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GATA-binding protein-3 regulates T helper type 2 cytokine and *ifng* loci through interaction with metastasis-associated protein 2

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Summary

GATA-binding protein-3 (GATA-3) regulates the T helper type 2 (Th2) cytokine locus through induction of chromatin remodelling. However, the molecular mechanism for this is poorly understood. To understand this mechanism better, we screened GATA-3 interacting proteins using affinity purification and mass spectrometry. We found that GATA-3 bound to metastasis-associated protein 2 (MTA-2), a component of the NuRD chromatin remodelling complex. GATA-3 and MTA-2 in turn bound to several regulatory regions of the Th2 cytokine locus and the *ifng* promoter. Cell transfection assay showed that MTA-2 acted as an antagonist with GATA-3 in the expression of Th2 cytokines, but co-operated with GATA-3 in the repression of the *ifng* gene expression. These results suggest that GATA-3 interacts with MTA-2 to co-ordinately regulate Th2 cytokine and *ifng* loci during T helper cell differentiation.

Keywords: chromatin remodelling; differentiation; GATA-3; interaction; metastasis-associated protein-2; T helper type 2

Introduction

CD4 T cells play essential roles in the activation and regulation of immune responses. Naive CD4 T cells differentiate into T helper type 1 (Th1), Th2 and Th17 cells upon antigenic challenge.[1–5] The Th1 cells produce interferon-γ (IFN-γ), activate macrophages and mediate cellular immunity; Th2 cells produce interleukin-4 (IL-4), IL-5 and IL-13, stimulate B cells to produce antibodies, and mediate humoral immunity; and Th17 cells produce IL-17A and IL-17F, mediate immunity against extracellular bacteria, and induce inflammation. Both Th1 and Th17 cells cause autoimmunity and Th2 cells are responsible for allergy and asthma.

The Th2 cytokine locus has been extensively investigated to elucidate the gene expression and epigenetic mechanisms underlying cell differentiation. The Th2 cytokine locus containing the *il4*, *il5 and il13* genes is regulated by many regulatory elements such as enhancers, a silencer and a locus control region (LCR).[6,7] Conserved non-coding sequence-1 (CNS-1)/HSS, HSV/CNS-2, and IE/HSII have been shown to be enhancers, and HSIV has been shown to be a silencer.[6,7] The Th2 LCR has been demonstrated to be a co-ordinate regulator of the Th2 cytokine locus in a study using transgenic mice carrying bacterial artificial chromosome (BAC) DNA containing an endogenous configuration of the Th2 cytokine locus.[8] The Th2 LCR is composed of four DNase I hypersensitive sites, namely RHS4, RHS5, RHS6 and RHS7.[9] Deletion of RHS7 causes great reduction of IL-4 and IL-13 in Th0 conditions and mild reduction of these cytokines in Th2 conditions.[10] The Th2 LCR has been shown to interact with promoters of Th2 LCR through long-range chromosomal interactions.[11] The Th2 cytokine locus undergoes epigenetic changes upon Th2 cell differentiation to accommodate the high-level expression of Th2 cytokine genes and to transmit the committed cell fate to daughter cells. These changes include DNase I hypersensitivity, histone modification and DNA methylation.[6,7]

GATA-binding protein-3 (GATA-3) has been shown to be the essential transcription factor for Th2 differentiation. GATA-3 is selectively expressed in Th2 cells and is necessary and sufficient for Th2 cell differentiation, as shown by a transgenic approach.[12] Conditional deletion of the *gata3* gene in the mouse genome causes a severe defect of Th2 cell differentiation *in vivo*,[13,14] confirming the essential role of GATA-3 in this process. Moreover, introduction of exogenous GATA-3 into Th1 cells causes IL-4 expression through induction of chromatin remodelling independent of IL-4 and signal transducer and activator of trasncription 6.[15–17] However, the detailed mechanism of GATA-3 in chromatin remodelling and regulation of the Th2 cytokine locus is poorly understood.

Metastasis-associated protein 2 (MTA-2) is a member of the MTA family of transcriptional co-repressors that function in histone deacetylation.[18] It is a component of the nucleosome remodelling histone deacetylase (NuRD) complex, and has been shown to positively regulate histone deacetylase activity of the complex.[18] Expression of MTA-2 enhances p53 deacetylation and strongly represses p53-dependent transcriptional activation.[19] The MTA-2 has been shown to interact with estrogen receptor α and repress its activity, possibly through deacetylation.[20] Although all MTA family proteins are found in NuRD complexes, these proteins form distinct complexes and are thought to target different sets of promoters.[18,21]

In this study, we investigated the role of GATA-3 in the regulation of Th2 cytokine and *ifng* loci. We found that GATA-3 interacts with MTA-2, which is a component of the NuRD chromatin repression complex and has been shown to be involved in *il4* gene expression. GATA-3 and MTA-2 bind to several regulatory regions of the Th2 cytokine locus mutually exclusively and to the *ifng* promoter simultaneously in Th2 cells. The MTA-2 negatively regulated the transactivation activity of GATA-3 at *il4* promoter, but co-operated with GATA-3 for repression at the *ifng* promoter. These results suggest that GATA-3 interacts with MTA-2 in the Th2 cytokine and *ifng* loci for the regulation of these loci.

Materials and methods

Affinity purification and mass spectrometry

HEK293T cells in a 10-cm plate were transfected with pcDNA3-HA–GATA-3 or with the empty pcDNA3 vector. After 48 hr of transfection, cell lysates were passed through the haemagglutinin (HA) -affinity column (Roche, Mannheim, Germany). The column was extensively washed, and then Th2 nuclear extracts were passed through the column again. After several washings, bound HA–GATA-3 protein complexes were eluted using HA-peptide (Roche), following which elutes were analysed by tandem spectrometry (MS/MS).

In vitro differentiation of CD4 T cells

CD4 T cells were enriched from spleen cells from C57BL/6 mice by negative selection through depletion using anti-major histocompatibility complex class II (M5/115), anti-NK1.1 (HB191), and anti-CD8 (T1B105) monoclonal antibodies, followed by depletion with a mixture of magnetic beads conjugated to anti-rat immunoglobulin and anti-mouse immunoglobulin antibodies (Perseptive Biosystems, Framingham, MA). Naive CD4 T cells were sorted based on the surface markers, $CD4^{high}$ and $CD62L^{high}$. These cells (1×10^6) were then stimulated with 10 μg/ml plate-bound anti-CD3 (2C11), 2 μg/ml soluble anti-CD28, and 20 U/ml IL-2 in 5 ml of RPMI-1640 medium with 5% fetal calf serum (Invitrogen, Carlsbad, CA) and penicillin/streptomycin. For Th1 polarization, 3·5 ng/ml IL-12 and anti-IL4 (11B11) were added, and for Th2 polarization, 1000 U/ml IL-4 and the XMG1.2 (anti-IFN-γ) antibody were added to the same culture setting. After 4 days the cells were washed and re-stimulated with 0·5 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin for 4 hr.

Chromatin immunoprecipitation (ChIP)

Naive CD4 T cells were stimulated under Th1 or Th2 polarizing conditions as described above. The Th1 or Th2 cells (1×10^6 to 2×10^6) were cross-linked with 1% formaldehyde and quenched with 0·125 M glycine. Cells were lysed with lysis buffer [50 mM Tris–HCl, pH 8·1, 1% sodium dodecyl sulphate (SDS), 10 mM ethylenediamine tetraacetic acid (EDTA)], and sonicated at the high power setting for 15 min using a Bioruptor sonicator (Diagenode, Liege, Belgium). Using these conditions, the average DNA fragment size was approximately 500 base pairs. Cell extracts were pre-cleared with protein A–agarose/salmon sperm DNA (Millipore, Billerica, MA), and incubated with either anti-GATA-3 (Santa Cruz Biotechnology, Santa Cruz, CA; sc-268), anti-MTA-2 (Santa Cruz, 28731), or rabbit immunoglobulin G (IgG; Santa Cruz, sc-2027) as a negative control. Antibody-bound chromatin was precipitated by protein A–agarose, washed and eluted with elution buffer (0·1 M sodium bicarbonate, 1% SDS). The chromatin was reverse cross-linked by incubating at 65° for 4 hr, followed by protease K treatment (100 ng/ml). The amount of precipitated DNA was quantified by real-time polymerase chain reaction (PCR) using the primers listed in Table 1.

Double ChIP

The first-round ChIP was carried out as described above using the anti-GATA-3 antibody. The cross-linked DNA–protein complex was briefly washed, and eluted with 10 mM dithiothreitol (DTT) at 37° for 1 hr. The elute was then diluted 50-fold in a ChIP buffer (0·01% SDS, 1·1% TX-100, 1·2 mM EDTA, 16·7 mM Tris–HCl pH 8·1, 167 mM NaCl), and then a second-round ChIP was performed with anti-MTA-2 or the control IgG antibody. Chromatin was collected with protein A/G–agarose, washed, and eluted with sodium bicarbonate–SDS, and the cross-linked DNA was reversed, which was followed by protease K treatment. Precipitated DNA was quantified by real-time PCR as described above.

Co-immunoprecipitation

The Th2 cells were stimulated for 4 days as described above. The Th2 cell lysates were made in a lysis buffer,

Table 1. Primers for chromatin immunoprecipitation

Primer name	Sequence
IL-4 promoter Fw	GCAGGATGACAACTAGCTGGG
IL-4 promoter Rv	ACGGCACAGAGCTATTGATGG
IL-5 promoter Fw	TTTCCTCAGAGAGAGAATAAATTGCTT
IL-5 promoter Rv	GCTGGCCTTCAGCAAAGG
IL-13 promoter Fw	GGGCAGGTGAGTATCAGTCTA
IL-13 promoter Rv	GTCTATATCCCTCCCACTCGT
CNS-1 Fw	TTCTCGGCTCCACCCAAAAG
CNS-1 Rv	TGCCTGCGTCACCTCTG
CNS-2 Fw	ATCAGAACATCACTCGTCT
CNS-2 Rv	ATGCCAACCTGAAGAACTAAC
RHS4 Fw	GGATCTGATGCCCTCTTCTG
RHS4 Rv	AGTTTCTCTGTGTAGCCCTGTCT
RHS5 Fw	GGGGAATGCCTTGATACCAC
RHS5 Rv	AGAAACCCCACAGACTCCACTT
RHS6 Fw	CAGAGATGAGCGTGGGTTAGAA
RHS6 Rv	CCCAGTGGTAGGAAGGCAAGTTT
RHS6.5 Fw	CCTATCTGCGCTTTCACAGAACCC
RHS6.5 Rv	CCACAGCCTCTCCTGTATGACCTT
RHS7 Fw	ACCCTTGTTGCATCTCACTCATA
RHS7 Rv	CTAGGAAAACTTAAAACATCAACCA
IFN-γ protomer Fw	CGGGGCTGTCTCATCGTC
IFN-γ protomer Rv	CTCGGGATTACGTATTTTCACAA
Myc-1	TCCAGGGTACATGGCGTATTG
Myc-2	TCTGCTTTGGGAACTCGGGA

and then pre-cleared with control IgG followed by protein G treatment. Pre-cleared lysates were incubated overnight at 4° with monoclonal anti-GATA-3, polyclonal anti-MTA-2, anti-acetylated lysine (Santa Cruz, sc-32268) or normal IgG, and then protein G beads were added, followed by incubation for an additional 2 hr. Immunocomplexes were extensively washed and then were resuspended in an SDS loading buffer. Immunoblot analysis was performed as described below.

Immunoblotting

Proteins were resolved by 10% SDS–PAGE and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% skim milk Tris-buffered saline with Tween (TBST), and incubated 1 hr at room temperature. The membrane was then probed with a rabbit polyclonal antibody against MTA-2 (Santa Cruz, 28731) or a mouse monoclonal antibody against β-actin (Santa Cruz, sc-47778) and GATA-3 (Santa Cruz, sc-268) diluted 1 : 100 in TBST overnight at 4°. After this, horseradish peroxidase-conjugated antibody against rabbit, mouse or goat IgG was added (Bethyl Laboratories, Inc., Montgomery, TX), diluted 1 : 2000 in 5% skim milk TBST for 1 hr at room temperature. Chemiluminescence was detected on an X-ray film after treating with enhanced chemiluminescence solution.

Cell transfection and dual luciferase assay

Expression vectors for GATA-3 and MTA-2 were constructed from the CMV-base expression vector (pCMV-SPORT6). Cell transfection to EL4, a mouse thymoma cell line, and measurement of dual luciferase was performed as previously described with minor modifications.[9] Five million EL4 cells were resuspended in 400 μl Opti-MEM (Invitrogen) and transferred to a 0·4-cm cuvette (Bio-Rad); expression vectors, reporter plasmids and *Renilla* luciferase reporter plasmid were added to the cuvette. Cells were electroporated using a Bio-Rad Gene Pulse set at 950 μF and 280 V. Transfected cells were allowed to recover overnight in complete medium, and were then stimulated with 0·5 ng/ml PMA and 1 μM/ml ionomycin for 4 hr. Cells were then harvested and cell extracts were made. Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Transfection efficiency was normalized by dividing firefly luciferase activity by *Renilla* luciferase activity.

Cell transfection and quantitative reverse transcription-PCR

EL4 cells were transfected by electroporation as described above. After 2 days, cells were stimulated with 0·5 ng/ml PMA and 1 μM/ml ionomycin for 4 hr. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen). Complementary DNA was synthesized using SuperScript II reverse transcriptase and oligo-dT (Invitrogen) according to the manufacturer's protocol. Quantitative PCRs were performed with real-time fluorogenic 5′-nuclease PCR using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequences used for quantitative PCR were as follows: *il4* sense: 5′-AGATCATCGGCATTTTGAACG-3′, *il4* anti-sense: 5′-TTTGGCACATCCATCTCCG-3′, *il4* probe: (FAM)-5′-TCACAGGAGAAGGGACGCCATGC-3′-(Tamra); *ifng* sense: 5′-GGATGCATTCATGAGTATTGC-3′, *ifng* anti-sense: 5′-CCTTTTCCGCTTCCTGAGG-3′, *ifng* probe: (FAM)-5′-TTTGAGGTCAACAACCCACAGGTC-CA-3′-(Tamra); *hprt* sense: 5′-CTGGTGAAAAGGACCT-CTCG-3′, *hprt* anti-sense: 5′-TGAAGTACTCATTATAG-TCAAGGGCA-3′, *hprt* probe: (FAM)-5′-TGTTGGATA-CAGGCCAGACTTTGTTGGAT-3′-(Tamra).

Small interfering RNA-mediated knockdown

Exponentially growing EL4 cells (1×10^7) were resuspended in 400 μl Opti-MEM (Invitrogen) and transferred to a 0·4-cm cuvette (Bio-Rad). Thirty microlitres of control or gata3 small interfering RNA (siRNA; stock concentration 100 μM) (Bioneer, Daejeon, Korea) was added to the cuvette. Cells were electroporated using a Bio-Rad Gene

Pulse set at 950 μF and 250 V. Cells were harvested for experiments after 24–36 hr of electroporation. The gata3 siRNA is a mixture of three kinds of double-stranded RNA. The sequences gata3 siRNA are as follow. gata3-1 (sense): 5′-GACGGAAGAGGUGGACGUA(dTdT)-3′; gata3-1 (anti-sense): 5′-UACGUCCACCUCUUCCGUC(dTdT)-3′; gata3-2 (sense): 5′-UCGUACAUGGAAGCUCAGU(dTdT)-3′; gata3-2 (anti-sense): 5′-ACUGAGCUUCCAUGUACG-A(dTdT)-3′; gata3-3 (sense): 5′-GAUUUCAGAUCUGGGC-AAU(dTdT)-3′; gata3-3 (anti-sense): 5′-AUUGCCCAG-AUCUGAAAUC(dTdT)-3′. The sequences of control siRNA are as follows. Control (sense): 5′-CCUACGCCACCAAUU-UCGU(dTdT)-3′; control (anti-sense): 5′-ACGAAAUUG-GUGGCGUAGG(dTdT)-3′.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Differences between groups were determined by a Student's *t*-test.

Results

Screening of GATA-3-interacting proteins

To investigate the molecular mechanism of GATA-3 in the regulation of Th2 cytokine and *ifng* loci, we searched for GATA-3-interacting proteins. We overexpressed HA-tagged GATA-3 in 293T cells. Cell extracts from these cells were passed through an HA-affinity column. Then, Th2 cell extracts were passed through this column. After washing and elution, GATA-3-interacting proteins were analysed by MS/MS spectrometry. As the profile of GATA-3-interacting proteins is huge, we narrowed down the list to transcription factors and chromatin-remodelling factors (Table 2). Among the GATA-3-interacting proteins, we were particularly interested in MTA-2 and selected it for subsequent study, because MTA-2 has been shown to be involved in *il4* transcription and chromatin regulation.[22]

GATA-3 interacts with MTA-2

We confirmed the binding of GATA-3 with MTA-2 by co-immunoprecipitation. We made cell extracts from *in vitro*-stimulated Th2 cells from C57/BL6 mice, and immunoprecipitated with either the anti-GATA-3 or anti-MTA-2 antibody, then immunoblotted the anti-MTA-2 or anti-GATA-3 antibody, respectively. GATA-3 and MTA-2 co-immunoprecipitated with either the anti-GATA-3 or anti-MTA-2 antibody (Fig. 1a,b), indicating that these proteins interact with each other, which validated our affinity purification and MS/MS data.

We next examined the relative amount of MTA-2 between Th1 and Th2 cells. We prepared cell extracts

Table 2. GATA-3 interacting proteins

Primer name	Score
ErbB3-binding protein 1	60·17
RCC1-like	50·25
DEK oncogene (DNA binding)	40·18
Pigpen	30·20
Retinoblastoma binding protein 7	20·20
Absent, small, or homeotic discs 1	10·19
Heterogeneous nuclear ribonucleoprotein D-like	10·18
Low-density lipoprotein receptor-related protein 1	10·17
Ewing sarcoma breakpoint region 1	10·17
Staphylococcal nuclease domain containing 1	10·16
General transcription factor IIF, polypeptide 1	10·16
Bromodomain containing 9	10·15
Methyltransferase like 8 isoform α	10·15
DEAH (ASP-Glu-Ala-His) box polypeptide 9	10·15
RAD51-like 3	10·15
Protein arginine *N*-methyltransferase 5	10·14
Guanylate binding protein family, member 6	10·14
ZXD family zinc finger C isoform 1	10·13
Zinc finger potein 198	10·13
DEAH (Asp-Glu-Ala-His) box polypeptide 29	10·12
Predicted: similar to cyclin-dependent kinase inhibitor 3 isoform 5	10·12
Retinoblastoma binding protein 6 isoform 1	10·12
Ovarian zinc finger protein	10·12
Metastasis-associated gene family, member 2	10·12
Thyroid hormone receptor beta isoform 2	10·12
Lens epithelium-derived growth factor	10·12

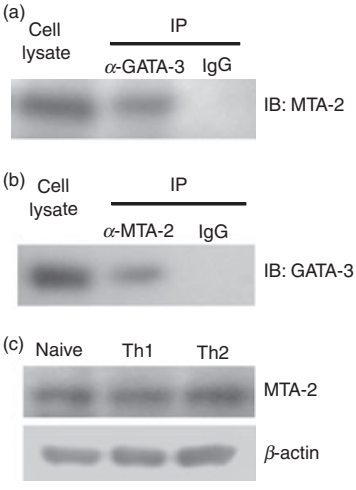

Figure 1. Interaction between GATA-3 and metastasis-associated protein 2 (MTA-2) (a, b) and expression of MTA-2 (c). (a, b) T helper type 2 (Th2) cell extracts were immunoprecipitated with either the anti-GATA-3 or anti-MTA-2 antibody. The precipitated proteins were analysed by immunoblotting with the anti-MTA-2 (a) or anti-GATA-3 (b) antibody. (c) Expression of MTA-2 protein in naive, Th1 and Th2 cells was measured by immunoblotting.

from Th1 and Th2 cells and measured the relative amount of MTA-2 protein by immunoblotting. The amount of MTA-2 protein was comparable between Th1 and Th2 cells (Fig. 1c).

Acetylation of GATA-3 at the lysine residues has been shown to affect the function of GATA-3, in particular, in T-cell survival and homing to secondary lymphoid tissues.[23] As the NuRD complex has deacetylase activity,[18] we examined whether the acetylation status of GATA-3 can affect the binding with MTA-2. We found that an acetylated protein the same size as GATA-3 was co-immunoprecipitated with MTA-2, suggesting indirectly that acetylated GATA-3 may bind to MTA-2 (Fig. S1).

GATA-3 and MTA-2 bind to several regulatory regions of Th2 cytokine locus and the *ifng* promoter

To investigate the role of GATA-3 and MTA-2 in the regulation of the Th2 locus, we examined the binding of GATA-3 and MTA-2 in several regulatory regions of the Th2 cytokine locus and *ifng* promoter by chromatin immunoprecipitation. We isolated splenic naive CD4 T cells from C57BL/6 mice and stimulated them *in vitro* in either Th1 or Th2 polarizing conditions. Cells were cross-linked and sonicated, and the chromatin was immunoprecipitated with either an anti-GATA-3 or anti-MTA-2 antibody. GATA-3 bound to Th2 LCR (RHS4, RHS5, RHS6, and RHS7), the promoters of *il4*, *il5* and *il13* genes, and enhancers (CNS-1 and CNS-2/HSV) in a Th2-specific manner (Fig. 2). This result shows that GATA-3 binds to the Th2 cytokine locus globally and to Th2 specifically.

The MTA-2 also bound to Th2 LCR (RHS4, RHS5, RHS6, and RHS7) and promoters of Th2 cytokine genes, and enhancers (CNS-1/HSS, CNS-2/HSV) (Fig. 2). However, in contrast to GATA-3, MTA-2 bound to these regions in a Th1-specific manner (Fig. 2). Therefore, the overall binding of MTA-2 and GATA-3 on the Th2 cytokine locus was mutually exclusive (Fig. 2).

Interestingly, both GATA-3 and MTA-2 bound to the promoter of the *ifng* gene in Th2 cells (Fig. 2). The simultaneous binding of GATA-3 and MTA-2 on the *ifng* promoter was confirmed by a double-chromatin immunoprecipitation experiment. Chromatin from Th1 or Th2 cells was first immunoprecipitated with an anti-GATA-3 antibody, and the bound antibody was detached from the chromatin by treating with DTT. The eluted chromatin was then immunoprecipitated with the anti-MTA-2 antibody. The result confirms that GATA-3 and MTA-2 bound to the *ifng* promoter simultaneously in Th2 cells (Fig. 3).

Next, we examined whether the binding of MTA-2 to *ifng* promoter is dependent on GATA-3. For this purpose, we used siRNA-mediated reduction (knockdown) of GATA-3 protein in EL4 cells. We transfected *gata3* siRNA into EL4 cells and measured the protein level of GATA-3 by immunoblotting (Fig. 4a). Treatment with *gata3* siRNA led to a significant reduction of GATA-3 protein level in EL4 cells (Fig. 4a). The expression of *ifng* gene was increased by treatment with *gata3* siRNA (Fig. 4b), consistent with the previous reports.[13,14] Interestingly, the

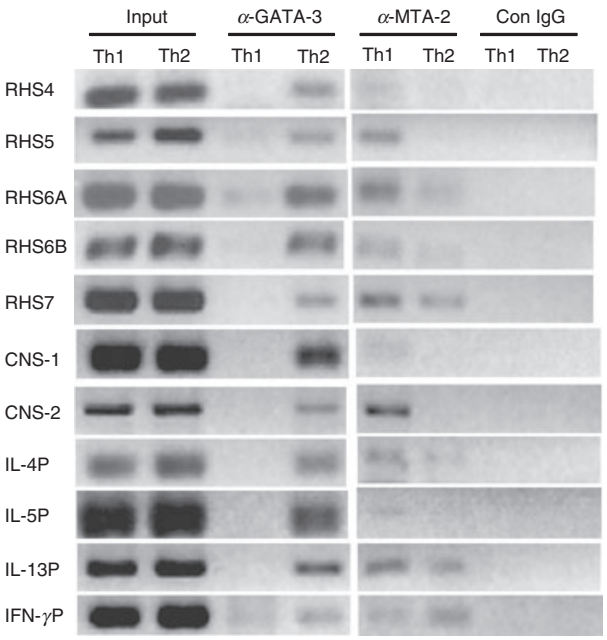

Figure 2. Chromatin immunoprecipitation with anti-GATA-3 and anti-metastasis-associated protein 2 (MTA-2). Chromatin from T helper type 1 (Th1) and Th2 cell extracts was immunoprecipitated with anti-GATA-3, anti-MTA-2 antibody or control immunoglobulin G (IgG) as described in the Methods. The amount of precipitated DNA containing several regulatory regions of the Th2 cytokine locus and *ifng* promoter was quantified by polymerase chain reaction.

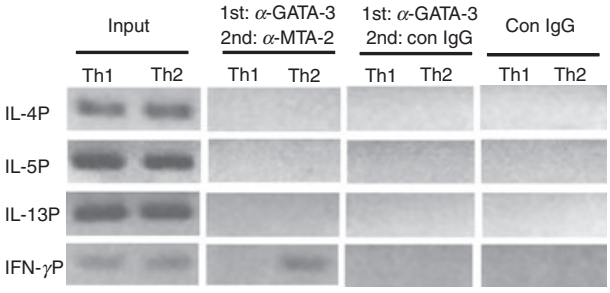

Figure 3. Double chromatin immunoprecipitation (ChIP) assay of the promoters of T helper type 2 (Th2) cytokines and *ifng*. Chromatin from Th1 or Th2 cells was first immunoprecipitated with the anti-GATA-3 antibody, and the bound antibody was detached from the chromatin by treating with dithiothreitol. The eluted chromatin was immunoprecipitated again, this time with the anti-metastasis-associated protein 2 (MTA-2) or control immunoglobulin G antibody. The amount of precipitated DNA of the promoters Th2 cytokine and *ifng* genes was quantified by polymerase chain reaction.

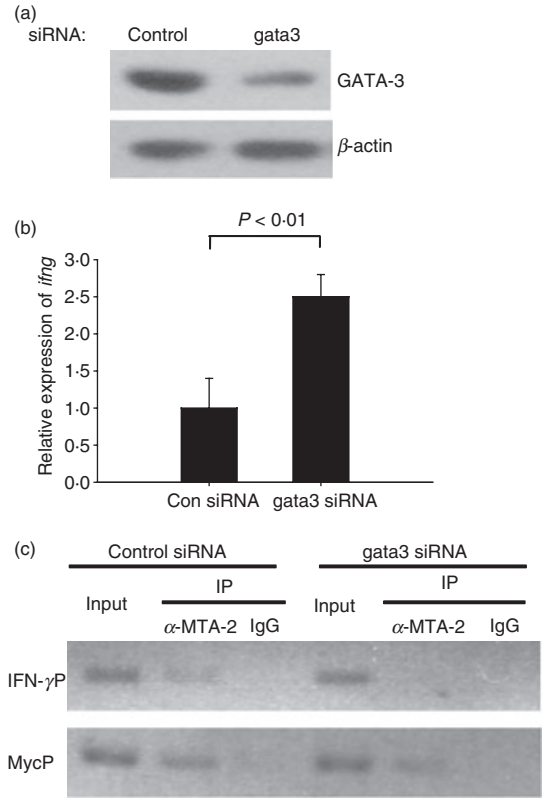

Figure 4. GATA-3-dependent binding of metastasis-associated protein 2 (MTA-2) on *ifng* promoter. (a) *gata3* small interfering RNA (siRNA) -mediated knockdown of GATA-3. EL4 cells were transfected with control or *gata3* siRNA. GATA-3 protein level was measured by immunoblotting. (b) The effect of *gata3* siRNA on the expression of *ifng* gene. EL4 cells were transfected with control or *gata3* siRNA. RNA was isolated and the expression of *ifng* gene was measured by quantitative reverse transcription–polymerase chain reaction. The relative amount of *ifng* transcript was measured and normalized to *hprt* transcript levels. Bars are shown to indicate mean ± SD ($n = 3$). Statistical difference between groups was analysed by Student's *t*-test. (c) EL4 cells were transfected with control or gata3 siRNA. MTA-2 binding to the *ifng* promoter (IFN-γP) and the *myc* promoter (MycP) was measured by chromatin immunoprecipitation using anti-MTA-2, or control immunoglobulin G antibody.

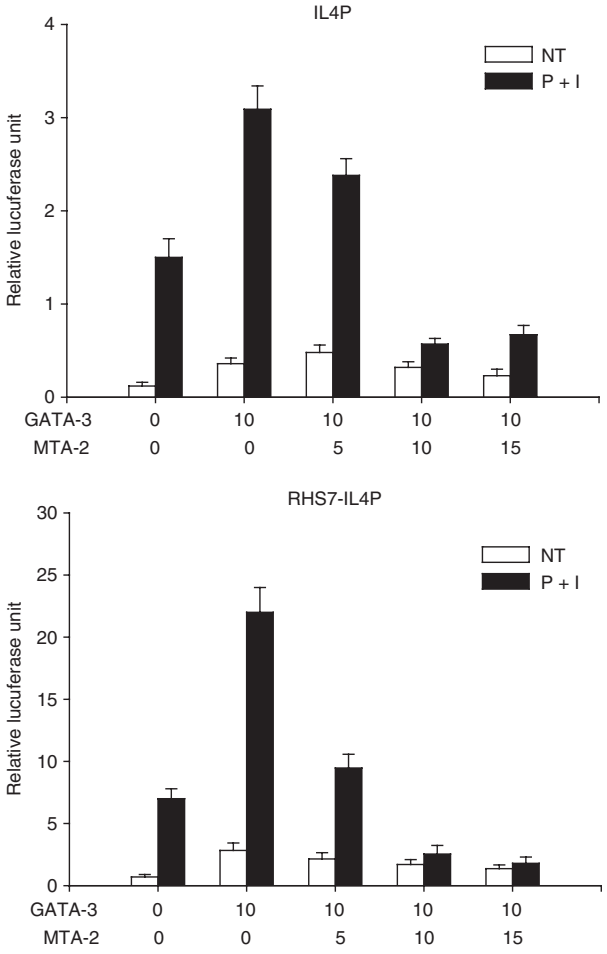

Figure 5. Reporter transfection assay. Expression vectors of GATA-3 (0, 10 μg) and/or metastasis-associated protein 2 (MTA-2; 0, 5, 10, 15 μg) were transfected by electroporation into EL4 cells with reporter constructs (IL-4P or RHS7-IL-4P) and left overnight. Cells were then stimulated with phorbol 12-myristate 13-acetate (0·5 ng/ml) and ionomycin (1 μM) for 4 hr, and luciferase activity was measured. Transfection efficiency was normalized by dual luciferase activity. Bars are shown to indicate mean ± SD ($n = 3$). Experiments were performed three times with similar results. NT, no treatment; P + I, PMA + ionomycin.

binding of MTA-2 to *ifng* promoter was abolished by *gata3* siRNA (Fig. 4c). However, the binding of MTA-2 to *myc* promoter, which has been shown previously[24,25] but has not been shown to have any relevance to GATA-3, was not affected by *gata3* siRNA (Fig. 4c). These results strongly suggest that the binding of MTA-2 to *ifng* promoter is specifically dependent on GATA-3.

MTA-2 antagonized the transactivational activity of GATA-3

We also examined whether MTA-2 affects the functional activity of GATA-3. The GATA-3 expression vector was transfected with reporter constructs that contain IL4P-luciferase (IL4P) or RHS7-IL4P-luciferase (IL4P-RHS7).[9] Introduction of GATA-3 transactivated the transcription of the reporter gene about two-fold in IL4P and about three-fold in RHS7-IL4P constructs after treatment with PMA + ionomycin (Fig. 5). These results suggest that the *il4* promoter and RHS7 are GATA-3 responsible elements, and are consistent with the ChIP data indicating that GATA-3 bound to these regions (Fig. 2). Co-expression of MTA-2 with GATA-3 dose-dependently decreased the transactivational activity of GATA-3 both in IL4P and RHS7 constructs (Fig. 5). These results suggest that MTA-2 is directly involved in the repression of transactivational activity of GATA-3 at the *il4* promoter and RHS7 regions.

MTA-2 repressed the expression of endogenous *il4* and *ifng* genes

We further examined the function of GATA-3 and MTA-2 in the expression of *il4* and *ifng* at the endogenous loci. We transfected the expression vectors of GATA-3 and/or MTA-2 into EL4 cells, and measured the expression of endogenous *il4* and *ifng* genes by quantitative reverse transcription-PCR. Over-expression of GATA-3 was found to enhance the expression of the endogenous *il4* gene about two-fold in stimulated EL4 cells (Fig. 6). This enhancement was inhibited by co-expression of MTA-2 (Fig. 6), confirming that MTA-2 antagonizes the function of GATA-3 at the endogenous *il4* promoter.

Over-expression of GATA-3 did not affect the expression of the endogenous *ifng* gene (Fig. 6). However, over-expression of MTA-2 inhibited the expression of *ifng* about two-fold (Fig. 6). Interestingly, the co-expression of MTA-2 and GATA-3 synergistically repressed the *ifng* expression (Fig. 6), suggesting that MTA-2 and GATA-3 may co-operate at the *ifng* promoter to repress the expression of the *ifng* gene. This result is consistent with the simultaneous binding of GATA-3 and MTA-2 at the *ifng* promoter (Fig. 3). Taken together, these results suggest that MTA-2 has repressive function at both *il4* and *ifng* loci.

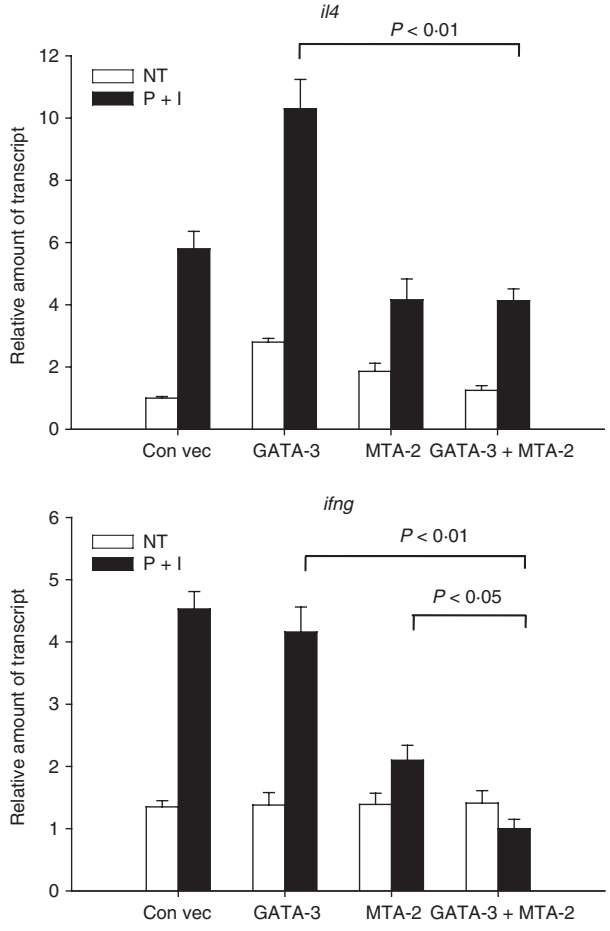

Figure 6. Effect of GATA-3 and metastasis-associated protein 2 (MTA-2) on the expression of endogenous *il4* and *ifng* genes. Expression vectors of GATA-3 (10 μg) and/or MTA-2 (10 μg) were transfected by electroporation into EL4 cells. After 2 days, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 0·5 ng/ml) and ionomycin (1 μM) for 4 hr, and total cellular RNA was isolated. The relative amount of *il4* and *ifng* transcript was measured and normalized to *hprt* transcript levels using quantitative reverse transcription polymerase chain reaction. Bars are shown to indicate mean ± SD (n = 3). Statistical difference between groups was analysed by Student's t-test. Experiments were performed three times with similar results. NT, no treatment; P + I, PMA + ionomycin.

Discussion

In this study, we searched for the molecular mechanism of GATA-3 action in the regulation of the Th2 cytokine and *ifng* loci. We found that GATA-3 interacts with MTA-2, a component of the NuRD chromatin remodelling complex. GATA-3 and MTA-2 bound to several regulatory regions of the Th2 cytokine locus and the *ifng* promoter. GATA-3 and MTA-2 antagonized in the regulation of the Th2 cytokine locus, but co-operated in the repression of *ifng* promoter, suggesting that GATA-3 may induce chromatin remodelling through interaction with MTA-2 during Th cell differentiation.

GATA-3 has been shown to be the critical regulator of Th2 cell differentiation. GATA-3 is selectively expressed in differentiating Th2 cells, and is necessary and sufficient for Th2 differentiation, as shown by transgenic and antisense experiments.[12] Conditional GATA-3 knockout mice showed dramatic reduction of Th2 cytokines, confirming the essential role of GATA-3 in Th2 cell differentiation.[13,14] It has been shown that Th2 cell differentiation accompanies chromatin remodelling, including histone modification, DNA methylation and DNase I hypersensitivity in the Th2 cytokine locus.[6,7] Retroviral introduction of GATA-3 into developing Th1 cells induced Th2 cytokine expression and chromatin structural changes,[15–17] suggesting that GATA-3 is involved in inducing chromatin remodelling. However, the detailed mechanism through which GATA-3 induces this change is poorly understood. In this study, we examined the molecular mechanism of GATA-3 in the regulation of the Th2 cytokine locus. We found that GATA-3 interacts with MTA-2. GATA-3 and MTA-2 bound to several regions of the Th2 cytokine locus mutually exclusively in Th1 and Th2 cells, and they antagonized the regulation of the *il4* gene. However, this antagonism did not occur in the regulation of *ifng* gene expression. Instead, both GATA-3 and MTA-2 bound to the *ifng* promoter preferentially in Th2 cells. Surprisingly, within one and the same Th2 cell, GATA-3 and MTA-2 associated in the *ifng* locus, but not in the Th2 cytokine locus. The reason for this discrepancy is not

clear and may be a consequence of a contribution of other differentially recruited proteins, the identity of which is currently not clear.

MTA-2 knockout (KO) mice have been shown to undergo abnormal T-cell activation and proliferation, and to develop lupus-like autoimmune disease.[22] The Th2 polarized cells from MTA-2 KO mice have been shown to produce increased amounts of both IL-4 and IFN-γ compared with those from wild-type mouse, but Th1 polarized cells from MTA-2 KO mice have been shown to produce comparable amounts of these cytokines. This result suggests that MTA-2 have inhibitory effects on the expression of IL-4 and IFN-γ in Th2 cells. This is consistent with our findings that MTA-2 inhibits the expression of both *il4* and *ifng* genes, and that GATA-3 and MTA-2 antagonize the regulation of Th2 cytokine genes.

GATA-3 has been shown to interact with several transcription factors, including repressor of GATA (ROG), friend of GATA (FOG), MAD homologue 3 (Smad), spleen focus forming virus proviral integration oncogene spi1 (PU.1), T-box protein expression T cells (T-bet), lymphoid enhancer factor 1 (LEF-1), and Pias1. The over-expression of ROG suppresses GATA-3-dependent transactivation and Th2 cell differentiation.[26] Forced expression of FOG-1 significantly repressed the transcriptional activity of GATA-3, the production of Th2 cytokines, and the differentiation of Th2 cells *in vitro*.[27] PU.1 suppresses Th2 cytokine production from the Th2 cells through the inhibition of GATA-3 binding to the HSVa enhancer.[28] T-bet mediates the inhibitory effect on *il5* promoter activity by interacting with GATA-3.[29] High-mobility group (HMG) box type transcription factor, lymphoid enhancer factor 1 (LEF-1) has been shown to interact with GATA-3 and suppress the function of GATA-3.[30] Transcriptional co-regulator Pias1 has also been found to interact with GATA-3, and increase its transcriptional activity.[31] In this study, we identified MTA-2 as a new partner of GATA-3, a transcriptional co-factor which is involved in chromatin remodelling. Hence, this study may provide a clue to search for a possible mechanism of GATA-3-mediated transcriptional regulation and chromatin remodelling. As our data show that MTA-2 recruited to *ifng* promoter in a GATA-3-dependent manner and that MTA-2 antagonized the function of GATA-3 at the Th2 cytokine locus, the function of GATA-3 in transcriptional regulation and chromatin remodelling may be involved in its interaction with MTA-2. Although MTA-2 has zinc finger domains similar to the GATA family of proteins, experimental evidence in support of direct DNA-binding activity of MTA proteins is lacking.[18,21] It therefore remains to study the detailed molecular mechanism of MTA-2 and GATA-3 interaction in the regulation of *il4* and *ifng* gene expression, in particular whether MTA-2 binds directly to DNA.

Previous studies have shown that GATA-1, the founding member of the GATA family, directly interacts with FOG-1,[32,33] and that FOG-1 recruits the NuRD complex, which includes MTA-2, to GATA-1/FOG-1 target genes through binding of N-terminal regions of FOG-1.[24,34,35] GATA-3 has also been shown to interact with FOG-1,[27] so there is a possibility that the interaction of GATA-3 with MTA-2 is also mediated by FOG-1. It will be interesting to study the involvement of FOG-1 in this interaction.

In conclusion, this study discovered that GATA-3 interacts with MTA-2, a chromatin remodelling factor, to regulate Th2 cytokine and *ifng* loci. This study describes a fundamental molecular mechanism of Th2 cell differentiation, and will provide valuable insight for finding strategies to treat Th2-related diseases such as allergy and asthma.

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Disclosures

The authors have no potential conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of the acetylation of GATA-3 on the interaction between GATA-3 and metastasis-associated protein 2 (MTA-2).

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