

Published in final edited form as:

Trends Immunol. 2011 April ; 32(4): 146–150. doi:10.1016/j.it.2011.01.006.

Regulating IL-9 transcription in T helper cells

Narayanan B. Perumal¹ and Mark H. Kaplan²

¹ School of Informatics, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202

² Department of Pediatrics, Herman B Wells Center for Pediatric Research² Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202

Abstract

T helper cells are critical for the development of immunity to infections and inflammatory disease. The acquisition of specific cytokine-secreting profiles, primed by the cytokine microenvironment, is required for effector function of Th cells. The most recent addition to the growing list of effector subsets are Th9 cells that secrete IL-9. In this article we propose a model for the transcriptional regulation of the *Il9* gene in IL-9-expressing T cells and the relatedness of this subset to other Th phenotypes. We suggest that transcription factors restricted to certain Th subsets, and common among several subsets, may play a role in the plasticity of Th9 cells.

Differentiation of Th subsets

The effector function of T helper subsets relies upon the production of particular cytokines. The cytokine-secreting potential of effector T helper subsets requires the activation and expression of transcription factors that promote the development of each subset. Differentiation is stimulated by the cytokine microenvironment and the activation of Signal Transducer and Activator of Transcription (STAT) proteins that initiate specific genetic programs (Fig. 1A). The subset-specific genetic programs include transcription factors required for the expression of cytokines and other genes that contribute to the effector function of each subset (Fig. 1A). While a single transcription factor is often called a “master regulator”, the appropriate development and function of Th subsets requires the coordinated activity of numerous transcription factors, some that are specific to a particular Th subset, and some that are shared among several subsets. The most recent addition to a growing continuum of Th subsets is the Th9 phenotype. These cells that develop *in vitro* in the presence of IL-4 and TGF- β , secrete high levels of IL-9, as well as IL-10, CCL17 and CCL22 [1–4]. However, the transcription factors regulating the hallmark cytokine IL-9 are not well defined. In this article we discuss factors that are specific to Th9 cells, or common among Th9 and other Th subsets, each of which may contribute to the expression of the *Il9* gene and the plasticity of phenotype between Th9 and other Th subsets.

Th9 cells: Regulating IL-9

Th9 cells are required for allergic inflammation and immunity to intestinal parasites [2,4] by virtue of the pleiotropic functions of IL-9 [5,6], as well as functions of chemokines and

Corresponding author: Kaplan, M.H. mkaplan2@iupui.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

potentially other mediators. The receptor-induced signals and downstream transcription factors that regulate IL-9 production are only beginning to be elucidated (Fig. 1B).

As mentioned already, Th9 cells develop in response to a balance of stimuli from TGF- β and IL-4. In the absence of IL-4, TGF- β promotes Treg development; in the absence of TGF- β , IL-4 leads to the development of Th2 cells (Fig. 1A). Thus, Th9 development likely requires signals from both receptors. In support of this, IL-4 activation of STAT6, and the STAT6 target gene GATA3 are both required for Th9 development, despite GATA3 being expressed in lower amounts in Th9 than in Th2 cells [3,4]. However, it is not clear whether GATA3 is the only relevant target of STAT6, nor is it clear how STAT6 or GATA3 function to promote Th9 development at the expense of Th2 cytokine production. GATA1 is known to promote *Il9* expression in mast cells [7], so it is possible that GATA3 binds directly to the *Il9* gene (Fig. 1B and 2). One factor that promotes the switch between the Th2 and Th9 phenotype is the ETS family transcription factor PU.1 [2]. PU.1 induces IL-9 production in cells cultured under Th2- or Th9-inducing conditions, and human or mouse T cells that are deficient in PU.1 expression have diminished IL-9 production. Because PU.1 binds directly to the *Il9* promoter (Fig. 2) to promote specific chromatin modifications, and PU.1 has been shown to interfere with GATA3 function [8,9], it might also be a determinant in altering the function of GATA3 between Th2 and Th9 cells. Importantly, PU.1 does not act alone. It has modest abilities to induce IL-9 when transduced in Th subsets other than Th2 or Th9 cells, and is expressed in iTreg that produce modest amounts of IL-9 compared to Th9 cells, together suggesting that an IL-4-induced factor is also important for IL-9 production.

Another transcription factor required for the development of IL-9-secreting T cells is IRF4 [10]. IRF4 binds directly to the *Il9* promoter and, like PU.1, is required for the development of allergic inflammation. However, unlike PU.1, IRF4 is required for the development of other Th subsets including Th2 and Th17 [11–13], both of which are required for allergic airway disease that develops following sensitization with ovalbumin and alum with subsequent ovalbumin challenge in the airways. Though IRF4 binds the *Il9* promoter, it also induces GATA3 expression during Th2 differentiation [13] and this function may impact Th9 development as well.

The regulation of *Il9* is likely to be as complex as that of other cytokine genes, requiring multiple cis-regulatory elements and specific trans-activating factors in addition to PU.1 and IRF4. A comparison of sequences [14] near the murine *Il9* gene with human and canine *IL9* identifies two conserved non-coding sequences (CNS) 5-prime of the *Il9* gene. CNS1 was previously identified in the promoter of the *Il9* gene [2] that bound PU.1 and IRF4 [2,10]. CNS2 was also previously identified between mouse and human sequences, but is not conserved with other species (Fig. 2). An additional CNS, ~6 kb upstream of the *Il9* gene is termed here CNS0 (Fig. 2). Sequence analysis [15,16] identifies binding sites for PU.1, IRF4, GATA3, NFAT proteins and STAT proteins (N4 spacing, meaning that there are 4 nucleotides between the TTC and GAA inverted repeats of the consensus binding site) in CNS1 (Fig. 2). Importantly, the PU.1 and IRF4 sites are not directly adjacent and are unlike the composite sites that have been identified in other target genes of both factors [17]. Biologically confirmed sites for AP-1 and NF- κ B are also present in CNS1 [18,19] (Fig. 2). NFAT proteins are also likely involved because IL-9 production is sensitive to cyclosporine A, an inhibitor of calcineurin that is required for NFAT nuclear translocation [20]. Potential sites for many of these factors are identified in CNS0, including a consensus STAT site (N3 spacing). A possible candidate for binding to this site is STAT1, which is activated by IFN γ , a cytokine known to negatively influence IL-9 production [1]. TGF- β , in addition to the programming of the IL-9 gene, has acute co-stimulatory effects in combination with TCR stimulation [2]. The downstream signaling pathways responsible for TGF- β activity, apart from the induction of PU.1 expression, are still not clear, though one report suggests that *Il9*

expression is normal in T cells deficient in Smad2 and Smad3 [21]. Additional work is needed to determine the function of these motifs in *Il9* regulation.

A lineage, a transient phenotype, or just one cytokine of many

Fifteen years ago T helper subsets were thought to acquire stable phenotypes after multiple rounds of stimulation [22], and this paradigm persisted well into the 21st century. However, as more subsets of T cells were discovered, it became clear that there is much greater plasticity of cytokine-secreting potential in Th subsets than was initially appreciated. Many of the initial descriptions of Th17 cells suggested that they are unstable and might be an intermediate in the transition to an IFN γ -secreting phenotype [23–27]. Indeed, purification of IL-17-secreting cells, and lineage tracing, demonstrated that IL-17-secreting cells can become IFN γ -secreting cells [24,28–30]. This flexibility was correlated with chromatin configurations that remained poised to acquire expression patterns associated with other lineages [31,32]. However, even cells that repress other lineage transcription factors and cytokines, can alter their cytokine-secreting phenotype. Th cells that secrete IFN γ , IL-4 or IL-17 can acquire attributes of T follicular helper cells [33–35]. Even Th2 memory cells are not entirely stable and are able to produce IFN γ after exposure to a new cytokine environment [36,37]. Together, these observations suggest that few Th cell subsets are completely fixed and with appropriate signals, cells may acquire new phenotypes.

This model of flexibility in programming still presumes that one polarized phenotype switches to another polarized phenotype. However, a number of recent reports have described the development of effector T cells that simultaneously have attributes of two effector subsets. Cells that display features of Th1 and Th17 cells, including the expression of both IFN γ and IL-17, are observed in patient samples, from mice with inflammatory disease, and in cultures of primary cells [38–41]. At least some of these cells can express both ROR γ t and T-bet, the transcription factors that respectively promote the development of Th17 and Th1 cells [41]. The in vivo microenvironment during a viral infection can initiate the conversion of Th2 cells to IFN γ -secreting Th1 cells that still retain some aspects of the Th2 phenotype resulting in co-expression of T-bet and GATA3 [37]. Pro-allergic cells that express both IL-4 and IL-17 in patient samples and mouse models of allergic inflammation are a result of cells that express GATA3 and ROR γ t, the transcription factors that respectively promote the development of Th2 and Th17 cells [42,43]. Most recently, it was shown that Th cells that are polarized to an IL-17-secreting phenotype in the absence of TGF- β acquire expression of ROR γ t and T-bet [44]. Together these observations suggest that in addition to highly polarized Th phenotypes, there are intermediate or bi-functional subsets that arise from the co-expression of multiple “lineage-defining” transcription factors.

Th9 cells may be another example of bi-functional Th cells. The derivation of Th9 cells by adding only TGF- β to Th2 cells [4], suggests that there is plasticity, perhaps based on the induction of PU.1, between these subsets. Other Th subsets can also express some amount of IL-9, though the absolute amounts might be considerably less than those produced by polarized Th9 cells [45,46]. Treg and Th17 cells may produce IL-9, and separation of Th17 cultures into IL-17-positive and negative cells demonstrated equivalent amounts of IL-9 in each population, suggesting that IL-17 and IL-9 can be co-produced [45,46]. In Th2 cultures, *Il9* expression is found in IL-4-low expressing cells, and Th9 cultures contain some IL-4-IL-9-double-positive cells, showing that IL-9 can coexist with IL-4 [2]. Th9 cells also produce IL-10, though IL-9 and IL-10 do not seem to be co-regulated as part of the same genetic program [2]. The basis for some of these mixed phenotypes may rely upon the relative expression of fate-determining transcription factors. For example, PU.1 is expressed with GATA3 in Th2 and Th9 cells. Although PU.1 inhibits at least some functions of

GATA3 in both cell types, one can imagine overlapping gradients of GATA3 and PU.1 expression where each combination might lead to subtly different cellular phenotypes.

The stability of IL-9-secreting cells is still not well documented. Allergic airway disease caused by adoptive transfer of Th9-polarized cells can be blocked by anti-IL-9, suggesting that the effector phenotype is maintained in vivo [10]. In contrast, adoptive transfer of Th9 cells in inflammatory models results in disease without maintained expression of IL-9, or concomitant with acquisition of potential for secreting other cytokines [47,48]. In vitro, the Th9 phenotype can be maintained through two rounds of culture, but polarized Th9 cells can also acquire other cytokine-secreting phenotypes [47].

The basis for the flexibility among these Th phenotypes might lie not in the factors that are different, but in the factors that are common among them. As noted above, IRF4 is expressed in Th1, Th2, Th9 and Th17 cells. C-maf is expressed in Th2, Th9 and Th17 cells, though a role for this factor in Th9 cells is still not clear. GATA3 is expressed in Th2 and Th9 cells. These similarities provide a backdrop for altering the expression of fate-determining factors and changing the cytokine-secreting potential of a Th cell. Thus, that chromatin is poised to allow the development of alternate lineages might be a reflection of the expression of factors that are common among Th phenotypes allowing a signal-induced change in the expression of lineage-specific transcription factors to shift genes from the poised to active state for polarized cytokine expression. The expression of common factors thus permits a restricted response to environmental signals that would need to alter the expression of only one or a few transcription factors to mediate the transformation into a new Th phenotype.

Concluding remarks

Our current understanding of Th differentiation has evolved beyond a simple one cytokine-one STAT-one phenotype paradigm. IL-4-induced STAT6 is critical for both Th2 and Th9 development. Moreover, Th2 development also requires STAT3 and STAT5, activated by additional cytokine signals [49,50], requirements that have not been completely tested in Th9 cells. The sharing of cytokine signals and transcription factors in the development of Th2 and Th9 cells suggests that these cells are not distinct phenotypes, but rather part of a continuum of phenotypes that is affected by the concentration of cytokines, both primary (IL-4, TGF- β) and secondary factors (IL-1, IL-25, IL-2, interferons), in the microenvironment, and are plastic enough to respond to changes in the cytokines present with altered cytokine-secreting potential.

Epigenetic programming of cytokine expression in T helper subsets

As T helper subsets differentiate, epigenetic changes occur at loci encoding cytokines that help to activate genes associated with one phenotype and repress genes associated with other Th subsets

Decreased DNA methylation and increased histone acetylation are generally associated with transcribed loci

Depending on the histone residue modified, histone methylation is associated with active or repressed genes

A gene that has both activating and repressive histone methylation marks is said to be poised, in that it is neither activated or repressed, but can respond to signals to become activated; this feature may be a component of the plasticity allowing one Th phenotype to become another

Outstanding Questions

Which transcription factors mediate acute induction of IL9 following T cell antigen receptor stimulation?

Which transcription factors work with PU.1 and IRF4 in programming the IL9 gene through chromatin modifications, ultimately promoting IL-9 production?

What is the ability of purified Th9 cells to acquire potential for secreting other cytokines?
How is the acquisition or loss of IL-9-secreting potential linked to the in vivo function of IL-9?

Acknowledgments

Preparation of this review was supported by Public Health Service Grant AI057459. We thank members of the Kaplan lab for their comments.

References

- Schmitt E, et al. IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *J Immunol.* 1994; 153 (9): 3989–3996. [PubMed: 7930607]
- Chang HC, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol.* 2010; 11 (6):527–534. [PubMed: 20431622]
- Dardalhon V, et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol.* 2008; 9 (12):1347–1355. [PubMed: 18997793]
- Veldhoen M, et al. Transforming growth factor-beta ‘reprograms’ the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol.* 2008; 9 (12):1341–1346. [PubMed: 18931678]
- Goswami R, Kaplan MH. A Brief History of IL-9. *J Immunol.* 2011 In press.
- Noelle RJ, Nowak EC. Cellular sources and immune functions of interleukin-9. *Nat Rev Immunol.* 2010; 10 (10):683–687. [PubMed: 20847745]
- Stassen M, et al. p38 MAP kinase drives the expression of mast cell-derived IL-9 via activation of the transcription factor GATA-1. *Mol Immunol.* 2007; 44 (5):926–933. [PubMed: 16650898]
- Chang HC, et al. PU.1 regulates TCR expression by modulating GATA-3 activity. *J Immunol.* 2009; 183 (8):4887–4894. [PubMed: 19801513]
- Chang HC, et al. PU.1 expression delineates heterogeneity in primary Th2 cells. *Immunity.* 2005; 22 (6):693–703. [PubMed: 15963784]
- Staudt V, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity.* 2010; 33 (2):192–202. [PubMed: 20674401]
- Ahyi AN, et al. IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. *J Immunol.* 2009; 183 (3):1598–1606. [PubMed: 19592658]
- Brustle A, et al. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol.* 2007; 8 (9):958–966. [PubMed: 17676043]
- Lohoff M, et al. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc Natl Acad Sci U S A.* 2002; 99 (18):11808–11812. [PubMed: 12189207]
- Ovcharenko I, et al. ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. *Nucleic Acids Res.* 2004; 32 (Web Server issue):W280–286. [PubMed: 15215395]
- Loots GG, Ovcharenko I. rVISTA 2.0: evolutionary analysis of transcription factor binding sites. *Nucleic Acids Res.* 2004; 32 (Web Server issue):W217–221. [PubMed: 15215384]

16. Bryne JC, et al. JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. *Nucleic Acids Res.* 2008; 36 (Database issue):D102–106. [PubMed: 18006571]
17. Escalante CR, et al. Crystal structure of PU.1/IRF-4/DNA ternary complex. *Mol Cell.* 2002; 10 (5): 1097–1105. [PubMed: 12453417]
18. Stassen M, et al. IL-9 and IL-13 production by activated mast cells is strongly enhanced in the presence of lipopolysaccharide: NF-kappa B is decisively involved in the expression of IL-9. *J Immunol.* 2001; 166 (7):4391–4398. [PubMed: 11254693]
19. Zhu YX, et al. Multiple transcription factors are required for activation of human interleukin 9 gene in T cells. *J Biol Chem.* 1996; 271 (26):15815–15822. [PubMed: 8663174]
20. Gessner A, et al. Differential regulation of IL-9-expression after infection with *Leishmania major* in susceptible and resistant mice. *Immunobiology.* 1993; 189 (5):419–435. [PubMed: 8125519]
21. Takimoto T, et al. Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. *J Immunol.* 2010; 185 (2):842–855. [PubMed: 20548029]
22. Murphy E, et al. Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *J Exp Med.* 1996; 183 (3):901–913. [PubMed: 8642294]
23. Bending D, et al. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J Clin Invest.* 2009
24. Lee YK, et al. Late developmental plasticity in the T helper 17 lineage. *Immunity.* 2009; 30 (1): 92–107. [PubMed: 19119024]
25. Martin-Orozco N, et al. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. *Eur J Immunol.* 2009; 39 (1):216–224. [PubMed: 19130584]
26. Mathur AN, et al. T-bet is a critical determinant in the instability of the IL-17-secreting T-helper phenotype. *Blood.* 2006; 108 (5):1595–1601. [PubMed: 16670261]
27. Shi G, et al. Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. *J Immunol.* 2008; 181 (10):7205–7213. [PubMed: 18981142]
28. Lexberg MH, et al. IFN-gamma and IL-12 synergize to convert in vivo generated Th17 into Th1/Th17 cells. *Eur J Immunol.* 2010; 40 (11):3017–3027. [PubMed: 21061434]
29. Lexberg MH, et al. Th memory for interleukin-17 expression is stable in vivo. *Eur J Immunol.* 2008; 38 (10):2654–2664. [PubMed: 18825747]
30. Stritesky GL, et al. IL-23 promotes maintenance but not commitment to the Th17 lineage. *J Immunol.* 2008; 181 (9):5948–5955. [PubMed: 18941183]
31. Mukasa R, et al. Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. *Immunity.* 2010; 32 (5):616–627. [PubMed: 20471290]
32. Wei G, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity.* 2009; 30 (1):155–167. [PubMed: 19144320]
33. King IL, Mohrs M. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med.* 2009; 206 (5):1001–1007. [PubMed: 19380638]
34. Reinhardt RL, et al. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol.* 2009; 10 (4):385–393. [PubMed: 19252490]
35. Zaretsky AG, et al. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med.* 2009; 206 (5):991–999. [PubMed: 19380637]
36. Adeeku E, et al. Flexibility accompanies commitment of memory CD4 lymphocytes derived from IL-4 locus-activated precursors. *Proc Natl Acad Sci U S A.* 2008; 105 (27):9307–9312. [PubMed: 18591677]
37. Hegazy AN, et al. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity.* 2010; 32 (1):116–128. [PubMed: 20079668]
38. Acosta-Rodriguez EV, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol.* 2007; 8 (6):639–646. [PubMed: 17486092]

39. Ivanov II, et al. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. 2006; 126 (6):1121–1133. [PubMed: 16990136]
40. Liu X, et al. Loss of STAT3 in CD4+ T cells prevents development of experimental autoimmune diseases. *J Immunol*. 2008; 180 (9):6070–6076. [PubMed: 18424728]
41. Annunziato F, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med*. 2007; 204 (8):1849–1861. [PubMed: 17635957]
42. Cosmi L, et al. Identification of a novel subset of human circulating memory. CD4(+) T cells that produce both IL-17A and IL-4. *J Allergy Clin Immunol*. 2010; 125(1):222–230. e221–224. [PubMed: 20109749]
43. Wang YH, et al. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic. allergic asthma. *J Exp Med*. 2010; 207 (11):2479–2491. [PubMed: 20921287]
44. Ghoreschi K, et al. Generation of pathogenic T(H)17 cells in the absence of TGF- β signalling. *Nature*. 2010; 467 (7318):967–971. [PubMed: 20962846]
45. Elyaman W, et al. IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc Natl Acad Sci U S A*. 2009; 106 (31):12885–12890. [PubMed: 19433802]
46. Nowak EC, et al. IL-9 as a mediator of Th17-driven inflammatory disease. *J Exp Med*. 2009; 206 (8):1653–1660. [PubMed: 19596803]
47. Tan C, et al. Antigen-Specific Th9 Cells Exhibit Uniqueness in Their Kinetics of Cytokine Production and Short Retention at the Inflammatory Site. *J Immunol*. 2010; 185:6795–6801. [PubMed: 20971929]
48. Jager A, et al. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol*. 2009; 183(11):7169–7177. [PubMed: 19890056]
49. Stritesky GL, et al. The transcription factor STAT3 is required for T helper 2 cell development. *Immunity*. 2011 In press.
50. Zhu J, et al. Stat5 activation plays a critical role in Th2 differentiation. *Immunity*. 2003; 19 (5): 739–748. [PubMed: 14614860]

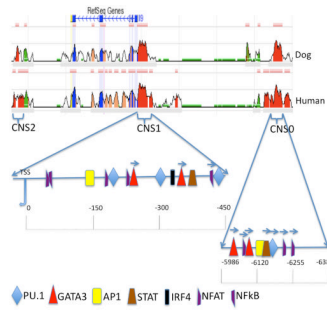


Figure 2. Conservation analysis and motif prediction in mouse *Il9* regulatory regions

(Top) Screen capture from the ECR Browser web site (<http://ecrbrowser.dcode.org/>) of the mouse *Il9* gene with evolutionary conserved regions (ECR) in the canine and human genomes. The mouse *Il9* gene with five exons is transcribed from the Crick strand (right to left) starting at 56,583,606 of chromosome 13 (mm9 reference genome). A region from -6.9 kb with respect to the gene or transcription start site (TSS) to $+2.7$ kb relative to the gene end is shown. The peaks in red show comparative pair wise ECR between mouse and human or canine sequences. (Bottom) Transcription factor binding sites predicted using the rVista and JASPAR software, as well as manual inspection and previously identified sites, are shown in the CNS0 and CNS1 regions. The rVista (<http://rvista.dcode.org/>) tool was employed to predict motifs from the phylogenetically conserved regions observed in the ECR Browser while for JASPAR (<http://jaspar.genereg.net/>) the individual species-specific CNS regions were uploaded as input. The locations of transcription factor motifs are shown mapped in scale relative to the TSS with the start of the CNS0 conserved region at 56,589,592; the motifs in the anti-sense (Watson) strand are marked with an arrow above the site.