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Review

The MTERF family proteins: Mitochondrial transcription regulators and beyond

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ABSTRACT

The MTERF family is a wide protein family, identified in Metazoa and plants, which consists of 4 subfamilies named MTERF1–4. Proteins belonging to this family are localized in mitochondria and show a modular architecture based on repetitions of a 30 amino acid module, the mTERF motif, containing leucine zipper-like heptads. The MTERF family includes the characterized transcription termination factors human mTERF, sea urchin mtDBP and *Drosophila* DmTTF. *In vitro* and *in vivo* studies show that these factors play different roles which are not restricted to transcription termination, but concern also transcription initiation and the control of mtDNA replication. The multiplicity of functions could be related to the differences in the gene organization of the mitochondrial genomes. Studies on the function of human and *Drosophila* MTERF3 factor showed that the protein acts as negative regulator of mitochondrial transcription, possibly in cooperation with other still unknown factors. The complete elucidation of the role of the MTERF family members will contribute to the unraveling of the molecular mechanisms of mtDNA transcription and replication.

1. The MTERF protein family

Oxidative phosphorylation is a fundamental process for eukaryotic cells. It takes place in mitochondria and is carried out by an enzymatic apparatus consisting of polypeptides encoded by both nuclear and mitochondrial genes. The production and assembling of these subunits require the coordinated expression of the two genomes that is based on signalling pathways between nucleus and cytoplasm [for review see refs. 1,2]. A consistent amount of studies has been focused on the mechanisms of transcription of mtDNA encoded genes. Although the emerging picture is far to be exhaustive, we have now reached a good level of knowledge that highlights some peculiar aspects of this process [for review see refs. 3,4]. The basal transcription apparatus consists of a phage-like mitochondrial RNA polymerase (mtRNAP) and the activation factors TFAM and TFB1/2M. Recently, several studies have been focused on the members of the MTERF protein family, whose founder member is human mTERF, the first characterized mitochondrial transcription termination factor. MTERF proteins have been identified in Metazoa and plants, but not in fungi. The evolutionary relationships of MTERF proteins have been described in Metazoa [5]; in vertebrates three mTERF paralogues have been found which, together with mTERF, identify four sub-families, named MTERF1 through MTERF4. Sub-families MTERF1, which includes the termination factor mTERF, and MTERF2 are unique to vertebrates; sub-families MTERF3 and MTERF4 also include members from insects and worms, thus probably representing the ancestral MTERF genes in Metazoa. Some other MTERF proteins do not apparently belong to any of the defined sub-families (see 2.4).

The characterized proteins of this family are localized in mitochondria and have a modular architecture due to the repetition of a variable number of a 30 amino acid motif named mTERF motif [6]. The distinctive feature of this motif is the conservation of a proline residue at position 8, and of a leucine or another hydrophobic amino acid at positions 11, 18 and 25; this suggests the existence of three leucine zipper (LZ)-like heptads X_3LX_3 inside the mTERF motif.

This paper focuses on the MTERF family members in animal organisms. We will review the structure and function of the characterized proteins of this family and will also include some unpublished results from our laboratory. All together, the information here reported brings new insights into the mitochondrial transcription regulation and also show that some proteins of this family serve multiple roles, possibly related to the peculiar organization of the mitochondrial genomes in different organisms.

2. The mitochondrial transcription termination factors

2.1. mTERF, the human transcription termination factor

Human mTERF is a 342-amino acid protein (mature form) that was shown to arrest *in vitro* mitochondrial RNA polymerase (mtRNAP) progression by specifically binding a 28 nt sequence located immediately

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downstream of the 3′ end of the 16S rRNA gene, within the $tRNA^{Leu(UUR)}$ gene [7,8]. On the basis of such properties mTERF was proposed to control mtDNA transcription. In mammals H-strand transcription proceeds through two partially overlapping units. One of them, the ribosomal unit, starts at the initiation site H1 and ends at the 3′ end of the 16S rRNA gene. It is responsible for the synthesis of the two rRNAs, of $tRNA^{Phe}$ and $tRNA^{Val}$. The second transcription unit begins at the initiation site H2 and produces a polycistronic molecule covering almost the entire H-strand. It was reported that H1 initiation site is more frequently used than H2 site, resulting in a higher rate of rRNAs transcription [9,10]. Transcription of the ribosomal unit is linked to a termination event operated by mTERF through its binding to the 3′ end of the 16S rRNA gene. This would account for the higher (40–50 folds) level of rRNAs with respect to mRNAs. Asin-Cayuela et al. [11] demonstrated that recombinant mTERF, bound to its canonical binding site, is able to promote termination in a bidirectional way, with an even higher efficiency when the mtRNAP transcribes in the L-strand direction. This event is consistent with the absence of genes downstream of the factor binding site.

Recent evidence indicates that the role of mTERF is probably more complex than initially thought. By using an artificial rDNA template bearing the termination and H1 initiation transcription sites, Martin et al. [12] showed that native mTERF causes not only transcription arrest but also transcription activation. This is due to the ability of one single mTERF molecule to simultaneously bind the well-known termination site and a novel site placed in the H1 region. Such double interaction causes the looping-out of the rDNA that would promote the recycling of the transcription machinery through the direct delivery of the mtRNAP from the termination site to the H1 initiation site. This model would account for the higher rate of transcription of rDNA compared to mRNA genes, thus providing the basic molecular mechanism for the regulation of the rRNA/mRNA ratio.

In spite of the plenty of information obtained by *in vitro* studies, there are no *in vivo* experimental evidences supporting the termination role of mTERF. In fact, *in vivo* alteration of mTERF level does not seem to affect the content of mitochondrial transcripts [13]. Information on the *in vivo* role of mTERF are not even provided by the analysis of cells harbouring the A3243G MELAS mutation. This nucleotide substitution, which falls within the mTERF binding site, was shown *in vitro* to reduce the affinity of mTERF to DNA and to cause a decrease in termination efficiency [14]. However, *in vivo* footprinting analysis of mutant and wild type mtDNAs did not reveal any difference in the occupancy of mTERF binding site [15]. This finding explains why in MELAS mutation carrying cells the ratio between transcripts mapping upstream and downstream of the termination site remains unchanged [16]. Recent evidences indicate that mTERF, in addition to its role in transcription, may play a role in modulating mtDNA replication. In human cultured cells over-expressing mTERF, the protein level modulates replication pausing at the canonical binding site as well as at novel contacted sequences [13]. These observations represent open questions on the role of human mTERF, which still await definite answers.

2.2. mtDBP, the sea urchin transcription termination factor

The different mtDNA gene organization in invertebrates compared to vertebrates prompted interest on the basic transcription mechanisms and on the functioning of the transcription factors, which might have evolved to meet structural changes occurred in the genomes during evolution. In particular, many studies have been focused on two experimental systems: the sea urchin and the *Drosophila melanogaster*.

mtDNA from sea urchin *Paracentrotus lividus* contains the same genes found in vertebrates but with a different arrangement [17]. The ribosomal genes are separated by a region of 3.3 kbp containing a cluster of 15 tRNA genes and the genes for ND1, ND2; the main non-coding region, NCR, is very short (132 bp) and is located in the tRNA gene cluster downstream of the 12S rRNA gene. In this organism mitochondrial transcription seems to occur via multiple and partially overlapping transcription units [18], but the precise initiation sites have not been defined to date.

We characterized a 348-amino acid DNA binding protein, mtDBP, which binds with high affinity two sites of sea urchin mtDNA. The first is located in the NCR at the 3′ end of the short D-loop structure, the second comprises the 3′ ends of the oppositely transcribed ND5 and ND6 genes [19]. The observation that mtDBP displays a significant homology with human mTERF suggested that the sea urchin protein could possess a transcription termination activity [20]. This hypothesis was demonstrated by an *in vitro* transcription assay performed in the presence of the recombinant sea urchin proteins mtDBP and mtRNAP [21]. In this reconstituted system, termination of transcription in the NCR occurred in an mtDBP-dependent manner when the enzyme approached the protein binding site in the direction of L-strand transcription (that is, the direction of synthesis of the H-strand primer). Instead, when the polymerase encountered the protein binding site in the opposite direction, termination occurred independently of the presence of mtDBP, within the protein binding site (Fig. 1A). The conclusions are that sea urchin mtDBP acts as a polar termination factor and that mitochondrial transcription termination may take place by two alternative modes based on protein-dependent and sequence-dependent mechanisms.

The observation that the mtDBP target site in the NCR coincides with the 3′ end of the small sea urchin D-loop arose the question whether mtDBP might control the expansion of the D-loop. Loguercio Polosa et al. [22] demonstrated that the protein has also a contrahelicase activity, thus suggesting a role as negative regulator of replication. Furthermore, it was observed that H-strand readthrough transcription co-existing with the event of sequence-dependent termination at mtDBP binding site causes dislodging of mtDBP (Fig. 1B); this event, in turn, would cause abrogation of helicase impairment and possible resumption of mtDNA replication. Therefore, it is likely that mtDBP is the molecular device that regulates a possible interplay between mtDNA transcription and replication in sea urchin mitochondria.

2.3. DmTTF, the Drosophila transcription termination factor

Drosophila melanogaster mtDNA has a size of about 19.5 kDa, due to a large non-coding region of about 4.6 kbp mainly composed by A and T [23]. Its gene organization is markedly different from that of human and sea urchin genomes; in fact the genes on *Drosophila* mtDNA are almost equally distributed between the two strands and form four clusters that are located alternatively on the two strands (Fig. 2).

Early mapping data of discrete and precursor mitochondrial RNAs suggested that *Drosophila* mtDNA could be transcribed by multiple transcription units starting in correspondence of the 5′ end of the gene blocks [24]. In addition, several transcription termination sites at the end of these blocks were hypothesized. We have identified and characterized DmTTF, a *Drosophila* homologue of human mTERF and sea urchin mtDBP [25]. The protein binds two homologous short non-coding sequences located at the end of blocks of genes transcribed in opposite direction. One sequence is placed between $tRNA^{Glu}$ and $tRNA^{Phe}$ genes; the other is placed between $tRNA^{Ser(UCN)}$ and ND1 genes. Interestingly, the location of these sequences coincides with two transcription termination sites previously predicted [24]. All these observations suggested for DmTTF a role as transcription termination factor. Such function was confirmed by assessing the capability of the recombinant protein to arrest *in vitro* mitochondrial transcription in the presence of human mtRNAP and a chimeric DNA template containing human HSP promoter and DmTTF binding site [26].

The role of DmTTF was subjected to a detailed *in vivo* analysis by our group. As a first step in this investigation, we analysed a DmTTF knock-down phenotype produced in D.Mel-2 cells by RNA interference

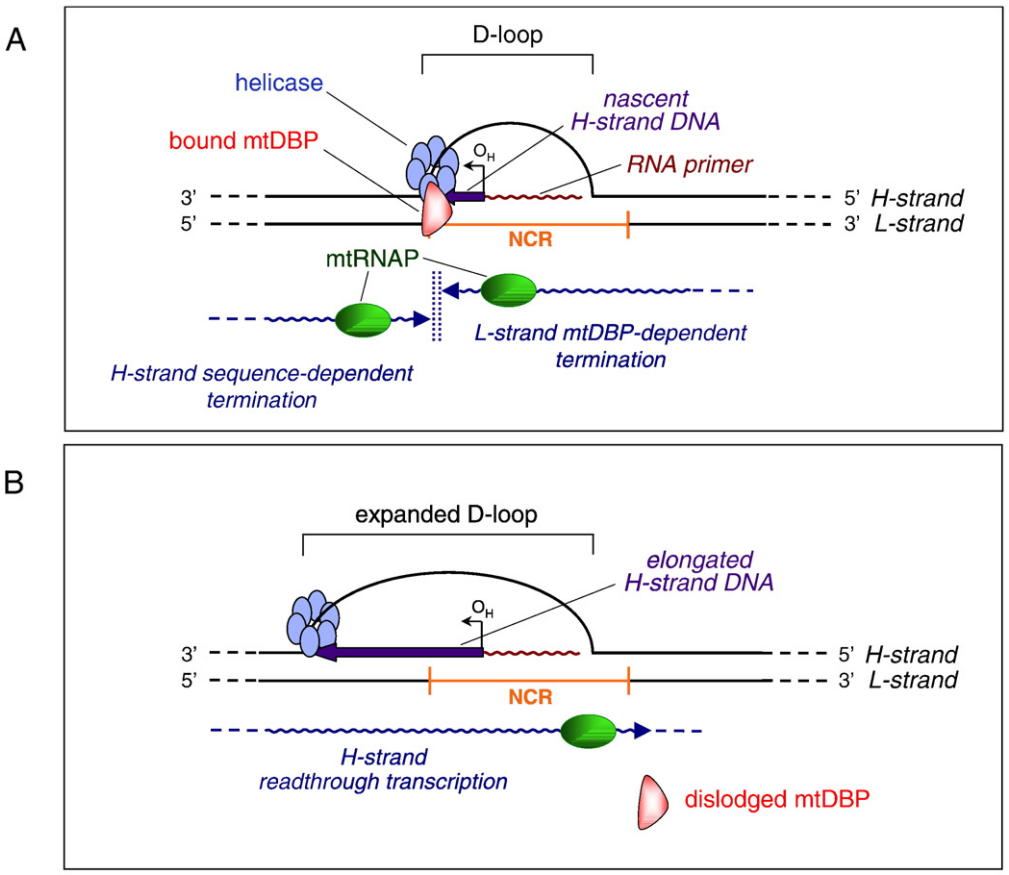

Fig. 1. Schematic diagram representing the role of mtDBP in transcription and replication of sea urchin mtDNA. (A) The region of *P. lividus* mtDNA containing the main non-coding region (NCR) with bound mtDBP is shown. Arrowed wavy lines below the scheme represent transcripts arrested by sequence-dependent and mtDBP-dependent termination events, with points of transcription arrest indicated by vertical dotted lines. The scheme also shows that H-DNA synthesis is arrested by the contrahelicase activity of DNA-bound mtDBP, thus forming the triple-stranded structure of the D-loop. The nascent chain is composed of the RNA primer (70–80 nt) and a short DNA tract (about 20 nt). (B) H-strand transcription through mtDBP–DNA complex (readthrough transcription) in the opposite direction of H-strand replication causes dislodging of mtDBP; this relieves the block to helicase, H-DNA synthesis is restarted and the D-loop is expanded.

(RNAi) [27]. RNase protection experiments indicated that DmTTF depletion did not affect the 3′ end formation of the $tRNA^{Ser(UCN)}$ molecule that maps just upstream of one of the protein binding sites. This implies that the termination factor is not involved in the generation of the 3′ end of transcripts.

A detailed characterization of the steady-state level of sense and antisense transcripts by Real Time RT-PCR showed that in knock-down cells the level of the transcripts located downstream of DmTTF binding sites was higher than in the control (see Fig. 2), a result in agreement with the role of DmTTF as transcription termination factor. This finding strongly indicates that DmTTF depletion relieved the block to transcription and confirms that *in vivo* the protein bound to both sites arrests the progression of mtRNAP moving on both strands. Surprisingly, DmTTF depletion decreased the level of transcripts of genes mapping between the AT-rich region and the protein binding sites (see Fig. 2). An interesting interpretation of this result is that DmTTF, similarly to mTERF, may also act as transcription activation factor, so that its depletion decreases transcription of those genes located downstream of the transcription promoters that are presumably placed in the AT-rich region. Alternatively, the decrease of transcription could be due to a reduced availability of mtRNAP molecules that are engaged in aberrant transcription extending beyond the termination sites.

We found that in D.Mel-2 RNAi cells DmTTF depletion did not alter mtDNA copy number; however, a direct involvement of the protein in mtDNA replication cannot be completely ruled out since the duration and penetrance of RNAi in our system could not be sufficient to reveal differences in mtDNA level, given the lower turnover rate of mtDNA as compared to mitochondrial RNAs.

Cotney et al. found that TFB2M over-expression in HeLa cells was accompanied by an increase in the level of TFB1M [28]. This observation suggests that perturbing the level of a factor could in principle induce an alteration in the level of other factors participating at the same process. Therefore, to confirm that all the described consequences of DmTTF depletion on mtDNA transcription were primary effects, we measured the level of transcription initiation factors TFAM and TFB2M in DmTTF RNAi cells by Real Time RT-PCR. The obtained results (data not shown) indicate that DmTTF knock-down does not alter the content of the initiation factors mRNAs.

The variation of mitochondrial transcript level caused by DmTTF depletion prompted us to investigate whether this alteration could be paralleled by a variation in the rate of synthesis of the corresponding polypeptides. To address this issue, we firstly measured by Real Time RT-PCR in DmTTF RNAi cells the level of the factor TFB1M that is known to influence mitochondrial protein synthesis in *Drosophila* due to its RNA methyltransferase activity [29]. We found no alteration of the TFB1M mRNA level (data not shown). Next, we used [S^{35}]-methionine pulse-labelling of mitochondrial polypeptides in D.Mel-2 DmTTF-depleted cells, whose results are shown in Fig. 3A. It appears that all the 13 mitochondrial translation products were detected by SDS gel electrophoresis in knock-down as well as in control cells, indicating that DmTTF depletion caused no qualitative alteration in the profile of mitochondrial polypeptides. However, the *de novo* mitochondrial protein synthesis was substantially affected on a

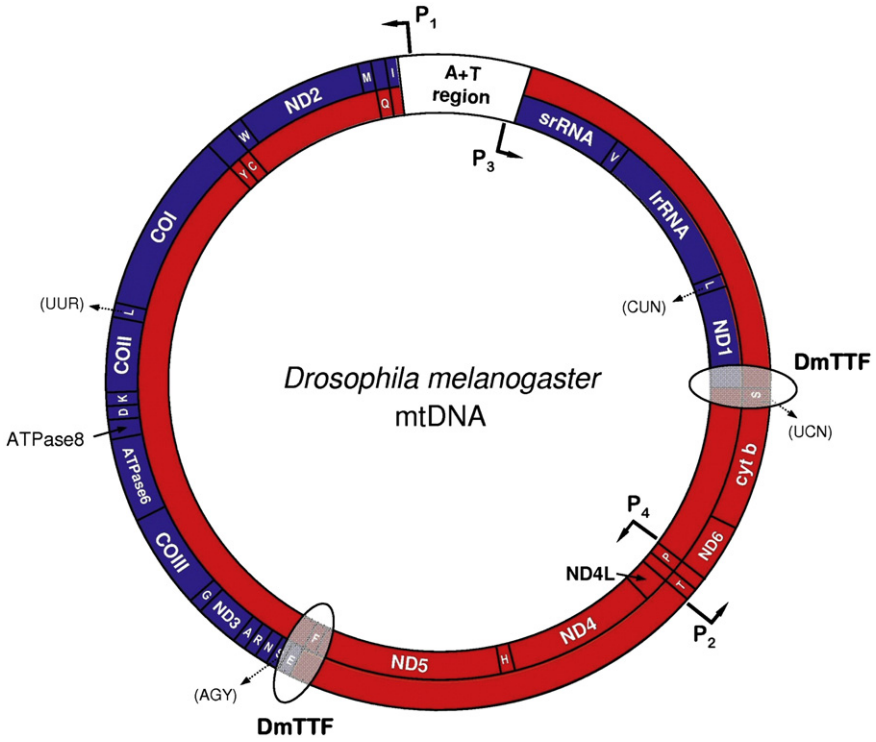

Fig. 2. Genetic map of *Drosophila melanogaster* mtDNA describing the effect of DmTTF depletion on mitochondrial transcription. The genes and the large non-coding A + T region are shown. The two ellipses indicate the bound DmTTF. In blue are shown the regions of mtDNA whose corresponding transcripts decrease in their level following DmTTF knock-down; in red are shown those regions whose transcript levels increase following DmTTF knock-down. The putative transcription promoters, indicated as P1, P2, P3 and P4, are shown on the molecule, with the arrows indicating transcription direction.

quantitative basis, with the labelling of six polypeptides decreasing by a factor of about 2, and the labelling of four polypeptides increasing by a factor ranging approx. from 1.3 to 3 (Fig. 3B). The polypeptides whose synthesis is mostly increased were ATP6 and ND4L, showing a 3.1- and 3.3-fold variation, respectively. Only for three polypeptides the labelling remained almost unchanged.

By comparing the level of *de novo* translation products with that of the respective mRNA levels in knock-down cells [see ref. 27], we

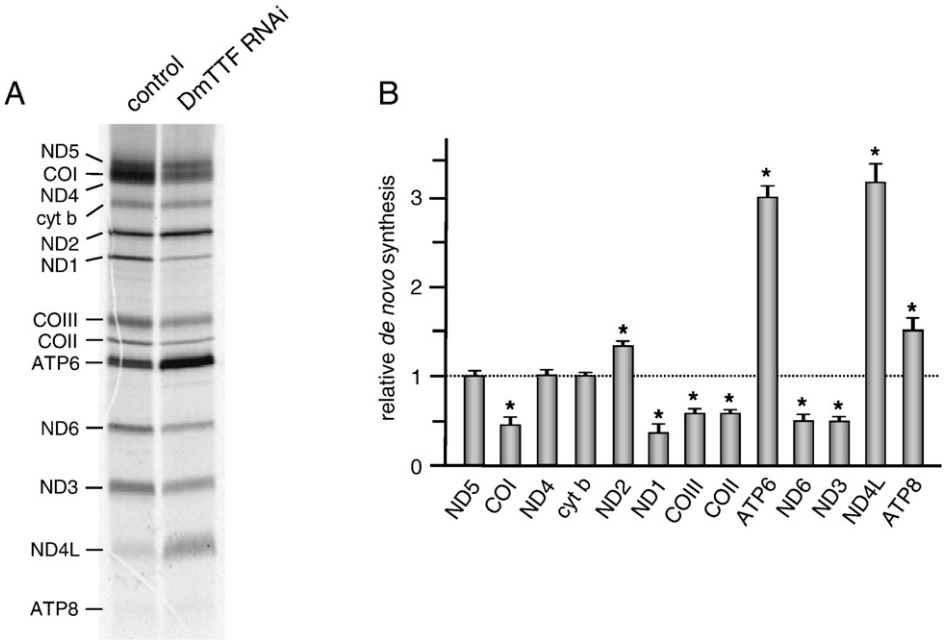

Fig. 3. Effect of DmTTF depletion on mitochondrial protein synthesis. (A) Mitochondrial translation products were labelled by incubating control and DmTTF RNAi D.Mel-2 cells with L-[^{35}S]-methionine for 90 min in the presence of emetine and cycloheximide as reported in [6]. Total cell proteins were fractionated on 15–20% exponential gradient SDS-polyacrylamide gel. Following phosphorimaging detection, the gel was stained with Coomassie Brilliant Blue for normalization (not shown). (B) Histogram showing the quantification of *de novo* polypeptide synthesis in RNAi cells relative to control cells, fixed as 1-value. Data represent the average of three independent experiments; standard deviations are indicated on the top of the bars. Statistically significant differences (paired two-tailed Student's *t*-test, $P<0.05$) are marked with an asterisk.

found that in general the decline in polypeptide synthesis was associated with a decrease in the level of the corresponding mRNAs. Instead, no apparent correlation was found for those polypeptides whose transcript level remained unchanged or were enhanced. It is likely that alteration of mitochondrial transcription induced by DmTTF depletion causes an imbalance of the relative mRNA translation. Imbalanced efficiency of mitochondrial protein synthesis was also observed by Cotney et al. in HeLa cells over-expressing TFB2M factor [28].

As a further approach in the *in vivo* study of DmTTF role, we monitored the effects of DmTTF over-expression on mitochondrial gene expression. The protein was over-expressed 10-fold in D.Mel-2 cells (Fig. 4A). The effect of such alteration on the level of mitochondrial transcripts was analysed by measuring the content of ND5 and cyt *b* mRNAs, two transcripts mapping downstream of DmTTF binding site on either strand (see Fig. 2). As expected, DmTTF over-expression diminished the level of both mRNAs, in agreement with the role of the protein as transcription terminator (Fig. 4B). Furthermore, pulse-labelling of mitochondrial polypeptides showed that the *de novo* synthesis was decreased for almost all polypeptides, including ND5 and cyt *b* (Fig. 4C, D). The described effects appear to be driven by DmTTF over-expression since no variation in the level of TFAM, TFB2M and TFB1M mRNAs was found (data not shown).

In a previous work [27] we proposed two models for *Drosophila* mtDNA transcription. One model assumed that transcription depends on one promoter only for each strand (P1 and P3, Fig. 2) located in the AT-rich region, with the two DmTTF–DNA complexes functioning mainly as attenuators. According to the other model, instead, transcription of each strand should require at least two promoters, each located at the 5′ end of each gene cluster (P1 + P2 and P3 + P4, Fig. 2); in this case DmTTF bound to $tRNA^{Ser(UCN)}$/ND1 site would function as terminator in both directions, while at the $tRNA^{Glu}$/$tRNA^{Phe}$ site the factor would act as terminator in one direction and as attenuator in the other. The here reported finding that the levels of cyt *b* and ND5 mRNAs, and presumably of ND6 and ND4 mRNAs, decreased following DmTTF over-expression suggests that they are transcribed from promoters P1 and P3, according to the first transcription model.

2.4. Structural and evolutionary analysis of the transcription termination factors

Interesting considerations regarding transcription termination factors derive from the analysis of the phylogenetic tree of MTERF family proposed by Linder et al. [5]. Although sea urchin mtDBP and *Drosophila* DmTTF were not included in any MTERF sub-family, they seem to be evolutionary more close to MTERF1 and MTERF2 than

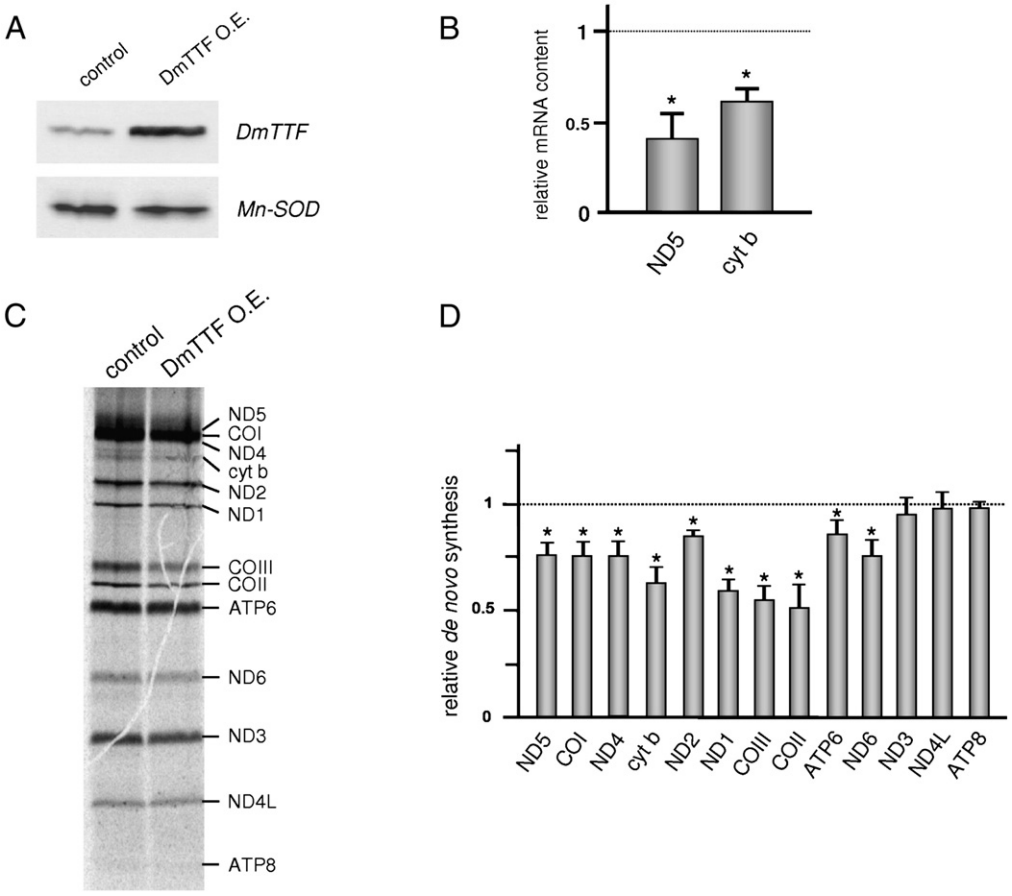

Fig. 4. Effects of DmTTF over-expression. (A) Immunoblot analysis of D.Mel-2 mitochondrial lysate. About 10^7 cells were cultured in antibiotic-free medium for 16 h prior to lipid-mediated transfection. Ten μg of plasmid pMt/Hy (control) or pMt/DmTTF/Hy (over-expression) were transfected in the presence of 50 μl of Cellfectin (Gibco-Invitrogen) and serum- and antibiotic-free medium according to the manufacturer's instructions. Cells were incubated for 24 h at 27 °C. After incubation, cells were induced with 500 μM $CuSO_4$ for 24 h at 27 °C. Two hundred μg of mitochondrial proteins were fractionated on a 12% SDS-polyacrilamide gel, transferred to PVDF membrane and incubated with polyclonal antibodies against DmTTF or against Mn-SOD (StressGen) for normalization. (B) Effect of DmTTF over-expression on the level of mitochondrial transcripts. After induction total RNA was extracted from control and DmTTF over-expressing cells. Relative quantification of mitochondrial ND5 and cyt *b* transcripts was performed by Real Time RT-PCR as described [27]. Bars indicate the relative content of transcripts in over-expressing with respect to control cells, fixed as 1-value. Standard deviations are indicated on the top of the bars and statistically significant differences ($P<0.05$) are marked with an asterisk. (C) Effect of DmTTF over-expression on mitochondrial protein synthesis, carried out as reported in Fig. 3A. (D) Histogram showing the quantification of *de novo* polypeptide synthesis in DmTTF over-expressing cells relative to control cells, fixed as 1-value. Data represent the average of three independent experiments; standard deviations are indicated on the top of the bars. Statistically significant differences ($P<0.05$) are marked with an asterisk.

MTERF3 and MTERF4 sub-families. Nevertheless, mTERF, mtDBP and DmTTF are less clustered than the members of MTERF3 sub-family; actually, multialignment of the amino acid sequence of the three termination factors shows 5% amino acid identity and 28% amino acid similarity that are quite low if compared with that of members of MTERF3 sub-family (see below). A database searching for DmTTF orthologues in other insects revealed intriguing features. In particular, the termination factor in the beetle *Tribolium castaneum* displays three deletions of about 20 residues, almost evenly distributed along the entire molecule. On the contrary, the factor from several mosquito species is about 200-amino acid longer than DmTTF, due to large insertions in the N-terminal portion of the protein.

These observations support the notion that the primary structure of the transcription termination factors is quite variable. Nevertheless, all these proteins display the conserved mTERF motifs that are typical of the MTERF family members. Fig. 5A, B shows the salient features of the motifs in human mTERF, sea urchin mtDBP and *Drosophila* DmTTF. All the three proteins contain a variable number of the mTERF motifs with some of them placed in corresponding position. The sequence alignment of the motifs shows, as already reported in this review, the conservation of a proline in position 8 and the presence of three LZ-like heptads (Fig. 5C). An extensive characterization of human mTERF performed by Attardi's group several years ago [8] pointed out the existence of LZ heptads and two N- and C-terminal basic domains; the leucine-zipper motifs were suggested to establish intramolecular interactions aimed at bringing the basic domains in close contact with DNA. A similar arrangement was also suggested for mtDBP; DmTTF, instead, lacks evident terminal basic domains despite the presence of the LZ motifs. It cannot be excluded that the LZ heptads of the termination factors, besides stabilizing the tertiary structure of the protein, could also be used to establish interactions with other factors.

2.5. The transcription termination factors: multiple function proteins

The emerging picture from the above reported observations highlights a structural variability of the transcription termination factors, which underlines their functional flexibility. Termination factors exert different roles in the transcription process. In particular, mTERF controls termination of transcription of the H-strand rDNA unit [7,8] and contributes to determine the higher level of rRNAs with respect to mRNAs; moreover the protein could also be responsible for L-strand transcription termination at the same canonical binding site [11]. Contrary to human mTERF, the invertebrate proteins mtDBP and DmTTF do not bind at the 3′ end of the ribosomal genes and do not seem to directly regulate rRNA level nor the 3′ end formation of the transcripts [19–21,25–27]. In invertebrates it is likely that termination factors act mainly in coordinating the passage of the transcription machineries moving on opposite strands; this would serve to avoid forced transcription pausing due to head-on collisions of the machineries, thus safeguarding the integrity of the genome [30–32]. It cannot be excluded, however, that such a role might also be played by the human factor mTERF.

In addition, the termination factors seem to perform a role in initiation of transcription. In particular, as already mentioned, mTERF seems to control transcription initiation by means of the looping mechanism [12]. Transcript quantification in knock-down cells

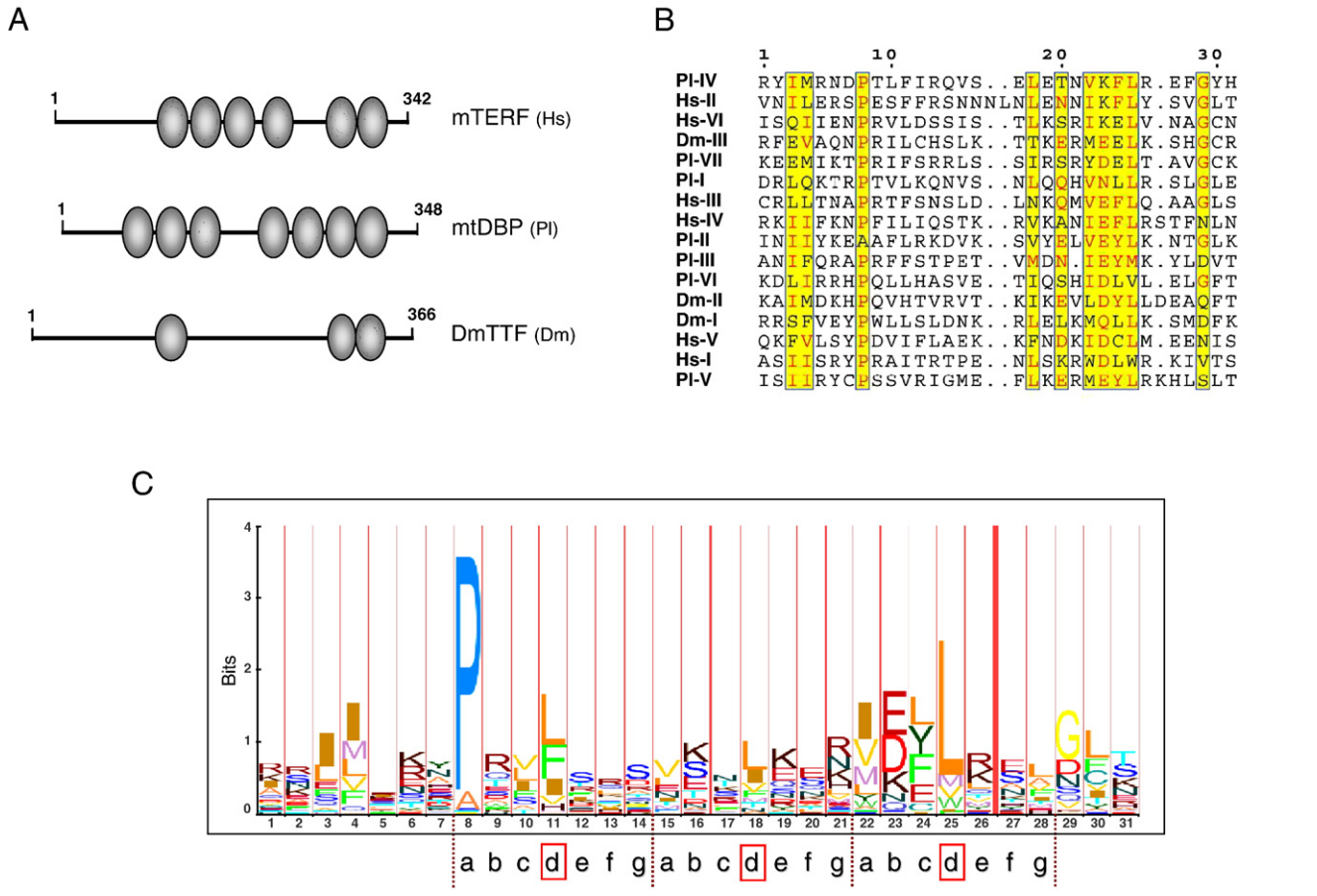

Fig. 5. Alignment of the mTERF motifs in the transcription termination factors mTERF, mtDBP and DmTTF. (A) Diagram showing the modular architecture of human mTERF, sea urchin *P. lividus* mtDBP and *Drosophila* DmTTF, according to SMART tool (http://smart.embl-heidelberg.de/). The position of the mTERF motifs on the sequences is indicated with ellipses. (B) Multiple alignments of the mTERF motifs obtained with ClustalW2 (PBIL). Initials of species name, together with the number indicating the progressive position of the mTERF motif, are reported to the left. Similar amino acids are in bold, conserved blocks are shaded in yellow. (C) Graphical representation, as HMM sequence logo, of the aligned mTERF motifs shown in (B). The amino acid positions in the putative leucine zippers (a–g) are also indicated.

suggests that also DmTTF is implicated in activating transcription initiation. This could be achieved through a DNA looping mechanism involving the simultaneous interaction of DmTTF with one canonical binding site and a still unknown site placed in proximity of the promoters in the AT-region. The existence of two already defined binding sites prompted us to investigate the possibility that they could be involved in DNA looping by DmTTF, although this loop would not be directly linked to transcription initiation. *In vitro* experiments (not shown) clearly demonstrate that DmTTF is not able to simultaneously bind the two sites.

The multiplicity of functions of transcription termination factors is further underlined by the finding that the human and sea urchin proteins play roles in mitochondrial replication, namely, mtDBP in the negative control of the D-loop expansion [22] and mTERF in the regulation of replication pausing [13].

3. The mitochondrial transcription repressors

MTERF3 is the other sub-family of the MTERF family whose protein members have been quite extensively characterized. In a recent report Park et al. [33] provided information on the mammalian MTERF3. The mature protein, about 350 amino acids, was shown to localize to mitochondria. MTERF3 is an essential protein because homozygous MTERF3 knock-out mouse embryos died in the midgestation. Tissue-specific gene inactivation produced vital mice, though with a shortened life span, and differently affected skeletal muscle and heart causing only in the latter evident mitochondrial dysfunction. In particular, an aberrant mtDNA transcription mainly consisting in an increased initiation at both promoters HSP and LSP was observed. Moreover, it was demonstrated by ChIP experiments that MTERF3 binds the mtDNA promoter region. All together, these data suggested for MTERF3 a role as mitochondrial transcriptional repressor. In the absence of information on a sequence specific binding of the protein to mtDNA, it can be hypothesized that MTERF3 function is mediated by interactions with the identified transcription initiation factors as well as with still unknown proteins.

Our work in *Drosophila* has provided further interesting information on MTERF3. We characterized the *Drosophila* homologue D-MTERF3, a protein of 323 residues, which localizes to mitochondria [6]. Preliminary experiments aimed at determining its sub-mitochondrial localization showed that the behaviour of D-MTERF3 is typical of a peripheral membrane protein. On the other hand we cannot rule out that the protein, besides being linked to the inner membrane, is able to interact with the DNA as reported for the mammalian homologue MTERF3 [33].

To obtain insights into the function of D-MTERF3, we analysed the effect of its over-expression and depletion on mitochondrial functions. Fig. 6A shows that over-expression of the protein resulted in an increased level of 40-fold. We analysed in D-MTERF3 over-expressing cells the level of three mitochondrial transcripts, srRNA, COI and ND2, which map on both strands immediately downstream of the AT-rich control region. As revealed by Real Time RT PCR analysis, the level of the transcripts was decreased in the range of 0.2–0.6 fold (Fig. 6B); no noticeable effect was detected on the level of mitochondrial factors TFAM, TFB1M, TFB2M (Fig. 6C), nor on mtDNA copy number (data not shown), thus suggesting that the observed alterations of the mitochondrial transcripts are directly linked to D-MTERF3 over-expression. These results tend to propose for the *Drosophila* factor a role as negative regulator of mitochondrial transcription in agreement with that of mammalian MTERF3.

In regard to the effect of D-MTERF3 depletion on the mitochondrial functions, we reported in a previous work that it did not influence the level of several sense and antisense mitochondrial transcripts. D-MTERF3 knock-down, however, produced an overall decrease in *de novo* synthesis of mitochondrial polypeptides, with ND1 being the most affected [6]. Here we measured the level of the mRNAs for factors TFAM, TFB2M and TFB1M also in D-MTERF3 depleted cells. Fig 6C shows that protein depletion was associated to a decrease in the level of TFAM and TFB1M, whereas that of TFB2M remained unchanged; TFAM decrease was also confirmed by immunoblotting (data not shown). Therefore, a tentative explanation of the general decline in mitochondrial protein synthesis observed in D-MTERF3 knock-down

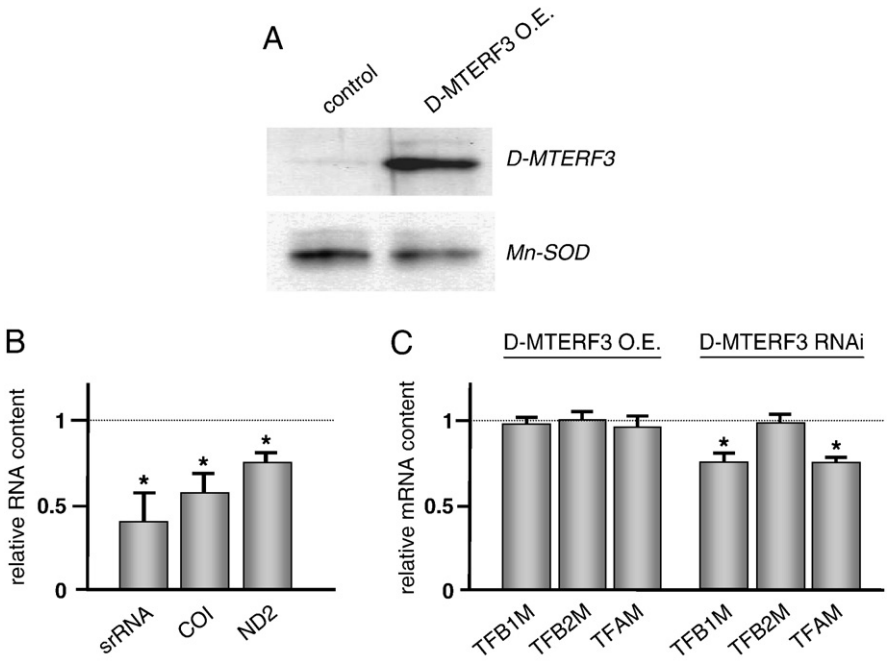

Fig. 6. Effect of over-expression and knock-down of D-MTERF3. (A) Immunoblot analysis of mitochondrial lysate from D.Mel-2 cells carrying pMt/Hy (control) or pMt/D-MTERF3/Hy (over-expression). Cells were cultured and processed as described in the legend to Fig. 4A. Antibodies were against D-MTERF3 or against Mn-SOD. (B) Effect of DmTTF over-expression on the level of mitochondrial transcripts. Total RNA was extracted from control and D-MTERF3 over-expressing cells and relative quantification of mitochondrial transcripts were carried out as reported in the legend to Fig. 4B. (C) Relative abundance of mRNAs for TFB1M, TFB2M and TFAM in D-MTERF3 over-expressing or RNAi cells with respect to control cells, fixed as 1-value. Standard deviations are indicated on the top of the bars; statistically significant differences ($P<0.05$) are marked with an asterisk.

cells could be the decrease in the level of TFB1M, a factor that is implicated in translation efficiency [29]. The unchanged level of the mitochondrial transcripts might be ascribed to the down-regulation of TFAM in D-MTERF3 depleted cells. Since TFAM is an activator of transcription [3,4], its diminished level could have counterbalanced the enhanced transcription caused by the removal of repressor D-MTERF3.

MTERF3 proteins are widespread in Metazoa as they can be clearly detected in worms, insects, echinoderms and vertebrates. They display a higher conservation of primary structure with respect to that of the transcription termination factors. In fact, multialignment of human, sea urchin and *Drosophila* MTERF3 shows 20% amino acid identity and 32% similarity. Moreover, the conservation of five mTERF motifs in corresponding position is evident going from *C.elegans* to human [6]. These observations suggest that MTERF3 is subjected to stronger functional constraints than the termination factors. Though the function of MTERF3 has been studied in mammalian and *Drosophila* only, it is possible to speculate that the sequence conservation of this factor implies a similar function in all Metazoa. It would result that, in spite of the wide variation in the gene arrangement, all the mitochondrial genomes seem to require the transcription repressor. Since MTERF3 function could need protein–protein interactions, it is possible that the mTERF motifs of the repressor serve to contact other proteins that could mediate binding of MTERF3 to the promoter regions.

4. Conclusions and perspectives

The here reported evidence highlights a relevant role of the MTERF family proteins in mitochondrial gene expression. Members of this family include the transcription termination factors that may perform multiple roles. The information about MTERF3 outlines a role of negative regulator of mitochondrial transcription; this knowledge adds a new component to the transcription machinery and suggests also the involvement of yet unknown regulatory factors as possible interacting partners of MTERF3. Therefore, the emerging scenario is that mtDNA expression could be finely tuned in response to cellular demands by the coordinated action of the activating factors TFAM and TFB1/2M [3], the repressor MTERF3 and the termination factor.

Still obscure is the role of MTERF2 and MTERF4. Preliminary reports from the laboratory of Gustafsson suggest that human MTERF4 binds to the D-loop region and interacts with a mitochondrial protein containing a RNA methyltransferase domain [34]. MTERF2 (mitochondrial transcription termination factor-like or mTERF.D3) was partially characterized by the groups of Chen and Moraes [35,36]. The human protein displays 29% amino acid identity and 52% similarity with mTERF. The two factors show an opposite expression behaviour to serum: MTERF2 is inhibited by addition of serum to serum-starved cultured cells, whereas mTERF is rather induced. Moreover, immunohistochemistry experiments and sub-cellular fractionation analysis showed that MTERF2 is a mitochondrial matrix protein and is associated with the inner membrane. The complete elucidation of the role of the MTERF family members will contribute to the unraveling of the molecular mechanisms of mtDNA transcription and replication.

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