1640 medium (Invitrogen). Transfections were carried out using Lipofectamine and Plus Reagent (Invitrogen) following the manufacturer's instructions. Reporter assays were performed as described previously [23]. Briefly, cells were typically cotransfected with 0.5  $\mu$ g of a luciferase reporter plasmid, 0.4  $\mu$ g of an expression plasmid and 0.5  $\mu$ g of pCMV-lacZ (Invitrogen) encoding  $\beta$ -galactosidase as internal control for normalization of transfection efficiency [29]. At 24 h post-transfection, cells were treated with PMA (25 ng/ml) and ionomycin (1  $\mu$ M) for 24 h. Each transfection was performed in duplicate dishes and repeated at least three times. The means  $\pm$  standard deviation (s.d.) of three experiments are presented.

#### Isolation of primary human CD4<sup>+</sup> T cells

Human PBMCs were separated from heparinized peripheral blood from healthy volunteer donors with lympholyte-H (Cedarlane Laboratories) according to manufacturer's instructions. CD4<sup>+</sup> T cells were isolated from PBMCs using Dynabeads CD4<sup>+</sup> lymphocyte negative isolation kit (Invitrogen DYNAL). Isolated CD4<sup>+</sup> T cells were checked for viability (>95%) and subcultured in RPMI-1640 supplemented with L-glutamine, 50  $\mu$ g/ml gentamycin and 10% heat-inactivated FBS (Invitrogen).

#### Cytokine ELISAs

IFN- $\gamma$  in cell culture medium of transfected Jurkat T cells was measured using an ELISA kit (R&D Systems) according to manufacturer's instructions. In assays where endogenous Prox1 was knocked down by siRNA, transfected Jurkat T cells were first sorted by FACS on a BD FACSAria and cells expressing GFP encoded by the pSuper vector or pSuper-Prox1-1830 were used for subsequent treatment and IFN- $\gamma$  measurement.

#### RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) following manufacturer's instructions. PCR was performed using the following primer sets (forward/reverse). Prox1: 5'-AAC TAG GGA TAC CAC GAG TC-3'/5'-CTT CAC TAT CCA GCT TGCAG-3'; IFN- $\gamma$ : 5'-TTC AGC TCT GCA TCG TTT TG-3'/5'-CTC TTT TGG ATG CTC TGG TC-3'; IL-2: 5'-ACC TCA ACT CCT GCC ACA AT-3'/5'-GCA CTT CCT CCA GAG GTT TG-3';  $\beta$ -actin: 5'-CAA CTC CAT CAT GAA GTG TGA CG-3'/5'-ACT CGT CAT ACT CCT GCT TGC-3'.

#### GST pull-down assays

<sup>35</sup>S-methionine-labeled proteins were produced *in vitro* using TNT Quick Coupled Transcription/Translation System (Promega) according to manufacturer's instructions. GST fusion protein expression in *Escherichia coli* BL21(pLys) (Novagen) was induced by 0.1 mM

isopropyl- $\beta$ -D-thiogalactoside for 4 h at 37 °C. Purification of GST fusion proteins and pull-down assays were performed essentially as described previously [23]. Each pull-down assay was carried out with 2  $\mu$ g of a GST fusion protein and 10  $\mu$ l of a labeled protein.

#### Co-immunoprecipitation

HEK293T cells were cotransfected with the expression plasmids of Prox1 and Flag-PPAR $\gamma$  using the calcium-phosphate precipitation method (7.5  $\mu$ g DNA/100 mm dish). Co-immunoprecipitation was performed as described previously [23]. Immunoblotting of Prox1 and Flag-PPAR $\gamma$  (1/20 input) was performed using rabbit anti-Prox1 antibody (1:2 500 dilution, Upstate) and mouse anti-Flag M2 monoclonal antibody (1:5 000 dilution, Sigma) as primary antibody, respectively. HRP-labeled porcine anti-rabbit IgG (1:1 000, DAKO) or rabbit anti-mouse IgG (1:1 000, DAKO) was used as secondary antibody.

#### Chromatin immunoprecipitation (ChIP)

ChIP assays were performed following a published protocol [30]. Briefly, chromatin was sheared by sonication (3 times, 10 s on, 60 s off). Precleared extracts were immunoprecipitated with rabbit anti-Prox1 antibody or rabbit IgG (Sigma) at 4 °C overnight. DNA was isolated from precipitated complexes and analyzed by PCR using primers for human IFN- $\gamma$  promoter (corresponding to -154 to +131): 5'-CTAACT ACA ACA CCC AAA TGC CAC-3'/5'-CAT CGT TTC CGA GAG AAT TAA GCC-3'. An aliquot of total input nuclear extract was used as loading control.

#### Acknowledgments

The authors are grateful to Dr Robyn Cunard (University of California and Veterans Affairs San Diego Healthcare System) and Dr Christopher B Wilson (University of Washington) for kindly providing mouse and human IFN- $\gamma$  promoters, respectively. This work is supported by National Basic Research Program of China (973 Program) (2005CB522402), National Natural Science Foundation of China (30393112, 30670434) and Science and Technology Committee of Shanghai Municipality (044107019, 06QH14014, 07DJ14006).

#### References

- 1 Di Marzio P, Puddu P, Conti L, Belardelli F, Gessani S. Interferon gamma upregulates its own gene expression in mouse peritoneal macrophages. *J Exp Med* 1994; **179**:1731-1736.
- 2 Williams CM, Coleman JW. Induced expression of mRNA for IL-5, IL-6, TNF-alpha, MIP-2 and IFN-gamma in immunologically activated rat peritoneal mast cells: inhibition by dexamethasone and cyclosporin A. *Immunology* 1995; **86**:244-249.
- 3 Lamkhouiou B, Gounni AS, Aldebert D, *et al.* Synthesis of type 1 (IFN gamma) and type 2 (IL-4, IL-5, and IL-10) cytokines by human eosinophils. *Ann N Y Acad Sci* 1996; **796**:203-208.
- 4 Howie SE, Aldridge RD, McVittie E, Forsey RJ, Sands C, Hunter JA. Epidermal keratinocyte production of interferon-gamma immunoreactive protein and mRNA is an early event in allergic contact dermatitis. *J Invest Dermatol* 1996; **106**:1218-1223.

- 5 Li L, Young D, Wolf SF, Choi YS. Interleukin-12 stimulates B cell growth by inducing IFN- $\gamma$ . *Cell Immunol* 1996; **168**:133-140.
- 6 Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon- $\gamma$ : an overview of signals, mechanisms and functions. *J Leukoc Biol* 2004; **75**:163-189.
- 7 Schoenborn JR, Wilson CB. Regulation of interferon- $\gamma$  during innate and adaptive immune responses. *Adv Immunol* 2007; **96**:41-101.
- 8 Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000; **100**:655-669.
- 9 Sica A, Dorman L, Viggiano V, et al. Interaction of NF- $\kappa$ B and NFAT with the interferon- $\gamma$  promoter. *J Biol Chem* 1997; **272**:30412-30420.
- 10 Ye J, Cippitelli M, Dorman L, Ortaldo JR, Young HA. The nuclear factor YY1 suppresses the human gamma interferon promoter through two mechanisms: inhibition of AP1 binding and activation of a silencer element. *Mol Cell Biol* 1996; **16**:4744-4753.
- 11 Sweetser MT, Hoey T, Sun YL, Weaver WM, Price GA, Wilson CB. The roles of nuclear factor of activated T cells and ying-yang 1 in activation-induced expression of the interferon- $\gamma$  promoter in T cells. *J Biol Chem* 1998; **273**:34775-34783.
- 12 Thierfelder WE, van Deursen JM, Yamamoto K, et al. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 1996; **382**:171-174.
- 13 Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 1996; **382**:174-177.
- 14 Penix LA, Sweetser MT, Weaver WM, Hoeffler JP, Kerppola TK, Wilson CB. The proximal regulatory element of the interferon- $\gamma$  promoter mediates selective expression in T cells. *J Biol Chem* 1996; **271**:31964-31972.
- 15 Zhang F, Wang DZ, Boothby M, Penix L, Flavell RA, Aune TM. Regulation of the activity of IFN- $\gamma$  promoter elements during Th cell differentiation. *J Immunol* 1998; **161**:6105-6112.
- 16 Cunard R, Eto Y, Muljadi JT, Glass CK, Kelly CJ, Ricote M. Repression of IFN- $\gamma$  expression by peroxisome proliferator-activated receptor gamma. *J Immunol* 2004; **172**:7530-7536.
- 17 Zhang X, Rodriguez-Galán MC, Subleski JJ, et al. Peroxisome proliferator-activated receptor- $\gamma$  and its ligands attenuate biologic functions of human natural killer cells. *Blood* 2004; **104**:3276-3284.
- 18 Oliver G, Sosa-Pineda B, Geisendorf S, Spana EP, Doe CQ, Gruss P. Prox 1, a prospero-related homeobox gene expressed during mouse development. *Mech Dev* 1993; **44**:3-16.
- 19 Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell* 1999; **98**:769-778.
- 20 Wigle JT, Chowdhury K, Gruss P, Oliver G. Prox1 function is crucial for mouse lens-fibre elongation. *Nat Genet* 1999; **21**:318-322.
- 21 Liu YW, Gao W, Teh HL, Tan JH, Chan WK. Prox1 is a novel coregulator of Fflb and is involved in the embryonic development of the zebra fish interrenal primordium. *Mol Cell Biol* 2003; **23**:7243-7255.
- 22 Sosa-Pineda B, Wigle JT, Oliver G. Hepatocyte migration during liver development requires Prox1. *Nat Genet* 2000; **25**:254-255.
- 23 Qin J, Gao DM, Jiang QF, et al. Prospero-related homeobox (Prox1) is a corepressor of human liver receptor homolog-1 and suppresses the transcription of the cholesterol 7- $\alpha$ -hydroxylase gene. *Mol Endocrinol* 2004; **18**:2424-2439.
- 24 Steffensen KR, Holter E, Bavner A, et al. Functional conservation of interactions between a homeodomain cofactor and a mammalian FTZ-F1 homologue. *EMBO Rep* 2004; **5**:613-619.
- 25 Truneh A, Albert F, Golstein P, Schmitt-Verhulst AM. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature* 1985; **313**:318-320.
- 26 Penix L, Weaver WM, Pang Y, Young HA, Wilson CB. Two essential regulatory elements in the human interferon gamma promoter confer activation specific expression in T cells. *J Exp Med* 1993; **178**:1483-1496.
- 27 Shnyreva M, Weaver WM, Blanchette M, et al. Evolutionarily conserved sequence elements that positively regulate IFN- $\gamma$  expression in T cells. *Proc Natl Acad Sci USA* 2004; **101**:12622-12627.
- 28 Harvey NL, Srinivasan RS, Dillard ME, et al. Lymphatic vascular defects promoted by Prox1 haploinsufficiency cause adult-onset obesity. *Nat Genet* 2005; **37**:1072-1081.
- 29 Sambrook J, Russell DW. *Molecular Cloning*. 3rd Edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 2001.
- 30 McCabe CD, Innis JW. A genomic approach to the identification and characterization of HOXA13 functional binding elements. *Nucleic Acids Res* 2005; **33**:6782-6794.