Distal regulation of the interferon gamma locus

By

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To my Mother

To my Father

To my Brother

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BAC: bacterial artificial chromosome

IFNG: Interferon-gamma

CNS: conserved noncoding sequence

H3K3me1/2/3: Histone 3 Lysine 4 methyl

HAT: Histone acetyltransferase

HDAC: Histone deacetyltransferase

GWAS: Genome wide association study

IFNG: Interferon gamma

Th: T helper

IL: Interleukin

NK: Natural Killer

NKT: Natural Killer T

DC: dendritic cell

Pol II: Polymerase II

CTCF: CCCTC binding factor

H3K9Me3: Histone-3 Lysine 9 tri-methylation

STAT: Signal activator and transducer of transcription

CHAPTER I

Introduction

The vast majority of the mammalian genome does not encode a transcribed message. This noncoding portion of the genome was once considered to be "junk" DNA, but is now understood to be critically important to human health and gene regulation. The importance of non-coding DNA has been illustrated by three observations: Firstly, the majority of the conserved elements in the genome are noncoding, implying necessity for fitness. Secondly, the noncoding section of the genome is actively marked in cell-type and stimulus-specific patterns by covalent histone modifications, patterns of DNase hypersensitivity, transcription factor binding and binding of proteins critical for transcription such as polymerase II (Pol II). This observation implies a global importance for gene regulation. Thirdly, mutations in the noncoding sections of the genome are associated with wide ranging human and animal traits and deletion of non-coding elements has been mechanistically linked to gene regulation. These three observations have led to the belief that understanding the noncoding section of the genome will be critical for understanding both gene regulation and for understanding human diseases which results from noncoding mutations.

In metazoans, developmentally regulated genes are thought to require cis-regulatory elements for faithful developmental decisions. For example, the gene interferon gamma (IFNG) must be expressed in T helper (Th) 1 cells, but actively repressed in alternative Th lineages such as Th2 cells. Transgenic mice carrying an 8.6 kb transgene of the

human *IFNG* gene fail to properly repress *IFNG* in Th2 cells. However, transgenic mice carrying an 190 kb transgene do. This has led to the hypothesis that *IFNG* requires cis-regulatory elements for proper developmental decisions. Interferon gamma (IFN-γ) signaling is critically involved in the host defense against intracellular pathogens. Individuals and animals that have defects in IFN-γ signaling pathways develop severe immunodeficiency, often succumbing to mycobacterial infection (1). Common polymorphisms associated with IFN-γ signaling are associated with an increasingly large array of diseases such as coronary artery disease (2), cervical cancer (3) and ulcerative colitis (4). As such, understanding IFN-γ signaling and *IFNG* regulation is important to understanding human health. In addition, understanding *IFNG* regulation provides much needed functional understanding of the noncoding segment of the human genome.

Distal regulation

Human disease and noncoding segments of the genome

A variety of human genetic diseases, as well as everyday polymorphisms, result from mutations in distal regulatory elements (5). Also, the majority of trait associated polymorphisms by genome wide association studies (GWAS) reside in noncoding segments of the human genome (6). Specific examples of distal regulation and human disease include cis-regulatory regions which have been implicated in both the repression and activation of genes involved in human disease. For a gain-of-function example, a mutation 1 megabase from the Sonic Hedge Hog (*SHH*) promoter has been shown to cause preaxial polydactyly (7). In these individuals, too much *SHH* is expressed in the

developing limb bud and an extra thumb develops. For a loss-of-function example, mutations in beta globin or alpha globin loci distal regulatory elements have been linked to thalassemia (8, 9). This was first demonstrated in 1983 when a thalassemia patient was discovered to have a long-range deletion near the beta globin locus. This chromosome did not express beta globin, and had hallmarks of a "closed" chromatin state (CpG methylation and a lack of DNase hypersensitivity) (8). The thalassemia chromatin accessibility defect arises specifically in erythroid cells, showing human cis-regulatory elements provide cell-type specific chromatin accessibility (10). In transgenic murine models, the thassalemia defect was found to be a deletion of a locus control region (LCR) which is necessary for both high levels of expression and transgene-copy number dependent expression of a human β-globin transgene (11). Distal elements have also been implicated in oncogenesis. Some instances of Burkitt's lymphoma result from a chromosomal translocation that causes an immunoglobulin heavy chain distal element to promote expression of the c-myc oncogene (12). Many other diseases have been linked to long-range elements (reviewed in ref (5)). As polymorphism analysis moves from analyzing just the core elements of a gene to analyzing the whole genome, many more will be characterized.

Long-range regulation

Arguably, the most intensely studied loci to date have been the alpha globin locus and the beta globin locus. Both of these loci contain multiple genes expressed in order during development, from the gene closest to the locus control region to the gene farthest away from the LCR (13, 14). The beta-globin LCR contains five DNase hypersensitivity sites

(six in the mouse) called 5'HS1-HS5. All of the five HS sites are necessary for tissue-specific transcription, but each has unique sequence composition and function(s). For instance, 5'HS5 is thought to insulate the spreading of neighboring heterochromatin (15), while 5'HS2 alone is able to confer tissue-appropriate expression *in vivo* (16). Similarly in the alpha-globin locus, only one element, a hypersensitivity site 40 kb from the promoter (HS-40), has been shown to be able to enhance alpha globin expression *in vivo* (17).

In cell based *in vitro* transcription assays, locus control regions are position independent, but copy number dependent. However, *in vivo* both position and orientation of locus control regions are critical for proper expression (18-21). For example, inverting the sequence of the beta globin LCR abolishes all transcription from the locus (21). In the *Hox* gene model, the different genes in the locus are thought to compete for LCR enhancers during development. So, the closest genes are first looped into close proximity to LCR enhancers and are expressed earliest. Experimental removal of "spacer" genes from the Hox locus results in aberrant *Shh* expression (activated by late Hox genes) and deformed limb growth (22). The reports on how such distal elements establish higher order chromatin structure have not been conclusive. In the human growth hormone locus, a major control element, HS1, is necessary for histone acetylation and phosphorylated RNA pol II binding throughout the locus, but not chromosome looping (19, 23, 24). Histone tail acetylation across loci is associated with transcriptionally active loci. However, when a transcription termination site is placed between HS1 and the genes it regulates, looping and transcription is disrupted, but histone acetylation is not (19). For the alpha globin locus, deletion of the LCR in a humanized BAC model abolishes

transcription but not histone acetylation (25). This LCR deletion was intended to mimic that of a naturally occurring Spanish thalassemia patient with a "closed" chromatin state. However, when the actual chromosome from the patient was introduced into a mouse cell line, histone acetylation was still absent (26).

In a study associating expression variability with polymorphism distances, the strength of a promoter was found to be cell-type specific but also related to the distance from the regulated gene (27). However, developmentally regulated genes often have distal cis-regulatory elements located up to a megabase away from their transcription start site (7) or can even be located on different chromosomes (28). These distant regulatory elements are thought to function with the assistance of both intra- and inter-chromosomal interactions. In mammals, the protein CCCTC binding factor (CTCF) is thought to provide a variety of functions, including the capability to bind at multiple regions of the genome and to form chromatin loops (29). Formation of such complexes is facilitated by the ring-forming protein cohesin (30). A relevant question is the exact function of CTCF binding regions in the genome. CTCF has been described as a transcriptional activator (31), insulator (32) and a repressor (33). However, most of these functional results, especially insulator studies, come from reporter assay systems, which do not take genomic context into account. In a genomic context, experimental mutation of a CTCF site in the β-globin locus showed effects on chromatin looping and accessibility, but not on expression of the β-globin gene (34). Recently, from chromatin immunoprecipitation followed by sequencing (ChIP-seq) data, CTCF peaks were found to separate active and repressive chromatin domains (35). However, this separation was found at only a small fraction of CTCF binding sites. In fact, barrier function may be achieved by association

with additional accessory effector proteins rather than by CTCF itself. As such, the functional role for specific CTCF binding sites remains to be determined.

The Histone Code

Eukaryotic DNA is wrapped around an octomer of a core comprising a heterodimer of four proteins: Histone 2A (H2A), H2B, H3 and H4. These histone proteins contain flexible N-terminal tails that are subject to post-translational covalent modification (36). These post-translational modifications form extensive reproducible patterns in a cell-type and stimulus-specific fashion leading to the hypothesis that there is a 'histone code' written and read as a means to orchestrate gene transcription (37, 38). Much work has gone into mapping histone tail modifications. A pattern has emerged which has allowed the classification of chromatin into several states based upon local histone modifications. In this model, mono-, di- and trimethylation of histone 3 lysine 9 (H3K9Me3), H3K9Me2 and H3K27me1/2/3 are associated with transcriptional repression or silent enhancers (39). In contrast, most other histone marks, as well as the unstable histone variant H2A.Z, are associated with either transcriptional activation or active/poised enhancers and promoters. For example, a strong, active promoter is associated with the histone mark H3K4Me3, while a poised or weak enhancer is marked with H3K4Me1 (40). Similarly, the histone acetylation marks are associated with active gene areas.

In addition to mapping histone marks, substantial work has gone into understanding the proteins that read and write the 'histone code.' Histone acetyltransferases (HATs) such as Tip60 or p300 promote the acetylation of histones, while histone deaceacetylases (HDAC) such as HDAC6 remove histone acetylation marks. Histone methylation marks

have a unique set of enzymes involved in the formation and reading of the marks, such as G9a, which catalyzes formation of the repressive H3K9Me2 mark (41) and LSD1, which removes the H3K9Me2 histone mark (42). These observations have led to the hypothesis that the histone code is written and erased in response to developmental cues or in response to extracellular stimuli. Interestingly, upon transcriptional activation both HAT and HDAC recruitment is seen at active genes and enhancers, leading to the hypothesis that gene transcription is not only accompanied the accumulation of activating acetylation histone marks, but is accompanied by a balance between acetylation and deacetylation to achieve proper levels of transcription (43). Indeed, treatment with a histone deacetylase inhibitor promotes the transcription of poised genes, showing that histone deacetylase recruitment is a critical part of regulating gene transcription (44).

Bacterial artificial chromosomes

Bacterial artificial chromosomes represent a useful methodology to study cis-regulation. BAC technology involves the cloning of a large segment, up to 300 kb, of a genome into a bacterial artificial chromosome backbone. The BAC can then be used for sequencing, used as a large transgene, or other purposes. The particularly large size of the BAC is often thought to provide better protection against transgene integration effects when integrated into mammalian genomes, and also provides a sufficiently large template to encapsulate complete genomic loci (45). Further, because the initial mouse and human genomes were sequenced using BAC libraries, the sequences of individual BACs are typically known in advance. Growth of BAC transgenes in *E. coli* allows easy experimental manipulation such as the mutation or removal of a cis regulatory element,

changing order of cis regulatory elements, or altering distance between cis regulatory elements. This strategy of using BAC transgenes with or without cis-regulatory elements has been successfully employed in the study of numerous loci such as the beta-globin locus, the *IL10* locus or the human growth hormone locus (46). Alternatively, BAC technology is commonly used as a method to analyze hard-to-detect proteins by replacing coding regions of genes with reporter genes (47) or to recreate transgenic viral genomes (48).

Key observations about the nature of cis-regulation have been made using BAC transgenic animals. In initial studies of the beta-globin locus, BAC transgenes were used to define a locus control region as a region which provides a high-level of copy-number dependent and position independent expression (11). In later studies, examining individual cis-regulatory elements within the beta-globin LCR has expanded the function of individual LCR elements. These studies demonstrate that individual cis-regulatory elements each have distinct roles in establishing chromosome accessibility, position-independent expression, nuclear positioning and initiation of transcription (15, 26). BAC transgenic systems have also been critical in determining the functional outcomes of the removal of cis-regulatory elements from a gene locus. In studies of the *HoxD* locus, inclusion or removal of cis-regulatory elements results in tissue-specific loss of gene expression (49). In the same study, and in studies of the beta-globin LCR, inversion of cis-regulatory elements resulted in loss of BAC transgene expression, showing genomic order is critical for proper gene expression (21, 49).

In addition to dissecting the roles of cis-regulatory elements, BAC transgenic systems have been used to examine species-specific expression patterns. In a key study of the

alpha-globin locus, a BAC transgene has been inserted into its proper genomic location by replacing the endogenous locus. In this study, expression of the human BAC transgene is cell-type specific and dependent upon cis-regulatory elements but expression levels are reduced to 40% of the expression level of the endogenous gene (50). Further, homozygous mice with the alpha globin BAC transgene knock-in were not viable. These results were interpreted as representing subtle differences in transcription factor usage across species, which give rise to critical outcomes in terms of proper levels of gene expression. In other models, use of BAC transgenes from alternate species gives rise to species-specific gene expression (49). For example, a puffer-fish BAC of the *HoxD* locus drives high-levels of tissue-specific expression but does not drive gene expression in the developing mouse limb bud. Approaches to determine species-specific tissue expression and the functional roles of cis-regulatory elements have been combined. Using tissue-specific gene expression from BACs with varying species of origins, combined with cis-regulatory element deletions, a mammal-specific *Tbx4* cis-regulatory element has been determined. This regulatory element is non-essential for viable mice but is needed for proper limb formation. In comparison, a vertebrate-specific *Tbx4* regulatory element was required for viability (51).

A key step in the use of BAC transgenes is the preparation and analysis of transgenic mice. Because of the particularly large nature of the BAC transgene, three problems arise. First, the large BAC plasmid shears easily. Parameters which determine stability and shearing of BAC transgenes negatively impact the rate of creation of transgenic pups. As such, improper buffer choice or improper BAC plasmid concentration negatively impacts successful creation of transgenic pups. However methods of purifying BAC transgenes,

such as cesium chloride or ion exchange columns, and physical characteristics such as BAC size do not correlate with the creation of successful transgenic mice (52). Second, because of the large size of the BAC plasmid partial integration effects are common (53). Because of the potential for partial integration effects, it is critical to check that transgenes have achieved full integration before lines can be analyzed. Proper integration can be easily checked by quantitative PCR. Third, copy number and full-transgene genotype must be assessed throughout generations to ensure that independent association does not occur as a result of a recombination event.

Interferon gamma

CD4 cell development

The adaptive immune system must respond to varying classes of challenges such as intracellular infections, infections by extracellular bacteria and fungi, infections by parasitic worms and autoimmunity. These responses are orchestrated by distinct lineages of CD4+ T cells termed Th1, Th17, Th2 and T regulatory cells (54). The primary function of the T helper cell is to "help" orchestrate the immune system to produce an appropriately tailored adaptive immune response, and elicit the same response for subsequent re-infections. This is achieved by creating T helper subsets, which produce tailored cytokine profiles. These cytokines are, in turn, recognized by other cells in the immune system and body. Each cytokine profile has unique roles. For example, the Th2 profile produces interleukin (IL)-4, IL-5 and IL-13. These cytokines are recognized by B

cells to promote antibody class switching to IgG1, IgE and IgA, and also by epithelial cells to promote the production of mucus and constriction.

The Th1 response is characterized by the production of IFN-γ. IFN-γ is also produced by CD8+ cytotoxic T cells and natural killer (NK) cells. Every nucleated cell in the body has an IFN-γ receptor, and the capability to signal through STAT1 upon IFN-γ stimulation. However, the downstream effects of STAT1 signaling vary with cell type and circumstances. IFN-γ signaling is most famous for its ability to activate macrophages to destroy phagocytosed cells, but IFN-γ can also elicit a pro-inflammatory response, halt cellular proliferation, promote expression of MHC molecules, promote nitric oxide production and promote class-switching to IgG subclasses. Most of these responses have the common effect of halting intracellular infections. Class-switching to IgG2 assists in opsonization of small immunogens, which allows for efficient complement fixation and efficient recognition by effector immune cells.

The cytokine profile of the Th17 response is far more heterogeneous (54). Human Th17 cells are characterized by the production IL-17A and IL-17F, but can also produce IL-22 and IL-26 (55). The production of IL-22 and IL-26 can also be made without IL-17A/F by Th22 cells. However, a common response downstream of receptor ligation seems to be STAT3 signaling, which results in characteristic production of antimicrobial peptides and recruitment of neutrophils. Human Th17 cells can also produce IFN-γ. In human Th17 cells, IL-17 and IL-22 are preferentially expressed by IFN-γ negative cells while IL26 is preferentially expressed by IFN-γ positive, IL-17A negative cells (56).

The developmental decisions of different T helper subsets stem from the instruction of cytokines received during T cell stimulation. For example, IL-12 is critical for Th1

differentiation by the signaling of STAT4 and subsequent upregulation of T-bet (57). In addition to IL-12 signaling, IFN-γ and IL-2 act as both autocrine and paracrine cytokines to promote Th1 development (58) by STAT1 and STAT5A/B signaling, respectively (59). Among other cytokines, IL-23 is critical for Th17 differentiation and inhibiting Th1 differentiation by signaling through STAT3 (60). Although IL-23 is unique from IL-12, they share a common evolutionary ancestor (61). IL-12 and IL-23 are separate heterodimers, which share a subunit, IL-12p40. The other IL-12 and IL23 subunits are encoded on a common locus. The receptors for IL-12 and IL-23 also are separate heterodimers sharing one common subunit and a separate common locus.

CD8+ cytotoxic T cells are an adaptive immune subset of the T cell lineage (Figure 1-1). Like mature Th1 cells, mature CD8+ T cells are able to rapidly produce the cytokine IFN-γ upon stimulation with cognate peptide-loaded major histocompatability complex (MHC) or simultaneous stimulation with IL-12 and IL-18 (62). Unlike CD4 T helper cells, which recognize peptide presented in MHC class two, CD8 cells recognize peptide presented by MHC class one. MHC class one is expressed on every nucleated cell and, as such, the chief function of CD8 cytotoxic T cells is to recognize infected cells which are presenting peptide on their MHC class one. In addition to IFN-γ, CD8 T cells have other effector functions, such as production of perforin, granzyme B and the ability to directly signal apoptosis by Fas-Fas ligand interactions (63).

CD4+ T helper T cells and CD8+ cytotoxic T cells develop from T cell precursors in the thymus microenviroment. In the thymus, both CD4+ and CD8+ subsets develop after successful recombination of both alpha and beta chains of the T cell receptor, allowing the recognition of distinct MHC-presented peptides by different T cell clones. A separate

branch of T cells, the natural killer T cell, also develops from a T cell precursor in the thymus, and has a semi-invariant T cell receptor which targets glycolipid antigens. Like conventional T cells, upon instruction in the periphery, the NKT cell can release cytokines such as IFN-γ. Outside of the T cell lineage, two main lines of lymphocytes develop in the bone marrow, natural killer cells and B cells. Natural killer cells exit the bone marrow microenviroment as innate immune cells, and are able to produce cytokines, such as IFN-γ, upon stimulation (64). B cells, like T cells, are adaptive immune cells, but do not have a principle role producing IFN-γ.

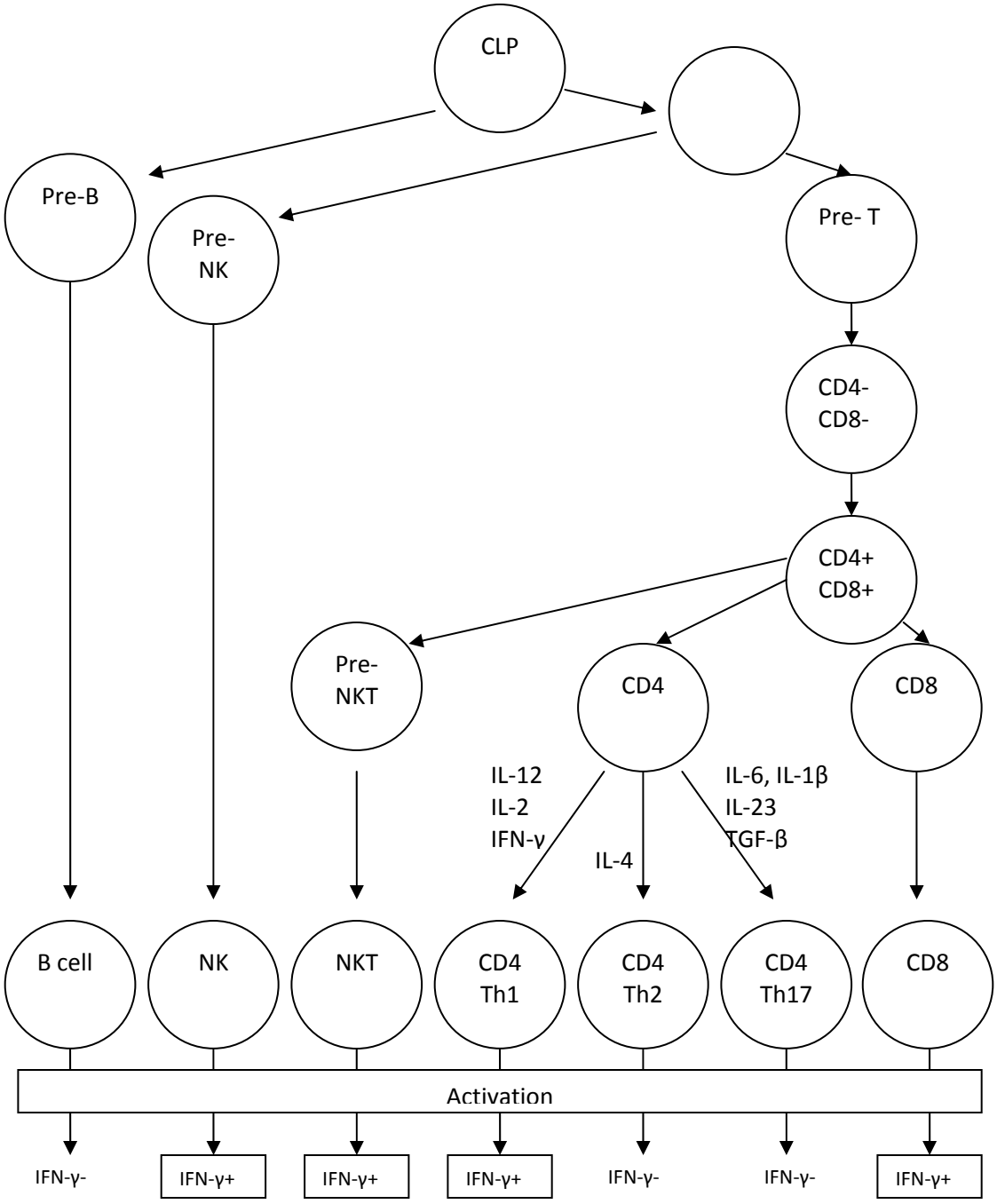

Figure 1-1. IFN-γ in lymphocyte cell differentiation. Lineages of the lymphocyte tree are shown as they relate to IFN-γ production. Cell types which are predominantly interferon gamma positive after activation are shown. Polarizing cytokines in T cell differentiation are shown to illustrate culture conditions. CP: Common precursor.

The interferon gamma gene locus

Interferon gamma is expressed in various cell types during the terminal stages of their differentiation processes in an activation-dependent manner. For example, when antigen-presenting cells stimulate naïve CD4+ T cells under Th1 conditions, these cells undergo a differentiation process resulting in an active interferon gamma locus (65). Shortly after initial stimulation there is a short period of diminutive IFN-γ expression in both Th1 and Th2 cells (28). After that point there is a period of one or two days without transcription followed by a burst of interferon gamma production only in the Th1 polarized cells. After that point, antigenic restimulation, or stimulation with appropriate cytokines, will produce a rapid and robust interferon gamma response. Thus, following the initial stimulation, the *Ifng* locus undergoes an epigenetic modification, which allows more rapid and robust expression upon subsequent restimulations (66). During this transition, the Th1 locus develops long range histone marks associated with transcriptionally permissive areas of the chromatin, such as H4 acetylation, and develops a pattern of long-range DNase hypersensitivity (67, 68) .

During the primary stimulation, IL-12 stimulation triggers the transcription factor STAT4 to induce transcription of a second transcription factor, T-bet (65). T-bet is necessary and sufficient for *Ifng* expression in activated CD4+ T-cells (69). During the primary stimulation, *Ifng* activation is T cell receptor (TCR) dependent. TCR stimulation, along with stimulation through costimulation molecules, triggers the downstream transcription factors c-Rel (of the Nf-κB complex), AP-1 and NFAT (70). These three transcription factors stimulate *Ifng* transcription. During secondary stimulation, *Ifng* transcription can be stimulated by TCR engagement, or by IL-18 and IL-12 cytokine co-

stimulation (71). IL-18 signaling through the adaptor molecule Myd88 triggers NF-κB activity (72).

The exact size of the human *IFNG* or mouse *Ifng* locus is unclear. In addition to the promoter, six regulatory regions have been well characterized (Figure 1-2). There are three sites within introns of *IFNG* known to have enhancer activity (68). The genomic region outside of the *IFNG* gene coding region contains numerous conserved noncoding sequences (CNS) that are marked with lineage-specific histone modifications (73). Because these sequences are conserved throughout evolution they should theoretically have regulatory function critical for fitness of the organism. Indeed, numerous CNS have been shown to regulate gene expression in reporter assays. Of the various mouse conserved noncoding sequences (mCNSs) implicated in regulating the mouse *Ifng* gene, the mCNSs at -22 kb, -6 kb, and +18-20 kb have been best characterized. Each of these three CNS has a distinct regulatory function.

Mouse CNS-22 is a T-bet binding element necessary for production of an *Ifng*-BAC transgenic Thy1.1 reporter gene in T-cells and natural killer cells (74). Mouse CNS-22 functions as a strong T-bet dependent enhancer in luciferase based assay systems (67). The -22 kb site does not have a DNase hypersensitivity site in naïve CD4+ cells and is maintained in a histone-tail hypoacetylated state from a histone deacetylase bound to Sin3a (44). Upon Th1 polarization T-bet replaces Sin3a and the -22 kb site develops H4 acetylation and a very strong hypersensitivity site. The site also develops histone marks associated with transcriptionally permissive chromatin in Th2 cells. Because the site contains a permissive chromatin environment in both Th1 and Th2 cells, and strongly reacts to T-bet in an activation-independent manner, it is theorized that mCNS-22

functions to create an environment favorable to transcription in Th1 cells. In contrast, it is not known if a similar function exists for the human equivalent of mCNS-22, CNS-16.

Mouse CNS-6 (also termed CNS1) possesses a DNase hypersensitivity site in naive T-cells (67), suggesting it is a site critical for early events in *Ifng* remodeling. The transcription factors T-bet, STAT5, NFAT1 and –theoretically- AP1 bind to the -6 kb site (75-77). Mouse CNS-6 is thought to play a role in an early event in T-cell differentiation: within 24-72 hours of Th1 cell development a Jak3-dependent cytokine signal, probably IL-2, stimulates recruitment of STAT5 to the CNS-6 site (76). By 72 hours after Th1 differentiation the chromatin containing CNS-6 has looped into the promoter, possibly explaining why T-bet is known to bind to both sites (78). An alternative hypothesis could be made based upon the observation that the chromatin loop does not occur until later in T cell development. In this model, mCNS-6 functions later in T cell development, which is made possible by the action of STAT5 early in Th1 cell development. How the -6 kb site functions in differentiated Th1 cells is unclear. *In vitro* promoter assays have been contradictory. Some reports show T-bet dependent enhancer activity (75), while some papers describe T-bet independent activity (67). Similarly, DNase hypersensitivity has (75) and has not (67) been reported at mCNS-6 in a differentiated Th1 cell.

Mouse CNS+18-20 (also called CNS2) is similar to mCNS-22 in that it does not develop DNase hypersensitivity until Th1 or Th2 polarization (67), suggesting a role after the initial differentiation signal. Unlike these other two well-characterized CNS elements, CNS+18-20 does not have intrinsic *in vitro* enhancer activity. Rather, mCNS+18-20 has been shown to augment enhancer activity of mCNS+6 (79). Along these lines, the chromatin containing mCNS+18-20 loops into the interferon gamma promoter upon Th1

differentiation (28). From these data it appears mCNS+18-20 functions by enhancing the activity of mCNS-6 after the Th1 differentiation signal. If mCNS+18-20, or the human homolog at CNS+20 has an individual function remains to be determined.

In a naïve CD4+ T cell, the *Ifng* locus localizes to the peripheral portion of the nucleus, in close physical contact with the *Il4* locus (28). This interaction of alternatively expressed genes has been deemed a "poised chromatin hub." Upon Th1 stimulation the *Ifng* locus detaches from the *Il4* locus, but remains in the nuclear periphery (80). This is in stark contrast to most mammalian genes which adopt a central location in the nucleus upon transcriptional activation (81). It is unknown if the *Ifng* locus occupies foci of active RNA polymerase II called "transcription factories" before or after Th1 stimulation.

The studies on different CNS within the interferon gamma locus suggest individual CNS have distinct regulatory functions, which come together to drive proper regulation of a gene. It is unknown how diverse the function of long-range regulatory regions may be. Studies on the *Ifng* locus have been largely descriptive, and the one study that deleted CNS-22 i*n vivo* did not establish a mechanism by which this CNS functioned (74). How these individual CNS cooperate to drive cell-type specificity, proper histone modifications, nuclear localization and chromosomal looping, and transcription factor occupancy throughout the loci is unknown.

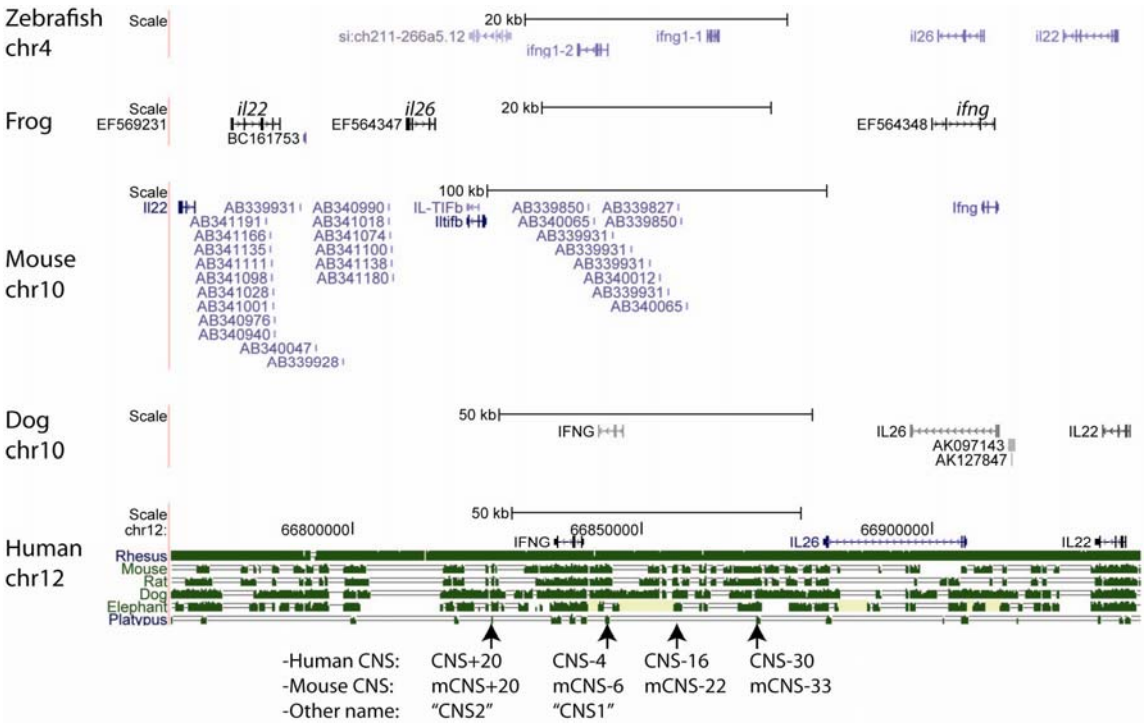

Figure 1-2 The *IFNG* locus.

Alignments of the human *IFNG* locus were made between humans, dogs, mice, frogs and zebrafish using the UCSC genome browser. Relative locations of human CNS+20, CNS-4, CNS-16 and CNS-30 are shown, as well as names for the mouse homologs and other names given to each CNS given in the literature. The mouse *Ifng* locus contains *Iltifb*, which is a full-length *Il22* duplication. In addition, retrotransposed pseudogenes with homology to *Cdc5l* are shown annotated on the mouse genome. The zebrafish genome contains at least one interferon gamma duplication, *ifng 1-1 (82)*. The predicted transcript *si:ch211-266a5.12* shows high homology to *ifng 1-2*, but expression has not been determined *in vivo*. Green hash marks are conserved sequences between humans and listed species

Interferon gamma and disease

Interferon gamma signaling pathways are thought to be critically involved in the host defense against intracellular pathogens. Collectively, mutations resulting in a lack of interferon-gamma signaling are termed "interferon-gamma deficiency." Commonly, interferon gamma deficiency is observed when the interferon gamma receptor is mutated, however mutations in STAT signaling pathways and the interferon gamma gene have been observed. Typically, interferon gamma deficiency is associated with susceptibility to mycobacterial infection (1) including disease and death from bacillus calmette-guerin (BCG), which is used as an attenuated vaccine to protect against *Mycobacterium tuberculosis* infection. In addition to susceptibility to mycobacterial infection, interferon gamma deficiency is associated with potentially fatal susceptibility to intracellular bacterial infections such as *Listeria monocytogenes* (83) as well as viral susceptibility, (84). Partial defects in interferon gamma signaling have been observed, and are again associated with BCG susceptibility and persistence of *tuberculosis* infection (85).

Evidence that IFN-γ is critical for murine health is, like for humans, overwhelming. Like humans with interferon gamma deficiency, mouse *Ifng* knockout models show susceptibility to M*ycobacterium tuberculosis* infection (86). Mice lacking interferon gamma are deficient in the host response to diseases other than viruses and intracellular bacteria. For example, interferon gamma knockout mice have altered immune responses to various cancers (87), and are susceptible to malarial infection (88). Another telling aspect of the relationship between interferon gamma and health are relationships made between murine models of autoimmunity and interferon gamma deficiency. In mouse

models, depletion of interferon gamma with neutralizing antibodies protects against colitis. However, in experimental systems where interferon gamma knockout CD4 cells are adoptively transferred into a host lacking T cells, colitis is maintained (89). These results suggest that interferon gamma is necessary for autoimmune colitis but that interferon gamma expression by CD4+ cells is not. As such, it is possible that interferon gamma produced by other lymphoid cells than CD4+ T cells contributes to disease pathogenesis. Interferon gamma knockouts have also been shown more susceptible to collagen-induced arthritis (90). As such, interferon gamma is clearly involved in autoimmune processes. In humans, an interferon gamma blocking antibody is safe and clinically effective in the treatment of Crohn's disease, but trials have not met clinical end points (91, 92). As such understanding interferon gamma regulation and variation in humans and its role in human autoimmune diseases provides a promising therapeutic option.

Specific Aims

Summary

Upon encounter with antigen a naïve CD4+ T-cell may be stimulated to differentiate into a Th1 cell or a Th2 cell. Th1 cells must express interferon-gamma (IFN-γ) while Th2 cells must not. *IFNG* spans 5 kb of genomic DNA, but is surrounded by up to 200 kb of evolutionarily-conserved, non-coding sequences (CNS). An 8.6 kb *IFNG* transgene does not confer Th1/Th2 selectivity. By using a transgenic bacterial artificial chromosome model, we have demonstrated that the much larger 200 kb region is sufficient to confer

Th1/Th2 selectivity. It is unknown why *IFNG* requires 200 kb of regulatory sequences and space. We hypothesize that appropriate timing and quantity of IFN-γ production is regulated by the coordinated action of numerous distal regulatory sequences. The long-term goal of this project is to determine how long-range distal elements regulate expression of a gene. The goal of this project is to determine the mechanism and function of *IFNG* distal regulatory elements. To address the above hypothesis and project goals I propose the following two aims:

Aim 1. To determine the necessary function of *IFNG* distal regulatory elements

Previous studies have identified multiple conserved noncoding sequences of the mouse *Ifng* locus which are sufficient for enhancer activity in cell-based assays. These studies do not directly address biology of the human *IFNG* locus or address necessary function in a genomic setting. Specifically, two hypothesizes could be made: One, all *IFNG* enhancers have the same necessary function; or two, each *IFNG* enhancer has a cell-type and stimulus specific necessary function. We hypothesize that multiple distal regulatory elements in the *IFNG* locus elements exist, in part, to accommodate cell-type and stimulus specific diversity. BAC transgenes will be created lacking CNS-77, CNS-30, CNS-16, CNS-4 and CNS+20. BAC transgene expression will be measured from developing and mature CD4+ Th1 and Th2 cells, CD8+ T cells, NK cells and NKT cells under T cell receptor signaling conditions or IL-12 and IL-18 signaling conditions. The results of this aim will determine how multiple distal regulatory elements determine cell and stimulus specific selectivity of *IFNG* expression and provide insight into how the immune system coordinates proper cytokine expression across many cell types.

Aim 2. To determine how distal regulatory elements determine covalent histone modifications

The interferon gamma locus develops cell-type specific covalent histone tail modifications, and recruits chromatin effector proteins in a cell-type and stimulus dependent manner. It is unknown if long-range distal elements are involved in, and regulate covalent histone modifications throughout the *IFNG* locus. Our preliminary data identify two distal elements which regulate interferon gamma transcription. Activating distal elements, such as CNS-30, are hypothesized to function in the recruitment of transcription factors to the *IFNG* locus, and to provide a chromatin environment permissive to gene expression. We hypothesize that distal regulatory elements establish the higher chromosomal structure of the *IFNG* locus. Chromatin immunoprecipitation (ChIP) will be used to determine the impact the BAC deletions have on histone modifications through the *IFNG* locus, as well as specifically testing the hypothesis that CNS-30 directs transcription factors to *IFNG*. The results of these experiments will elucidate how distal regulatory elements function to establish higher order chromatin structure.

CHAPTER II

Distal regions of the human *IFNG* locus direct cell-type specific expression

Abstract

Genes, such as *IFNG*, which are expressed in multiple cell lineages of the immune system, may employ a common set of regulatory elements to direct transcription in multiple cell types or individual regulatory elements to direct expression in individual cell lineages. By employing a BAC transgenic system, we demonstrate that *IFNG* employs unique regulatory elements to achieve lineage specific transcriptional control. Specifically, a one 1-kb element 30 kb upstream of *IFNG* activates transcription in T cells and NKT cells but not NK cells. This distal regulatory element contains a Runx3 binding site in Th1 cells, and is needed for RNA polymerase II recruitment to *IFNG*, but not absolutely required for histone acetylation of the *IFNG* locus. These results support a model whereby *IFNG* utilizes *cis*-regulatory elements with cell-type restricted function.

Introduction

Cell lineage-specific gene expression is a central challenge of multi-cellular life. Cell-type specific, developmentally regulated genes may be expressed in just one cell type or in multiple cell lineages. Developmentally regulated genes expressed in multiple lineages must be responsive to different arrays of stimuli, transcription factors, and chromatin

environments present in the varying cell types in which they are expressed. Conversely, expression must be actively restricted to appropriate cell lineages. In metazoans, lineage-specific expression is thought to be conferred by the use of distal regulatory elements (93, 94). However, mechanisms by which distal regulatory elements direct lineage specific gene expression are largely unknown. One possibility is that developmentally regulated genes employ a single common set of regulatory elements to direct transcription in all cell types expressing these genes. An alternate possibility is that genes employ unique cell-type specific distal regulatory elements to achieve cell-type specific expression.

IFN-γ is a cytokine produced by select cells of the innate and adaptive immune system. IFN-γ is most notably produced by CD4+ T-helper 1 cells, but is also produced by CD8+ cytotoxic T cells, natural killer cells, Natural Killer T (NKT) cells, macrophages and dendritic cells. IFN-γ must not be transcribed in other cell types, such as Th2 cells, developing T cells, and cells outside the immune system. A multitude of transcription factors have been implicated in regulating *Ifng,* including T-bet, STAT4, Runx3, GATA3 and Hlx (59). The transcription factor T-bet is necessary and sufficient for production of IFN-γ by CD4+ T cells (69). CD8+ T cells and natural killer cells express a second T-box containing transcription factor called eomesodermin (95). Because of this second transcription factor, CD8+ T cells do not show the strict reliance on T-bet for *Ifng* transcription under *in vitro* conditions as do CD4+ T cells (96, 97). These differences in T-box proteins show that transcription factor regulation of *Ifng* varies among the different cell types which produce IFN-γ.

Mice carrying an 8.6 kb human *IFNG* transgene express high levels of human IFN-γ in both Th1 and Th2 cells. Th2 cells should not express IFN-γ. In contrast, a 190

kb bacterial artificial chromosome transgene with the human *IFNG* gene, and 90 kb of both upstream and downstream sequences does recapitulate Th1/Th2 selective expression (98). This indicates that one or more distal regulatory elements are critical for cell-type specific regulation of *IFNG*. Regulatory elements within the *Ifng* locus (67, 75, 79, 99), and other developmentally regulated loci (93), may exist within distal conserved non-coding sequences. For example, a CNS located -22 kb from the mouse *Ifng* transcription start site (CNS-22) is critical for mouse IFN-γ production in T cells and NK cells (74). Other work has shown that the CNS within the *Ifng* locus acquire cell-type specific histone marks which correlate with IFN-γ expression in Th1 cells, and histone marks which correlate with repression in Th2 cells (59). However, these studies do not explain how distal regulatory elements within the *Ifng* locus confer cell-type specific expression. They also do not explain why *Ifng* possesses a large *cis*-regulatory region and requires numerous transcription factors for proper regulation. Because the *IFNG* promoter alone does not confer cell-type specific expression, we sought to determine if a single distal CNS within the *IFNG* locus dictates cell-type specific expression in all cell types expressing *IFNG* or if separate CNS are employed to direct *IFNG* transcription by distinct cell lineages.

To determine the role of distal regulatory elements within the *IFNG* locus, we created transgenic mice carrying a 190 kb transgene of the human *IFNG* locus. Activity of the BAC transgene mirrored many aspects of regulation of the endogenous gene including Th1/Th2 selectivity and reliance upon T-bet and Stat4 for expression of human IFN-γ in CD4+ cells and CD8+ cells. To determine which regions of the 190 kb BAC transgene contain distal regulatory elements, we pursued an unbiased deletion strategy.

We created 4 new BAC transgenic mice with 40 kb deletions of upstream or downstream sequences of the *IFNG* locus. Using the larger deletions as a guide, we created BAC transgenic mice with 1 kb deletions of individual CNS. We found that a CNS located at -30 kb, relative to the transcription start site, is necessary for human IFN-γ production in T cells and NKT cells, but not NK cells. This CNS binds the transcription factor Runx3 and is necessary to recruit RNA polymerase II (RNAP II) to *IFNG* in T cells, but not absolutely required for establishment of histone acetylation across the *IFNG* locus. Together these results show that distal CNSs of a human gene have cell-type restricted function.

Materials and Methods

Mice.

C57BL/6, C57BL/6.*Stat4* -/- mice were obtained from Jackson Laboratory (Bar Harbor, ME), bred in the Vanderbilt University animal facilities, and used between 4-5 weeks of age. *Tbx21* (T-bet) -/- mice have been previously described (69, 98). Research using mice complied with all relevant institutional and federal guidelines and policies.

Cell Purification and Cultures.

CD4+ and CD8+ T cells were purified from splenocytes by negative selection as previously described (98). Purified CD4+ or CD8+ T cells, 1 x 10^5 cells/ml, were stimulated with immobilized anti-CD3 (2C11, ATCC, Manassas, VA) and irradiated antigen presentation cells (1 x 10^6 cells). IL-12 (5 ng/ml) and anti-IL-4 (11B11, ATCC)

were added to cultures to generate Th1/Tc1 effector cells and IL-4 (5 ng/ml) and anti-IFN-γ (10 μg/ml, R4-642, ATCC) were added to generate Th2/Tc2 cultures. Anti-IFN-γ was omitted from Th2 cultures used for ELISA assays. Assays were preformed at day 3 to measure the primary response. To analyze the effector response, T cells were cultured for 5 days, harvested and re-stimulated with plate bound anti-CD3 alone, a mock treatment, 10 ng/ml IL-12, 10 ng/ml IL-18, or 10 ng/ml IL-12 and 10 ng/ml IL-18. Anti-CD3 coated plates were prepared by treating tissue culture plates with a 10 μg/ml anti-CD3 solution overnight at 4ºC. Splenic CD8+ T cells were also purified from OT-1 transgenic mice and stimulated with antigen peptide, irradiated spleen cells and IL-2 as outlined in the results section. NK cells and dendritic cells were isolated by negative selection and macrophages were isolated by positive selection using magnetic sorting (Miltenyi Biotech), according to manufacturer's instructions. NK cells, macrophages and dendritic cells were stimulated as described in the text for 48 hours. For NKT cultures, $5X10^5$ splenocytes were stimulated with 100 ng/ml αGalCer (kindly provided by Laura Gordy). Cultures were harvested after 48 hours. IFN-γ assays were performed by ELISA with mAbs recommended by BD Pharmingen.

Intracellular cytokine staining.

T cells were harvested on day four and restimulated with 50 ng/ml PMA and 1 μM ionomycin for two hours followed by addition of GolgiPlug (BD) and culture for an additional four hours. For macrophage and dendritic cell experiments, mechanically disrupted spleens were stimulated with PMA/ionomycin for five hours, briefly resuspended in water to lyse red blood cells, and processed for intracellular cytokine

staining. Cells were washed twice in ice-cold FACS buffer (2% FCS, 0.1% NaN_3 in PBS) and labeled with fluorescent antibodies as outlined in the results section for 30 minutes. Cells were permeabilized and fixed with Cytofix/Cytoperm (BD) according to the manufacturer's instructions. Flow Cytometry experiments were performed in the VUMC Flow Cytometry Shared Resource. The VUMC Flow Cytometry Shared Resource is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404). T cell cultures were gated on CD4+ or CD8+ subsets for analysis.

Preparation of transgenic reporter lines.

The human *IFNG* BAC clone RPCI11-444B24 (Invitrogen, Carlsbad, CA) was moved into the EL250 *E. coli* strain to allow controlled recombination and FLP protein expression (100). To mediate deletions with the BAC, 50-nucleotide homology arms were designed to flank segments of the BAC. Long PCR primers containing 5' homology arms were synthesized (Table 2-1) and used to PCR-amplify a bacterial tetracycline resistance cassette flanked by FRT sites as previously described (101). This created "deletion cassettes" that were transformed into recombination-competent EL250/BAC cells (100). Tetracycline-resistant colonies were screened by PCR across recombination junctions and restriction digests were performed to verify predicted recombinations for each BAC deletion. The cassette was deleted by inducing FLP expression, leaving behind only a 34-bp FRT sequences in place of the deleted material. Final verification of correct modification and absence of unwanted alterations occurring during the modification

process was confirmed by additional PCR analysis, restriction enzyme digestion and pulsed-filed gel electrophoresis, and direct BAC sequencing.

To prepare BAC clone DNA for microinjection, BACs were purified using Nucleobond Giga-prep columns (Clontech) followed by treatment with Plasmid Safe DNase to remove sheared DNA (Epicenter). The remaining circular BAC DNA was desalted using an Amicon 100 centrifugal filtration column. Transgenic mice were prepared by standard pronuclear injection using circular BAC DNA, which is effective for generating transgenic founders (101). Positive founder mice were identified by PCR analysis of tail DNA using appropriate primers for the human *IFNG* gene. We also used PCR with human-specific primers at various points across the human *IFNG* BAC sequence to verify that all regions of the BAC transgenes were still present after integration into genomic DNA.

Chromatin Immunoprecipitation.

ChIP assays were preformed according to Millipore's online protocol with minor variations: cells were harvested by centrifugation; each protein A Agarose-antibody/chromatin wash was performed two times for ten minutes each and DNA was isolated by phenol-chloroform extraction. Immunoprecipitations were performed using either an anti-acetyl H4 antibody (Upstate 06-866), anti-H3k9Me2 (ab-1220), anti-Pol II (SantaCruz Biotech 899X), anti-Runx3 (Active Motif 39301) or normal rabbit IgG control. Significance by ANOVA was determined using PrismGraph software. For determining significance at specific genomic locations, a student's *t*-test was preformed with Bonferroni's inequality applied to the calculated probability.

Results

Stat4 and T-bet dependence of reporter gene expression in T cells

To initiate these studies, we examined activity of the 190 kb *IFNG*-BAC transgene in wildtype, *Stat4* -/- and *Tbx21* -/- CD4+ T cells. CD4+ T cells were cultured for three days with plate bound anti-CD3 under Th1 or Th2 conditions (primary stimulation). To measure secondary, effector responses, cells were cultured for five days, harvested and restimulated with plate bound anti-CD3 alone for an additional two days. We found that absence of either Stat4 or T-bet markedly reduced the activity of the *IFNG*-BAC transgene in Th1 cells during either primary or secondary, effector responses (Figure 2-1, left panel). Production of human IFN-γ by BAC transgenic CD4+ T cells was reduced by T-bet or Stat4 deficiency to a similar degree as production of murine IFN-γ (Figure 2-1, right panel). These results demonstrate that the *IFNG*-BAC transgene exhibits the same critical T-bet and Stat4 requirements for activity in the CD4 T cell lineage, as does the endogenous *Ifng* gene.

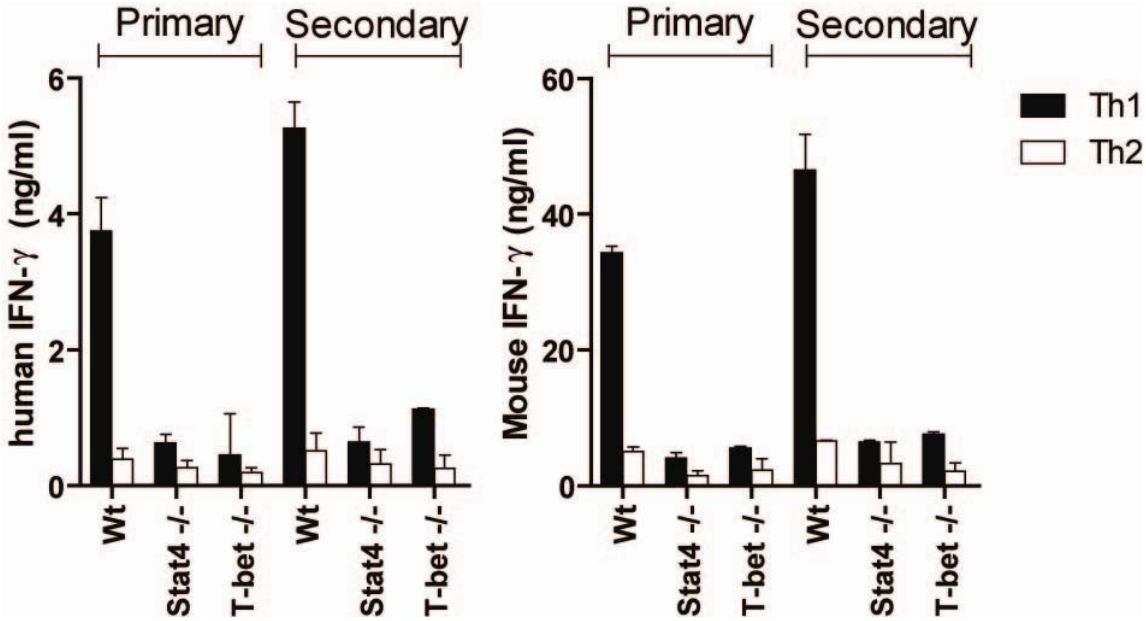

Figure 2-1 Expression of human IFN-γ from a 190 kb *IFNG* BAC reporter transgene in CD4+ T cells is Stat4 and T-bet dependent.
Transgenic CD4+ T cells from C57/BL6 (Wt), C57/BL6.*Tbx21* -/- (T-bet -/-), or C57/BL6.Stat4 -/- mice were cultured under Th1 or Th2 conditions for three days (primary stimulation) or five days. After 5 days, cultures were harvested and re-stimulated with plate-bound anti-CD3 only for 2 days (secondary stimulation). Levels of human IFN-γ (left panel) and of murine IFN-γ (right panel) were determined by ELISA.

To determine if the 190 kb *IFNG*-BAC transgene displays cell-type specific dependence upon the transcription factor T-bet, we next examined its expression in CD8+ T cells. We cultured wildtype, T-bet -/- and Stat4 -/- BAC transgenic CD8+ T cells with anti-CD3 and antigen presenting cells under Th1 or Th2 polarizing conditions. Similar to CD4+ T cells, absence of Stat4 reduced the ability of CD8+ T cells to produce human IFN-γ. Unlike the CD4+ T cells, transgenic CD8+ cells were able to produce human IFN-γ in a T-bet -/- background (Figure 2-2A). These results are consistent with our earlier observations that T-bet does not influence endogenous IFN-γ production in CD8+ T cells that have been stimulated by polyclonal activation with anti-CD3 (65, 95, 102). T-bet -/- CD8+ T cells stimulated *in vivo*, or *in vitro* with antigen specific stimulation produce significantly reduced amounts of IFN-γ when compared to WT CD8+ T cells (96, 97). *IFNG*-BAC transgenic mice were backcrossed onto either wild type OT-1 TCR or T-bet -/- OT-1 TCR backgrounds. CD8+ T cells were stimulated with the OT-I ovalbumin peptide, SIINFEKL, antigen presenting cells and IL-2 for five days. After five days, cultures were harvested and restimulated with SIINFEKL peptide and irradiated spleen cells. Cultures were harvested 2 days later and levels of human and murine IFN-γ were determined by ELISA. Under this antigen-specific stimulation, transgenic CD8+ T cells produced markedly reduced levels of murine and human IFN-γ in the T-bet deficient background (Figure 2-2B). Taken together, these results demonstrate that the *IFNG*-BAC transgene exhibits the same Stat4 and T-bet dependency as the endogenous *Ifng*-gene in CD8+ T cells.

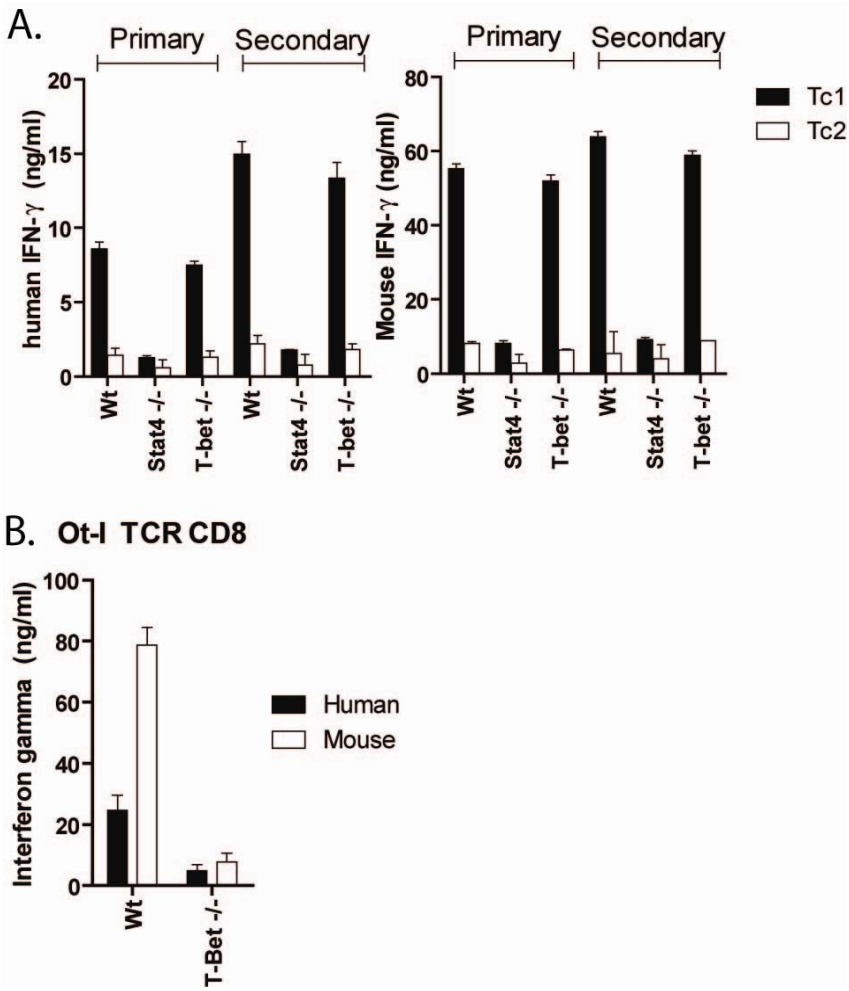

Figure 2-2 Regulation of *IFNG*-BAC transgene activity in CD8+ T cells.
(A) CD8+ T cells were purified from *IFNG* BAC positive wild type, Stat4 -/-, or T-bet -/- mice and were cultured under Th1 or Th2 polarizing conditions as in Figure 1. Cultures were harvested after 3 d (primary responses) or 2 d after re-stimulation with anti-CD3 (effector responses) and were analyzed for levels of human or mouse IFN-γ by ELISA. (B) CD8+ T cells were also purified from *IFNG* BAC positive wildtype or T-bet -/- OT-I TCR transgene positive mice and stimulated with peptide antigen, APC and IL-2. After 5 d, cultures were harvested and re-stimulated with peptide and APC. After an additional 2 d, cultures were harvested and IFN-γ ELISA assays performed.

Taken together, our results demonstrate that the *IFNG*-BAC transgene is sufficient to direct a) high level expression, b) antigen-induced expression, c) T-bet and Stat4 dependency, and d) T1/T2 selective expression of human IFN-γ. Importantly, our results demonstrate that the *IFNG*-BAC transgene recapitulates key regulatory features of *Ifng* transcription. To identify key distal regulatory elements within the *IFNG*-BAC, we next performed a deletion analysis of the *IFNG*-BAC.

40 kb regulatory regions within the 190 kb *IFNG* BAC transgene

To identify elements within the 190 kb BAC transgene essential to drive faithful production of human IFN-γ, we first pursued an unbiased deletion strategy. Positions of individual conserved non-coding sequences within the 190 kb *IFNG* locus are shown in figure 2-3. While the distance between each CNS varies slightly among species, the order of individual CNS across the locus is absolutely conserved (Figure 2-3 bottom). It is unknown how many CNSs across the 190 kb locus participate in regulating *IFNG* transcription. We divided the transgene into four ~40 kb blocks for analysis, Δ1-4 (Figure 2-3, Table 2-1). We constructed four deletions in the BAC representing each of these 40 kb blocks, and prepared transgenic mice. We prepared a fifth 80 kb BAC deletion lacking the Δ3/4 region (Figure 2-3). We were able to leave the human homologs to mouse CNS-22 (74), CNS-6 (37, 75) and CNS+18-20 (79), which have been studied in various reports, in the undeleted region. This way all deletions resulted in removal of novel regulatory elements.

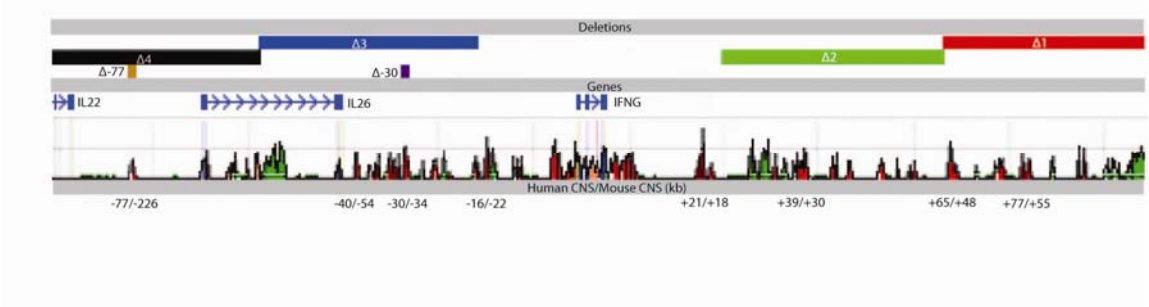

Figure 2-3 Deletions made in this study.
Positions of 40 kb deletions from the *IFNG*-BAC transgene are shown: red Δ1, green Δ2, blue Δ3, black Δ4, and small 1 kb CNS deletions, brown Δ-77, purple Δ-30. Relative positions of human *IFNG*, *Il26*, and *Il22* in the *IFNG*-BAC transgene are shown below. Evolutionary Mouse-Human sequence conservation, locations of CNS (red, > 70% sequence conservation between mouse and human spanning ≥ 200 bp) and locations of transposons (green) within the 190 kb human *IFNG*-BAC transgene as identified using the dcode website (www.dcode.org).

Table 2-1. Deletion locations

The locations of the BAC deletions, and the entire BAC transgene are presented bellow. Note: Fig 2-3 is "flipped" in regard to absolute genomic location, so that the beginning of the IFNG gene appears on the left of the figure. Genomic locations are based on March 2006 (NCBI36/hg18) assembly.

Deletion	Location
190 kb BAC	chr12:66740833-66931792
Δ1	chr12:66740833-66775765
Δ2	chr12:66775767-66814449
Δ3	chr12:66857579-66895638
Δ4	chr12:66895640-66931792
Δ-30	chr12:66869614-66870500
Δ-77	chr12:66917565-66918546
IFNG	chr12:66834817-66839788

To determine which 40 kb blocks regulate *IFNG* transcription, CD4+ T cells harboring the different transgenes were cultured under Th1 or Th2 conditions. Cultures were harvested after primary or secondary stimulation and human and murine IFN-γ levels were determined by ELISA. Removal of Δ1 (+59 kb to +93 kb relative to the human *IFNG* transcriptional start site) or Δ2 (+20 to +59 kb) did not affect human IFN-γ production by either primary or effector CD4+ T cell cultures (Figure 2-4A). In contrast, the Δ3/4 (-92 to -18 kb) deletion caused a dramatic loss of human IFN-γ production after primary stimulation, and a complete loss of human IFN-γ production after secondary stimulation of effector cell cultures (Figure 2-4B). Results for Δ3 (-56 to -18 kb) mice were similar to Δ3/4 mice (not shown), and as such Δ3/4 mice were analyzed further, rather than Δ3 mice, to rule out cooperativity effects between the truncated, non-expressed *IL22*, and its regulatory elements, and *IFNG* regulatory elements. Deletion of only Δ4 (-92 to -56 kb) resulted in an increase in human IFN-γ production in both primary and effector cell cultures (Figure 2-4B). Levels of murine IFN-γ were unchanged in cultures with the different BAC transgenes (Figure 2-4C). We also determined human and mouse IFN-γ levels by ELISA from CD8+ T cells cultured under Ct1 or Ct2 conditions. Like CD4+ T cells, deletion of Δ4 resulted in an increase in human IFN-γ in both primary and effector CD8+ cell culture supernatants (Figure 2-4D). The Δ4 deletion removed the *IL26* gene, which is a pseudogene in the mouse, and the 3' end of *IL22* contained on the *IFNG*-BAC.

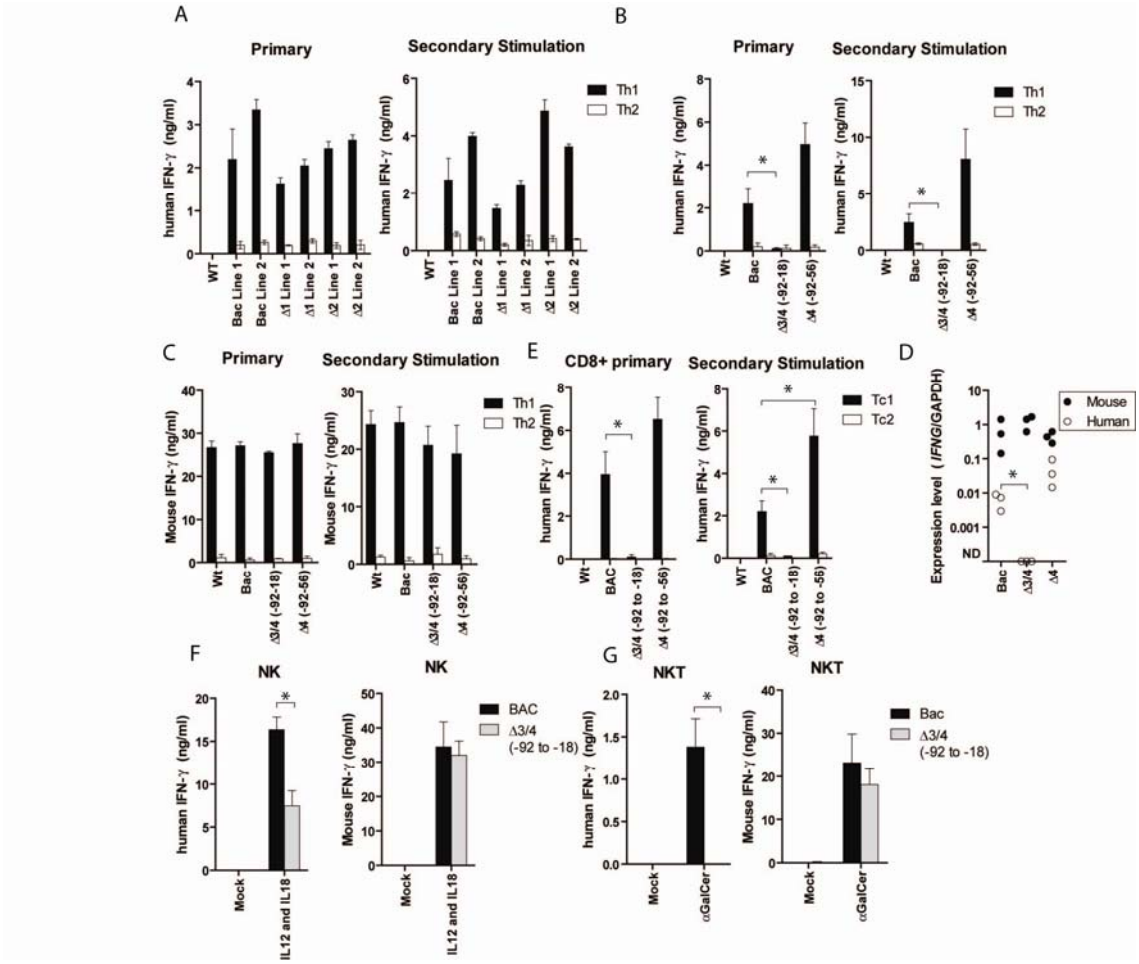

Figure 2-4 Functional consequences of large 40 kb deletions from the 190 kb human *IFNG*-BAC transgene.

(A-D) Transgenic CD4+ T cells were cultured under Th1 or Th2 conditions as in figure 1. (A) Human IFN-γ levels were determined for *IFNG*-BAC, Δ1 and Δ2 CD4+ T cells, (B) determined for *IFNG*-BAC, Δ3/4 and Δ4 CD4+ T cells or (C) murine IFN-γ levels were determined for *IFNG*-BAC, Δ3/4 and Δ4 CD4+ T cells. (D) *IFNG*-BAC, Δ3/4 and Δ4 CD4+ T cells were cultured under Th1 conditions for three days and mRNA levels were determined by quantitative PCR. Data points represent results of independent experiments. (E) CD8+ T cells were cultured under Tc1 or Tc2 conditions as in Figure 2 and human IFN-γ levels were determined by ELISA. (F) Freshly isolated NK cells were stimulated with nothing, IL-12, IL-18, or IL-12 + IL-18. After 48 hours culture IFN-γ levels were determined by ELISA. (G) *IFNG*-BAC or Δ3/4 BAC transgenic splenocytes were stimulated with αGalCer for two days. IFN-γ levels were determined by ELISA. (All except D) Results are averages of at least three independent experiments. Error bars are s.e.m. * $p < 0.05$

To observe the effect of these deletions at the transcript level, we verified our results by quantitative PCR of cDNA made from day three Th1 cultures (Fig 2-4E). Taken together, these results identify two novel 40 kb regions which regulate *IFNG* transcription in T cells. The -56 to -18 kb region contains elements necessary for human IFN-γ production, while the -92 to -56 kb region represses *IFNG* transcription. Notably, none of these large deletions resulted in increased production of human IFN-γ in Th2 cells. This phenotype is predicted based upon observations that mice carrying only an 8.6 kb *IFNG* transgene fail to repress human IFN-γ in Th2 cells (98). These results suggest that additional regulatory element(s) exist within the undeleted regions, -18 kb to +20 kb from the *IFNG* transcription start site, which govern Th1/Th2 selectivity.

The -92 to -18 kb region is dispensable for IFN-γ production in innate immune cells

To determine if function of regulatory regions within the -92 to -18 kb region is T cell specific, we isolated NK cells from BAC transgenic mice or Δ3/4 BAC transgenic mice and stimulated them with IL-12 and IL-18. To our surprise, in contrast to Δ3/4 BAC transgenic effector Th1 and Tc1 cells, Δ3/4 BAC transgenic NK cells robustly produced human IFN-γ (Figure 2-4F). To determine if dependence upon the -92 to -18 kb region is T cell intrinsic, regardless of expression of NK cell receptors, we examined human IFN-γ expression in Natural Killer T (NKT) cells. NKT cells are cells of the T cell lineage which express a semi-invariant T cell receptor, express natural killer cell surface markers and respond to glycolipid antigens (103, 104). NKT cells are the sole cell population stimulated by the NKT cell ligand alpha-galactosylceramide (αGalCer). Δ3/4 or BAC transgenic splenocytes were stimulated with the NKT cell ligand αGalCer. After two

days, human IFN-γ levels were determined. BAC transgenic, but not Δ3/4, NKT cells produced human IFN-γ in response to αGalCer (Figure 2-4G). Taken together, these results demonstrate that the -92 to -18 kb region is necessary in T cells and NKT cells, but not necessary in innate immune cells such as NK cells.

CNS-30 is necessary for human IFN-γ production by T cells

Having identified two large distal regions within the human *IFNG*-BAC transgene that differentially regulated *IFNG* transcription, we sought to identify specific CNS within these regions that recapitulated regulatory properties of these larger genomic regions. To identify the regulatory element responsible for the loss of human IFN-γ production seen in Δ3/4 BAC transgenic T cells, we focused on a conserved noncoding sequence located at -30 kb (CNS-30) from the *IFNG* promoter. The mouse homolog of CNS-30, located in the mouse genome at -34 kb, becomes histone acetylated in T cells, but not in freshly isolated NK cells and cultured NK cells. We examined mouse CNS-34 for conserved binding sites for transcription factors known to regulate *Ifng.* We identified two conserved AML (also known as Runx) binding sites within the CNS. Runx3 has been shown to be necessary for sufficient *Ifng* production in T cells (105), but not necessary for *Ifng* production in NK cells (106). To verify Runx3 binding to CNS-34, we cultured CD4+ T cells for three days under Th1 or Th2 conditions and preformed chromatin immunoprecipitation (ChIP). We observed Runx3 binding predominantly to mouse CNS-34 and to the *Ifng* promoter in CD4+ Th1 cells (Fig 2-5A). Considering these properties, we reasoned that CNS-30 may be the site within the -56 to -18 kb region required for

efficient *IFNG* transcription. We prepared a new BAC construct harboring a 1 kb deletion of a CNS-30 and produced new transgenic mice (Δ-30, Figure 2-3).

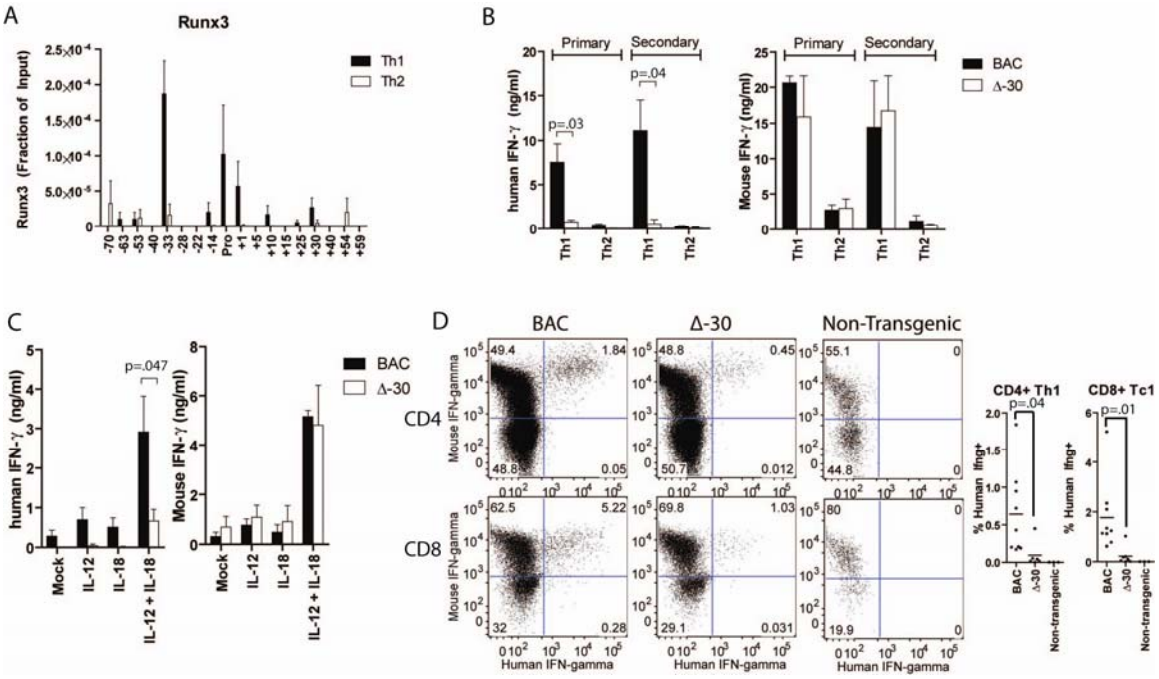

Figure 2-5. CNS-30 is necessary for human IFN-γ production by T-cells.
Cells were cultured from *IFNG*-BAC, Δ3/4 BAC or Δ-30 BAC transgenic mice. (A) CD4+ T cells were cultured for three days under Th1 or Th2 conditions and processed for ChIP assays with antibodies specific for Runx3 or IgG. Values for IgG controls were subtracted from means. (B) CD4+ T cells were cultured under Th1 or Th2 polarizing conditions. Levels of human and mouse IFN-γ were determined by ELISA. (C) Effector Th1 cells were stimulated with nothing, IL-12, IL-18, or IL-12 + IL-18. After 48 hours culture IFN-γ levels were determined by ELISA. (D) CD4+ and CD8+ T cells were cultured for four days under Th1 conditions, restimulated with PMA/Ionomycin and analyzed for intracellular cytokine staining. Cells are gated on CD4+ or CD8+ populations. Results of individual experiments are shown.

Δ-30 or *IFNG*-BAC CD4+ and CD8+ transgenic cells were cultured for primary and secondary stimulation as described above. We observed a dramatic reduction in human IFN-γ production by Δ-30 T cells in both the primary and the secondary Th1 cultures (Figure 2-5B). We next determined if CNS-30 had an agonist-specific role. We harvested day 5 effector Th1 cells and restimulated them with either plate bound anti-CD3 or IL-12 and IL-18. Loss of human IFN-γ was evident in effector Th1 cells stimulated with anti-CD3 and cells stimulated with IL-12 and IL-18 (Figure 2-5C). Our results were not due to differences in culture conditions as murine IFN-γ levels were unchanged (Figure 2-5 B, C). We next sought to distinguish if CNS-30 regulated the percentage of T cells which became IFN-γ producers, and thus by definition Th1 cells, or the amount of IFN-γ produced on a per-cell basis. We restimulated day four Δ-30 or *IFNG*-BAC Th1 cells with PMA/Ionomyocin and measured mouse and human IFN-γ levels by intracellular cytokine staining (Figure 2-5D). The Δ-30 deletion reduced the overall percentage of CD4+ T cells that became human IFN-γ positive. As such, our data indicated that CNS-30 is necessary for human IFN-γ production in Th1 cells in response to both T cell receptor stimulation and IL-12 and IL-18 costimulation.

CNS-30 is dispensable for IFN-γ production in innate immune cells

We next isolated NK cells from BAC transgenic mice or Δ-30 BAC transgenic mice and stimulated them with IL-12, IL-18 or a combination of both cytokines. Like transgenic NK cells lacking the Δ3/4 (-92 to -18 kb) region, Δ-30 natural killer cells were able to robustly produce human IFN-γ (Figure 2-6A). We also measured *IFNG* mRNA

levels in CD4+ Th1 cells after TCR stimulation and NK cells after IL-12 and IL-18 stimulation. We observed a ten-fold reduction of human *IFNG* mRNA in Δ-30 T-cells, relative to *IFNG*-BAC transgenic T cells, but equivalent production of *IFNG* mRNA by NK cells from the two transgenic lines (Figure 2-6B). These results demonstrate that CNS-30 is a regulatory element necessary for human *IFNG* transcription in T cells, but is dispensable for transcription in NK cells.

Natural killer cells are members of the innate immune system which arise from the lymphoid cell lineage. To determine if CNS-30 was necessary for sufficient IFN-γ production in innate immune cells of the myeloid cell lineage, we examined human IFN-γ expression in macrophages and dendritic cells. For macrophage isolation, we purified CD11b+ cells and for dendritic cell isolation we purified CD11c+ cells. Δ-30, Δ3/4 and BAC transgenic CD11b+ and CD11c+ cells were isolated and stimulated with IL-12 and IL-18. Like natural killer cells, transgenic Δ-30 and Δ3/4 CD11b+ and CD11c+ cells were able to produce human IFN-γ at levels comparable to the complete *IFNG*-BAC transgenic controls (Figure 2-6C). We purified CD11c+, CD8+ cells and measured supernatant IFN-γ levels after 48 hours of IL-12 and IL-18 stimulation. We did not detect human IFN-γ in these culture conditions. Human IFN-γ expression in our *ex vivo* culture assay was stimulation dependent, and we did not observe spontaneous IFN-γ production.

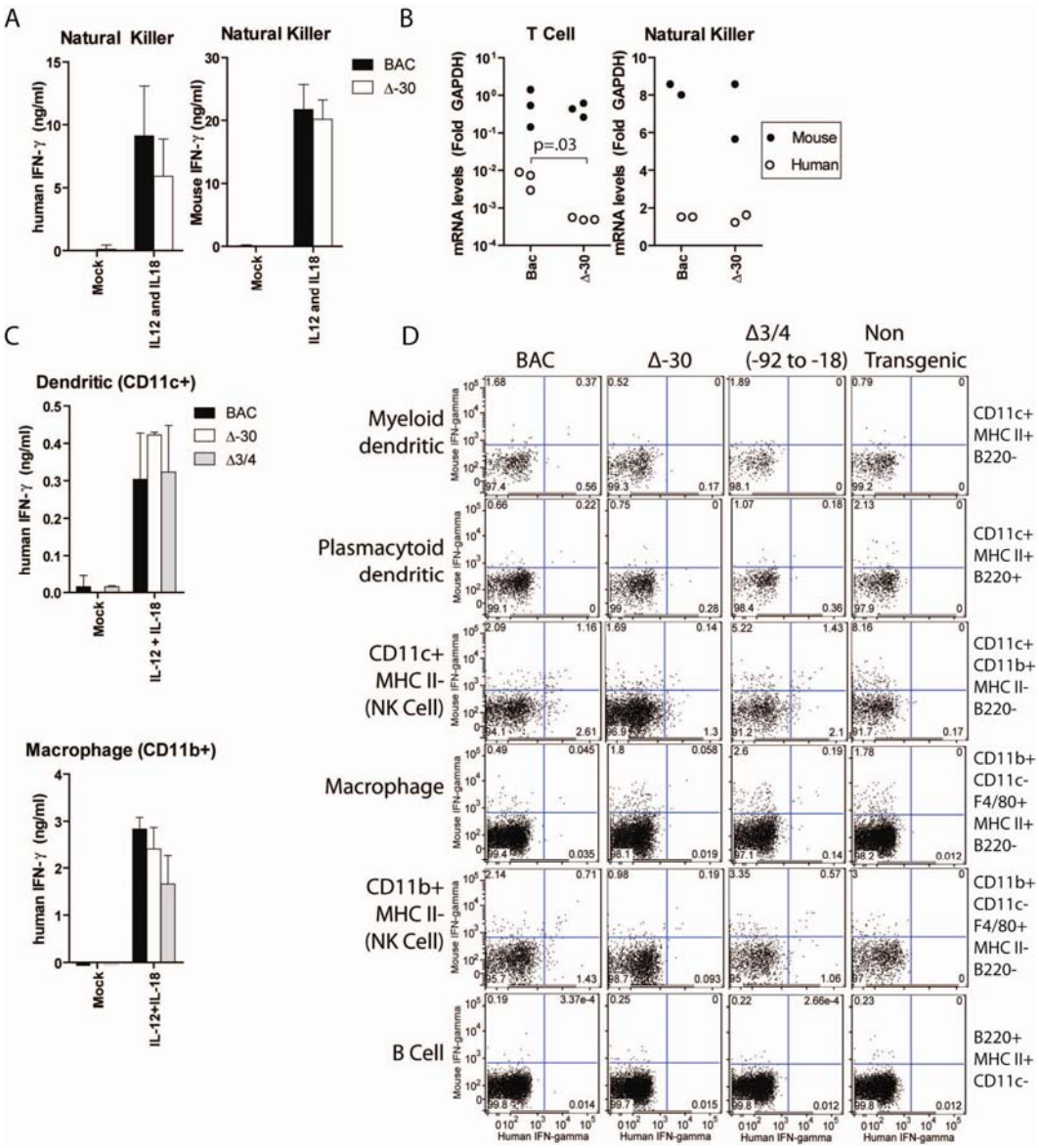

Figure 2-6 CNS-30 is not necessary for human IFN-γ production in NK cells, macrophages and dendritic cells

(A) Freshly isolated NK cells were stimulated with nothing, IL-12, IL-18, or IL-12 + IL-18. After 48 hours culture IFN-γ levels were determined by ELISA. (B) *IFNG*-BAC and Δ-30 CD4+ T cells were cultured under Th1 conditions for three days (left panel) or NK cells were cultured for two days with IL-12 and IL-18 (right panel) and mRNA levels were determined by quantitative PCR. Data points represent results of individual experiments. (C) Freshly isolated macrophages or dendritic cells were cultured for two days stimulated with nothing, or with IL-12 and IL-18 and culture fluids were harvested and analyzed for IFN-γ levels by ELISA. Results are averages of at least three independent experiments. Error bars are s.e.m. (D) Whole spleen was stimulated with PMA/Ionomycin and analyzed for intracellular cytokine staining.

To determine which CD11b+ and CD11c+ cell populations produced human IFN-γ, we stimulated *IFNG*-BAC, Δ3/4, Δ-30 and non-transgenic splenocytes with PMA/Ionomycin and determined human and mouse IFN-γ expression by intracellular cytokine staining. After excluding dead cells by forward and side scatter, we characterized cell populations based on MHC II, B220, CD11b, CD11c and F4/80 staining (Figure 2-6). The NK1.1 surface marker was not used to distinguish NK cells because these experiments were performed on a BALB/C background. Among CD11b+ and CD11c+ populations, IFN-γ expression was low in MHC II+ dendritic cell and macrophage populations. However, human IFN-γ was detected among MHC II-, CD11b+, CD11c+, B220- and MHC II-, CD11b+, CD11c-, B220-, F4/80+ populations (Figure 2-8D). These MHC II- populations have been described as an IFN-γ producing NK cell subset (107). Unlike CD4+ and CD8+ T cells, in which human IFN-γ was almost always co-expressed with mouse IFN-γ (Figure 2-5D), human IFN-γ expression in CD11b+ and CD11c+ cells was predominantly in the mouse IFN-γ negative cell population. Human IFN-γ staining was comparable between *IFNG*-BAC and Δ3/4 CD11b+ and CD11c+ cells. Thus, unlike T cells and like NK cells, CD11b+ and CD11c+ cells do not require either CNS-30 or the larger Δ3/4 genomic region for sufficient IFN-γ production.

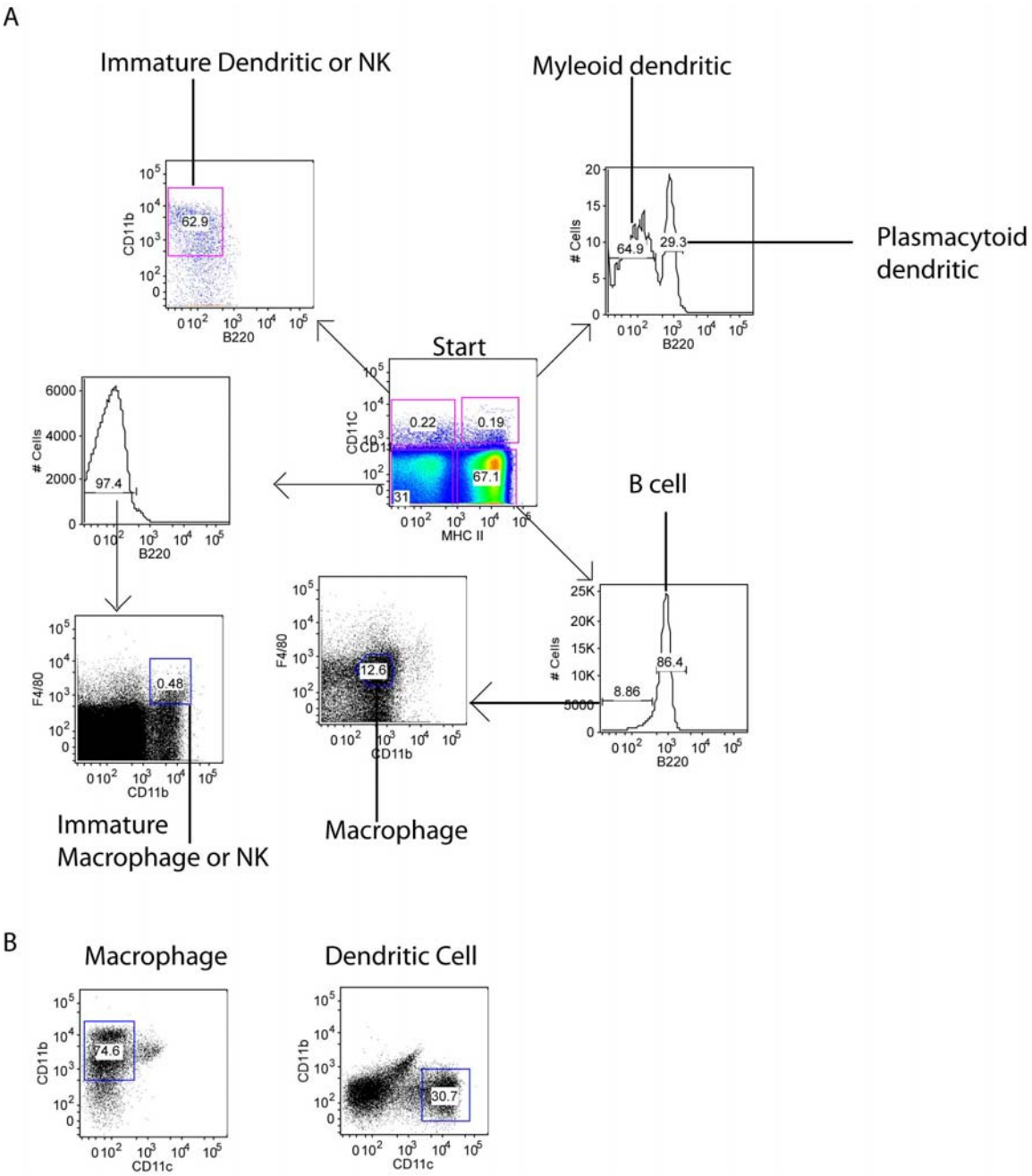

Figure 2-7 Intracellular cytokine staining (A) (Left) Representative data for intracellular cytokine staining in Figure 2-6. After red blood cell lysis and PMA/Ionomyocin stimulation, whole splenocytes were subject t o intracellular cytokine staining. Cells were gated on B220, MHC II, F4/80, CD11b and CD11c. IFNG- BAC splenocytes are used as the example cell population. (B) Representative cell purities for ex vivo cultures used in Figure 2-5C

Copy Number Variation

We also determined if human IFN-γ production varied with relative transgene copy number. We assessed transgene copy number by standard techniques using quantitative PCR and genomic primer pairs positioned across both the human BAC transgene and the endogenous murine locus in the different transgenic lines. Because we did not have variation in copy number between *IFNG*-BAC transgenic lines, we selected mice carrying an *IFNG*-BAC transgene where we had removed CNS-77 (Δ-77). We selected Δ-77 BAC transgenic lines for our analysis since these had the greatest variation in transgene copy number (Figure 2-8). Since we were unable to determine absolute copy number by this approach, results are expressed as copy number relative to the *IFNG*-BAC transgenic line. Flow cytometry and intracellular cytokine staining were employed to determine levels of human and murine IFN-γ producing cells in Δ-77 BAC transgenic CD8+ T cells cultured under Th1 polarizing conditions from three separate founder lines. This analysis demonstrated that, among the three Δ-77 BAC transgenic lines, human IFN-γ production by the Δ-77 BAC transgenic lines was proportional to transgene copy number.

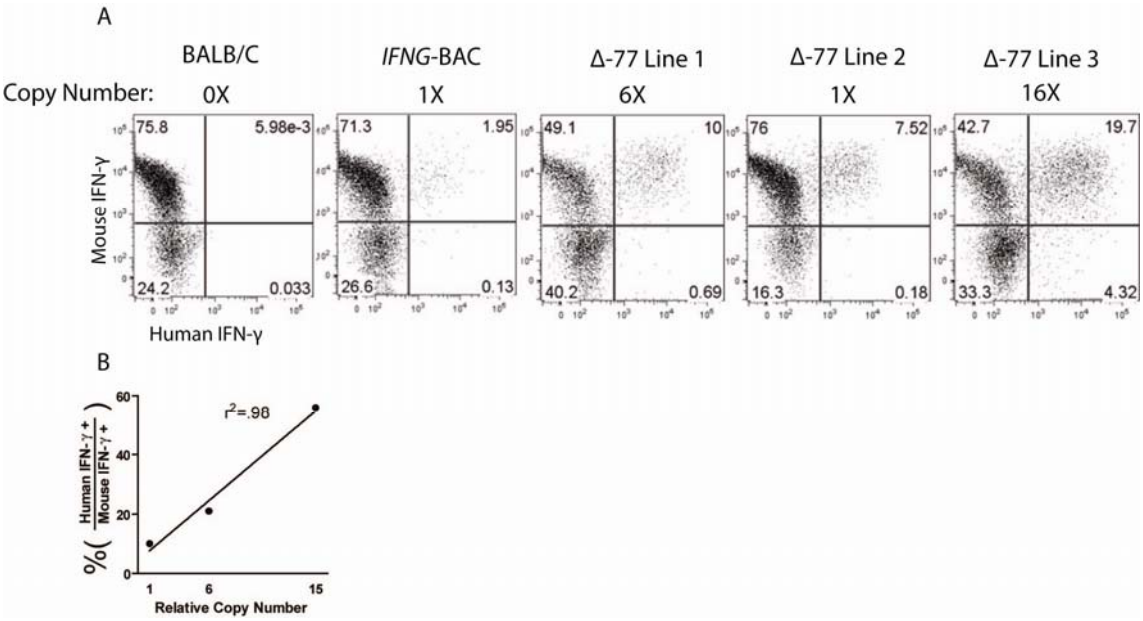

Figure 2-8 Copy number dependent expression

(A) Relative copy number of three different -77 lines was determined by quantitative PCR analysis of genomic DNA. CD8+ T cells were cultured for 4 days under Th1 polarizing conditions and were analyzed for IFN- levels by intracellular cytokine staining. Cells were gated on CD8+ cell populations. (B) Replicates were subjected to a linear regression analysis.

CNS-30 is required for RNAP II recruitment, but not histone acetylation

In eukaryotes, DNA is wrapped around packaging histone proteins. These histones are subject to a series of enzymatically-catalyzed chemical modifications that produce a 'histone code' of epigenetic information above the genetic code (38). In mice, CNSs within the *Ifng* locus are marked by histone-4 tail acetylation (H4Ac) during Th1 development by a Stat4 and T-bet dependent mechanism (44, 59, 73, 77, 108). H4Ac is correlated with transcriptionally permissive areas of chromatin (37). To determine if the human *IFNG*-BAC transgenic locus also acquired H4Ac marks and the role of distal regulatory elements within the *Ifng* locus in establishing the histone code, we cultured CD4+ T cells from BAC transgenic mice for three days under Th1 conditions and performed ChIP assays. Under these conditions the endogenous mouse locus developed extensive, long-range H4Ac marks (Figure 2-9). We were able to detect H4Ac marks across the human locus in *IFNG*-BAC transgenic Th1 cells, although at reduced levels than observed at the endogenous mouse locus (Figure 2-9), consistent with reduced *IFNG* expression levels (Figures 2-1 and 2-2).

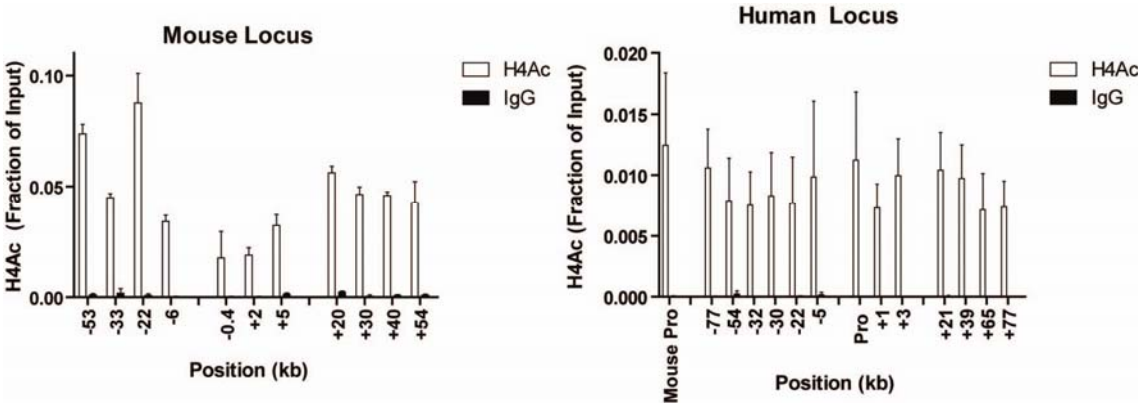

Figure 2-9 Relative aceylation between the endogenous mouse *Ifng* locus and the human *IFNG* locus.

IFNG-BAC CD4+ T cells were cultured for three days under Th1 conditions and processed for chromatin immnocprecipitation assays with antibodies specific for normal rabbit IgG or H4Ac marks. Results are presented as fraction of input of DNA as determined from a standard curve and the average of three independent experiments. Error bars are s.d.

We performed H4Ac ChIP assays on day 3 CD4+ Th1 cells from Δ4, Δ3/4, Δ-30 and *IFNG*-BAC transgenic mice. Deletion of any of the distal regulatory regions examined did not abolish H4 acetylation at the remaining CNS across the human *IFNG* locus (Figure 2-10A). While a paired-ANOVA test demonstrated significant difference between the H4Ac values between the different groups ($p < .01$), a control *t*-test did not identify any significance at any exact genomic position. Further, H4Ac levels between BAC transgenic lines in CD4+ Th1 cells did not absolutely correlate with human *IFNG* mRNA levels in day three Th1 cells. For example, the Δ3/4 deletion did not significantly affect H4Ac at any specific location but completely abrogated human *IFNG* mRNA production by day three Th1 cells. Conversely, the Δ4 deletion resulted in up to a ten-fold increase in human *IFNG* mRNA, compared to *IFNG*-BAC transgenic controls. But, H4Ac levels across the *IFNG* locus in Δ4 Th1 cells were not ten fold above H4Ac levels in BAC transgenic Th1 cells (Figure 2-4F).

We next sought to determine if distal regulatory elements functioned to remove a repressive histone mark. The di-methyl histone-three, lysine 9 histone mark is thought to mark gene repression in mammalian euchromatin (37). CD4+ T cells develop this mark during both Th1 and Th2 cell polarization, but subsequently lose the mark after three days of Th2 polarization (108). We preformed ChIP assays from day three Δ3/4 or *IFNG*-BAC Th1 cultures. H3K9Me2 levels did not vary significantly ($p=0.13$ by ANOVA) between Δ3/4 and *IFNG*-BAC T cells (Figure 2-10B). While these negative results do not rule out a role in establishing histone acetylation and methylation throughout the *IFNG* locus, we next considered that the -92 to -18 kb (Δ3/4) region and specifically CNS-30 functioned to recruit components necessary for transcription to *IFNG*. To test this

hypothesis, we preformed ChIP assays for RNAP II on day three Δ3/4, Δ-30 and *IFNG*-BAC Th1 and Th2 cells. RNAP II was recruited predominantly to the *IFNG*-BAC promoter, CNS-16, and CNS-30 in Th1 cells. In contrast, RNAP II recruitment to the *IFNG* promoter and the CNS-16 sites was significantly reduced in Δ3/4 and Δ-30 Th1 cells, compared to *IFNG*-BAC Th1 cells (Fig 2-10C). Taken together, these results indicate that CNS-30 is necessary for efficient RNAP II recruitment to the *IFNG* locus in Th1 cells, but is not absolutely necessary to establish histone 'marks' across the locus.

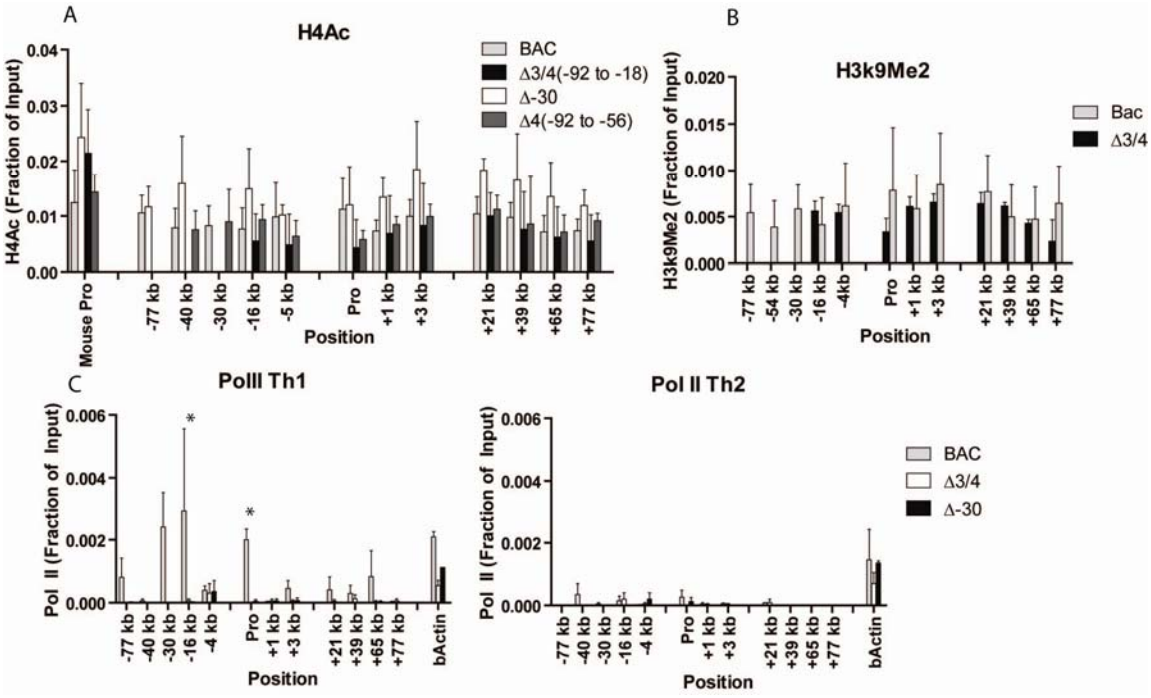

Figure 2-10. CNS-30 is necessary for Pol II recruitment to the *IFNG* locus.
Δ3/4, Δ-30, Δ4 or *IFNG*-BAC CD4+ T cells were cultured for three days under Th1 conditions and processed for ChIP assays with antibodies specific for normal rabbit IgG or (A) H4Ac marks (B) H3K9Me2 marks (C) Pol II. Results are presented as fraction of input of DNA as determined from a standard curve and are the average of three independent experiments. Values for IgG controls were less than 0.001 and were subtracted from means. Error bars are s.d. Significance was determined by a Control *t*-test and $P < 0.025$

Discussion

Results presented here demonstrate that the *IFNG*-BAC transgene recapitulates many functions of the endogenous *Ifng* gene. These include high level expression, Th1/Th2 and Tc1/Tc2 selective expression, requirement for T-bet by CD4 and CD8 cells to express *IFNG*, responsiveness to IL-12 and IL-18 stimulation in both T cells and NK cells, and responses to antigen stimulation by effector CD8 T cells via a T-bet dependent mechanism. Thus, the *IFNG*-BAC transgene is capable of integrating the multiplicity of signals required for both differentiation and expression of effector function by three distinct cell lineages independent of its position of integration in the genome.

There are two known distal regulatory elements necessary for sufficient IFN-γ production. Yet, their function in CD4+ T cells is non-redundant. Human CNS-16 (mouse CNS-22) is a major binding site for the transcription factor T-bet (74). Human CNS-30 (mouse CNS-34) binds the transcription factor Runx3. A separate group has also identified CNS-34 as a site which binds a Runx family cofactor, CBFβ, and demonstrated that inhibition of Runx family members by a dominant negative approach markedly diminishes IFN-γ production in CD4+ Th1 cells (109). CNS-30 is necessary for efficient RNAP II recruitment to *IFNG*, but not absolutely required for histone acetylation throughout the interferon-gamma locus. Thus, a high level of cooperativity is needed among distal regulatory elements, which bind separate transcription factors, to achieve proper transcriptional control. In this model, T-bet binds to CNS-16 and provides initial chromatin remodeling of the *Ifng* locus in order to provide locus accessibility to Runx3 and other transcription factors such as Hlx (105). Second, binding of these auxiliary

transcription factors to their own distal regulatory elements are necessary to promote recruitment of members of the basal transcription complex, such as RNAP II to the locus.

Removal of distal regulatory elements from -18 to -92 kb of the *IFNG* transcription start site does not completely eliminate *IFNG* locus H4Ac, even though removal of these regulatory elements markedly interferes with *IFNG* transcription. Other groups have proposed that CNS-4 (99), or the mouse homolog CNS-6 (76), responds to an early signal in effector Th1 cell differentiation to promote histone acetylation throughout the interferon-gamma locus. Two recent studies have shown CNS located -63 kb, +1.5 kb and +119 kb from the *IFNG* transcription start site participate in formation of chromatin loops by binding the chromatin insulator CTCF (110, 111). In this model, CNS-63 and CNS+119 shield the *IFNG* locus from neighboring chromatin and regulatory elements. However, Δ4 transgenic T-cells, which lack both CNS-63 (within Δ4) and CNS+119 (not in the *IFNG*-BAC transgene), produce robust quantities of human IFN-γ in a Th1/Th2 specific and stimulus-specific manner, demonstrating that these distal CTCF binding sites are not required for cell-type and stimulus specific gene expression.

CNS-30 is a distal regulatory element necessary for human IFN-γ production by T-cells and NKT cells, but not natural killer cells. The mouse homolog of human CNS-30, CNS-34, develops histone acetylation upon Th1 differentiation, but is not acetylated in freshly isolated or cultured NK cells (77). CNS-34 binds the transcription factor Runx3, which is necessary for sufficient *Ifng* production in Th1 cells (105), but not in NK cells (106). In contrast to the cell-type specific function of CNS-34, previous studies have identified mouse CNS-22 (the homolog to human CNS-16) as a distal regulatory element necessary for IFN-γ production in T cells and NK cells (74). Unlike CNS-34, CNS-22 is

acetylated in NK cells (77). As such, CNS-22 is a distal regulatory element necessary for IFN-γ production in cells which participate in both innate and adaptive immunity, while CNS-34 (human CNS-30) is a regulatory element only necessary in T cells, but is dispensable in innate immune cells (Figure 2-11).

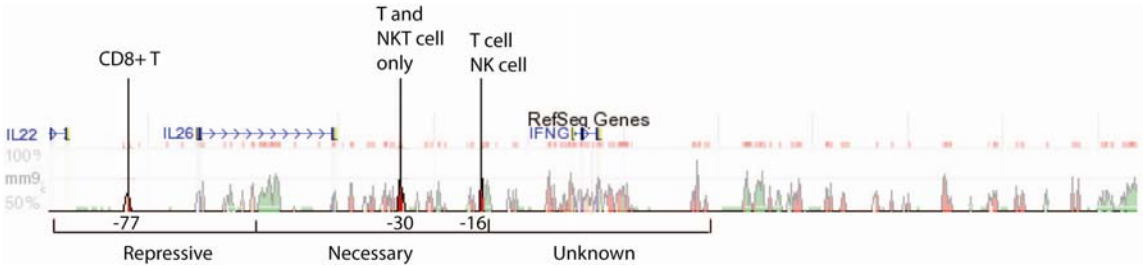

Figure 2-11 Regulatory regions within the IFNG locus possess diverse functions and display cell type utilization.
The -92 to -56 kb region contains repressive distal regulatory elements, while the -56 to -16 kb region contains necessary distal regulatory elements. In contrast, CNS-30 is a distal regulatory element necessary in T cells, including NKT cells, but not natural killer cells, macrophages and dendritic cells. The mouse homolog of CNS-16 is necessary for IFN-g production by T cells and NK cells (Hatton et al, Immunity 2006). Evolutionary mouse Human sequence conservation within the 190 kb human IFNG-BAC transgene is shown as identified using the dcode website (www.dcode.org).

The ability of myeloid cells to express interferon gamma is controversial (112). In the mouse, the identity of which exact dendritic cell subset produces IFN-γ has not been consistent throughout various reports (113-115). Further, mouse CD11c+ and CD11b+ NK cell subsets have made the identification of an IFN-γ producing dendritic cells difficult (107, 116). To our knowledge, human IFN-γ producing dendritic cells have not been described. Therefore, to understand the possible extent for cell-type specific *cis*-regulatory elements, we determined which *IFNG*-BAC CD11c+ and CD11b+ cells were capable of producing human IFN-γ. Between *IFNG*-BAC transgenic CD11b+ and CD11c+ populations, IFN-γ expression is detectable within the MCH II- NK cell populations. Despite PMA/Ionomycin stimulation, human IFN-γ was close to background in MHC II+ populations. Human IFN-γ expression was very low in CD11c+, B220+, MHC II+ cells, which contain "Interferon-Producing Killer Dendritic Cells" (115). We also did not detect human IFN-γ among CD11c+, CD8+ cells, which have been reported to produce IFN-γ (113). Therefore, human IFN-γ, in *IFNG*-BAC mice, is expressed in T cells, NKT cells and NK cells. Unlike in CD4+ and CD8+ T cells, in MHC II-, CD11b+, CD11c+ NK populations, human IFN-γ is predominantly expressed in the mouse IFN-γ negative population. These results further substantiate cell-type specific regulation of *IFNG*.

Mice carrying *IFNG*-BAC transgenes without the +20 kb to +93 kb regions (Δ1 and Δ2 mice) were capable of producing human IFN-γ in CD4+ and CD8+ T cells. Because these areas contain conserved non-coding sequences, which are by definition implicated in fitness, we consider it likely the +20 kb to +93 kb region has a role in regulating *IFNG*. Future work will focus on the +20 kb to +93 kb region. It is possible

that there are elements within this region with cell-type specific roles in NK cells, where the -92 to -18 kb region is dispensable. Alternatively, there may be unique roles for regulatory elements *in vivo*. For example, CNSs within the IFNG locus could have tissue specific, or even pathogen specific, function. Both spontaneous and antigen-specific human IFN-γ expression in endogenous tissues will be examined in future studies.

These results demonstrate that distal regulatory elements of a human gene function in a cell-type specific manner. This attribute of human gene regulation is recapitulated in the mouse genome. In the mouse Th2 cytokine locus, CNS-1 is necessary for IL-4 production in Th2 cells but not in mast cells (117). Other regulatory elements within the IL-4 locus do not show cell type specificity (118). Thus, different distal regulatory elements have varying cell-type specific functions (*IFNG* CNS-30 and mouse Th2 CNS-1) or alternatively have general function (mouse *Ifng* CNS-22 (74)). This modular use of distal regulatory elements in the mammalian Th2 cytokine and *IFNG* loci is similar to what has been found in studies of *Drosophila* promoters, where modular elements of gene promoters can function independently to direct cell-type specific expression (119, 120). Thus, metazoan developmentally regulated genes, in which expression is restricted to more than one cell-type, utilize modular distal regulatory elements with cell-type specific regulatory elements in possible conjunction with regulatory elements that function in multiple cell types.

CHAPTER III

Expression of *IFNG* and *IL26* requires shared and distinct cis-regulatory elements

Abstract

Although synteny of *IFNG* and *IL26* genes is preserved from zebrafish to humans, these genes are expressed by opposing CD4 T helper cell lineages. We show that the mouse *Ifng* locus lacks *Il26* due to the insertion and serial duplication of a *Cdc5l* retrotransposon pseudogene. We employed a bacterial artificial chromosome transgenic system to define functional requirements for correct expression of *IFNG* and *IL26*. Surprisingly, both *IFNG* and *IL26* display Th1/Th2 selective expression, have promoters that bind STAT4, possess promoter T-box binding sites and share functional requirements for cis-regulatory elements, CNS-30 and CNS-4. Th17 differential expression is achieved because both genes also employ unique cis-regulatory elements, CNS-16 for *IFNG* regulation and CNS-77 for *IL26* regulation. We assessed necessary roles for each CNS: CTCF binding CNS+120 and CNS+20 do not have roles in the IFNG-BAC transgenic system. CNS-4 is needed only for Th1 and NK cell effector function. CNS-16 is an IFNG repressive element, but only utilized in T cell receptor signaling. As such, we establish one *IFNG/IL26* locus where correct expression of each gene is established by the collaborative roles of many non-redundant cis-regulatory elements.

Introduction

The adaptive immune system must respond to varying classes of challenges such as intracellular infections or infections by extracellular bacteria and fungi. These responses are orchestrated by CD4+ Th1 or Th17 cells, respectively (54). The Th1 response is characterized by the production of interferon gamma (*IFNG*) by Th1 cells, CD8+ cytotoxic T cells and natural killer (NK) cells. The cytokine profile of the Th17 response is far more heterogeneous (54). Human Th17 cells are characterized by the production interleukin 17, but also produce IL-22 and IL-26 (55). Human Th17 cells also produce IFN-γ. In human Th17 cells, IL17 and IL22 are preferentially expressed by IFN-γ negative cells while IL26 is preferentially expressed by IFN- γ positive, IL-17F negative cells (56).

The *IFNG* gene borders the two genes *IL26* and *IL22*. This synteny is conserved from humans to *Xenopus* and zebrafish (82, 121). However, *IL26* and *IL22* are both IL10 family members and are not structurally related to IFNG implying they possess distinct functions. The function of *IL22* is established and this cytokine plays a key role in epithelial defense (122, 123). The function of *IL26* is often assumed to be similar. However, it is known that the IL-26 protein binds to a unique receptor complex (124, 125). In immortalized epithelial cell culture, IL-26 is thought to promote inflammatory cytokine expression (126). Polymorphisms within the *IFNG/IL26* region are associated with increased risk of ulcerative colitis by genome wide association (4) and *IL26* and *IL22* are expressed at high levels in colonic and psoriatic sites of inflammation (55, 126), implicating a central role in inflammatory diseases of the human epithelium. However, mice lack an *Il26* gene and its function, *in vivo*, is unknown.

In human Th1 cells, the *IL26* gene loops close into the *IFNG* gene (30), and in murine Th1 cells, the *Il26* pseudogene loops into the *Ifng* gene (111). These data raise the possibility of one common *IFNG*/*IL26* locus, similar to the Th2 locus or the *IL17A/F* locus. However, an *IFNG/IL26* locus would be a Th1/Th17 locus and not explained with current understanding of *IFNG* cis-regulation. *IFNG* depends upon noncoding sequences for both cell-type and stimulus specific expression. *IFNG* is expressed when mature Th1 cells receive T-cell receptor signaling (72, 127). In direct contrast, CD4+ Th2 cells or Th17 cells must specifically repress *IFNG* transcription in response to identical T-cell receptor signaling. Transgenic mice carrying an 8.6 kb *IFNG* reporter transgene fail this developmental decision (98). In contrast mice carrying a 190 kb transgene correctly recapitulate repression in Th2 cells showing that *IFNG* requires cis-regulatory elements for cell-type selective expression. A 190 kb *IFNG* transgene also recapitulates other key aspects of *IFNG* regulation, such as stimulus-specific expression following coordinated stimulation by IL-12 and IL-18 (Chapter II), and specific expression in cytotoxic CD8+ T cells and natural killer cells. While regulation of *IFNG* requires cis-regulatory elements, precisely which cis-regulatory elements are required for individual aspects of *IFNG* regulation is incompletely understood.

Transcription factors T-bet (74, 77), STAT4 (77), STAT5 (76, 99), NF-κB family members (128), and Runx3 (129) positively regulate *IFNG* expression and directly bind to distinct conserved non-coding sequences of the mouse *Ifng* locus in a Th1 and stimulus dependent manner. Transcription factor binding is accompanied by Th1-specific covalent histone modifications at conserved noncoding sequences (67, 73, 128). These observations have led to the hypothesis that proper regulation of interferon gamma is

conferred by transcription factor interactions with CNS. In transgenic model systems, a CNS -30 kb (CNS-30) from the *IFNG* start site is needed for *IFNG* production in T cells (129), and a mouse CNS homolog to CNS-16 is needed for reporter expression from the mouse *Ifng* locus (74). In transient reporter assay systems mouse homologs of CNS+20, CNS-4, CNS-16 and CNS-30 all display enhancer activity (67, 75, 79). Lastly, CNS at +120 and -63 bind the chromatin looping/insulator protein CTCF (30) and are sites of chromatin looping of the *IFNG* locus. However, our understanding of function in the setting of an intact genome is incomplete. Two non-exclusive hypotheses could be made about CNS function. First, CNS may have redundant function where each CNS is necessary for a fraction of *IFNG* expression in all responder cell types in response to diverse stimuli. An alternative hypothesis is that CNSs possess unique functions such that each individual CNS provides a unique contribution to developmental decisions and stimulus-specificity to achieve proper *IFNG* transcriptional regulation.

To define CNS function within the *IFNG/IL26* locus we employed an *IFNG*-bacterial artificial chromosome model system (98, 129). In this model, mice are created with a transgene that contains *IFNG*, *IL26* and surrounding regulatory regions with or without a specific CNS. The normal mouse production of IFN-γ is not affected, and serves as a control. IL26 is expressed by IFNG-BAC Th17 cells, but also displays Th1/Th2 selective expression. We find both that individual CNS's confer very selective aspects to *IFNG/IL26* cis-regulation and for some stages of *IFNG* expression there is functional overlap between CNSs. We also find that activity of individual CNS is required for expression of both *IFNG* and neighboring *IL26*. Thus, two adjacent and oppositely expressed genes can share a single CNS.

Materials and Methods

Mice and Preparation of transgenic reporter lines

C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME), housed in the Vanderbilt University animal facilities, and used between 4-5 weeks of age. Preparation of human *IFNG*-BAC transgenic lines was preformed as described in chapter II. Briefly, human *IFNG*-BAC CTD-3002C24 was used to make 210 kb *IFNG*-BAC mice. Alternatively, CTD-3002C24 was moved into EL250 *E. coli* and CNS deletion was achieved using homologous recombination followed by FRT-mediated removal of the selection marker. Targeting primers are described in Table 3-1. Transgenic mice were prepared by standard pronuclear injection using circular BAC DNA, which is effective for generating transgenic founders (101). We used human-specific PCR primers to verify insertion integrity of the different *IFNG*-BAC transgenes. Lines without full-length insertions were excluded from analysis. All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

	Primer name / Plasmid name	Targeting Primer sequence	Location (hg18)	Mouse homolog (mm8)
Δ-16	Δ -16 Forward	GCAGCAAATCAGCATAGCACACATTTA CCTATGTAACAAACCTGTACATCGAAG TTCCTATTCTCTAGAAAGTATAGGAACT TCAGATCTATGATTCCCT	chr12: 66855250-66856250	chr10: 117822073-117822727
	Δ -16 Reverse	TGAGAAAAACATCTGGTAAGGTTATTC AGACCACTCAGCTTCATATGGATGAAG TTCCTATACTTTCTAGAGAATAGGAACT TCAAGCTTATGATGATGA		
Δ-4	Δ -4 Forward	CCAACCTGACTTCATGGACATGCAACC TGTGCAGTCTGACAGGACCCTGAGAA GTTCCTATTCTCTAGAAAGTATAGGAA CTTCAGATCTATGATTCCCT	chr12: 66843300-66844299	chr10: 117838122-117839078
	Δ -4 Reverse	TGATTGTACTGTGTAAGTAGCATACCT TCTATATTCTTTGAACCACTCCTGAAGT TCCTATACTTTCTAGAGAATAGGAACTT CAAGCTTATGATGATGA		
Δ+20	Δ +20 Forward:	CCACAGAACCTGAAGCCTCCCCCTGG AAACTTGATTATTTCAGAATCAAGGAA GTTCCTATTCTCTAGAAAGTATAGGAA CTTCAGATCTATGATTCCCT	chr12: 66855250-66856250	chr10: 117822073-117822727
	Δ +20 Reverse	GTGGGTTAGGGGCACCAGGAATTCTG GGTAATTGGGGCTTAGCTATTTTTGAA GTTCCTATACTTTCTAGAGAATAGGAA CTTCAAGCTTATGATGATGA		
Δ-30	Δ -30 Forward	TATAATTTAGAGTTAGCCTTTGAAATAA CTTATAGAAAGCATTAATTGATGAAGTT CCTATTCTCTAGAAAGTATAGGAACTT CAGATCTATGATTCCCT	chr12: 66869614-66870500	chr10: 117809759-117810486
	Δ-30 Reverse	ATGAAGCAGAGCTAGAAAAGTGTATTA TTAATGAAGAAGAAGAAAAACAAGAAG TTCCTATACTTTCTAGAGAATAGGAACT TCAAGCTTATGATGATGA		
Δ-77	Δ -77 Forward	CATCCAAATAAAATCTGACCTTGTGTTT TCAAAGCCCATCCCACAGGATCGAAGT TCCTATTCTCTAGAAAGTATAGGAACTT CAGATCTATGATTCCCT	chr12: 66917565-66918546	chr10: 117616708-117617782
	Δ -77 Reverse	CCCTATTTACTCTTCTTCCCCCTTCCAT TTCCCGCCCCTCCCCAACTCCCGAAGT TCCTATACTTTCTAGAGAATAGGAACTT CAAGCTTATGATGATGA		
190 kb BAC	RPCI11-444B24		chr12: 66740833-66931792	chr10: 117609673-117903582
210 kb BAC	CTD-3002C24		chr12: 66705776-66923225	chr10: 117614646-117925027

Table 3-1. Primer sequences used to prepare BAC deletions and deletion locations.
The primers used for amplifying FRT-Tet-FRT cassettes for deletion engineering and the locations of the BAC deletions and the entire BAC transgenes are listed below. The first 50 nt at the 5' end of each primer are homology sequences for recombination, while the remaining 3' nt are the annealing sequences for PCR amplification of the FRT-Tet-FRT cassette. Please note, figures are "flipped" in regard to absolute genomic location so that the beginning of the IFNG gene appears on the left of the figure. Genomic locations are based on March 2006 (NCBI36/hg18) assembly.

Cell Purification and Cultures

CD4+ and CD8+ T cells were purified from splenocytes by positive selection per manufactures instructions (Miltenyi Biotec). T cells were cultured with plate bound 1 μg/ml anti-CD3 (hybridoma 2C11, American Type Culture Collection, Manassas, VA) and anti-CD28 (hybridoma 2C11, ATCC, Manassas, VA). For Th1/Tc1 cultures, cells were cultured with 10 ng/ml IL-12 and 10 μg/ml anti-IL4 (11B11 hybidoma, ATCC). For Th2 cultures, CD4+ T cells were cultured with 20 ng/ml IL-4 and 10 μg/ml anti-IFN-γ (hybridoma, ATCC). For Th17 cultures, CD4 cells were cultured with 10 ng/ml IL-1β, 20 ng/ml IL-6, 10 ng/ml IL-23, 1 ng/ml TGFβ and 10 μg/ml anti-IFN-γ. For assays of resting cells, cultures were harvested on after three days and re-plated in IL-2 containing media for two additional days. Rested cells were restimulated with plate bound anti-CD3, 10 ng/ml IL-12 and 10 ng/ml IL-18, 10 ng/ml IL-12 and 10 ng/ml IL-2, or 50 ng/ml PMA and 1 μM ionomycin. DX5+ NK cells were purified from spleen by positive selection (Miltenyi Biotech).

Blood was obtained from healthy donors. All participants provided written informed consent and studies were approved by the Vanderbilt Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were purifed by centrifugation in a Ficoll–Hypaque gradient (GE Healthcare Systems). Cell sorting for CD4+ cells was preformed using positive selection as per manufactures' instructions (Miltenyi Biotech). Human cells were cultured with plate-bound anti-CD3 (OKT3 clone, ATCC), human polarizing cytokines and processed for analysis identical to murine cultures conditions.

Intracellular cytokine staining

T cell cultures were harvested on day four and restimulated with 50 ng/ml PMA and 1 μM ionomycin for two hours followed by addition of GolgiPlug (130) and culture for an additional four hours. Cells were washed twice in ice-cold FACS buffer (2% FCS, 0.1% NaN_3 in PBS) and labeled with fluorescent antibodies for 30 minutes as outlined in the results section. Cells were permeabilized and fixed with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Flow cytometric experiments were performed in the VUMC Flow Cytometry Shared Resource. T cell cultures were gated on CD4+ or CD8+ subsets for analysis.

Quantitative RT-PCR

Total RNA was purified from restimulated Th1, Th2 and Th17 cultures and cDNA was synthesized using Super Script III First-strand synthesis, as per manufacture's instruction (Invitrogen). Specific transcripts were quantified using TaqMan pre-made assays as per manufacture's instructions (Applied Biosystems). Relative change in transcript levels were determined using the delta Ct method.

Transient transfection assays

The *IFNG*-luciferase plasmid was purchased from Addgene, plasmid number 17598. CNS-77 or ΔNF-κB CNS-77 was PCR amplified from RPCI11-444B24 (Invitrogen, Carlsbad, CA) and moved into a Pac I site of the *IFNG*-luciferase plasmid. Jurkat cells were transfected with 5 μg plasmid by electroporation. After an overnight rest, cells were stimulated with PMA/ionomyocin for 24 hours. Primary splenocytes were transfected

with 2.5 μg of luciferase plasmids using Amaxa mouse T cell nucleofector kits (Lonza). Cells were rested 3 hr and stimulated over night with 2 μg/ml plate-bound anti-CD3, 2 μg/ml anti-CD28, 10 ng/ml IL-12 and IL-2.

Conservation and chromatin mapping

Mapping of ChIP-seq and DNase-seq was performed using the UCSC genome browser (http://genome.ucsc.edu). Data were acquired from previous experiments on whole CD4+ T cells (37, 39, 43, 128, 131) using the Geo database (http://www.ncbi.nlm.nih.gov/geo/) and (http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.aspx) for histone methylation marks. Multi-species locus conservation and alignments were mapped using the UCSC browser alignments, hg18. Transcription factor binding site prediction and genome alignments were preformed using the rVISTA Mulan program (132) (http://mulan.dcode.org/) with pre-aligned genomes. Comparison of synteny was performed using the rVISTA Mulan interface, with previously aligned sequences downloaded from the UCSC genome browser.

Results

IL26 and IFNG share conserved promoter elements and STAT4 binding sites

The *IFNG* and *IL26* gene locus is conserved in most species, but *Il26* is absent in C57BL/6 mice. Mice are important models of human immunology, so we first determined the nature of the *Il26* disruption in C57BL/6 mice. Analysis of sequence

conservation showed that the *IL26* sequence was conserved throughout mammals, including mice (Figure 3-1). When we compared conservation of genomic order, we observed that in C57BL/6 mouse genome the *Il26* region was disrupted due to a large duplication and inversion of the *Il22* gene, *Il26* start site and surrounding regulatory region (Fig 3-2, Figure 3-1A). Bordering the *Il22* duplicated region, we found a mouse-specific intrachromosomal crossover event (Figure 3-2), with serial duplications of all but the first 150 base pairs of the cell division cycle 5-like (*Cdc5l*) exons and L1 transposable elements. These processed pesudogenes are thought to arise when a mature transcript becomes incorporated into an L1 transposable element, and are typically not expressed (133). The *Il22* duplication and serially duplicated *Cdc5l* processed pesudogenes were not found in the rat genome and the *Cdc5l* pseudogenes showed up to 97.8% sequence identity to the mouse *Cdc5l* transcript but only up to 94.3% identity to the rat *Cdc5l* transcript (Figure 3-2) suggesting a mouse-specific chromosomal disruption. These data were consistent with a duplication of *Il22* in some strains of mice, including C57BL/6 but not BALB/c and parental DBA/2 (134). However, a sequence gap in the published rat genome covering the *Il26* region prevented further analysis. Mouse CNS-54 was homologous to the end of the human *IL26* gene. As such, the *Il26* pseudogene gapped the duplication and cross-over event, leaving two oppositely facing start sites and numerous *Cdc5l* processed pseudogenes.

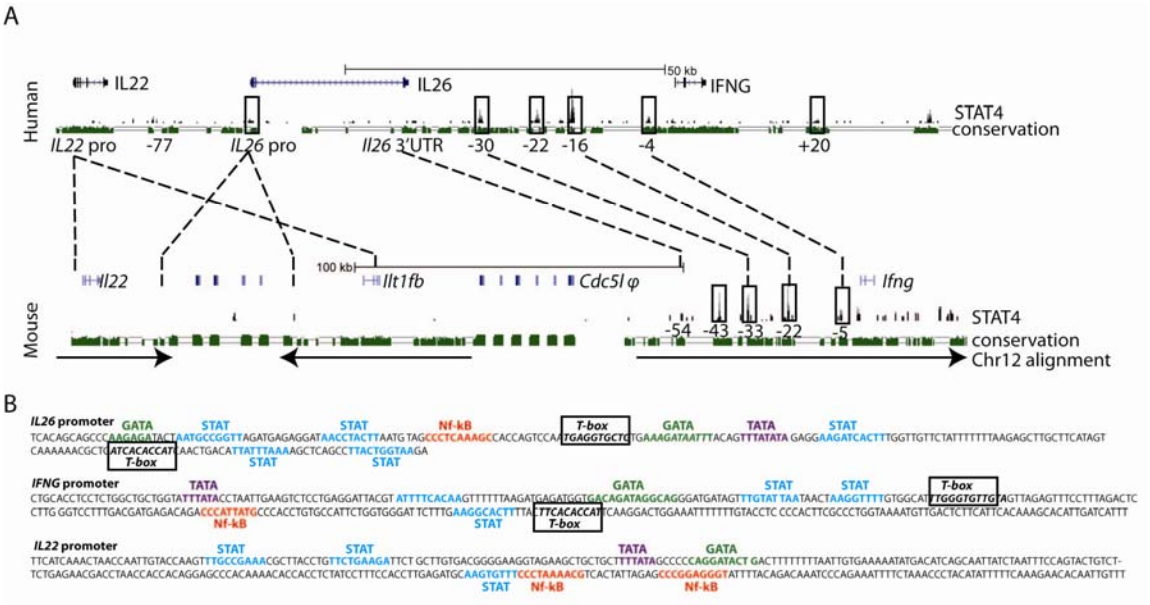

Figure 3-1 Comparison of human and mouse interferon gamma loci.

A, Locations of *IFNG* locus genes and STAT4 binding (135) were compared between the (Top) human locus and (Bottom) C57BL/6 mouse locus. Alignments were performed using pre-aligned data from the UCSC genome browser. Gene and STAT4 binding homologs are connected by dashed lines (Middle) and alignments from mouse chromosome 10 to human chromosome 12 are depicted by arrows (Bottom). STAT4 binding sites are boxed and listed as kb location relative to *IFNG* or *Ifng*. B, Transcription factor binding sites in human *IFNG* locus genes. Transcription factor binding consensus.

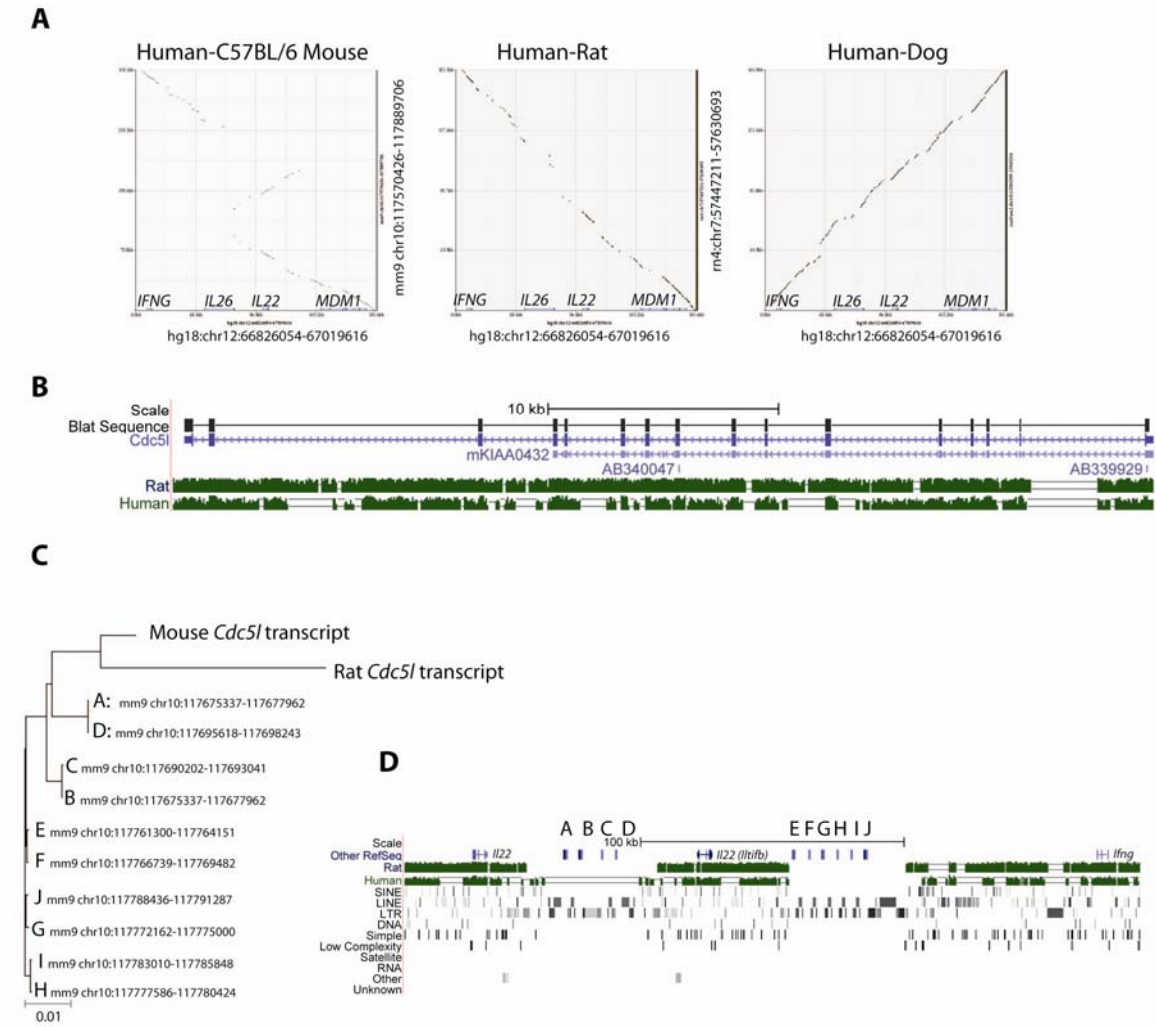

Figure 3-2 Comparison of the mouse *Ifng* locus duplications and the *Cdc5l* transcript. (A) Conservation of synteny was determined using the rVISTA Mulan program and Pipmaker. Alignments were uploaded from the UCSC genome browser database. The X-axis represents position on the human genome, and Y-axis represents position on the compared genome. (B) A BLAT search using DNA from the highly-conserved repetitive region within the mouse *Ifng* locus (mm9 chr10:117788436-117791287) showed high homology to the mouse *Cdc5l* exons, but not the *Cdc5l* intron regions. (C) Each serially duplicated *Cdc5l* transcript within the *Ifng* genomic region was aligned to the rat and mouse *Cdc5l* transcripts using MAFT version 6. Comparing alignment to genomic position (D) the *Cdc5l* insertions showed close sequence identity the mouse *Cdc5l* gene. Multiple duplication events appear to have occurred, with an ancestral "A" and "B" site duplication, followed by "C" and "D". A large duplication, probably involving the duplication of *Il22* to create *Iltifb* and the E through J sites was recent, consistent with strain-specific *Il22* duplications. The *Cdc5l* repetitive duplications are flanked by ERV1 and musHAL1 retroviral transposable elements. These data suggested that the *Cdc5l* transcript was incorporated into a retroviral transposable element after the rat-mouse split and repeatedly duplicated throughout the mouse lineage.

To further compare the human and mouse interferon gamma loci, we mapped STAT4 binding in IL-12 stimulated mouse and human Th1 cells using published ChIP-seq data (135). In humans, STAT4 binding was observed at both the *IL26* and *IFNG* promoters, but not the *IL22* promoter (Figure 3-1A). STAT4 binding was also observed at established human cis-regulatory elements such as CNS-16, as well as a novel site at -22 kb which we name human CNS-22 (hCNS-22) to avoid confusion with the mouse homolog of CNS-16. In mice, STAT4 binding was seen at previously established mouse conserved noncoding sequences (mCNSs) such as mCNS-5, mCNS-22 and mCNS-33 and also mCNS-43, which did not have a human STAT4 binding counterpart. In addition, STAT4 binding was not observed at homologs of the *IL26* promoter in mice. We next determined conserved transcription factor binding sites between humans and rabbits. We were able to identify conserved T-box (T-bet), STAT and AML1 (Runx3) transcription factor binding sites at previously established CNS, such as CNS-16 and the *IFNG* promoter, as well as at the *IL26* promoter. In contrast, we did not observe conserved T-box binding sites at the *IL22* promoter. To determine if the human *IL26* promoter contained the same transcription factor binding sites as the *IFNG* or *IL22* promoters, we examined the human *IFNG*, *IL26* and *IL22* promoters for T-box binding sites. We found T-box binding sites at the *IL26* and *IFNG* but not *IL22* promoters (Figure 3-1B). Commonality between *IFNG* and *IL26,* such as STAT4 binding, T-bet binding sites and shared genomic space suggested *IL26* might exhibit Th1-like regulation, which we examined next.

IL26 is expressed from the IFNG-BAC transgenes

To determine regulation of *IL26* we employed an *IFNG*-BAC transgenic model. *IFNG*-BAC transgenes used in this study contain both *IFNG* and *IL26* genes and surrounding regulatory regions. We used two transgenic lines, a 190 kb *IFNG*-BAC transgene and a larger 210 kb *IFNG*-BAC transgene. We sought to determine if the *IFNG*-BAC transgene was sufficient to direct *IL26* expression in CD4+ cells. Intracellular cytokine immunoassays using IL-26 antibodies resulted in universal background staining of all cells tested in multiple strains of mice and types of human cells, so we focused on transcript measurements. We cultured *IFNG*-BAC CD4+ cells under Th1, Th2 or Th17 polarizing conditions for three days followed by a two day rest in IL-2. At day five, cells were restimulated for five hours with PMA/ionomycin and transcript levels were determined by quantitative PCR. Elevated levels of *IL26* were expressed in cells cultured under both Th1 and Th17 conditions relative to cells cultured under Th2 conditions (Figure 3-3A). Interestingly, expression of *IL26* showed Th1/Th2 selectivity. *IL26* transcript levels produced by either 190 kb or 210 kb *IFNG*-BAC transgenic Th1 cells were ~5,000 fold lower than *IFNG* transcript levels (Figure 3-3B). For comparison, we also cultured human CD4+ cells from PBMC under identical Th1 and Th2 polarizing conditions. Under these conditions, *IL26* displayed identical Th1/Th2 selectivity to *IFNG*. Transcript levels of *IL26* in human CD4 cells were ~2,500 fold lower than *IFNG* message. As such, the *IFNG*-BAC transgene was sufficient to recapitulate certain features of *IL26* transcriptional regulation as seen in human CD4 cells, such as T helper cell selectivity. To determine if *IL26* expression impacted intestinal physiology,

we performed histological analysis of the colon and found no signs of spontaneous inflammation or abnormality in 190kb *IFNG*-BAC mice up to 6 months of age. Upon oral challenge with dextran sulfate sodium, a chemical irritant, transgenic mouse weight and measures of inflammation were indistinguishable from controls. As such, we focused on regulation of *IFNG* and *IL26*.

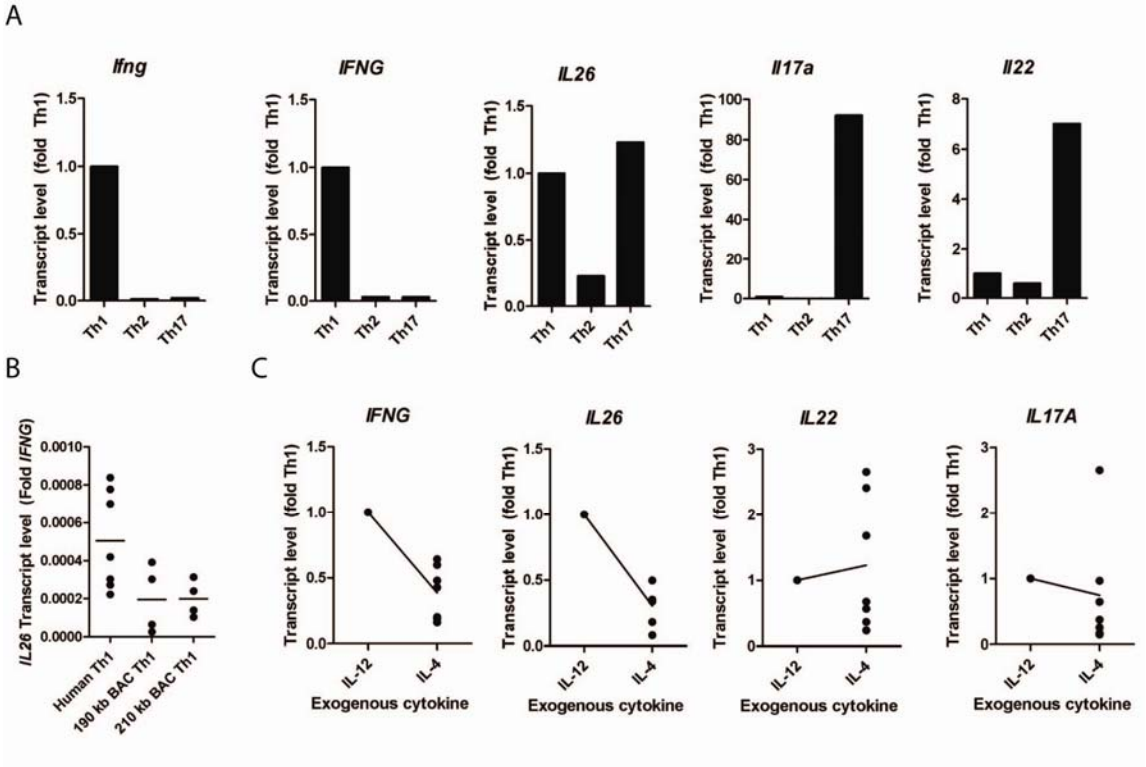

Figure 3-3 *IL26* is expressed from the *IFNG*-BAC transgene.
Expression levels of transcripts were determined after culture for three days with anti-CD3/CD28 and polarizing cytokines, two days rest in IL-2 media, and PMA/ionomycin restimulation. A, Transcript levels from 190 kb *IFNG*-BAC transgenic Th1, Th2 or Th17 cells. B, Comparison of *IL26* expression in polarized human Th1 cells and *IFNG*-BAC transgenic Th1 cells. C, Cytokine transcript levels from Th1/Th2 polarized human CD4+ cells.

Creation and analysis of ΔCNS-16, ΔCNS-4, ΔCNS+20 transgenic mice

Our BAC transgene correctly recapitulates developmental and signaling-dependent expression of *IFNG* (chapter II). Based upon our conservation mapping to known mouse regulatory regions, we hypothesized that transcriptional regulation of *IFNG* and *IL26* was conferred by CNS-77, CNS-30, CNS-16, CNS-4 and CNS+20. To test this hypothesis, we prepared new 210 kb BAC constructs with 1 kb deletions of CNS-4 and CNS-16 and a 3 kb deletion of CNS+20. We named these transgenic mice ΔCNS+20, ΔCNS-16 and ΔCNS-4 mice. We have previously described mice lacking CNS-30 or CNS-77 from the 190 kb *IFNG*-BAC (129). Each new line had a copy number of two, equivalent to the copy number of the intact BAC transgene. Transgenic mice displayed no overt abnormalities, were born at Mendelian ratios and gained weight at an appropriate rate. Mouse IFN-γ production was consistent among different transgenic lines and not affected by the transgene. We analyzed transgenic 210 kb *IFNG*-BAC, ΔCNS-16, ΔCNS-4 and ΔCNS+20 cells to determine if deletion of any cis-regulatory element resulted in loss of appropriate cell-type or stimulus specific expression of *IFNG* or *IL26*.

CNS-4 is necessary for IFN-γ production

We first assayed for a role for CNS-4 in *IFNG* and *IL26* production. We cultured 210 kb *IFNG*-BAC or ΔCNS-4 mice for 3 days with anti-CD3 and anti-CD28 under Th1 or Th2 polarizing conditions. At day 3, we measured culture levels of human (left panels) and mouse (right panels) IFN-γ. Mouse IFN-γ did not vary among culture conditions. Loss of CNS-4 did not result in a significant decrease in human IFN-γ production after

anti-CD3/CD28 stimulation of primary Th1 cultures (Figure 3-4A). We next tested a physiological stimulus by backcrossing mice onto an OT-II background, in which the CD4+ T cell receptor recognizes a fixed OVA323–339 epitope. We cultured transgenic CD4+ T cells with OVA323-339 loaded APC. Loss of CNS-4 did not result in a significant decrease in human IFN-γ production (Figure 3-4B). We next determined if CNS-4 regulated long-term stability of human IFN-γ expression. We cultured CD4+ T cells for 3 days under Th1 polarizing conditions and for 2 additional days in IL-2. On day 5, we restimulated resting Th1 cells with varying concentrations of anti-CD3. Human IFN-γ was only detectable in CNS-4 Th1 cultures stimulated with high concentrations of anti-CD3 (Figure 3-4C). Similarly, ΔCNS-4 Th1 cells did not produce human IFN-γ after restimulation with IL-12 and IL-18 (Figure 3-4D). Human IFN-γ was also not detectable in primary cultures of ΔCNS-4 DX5+ NK cells stimulated with IL-12 and IL-18 (Figure 3-4E). These results were consistent with a requirement for CNS-4 for IFN-γ production dependent upon either signal strength and/or stimulation of differentiated effector cells. We next restimulated ΔCNS-4 or 210 kb *IFNG*-BAC Th1 cells and Th17 cells with PMA/Ionomycin and measured *IL26* transcript levels. ΔCNS-4 *IL26* transcript levels were low in both Th1 cells and Th17 cells, consistent with a model in which CNS-4 is required for stable *IFNG* and *IL26* expression by differentiated effector cells. Thus, we reasoned CNS-4 might recruit histone acetyltransferases (HAT) and deacetyltransferases to establish the epigenetic stability of *IFNG*. We mapped HAT and HDAC binding using published ChIP-seq data (43). Indeed, both HAT and HDAC were recruited to CNS-4 upon CD4+ T cell activation (Figure 3-4F).

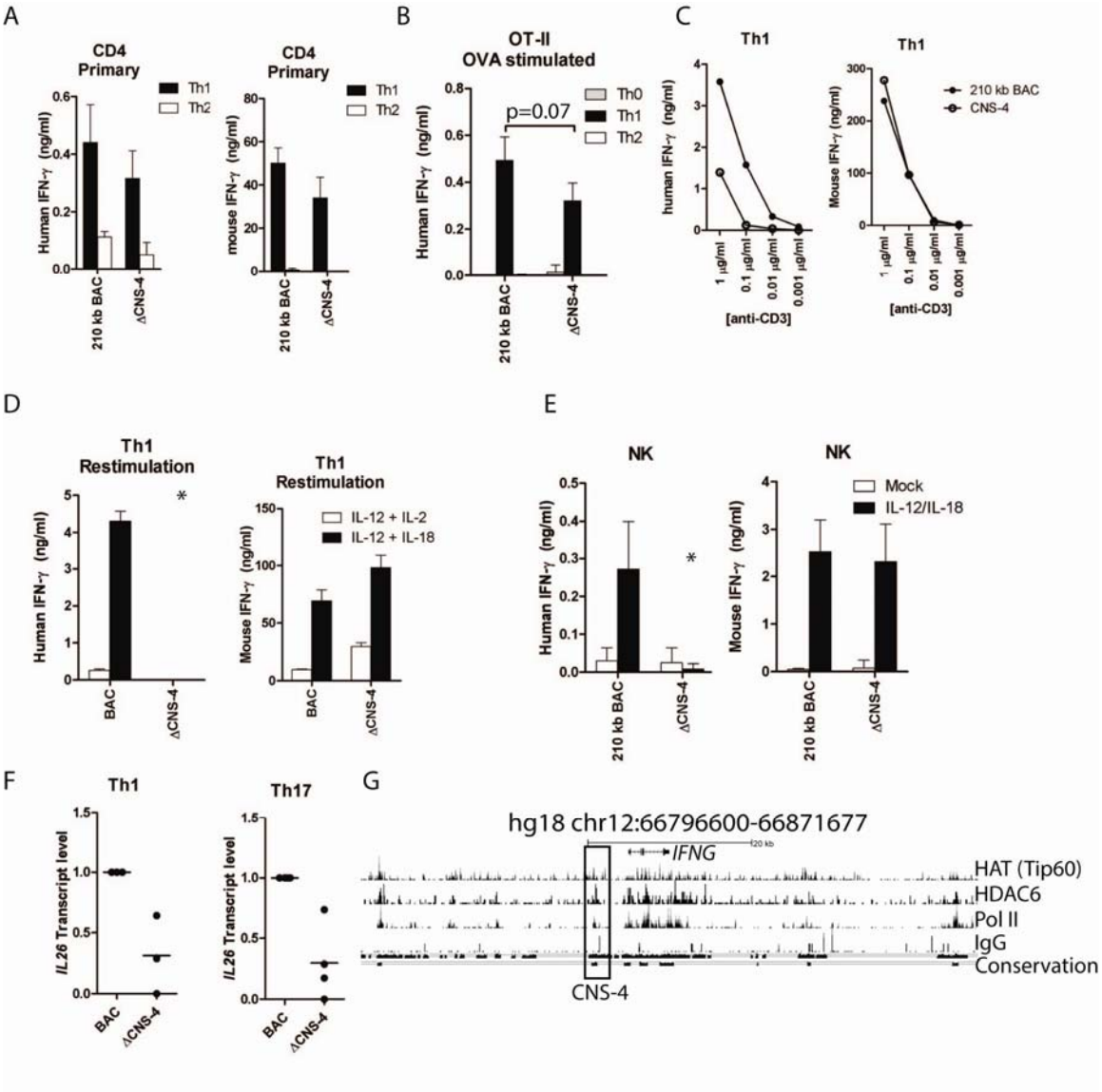

Figure 3-4 CNS-4 is required for IFN-γ production by differentiated effector cells.
A, B, IFN-γ levels of transgenic CD4+ cultured for three days under polarizing conditions with A, anti-CD3 and anti-CD28 stimulation or B, OT-II double transgenic mice stimulated with OVA323-339 and APC. C, Rested Th1 cells were restimulated with varying concentrations of anti-CD3 for two days. D, Rested Th1 cells were restimulated with IL-12 and IL-18, or IL-12 and IL-2 for two days. E, Primary DX5+ cells were stimulated with IL-12 and IL-18 for two days. A-E, Representative experiments are shown and error bars are s.d. * $p < .01$. F, Resting Th1 or Th17 cells were restimulated with PMA/Ionomycin and transcript levels were quantified relative to BAC controls. Results are biological replicates. G, Relative locations of Pol II, HDAC and HAT binding (43) and mouse-human (conservation top) or platypus-human (conservation bottom) conservation were downloaded and plotted by the UCSC genome browser.

T cell receptor signaling-mediated repression of IFNG is defective in CNS-16 mice.

We next determined the role of CNS-16 in *IFNG* and *IL26* regulation. Given known activity of the mouse homologue, mCNS-22, we expected CNS-16 to be absolutely necessary for *IFNG* production (74). However, in primary cultures, removal of CNS-16 resulted in a marked increase in human IFN-γ levels in both Th1 and Th2 cell cultures compared to the 210 kb *IFNG*-BAC transgene (Figure 3-5A). Removal of CNS-16 also resulted in a gain-of-function phenotype in primary cultures of CD8+ T cells (Figure 3-5B). Upon restimulation of IL-2 rested Th1 cells, levels of human IFN-γ levels were dependent upon concentrations of anti-CD3 stimulus indicating that loss of CNS-16 did not produce stimulus-independent expression and was epigenetically stable (Figure 3-5C). We next tested stimulus-dependence. In contrast to T-cell receptor signaling, expression of human IFN-γ in response to IL-12 and IL-18 stimulation was not affected by the CNS-16 deletion (Figure 3-5D). Similarly, the Δ-16 CNS deletion did not affect human IFN-γ production by NK cells stimulated with IL-12/IL-18 (Figure 3-5E). When we analyzed *IFNG* and *IL26* transcript levels in PMA/Ionomycin restimulated Th1 and Th17 cells, we confirmed a relative increase in *IFNG* transcript levels from CNS-16 mice, compared to controls (Figure 3-5E). However, *IL26* transcript levels were not different between CNS-16 and 210 kb *IFNG*-BAC cultures (Figure 3-5 E, F). Single cell analysis of PMA/Ionomycin restimulated cells showed an increase in the percentage of human IFN-γ+ cells in ΔCNS-16 CD4 and CD8 cells, relative to controls (Fig 3-6). To summarize, deletion of CNS-16 from the 210 kb *IFNG* transgene resulted in a gain-of-*IFNG* expression phenotype in CD4+ Th1, Th2, Th17 and CD8 cells which was dependent upon T cell receptor signaling. We reasoned repression may involve a cell-

type independent repressor of *IFNG*. The transcription factor YY1 is a constitutively expressed situational repressor or activator of IFN-γ (136, 137). We mapped binding of YY1 in resting CD4 cells and STAT4 and STAT5 binding in activated CD4 cells using published ChIP-seq data (131, 135). Indeed, while multiple CNS bound STAT proteins, YY1 binding in resting CD4 cells was restricted to CNS-16 (Figure 3-5H).

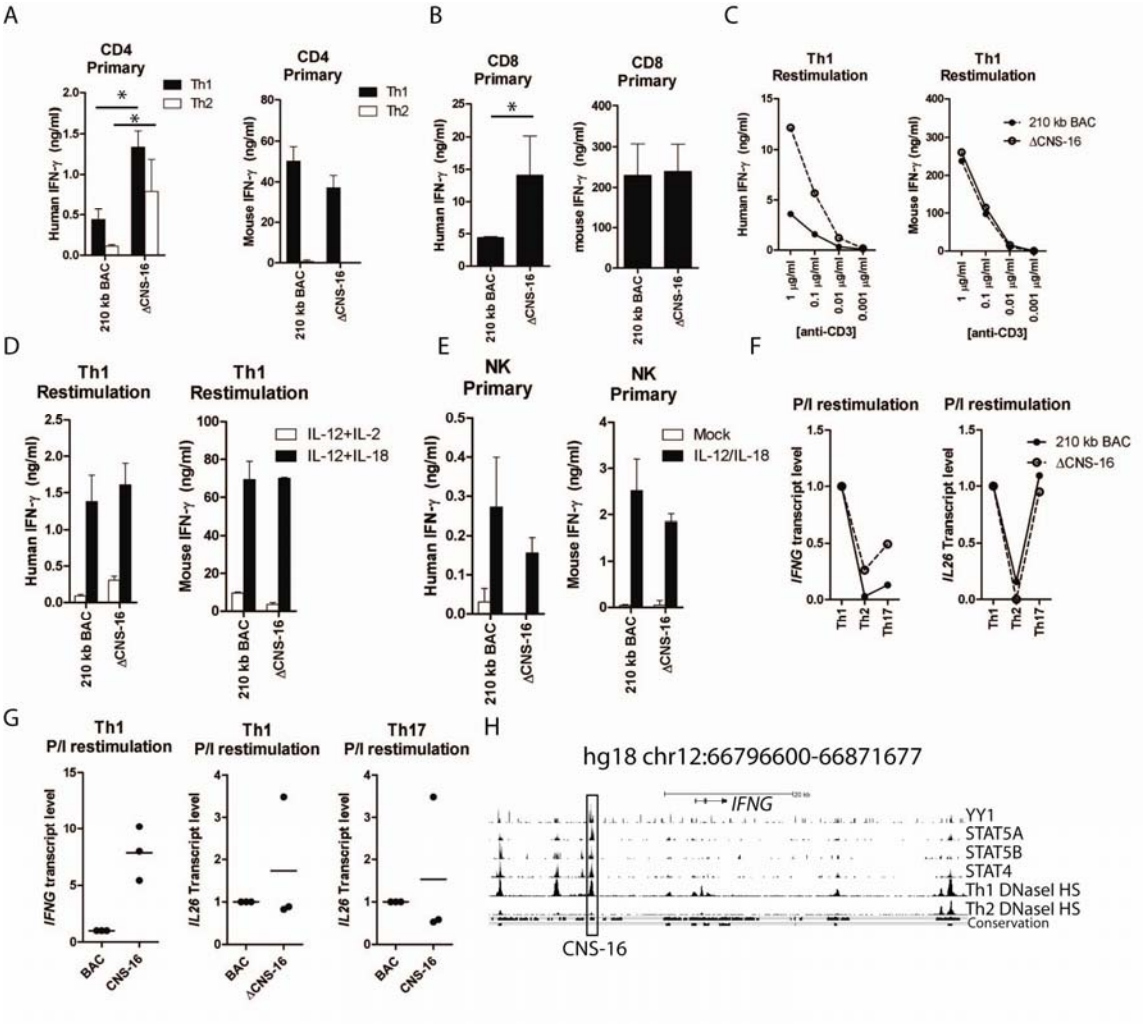

Figure 3-5 Loss of CNS-16 impairs Th2-mediated IFNG repression.
A, B, IFN-γ levels of transgenic CD4+ or CD8+ T cells cultured for three days under polarizing conditions. C, Rested Th1 cells were restimulated with varying concentrations of anti-CD3 for two days. D, Rested Th1 cells were restimulated with IL-12 and IL-18, or IL-12 and IL-2 for two days. E, Primary DX5+ cells were stimulated with IL-12 and IL-18 for two days. A-E, Human and murine IFN-γ levels in cultures were determined by ELISA. Error bars are s.d. F, G, Resting Th1, Th2 or Th17 cells were restimulated with PMA/Ionomycin and transcript levels were quantified relative to F, Th1 cultures or G, 210 kb *IFNG*-BAC controls. H, Relative locations of STAT4, STAT5A, STAT5B (128) and YY1 binding (131), Th1/Th2 DNaseI-HS (UW ENCODE) and mouse-human (conservation top) or platypus-human (conservation bottom) conservation were downloaded and plotted by the UCSC genome browser.

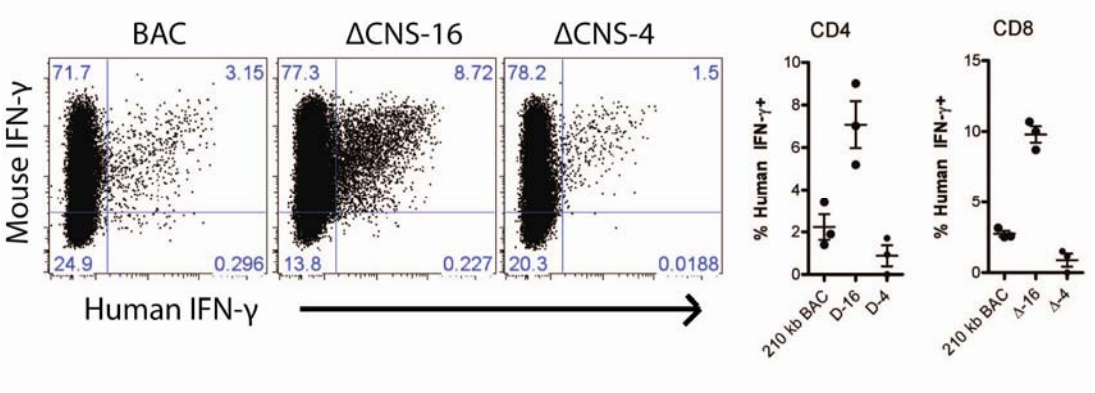

Figure 3-6 Intracellular cytokine staining of CNS-16 and CNS-4 T cell cultures.
Day five CD4+ Th1 cells or CD8+ Tc1 cells were restimulated for five hours with PMA/Ionomyicin and IFN-γ levels were determined by intracellular cytokine staining. Data points are independent experiments.

190 kb, 210 kb, and ΔCNS+20 *IFNG*-BAC mice express *IFNG* equivalently

We next compared mice containing the 190 kb IFNG-BAC transgene and the 210 kb IFNG-BAC transgene. The two transgenes differ primarily by the absence of a +120 kb CCCTC binding factor site in the 190 kb IFNG-BAC. We mapped locus CTCF sites to our *IFNG*-BAC transgene using published ChIP-Seq data (39) (Figure 3-7A). Culture levels of human IFN-γ and murine IFN-γ did not differ between 190 kb and 210 kb *IFNG*-BAC transgenic CD4+ or CD8+ T cell cultures containing either two or three CTCF binding sites, respectively (Figure 3-7B, 5C). Both 190 kb and 210 kb *IFNG*-BAC transgenes demonstrated Th1/Th2 selectivity but neither was expressed at equivalent levels to the endogenous mouse *IFNG* gene (Figure 3-7C). Similarly, the ΔCNS+20 deletion did not affect IFN-γ production by either T cells or NK cells (Figure 3-8). As such, we were unable to demonstrate a functional role for CNS+20 or the +120 kb CTCF site in our transgenic *IFNG*-BAC model system.

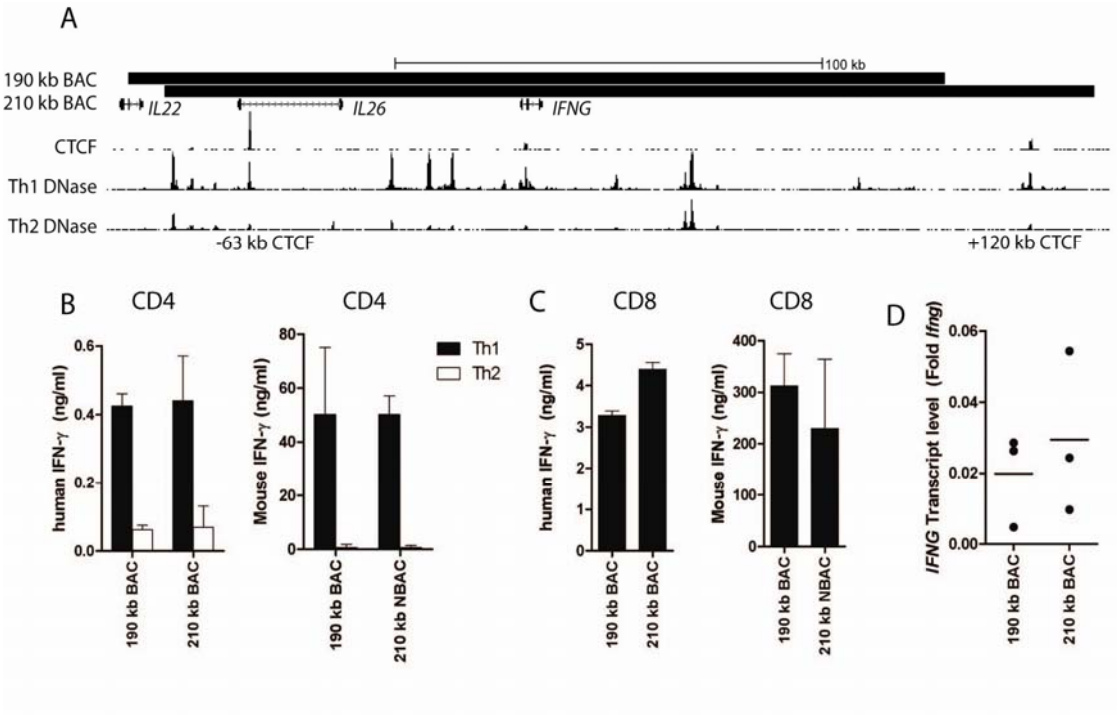

Figure 3-7 Two *IFNG*-BAC transgenes express *IFNG* equivalently.
A, Relative locations of the 190 kb *IFNG*-BAC transgene and 210 *IFNG*-BAC transgenes used in this study, Th1/Th2 DNaseI HS-seq and resting CD4+ T cell CTCF ChIP-seq were mapped using the UCSC genome browser. B, 190 kb or 210 kb *IFNG*-BAC transgenic CD4+ T cells were cultured for three days with anti-CD3/CD28 under Th1 or Th2 polarizing conditions. At day three human and mouse IFN-γ levels in cultures were determined by ELISA. C, Human and mouse IFN-γ levels from day three CD8+ Tc1 cultures. D, CD4+ Th1 cells were cultured for three days, rested for two days in IL-2 media and restimulated with PMA/Ionomycin. Transcript levels were determined by qPCR after cDNA synthesis.

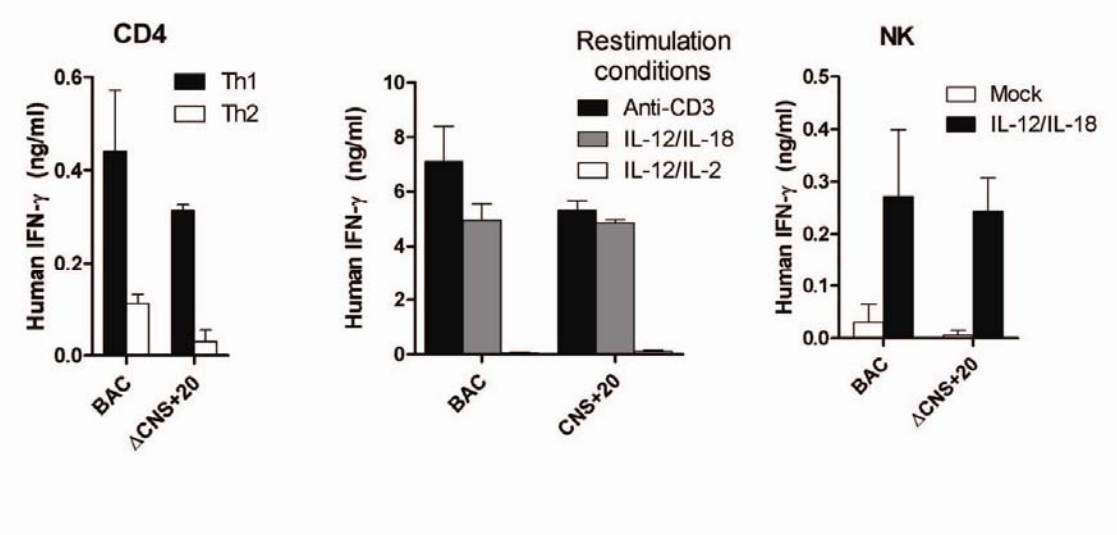

Figure 3-8 Removal of CNS+20 does not result in a significant change in human IFN-γ levels

(A) IFN-γ levels of transgenic CD4+ cells cultured for three days under polarizing conditions. (B) Rested Th1 cells were restimulated with varying concentrations of anti-CD3, IL-12 and IL-18, or IL-12 and IL-2 for two days. (C) Primary DX5+ cells were stimulated with IL-12 and IL-18 for two days

CNS-77 regulates IL26 but not IFNG

To complete our functional analysis of the *IFNG/IL26* locus, we asked whether *IL26* has unique regulatory elements separate from *IFNG* regulatory elements. We returned to transgenic mice lacking ΔCNS-77 from the 190 kb *IFNG*-BAC transgene (ΔCNS-77 mice) created previously (Chapter II). We also examined ΔCNS-30 mice, which lack CNS-30 and express human *IFNG* at ten fold reduced levels to 190 kb *IFNG*-BAC transgenic T cells. The CNS-77 deletion was identified by conservation between humans and mice. However, mapping of covalent histone modifications associated with active enhancers such as histone-3 lysine 4-methyl (H3K4Me1), H3K4Me2 and H3K4Me3 identified two potential regulatory sites on both sides of the CNS-77 deletion (Figure 3-9). To validate CNS-77 itself as an enhancer we cloned the CNS-77 region into a well-characterized *IFNG*-luciferase vector (138). We transfected the *IFNG*-luciferase vector with or without CNS-77 into primary BALB/C splenocytes, cultured with anti-CD3/CD28 for three days and measured luciferase activity after PMA/Ionomycin restimulation (Figure 3-9C). Inclusion of CNS-77 resulted in a relative increase in luciferase activity demonstrating the presence of enhancer function. Next, we identified two NF-κB binding sites within CNS-77. To test for NF-κB dependence, we removed one of these binding sites in a new CNS-77 *IFNG*-luciferase construct. We transfected Jurkat T cells with the three different luciferase constructs and, after recovery, restimulated with PMA/ionomycin. Deletion of one NF-κB site abrogated enhancer function (Figure 3-9D). Having Nf-kB dependent established enhancer potential, we continued with our analysis of ΔCNS-77 mice to determine function in a genomic setting.

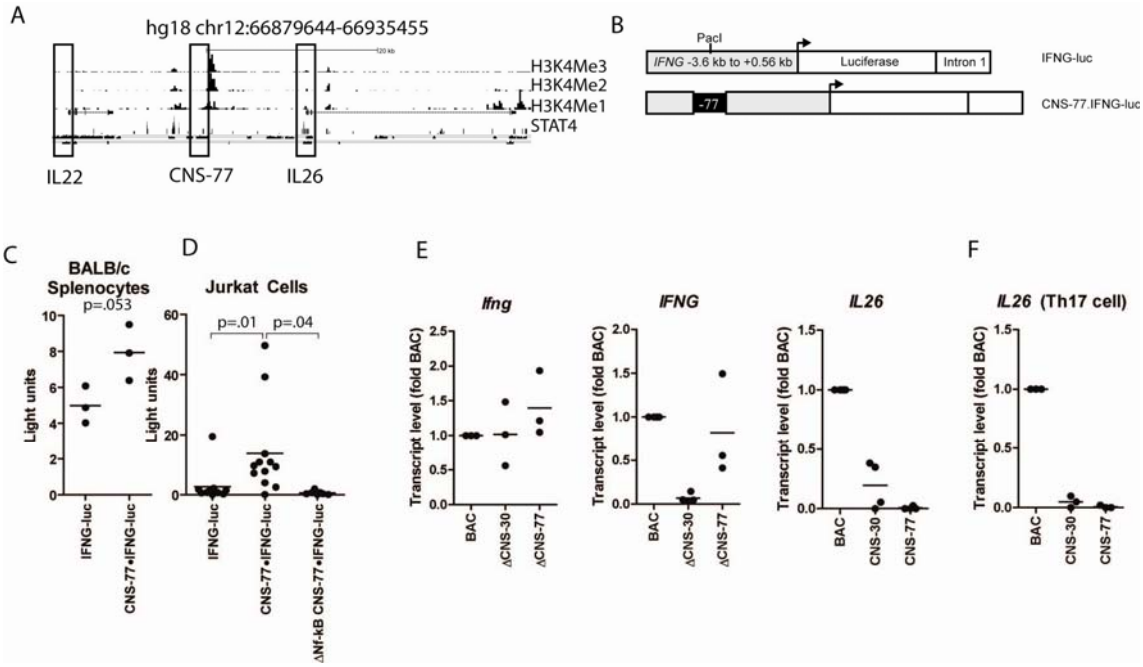

Figure 3-9 *IL26* expression in ΔCNS-77 and ΔCNS-30 CD4 cells

A, Locations of genes, covalent histone marks (37) and STAT4 binding (128) sites were downloaded and mapped using the UCSC genome browser. B, Diagram of *IFNG*-luciferase reporter plasmids. C, BALB/C splenocytes were transfected with *IFNG*-luc or CNS-77•*IFNG*-luc reporter plasmids. Luciferase activity was determined after 24-hour stimulation with anti-CD3 and anti-CD28. D, Jurkat cells were transfected with luciferase constructs and after overnight rest stimulated with PMA/ionomycin for four hours and luciferase activity was determined. Data points are biological replicates of seven independent experiments. E, Rested 190 kb *IFNG*-BAC, ΔCNS-30 or ΔCNS-77 *IFNG*-BAC CD4+ Th1 cells were restimulated with PMA/ionomycin and transcript levels were determined by qPCR. F, Transcript levels of *IL26* in Th17 cells. Data points are biological replicates of three experiments.

To determine if either CNS-77 or CNS-30 was required for *IL26* expression from the 190 kb *IFNG*-BAC we restimulated resting 190 kb *IFNG*-BAC, ΔCNS-30 or ΔCNS-77 Th1 and Th17 cells with PMA/Ionomycin and measured transcript levels (Figure 3-9 E & F). As previously reported, CNS-30 was required for *IFNG* transcription in restimulated Th1 cells. CNS-30 was also required for *IL26* transcription by both Th1 cells and Th17 cells. Deletion of CNS-77 did not alter *IFNG* transcript levels in Th1 or Th17 cells, but removal of CNS-77 resulted in a marked loss of *IL26* transcripts. Combining these results with results from CNS-4, CNS-16 and CNS+20 deletions, we conclude that CNS-4 and CNS-30 deletions had multi-gene phenotypes, impacting both *IFNG* and *IL26* expression. CNS-77 and CNS-16 deletions had single-gene phenotypes, regulating just *IL26* or *IFNG*, respectively.

Discussion

Here we have determined the functional roles of cis-regulatory regions throughout the *IFNG*/*IL26* locus using a BAC transgenic system (Figure 3-10). We have demonstrated that *IFNG* and *IL26* share common regulatory elements in surprisingly both Th1 cells and Th17 cells, demonstrated that loss of one distal CTCF site in the *IFNG* locus is not required for efficient transcription, and defined specific functional roles of conserved *IFNG/IL26* regulatory regions. We demonstrate that CNS-4, CNS-16, and CNS-30 play key roles in regulating *IFNG* expression. We show that IFN-γ production in response to either T cell receptor signals or cytokine signaling requires distinct cis-regulatory elements. Th1/Th2 specificity is not solely dependent upon activating Th1 enhancers but also requires Th2 specific repressors, as evidenced by a lack of complete *IFNG* repression in Th2 cells lacking CNS-16. We have identified cis-regulatory elements necessary for only *IFNG* or *IL26* expression and alternatively identified cis-regulatory elements whose function is shared between both *IFNG* and *IL26*.

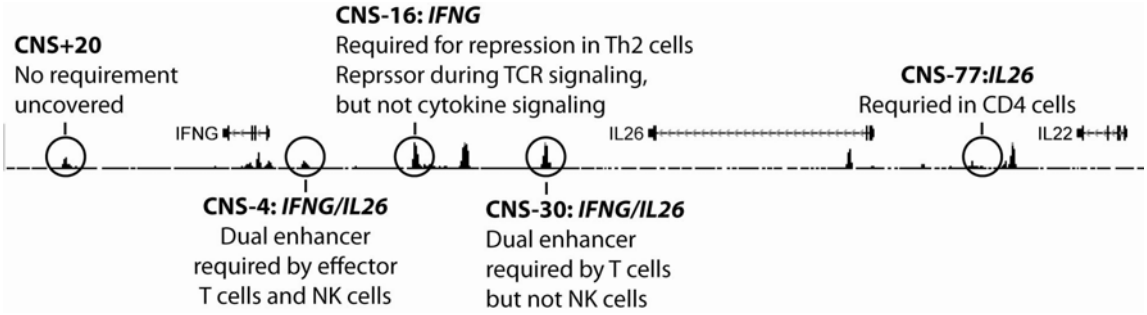

Figure 3-10 Summary of *IFNG*-BAC CNS deletion phenotypes.
Plotted are relative locations of Th1 DNase-HS sites, labeled with function in an *IFNG*-BAC transgene. Each CNS is listed with what gene it is required for, and a short description of what cell type and/or stimulus it is required for. The summary for CNS-30 includes previous results (129).

A particularly novel finding is that expression of both *IL26* and *IFNG* requires CNS-30 and CNS-4 and both show Th1/Th2 selectivity, establishing an *IFNG*/*IL26* gene locus. The IL26 promoter closely resembles the *IFNG* promoter, but not the IL22 promoter in that the *IL26* promoter contains T-box binding sites and is a site of STAT4 recruitment. However, *IL26* also exhibits distinct expression from *IFNG* and is upregulated in Th17 cells. Distinct regulation of *IFNG* and *IL26* is accompanied by distinct cis-regulatory elements for each gene. *IFNG* requires CNS-16 for Th1-specific expression and *IL26* requires CNS-77 for transgenic expression in both Th1 and Th17 cell types. How the *IFNG*/*IL26* locus integrates information for coordinated repression and/or activation of *IFNG* and *IL26* will be a subject of future investigation. Expression of *IL26* in both human and mouse T cells was low. Further, currently available reagents prevented meaningful analysis of IL-26 protein expression. Therefore, it is not clear if there are Th1 IL-26+ cells and/or Th17 IL-26+ cells. It is also currently not clear exactly what specific cell types, if any, express *IL26* at abundant levels. Further, the in vivo function of *IL26* is currently unknown due, in part, to absence of mouse models. The *IL26* and *IFNG* locus is a candidate locus for ulcerative colitis risk (4). However, this risk association has not been mechanistically linked to either gene. Further, transgenic expression of human *IL26* does not cause spontaneous inflammation in a mouse model, or impact severity of DSS-induced colitis. Future studies will also focus on determining the function of *IL26* in the immune system.

One surprising finding is the requirement of CNS-16 for T cell-receptor mediated repression of *IFNG* in Th2 cells. A similar phenotype is seen in transgenic mice carrying only an 8.6 kb *IFNG* transgene, in which Th2 cells do not repress *IFNG* in response to T

cell-receptor signaling (98). One hypothesis is repressive function is conferred by the recruitment of YY1, which represses *IFNG* expression (137). Alternatively, CNS-16 may function to recruit repressive HDAC proteins. In mice, recruitment of HDACs to the CNS-16 homolog is critical for proper suppression of *Ifng* (44). However, in a similar BAC transgenic system, the murine CNS-16 homolog is absolutely necessary for transcription of an *Ifng*-BAC transgene reporter (74). Given that CNS-16 is not conserved outside of non-placental mammals and BAC transgenic expression of *IFNG* is possible without CNS-16, CNS-16 is not universally required for interferon gamma expression. Instead, the CNS-16 homolog STAT4 binding potential may be more critical in the C57BL/6 mouse *Ifng* locus than it is in the human locus. Our chromatin mapping and conservation analysis reveals a multitude of differences between the mouse and human locus, possibly explaining the differences in phenotypes. The human but not mouse locus contains hCNS-22 (Figure 3-1), which may provide additional function in the human genome relative to the murine genome. The mouse *Ifng* locus also contains wide-ranging structural variation including serial duplications of the *Cdc5l* processed pseudogene. Retrotransposons are well known to have regulatory effects on nearby protein-coding genes (139). The C57BL/6, but not BALB/c, genome also contains a large duplication and inversion of *Il22* and the *Cdc5l* pseudogenes (134). Given these differences, comparative genomics between the C57BL/6 and BALB/c mouse *Ifng* loci and human *IFNG*/*IL26* loci will likely provide fruitful insights into the role of species-specific regulatory regions and structural variation.

Although CNS-77, CNS-30, CNS-16 and CNS-4 exhibit necessary functions in regulation of *IFNG/IL26* expression in our *IFNG*-BAC transgenic system, *IFNG/IL26*

expression does not exhibit similar requirements for CNS+20 or CNS+120. Both CNS+20 and CNS+120 are well conserved throughout mammals, implying function. CNS+120 is thought to facilitate three-dimensional organization of the *IFNG* locus via CTCF binding. CTCF has been described as a transcriptional activator (31), insulator (32) and repressor (33). However, most of these functional studies come from reporter assay systems which do not take genomic context into account and a relevant question is the exact function of CTCF binding sites in the genome. In a genomic context, experimental mutation of a single CTCF site in the β-globin locus affects chromatin looping and accessibility, but not expression of the β-globin gene (34). Arguing against an insulating role of the +120 CTCF site in the *IFNG* locus are previous experiments reporting copy-number dependence for 190 kb *IFNG*-BAC transgenes lacking the +120 CTCF site (Chapter II), and equivalent expression between 190 kb and 210 kb *IFNG* BAC transgenes. Another hypothesis would be that the +120 kb and -63 kb CTCF sites serve to bring the *IFNG* locus into close physical location with other genes in the surrounding regions and may play a role in co-regulation of *IFNG* and *IL26* or may govern interactions between the *Ifng* locus and the *Il4* locus (28). Like CNS+120, CNS+20 does not have an appreciable role in our model system. In *IFNG*-luciferase systems, CNS+20 has enhancer potential (67, 79), but CNS+20 may only have necessary functions observable under suboptimal signaling conditions in cooperation with CNS-4. To analyze this hypothesis, future studies will focus on experiments, *in vivo,* to determine the roles of cis-regulatory elements in the context of infections, which suppress the immune system, as well as determining expression when specific transcription factors are absent.

Despite recent advances in the ability to identify cis-regulatory element location, it is difficult to answer basic questions about function of a cis-regulatory element such as what gene it regulates, in what cell type and whether it plays a repressor or activator role. *IFNG*/*IL26* regulatory elements are necessary for functionally redundant roles and also necessary for highly specialized roles. For example, there are cell-type specific cis-activating elements, such as CNS-30 (Chapter II), and stimulus specific repressors, such as CNS-16. Consistent with these results, a growing body of evidence suggests that cis-regulation is necessary for very exact cell and stimulus specific roles (27, 140). Alternatively, we also identified redundant roles for cis-regulation. CNS-30 and CNS-4 both recruit Pol II and are both required for *IFNG*/*IL26* production in mature T cells. Consistent with these results, studies in *Drosophila* demonstrate that redundant enhancers provide phenotypic robustness under suboptimal conditions (141). Lastly, we find evidence of shared requirements for individual cis-regulatory elements between adjacent genes. As such, human cis-regulatory elements may be necessary for a variety of different functions, not currently predictable without experimental testing. The multiplicity of potential cis-regulatory functions may explain why it has been difficult to link trait associated human single polymorphisms with function (6). Our results show a surprisingly wide breath of function that cis-regulatory elements possess, and illustrate the great diversity of phenotypes that can result from cis-regulatory mutations in humans.

CHAPTER IV

Synopsis and conclusion

Conclusions

In this work I show that *IFNG* cis-regulatory elements have highly specialized roles, each necessary for a unique functional requirement. I also redefine the *IFNG* locus and show that it is part of a larger *IFNG/IL26* locus whereby both genes utilize common regulatory elements to produce shared patterns of expression.

The major finding of chapter II is that utilization of *IFNG* distal regulatory elements is cell-type specific. Natural killer cells and cells of the T cell lineage do not have similar requirements for *IFNG* expression from an *IFNG*-BAC transgene. This phenotype is observed in two instances. First, expression of *IFNG* in T cells is predominately restricted to mouse IFN-γ positive cells, while in natural killer cells human IFN-γ expression is observed in mouse IFN-γ negative cells and mouse IFN-γ positive cells. A second way in which expression of *IFNG* differs between T cells and NK cells is that T cells require CNS-30 for proper expression of human IFN-γ. In contrast, NK cells do not require CNS-30.

A second important finding of chapter II is the mechanism of action of CNS-30. Transcriptional regulation can occur at several stages including alterations in chromosomal accessibility, transcription factor recruitment, polymerase recruitment and polymerase initiation and elongation. It was our assumption that CNS-30 would function

to promote chromosome accessibility. However, removal of CNS-30 from an IFNG-BAC transgene does not change locus acetylation or H3K9me2, compared to controls. Instead, removal of CNS-30 completely prevents recruitment of polymerase showing that CNS-30 plays a major role in late stages in the activation of *IFNG*. The requirement for Pol II binding correlates with Runx3 binding at CNS-30 and, as shown in chapter III, STAT4 and STAT5B binding at CNS-30 in activated T cells. As such, the coordinated action of Th1-promoting transcription factor binding at this distal regulatory element serves to permit RNA Pol II recruitment to this CNS as well as to the *IFNG* promoter.

The major finding of chapter III is that some *IFNG* cis-regulatory elements are not redundant. We had expected to find a series of cis-regulatory elements all necessary for *IFNG* expression. Instead, we found several cis-regulatory elements, which do not affect human IFN-γ expression from our *IFNG*-BAC model system, such as CNS+20 and CTCF binding CNS+120. Other elements have very selective functions, such as CNS-4, which promotes epigenetically stable expression of *IFNG* in mature T cells and natural killer cells. Loss of CNS-16 produces a phenotype opposite of expectations and results in aberrantly high human IFN-γ expression in T cells stimulated with T-cell receptor signaling, but not T cells or NK cells stimulated with IL-12 and IL-18. Functionally, CNS-16 appears to play a necessary role to actively repress *IFNG* expression in effector Th2 cells. This model of highly specific distal regulatory elements is very consistent with the view of cis-regulation in evolution, where the acquisition of necessary cis-regulatory elements occurs when new functions are acquired and thus cis-regulatory elements have very fine-tuned roles.

A subsequent finding of chapter III is that *IFNG* and *IL26* share common regulatory elements and expression, but also have distinct regulatory elements and distinct expression profiles. For example, both *IFNG* and *IL26* exhibit relatively high expression in Th1 cells compared with Th2 cells. However, only *IL26* is also expressed at relatively high levels in Th17 cells while *IFNG* is repressed in *in vitro* generated murine Th17 cells. Both genes contain similar promoters, with T-box and STAT binding sites. Further, the co- and differential expression is in part achieved by shared cis-regulatory elements and distinct cis-regulatory elements. In CD4+ T cells, CNS-77 is required for *IL26* expression but not *IFNG* expression. Similarly, removal of CNS-16 from the IFNG-BAC transgene results in high *IFNG* expression but not high *IL26* expression in response to PMA/Ionomycin stimulation. In contrast, both elements share CNS-4 and CNS-30 because removal of either from the IFNG-BAC transgene prevents expression of both *IL26* and *IFNG*.

Future Directions

Distal regulation of Interferon Gamma

Distal regulation of the IFNG locus varies between natural killer cells and T cells. This result is expected in a model of functionally specific cis-regulatory elements, but mechanistically unclear. It may be possible to understand the differences in transcriptional regulation between natural killer cells and T cells from two approaches. Firstly, using BAC model systems additional cis-regulatory elements can be discovered and their roles in natural killer cells versus other T cells can be probed. This may be

promising in the +40 to +120 kb region of the IFNG-BAC transgene, which does not appear to be required for expression in T cells. This first approach stems from the hypothesis that natural killer cells utilize distinct cis-regulatory elements for proper transcription and could be verified mechanistically by examining chromatin accessibility and transcription factor recruitment to the IFNG locus. If true, natural killer cells should have distinct patterns of DNase I hypersensitivity, transcription factor and Pol II recruitment and covalent histone modifications.

However, a second possible hypothesis may also explain why natural killer cells and T cells have different functional requirements for *IFNG* distal regulatory elements. In this hypothesis, the transcription factor population required to promote cytokine gene expression is fundamentally different in natural killer cells and T cells due to broad chromatin regulation. This hypothesis is supported by the observation that mouse natural killer cells express high levels of both *Ifng* and transgenic *IFNG* transcript, and other reports detail post-transcriptional regulation of cytokine production in natural killer cells (142). Under this hypothesis, a repressive transcription factor or chromatin regulation protein could be differently modified or expressed in T cells and natural killer cells, allowing easier expression in NK cells. If true, many observations such as DNaseI hypersensitivity and transcription factor binding may be different in NK cells than in T cells. So, a transgenic approach to test sufficiency rather than necessity may be appropriate. Further, analysis of protein expression and modification by mass spectrometry approaches will provide insight into effector protein differences. If natural killer cells have fundamentally different requirements for gene expression, understanding these differences will provide needed basic insight into how gene expression is portioned

between the innate immune system, which does not need to avoid autoimmunity, and the adaptive immune system, which does.

Reports on the relationship between cis-regulatory elements and chromatin accessibility, Pol II recruitment and transcription factor recruitment have varied based upon experimental system and gene locus, as described in chapter I. In the IFNG-BAC transgenic system, CNS-30 is required for Pol II recruitment, but not required for locus histone tail acetylation or methylation. Using transgenic mice lacking CNS-30, CNS-16, CNS-4 and CNS+20 the relationship between distal regulation and effector protein recruitment and chromatin accessibility of the *IFNG* locus can be studied in fine detail. For experimental purposes transcription can be broken into four broad stages: chromatin accessibility, transcription factor recruitment, polymerase recruitment, and polymerase initiation and elongation. A fifth very poorly understood stage of nuclear positioning could also be added. Using chromatin immunoprecipitation techniques, the relationship between distal regulatory elements to each of these first four stages could be tested with the IFNG-BAC transgenes. For example, CNS-4 seems required for IFNG expression after primary stimulation. If CNS-4 is required for establishing an epigenetically permissive *IFNG* locus then histone acetylation or *IFNG* promoter H3K4Me3 marks may be absent in ΔCNS-4 Th1 cells. Secondly, using the two different 190 kb and 210 kb IFNG-BAC transgenes the relationship between CTCF binding sites, chromatin looping, insulation and polymerase initiation can be probed. Finally, the relationship between cis-regulation and location in the nucleus is not clear. Using the different 190 kb and 210 kb BAC transgenes, and CNS deletions, 3D FISH technology could be used to determine if

transgene incorporation in to transcription factory 'hubs' depends upon distal CTCF sites and/or *IFNG* regulatory elements.

Interleukin 26

The mouse *Ifng* locus is interrupted with serially duplicated *Cdc5l* retrotransposed pseudogenes. These pseudogenes are evolutionarily recent, with higher homology to the mouse than rat *Cdc5l* parent transcript. The *Il22* gene and surrounding region is duplicated in some strains of mice, but not others. This duplication, by homology, contains *Cdc5l* pseudogenes. This would theoretically create varying amounts of *Cdc5l* pseudogene duplications in different strains of mice. A congenic approach could provide insights into the role of structural variation and retrotransposed elements in the local chromatin environment. Besides implications for mouse models of *Ifng* expression, structural variation has been implicated in many different oncogenic events and retrotransposition events are known to impact local chromatin environments by recruiting repressive histone modifying proteins. An *Ifng* locus congenic, or BAC-transgenic model of the *Ifng* locus with and without *Cdc5l* repetitive elements would be an excellent model to understand structural variation and the role retrotransposed pseudogenes play in long-range regulation and the establishment of proper epigenetic states. Of note, mice with theoretically few *Cdc5l* pseudogenes, like BALB/c have low IFN-γ expression, while strains with theoretically high *Cdc5l* pseuodgene counts, like C57BL/6 have high IFN-γ expression.

Mice lack *Il26* and this may explain why little is known about the function of IL-26 in both the innate and adaptive arms of the immune system. However, many genes are

lacking in mice and little may be known about IL-26 because IL-26 may be vestigial or of little importance in higher mammals. Arguing against an unimportant role in the immune system is the fact that IL-26 is conserved throughout the metazoan tree, arguing for some essential function. In tissue culture models, recombinant IL-26 promotes IL-8 and anti-microbial peptide synthesis and release. Further, IL-26 is highly expressed in ulcerative colitis inflamed lesions. However it is still unclear which cell types in response to which stimuli produce the IL-26 protein. While the gene shares regulation with *IFNG*, it does not share high expression with *IFNG* in Th1 cells. Possibly, select tissues other than mature Th1 or Th17 cells may express IL-26. For example, inflamed epithelial cells produce IL-22 and may express IL26. It may be possible to better understand IL-26 biology with the BAC transgene system we have developed. It would first be important to establish that the human IL-26 protein is functional in the mouse by establishing expression of the IL-26 receptor in the mouse, and if recombinant human IL-26 can activate the mouse IL-26 receptor. In a DSS colitis experiment, the IFNG-BAC transgene provided protection from DSS in initial experiments, but not upon subsequent experiments. With refinement of experimental procedures, or with alternative models of colitis and inflammation, a role of IL26 in the immune system could be elucidated.

Human Polymorphisms

In a recent genome wide association study, an ulcerative colitis associated single nucleotide polymorphism was identified on the *IFNG* locus. The more probable association was found in a region not containing any of the known *IFNG* regulatory elements established in my BAC transgenic model, or studied in mouse homologs.

Instead, the region appears devoid of IFN-γ cis-regulatory elements, other than one conserved noncoding region at +80 kb from the *IFNG* start site (Figure 4-1). CNS+80 shows Th1 specific DNaseI HS, as well as STAT4 and STAT5B binding. However, CNS+80 is 30 kb from the GWAS-identified rs158744. Linkage disequilibrium is low, and the HapMap CEU r squared value for this region does not exceed 0.5. Further, rs1558744 does not have a high r squared value for any region in the established *IFNG* regulatory regions. As such, further characterization of CNS+80 and its relationship to rs158744 is needed, as well as associative studies between the UC-associated polymorphisms and cytokine gene expression. Cis-regulation is notorious for jumping genes, and even acting on opposing chromosomes, making analysis difficult. However, there is a long and controversial relationship between IFN-γ and inflammatory bowel disease. As such, the relationship between rs158744 and *IFNG* expression needs to be examined further.

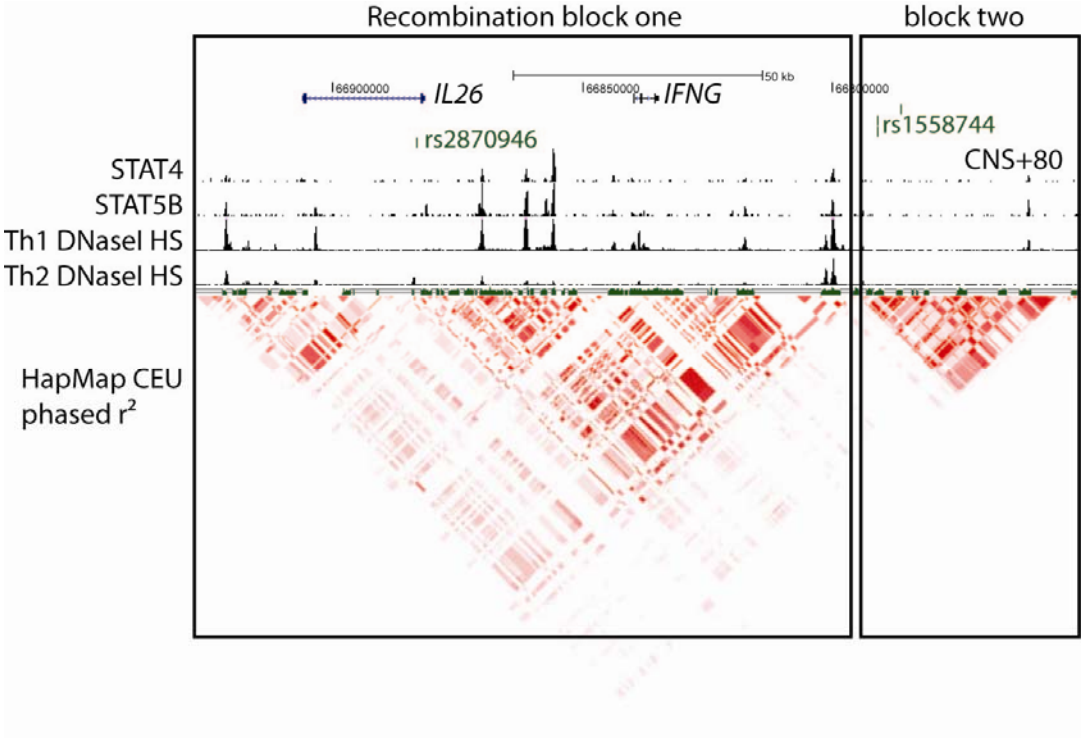

Figure 4-1. The interferon gamma locus and genome wide association studies
STAT4, STAT5B, Th1 DNaseI HS, Th2 DNaseI HS and HapMap CEU phased r^2 values were plotted using the UCSC genome browser. Labeled SNPs are associated with ulcerative colitis by a genome wide association study by Sileverberg et al. While the SNP rs2870946 is within high linkage disequilibrium to most of the know interferon gamma locus, SNP rs1558744 is separated from the known IFNG locus by a recombination hot spot.

In preliminary experiments, genotype of rs158744 correlated with *IFNG* transcript level in both cultures of peripheral Th1 cells and in peripheral blood mononuclear cells. Increasing copies of the rs1558744 minor allele correlate with increasingly lower levels of *IFNG* transcript. As such, if rs158744 genotype can be mechanistically linked to a CNS+80 defect then a role of *IFNG* cis-regulation in ulcerative colitis pathogenesis can be established. Because rs158744 is common and because human T cells can be easily grown and manipulated *ex vivo*, a CNS+80 defect would be an ideal model system to study distal regulation in humans. A growing observation in the field of human genetics is that the majority of the common variation associated with disease lies in the noncoding segment of the genome (6). As such, understanding the non-coding segment of the genome will be critical for the development of novel therapeutics, tailored medicine and biomarkers. *IFNG* CNS+80 would be a prime example, as IFN-γ expression will have roles in many aspects of the immune system and is a viable therapeutic target. Further, as analysis of genetic susceptibility goes from common variants to uncommon and rare variants, more and more cis-regulatory elements are likely to be discovered. Understanding cis-regulation from a functional standpoint will be, and is currently, absolutely necessary to interpret human disease risk and thus treatment.

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