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Regulation of cooperative function of the *Il12b* enhancer and promoter by the interferon regulatory factors 3 and 5

Ryuji Koshiba [a,b,1], Hideyuki Yanai [a,b,1], Atsushi Matsuda [a], Ayana Goto [a,b], Akira Nakajima [a,2], Hideo Negishi [a,b], Junko Nishio [a,b], Stephen T. Smale [c], Tadatsugu Taniguchi [a,b,*]

[a] Department of Molecular Immunology, The University of Tokyo, Komaba 4-6-1, Meguro-Ku, Tokyo 153-8505, Japan
[b] Core Research for Evolution Science and Technology, Japan Science and Technology Agency, Chiyoda-Ku, Tokyo 102-0075, Japan
[c] Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, CA 90095, USA

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ABSTRACT

The regulation of the *Il12b* gene, encoding the shared p40 subcomponent for IL-12 and IL-23, is critical for innate immune responses and subsequent T cell polarization. This gene is robustly induced upon Toll-like receptor (TLR) stimulation, wherein an enhancer located 10 kb upstream of the transcription start site is required for promoter activity; however, the underlying mechanisms that regulate this enhancer in cooperation with the promoter has remained elusive. We show here that the *Il12b* enhancer contains functional ISREs for recognition by interferon regulatory factors (IRFs), and provide evidence that TLR-activated IRF5 mediates cooperativity of the enhancer with the promoter which also contains ISREs. By contrast, IRF3 activated by cytosolic RIG-I-like receptor (RLR) signaling binds to these ISREs and causes gene suppression. Consistently, IRF5 binding is accompanied with chromatin remodeling of both regulatory regions and the formation of a productive transcriptional complex containing other transcription factors, whereas these events are inhibited by IRF3 binding. We show that the ISREs embedded in the enhancer are indeed critical for its activation by IRF5. We also adduce evidence that the 5′ sequences of the enhancer and promoter ISREs, all of which deviate from consensus ISREs, critically affect the function of IRF3. The dual commitment of these IRFs in the regulation of the *Il12b* enhancer and promoter is unique and may have implications for understanding the evolution of this gene.

1. Introduction

Complex gene regulation networks operate to ensure appropriate responses against distinct pathogens. Infection by pathogens triggers the activation of several types of signal transducing innate receptors, including membrane-bound Toll-like receptors (TLRs) and cytosolic receptors such as retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) [1,2]. While the nuclear factor κB (NF-κB) is widely involved in the regulation of the target genes induced upon activation of these receptors [3,4], the interferon (IFN) regulatory factor (IRF) family of transcription factors also play critical roles in these gene regulatory networks [1,5].

The *Il12b* gene, which encodes the interleukin (IL)-12p40 subunit that is shared by IL-12 and IL-23, has been one of the best studied target genes induced in antigen-presenting cells (APCs) by TLR signaling in the context of the innate receptor-mediated instruction of T cell polarization against bacterial infections [6,7]. Indeed, the TLR-induced IL-12 and IL-23 is critical for the differentiation of naive T cells into T helper type 1 (Th1) cells and the maintenance of IL-17-producing Th17 cells, respectively [6–8]. The mouse *Il12b* promoter contains several *cis*-elements that recruit a number of transcription factors in response to TLR signaling, including IRF5, NF-κB, and CCAAT/enhancer-binding protein (C/EBP) [9,10].

The *Il12b* gene is also controlled by a TLR-inducible enhancer region located 10 kb upstream of the transcription start site; however, the underlying regulatory mechanism(s) for how the enhancer cooperates with the promoter still remains to be understood. TLR-induced gene transcription is accompanied with nucleosome remodeling and recruitment to the enhancer of octamer-binding transcription factor 1 (Oct-1) and Oct-2, which are constitutively expressed in APCs; hence, enhancer activation may involve binding of an unidentified, TLR-inducible transcription factor(s) that facilitate the recruitment of the Oct proteins [11].

We have shown that IRF5 upon activation by TLR signaling binds to the IRF-binding sites (IFN-stimulated response elements; ISREs) of the *Il12b* promoter, and is indispensable for the TLR-mediated activation of the *Il12b* gene [12]. More recently, we have

* Corresponding author at: Department of Molecular Immunology, The University of Tokyo, Komaba 4-6-1, Meguro-Ku, Tokyo 153-8505, Japan.
E-mail address: tada@m.u-tokyo.ac.jp (T. Taniguchi).
[1] These authors contributed equally to this work.
[2] Present address: AK project, Graduate School of Medicine, Kyoto University, Yoshida Sakyo-ku, Kyoto 606–8501, Japan.

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shown that IRF3 activated by RLR signaling dominantly binds to the ISREs of the *Il12b* promoter over TLR-activated IRF5, thereby selectively suppressing the TLR-mediated gene induction and subsequent Th1- and Th17-type T cell responses [13]. However, the molecular basis of how the RLR-activated IRF3 functions as activator for type I IFN genes on the one hand and exerts a suppressive role on the *Il12b* promoter on the other remains unknown.

In this study, we identify new IRF-binding ISREs within the *Il12b* enhancer region and demonstrate that upon TLR stimulation IRF5, C/EBP and Oct transcription factors bind to the enhancer. We also show that, similar to the *Il12b* promoter, RLR-activated IRF3 dominantly binds to the enhancer in lieu of IRF5 and interferes with the enhancer binding of C/EBP and Oct factors. As such, IRF5 binding to ISREs of the *Il12b* enhancer and promoter results in cooperative activation of the gene, whereas IRF3 binding results in gene suppression. We also adduce evidence for the critical function of the unique ISRE sequences embedded in the *Il12b* enhancer/promoter and that the N-terminal DNA binding region (DBR) of IRF3 dictate the functional fate of this transcription factor on the *Il12b* gene. We discuss our findings in terms of the unique evolution of the regulatory mechanism of the *Il12b* gene by these IRFs.

2. Materials and methods

2.1. Reporter assay

HEK293T cells (3.0×10^4 cells) seeded in 48-well plates were transiently cotransfected with 5 ng of a *Renilla* luciferase plasmid and 100 ng of a reporter plasmid together with expression plasmids for IRF3–5D, IRF5A, IRF5N3C, IRF3N5C and/or c-Rel using X-tremeGENE 9 reagent (Roche). At 36 h after transfection, cells were harvested and luciferase activities were measured using the dual-luciferase reporter assay system (Promega). In all cases, the obtained data were normalized for transfection efficiency by dividing firefly luciferase activity by *Renilla* luciferase activity. Other materials and methods are in the Supplementary Information.

3. Results

3.1. Binding of IRFs to the Il12b enhancer upon TLR or RLR activation

It was shown previously that a 105-bp segment of DNA located 10 kb upstream of the transcription start site of the *Il12b* gene functions as a TLR-inducible enhancer [11]. Upon inspection of the enhancer sequence, we identified three new putative ISREs, which we termed e-ISRE1, e-ISRE2 and e-ISRE3; accordingly, we refer to the ISREs within the promoter as p-ISRE1 and p-ISRE2 throughout (Fig. 1A). We then examined by ChIP assay the binding of IRFs and other transcription factors to the enhancer region in mouse peritoneal macrophages following stimulation with either LPS for TLR4 activation or poly(I:C) for RLR activation. Interestingly, TLR4 stimulation resulted in the recruitment of IRF5, C/EBPβ and Oct-1/-2 transcription factors to the enhancer (Fig. 1B and Supplementary Fig. 1A). On the other hand, IRF3 was recruited in lieu of IRF5 upon RLR stimulation, but not C/EBPβ or Oct-1/-2, suggesting that the binding of IRF3 results in an abortive assembly of transcription factors on the enhancer (Fig. 1B and Supplementary Fig. 1A). Furthermore, when cells are stimulated by poly(I:C) followed by LPS stimulation, IRF3 binding is predominant over IRF5 and LPS-induced C/EBPβ and Oct-2 binding is attenuated (Fig. 1C and Supplementary Fig. 1B). These observations are reminiscent of the *Il12b* promoter regulation by these IRFs in that the IRF3 transcription factor activated by RLR stimulation functions as a suppressor of the promoter, where it interferes with the productive assembly of transcription factors that is otherwise induced by TLR signaling [13]. Consistent with this, a restriction enzyme accessibility assay revealed that the induction of chromatin

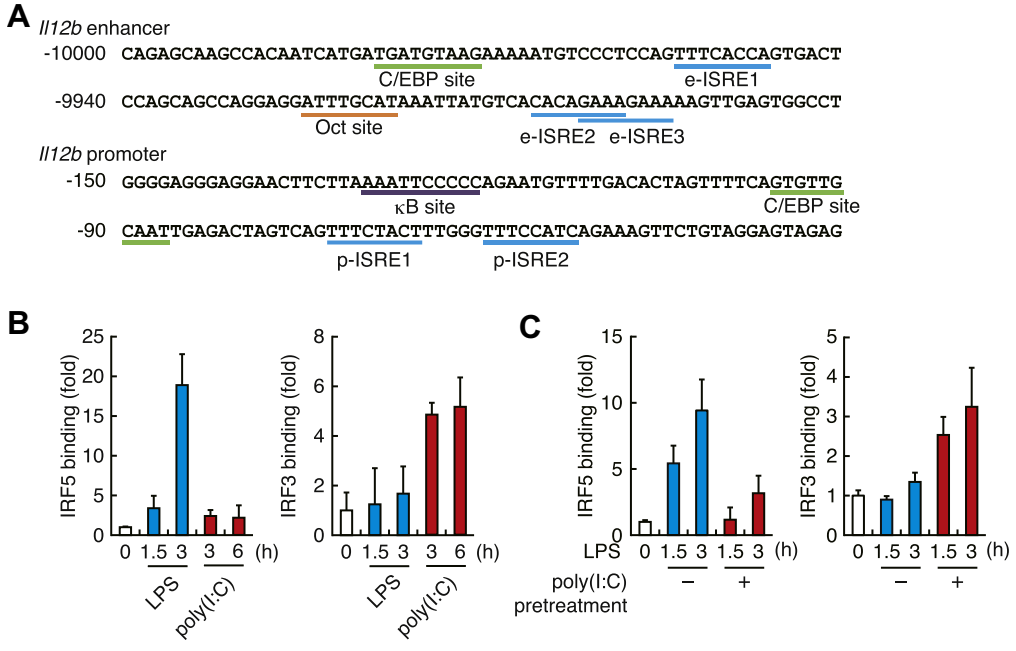

Fig. 1. Binding of IRFs to *Il12b* enhancer upon TLR or RLR activation (A) Schematic illustration of the murine *Il12b* enhancer and promoter regions, with three putative ISREs (e-ISRE1, e-ISRE2 and e-ISRE3; blue color) and two ISREs (p-ISRE1 and p-ISRE2; blue color), respectively, are shown. Oct (orange), C/EBP (green) and κB (purple)-binding sites are also shown. Numbers show the distance from the transcription start site of the *Il12b* gene. (B) Peritoneal macrophages from C57BL/6 mice were stimulated with poly(I:C) (5 μg/ml; red color) or LPS (100 ng/ml; blue color) for the indicated time periods. (C) Peritoneal macrophages from C57BL/6 mice were stimulated for the indicated time periods with LPS alone (100 ng/ml; blue color) or LPS with poly(I:C) (5 μg/ml; red color) pretreatment for 2 h. ChIP assay was performed using anti-IRF5 or anti-IRF3 antibody. Results shown are means ± SD of three independent experiments.

remodeling that occurs on both the enhancer and promoter regions upon TLR stimulation [11] is not observed upon RLR stimulation (Supplementary Fig. 1C).

3.2. Regulation of the Il12b enhancer by IRFs

The above observations suggest that, similar to the *Il12b* promoter, TLR-activated IRF5 also activates the enhancer through binding to the ISREs, whereas the binding of the RLR-activated IRF3 suppresses the IRF5-mediated activation. To test this, we carried out a transient reporter assay. Because it has been shown that the enhancer region confers an increased *Il12b* promoter activity when synthetically attached to the promoter [11], we first examined whether IRF5 contributes to the reporter gene that is driven by the enhancer-attached promoter using the reporter plasmid, eWT-pWT (depicted in Supplementary Fig. 2A). As reported previously, expression of IRF5A, a constitutive active type isoform of IRF5 [13], activated the reporter gene driven by the *Il12b* promoter alone (pWT) in HEK293T cells (Fig. 2A). Interestingly, the IRF5-dependent activation is dramatically enhanced by the 105-bp enhancer DNA segment (eWT-pWT) (Fig. 2A); this enhancement requires p-ISREs, as mutations of these ISREs (eWT-pM) result in the reduction of IRF5-mediated reporter gene activation (Supplementary Fig. 2A; right panel).

To examine the involvement of the three putative ISREs of the enhancer, we introduced mutations at these ISRE site(s) within the reporter gene (depicted in Fig. 2B). As shown in Fig. 2C, IRF5A-driven activation of the enhancer-promoter hybrid was partially diminished in eM1-pWT and eM2-pWT mutants, which carry mutations in e-ISRE1 and e-ISRE2, respectively, but not in eM3-pWT mutant with mutations in e-ISRE3. Further, the level of gene activation for eM1M2-pWT carrying mutations in both e-ISRE1 and 2 was nearly the same as that for the promoter alone (Fig. 2C). These observations indicate that these two e-ISREs, e-ISRE1 and e-ISRE2, are responsible for the IRF5-mediated gene activation in cooperation with its cognate promoter.

To examine the role of these ISREs in the context of a different enhancer-promoter construct, we prepared a reporter construct, termed eWT-minP, in which the enhancer was fused with a minimal promoter (minP), wherein essentially a TATA box serves as an acting *cis*-element. The eWT-minP was also activated by IRF5A in a dose dependent manner, albeit much weaker than the reporter gene containing the *Il12b* promoter described above (by about 20-fold) (see Fig. 2C and Supplementary Fig. 2B). This activation was diminished in reporter constructs that harbor e-ISRE mutations (Supplementary Fig. 2B). Thus, these data support the notion that IRF5 binds to the enhancer ISREs and augments the promoter activation of the gene.

We have previously shown that RLR-activated IRF3 binds to promoter of the *Il12b* gene and suppresses TLR-activated gene transcription by IRF5 [13]. In view of our ChIP assay data showing dominant binding of the RLR-activated IRF3 over TLR-activated IRF5 to the *Il12b* enhancer (Fig. 1C), we considered that IRF3 may also suppress the enhancer activity. To test this notion, we examined the effect of IRF3–5D, a constitutively active mutant of IRF3 [14], on the IRF5A-mediated enhancer activation in HEK293T cells using eWT-pWT or eWT-minP. As shown in Fig. 2D, IRF5A-mediated activation of the eWT-pWT reporter gene was inhibited by increasing the amount of IRF3–5D expression vector; a similar observation was also made with eWT-minP (Supplementary Fig. 2C). Of note, the suppressive effect of IRF3 is specific for IRF5 because IRF3–5D did not interfere with c-Rel-mediated activation of enhancer-promoter reporter gene (Supplementary Fig. 2D). These data further indicate that IRF3 binds to the e-ISREs where it inhibits the IRF5-mediated activation of the enhancer.

3.3. Elements within the IRFs and enhancer/promoter ISREs for the fate determination of the Il12b gene

Why IRF3, a well known transcriptional activator for the *Ifnb1* gene [5], functions as a suppressor on the *Il12b* enhancer and promoter, both of which are activated by IRF5? These two IRFs

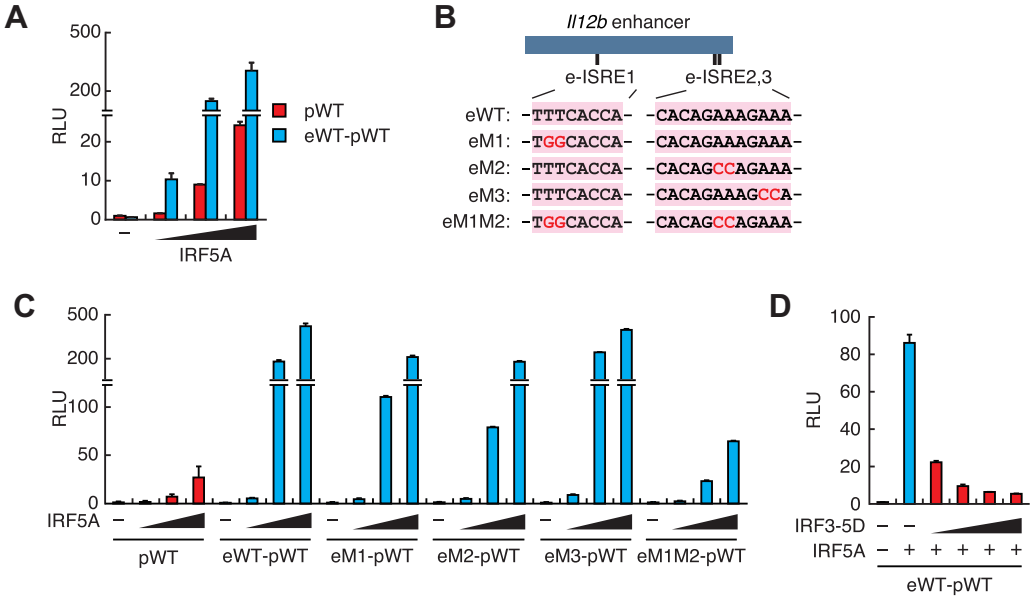

Fig. 2. Regulation of *Il12b* enhancer activity by IRFs (A) HEK293T cells were transiently cotransfected with pWT or eWT-pWT of pIL-12p40–Luc reporter and the expression vector for IRF5A (2, 10 or 50 ng). (B) A schematic illustration of ISRE sequence in the *Il12b* enhancer (eWT) is shown. Mutated sequence(s) is shown in red color (eM1, eM2, eM3 and eM1M2) (C) HEK293T cells were transiently cotransfected with pWT (red), eWT-pWT (blue), eM1-pWT (blue), eM2-pWT (blue), eM3-pWT (blue), or eM1M2-pWT (blue) of pIL-12p40–Luc reporter and the expression vector for IRF5A (2, 10 or 50 ng). (D) HEK293T cells were transiently cotransfected with eWT-pWT of pIL-12p40–Luc reporter and the expression vector for IRF5A alone (10 ng; blue color), or IRF5A with IRF3–5D expression vector (2, 10, 50 or 100 ng; red color). Luciferase activity was measured. All results shown are means ± SD of three independent experiments.

are distantly related within the IRF family, wherein their N-terminal DBRs and C-terminal regions which may associate with other transcription factors [15], respectively shows homology of 36% and 23% [16]. To examine which of the two regions dictate the 'fate' of these IRFs, we prepared the expression vectors for chimeric proteins termed IRF5N3C (IRF5 DBR with IRF3–5D C-terminal region) and IRF3N5C (IRF3 DBR with IRF5A C-terminal region), and then subjected them to reporter gene assays (Fig. 3A, B). Perhaps surprisingly, it was found that IRF5N3C but not IRF3N5C functions as activator for enhancer or promoter of the *Il12b* gene (Fig. 3B; left and middle panels). Furthermore, IRF5N3C expression also resulted in an efficient activation of the eWT-pWT reporter gene, the expression of which is driven by the enhancer and promoter (Fig. 3B; right panels), whereas IRF3N5C expression resulted in suppression of these reporter gene activities by IRF5A (Fig. 3C and Supplementary Fig. 3A). Of interest, IRF3N5C is still capable of activating the *Ifnb1* promoter as judged by a reporter gene assay (Supplementary Fig. 3B). Thus, these data suggest that N-terminal DBRs of the IRFs dictate the transcriptional fate of its function on the *Il12b* enhancer and promoter.

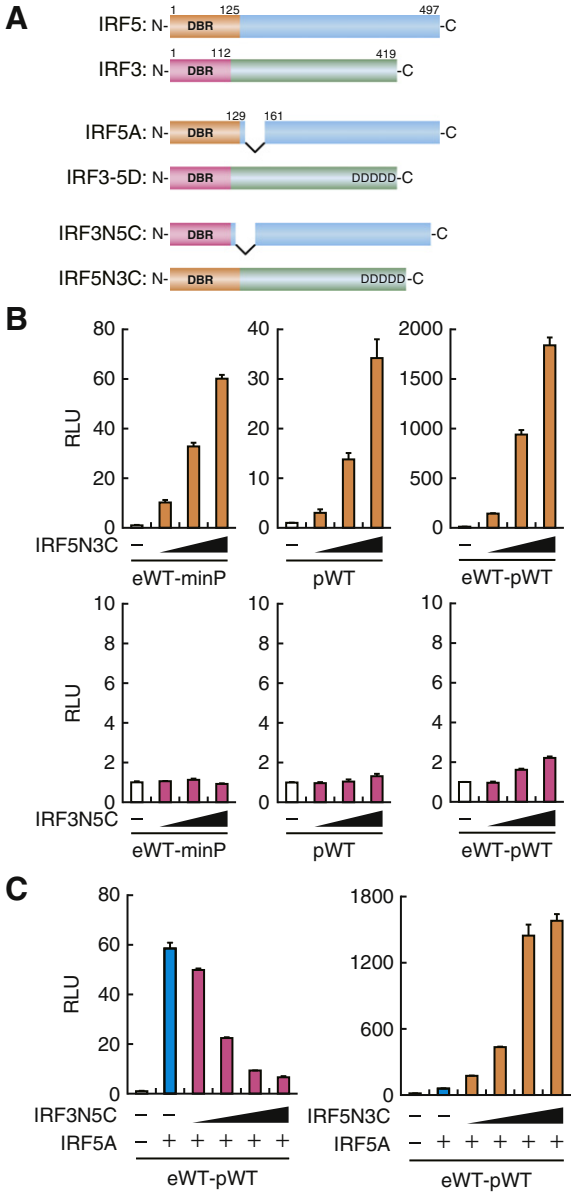

Fig. 3. The role of chimeric IRFs for the regulation of *Il12b* enhancer-promoter activity (A) A schematic illustration of chimeric IRF3N5C and IRF5N3C proteins is shown. DBRs for IRF5 and IRF3 are shown in orange and pink colors, respectively. IRF5A lacks amino acids (129–161) of IRF5 [13]. IRF3–5D is a mutant of IRF3 whose five serine and threonine residues (S388, S390, S394, T396 and S397) in C-terminal region are substituted by five aspartic acids (DDDDD). (B) HEK293T cells were transiently cotransfected with eWT-minP, pWT or eWT-pWT of pIL-12p40–Luc reporter and the expression vector for IRF5N3C (2, 10 or 50 ng; upper panels, orange color) or IRF3N5C (2, 10 or 50 ng; lower panels, pink color). (C) HEK293T cells were transiently cotransfected with eWT-pWT of pIL-12p40–Luc reporter and the expression vector for IRF5A alone (10 ng; blue color), or IRF5A with IRF3N5C (2, 10, 50 or 100 ng; left panel, pink color) or IRF5N3C (2, 10, 50 or 100 ng; right panel, orange color). Luciferase activity was measured. All results shown are means ± SD of three independent experiments.

These observations prompted us to examine the possibility that the *Il12b* ISREs may intrinsically induce a conformational change of the bound IRF3. Interestingly, when a sequence alignment of the *Il12b* ISREs with ISREs of other genes known to be activated by IRF3 was performed, all *Il12b*-derived ISREs have deviations from the consensus ISRE sequence, 5′-AANNGAAA-3′, specifically at the 5′AA sequence (Fig. 4A). It has been shown that this AA sequence interacts with the His40 residue within loop 1 (L1 loop) of IRF3, whereas the downstream GAAA sequence interacts with the Asn79, Arg81 and Ser82 residues within α-helix 3 motif [17]. To examine further whether these deviations are critical for the action of IRF3, we modified the ISREs of the *Il12b* enhancer and/or promoter in the above-described reporter genes to either pAA, eAA-minP or eAA-pAA (depicted in Fig. 4B) and subjected each to the reporter gene assay. Interestingly, the pAA reporter gene, which is still activated by IRF5A (Supplementary Fig. 4; left panel), is also activated by IRF3–5D or IRF3N5C (Fig. 4C; left panels). Although we could not detect activation of eAA-minP by these IRFs, which we surmise is due to a weak activity of this promoter (Fig. 4C; middle panels), we found a marked increase of the reporter gene activation levels, approximately by 5-to 10-fold for both IRF3–5D and IRF3N5C, in eAA-pAA as compared to pAA (Fig. 4C; left and right panels). This indicates a contribution of the mutated enhancer ISREs activated by these IRFs. These observations suggest that the 5′ di-nucleotide sequences of the e-ISREs and p-ISREs within the *Il12b* gene indeed dictate, at least in part, the fate of gene regulation by IRF3. On the other hand, in view of the fact that the activation levels of these reporter genes by IRF3–5D or IRF3N5C are significantly lower than those by IRF5A (see the right panels of Fig. 4C and Supplementary Fig. 4), additional sequence elements are also involved in the regulation of the IRF3 activity on the *Il12b* enhancer and promoter.

4. Discussion

In this study, we provided evidence that IRF5 is involved in a TLR-mediated activation of the *Il12b* gene enhancer. The ChIP assay revealed that TLR-activated IRF5 binds to *Il12b* enhancer region and this binding was accompanied by the binding of the C/EBPβ, Oct-1 and Oct-2 transcription factors, while binding of RLR-activated IRF3 to this enhancer resulted in the inhibition of binding of other transcription factors. These findings are reminiscent of the regulation of the promoter by these IRFs, wherein RLR-activated IRF3 inhibits the binding of TLR-activated IRF5, thereby attenuating *Il12b* gene expression [13]. Here, we provide additional mechanistic insight, wherein both enhancer and promoter are similarly regulated by these IRFs for the outcome of the gene transcription.

By employing reporter gene assay, we identified two functional ISREs, termed e-ISRE1 and e-ISRE2, within the enhancer region and adduced evidence that these ISREs indeed cooperate with promoter ISREs for the IRF5-mediated activation. We also provided evidence that IRF3 acts on both the enhancer and promoter to suppress IRF5-mediated gene expression. Although a more detailed description of the mechanism for how enhancer and promoter

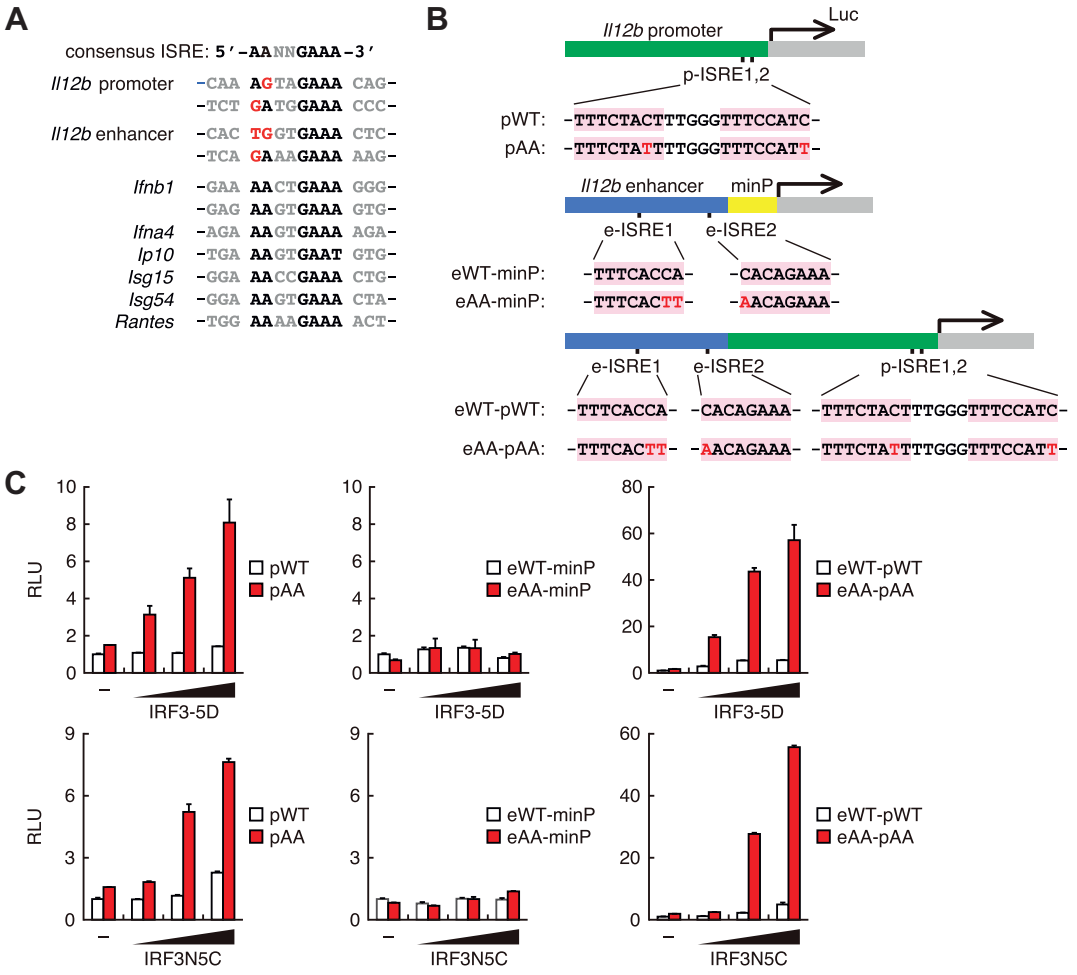

Fig. 4. IRF3 function is dictated by ISRE sequences of the *Il12b* enhancer and promoter. (A) Comparison of the IRSE sequences on IRF3 target genes. Non-conserved AA sequences of the *Il12b* enhancer and promoter ISREs are shown in red color. (B) A schematic view of *Il12b* enhancer (blue), promoter (green) or enhancer-promoter reporter gene containing ISRE mutations (red) is shown. Minimal promoter (minP) and a luciferase reporter gene (Luc) are shown in yellow and gray colors, respectively. (C) HEK293T cells were transiently cotransfected with each pIL-12p40–Luc reporter gene in B and the expression vector for IRF3–5D (2, 10 or 50 ng; upper panels) or IRF3N5C (2, 10 or 50 ng; lower panels). Luciferase activity was measured. Results shown are means ± SD of three independent experiments.

ISREs are regulated in the context of chromosomal gene regulation is required, our ChIP and chromatin remodeling data are consistent with a model for the dual requirement of the ISREs of the *Il12b* enhancer and promoter. Thus, our results imply that TLR signaling activates the *Il12b* gene by means of forming an enhanceosome complex, wherein IRF5 binding to the enhancer and promoter contributes to the formation of a larger transcriptional complex that includes other transcription factors such as c-Rel, C/EBPβ and Oct-1/-2 [9–11].

Similar to the *Il12b* promoter, the enhancer appears also to be negatively regulated by RLR-activated IRF3. Indeed, RLR stimulation resulted in the dominant binding of IRF3 over IRF5, accompanied with the loss of C/EBPβ and Oct-2 recruitment as revealed by ChIP assay (Fig. 1C and Supplementary Fig. 1B), and suppression of the IRF5-mediated activation of the *Il12b* promoter and enhancer by IRF3 by reporter gene analysis (Fig. 2D and Supplementary Fig. 2C).We also showed that the N-terminal DBR of IRF3 is responsible for dictating the fate of this transcription factor on the *Il12b* enhancer and promoter. IRF3–5D chimeric IRF composed of the N-terminal DBR of IRF3 and C-terminal region of IRF5 functions as a transcriptional suppressor (Fig. 3C; left panel), whereas the reverse chimeric protein, IRF5N3C, functions as activator (Fig. 3B; upper panels). From this, we focused on the ISRE sequences embedded in the enhancer and promoter of this gene and found unique deviations in all ISREs at the 5′ AA sequence that would interact with the conserved His40 residue within L1 loop of the IRF3 protein (Fig. 4A). The results of our reporter gene assay showed that the enhancer and promoter could be converted from 'IRF3-repressible' to 'IRF3-activatable' when these deviated sequences were converted to the "consensus" AA sequence. Although it is likely that other sequences also affect the fate of IRF3 on this gene for the endogenous gene regulation, we believe this sequence deviation explains, at least in part, the unique function of IRF3, which otherwise activates many target genes such as type I IFN genes [5].

We infer that IRF3 interactions with ISREs containing the "deviated" 5′ AA sequence will undergo a conformational change that is different from IRF3 bound to the "consensus" AA sequence. This conformational change may render IRF3 to induce a transcriptionally inactive state. In this regard, it is interesting to note that DNA sequence-induced conformation change of a transcription factor has been suggested previously for several genes [18–20], and transcription factor conformation when bound to a specific DNA site determines its activation potential [21]. Thus, our results may also indicate a DNA sequence-instructed regulation of a transcription factor for its function. This issue obviously needs further elaboration in the context of endogenous gene regulation.

Finally, the present study raises an interesting evolutional issue regarding the *Il12b* enhancer and promoter ISREs. These ISRE

sequences are, in fact, well conserved among mammalian species, which indicates a biological significance of this conservation (Supplementary Fig. 5). Since IL-12p40 induction contributes to differentiation of Th1 and Th17 inflammatory T cells [6–8], we speculate that the RLR-mediated suppression of the *Il12b* gene via IRF3 during viral infections may be a means by which to attenuate excessive inflammatory responses often caused by viruses. Of further note, there is ample evidence for the involvement of type I IFN signaling to the development or exacerbation of autoimmune responses that involves inflammatory T cells [22,23]. Thus, the RLR-activated IRF3, required for anti-viral type I IFN responses on one hand, may serve to suppress the development of excessive inflammatory T cells on the other, by suppressing *Il12b* gene expression. Although further elaboration will be required, this scenario offers an explanation for the conservation of the "deviated" AA sequence embedded in the *Il12b* enhancer and promoter regions during evolution. It will be interesting to generate mice carrying "genuine" AA sequences in lieu of the "deviated" sequences of the *Il12b* gene and examine the immune responses upon viral and/or bacterial infections. In conclusion, our present study offers new insights into the complex regulatory mechanism operating for the *Il12b* gene transcription by IRFs and their target *cis*-elements.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.006.

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