Dual targeting of isoleucyl-tRNA synthetase in *Trypanosoma brucei* is mediated through alternative *trans*-splicing

Jochen Rettig¹, Yimu Wang², André Schneider¹,* and Torsten Ochsenreiter²,*

¹Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3 and ²Institute of Cell Biology, University of Bern, Baltzerstrasse 4, 3012 Bern, Switzerland

Received July 21, 2011; Revised September 7, 2011; Accepted September 11, 2011

ABSTRACT

Aminoacyl-tRNA synthetases catalyze the aminoacylation of tRNAs with their cognate amino acids. They are an essential part of each translation system and in eukaryotes are therefore found in both the cytosol and mitochondria. Thus, eukaryotes either have two distinct genes encoding the cytosolic and mitochondrial isoforms of each of these enzymes or a single gene encoding dually localized products. Trypanosomes require *trans*-splicing of a cap containing leader sequence onto the 5′-untranslated region of every mRNA. Recently we speculated that alternative *trans*-splicing could lead to the expression of proteins having aminotermini of different lengths that derive from the same gene. We now demonstrate that alternative *trans*-splicing, creating a long and a short spliced variant, is the mechanism for dual localization of trypanosomal isoleucyl-tRNA synthetase (IleRS). The protein product of the longer spliced variant possesses an amino-terminal presequence and is found exclusively in mitochondria. In contrast, the shorter spliced variant is translated to a cytosol-specific isoform lacking the presequence. Furthermore, we show that RNA stability is one mechanism determining the differential abundance of the two spliced isoforms.

INTRODUCTION

Most organisms employ 20 functionally distinct aminoacyl-tRNA synthetases (generic: aaRS; specific enzyme: three letter code for amino acid followed by RS) to charge their tRNAs with the cognate amino acid. In eukaryotes, aaRSs are required for both cytosolic and mitochondrial translation. With the exception of plants, which additionally contain plastids, eukaryotes are therefore expected to have two sets of aaRSs, one cytosolic and one targeted to the mitochondria (1). In many organisms, however, some of the aaRSs are dually targeted and thus must have evolved mechanisms to become localized to the cytosol and mitochondria using only the information encoded in a single gene. Trypanosomatids represent an extreme example where the genome encodes only 23 aaRSs (2) indicating that all but six aaRSs likely require dual targeting.

Unlike other eukaryotes trypanosomatids lack mitochondrial tRNA genes and therefore need to import all of their organellar tRNAs from the cytosol (3). The extensive dual targeting of trypanosomatid aaRSs therefore correlates with the presence of eukaryotic-type mitochondrial tRNAs that have been imported from the cytosol.

Dual localization of proteins increases their cellular functions without increasing the number of genes and is widespread within eukaryotes (4). Dual targeting mechanisms include: (i) alternative transcription start sites that lead to cytosolic and mitochondrial isoforms of the protein as has been described for the yeast ValRS and HisRS (5,6); (ii) alternative *cis*-splicing as is the case for the Ca²⁺/calmodulin-dependent protein kinase, which is dually targeted to the nucleus and the cytosol (7); (iii) alternative translation initiation at two different AUG start codons within one mRNA as it has been shown for the modification enzyme Mod5 in yeast (8) and the DNA polymerase g2 in plants (9), (iv) ambiguous import signals, which are used to achieve dual localization to plastid and mitochondria in the case of some plant aaRSs (10) and (v) retrograde movement of proteins after partial mitochondrial import as described for the yeast fumarase (11).
Dual localization of proteins has also been described in protozoa. In the apicomplexan parasite *Toxoplasma gondii* a superoxide dismutase is dually localized to the mitochondrion and the apicoplast, the mechanism, however, remains enigmatic (12). For the *T. gondii* hypoxanthine–xanthine–guanine phosphoribosyltransferase, which is found in the inner membrane complex and the cytosol, the mechanism for dual localization has been shown to involve alternative cis-splicing (13). In trypanosomatids only a few examples of dually targeted proteins are known. They include the phosphodiesterase TbrPDEB2, which localizes to the cytosol and flagellum (14), the LYT1 protein in *Trypanosoma cruzi*, which localizes to the plasma membrane and to the base of the flagellum (15) and glutaminyl- and glutamyl-tRNA synthetases whose cytosolic and mitochondrial activities are encoded by a single gene (16). In all cases, however the mechanism of dual localization is not well understood.

The majority of protein-coding genes in trypanosomatids are transcribed polycistronically by RNA Polymerase II (17,18). Maturation of mRNAs requires trans-splicing of a 39-nt capped leader sequence to the 5' (17,18). Maturation of mRNAs requires trans-splicing of a 39-nt capped leader sequence to the 5'-end of the transcript followed by polyadenylation of the 3'-end (19,20). Recently, it was shown that trans-splicing produces a large number of spliced isoforms many of which change in abundance during the life cycle of the parasite (21–23). The functional consequence of the alternative trans-spliced isoforms, although speculated on, has not been experimentally tested.

Here, we demonstrate for the first time how alternative trans-splicing determines the dual localization of a trypanosomal aaRS to the cytosol and the mitochondrion.

**Experimental Procedures**

**Bioinformatics**

The alignment from 38 taxa was done using T-Coffee (24). After alignment of the sequences, ambiguous regions with gaps were removed by hand such that no gap positions were allowed in the final alignment. All regions with contiguous non-conserved positions larger than eight were also removed. The phylogenetic reconstruction of the IleRS was done using the maximum likelihood method as implemented in the PhyML program (v3.0 aLRT) (25). Reliability for the branching order was assessed using the aLRT test (26). All other informatics analysis were done using Bioperl and the EMBOSS software package (27,28).

**Cell culture**

Procyclic *Trypanosoma brucei* strain 29–13 and the corresponding transgenic cell lines were grown in SDM79 supplemented with 15% FCS, 25 μg/ml hygromycin, 15 μg/ml G-418, 10 μg/ml blasticidin, at 27°C, and harvested at 1.0–3.5 × 10⁷ cells per ml. Procyclic *T. brucei*, strain Lister 427 was grown in SDM79 supplemented with 10% FCS.

**Transgenic cell lines**

RNAi cell lines were produced by using pLew100-based stem loop constructs containing the puromycine resistance gene (30,31). As inserts we used a 473-bp fragment (nucleotides 2013–2486 of the ORF Tb927.10.9190) for the ORF-IleRS RNAi cell line and a 448-bp fragment (nucleotides 307–141 of ORF Tb927.10.9190) for the 5'-UTR-IleRS RNAi cell line. Tagging of IleRS gene with a triple HA-epitope was done as reported previously (32). The resistance marker of pMOTag3H was exchanged for a blasticidin resistance cassette. Transfection of *T. brucei* and selection with antibiotics, cloning and induction with tetracycline was done as described previously (33).

**Cell fractionation by digitonin**

Fractionation of the HA-tagged IleRS-expressing cells was done by digitonin extraction and subsequent centrifugation as described previously (34). Total, cytosolic and mitochondrial fractions corresponding to 6 × 10⁶ cell equivalents were separated by SDS–PAGE and analyzed by immunoblotting.

**Acid gel analysis of acylated and deacylated tRNA_{ile}**

Purification of the crude mitochondrial fraction was done by a modification of the digitonin extraction protocol described above, but including an RNase A treatment of the isolated organelles essentially as described previously (35). RNA was isolated as described in (36). Total RNA corresponding to 1 × 10⁷ cell equivalents and isolated mitochondrial RNA corresponding to 3.8 × 10⁸ cell equivalents was separated on a long acidic gel in order to discriminate acylated from deacylated tRNAs (34,37). The tRNA containing region was analyzed by northern blotting using a labeled oligonucleotide (tRNA_{ile}, 5'TGC TCCGGCGGGGTCGAA3'; tRNA_{Met-e}, 5'GACTGCG CCACGCTCGC3') as described previously (35).

**mRNA decay assay and northern blotting**

For the mRNA decay assay cells were grown to a density of 5 × 10⁶ cells/ml and treated with 5 μg/ml of actinomycin D. A total of 10⁸ cells were harvested after 0, 30, 60, 90 min, and re-suspended in Trizol (Invitrogen, 1 ml/1 × 10⁸ cells). The RNA was purified by ethanol precipitation. The resulting fraction was treated with DNaseI, extracted with phenol/chloroform and precipitated with ethanol before it was stored at −80°C for subsequent northern blot analysis. For qPCR 1 μg RNA was used to synthesize cDNA using random hexamer primers with the DyNAmo™ cDNA Synthesis Kit (Finnzymes). A total 30 ng of random hexamer cDNA from each time point was analyzed (in triplicates) using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems). Relative amounts of both the long (forward: 5'CGAAG TTGGAGTAAATAT3'; reverse: 5'GATGTCAAGCA ACAATGTATA3') and short (forward: 5'TTGAATGC ATCGATATCCT3'; reverse: 5'TGTATATATTGCA TCAAAAGA3') Ile-RS spliced variants were calculated and normalized to 18S rRNA (primer forward: 5'GGGA
The assay was performed on four biological replicates. For the RNAi experiments and the detection of tRNAs, cells were grown to 1/10^7 cells/ml. For northern blots, 10–20 μg of total cellular RNA was resolved for 4–5 h at 100 V on a 1–1.4% polyacrylamide gel. The RNA was transferred to nitrocellulose membrane (Roche) and UV cross-linked. Subsequently, the membrane was incubated overnight with an α-32P-CTP labeled probe. Next, the membrane was washed, exposed to a phosphor imager screen and analyzed with a Storm 820 Phosphorimager (Amersham Biosciences). To normalize for equal loading of the samples the membrane was stripped by boiling in 0.1% SDS to remove the previous probe and then re-hybridized to a γ-32P-ATP-labeled 18S rDNA oligonucleotide.

RESULTS

A single IleRS gene gives rise to two differentially trans-spliced mRNAs

The analysis of the T. brucei genome revealed a single isoleucyl-tRNA synthetase (IleRS) gene (Tb927.10.9190) of the eukaryotic type (Figure 1A). Previous expression profiling by spliced leader trapping [SLT, (22)] predicted two alternative trans-spliced variants of the T. brucei IleRS. The more abundant, shorter splice variant excluded translation from the annotated first AUG of the predicted ORF but would allow use of the second AUG, 44-nt downstream of the second splice acceptor site (Figure 1B). The longer splice variant, on the other hand, would permit use of the first AUG as the translation start. ClustalW alignment of the amino-terminal regions of the trypanosomal IleRS from T. brucei and T. cruzi with other cytosol-specific eukaryotic IleRS from the corresponding phylogenetic tree.
IleRS is essential in insect form *T. brucei*

In order to study the function of the trypanosomal IleRS a RNAi cell line targeting the region of the ORF that is common between the two splice variants was produced. The cell line was termed ORF-IleRS RNAi; induction with tetracycline led to a significant growth defect after 2 to 3 days, which was concomitant with the loss of both IleRS mRNA splice variants (Figure 3B). Immunoblot analysis of an ORF-IleRS RNAi cell line that simultaneously expresses the HA-tagged IleRS shows that both isotypes of the protein become ablated in the cytosolic and the mitochondrial fractions of induced cells. The abundance of marker proteins was not affected, however (Figure 3C). To determine the biochemical phenotype of the ORF-IleRS RNAi cell line, we isolated total and mitochondrial RNA from uninduced and induced cells. The RNAs were resolved on long acidic urea polyacrylamide gels to determine the ratio of uncharged to charged tRNAs\(^{\text{Ile}}\)\(^{\text{charged}}\)\(^{\text{charged}}\). The corresponding northern blot in Figure 3D shows that simultaneous ablation of both IleRS isotypes results in the accumulation of uncharged tRNAs\(^{\text{Ile}}\) in the cytosol as well as the mitochondrion. In summary these results show that the single trypanosomal IleRS gene is essential for normal growth and for charging of cytosolic and mitochondrial tRNAs\(^{\text{Ile}}\).

Long splice variant of the IleRS gene encodes the mitochondrial isoform

In order to test the hypothesis that the longer splice variant encoded the mitochondrially targeted IleRS a RNAi cell line targeting the 5'-untranslated region (5'-UTR) specific to the long splice variant was produced. The cell line was termed 5'-UTR-IleRS RNAi. In contrast to the ORF-IleRS RNAi cell line, induction with tetracycline did not cause a growth defect (Figure 4A), although the long splice variant IleRS mRNA was specifically lost after 2 days as shown by northern blotting (Figure 4B). Immunoblotting of total cellular and mitochondrial extracts from uninduced and induced 5'-UTR-IleRS RNAi cell lines that simultaneously express HA-tagged IleRS showed a loss of the mitochondrially localized larger protein isoform, while the abundance of the cytosol-specific smaller protein isoform remained unchanged (Figure 4C). This finding was supported by the observation that uncharged tRNAs\(^{\text{Ile}}\) accumulated specifically in the mitochondrial fraction (Figure 4D). Thus, these results show that the long splice variant encodes an active isoform of IleRS that specifically localizes to mitochondria.

Differential abundance of the IleRS splice isoforms and their mRNA stability

Depending on the method used we found the short splice variant to be three to four times more abundant than the long splice variant (northern 3.93 ± 0.63, standard error of the mean, SEM; SLT 3.25). In order to test the hypothesis that differential stability of the alternatively spliced IleRS mRNAs determined their differential abundance we measured mRNA stability using qPCR (Figure 5 and extensions and is with 123.5 kDa, about 2.4 kDa smaller than the processed mitochondrial isoform.

**Differential localization of the two IleRS isoforms**

To determine the localization of the trypanosomal IleRS we prepared a transgenic cell line that constitutively expresses a variant of IleRS containing a triple hemagglutinin (HA) tag at its carboxy-terminus. Immunofluorescence microscopy showed that the tagged protein was distributed throughout the cell (Figure 2A). Immunoblot analysis using anti-HA antibody revealed two bands of ~125 kDa, corresponding to the predicted size of the two IleRS isoforms in the total cellular protein fraction (Figure 2B). Biochemical fractionation by digitonin extraction and subsequent centrifugation of cells expressing the tagged IleRS revealed a co-fractionation of the larger isoform with the mitochondrial heat shock protein 70 (mHSP70), while the smaller isoform co-fractionated with the cytosol-specific eukaryotic elongation factor 1a (eEF1a; Figure 2B). In summary these results show that the large isoform of IleRS is exclusively mitochondrial, whereas the smaller one is found only in the cytosol.

**Figure 2.** The two trypanosomal IleRS isoforms are differentially localized. (A) Immunofluorescence of a *T. brucei* cell line expressing IleRS carrying a carboxy-terminal triple HA-tag. The cells were stained for DNA using 4′-6-diamidino-phenylindole, for a subunit of ATPase serving as a mitochondrial marker and with a monoclonal antibody recognizing the HA-epitope. The outlines of the cells have been traced in the phase contrast channel and are projected on the fluorescent pictures. (B) Immunoblot analysis of total cellular (T), crude mitochondrial (M) and crude cytosolic extracts (C) for the presence of HA-tagged IleRS. Eukaryotic elongation factor 1-a (eEF1a) and mitochondrial heat shock protein 70 (mHSP70) served as cytosolic and mitochondrial marker, respectively.

Differential abundance of the IleRS splice isoforms and their mRNA stability

Depending on the method used we found the short splice variant to be three to four times more abundant than the long splice variant (northern 3.93 ± 0.63, standard error of the mean, SEM; SLT 3.25). In order to test the hypothesis that differential stability of the alternatively spliced IleRS mRNAs determined their differential abundance we measured mRNA stability using qPCR (Figure 5 and...
Figure 3. IleRS is essential for growth of procyclic *T. brucei* and is responsible for the formation of isoleucyl-tRNA<sub>Ile</sub> in the cytosol and the mitochondria. Analysis of the RNAi cell line targeting the ORF common to both IleRS splice variants (ORF-IleRS). (A) Growth curve in presence and absence of tetracycline (±Tet). (B) Northern blot of total RNA from uninduced and induced cells probed for IleRS mRNAs 2 days post-tetracycline induction; rRNAs were used as loading control. (C) Western blot analysis detecting HA-tagged IleRS. Extracts from total cellular (T), crude mitochondrial (M), and crude cytosolic protein (C) were prepared on Day 3 post-induction with tetracycline; The mitochondrial heat shock protein (mHSP70) and the cytosolic elongation factor 1 alpha (eEF1a) serve as mitochondrial and cytosolic markers, respectively. (D) Northern blot analysis probed for tRNA<sub>Ile</sub> of total and mitochondrial RNA 5 days post-tetracycline induction. The RNA fractions were resolved on a long acidic urea gel to separate aminoacylated (acyl.) from deacetylated (deac.) tRNAs; (d) chemically deacetylated total RNA sample probed for tRNA<sub>Ile</sub> (Ile) and elongator tRNA<sub>Met</sub> (Met-e).

Figure 4. The long isoform of IleRS is responsible for Isoleucyl-tRNA<sub>Ile</sub> formation in the mitochondria. Analysis of the RNAi cell line targeting the 5′-UTR specific to the long splice variant (5′-UTR-IleRS). (A) Growth curve in the presence and absence of tetracycline (±Tet). (B) Northern blot of total RNA probed for IleRS mRNAs 2 days post-tetracycline induction; rRNAs were used as loading control. (C) Western blot analysis detecting HA-tagged IleRS. Extracts from total cellular (T), crude mitochondrial (M) and crude cytosolic protein (C) were prepared on Day 3 post-induction with tetracycline; The mitochondrial heat shock protein (mHSP70) and the cytosolic elongation factor 1 alpha (eEF1a) serve as mitochondrial and cytosolic markers, respectively. (D) Northern blot probing for tRNA<sub>Ile</sub> using total and mitochondrial RNA isolated 5 days post-induction. The RNA fractions were resolved on a long acidic urea gel to separate aminoacylated (acyl.) from deacetylated (deac.) tRNAs; (d) chemically deacetylated total RNA sample probed for tRNA<sub>Ile</sub> (Ile) and elongator tRNA<sub>Met</sub> (Met-e).
splicing of mRNA precursors during the mRNA decay process we pre-incubated the cells with the splicing inhibitor sinefungin ([41]; Enzo Life Sciences). Under these conditions the above described faster decay of the long splice variant was confirmed (t50 long: 42.6 ± 4.8 SD; short 64.8 ± 6.6 SD; Supplementary Figure S3). In conclusion we could demonstrate an increased turnover of the long splice variant compared to the short splice variant that in part explained the differential in vivo abundance of the two splice isoforms.

DISCUSSION

Dual localization of aaRSs has been reported in a number of organisms including yeast and plants (5,6,10); however, this is the first report identifying differential trans-splicing as the underlying mechanism. We have shown that RNAi against the ORF of the trypanosomal IleRS mRNAs causes growth arrest and leads to the loss of both spliced variant mRNAs and the corresponding protein isoforms. As a consequence it also leads to an accumulation of uncharged tRNA\textsuperscript{Ile} in the cytosol and the mitochondrial. RNAi targeting the 5’-UTR of the longer splice variant, on the other hand, resulted in a specific loss of the long variant mRNA and the mitochondrial isoform of IleRS. In agreement with the loss of mitochondrial IleRS we detected an accumulation of uncharged tRNA\textsuperscript{Ile} in the mitochondrion, while the cytosolic tRNA\textsuperscript{Ile} population remained unaffected. Despite the loss of charged tRNA\textsuperscript{Ile} in the mitochondrion we did not detect any growth defect. This was surprising since mitochondrial translation is expected to be essential in insect stage T. brucei (34); however, it does not affect our conclusions regarding the role of trans-splicing for dual targeting. Possible explanations for the lack of a growth defect are (i) that the small amount of mitochondrial IleRS that is still present in induced RNAi cells is sufficient to allow for normal growth and/or (ii) that mitochondrial translation is supported by import of tRNA\textsuperscript{Ile} that has been aminoacylated in the cytosol. In the latter case the tRNA could only be used for one round of translation elongation, a scenario that appears to apply to the apicomplexan T. gondii, which lacks mitochondrial aaRSs altogether (42).

The differential abundance of the two splice variants of the IleRS mRNA was reflected in the amount of protein detected in the cytosol and the mitochondrion. RNA stability measurements of the short splice variant showed a biphasic decay pattern, which consequently could not be displayed in a single half-life. We thus decided to analyze the time from addition of actinomycin D to the point where 50% of the transcript had decayed. Using this approach, we could show that the short splice variant is significantly more stable than the long splice variant. Thus the difference in steady state transcript abundance is due, at least in part, to decreased stability of the longer splice variant, although we cannot exclude that other RNA processing events, including splicing, are also involved in the differential abundance of the two splice isoforms.

Since the only obvious difference between the two transcripts is the 5’-UTR we hypothesize that there is a destabilizing and/or stabilizing element in this region; however, additional work is required to define the postulated sequence elements.

The evolution of dual targeting of aaRSs, and the dual targeting of cytosolic tRNAs give interesting insights into the evolution of mitochondrial tRNA import. Trypanosomatids are excellent examples for this, since essentially all aaRSs are dually targeted, and all mitochondrial tRNAs are imported from the cytosol. The bacterial endosymbiots that gave raise to mitochondria would have had a complete set of aaRSs implying that early eukaryotes (at least temporarily) had two complete sets of these enzymes (1). During the transformation of the endosymbiont into an organelle most of its genes were lost or transferred to the nuclear genome (43). In one possible scenario some cytosolic aaRSs may then have acquired the capability to be dually targeted to the cytosol and the mitochondrion. As a consequence loss of the bacterial-type aaRS genes could be tolerated, provided that the dually targeted aaRSs were capable of aminoacylating cytosolic and mitochondrial tRNAs. In a subsequent step the cells evolved mechanisms to import...
the cytosolic tRNAs, which consequently would have led to the complete loss of bacterial type tRNA genes as is the status quo in trypanosomes today. However, the order of events is unknown and it is equally possible that the loss of mitochondrial tRNA genes and the import of the corresponding cytosolic tRNAs might have preceded the evolution of dual targeting of aaRSs.

Here, we show that alternative trans-splicing is one mechanism by which dual targeting of the aaRSs has evolved in T. brucei. Recent genome-wide transcriptome studies revealed widespread alternative trans-splicing events in trypanosomatids including T. brucei, T. cruzi and Leishmania [44; Ochsenreiter unpublished]. This is not too surprising since the minimal requirements for trans-splicing are quite relaxed (45). A polypyrrimidine tract (Poly Y) of at least 10 nt followed by a downstream (+5–20 nt) splice acceptor dinucleotide (mostly AG) seems to be sufficient to recruit the splicing machinery and trans-splice the 39-nt leader sequence to the 5’-end of the mRNA. In the genomes of T. brucei (21–23) and its sister species T. cruzi (Ochsenreiter unpublished) the average number of alternative splice acceptor sites ranges from two to three per gene. Thus, alternative trans-splicing is a particularly suitable mechanism to achieve dual localization of trypanosomal proteins. We therefore suggest that for most trypanosomal tRNA/aaRS pairs, due to the ease with which it can evolve, dual targeting of the enzymes by differential trans-splicing may have preceded import of tRNAs and thus have been the driving force for the subsequent loss of mitochondrial tRNA genes.

According to this scenario we would expect differential trans-splicing to be a mechanism by which several trypanosomal aaRSs are dually targeted. In total there are 17 trypanosomal aaRSs that are predicted to be dually localized. In the present study, we show that for IleRS differential trans-splicing creates a long variant carrying a presequence that is imported into mitochondria and a shorter more abundant variant that remains in the cytosol. A bioinformatic analysis of data published by three different groups shows that a similar situation might apply to AsnRS, ProRS, GluRS and GlnRS [Supplementary Table S1, (21–23)]. Moreover, we find a second set of two aaRSs whose mRNAs are also differentially trans-spliced but where the major splice site is upstream of the first AUG. In these cases the longer transcripts encode the cytosolic proteins, whereas the shorter versions use the second AUG for translation initiation, uncovering a MTS which is masked in the long form (Supplementary Table S1). Even though mitochondrial presequences can, in principle, be predicted bioinformatically we find a predicted presequence only for the short version of the SerRS. However, this might be explained by the fact that trypanosomal proteins often have very short presequences that cannot be reliably predicted (46).

While more experiments are needed, these results suggest that in trypanosomes differential trans-splicing is an important mechanism by which aaRSs are dually targeted. Thus, the extensive dual targeting of trypanosomal aaRSs might have been a factor that contributed to the complete loss of mitochondrial tRNA genes that is compensated for by import of cytosolic tRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3, Supplementary Table 1.

ACKNOWLEDGEMENTS

The authors would like to thank Isabel Roditi for critically reading the article.

FUNDING

Swiss National Science Foundation (SNF) (3103A_121937 to J.R., A.S.); SNF (3103A_125194 to Y.W., T.O.). Funding for open access charge: Swiss National Science Foundation.

Conflict of interest statement. None declared.

REFERENCES


