JOURNAL OF BACTERIOLOGY, Nov. 1994, p. 6631–6635
0021-9193/94/$04.00+0

Vol. 176, No. 21

Cloning, Nucleotide Sequence, and Regulation of *Schizosaccharomyces pombe thi4*, a Thiamine Biosynthetic Gene

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Received 8 June 1994/Accepted 31 August 1994

***thi4* mutants of *Schizosaccharomyces pombe* exhibit defective thiamine biosynthesis, and *thi4* mutations define a gene which is believed to be involved in the phosphorylation of 4-amino-5-hydroxymethyl-2-methylpyrimidine or 5-(2-hydroxyethyl)-4-methylthiazole and/or in the coupling of the two phosphorylated precursors to thiamine monophosphate (A. M. Schweingruber, J. Dlugonski, E. Edenharter, and M. E. Schweingruber, Curr. Genet. 19:249–254, 1991). The *thi4* gene was cloned by functional complementation of a *thi4* mutant and physically mapped on the left arm of chromosome I close to the genetic marker *gln1*. The *thi4*-carrying DNA fragment shows an open reading frame encoding a protein of 518 amino acids and a calculated molecular mass of 55.6 kDa. The appearance of *thi4* mRNA is strongly repressed by thiamine and to a lesser extent by 5-(2-hydroxyethyl)-4-methylthiazole. *thi4* mRNA production is under the control of the *thi1* gene-encoded transcription factor and of the negative regulators encoded by genes *tnr1*, *tnr2*, and *tnr3*. *thi4* is expressed and regulated in manners similar to those of other *S. pombe* genes involved in thiamine metabolism, including *thi2*, *thi3*, and *pho4*.**

Thiamine (vitamin B_1) consists of a pyrimidine moiety and a thiazole moiety. The two halves of the molecule, 4-amino-5-hydroxymethyl-2-methylpyrimidine and 5-(2-hydroxyethyl)-4-methylthiazole, are synthesized by separate pathways. These precursors are further phosphorylated to hydroxymethylpyrimidine pyrophosphate and thiazole phosphate, and by the action of thiamine phosphate pyrophosphorylase, they are coupled to thiamine monophosphate (TMP), which is further converted to thiamine diphosphate (TDP) (for a review, see reference 22). TDP is an essential cofactor for several enzymes, including transketolase, pyruvate dehydrogenase, pyruvate decarboxylase, and α-ketoglutarate dehydrogenase (for a review, see reference 6). The biosynthetic pathway of TDP and its regulation are still poorly understood. In *Escherichia coli*, 11 genes are known to be involved in TDP biosynthesis (for a review, see reference 2). Five genes encoding kinases involved in TDP biosynthesis have been located at positions 10, 25, and 46 min of the *E. coli* genetic map. Five other genes of still unknown function in the synthesis of the pyrimidine and thiazole moieties map at position 90 min and possibly form an operon (21).

To understand the biosynthesis of TDP and its genetic regulation in a eucaryotic organism, we are working with the fission yeast *Schizosaccharomyces pombe*. In this organism, thiamine is not only essential for growth but also acts as a regulator of mating (19). Three loci encoding thiamine biosynthetic genes have been identified so far in this organism (17). *thi2* and *thi3* (allelic to *nmt1*) are involved in the synthesis of the thiazole and pyrimidine moieties, respectively. The *thi4* gene is defined by mutants that require thiamine but do not grow in the presence of 4-amino-5-hydroxymethyl-2-methylpyrimidine and 5-(2-hydroxyethyl)-4-methylthiazole. Its product therefore has to be responsible for the phosphorylation of one or both of the precursors and/or the coupling of the two phosphorylated moieties to TMP.

Genes *thi2* and *thi3* have been cloned and sequenced, and their expression has been shown to be repressed by thiamine (13, 18a, 23). At least four regulatory genes, *thi1*, *tnr1*, *tnr2*, and *tnr3* are responsible for the regulation of *thi2*, *thi3*, and *pho4*. *pho4* is also repressed by thiamine and believed to code for a thiamine phosphate phosphatase (17, 18, 20, 23). *tnr1*, *tnr2*, and *tnr3* act negatively on the expression of these genes, whereas *thi1* acts as an activator and has recently been shown to encode a Cys_6-zinc finger-containing transcription factor (5). In this communication, we report the isolation, physical mapping, and sequence of the *thi4* gene and show that its expression is repressible by thiamine and under the control of the regulatory genes *thi1*, *tnr1*, *tnr2*, and *tnr3*.

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MATERIALS AND METHODS

Strains and media. *S. pombe thi4-4 ura4-D18* h^- and the heterothallic wild-type strains 972 h^- and 975 h^+ are from our collection in Berne. The *pho1-44* deletion strain and the *thi1-1*, *tnr1-18*, *tnr2-2*, and *tnr3-5* regulatory mutants have been described by Schweingruber et al. (18). They were cultivated in liquid (YEL) or solid (YEA) yeast extract medium or in liquid (MM) or solid (MMA) minimal medium as previously described (17). The supplements 4-amino-5-hydroxymethyl-2-methylpyrimidine and 5-(2-hydroxyethyl)-4-methylthiazole (sometimes just referred to as pyrimidine and thiazole moiety) were kindly supplied by Gerard Moine, Hoffmann-La Roche, Basel, Switzerland.

Molecular cloning. The partial *Sau*3A genomic library ligated in the shuttle vector pFL20 (11) described previously (4) and provided by Louise Clarke (Santa Barbara, Calif.) was used to clone the *thi4* gene. Strain *thi4-4 ura4-D18* h^- was transformed by the alkali cation method (10) and plated on MMA. The growing Ura^+ colonies were replica plated on MMA. By this second transfer to MMA, accumulated intracellular thiamine is exhausted, and only thiamine prototrophic colonies are able to grow. Plasmids recovered from transformants were propagated in *E. coli* XL1-blue (3). Standard methods were used for restriction endonuclease digestion, ligation, and transformation in *E. coli* (15). Enzymes were purchased from Boehringer Mannheim.

Genetic techniques and physical mapping. Standard genetic techniques for *S. pombe* have been described elsewhere (8). Genes *thi2*, *thi3*, and *thi4* were physically mapped by using P1 and cosmid libraries covering the complete *S. pombe* genome (9, 12). Gene *ura4* was taken as a known reference probe. Nylon filters spotted with the genome fragments were kindly provided by Elmar Maier (Imperial Cancer Research Fund, London, England) and hybridized with the probes mentioned above as previously described (9). The probes used to map *thi2* and *thi3* were the 1.8-kb *Sal*I-*Cla*I fragment given in Fig. 1 of reference 23 and the 0.9-kb *Hin*dIII-*Bam*HI fragment shown in Fig. 4A of reference 13. The *thi4* probe is shown in Fig. 1 of this report.

Northern (RNA) blot analyses. Cells were grown in MM and MM supplemented with 1 μM thiamine, 3.6 μM 5-(2-hydroxyethyl)-4-methylthiazole, or 3.6 μM 4-amino-5-hydroxymethyl-2-methylpyrimidine to an A_{595} of 0.9 to 1.1. Total RNA was extracted, separated by the glyoxal method, blotted, and hybridized as previously described (18).

DNA sequencing. DNA sequences were determined from denatured, double-stranded templates by the dideoxy-chain termination method (16) with modified T7 polymerase and [^{35}S]dATP, using the Sequenase II kit (United States Biochemicals, Cleveland, Ohio) following the manufacturer's instructions. [α-^{35}S]dATP (1,190 Ci mmol^{-1}) was from New England Nuclear (Boston, Mass.). Custom-made oligonucleotide primers were synthesized in our laboratory. DNA and protein sequences were analyzed with the University of Wisconsin Genetics Computer Group program (7). The *thi4* sequence was compared with sequences from the GenBank/EMBL database using the program FASTA.

Nucleotide sequence accession number. The GenBank/EMBL nucleotide sequence accession number of the *S. pombe* *thi4* gene is X78824.

RESULTS AND DISCUSSION

Molecular cloning and physical mapping. The *thi4* gene was cloned by transforming an *S. pombe thi4-4 ura4-D18* h$^-$ strain with a *Sau*3A partial genomic library and by selecting transformants prototrophic for uracil and thiamine as described in Materials and Methods. Plasmid pTH40 was recovered from a transformant and amplified in *E. coli* XL1-blue. It revealed an insert of 13.3 kb, and the restriction map of a 4.0-kb subfragment is shown in Fig. 1. The 2.5-kb *Sph*I-*Nhe*I fragment of this insert complemented the thiamine auxotrophic phenotype of the *thi4-4* mutant with a frequency similar to that of the original plasmid. Thiamine prototrophic transformants were tested for the stability of the Thi$^+$ phenotype. Eight stable transformants were crossed with the wild-type strain 975 h$^+$. Of 10,096 colonies examined, no thiamine auxotrophic recombinants could be recovered. This provides evidence that the 2.5-kb *Sph*I-*Nhe*I fragment integrated at or close to the *thi4* locus and therefore contains the *thi4* gene.

With the 0.8-kb *Pvu*II fragment (Fig. 1) as a probe, *thi4* was physically mapped to the left arm of chromosome I between the genetic marker *gln1* and probe 7a7 of the physical map given in Fig. 2 by Hoheisel et al. (9). To test whether *thi4* is linked to the other known genes in thiamine biosynthesis, *thi2* and *thi3* were also mapped (for probes, see Materials and Methods). *thi2* can be localized on the right arm of chromosome II at probe 1g9 on the left of *nda3*, and *thi3* can be localized on the right arm of chromosome III between the genetic markers *adh1* and *ade5* at probe 23g1.

DNA sequence analysis. The sequence of the *thi4*-complementing *Sph*I-*Nhe*I fragment was determined, and additional upstream sequences were read directly from pTH40. The only open reading frame with reasonable length is that given in Fig. 2. This open reading frame encodes a protein of 518 amino acids with a predicted molecular mass of 55.6 kDa. We assume that it represents the *thi4* gene. Its expression does not affect the expression of other thiamine metabolic genes such as *thi2*, *thi3*, and *pho4* and is therefore most unlikely a regulatory gene (our unpublished results). According to the growth requirements of *thi4* mutants (17), it most probably codes for an enzyme with one or several of the following activities: hydroxymethylpyrimidine kinase (EC 2.7.1.49), phosphomethylpyrimidine kinase (EC 2.7.4.7), hydroxyethylthiazole kinase (EC 2.7.1.50), and thiaminephosphate pyrophosphorylase (EC 2.5.1.3).

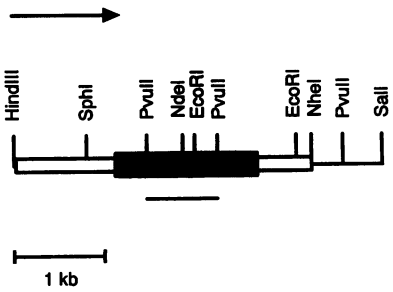

FIG. 1. Restriction map of *thi4*-containing 4.0-kb DNA fragment. The sequenced region is given as an open box. The black region denotes the *thi4* open reading frame, and the arrow indicates the direction of transcription. The fragment between the two unique restriction sites *Nhe*I and *Sph*I was used to integrate *thi4* into the genome of *S. pombe*. The underlined *Pvu*II fragment was used as the hybridization probe in Northern blots.

GenBank/EMBL database search revealed that the 223 N-terminal amino acids of the Thi4 protein show significant sequence homology with the whole ThiE protein of *E. coli* (21). This protein has an unknown function in the synthesis of 5-(2-hydroxyethyl)-4-methylthiazole. The sequences exhibit 26.4% identity and 52.4% similarity (Fig. 3). The meaning of this homology is not clear. In catalyzing an unknown reaction in the synthesis of the thiazole moiety of thiamine (21), it is possible that the ThiE protein can bind thiazole or thiazole-phosphate as an allosteric inhibitor or contains a sequence necessary for substrate and/or product interactions that can recognize the whole molecule or part of the thiazole molecule. Such sequences might have been conserved in functionally related eucaryotic enzymes and may be present in *S. pombe* *thi4*.

The putative promoter contains two canonical TATA boxes at the positions −202 to −196 and −363 to −358 relative to the start codon ATG. The *thi4* promoter does not reveal potential regulatory sequences shared with the *thi2*, *thi3*, or *pho4* gene.

Regulation of *thi4* expression. We examined the expression of the *thi4* gene in response to thiamine and its biosynthetic precursors. RNA extracted from wild-type strain 972 h$^-$ grown in MM and MM supplemented with thiamine, 4-amino-5-hydroxymethyl-2-methylpyrimidine, or 5-(2-hydroxyethyl)-4-methylthiazole was subjected to Northern blot analysis by using the 0.8-kb *Pvu*II fragment (Fig. 1) as a probe. Repression of *thi4* expression by thiamine is strong (Fig. 4). Furthermore, 5-(2-hydroxyethyl)-4-methylthiazole significantly represses *thi4* expression, although repression is less than that seen with thiamine. 4-Amino-5-hydroxymethyl-2-methylpyrimidine has no effect on the *thi4* mRNA level. By comparing Northern

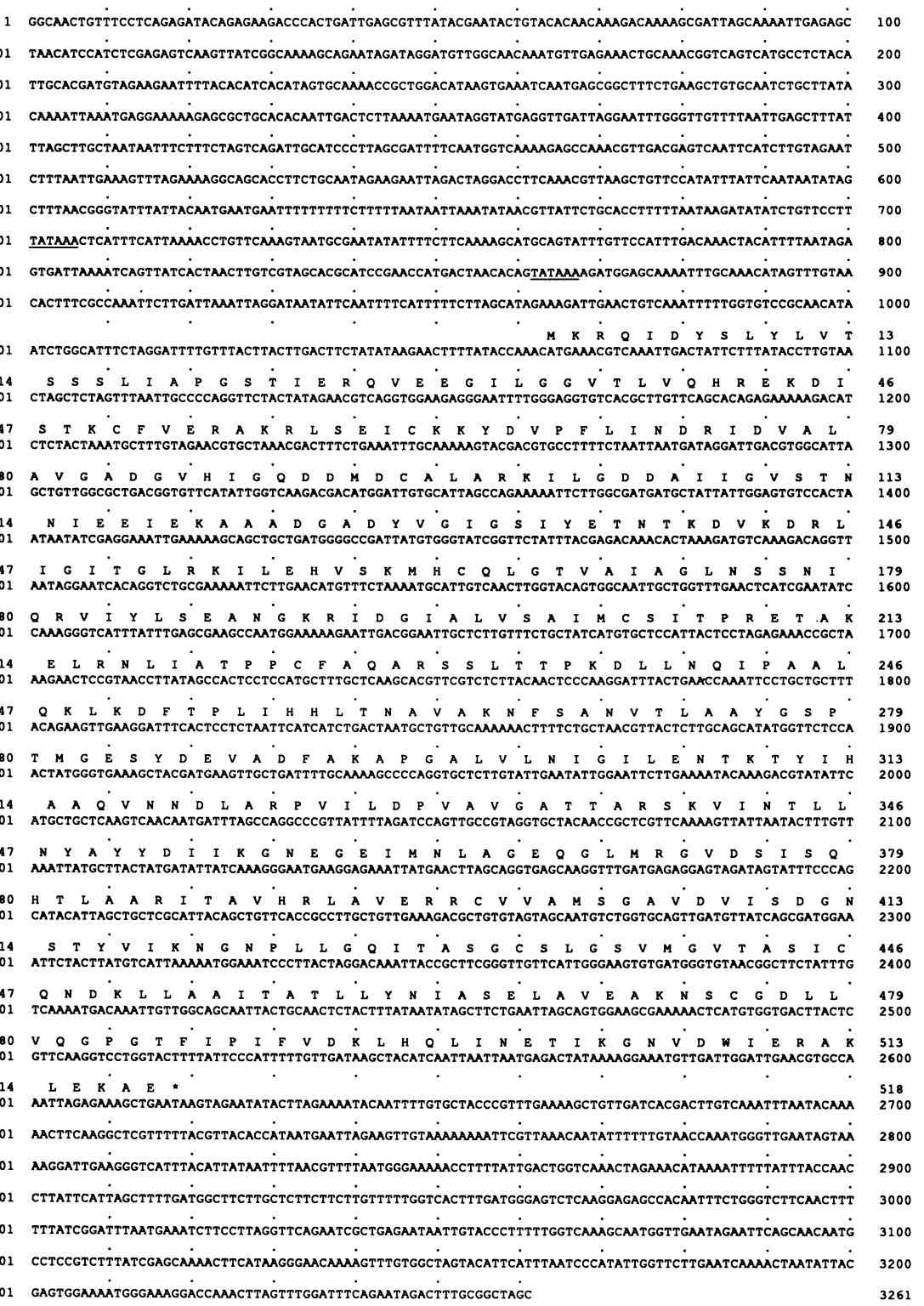

FIG. 2. Nucleotide sequence of the *thi4* gene and its deduced amino acid sequence. The sequence was determined as described in Materials and Methods. Both strands were sequenced. The nucleotide sequence of the noncoding strand is shown. Potential TATA boxes are underlined.

blots, we find that *thi4* is transcribed in the same order of magnitude as that of *thi2* and *thi3* (our unpublished results). *thi3* (*nmt1*) is one of the most strongly expressed genes known so far in *S. pombe*, its expression being about six times higher than that of the strongly expressed *adh* gene (1).

To examine whether *thi4* expression is under the control of the regulatory genes *tnr1*, *tnr2*, *tnr3*, and *thi1*, RNA from representative strains grown in the presence and absence of thiamine was extracted and probed for *thi4* RNA (Fig. 5). The *tnr1* strain shows almost no repression by thiamine, and in *tnr2*

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thi4   4 QIDYSLYLVTSSSLIAPGSTIERQVEEGILGGVTLVQHREKDISTKCFVE 53
         | |:.  :. .|:|.:...:::: :|  : :||  :| | ||  ... .
thiE   3 QPDFPP.VPFRSGLYPVVDSVQW.IERLLDAGVRTLQLRIKDRRDEEVEA 50

thi4  54 RAKRLSEICKKYDVPFLINDRIDVALAVGADGVHIGQDDMDCALARKILG 103
           .   .:.::|:..::|||   :|:  .| |||:||:|::..  . | :
thiE  51 DVVAAIALGRRYNARLFINDYWRLAIKHQAYGVHLGQEDLQATDLNAIRA 100

thi4 104 DDAIIGVSTNNIEEIEKAAADGADYVGIGSIYETNTKDVKDRLIGITGLR 153
         .:  :||||::  ||: | |. :.|:::| ::.|.||::..  |:..|
thiE 101 AGLRLGVSTHDDMEIDVALAARPSYIALGHVFPTQTKQMPSAPQGLEQLA 150

thi4 154 KILEHVSKMHCQLGTVAIAGLNSSNIQRVIYLSEANGKRIDGIALVSAIM 203
         : :|::.    :..||||:|:. .. . ||      :. :::||:||||
thiE 151 RHVERLA....DYPTVAIGGISLARAPAVI......ATGVGSIAVVSAIT 190

thi4 204 CSITPRETAKELRNLIATPP 223
         . . | .. :| :: :...
thiE 191 QAADWRLATAQLLEIAGVGD 210
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FIG. 3. Sequence similarity between the 223 N-terminal amino acids of the *S. pombe* Thi4 and the entire *E. coli* ThiE protein. Sequence identities are indicated by vertical bars, strong similarities are indicated by two dots, and weak similarities are indicated by one dot. The two sequences were aligned by using the program BESTFIT; the gap weight was 3.0, and gap length weight was 0.1.

and *tnr3* mutants, repression of *thi4* by thiamine is clearly impaired. In *thi1* mutants, *thi4* mRNA is repressed; a weak *thi4* band is visible in the absence of thiamine only if the blot is overexposed.

Taken together, the expression studies from the genes *thi2*, *thi3*, *thi4*, and *pho4* illustrate that thiamine metabolism, and in particular thiamine biosynthesis, is highly regulated in *S. pombe*. Recently, a strong regulation of a thiamine biosynthetic gene has also been demonstrated in *Saccharomyces cerevisae* (14), indicating that tight genetic regulation of thiamine biosynthesis in eucaryotic organisms is not *S. pombe* specific. Regulation of thiamine biosynthesis may be important not only for economic reasons for the cell but also for metabolic control. Thiamine being involved as a cofactor (in the form of TDP) of enzymes in the citric acid cycle, glycolysis, and the pentose phosphate cycle, these pathways can potentially be coordinately controlled by the levels of intracellular thiamine. The studies further show that in addition to the common regulation observed by thiamine and the regulatory genes *thi1*, *tnr1*, *tnr2*, and *tnr3*, expression of the genes *thi2*, *thi3*, *thi4*, and *pho4* is also to some extent individually regulated. For example, *thi2* and *thi3* are more strongly repressed by thiamine and *thi1* mutations than *pho4* and *thi4*, and *pho4* is regulated only by thiamine, whereas *thi2* and *thi4* are also slightly repressed by the thiazole moiety and *thi3* by the pyrimidine moiety (18, 23; unpublished results).

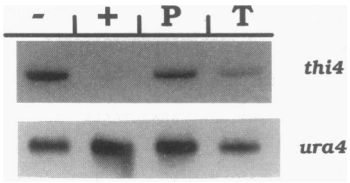

FIG. 4. Northern blot analysis of *thi4* mRNA from cells grown in the presence and absence of thiamine and its precursors. The wild-type cells were grown in MM (−) and MM containing 1 μM thiamine (+), 3.6 μM 4-amino-5-hydroxymethyl-2-methylpyrimidine (P), or 3.6 μM 5-(2-hydroxyethyl)-4-methylthiazole (T). As a control, the RNA was probed with *ura4*, which is not regulated by thiamine or its precursors. Overexpressing the blot revealed a weak band for the *thi4* probe in the + lane.

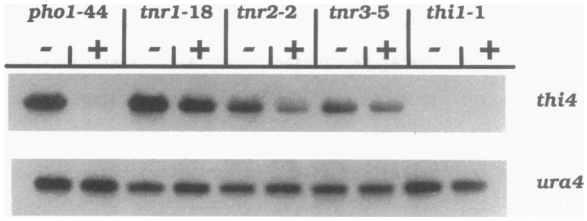

FIG. 5. Northern blot analysis of *thi4* mRNA in *tnr1*, *tnr2*, *tnr3*, and *thi1* mutants. *tnr1*, *tnr2*, and *tnr3* mutant strains were grown in MM (−) and MM containing 1 μM thiamine (+). *thi1-1* as a thiamine auxotroph was grown in MM containing 40 nM thiamine (−) (this is a nonrepressing concentration but allows growth) and in MM containing 1 μM thiamine (+). *pho1-44* is the parental strain from which all mutants are derived. As a control, the RNA was probed with *ura4*.

ACKNOWLEDGMENTS

We thank L. Clarke for the *Sau*3A genomic library in pFL20, G. Moine for the generous supply of 4-amino-5-hydroxymethyl-2-methylpyrimidine and 5-(2-hydroxyethyl)-4-methylthiazole, E. Maier for the filters spotted with the genome fragments, and W. Wilson for comments on the manuscript.

This study was supported by the Swiss National Foundation.

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