Supplementary Information

"Long-range genomic interactions epigenetically regulate the expression of a cytokine receptor"

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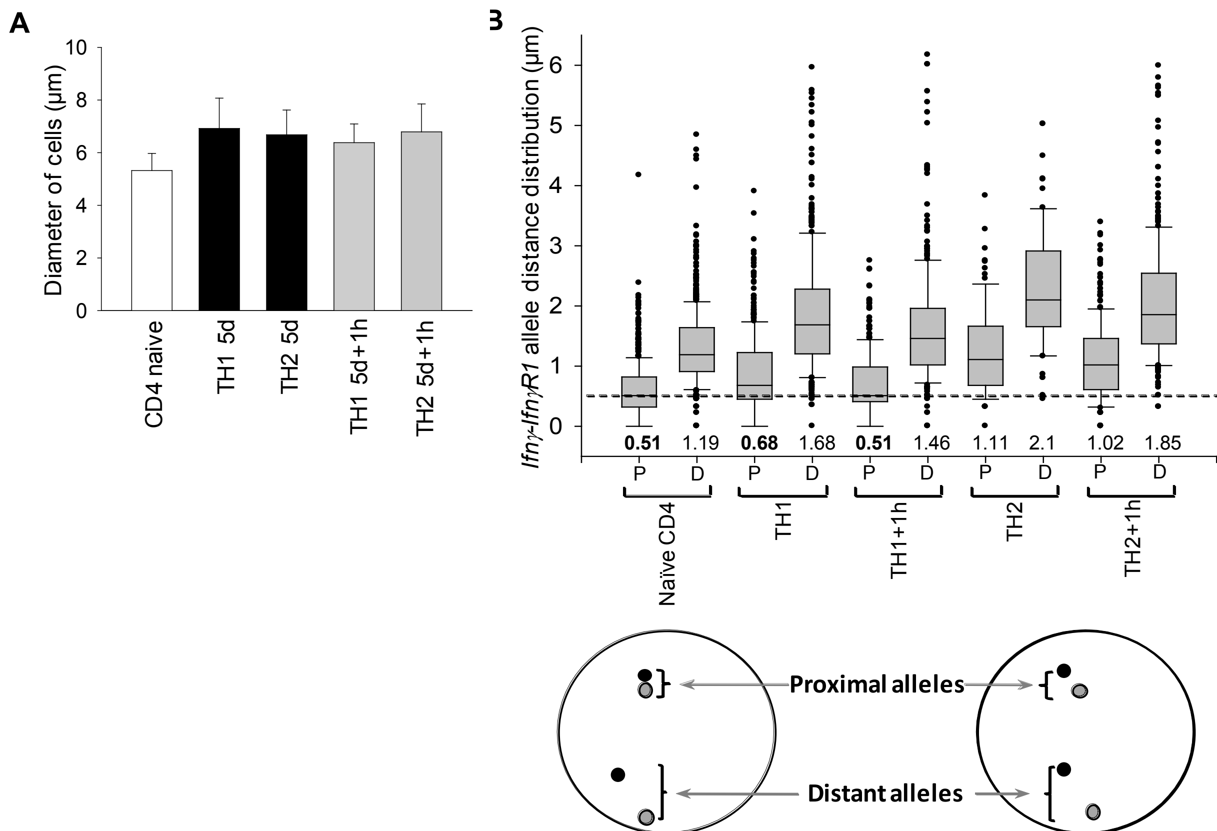
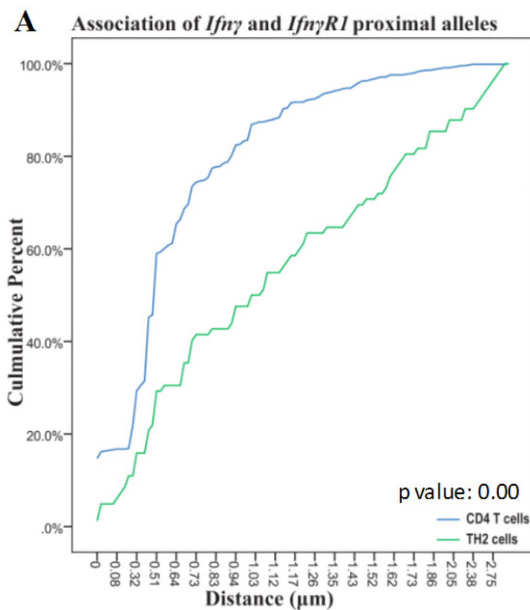
Supplementary Figure 1

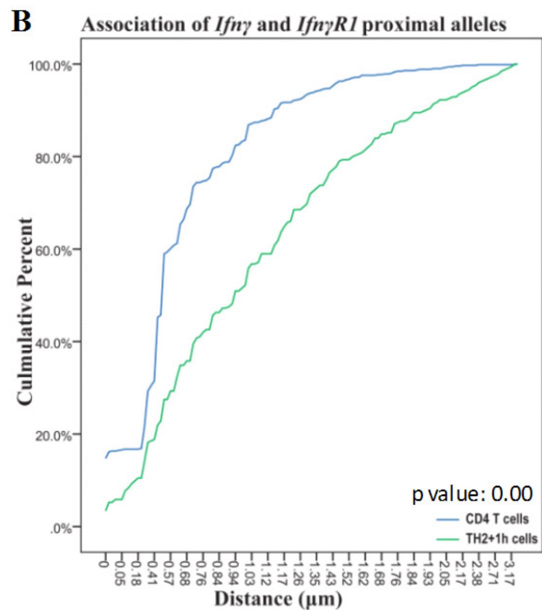
Statistically significant *Ifnγ-IfnγR1* allele colocalization in specific $CD4^+$ T cell lineages. In the DNA-FISH experiments presented in Figure 1D for each cell we have scored (total number of cells: 5568), we measured the longest diameter of each cell, the distance of the two most proximal signals for the *Ifnγ-IfnγR1* alleles/signals (taken from the center of one *Ifnγ* signal to the center of the proximal *IfnγR1* signal) and finally the distance of the *Ifnγ-IfnγR1* distant alleles/signals. The measurements were performed in three dimensional cell nuclei using the Volocity software. **(A)** The average diameter of the cells we have scored is 5.32±0.65 μm for non-differentiated $CD4^+$ cells, 6.9±1.15 μm for TH1 cells, 6.68±0.94 μm for the TH2 cells, 6.38±0.71 μm for the restimulated TH1 cells (TH1+1) and 6.79±1.06 μm for the restimulated TH2 cells (TH2+1). We found that the TH2 and restimulated TH2 cells have lower levels of *Ifnγ-IfnγR1* colocalization not due to the increase of their nuclear size since they show similar nuclear sizes to the TH1 cell lineages. **(B)** Quantitative analysis of the *Ifnγ-IfnγR1* allele distance distribution of all the cells scored for DNA-FISH in Figure 2. The numbers denote the median values of all the distances of *Ifnγ-IfnγR1* alleles measured for each cell type. P indicates the measurements of the *Ifnγ-IfnγR1* proximal alleles and D indicates the measurements for the *Ifnγ-IfnγR1* more distant alleles (see graph).

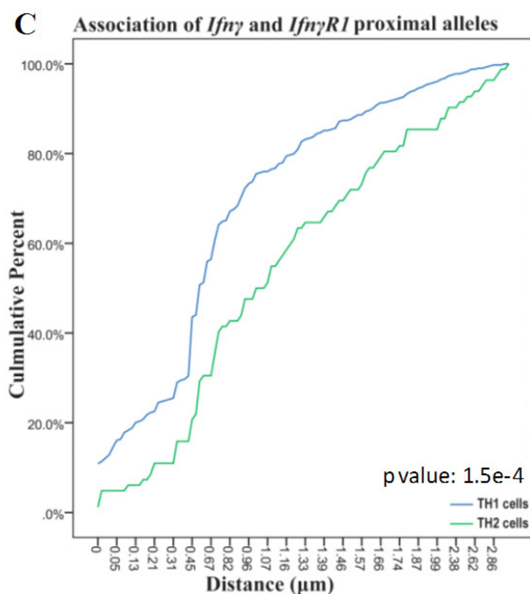


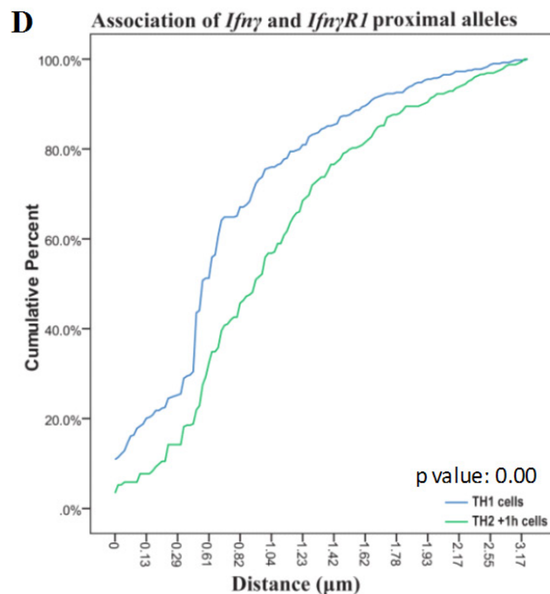
Supplementary Figure 2

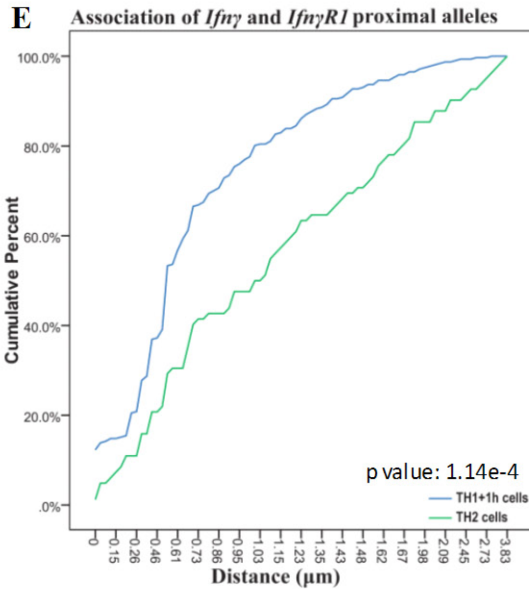
Kolmogorov-Smirnov test. Statistical analysis has been performed for the data presented in Supplemental Figure 1B. The statistical significance of pairwise distributions' dissimilarity was assessed with the nonparametric two-sample Kolmogorov-Smirnov (KS) test. There is a statistically significant difference in the distribution of the *Ifnγ-IfnγR1* proximal alleles between the non-differentiated $CD4^+$ and cells of the TH2 cell lineage (A, B), TH1 or restimulated TH1 cells and the TH2 or restimulated TH2 cells respectively (C-F). Non-differentiated $CD4^+$ and cells of the TH1 cell lineage have similar distributions for the distances of the *Ifnγ-IfnγR1* proximal alleles (G-J). The X axis represents the allele distance measured and the Y axis represents cumulative percent. N=5568 cells. The reported p values were calculated with the IBM SPSS19 software package. A P value of 0.00 denotes a value of probability for rejecting the *null* hypothesis close to 0.00 with statistical significance $p<0.001$.

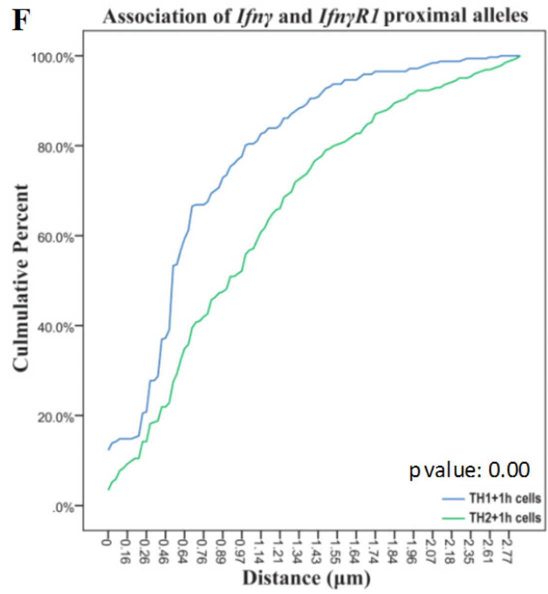


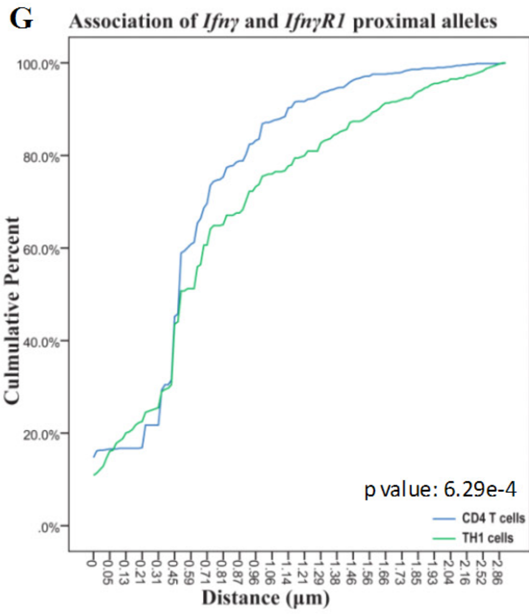


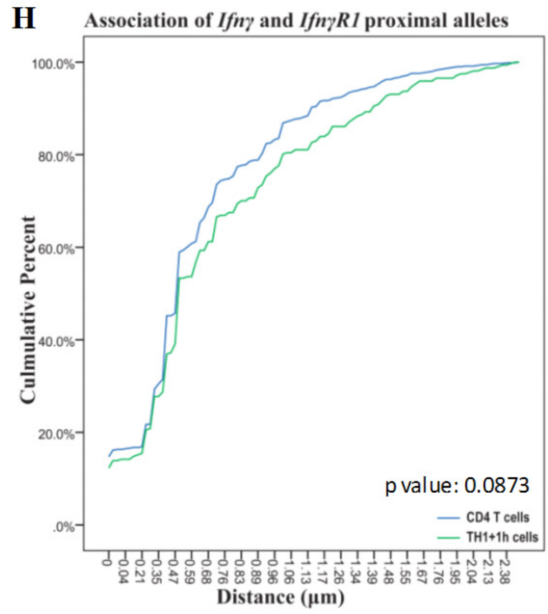


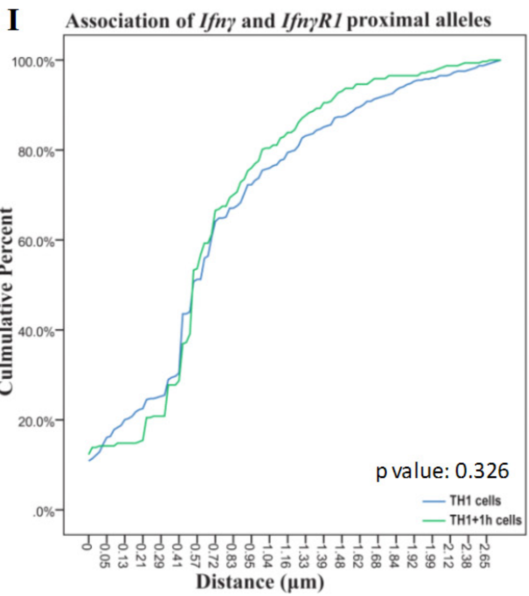


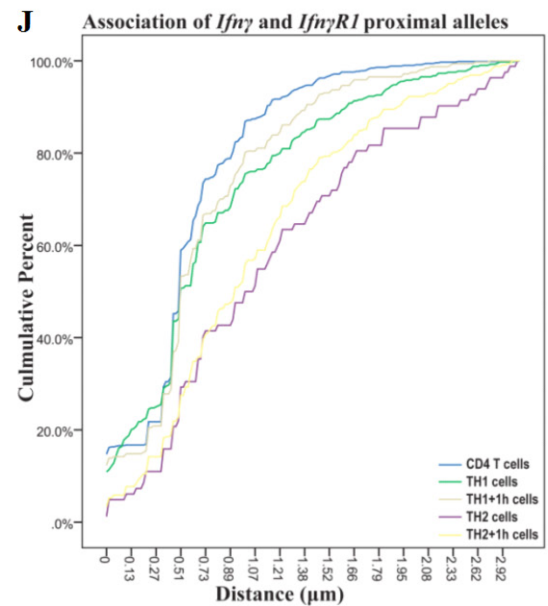

E
Association of Ifnγ and IfnγR1 proximal alleles
Cumulative Percent
100.0%
80.0%
60.0%
40.0%
20.0%
.0%
p value: 1.14e-4
TH1+1h cells
TH2 cells
0 0.15 0.26 0.46 0.61 0.73 0.86 0.95 1.03 1.15 1.23 1.35 1.43 1.48 1.62 1.67 1.98 2.09 2.45 2.73 3.83
Distance (μm)


F
Association of Ifnγ and IfnγR1 proximal alleles
Culmulative Percent
100.0%
80.0%
60.0%
40.0%
20.0%
.0%
p value: 0.00
TH1+1h cells
TH2+1h cells
0 0.16 0.26 0.46 0.64 0.76 0.89 0.97 1.14 1.21 1.34 1.43 1.55 1.64 1.74 1.84 1.96 2.07 2.18 2.35 2.61 2.77
Distance (μm)


G
Association of Ifnγ and IfnγR1 proximal alleles
Culmulative Percent
100.0%
80.0%
60.0%
40.0%
20.0%
.0%
p value: 6.29e-4
CD4 T cells
TH1 cells
0 0.05 0.13 0.21 0.31 0.45 0.59 0.71 0.81 0.87 0.96 1.06 1.14 1.21 1.29 1.38 1.46 1.56 1.66 1.73 1.85 1.93 2.04 2.16 2.52 2.86
Distance (μm)

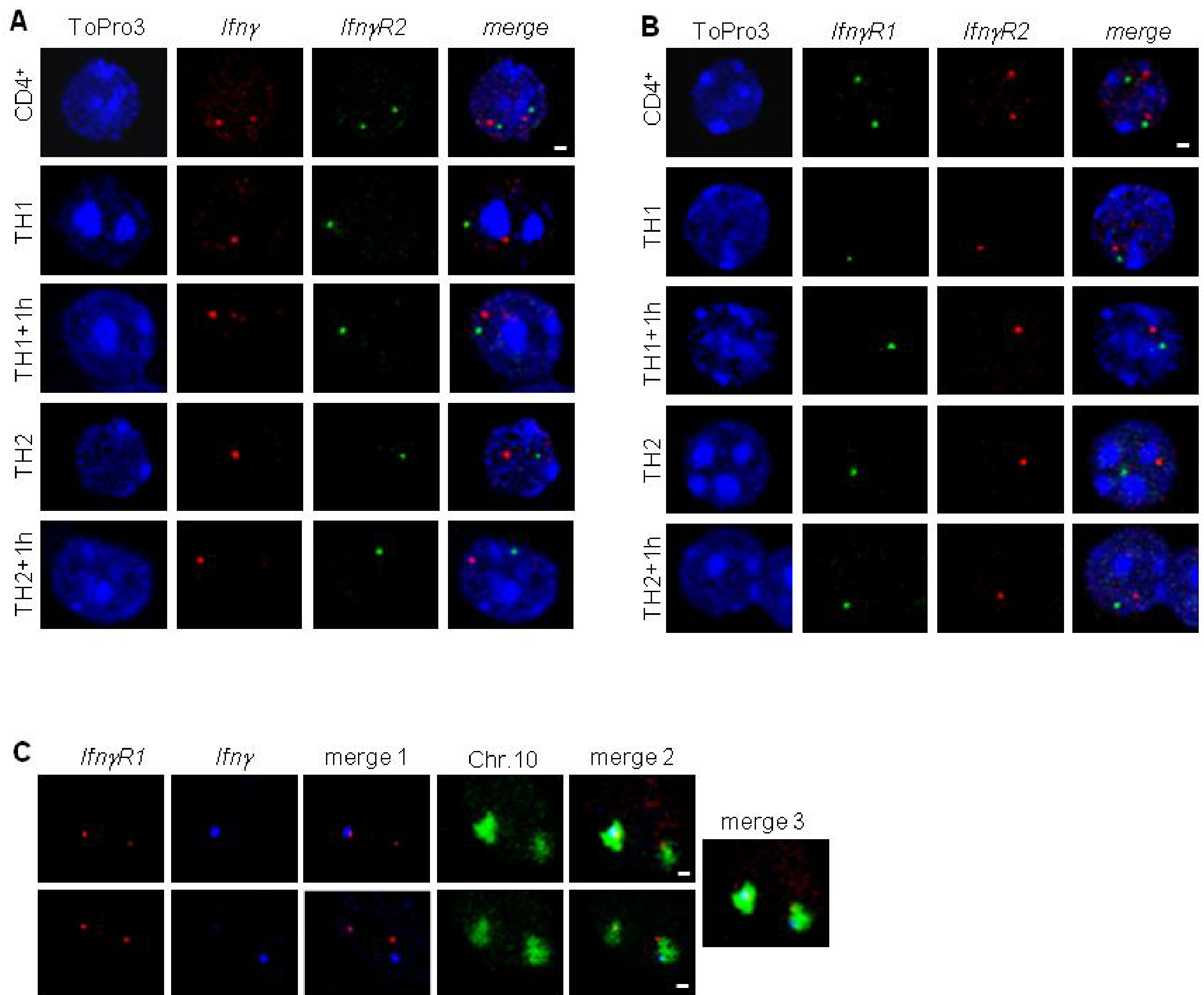

H
Association of Ifnγ and IfnγR1 proximal alleles
Culmulative Percent
100.0%
80.0%
60.0%
40.0%
20.0%
.0%
p value: 0.0873
CD4 T cells
TH1+1h cells
0 0.04 0.21 0.35 0.47 0.59 0.68 0.76 0.83 0.89 0.96 1.06 1.13 1.17 1.26 1.34 1.39 1.48 1.55 1.67 1.76 1.95 2.04 2.13 2.38
Distance (μm)


I
Association of Ifnγ and IfnγR1 proximal alleles
Culmulative Percent
100.0%
80.0%
60.0%
40.0%
20.0%
.0%
p value: 0.326
TH1 cells
TH1+1h cells
0 0.05 0.13 0.21 0.29 0.41 0.57 0.72 0.83 0.95 1.04 1.16 1.33 1.39 1.48 1.62 1.68 1.84 1.92 1.99 2.12 2.38 2.65
Distance (μm)


J
Association of Ifnγ and IfnγR1 proximal alleles
Culmulative Percent
100.0%
80.0%
60.0%
40.0%
20.0%
.0%
CD4 T cells
TH1 cells
TH1+1h cells
TH2 cells
TH2+1h cells
0 0.13 0.27 0.51 0.73 0.89 1.07 1.21 1.38 1.52 1.66 1.79 1.95 2.08 2.33 2.62 2.92
Distance (μm)

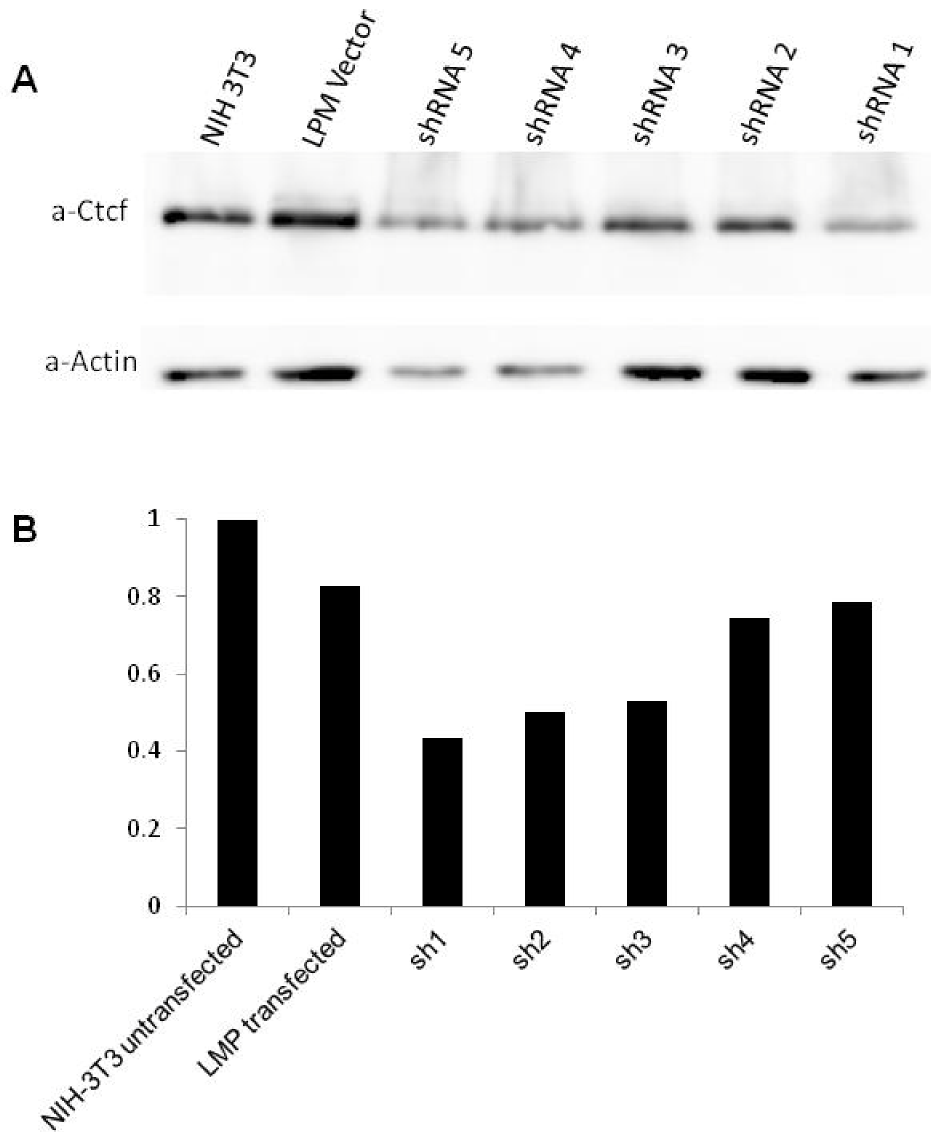
Supplementary Figure 3

DNA-FISH and chromosome painting for the assessment of intra- and inter-chromosomal interactions. (A) DNA-FISH for the *Ifnγ* (labeled with spectrum orange) and *IfnγR2* (labeled with spectrum Green) gene loci. 1338 cells have been scored totally in 2-5 independent experiments for each cell type. (B) DNA-FISH for the *IfnγR1* (labeled with spectrum green) and *IfnγR2* (labeled with spectrum orange) gene loci. 1378 cells scored totally in 2-5 independent experiments for each cell type. (C) Chromosome painting in non-differentiated $CD4^{+}$ cells for mouse chromosome 10 (FITC) in combination with DNA-FISH for the *Ifnγ* (labeled with Alexa Fluor-647 dCTP and pseudocolored blue) and *IfnγR1* (labeled with spectrum orange) gene loci. Top and bottom panels are representative images of two different focal plains of the same cell. Scale bar in all images is 1μm. DNA was counterstained with ToPro3 and pseudocolored blue.



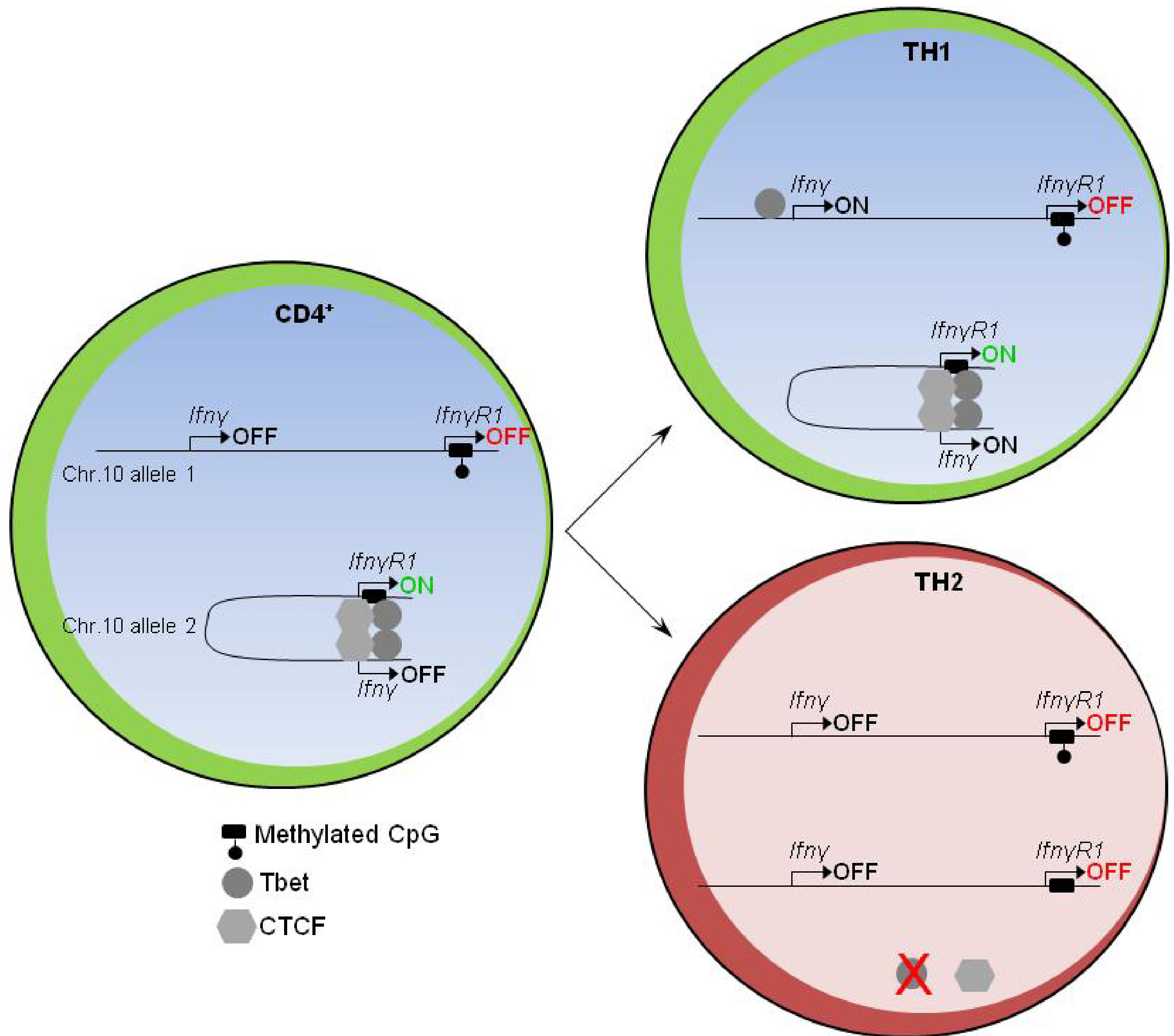
Supplementary Figure 4

Retroviral transduction of short hairpin RNAs (shRNAs) targeting CTCF. (A) Western blot analysis for the expression of CTCF in NIH-3T3 cells before and after the retroviral transduction of five different shRNAs targeting CTCF. LMP: retroviral transduction of an empty vector. (B) Densitometric analysis using the software Image J for the western blot presented in A. Correction is performed using the expression of Actin.



Supplementary Figure 5

Suggested model for the *IfnγR1* transcriptional regulation in $CD4^+$ T cell lineages.



Supplementary Methods

Mice and $CD4^+$ T cell isolation

All experiments were conducted according to institutional guidelines upon ethical committee approval. Peripheral $CD4^+$ cells were isolated from spleen and lymph nodes of 4-6 week-old C57BL/6 mice by positive selection with CD4 MicroBeads and MACS columns (Miltenyi Biotech). Differentiation of naive $CD4^+$ T cells was carried out by stimulation with plate bound αCD3 and αCD-28 antibodies for 4-5 days in CLICK's medium (SIGMA) supplemented with 100µg/ml Penicillin/Streptomycin, 0.05mM β-mercaptoethanol, 2mM L-Glutamine and 25mM HEPES pH 7.4. For TH1 cells, the medium was supplemented with 20 units/ml IL-2, 3.5 µg/ml IL-12 (p70) and 10 mg/ml 11B11 (αIL4, BD Pharmingen). For TH2 cells, 20-50 units/ml of IL-2, 10 µg/ml IL-4 (BD Pharmingen) and 10 mg/ml XMG1.2 (αIFNγ, BD Pharmingen) have been used.

RNA preparation, reverse transcription

Whole cell RNA was prepared using the Trizol reagent (GibcoBRL) following the manufacturer's instructions. 2 µg RNA were reverse transcribed using the iScript Select cDNA Synthesis Kit (BIO-RAD). 10% of the cDNA produced was used for quantitative PCR in an Opticon Real Time PCR instrument creating standard curves.

Primer and probe sets for the analysis of the *IfnγR1* and *IfnγR2* genes have been purchased from Applied Biosystems and are the following:

IfnγR1: MmUU599890_m2, *IfnγR2*: MmUU492626_m1

For the rest we have used custom made primer and probe sets as follows:

HPRT sense: 5'-CTGGTGAAAAGGACCTCTCG-3'
HPRT antisense: 5'-TGAAGTACTCATTATAGTCAAGGGCA-3'
HPRT probe: VIC-5'-TGTTGGATACAGGCCAGACTTTGTTGGAT-3'-(TAMRA)
IL-4 sense: 5' AGATCATCGGCATTTTGAACG 3'
IL-4 antisense: 5' TTTGGCACATCCATCTCCG 3'
IL-4 probe: (FAM)-5'TCACAGGAGAAGGGACGCCATGC-3'-TAMRA
IFNγ sense: 5'-GGATGCATTCATGAGTATTGC-3'
IFNγ antisense: 5' CCTTTTCCGCTTCCTGAGG 3'
IFNγ probe: FAM-5'-TTTGAGGTCAACAACCCACAGGTCCA-3'-(TAMRA)
mCTCF sense: 5'-GGAAGGACTGCTGTCTGAGG-3'
mCTCF antisense: 5'-TTCTGAATGCTCTGCCACAC-3'

***IfnγR* cloning**

For the isolation and cloning of the *IfnγR* genes we have used the following primers:

IfnγR1 sense: 5'-CGCGGGTCCCCTGTCAGAGGT-3'
IfnγR1 antisense: 5'-TTAGGACAGCTCCTGGGCCTC-3'
IfnγR2 sense: 5'-TGCGAGTCTGAGCGGCGTCCA-3'
IfnγR2 antisense: 5'-TCACGGTGTTTGGAGCACATC-3'

The PCR products have been cloned in the pCR2.1 vector and sequenced.

3C assay

The restriction enzyme BglII (NEB) was used to generate chromatin fragments of appropriate size with an average of 10 kb or less. Control templates, with all possible ligation products present, were prepared using BAC clone DNA spanning the *Ifnγ*, *IfnγR1* and *IfnγR2* gene loci. The BAC clone DNA was digested with 200U BglII for 5 hours at 37°C, phenol/chloroform extracted and ethanol precipitated. DNA fragments were ligated with T4 DNA ligase (NEB) at a concentration of 300ng/µl. For all the PCR signals that were considered positive the bands were isolated, gel extracted (Qiaquick, Qiagen), cloned in a TA vector (TA cloning Kit, Invitrogen) and sequenced to confirm the presence of the sequences of two different restriction enzyme fragments ligated in a BglII site.

We have used the following primers for the 3C analysis:

NAME	SEQUENCE 5'-3'	Primer ID
Ifnγ R1.S	CAACCCCAGAGAAGTGTGTGT	551
Ifnγ R1.A	ACAGTCCTTGAGAAGTGCTGC	552
Ifnγ R2.S	CACCCACGTAAGATGCTGTCT	553
Ifnγ R2.A	CTACACTCACTGCAGCTCCCT	554
Ifnγ.S	TGGGTAACTAGGCAACAGTGG	555
Ifnγ.A	TAACACCTGATGACGGGGAC	556
Ifnγ-34.S	CAGGGAGACGTCACTCCAGTA	559
Ifnγ-34.A	ACGAAATCCTCCCTATCCAGA	560
Ifnγ-22.S	GGTTCCATATTGAGGAAAGGC	561
Ifnγ-22.A	CACTCCAGCCATGTAATCCAG	562
Ifnγ CNS1.S	TTTCTCCTCGGAGAGATGGAT	563
Ifnγ CNS1.A	CAACCAAGCTAGTGACGCTGT	564
Ifnγ CNS2.S	ATCATGGAGTGATGTTCTGGG	565
Ifnγ CNS2.A	GATACTGCTCACCCTGAGGAA	566
Ifnγ+29.S	AGTCCATTGCAGTTTCTCCCT	567
Ifnγ+29.A	TACCTTCCTGTCCCTTCAGGT	568
Ifnγ+46.S	CTTTGGTCTGACATGGCTTTG	569
Ifnγ+46.A	GCTCTGCTAATGCCTCAGTTG	570
Ifnγ+55.S	ATGACCAGCTGATGGCTTCTA	571
Ifnγ+55.A	CAAACTGGGCTCAGAGTGAAC	572

The following combinations of primer sets have been used for the 3C analysis for the *Ifnγ* locus, *IfnγR1* and *IfnγR2* gene promoters in pairwise combinations:

3C for *IfnγR1-Ifnγ*		
PRIMER COMBINATION	**PCR PRODUCT SIZE (bp)**	***Ifnγ* element**
551+559	365	**-34**
551+560	328	
552+559	388	
552+560	351	
551+561	204	**-22**
551+562	810	
552+561	227	
552+562	833	
551+563	1564	**CNS1**
551+564	255	
552+563	1587	
552+563	278	
551+555	246	***Ifnγ* gene**
551+556	314	
552+555	269	
552+556	337	
551+565	206	**CNS2**
551+566	384	

552+565	229	
552+566	407	
551+567	811	**+29**
551+568	255	
552+567	834	
552+568	278	
551+569	557	**+46**
551+570	214	
552+569	580	
552+570	237	
551+571	609	**+55**
551+572	517	
552+571	638	
552+572	540	

3C for *IfnγR2-Ifnγ*		
3C PRIMER COMBINATION	**PRODUCT SIZE (bp)**	***Ifnγ* element**
553+559	813	**-34**
553+560	777	
554+559	870	
554+560	834	
553+561	652	**-22**
553+562	1259	
554+561	709	
554+562	1316	
553+563	2012	**CNS1**
553+564	704	
554+563	2069	
554+563	761	
553+555	694	***Ifnγ* gene**
553+556	763	
554+555	751	
554+556	820	
553+565	654	**CNS2**
553+566	833	
554+565	711	
554+566	890	
553+567	1259	**+29**
553+568	704	
554+567	1316	
554+568	761	
553+569	1005	**+46**
553+570	663	
554+569	1062	
554+570	720	
553+571	1057	**+55**

553+572	966	
554+571	1114	
554+572	1023	

3C for *IfnγR1-IfnγR2*		
3C PRIMER COMBINATION	**PRODUCT SIZE (bp)**	**element**
551+553	539	**Promoters**
551+554	596	
552+553	569	
552+554	625	

Chromatin immunoprecipitation

Soluble chromatin solution was prepared with urea gradient ultracentrifugation. 20µg of chromatin were used for precipitation using 5µg agarose-conjugated rabbit polyclonal CTCF (sc-28198, Santa Cruz) or T-bet (sc-21749, Santa Cruz) antibodies. 10% of the immunoprecipitated DNA has been used in quantitative PCR by creating standard and melting curves and data have been corrected for input chromatin. The following primers have been used for the amplification of the *IfnγR1* regions in the chromatin immunoprecipitation experiments:

*IfnγR1*Prom sense: 5'-GACCCGCCAAGACACGCCTC-3'
*IfnγR1*Prom antisense: 5'-CGGAGCCTCACGGGGCAAAG-3'
IfnγR1-Intron3 sense: 5'-GTCCAAGGCTTTCAGCTCTTT-3'
IfnγR1-Intron3 antisense: 5'-GAACTAGGAAGGGAAGCTGGA-3'
IfnγR1-Intron6 sense: 5'-ATCCCATCCCCATTATAGCAG-3'
IfnγR1-Intron6-6 antisense: 5'-CCTCAGGATCAGTGTTTGGAA-3'
IfnγR1-(+20kb) sense: 5'-TGACTCGGATAACAAAGGCAG-3'
IfnγR1-(+20kb) antisense: 5'-ACTGCCTCACCTCTGTCATGT-3'

Methylation-Sensitive PCR

Genomic DNA was treated with bisulfite and analyzed by methylation-sensitive PCR according to the CpG WIZ kit instructions (Chemicon). The following primer sets have been utilized to detect the methylated (M) and non-methylated (U) *IfnγR1* regions upon bisulfite treatment:

Primer ID	sequence 5' → 3'	PCR product size
Sense _M_IFNγR1 intron 1	GTATTGAGGATTTTGAGTTTTTTTC	209
Antisense _M_IFNγR1 intron 1	CAAACCACAAATTTATATTATATCGAC	
Sense _U_IFNγR1 intron 1	TATTGAGGATTTTGAGTTTTTTTG	206
Antisense _U_IFNγR1 intron 1	AACCACAAATTTATATTATATCAAC	
Sense _M_IFNγR1 PROM 1	TTTATTTTAGATTCGTTAAGATACGT	114
Antisense_M_IFNγR1 PROM 1	CAAAAAAACGAAACCAATACG	
Sense_U_IFNγR1 PROM 1	TTTATTTTAGATTTGTTAAGATATGT	117
Antisense_U_IFNγR1 PROM 1	AACCAAAAAAACAAAACCAATACAC	
Sense _M_IFNγR1 PROM 2	TTAATAAATAAAATTATCGAACGAACGT	177
Antisense _M_IFNγR1 PROM 2	AAAAAACACTATAAAAACAATATCGAT	

Sense_U_IFNγR1 PROM 2	TAATTTAATAAATAAAATTATTGAATGAAT	182
Antisense_U_IFNγR1 PROM 2	AAAAAAACACTATAAAAACAATATCAAT	

Chromosome Painting

Cell fixation and DNA BAC probe preparation was performed as for the DNA-FISH. FITC-labeled Paint of mouse chromosome 10 (Cambio) was warmed at 37°C, added to the probe mix with 9μl of chromosome paint hybridization mix and incubated at 37°C for 10 min. Probes were denatured for 10 minutes at 70°C, followed by a 30 minutes incubation at 37°C. Cells were dehydrated using an ethanol series, briefly dried at 37°C and incubated with denaturation buffer (70% deionized formamide, 2xSSC, pH 7.2) for 5 minutes at 73°C. Another cycle of ethanol dehydration was performed and coverslips were incubated with the probe mix at 37°C overnight in a moist chamber. Three washes followed with 50% formamide/2xSSC at 42°C for 8 minutes, three times with 0.1xSSC at 60°C for 10 minutes and once with 0.1% Tween-20/4xSSC at 42°C for 10 minutes. Cells were mounted in Prolong Gold antifade reagent with DAPI (Invitrogen).

Microscopy and image analysis

FISH signals were examined on a Zeiss-Biorad confocal microscope unit and image stacks were captured on a CCD camera with a step of 300nm. The distances of DNA-FISH signals were further analyzed using the Volocity image analysis software (Improvision) by two independent investigators.

Statistical analysis

Two-sample Kolmogorov-Smirnov test was used to assess the statistical significant differences between groups of DNA FISH and Immuno-DNA FISH data. The analysis was carried out using the IBM SPSS Statistics 19 statistical software.

5-Aza-2´-deoxycytidine treatment

CD4+ T cells were isolated and placed under TH2 differentiation conditions for 4 days as described and were treated with 5-Aza-2´-deoxycytidine (Sigma, A3656-5MG) every 24hrs. Cells were treated under the same conditions with DMSO (0μM Aza), as a control.

Mouse Embryonic Fibroblasts

Pregnant female mice C57/Black6 were sacrificed at day 13 of gestation and the embryos were dissected out. The soft organs and viscera of the embryos were dissected out and the remaining carcasses were incubated in 1x PBS supplemented with 0,25% Trypsin and 0,5mM EDTA overnight at 4°C . Following dissolution of the embryos with pipetting, they were placed approximately 1 embryo per 50mL of culture media (DMEM) in a T175 flask. Cells were incubated at 37°C, 5% CO_2 until confluency.

Retroviral stock production and infection

CTCF-shRNA oligonucleotides were amplified with PCR and were cloned into the NSCV-LTRmiR30-PIG (LPM) vector. Plat-E cells were transfected with the recombinant vectors using Lipofectamin 2000 (Invitrogen, Cat no. 11668-027). The supernatants containing the retroviruses were collected at day 3 and day 4 of transfection, filtered with 0,45μm filter and were used immediately. 10μg/μl of Polybrene (Millipore, TR-1003-G) was added per 1mL of retrovirus containing medium prior to infection. $CD4^{+}$ T cells were isolated and placed under TH1 differentiation conditions. After 6hrs of differentiation the medium was replaced by the retrovirus containing medium. Cells were centrifuged at 30 ^{o}C, 1400rpm for 2hr and the medium was replaced once more with the initial medium for TH1 polarization. The same procedure was repeated after 24 hrs. Following two rounds of infection, cells were cultured for at least 72 hrs under differentiation conditions and then harvested for further experimental procedures.

The following oligonucleotides have been used for the production of the CTCF shRNAs (numbers denote the position of the shRNA relatively to the CTCF mRNA transcription start site). Sequences are given from the 5' to the 3' end:

sh1-3134
TGCTGTTGACAGTGAGCGAACAGACTTAGTGGTATGTAAATAGTGAAGCCACAGATGT
ATTTACATACCACTAAGTCTGTGTGCCTACTGCCTCGGA
Sh2-1179
TGCTGTTGACAGTGAGCGCACAGTGACCCTCCTGAGGAATTAGTGAAGCCACAGATGT
AATTCCTCAGGAGGGTCACTGTTTGCCTACTGCCTCGGA
Sh3-1680
TGCTGTTGACAGTGAGCGATGTGATGCTGTGTTTCATGAGTAGTGAAGCCACAGATGT
ACTCATGAAACACAGCATCACAGTGCCTACTGCCTCGGA
Sh4-1762
TGCTGTTGACAGTGAGCGAAGTGTGATTATGCTTGTAGACTAGTGAAGCCACAGATGT
AGTCTACAAGCATAATCACACTGTGCCTACTGCCTCGGA
Sh5-2768
TGCTGTTGACAGTGAGCGCGCCCTCTCTCTTGATCGTAAATAGTGAAGCCACAGATGTA
TTTACGATCAAGAGAGAGGGCTTGCCTACTGCCTCGGA