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MafT, a new member of the small Maf protein family in zebrafish☆

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Abstract

Small Maf proteins play critical roles on morphogenesis and homeostasis through associating with CNC proteins. To date, three small Maf proteins, MafF, MafG, and MafK, have been reported in vertebrates, which share redundant functions. In this study, we tried to identify and characterize small Maf proteins in zebrafish to elucidate their conservation and diversity in the fish kingdom. We identified homolog genes of MafG and MafK but not MafF in zebrafish, indicating the former two are conserved among vertebrates. In addition, a novel type of small Maf protein MafT was identified. MafT protein bound MARE sequence as a homodimer or heterodimers with zebrafish Nrf2 or p45 Nfe2. Co-overexpression of MafT and Nrf2 synergistically activated MARE-mediated gene expression in zebrafish embryos. These results indicated that MafT is a new member of small Maf proteins and involved in the Nrf2-dependent gene regulation in cellular defense system.

Keywords: Diethylmaleate; Heterodimer; Luciferase assay; Nrf2; p45; Nfe2; Phase 2 induction; Radiation hybrid mapping; Small Maf protein; Transcription; Zebrafish

The induction of phase 2 enzyme genes is an important regulatory response for cytoprotection against electrophilic insults and reactive oxygen species [1]. Expression of phase 2 detoxifying enzymes and antioxidant proteins is strongly induced upon exposure to low levels of electrophiles or oxidative stress. Activation of the cellular defense system by phase 2 inducers renders cells more resistant to the potential challenges of a subsequent stress. This coordinated response is regulated through a *cis*-acting element called the antioxidant responsive element (ARE) or electrophile responsive element (EpRE) within the regulatory region of each gene. Analysis of gene disruption in mice has revealed that the transcription factor Nrf2 plays central roles in the ARE/EpRE-mediated transcriptional induction [2,3].

Nrf2 belongs to the family of Cap'n'collar (CNC)-type basic region-leucine zipper (bZIP) proteins, which includes p45 NF-E2, Nrf1, Nrf3, Bach1, and Bach2 [4]. Activation of Nrf2 is regulated in several steps: nuclear translocation, protein stabilization, and DNA binding [5]. Keap1, a member of the Kelch family of proteins, regulates the former two steps [6,7]. In the absence of phase 2 inducers, Nrf2 associates with Keap1 in the cytoplasm and is rapidly degraded by the ubiquitin–proteasome pathway, but upon the addition of phase 2 inducers, Nrf2 is stabilized, translocates into nuclei, and conducts the activation of target gene transcription [6,8]. Control of DNA binding is also critical for the Nrf2 functions, since Nrf2 cannot bind to the ARE/EpRE as a monomer or a homodimer, but Nrf2 must heterodimerize with one of the small Maf proteins for DNA binding and transactivation [9,10].

Seven members of Maf proteins have been identified in vertebrates, which are divided into the large and small Maf subfamilies [4]. The large Maf proteins include c-Maf [11], MafB [12], Nrl [13], and L-maf/MafA/S-maf

☆ *Abbreviations:* ARE, antioxidant responsive element; bZIP, basic region-leucine zipper; CNC, cap'n'collar; cR, centiRays; CSKN1E, casein kinase I epsilon isoform; DEM, diethylmaleate; EH, extended homology; EMSA, electrophoretic mobility shift assays; EpRE, electrophile responsive element; EST, expressed sequence tags; LFNG, lunatic fringe; LG, linkage group; MARE, Maf recognition element; RT-PCR, reverse transcriptase-polymerase chain reaction.

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[14–16], which all contain an N-terminal acidic domain that serves as a transactivation domain. Rest of the members, MafF, MafG, and MafK, constitute the small Maf protein family that possesses a bZIP motif mediating the DNA binding and dimer formation in common, but lack any recognizable transcriptional effector domains [10,17]. Both large and small Maf proteins commonly recognize a specific palindromic sequence TGCTGA(C/G)TCAGCA, called MARE (Maf recognition element) [18]. Even though the small Maf proteins do not have a transactivation domain, they affect transcription either by forming heterodimers with CNC proteins [9,18–22] or homodimers that can displace active MARE binding factors [18].

MafF, MafG, and MafK were originally identified in chicken, while mammalian homologs of these were later identified in both mouse and human, suggesting that these three paralogs are conserved among vertebrates [21,23–28]. Remarkable similarities in amino acid sequences among the three small Maf proteins have led us to speculation regarding their functional redundancy. Indeed, in vitro analyses revealed that MafF, MafG, and MafK are functionally interchangeable [10,18] and transgenic overexpression of MafK proteins could compensate for the loss of MafG in gene knockout line of mice [29].

On the other hand, null mutant mice of either *mafK* or *mafF* [26,27,30] showed no apparent phenotype, while *mafG* mutant mice displayed both mild neurological and hematological phenotypes [26]. Each small Maf gene is expressed in overlapping but distinct tissue distribution pattern during development [17,26,27], which may be the reason why disruption of the single gene *mafG* showed specific phenotypes. The *mafG::mafK* compound mutants displayed far more severe phenotypes than did mutants with the *mafG* mutation alone, implying a functional redundancy of small Maf proteins and an importance of their gene dosage [31,32]. In support of the Maf dosage hypothesis, transgenic overexpression of MafK in mice severely affected T cell proliferation and function [33], and elevation not only reduction of the small Maf protein abundance caused severe defects in megakaryopoiesis [29].

Recently, we identified both Nrf2 and Keap1 in zebrafish, demonstrating that the Nrf2–Keap1 system also regulates the expression of cytoprotective genes in fish [34]. Although there has been no report for fish small Maf proteins, four members of the large Mafs have been isolated in zebrafish [16,35,36] in addition to another member of zebrafish CNC protein p45 NF-E2 [37]. These information strongly imply that small Maf proteins may also be present in fish. Therefore, it has been of interest to know whether fish has all three small Maf proteins or not. In this study, we tried to isolate small Maf proteins in zebrafish and compare their structures with those of higher vertebrates. We identified cDNAs for four small Maf proteins in zebrafish, one MafK, and two MafG homologs in addition to a novel subtype MafT, which appears to exist specifically in fish. Interestingly, we could not find a MafF homolog in zebrafish, suggesting that both MafK and MafG are conserved among vertebrates, but not MafF is conserved in fish.

Materials and methods

Isolation of cDNAs. cDNA clones encoding zebrafish small Maf proteins were prepared by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from zebrafish day 4 larvae and specific primers. The primers have the following sequences: *mafk*, GGGTCG ACGACAGATTTTTGAAGAGTTCTG and 5′-GGTCTAGAGAGC ATCTCAGATTCAGATTC; *mafg1*, 5′-GGGTCGACGATTCAGAC TGTCATCTTGTG and 5′-GGTCTAGAGATGAACGACCCTGTG CTTG; *mafg2*, 5′-GGGTCGACGTTTTGCAGATCTGCGTGGC and 5′-GGTCTAGACTGCAGCTCTATGGTGCAC; and *maft*, 5′-G GGGATCCATGACTTCAGACGGCAGAG and 5′-GGGTCGACA AGCCTTCCAGCTCACGC. Several independent clones for each small Maf gene were isolated and analyzed to eliminate PCR errors. For *maft*, a cDNA library of 15–19-h stage zebrafish [38] was screened using a partial cDNA fragment as a probe to isolate full-length clone. Expression of small Maf genes was analyzed by RT-PCR as described previously [34] using following primers: *mafk*, 5′-GGGGATCCGCCAT GACGACTCATTTTAAAGC and 5′-GGTCTAGACTACGATTG TGCTGAAAAGG; *mafg1*, 5′-GGGGATCCAGGTGGAGAAGCTC GCCTC and 5′-GGCTCGAGCATTATGACCGTGCTTCTG; *mafg2*, 5′-CATGACGACCACTAATAAAGG and 5′-CTACTAAGACCTG GCGTCG; *maft*, 5′-GGGGATCCATGACTTCAGACGGCAGAG and 5′-GGGTCGACAAGCCTTCCAGCTCACGC; and *ef1α*, 5′-GCC CCTGCCAATGTA and 5′-GGGCTTGCCAGGGAC.

Fish and inducer treatment. Zebrafish embryos were obtained by natural mating. For induction studies, fish were placed in culture dishes or in tanks containing 100 μM diethylmaleate (DEM).

Plasmid construction. The plasmids pCS2mafG1, pCS2HAmafG2, pCS2mafK, pCS2mafT, and pCS2nfe2 were constructed by subcloning cDNAs for the open reading frame regions of zebrafish *mafg1*, *mafg2*, *mafk*, *maft*, and *nfe2* [37], respectively, into pCS2+ vector. In the case of pCS2HAmafG2, cDNA corresponding to HA-tag (YPYDVPDYA) was inserted after initiation ATG sequence. Other plasmids used in this study (pCS2nrf2 and pRBGP2) were described previously [18,34].

Luciferase assay. Luciferase assay was performed as described previously [34] with a slight modification. Briefly, 50 pg of the circular reporter constructs was injected alone or with synthetic capped RNAs for overexpressing transcriptional factors into embryos at the one-cell stage. Embryos were harvested at midgastrula and the luciferase activity in five embryos for each condition was determined. All luciferase activities were analyzed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. Injection efficiencies were routinely normalized to the activity of a *Renilla* luciferase expression plasmid, pRL-TK. At least three independent experiments, each carried out in duplicate, were performed.

Electrophoretic mobility shift assays. Proteins were prepared by in vitro transcription and translation reactions using TNT wheat germ extract (Promega). A single MARE containing oligonucleotide (5′-TCGAGCTCGGAATTGCTGACTCATCATTACTC, identical to #25 nucleotide in Kataoka et al. [10]) was prepared by annealing synthetic oligonucleotides and ^{32}P-labeled using Rediprime II DNA Labelling System (Amersham Biosciences). Proteins were incubated with 2 fmol of ^{32}P-labeled probe at room temperature for 30 min in 20 μl of electrophoretic mobility shift assay (EMSA) binding buffer [20 mM Hepes–HCl (pH 7.6), 60 mM KCl, 4% Ficoll (4×10^5), 1 μg

poly(dI–dC), and 0.66 mM dithiothreitol]. Complexes were resolved by electrophoresis on a 4% polyacrylamide gel.

Radiation hybrid mapping. Radiation hybrid mapping using panel LN54 was performed as described in Hukriede et al. [39] using specific primers for each small Maf gene. Sequences of each primer are following: *mafk*, 5′-TTGACCAAGGAAGACGTGG and 5′-CTGTGATTGGC AGACTTGAC; *mafg1*, 5′- GAGAGCTGAATCAGCACTTG and 5′-CAGCAACTTTGCCTGGTATG; *mafg2*, 5′-CCGAGTCAAGCGCG TAACG and 5′-AGACCTGGCGTCGGTCTTG; *maft*, 5′-AGGTAC AGAAGCTGAAGCAG and 5′-CTTGACTATGGTGATGACGG; and *cskn1e*, 5′- CTCTAGCAGAACAGCTGAGG and 5′- CTCACC AGACTGAGGTACAC.

Results and discussion

Isolation of four zebrafish small Maf cDNAs

We found four candidate genes for zebrafish homologs of small Maf proteins in the zebrafish genomic DNA (http://www.ensembl.org/Danio_rerio/) and expressed sequence tag (EST) databases (http://www.ncbi.nlm.nih.gov/dbEST/index.html). Based on the sequence information, full-length cDNA clones, *mafk*, *mafg1*, *mafg2*, and *maft* (see below for assignment and naming), were isolated by RT-PCR using total RNA from Day 4 larvae or by cDNA library screening. The percentage identity of deduced amino acid sequences between zebrafish and mouse MafK [23] was 82%, and those between zebrafish MafG1 or MafG2 and mouse MafG [26] were 88% and 85%, respectively. These results indicate that both MafK and MafG are highly conserved in the fish. The high similarity between MafG1 and MafG2 (88% identity) also suggests that the *mafg* locus was duplicated in the course of fish evolution. Fig. 1A shows multiple alignments of vertebrate small Maf proteins. From this comparison, four highly conserved regions emerge among vertebrate small Maf proteins. The extended homology (EH) and basic regions that are essential for binding to the MARE sequences [10,13,40] are conserved among the large Maf proteins. On the contrary, the other two conserved amino acid stretches, N-terminal KALKVK and C-terminal SVITIVK, were characteristic for small Maf proteins, although their functions remain to be characterized.

Sequence comparison reveals the presence of new small Maf protein MafT

An important observation was that deduced amino acid sequences of the fourth clone showed relatively low homology to all mouse small Mafs (MafK 58%, MafG 54%, and MafF 57%), suggesting that this small Maf clone may belong to a novel subfamily of small Maf proteins. While we could find a gene encoding highly homologous protein to this new small Maf in the fugu genomic DNA database (83% identity, clone number M000373 in http://fugu.hgmp.mrc.ac.uk/Analysis/), we could not find such gene in mouse, human or other vertebrate databases, implying that this subfamily is specific for teleost. We therefore named this new member of small Maf protein as MafT (small Maf in Teleost). In reverse, we could find any homologous genes to MafF neither in zebrafish nor in fugu databases (data not shown). Thus, mammals and birds have only MafF, while fish has only MafT. These results allow us to speculate that these two small Maf subfamilies might be derived from the common MafF/MafT ancestor. While genetic distance of MafF and MafT was considerably far in the phylogenetic tree (Fig. 1B), it is also possible that these two subfamilies emerged independently.

Genetic mapping of zebrafish small Maf genes

We mapped zebrafish genes for four small Mafs on the linkage map by using LN54 hybrid panel [39]. Table 1 shows the results of gene mapping. Both *mafk* and *mafg1* were mapped to linkage group 3 (LG3), 388.04 and 503.01 centiRays (cR) from the most terminal markers in each linkage group, respectively. These loci are very close to the map positions of *lfng* and *axin2*, zebrafish homologs of human lunatic fringe (*LFNG*) and *AXIN2*. Since human *MAFK* and *MAFG* are localized in close proximity of *LFNG* and *AXIN2* loci as is the case for zebrafish genes, these results argue for the presence of strong synteny between zebrafish and human genes. *mafg2* was mapped to LG11, where synteny seems not to exist with human *MAFG* or other small Maf genes. The result suggests that *mafg2* is a second MafG homolog that emerges from the fish-specific evolution. *maft* is mapped on LG12 192.54 cR from the terminal, where no adjacent gene has been mapped. Importantly, we found a 194 kb length contig of fugu genomic DNA containing puffer fish homolog of MafT gene (M000373) and a homolog for epsilon isoform of human casein kinase I (CSKN1E) gene. Human *CSKN1E* localizes on 22q13.1 where *MAFF* is also localized. We isolated a partial cDNA for the zebrafish CSKN1E homolog (*cskn1e*) based on information of the EST database (DDBJ/EMBL/GenBank Accession No. CF998445) and mapped its position. As expected, *cskn1e* was mapped to the identical position with *maft* under current resolution. These results thus support our contention that MafT and MafF are derived from a common MafF/MafT ancestor, albeit their sequences are diverged significantly during the molecular evolution.

Expression profiles of small Maf mRNAs in zebrafish

It has been shown that mouse *mafF*, *mafG*, and *mafK* exhibited distinct temporal expression profiles in developing embryos [26,27]. They also show different tissue-specific expression patterns in adult mice [27,31]. In addition, the expression of small Mafs in human and

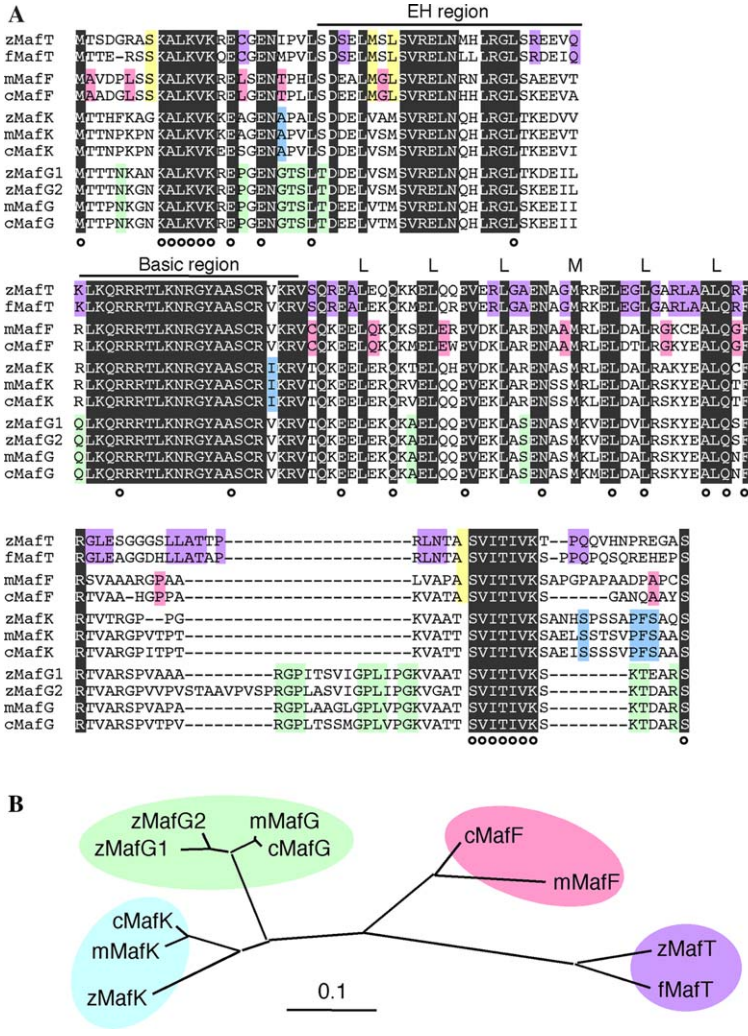

Fig. 1. Comparison of the vertebrate small Maf proteins. (A) Sequence alignment of various small Maf proteins. Conserved amino acids among all small Maf proteins are exhibited by white characters with black background, and those among only MafT, MafF, MafK, and MafG subfamilies are highlighted in purple, pink, blue and green, respectively. Amino acids highlighted in yellow indicate those conserved among MafT and MafF proteins. Open circles denote amino acids conserved among small Maf but not large Maf proteins. Heptad repeats of L or M indicate the leucine zippers. Nucleotide sequence data of *mafg1*, *mafg2*, *mafk*, and *maft* have been deposited in the DDBJ/EMBL/GenBank databases with accession numbers AB167540, AB167541, AB167542, and AB167543, respectively. (B) Phylogenetic tree of small Maf proteins. c, chicken; f, fugu; m, mouse; and z, zebrafish. Scale bar, genetic distance.

Table 1
Genetic mapping and conserved syntenies of small Maf genes

Zebrafish genes	Map positions	Human genes	Map positions
mafk	LG3-388.04 cR	*MAFK*	7p22
lfng	LG3-379.07 cR	*LFNG*	7p22
mafg1	LG3-503.01 cR	*MAFG*	17q25
axin2	LG3-501.1 cR	*AXIN2*	17q23-q24
mafg2	LG11-473.32 cR		
maft	LG12-192.54 cR	*MAFF*	22q13.1
csnk1e	LG12-192.54 cR	*CSNK1E*	22q13.1

Map positions for zebrafish *lfng* and *axin2* were cited from ZFIN Genetic Maps (http://zfin.org/cgi-bin/mapper_select.cgi), and those for human genes from NCBI human genome resources (http://www.ncbi.nlm.nih.gov/mapview/).

chicken was also demonstrated to be tissue-specific [17,21,24,25,28]. The difference in small Maf gene expression profiles led us to speculate the presence of significant variations in the regulatory mechanisms between the known small Maf genes and that of MafT. Thus, it is of interest to analyze the expression profiles of small Maf genes in zebrafish.

We therefore investigated tissue distribution of the small Maf mRNAs in adult fish. Total RNA fractions were prepared from various tissues in 7-month-old zebrafish and analyzed by RT-PCR (Fig. 2A). Amount of cDNA was standardized with the expression level of *ef1α*, a gene encoding a widely expressed translational elongation factor. While *maft* was expressed ubiquitously, its expression was relatively abundant in the

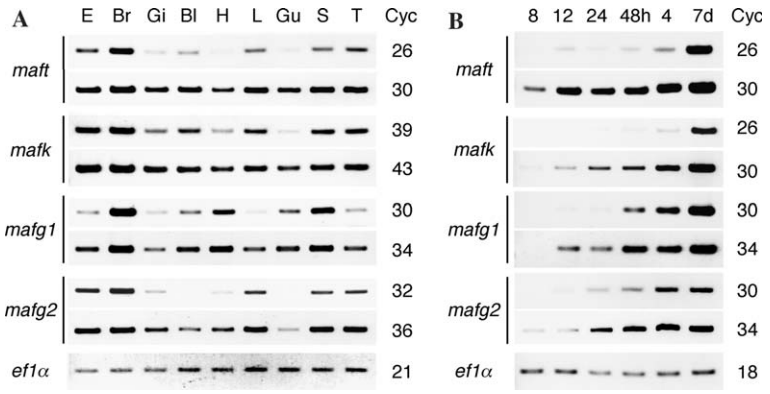

Fig. 2. Expression of small Maf mRNAs in zebrafish. Total RNA isolated from 7-month-old adult tissues (A) or whole body of embryos or larvae at the indicated developmental stages (B) was analyzed by RT-PCR using specific primers for each small Maf gene. Expression of *ef1α* was used to standardize amount of cDNA. The numbers indicate reaction cycles (Cyc) performed in the PCR. E, eye; Br, brain; Gi, gill; Bl, bladder; H, heart; L, liver; Gu, gut; S, spleen; and T, testis.

brain but scarce in the gill, heart, and gut. Expression profiles of *mafk* and *mafg2* were similar to *maft*, except a weak expression in the bladder in the case of *mafg2*. *mafg1* was also expressed ubiquitously, but of relatively high level in the heart and gut, and low level in the liver and testis in comparison with other small Maf genes.

We also examined the expression of zebrafish small Maf genes at embryonic and larval stages. RT-PCR analyses demonstrated that all Maf genes were expressed in every tested stage, but only at low level before hatching (48–72 h) during early embryogenesis (Fig. 2B).

Inducible expression of small Maf genes by DEM

In human cells or mouse tissues, the expression of small Maf genes is induced by the treatment of cells with phase 2 inducers or electrophiles [41,42]. To examine whether these inducers can also activate the expression of zebrafish small Maf genes, we analyzed expression of the small Maf genes in adult gill and whole body of larvae after treatment with DEM, a potent inducer of phase 2 enzyme genes not only in mammals but also in zebrafish [34]. Fig. 3 shows that the expression of *maft* and *mafg1* was induced by the DEM treatment. This result indicates that regulatory mechanisms of small Maf genes are conserved among vertebrate. It is also consistent with the previous report, in that the induction level of each small Maf gene was varied among human cell lines [42]. While we could not find a significant difference in responsiveness to DEM between adult and larvae, *maft* in zebrafish showed highest induction level among small Maf genes. This observation shows very good agreement with that of *MAFF* in human cells [42].

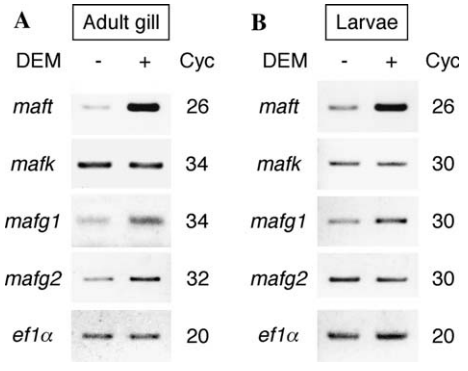

Fig. 3. Induction of small Maf genes after DEM treatment. Total RNA from adult gill (A) or whole body of larvae (B) was prepared after treatment with 100 μM DEM for 6 h and analyzed by RT-PCR using specific primers for small Maf genes.

Activity of MafT protein to heterodimerize with CNC proteins and bind to DNA

We then carried out to assess the ability of MafT and other zebrafish small Maf proteins forming homodimers or heterodimers with the known members of the zebrafish CNC proteins, i.e., Nrf2 and p45 Nfe2 [34,37], exploiting a single MARE containing oligonucleotide [10] as a probe (Fig. 4). Formation of homodimers was observed in the case of MafT and MafK, but it was not obvious for MafG1 nor MafG2 (Fig. 4, lanes 4, 7, 10, and 13, arrow). The weakened activity of MafG1 and MafG2 to form homodimers may be due to insolubility of homotypic full-length proteins under the conditions used for EMSA, since this was also observed when we examined mouse or chicken MafG proteins (data not shown) [10]. When we tested heterodimer formation of zebrafish small Maf proteins with CNC proteins, appearance of slower migrating bands was found upon the addition of Nrf2 to the reaction mixture (Fig. 4, closed arrowheads). These bands correspond to Nrf2-small Maf heterodimers, since specific antibodies for Nrf2 and small Mafs abolished the bands (data not shown). Similarly, an additional band to the homodimer band was observed after combining p45 Nfe2 to the reaction

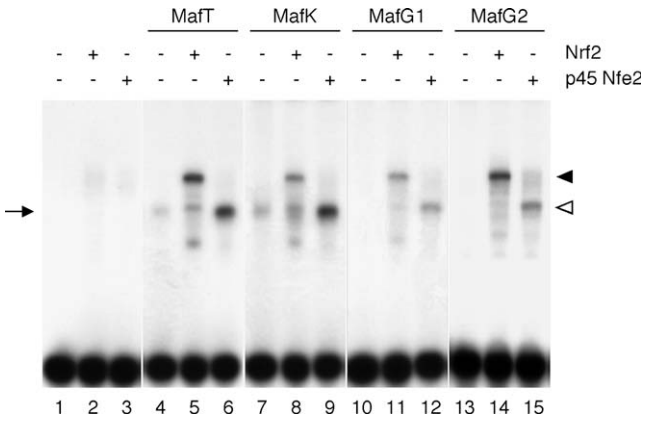

Fig. 4. DNA binding and interaction with CNC proteins of zebrafish small Maf proteins. Autoradiographic image of EMSA with the single MARE containing probe. Binding reactions were carried out with in vitro translated proteins as indicated. Arrow and arrowheads indicate complexes containing small Maf homodimers and small Maf-CNC protein heterodimers, respectively.

mixture (Fig. 4, open arrowheads). We confirmed that this additional band corresponded to the complex containing small Maf-p45 Nfe2 heterodimers by super-shift analyses using FLAG-tagged protein of p45 Nfe2 and antibodies against FLAG and small Maf proteins (data not shown). These results thus indicate that the properties of MafT are similar to those of other small Mafs.

MafT and other small Maf proteins of zebrafish enhance transactivation activity of Nrf2

MafF, MafK, and MafG have been shown to act as transcriptional repressors, when each of them was force expressed in cultured cells [10,18,23]. The repression was brought about by the lack of transactivation domains in the small Maf proteins, so that homodimers of small Maf proteins tend to inhibit binding of CNC transactivators to the MARE sequences. In order to elucidate whether MafT has any hidden transactivation domains, we analyzed transregulation activity of MafT in zebrafish embryos by reporter gene assays. To this end, luciferase gene fused to 3× MARE sequences of chicken β-globin enhancers was used as a reporter (pRBGP2) [18]. After co-injection of the reporter construct with MafT mRNA into zebrafish embryos at the one-cell stage, luciferase activity of the whole cell extract was measured at mid-gastrula. As shown in Fig. 5A, overexpression of MafT shows no increase of the luciferase activity. Similar results were obtained when amount of injecting mRNA was elevated to 50 pg (data not shown). These results thus indicate that MafT does not contain any canonical transactivation domains.

We next examined the effect of MafT on transactivation activity of Nrf2. Overexpression of Nrf2 alone strongly activated the expression of the reporter gene (Fig. 5A), as we previously described [34]. When MafT was co-expressed with Nrf2, the luciferase activity was further activated by nearly 10-fold (Fig. 5B). To elucidate whether this activity was MafT specific, we also examined the effect of MafK, MafG1, and MafG2 on the Nrf2 transactivation activity and found that all these proteins can activate the Nrf2 activity (Fig. 5C).

When amount of co-injecting MafT mRNA was increased to 250 pg, relative activity of luciferase reduced to approximately 1/15 of that in embryos injected with Nrf2 mRNA alone. The high expression level of MafT may suppress MARE-dependent transcription, probably due to forming non-transactive MafT–MafT homodimers and competing with heterodimeric transactivators' binding to MARE. These results strongly support our notion that the balance between small Maf proteins and Nrf2 or other CNC proteins determines the output of transcription [29].

Some previous reports using the culture cell-transfection systems showed that the addition of small Maf proteins provokes only repression of the Nrf2 activity

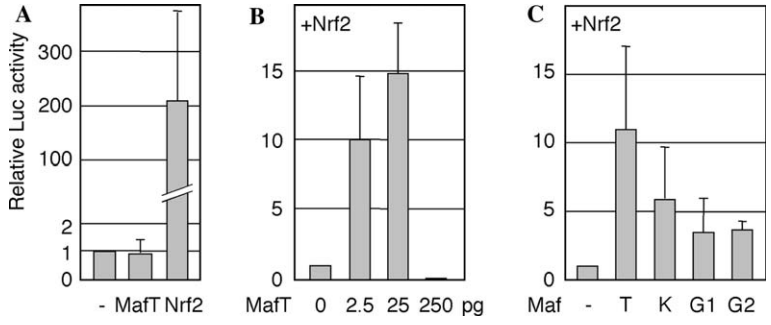

Fig. 5. MafT and other small Maf proteins enhance transactivation activity of Nrf2. (A) Fifty picograms of reporter constructs was co-injected with 12.5 pg MafT or 50 pg Nrf2 mRNAs into embryos at the one-cell stage and luciferase (Luc) activity was analyzed at midgastrula. Luciferase activity in the absence of MafT or Nrf2 (denoted as −) was set at 1. (B) Effect of co-overexpression of MafT on the Nrf2 activity. Indicated amount of MafT mRNA was co-injected with 50 pg Nrf2 mRNA and reporter constructs into zebrafish embryos. Luciferase activity in the embryos overexpressing only Nrf2 was set at 1. (C) Effect of co-overexpression of MafK, MafG1 or MafG2 on the Nrf2 activity. Five picograms each of small Maf mRNA was co-injected with 50 pg Nrf2 mRNA and reporter constructs into zebrafish embryos. The results of more than three independent experiments are shown, each carried out in duplicate. Standard deviation values are shown by bars.

[24,43–45]. In contrast, this study explicitly demonstrates the activation phase of Nrf2 activity by small Maf proteins. One plausible explanation for the difference is to assume the distinct abundance of small Maf and Nrf2 in the nucleus of culture cells and zebrafish embryos. Whereas small Maf proteins translocate quickly into the nucleus [4], Nrf2 localizes in cytoplasm with binding to Keap1 [6,7] and is degraded rapidly by proteasome [8] without stimuli of electrophiles. Thus, the expression level of small Maf proteins in nuclei of conventional culture cells may be relatively abundant compared to Nrf2, so that further addition of small Mafs to the cells does not activate the reporter gene transcription. In contrast, in early zebrafish embryos the expression level of all small Maf proteins is quite low compared with later stages (see Fig. 2B). Therefore, the expression of small Maf proteins effectively supplied partner molecules for Nrf2 and the effect of small Mafs was detectable. The experimental system utilizing the zebrafish embryos thus provides an excellent model system to assess the transregulatory activity of small Maf proteins in vivo. Zebrafish system provides a powerful tool for the analysis of gene regulation, such as external fertilization, transparent embryos, and application of random mutagenesis and screening techniques. New aspects of Maf and CNC proteins may emerge from future zebrafish analyses.

Concluding remarks

In this report, we identified MafT as a new member of small Maf proteins. This study also suggests that small Maf proteins are important intrinsic partners for Nrf2. Among many other questions still unanswered, the following question is intriguing as to why does fish develop its original subtype MafT? Searching for small Maf genes in the fugu genomic DNA database demonstrates that fugu also has MafG and MafK in addition to MafT, but not MafF, suggesting the conservation of MafG and MafK, but not MafF and MafT among the vertebrate (data not shown). As the mouse *mafF* gene is strongly expressed in the lung, a tissue which fish does not have [27], one simple hypothesis is that MafT was specialized for water-living organism. However, expression of zebrafish *maft* in the gill or bladder was relatively weak compared to those of other tissues. Thus, the question remains to be elucidated.

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