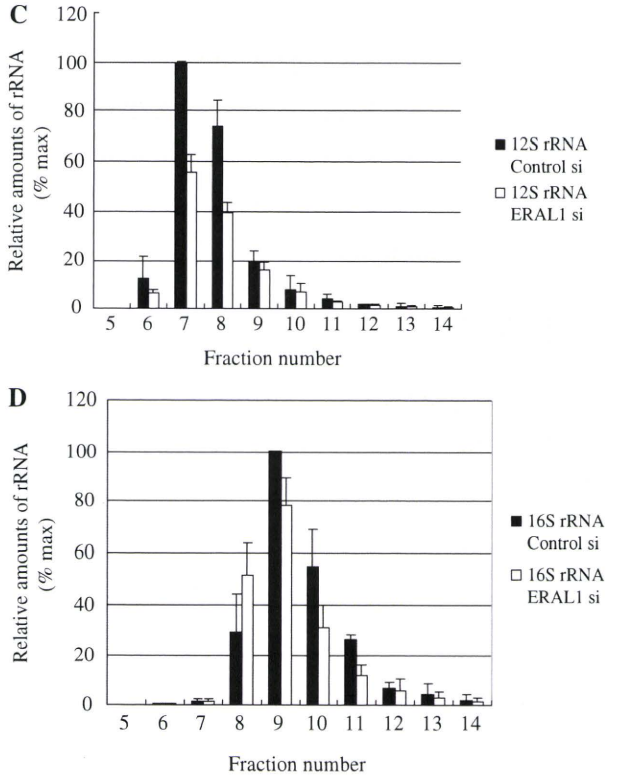

Figure 6. Continued.

collapse of mitochondrial membrane potential. In subsequent experiments, therefore, the production of oxidants was tested by a redox-responsive fluorescent dye H_2DCFDA to examine whether ERAL1 siRNA knockdown led to the generation of ROS. A statistically significant increase in the fluorescence over the control was seen at 72 h after the siRNA transfection (Figure 7).

Cell growth retardation

In order to investigate the effect of the elimination of ERAL1 on the cell growth, the number of living cells in each clone was monitored using a cell counter (Figure 8A, upper panel). Up to 2 days after siRNA transfection, there was no significant difference in the growth or morphology of the cells between ERAL1 siRNA and control siRNA transfectants. However, the increase in living cells was markedly suppressed at Day 3 in ERAL1 siRNA-treated cells and this was accompanied by an increment in the population of round and dead cells. These results indicate that ERAL1 is important for the normal cell proliferation and its loss affects mitochondrial functions. We cultured the cells in glucose-free media instead supplemented with galactose to force dependence on oxidative phosphorylation. Even in the galactose culture media, the inhibitory effect of ERAL1 depletion on the cell proliferation was little changed (Figure 8A, lower panel), raising the possibility that the inhibition of the cell proliferation is not much dependent on the mitochondrial dysfunction.

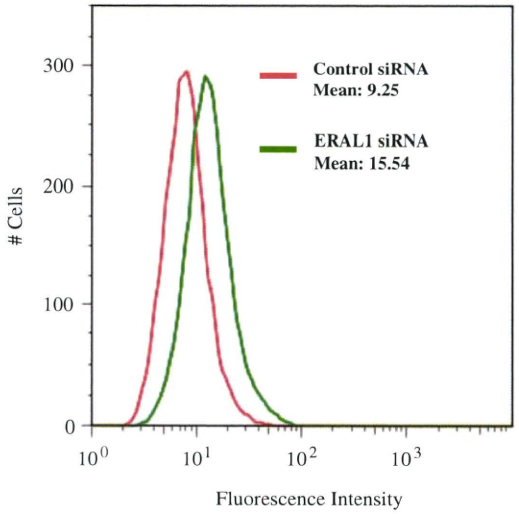

Figure 7. Increased ROS production by ERAL1 siRNA-mediated knockdown. HeLa cells were transfected with control (Green) and ERAL1 siRNAs (Red). After 72 h, the cells were treated 10 μM H_2DCFDA for 30 min and were subjected to FACS analysis for quantitative estimation of ROS.

Induction of apoptosis in ERAL1 knockdown HeLa cells

The increase in the apparently apoptotic cells as described earlier prompted us to confirm apoptotic cell death by examining caspase 3 activation. After the treatment of ERAL1 siRNA for 72 h, the cells were lysed and subjected to western blotting. Cleaved caspase 3 or activation of caspase 3 was observed in the ERAL1 knockdown cells (Figure 8B).

DISCUSSION

Here we have shown that ERAL1 is a mitochondrial ribosome-associated factor and is important for mitochondrial function and cell viability based on the following evidence; (i) ERAL1 is a mitochondrial matrix protein, (ii) ERAL1 is associated with small mitoribosomes including the 12S rRNA, (iii) depletion of ERAL1 impairs the assembly of the small subunit of mitoribosome, inhibits the mitochondrial translation, decreases mitochondrial membrane potential and increases ROS generation and (iv) depletion of ERAL1 severely retards cell growth. One of primary roles of ERAL1 may be in the formation of functional ribosomal small subunits and might be involved in mitochondrial biogenesis and associated with the mitochondrial ribosome during the assembly, rRNA folding and RNA modification.

A direct link between mitochondrial transcription and translation has been demonstrated in *Saccharomyces cerevisiae*. An amino-terminal domain of mitochondrial RNA polymerase (Rpo41p) is a binding site for Nam1p that is proposed to deliver newly synthesized RNAs to the inner mitochondrial membrane and promote subsequent interactions between gene specific translational activators and ribosomes. Thus the processes of transcription and

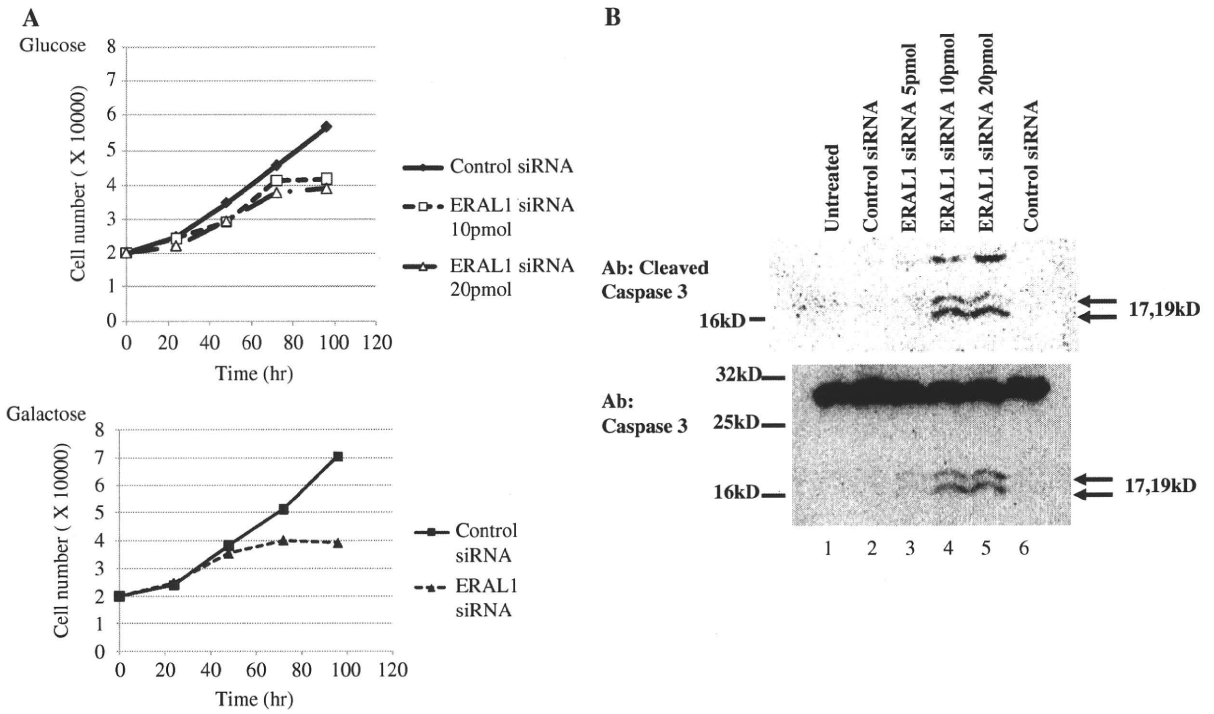

Figure 8. Depletion of ERAL1 caused a growth defect and increased apoptosis. (**A**) Proliferation rate of siRNA-transfected HeLa cells. The cells were cultured with a normal glucose medium (upper) or a galactose medium (lower). HeLa cells were transfected with control and ERAL1 siRNAs at Day 0. At indicated time, the cells were harvested and counted by a cell counter. (**B**) HeLa cells were transfected with control and ERAL siRNAs for 72 h. Untransfected (lane1) and transfected HeLa cells were lysed and immunoblotted by anti-cleaved caspase 3 (upper panel) and anti-caspase 3 (lower panel) antibodies, respectively.

translation are physically and functionally coupled in *S. cerevisiae* (26,27).

Proteins co-immunoprecipitated with ERAL1 included putative nucleoid proteins in addition to mitochondrial ribosomal proteins and several translation-associated proteins in HeLa cells (Table 1). Nucleoids are dynamic structures containing mtDNA and proteins, which are involved in maintenance, replication and transcription of mtDNA (13). Coupling of transcription and translation in human mitochondria has been suggested (3). There has been reported interaction of mitochondrial RNA polymerase with a mitoribosomal protein MRPL12. This interaction was shown *in vitro* to stimulate transcription (28). In a recent report on proteins associated with mitochondrial nucleoids, 15 mitoribosomal proteins were identified along with assorted factors that also function in protein synthesis (29). These results raise the possibility that mitochondrial ribosomal proteins and associated factors are coupled with transcription via transcription-related factors such as TFAM also in higher eukaryotes.

Gohda *et al.* (21) reported that depletion of chicken ERA protein diminished the growth rate of the cell, accompanied by an accumulation of apoptosis cells. The analysis of cell cycle indicates that the elimination of chicken ERA caused arrest at G1 phase but not at M phase, which suggests a distinct role of chicken ERA in the cell cycle progression from that of bacterial Era protein (21,22). They showed that chicken ERA protein was localized in cytosol in DT40 cells and bound to RNA homopolymer (poly-U). Our experiment showed that human ERAL1 was localized at mitochondrial matrix and associated directly or indirectly with 12S mitochondrial rRNA. Depletion of human ERAL1 also diminished the growth rate of HeLa cell probably by apoptosis. In a eukaryotic cell, ERAL1 may be involved in cell viability and apoptosis at least in part through the mitochondrial function.

The human mitochondrial ribosome recycling factor is essential for cell viability (30). Rorbach *et al.* (30) cloned the putative mitochondrial recycling factor, mtRRF and showed that the protein could associate with mitoribosomes. Depletion of mtRRF was lethal, causing initially mitochondrial dysmorphism, aggregation of mitoribosome, elevated mitochondrial ROS and loss of OXPHOS complexes. The phenotypes caused by ERAL1 depletion resemble to those by mtRRF, suggesting that ERAL1 and mtRRF share the some functions or cooperate in mitochondrial ribosomes.

Mitochondrial nucleoids include not only factors involved in replication and transcription but also structural proteins required for mtDNA maintenance. Recently, Wang and Bogenhagen (31) reported that EF-Tu, LRP130, ATAD3 and DHX30 were TFAM- and mtSSB-associated proteins. EF-Tu is a translation elongation factor with significant homology to its prokaryote counterparts. Previously we have isolated several proteins such as ERAL1, EF-Tu, LRP130 and Hsp60 by co-immunoprecipitaion with TFAM (17). Thus, the

nucleoides harbour the proteins related to translation in addition to transcription, replication and mtDNA maintenance.

In *E. coli* RbfA (ribosome-binding factor A) is essential for cell growth at low temperature (32,33). RbfA is a bacterial cold shock response protein, required for an efficient processing of the 5′ end of the 16S ribosomal RNA during assembly of the small (30S) ribosomal subunit. RbfA binds to the 30S subunit in a position overlapping the binding site of the A and P sites for tRNAs. RbfA has an important role in maturation of the 30S subunit and is involved in translational advantage under conditions of cold shock. It is demonstrated that Era and RbfA have an overlapping function that is essential for the ribosome biogenesis (32). Mammalian ERAL1 is also likely to bind 12S rRNA, is associated with the small subunit of mitochondrial ribosome, and is involved in the ribosomal assembly. Considering the importance of ERAL1 for cell viability, it might be also involved in stress response like RbfA.

Genetic investigation of patients with defective mitochondrial translation led to the discovery of novel mutations in EFG1 in one affected baby and in the mitochondrial EF-Tu in another one (34). Both patients were affected by severe lactic acidosis and rapidly progressive, fatal encephalopathy. The EFG1-mutant patient had early-onset Leigh syndrome, whereas the EF-Tu mutant patient had severe infantile macrocystic leukodystrophy with micropolygyria. ERAL1 is a mitoribosomal protein which is associated with a small subunit of ribosome. In our immunoprecipitation analysis, we showed the interaction of ERAL1 with EF-Tu. ERAL1 might be involved in the translation reaction itself in addition to the assembly of mitoribosomes.

DAP3, which exists in the mitochondrial matrix, is involved in apoptosis (35). DAP3 has been reported to be involved in both γ-interferon and tumour necrosis factor-α-induced apoptosis as well as staurosporine-induced mitochondrial fragmentation (36–38). hNOA1 (human nitric oxide-associated protein 1) interacts with complex I and DAP3 and regulates mitochondrial respiration and apoptosis (39). ERAL1 is also associated with DAP3 and depletion of ERAL1 causes apoptosis. Accordingly, ERAL1 could be involved in apoptosis through the DAP3 interaction.

So far, DAP3 has been the only GTP-binding protein found in the small subunit of the mammalian and yeast mitochondrial ribosomes (40). This protein accounts for the highly specific GTP-binding affinity of small subunits of mitochondrial ribosomes. An immuno-electron microscopic study showed that DAP3 is localized to the base of the lower lobe of the small subunit on the solvent side of the ribosome (41). ERAL1 is associated with the small ribosomal subunit and has a putative GTPase domain. There is the possibility that ERAL1 and DAP3 coordinately regulate the mitochondrial translation and apoptosis.

DAP3 is located at the lower bottom of the 28S subunit and it is not likely that it would interact with a protein that would bind to the platform of the small subunit. In our experiment by using the sucrose density gradient centrifugation, ERAL1 and DAP3 distributed broadly also in lighter fractions through fractions 1–6. However the distribution of other small subunit protein such as MRPS22 and large subunit protein MRPL3 were located through fractions 9–11. The RNA distribution analysis also showed that the ribosomes were located to fractions 7–9, raising the possibility that some DAP3 protein do not associate with the small ribosomal subunits and conducts another function for example as a death associated protein. We also observed that ERAL1 was associated with DAPK2 (death-associated protein kinase 2) (Table 1). We speculate that putative free DAP3 binds to free ERAL1 in mitochondria and the two proteins work cooperatively for apoptosis and/or other functions irrespective of the mitochondrial respiratory chain function. In this respect, it is noteworthy that the replacement of glucose by galactose in the culture medium did not augment the growth retardation induced by the ERAL1 siRNA (Figure 8A)

Several mitochondrial ribosome-related genes are known to be associated with mitochondrial disease and diseases characterized by reduced energy metabolism. For example, Weraarpachai *et al.* (42) have reported that TACO1 protein is a translational activator of COX I. Mutation of the TACO1 gene leads to cytochrome *c* oxidase deficiency and resultantly to late onset Leigh syndrome. The TACO1 protein is associated with EF-Ts and involved specifically in the COX I synthesis.

LRPPRC (LRP130), which is a yeast translational activator of Pet309 (43), was coimmunoprecipitated with ERAL1 (Table1). Pet309 is a mitochondrial protein needed for the proper splicing and translational initiation of mtDNA-encoded COX mRNA (43). These proteins contain PPR motifs that consist of degenerate sequences of 35 amino acids and form antiparallel α helices. PPR motif proteins are thought to be involved in post-translational mRNA metabolism, especially in mitochondria and chloroplasts (44,45). Mutations in LRPPRC are found in French–Canadian individuals with Leigh syndrome harbouring an isolated COX deficiency (46). LRPPRC is reported to be involved in the stabilization of mRNAs for both COX I and COX III in mammals, without directly affecting their translation, suggesting that this protein is involved in processing of the primary RNA unit or in stabilizing mature transcripts with a very limited sequence selectivity (47). Mitochondrial ribosomal proteins and translation related proteins should be considered as important candidates for mitochondrial disease, especially when mtDNA mutations have been ruled out (47). Mutation of ERAL1 also could be a cause of some types of mitochondrial disease.

ERAL1 may be involved in diverse functions, such as mitoribosome assembly, mitochondrial translation, mitochondrial transcription, proliferation, apoptosis and so on, as a mitoribosome-associated protein and/or free protein.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We wish to thank Ms Kawabe for helping the manuscript preparation. The authors would like to acknowledge the technical expertise of the Support Center for Education and Research, Kyushu University.

FUNDING

Ministry of Education, Science, Technology, Sports and Culture of Japan, Grants-in-Aid for Scientific Research (19209019 and 21590337). Funding for open access charge: Ministry of Education, Science, Technology, Sports and Culture of Japan.

Conflict of interest statement. None declared.

REFERENCES

1. Kang,D. and Hamasaki,N. (2005) Mitochondrial transcription factor A in the maintenance of mitochondrial DNA: overview of its multiple roles. *Ann. NY Acad. Sci.*, **1042**, 101–108.
2. Asin-Cayuela,J. and Gustafsson,C.M. (2007) Mitochondrial transcription and its regulation in mammalian cells. *Trends Biochem. Sci.*, **32**, 111–117.
3. Falkenberg,M., Larsson,N.G. and Gustafsson,C.M. (2007) DNA replication and transcription in mammalian mitochondria. *Annu. Rev. Biochem.*, **76**, 679–699.
4. Pel,H.J. and Grivell,L.A. (1994) Protein synthesis in mitochondria. *Mol. Biol. Rep.*, **19**, 183–194.
5. Rorbach,J., Soleimanpour-Lichaei,R., Lightowlers,R.N. and Chrzanowska-Lightowlers,Z.M. (2007) How do mammalian mitochondria synthesize proteins? *Biochem. Soc. Trans.*, **35**, 1290–1291.
6. O'Brien,T.W. (2002) Evolution of a protein-rich mitochondrial ribosome: implications for human genetic disease. *Gene*, **286**, 73–79.
7. Kenmochi,N., Suzuki,T., Uechi,T., Magoori,M., Kuniba,M., Higa,S., Watanabe,K. and Tanaka,T. (2001) The human mitochondrial ribosomal protein genes: mapping of 54 genes to the chromosomes and implications for human disorders. *Genomics*, **77**, 65–70.
8. Coenen,M.J., Antonicka,H., Ugalde,C., Sasarman,F., Rossi,R., Heister,J.G., Newbold,R.F., Trijbels,F.J., van den Heuvel,L.P., Shoubridge,E.A. *et al.* (2004) Mutant mitochondrial elongation factor G1 and combined oxidative phosphorylation deficiency. *N. Engl. J. Med.*, **351**, 2080–2086.
9. Antonicka,H., Sasarman,F., Kennaway,N.G. and Shoubridge,E.A. (2006) The molecular basis for tissue specificity of the oxidative phosphorylation deficiencies in patients with mutations in the mitochondrial translation factor EFG1. *Hum. Mol. Genet.*, **15**, 1835–1846.
10. Miller,C., Saada,A., Shaul,N., Shabtai,N., Ben-Shalom,E., Shaag,A., Hershkovitz,E. and Elpeleg,O. (2004) Defective mitochondrial translation caused by a ribosomal protein (MRPS16) mutation. *Ann. Neurol.*, **56**, 734–738.
11. Emdadul Haque,M., Grasso,D., Miller,C., Spremulli,L.L. and Saada,A. (2008) The effect of mutated mitochondrial ribosomal proteins S16 and S22 on the assembly of the small and large ribosomal subunits in human mitochondria. *Mitochondrion*, **8**, 254–261.
12. Chen,X.J. and Butow,R.A. (2005) The organization and inheritance of the mitochondrial genome. *Nat. Rev. Genet.*, **6**, 815–825.
13. Kucej,M. and Butow,R.A. (2007) Evolutionary tinkering with mitochondrial nucleoids. *Trends Cell Biol.*, **17**, 586–592.
14. Parisi,M.A. and Clayton,D.A. (1991) Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. *Science*, **252**, 965–969.
15. Ohno,T., Umeda,S., Hamasaki,N. and Kang,D. (2000) Binding of human mitochondrial transcription factor A, an HMG box protein, to a four-way DNA junction. *Biochem. Biophys. Res. Commun.*, **271**, 492–498.
16. Shadel,G.S. (2008) Expression and maintenance of mitochondrial DNA: new insights into human disease pathology. *Am. J. Pathol.*, **172**, 1445–1456.
17. Kanki,T., Ohgaki,K., Gaspari,M., Gustafsson,C.M., Fukuoh,A., Sasaki,N., Hamasaki,N. and Kang,D. (2004) Architectural role of mitochondrial transcription factor A in maintenance of human mitochondrial DNA. *Mol. Cell. Biol.*, **24**, 9823–9834.
18. Sullivan,S.M., Mishra,R., Neubig,R.R. and Maddock,J.R. (2000) Analysis of guanine nucleotide binding and exchange kinetics of the Escherichia coli GTPase Era. *J. Bacteriol.*, **182**, 3460–3466.
19. Meier,T.I., Peery,R.B., McAllister,K.A. and Zhao,G. (2000) Era GTPase of Escherichia coli: binding to 16S rRNA and modulation of GTPase activity by RNA and carbohydrates. *Microbiology*, **146**, 1071–1083.
20. Sharma,M.R., Barat,C., Wilson,D.N., Booth,T.M., Kawazoe,M., Hori-Takemoto,C., Shirouzu,M., Yokoyama,S., Fucini,P. and Agrawal,R.K. (2005) Interaction of Era with the 30S ribosomal subunit implications for 30S subunit assembly. *Mol Cell*, **18**, 319–329.
21. Gohda,J., Nomura,Y., Suzuki,H., Arai,H., Akiyama,T. and Inoue,J. (2003) Elimination of the vertebrate Escherichia coli Ras-like protein homologue leads to cell cycle arrest at G1 phase and apoptosis. *Oncogene*, **22**, 1340–1348.
22. Akiyama,T., Gohda,J., Shibata,S., Nomura,Y., Azuma,S., Ohmori,Y., Sugano,S., Arai,H., Yamamoto,T. and Inoue,J. (2001) Mammalian homologue of *E. coli* Ras-like GTPase (ERA) is a possible apoptosis regulator with RNA binding activity. *Genes Cells*, **6**, 987–1001.
23. Shibahara,K., Uchiumi,T., Fukuda,T., Kura,S., Tominaga,Y., Maehara,Y., Kohno,K., Nakabeppu,Y., Tsuzuki,T. and Kuwano,M. (2004) Targeted disruption of one allele of the Y-box binding protein-1 (YB-1) gene in mouse embryonic stem cells and increased sensitivity to cisplatin and mitomycin C. *Cancer Sci.*, **95**, 348–353.
24. Uchiumi,T., Fotovati,A., Sasaguri,T., Shibahara,K., Shimada,T., Fukuda,T., Nakamura,T., Izumi,H., Tsuzuki,T., Kuwano,M. *et al.* (2006) YB-1 is important for an early stage embryonic development: neural tube formation and cell proliferation. *J. Biol. Chem.*, **281**, 40440–40449.
25. Ohgaki,K., Kanki,T., Fukuoh,A., Kurisaki,H., Aoki,Y., Ikeuchi,M., Kim,S.H., Hamasaki,N. and Kang,D. (2007) The C-terminal tail of mitochondrial transcription factor a markedly strengthens its general binding to DNA. *J. Biochem.*, **141**, 201–211.
26. Rodeheffer,M.S., Boone,B.E., Bryan,A.C. and Shadel,G.S. (2001) Nam1p, a protein involved in RNA processing and translation, is coupled to transcription through an interaction with yeast mitochondrial RNA polymerase. *J. Biol. Chem.*, **276**, 8616–8622.
27. Wang,Y. and Shadel,G.S. (1999) Stability of the mitochondrial genome requires an amino-terminal domain of yeast mitochondrial RNA polymerase. *Proc. Natl Acad. Sci. USA*, **96**, 8046–8051.
28. Wang,Z., Cotney,J. and Shadel,G.S. (2007) Human mitochondrial ribosomal protein MRPL12 interacts directly with mitochondrial RNA polymerase to modulate mitochondrial gene expression. *J. Biol. Chem.*, **282**, 12610–12618.
29. Bogenhagen,D.F., Rousseau,D. and Burke,S. (2008) The layered structure of human mitochondrial DNA nucleoids. *J. Biol. Chem.*, **283**, 3665–3675.
30. Rorbach,J., Richter,R., Wessels,H.J., Wydro,M., Pekalski,M., Farhoud,M., Kuhl,I., Gaisne,M., Bonnefoy,N., Smeitink,J.A. *et al.* (2008) The human mitochondrial ribosome recycling factor is essential for cell viability. *Nucleic Acids Res.*, **36**, 5787–5799.
31. Wang,Y. and Bogenhagen,D.F. (2006) Human mitochondrial DNA nucleoids are linked to protein folding machinery and metabolic enzymes at the mitochondrial inner membrane. *J. Biol. Chem.*, **281**, 25791–25802.
32. Inoue,K., Chen,J., Tan,Q. and Inouye,M. (2006) Era and RbfA have overlapping function in ribosome biogenesis in Escherichia coli. *J. Mol. Microbiol. Biotechnol.*, **11**, 41–52.

33. Datta,P.P., Wilson,D.N., Kawazoe,M., Swami,N.K., Kaminishi,T., Sharma,M.R., Booth,T.M., Takemoto,C., Fucini,P., Yokoyama,S. *et al.* (2007) Structural aspects of RbfA action during small ribosomal subunit assembly. *Mol. Cell*, **28**, 434–445.
34. Sasarman,F., Antonicka,H. and Shoubridge,E.A. (2008) The A3243G tRNALeu(UUR) MELAS mutation causes amino acid misincorporation and a combined respiratory chain assembly defect partially suppressed by overexpression of EFTu and EFG2. *Hum. Mol. Genet.*, **17**, 3697–3707.
35. Berger,T., Brigl,M., Herrmann,J.M., Vielhauer,V., Luckow,B., Schlondorff,D. and Kretzler,M. (2000) The apoptosis mediator mDAP-3 is a novel member of a conserved family of mitochondrial proteins. *J. Cell Sci.*, **113**, 3603–3612.
36. Kim,H.R., Chae,H.J., Thomas,M., Miyazaki,T., Monosov,A., Monosov,E., Krajewska,M., Krajewski,S. and Reed,J.C. (2007) Mammalian dap3 is an essential gene required for mitochondrial homeostasis in vivo and contributing to the extrinsic pathway for apoptosis. *FASEB J.*, **21**, 188–196.
37. Kissil,J.L., Deiss,L.P., Bayewitch,M., Raveh,T., Khaspekov,G. and Kimchi,A. (1995) Isolation of DAP3, a novel mediator of interferon-gamma-induced cell death. *J. Biol. Chem.*, **270**, 27932–27936.
38. Mukamel,Z. and Kimchi,A. (2004) Death-associated protein 3 localizes to the mitochondria and is involved in the process of mitochondrial fragmentation during cell death. *J. Biol. Chem.*, **279**, 36732–36738.
39. Tang,T., Zheng,B., Chen,S.H., Murphy,A.N., Kudlicka,K., Zhou,H. and Farquhar,M.G. (2009) hNOA1 interacts with complex I and DAP3 and regulates mitochondrial respiration and apoptosis. *J. Biol. Chem.*, **284**, 5414–5424.
40. Cavdar Koc,E., Ranasinghe,A., Burkhart,W., Blackburn,K., Koc,H., Moseley,A. and Spremulli,L.L. (2001) A new face on apoptosis: death-associated protein 3 and PDCD9 are mitochondrial ribosomal proteins. *FEBS Lett.*, **492**, 166–170.
41. O'Brien,T.W., O'Brien,B.J. and Norman,R.A. (2005) Nuclear MRP genes and mitochondrial disease. *Gene*, **354**, 147–151.
42. Weraarpachai,W., Antonicka,H., Sasarman,F., Seeger,J., Schrank,B., Kolesar,J.E., Lochmuller,H., Chevrette,M., Kaufman,B.A., Horvath,R. *et al.* (2009) Mutation in TACO1, encoding a translational activator of COX I, results in cytochrome c oxidase deficiency and late-onset Leigh syndrome. *Nat. Genet.*, **41**, 833–837.
43. Manthey,G.M., Przybyla-Zawislak,B.D. and McEwen,J.E. (1998) The Saccharomyces cerevisiae Pet309 protein is embedded in the mitochondrial inner membrane. *Eur. J. Biochem.*, **255**, 156–161.
44. Lurin,C., Andres,C., Aubourg,S., Bellaoui,M., Bitton,F., Bruyere,C., Caboche,M., Debast,C., Gualberto,J., Hoffmann,B. *et al.* (2004) Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *Plant Cell*, **16**, 2089–2103.
45. Delannoy,E., Stanley,W.A., Bond,C.S. and Small,I.D. (2007) Pentatricopeptide repeat (PPR) proteins as sequence-specificity factors in post-transcriptional processes in organelles. *Biochem. Soc. Trans.*, **35**, 1643–1647.
46. Mootha,V.K., Lepage,P., Miller,K., Bunkenborg,J., Reich,M., Hjerrild,M., Delmonte,T., Villeneuve,A., Sladek,R., Xu,F. *et al.* (2003) Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics. *Proc. Natl Acad. Sci. USA*, **100**, 605–610.
47. Xu,F., Morin,C., Mitchell,G., Ackerley,C. and Robinson,B.H. (2004) The role of the LRPPRC (leucine-rich pentatricopeptide repeat cassette) gene in cytochrome oxidase assembly: mutation causes lowered levels of COX (cytochrome c oxidase) I and COX III mRNA. *Biochem. J.*, **382**, 331–336.

Biochimica et Biophysica Acta 1803 (2010) 931–939

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Mitochondrial single-stranded DNA binding protein is required for maintenance of mitochondrial DNA and 7S DNA but is not required for mitochondrial nucleoid organisation

Heini Ruhanen [a,1], Sarah Borrie [a,1], Gyorgy Szabadkai [b,e], Henna Tyynismaa [a], Aleck W.E. Jones [b], Dongchon Kang [c], Jan-Willem Taanman [d,e], Takehiro Yasukawa [a,e,*]

[a] The Wolfson Institute for Biomedical Research, University College London, Gower Street, London, WC1E 6BT, UK
[b] Department of Cell and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, UK
[c] Department of Clinical Chemistry and Laboratory Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan
[d] Department of Clinical Neurosciences, Institute of Neurology, University College London, Rowland Hill Street, London, NW3 2PF, UK
[e] Consortium for Mitochondrial Research (CfMR), University College London, UK

ARTICLE INFO

Article history:
Received 1 December 2009
Received in revised form 31 March 2010
Accepted 20 April 2010
Available online 28 April 2010

Keywords:
Single-stranded DNA binding protein
mtSSB
Mitochondrial DNA
7S DNA
DNA replication
Mitochondrial nucleoid

ABSTRACT

Single-stranded DNA binding protein (SSB) plays important roles in DNA replication, recombination and repair through binding to single-stranded DNA. The mammalian mitochondrial SSB (mtSSB) is a bacterial type SSB. *In vitro*, mtSSB was shown to stimulate the activity of the mitochondrial replicative DNA helicase and polymerase, but its *in vivo* function has not been investigated in detail. Here we studied the role of mtSSB in the maintenance of mitochondrial DNA (mtDNA) in cultured human cells. RNA interference of mtSSB expression in HeLa cells resulted in rapid reduction of the protein and a gradual decline of mtDNA copy number. The rate of mtDNA synthesis showed a moderate decrease upon mtSSB knockdown in HeLa cells. These results confirmed the requirement of mtSSB for mtDNA replication. Many molecules of mammalian mtDNA hold a short third strand, so-called 7S DNA, whose regulation is poorly understood. In contrast to the gradual decrease of mtDNA copy number, 7S DNA was severely reduced upon mtSSB knockdown in HeLa cells. Further, 7S DNA synthesis was significantly affected by mtSSB knockdown in an oseteosarcoma cell line. These data together suggest that mtSSB plays an important role in the maintenance of 7S DNA alongside its role in mtDNA replication. In addition, live-cell staining of mtDNA did not imply alteration in the organisation of mitochondrial nucleoid protein-mtDNA complexes upon mtSSB knockdown in HeLa cells. This result suggests that the presence of 7S DNA is not crucial for the organisation of mitochondrial nucleoids.

1. Introduction

Human mitochondria maintain closed-circular, double-stranded DNA molecules of 16,569 base pairs, the mitochondrial DNA (mtDNA). In contrast to diploid nuclear DNA, mtDNA is a multi-copy genome, and typically exists as 10^3–10^4 copies per cell. It encodes 13 subunits of the oxidative phosphorylation complexes, along with 22 transfer RNAs and 2 ribosomal RNAs for translation of the subunits within mitochondria. It is generally accepted that *in vivo* several mtDNA molecules are held together in large protein-DNA complexes, the mitochondrial nucleoids [1]. The importance of mtDNA maintenance can be recognised by the fact that mtDNA mutations and copy number depletion are responsible for human pathology. Such mtDNA abnormalities impair the oxidative phosphorylation system and result in a variety of clinical symptoms, which frequently affect the neuronal system, and cardiac and skeletal muscle [2–4]. Also, the accumulation of somatic mtDNA mutations was shown to accelerate ageing in mice [5,6] and large-scale heteroplasmic mtDNA deletion to cause male infertility [7].

DNA replication is operated by the coordinated action of multiple proteins. In the process of genome duplication, the parental double-stranded (ds) DNA is unwound to single-stranded (ss) intermediates, and nascent strands are synthesised using the unwound ssDNA parental strands as templates [8]. Among the factors for DNA replication, single-stranded DNA binding proteins (SSB), which exist throughout the kingdoms of life, associate with ssDNA in a non sequence-specific manner to provide protection to ssDNA against degradation and to maintain DNA in its functionally active single-stranded state [9–11]. In addition to this well-recognised function,

* Corresponding author. The Wolfson Institute for Biomedical Research, University College London, Gower Street, London, WC1E 6BT, UK. Tel.: +44 20 7679 6613; fax: +44 20 7209 0470.
E-mail address: t.yasukawa@ucl.ac.uk (T. Yasukawa).
[1] The first two authors contributed to this work equally.

0167-4889/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2010.04.008

SSB is also known to interact with DNA replication factors, as well as those for DNA repair and recombination. In the case of eubacterial SSB proteins, the C-terminal region is involved in such interactions, through which SSB stimulates the activity of interacting proteins. SSB is thus not only a protector of ssDNA stretches but also actively involved in DNA transaction processes through protein–protein interactions [10,11].

SSB also exists in eukaryotic mitochondria [12]. cDNA analysis revealed that human mitochondrial SSB (*Hs* mtSSB) is translated as a 148 amino acid polypeptide, the first 16 amino acids of which were proposed to be removed after import of this protein into mitochondria, resulting in a mature protein of 132 amino acids [13]. mtSSB is distinct from nuclear SSB, the replication protein A (RPA), but similar to homotetrameric eubacterial SSBs, such as *Escherichia coli* SSB. Despite the relatively low sequence identity between *Hs* mtSSB and *E. coli* SSB [13], they share close similarity in homotetrameric structure [14,15] and biophysical properties in binding to ssDNA [16]. *Hs* mtSSB lacks the C-terminal region of *E. coli* SSB which is required for the interaction with the factors for DNA metabolism [10]. Although this suggests that *Hs* mtSSB cannot interact with other proteins, *Hs* mtSSB was shown to have a stimulatory effect on the unwinding activity of the mitochondrial DNA helicase, Twinkle [17]. This stimulation of Twinkle was not observed with *E. coli* SSB, and thus a direct interaction between mtSSB and Twinkle was presumed [17]. Furthermore, mtSSB considerably enhanced the mitochondrial DNA polymerase γ (polγ)-dependent DNA synthesis reaction in the presence of Twinkle *in vitro* [18]. Also, *in vitro* studies with *Drosophila* (*Ds*) mtSSB proposed that this protein increases the processivity of *Ds* polγ by stimulating the initiation of DNA synthesis, and an interaction of *Ds* mtSSB with *Ds* polγ was assumed [19,20]. A fly mutant lacking mtSSB protein expression showed drastic mtDNA depletion and cell proliferation failure during development [21]. In *Saccharomyces cerevisiae*, the mtSSB gene *RIM1* is essential for the maintenance of mtDNA [22]. These reports all support the idea that mtSSB is an indispensable protein for mtDNA maintenance. Curiously, quantification of *Hs* mtSSB in cultured cells revealed that mtSSB protein is approximately 3000 fold more abundant than mtDNA molecules [23]. The large excess of mtSSB over mtDNA raises the question as to whether the expression level of mtSSB is tightly regulated for replication and maintenance of mtDNA.

A certain proportion of mtDNA molecules in mammals hold a short third strand which forms a triple-stranded region, the D-loop [24,25]. The third strand, called 7S DNA, is positioned in the major non-coding region of mtDNA, and is approximately 650 bases long in humans [26]. Although 7S DNA was discovered about four decades ago [24,25], its function is still not clear. It was proposed to be a primer for the heavy (H) strand replication under the strand-displacement mtDNA replication model or to be an aborted product of nascent H strand synthesis [26]. A role in the regulation of mitochondrial transcription was also suggested [26]. Recently, the D-loop was proposed to serve as the binding centre of proteins that regulate the dynamics of mitochondrial nucleoids [27,28]. In addition to the unclear role of 7S DNA, nearly nothing is known about its regulation. For example, it is not understood how the abundance of 7S DNA is determined and monitored.

Here we investigated the role of mtSSB in the mitochondria of cultured human cells. Knockdown of mtSSB expression by siRNA transfection in HeLa cells resulted in a moderate reduction of mtDNA copy number, which was confirmed to be due to a decreased activity of mtDNA synthesis. Furthermore, reduction of mtSSB protein level caused a rapid and severe decrease of 7S DNA in HeLa cells. Pulse-labelling of newly synthesised mtDNA in the thymidine kinase 1-deficient osteosarcoma cell line 143B [TK$^-$] suggested that the synthesis of 7S DNA was significantly affected when mtSSB was silenced in this cell line. Here we propose that mtSSB is involved in the regulation of the mtDNA D-loop by playing an important role in the synthesis of 7S DNA, an activity which appears to be distinct from its role in normal mtDNA replication.

2. Materials and methods

2.1. Cell culture and RNA interference

HeLa cells were cultured in DMEM supplemented with 10% FBS and penicillin–streptomycin. Transfection of cells with short double-stranded RNA (dsRNA) was performed using Lipofectamine 2000 reagent (Invitrogen) according to the method described previously [28] with modifications. Briefly, cells were plated on the day previous to the day of transfection, and the transfection was performed with either scrambled control dsRNA (AllStars Negative Control siRNA from Qiagen) or mtSSB-specific dsRNA (iGENE Therapeutics, Japan) at a concentration of 1 nM in the transfection medium Opti-MEM (Invitrogen) on "Day 0". Four hours after addition of the transfection medium, an equal volume of medium consisting of DMEM with 30% FBS and penicillin–streptomycin was added. Twenty-four hours later, the medium was replaced with the standard medium. Three days post-transfection, cells were harvested for the subsequent analyses. When time-course assays were performed, multiple transfections were performed at Day 0 and cells were harvested daily. The concentration of dsRNA in the time course was also 1 nM except for one set of assays with 0.5 nM. The sequence of human mtSSB-specific dsRNA was 5′-GCGACAAGCAACAACAAUC-3′ (sense) and 3′-GAUU-GUUGUUGCUUGUCGCCU-5′ (antisense).

In addition, the thymidine kinase 1-deficient osteosarcoma cell line, 143B [TK$^-$] [29] was used for bromo-2′-deoxyuridine (BrdU) labelling of mtDNA (see Section 2.5).

2.2. Western blot analysis

Cells were lysed with cell lysis buffer consisting of PBS, 1% SDS and protease inhibitor cocktail (Complete, EDTA-free Protease Inhibitor Cocktail from Roche Diagnostics), followed by sonication and centrifugation of the lysate at 13,000 rpm, after which the supernatant was separated from any debris. The concentration of the total cellular protein was determined using the BCA protein assay kit (Thermo Scientific). Equal amounts of protein were electrophoresed in SDS-polyacrylamide gels, and the proteins were blotted onto PVDF membrane (Millipore). Immunodetection of mtSSB, the accessory subunit of polγ (polγB) and mitochondrial transcription factor A (TFAM) was carried out using previously described primary antibodies for the above proteins [23,30]. The secondary antibody used was anti-rabbit HRP conjugate. For tubulin detection, anti-tubulin antibody (Abcam) and anti-mouse HRP conjugate were used. The immunodetection of the protein bands was performed using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) by exposing the membranes to Amersham Hyperfilm ECL (GE Healthcare). The western blot images were scanned and densitometric analysis of the immunodetected protein bands was performed using ImageJ.

2.3. Total DNA preparation, RT-qPCR and Southern hybridisation

Total DNA was prepared from cells using either DNeasy Blood & Tissue Kit (Qiagen) or a conventional DNA preparation method described in [28] with modifications. In the latter method, cells were lysed with cell lysis buffer (75 mM NaCl, 50 mM EDTA, 10 mM Hepes-NaOH (pH7.2), 1% SDS and 0.2 mg/ml proteinase K) and the lysate was incubated at 50 °C for 1 hour. An equal volume of 2-propanol was then mixed with the lysate, and DNA was pelleted by centrifugation. The pellet was rinsed with 70% ethanol, air-dried and dissolved in 10 mM Hepes-NaOH (pH 7.2). In some instances, the extraction steps with phenol and chloroform/isoamyl-alcohol were combined.

Quantification of mtDNA copy number was carried out using a real-time quantitative PCR (RT-qPCR) method with Dynamo SyBR Green qPCR kit (New England Biolabs). The primer set for the amplification of a region in human mtDNA was 5′-CCTGACTCCTACCCCTCACA-3′ and 5′-

ATCGGGTGATGATAGCCAAG-3′ and that of beta-actin, used as a nuclear gene standard, was 5′- TCACCCACACTGTGCCCATCTACGA-3′ and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′. PCR was performed using Mastercycler ep Realplex (Eppendorf) with 40 cycles of 95 °C for 15 s, 56 °C for 15 s and 72 °C for 20 s. The mtDNA content in each sample was normalised to the beta-actin gene content.

Southern blot analysis of mtDNA was carried out with total DNA prepared by the conventional DNA preparation method. To release 7S DNA from mtDNA, heat denaturation of DNA was performed in buffer containing 25 mM EDTA at 90 °C for 3 min and the incubation was terminated by transferring the sample tubes into crushed ice. To linearise the genome-length mtDNA, total DNA was digested with restriction enzyme *Pvu*II (New England Biolabs). The processed DNA samples were then electrophoresed in 0.8–1% agarose gels, and Southern hybridisation was performed as described previously [31]. The gels were blotted onto Zeta-Probe Membrane (Bio-Rad Laboratories), followed by fixation of the blotted DNA by UV cross-linking. A radiolabelled probe was then hybridised to the blotted membranes at 65 °C in 0.25 M sodium phosphate (pH 7.2), 7% SDS overnight, and the membranes were washed with 150 mM NaCl, 15 mM sodium citrate buffer (pH 7.0) (1×SSC) and then 1×SSC with 0.1% SDS a few times each. The membranes were exposed to phosphor imaging plates for visualisation of the bands and quantification of the band intensity performed with a Typhoon instrument (GE Healthcare). The radiolabelled DNA probe was produced with Ready-To-Go DNA Labelling Beads (-dCTP) (GE Healthcare) and [α-^{32}P] dCTP (3000 Ci/mmol, from PerkinElmer or Hartmann Analytic) using a PCR fragment of the mtDNA region spanning from nucleotide 16,341 to 151 that covers part of D-loop region. The primers used for the production of the PCR fragment were 5′-TTACAGTCAAATCCCTTCTCGTCC-3′ and 5′- GGATGAGGCAGGAATCAAAGACAG-3′ [31].

2.4. In cell radioactive mtDNA synthesis assay

Synthesis of mtDNA was observed in living cells by measuring the incorporation of [methyl-^{3}H] thymidine into mtDNA as described previously [32] with some modifications. Scr dsRNA or mtSSB dsRNA was transfected into HeLa cells in 12-well plates in duplicate and the cells were incubated for 3 days as described above. After 2 days of transfection, 4 μg/ml aphidicolin with or without 25 μM 2′,3′-dideoxycytidine (ddC) was added to the medium. Then, for the last 22 hours, cells were treated with 5 μCi/well [methyl-^{3}H] thymidine (MP Biomedicals). After labelling of mtDNA, cells were washed three times with PBS and lysed overnight in 0.25 M NaOH. A fraction of lysed cells was spotted onto DE81 anion exchange paper (Whatman) in triplicate. The anion exchange paper was then washed three times with 2×SSC and once with ethanol to remove unincorporated [methyl-^{3}H] thymidine. Finally, the anion exchange paper was dried and the radioactivity on the paper was measured by liquid scintillation counting. The amount of protein in HCl-neutralised lysates was determined with the BCA Protein Assay kit. The ddC-sensitive [methyl-^{3}H] thymidine incorporation was calculated per μg of protein.

2.5. In cell BrdU labelling of newly synthesised mtDNA and Southwestern analysis using 143B [TK^{-}] cells

Prior to the BrdU labelling, 143B [TK^{-}] cells were transfected with either control siRNA or mtSSB-specific dsRNA at a concentration of 1nM and cultured for approximately three days. Then, the cells were incubated in culture medium containing 100 μM BrdU at the indicated duration to label mtDNA. After the incubation, cells were lysed with the cell lysis buffer and total DNA was prepared from the cells by the conventional DNA preparation method as described in Section 2.3. Release of 7S DNA from mtDNA and generation of the linear genome-length mtDNA was performed essentially as described in Section 2.3 except that the restriction enzyme used for the linearisation was *Nae*I (New England Biolabs). The processed DNA samples were then electrophoresed in 0.8–1% agarose gels and Southwestern blotting was performed [29] with some modifications. Briefly, the gels were treated once with 0.258 N HCl, then twice with 0.5 M NaOH, 1.5 M NaCl and finally twice with 0.5 M Tris–HCl, 1.5 M NaCl (pH 7.4), and then blotted onto Hybond N^{+} membrane (GE Healthcare) and the blotted DNA was fixed by UV cross-linking. After blocking procedure, immunodetection of the BrdU-labelled DNA was performed with 1:1000 dilution of anti-BrdU (Becton Dickinson Immunocytometry Systems) for the primary antibody and 1:5000 dilution of anti-mouse HRP conjugate (DakoCytomation) for the secondary antibody. Visualisation of the DNA bands was performed using Amersham ECL Plus Western Blotting Detection Reagents by exposing the membranes to Amersham Hyperfilm ECL.

2.6. Live-cell imaging

For visualisation of mtDNA and the mitochondrial network, HeLa cells were seeded on glass coverslips and dsRNA transfection performed as described in 2.1. For mtDNA staining, cells were loaded with 0.5 μl/ml of PicoGreen solution (Quant-iT PicoGreen DS DNA assay, Invitrogen P7589) in a modified Krebs buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM K_2HPO_4, 1 mM $CaCl_2$, 5.5 mM glucose, 20 mM HEPES, pH 7.4) for 1 h at 37 °C. For the final 20 min of staining with PicoGreen, 50 nM Mitotracker Deep Red (Invitrogen, M22426) was added to the solution to visualise the mitochondrial network. After loading, cells were washed twice with Krebs buffer, and the coverslip was mounted in an imaging chamber in dye free Krebs buffer. Loaded coverslips were imaged immediately on a Zeiss LSM 700 confocal microscopy system (Carl Zeiss, Jena). Z-series of images were acquired with a 63×, 1.4 NA objective, using the 488 nm and 633 nm excitation wavelengths of a diode laser for PicoGreen and MitoTracker Deep Red respectively, in order to avoid any spectral overlap. PicoGreen intensities were obtained after maximal projection and thresholding of the images over the nuclear and mitochondrial regions of the cell, using MetaMorph 5.0 software (Molecular Devices).

3. Results

3.1. RNA interference of mtSSB expression decreases mtDNA copy number moderately and causes a severe reduction in the level of 7S DNA in HeLa cells

To study the involvement of mtSSB protein in mtDNA maintenance, knockdown of mtSSB was performed using RNA interference technique on cultured human HeLa cells. Three days after transfection with short dsRNA targeting mtSSB mRNA, a significant reduction of mtSSB protein was observed by western blot analysis (Fig. 1A), confirming effective knockdown of the target protein with the mtSSB-targeted dsRNA used. The mtSSB protein level was estimated to be approximately 20% in mtSSB dsRNA-treated cells as compared to that in the scramble (Scr) dsRNA-treated cells three days after transfection. Under these conditions, RT-qPCR revealed that the mtDNA copy number in the mtSSB dsRNA-treated cells was decreased moderately, by approximately 40%, as compared to that in Scr dsRNA-treated cells (Fig. 1B).

In an *in vitro* assay where the release of 7S DNA from mtDNA was induced either by recombinant TFAM or 1-methyl-4-phenylpyridinium (MPP^{+}), pre-treatment of isolated mtDNA with recombinant mtSSB prevented the 7S DNA release [23]. This prompted us to analyse whether mtSSB is involved in the regulation of 7S DNA in living cells. Total DNA prepared from mtSSB dsRNA- or Scr dsRNA-transfected HeLa cells was heat-denatured to release 7S DNA from mtDNA and subjected to Southern hybridisation. While 7S DNA was readily visible

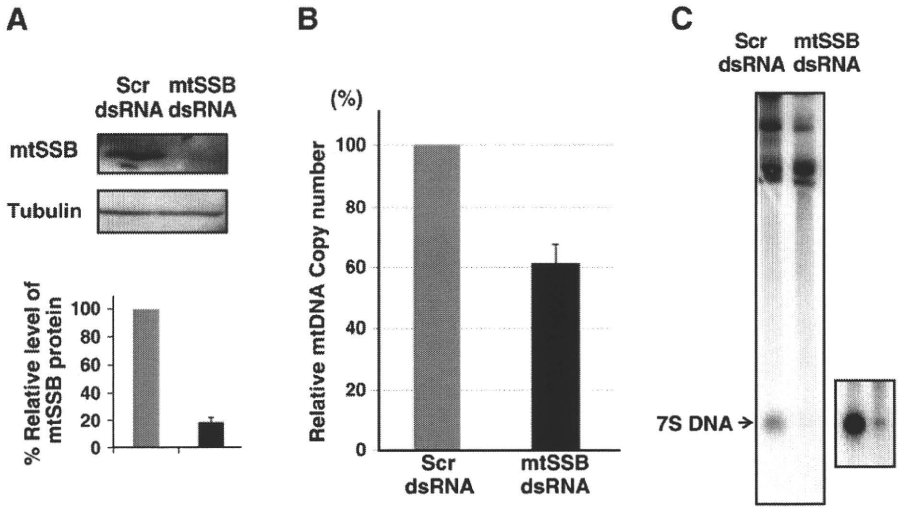

Fig. 1. Analysis of mtSSB levels and changes in mtDNA upon dsRNA knockdown. HeLa cells were transfected with either scramble (Scr) dsRNA or mtSSB-targeted (mtSSB) dsRNA and incubated for 3 days. A. mtSSB levels were analysed in total cellular lysate by western blotting. Tubulin was used as the loading control for mtSSB detection. The graph below the western blot panels shows the estimate of the mtSSB levels 3 days after dsRNA transfection using four selected western blot results from the single time point transfections and the time-course assays (see Fig. 3). In each experiment, the intensity of the mtSSB band was normalised by that of the tubulin band in each lane. The mtSSB level in Scr dsRNA-treated samples was expressed as 100 and the relative level of mtSSB in mtSSB dsRNA-treated samples was calculated and the mean with S.E.M. is shown. B. The relative copy number of mtDNA was analysed using RT-qPCR. The value of mtDNA content was normalised against beta-actin gene content. The relative mtDNA copy number in Scr dsRNA-treated cells was expressed as 100 in each experiment and that in mtSSB dsRNA-treated cells was expressed relative to this. The graph gives the average of the results from three independent transfection experiments with S.E.M. C. Southern hybridisation analysis of 7S DNA. Total DNA was prepared from Scr dsRNA-treated cells and mtSSB dsRNA-treated cells and an equal amount of the DNA was subjected to heating, followed by agarose gel electrophoresis and Southern hybridisation using a radiolabelled probe covering the D-loop region of mtDNA. The right panel shows the digitally intensified image of the 7S DNA region of the left panel. The band corresponding to 7S DNA is indicated by an arrow. Bands in high molecular weight range are denatured genome-length mtDNA [44].

in Scr dsRNA-treated cells, they were scarcely detectable in mtSSB dsRNA-treated cells, indicating that a severe reduction in 7S DNA was induced by mtSSB knockdown (Fig. 1C).

3.2. Activity of mtDNA replication is decreased upon mtSSB knockdown in HeLa cells

To examine whether the reduction of the mtDNA copy number in mtSSB dsRNA-transfected cells (Fig. 1) was caused by a decrease in the activity of mtDNA synthesis, we quantified the incorporation of [methyl-^{3}H] thymidine into mtDNA on the third day of dsRNA transfection in the presence of aphidicolin, an inhibitor of nuclear DNA polymerases using HeLa cells [32]. The same assay was performed in parallel in the presence of 2′ 3′-dideoxycytidine (ddC), a widely-used inhibitor of polγ [33] in the culture medium. The amount of incorporated [methyl-^{3}H] thymidine into DNA in the presence of aphidicolin and ddC was subtracted from the value obtained in the corresponding assay with aphidicolin but without ddC to exclude any residual incorporation of [methyl-^{3}H] thymidine into nuclear DNA from the data for *de novo* mtDNA synthesis.

Using this assay system, it was revealed that incorporation of [methyl-^{3}H] thymidine into mtDNA of mtSSB dsRNA-treated cells was inhibited by approximately 60% as compared to that of Scr dsRNA-treated cells on the third day of dsRNA transfection (Fig. 2). Because the mtDNA copy number was decreased by approximately 40% after 3 days of treatment with mtSSB dsRNA (Fig. 1), the mtDNA synthesis result suggests that the activity of mtDNA replication was slightly but clearly decreased when mtSSB was knocked down in HeLa cells.

3.3. Decrease of 7SDNA follows the changes in mtSSB protein level in HeLa cells

To investigate how the changes in the expression levels of mtSSB influence the mtDNA copy number and the abundance of 7S DNA in HeLa cells, these parameters were monitored in a time-course manner. Western blot analysis of mtSSB revealed that its expression levels were already reduced after one day of dsRNA transfection (Fig. 3A). The copy number of mtDNA in Scr dsRNA- and mtSSB dsRNA-treated cells was quantified using RT-qPCR. The changes of the mtDNA copy number upon the dsRNA transfection were expressed as the percentage of mtDNA content in mtSSB dsRNA-treated cells relative to that in Scr dsRNA-treated cells at each day (Fig. 3B). This value decreased gradually from day 1 to day 3, which is consistent with the moderately reduced mtDNA replication activity observed in the *in cell* mtDNA replication assay (Fig. 2). To analyse the changes in the abundance of 7S DNA accurately, we examined the 7S DNA amount against the genome-length mtDNA molecules in the DNA samples that were prepared from Scr dsRNA- and mtSSB dsRNA-transfected HeLa cells as follows. An equal amount of total DNA from a sample was either heat-denatured to have fully released 7S DNA or digested with *Pvu*II to produce linearised genome-length mtDNA

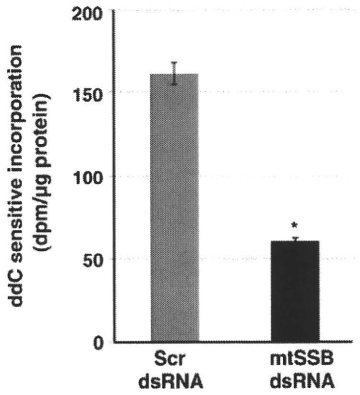

Fig. 2. Measurement of the mitochondrial DNA synthesis activity. On the third day after transfection of HeLa cells with either Scr dsRNA or mtSSB dsRNA, *de novo* mtDNA synthesis was examined by quantification of the ddC-sensitive incorporation of [methyl-^{3}H] thymidine into mtDNA over a 22-hour duration in the presence of aphidicolin. Data are shown as disintegrations per minute incorporated per μg protein, and are the mean of 4 independent transfection assays with S.E.M. (*$p<0.0001$).

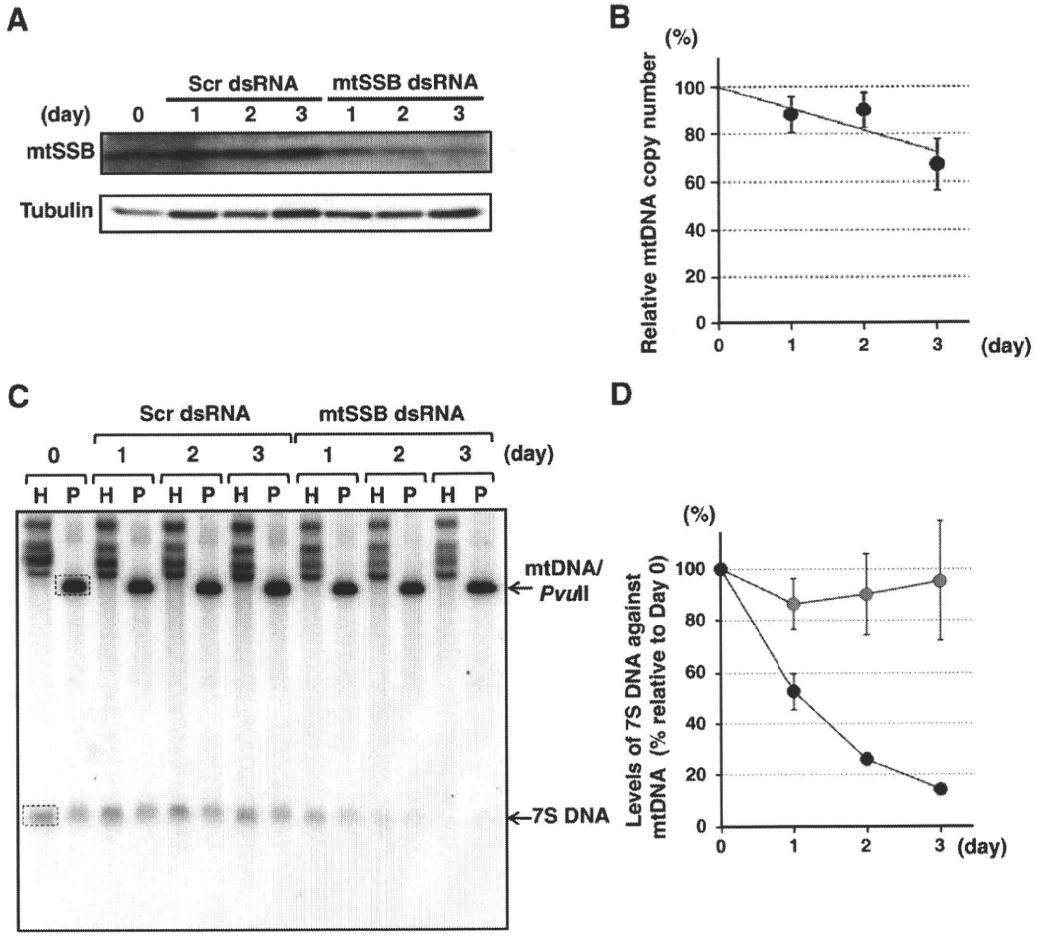

Fig. 3. Time-course analysis of mtSSB protein level (A), mtDNA copy number (B) and 7S DNA level (C and D). dsRNA transfection of HeLa cells was performed on Day 0. The following days (from Day 1 to Day 3) indicate the duration after the transfection. Cells harvested on Day 0 were not transfected with dsRNA. At each day, total cellular lysate and DNA were prepared from the cells. A. Western blot analysis of mtSSB and Tubulin. B. The relative copy number of mtDNA in mtSSB dsRNA-treated cells against Scr dsRNA-treated cells. The mtDNA content of mtSSB dsRNA-treated cells were divided by that of Scr dsRNA-treated cells at each day and plotted as percentage value. C. Southern hybridisation analysis of 7S DNA. Total DNA was either subjected to heat denaturation (lanes H) or digested with *Pvu*II (lanes P) and electrophoresed next to each other. The bands corresponding to the linearised genome-length mtDNA (mtDNA/*Pvu*II) and 7S DNA are indicated by arrows. D. Quantification of 7S DNA against the genome-length mtDNA. The intensity of the band corresponding to 7S DNA in lane H against that to the linearised mtDNA in lane P at Day 0 (the bands are indicated by dotted square boxes in C) was expressed as 100 and the relative values of 7S DNA in lanes H against the linearised mtDNA in lanes P at each day and each dsRNA treatment were plotted (gray circles for Scr dsRNA-treated cells and black circles for mtSSB dsRNA-treated cells). The graphs in B and D were produced from 4 independent transfection experiments, three with 1 nM dsRNA and one with 0.5 nM dsRNA with S.E.M. The images in A and C are typical examples of these experiments.

molecules. The ratio of 7S DNA to genome-length mtDNA at each time point and with each dsRNA treatment was then determined. In contrast to the changes in mtDNA copy number, the level of 7S DNA against the mtDNA molecules appeared to be influenced directly by the expression level of mtSSB; one day after transfection it had already fallen significantly and decreased sharply in the following days in mtSSB dsRNA-treated cells, while the level of 7S DNA against the genome-length mtDNA in Scr dsRNA-treated cells remained more or less constant from day 1 to day 3 (Fig. 3C and D). Further, taking into consideration the fact that the copy number of mtDNA declined in mtSSB-knockdown cells, the decrease in the absolute amount of 7S DNA in these cells was extremely severe.

Since 7S DNA was reported to have a high turnover rate in mouse cells [34], the loss of 7S DNA observed above could be the consequence of rapid decay of 7S DNA over the reduced mtDNA synthesis activity. To examine this possibility, inhibition of mtDNA synthesis by ddC was performed using HeLa cells. The primary mode of action of ddCTP in the inhibition of mtDNA synthesis is believed to be through decreasing the processivity of polγ [35]. When the copy number of mtDNA in cells cultured in medium containing ddC was reduced as compared to that in the control cells cultured in normal medium (Supplementary Fig. 1A), the levels of 7S DNA against the genome-length mtDNA did not show a decrease in the ddC-treated cells at day 3 of the treatment (Supplementary Fig. 1B and 1C). This result suggests that the reduction of the 7S DNA observed upon silencing of mtSSB expression was directly caused by the knockdown of mtSSB and not by the reduced activity of mtDNA synthesis due to the mtSSB knockdown.

In addition, since no significant shorter mtDNA molecules were detected below the genome-length mtDNA band in the mtSSB dsRNA-treated samples, reduction of mtSSB levels did not compromise mtDNA integrity in HeLa cells.

3.4. Mitochondrial proteins known to influence the 7S DNA levels are unchanged in HeLa cells

Previously, mtDNA copy number and the level of 7S DNA were shown to be influenced by the modulation of the expression levels of the mitochondrial transcription factor A, TFAM [36,37] and the accessory subunit of polγ, polγB [28]. Thus we investigated whether there were any changes in the levels of these proteins in HeLa cells under the experimental conditions used here. As shown in Fig. 4, the levels of TFAM and polγB in Scr dsRNA- and mtSSB dsRNA-transfected

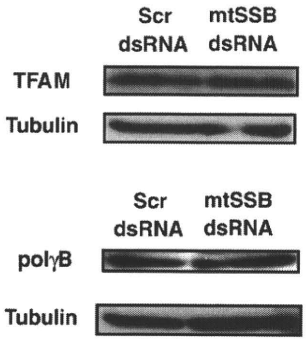

Fig. 4. Western blot analysis of TFAM and polγB. Total cellular lysate of HeLa cells cultured for 3 days after dsRNA transfection was subjected to analysis of protein levels of TFAM and polγB. Reprobing with Tubulin on the respective membranes was performed as the loading control.

cells were similar, suggesting that the loss of 7S DNA was the direct result of the reduction in mtSSB level.

3.5. Knockdown of mtSSB significantly affects the synthesis of 7S DNA in 143B [TK⁻] cells

To investigate how the reduction of the mtSSB level resulted in the loss of 7S DNA, pulse-labelling of newly synthesised mtDNA was performed using the thymidine kinase 1-deficient osteosarcoma cell line, 143B [TK⁻]. Since this cell line has thymidine kinase 2 which is localised to mitochondria, BrdU is incorporated specifically into mtDNA when BrdU is added to the cell culture medium [29]. The labelled mtDNA can be visualised with Southwestern blotting.

Approximately 3 days after 143B [TK⁻] cells were transfected with either Scr dsRNA or mtSSB dsRNA, they were incubated in medium containing BrdU at the indicated duration and harvested. To analyse the genome-length mtDNA, the samples were linearised with *Nae*I. The recognition sequence of this enzyme does not contain thymidine where BrdU could be incorporated. A band at the expected size of the genome-length mtDNA was observed and its intensity increased in the time dependent manner (Fig. 5A). For 7S DNA detection, the samples were heat-denatured prior to gel electrophoresis and the

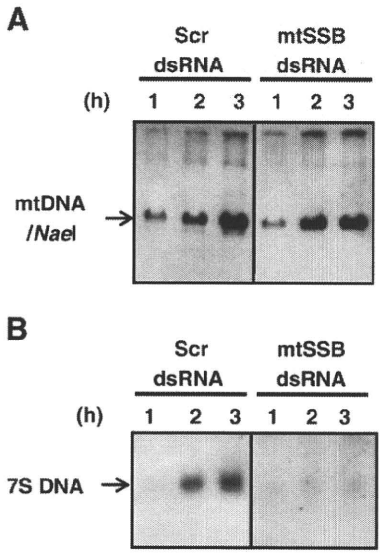

Fig. 5. Southwestern blot analysis of newly synthesised mtDNA. Pulse-labelling of mtDNA with BrdU was performed using 143B [TK⁻] cells at the indicated duration (1–3 h) after the transfection with either Scr dsRNA or mtSSB dsRNA. Total DNA prepared from the cells was subjected to Southwestern blot analysis. A. Detection of the BrdU-labelled genome-length mtDNA digested with *Nae*I. B. Detection of BrdU-labelled 7S DNA released by heat denaturation treatment.

expected size band for 7S DNA was observed in Scr dsRNA-treated cells (Fig. 5B). While the bands for the genome-length mtDNA in mtSSB dsRNA-treated cells showed similar intensity to those in Scr dsRNA-treated cells in the labelling duration used, the labelling of 7S DNA was much poorer in the former (Fig. 5). This result suggested that the knockdown of mtSSB directly suppressed the synthesis of 7S DNA.

3.6. mtSSB reduction does not affect the organisation of mitochondrial nucleoids in HeLa cells

To investigate the effect of mtSSB knockdown and 7S DNA depletion on the organisation of mitochondrial nucleoids, staining of mtDNA with the DNA intercalating dye PicoGreen was performed in living HeLa cells. Cells were stained three days after the dsRNA transfection and observed with confocal microscopy. As is evident in Fig. 6A, mitochondrial nucleoids in mtSSB dsRNA-treated cells exhibited less intense staining with PicoGreen as compared to those in Scr dsRNA-treated cells. Since it has been proposed that the staining of mitochondrial nucleoids by PicoGreen is influenced by the copy number of mtDNA [38] and the topological state of mitochondrial nucleoids [1,27], quantitative analysis of the fluorescent signal of the nucleoids was performed. We quantified the nucleoid fluorescent intensities in at least 20 cells at each measurement, and obtained the mean of the sum of all nucleoid fluorescent intensities per cell. A similar calculation was performed to obtain the mean of the fluorescent intensity of the nucleus in each cell. The ratio of the mean value of total nucleoid fluorescent intensity to the mean value of nuclear fluorescent intensity was then calculated for each cell to obtain the 'relative fluorescent intensity of mitochondrial nucleoid'. This value was used for the subsequent quantitative examination of mitochondrial nucleoids. Six sets of analyses with both Scr dsRNA and mtSSB dsRNA were performed (three sets per dsRNA under two independent transfection trials), and the mean of the six 'relative nucleoid fluorescent intensity' for each condition was calculated (Fig. 6B). The mean value for mtSSB dsRNA-treated cells was approximately 30% less than Scr dsRNA-treated cells. Since this value is in good correlation with the reduction of mtDNA copy number determined by RT-qPCR (Fig. 1) [38], the result suggested that no alteration in the organisation of mitochondrial nucleoids was induced upon knockdown of mtSSB and concomitant depletion of 7S DNA.

Mitotracker staining showed no apparent difference between the mtSSB dsRNA-treated cells and Scr dsRNA-treated cells. This suggested no influence on mitochondrial morphology of mtSSB knockdown (Fig. 6).

4. Discussion

4.1. Necessity of mtSSB for mtDNA replication

The molecular ratio of mtSSB and mtDNA was estimated to be 3000:1 [23], but it is not known whether such a large excess of mtSSB is a critical factor for mtDNA replication to maintain the copy number of mtDNA. We examined this point by knocking down mtSSB levels with the dsRNA transfection in cultured human HeLa cells, and observed that the mtDNA copy number declined gradually. Because the decrease in the *de novo* mtDNA synthesis is slightly more pronounced than the decrease in mtDNA copy number in HeLa cells, these data suggest that the reduction in mtDNA copy number is a consequence of moderately decreased activity of DNA synthesis in mitochondria due to the insufficient supply of mtSSB to the replication machinery. We thus propose that the high level of mtSSB is not an absolute requirement of the DNA synthesis but is to assure the mitochondrial replication system maintains the required rate of mtDNA synthesis, presumably through the efficient and complete binding of mtSSB to the ssDNA region at the replication

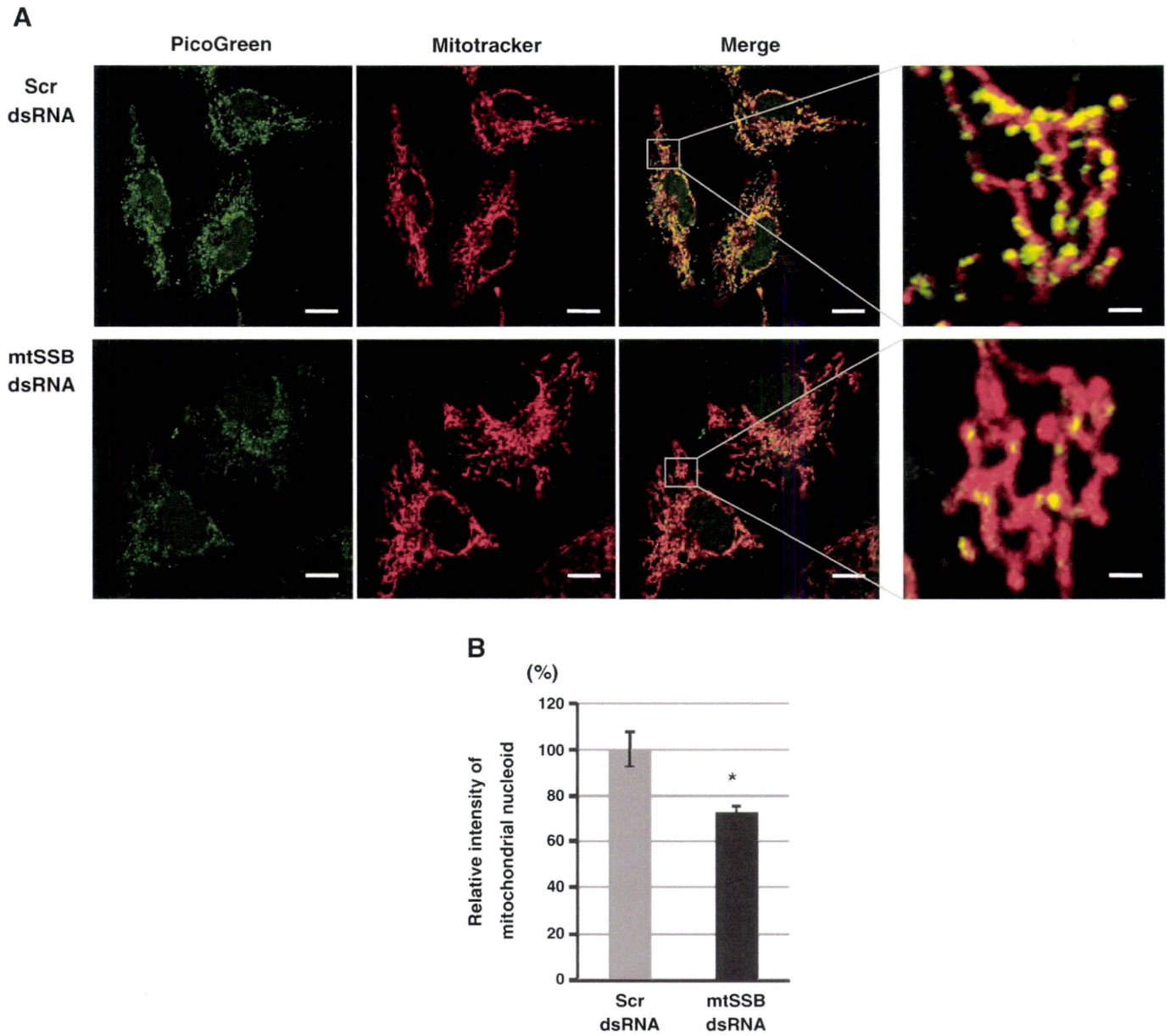

Fig. 6. Confocal analysis of mitochondrial nucleoids. A. Live-cell staining with PicoGreen and Mitotracker, 3 days after dsRNA transfection of HeLa cells. Cells were imaged as described in Section 2. 6. Representative images of Scr dsRNA- and mtSSB dsRNA-transfected cells are shown. Scale bars are 20 μm and 2 μm on the low and high magnification images, respectively. B. Quantitative analysis of the PicoGreen signal from mitochondrial nucleoids. The mean fluorescent intensity of mitochondrial nucleoids was normalised to the fluorescent signal from the nucleus (see main text), and the subsequent ratio expressed as a percentage of the ratio in Scr dsRNA-treated cells. The data are presented as mean ratio ±s.e.m ($n=6$) ($^*p=0.015$). Mitochondrial nucleoid foci with weak PicoGreen staining were hindered by the Mitotracker signal in Merge images, particularly in mtSSB dsRNA-treated cells.

fork. Its role in replication would thus be to protect the ssDNA from degradation, to prevent it from forming secondary structure and to interact with Twinkle and polγ to ensure their processivity.

As mentioned above, the [methyl-^{3}H] thymidine labelling using HeLa cells showed reduced activity of mtDNA synthesis upon mtSSB knockdown. On the other hand, BrdU incorporation into the genome-length mtDNA in 143B [TK$^-$] cells was similar between the cells treated with Scr dsRNA and mtSSB dsRNA, though the relative mtDNA copy number in the mtSSB dsRNA-treated cells was roughly 30% of that in Scr dsRNA-treated cells at the time of the labelling (data not shown). The reason why the latter labelling experiment gave such a result is not clear. It could be due to the large difference in the duration of labelling; 22 h for the HeLa cell labelling compared with only a few hours for the 143B [TK$^-$] cell labelling. It might be difficult to analyse the rate of mtDNA synthesis over such a short duration under our experimental conditions of the BrdU labelling. Another possibility could be that polγ misincorporates BrdUTP into DNA more frequently when mtSSB level is unusually low.

4.2. Requirement of mtSSB for 7S DNA maintenance

In the current work, we showed that the knockdown of mtSSB caused a rapid and severe loss of 7S DNA in HeLa cells. It was fortunate that the decay profiles of the relative mtDNA copy number and the 7S DNA level showed a clear difference in the dsRNA transfection experiments with HeLa cells. Since the inhibition of mtDNA synthesis by ddC treatment did not result in such reduction of 7S DNA, the decreased rate of mtDNA synthesis is unlikely to explain the loss of 7S DNA when mtSSB was knocked down in HeLa cells. The result implies a certain specific role of mtSSB on the maintenance of 7S DNA. To

investigate how mtSSB is involved in this maintenance apart from its role in normal DNA replication pulse-labelling of newly synthesised mtDNA was performed utilising the 143B [TK$^-$] cell line. 7S DNA was found to be poorly labelled in the mtSSB dsRNA-transfected cells, suggesting that mtSSB plays an important role in the synthesis of 7S DNA.

mtSSB has been shown to remain bound at the D-loop after a purification procedure of mtDNA-protein complex that included extraction with strong detergent (1% SDS) and dialysis with high concentration salt (0.5 M NaCl) [39]. Further, the mtSSB bound to the D-loop in the preparation inhibited branch migration of 7S DNA from the genome-length mtDNA [39]. Using purified mtDNA molecules and recombinant mtSSB, the ability of mtSSB to prevent the release of 7S DNA from mtDNA despite the promotion of release by TFAM or MPP$^+$ was demonstrated [23]. Although these results implied that mtSSB could play a key role in stabilising the D-loop structure *in vivo*, it remained possible that mtSSB non-specifically bound to the exposed ssDNA in D-loop region after the actual D-loop binding proteins were stripped off in the above mentioned studies [23,39]. The data obtained in this study suggested that mtSSB is crucially required for the 'synthesis step' of 7S DNA, to maintain the normal level of 7S DNA. This idea does not contradict with the report in which 7S DNA was shown to be high turnover molecules [34]. It is possible to speculate that mitochondria may have different DNA synthesis mechanisms, one for 7S DNA synthesis and the other(s) for replication of the genome-length mtDNA molecules. Our data implied that mtSSB is involved in both of them, and the maintenance of the normal level of mtSSB appears to be more significant to the former.

Recently, two mitochondrial proteins, ATAD3 and polγB were reported to be capable of binding to the D-loop, and modulation of their expression levels in cultured cells influenced the organisation of mitochondrial nucleoids [1,27,28]. It was thus proposed that the D-loop acts as a protein recruitment centre for the regulation of nucleoid dynamics and that these proteins are involved in the regulation. Staining of mitochondrial nucleoids in living HeLa cells using PicoGreen fluorescent dye suggested no appreciable change in the organisation of the nucleoids upon knockdown of mtSSB and the concomitant loss of 7S DNA (Fig. 6). Our result thus suggested that the presence of 7S DNA is not essential for the maintenance of normal mitochondrial nucleoid organisation.

4.3. Possible association of mtSSB in human diseases

It is known that mutations in mtDNA maintenance genes can give rise to autosomally inherited disorders with secondary mtDNA instability, such as depletion and deletion, and the disorders are recognised as a large group of human diseases [40,41]. It was pointed out that several other known or unknown proteins should have pathogenic mutations in mtDNA depletion syndromes (MDS) because the reported mutations cannot account for all the MDS cases [41]. As far as we are aware, there is currently no literature reporting a mutation on mtSSB gene associated with MDS, unlike the genes for the catalytic subunit of Polγ (PolγA) and Twinkle [41–43]. It could be possible that an unknown mutation in the mtSSB gene that results in disruption of the binding of mtSSB to DNA causes mtDNA depletion in some MDS case. Alternatively, depletion of mtDNA could occur if the gene expression of mtSSB was significantly down-regulated. In support of this idea, the absence of mtSSB protein caused mtDNA depletion in fly larval stage and in yeast [21,22].

Acknowledgements

We thank Dr Hiroshi Sembongi for his helpful advice, Profs Michael R. Duchen and Linda Partridge for the use of confocal microscopy and Dr Pedro Echave for technical assistance. This work was supported by the Biotechnology and Biosciences Research Council David Phillips Fellowship and University of London Central Research Fund (to T.Y.), the Academy of Finland and EMBO Long-Term Fellowship (to H.T.), Parkinson's disease Society (Project Grant G-0905 to G.S.), Medical Research Council PhD Fellowship (to A.J.), Grants-in-Aid for Scientific Research from the Ministry of Science, Education, Sports and Culture of Japan (to D.K.), and the Association Française contre les Myopathies grant 14343 (to J-W.T.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2010.04.008.

References

[1] I.J. Holt, J. He, C.C. Mao, J.D. Boyd-Kirkup, P. Martinsson, H. Sembongi, A. Reyes, J.N. Spelbrink, Mammalian mitochondrial nucleoids: organizing an independently minded genome, Mitochondrion 7 (2007) 311–321.
[2] I.J. Holt, Ed., Genetics of Mitochondrial Diseases, Oxford University Press, Oxford, 2003.
[3] M.C. Brandon, M.T. Lott, K.C. Nguyen, S. Spolim, S.B. Navathe, P. Baldi, D.C. Wallace, MITOMAP: a human mitochondrial genome database—2004 update, Nucleic Acids Res. 33 (2005) D611–D613.
[4] A.H. Schapira, Mitochondrial disease, Lancet 368 (2006) 70–82.
[5] A. Trifunovic, A. Wredenberg, M. Falkenberg, J. Spelbrink, A. Rovio, C. Bruder, M. Bohlooly-Y, S. Gidlof, A. Oldfors, R. Wibom, J. Tornell, H. Jacobs, N. Larsson, Premature ageing in mice expressing defective mitochondrial DNA polymerase, Nature 429 (2004) 417–423.
[6] G.C. Kujoth, A. Hiona, T.D. Pugh, S. Someya, K. Panzer, S.E. Wohlgemuth, T. Hofer, A.Y. Seo, R. Sullivan, W.A. Jobling, J.D. Morrow, H. Van Remmen, J.M. Sedivy, T. Yamasoba, M. Tanokura, R. Weindruch, C. Leeuwenburgh, T.A. Prolla, Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging, Science 309 (2005) 481–484.
[7] K. Nakada, A. Sato, K. Yoshida, T. Morita, H. Tanaka, S. Inoue, H. Yonekawa, J. Hayashi, Mitochondria-related male infertility, Proc. Natl Acad. Sci. USA 103 (2006) 15148–15153.
[8] A. Kornberg, T.A. Baker, DNA Replication (2nd edn), University Science Books, Sausalito, Calfornia, 1992.
[9] R.R. Meyer, P.S. Laine, The single-stranded DNA-binding protein of *Escherichia coli*, Microbiol. Rev. 54 (1990) 342–380.
[10] R.D. Shereda, A.G. Kozlov, T.M. Lohman, M.M. Cox, J.L. Keck, SSB as an organizer/mobilizer of genome maintenance complexes, Crit. Rev. Biochem. Mol. Biol. 43 (2008) 289–318.
[11] P.E. Pestryakov, O.I. Lavrik, Mechanisms of single-stranded DNA-binding protein functioning in cellular DNA metabolism, Biochemistry (Mosc) 73 (2008) 1388–1404.
[12] L.S. Kaguni, DNA polymerase gamma, the mitochondrial replicase, Annu. Rev. Biochem. 73 (2004) 293–320.
[13] V. Tiranti, M. Rocchi, S. DiDonato, M. Zeviani, Cloning of human and rat cDNAs encoding the mitochondrial single-stranded DNA-binding protein (SSB), Gene 126 (1993) 219–225.
[14] G. Webster, J. Genschel, U. Curth, C. Urbanke, C. Kang, R. Hilgenfeld, A common core for binding single-stranded DNA: structural comparison of the single-stranded DNA-binding proteins (SSB) from E. coli and human mitochondria, FEBS Lett. 411 (1997) 313–316.
[15] C. Yang, U. Curth, C. Urbanke, C. Kang, Crystal structure of human mitochondrial single-stranded DNA binding protein at 2.4 A resolution, Nat. Struct. Biol. 4 (1997) 153–157.
[16] U. Curth, C. Urbanke, J. Greipel, H. Gerberding, V. Tiranti, M. Zeviani, Single-stranded-DNA-binding proteins from human mitochondria and *Escherichia coli* have analogous physicochemical properties, Eur. J. Biochem. 221 (1994) 435–443.
[17] J.A. Korhonen, M. Gaspari, M. Falkenberg, TWINKLE Has 5′–>3′ DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein, J. Biol. Chem. 278 (2003) 48627–48632.
[18] J.A. Korhonen, X.H. Pham, M. Pellegrini, M. Falkenberg, Reconstitution of a minimal mtDNA replisome in vitro, EMBO J. 23 (2004) 2423–2429.
[19] A.J. Williams, L.S. Kaguni, Stimulation of Drosophila mitochondrial DNA polymerase by single-stranded DNA-binding protein, J. Biol. Chem. 270 (1995) 860–865.
[20] C.L. Farr, Y. Wang, L.S. Kaguni, Functional interactions of mitochondrial DNA polymerase and single-stranded DNA-binding protein. Template-primer DNA binding and initiation and elongation of DNA strand synthesis, J. Biol. Chem. 274 (1999) 14779–14785.
[21] D. Maier, C.L. Farr, B. Poeck, A. Alahari, M. Vogel, S. Fischer, L.S. Kaguni, S. Schneuwly, Mitochondrial single-stranded DNA-binding protein is required for mitochondrial DNA replication and development in *Drosophila melanogaster*, Mol. Biol. Cell 12 (2001) 821–830.
[22] E. Van Dyck, F. Foury, B. Stillman, S.J. Brill, Single-stranded DNA binding protein required for mitochondrial DNA replication in *S. cerevisiae* is homologous to *E. coli* SSB, EMBO J. 11 (1992) 3421–3430.

[23] C. Takamatsu, S. Umeda, T. Ohsato, T. Ohno, Y. Abe, A. Fukuoh, H. Shinagawa, N. Hamasaki, D. Kang, Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein, EMBO Rep. 3 (2002) 451–456.
[24] A. Arnberg, E.F. van Bruggen, P. Borst, The presence of DNA molecules with a displacement loop in standard mitochondrial DNA preparations, Biochim. Biophys. Acta 246 (1971) 353–357.
[25] H. Kasamatsu, D.L. Robberson, J. Vinograd, A novel closed-circular mitochondrial DNA with properties of a replicating intermediate, Proc. Natl Acad. Sci. USA 68 (1971) 2252–2257.
[26] D.A. Clayton, Replication of animal mitochondrial DNA, Cell 28 (1982) 693–705.
[27] J. He, C.C. Mao, A. Reyes, H. Sembongi, M. Di Re, C. Granycome, A.B. Clippingdale, I.M. Fearnley, M. Harbour, A.J. Robinson, S. Reichelt, J.N. Spelbrink, J.E. Walker, I.J. Holt, The AAA + protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization, J. Cell Biol. 176 (2007) 141–146.
[28] M. Di Re, H. Sembongi, J. He, A. Reyes, T. Yasukawa, P. Martinsson, L.J. Bailey, S. Goffart, J.D. Boyd-Kirkup, T.S. Wong, A.R. Fersht, J.N. Spelbrink, I.J. Holt, The accessory subunit of mitochondrial DNA polymerase gamma determines the DNA content of mitochondrial nucleoids in human cultured cells, Nucleic Acids Res. 37 (2009) 5701–5713.
[29] J. Magnusson, M. Orth, P. Lestienne, J.W. Taanman, Replication of mitochondrial DNA occurs throughout the mitochondria of cultured human cells, Exp. Cell Res. 289 (2003) 133–142.
[30] X. Cheng, T. Kanki, A. Fukuoh, K. Ohgaki, R. Takeya, Y. Aoki, N. Hamasaki, D. Kang, PDIP38 associates with proteins constituting the mitochondrial DNA nucleoid, J. Biochem. 138 (2005) 673–678.
[31] T. Yasukawa, M.Y. Yang, H.T. Jacobs, I.J. Holt, A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA, Mol. Cell 18 (2005) 651–662.
[32] M.E. Gegg, J.M. Cooper, A.H. Schapira, J.W. Taanman, Silencing of PINK1 expression affects mitochondrial DNA and oxidative phosphorylation in dopaminergic cells, PLoS ONE 4 (2009) e4756.
[33] M.V. Simpson, C.D. Chin, S.A. Keilbaugh, T.S. Lin, W.H. Prusoff, Studies on the inhibition of mitochondrial DNA replication by 3′-azido-3′-deoxythymidine and other dideoxynucleoside analogs which inhibit HIV-1 replication, Biochem. Pharmacol. 38 (1989) 1033–1036.
[34] D. Bogenhagen, D.A. Clayton, Mechanism of mitochondrial DNA replication in mouse L-cells: kinetics of synthesis and turnover of the initiation sequence, J. Mol. Biol. 119 (1978) 49–68.
[35] T.A. Brown, D.A. Clayton, Release of replication termination controls mitochondrial DNA copy number after depletion with 2′, 3′-dideoxycytidine, Nucleic Acids Res. 30 (2002) 2004–2010.
[36] J.L. Pohjoismaki, S. Wanrooij, A.K. Hyvarinen, S. Goffart, I.J. Holt, J.N. Spelbrink, H.T. Jacobs, Alterations to the expression level of mitochondrial transcription factor A, TFAM, modify the mode of mitochondrial DNA replication in cultured human cells, Nucleic Acids Res. 34 (2006) 5815–5828.
[37] K. Ohgaki, T. Kanki, A. Fukuoh, H. Kurisaki, Y. Aoki, M. Ikeuchi, S.H. Kim, N. Hamasaki, D. Kang, The C-terminal tail of mitochondrial transcription factor a markedly strengthens its general binding to DNA, J. Biochem. (Tokyo) 141 (2007) 201–211.
[38] N. Ashley, D. Harris, J. Poulton, Detection of mitochondrial DNA depletion in living human cells using PicoGreen staining, Exp. Cell Res. 303 (2005) 432–446.
[39] G.C. Van Tuyle, P.A. Pavco, Characterization of a rat liver mitochondrial DNA-protein complex. Replicative intermediates are protected against branch migrational loss, J. Biol. Chem. 256 (1981) 12772–12779.
[40] W.C. Copeland, Inherited mitochondrial diseases of DNA replication, Annu. Rev. Med. 59 (2008) 131–146.
[41] S. Alberio, R. Mineri, V. Tiranti, M. Zeviani, Depletion of mtDNA: syndromes and genes, Mitochondrion 7 (2007) 6–12.
[42] A.H. Hakonen, P. Isohanni, A. Paetau, R. Herva, A. Suomalainen, T. Lonnqvist, Recessive Twinkle mutations in early onset encephalopathy with mtDNA depletion, Brain 130 (2007) 3032–3040.
[43] E. Sarzi, S. Goffart, V. Serre, D. Chretien, A. Slama, A. Munnich, J.N. Spelbrink, A. Rotig, Twinkle helicase (PEO1) gene mutation causes mitochondrial DNA depletion, Ann. Neurol. 62 (2007) 579–587.
[44] M.Y. Yang, M. Bowmaker, A. Reyes, L. Vergani, P. Angeli, E. Gringeri, H.T. Jacobs, I.J. Holt, Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication, Cell 111 (2002) 495–505.

nature genetics

Variation in *TP63* is associated with lung adenocarcinoma susceptibility in Japanese and Korean populations

Daiki Miki[1,2], Michiaki Kubo[3], Atsushi Takahashi[4], Kyong-Ah Yoon[5], Jeongseon Kim[5], Geon Kook Lee[5], Jae Ill Zo[5], Jin Soo Lee[5], Naoya Hosono[3], Takashi Morizono[6], Tatsuhiko Tsunoda[6], Naoyuki Kamatani[4], Kazuaki Chayama[2], Takashi Takahashi[7], Johji Inazawa[8], Yusuke Nakamura[1] & Yataro Daigo[1,9,10]

Lung cancer is the most common cause of death from cancer worldwide, and its incidence is increasing in East Asian and Western countries. To identify genetic factors that modify the risk of lung adenocarcinoma, we conducted a genome-wide association study in a Japanese cohort, with replication in two independent studies in Japanese and Korean individuals, in a total of 2,098 lung adenocarcinoma cases and 11,048 controls. The combined analyses identified two susceptibility loci for lung adenocarcinoma: *TERT* (rs2736100, combined $P = 2.91 \times 10^{-11}$, odds ratio (OR) = 1.27) and *TP63* (rs10937405, combined $P = 7.26 \times 10^{-12}$, OR = 1.31). Fine mapping of the region containing *TP63* showed that a SNP (rs4488809) in intron 1 of *TP63* showed the most significant association. Our results suggest that genetic variation in *TP63* may influence susceptibility to lung adenocarcinoma in East Asian populations.

Primary lung cancer is a leading cause of death from cancer in most countries[1,2]. Lung cancer comprises various types of histology that are often divided into two main types, non–small-cell lung cancer and small-cell lung cancer. Each type has different pathophysiological and clinical features, suggesting that their mechanisms of carcinogenesis differ[3]. Adenocarcinoma accounts for about 50% of all histological types of lung cancer, and its incidence is increasing in East Asian and Western countries[4–14].

Previous studies have suggested that inherited genetic factors have a significant role in lung cancer development[15,16]. Genome-wide association studies (GWAS) of lung cancer in populations of European ancestry have identified the 15q24–25.1 region to be the most significantly associated region; this region contains genes that encode nicotinic acetylcholine receptor subunits (*CHRNA5*, *CHRNA3* and *CHRNA4*)[17–22]. Other GWAS of lung cancer identified two associated risk loci at 5p15.33 (*TERT-CLPTM1L*) and 6p21.33 (*BAT3-MSH5*)[20–22]. These signals at 5p15, 6p21 and 15q25 have been refined in European populations by a GWAS that searched for inherited susceptibility to specific histological types of lung cancer. In this GWAS meta-analysis, rs2736100 (in *TERT*) was indicated to be associated with risk of lung adenocarcinoma, and no additional loci were shown to reach genome-wide significance[23]. Because most of the current GWAS on lung cancer have been conducted in European populations with a full range of different histological types of lung cancer, GWAS that focus on individual histological groups with archived clinicopathological features are required. The allelic frequencies of the strong susceptibility locus at 15q25 in European populations are very rare in Asian populations. The risk-associated SNPs in the 15q25 locus that were found in European populations were not replicated in Chinese populations, whereas other common variants in 15q25 were identified as susceptibility loci[24]. These findings suggest that there may be a significant difference in lung cancer susceptibility loci between European and Asian populations. Here, we investigated susceptibility loci for lung adenocarcinoma in Japanese and Korean populations.

We conducted a GWAS to identify genes related to lung adenocarcinoma susceptibility in 1,004 Japanese individuals with lung adenocarcinoma and 1,900 without cancer. These samples were genotyped using the Illumina Human610-Quad BeadChip in cases and the Illumina HumanHap550v3 BeadChip in controls. Genotype concordance between these two BeadChips was 99.99% among 182 duplicate samples, indicating a low possibility of genotype error. After we applied stringent quality control criteria, we carried out association analysis in 432,024 autosomal SNPs that were available on both BeadChips. Principal component analysis showed no population substructure in our population (**Supplementary Fig. 1a**). In addition, a quantile-quantile plot using the results of a Cochran-Armitage test showed that the inflation factor, λ, was 1.04, indicating a low possibility of false-positive associations resulting from population stratification (**Supplementary Fig. 1b**).

[1]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan. [2]Department of Medical Molecular Science, Hiroshima University, Hiroshima, Japan. [3]Laboratory for Genotyping Development, Center for Genomic Medicine, RIKEN, Yokohama, Japan. [4]Laboratory for Statistical Analysis, Center for Genomic Medicine, RIKEN, Yokohama, Japan. [5]Research Institute and Hospital, National Cancer Center, Goyang, Gyeonggi, Korea. [6]Laboratory for Medical Informatics, Center for Genomic Medicine, RIKEN, Yokohama, Japan. [7]Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan. [8]Department of Molecular Cytogenetics, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan. [9]Department of Medical Oncology, Shiga University of Medical Science, Otsu, Japan. [10]Cancer Center, Shiga University of Medical Science Hospital, Otsu, Japan. Correspondence should be addressed to Y.D. (ydaigo@ims.u-tokyo.ac.jp).

Received 12 March; accepted 26 August; published online 26 September 2010; doi:10.1038/ng.667

Table 1 Summary of GWAS and replication studies

SNP	Gene	Study	Allele [1/2]	Case 11	Case 12	Case 22	Control 11	Control 12	Control 22	MAF Case	MAF Control	OR (95%CI)[a]	P^b	P^c_{het}
rs10937405	*TP63*	GWAS	[T/C]	78	400	526	194	854	852	0.277	0.327	1.27 (1.13–1.43)	8.28×10^{-5}	
		1st replication (Japanese)		40	207	276	804	3437	3437	0.274	0.329	1.29 (1.12–1.49)	2.71×10^{-4}	
		2nd replication (Korean)		32	227	301	181	605	666	0.260	0.333	1.42 (1.22–1.66)	1.06×10^{-5}	
		Combined replication studies[d]										1.35 (1.22–1.50)	1.47×10^{-8}	0.37
		Combined all studies[e]										1.31 (1.22–1.42)	7.26×10^{-12}	0.50
rs2736100	*TERT*	GWAS	[A/C]	291	498	215	696	890	314	0.462	0.399	1.29 (1.16–1.44)	5.20×10^{-6}	
		1st replication (Japanese)		157	273	95	2830	3664	1182	0.441	0.393	1.22 (1.08–1.38)	1.92×10^{-3}	
		2nd replication (Korean)		174	277	106	567	692	199	0.439	0.374	1.31 (1.14–1.51)	1.41×10^{-4}	
		Combined replication studies[d]										1.26 (1.15–1.38)	1.48×10^{-6}	0.46
		Combined all studies[e]										1.27 (1.19–1.37)	2.91×10^{-11}	0.71

MAF, minor allele frequency; OR, odds ratio; CI, confidence interval. Odds ratios and *P* values for independence test were calculated by the Mantel-Haenszel method.
[a]Odds ratios of risk allele from two-by-two allele frequency table. [b]*P* value of Cochran-Armitage trend test. [c]Result of Breslow-Day test. [d]Meta-analysis of two replication studies. [e]Meta-analysis of all three studies.

No SNP reached a genome-wide significance level of association ($P < 1 \times 10^{-7}$) in our GWAS (**Supplementary Fig. 1c**). Next, we conducted a replication study using two sample sets: 525 lung adenocarcinoma cases and 7,678 controls for the Japanese set and 569 lung adenocarcinoma cases and 1,470 controls for the Korean set. For the first replication study, we selected 50 SNPs with $P < 1 \times 10^{-4}$ in our GWAS after excluding 12 SNPs with $r^2 \geq 0.8$ in the same locus. As a result, we successfully genotyped all 50 SNPs using the multiplex PCR-based Invader assay and five of them showed $P < 0.05$ with the same risk allele direction (**Supplementary Table 1**). In the second replication study, we genotyped these five SNPs and found two (rs10937405 and rs2736100) that showed $P < 0.05$ (**Table 1**). When we combined the results of the two replication studies using the Mantel-Haenszel method, we found that these two SNPs showed a significant association with lung adenocarcinoma after Bonferroni correction (calculated as $P < 0.05/50$). The first SNP, rs2736100, was located in intron 2 of *TERT*, where we replicated previous findings (combined $P = 2.91 \times 10^{-11}$, OR = 1.27); rs10937405 (combined $P = 7.26 \times 10^{-12}$, OR = 1.31) was located in intron 1 of *TP63* on chromosome 3q28. As shown in **Table 1**, ORs were similar across the three studies and we observed no heterogeneity.

We next analyzed the effect of rs10937405 according to gender and smoking behavior (**Supplementary Table 2**). After combined analysis by the Mantel-Haenszel method, this SNP tended to have higher OR for females than males, but we found no clear trend for smoking behavior. When we analyzed the combined effect of *TP63* and *TERT*, we found that these two susceptibility genes showed a synergistic effect on the risk of lung adenocarcinoma (OR = 4.26; **Supplementary Table 3**).

We also checked the association of other previously reported loci for lung cancer (**Supplementary Table 4**). Although we could not test the association of several SNPs in the loci on chromosomes 6p21.33, 6p22.1, 15q25.1 and 19q13.41 because their minor allele frequencies (MAFs) were too low or zero, our GWAS replicated three loci: rs9840545 in chromosome 3q26.32 ($P = 0.0242$), rs401681 in *CLPTM1L* ($P = 0.0106$) and rs3749971 in *OR12D3* ($P = 0.0241$).

In this study, we identified a new susceptibility locus for lung adenocarcinoma in intron 1 of *TP63* (rs10937405, $P = 7.26 \times 10^{-12}$). *TP63* contains 14 exons and spans about 300 kb. When we constructed linkage disequilibrium (LD) blocks and a $-\log_{10}$ (*P* value) plot around *TP63* using GWAS data, we found that the most associated SNP (rs10937405) represented an LD block that spanned from the upstream exon 1 to intron 1 of *TP63*. This SNP is included in the TP63 isoforms of TAp63 that are transcribed using a promoter upstream of exon 1, but not in other isoforms (ΔNp63) that are regulated by another promoter in intron 3 (ref. 25). To identify causative SNPs, we performed fine mapping of a genomic region containing approximately 200 kb from 190.7 to 190.9 Mb on chromosome 3 using GWAS case-control samples (**Fig. 1**). We used Haploview software to select 64 tag SNPs ($r^2 > 0.9$) on the basis of HapMap Japanese JPT data. We successfully genotyped 63 tagging SNPs in addition to the 45 SNPs already genotyped in GWAS (**Supplementary Table 5**), and we identified three SNPs (rs4488809, rs9816619 and rs4600802) that showed stronger association than rs10937405. Among them, rs4488809 (located 27 kb upstream of the landmark SNP) showed the strongest association. These SNPs were located in neighboring LD blocks and both blocks were located in intron 1 of *TP63* (**Fig. 1**).

Because three other LD blocks upstream of these blocks shared relatively high *D′* values with these blocks, we resequenced a 75-kb region from 190.805 to 190.880 Mb on chromosome 3 using genomic DNA from 96 individuals with lung adenocarcinoma. We identified 11 new SNPs in addition to the 162 known SNPs registered in the dbSNP database and genotyped 4 new SNPs with a MAF ≥ 0.05. However, none of the SNPs showed stronger association than

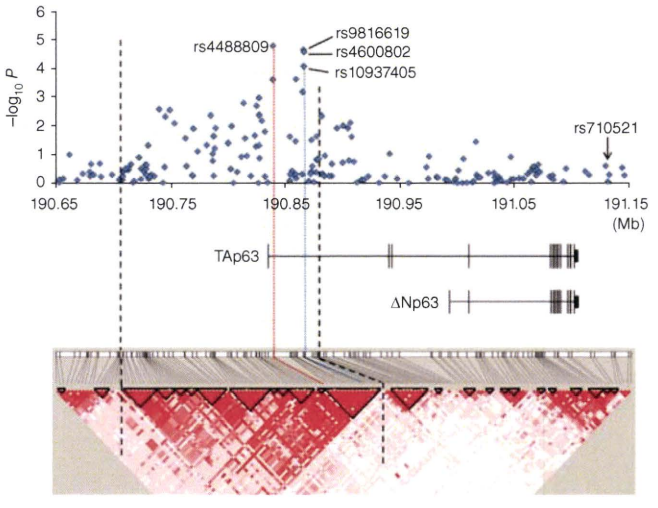

Figure 1 Case-control association plots, LD map and genomic structure of the *TP63* region in chromosome 3q28. The candidate region is shown between two black dotted lines. Fine mapping was performed in the region from 190.7 to 190.9 Mb. Blue diamonds represent $-\log_{10}P$ obtained from the GWAS and fine mapping using GWAS samples. The LD map based on *D′* values was drawn using the genotype data of the cases and controls in GWAS samples. Red dotted line, rs4488809; blue dotted line, landmark SNP (rs10937405); black arrow, rs710521, which shows a significant association with urinary bladder cancer.

rs4488809. We also performed haplotype analysis to investigate the effect of combinations of the four SNPs that were highly associated with lung adenocarcinoma susceptibility; however, no haplotype showed stronger association than the single-marker association of rs4488809 (**Supplementary Fig. 2a**). Although further functional analysis is needed to clarify which SNP is the true causative variant for lung adenocarcinoma, rs4488809 may be a candidate of functional significance.

Recent GWAS for lung cancer and follow-up studies using samples from more than 10,000 samples, mainly of European ancestry, have identified three lung cancer susceptibility loci: 15q24-25.1, which contains genes that encode nicotinic acetylcholine receptor subunits; 5p15.33 (rs2736100, *TERT*; rs401681, *CLPTM1L*); and 6p21.33 (*BAT3–MSH5*)[17–23]. The reasons for the differences between their results and ours might include the existence of unknown causative variant(s) at the same loci that emerged after the European-Asian split and insufficient statistical power due to the difference in MAF between the populations. For example, rs3117582 and rs3131379 in 6p21.33 were not polymorphic in the Japanese population (**Supplementary Table 4**). This may be a case in which the susceptibility variants emerged after the European-Asian split. On the other hand, SNPs in 15q25.1 showed large differences in MAFs between Japanese and European populations. If the true causative variants are linked to rs8034191 or rs1051730, it will be difficult to replicate the association in the Japanese population because of insufficient statistical power due to the low MAF of these SNPs (0.011). Regarding the *TP63* locus, the MAF of the landmark SNP (rs10937405) was similar between European and Japanese populations: MAF = 0.367 in CEU and MAF = 0.322 in JPT in the HapMap database. To investigate whether *TP63* could be a susceptibility locus in European populations, we searched the genotype data of the public lung cancer GWAS dataset from the International Agency for Research on Cancer (IARC)[17]. Genotype data for rs10937405 were not available in the IARC dataset; however, we obtained the genotype data for the alternative SNP, rs4396880, which is highly associated with rs10937405 (r^2 = 0.89 in CEU and 0.95 in JPT in the HapMap database). There was a weak association between rs4396880 and lung cancer in Central European populations with the same risk allele direction (P = 0.0297, OR = 1.11, 95% CI = 1.01–1.21). Although no SNP in this locus was listed in the top 200 SNPs in the meta-analysis of GWAS[23], these data suggest that genetic variation in *TP63* may also influence the susceptibility to lung cancer in European populations.

TP63 (also known as *p63*) is a member of the tumor suppressor *TP53* (also known as *p53*) gene family, which is involved in development, differentiation and response to cellular stress. *TP53* encodes a transcription factor that is essential for the prevention of cancer formation[25]. *TP63* is expressed mainly as two isoforms, the TA and N-terminal-truncated (ΔN) forms. TAp63 isoforms can also transactivate *TP53* target genes[26]. TAp63 isoforms are transcribed using a promoter located upstream of exon 1, whereas expression of the ΔNp63 isoforms are regulated by another promoter in intron 3 (ref. 25). TP63 is induced after exposure of cells to DNA damage[27,28]. Accumulation of DNA damage and lack of response to genotoxic stress contribute to an earlier step in carcinogenesis. Because possible candidate SNPs are located in intron 1 of *TP63*, which encodes TAp63 isoforms, we suggest that one or more of these SNPs may have a functional role in the regulation of *TP63* gene expression. rs710521, located near *TP63*, shows a genome-wide significant association with urinary bladder cancer[29]. Because lung and urinary bladder cancers share common environmental risk factors, such as smoking, we investigated the correlation between SNP rs710521 and those identified in our lung adenocarcinoma study. However, rs710521 was not associated with SNPs identified in our study or with lung adenocarcinoma (P = 0.242; **Fig. 1** and **Supplementary Fig. 2b**).

In summary, our data suggest that *TP63* is a candidate susceptibility gene for lung adenocarcinoma in Japanese and Korean populations. Further functional studies are necessary to clarify the mechanisms by which TP63 influences susceptibility to lung adenocarcinoma. Additional studies on other ethnic populations will also provide detailed information on the genetic etiology and heterogeneity of lung cancer.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank the staff of the Laboratory for Genotyping Development, Center for Genomic Medicine, RIKEN and the Human Genome Center, Institute of Medical Science, The University of Tokyo for their contribution to SNP genotyping. We also thank members of the BioBank Japan project, the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan, and Research Institute and Hospital, National Cancer Center, Korea for supporting our study. Y.D. is a member of the Shiga Cancer Treatment Project supported by Shiga Prefecture (Japan). This work was conducted as a part of the BioBank Japan Project and supported by the Ministry of Education, Culture, Sports, Sciences and Technology of the Japanese government. Management of second replication samples in Korea was supported by grants 0710221 and 0940620 from the National Cancer Center, Korea.

AUTHOR CONTRIBUTIONS

Y.N. conceived the study; D.M., M.K., Y.N. and Y.D. designed the study; D.M., N.H., M.K. and Y.D. performed genotyping; D.M., M.K., Y.N. and Y.D. wrote the manuscript; A.T., T.M., T. Tsunoda and N.K. performed data analysis at the genome-wide phase; Y.N. and M.K. managed DNA samples belong to BioBank Japan; K.-A.Y., J.K., G.-K.L., J.I.Z. and J.S.L. managed second replication samples in Korea; D.M. and Y.D. summarized the results; Y.N., T. Takahashi, K.C., J.I. and Y.D. obtained funding for the study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturegenetics/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Jemal, A. *et al.* Cancer statistics, 2009. *CA Cancer J. Clin.* **59**, 225–249 (2009).
2. Parkin, D.M., Bray, F., Ferlay, J. & Pisani, P. Global cancer statistics, 2002. *CA Cancer J. Clin.* **55**, 74–108 (2005).
3. Daigo, Y. & Nakamura, Y. From cancer genomics to thoracic oncology: discovery of new biomarkers and therapeutic targets for lung and esophageal carcinoma. *Gen. Thorac. Cardiovasc. Surg.* **56**, 43–53 (2008).
4. Toyoda, Y., Nakayama, T., Ioka, A. & Tsukuma, H. Trends in lung cancer incidence by histological type in Osaka, Japan. *Jpn. J. Clin. Oncol.* **38**, 534–539 (2008).
5. Sobue, T. *et al.* Trend of lung cancer incidence rate by histological type: a population-based study in Osaka, Japan. *Jpn. J. Cancer Res.* **90**, 6–15 (1999).
6. Thun, M.J. *et al.* Cigarette smoking and changes in the histopathology of lung cancer. *J. Natl. Cancer Inst.* **89**, 1580–1586 (1997).
7. Devesa, S.S., Bray, F., Vizcaino, A.P. & Parkin, D.M. International lung cancer trends by histological type. *Int. J. Cancer* **117**, 294–299 (2005).
8. Janssen-Heijnen, M.L. & Coebergh, J.W. The changing epidemiology of lung cancer in Europe. *Lung Cancer* **41**, 245–258 (2003).
9. Yang, C.H. EGFR tyrosine kinase inhibitors for the treatment of NSCLC in East Asia: present and future. *Lung Cancer* **60** (Suppl. 2), S23–S30 (2008).
10. Jee, S.H., Kim, I.S., Suh, I., Shin, D. & Appel, L.J. Projected mortality from lung cancer in South Korea, 1980–2004. *Int. J. Epidemiol.* **27**, 365–369 (1998).
11. Liam, C.K., Pang, Y.K., Leow, C.H., Poosparajah, S. & Menon, A. Changes in the distribution of lung cancer cell types and patient demography in a developing multiracial Asian country: experience of a university teaching hospital. *Lung Cancer* **53**, 23–30 (2006).
12. Fukuoka, M. *et al.* Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J. Clin. Oncol.* **21**, 2237–2246 (2003).

13. Huang, S.F. *et al.* High frequency of epidermal growth factor receptor mutations with complex patterns in non-small cell lung cancers related to gefitinib responsiveness in Taiwan. *Clin. Cancer Res.* **10**, 8195–8203 (2004).
14. Marchetti, A. *et al.* EGFR mutations in non-small-cell lung cancer. *J. Clin. Oncol.* **23**, 857–865 (2005).
15. Matakidou, A., Eisen, T. & Houlston, R.S. Systematic review of the relationship between family history and lung cancer risk. *Br. J. Cancer* **93**, 825–833 (2005).
16. Zhang, Y. *et al.* Family history of cancer and risk of lung cancer among nonsmoking Chinese women. *Cancer Epidemiol. Biomarkers Prev.* **16**, 2432–2435 (2007).
17. Hung, R.J. *et al.* A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. *Nature* **452**, 633–637 (2008).
18. Amos, C.I. *et al.* Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. *Nat. Genet.* **40**, 616–622 (2008).
19. Thorgeirsson, T.E. *et al.* A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature* **452**, 638–642 (2008).
20. McKay, J.D. *et al.* Lung cancer susceptibility locus at 5p15.33. *Nat. Genet.* **40**, 1404–1406 (2008).
21. Wang, Y. *et al.* Common 5p15.33 and 6p21.33 variants influence lung cancer risk. *Nat. Genet.* **40**, 1407–1409 (2008).
22. Broderick, P. *et al.* Deciphering the impact of common genetic variation on lung cancer risk: a genome-wide association study. *Cancer Res.* **69**, 6633–6641 (2009).
23. Landi, M.T. *et al.* A genome-wide association study of lung cancer identifies a region of chromosome 5p15 associated with risk for adenocarcinoma. *Am. J. Hum. Genet.* **85**, 679–691 (2009).
24. Wu, C. *et al.* Genetic variants on chromosome 15q25 associated with lung cancer risk in Chinese populations. *Cancer Res.* **69**, 5065–5072 (2009).
25. Moll, U.M. & Slade, N. p63 and p73: roles in development and tumor formation. *Mol. Cancer Res.* **2**, 371–386 (2004).
26. Flores, E.R. The roles of p63 in cancer. *Cell Cycle* **6**, 300–304 (2007).
27. Katoh, I., Aisaki, K., Kurata, S., Ikawa, S. & Ikawa, Y. p51A (TAp63gamma), a p53 homolog, accumulates in response to DNA damage for cell regulation. *Oncogene* **19**, 3126–3130 (2000).
28. Petitjean, A. *et al.* Properties of the six isoforms of p63: p53-like regulation in response to genotoxic stress and cross talk with DeltaNp73. *Carcinogenesis* **29**, 273–281 (2008).
29. Kiemeney, L.A. *et al.* Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. *Nat. Genet.* **40**, 1307–1312 (2008).

ONLINE METHODS

Samples. Characteristics of each case-control group are shown in **Supplementary Table 6**. Case and control samples used in this study for the Japanese population were obtained from BioBank Japan at the Institute of Medical Science, The University of Tokyo[30]. From the registered samples in BioBank Japan, we selected 1,551 individuals who were pathologically diagnosed as having lung adenocarcinoma. We used 1,026 samples as a set for the GWAS and an independent 525 samples as a set for the first replication study. The control groups included 994 samples for the GWAS and an independent 7,678 samples as a set for the first replication study. All the control samples were GWAS samples for other diseases in the BioBank Japan project. We excluded control samples that were registered for any cancer. We also obtained 906 Japanese control DNAs that were analyzed in the GWAS from volunteers without cancer from the Osaka-Midosuji Rotary Club, Osaka, Japan. Lung adenocarcinoma cases and controls obtained for the second replication study in the Korean population (n = 569 and 1,470, respectively) were obtained from the National Cancer Center, Korea. All participants provided written informed consent. This project was approved by the ethical committees of each participating institution.

SNP genotyping. Genomic DNA was extracted from peripheral blood leukocytes using a standard method. For the GWAS, we genotyped 1,026 cases using Illumina HumanHap610-Quad BeadChip and 1,900 controls using the Illumina HumanHap550v3 Genotyping BeadChip. After we excluded 22 samples with call rate <0.98, we applied SNP quality control (call rate ≥0.99 in both cases and controls, a MAF ≥0.01 and a P value in the Hardy-Weinberg equilibrium test of $\geq 1.0 \times 10^{-6}$ in controls): 432,024 SNPs in autosomal chromosomes passed the quality control filters and were further analyzed. We used genome-wide screening data for other diseases (chronic hepatitis B, keloid, drug rash, febrile seizure and pulmonary tuberculosis) as controls for the GWAS, which were genotyped using the Illumina HumanHap550v3 Genotyping BeadChip. For controls for the first replication study in the Japanese population, we also used genome-wide screening data for diabetes, peripheral artery disease, arrhythmias, stroke and myocardial infarction, which were genotyped using the Illumina HumanHap610 Genotyping BeadChip and applied the same quality control criteria as for the GWAS.

We used multiplex-PCR based Invader assays (Third Wave Technologies) for the replication studies of Japanese and Korean populations and also for fine mapping[31].

Fine mapping and resequencing. We performed fine mapping using all case and control samples in GWAS. Haploview was used to select tag SNPs with a pairwise $r^2 > 0.90$ and a MAF ≥0.05 on the basis of HapMap JPT data. Resequencing of candidate regions was performed in 96 lung adenocarcinoma cases using an ABI3730 Genetic Analyzer.

Statistical analysis. In GWAS and the first and second replication studies in Japanese and Korean populations, the statistical significance of the association with each SNP was assessed using a 1-degree-of-freedom Cochran-Armitage trend test. OR and CI were calculated from a two-by-two allele frequency table. Combined analysis was performed using the Mantel-Haenszel method. Heterogeneity among studies was examined using the Breslow-Day test. We used Haploview software to analyze the association of haplotypes and LD values between *TP63* and SNPs.

Software. For general statistical analyses, we used the R statistical environment version 2.6.1 or PLINK1.03 (ref. 32). To draw the LD map and analyze the association of haplotypes, we used Haploview software[33].

URLs. PLINK1.03, http://pngu.mgh.harvard.edu/~purcell/plink/; R statistical environment, http://www.cran.r-project.org/; IARC lung cancer database, http://www.cng.fr/prog_cancergenomics/lung_cancer.html.

30. Nakamura, Y. The BioBank Japan project. *Clin. Adv. Hematol. Oncol.* **5**, 696–697 (2007).
31. Ohnishi, Y. *et al.* A high-throughput SNP typing system for genome-wide association studies. *J. Hum. Genet.* **46**, 471–477 (2001).
32. Purcell, S. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–575 (2007).
33. Barrett, J.C., Fry, B., Maller, J. & Daly, M. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263–265 (2005).

nature genetics

Genome-wide association study identifies five new susceptibility loci for prostate cancer in the Japanese population

Ryo Takata[1,2], Shusuke Akamatsu[1,3], Michiaki Kubo[4], Atsushi Takahashi[5], Naoya Hosono[4], Takahisa Kawaguchi[6], Tatsuhiko Tsunoda[6], Johji Inazawa[7], Naoyuki Kamatani[5], Osamu Ogawa[3], Tomoaki Fujioka[2], Yusuke Nakamura[8] & Hidewaki Nakagawa[1]

Prostate cancer is one of the most common malignancies in males throughout the world[1], and its incidence is increasing in Asian countries. We carried out a genome-wide association study and replication study using 4,584 Japanese men with prostate cancer and 8,801 control subjects. From the thirty-one associated SNPs reported in previous genome-wide association studies in European populations, we confirmed the association of nine SNPs at $P < 1.0 \times 10^{-7}$ and ten SNPs at $P < 0.05$ in the Japanese population. The remaining 12 SNPs showed no association ($P > 0.05$). In addition, we report here five new loci for prostate cancer susceptibility, at *5p15* (λ-corrected probability $P_{GC} = 3.9 \times 10^{-18}$), *GPRC6A/RFX6* ($P_{GC} = 1.6 \times 10^{-12}$), *13q22* ($P_{GC} = 2.8 \times 10^{-9}$), *C2orf43* ($P_{GC} = 7.5 \times 10^{-8}$) and *FOXP4* ($P_{GC} = 7.6 \times 10^{-8}$). These findings advance our understanding of the genetic basis of prostate carcinogenesis and also highlight the genetic heterogeneity of prostate cancer susceptibility among different ethnic populations.

Asian populations have the lowest incidence and mortality rate of prostate cancer in the world, but the incidence of prostate cancer is rapidly increasing in Japan and other Asian countries[2,3]. Although the mechanisms by which prostate cancer develops and progresses are not clear, there is considerable evidence that genetic factors are important in its etiology[4,5]. A positive family history of prostate cancer is one of the most important risk factors for prostate cancer[4], and twin studies have indicated that the contribution of genetic factors to the development of prostate cancer is larger than in other common human tumors[5].

Recent genome-wide association studies (GWAS) have identified common variants at multiple loci that have moderate effects on prostate cancer risk[6–15]. Nearly 30 independent loci have been indicated to be associated with prostate cancer risk. However, GWAS for prostate cancer have been undertaken exclusively among European populations[16]. Further GWAS of Asian and other ethnic populations are required to identify additional susceptibility loci for prostate cancer and to enhance the understanding of the complex genetic effect on prostate carcinogenesis.

To identify genetic factors that confer risk of prostate cancer in the Japanese population, we carried out a GWAS using 1,583 Japanese men with prostate cancer and 3,386 control subjects (**Supplementary Fig. 1** and **Supplementary Table 1**). These subjects were genotyped using Illumina Human610-Quad BeadChip for the men with prostate cancer and Illumina HumanHap550v3 BeadChip for the control subjects, and the concordance between the genotypes determined by each of these platforms among 182 duplicated samples was 0.99998. We conducted association analysis for 510,687 SNPs that were commonly available on both BeadChips. Principal component analysis (PCA) showed that all cases and controls were clustered in the Asian population (**Supplementary Fig. 2a**). A quantile-quantile plot of the distribution of test statistics for the comparison of genotype frequencies in patients with prostate cancer versus control subjects showed that the genomic inflation factor lambda (λ) was 1.07, and the λ-value adjusted by sample size[17], $\lambda_{1,000}$, was 1.03 (**Supplementary Fig. 2b**). To further exclude the possibility of population substructure for our ethnic specific study and its influence on our GWAS results, we performed PCA again using only HapMap JPT and CHB as the references, and we found that almost all of the subjects fell into the known two main clusters of the Japanese population[18] (**Supplementary Fig. 2c**). Then we evaluated the genomic inflation factor using only the largest cluster on this PCA, but the inflation factor was not improved (**Supplementary Fig. 2d**; $\lambda = 1.06$, $\lambda_{1,000} = 1.03$), indicating that there is unlikely to be population substructure in our GWAS samples. Therefore, genotype misclassification and population substructure could not be the cause of the difference in the inflation factor.

[1]Laboratory for Biomarker Development, Center of Genomic Medicine, RIKEN, Tokyo, Japan. [2]Department of Urology, Iwate Medical University, Morioka, Japan. [3]Department of Urology, Graduate School of Medicine, Kyoto University, Kyoto, Japan. [4]Laboratory for Genotyping Development, Center of Genomic Medicine, RIKEN, Yokohama, Japan. [5]Laboratory for Statistical Analysis, Center of Genomic Medicine, RIKEN, Yokohama, Japan. [6]Laboratory for Medical Informatics, Center of Genomic Medicine, RIKEN, Yokohama, Japan. [7]Department of Molecular Cytogenetics, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan. [8]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. Correspondence should be addressed to H.N. (hidewaki@ims.u-tokyo.ac.jp).

Received 26 February; accepted 6 July; published online 1 August 2010; doi:10.1038/ng.635