Supplementary material

Supplementary Results

Altered nuclear and mitochondrial parameters after cell cycle blockage by double thymidine.

Treatment with double thymidine resulted in about 2-fold higher levels of PCNA immunostaining and at least 2 to 4-fold higher levels of mREP (Supplementary Figure S5A) indicating enhanced activity of DNA synthesis both at the nuclear and organelle level compared to serum starvation. Higher levels of mREP were associated with larger amounts of mtDNA (about 5-folds) as it appeared from q-PCR analysis (Supplementary Figure S5A), although the mitochondrial mass (TOM22 immunolabeling, Figure 5C) was not significantly altered compared to serum starvation (Figure 2E). Interestingly, the ratio of 7S DNA to mtDNA was globally close to 1 (from 0.71 to 1.38) in all phases of the cell cycle after serum starvation whereas it was always >1 (from 1.22 to 1.85) after double thymidine treatment (Supplementary Figure S5A), indicating that in the latter only a minority of events of initiation of replication extend beyond the 7S region. Finally, mTRANS levels were 2 to 4-fold larger after double thymidine treatment than after serum starvation (Supplementary Figure S5B); this was also the case for mitochondrial transcripts assessed by RT-qPCR (about 15-fold higher levels of *16S* and *CytB* in G1, and until 38-fold higher levels of *16S* during the S-phase), indicating that not only DNA synthesis but also transcript levels globally increased in mitochondria after double thymidine treatment.

Altered mitochondrial parameters after cell cycle blockage by nocodazole

Nocodazole arrests cells in G2/M. During blockage of the cell cycle by nocodazole the mitochondrial mass measured by TOM22 immunofluorescence was in certain phases about 2-

fold greater than during blockage with serum starvation (Figure 6C and Figure 2E, respectively) and about 50% more than during blockage with double thymidine, Figure 5C. These data are in agreement with the 20 % increase in the mt mass/cell after treatment with nocodazole for 14-15h (but not at shorter times, 8h), measured by Mito tracker Green FM, and flow cytometry by an acridine orange derivative, NAO (37).

The mitochondrial transcripts, measured by mTRANS labelling were about 2.5-fold higher after cell cycle arrest with nocodazole than with the other treatments (Figure 6F and Supplementary Figure S5B). mTRANS levels remained high during all the cell cycle, with the exception of the G2/M phases. After nocodazole treatment, the levels of mtDNA were also significantly higher than after serum starvation (5 to 22-fold in the D-loop region, Supplementary Figure S5A, and 5.7-fold at the 12S locus in the S-phase), and about 2-fold higher than after synchronization by double thymidine (but 4.4-fold higher in G1/S). Interestingly, after treatment with nocodazole the mtDNA content (grey columns, Supplementary Figure S5A) was highly variable during the cell cycle, although to a lesser extent than 7S (white columns, Supplementary Figure S5A), with about a 3-fold decrease at the end of G1 and the highest values at the end of the S-phase. In contrast, after release from serum starvation the mtDNA content decreased once, sharply, (Figure 3E), as reported (1), then it slightly increased until the S-phase and again in G1. Nevertheless under these conditions the mtDNA content remains globally comparable during the cell cycle in agreement with previous findings (27). Thus, differently from serum starvation, nocodazole treatment globally alters the mtDNA content, transcript levels, and the mitochondrial mass, and this is also the case within the various phases of the cell cycle.

Supplementary Reference

1. Xiong, W., Jiao, Y., Huang, W., Ma, M., Yu, M., Cui, Q. and Tan, D. (2012) Regulation of the cell cycle via mitochondrial gene expression and energy metabolism in HeLa cells. *Acta Biochim Biophys Sin (Shanghai)*, **44**, 347-358.

Supplementary Figure legends

Figure S1. Variation in the mitochondrial mass is not necessarily correlated with the cell size. (A) Graph represents mitochondrial mass (TOM22 immunofluorescence) and cell surface (square pixels) variation, in percentage, at different phase of the cell cycle compared to G0. The cell cycle was synchronized with serum starvation. Red arrows indicate conditions where mitochondrial mass variation does correlated with changes in the cell size. **(B)** The same analysis as in panel A, performed with cell cycle synchronized with double thymidine and, **(C)** with nocodazole. With double thymidine synchronization the percentages of TOM22 and cell surface appear to display similar profiles during the cell cycle, however note that in several cases (i.e. 5h and 16h) significant increases of the mitochondrial mass takes place at unchanged cell surface. Note also that with all synchronization procedures the cell surface is severely reduced in G2/M (and when identified, in events B), as expected for cells undergone mitosis, and this is also the case for the mitochondrial mass, indicating redistribution of mitochondria in the two daughter cells. **(D)** Absolute values of TOM22 fluorescence intensity and cell surface (square pixels, ImageJ, Area Fraction) for conditions shown in panels A-C. N=30 cells, from three independent experiments. Standard error of the mean is also indicated. Absence of direct correlation between mitochondrial mass (TOM22 immunofluorescence) and cell length (μm of the longest axes) was also observed, not shown. TOM22 fluorescence intensity quantification (absolute values) were shown in Figure 2E (serum starvation), Figure 5C (double thymidine), and Figure 6C (nocodazole).

Figure S2. Immunofluorescence of nucleoid makers TFAM and Polγ during the cell cycle synchronized by serum starvation. 3D-reconstruction of HeLa cells immunolabelled with **(A)** Polγ (green) and **(B)** TFAM (green) during the cell cycle at times indicated on top. Scale bar = 10μm. Quantification of the intensity of fluorescence of Polγ and of TFAM are reported in Figure 3C and Figure 4C, respectively.

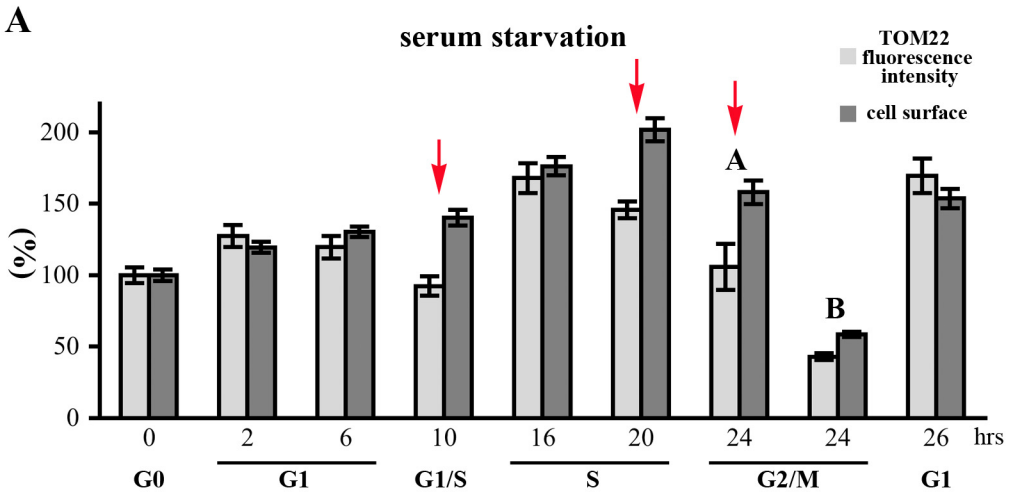
Figure S3. Proportion of mREP and mTRANS labelling in mitochondria during the cell cycle synchronized by serum starvation. (A) Proportion of mitochondria engaged in mtDNA initiation of replication during the cell cycle evaluated by percentage of mREP signal (initiation of mtDNA replication) relative to mitochondrial mass (immunolabeling with TOM22). The high levels of mREP/TOM22 at G2/M are largely due to decreased mitochondrial mass probably associated to decreased cell size, in particular at events "B", which likely represent cells undergone mitosis, rather than to an increase in the mREP signal itself. **(B)** Proportion of mitochondria engaged in mtDNA transcription during the cell cycle evaluated by percentage of mTRANS signal (mtDNA transcription) relative to mitochondrial mass. The high levels of mTRANS/TOM22 at G2/M are largely due to decreased mitochondrial mass probably associated to decreased cell size, rather than to an increase in the mREP signal itself.

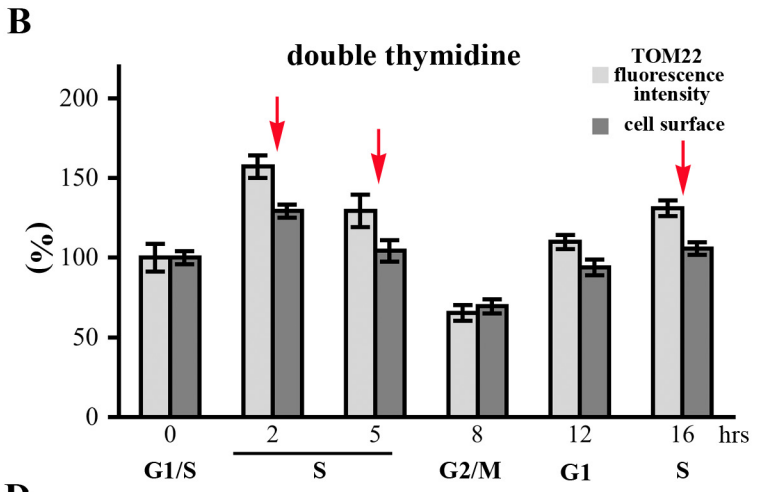
Figure S4. Immunostaining of nuclear PCNA and mitochondrial TOM22 during the cell cycle synchronized by double thymidine and nocodazole. (A) Synchronization by double thymidine. Left panels: 3D-reconstruction of HeLa cells labelled with anti-PCNA (red) at the indicated times after release from the treatment identifies the phase of cell cycle. S-phase occurs at 2h-5h, and a second S-phase appears at 16h. With the other synchronization

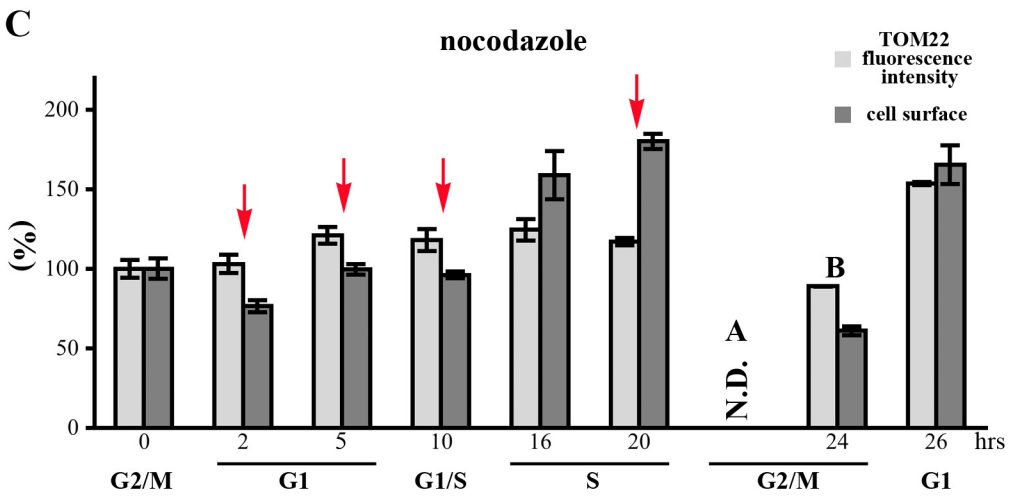
procedures a single S-phase was detected in the time intervals analysed. Quantification of PCNA immunofluorescence is shown in Figure 5B. Right panels: 3D-reconstruction of HeLa cells labelled with anti-TOM22 (green) at the indicated times after release from double thymidine treatment. Quantification of TOM22 immunofluorescence is shown in Figure 5C. Scale bar=10 μm. **(B)** Synchronization by nocodazole. Left panels: 3D-reconstruction of HeLa cells labelled with anti-PCNA (red) at the indicated times after release from the treatment identifies the phases of cell cycle. Quantification of PCNA immunofluorescence is shown in Figure 6B. Right panels: 3D-reconstruction of HeLa cells labelled with anti-TOM22 (green) at the indicated times after release from nocodazole treatment. Quantification of TOM22 immunofluorescence is shown in Figure 6C. Scale bar =10 μm.

Figure S5. Comparison of replication and transcription related parameters during the cell cycle using different synchronization procedures. (A) Comparison of DNA replication parameters with three different synchronization procedures. Relative fluorescence intensity levels of PCNA and mREP, and mtDNA content (including 7S DNA) at various times after release from serum starvation, double thymidine, or nocodazole treatment are normalized to serum starvation. For each condition, the phase corresponding to cell cycle arrest is indicated on top of the first column in the left panel. On the Y-axis, relative values are indicated (PCNA 1=200k; mREP 1=100k; mtDNA content, measured as in (27); 1= value 50); the same scales are used for the three conditions to underscore quantitative differences. X-axis is shifted to place S-phases at the same position for the three conditions. PCNA and mREP values are from Figure 2B and Figure 3B, respectively (serum starvation), and from Figure 5 B,C (double thymidine) and Figure 6 B,C (nocodazole). **(B)** Comparison of mtDNA transcription with three different synchronization procedures. Relative fluorescence intensity levels of mTRANS and expression levels of TFAM at various times after release from serum

starvation, or double thymidine, or nocodazole treatments, are normalized to serum starvation. Indications as in panel A. On the Y-axis, relative values are indicated (mTRANS 1=100k; TFAM 1=200k); mTRANS and TFAM values are from Figure 4 (serum starvation) and Figure 5 (double thymidine) and 6 (nocodazole).







D

serum starvation				
hrs	TOM22 fluorescence intensity	Cell surface	TOM22 SEM	Surface SEM
0	310321	27371	17032	1083
2	395586	32703	23761	1022
6	371038	35702	24387	1017
10	286580	38352	21183	1491
16	521109	48236	32241	1780
20	452192	55199	17719	2231
24_A	328001	43251	49830	2257
2A_B	133253	16045	7371	512
26	525971	42033	37115	1878

nocodazole				
hrs	TOM22 fluorescence intensity	Cell surface	TOM22 SEM	Surface SEM
0	929629	82664	52080	5306
2	958374	63294	53703	3156
6	1125519	82352	49243	2780
10	1097473	79468	63499	1758
16	1157535	131317	62752	12426
20	1087999	148940	21480	3934
24_A	ND	ND	ND	ND
2A_B	826969	50497	29700	2299
26	1427840	136822	145172	10055

double thymidine				
hrs	TOM22 fluorescence intensity	Cell surface	TOM22 SEM	Surface SEM
0	378103	48459	33405	1985
2	594234	62628	26444	2034
5	489354	50575	38165	3285
8	246900	33674	18500	2175
12	415427	45550	16488	2377
16	495428	51239	18486	1951

Figure S1

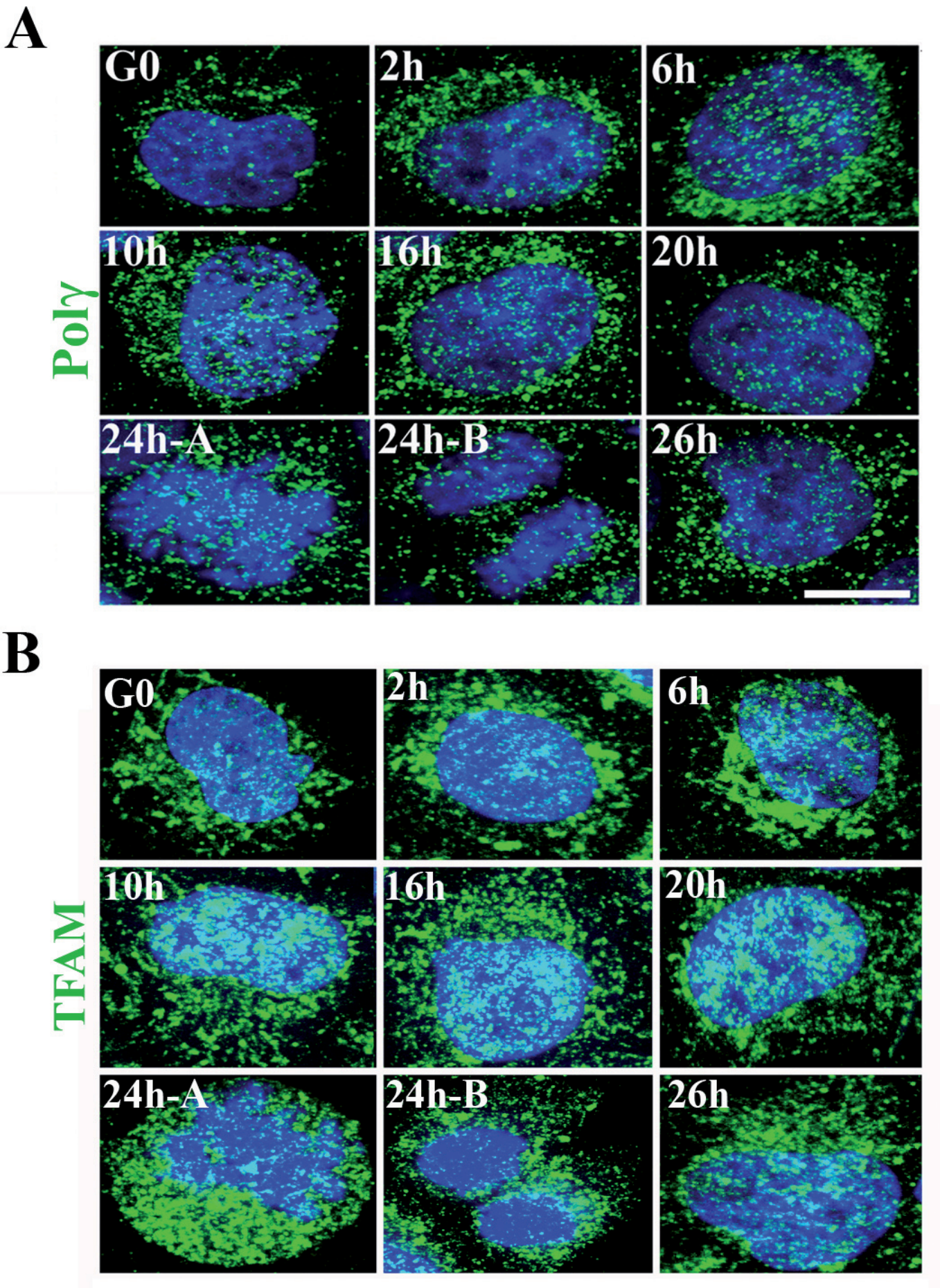

A
G0
2h
6h
10h
16h
20h
Polγ
24h-A
24h-B
26h
B
G0
2h
6h
10h
16h
20h
TFAM
24h-A
24h-B
26h

Figure S2

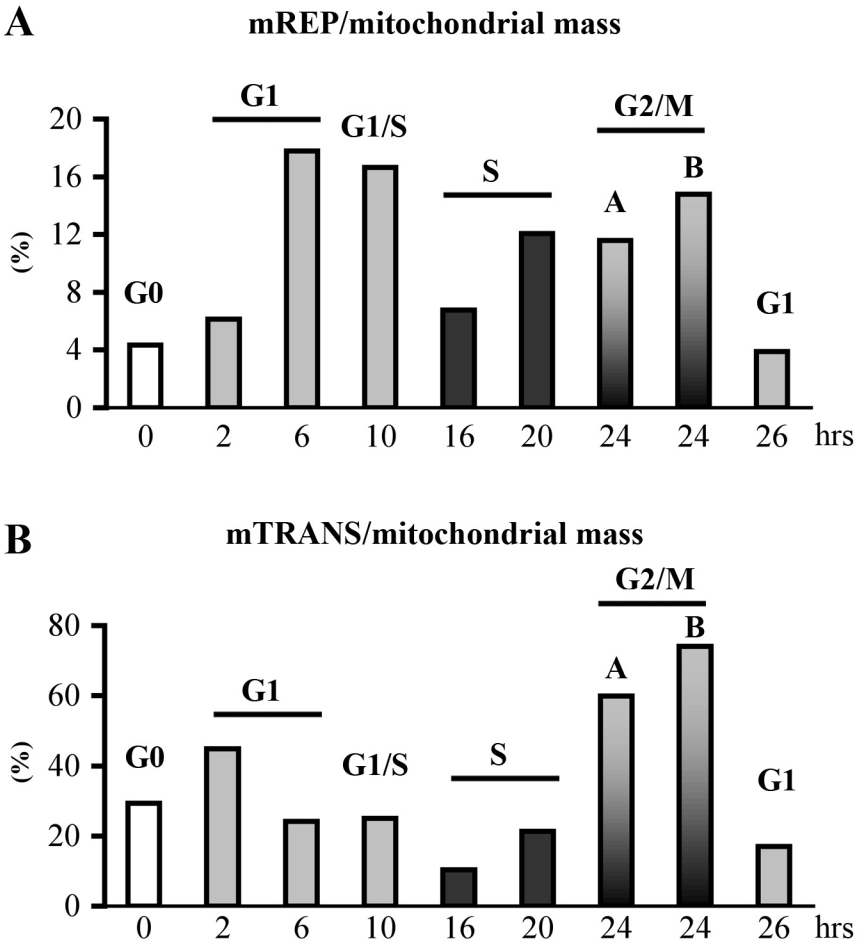

A
mREP/mitochondrial mass
G1
G1/S
S
G2/M
A
B
G0
G1
(%)
20
16
12
8
4
0
0
2
6
10
16
20
24
24
26
hrs
B
mTRANS/mitochondrial mass
G2/M
B
A
G1
G0
G1/S
S
G1
(%)
80
60
40
20
0
0
2
6
10
16
20
24
24
26
hrs

Figure S3

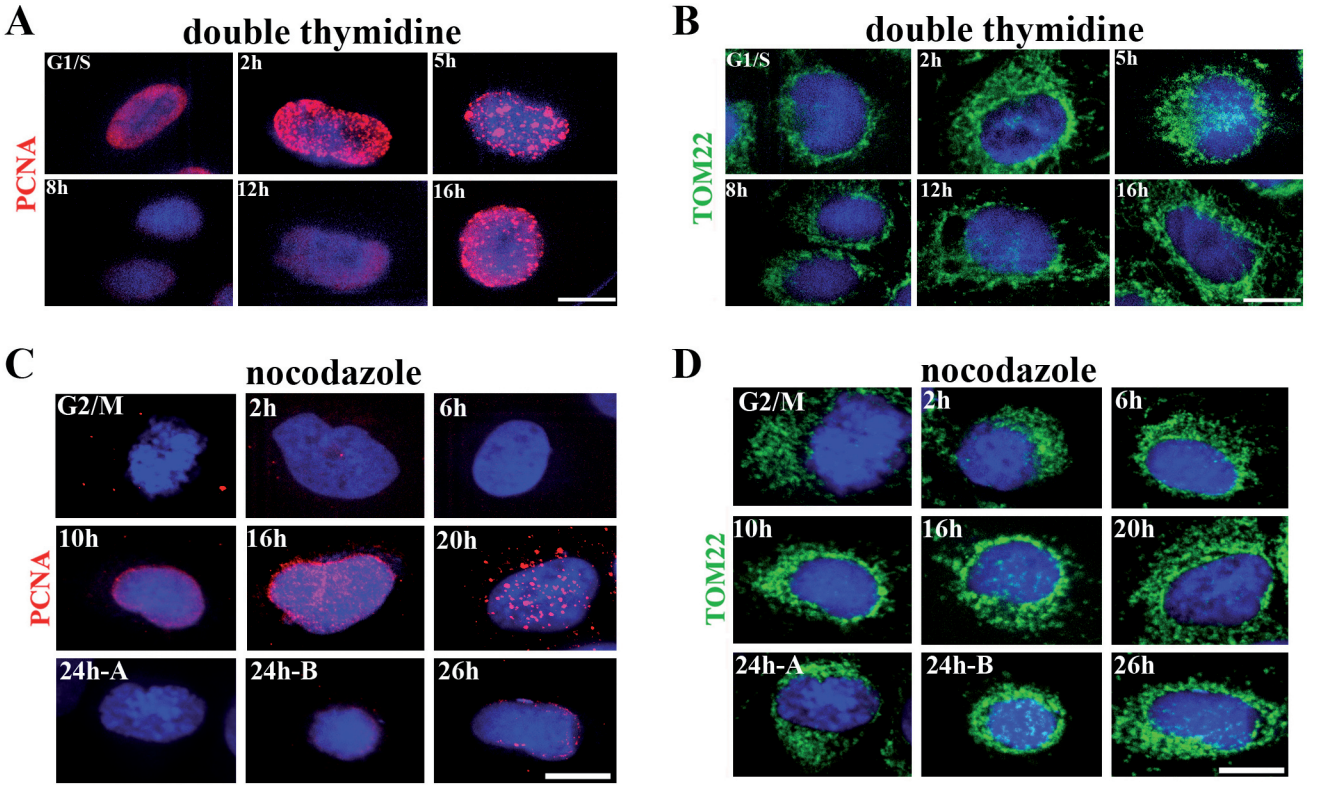

A
double thymidine
G1/S
2h
5h
PCNA
8h
12h
16h
B
double thymidine
TOM22
C
nocodazole
G2/M
2h
6h
PCNA
10h
16h
20h
24h-A
24h-B
26h
D
nocodazole
TOM22

Figure S4

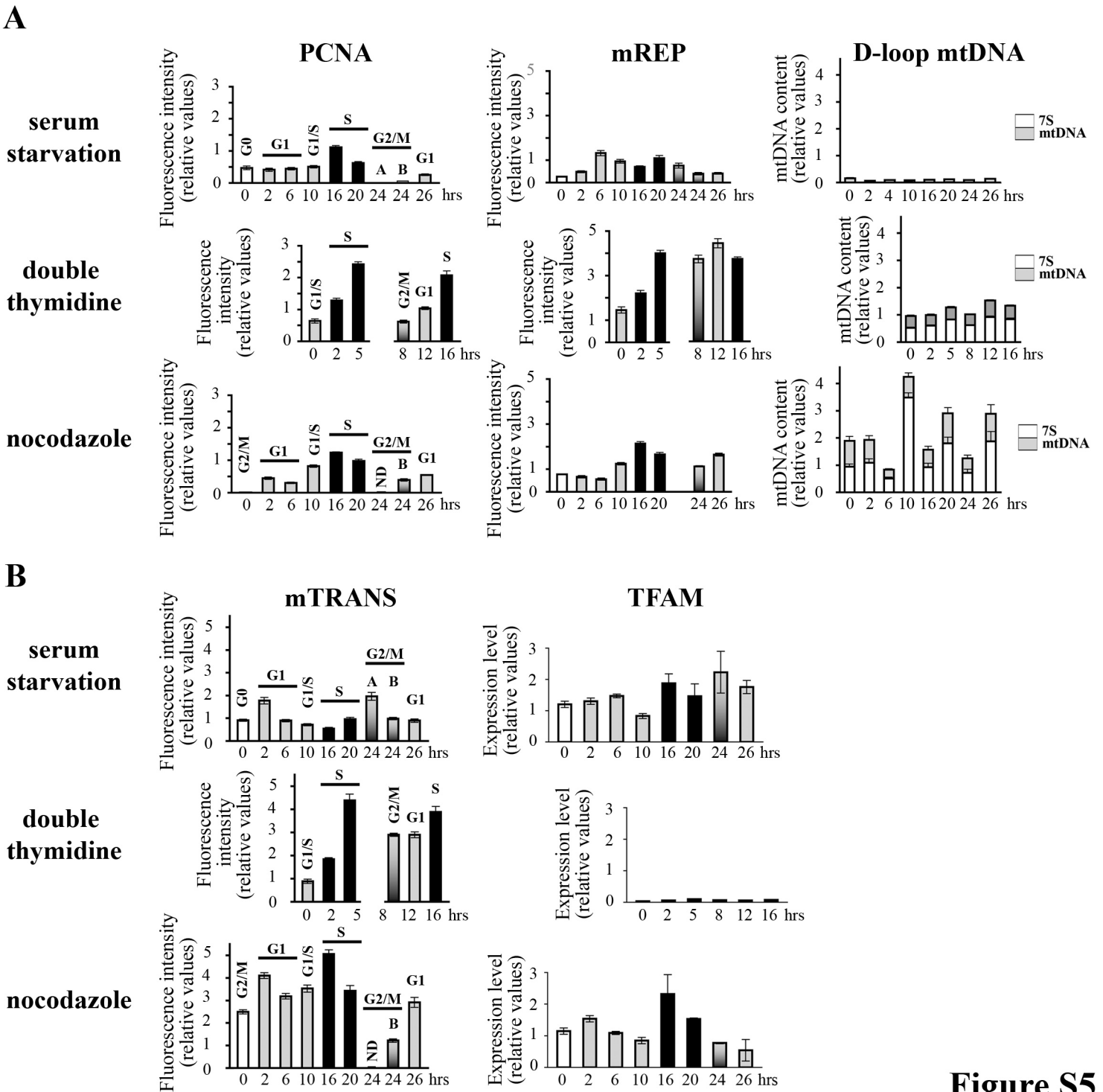

A
PCNA
mREP
D-loop mtDNA
serum starvation
double thymidine
nocodazole
Fluorescence intensity (relative values)
mtDNA content (relative values)
7S
mtDNA
G0
G1
G1/S
S
G2/M
A
B
ND
hrs
B
mTRANS
TFAM
Expression level (relative values)

Figure S5

Probe	start	end	size
1	1905	2866	961
6	7400	8518	1118
11	13416	14836	1420
mREP	446	544	98

mTRANS	probes 1, 6, 11

human mt genome start	1
human mt genome end	16568

Table S1. Coordinates of the probes.
The start and end points of probes used for FISH experiments are given on the mitochondrial DNA (NC_012920,GenBank, was used as reference). This mtDNA is a circular molecule of 16568 bp in size according to the reference above. Mix of more than one probe and their composition are indicated in individual panels below. All probes are oriented in the direction of transcription of the H strand, with the exception of probe ND6 that is in the inverse orientation (transcription on the L strand).

RT-qPCR primers

Probe	forward primer	reverse primer	reference
TBP	CTCACAGGTCAAAGGTTTAC	GCTGAGGTTGCAGGAATTGA	*Mercy et al. 2005. FEBS Journal 272(19):*
12S (1)	CTGCTCGCCAGAACACTACG	TGAGCAAGAGGTGGTGAGGT	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
12S (2)	AAACTGCTCGCCAGAACACT	CATGGGCTACACCTTGACCT	*Uchuimi et al. 2010. NAR 38(16)*
16S (1)	GTATGAATGGCTCCACGAGG	GGTCTTCTCGTCTTGCTGTG	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
16S(2)	GCTAAACCTAGCCCCAAACC	TTGGCTCTCCTTGCAAAGTT	*Uchuimi et al. 2010. NAR 38(16)*
ND1	TGGCCAACCTCCTACTCCTC	ATGGCGTCAGCGAAGGGTTG	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
ND2	ACTGCGCTAAGCTCGCACTG	ATTATGGATGCGGTTGCTTG	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
COI	ACCCTAGACCAAACCTACGC	TAGGCCGAGAAAGTGTTGTG	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
COII	ACAGATGCAATTCCCGGACG	GGCATGAAACTGTGGTTTGC	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
ATP8	ATGCCCCAACTAAATACT	TTGTGGGGGCAATGAATG	*Uchuimi et al. 2010. NAR 38(16)*
ATP6	CCCACTTCTTACCACAAGGC	GTAGGTGGCCTGCAGTAATG	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
COIII	ACTTCCACTCCATAACGCTC	TGGCCTTGGTATGTGCTTTC	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
ND3	CTACCATGAGCCCTACAAAC	ACTCATAGGCCAGACTTAGG	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
ND4L	TATCGCTCACACCTCATATC	AGGCGGCAAAGACTAGTATG	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
ND4	ACAAGCTCCATCTGCCTACG	TTATGAGAATGACTGCGCCG	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
ND5	GGTTTCATCCTCGCCTTAGC	ACCTAATTGGGCTGATTTGC	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
CYTB	CTCCCGTGAGGCCAAATATC	GAATCGTGTGAGGGTGGGAC	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
ND6	ATTGGTGCTGTGGGTGAAAG	GGATCCTCCCGAATCAACCC	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
NRF1	GGAGTGATGTCCGCACAGAA	CGCTGTTAAGCGCCATAGTG	*Savagner et al. 2003. Biochem Biophys Res Com310(3)*
POLG	GAGAAGGCCCAGCAGATGTA	ATCCGACAGCCGATACCA	*Setzer et al. 2008. Am J. Pathol. 172(3)*
TFAM	GACTTCTGCCAGCATAATAC	GAGTTCTGCCTGCTTTATG	*Piechota et al. 2006. Acta Biochim Pol.53(3)*

qPCR primers

Probe	forward primer	reverse primer	reference
18S	GAGAAACGGCTACCACATCC	GCCTCGAAAGAGTCCTGTAT	*Suissa et al. 2009. PLoS Genetics 5(5):e1000474*
12S	GCTCGCCAGAACACTACGAG	CAGGGTTTGCTGAAGATGGC	*Parone et al. 2008. PLoS One 3(9):e3257*
A	GTGGCTTTGGAGTTGCAGTT	-	*Antes et al. 2011. NAR 38(19):6466-6476*
B1	-	CAGCCACCATGAATATTGTAC	*Antes et al. 2011. NAR 38(19):6466-6476*
B2	-	GAAGCAGATTTGGGTACCAC	*Antes et al. 2011. NAR 38(19):6466-6476*

Table S2. RT-qPCR and qPCR primers. The sequence of forward and reverse primers for RT-qPCR (upper panel) and qPCR (lower panel) is indicated after the name of the probe that also indicats the gene analysed. Number in parenthesis indicate different sets used to test the same gene. The pair A-B1 amplifies a mtDNA region included in 7S, while the pair A-B2 amplifies a region beyond 7S in the direction of the H-strand (see scheme in Fig. 2E). Reference is indicated in the last column.