Short Communication

Oleg Georgiev, Viola Günther, Kurt Steiner, Katharina Schönforth and Walter Schaffner*

The legless lizard *Anguis fragilis* (slow worm) has a potent metal-responsive transcription factor 1 (MTF-1)

Abstract: The metal-responsive transcription factor-1 (MTF-1) is a key regulator of heavy metal homeostasis and detoxification. Here we characterize the first MTF-1 from a reptile, the slow worm *Anguis fragilis*. The slow worm, or blind worm, is a legless lizard also known for its long lifespan of up to several decades. *Anguis* MTF-1 performs well and matches the strong zinc and cadmium response of its human ortholog, clearly surpassing the activity of rodent MTF-1s. Some amino acid positions critical for metal response are the same in humans and slow worm but not in rodent MTF-1. This points to a divergent evolution of rodent MTF-1, and we speculate that rodents can afford a less sophisticated metal handling than humans and (some) reptiles.

Keywords: cadmium toxicity; longevity; metal homeostasis; metal regulatory transcription factor; nuclear export signal (NES); zinc-induced transcription.

*These authors contributed equally to this work.

*Corresponding author: Walter Schaffner, Institute of Molecular Life Sciences, University of Zürich, CH-8057 Zürich, Switzerland, e-mail: walter.schaffner@imls.uzh.ch

Oleg Georgiev, Viola Günther, Kurt Steiner and Katharina Schönforth: Institute of Molecular Life Sciences, University of Zürich, CH-8057 Zürich, Switzerland

The terms slow worm, and especially blind worm, are misnomers: *Anguis fragilis* is neither a worm nor is it blind (Petzold, 1971; Böhme, 1981; Völk and Alfermann, 2007; Geiser et al., 2013). It is in fact a legless lizard of some 40 cm in length, with a wide distribution centered in the temperate climate zone of Europe (German: Blindschleiche; French: orvet; Italian: orbetto; Spanish: culebra de cristal). The erroneous label ‘blind’ stems from the old Germanic *plintsľicho*/blendschleiche (‘shiny creeper’) and refers to its skin that appears to glisten as if polished, unlike the scaly, more coarse skin of a snake. In contrast to the snakes, which lack eyelids, *Anguis* has eyelids which give its face a more familiar, seemingly friendly look. Perhaps unaware of these differences, Carl von Linné classified *Anguis* as a snake; a ‘fragile’ snake, in reference to the typical ability of lizards to shed their tail when attacked by a predator. *Anguis* lives a secluded life in meadows, underbrush and gardens. A surprisingly long lifespan of several decades has been documented, but in the wild only a few can expect to live that long; besides losses caused by predators, including domestic cats, slow worms often fall victim to road traffic. Unlike other European lizards, which are adept insect hunters, *Anguis* mostly feeds on slow prey such as small slugs, worms and woodlice.

Some plants and mushrooms accumulate heavy metals and snails have been reported to have a special metallothionein to cope with cadmium load (Palacios et al., 2011). Along the same vein, woodlice are able to accumulate high amounts of heavy metals (Hopkin and Martin, 1982). Therefore we speculated that *Anguis fragilis* might be particularly well-equipped to cope with heavy metal fluctuations and focused our attention on the transcription factor MTF-1 (metal-responsive transcription factor-1, also referred to as metal-regulatory transcription factor-1, or metal response element-binding transcription factor-1).

MTF-1 is a main regulator of heavy metal homeostasis in vertebrates (reviewed in Laity and Andrews, 2007; Günther et al., 2012b), and a MTF-1 ortholog has also been characterized in the fruit fly *Drosophila melanogaster* (Zhang et al., 2001; Egli et al., 2003; Balamurugan et al., 2004). MTF-1 binds via multiple zinc fingers to its cognate DNA motif, termed the metal response element (MRE) (Stuart et al., 1985; Wang et al., 2004a). MREs share the core sequence TGCRNC and are usually found in multiple copies in the promoter/enhancer region of target genes. Depending on the type of metal insult, *Drosophila* MTF-1 preferentially binds to specific variants of the MRE sequence (Sims et al., 2012). The best characterized target genes of MTF-1 encode the metallothioneins, a family of
small, cysteine-rich proteins that bind and sequester a variety of heavy metals (Vašák and Meloni, 2011). Mice with a targeted disruption of the MTF-1 gene die early in gestation because of liver degeneration (Günes et al., 1998; Wang et al., 2004b) but whether this is caused by a defect in metal homeostasis or another function remains to be seen. In *Drosophila*, MTF-1 is dispensable for life under normal laboratory conditions but essential to cope with environmental fluctuations of metal concentrations (Egli et al., 2003).

Here we characterize the first reptilian MTF-1, from the long-lived lizard *Anguis fragilis*. We show that its inducibility upon zinc and cadmium load is as high as that of human MTF-1, and that it clearly outperforms the rodent MTF-1s of mouse and capybara. To obtain *Anguis* MTF-1, a cDNA library was generated from a slow worm tissue sample and screened with primers specific for vertebrate MTF-1s. The cDNA coding sequence was assembled and compared to orthologs of other vertebrates, including human, mouse, capybara and pufferfish fugu (Brugnera et al., 1994; Auf der Maur et al., 1999; Lindert et al., 2008), and of the New World lizard *Anolis carolinensis* (Alföldi et al., 2011; Eckalbar et al., 2013). *Anguis* MTF-1 is a typical vertebrate MTF-1, which not unexpectedly is most closely related to the other lizard MTF-1. The zinc finger region and other domains of functional importance that had been characterized in human MTF-1 are strongly conserved in the slow worm. Of note, the human nuclear export signal (NES), which is embedded in the acidic activation domain (Saydam et al., 2001; Lindert et al., 2009), is even more similar to the corresponding reptilian sequence of *Anguis* (and *Anolis*) than to the one of the house mouse (Figure 1). This region is particularly important for metal inducibility (Lindert et al., 2009). Moreover, the overall length of slow worm MTF-1 (738 aa) is similar to *Anolis* (754 aa) and human (753 aa) and thus clearly different from MTF-1 in the mouse (675 aa) and another rodent, the South American capybara *Hydrochoerus hydrochaeris* (638 aa) (Lindert et al., 2008). The ‘cysteine cluster’ CQCQCAC, which is indispensable for MTF-1 activity (Chen et al., 2004; He and Ma, 2009; Günther et al., 2012a) (Figure 1), is also perfectly conserved in *Anguis*.

To determine the activity of *Anguis* MTF-1 we used the well-characterized Dko7 murine cell line, which carries a targeted disruption of the MTF-1 gene (Heuchel et al., 1994). These cells were shown before to work well with other transfected vertebrate MTF-1s (Brugnera et al., 1994; Auf der Maur et al., 1999; Lindert et al., 2008). An expression clone of *Anguis* MTF-1 was tested together with human, mouse, capybara and pufferfish MTF-1 in

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**Figure 1** Schematic overview of MTF-1 with functionally important domains.
Shown here is the domain structure of human MTF-1, which has been studied most extensively. The acidic activation domain, which also includes the nuclear export signal (NES) is known to be essential for activity and metal responsiveness, whereby critical amino acids overlap the NES motif. The NES sequences of human, mouse (*Mus musculus*), capybara (*Hydrochoerus hydrochaeris*), slow worm (*Anguis fragilis*), green anole (*Anolis carolinensis*), and pufferfish fugu (*Takifugu rubripes*) are aligned, with divergent amino acid positions indicated in bold red. Note that in this particular region, human MTF-1 is more closely related to reptile than to rodent (mouse, capybara) MTF-1, which is functionally significant [see Figure 4 and Lindert et al. (2009)]. Other domains of functional importance are also indicated (Günther et al., 2012b). For the cloning of *Anguis fragilis* MTF-1, tissue was retrieved from a roadkill in Weiningen/Zürich, Switzerland, and RNA was isolated using Trizol (Invitrogen) following the supplier’s recommendations. MTF-1 cDNA was assembled from subsegments obtained by the Smart Race cDNA Amplification Kit (Clontech, Saint-Germain-en-Laye, France) and inserted into the expression vector driven by the human cytomegalovirus enhancer-promoter as described (Heuchel et al., 1994).
Vertebrate MTF-1s differ in their transcriptional response to metals.

(A) Comparison of the transcription-stimulating activity of human, slow worm (*Anguis fragilis*), pufferfish, mouse and capybara MTF-1. 0.5 μg of the respective MTF-1 expression plasmids were transfected into MTF-1 knockout cells (Dko7) (Heuchel et al., 1994), together with 10 μg 4xMREd reporter plasmid and 5 μg reference plasmid per 100 mm dish. If indicated, cells were treated with 100 μM ZnSO$_4$ or 30 μM CdCl$_2$ for 4 h. Reporter and reference transcripts were quantified by the S1 nuclease assay (Weaver and Weissmann, 1979; Westin et al., 1987). The experiment was done in triplicate (bars); gel bands of one representative experiment are shown below.

(B) To reveal potential differences in transcriptional activation between human and *Anguis* MTF-1, transfected Dko7 cells were treated with 50, 100 or 200 μM ZnSO$_4$ or 25, 50 or 100 μM CdCl$_2$ for 4 h (left graph), or 25, 50 or 100 μM ZnSO$_4$ or 10, 25 or 50 μM CdCl$_2$ for 20 h (right graph).

(C) Transcriptional activity of human and *Anguis* MTF-1 in transfected Dko7 cells kept at 37°C or 30°C. White bars, no metal treatment; grey bars, cells exposed to 100 μM ZnSO$_4$ for 4 h.
cells exposed to zinc or cadmium. As shown in Figure 2A, both human and Anguis MTF-1 responded strongly to both metals, more than the rodent MTF-1s. In another experiment with human and Anguis MTF-1, exposure to different metal concentrations was extended to 20 h; again the response of both MTF-1s was similar (Figure 2B). Because Anguis is poikilothermic and occasionally exposes itself to sunshine, its body temperature can vary greatly but is typically lower than that of mammals. Therefore we wondered if a test at 30°C, rather than the standard 37°C, would reveal a difference between human and Anguis MTF-1. This was however not the case: also at the lower temperature both MTF-1s behaved the same (Figure 2C).

To verify the production of MTF-1 in the knockout host cell, we performed an electrophoretic mobility shift assay (Figure 3A). The level of DNA-bound protein is highest for the two rodent MTF-1s, particularly striking in the case of mouse MTF-1, less so in capybara, and is similar between human and Anguis. We have noted before that transfected mouse MTF-1 is highly expressed in several cell lines, but at the same time is less metal-responsive than MTF-1 of human or pufferfish. This was the case irrespective of whether activity was tested in mouse or human cells. Therefore we asked whether an excessive amount of mouse MTF-1 produced by the transfected cell, quenches metal inducibility via some auto-inhibitory feedback mechanism, such as titration of an essential cofactor. This was tested by performing a dilution series of the transfected MTF-1 expression plasmid. However, as shown in Figure 3B, the weak metal inducibility is an intrinsic feature of mouse MTF-1, irrespective of its expression level. It should nevertheless be pointed out that the mouse is not defenseless against heavy metal load. In vivo, metallothionein gene transcription, which

![Image](image_url)

**Figure 3** MTF-1 expression level hardly affects inducibility.
(A) Electrophoretic mobility shift assay of human, slow worm (Anguis fragilis), mouse, capybara and pufferfish MTF-1. For binding reactions a radioactively labeled DNA probe containing an MTF-1 binding site was incubated with nuclear extracts (Schreiber et al., 1989) from mouse MTF-1 knockout cells. These had been transfected with the respective expression plasmids and, if indicated, treated with 100 μM ZnSO4 for 4 h prior to extract preparation. First lane: free probe without nuclear extract added; second lane: nuclear extract of untransfected MTF-1 knockout cells. The electrophoretic binding assays were done according to (Westin and Schaffner, 1988). (B) Human and mouse MTF-1 retain their characteristic inducibility at different MTF-1 concentrations. Dko7 cells were transfected with decreasing amounts of either human or mouse MTF-1 expression plasmid, with other conditions as described for Figure 2, and reporter gene transcripts were quantified by S1 nuclease protection.
strictly depends on MTF-1, is substantially induced by cadmium and zinc. What might be missing is a level of functional redundancy that is typical for many biological responses. In MTF-1, the zinc fingers are involved in metal response (Bittel et al., 1998; Zhang et al., 2003) and in humans (and presumably in Anguis) – but not in rodents – the acidic activation domain can independently respond to metals (Lindert et al., 2009). Because the difference in metal response between human and mouse MTF-1 has been mapped to the nuclear export signal/acidic activation domain (Lindert et al., 2009), we compared the activity of the NES motif of Anguis with that of its human and mouse counterparts and with selected point mutants. As shown in Figure 4, the NES domains

![Figure 4](image-url)

Figure 4  In contrast to mouse MTF-1, reptile and human MTF-1 harbor a strong nuclear export (NES) function. The NES region of the slow worm Anguis fragilis and corresponding regions of human and mouse (plus two point mutants to make the mouse NES in part more human and/or Anguis-like) were fused to an inert reporter protein and tested for nuclear export function according to (Saydam et al., 2001). NES motifs (see also Figure 1) were: (A) slow worm Anguis fragilis; (B) mouse; (C) human; (D) negative control of reporter protein without NES motif; (E) mouse NES mutation Y→C (SLCLSELGLLST); (F) mouse NES mutation L→M (SLYSELGLMST).
of both *Anguis* and human led to the nuclear export of a fused reporter protein. Remarkably, the putative NES of the mouse failed, as did two single point mutations in mouse NES which partly reconstituted a human and/or *Anguis* NES sequence.

Do these findings mean that metal inducibility is coupled to nuclear export function, such that MTF-1 must shuttle between nucleus and cytoplasm? This scenario is likely an oversimplification, as our previous work has shown that upon inhibition of nuclear export by the drug LMB, human MTF-1 is confined to the nucleus but still retains a large part – though not all – of its metal inducibility (Lindert et al., 2009). One also has to keep in mind that the NES motif overlaps with the acidic activation domain, thus a change in one might affect the other. In any case we find it striking that at some amino acid positions critical for metal responsiveness, human and many other mammalian MTF-1s are more similar to reptile MTF-1 than to MTF-1 of mouse and capybara. After all, the mammalian line diverged from the reptiles more than 300 million years ago. We also noted that in rodents which tend to be short-lived, MTF-1 is severely truncated at the C-terminus, indicating that it has evolved faster than in other mammals. We thus speculate that rodents can afford a less sophisticated handling of heavy metals than some longer-lived species.

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**References**


