

c-Maf is essential for the F4/80 expression in macrophages *in vivo*

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Running Title: c-Maf is a regulator of F4/80

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Abstract

c-Maf, which is one of the large Maf transcription factors, can bind to Maf recognition element (MARE) and activates transcription of target genes. Although c-Maf is expressed in macrophages and directly regulates the expression of interleukin-10, detailed information regarding its function in the null mutant phenotype of tissue macrophages remain unknown. In this study, we demonstrated that c-Maf is specifically expressed in the F4/80 positive fetal liver and adult macrophages. The expression of F4/80, which is a tissue macrophage-specific seven trans-membrane receptor, was dramatically suppressed in the c-Maf-deficient macrophage, whereas the expression of Mac-1 was not affected, suggesting that c-Maf is not necessary for the lineage commitment of macrophages. Luciferase reporter and electrophoretic mobility shift assay showed that c-Maf directly regulates the expression of F4/80 by interacting with the half-MARE site of the F4/80 promoter. These results suggest that c-Maf is required for the F4/80 expression in macrophages *in vivo*.

Introduction

The c-Maf is a large Maf transcription factor and a cellular homolog of *v-Maf* that was isolated from a musculoaponeurotic fibrosarcoma of chicken and identified as the trans-forming gene of the avian retrovirus AS42 [1]. The large Maf proteins, Mafa, Mafb, c-Maf and Nrl, contain an acidic domain that promotes transcriptional activation, and a basic region and an amphipathic helix (bZip) domain that mediate DNA binding to Maf recognition elements (MAREs) [2-4] and dimer formation, respectively. Each large Maf protein has been shown to play key roles in cellular differentiation [1, 5-11].

c-Maf is expressed in lens fiber cells [9], central nervous system [12], chondrocytes [12, 13], T helper cells [14], and macrophages [15, 16]. c-Maf-deficient mice (*c-Maf*^{-/-}) exhibited abnormal lens development [9], impaired expression of IL-4 and IL-21 in the T helper cells [14, 17] and suppressed expression of IL-10 in the LPS-stimulated macrophages [15, 16]. c-Maf directly interacts with Ets-1 and c-Myb, and this transcriptional complex is active in early

myeloid cell differentiation [18]. However, detailed characterization of the c-Maf function in tissue macrophages still remains unknown.

We previously reported that in the F4/80 promoter region there is a highly-conserved half-MARE site, which is essential for the transcriptional activation of F4/80 gene by Maf_b, other member of the large Maf transcription factor family [10]. F4/80 was established as one of the most specific cell-surface markers for the murine tissue macrophages [19, 20]. Recently, it was reported that F4/80 was crucial for the generation of the antigen specific regulatory T cell that is essential for peripheral tolerance induction [21].

In the present report, we analyzed *c-Maf*^{-/-} mice and demonstrated that c-Maf is predominantly expressed in the F4/80-positive fetal liver macrophages, and is essential for the F4/80 expression. However, expression of Mac-1 was not altered in *c-Maf*^{-/-} fetal liver macrophage in comparison with that of the wild-type (WT) control. The results obtained from the luciferase reporter assay as well as EMSA showed that c-Maf bound to the MARE site of the F4/80 promoter region. These data suggest that c-Maf is required for the expression of F4/80 *in vivo* as same as

Mafb [10]. Regulation of F4/80 by the large Maf transcription factors might control the induction of antigen specific peripheral tolerance.

Materials and methods

Animals. *c-Maf*^{-/-} mice were originally generated with a 129/Sv background [9] and have been backcrossed to C57BL/6J for more than seven generations.

Genotypes were determined by PCR analysis of tail DNA. The nucleotide sequences of the primers used for the PCR analysis are follows: *c-Maf* 1829s (orf)

5'-CTGCCGCTTCAAGAGGGTGCAGC-3', *c-Maf* 3' (orf)

5'-TCGCGTGTCACTCACATG-3', *c-Maf* geno 5' (lacZ)

5'-TGTTACGTTTCGAGCTTTC-3', β -gal 3'(*c-Maf*^{-/-})

5'-GATTAAGTTGGGTAACGCC-3'. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba.

Reconstitution of hematopoietic cells with c-Maf-deficient fetal liver cells. The donor cells for hematopoietic reconstitution were prepared from E14.5 fetal livers of WT and *c-Maf*^{-/-} (C57BL/6J-Ly5.1) mice. Suspensions of single fetal liver cells

were prepared by mechanical disruption, grinding with a syringe insert against a 35 μm nylon cell strainer (BD Biosciences, San Jose, CA). The donor cells were injected into the tail vein of 8-10 weeks old C57BL/6J-Ly5.2 mice, previously exposed to X-ray in doses of 1000R. The reconstitution efficiency was checked by FACS by monitoring the Ly5.2/5.1 ratio of peripheral blood cells. Mice showing more than 90% reconstitution were used for analysis.

Immunohistochemical analysis. E14.5 embryos were fixed in 4% paraformaldehyde, and several 4 μm frozen sections were cut. Frozen sections were washed in PBS, and the intrinsic peroxidase activity was blocked with 3% H_2O_2 . After blocking the sections for 1 hr in PBS containing 10% goat serum, 2% skim milk and 0.2% polyoxyethylene sorbitan monolaurate, they were incubated with the anti-c-Maf antibody (1:1000; Bethyl, Montgomery, TX) overnight at 4°C. The slides were further incubated with EnVision⁺ System Labeled Polymer-HRP Anti-rabbit antibody (Dako Cytomation, Glostrup, Denmark) and substrate DAB (Nichirei Bioscience Inc., Tokyo, Japan). Next, they were treated with rat anti-F4/80 antibody

(1:1000; Serotec, Oxford, UK), incubated with anti-rat IgG alkaline phosphatase (1:300; Jackson ImmunoResearch, West Grove, PA), and then stained with using the BCIP/NBT substrate system (Dako Cytomation).

Real time RT-PCR analysis of fetal liver macrophages. Macrophages were prepared from E14.5 fetal livers of WT and *c-Maf*^{-/-} mice. Suspensions of single fetal liver cells were prepared as described above. Mac-1 positive cells were labeled with Mac-1⁺ magnetic beads to separate macrophages by MACS™ system (Milteneu Biotech, Bergisch-Gladbach, Germany). Total RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). The *c-Maf*, *Mafb* and *F4/80* mRNA levels were examined by real time RT-PCR using the Thermal Cycler Dice Real Time System (Takara Bio, Shiga, Japan). This procedure enabled standardization of initial mRNA content of cells relative to the amount of *Hprt* mRNA. The following specific primers were used for PCR; *F4/80*-forward: 5'-CCCAGCTTCTGCCACCTGCA-3', *F4/80*-reverse: 5'-GGAGCCATTCAAGACAAAGCC-3', *c-Maf*-forward:

5'-CTGCCGCTTCAAGAGGGTGCAGC-3', *c-Maf*-reverse:
5'-GATCTCCTGCTTGAGGTGGTC-3', *Mafb*-forward:
5'-TGAGCATGGGGCAAGAGCTG-3' *Mafb*-reverse:
5'-CCATCCAGTACAGGTCCTCG-3' *Hprt*-forward:
5'-TTGTTGTTGGATATGCCCTTGACTA-3', *Hprt*-reverse:
5'-AGGCAGATGGCCACAGGACTA -3'

Flow cytometric analysis. The single cell suspension of fetal liver or adult bone marrow cells was prepared from each genotype of littermates. Flow cytometric analysis was performed by using PE and APC-conjugated anti-Mac-1 (Pharmingen, San Diego, CA), anti-F4/80 (Serotec) antibodies were used on a DB LSR (Becton Dickinson, Mountain View, CA). The data were analyzed using Flowjo software.

Macrophage colony assay. E12.5 fetal liver cells were seeded into methylcellulose medium (Methocult Stem Cell Technologies, Vancouver, BC) with IL-3 (20 ng/ml)

and GM-CSF (10 ng/ml). Seven days later, macrophage colonies were enumerated under an inverted microscope.

Plasmid construction and transient transfection assay. The pGL3Basic-based mouse F4/80 promoter luciferase plasmid construct was reported previously [22].

The -253+mut/Luc was created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the following PCR primers; 5' CACCTCTGATGGTGGCAATCTCTTCGAAAGCTGCTGGGCTCAGTC 3' and 5' GACTGAGCCCAGCAGCTTTTCGAAGAGATTGCCACCATCAGAGGTG 3'.

The italicized sequences represent the mutated site. For transient transcription, the c-Maf expression plasmid pEFX3-FLAG-c-Maf, construction of which was previously described [23] was used. Reporter and effector plasmids were transfected into the RAW264.7 cells, luciferase assays were performed as described [10].

Electrophoretic mobility shift assay (EMSA). c-Maf and mock-control proteins were

prepared from the 293T cells that were transiently transfected with either the expression plasmid pEFX3-FLAG-Mafb or the mock vector pEFX3-FLAG. Nuclear extracts were prepared using a NucBuster Protein Extraction Kit (Novagen, Madison, WI). EMSA and competitive EMSA were performed according to the manufacturer's protocol using a LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL). The following oligonucleotides were used as probes: Consensus MARE, 5'-AGCTCGGAATTGCTGACTCATCATTACTC-3' (biotin labeled or non-labeled), 5'-GAGTAATGATGAGTCAGCAATTCCGAGCT-3', Wild-type competitor, 5'-CTCATGGTGGCAACTCAGCAGAAAGCTGCT-3', 5'-AGCAGCTTTCTGCTGAGTTGCCACCATGAG-3', Mutant competitor, 5'-CTCATGGTGGCAACGACTACTAAAGCTGCT-3', 5'-AGCAGCTTTAGTAGTCGTTGCCACCATGAG-3'.

Statistical analysis. The results were recorded as mean \pm standard error of the mean.

Data were analyzed by *t*-test to determine statistically significant difference.

Result

c-Maf is specifically expressed in the fetal liver macrophages. *c-Maf*^{-/-} mice were originally generated with a 129/Sv background, died within a few hours after birth [9]. Unexpectedly, *c-Maf*^{-/-} mice with C57BL/6 genetic background, *c-Maf*-deficient mice showed embryonic lethality around e15.5 (data not shown). Therefore, to elucidate the physiological significance of *c-Maf* in hematopoietic cells, we examined fetal liver cells at stage E14.5. To identify which fetal liver cells express *c-Maf*, we performed immunohistochemical analysis of the E14.5 fetal liver using the anti-*c-Maf* and anti-F4/80 antibodies. Interestingly, *c-Maf* immunoreactivity was specifically observed in the F4/80-positive tissue macrophages (Fig. 1A). To confirm this macrophage-specific *c-Maf* expression, we next purified both Mac-1-positive and -negative cells from E14.5 fetal liver using magnetic beads, and examined expression of the *c-Maf*, *Mafb* and *F4/80* mRNAs using real time RT-PCR. Consistent with the immuno-histochemistry results, real time RT-PCR analysis also demonstrated that the *c-Maf*, *Mafb* and *F4/80* mRNAs

were expressed more abundantly in the Mac-1-positive cells than in the Mac-1-negative cells (Fig. 1B). Thus, these results suggest that in addition to Mafb [10], c-Maf is also predominantly expressed in the F4/80-positive fetal liver macrophages.

c-Maf is essential for F4/80 expression in fetal liver macrophages. To gain insight into the physiological function of c-Maf in macrophages, we next examined the expression of F4/80 in the E14.5 *c-Maf*^{-/-} fetal liver macrophages by immunohistochemistry. Surprisingly, the number of F4/80 immunoreactivity-positive cells in the *c-Maf*^{-/-} fetal liver dramatically decreased as compared to the WT control (Fig. 2A). Consistent with this observation, FACS analysis of the fetal liver cells also showed a significant reduction of F4/80-positive cells in the *c-Maf*^{-/-} fetal liver (0.3 %; Fig. 2B.R1) than in the WT fetal liver (1.05 %; Figs. 2B.R1 and 2C). On the other hand, Mac-1-positive and F4/80-negative fractions were increased in the *c-Maf*^{-/-} fetal liver cells (Figs. 2B.R2 and 2C). Moreover, real time RT-PCR analysis showed about 3-fold reduction in

the expression of F4/80 mRNA in the *c-Maf*^{-/-} Mac-1-positive fraction (Fig. 2D, left panel). We also observed a slight change in the expression level of the *Mafb* mRNA in *c-Maf*^{-/-} (Fig. 2D, middle panel). However, expression of *c-Maf* mRNA was not observed in *c-Maf*^{-/-} (Fig. 2D, right panel). To determine the cause for this reduction, we performed a colony assay using the WT and *c-Maf*^{-/-} fetal liver cells in the presence of IL-3 and GM-CSF, and found virtually no difference between the number of macrophage colonies formed as a result of this treatment (Fig. 2E). Hence, these results also suggest that c-Maf is required for the maturation of macrophages or expression of F4/80 in fetal liver cells *in vivo*, but is not essential for the macrophage lineage commitment.

c-Maf is also essential for F4/80 expression in adult bone marrow macrophages.

Since *c-Maf*^{-/-} mice with C57BL/6 background are embryonic lethal around e15.5, we could not analyze what functional role c-Maf might play in F4/80 expression in adult macrophage. To circumvent this problem, analysis was carried out after reconstitution of hematopoietic cells using the donor cells prepared from E14.5

fetal livers of WT and *c-Maf*^{-/-} mice. As shown in Figure 3, F4/80 positive cells significantly decreased in the bone marrow of the mouse reconstituted with *c-Maf*^{-/-} fetal liver cells (F4/80+Mac-1-: 2.58%, F4/80+Mac-1+: 4.75%) than the mouse reconstituted with the WT fetal liver cells (F4/80+Mac-1-: 13.1%, F4/80+Mac-1+: 8.25%). In contrast, the percentage of Mac-1 positive cells in WT (F4/80-Mac-1+: 21.1%) and *c-Maf*^{-/-} (F4/80-Mac-1+: 20.3%) remained virtually same. These results suggest that c-Maf is also indispensable for the total expression of F4/80 in adult mice.

c-Maf directly activates F4/80 expression. We have previously reported that the *Mafb* is required for F4/80 expression in fetal liver macrophages, and that the *F4/80* reporter was activated by exogenous *Mafb* expression in the macrophage cell line RAW264.7 [10]. We found that F4/80 promoter region contains a highly conserved MARE site (Fig. 4A). Therefore, we hypothesized that c-Maf could directly bind to the MARE site and activate *F4/80* gene expression. To prove this hypothesis, we transfected the promoter-firefly luciferase reporter plasmid

construct, which contained the 253-bp *F4/80* promoter region, along with the c-Maf expression plasmid (pEFX3-FLAG-c-Maf) into the RAW264.7 cell line. As expected, the *F4/80* promoter reporter was activated by co-expression of c-Maf in a dose dependent manner (Fig 4B). However, neither the luciferase reporter plasmid construct that contained the 117-bp truncated, MARE-less *F4/80* promoter nor the luciferase reporter plasmid constructs that contained mutations in the half-MARE site of the *F4/80* promoter (253 bp mut) was activated by c-Maf (Fig. 4B). EMSA demonstrated that the non-labeled MARE probe competed out the binding of c-Maf to the biotin-labeled consensus MARE probe, whereas the mutant MARE probe did not (Fig. 4C). These results suggest that c-Maf could directly regulate the expression of *F4/80* by interacting with the highly conserved half-MARE site in its promoter region.

Discussion

There are several reported studies examining the function of c-Maf in macrophages. It was shown that c-Maf interacts with Ets-1 and c-Myb and the complex can induce differentiation of human myeloid cell line [18]. Cao et al found that c-Maf binds to the IL-10 promoter and activates its expression, and also demonstrated that LPS stimulated macrophages from fetal liver of *c-Maf*^{-/-} fail to secrete IL-10 [15, 16]. These observations suggested that c-Maf might play a crucial role in differentiation and function of macrophages. However, detail contribution of c-Maf in macrophage differentiation and function still remained unclear. In this study, we demonstrated that the c-Maf is required for the expression of F4/80 in macrophages, and our results using the reporter constructs suggest that c-Maf directly regulates the expression of F4/80 by binding to the half-MARE of the F4/80 promoter.

The F4/80 is a seven trans-membrane receptor, which was shown as one of the most specific cell-surface markers for the murine macrophages [19, 20]. A

recent study showed that F4/80 is required for the differentiation of antigen-specific CD8⁺ Treg cells, a process that requires direct cellular interaction of CD1d⁺ APCs with NK-T cells [21]. Although the F4/80 protein seems to have a biologically important function, information regarding its regulation at the transcriptional stage are lacking. The F4/80 has been reported to be highly and constitutively expressed in most resident tissue macrophages, including the red pulp macrophages in the spleen, microglia in the brain, Kupffer's cells in the liver and Langerhans' cells in the skin [24]. On the contrary, F4/80 is expressed at lower levels in activated macrophages isolated from *bacilli Calmette-Guerin*-infected animals [25]. Similarly, F4/80 expression is down regulated in IFN- γ treated macrophages [26]. These observations suggested that the expression of F4/80 might be under flexible transcriptional regulatory control for appropriate immunological response. We have previously demonstrated that other member of the large Maf transcription factors, Mafb, directly regulates F4/80 expression in E14.5 fetal liver, P0 spleen and cultured macrophage under non-adherent culture condition. Our results showed that the c-Maf is expressed specifically in the F4/80 positive cells and is also required

for F4/80 expression (Fig. 2A and B). Furthermore, the observed reduction in F4/80 expression in *c-Maf*^{-/-} E14.5 fetal liver macrophages is more than that was previously observed in *Mafb*^{-/-} [10]. Taken together, these results suggest that c-Maf might be involved in regulating F4/80 expression. Our results are in good agreement with this idea, as we have found a half-MARE site in the 5'-flanking region of the *F4/80* gene. This half-MARE site is highly conserved among mammals (Fig. 3A). We have demonstrated in this paper that the c-Maf binds to the half-MARE site and activates the *F4/80* promoter (Fig. 3B and C). Since we have also found that the *Mafb* could bind to this half-MARE site (data not shown), we suggest that the F4/80 expression in macrophages might be cooperatively regulated *in vivo* by c-Maf and *Mafb* through the half-MARE of the *F4/80* promoter region.

It is noteworthy that the expression pattern of F4/80 is different between the c-Maf- and *Mafb*-deficient macrophages *in vitro*. In the presence of M-CSF, *Mafb* is indispensable for F4/80 expression under non-adherent culture condition, but not under adherent culture condition [10]. The expression of F4/80 is, however, not reduced in *c-Maf*^{-/-} macrophages under both adherent and non-adherent culture

conditions (data not shown). Our results indicate that in the presence of M-CSF the expression of *Mafb* is up-regulated in the *c-Maf*^{-/-} macrophages, which in turn could compensate for the deficiency of c-Maf. In contrast, c-Maf is not up-regulated in *Mafb*-deficient macrophages, and these macrophages show clear phenotype under non-adherent culture condition. Based on these results, we suggest that c-Maf and *Mafb* might be the main transcription factors for the induction of *F4/80* expression under non-adherent culture condition, but not under adherent culture condition. Binding sites for the transcription factors PU.1, AP-1 and C/EBP were also found in the *F4/80* promoter region [22]. Studies showing that PU.1 and AP-1 could dimerize with large Mafs [27, 28], and that C/EBP binding activity was affected by c-Maf [15] tend to suggest that the regulation of *F4/80* expression might be complicated. Further analysis are needed to elucidate the detail mechanism.

In summary, we found that in addition to *Mafb*, the c-Maf is a key regulator for *F4/80* expression in fetal liver and adult macrophages. It would be interesting to generate c-Maf and *Mafb* double mutant mice for an understanding of the

mechanisms involved in regulating the F4/80 expression and peripheral tolerance.

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Figure legend

Figure 1. c-Maf is specifically expressed in the fetal liver macrophages. (A) Immunohistochemical analysis of wild-type (WT) E14.5 fetal liver using the anti-c-Maf and anti-F4/80 antibodies. c-Maf is expressed specifically in F4/80-positive macrophages. Brown and Purple staining indicate the presence of c-Maf and F4/80, respectively. Scale bar: 40 μm .

(B) Real time RT-PCR analysis. *F4/80* and *c-Maf* mRNAs are expressed more abundantly in the Mac-1-positive cells than in the Mac-1-negative cells. *Mafb* is also predominantly detected in the Mac-1-positive cells. *: P < 0.01

Figure 2. F4/80 expression is suppressed in *c-Maf*-deficient macrophages from fetal liver. (A) Immunohistochemical analysis of the WT and *c-Maf*^{-/-} fetal livers shows reduced expression of F4/80 in *c-Maf*^{-/-}. Brown staining indicates the presence of F4/80. Scale bars: 200 μm . (B) Single cell suspensions were prepared from the livers of the WT, heterozygous (*c-Maf*^{+/-}) or *c-Maf*^{-/-} mutant embryos at

stage E14.5. The cells were stained with PE-conjugated anti-F4/80 and APC-conjugated anti-Mac-1 antibodies, and then analyzed by flow cytometer. R1: F4/80-positive population of macrophages and R2: Mac-1-positive population of macrophages. (C) Quantification of the FACS analysis shows that the F4/80-positive population (gated as R1) is significantly decreased and the Mac-1 single positive population (gated as R2) is increased in *c-Maf*^{-/-}. Four embryos were analyzed in each genotype. (D) Real time RT-PCR analysis of Mac-1 positive fraction from the WT (n = 6) and *c-Maf*^{-/-} (n = 4) fetal liver cells: lower levels of F4/80 mRNA is observed in *c-Maf*^{-/-} compared with that in the WT. (E) The number of macrophage colonies induced by IL-3 and GM-CSF: control WT (n = 3) and *c-Maf*^{-/-} (n = 6). E14.5 fetal liver cells were treated with IL-3 and GM-CSF as described in the Materials and methods. *: P < 0.05. **: P < 0.01. n.d.: not detected.

Figure 3. Flow cytometric analysis of bone marrow cells from adult mice reconstituted with *c-Maf*^{+/-} and *c-Maf*^{-/-} E14.5 fetal liver cells. (A) FACS profile of F4/80/Mac-1 myelomonocytic populations from bone marrow. (B) Quantification

of F4/80 positive cells and Mac-1 positive cells from *c-Maf*^{+/-} (n = 3) and *c-Maf*^{-/-} (n = 3). *: P < 0.05.

Figure 4. Identification of c-Maf-responsive region in the *F4/80* promoter.

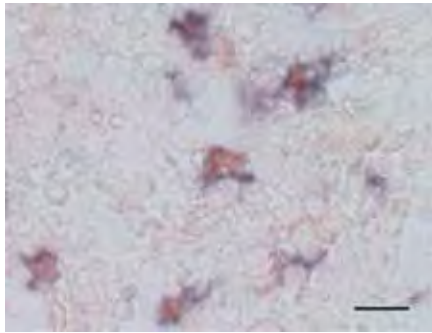
(A) Analysis of the 5'-flanking region of the *F4/80* gene by the Ensembl Genome Database website identified highly conserved mammalian c-Maf binding (half-MARE) site (TCAGCA + AT rich) and C/EBP binding site (RTTGCGYAA_Y, where R = A or G, and Y = C or T). (B) Reporter assay. The macrophage cell line RAW264.7 was co-transfected with an indicated *F4/80* promoter-reporter plasmid construct (wild-type: 253 bp, MARE-deleted: 117 bp, MARE containing mutations: 253 bp mut) along with the c-Maf expression plasmid, and the relative luciferase activity was measured as described in Materials and methods. The relative luciferase activities of the reporter plasmids are shown on the right hand panel and are presented as values relative (-fold) to that of the cells transfected with the empty reporter vector (0 ng) and the c-Maf expression plasmid. Error bars; SEM. (C) c-Maf and mock-control proteins were prepared from the 293T cells transfected

with the c-Maf and the mock expression plasmids, respectively, and were used in EMSA as described in Materials and methods. Lane 1: biotin labeled MARE probe. Lanes 2-9: c-Maf protein, indicated biotin labeled MARE (WT or mutant) probe, and increasing amounts of the unlabeled competitor oligonucleotide. Lane 10: mock protein and biotin labeled MARE probe.

Figure 1

A

c-Maf/F4/80



B

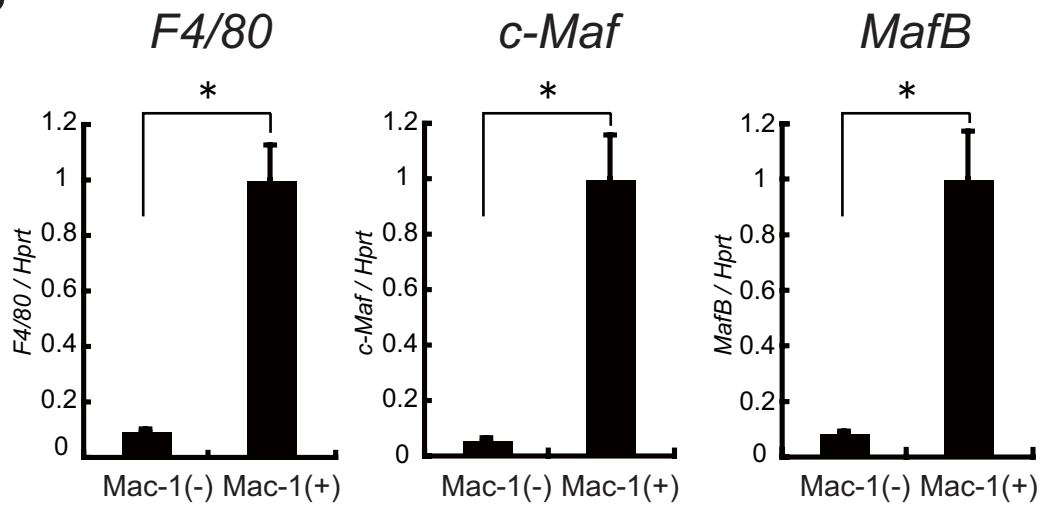


Figure 2

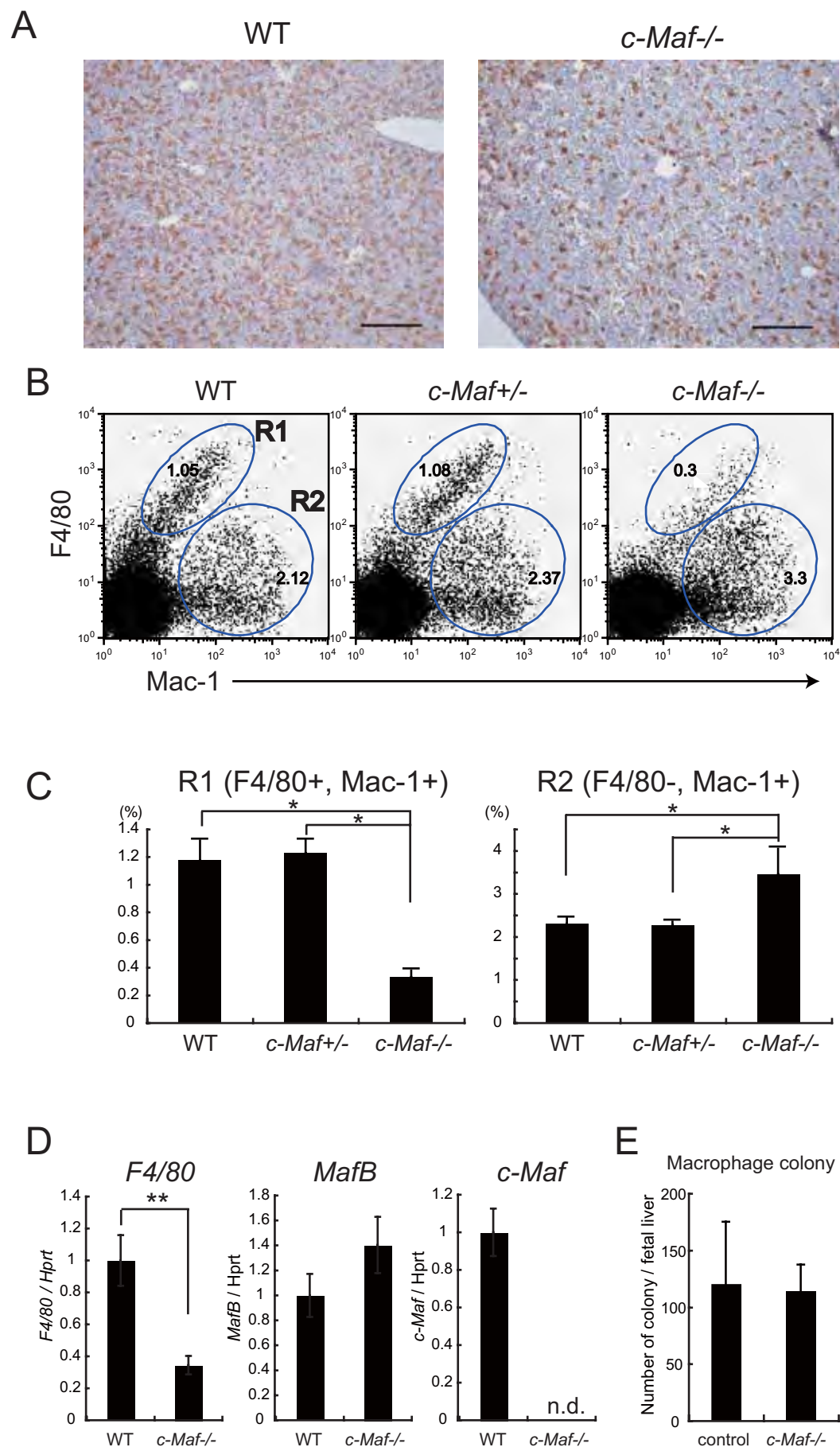


Figure 3

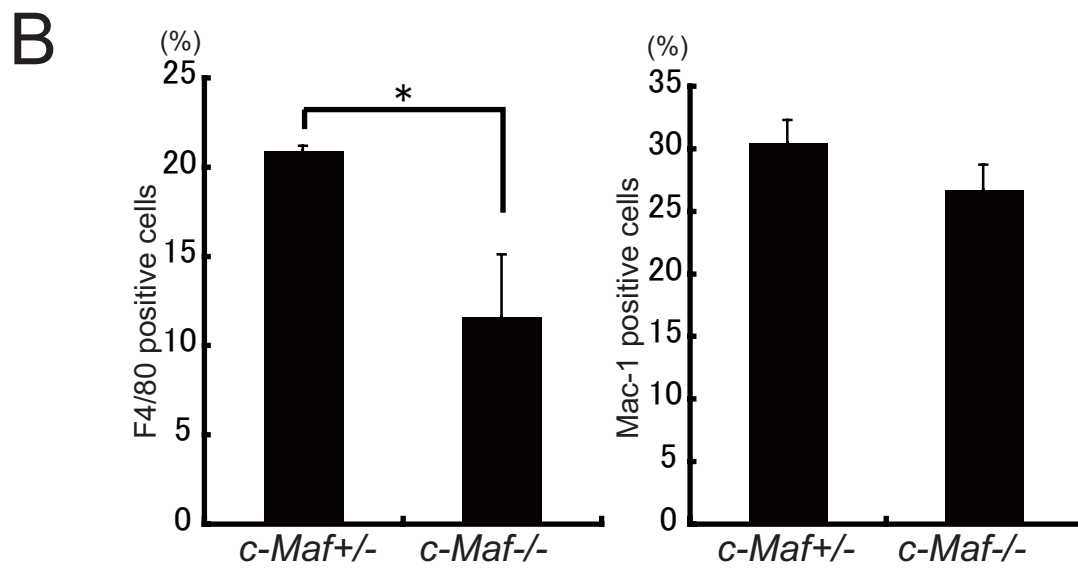
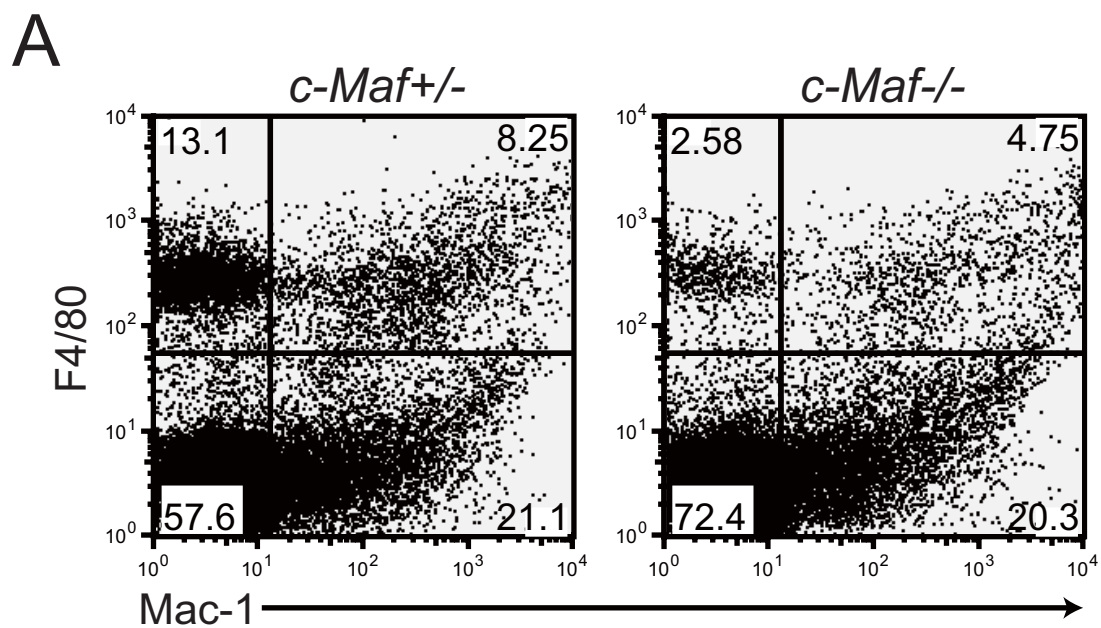
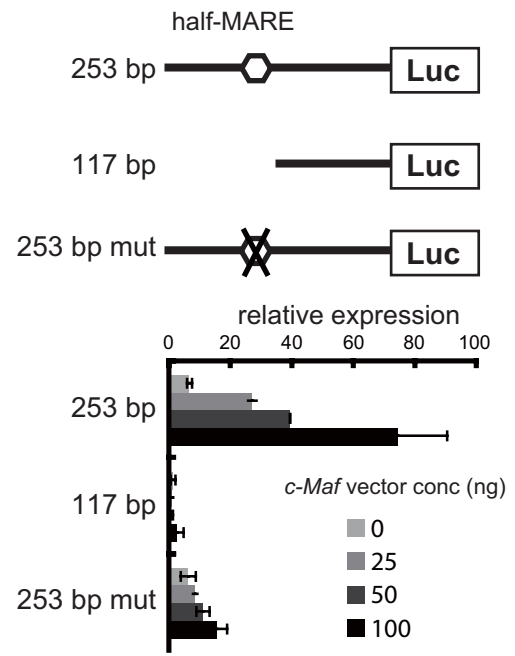


Figure 4

A

		half-MARE <u>TCAGCA+AT rich</u>	C/EBP <u>RTTGCGYAA</u>	
human	-149	---TTGCAGCAAG TCAGCAAAA GGGCTATGATGA--CCAGGCAGCATTGAGCAACTCTG		-94
mouse	-136	TGATGGTGGCAACT TCAGCAGAAA GCTGCTGGGCTCAGTCTGGCTTTGTTGAGCAACCCTG		-76
chimpanzee	-149	---TTGCAGCAACT TCAGCAAAA GGGCTATGATGA--CCAGGCAGCATTGAGCAACTCTG		-94
dog	-152	---TAGCAGCAAT TCAGCAAAA AAGCTGTCTTGC--TCTGGCGGCATTGAGCAATTCCG		-97
dolphin	-152	GAGTGGAGGCAACT TCAGCAAAA GGGCCATGATGC--CTTGGGTGCATTGAGCAATGCTG		-92
elephant	-150	TAGTGAAGGGGACT TCAGCAGAAA GGGCTCTGATTC--TCTGGCATCATTGAGCTACCGTG		-92
		* * * * * * * * * *	* * * * * * * *	

B



C

