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Two major branches of anti-cadmium defense in the mouse: MTF-1/metallothioneins and glutathione

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ABSTRACT

Metal-responsive transcription factor 1 (MTF-1) regulates expression of its target genes in response to various stress conditions, notably heavy metal load, via binding to metal response elements (MREs) in the respective enhancer/promoter regions. Furthermore, it serves a vital function in embryonic liver development. However, targeted deletion of *Mtf1* in the liver after birth is no longer lethal. For this study, *Mtf1* conditional knockout mice and control littermates were both mock- or cadmium-treated and liver-specific transcription was analyzed. Besides the well-characterized metallothionein genes, several new MTF-1 target genes with MRE motifs in the promoter region emerged. MTF-1 is required for the basal expression of selenoprotein W, muscle 1 gene (*Sepw1*) that encodes a glutathione-binding and putative antioxidant protein, supporting a role of MTF-1 in the oxidative stress response. Furthermore, MTF-1 mediates the cadmium-induced expression of N-myc downstream regulated gene 1 (*Ndrg1*), which is induced by several stress conditions and is overexpressed in many cancers. MTF-1 is also involved in the cadmium response of cysteine- and glycine-rich protein 1 gene (*Csrp1*), which is implicated in cytoskeletal organization. In contrast, MTF-1 represses the basal expression of *Slc39a10*, a putative zinc transporter. In a pathway independent of MTF-1, cadmium also induced the transcription of genes involved in the synthesis and regeneration of glutathione, a cadmium-binding antioxidant. These data provide strong evidence for two major branches of cellular anti-cadmium defense, one via MTF-1 and its target genes, notably metallothioneins, the other via glutathione, with an apparent overlap in selenoprotein W.

INTRODUCTION

All organisms have evolved mechanisms to cope with a variety of stress situations. One type of stress response is triggered by heavy metals, such as zinc, copper and cadmium (for convenience, the terms zinc, copper and cadmium are also used here to denote Zn^{2+}, Cu^{2+} and Cd^{2+}, respectively). Metallothioneins (MTs), small, cysteine-rich proteins, play an important role in metal homeostasis and detoxification due to their ability to bind different heavy metal ions (1–3). In the mouse, there are four metallothionein genes, designated as *Mt1* to *Mt4*. Basal, as well as heavy metal-induced, expression of *Mt1* and *Mt2* is mediated by metal-responsive transcription factor 1 (MTF-1) (4–7). This zinc finger protein recognizes short *cis*-acting DNA sequences, termed metal response elements (MREs; core consensus sequence TGCRCNC), which are present in the promoters of target genes (8,9). MTF-1 is conserved in evolution, and homologs have been characterized in the mouse (10), humans (11), Drosophila (12–14) and fish (15,16).

The role of MTF-1 has been studied most extensively in the mouse. Besides coping with heavy metal load, MTF-1 can also mediate the induction of *Mt* genes in response to other stress situations, such as oxidative stress (5,17) and hypoxia (18). In addition, it is required for the metalloregulation of *Znt1*, encoding the major plasma membrane-localized zinc efflux transporter (19), the hypoxic/anoxic induction of the gene for placental growth factor (*Plgf*), an angiogenic protein of the vascular endothelial growth factor (VEGF) family (20), and has recently been invoked in tumor development (21,22). Furthermore, MTF-1 has an essential function during embryogenesis: targeted disruption of *Mtf1* results in embryonic lethality around 14 days *post coitum* due to hepatocyte necrosis (23). In contrast, mice with null mutations for the stress-inducible metallothionein genes (*Mt1* and *Mt2*) are viable, though sensitive to cadmium (24,25), indicating that additional important MTF-1 target genes are involved in the lethal phenotype. With the Cre-*loxP* conditional knockout technique, it is possible to circumvent the embryonic lethal phenotype of conventional *Mtf1* knockout mice. Previous experiments with this technique revealed that deletion of

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Mtf1 from the liver after birth is no longer lethal under non-stress conditions (26).

For this study, an inducible, liver-specific *Mtf1* knockout mouse line was generated to perform a search for MTF-1 target genes and cadmium-inducible genes in the adult liver. A number of target gene candidates emerged upon a transcriptome analysis of mock- and cadmium-treated *Mtf1* conditional knockout mice and control littermates and several of these were confirmed by semiquantitative RT–PCR. Besides the stress-inducible metallothionein genes that were already known as target genes of MTF-1, we find that MTF-1 is important for basal liver expression of the gene for selenoprotein W, muscle 1 (*Sepw1*) as well as for cadmium-induced expression of N-myc downstream regulated gene 1 (*Ndrg1*) and the gene encoding cysteine- and glycine-rich protein 1 (*Csrp1*). In addition, MTF-1 appears to repress the expression of solute carrier family 39, member 10 gene (*Slc39a10*), which encodes a putative metal ion transporter. In an MTF-1-independent transcriptome response, several genes involved in glutathione metabolism are induced. Further studies confirmed a dual anti-cadmium defense, one via glutathione and another one via MTF-1 and its target genes, including metallothioneins.

MATERIALS AND METHODS

Generation of *Mtf1* conditional knockout mice and liver-specific deletion

Mtf1 conditional knockout mice were generated in collaboration with Dr Michael Leviten (San Carlos, CA). Two genomic clones containing exons 3 to 6 of *Mtf1* were used to construct a gene targeting vector for homologous recombination (Supplementary Data). A neomycin resistance cassette (PGK-neo) flanked by two *loxP* sites was cloned into the SacI site 5′ of exon 3 of *Mtf1*, the third *loxP* site was cloned into the ScaI site 3′ of exon 4. A thymidine kinase (TK) cassette was inserted in the HpaI site 3′ of exon 6. 129 ES cells were electroporated with the linearized targeting vector, selected in the presence of G418 and FIAU, and screened for correct integration events by PCR and Southern blot analysis. Transient expression of Cre recombinase led to removal of the PGK-neo cassette, and mice carrying the modified $Mtf1^{loxP}$ allele were generated by injection of positive clones into C57Bl/6 blastocysts and subsequent crosses. Homozygous conditional knockout animals ($Mtf1^{loxP/loxP}$) were crossed with the Cre recombinase transgenic line *Mx-cre* (a gift from Prof. Michel Aguet) to obtain an inducible, liver-specific *Mtf1* knockout line. The mice were genotyped by PCR using the following primers (Microsynth): Cre recombinase: 5′-CTATCCAGCAACATTTGGGCCAGC-3′; 5′-CCAGGTTACGGATATAGTTCATGAC-3′, $Mtf1^{loxP}$ or wild-type allele: 5′-CACACCCAGTTTGTGTATGTCTTC-3′; 5′-CAGTCACAAGCAAATTACCAAACACTGCC-3′.

Animal treatment

At 8 weeks of age, male $Mtf1^{loxP/loxP}$ mice harboring the *Mx-cre* transgene ($Mtf1^{loxP/loxP}$ *Mx-cre*, abbr.: $Mtf1^{Mx\text{-}cre}$) and control littermates without transgene ($Mtf1^{loxP/loxP}$, abbr.: $Mtf1^{loxP}$) received four intraperitoneal injections each of 300 μg synthetic double-stranded RNA polyinosinic–polycytidylic acid [pI–pC; Sigma; in a volume of 60 μl phosphate-buffered saline (PBS)] at 3 day intervals. Only in the case of DNA-binding studies with MRE sequences from MTF-1 target gene candidates, the control mice received no pI–pC injections. For experiments with metal treatment, mice received 2 days after the last pI–pC treatment a subcutaneous (s.c.) injection of either 20 μmol/kg body weight $CdSO_4$ (2 mM $CdSO_4$ in H_2O; cadmium treatment) or 10 ml/kg body weight H_2O (mock treatment) 6 h before sacrificing them.

Microarray analysis and data processing

Total RNA was isolated from liver tissue of pI–pC-induced, mock- or cadmium-treated $Mtf1^{Mx\text{-}cre}$ and $Mtf1^{loxP}$ mice ($n = 3$ per genotype and respective treatment; all male) essentially as described by Chomczynski and Sacchi (27).

Gene expression analysis was performed in the Functional Genomics Center Zurich using GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix) according to the manufacturer's instructions and the following reagents and conditions. cDNA was synthesized with SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen), using 15 μg total RNA. *In vitro* transcription was performed with BioArray™ High Yield™ RNA Transcript Labeling Kit (Enzo) and 3.5 to 6 μg of each cDNA. Clean-up of both cDNA and cRNA samples was done using GeneChip® Sample Clean-up Module (Affymetrix). For the automated washing and staining in the Affymetrix fluidics station 450, the protocol EukGE-Ws2v4_450 was used. The probe arrays were scanned with the Affymetrix GS 3000 scanner. Raw data are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress, accession number E-MEXP-438).

Data analysis was performed with GeneSpring 6.1 software (Silicon Genetics), applying a significance level $P \leqslant 0.05$. Furthermore, multiple testing correction was used in addition to obtain cadmium-responsive, MTF-1-independent genes. Genes were considered to be differentially expressed if there was at least a 2-fold difference in expression levels of the compared experimental groups (genotype and respective treatment). The result was considered reliable if signal values for the respective gene were scored 'present' at least for all mice in one experimental group or for two mice in each of two groups.

To screen for the presence of the MRE core consensus sequence TGCRCNC in the promoter region, the upstream sequences of the respective genes were obtained from the University of California Santa Cruz (UCSC) Genome Browser Database (http://genome.ucsc.edu; October/November 2004) (28).

RT–PCR

All RT–PCRs were performed with QIAGEN® OneStep RT–PCR Kit (QIAGEN) according to the manufacturer's instructions, using 150–200 ng DNase I-digested total RNA (RNA isolation see microarray analysis). The reactions were carried out using the following primers: *Csrp1*: 5′-TTCCGATGTGCCAAGTGTGGC-3′; 5′-AGTAGAGAGTGGACATTCAGC-3′, hypoxanthin-guanin-phosphoribosyltransferase (*Hprt*): 5′-GCTGGTGAAAAGGACCTCTCG-3′; 5′-CCACAGGACTAGGACACCTGC-3′, *Mtf1*: 5′-GTGACTTTTGAGACTGTACTGAGTG-3′; 5′-CATGCCAAGAAACAT-

TGAAGGTG-3′, *Ndrg1*: 5′-AGATACACAACAACGTG-GAGG-3′; 5′-TGTGCGAGCGGCTTCGGGGGC-3′, *Sepw1*: 5′-TAGAGGCAGGGTCCTGAAAGC-3′; 5′-ACACCTG--ACACCTGGAAACATGGCTGCC-3′, *Slc39a10*: 5′-GCT-GTGGCTGGTAGTAAAAGC-3′; 5′-GTGGCATGGGATG-TAAACAGC-3′.

S1 nuclease mapping of transcripts (S1 analysis)

S1 analysis was performed as previously described (29), using 100 μg DNase I-digested total RNA (RNA isolation see microarray analysis). The gels were developed using PhosphorImager (Molecular Dynamics). S1 analysis was done with the following ^{32}P-labeled oligonucleotides: *Hprt* S1: 5′-TCTTCAGTCTGATAAAATCTACAGTCATAGGAAT-GGATCTATCACTATTTCTATTCAGTGATTACATTAA-AG-3′, *Sepw1* S1: 5′-TTCAACCGGGAACACCTGGAA-ACATGGCTGCCTGTCTTCTTGAAGTCTTGAGGTGGAA-AGGGAAAGCAAAGCAGGAGGGTTTTCCCACCC-3′.

Electrophoretic mobility shift assay (EMSA)

Protein was extracted from liver tissue with T-PER™ Tissue Protein Extraction Reagent (Pierce) according to the manufacturer's instructions, using a 1:10 ratio of mouse tissue (mg) to T-PER reagent (μl).

EMSA was essentially performed as previously described (10). All binding reactions were carried out by incubating 2–5 fmol ^{32}P-end-labeled double-stranded oligonucleotides with 100 to 130 μg liver protein extract. For competition experiments, 5 pmol of unlabeled competitor oligonucleotide was added to the binding reaction before addition of the extract. All EMSA gels were developed using PhosphorImager (Molecular Dynamics). The following oligonucleotides were annealed and used for the reactions: *Csrp1* MRE1: 5′-GGAAA-CAAAACGGCGCGCACTCCGGCGC-3′; 5′-GGCTGCGC CGGAGTGCGCGCCGTTTTGT-3′, *Csrp1* MRE2: 5′-TGTTGTGGTGCAGTGTGCAAAGCCTAC-3′; 5′-ACCAG-TAGGCTTTGCACACTGCACCAC-3′, *Csrp1* MRE3: 5′-GAGATCGCCATAGGGTGCAAAGAGAAG-3′; 5′-GTGA-CTTCTCTTTGCACCCTATGGCGA-3′, *Csrp1* MRE4: 5′-TGTCTTATTCTGGAGTGCAAGTTAGTC-3′; 5′-AGGG-GACTAACTTGCACTCCAGAATAA-3′, Gal4: 5′-TCCG-GAGGACTGTCCTCCGG-3′; 5′-GCCGGAGGACAGTCC-TCCGG-3′, MREd [MRE derived from mouse *Mt1* promoter (10)]: 5′-CGAGGGAGCTCTGCACTCCGCCCGAAAAG-TG-3′; 5′-TCGACACTTTTCGGGCGGAGTGCAGAGCTC-CCTCGAGCT-3′, MRE-s [synthetic MRE consensus sequence (10)]: 5′-CGAGGGAGCTCTGCACACGGCCCGAAAAG-TG-3′; 5′-TCGACACTTTTCGGGCCGTGTGCAGAGCTC-CCTCGAGCT-3′, *Ndrg1* MRE1: 5′-CAGCCCAGG-CAGGGTGCAGCACGAG-3′; 5′-CCGCCTCGTGCTGCA-CCCTGCCTGG-3′, *Ndrg1* MRE2: 5′-CACACGTTCGCTG-CACACGCCGCGG-3′; 5′-GGGACCGCGGCGTGTGCAG-CGAACG-3′, *Ndrg1* MRE3,4: 5′-GGAGTCCTTATGCACA-CGCGCACGAGCGCGCACGGGCAC-3′; 5′-TGGTGTGC-CCGTGCGCGCTCGTGCGCGTGTGCATAAGGAC-3′, *Sepw1* MRE1: 5′-GAGGCAGTCGGCTGTGCGCACGG-CCCCACGCTC-3′; 5′-CTCTGAGCGTGGGGCCGTGCGC-ACAGCCGACTGC-3′, *Sepw1* MRE2: 5′-ATGGTTTTGGG-GGTGCGCAGGGGGTCTG-3′; 5′-CGACAGACCCCCTG-CGCACCCCCAAAAC-3′, *Slc39a10* MRE1: 5′-GAATAC-ACGACTGGGTGCAGCCGGGGTTTGG-3′; 5′-GGTAC-CAAACCCCGGCTGCACCCAGTCGTGTA-3′, *Slc39a10* MRE2: 5′-GCGGAGAGGAGATGCACACGGCACTCG-3′; 5′-CACTCGAGTGCCGTGTGCATCTCCTCT-3′, Specificity protein 1 (Sp1) binding sequence (10): 5′-CGAGGCCC-CGCCCAG-3′; 5′-TCGACTGGGCGGGGCCTCGAGct-3′.

Cell culture

Primary embryonic fibroblasts were isolated from a 12.5 day old *Mtf1*^{loxP} mouse embryo and grown in DMEM supplemented with 10% fetal bovine serum (ICN), 100 U/ml penicillin–streptomycin (Gibco BRL) and 2 mM L-glutamine (Gibco BRL). 100 mm plates with primary cells were transfected with 10 μg of an expression plasmid coding for simian virus 40 (SV40) large T antigen driven by the cytomegalovirus (CMV) promoter, using lipofectamine™ reagent (Invitrogen) according to the manufacturer's instructions. Cell foci were isolated and the immortalized mouse embryonic fibroblast cell line ckoC was derived from one of them. The *Mtf1*^{loxP} genotype of this line as well as the genomic integration of the T antigen were confirmed by PCR. 100 mm plates with these cells were further transfected by the calcium phosphate method (30) with 19.6 μg of an expression plasmid for Cre recombinase driven by the CMV promoter and 0.4 μg of an expression plasmid for the neomycin resistance gene under the control of the TK promoter. Stably transfected cells were selected in the presence of 0.4 μg/μl G418 (Calbiochem), isolated clones of resistant cells were harvested and grown independently, and the expression of Cre recombinase and excision of exons 3 and 4 of *Mtf1* were analyzed by RT–PCR. The cell lines delC19, delC21 and delC23 with a deletion of *Mtf1* were chosen for further experiments.

Cytotoxicity assay

Samples of 1×10^4 cells/well were plated in 96-well tissue culture plates and allowed to adhere for 24 h. The cells were then pre-incubated for 24 h in medium containing 0, 5, 10, 25 or 50 μM L-buthionine-[*S*,*R*]-sulfoximine (BSO) (Sigma), a drug that inhibits glutathione synthesis (31). Later, cells were exposed to 0, 5, 10 or 20 μM $CdCl_2$ in the specified pre-incubation medium for an additional 24 h. Cytotoxicity was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid)-based Cell Proliferation Kit I (Roche) according to the manufacturer's instructions.

RESULTS

Generation of an inducible, liver-specific *Mtf1* knockout mouse line

Using a homologous recombination strategy, mice were obtained with a modified *Mtf1* allele *Mtf1*^{loxP} where exons 3 and 4, encoding four of the six zinc fingers of the DNA-binding domain, are flanked by *loxP* sites (Figure 1a). Mice homozygous for the *Mtf1*^{loxP} allele were further crossed with animals of the Cre recombinase transgenic line *Mx-cre*. Cre recombinase is expressed in this line under the control of the mouse *Mx1* gene promoter, which is inducible by administration of interferon alpha or beta, or synthetic double-stranded RNA pI–pC (32). Cre-mediated deletion was reported to be

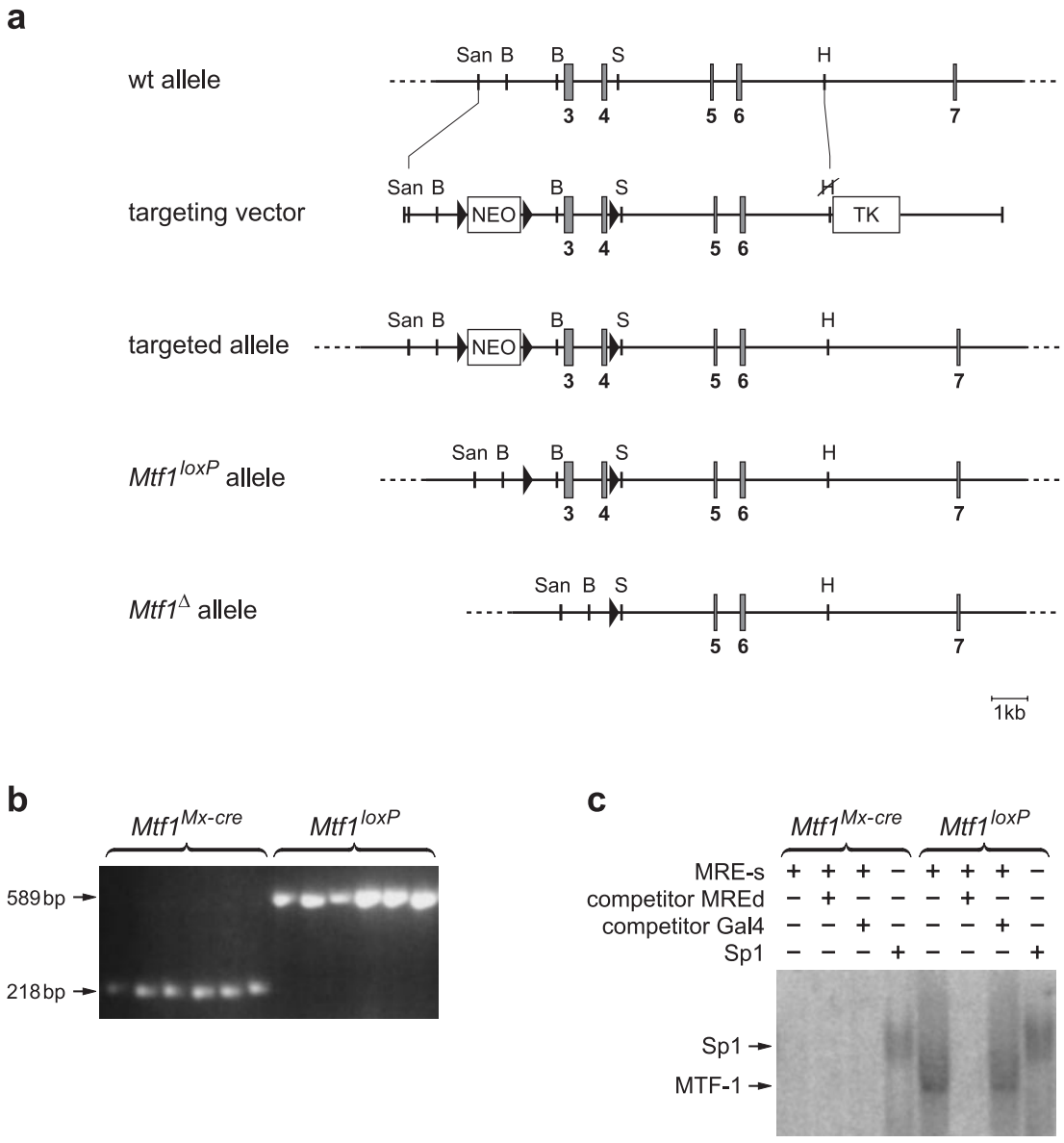

Figure 1. Deletion of *Mtf1* in adult mouse liver. (**a**) Generation of *Mtf1* conditional knockout mice. The targeted allele was obtained by homologous recombination of wild-type (wt) allele and targeting vector in ES cells. Removal of the neomycin cassette (NEO) by Cre recombinase led to the conditional knockout allele $Mtf1^{loxP}$. Conditional Cre-mediated deletion of exons 3 and 4 ($Mtf1^{\Delta}$) results in loss of function via loss of an essential part of the DNA-binding domain and the generation of a new stop codon right after exon 2. Exons 3 to 7 of *Mtf1* are indicated by grey boxes, *loxP* sites by black triangles. TK, thymidine kinase cassette. Restriction enzymes: San, SanDI; B, BbvCI; S, SrfI; H, HpaI. The HpaI site indicated by the crossed H was lost during the cloning procedure for the targeting vector. (**b**) RT–PCRs with total liver RNA from pI–pC-induced male $Mtf1^{Mx\text{-}cre}$ or $Mtf1^{loxP}$ mice. The used primer pair results in products of 589 bp and 218 bp with full-length mRNA and mRNA without exons 3 and 4, respectively. (**c**) EMSA with liver protein extract of a pI–pC-induced male $Mtf1^{Mx\text{-}cre}$ or $Mtf1^{loxP}$ mouse. MTF-1 protein–DNA complex formation was tested with ^{32}P-labeled MRE consensus oligonucleotide MRE-s. Specificity of binding was verified with excess of unlabeled competitor MREd or unrelated Gal4 oligonucleotide; Sp1 bandshifts with ^{32}P-labeled Sp1 consensus oligonucleotide were included as a loading control.

complete in the liver, while varying in other tissues, ranging from 94% in spleen to 8% in brain (32). After Cre-mediated deletion of exons 3 and 4, which results in a frameshift and premature translation stop, no functional MTF-1 protein can be produced.

For induction of Cre recombinase, *Mtf1* conditional knockout mice harboring the *Mx-cre* transgene ($Mtf1^{Mx\text{-}cre}$) received four intraperitoneal pI–pC injections at 3 day intervals (pI–pC induction); control littermates without transgene ($Mtf1^{loxP}$) received similar injections. Using RT–PCRs (Figure 1b), a shortened product was obtained with RNA from $Mtf1^{Mx\text{-}cre}$ livers, indicating a successful excision of exons 3 and 4 of *Mtf1* in these animals. On close examination, a very faint band similar in size to full-length signal was also observed in those mice, probably due to a low amount of residual full-length *Mtf1* mRNA. The level of functional MTF-1 protein was examined by EMSA (Figure 1c): MTF-1 protein–DNA complex was detectable with liver protein extract from an $Mtf1^{loxP}$ control mouse, but no functional MRE-binding protein was observed with an $Mtf1^{Mx\text{-}cre}$ sample. Thus, deletion of exons 3 and 4 of *Mtf1* in the liver of $Mtf1^{Mx\text{-}cre}$ mice was virtually complete. All examined liver-specific knockout

mice were viable under laboratory conditions and appeared normal.

MTF-1 target gene search

For the identification of MTF-1 target genes, we compared the liver transcript profiles of mice with and without functional *Mtf1* gene that had been mock-treated or exposed to cadmium ($n = 3$ per genotype and respective treatment).

In a first screen, the transcripts were analyzed with a differential display-based method, called amplification of double-stranded cDNA end restriction fragments (ADDER) (33). Thereby an overwhelming number of signals was obtained for the two stress-inducible metallothioneins (*Mt1* and *Mt2*), due to the abundance of their transcripts both in mock-treated and especially in cadmium-treated livers that harbored a functional MTF-1 gene (data not shown). This result confirmed the importance of MTF-1 for both basal and metal-induced expression of metallothionein genes.

In a second approach, the gene expression profile in livers of the above mentioned mice was compared by Affymetrix GeneChip® Mouse Genome 430 2.0 Arrays (Table 1). When analyzing the probe array data of livers from mock-treated $Mtf1^{Mx\text{-}cre}$ and $Mtf1^{loxP}$ mice, an at least 2-fold, reliable downregulation of expression was detected in $Mtf1^{Mx\text{-}cre}$ livers for 13 Affymetrix GeneChip® probe sets corresponding to 11 characterized genes (Table 1, a). Seven of these genes contain one or more MRE core consensus sequence TGCRCNC within a segment of 1000 bp upstream of the transcription start. For 26 probe sets corresponding to 24 different characterized genes, a 2-fold or higher, reliable upregulation was detected in $Mtf1^{Mx\text{-}cre}$ livers (Table 1, b); 17 of these 24 genes contain MRE core consensus sequences in the upstream region. The data set for livers of cadmium-treated $Mtf1^{Mx\text{-}cre}$ and $Mtf1^{loxP}$ mice revealed an at least 2-fold, reliable downregulation in $Mtf1^{Mx\text{-}cre}$ livers for 21 probe sets corresponding to 16 different characterized genes (Table 1, c); 10 of these contain MRE core consensus sequences in their upstream region. For 9 probe sets corresponding to 9 different characterized genes, an at least 2-fold, reliable upregulation was detected (Table 1, d); five of them contain MRE motifs. In addition to characterized genes, ESTs and RIKEN cDNA sequences were also found in the comparison of $Mtf1^{Mx\text{-}cre}$ and $Mtf1^{loxP}$ livers to be differentially expressed (Supplementary Table 1). Downregulation of *Mt1* and *Mt2* was detected in $Mtf1^{Mx\text{-}cre}$ livers for both conditions (though the level of significance for the downregulation of *Mt1* in mock-treated animals was above 0.05; data not shown).

For all MTF-1 target genes characterized so far, such as *Mt1*, *Mt2* and *Znt1*, MTF-1 exerts its transcriptional activation activity via standard MRE sequences located proximal to the transcription start (4,5,8,18,19). Even a specific search for MTF-1 binding sites by selection from a pool of double-stranded oligonucleotides with random sequences yielded no new binding motif for MTF-1 in addition to the known MREs (34). Thus, an MRE sequence is to date the only indication for a direct MTF-1 target gene, and four MRE-containing target gene candidates were further analyzed.

Basal expression of *Sepw1* depends on MTF-1

Sepw1 was found in microarray analysis to be significantly downregulated in livers from cadmium- and mock-treated $Mtf1^{Mx\text{-}cre}$ mice (Table 1, a and c). SEPW1 is a selenocysteine-containing protein that binds glutathione (35) and is thought to act as an antioxidant *in vivo* (36).

Sepw1 expression in livers of pI–pC-induced, mock- or cadmium-treated $Mtf1^{Mx\text{-}cre}$ and $Mtf1^{loxP}$ mice was further analyzed by semiquantitative RT–PCRs and S1 analysis (Figure 2a and b). In accordance with microarray data a slight, if any, upregulation of *Sepw1* transcription was observed in livers from $Mtf1^{loxP}$ mice upon cadmium treatment. The basal level was reduced in livers from mock- and cadmium-treated $Mtf1^{Mx\text{-}cre}$ mice, indicating that MTF-1 is important for the basal expression of *Sepw1*.

Three MRE core consensus sequences were found in the region upstream of the mouse *Sepw1* transcription start (Figure 2c). Two of them in opposite orientation overlap almost completely proximal to the transcription start (MRE1, −40 bp), the third one is located further upstream (MRE2, −527 bp). Specific binding of MTF-1 to *Sepw1* MRE1 but not MRE2 oligonucleotide was observed in EMSA with liver protein extract from an $Mtf1^{loxP}$ control mouse (Figure 2d). As a control, no binding to MRE1 was detected with extract from a pI–pC-induced $Mtf1^{Mx\text{-}cre}$ mouse, confirming that the bandshift was indeed dependent on the presence of MTF-1.

Cadmium response of *Ndrg1* depends on MTF-1

Ndrg1 was significantly downregulated in microarrays of liver transcripts from cadmium-treated $Mtf1^{Mx\text{-}cre}$ mice compared to similarly treated $Mtf1^{loxP}$ control mice (Table 1, c). *Ndrg1* probably has some role in stress response since various stimuli, including hypoxia and nickel compounds, activate expression of rodent *Ndrg1* and/or its human ortholog (37–40).

The *Ndrg1* microarray results were confirmed with semiquantitative RT–PCRs (Figure 3a): for $Mtf1^{loxP}$ control livers, a clear increase of *Ndrg1* expression was observed after cadmium exposure; in livers from $Mtf1^{Mx\text{-}cre}$ mice, this cadmium response was not detectable, while basal expression was similar to controls. This indicates that cadmium-induced expression of *Ndrg1* depends on MTF-1.

Five MRE core consensus sequences are located upstream of the mouse *Ndrg1* transcription start (Figure 3b). Four of them are clustered (MRE1 to MRE4, −138 to −332 bp), the fifth one is located farther upstream (MRE5, −883 bp). EMSA was performed to test whether MTF-1 is interacting with some or all of the four proximal MRE sequences (Figure 3c). Separate oligonucleotides were tested for MRE1 and MRE2, whereas one oligonucleotide spanning both sequences was used for MRE3 and MRE4 (MRE3,4). No complex was seen with MRE1, but specific MTF-1 complexes were observed for both the MRE2 and MRE3,4 oligonucleotides with liver protein extract from an $Mtf1^{loxP}$ mouse. As expected, no bandshift was observed with protein extract from a mouse lacking MTF-1 ($Mtf1^{Mx\text{-}cre}$).

Cadmium response of *Csrp1* depends on MTF-1

Csrp1 was found in microarray analyses to be significantly downregulated in cadmium-treated $Mtf1^{Mx\text{-}cre}$ mice compared to $Mtf1^{loxP}$ mice (Table 1, c). CSRP1 is a member of the evolutionary conserved CRP family of proteins that have

Table 1. Comparison of liver gene expression for pI–pC-induced *Mtf1*$^{Mx\text{-}cre}$ and *Mtf1*^{loxP} mice (up- or downregulation at least 2-fold, $P \leqslant 0.05$)

Gene symbol	Gene title	Relative activity *Mtf1*^{loxP} −Cd	*Mtf1*^{loxP} +Cd	*Mtf1*$^{Mx\text{-}cre}$ −Cd	*Mtf1*$^{Mx\text{-}cre}$ +Cd	MRE-s (−1000 bp)	*P*-value
(a) Genes downregulated in mock-treated *Mtf1*$^{Mx\text{-}cre}$ mice							
Mt2	Metallothionein 2	1	3.816	0.135	0.381	6	0.045
Myom2	Myomesin 2	1	0.845	0.219	0.844	0	0.044
Sepw1[b]	Selenoprotein W, muscle 1	1	1.404	0.401	0.418	3	0.020
Ppab2b	Phosphatidic acid phosphatase type 2B	1	0.947	0.408	0.430	0	0.048
Cyp39a1	Cytochrome P450, family 39, subfamily a, polypeptide 1	1	0.501	0.415	0.368	1	0.050
Sult5a1	Sulfotransferase family 5A, member 1	1	0.432	0.432	0.754	0	0.015
Slc25a25	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25	1	0.348	0.465	0.350	1	0.003
Pdxk	Pyridoxal (pyridoxine, vitamin B6) kinase	1	1.226	0.474	0.878	1	0.039
Usp24[b]	Ubiquitin specific protease 24	1	0.885	0.487	0.537	0[a]	0.011
Ttc4	Tetratricopeptide repeat domain 4	1	1.053	0.498	0.442	1	0.004
Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit	1	1.433	0.498	0.846	1	0.043
(b) Genes upregulated in mock-treated *Mtf1*$^{Mx\text{-}cre}$ mice							
Slc39a10[b]	Solute carrier family 39 (zinc transporter), member 10	1	0.563	6.383	4.225	1	0.017
Gzmb	Granzyme B	1	1.987	4.294	2.975	0	0.040
Cyp2b9	Cytochrome P450, family 2, subfamily b, polypeptide 9	1	1.510	3.110	3.518	1	0.035
Ier3	Immediate early response 3	1	5.211	2.999	5.231	1	0.048
Gzma	Granzyme A	1	1.856	2.856	2.531	2	0.035
Timp1	Tissue inhibitor of metalloproteinase 1	1	1.862	2.609	2.471	1	0.016
Fmn2	Formin 2	1	2.478	2.473	1.306	3	0.036
Pkm2	Pyruvate kinase, muscle	1	1.852	2.459	2.540	0	0.039
Arrdc3	Arrestin domain containing 3	1	2.014	2.351	2.201	1[a]	0.014
Ncald	Neurocalcin delta	1	1.613	2.308	1.863	1	0.002
Rap2b	RAP2B, member of RAS oncogene family	1	2.074	2.297	2.183	2	0.015
Mcm2	Minichromosome maintenance deficient 2 mitotin (*S.cerevisiae*)	1	1.133	2.221	1.508	1	0.003
Pla2g4a	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	1	1.343	2.218	1.951	3	0.024
Armcx2	Armadillo repeat containing, X-linked 2	1	1.403	2.176	2.131	0	0.015
Dck	Deoxycytidine kinase	1	1.458	2.158	1.735	2	<0.001
Ncf4	Neutrophil cytosolic factor 4	1	1.665	2.144	2.279	0	0.034
Mcm5	Minichromosome maintenance deficient 5 cdc46 (*S.cerevisiae*)	1	1.151	2.113	1.992	1	0.044
Serpinh1	Serine (or cysteine) proteinase inhibitor, clade H, member 1	1	1.472	2.096	2.108	1	0.042
Cyb561	Cytochrome b-561	1	2.549	2.095	1.536	1	0.023
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	1	1.982	2.087	2.661	1	0.017
Fcgr1	Fc receptor, IgG, high affinity I	1	1.343	2.086	1.374	0	0.035
Cotl1	Coactosin-like protein	1	1.184	2.080	1.554	0	0.045
Fgl2[b]	Fibrinogen-like protein 2	1	1.270	2.031	1.487	1	0.028
Il7	Interleukin 7	1	2.387	2.019	2.354	0	0.041
(c) Genes downregulated in cadmium-treated *Mtf1*$^{Mx\text{-}cre}$ mice							
Mt2	Metallothionein 2	1	3.816	0.135	0.381	6	0.009
Rxrg	Retinoid X receptor gamma	1	3.953	0.789	0.665	0	0.036
Mt1	Metallothionein 1	1	2.105	0.111	0.405	5	0.042
Ndrg1[c]	N-myc downstream regulated gene 1	1	4.456	1.208	1.297	5	0.021
Sepw1[b]	Selenoprotein W, muscle 1	1	1.404	0.401	0.418	3	0.007
Pdgfa	Platelet derived growth factor, alpha	1	3.219	1.540	0.983	1	0.041
Ctse	Cathepsin E	1	1.274	0.769	0.402	0	0.050
Rab7l1	RAB7, member RAS oncogene family-like 1	1	2.566	0.895	0.866	0	0.003
Csrp1[b]	Cysteine and glycine-rich protein 1	1	3.116	1.016	1.186	3	0.014
Paxip1	PAX interacting (with transcription-activation domain) protein 1	1	1.995	0.926	0.763	5	0.017
Ttc4	Tetratricopeptide repeat domain 4	1	1.053	0.498	0.442	1	0.012
Hipr1	Huntingtin interacting protein 1 related	1	1.399	0.953	0.623	3	0.020
Ppap2b	Phosphatidic acid phosphatase type 2B	1	0.947	0.408	0.430	0	0.045
H2afv	H2A histone family, member V	1	1.207	0.565	0.555	1	0.041
Gsto1	Glutathione-S-transferase omega 1	1	1.321	0.507	0.626	0	0.002
Mpra	Membrane progestin receptor alpha	1	1.066	0.862	0.515	0	0.025
(d) Genes upregulated in cadmium-treated *Mtf1*$^{Mx\text{-}cre}$ mice							
Slc39a10	Solute carrier family 39 (zinc transporter), member 10	1	0.666	6.803	3.860	1	0.029
Ubc	Ubiquitin C	1	0.792	1.367	2.065	0	0.006
Plk3	Polo-like kinase 3 (Drosophila)	1	0.819	0.741	2.010	0	0.027
Smad7	MAD homolog 7 (Drosophila)	1	0.630	1.011	1.451	1	0.041
Zfp617	Zinc finger protein 617	1	0.834	1.043	1.827	2	0.031
Zfp535	Zinc finger protein 535	1	0.571	1.045	1.218	3	0.026
Pparg	Peroxisome proliferator activated receptor gamma	1	1.608	1.269	3.295	0	0.005
Idb3	Inhibitor of DNA-binding 3	1	1.470	1.451	2.961	4	0.015
Lpl	Lipoprotein lipase	1	1.377	1.643	2.760	0	0.042

The animals had obtained either mock s.c. injections (−Cd) or s.c. injections with 20 μmol/kg body weight $CdSO_4$ (+Cd) 6 h before sacrificing them. The expression values for each gene are given as mean value of three animals per group, normalized to the mean value of group *Mtf1*^{loxP} −Cd (relative activity). Grey shading indicates the two groups of animals compared, relative activities for the other two groups are shown for a complete overview. The number of MRE core consensus sequences TGCRCNC in a region of 1000 bp upstream from the annotated transcription start is indicated.

[a]Only incomplete region up to −1000 bp from transcription start is available in database.

[b]Mean values of two independent Affymetrix probe sets.

[c]Mean value of four independent Affymetrix probe sets.

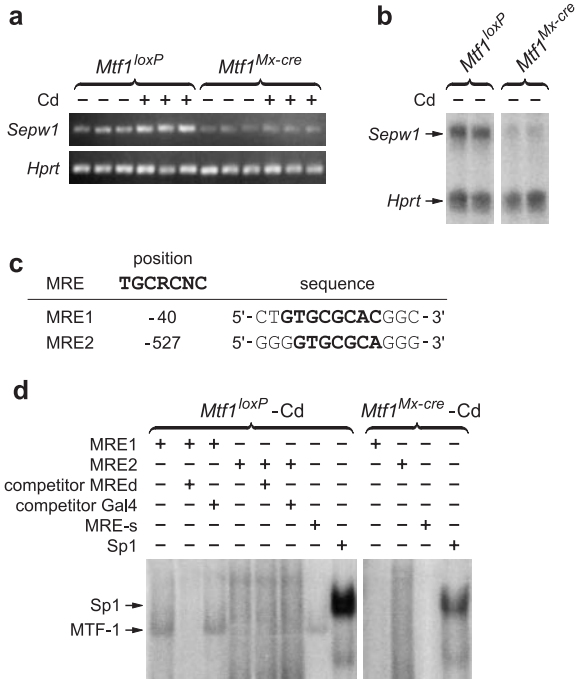

Figure 2. *Sepw1* basal expression depends on MTF-1. (**a**) Semiquantitative RT–PCRs for *Sepw1* mRNA using total liver RNA from pI–pC-induced male $Mtf1^{Mx\text{-}cre}$ or $Mtf1^{loxP}$ mice. The animals had obtained either mock s.c. injections (−Cd) or s.c. injections with 20 µmol/kg body weight $CdSO_4$ (+Cd) 6 h before sacrificing them. RT–PCRs for *Hprt* mRNA were used as internal control to adjust the amount of total RNA used. (**b**) S1 analysis for *Sepw1* mRNA with RNA described in (a), using a ^{32}P-labeled *Sepw1* S1 probe. A ^{32}P-labeled S1 probe for *Hprt* mRNA was used to adjust the amount of RNA used. (**c**) MRE core consensus sequences TGCRCNC (bold letters) and flanking sequences found in a region of 1000 bp upstream from *Sepw1* transcription start; the position of each core sequence is indicated. (**d**) EMSA with liver protein extracts of a male $Mtf1^{loxP}$ or a pI–pC-induced, male $Mtf1^{Mx\text{-}cre}$ mouse, both mock-treated. MTF-1 protein–DNA complex formation was tested with ^{32}P-labeled *Sepw1* MRE1 or MRE2 oligonucleotide, respectively. Specificity of binding was verified with excess of unlabeled competitor MREd or unrelated Gal4 oligonucleotide. ^{32}P-labeled MRE-s was included to indicate the position of an MTF-1-DNA complex; bandshifts for the common transcription factor Sp1 with ^{32}P-labeled Sp1 consensus oligonucleotide were obtained as protein loading control.

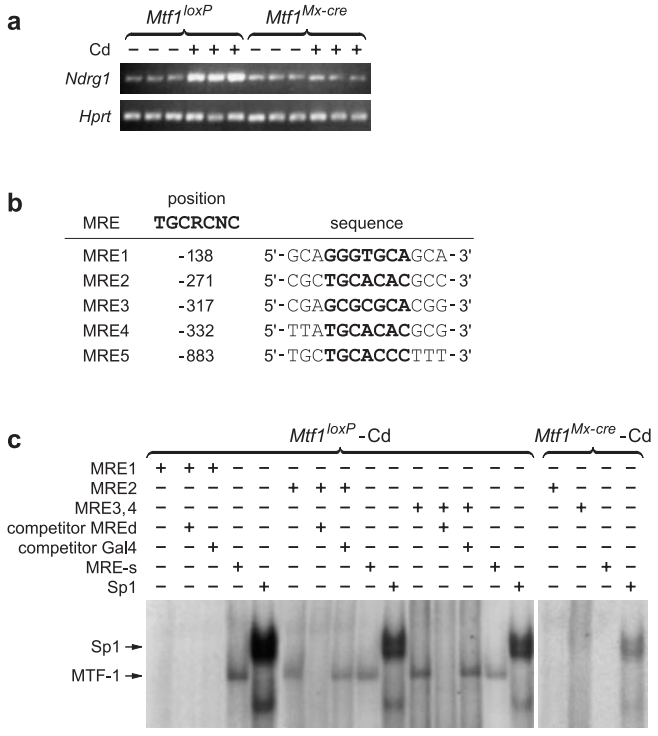

Figure 3. Cadmium response of *Ndrg1* depends on MTF-1. (**a**) Semiquantitative RT–PCRs for *Ndrg1* mRNA using total liver RNA from pI–pC-induced male $Mtf1^{Mx\text{-}cre}$ or $Mtf1^{loxP}$ mice. The animals had obtained either mock s.c. injections (−Cd) or s.c. injections with 20 µmol/kg body weight $CdSO_4$ (+Cd) 6 h before sacrificing them. RT–PCRs for *Hprt* mRNA were used as internal control to adjust the amount of total RNA used. (**b**) MRE core consensus sequences TGCRCNC (bold letters) and flanking sequences found in a region of 1000 bp upstream from *Ndrg1* transcription start; the position of each core sequence is indicated. (**c**) EMSA with liver protein extracts of a male $Mtf1^{loxP}$ or a pI–pC-induced, male $Mtf1^{Mx\text{-}cre}$ mouse, both mock-treated. MTF-1 protein–DNA complex formation was tested with ^{32}P-labeled *Ndrg1* MRE1 or MRE2 oligonucleotide, or a ^{32}P-labeled oligonucleotide including both MRE3 and MRE4 (MRE3,4). Specificity of binding was verified with excess of unlabeled competitor MREd or unrelated Gal4 oligonucleotide. ^{32}P-labeled MRE-s was included to indicate the position of an MTF-1-DNA complex; Sp1 bandshifts with ^{32}P-labeled Sp1 consensus oligonucleotide were obtained as protein loading control.

been implicated in myogenesis and cytoskeletal remodeling (41,42).

Semiquantitative RT–PCRs confirmed the microarray results, namely, that *Csrp1* expression is elevated in $Mtf1^{loxP}$ livers upon cadmium exposure (Figure 4a). In contrast, no cadmium response was detectable in livers from $Mtf1^{Mx\text{-}cre}$ mice, suggesting that MTF-1 is required for cadmium induction of *Csrp1*.

Three MRE core consensus sequences were found upstream of the *Csrp1* transcription start (MRE2 to MRE4, −56 to −366 bp), one was found immediately downstream (MRE1, +7 bp; Figure 4b). Specific binding of MTF-1 was observed with EMSA for MRE2 oligonucleotide and protein extract from an $Mtf1^{loxP}$ liver, but not an $Mtf1^{Mx\text{-}cre}$ liver extract lacking MTF-1, confirming the participation of MTF-1 in the complex (Figure 4c).

MTF-1 inhibits expression of *Slc39a10*

Slc39a10 was detected in microarray analysis to be significantly upregulated in livers from both mock- and cadmium-treated $Mtf1^{Mx\text{-}cre}$ mice compared to control animals (Table 1, b and d). SLC39 proteins are members of the Zrt- and Irt-like protein (ZIP) family of metal ion transporters that transport, with no known exception, metal ion substrates across cellular membranes into the cytoplasm (43,44).

In accordance with microarray data, semiquantitative RT–PCRs showed a downregulation of *Slc39a10* expression in livers of $Mtf1^{loxP}$ mice upon cadmium exposure. In samples from $Mtf1^{Mx\text{-}cre}$ mice, the basal expression was significantly increased; cadmium treatment still resulted in a decrease of *Slc39a10* expression (Figure 5a). It cannot be judged by this experiment whether the degree of cadmium-induced reduction of *Slc39a10* transcription was identical for $Mtf1^{Mx\text{-}cre}$ and $Mtf1^{loxP}$ mice or lower in the absence of MTF-1. In microarray analysis, the degree of the downregulation was either comparable to the one in control livers or lower, depending on the considered Affymetrix GeneChip® probe set (data not shown). The results indicate that MTF-1 is involved in repression of the basal expression of *Slc39a10*. It might also participate in

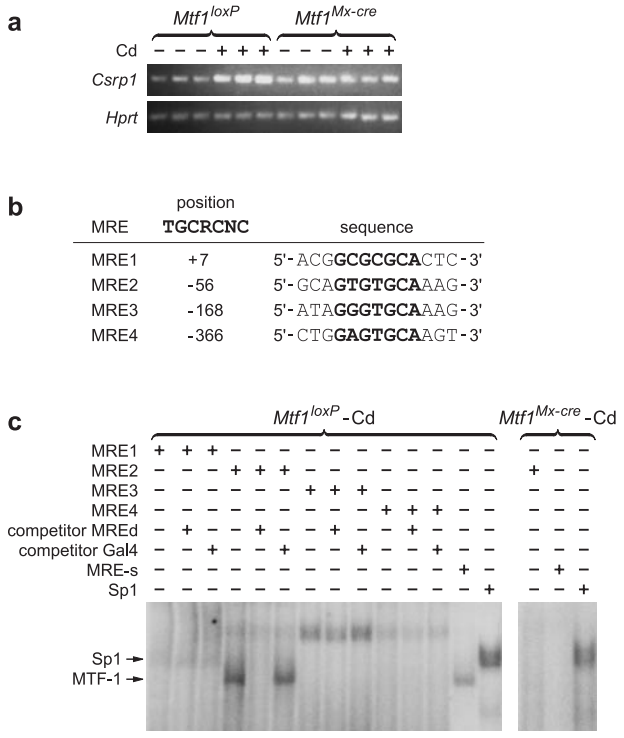

Figure 4. Cadmium response of *Csrp1* depends on MTF-1. (**a**) Semiquantitative RT–PCRs for *Csrp1* mRNA using total liver RNA from pI–pC-induced male $Mtf1^{Mx\text{-}cre}$ or $Mtf1^{loxP}$ mice. The animals had obtained either mock s.c. injections (−Cd) or s.c. injections with 20 µmol/kg body weight $CdSO_4$ (+Cd) 6 h before sacrificing them. RT–PCRs for *Hprt* mRNA were used as internal control to adjust the amount of total RNA used. (**b**) MRE core consensus sequences TGCRCNC (bold letters) and flanking sequences found in a region of 1000 bp upstream from *Csrp1* transcription start; the position of each core sequence is indicated. (**c**) EMSA with liver protein extracts of a male $Mtf1^{loxP}$ or a pI–pC-induced, male $Mtf1^{Mx\text{-}cre}$ mouse, both mock-treated. MTF-1 protein–DNA complex formation was tested with ^{32}P-labeled *Csrp1* MRE1, MRE2, MRE3 or MRE4 oligonucleotide, respectively. Specificity of binding was verified with excess of unlabeled competitor MREd or unrelated Gal4 oligonucleotide. ^{32}P-labeled MRE-s was included to indicate the position of an MTF-1–DNA complex; Sp1 bandshifts with ^{32}P-labeled Sp1 consensus oligonucleotide were obtained as protein loading control.

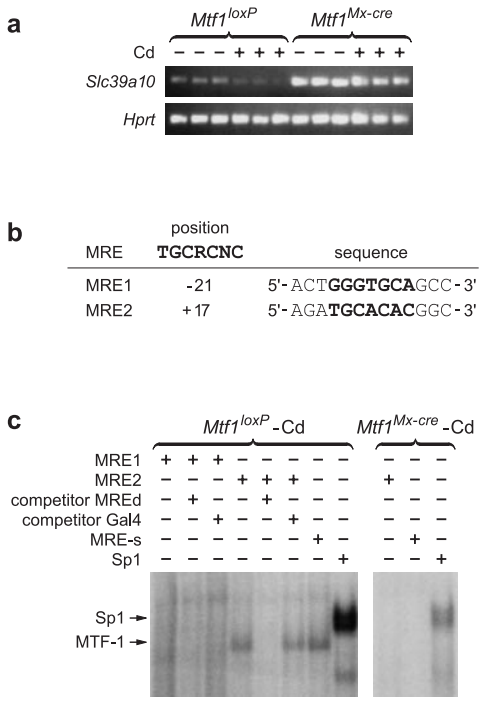

Figure 5. MTF-1 represses basal expression of *Slc39a10*. (**a**) Semiquantitative RT–PCRs for *Slc39a10* mRNA using total liver RNA from pI–pC-induced male $Mtf1^{Mx\text{-}cre}$ or $Mtf1^{loxP}$ mice. The animals had obtained either mock s.c. injections (−Cd) or s.c. injections with 20 µmol/kg body weight $CdSO_4$ (+Cd) 6 h before sacrificing them. RT–PCRs for *Hprt* mRNA were used as internal control to adjust the amount of total RNA used. (**b**) MRE core consensus sequences TGCRCNC (bold letters) and flanking sequences found in a region of 1000 bp upstream from *Slc39a10* transcription start; the position of each core sequence is indicated. (**c**) EMSA with liver protein extracts of a male $Mtf1^{loxP}$ or a pI–pC-induced, male $Mtf1^{Mx\text{-}cre}$ mouse, both mock-treated. MTF-1 protein–DNA complex formation was tested with ^{32}P-labeled *Slc39a10* MRE1 or MRE2 oligonucleotide, respectively. Specificity of binding was verified with excess of unlabeled competitor MREd or unrelated Gal4 oligonucleotide. ^{32}P-labeled MRE-s was included to indicate the position of an MTF-1–DNA complex; Sp1 bandshifts with ^{32}P-labeled Sp1 consensus oligonucleotide were obtained as protein loading control.

the cadmium response of this gene, but it is apparently not exclusively responsible.

One MRE core consensus sequence was found just upstream of the mouse *Slc39a10* transcription start (MRE1, −21 bp), another one directly downstream (MRE2, +17 bp; Figure 5b). Specific binding of MTF-1 was observed in EMSA analysis for MRE2 with liver protein extract from an $Mtf1^{loxP}$ but not from an $Mtf1^{Mx\text{-}cre}$ mouse, while no binding was detected with MRE1 (Figure 5c).

Cadmium-responsive, MTF-1-independent genes

Finally, we also identified a number of cadmium-responsive genes that were independent of MTF-1 presence, by comparing the probe array data of all cadmium-treated mice with the data of all mock-treated mice, irrespective of the genotype (Table 2). An at least 2-fold, reliable upregulation was observed after cadmium exposure for 31 probe sets corresponding to 21 different characterized genes (Table 2, a). For 2 probe sets corresponding to 2 characterized genes, an at least 2-fold downregulation was detected (Table 2, b).

Several genes involved in the metabolism of the antioxidant glutathione were found to be upregulated by cadmium exposure, namely the genes encoding the catalytic subunit of glutamate-cysteine ligase (*Gclc*) that is the rate limiting enzyme in *de novo* synthesis of glutathione (45); glutathione reductase 1 (*Gsr*), the reducing enzyme for oxidized glutathione (45); and glutathione-*S*-transferase, mu 4 (*Gstm4*), which is a member of the glutathione-*S*-transferase supergene family of detoxification enzymes (45). In all of these cases, induction was confirmed by semiquantitative RT–PCRs (data not shown). *Gclc*, also referred to as heavy chain subunit of gamma-glutamylcysteine synthetase (*Ggcs-hc*), had been discussed previously as a target gene of MTF-1 (6). Our expression data indicate that *Gclc* is induced by cadmium but, at least in the adult mouse liver, not dependent on MTF-1.

To analyze the role of the glutathione system in the cellular cadmium response, mouse embryonic fibroblasts with and without functional *Mtf1* were treated with cadmium in combination with BSO, a specific inhibitor of glutamate-cysteine ligase (31), and cell viability was assessed by a colorimetric assay based on the tetrazolium salt MTT (Figure 6). Increasing concentrations of BSO or cadmium alone were to some

Table 2. Comparison of liver gene expression for cadmium- and mock-treated mice (up- or downregulation at least 2-fold, $P \leq 0.05$)

Gene symbol	Gene title	Relative activity $Mtf1^{loxP}$ −Cd	$Mtf1^{loxP}$ +Cd	$Mtf1^{Mx\text{-}cre}$ −Cd	$Mtf1^{Mx\text{-}cre}$ +Cd	*P*-value
(a) Genes upregulated in cadmium-treated mice						
Cbr3	Carbonyl reductase 3	1	19.600	1.430	27.650	0.002
Npn3[a]	Neoplastic progression 3	1	12.907	1.045	12.460	0.002
Ddc	Dopa decarboxylase	1	7.166	1.776	7.633	0.024
Serpina9	Serine (or cysteine) proteinase inhibitor, clade A, member 9	1	6.345	0.998	9.455	0.041
Ppfibp2	Protein tyrosine phosphatase, receptor-type, F interacting, binding protein 2	1	3.771	1.167	5.279	0.004
Gclc	Glutamate-cysteine ligase, catalytic subunit	1	3.678	1.166	4.046	0.002
Pgd[a]	Phosphogluconate dehydrogenase	1	3.530	1.419	3.821	0.022
Ikbkg[b]	Inhibitor of kappaB kinase gamma	1	3.319	1.128	3.702	0.010
Txnrd1	Thioredoxin reductase 1	1	3.273	1.046	3.467	0.005
Kdelr2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	1	3.209	1.138	3.229	0.006
Ddit41	DNA-damage-inducible transcript 4-like	1	2.906	1.083	4.713	0.049
Aqp8	Aquaporin 8	1	2.810	1.505	3.528	0.049
Gstm4	Glutathione-S-transferase, mu 4	1	2.614	1.346	2.800	0.037
Bag3	Bcl2-associated athanogene 3	1	2.528	0.997	3.905	0.049
Gsr[c]	Glutathione reductase 1	1	2.376	1.224	2.611	0.010
Pir	Pirin	1	2.235	1.011	2.595	0.043
Htatip2	HIV-1 tat interactive protein 2, homolog (human)	1	2.130	1.069	2.585	0.035
Mocos	Molybdenium cofactor sulfurase	1	2.114	1.201	2.424	0.026
Abcc4	ATP-binding cassette, subfamily C, member 4	1	2.095	0.973	3.072	0.030
Rassf6	Ras associated (RalGDS/AF-6) domain family 6	1	2.056	1.087	2.930	0.030
Entpd5	Ectonucleoside triphosphate diphosphohydrolase 5	1	2.035	1.016	2.366	0.041
(b) Genes downregulated in cadmium-treated mice						
Sntg2	Syntrophin, gamma 2	1	0.400	1.000	0.400	0.027
G6pc	Glucose-6-phosphatase, catalytic	1	0.152	0.547	0.169	0.041

The animals had obtained either mock s.c. injections (−Cd) or s.c. injections with 20 μmol/kg body weight $CdSO_4$ (+Cd) 6 h before sacrificing them. The expression values for each gene are given as mean value of three animals per group, normalized to the mean value of group $Mtf1^{loxP}$ −Cd (relative activity).
[a]Mean values of three independent Affymetrix probe sets.
[b]Mean value of six independent Affymetrix probe sets.
[c]Mean value of two independent Affymetrix probe sets.

extent cytotoxic for the examined cell lines. Treatment with both BSO and cadmium resulted in an enhanced lethality particularly for the cells without functional *Mtf1*, indicating that a depletion of glutathione together with a lack of *Mtf1* impair an efficient anti-cadmium defense. Thus, adequate glutathione supply as well as MTF-1 and its target genes are essential for the survival of the cell under cadmium stress.

Besides genes related to the glutathione pathway, several other stress-related genes were upregulated upon cadmium exposure, including genes for thioredoxin reductase 1 (*Txnrd1*), one of the reducing enzymes of the antioxidant thioredoxin (46); KDEL endoplasmic reticulum protein retention receptor 2 (*Kdelr2*) participating in ER stress response (47); and the anti-apoptotic Bcl2-associated athanogene 3 (*Bag3*) involved in stress-induced apoptosis (48).

DISCUSSION

In this study, a virtually complete deletion of *Mtf1* in the liver of adult, pI–pC-induced $Mtf1^{Mx\text{-}cre}$ mice did not detectably affect the phenotype of the respective mice under non-stress conditions, confirming that MTF-1 is dispensable in the adult liver (26), in contrast to its essential role in embryonic liver development (23).

The comparison of gene expression in livers of mock- or cadmium-treated $Mtf1^{Mx\text{-}cre}$ and $Mtf1^{loxP}$ mice revealed several MTF-1 target gene candidates. Transcripts of the two stress-responsive metallothionein genes *Mt1* and *Mt2* were severely reduced, in support of a crucial role of MTF-1 for both basal and metal-induced expression of metallothioneins (4,6).

One of the newly found target genes is *Sepw1*. The exact molecular function of SEPW1 protein is unknown to date, but a role as antioxidant has been proposed due to its ability to bind glutathione (35). In accordance with this, ectopic expression of mouse *Sepw1* renders cells resistant to hydrogen peroxide, and this resistance is dependent on it binding glutathione (36). Furthermore, Amantana *et al*. (49) showed that expression of a reporter gene fused to a rat *Sepw1* promoter fragment can be induced in rat glial cells by copper and zinc, but not cadmium. This response was dependent on an overlapping-inverted MRE sequence located proximal to the rat *Sepw1* transcription start (49), even though initial studies failed to demonstrate MTF-1 binding to that sequence (50). Our expression and DNA-binding studies strongly suggest that MTF-1 is important for the basal expression of mouse *Sepw1* by binding to the corresponding overlapping-inverted MRE sequence.

Ndrg1, another interesting MTF-1 target gene was named N-myc downstream regulated gene 1 following the discovery that the transcription factor N-myc represses the expression of mouse *Ndrg1* (51). Transcription of *Ndrg1* and/or its human ortholog is induced by different physiological and cell stress conditions, such as androgens, nickel compounds, DNA damage and hypoxia (37–40,52,53). In addition, the protein is overexpressed in human cancers of many tissues, such as lung, liver, brain, breast, kidney and skin (40). Although *Ndrg1* and especially its human ortholog have been quite intensely studied, its function remains unclear; however, the

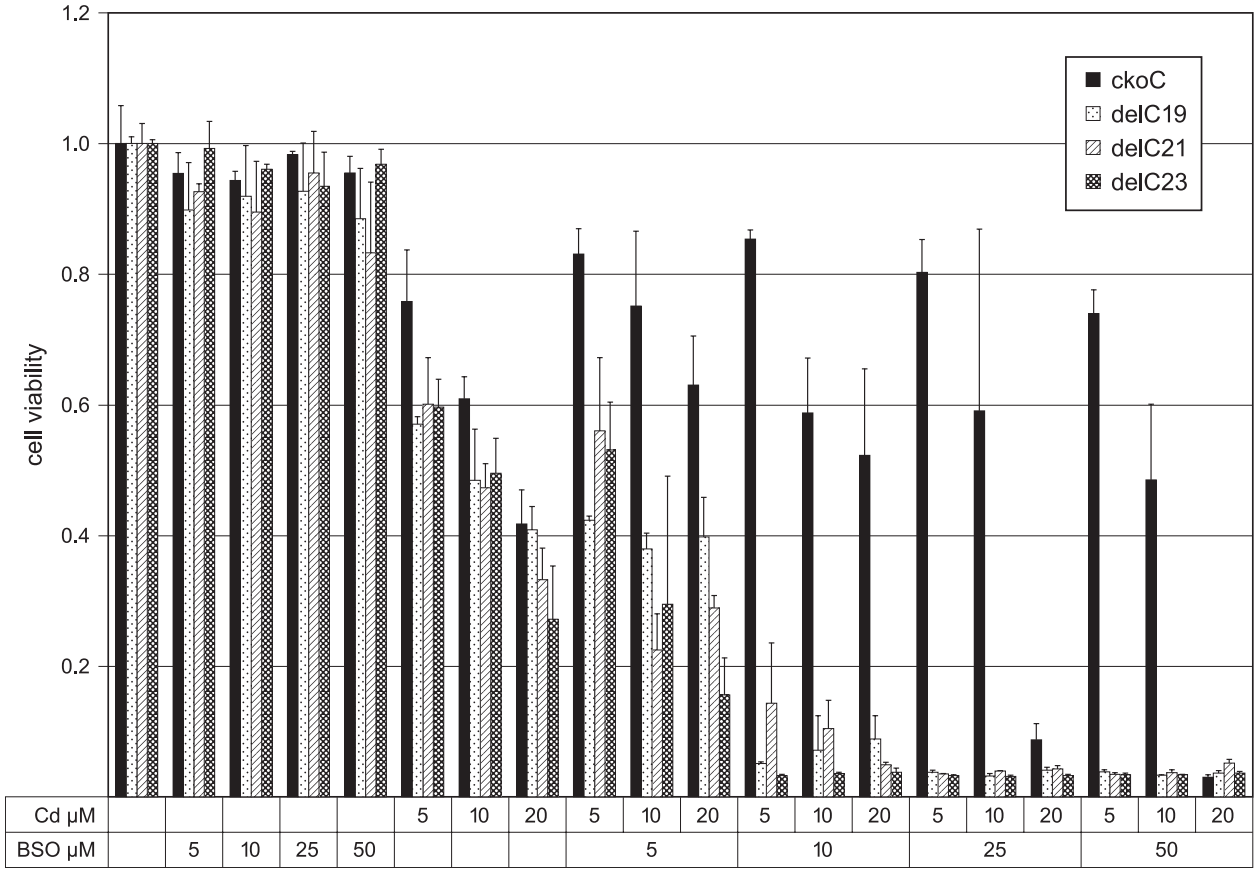

Figure 6. Cells with reduced glutathione level that also lack MTF-1 are hypersensitive to cadmium. The viability of cells was assessed with the so-called MTT assay. Mouse embryonic fibroblasts with (ckoC) and without (delC19, delC21 and delC23) functional *Mtf1* were compared. Cells were pre-incubated in medium containing 0, 5, 10, 25 or 50 µM BSO for 24 h and further exposed to 0, 5, 10 or 20 µM $CdCl_2$ (Cd) in the specified pre-incubation medium for an additional 24 h. Results are expressed as mean values ± SD ($n = 3$) normalized to the respective value of untreated cells.

induction by stimuli like nickel and hypoxia suggests an involvement in the cell stress response. Such a role is strongly endorsed by our finding that *Ndrg1* gene expression is also induced by cadmium, and that MTF-1 plays a crucial role in this induction.

In the case of *Csrp1*, expression analyses and DNA-binding studies indicate that MTF-1 is required for cadmium induction by binding to an MRE upstream of the transcription start. Studies with human, avian and chicken CSRP1 have shown that this protein is localized at adhesion plaques and in association with filamentous actin, and interacts with the adhesion plaque protein zyxin, as well as the actin-cross-linking protein alpha-actinin (54–57). The ability to bind these partners suggests a role in cytoskeletal organization (58). Exposure of cultured cells to cadmium causes a decrease in, and destruction of, cellular contact proteins and the actin cytoskeleton (59). In the proximal tubule cells of the rat kidney, a partial loss of actin and the actin-bundling protein villin is observed upon cadmium treatment, as well as the derangement and depolymerization of microtubules (60). Assuming that CSRP1 is important for the organization of cytoskeletal elements in the mouse, its upregulation by cadmium might protect the organism from damage of the cytoskeleton. Such a mechanism would expand the role of MTF-1 in stress response.

Our expression studies also suggest that MTF-1 represses basal transcription of *Slc39a10*, in contrast to its role as activator for the expression of other target genes like *Mt1*, *Mt2*, and *Znt1* (4,19). SLC39A10 is one of 14 mouse SLC39 members, which belong to the ZIP family of metal ion transporters (43,44). All members of the ZIP family characterized so far increase intracellular cytoplasmic metal ion concentrations by promoting extracellular and vesicular ion transport into the cytoplasm. ZIP proteins have been reported to be transporters of zinc, iron, manganese and/or cadmium (44,61–63). Although SLC39A10 is largely uncharacterized (44), it is referred to in several databases as putative zinc transporter. It has been previously shown that MTF-1 is important for both basal expression and metal induction of the mouse *Znt1* gene (19). ZnT proteins represent a different family of transporters that reduce intracellular cytoplasmic zinc by promoting zinc efflux from cells or into intracellular vesicles. Thus, members of the ZnT and ZIP family with zinc as predominant substrate have opposite roles in cellular zinc homeostasis (43). Assuming that SLC39A10 is indeed a zinc transporter, MTF-1 would control expression of two zinc transporters with antagonistic functions, namely, *Znt1* and *Slc39a10*. Specific binding of MTF-1 was observed for an MRE located just downstream of the *Slc39a10* transcription start. In a simple model, such a binding could interfere with the accessibility of the

transcriptional start site for RNA polymerase II and/or general transcription factors, thus preventing transcription initiation of the gene. Indeed, such a mechanism has been described in yeast for the zinc-responsive activator protein 1 (Zap1) and its target gene, zinc-regulated transporter 2 (*ZRT2*) (64). However, the inhibition of *Slc39a10* expression by MTF-1 may well be more complex than a competition for promoter binding. Independent of MTF-1, cadmium treatment also leads to downregulation of *Slc39a10* transcripts, suggesting that some other factor is mediating this response.

A previous target gene search for MTF-1 with mouse embryos of conventional *Mtf1* knockout phenotype revealed, besides metallothionein genes, the multifunctional alpha-fetoprotein (*Afp*) and the liver-enriched transcription factor CCAAT/enhancer binding protein alpha (*Cebpa*) as prime candidates (65). After an early onset during hepatogenesis, *Afp* expression is repressed postnatally and replaced by albumin (66). Thus, our adult $Mtf1^{Mx\text{-}cre}$ mice lacking MTF-1 were not suitable to analyze *Afp* expression. *Cebpa* is expressed in the adult liver as well as in other tissues (67), but the present microarray data revealed no significant expression differences in livers from adult $Mtf1^{loxP}$ and $Mtf1^{Mx\text{-}cre}$ mice (data not shown). Therefore, MTF-1 may affect *Cebpa* expression only during embryonal development, perhaps in combination with as yet unidentified factors.

The present study confirms and extends the role of MTF-1 as an important stress response regulator. We have identified and preliminarily characterized four target genes of MTF-1 in the adult mouse liver: in the case of *Sepw1*, MTF-1 is required to maintain basal expression, supporting a role of mouse MTF-1 in oxidative stress response. In addition, MTF-1 contributes to the cadmium-induced expression of *Ndrg1* and *Csrp1*. Furthermore, MTF-1 helps to repress the basal expression of *Slc39a10*, in contrast to its role as transcriptional activator for genes like *Mt1*, *Mt2* or *Znt1*. Thus the same transcription factor apparently serves as an activator or repressor, depending on the target gene.

The comparison of liver gene expression of cadmium- and mock-treated mice also revealed a number of genes that were responsive to cadmium exposure, independent of the presence or absence of MTF-1. Evidence suggests that the production of reactive oxygen species is a major effect of acute cadmium toxicity (68,69), and exposure of cultured cells or animals to cadmium is associated with depletion of reduced glutathione, lipid peroxidation and DNA damage (70–73). Oxidative stress and the subsequent restoration of cellular homeostasis have been shown to induce the expression of genes encoding acute-phase proteins and antioxidant enzymes (74). In mammals, cadmium tends to accumulate in the kidney and liver as a cadmium-metallothionein complex that has an extremely slow turnover (75,76). Furthermore, metallothioneins provide protection against oxidative stress (1,17). In addition to metallothioneins, glutathione was postulated as a first line of defense against cadmium toxicity (77). Glutathione efficiently complexes cadmium (78) and scavenges free radicals and other reactive oxygen species directly, and indirectly via enzymatic reactions (45). In such reactions, glutathione is oxidized and has to be regenerated by glutathione reductase. Also, glutathione-*S*-transferases mediate the conjugation of various electrophiles to glutathione. The observed cadmium-induced upregulation of *Gclc*, *Gsr* and *Gstm4* supports the importance of glutathione in the cellular cadmium response. The enhanced sensitivity to cadmium toxicity that we found for mouse embryonic fibroblasts upon a combination of *Mtf1* deletion and depletion of glutathione further corroborates the importance of MTF-1 and its target genes as well as reduced glutathione for an efficient anti-cadmium defense.

Our data provide strong evidence for at least two branches of cellular anti-cadmium defense, one via MTF-1 and its target genes, notably metallothioneins, the other via glutathione, with an apparent overlap in *Sepw1*.

SUPPLEMENTARY DATA

Supplementary data are available at NAR online.

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