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SHORT COMMUNICATION

MTF1 binds to metal-responsive element e within the *ATP7B* promoter and is a strong candidate in regulating the *ATP7B* expression

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Abstract

Wilson's disease is an autosomal recessive disorder resulting from copper excess. Some patients with clinical Wilson's disease symptoms exhibit no or only heterozygous pathogenic variants in the coding region of the disease-causing *ATP7B* gene. Therefore, the *ATP7B* promoter region is of special interest. Metal-responsive elements (MREs) located in the *ATP7B* promoter are promising motifs in modulating the *ATP7B* expression. We studied protein interaction of MREe, MREc, and MREd by electrophoretic mobility shift assays and revealed specific interactions for all MREs. We further narrowed down the specific binding site. Proteins potentially binding to the three MREs were identified by MatInspector analyses. Metal regulatory transcription factor 1 (MTF1) could be validated to bind to MREe by electrophoretic mobility shift assays. *ATP7B* promoter-driven reporter gene expression was significantly increased because of this interaction. MTF1 is a strong candidate in regulating the *ATP7B* expression through MREe binding.

KEYWORDS

metal regulatory transcription factor 1 (MTF1), metal-responsive element (MRE), Wilson's disease

1 | INTRODUCTION

Wilson's disease (WD) is an autosomal recessive disorder caused by alterations in the *ATP7B* gene, which encodes a copper-transporting ATPase. WD mainly presents with liver-specific as well as neurologic and/or psychiatric manifestation (Das & Ray, 2006). In some patients with clinical WD symptoms, no or only heterozygous pathogenic *ATP7B* variants are detectable. Detection rates range from 68.5% to 98% (Hedera, 2017). It has been shown that copper (Cu) is able to increase the *ATP7B* expression in the liver of pigs (Huang et al., 2015), rats (Bauerly, Kelleher, & Lönnerdal, 2005), and seabreams (Minghetti, Leaver, & George, 2010). Notably, the *ATP7B* promoter contains four metal-responsive elements (MREs): MREa, MREe, MREc, and MREd (Oh, Kim, Park, Hahn, & Yoo, 1999) (Figure 1; Supplementary Methods Figure S1). MREs are well-known regulatory motifs in the metallothionein promoter, which interact with transcription

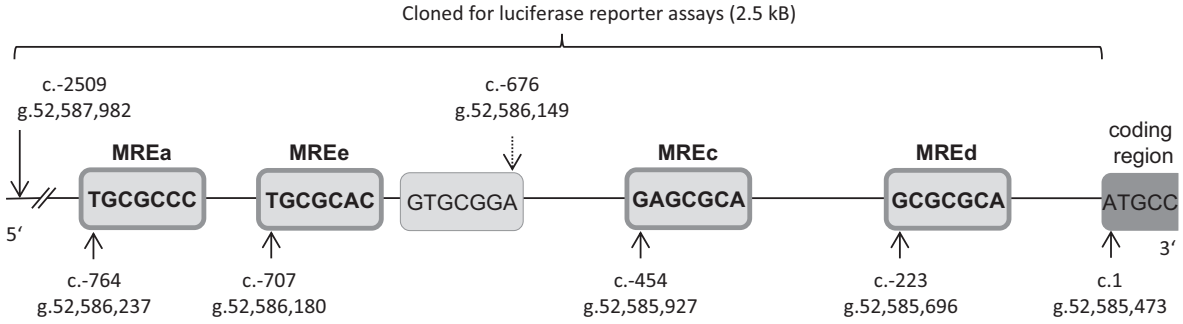

FIGURE 1 Localization of metal-responsive elements (MREs) in the *ATP7B* promoter. Variant g.52,586,149T > C reported by Chen et al. (2018) is marked by a dotted arrow. Positions refer to NC_000013.10 (NM_000053.3), *Homo sapiens*, chr.13, build GRCh37/hg19

factors (TFs) in a metal-dependent manner (Culotta & Hamer, 1989). For the *ATP7B* promoter, only one MRE-binding TF (MRE-TF), the Ku-protein, has been described. Ku has been shown to be necessary for the basal transcription of *ATP7B* by binding to MREa (Oh et al., 2002). Likewise, the remaining MREs are promising candidates in orchestrating *ATP7B* expression. Here, we investigate MREe, c, and d with respect to their role in *cis* regulation of *ATP7B*.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Hepatocellular carcinoma cell lines HepG2 and HLE were cultured in DMEM containing 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin at 37°C and 5% CO_2 in a humidified incubator (details in Supplementary Methods).

2.2 | Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific). 15 µg of nuclear extract or 50 ng of purified MTF1 protein (TP304861, Origene, Herford, Germany) were incubated with 20 fmol double-stranded, biotin-labeled oligonucleotides (details in Supplementary Methods).

2.3 | MatInspector analysis

To determine potential TF for the MREs, MatInspector analysis (Genomatix GmbH, München, Germany) was performed with unmutated EMSA oligonucleotides as input sequences (search for family matches, Matrix Family Library, Version 9.2; selected matrix groups: general core promoter elements and vertebrates; core similarity: 0.7; matrix similarity: optimized).

2.4 | Luciferase reporter assay

Luciferase reporter assays were performed using vectors containing 2.5 kB of the *ATP7B* promoter region with intact or mutated MREe, MREc, or MREd. Expression vectors harboring potential TF (pcDNA3) were cotransfected (details in Supplementary Methods).

3 | RESULTS

MREs are promising candidates for the *cis* regulation of *ATP7B*. We, therefore, analyzed the protein interaction of the three uninvestigated MREs (e, c, and d) by EMSA. We detected a specific protein interaction for all investigated MREs (Figure 2a). We then aimed to narrow down the protein-binding site of the MREs by sequentially preincubating nuclear protein extracts with different mutated unlabeled oligonucleotides before adding labeled wild-type oligonucleotides. A strongly shifted band indicates an important role in protein binding of a mutated nucleotide (Figure 2b–d; full-size EMSA in Supplementary Results Figure S1a–c). Nucleotides that turned out to be significantly involved in protein binding are shown in Figure 2e, marked in green. In summary, protein interactions for MREc and MREd were mainly located directly adjacent to the MRE consensus sequence (TGCRCN, marked with black boxes; Culotta & Hamer, 1989). For MREe, protein interaction was mainly established by the first four MRE consensus nucleotides (TGCR).

In silico analysis can be used to identify TFs for a known protein-binding site. We used MatInspector (Cartharius et al., 2005) to determine possible TFs for MREe, d, and c (Figure 2e and Supplementary Results Figure S1d). For each MRE, we selected one to two potential TFs for experimental validation. For MREe and MREc, we mainly selected TFs that matched the binding sites determined by EMSA (overlapping green and yellow nucleotides). For MREe, we chose MTF1. For MREc, we chose DMTE, which was, in contrast to most of the other

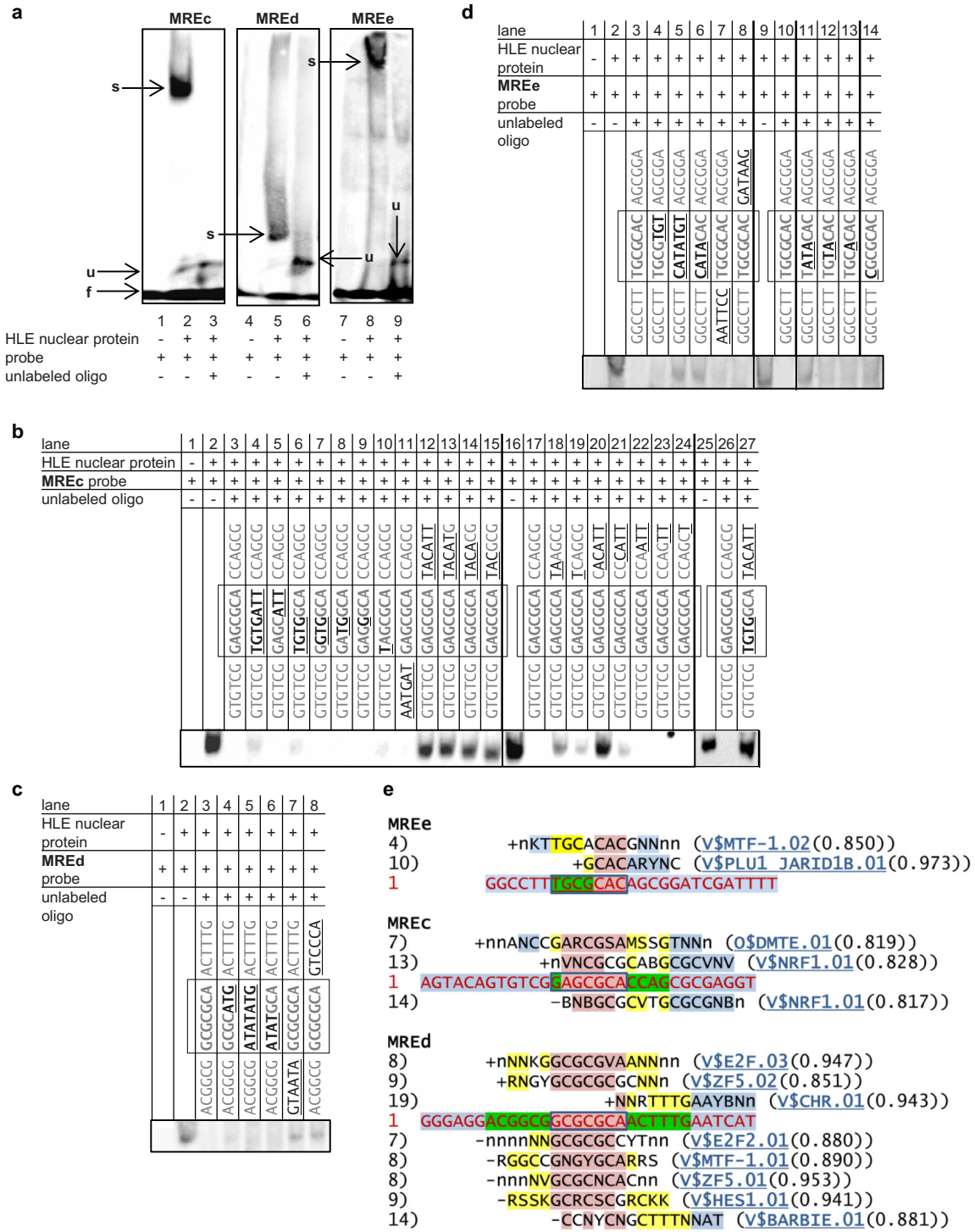

FIGURE 2 Metal-responsive elements (MRE)e, MREc, and MREd within the *ATP7B* promoter interact with proteins at a specific binding site. (a) Electrophoretic mobility shift assay (EMSA) showing protein binding (lanes 2, 5, 8) and binding specificity validation by preincubating nuclear extracts with unlabeled MRE oligonucleotides (lanes 3, 6, 9). (b–d) preincubation of nuclear extracts with mutated unlabeled oligonucleotides to narrow down protein-binding sites. Box: MRE consensus sequence, underlined: inserted mutations. (e) Transcription factors (TFs) predicted to bind MREs within the *ATP7B* promoter by MatInspector algorithm. IUPAC consensus sequences of predicted TF-binding sites (black letters) aligned with input sequence (EMSA oligonucleotides, red letters). Black boxes: MRE consensus sequence. Green: protein-binding sites determined in EMSA. Yellow: matches to determined binding sites. Pink: matches to MRE consensus sequence. Blue: matching bases outside of consensus sequence or determined protein-binding site. Numbers on the *left*: sequence start relative to input sequence. Numbers on the *right*: matrix similarity. TF above input sequence indicate matches on (+)-strand, below on (−)-strand. s, specific shift; u: unspecific shift; f, free probe

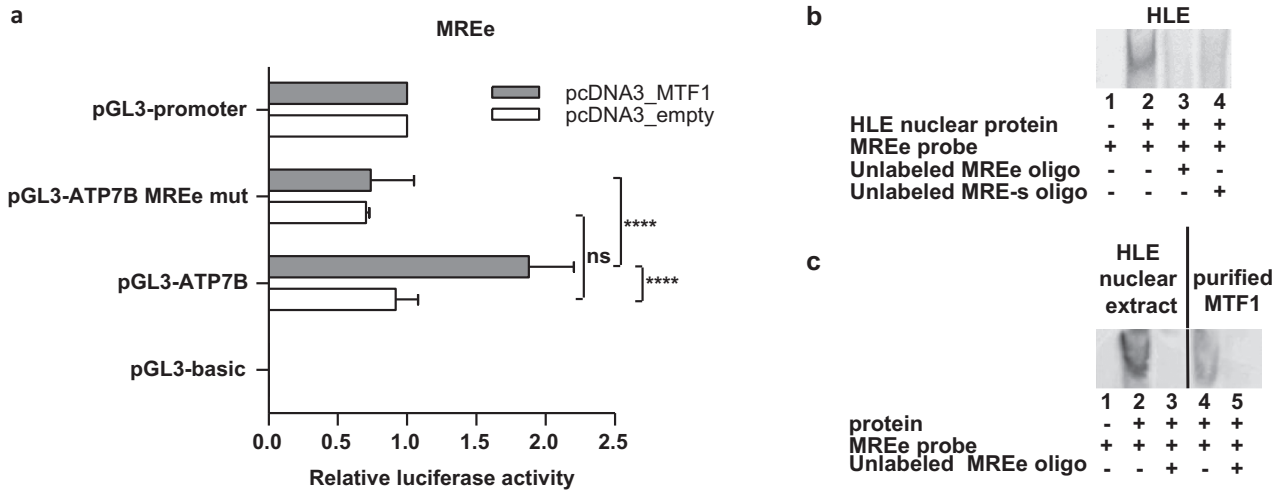

FIGURE 3 MTF1 binds to metal-responsive element e (MREe) within the *ATP7B* promoter and significantly increases *ATP7B* promoter-driven reporter gene expression. (a) 48 hours after HepG2 cotransfection with indicated pGL3 luciferase reporter vectors and pcDNA3 expression vectors firefly luciferase activity was measured and normalized to Renilla luciferase activity. One-way ANOVA/Bonferroni's multiple comparison test; ns, not significant; ****$P \leq 0.0001$. Error bars show standard deviation. Biological replicates $N = 3$. (b–c) Validation of MTF1/MREe binding within the *ATP7B* promoter through electrophoretic mobility shift assay. (b) Lane 4: HLE nuclear extract preincubated with an unlabeled oligonucleotide containing a known MTF1-binding site (MRE-s; Brugnera et al., 1994; Radtke et al., 1993). (c) Purified human MTF1 protein was used (lanes 4+5) instead of HLE nuclear extract (lanes 2+3)

candidates, not a protein but a DNA sequence motif. Because TAF9 and TAF6 have been identified as the subunits connecting to DMTE (Theisen, Lim, & Kadonaga, 2010), we included them for experimental validation. For MREd, whose binding sites determined by EMSA (green) were exclusively located outside the MRE consensus sequence (black boxes), MatInspector matches were mainly located within the MRE consensus sequence (pink). We chose two candidates from these matches, MTF1 and E2F2.

To investigate whether the selected TF candidates interact with the *ATP7B* promoter, we performed luciferase reporter assays. We used vectors with 2.5 kB of the *ATP7B* promoter cloned upstream of the luciferase reporter gene and then compared luciferase signals of the wild-type vector with that of the vector with mutated MRE-binding sites. The assays were performed under basal conditions or under coexpression of MRE-TF candidates. For MREc and MREd, no interaction with the predicted MRE-TFs was detected (Supplementary Results Figure S2). In contrast, MTF1 significantly increased luciferase activity ($P \leq 0.0001$), indicating an interaction with MREe, while mutated MREe reduced luciferase expression to the basal level ($P \leq 0.0001$) (Figure 3a). To validate MTF1-MREe binding, we performed different EMSA (Figure 3b+c; full-size EMSA in Supplementary Results Figure S3). First, we preincubated HLE nuclear protein extracts with unlabeled oligonucleotides containing a known MTF1-binding site (MRE-s; Brugnera et al., 1994; Radtke et al., 1993). Shift disappearing because of preincubation with MRE-s in our EMSA argues for MREe–MTF1 interaction (Figure 3b). We then used purified MTF1 protein instead of nuclear protein extract (Figure 3c), which also shifted the probe. Together, these results demonstrate that MTF1 bound to MREe within the *ATP7B* promoter and significantly increased *ATP7B* promoter-driven reporter gene expression.

4 | DISCUSSION

MTF1 regulates several genes in a metal-dependent manner or through oxidative stress. It is also essential for basal gene expression of some genes (Günther, Lindert, & Schaffner, 2012), The most prominent MTF1-regulated gene is the *metallothionein* gene. The *Drosophila DmATP7*, the human *ATP7B* and *ATP7A* orthologue, is another MTF1-regulated gene, which is involved in Cu homeostasis. This gene is expressed Cu-dependently in the larvae midgut mediated by MTF1 (Burke, Commons, & Camakaris, 2008), suggesting a role of MTF1 in regulating human *ATP7B*.

Chen et al. have recently reported on a patient with clinically diagnosed WD and a homozygous variant (chr13:g.52,586,149T > C; NC_000013.10,hg19) in the *ATP7B* promoter region (Chen et al., 2018). They found the affected site to be bound by MTF1 as well. Our promoter construct also includes this site (Figure 1, Supplementary Methods Figure S1), albeit we did not investigate it further. However, in our luciferase assay with MTF1 coexpression, mutation of MREe alone was already sufficient to reduce luciferase activity to basal level even with the site

g.52,586,149 still intact. Chen et al. have shown that variant g.52,586,149T > C has only led to a reduction of approximately 15%. Possibly, both sites are necessary for binding and activation, and disruption of either will reduce expression. The difference in the extent of reduction might be caused by the complete disruption of all seven nucleotides of our MTF1-binding site, whereas g.52,586,149T > C affects only one nucleotide.

Our current results should be complemented by further investigations. The MTF1-mediated signal increase in our luciferase reporter assay was clear, but modest (Figure 3a). Here, a positive control using an established MTF1-dependent promoter would be helpful to better assess the extent of the effect. Furthermore, the current results should be verified in a more physiological context. Evaluating the effects of the introduction of g.52,586,149T > C and/or mutation in MREe using CRISPR/Cas9 technology on ATP7B expression with and without copper treatment would help to gain more insight into physiological relevance. These investigations would have gone beyond the scope of this article and will be addressed in a subsequent study.

In summary, we show that MTF1 binds to MREe within the *ATP7B* promoter and is a strong candidate in regulating *ATP7B* gene expression. Our results argue for not solely focusing on *ATP7B* coding variants when investigating WD pathomechanisms, but to also include variants in the promoter region, especially the MTF1-binding sites, and mutations in or a deregulation of MTF1 or MREa-binding Ku protein itself.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Study design: BSk, NVN, AS, TI, BSc, E-DP, UB; data collection, analysis, and interpretation: AS, MS, BV, ER, NH, BSk; manuscript preparation: AS; critically reviewed by E-DP, UB, TI, ER, MS, BV, NH, BSc, NVN, and BSk.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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