Identification of thyroid hormone response elements in vivo using mice expressing a tagged thyroid hormone receptor α1

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INTRODUCTION

The importance of TH (thyroid hormone) for proper brain development and function is widely recognized [1,2]. It becomes most evident in congenital (cretinism) and developmental hypothyroidism [3]. Similarly, low maternal TH levels during pregnancy can interfere with normal brain development in the offspring [4].

Most of the defects arising from the developmental hypothyroidism result from the actions of unliganded (apo-) TRα1 (thyroid hormone receptor α1), the main TR isoform in the brain [5]. To understand the role of apo-TRα1 in brain development better, mice heterozygous for a point mutation in the ligand-binding domain of the receptor (TRα1 + m mice) have been generated [6]. Like in cretinism, these TRα1 + m mice present a plethora of brain defects, e.g. high anxiety, locomotor dysfunction and defects in the control of autonomic functions [7–9]. At the anatomical level, neuronal defects were observed in the hippocampus, cerebellum and cortex [9,10]. Recently, the first patients carrying a mutation in TRα1 were identified [11,12], who also display central defects, e.g. in memory function or the control of fine-motor movement. Thus, the findings in man and mice collectively underline the importance of TRα1 signalling for proper brain development – unfortunately, the underlying molecular mechanisms have remained largely enigmatic. To date, only a few target genes have been identified in the brain [13], severely hampering the understanding of defects caused by developmental hypothyroidism.

Several specific reasons account for this lack of knowledge in the field of TH action. First, the identification of TH target genes by in silico studies is severely complicated by the remarkable

Synopsis

TRα1 (thyroid hormone receptor α1) is well recognized for its importance in brain development. However, due to the difficulties in predicting TREs (thyroid hormone response elements) in silico and the lack of suitable antibodies against TRα1 for ChIP (chromatin immunoprecipitation), only a few direct TRα1 target genes have been identified in the brain. Here we demonstrate that mice expressing a TRα1–GFP (green fluorescent protein) fusion protein from the endogenous TRα locus provide a valuable animal model to identify TRα1 target genes. To this end, we analysed DNA–TRα1 interactions in vivo using ChIP with an anti-GFP antibody. We validated our system using established TREs from neurogranin and hairless, and by verifying additional TREs from known TRα1 target genes in brain and heart. Moreover, our model system enabled the identification of novel TRα1 target genes such as RNF166 (ring finger protein 166). Our results demonstrate that transgenic mice expressing a tagged nuclear receptor constitute a feasible approach to study receptor–DNA interactions in vivo, circumventing the need for specific antibodies. Models like the TRα1–GFP mice may thus pave the way for genome-wide mapping of nuclear receptor-binding sites, and advance the identification of novel target genes in vivo.

Key words: brain, hyperthyroidism, hypothyroidism, thyroid hormone receptor, thyroid hormone response element

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Abbreviations used: 3′ UTR, 3′-untranslated region; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; HCN2, hyperpolarization-activated cyclic nucleotide-gated ion channel 2; qRT–PCR, quantitative real-time PCR; RNF166, ring finger protein 166; RXR, retinoid X receptor; Sema3a, Semaphorin 3a; TH, thyroid hormone; TRE, thyroid hormone response element; TRα1, thyroid hormone receptor alpha 1; wt, wild-type.

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sequence variety in the structures of the TREs (thyroid hormone response elements) described in previous studies [14,15]. Secondly, the available antibodies for TRs are often unspecific and display strong cross-reactivity with cytosolic proteins. Consequently, the identification of TRα1 target cells and target genes in the brain is not accessible by techniques relying on antibody quality such as immunohistochemistry or ChIP (chromatin immunoprecipitation).

To overcome this problem, mice expressing a TRα1–GFP (green fluorescent protein) fusion protein from the endogenous TRα1 locus were generated recently [16]. The analysis of brains from these animals revealed TRα1 expression in almost all post-mitotic neurons, with an exclusively nuclear localization [16]. Given the specificity in the detection provided by the tagged TRα1, we explored if TRα1–GFP mice could be used for ChIP experiments using GFP antibodies, aiming at the identification of TRα1 target genes in vivo.

Here we show that TRα1–GFP binds to previously characterized as well as novel TREs of TH target genes in heart and brain, independently of ligand availability. Our candidates were characterized as well as novel TREs of TH target genes in heart and brain, independently of ligand availability. Our candidates were confirmed by electrophoretic mobility shift and reporter gene assays. As a proof of concept, we identify a novel TH target gene in the brain — the RNF166 (ring finger protein 166).

**EXPERIMENTAL**

**Animals and manipulation of TH status**

Animal care procedures were conducted according to the guidelines of the EEC (European Community Council Directives 86/609). Required animal permissions were obtained from the local ethical committee. 4–6 month-old mice homozygous for TRα1–GFP (gfp/gfp) (previously described in [16]) and wt (wild-type) mice were housed at 21°C on a 12 h light, 12 h dark cycle. If required the animals were treated to achieve a hypothyroid or hypothyroid status, respectively. For immunoprecipitation, 25 μl protease inhibitor cocktail and 5 μl PMSF (all provided by the manufacturer) for 30 min on ice. After centrifugation (10 min at 2100 g at 4°C) the precipitate was resuspended in shearing buffer (provided by the manufacturer), and the DNA was subsequently sheared by sonication (seven times, interval 0.5, 30 s sonication followed by 30 s break; Bioruptor Sonicator, Diagenode). The shearing efficiency was verified on an agarose gel, showing fragment sizes between 500 and 1000 bp. 10 μl of the sheared chromatin was used to determine ‘input DNA’ for normalization. For immunoprecipitation, 25 μl sheared chromatin (corresponding to ~7 μg DNA) was incubated with the GFP antibody (1:300, rabbit anti-GFP, abcam ab290). The precipitation, washing, reverse cross-linking and proteinase K incubation were conducted according to manufacturer’s manual. To recover the precipitated DNA fragments, the solution was incubated at 95°C for 3 h, and the DNA subsequently extracted twice with phenol–chloroform. The DNA was then precipitated with ethanol, washed and dissolved in 100 μl buffer containing 10 mM Tris and 1 mM EDTA. For chromatin from heart, the same procedure was applied on whole mouse hearts.

**qRT–PCR (quantitative real-time PCR)**

To quantify the amount of precipitated DNA, real-time PCR was conducted before (input) and after the ChIP using the primers listed in Table 1. The ratio between precipitated and input DNA was calculated for each TRα1–GFP animal to correct for differences in input DNA, yielding a percentage pulldown value. The same procedure was performed in wt animals to determine the unspecific pulldown by the GFP antibody (background). The ChIP experiments were independently performed in five pairs consisting of one TRα1–GFP and one wt animal each.

The results presented show the precipitation in five TRα1–GFP animals normalized against the corresponding wt brain from the same experiment. qRT–PCR was performed with the 7300 Real Time PCR System (Applied Biosystems) and the FastStart Universal SYBR Green PCR Master Mix (Roche) with 40 cycles of 95°C for 15 s and 62°C for 90 s. Specificity of amplification was verified by melting curve analyses.
### Table 1. List of TREs

The different TREs, their location within the respective gene relative to the translation start site (ATG), the primers used for the detection after ChIP and previously published characterizations. n.a., not applicable.

| Gene     | TRE                  | Location relative to ATG | Primer ChIP                                                                 | Literature                                      |
|----------|----------------------|--------------------------|-----------------------------------------------------------------------------|-------------------------------------------------|
| RC3      | GGATTAAGGGCCTCG       | −4192                    | Fwd_5′-GCAGGAAATGGAGATCAGAGG-3′<br>Rev_5′-GCATGGTGGTCGCTGCTG-3′            | Analogous to Arrieta et al. [18]                |
| Hairless TRE1 | CCCCCAAGATCGAGCAGGACA<br>GCCCGCGCGTTCC | −3523                    | Fwd_5′-TCTCGAGAGCTGGATCAGTCG-3′<br>Rev_5′-CATGACATCTGCTGCTG-3′            | Thompson et al. [19]                            |
| Hairless TRE2 | AGGCGATCGAGGACA       | −4220                    | Fwd_5′-TTCAGCTGCTGGAAGGATGG-3′<br>Rev_5′-GCCAAGTGGGCCAATCC-3′            |                                                  |
| HCN2     | TTGGTTTCTAAAGGCTCA<br>TGATTTTTGTTG          | −2557                    | Fwd_5′-GCAGGGAAGATGGCAGCACCC-3′<br>Rev_5′-GTTCACACCCGCTTTGGGA-3′          |                                                  |
| HCN4     | GGGAGTCCTGACGACT      | −508                     | Fwd_5′-TATGGCAACCGCGCGAGTCG-3′<br>Rev_5′-GGTGTGCTGGGCTGCTAGG-3′          | Perfect inverted repeat far upstream, but the gene also spreads over 200 kb |
| Sema3a   | AGGACATGACCT          | −20442                   | Fwd_5′-ACTCTGACCTCTCTCTAAGAG-3′<br>Rev_5′-AGGCTGAGCACAACCCAGGCT-3′         |                                                  |
| RNF166   | GGGACCCGGCGGGTTG       | +9241                    | Fwd_5′-GGTGTGCTGGTGTTGGAAGGG-3′<br>Rev_5′-AATGCTACCTGGAAGC-3′            |                                                  |
| F2T2 TRE | ATTGACCCACGCTGAGGAG<br>TCAATGTTATGAGGGAGG<br>AGCTGAGGTCAGAGAGAGGAAGAGGG | n.a.                      | n.a.                                                                       | Wallis et al. [16]                               |
| DR4-TRE (synthetic TRE) | TAAGGTCATTCTACGGACTCA<br>CTGATCACCTAAGGCTCA<br>CTTCAGAGTCCT | n.a.                      | n.a.                                                                       | Hofmann et al. [20]                              |
| Negative control | No TRE             | Genomic region on Chr 1 | Fwd_5′-GAAGACCTGCTGTTGGGCTTG-3′<br>Rev_5′-CTCACAGCTGTGCTCACG-3′          | Between the lysine-specific demethylase 5B and synaptotagmin 2 |

For gene expression analysis with qRT-PCR, total RNA was isolated from the cortex of juvenile wt mice (treated with T3 or untreated) according to manufacturer’s instructions (RNeasy Mini, Qiagen) and cDNA was subsequently synthesized from 4 μg RNA using oligo dT primers (Transcriptor First Strand cDNA Kit, Roche). The following primers were used to determine RNF 166 expression levels (spanning the intron between exon 5 and 6): fwd 5′-CGCAGCACAAGTTCTGCTCAG-3′ and rev 5′-TGCCTCAATGCTCAGAGGAGG-3′. A standard curve was used to correct for PCR efficiency, and the gene expression was normalized using HPRT (hypoxanthine–guanine phosphoribosyltransferase) as housekeeping gene. The expression was normalized using HPRT (hypoxanthine–guanine phosphoribosyltransferase) as housekeeping gene. The specificity of the reaction was confirmed with a melting curve analysis showing a single product. For statistical analysis, a two-tailed t-test was performed, and P-values <0.05 were considered significant.

### Cloning of TREs

Oligonucleotides containing the TREs were designed with KpnI and BglII overhangs for subcloning or EMSAs (electrophoretic mobility shift assays). For cloning, the oligonucleotides were heated to 90 °C for 5 min and subsequently cooled for annealing. The TREs were then ligated into the pG3 promoter luciferase vector (Promega) between the KpnI and BglII restriction sites using a Quick Ligation kit (New England BioLabs). Positive colonies were identified using restriction analysis and sequencing, and the plasmid DNA for transfection was prepared using the Maxi Prep Kit (Invitrogen).

### Luciferase reporter gene assay

Luciferase assays were conducted according to the method of Hofmann et al. [20] with minor modifications. HepG2 cells (Leibniz-Institut DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were diluted to a final concentration of 20000 cells per well in DMEM (Dulbecco’s modified Eagle’s medium)/F12 (Life Technologies) containing 10% (v/v) FBS (fetal bovine serum, Biochrom). Cells were transfected with 70 ng of reporter plasmid pGL3-Promoter (Promega) containing the TRE of interest or empty control, 5 ng pGL4.74 hRluc (Promega) and 25 ng of pRS-rTrx1 [21] using Fugene™ HD (Promega) during seeding in white 96-well plates (Nunc). As positive control, p(DR4)2-SV40-DNA (Promega) and 25 ng of pRS-rTrx1 [21] were used. After incubation under a 5% (v/v) CO₂ humidified atmosphere at 37 °C overnight the solution was replaced by DMEM (Biochrom), and the medium was removed again after 1 h. For the assay 100 nM T3 in DMEM was added to the cells and incubated
for 24 h. The medium was removed and cells were lysed (passive lysis buffer; Promega) by shaking for 15 min at room temperature (21°C). Luciferase assays were conducted using the luciferase assay system (Promega) and luminescence was measured with a Berthold Mithras luminometer (Berthold Technology). The results were normalized against the hRluc luminescence.

EMSA

Single-stranded oligonucleotide pairs (2.5 μg) were denatured at 95°C for 5 min and annealed by slow cooling. Annealed oligonucleotides (10 pmol) were labelled with 32P-dCTP (NEN 3000Ci/mmol; PerkinElmer) using Klenow DNA polymerase (Fermentas) to fill in the overhanging ends [22]. EMSA was conducted according to Wahlström et al. [23] with minor alterations. Receptor/DNA complex formation was done in an incubation buffer consisting of 4% Ficoll, 60 mM KCl, 1 mM EDTA, 10 mM Hepes pH 7.9 and a final concentration of 0.1 μg/μl of carrier poly dIdC DNA with 1–3 μg receptor-nuclear extracts (obtained from overexpressing of receptors in vaccinia virus, [23]). T3 was added to a final concentration of 250 nM to saturate the TRs, and incubated for 1 min on ice. The mixture was complemented with 1500–10000 cpm of labelled oligonucleotide and incubated for 15 min on ice. A 6% PAGE was pre-run for 60 min at 4°C (250 V). The buffer was changed, 10 μl of receptor/DNA complexes were loaded on to the gel and the gel was run for an additional 60–90 min. To visualize the band shift, the gel was fixed in 20% (v/v) methanol and 10% (v/v) acetic acid and dried at 80°C for 30 min. Radiolabelled bands were visualized with a phosphor imager.

RESULTS

To identify native TR-binding sites, we prepared chromatin from the TRα1 expressing tissues brain and heart of TRα1–GFP mice, and performed ChIP with an anti-GFP antibody. As controls we used chromatin from wt mice with the anti-GFP antibody—a procedure that is superior to other methods, which omit the primary antibody or use an unspecific primary IgG for control, as our approach generates the same background in sample and control. We initially tested the ChIP on previously characterized TREs from the known TH target genes neurogranin (RC3) and hairless [18] in brains of hypo-, eu- and hyper-thyroid adult mice (Figure 1A, Table 1). We detected TRα1–GFP binding on all three TREs; however, there was no significant difference when comparing the three TH levels (P > 0.05 for all genes). Low binding was detected with a fragment of genomic DNA that contained no TRE-like elements and served as a negative control (Table 1).

We then analysed chromatin from heart as a second tissue expressing predominately TRα1 [24]. Our results identified TRα1 binding to novel TREs in the promoter region of three known cardiac TH target genes (Figure 1B), namely Sema3a (Semaphorin 3a), and potassium/sodium HCN2 and HCN4 (hyperpolarization-activated cyclic nucleotide-gated ion channels 2 and 4). Interestingly, although the chromatin was prepared from heart, TRα1 binding also occurred on the TREs of RC3 and hairless—genes that have not been reported to be expressed in this tissue.

To confirm the results obtained by the ChIP, we used EMSA as an independent method for detecting TRα1 binding to TREs (Figure 2). We radioactively labelled the TREs from F2T2 (positive control, see [16]), hairless, Sema3a, HCN2 and HCN4, and

Figure 1  ChIP from brain and heart of TRα1–GFP mice

(A) ChIP from forebrain homogenates of hypo-, eu- or hyperthyroid TRα1–GFP mice with subsequent QPCR detection of the TREs from neurogranin (RC3) and hairless as well as a negative control (squares represent independent experiments, n = 5 per group, the mean is indicated by a horizontal line, no significant difference between the groups; P > 0.05). (B) ChIP from heart homogenates of euthyroid TRα1–GFP mice with subsequent QPCR detection of TREs from Sema3a, HCN2 and HCN4, including negative control as well as TREs from RC3 and hairless2 (squares represent independent experiments, n = 5 per group, the mean is indicated by a horizontal line).
analysed their interaction with TR, RXR (retinoid X receptor) or T3 by gel electrophoresis. Monomeric TR binding (indicated with #) was observed for all TREs when compared with controls, and all TREs except for hairless1 also displayed a shift caused by the TR/RXR heterodimer (indicated with *). The addition of T3 caused an additional minor shift of the TRE bound to TR or TR/RXR, due to a conformational change of the complex as described previously [22,23]. Collectively, the results of the EMSA confirm the TRα1 binding to the TREs identified in the ChIP, and underline that the TRα1–GFP-binding properties to TREs are comparable with those of wt TRα1.

To test whether the TREs are capable of functionally regulating gene expression, we used a luciferase reporter assay as described in detail previously [20]. All TREs including the novel ones (Sema3a, HCN2, HCN4 and hairless1) were capable of inducing luciferase expression upon T3 stimulation (Figure 3), with the strongest induction observed for the F2T2 control and the hairless TREs.

Since the in vitro studies substantiated that all TREs identified by ChIP assay were functional, we tested if this approach could also identify novel TH responsive genes. Therefore we conducted ChIP assays on chromatin from brains of TRα1–GFP mice, reversed the cross-linking and incubated the precipitated shared DNA fragments with DNA polymerase to fill in possible gaps and add an additional 5′-adenosine. After cloning the fragments into pGEM T-easy vector, we tested clones for positive inserts using restriction analysis and sequenced the fragments in random clones. The most frequent clone contained a region adjacent to the RNF166 gene, which was subsequently screened for putative TREs by in silico analysis. Surprisingly, we identified a possible TRE in the RNF166 genomic sequence coding for the 3′-UTR (3′-untranslated region) (Table 1). Subsequent ChIP analysis indeed confirmed TRα1 binding to this TRE in brain chromatin of hypo-, eu- and hyperthyroid mice (Figure 4A), with no significant binding differences between the three conditions. This result was confirmed by binding of the putative TRE to TR (#) and TR/RXR (*) in the EMSA (Figure 4B). The TRE was also found to be functional in regulating luciferase expression in vitro (Figure 4C). Consequently, we tested whether RNF166 would be regulated in vivo as well, and found a significant approx. 50% reduction of RNF166 mRNA levels in the cortex of T3-treated wt mice compared with untreated controls (Figure 4D), identifying RNF166 as a novel TH target gene.
DISCUSSION

The results presented in this study demonstrate that a transgenic mouse strain expressing a TR–GFP fusion protein represents a valuable tool to investigate previously described and novel TREs in chromatin, taking advantage of the high quality of GFP antibodies in the ChIP assays. Our approach was carefully validated using previously characterized TREs and complemented by studies employing EMSA or reporter gene assays.

Advantages and limitations of the TRα1–GFP ChIP assay

Compared with the frequently used in vitro systems, the TRα1–GFP animal model has several distinct advantages. First, it represents the exact in vivo situation of an intact animal and normal tissue with regard to the molecular repertoire of the cells. This is in stark contrast to the several cell line-based approaches used for the analysis of TH responsiveness, which often do not express TRs or required cofactors, or are poorly characterized with regard to their regulatory machinery. Consequently, they often have to be transfected to express the molecular components needed to obtain a detectable response to TH. This strategy usually results in supraphysiological expression levels leading to false positive results from TRs binding to DNA sites, which they might not necessarily occupy in vivo. Furthermore, transfected and proliferating cells often contain poorly chromatinized DNA, which could alter the accessibility of TREs on the DNA [26,27]. These epigenetic differences between tissue samples and cell culture-based studies constitute another cause for potentially discrepant findings as recently shown for the T3-effects on the amyloid precursor protein [28].

It needs to be acknowledged, however, that the levels of the fusion protein are also slightly elevated in TRα1–GFP mice [16]. This is an inevitable consequence of targeting the TRα1 locus, as it eliminates the alternative splicing variant TRα2 and therefore results in a mildly increased production of the TRα1–GFP, about 2.5-fold [16]. However, TRα1–GFP mice do not display obvious phenotypical defects resulting from impaired TRα1 signalling [16], suggesting that this mild elevation seems to be of negligible biological relevance. Moreover, these findings imply that the chimeraic TRα1–GFP receptor is functionally equivalent to the wt TRα1, which concurs well with the similar transactivation properties of both constructs in vitro [16]. In fact, previous in vitro studies convincingly demonstrated that the GFP tag does not interfere with T3 or TRE binding, transcriptional activation or subcellular localization of TH receptors [29,30].

Secondly, a distinct advantage of the TRα1–GFP mouse strain is the possibility to use high-quality GFP antibodies to precipitate the fusion protein. This option not only circumvents the problem of the well-known lack of specificity of commercially available TR antibodies, but it also allows the use of the same primary antibody in wt animals as ideal controls. In contrast to other methods that often omit the primary antibody or use a different IgG as control, this approach enables the detection and quantitative correction of the endogenous background effect of the primary antibody. Moreover, as the GFP tag is outside the
functional domains of the TR, it is not affected by any structural changes that occur upon ligand or cofactor binding, and the affinity of the antibody for the apo- or the holo-TR is probably identical. This is an absolute prerequisite for the analysis of TR–DNA interactions in dependence of the hormone availability [27]: if the affinity differed, false occupancy rates of the TREs would likely be obtained under hypo- or hyper-thyroid conditions.

Gene regulation by TH receptors

Our findings that TREs seem to be similarly occupied by the TRα1–GFP in the hypo-, hyper-, or eu-thyroid state is not surprising, as it concurs with the current model of TH gene regulation in the literature [14,31]. It is assumed that the availability of the hormone constitutes the major variable controlling the expression rate of target genes. For positively regulated genes, the model predicts that in hypothyroid conditions the apo-TR is bound to the TRE and associates with a co-repressor complex thereby suppressing target gene transcription. If TH becomes available and binds to the receptor, a conformational change is induced, the co-repressors are released and co-activators are recruited, thereby stimulating gene expression [14,31]. This model is supported by our data, showing a similar degree of TR binding in hypo-, eu- and hyper-thyroid states. However, as the ChiP procedure requires a strong fixation, which permanently crosslinks DNA and TR, it can only provide a snapshot of the highly dynamic events taking place in vivo.

Identification of novel TH target genes

As proof of principle, we demonstrate here that the TRα1–GFP mice are valuable for the identification of novel genes regulated by TH. We detected a TRα1-binding site in the RNF166 gene, which was biologically active as a functional TRE in a reporter gene assay. Although the genomic location coding for the 3′-UTR of RNF166 seems to represent an unusual binding site for a transcription factor, such a location for a regulatory site has been described previously in TH-regulated genes [32]. In fact, it is not uncommon that a genomic 3′-UTR region contains transcriptional regulatory elements, which can loop back to the promoter to regulate gene expression [33,34]. Interestingly, the location of the TRE also seems crucial for its regulatory effects: in vivo, we observe suppression of cortical RNF166 mRNA levels by T3, whereas in vitro when the TRE is located in the promoter region, a T3-dependent induction of luciferase expression is observed. Similar effects have been reported previously for a different TRE in the 3′-UTR [32], underlining that the relative location plays a decisive role for the type of regulation. However, the precise mechanisms underlying this positional effect remain yet to be elucidated. Also, as the function of RNF166 in vivo is poorly understood, we can only speculate that the protein could constitute a link between TH and the regulation of ubiquitin ligation [35]. Nevertheless, the example of RNF166 demonstrates that mice expressing a tagged nuclear receptor isoform can be used for identifying novel DNA-binding sites in vivo, while circumventing the need for specific antibodies or artificial expression systems. Moreover, with regard to TH action, our study opens the road for genome-wide analyses of TR-binding sites, e.g. by using ChiP-sequencing.

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AUTHOR CONTRIBUTION

Susi Dudazy-Gralla, Kristina Nordström, Peter Josef Hofmann and Dina Abdul Meseh performed the experiments. Susi Dudazy-Gralla, Peter Josef Hofmann, Lutz Schomburg, Björn Vennström and Jens Mitchell designed the experiments. Susi Dudazy-Gralla, Kristina Nordström, Peter Josef Hofmann, Lutz Schomburg, Björn Vennström and Jens Mitchell analysed the data. Susi Dudazy-Gralla, Peter Josef Hofmann, Björn Vennström and Jen Mittag drafted the paper; all authors discussed and corrected the paper.

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