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Twinkle helicase is essential for mtDNA maintenance and regulates mtDNA copy number

Henna Tyynismaa[1], Hiroshi Sembongi[2], Monika Bokori-Brown[2], Caroline Granycome[2], Neil Ashley[3], Joanna Poulton[3], Anu Jalanko[4], Johannes N. Spelbrink[5], Ian J. Holt[2] and Anu Suomalainen[1,6,*]

[1]Department of Neurology and Programme of Neurosciences, University of Helsinki, 00290 Helsinki, Finland, [2]Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, UK, [3]Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK, [4]Department of Molecular Medicine, National Public Health Institute, 00290 Helsinki, Finland, [5]Institute of Medical Technology and Tampere University Hospital, 33014 Tampere, Finland and [6]Department of Clinical Chemistry, Helsinki University Central Hospital, 00290 Helsinki, Finland

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Mechanisms of mitochondrial DNA (mtDNA) maintenance have recently gained wide interest owing to their role in inherited diseases as well as in aging. Twinkle is a new mitochondrial 5′–3′ DNA helicase, defects of which we have previously shown to underlie a mitochondrial disease, progressive external ophthalmoplegia with multiple mtDNA deletions. Mouse Twinkle is highly similar to the human counterpart, suggesting conserved function. Here, we have characterized the mouse *Twinkle* gene and expression profile and report that the expression patterns are not conserved between human and mouse, but are synchronized with the adjacent gene MrpL43, suggesting a shared promoter. To elucidate the *in vivo* role of Twinkle in mtDNA maintenance, we generated two transgenic mouse lines overexpressing wild-type Twinkle. We could demonstrate for the first time that increased expression of Twinkle in muscle and heart increases mtDNA copy number up to 3-fold higher than controls, more than any other factor reported to date. Additionally, we utilized cultured human cells and observed that reduced expression of Twinkle by RNA interference mediated a rapid drop in mtDNA copy number, further supporting the *in vivo* results. These data demonstrate that Twinkle helicase is essential for mtDNA maintenance, and that it may be a key regulator of mtDNA copy number in mammals.

INTRODUCTION

Defects in nuclear-encoded proteins involved in mitochondrial DNA (mtDNA) maintenance have recently been shown to be a frequent cause of inherited metabolic disorders (1), and also important in neurodegeneration and aging (2,3). Twinkle, a mtDNA helicase, is important for mtDNA maintenance, as its dominant mutations are associated with progressive external ophthalmoplegia with multiple mtDNA deletions (PEO, OMIM #157640). The disease manifests as myopathy, often affecting the extra-ocular muscles, and as exercise intolerance, occasionally accompanied by major depression (4,5). The molecular hallmark of the disease is the accumulation of multiple mtDNA deletions in post-mitotic tissues, namely, in the brain, the skeletal muscle and the heart. Autosomal dominant PEO is genetically heterogeneous, since in addition to Twinkle, the disease can be caused by mutations in adenine nucleotide translocator 1 and mtDNA polymerase gamma (POLG) (6,7).

The helicase motifs of Twinkle are highly homologous to the bacteriophage T7 gene 4 primase/helicase (T7gp4), which suggested that it might be the replicative helicase of mtDNA (8). *In vitro*, Twinkle displays 5′ → 3′ DNA helicase activity supporting its role in unwinding the mtDNA

*To whom correspondence should be addressed at: Biomedicum-Helsinki, r. c522B, University of Helsinki, Haartmaninkatu 8, 00290 Helsinki, Finland. Tel: +358 947171965/+358 405936386; Fax: +358 947171964; Email: anu.wartiovaara@helsinki.fi

replication fork (9). T7gp4 is functional as a hexameric ring and Twinkle also formed hexamers *in vitro* (8). The 15 mutations so far identified from the Twinkle-PEO patients are located at or near putative regions involved in subunit interactions of the hexameric helicase (8,10–12). The T7gp4 primase is poorly conserved in Twinkle, and no primase function for the protein has so far been identified. Twinkle co-localizes with mtDNA in mitochondrial nucleoids that are stable assemblies of nucleoproteins and mtDNA (8,13).

The mechanisms controlling mtDNA copy number are starting to unravel. Any factor necessary for mtDNA replication will reduce mtDNA copy number if scarce or absent. For example, lack of the mitochondrial transcription factor A (TFAM) or RNaseH1 results in mtDNA depletion and embryonic lethality in mice (14,15). However, overexpression of a protein involved in mtDNA metabolism does not always increase mtDNA copy number: an excess of the catalytic subunit of POLG does not alter the level of mtDNA in cultured cells (16), and in transgenic flies, it results in a drastic reduction of mtDNA (17). TFAM is thought to stabilize mtDNA, and its copy number follows mtDNA mass: overexpression of TFAM increases mtDNA copy number in a chicken lymphoma cell line (18) and transgenic mice (19). In *Drosophila melanogaster*, overexpression of transcription factor TFAM or B2 (mtTFB2) leads to an increase in mtDNA copy number (20). As mitochondrial transcription is required for the initiation of mtDNA replication, these transcription factors have been suggested to be the major mtDNA copy number regulators.

We studied the effects of inhibition of Twinkle in cell culture and overexpression in transgenic mice and provide the first functional evidence for Twinkle as the essential replication helicase of mtDNA and as an independent regulator of mtDNA copy number.

RESULTS

Characterization of mouse *Twinkle* gene and its expression profile

The mouse *Twinkle* (mTwinkle) gene extends over 4.8 kb of genomic DNA, located in the chromosomal band 19C3; a region syntenic to human chromosome 10q23.3. The mTwinkle gene, like its human counterpart hTwinkle (also known as C10orf2 or PEO1), comprises five exons, and the exon–intron boundaries are highly conserved between the species (Fig. 1A and C). Twinkle is arranged head-to-head with the MrpL43 gene with the open-reading frames separated by 768 and 735 bp in human and mouse, respectively (Fig. 1A). MrpL43 gene encodes a large 39S mitoribosome subunit protein.

Twinky, the alternative splice variant of hTwinkle, results from the use of a downstream exon 4 splice-donor site and leads to a 43-base-pair insertion between the regular exon 4–exon 5 sequence (8) (Fig. 1D). The insertion encodes for four unique amino acids followed by a premature stop codon (Fig. 1H). Only one mouse EST (BB002839) can be found from the EST database with putative Twinky stop codon, but this EST also contains most of the intron 4 sequence suggesting that if Twinky exists in the mice, the intron 4 is not excised (Fig. 1E). Twinky mRNA was undetectable on northern blots of mouse tissues using intron 4 as a probe (Fig. 2A), or by cDNA amplification (data not shown), whereas Twinkle was readily detected by both methods.

The mTwinkle expression was found to be ubiquitous with the highest levels in the liver, the heart and the kidney, using mTwinkle cDNA as probe for northern blots of mouse tissues (Fig. 2A). The skeletal muscle, the brain and the testis showed lower but detectable expression. Only one transcript of ~3.5 kb was visible; the open reading frame of Twinkle is 2.0 kb and the 5′UTR is 0.7 kb. The expression pattern of the murine MrpL43 was similar to that of mTwinkle (Fig. 2A).

Twinkle has been shown to be highly expressed in the skeletal muscle of humans (8). In addition, we found high expression of full-length Twinkle (~4 kb) and a putative splice variant of ~3 kb from the testis (Fig. 2C). The human MrpL43 expression pattern follows closely that of hTwinkle, with the highest expression in the skeletal muscle and the testis (Fig. 2B and C). Thus, Twinkle and MrpL43 expression appears to be co-regulated in mouse and humans.

Mouse and human Twinkle proximal 5′UTR sequences were studied for transcription factor binding sites with the MatInspector program. EST contigs identified ~600 bp of Twinkle 5′UTR, restricting the distance between the 5′UTRs of Twinkle and the MrpL43 to ~100 bp. Predicted SP1 and NRF2 transcription factor binding sites are located in this region (Fig. 1F).

Amino acid identity of hTwinkle and mTwinkle proteins is 89%. The conservation of the putative subunit interaction region is 100% as previously reported (8). The helicase domain identity is also high (94%), whereas the N-terminus of the protein has more amino acid differences with 85% identity.

Transgenic Twinkle overexpressor mice have an increased mtDNA copy number

To study the gene dosage effect of Twinkle, we generated two lines of transgenic mice (A and B), which overexpressed wild-type mTwinkle under a β-actin promoter. The transgene construct contained the cDNA + intron 4, allowing alternative splicing of the Twinky variant. The A mice had three to four transgene copies and the B mice one to two copies determined by semi-quantitative Southern blot (data not shown). We quantified Twinkle expression levels in transgenic muscle, heart and brain by comparing the transgene expression with the expression of the native allele using RT–PCR and solid-phase minisequencing (21). The A mice displayed a 6-fold increase of Twinkle mRNA in skeletal muscle and 8.4-fold increase in heart, whereas the B mice had a 1.5- and 4.2-fold increase in the respective tissues (Fig. 3A).

Increase in Twinkle mRNA level was accompanied by increase in mtDNA copy number (Fig. 3B and C). In the skeletal muscle of A mice, mtDNA level was 2.5 fold higher than in the control littermates ($P = 0.0001$). In the same tissue of B mice, the increase in Twinkle mRNA was less marked and there was no significant increase in mtDNA level (1.09, $P = 0.24$). In heart, both lines of mice displayed an increase in mtDNA copy number; 3-fold in A mice ($P = 0.003$) and 2-fold in B mice ($P = 0.006$). Although there was a clear

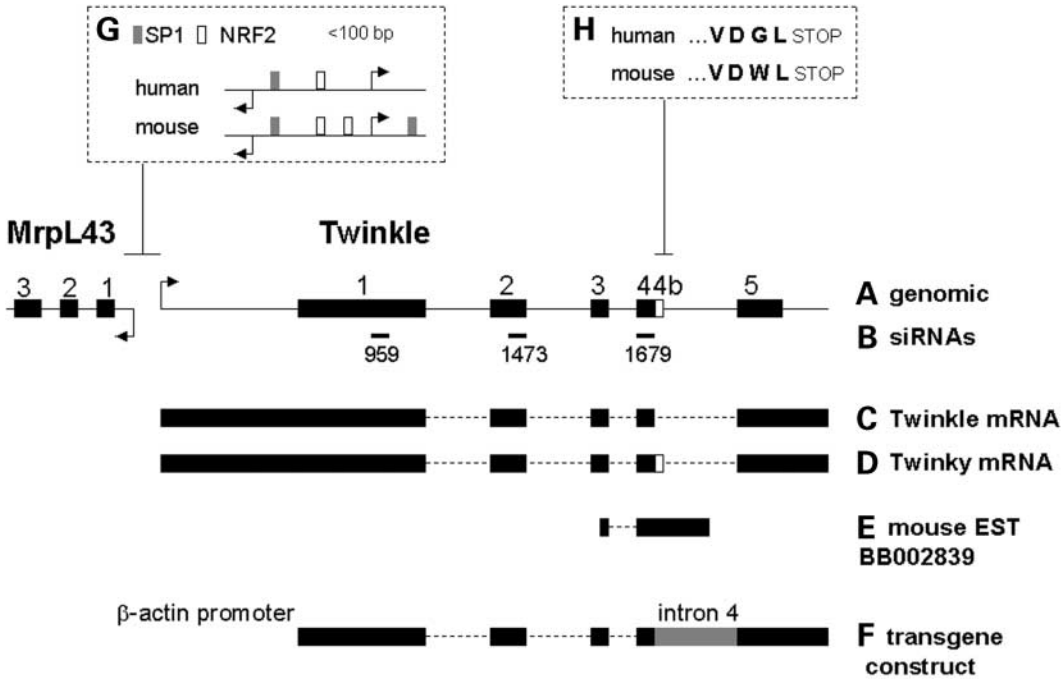

Figure 1. Alignment of Twinkle genomic structure and transcripts. Human Twinkle and mouse Twinkle have similar genomic structures and their chromosomal regions are conserved. (**A**) Twinkle is arranged head-to-head with MrpL43 gene. The exons 1–5 of Twinkle are marked with black boxes and the alternative exon 4B with white box. Arrows mark the start of transcripts Twinkle and MrpL43 as determined by the sequence of several ESTs. (**B**) Location of the three Twinkle dsRNAs 959, 1473 and 1679. (**C**) Structure of Twinkle messenger RNA. (**D**) Structure of the alternative splice variant Twinky messenger RNA. (**E**) Only one mouse EST (BB002839) is suggestive of existence of Twinky in mouse. (**F**) The transgene construct to overexpress Twinkle and Twinky in mouse consists of Twinkle mRNA and intron 4. (**G**) The ~100 bp between the putative transcription start sites of MrpL43 and Twinkle was predicted to contain SP1 and NRF2 transcription binding sites. (**H**) The conservation of four final amino acids of human and mouse Twinky protein suggests that the alternative splice variant Twinky exists in mouse.

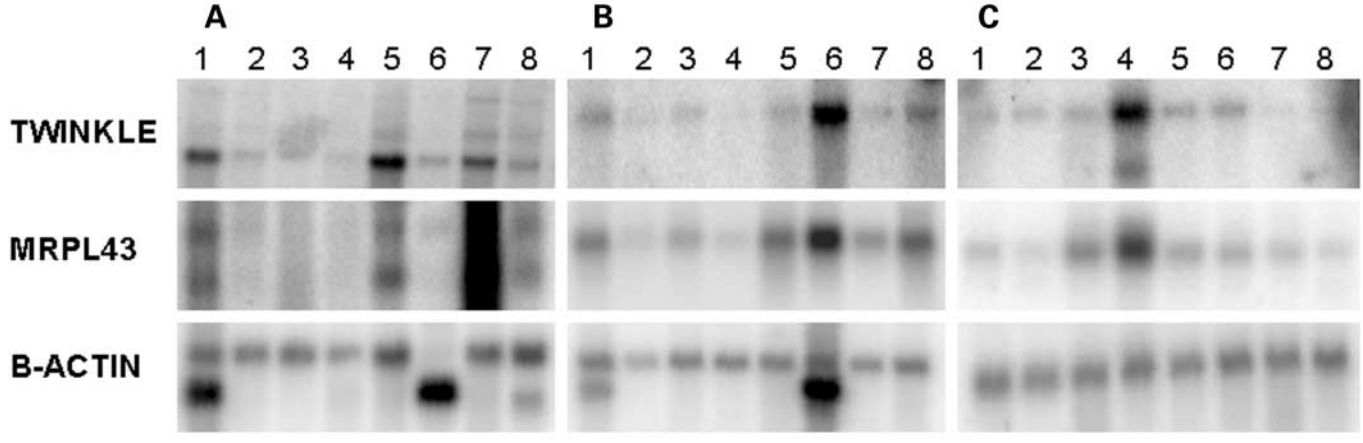

Figure 2. Twinkle and MrpL43 are co-expressed in a tissue-specific manner. Multiple northern blots of mouse and human tissues probed with Twinkle, MrpL43 and β-actin cDNAs. The mouse MRPL43 probe detects two alternative transcripts of MRPL43. (**A**) Mouse tissues: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis. (**B**) Human blot 1, tissues; 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas. (**C**) Human blot 2 tissues: 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; 8, leukocytes.

correlation between increased Twinkle mRNA and increased mtDNA copy number, the ratio was not one to one (Fig. 3D). For example, a 2.5-fold increase in mtDNA in the muscle of A mice depended on a 6-fold increase in Twinkle mRNA, whereas a 3-fold increase in heart mtDNA required a 8.4-fold increase in Twinkle mRNA. The F2 generation mice homozygous for the transgene had a higher mtDNA copy number than heterozygous mice (Fig. 3C); however, the small number of animals obtained was insufficient for statistical analysis. No detrimental effect of Twinkle overexpression was noted: the eldest mice are now 16 months of age and have developed normally to date. Moreover, histological analysis of

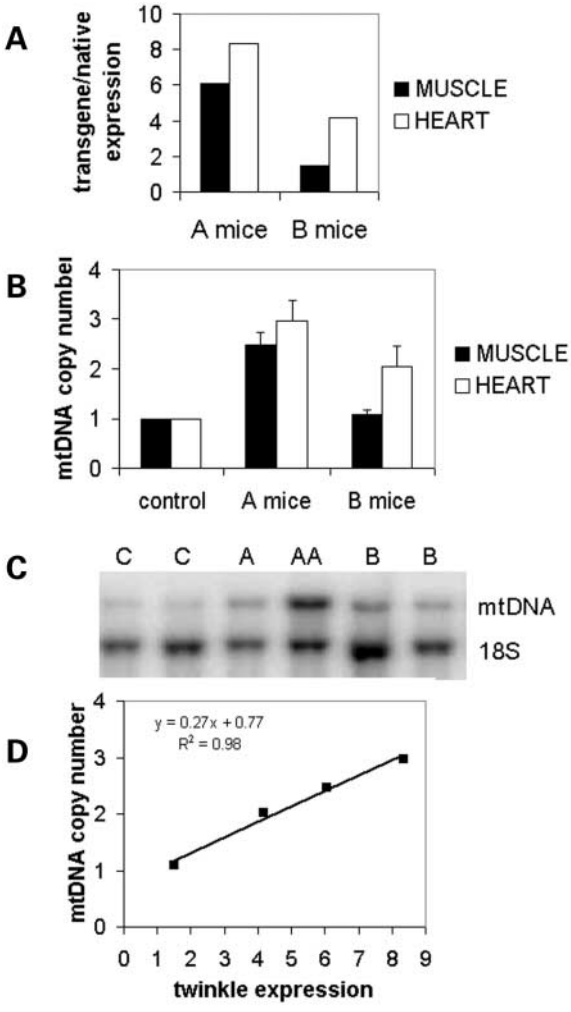

Figure 3. Twinkle overexpressor mice have increased mitochondrial DNA copy number. (**A**) Two lines of transgenic Twinkle overexpressor mice were produced (A and B) and the transgene expression levels in muscle and heart were determined by RT–PCR and solid-phase minisequencing comparing to the expression of native Twinkle allele. (**B**) mtDNA copy number was determined from the muscle and heart of A and B mice by Southern blotting comparing the signal of mtDNA with nuclear 18SrRNA gene. (**C**) Southern blot of heart mtDNA and 18SrRNA. C, control mouse; A, heterozygous mouse from line A; AA, homozygous F2 mouse from line A; B, heterozygous mouse from line B. (**D**) The heart and muscle mtDNA copy number of Twinkle overexpressor mice is plotted against Twinkle mRNA level. A regression line $y = 0.27x + 0.77$ is drawn to connect the plots. The correlation coefficient r^2 of the line was 0.98.

the tissues of transgenic mice revealed no morphological abnormalities of mitochondria (data not shown).

Twinkle RNAi reduces mitochondrial nucleoid signal and mtDNA copy number

As a prelude to analysing the effects of Twinkle RNA interference (RNAi), first we had to establish that short interfering RNAs (siRNAs) reduced Twinkle expression. To this end, human osteosarcoma (143B) cells were transfected with one of three synthetic double-stranded RNAs (959, 1473 or 1679, Fig. 1B) at a final concentration of 2, 5 or 10 nM;

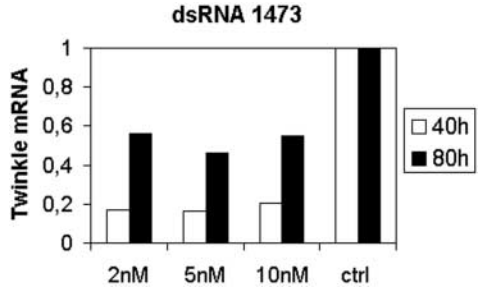

Figure 4. Short dsRNAs mediate RNAi of Twinkle mRNA. RNA of 143B cells transfected with siRNA 1473 was isolated 40 and 80 h after transfection and subjected to Q-PCR of Twinkle mRNA. Values are expressed as a percentage of Twinkle mRNA level in mock-transfected cells cultured concurrently.

RNA was extracted 40 and 80 h later and real-time PCR applied to quantify the level of Twinkle mRNA. All three siRNAs tested induced a decrease in Twinkle mRNA. The decrease was consistently more marked at 40 than 80 h, indicating that any effect of the siRNAs was likely to be transient. siRNA 1473 produced the most pronounced decrease in Twinkle mRNA: $<20\%$ of control values at 40 h, recovering to around 50% of control levels at 80 h (Fig. 4). An siRNA of 2 nM was close to the minimum dose capable of mediating RNA interference; therefore, 5 and 10 nM were adopted as the standard concentrations for all further experiments.

Human osteosarcoma cells transfected with 5 or 10 nM siRNA 1473 were examined by confocal microscopy at daily intervals for up to 5 days. Mitochondria were stained with a specific dye, Mitotracker Orange™ (Molecular Probes), and mtDNA was visualized after staining with PicoGreen™ (Molecular Probes) (N. Ashley *et al.*, manuscript in preparation). As with other mtDNA labelling methods, much mtDNA appears as distinct foci dispersed throughout the reticular mitochondrial network after PicoGreen staining (Fig. 5); this reflects the arrangement of mtDNA in multi-copy nucleoprotein complexes, or nucleoids (8,13,22,23).

Examination of 143B human cells treated with siRNA 1473 revealed a marked decrease in PicoGreen staining of mtDNA 96 h after transfection, compared with mock-transfected cells (Fig. 5 and Supplementary Material). Subsequently, time courses showed that the effect was apparent within 48 h of transfection. Although the decrease in signal persisted for a further 48 h ($t = 96$ h after transfection), we saw clear signs of recovery 120 h after transfection. Co-staining of cells with Mitotracker Orange indicated that no general disturbance of mitochondrial number or structure was apparent in the transfected cells (Fig. 5), suggesting that the effects of siRNA 1473 specifically related to changes in the level of Twinkle mRNA. A decrease in the intensity of PicoGreen-stained mtDNA was also observed with the other two siRNAs (1679 and 959) specific for Twinkle mRNA.

Fluorescence analysis indicated a substantial decrease in the intensity of individual nucleoids in the cells subjected to Twinkle RNAi (Fig. 6), indicating mtDNA copy number decrease per nucleoid in these cells. Estimation of mtDNA copy number by real-time PCR analysis demonstrated that this decrease correlated with a reduction in mtDNA copy

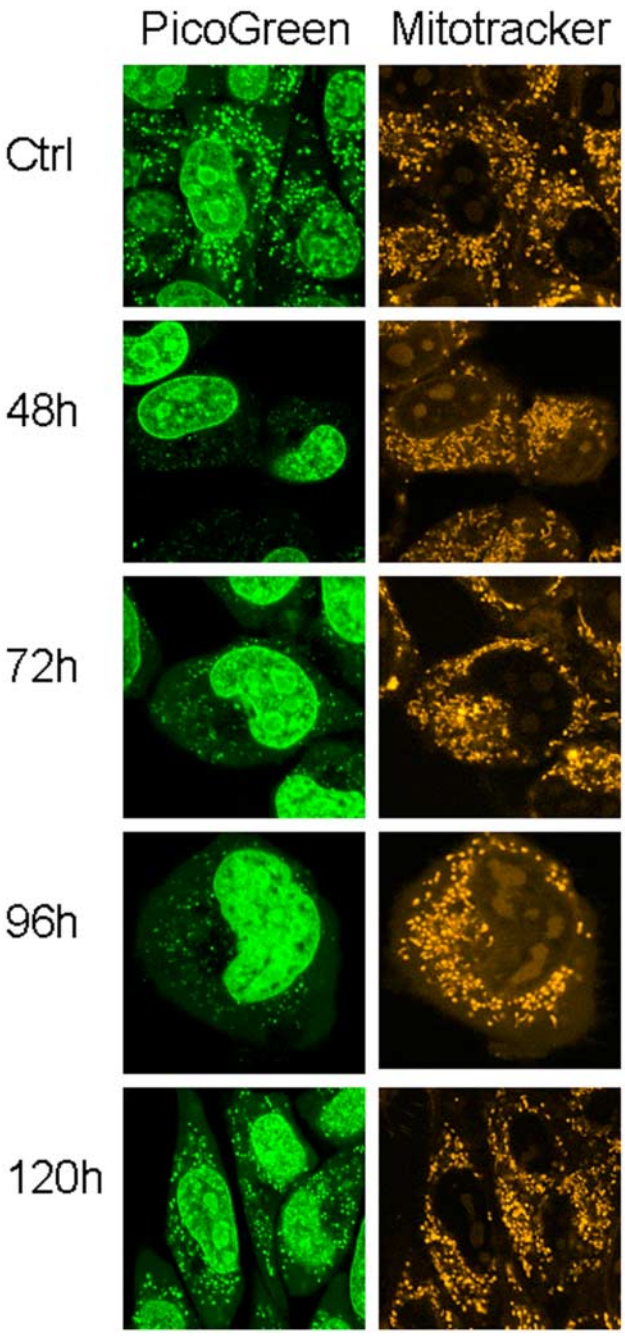

Figure 5. Twinkle RNAi reduces mitochondrial nucleoid signal in human cells. Untreated control cells and cells transfected with 5 nM dsRNA 1473 were stained with PicoGreen and Mitotracker Orange, after 48, 72, 96 or 120 h. Cells examined 24 h after transfection showed a small decrease in nucleoid signal (data not shown).

number (Fig. 7). The decrease reached a maximum of ~50% of control values 96 h after introduction of dsRNA. The mtDNA copy number recovered to three quarters of control levels 120 h after transfection (Fig. 7), in line with the increased intensity of PicoGreen staining documented earlier (Fig. 5).

Three dsRNAs to the mitochondrial import protein Tim17 produced a decrease in the target message of 50–70%, at a dose of 10 nM, without any discernible effect on mtDNA (Supplementary Material). In other control experiments, it was demonstrated that 10 and 30 nM dsRNAs to Tim17a had no significant effect on the level of Twinkle mRNA, and siRNA of Twinkle had no effect on another message whose product is implicated in mtDNA metabolism, the catalytic subunit of DNA POLG (Supplementary Material).

DISCUSSION

Here, we show that the new mtDNA helicase Twinkle is an essential component of the mtDNA maintenance apparatus. Inhibition of Twinkle expression in cultured cells resulted in rapid mtDNA depletion, showing that Twinkle is required for mtDNA maintenance. Furthermore, overexpression of Twinkle in transgenic mice led to considerable increase in mtDNA copy number, indicating that Twinkle is an independent copy number regulator. Previously, Twinkle was predicted to play an important role in mtDNA metabolism on the basis of its involvement in PEO with multiple mtDNA deletions and its co-localization with mtDNA in mitochondrial nucleoids (8). Subsequently, biochemical characterization of Twinkle *in vitro* demonstrated 5′–3′ DNA helicase activity on fork-like structures with both a 5′- and a 3′-single-stranded DNA overhang. The same study found that mitochondrial single-stranded DNA-binding protein (mtSSB) had a strong stimulatory effect on the unwinding activity of Twinkle (9). A recent report showed that Twinkle together with POLG and mtSSB form a minimal but sufficient set of proteins capable of synthesizing 16 kb lengths of DNA in a cell-free system (24). Our data establish Twinkle as a protein with an essential role in mtDNA maintenance and as an independent regulator of mtDNA copy number in cell culture and *in vivo*, properties consistent with its assignment as the replication helicase of mitochondria.

To characterize the mouse Twinkle gene in detail, we studied its genomic structure, expression profile and the conservation of genomic and protein sequences in human and mouse. In the genomes of both species, Twinkle lies adjacent to the mitoribosome subunit MrpL43 gene, separated by only ~0.7 kb. Mammalian nuclear genomes encode all of the ~80 mitochondrial ribosomal proteins (MRPs) which form the small (28S) and large (39S) subunits of the mitoribosome (25,26). MrpL43 is one of the few MRPs that lack a bacterial homologue. We studied the tissue expression of Twinkle and found that in human tissues it is highly expressed in the skeletal muscle and the testis, whereas in mouse the expression levels are highest in the liver, the kidney and the heart. These differences in the expression patterns could reflect species-specific variability in the general mitochondrial mass or the rate of mtDNA replication and turnover of the tissue and should be addressed in further studies. In addition, the possible consequences of the high Twinkle expression in human testis as well as its putative testis-specific splice variant remain to be studied in particular because mtDNA copy number is known to be downregulated during spermatogenesis. We additionally observed that Twinkle and MrpL43 are co-expressed in a species-specific manner, suggesting a shared promoter. Shared bidirectional promoters are common

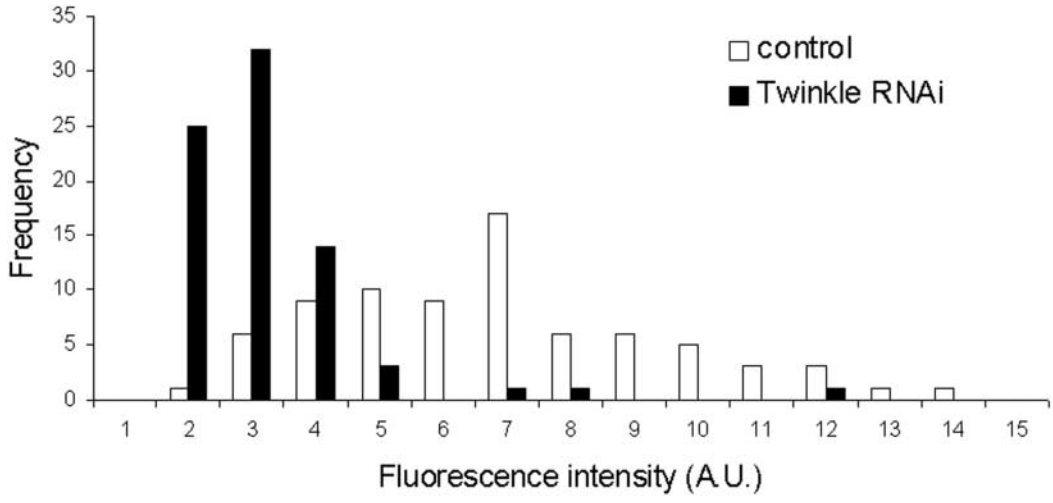

Figure 6. Twinkle RNAi induces a decrease in average mtDNA copy number per nucleoid based on integrated fluorescence of PicoGreen stained mtDNA. Quantitation of nucleoid signal. The data represents either mean pixel intensity (grey values) or corresponding integrated density (ID) (ID is the sum of the grey values selected, with background subtracted) per nucleoid.

among nuclear encoded mitochondrial proteins (27,28), suggesting that this is an efficient way of regulating expression of mitochondrial house-keeping genes. Owing to the co-ordinated expression of Twinkle and MrpL43, it is not certain which of the two transcripts is the most important in a given tissue.

We found that inhibition of Twinkle expression led to a rapid decrease in mtDNA level, but not to complete loss of mtDNA. This is to be expected; RNAi mediated by oligonucleotides typically produces a limited decrease in expression, knock-down rather than knock-out. Moreover, ~10% of cells showed no decrease in mitochondrial nucleoid signal, which was equivalent to the proportion of untransfected cells observed after the introduction of a GFP expression plasmid into the osteosarcoma cell line. Hence, the real fall in copy number due to Twinkle inhibition was slightly greater than estimated by quantitative PCR and the clear prediction is that cells devoid of Twinkle will rapidly lose all their mtDNA.

Twinkle co-localizes with TFAM and mtSSB in most nucleoids making it a potential stabilizer of the mitochondrial chromosome and nucleoid structure (13). The membrane-association of nucleoids may be absolutely required for transmission, and if their structure depends on the presence of Twinkle, loss of the protein could well prove catastrophic for mtDNA maintenance, irrespective of its role in replication. Enticingly, two groups have recently reported preliminary evidence of mammalian nucleoids being linked to components of the cytoskeleton or associated factors (22,29). However, the essential role of Twinkle in mtDNA homeostasis is most likely in mtDNA replication. *In vitro*, POLG alone is unable to use dsDNA as a template for DNA synthesis, but instead a functional interaction of POLG and Twinkle is needed (24). POLG overexpression alone does not increase mtDNA copy number (16,17). The findings of this report demonstrate a direct correlation between Twinkle expression and mtDNA copy number, and our firm conclusion is that Twinkle is required for mtDNA synthesis and is rate limiting for replication initiation. Hence, Twinkle fulfils the criteria for a licensing factor of mtDNA replication.

More than one pathway may be involved in mtDNA copy number regulation in different organisms and in the tissues of an individual. Previous studies showed that overexpression of mitochondrial transcription factors TFAM and mtTFB2 increase mtDNA levels in transgenic animals and cultured cells (18–20). In addition to its role in transcription, TFAM has been proposed as a mitochondrial histone and the increase in copy number by TFAM overexpression may be due to prolonged mtDNA turnover achieved through the histone-like direct binding and stabilization of mtDNA (19,20). MtTFB2 is proposed to be involved in the synthesis of RNA primers for mtDNA replication and, therefore, to be critical for replication initiation (20). However, TFAM imported into isolated rat liver mitochondria stimulates 7S DNA synthesis (30), a putative precursor of mtDNA replication (31). Therefore, both TFAM and mtTFB2 might increase mtDNAs via increased primer synthesis. MtDNA maintenance is also dependent on finely balanced nucleotide pools; increase in thymidine concentration is known to cause mtDNA depletion (32). Although a single master regulator of mtDNA copy number, common to all organisms or even to all tissues of an individual, probably does not exist, Twinkle appears to be a major player, as the 3-fold increase in mtDNA in mouse heart overexpressing Twinkle is the most substantial *in vivo* increase in mtDNA copy number reported to date.

Understanding mtDNA replication and defining the mtDNA maintenance machinery are important both for understanding mitochondrial disease pathogenesis and mammalian mtDNA homeostasis. Here, we have shown that Twinkle is essential for mtDNA maintenance in human cultured cells and that it has a novel role in the control of mtDNA copy number. Future studies are required to reveal the mechanisms of this

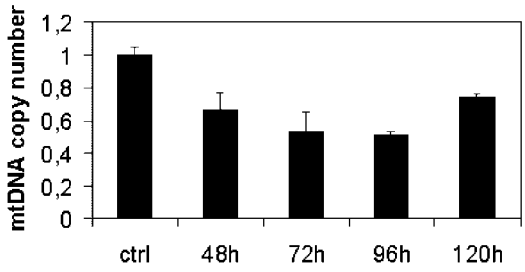

Figure 7. Twinkle RNAi induces a decrease in mtDNA copy number. Comparative Q-PCR of mtDNA and single copy nuclear gene (APP) is a measure of mtDNA copy number. The Q-PCR method was validated on cells without mtDNA (data not shown), and cells transiently depleted of much of their mtDNA (see Supplementary Material).

regulation and the relevance of the essential function to the pathogenesis of Twinkle-associated diseases of the muscle and nervous system.

MATERIALS AND METHODS

Electronic database search

Part of mTwinkle mRNA sequence was identified through BLAST searches of EST databases with the help of hTwinkle cDNA sequence (www.ncbi.nlm.nih.gov/BLAST/). The genomic sequence of mTwinkle gene was identified by BLAST comparison of mTwinkle cDNA and mouse genomic DNA (www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html). MatInspector (www.genomatix.de/software_services/software/MatInspector/MatInspector_stb.html) was used to analyse the transcription factor binding sites in the region between Twinkle and MrpL43 translational start codons.

RT–PCR

Total RNA isolated from 3T3 cells was reverse transcribed and amplified with Titan One Tube RT–PCR Kit (Roche) using primers A and B (see Supplementary Material for sequences). The PCR product was cloned to pCR2.1-TOPO vector (Invitrogen) and sequenced (submitted to NCBI: NM_153796).

Northern blot

The Twinkle, MrpL43 and β-actin expression patterns were analysed in different mouse and human tissues by probing multiple tissue northern blots (Clontech, BD Biosciences) with the corresponding cDNAs according to manufactures' instructions. The mTwinkle cDNA probe was constructed by RT–PCR as described earlier. The hTwinkle cDNA generation has been described previously (8). The mouse MrpL43 probe was generated from genomic DNA by PCR using primers C and D and the human MrpL43 probe with primers E and F. The manufacturer provided the β-actin probe. The hybridized blots were exposed to a Fuji Imaging Plate, and the plate was scanned using the Storage Phosphor acquisition mode of Typhoon 9600 (Amersham Biosciences).

Generation and genotyping of transgenic overexpressor mice

To be able to separate the transgenic Twinkle expression from the endogenous Twinkle, we introduced a 1077C > G silent point mutation into mTwinkle cDNA in pCR2.1-TOPO with two rounds of site-directed mutagenesis PCR using primers G and H. Intron 4 was amplified from genomic DNA with primers I and J, digested with *Kpn*I/*Blp*I and ligated to mTwinkle-TOPO vector to allow for the expression of splice variant Twinky. The mTwinkle cDNA + intron 4 was then digested with *Bgl*II and cloned to *Bam*HI site of pHBApr-1-neo (33). The final construct DNA was linearized with *Bgl*II and *Nde*I, gel purified with Qiaquick Gel Extraction kit (Qiagen), ethanol precipitated, dissolved in 10 mM Tris, 0.1 mM EDTA pH 7.5 and used in pro-nuclear microinjection of FVB/N mouse fertilized oocytes. The injected oocytes were transferred to pseudopregnant carrier females. Transgenic founder mice were identified by PCR amplification with primers K and L which result in a PCR product of 161 bp from the transgene and 339 bp from the endogenous Twinkle, and further confirmed together with the estimation of the transgene copy number by Southern blot analysis of *Bam*HI-digested DNA probed with a α-^{32}P-dCTP-randomly labelled (Rediprime II, Amersham Biosciences) mTwinkle cDNA probe. Germ line transmission was obtained by breeding to FVB/N mice. F1 mice heterozygous for the transgene were bred to produce homozygous F2 mice. The transgene copy number suggestive of homozygosity was detected by PCR amplification of genomic DNA with primers M and N, and detection of the level of 1077C > G silent change by solid-phase minisequencing using detection oligo O as previously described (34).

Transgene expression level determination

Transgene RNA levels in mouse tissues were determined by solid-phase minisequencing (21). Tissue samples for RNA analysis were snap-frozen in liquid nitrogen and stored at −80°C. RNA was extracted from muscle, heart, and brain samples with Rneasy Mini Kit (Qiagen). RT–PCR was performed with Titan One Tube RT–PCR Kit (Roche) using primers P and Q, and the 1077C > G silent change was detected with oligo O to distinguish the transgene transcript from endogenous transcript.

Mouse mtDNA quantification

Total DNA was isolated from mouse tissues by standard proteinase K and phenol–chloroform methods. Three micrograms of DNA was digested with *Sac*I restriction enzyme, run on agarose gel and blotted on Hybond-N+ membrane (Amersham Biosciences). The membrane was hybridized with P^{32}-labelled PCR-generated mouse mtDNA probe (with primers R and S) and 18S rRNA gene probe in pBR322 plasmid. Phosphoimager analysis was done with Typhoon 9400 (Amersham Biosciences) and mtDNA quantified against the signal levels of nuclear 18SrRNA gene utilizing the ImageQuant v5.0 software (Amersham Biosciences).

SiRNA

Double-stranded RNAs of 25 nt were purchased from iGENE (Tokyo, Japan). Three targets for the mtDNA helicase Twinkle were selected using a proprietary algorithm (iGENE, Fig. 1B). See Supplementary Material for sequences of dsRNA1: Twinkle1473 (oligo T), dsRNA2: Twinkle1679 (oligo U) and dsRNA3: Twinkle959 (oligo V). All dsRNAs had an AG 3′ overhang on the sense strand and an AU 3′ overhang on the antisense strand.

Cell culture

143B human osteosarcoma cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 110 mg/ml pyruvate and 10% fetal bovine serum. 143B cells were transfected with 0, 2, 5 or 10 nM (final concentration) of each Twinkle dsRNA with 3 μl of Lipofectamine2000 (Invitrogen) per 1 ml of OptiMEM, preincubated for 20 min at room temperature before addition to cells 50% confluent on a 35 mm plate.

Q-PCR of human Twinkle mRNA

Total RNA was isolated from 143B cells 40 and 80 h after transfection with dsRNA, mock transfected cells and untreated cells using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. cDNAs were synthesized via random hexamer primers and TaqMan reverse transcription reagents (Applied Biosystems), after RQ1 RNase-Free DNase (Promega) treatment. Q-PCR analysis for Twinkle mRNA was performed using forward primer W, reverse primer X and probe Y. GAPDH Q-PCR employed forward primer Z, reverse primer AA and probe AB as an internal control. ABI PRISM 7700 Sequence Detection System and AmpliTaq Gold DNA polymerase were used for all Q-PCR analyses according to the manufacturer's instructions. A standard curve was created using a dilution series (1/4, 1/16, 1/64, 1/256 and 1/512) of cDNA from untreated 143B cells. This produced a linear plot ($r^2 = 0.9964$) as expected (see Supplementary Material).

Confocal microscopy

143B cells transfected with 0, 5 or 10 nM Twinkle1473 dsRNA were stained with 3 μl/ml of PicoGreen reagent (Molecular Probes) and 500 nM of Mitotracker Orange (Molecular Probes) for 30 min at 37°C. Stained cells were observed using a Radiance2000 confocal microscopy system (BIORAD). PicoGreen excitation/emission wavelengths are 502/523 nm. Mitotracker Orange excitation/emission wavelengths are 551/576 nm. Images of the cells were captured by LaserSharp2000 software and modified by PhotoshopElement software. Tif images of the cells were processed using Scion Image Beta 4.02 for Windows XP to estimate fluorescence intensity.

Real-time PCR estimation of mtDNA copy number in human osteosarcoma cells

Total DNA samples were prepared from cells lysed in 500 μl 75 mM NaCl, 50 mM EDTA, 0.2% SDS pH 8.0. Cell lysates were incubated at 50°C for 2 h with 400 μg/ml Proteinase K. DNA was precipitated by the addition of an equal volume of isopropanol, pelleted by centrifugation at $8500g^{max}$ for 30 min, and dissolved in 100 μl of TE buffer pH 8.0. The mtDNA copy number of control and siRNA treated osteosarcoma cells was estimated by amplifying a portion of the cytochrome b (cyt b) gene of mtDNA and comparing it to the amplification profile of a nuclear single copy gene, Amyloid Precursor protein (APP). Primers for cyt b were forward AC, reverse AD and probe AE. APP primers were forward AF, reverse AG and probe AH. Other reagents, software and hardware were as for Twinkle mRNA RT–PCR.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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