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SHORT REPORT

Mitochondrial functions of RECQL4 are required for the prevention of aerobic glycolysis-dependent cell invasion

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ABSTRACT

Germline mutations in RECQL4 helicase are associated with Rothmund-Thomson syndrome, which is characterized by a predisposition to cancer. RECQL4 localizes to the mitochondria, where it acts as an accessory factor during mitochondrial DNA replication. To understand the specific mitochondrial functions of RECQL4, we created isogenic cell lines, in which the mitochondrial localization of the helicase was either retained or abolished. The mitochondrial integrity was affected due to the absence of RECQL4 in mitochondria, leading to a decrease in F_1F_0-ATP synthase activity. In cells where RECQL4 does not localize to mitochondria, the membrane potential was decreased, whereas ROS levels increased due to the presence of high levels of catalytically inactive SOD2. Inactive SOD2 accumulated owing to diminished SIRT3 activity. Lack of the mitochondrial functions of RECQL4 led to aerobic glycolysis that, in turn, led to an increased invasive capability within these cells. Together, this study demonstrates for the first time that, owing to its mitochondrial functions, the accessory mitochondrial replication helicase RECQL4 prevents the invasive step in the neoplastic transformation process.

KEY WORDS: RECQL4, Cell invasion, SOD2, SIRT3, OXPHOS

INTRODUCTION

Mutations in RecQ-like helicase 4 (*RECQL4*) lead to Rothmund-Thomson syndrome (RTS), RAPADILINO syndrome and Baller–Gerold syndrome. A substantial proportion of RTS patients and few RAPADILINO patients are susceptible to the development of lymphoma and osteosarcoma (Dietschy et al., 2007; Siitonen et al., 2009; Vargas et al., 1992). RECQL4 has a well-characterized role in the maintenance of the nuclear genome (Croteau et al., 2012b), and is involved in the initiation of DNA replication by enhancing the chromatin binding of DNA polymerase α (Matsuno et al., 2006; Sangrithi et al., 2005).

RECQL4 also localizes to the mitochondria (Croteau et al., 2012a; De et al., 2012), mediated through a validated N-terminal mitochondrial localization signal (MLS) (De et al., 2012). RECQL4 is involved in enhancing the processivity and polymerization of mitochondrial DNA (mtDNA) replication. Hence, absence of RECQL4 contributes to multiple mutations and polymorphisms over the entire mtDNA within cells of RTS patients (Gupta et al., 2014).

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Aerobic glycolysis positively influences multiple parameters of cancerous growth including raised biomass synthesis, and increased production of ATP and reactive oxygen species (ROS) (Warburg, 1956). Studies have shown that the presence of mutated mtDNA is associated with both solid tumors and leukemia (Fliss et al., 2000; He et al., 2003). Some of the mtDNA mutations also lead to increased ROS production (Ishikawa et al., 2008). We now demonstrate that lack of mitochondrial RECQL4 causes mitochondrial dysfunction, aerobic glycolysis and, thereby, imparts increased invasive potential to RTS patient cells.

RESULTS AND DISCUSSION

Lack of localization of RECQL4 to mitochondria compromises mitochondrial integrity

Our recent results (De et al., 2012; Gupta et al., 2014) led to the question whether the lack of mitochondrial localization of RECQL4 in RTS patients affects the functioning of the mitochondria and, thereby, contributes to the neoplastic transformation process. To answer this question, two cellular models were employed: (i) hTERT immortalized normal human fibroblasts GM07532 and RTS patient fibroblast cell lines AG03587, AG05013, AG05139, L9552914-J, B1865425K, D8903644-K; (ii) an isogenic system, wherein either *Aequorea coerulescens* (Ac)GFP-tagged wild-type RECQL4 or RECQL4 without the mitochondrial localization signal, i.e. RECQL4 (Δ84), were stably expressed in AG05013 cells. In contrast to cells expressing wild-type RECQL4, the localization of the helicase was completely nuclear in RECQL4 (Δ84) cells (Fig. 1A). The steady-state expression levels of the two proteins were comparable (Fig. 1B). Mitochondrial import assays carried out with purified and validated mitochondrial fractions (Fig. S1A) indicated that wild-type RECQL4, but not RECQL4 (Δ84), was resistant to trypsin treatment and was present within the mitoplast (Fig. 1C).

We observed a statistically significant reduction (20-25%) in the mtDNA copy number in the RTS patient fibroblasts (AG05013). This decrease in mtDNA copy number was due to the absence of mitochondrial localization of RECQL4 because cells expressing RECQL4 (Δ84) also showed a similar decrease (Fig. 1D). This defect in mtDNA copy number might be a reflection of the defect in mitochondrial mass and not due to any defect in the mtDNA itself. Hence, mitochondrial mass was measured by multiple methods – by determining the protein levels of various mitochondrial markers residing in different mitochondrial compartments (Fig. 1E) or by using two the dyes Mitotracker Red 580 (Fig. 1F,G, Fig. S1B,C) and Nonyl Acridine Orange (NAO) (Fig. S1D). The mitochondrial mass was found to be decreased in RTS patient fibroblasts and RECQL4 (Δ84) cells following application of any of these methods (Fig. 1E-G, Fig. S1B-D). Enhanced citrate synthase activity (a marker for

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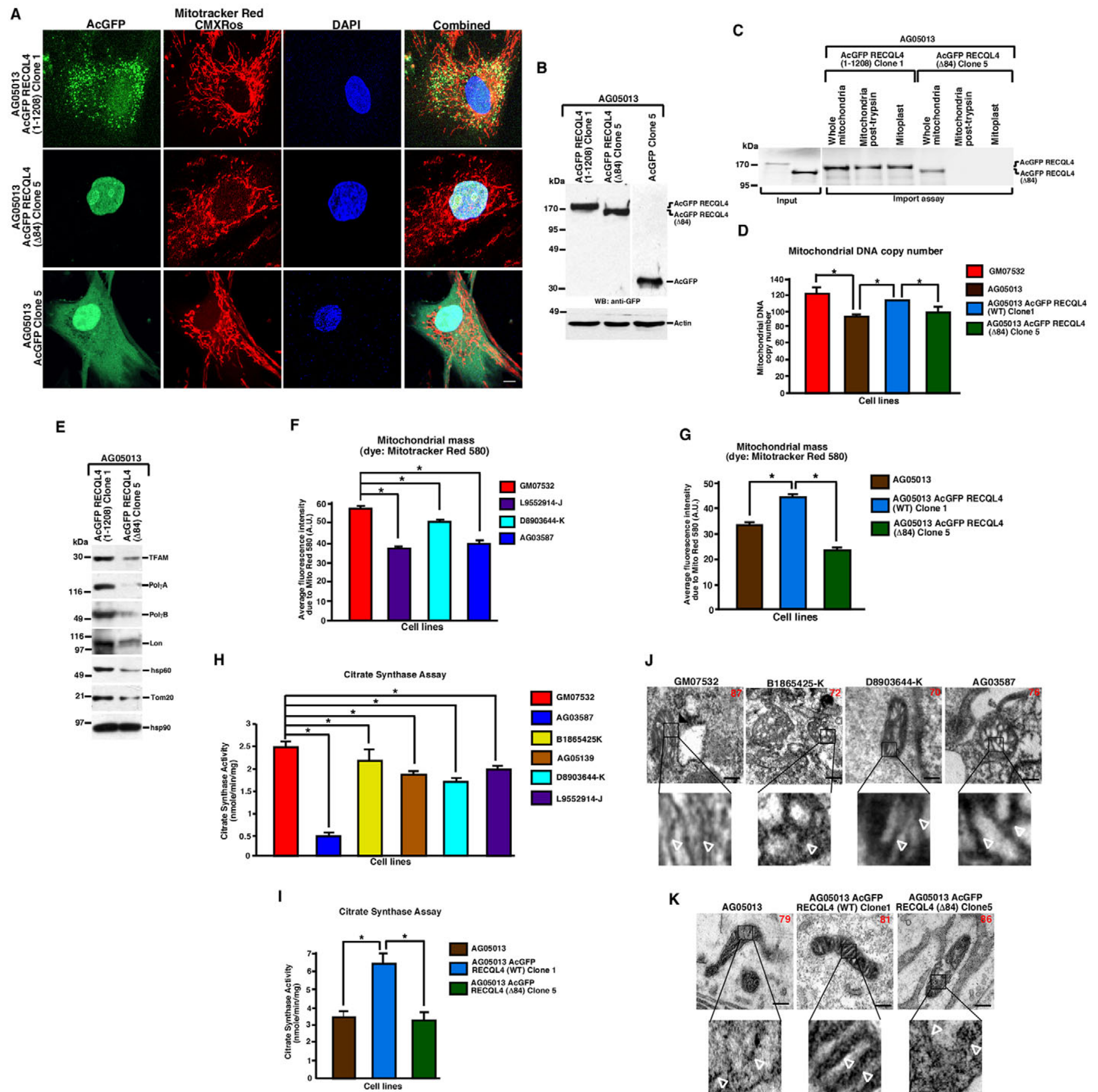

Fig. 1. Cells lacking RECQL4 have decreased mitochondrial integrity. (A,B) Characterization of isogenic cell lines. (A) Confocal microscopy images of isogenic cells stained with Mitotracker Red CMXRos. Scale bar, 5 µm. (B) Western blot analysis of the lysates obtained from isogenic cells, probed with antibodies against GFP and actin. (C) RECQL4 lacking mitochondrial localization signal cannot enter into mitochondria. Mitochondrial import assay was carried out using purified mitochondrial fractions with either wild-type RECQL4 or RECQL4 (Δ84). The import was analyzed by phosphorimaging. (D) Lack of RECQL4 decreases mitochondrial DNA copy number. Mitochondrial DNA copy number was determined in asynchronously growing GM07532, indicated RTS fibroblasts and isogenic cells. Data were analyzed by unpaired two-tailed Student's *t*-test, *$P\leq0.05$. (E-G) Lack of RECQL4 decreases mitochondrial mass. Mitochondrial mass was measured by determining the levels of mitochondrial markers in different mitochondrial compartments, namely Tom20 (an outer membrane protein), TFAM, PolγA, PolγB, Lon, Hsp60 (all mitochondrial matrix components). Hsp90 was used as a total protein loading control (E). Additionally, mitochondrial mass was also determined in asynchronously growing GM07532 cells, and the indicated RTS fibroblasts (F), and isogenic cells (G) after staining with Mitotracker Red 580. Data were statistically analyzed by using two-way ANOVA; * $P\leq0.05$. (H,I) Lack of RECQL4 leads to a decrease in mitochondrial matrix integrity. Activity of mitochondrial matrix enzyme, citrate synthase was measured in GM07532 (H) and indicated RTS fibroblasts, and isogenic cells (I). Data were analyzed using unpaired two-tailed Student's *t*-test; *$P\leq0.05$. (J,K) Absence of RECQL4 mitochondrial localization affects cristae ultrastructure. Transmission electron micrographs of mitochondria obtained from asynchronously growing cultures of GM07532, indicated RTS fibroblasts (J). Scale bar, 500 nm. (K) AG05013 and the indicated isogenic cells. Original magnification: 6500×. Enlarged images for each micrograph are also shown above. White arrowheads indicate cristae, numbers indicate the percentage of cells with the depicted phenotype. Error bars are mean±s.d.

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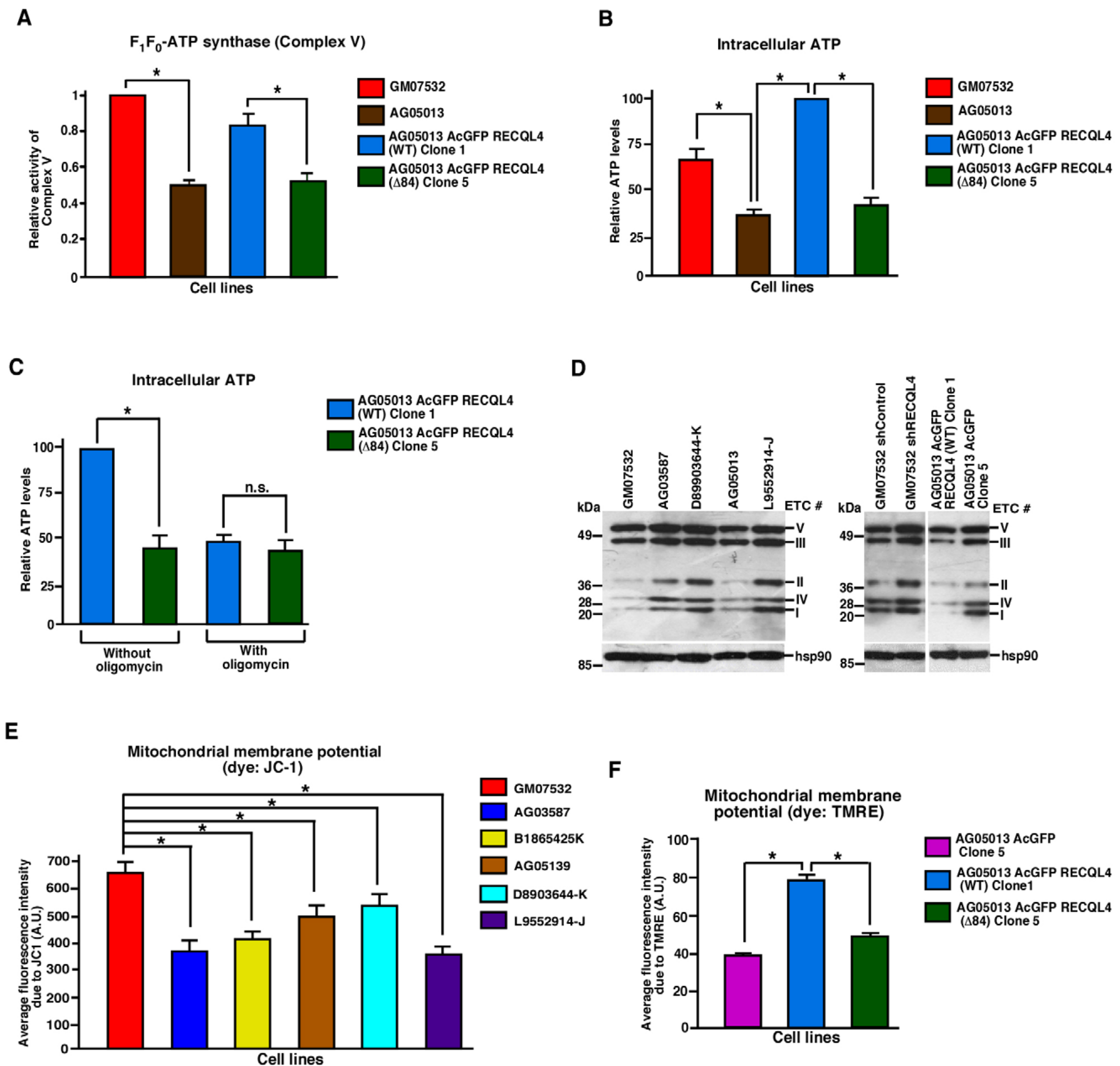

Fig. 2. Absence of mitochondrial localization of RECQL4 deregulates electron transport chain complex. (A) F_1F_0-ATP synthase (complex V) activity is decreased in absence of mitochondrial RECQL4. Complex V activity was measured from lysates obtained from asynchronously growing GM07532, indicated RTS fibroblasts and isogenic cells. The values are represented with respect to GM07532. Data were analyzed using unpaired two-tailed Student's *t*-test; * $P\leq0.05$. (B,C) Lack of mitochondrial localization of RECQL4 affects mitochondrial ATP. (B) Relative intracellular ATP levels were measured in asynchronously growing GM07532, indicated RTS fibroblasts and isogenic cells. (C) Same as in B, except that isogenic cells were either treated with vehicle or oligomycin. ATP levels were extrapolated from the luciferase values and are represented with respect to RECQL4 (WT) Clone 1. Data were analyzed by unpaired two-tailed Student's *t*-test; *$P\leq0.05$. n.s., not significant. (D) Protein levels of OXPHOS complex subunits are increased in absence of mitochondrial RECQL4. Lysates prepared from asynchronously growing GM07532 and indicated RTS fibroblasts (left), GM07532 shControl, GM07532 shRECQL4 and isogenic cells (right) were probed with OXPHOS antibody cocktail or hsp90. (E,F) Mitochondrial membrane potential is decreased in absence of RECQL4. Mitochondrial membrane potential was measured by JC-1 staining in asynchronously growing GM07532, indicated RTS fibroblasts (E) or by TMRE staining in asynchronously growing isogenic cells (F). Data were statistically analyzed by using two-way ANOVA with *$P\leq0.05$. A.U., arbitrary units. Error bars are mean±s.d.

mitochondrial matrix integrity) was observed only when wild-type RECQL4 was expressed in mitochondria (Fig. 1H,I). These changes in the mitochondrial integrity were reflected in altered mitochondrial ultrastructure, whereby, in contrast to GM07532, fibroblasts from RTS patients showed aberrant mitochondrial morphology, i.e. distorted cristae, that resulted in reduced cristae surface (Fig. 1J). In contrast to RECQL4 (Δ84) cells, cells expressing wild-type RECQL4 exhibited normal mitochondrial ultrastructure (Fig. 1K).

Compromised SIRT3 activity causes increased ROS generation in cells without mitochondrial RECQL4

Electron transport chain (ETC) complexes are involved in the transfer of electrons via redox reactions leading to the transfer of protons across a membrane. This creates an electrochemical proton gradient, which is the main source of ATP through oxidative phosphorylation (OXPHOS). In the absence of mitochondrial RECQL4, the deformed cristae (Fig. 1J,K) might result in sub-optimal functioning of OXPHOS. Activity assays carried out by using the isogenic cell

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lines indicated that the relative activity of either ETC complex I or ETC complex IV was not altered, irrespective of the status of RECQL4 (Fig. S1E,F). In contrast, activity of F_1F_0-ATP synthase (ETC complex V) was diminished in AG05013 cells. Restoring the expression of wild-type RECQL4, but not RECQL4 (Δ84), reinstated the activity of ETC complex V (Fig. 2A). Consequently, levels of intracellular ATP were higher in GM07532 and RECQL4 (WT) cells compared with those in AG05013 and RECQL4 (Δ84) cells (Fig. 2B). This difference in the levels of intracellular ATP was abolished when cells were grown in the presence of the F_1F_0-ATP synthase inhibitor oligomycin (Fig. 2C), indicating that lack of mitochondrial RECQL4 decreases the contribution of mitochondrial ATP production. Overexpression of the ETC complexes at protein level in cells that lack RECQL4 (Fig. 2D) seems to indicate a compensatory cellular response. In concordance with the results that indicate gross mitochondrial dysfunction (Fig. 1), membrane potential was reduced in RTS fibroblasts, and also in AG05013 cells that express either AcGFP or AcGFP RECQL4 (Δ84) (Fig. 2E,F, Fig. S1G,H). Consequently, mitochondrial ROS was increased in RTS patient cells (Fig. 3A) and in cells that lack mitochondrial RECQL4 (Fig. 3B, Fig. S1I). The increased level of ROS led to an increased number of carbonyl (CO) groups introduced into proteins by the oxidative reactions in cells that lack RECQL4 (Fig. 3C).

High levels of mitochondrial ROS in cells lacking mitochondrial RECQL4 led to the expectance of very low levels of SOD2 in these cells. However, increased SOD2 protein levels (Fig. 3D,E, Fig. S2A, B), but not enhanced levels of *SOD2* transcripts (Fig. S2C,D), were observed in both RTS patient cells and RECQL4 (Δ84) cells. SOD2 acetylated at lysine residue 68 (AcLys68 SOD2) is known to be catalytically inactive (Chen et al., 2011). Cells that lack mitochondrial RECQL4 and express increased AcLys68 SOD2 levels. Hence, lower ratios of SOD2 to AcLys68 SOD2 (SOD2: AcLys68 SOD2) were observed in cells not expressing wild-type RECQL4 (Fig. 3F,G, Fig. S2E,F). This indicates that the high levels of SOD2 in cells lacking mitochondrial RECQL4 is catalytically inactive. SIRT3 deacetylates and, thereby, activates SOD2, which – in turn – scavenges mitochondrial ROS (Miao and St. Clair, 2009). The amount of SIRT3 transcript or protein was unaltered, irrespective of whether mitochondrial RECQL4 was present or absent (Fig. S3A-C). However, reduction in SIRT3 activity was observed in RECQL4 (Δ84) and AcGFP cells (Fig. 3H) compared with that in cells expressing wild-type RECQL4, which possibly caused the accumulation of inactive SOD2.

Altered glucose metabolism in RECQL4 (Δ84) cells leads to increased cellular invasive potential

The above results indicate that, instead of depending on the oxidative phosphorylation process, cells that lack mitochondrial RECQL4 utilize the less efficient but faster aerobic glycolysis process to generate ATP through a glycolytic shift (Warburg, 1956). Indeed, in contrast to GM07532, all tested RTS patient cells had significantly increased glucose uptake (Fig. 4A), due to which activity of the glycolytic enzyme 6-phosphofructokinase (PFK) was enhanced in cells that did not express RECQL4 (Fig. 4B). Consequently, the excess of the glucose consumed is converted to lactate instead of pyruvate, thereby causing an increase in the lactate:pyruvate ratio in cells that lack mitochondrial localization of RECQL4 (Fig. 4C, Fig. S3D,E).

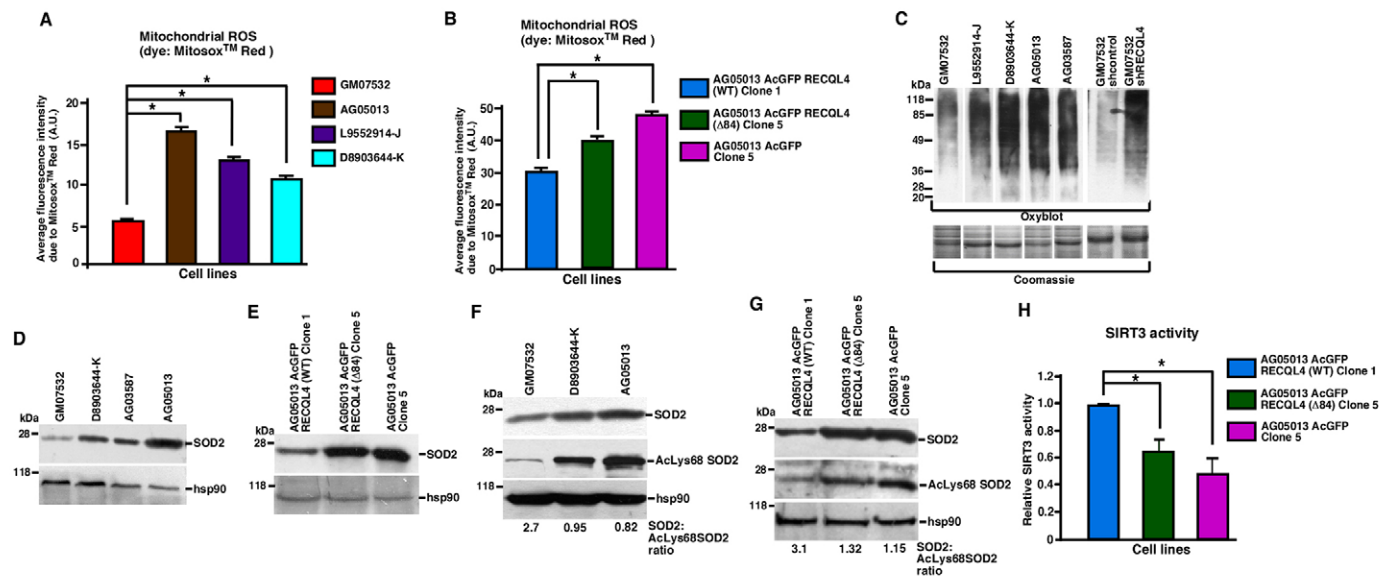

Fig. 3. Decreased SIRT3 activity causes the accumulation of inactive SOD2 in cells where RECQL4 cannot localize to the mitochondria. (A,B) Lack of mitochondrial localization of RECQL4 leads to an increase in the level of mitochondrial ROS. Mitochondrial ROS levels were measured by Mitosox™ Red staining in (A) asynchronously growing GM07532 and indicated RTS fibroblasts, or (B) in isogenic cells. Data were analyzed by unpaired two-tailed Student's *t*-test with * denoting $P \leq 0.05$. A.U. represents arbitrary units. (C) Lack of RECQL4 increases the ROS-dependent oxidative modification of proteins. Equal amounts of lysates (Coomassie staining, bottom) from GM07532, the indicated RTS fibroblasts, GM07532 shControl and GM07532 shRECQL4 cells post-derivatization were probed with an anti-DNP antibody (top). (D,E) SOD2 protein levels were increased when RECQL4 is not localized to mitochondria. Lysates from asynchronously growing (D) GM07532 and indicated RTS fibroblasts, and (E) isogenic cells were electrophoresed on SDS-PAGE gels. Western blot analysis was performed with antibodies against SOD2 and hsp90. (F,G) Acetylation of SOD2 at lysine 68 was enhanced in the absence of mitochondrial RECQL4. Lysates from asynchronously growing (F) GM07532, indicated RTS fibroblasts and (G) isogenic cells were electrophoresed on SDS-PAGE gels. Western blot analysis was performed with antibodies against SOD2, AcLys68 SOD2 and hsp90. The SOD2:AcLys68-SOD2 ratio is indicated at the bottom. (H) Activity of SIRT3 is decreased in the absence of mitochondrial RECQL4. The relative activity of SIRT3 in the lysates from asynchronously growing isogenic cells was measured. The values are represented with respect to those of RECQL4 (WT) Clone 1 cells. Data were analyzed by unpaired two-tailed Student's *t*-test with *$P \leq 0.05$. Error bars are mean±s.d.

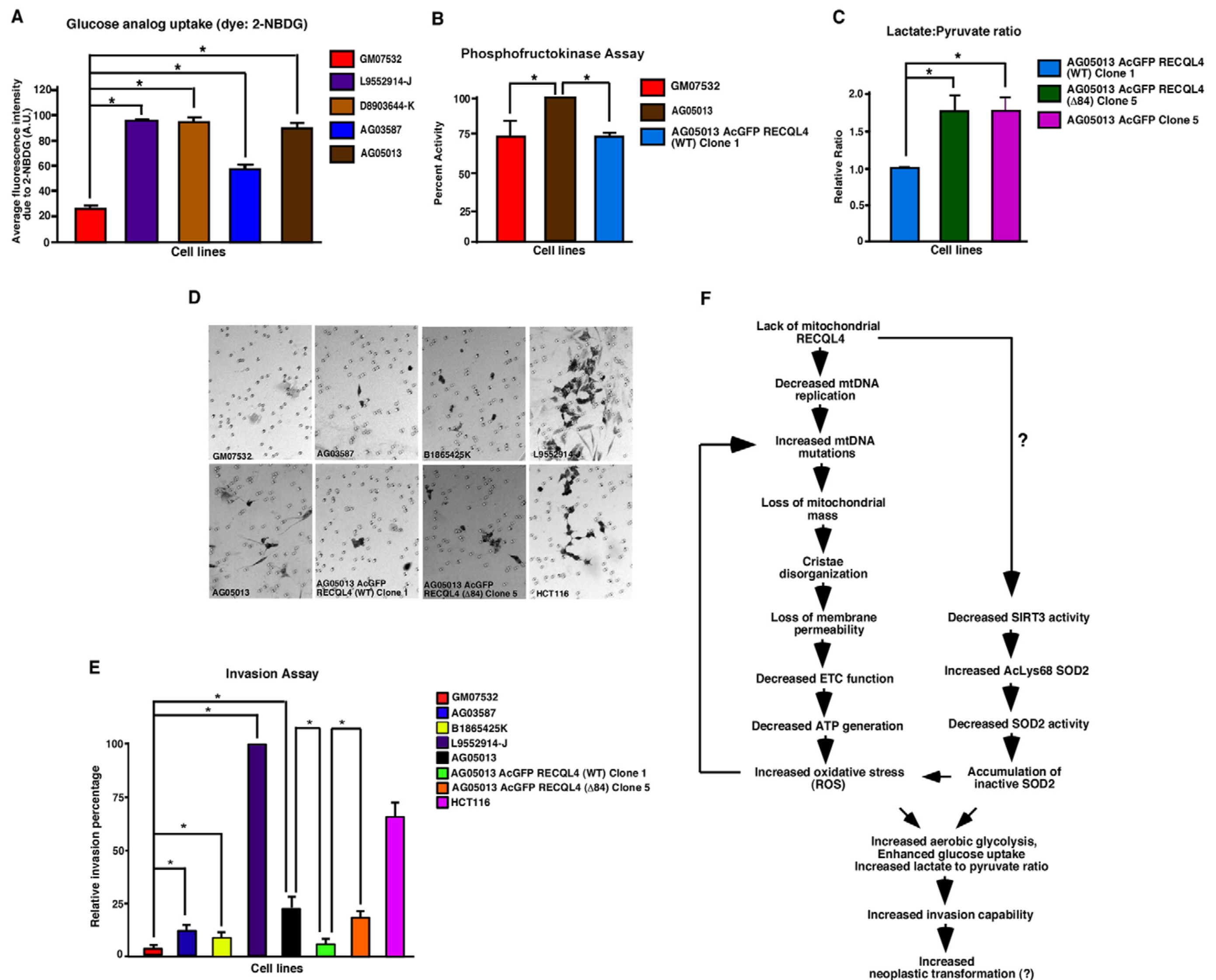

Fig. 4. Invasive capability of RTS patient cells depends on the absence of mitochondrial RECQL4. (A) Glucose analog (2-NBDG) uptake is enhanced in the absence of mitochondrial RECQL4. The uptake of glucose analog (2-NBDG) was measured in asynchronously growing GM07532 and indicated RTS fibroblasts. Data were analyzed by unpaired two-tailed Student's *t*-test with * denoting $P \leq 0.05$. A.U. represents arbitrary units. (B) The absence of mitochondrial RECQL4 enhances the activity of phosphofructokinase. Relative activity of phosphofructokinase in asynchronously growing GM07532, AG05013 and AG05013 AcGFP RECQL4 (WT) cells. The values are represented with respect to those of AG05013 cells. Data were analyzed by unpaired two-tailed Student's *t*-test with * denoting $P \leq 0.05$. (C) The lactate:pyruvate ratio is increased in cells where RECQL4 was not localized to mitochondria. Lactate and pyruvate assays were performed in asynchronously growing isogenic cells. The lactate:pyruvate ratio was obtained from the absolute lactate and pyruvate concentrations, and are represented with respect to RECQL4 (WT) Clone 1 cells. Data were analyzed by unpaired two-tailed Student's *t*-test with * denoting $P \leq 0.05$. (D,E) A lack of mitochondrial localization of RECQL4 enhanced invasion percentage of the cells. Matrigel invasion assays were performed in asynchronously growing GM07532 and indicated RTS fibroblasts, isogenic cells and HCT116 cells (positive control). Representative images for each cell line are shown in D. Quantification (E) represents the relative invasion percentage of the different cell lines with respect to that of L9552914-J (having maximum invasion percentage). Data were analyzed by unpaired two-tailed Student's *t*-test with * denoting $P \leq 0.05$. (F) Schematic diagram depicting how the lack of mitochondrial RECQL4 leads to invasion capability. Lack of mitochondrial RECQL4 leads to decreased mitochondrial mass and disorganized cristae, which results in the loss of membrane permeability, a decrease in ETC function, and ultimately causes a decrease in the levels of intracellular ATP. Hence, these cells show increased ROS levels. Paradoxically, the increased ROS in these cells coincides with the accumulation of high levels of catalytically inactive AcLys68 SOD2, which accumulates owing to the decreased levels of active SIRT3. These alterations lead to increased rates of aerobic glycolysis, which manifests as enhanced glucose uptake in the cells and a high lactate to pyruvate ratio. As a consequence, cells that lack mitochondrial RECQL4 have higher invasive potential, which might account for the cancer predisposition in RTS patients. Error bars are mean±s.d.

The augmented glucose uptake, increased PFK activity and accumulation of lactate in cells that lack mitochondrial RECQL4 should lead to the acidification of the cellular milieu, thereby providing a micro-environment conducive for the greater invasive capability of cells (Gerweck and Seetharaman, 1996). To test this hypothesis, *in vitro* matrigel invasion assays were carried out. Cells from RTS patients and from cells that express RECQL4 (Δ84) have increased invasive capability (Fig. 4D,E). Thus, by using isogenic cell lines in conjunction with immortalized RTS patient fibroblasts, we have determined that alterations in the mitochondrial bioenergetics occur when RECQL4 does not localize to the organelle, and this might contribute towards the neoplastic

transformation process, commonly observed in RTS patients (Fig. 4F).

It is worth emphasizing that the *RECQL4* gene is encoded in the nucleus and that its protein product has well-documented roles in the maintenance of multiple genome-repair processes within the nucleus (Croteau et al., 2012b). The role of RECQL4 in the maintenance of both the nuclear and mitochondrial genomes might, thus, be due to the cell-cycle-specific subcellular localizations of the protein (De et al., 2012). The fact that not all initially cytosolic RECQL4 localizes to the mitochondria (Fig. 1A) might indicate a non-nuclear, non-mitochondrial pool, possibly, with independent functions (Yin et al., 2004). We hypothesize that mitochondria are the primary site of RECQL4 function and the subsequent effect on nuclear genome maintenance occurs due to the effect of retrograde signaling from the mitochondria to the nucleus.

MATERIALS AND METHODS

Antibodies, cell lines, cell culture conditions

Antibodies used are listed in Table S1. hTERT immortalized normal human fibroblasts GM07532 and RTS fibroblasts, isogenic cell lines AcGFP-N1-RECQL4 (1-1208) Clone 1, AcGFP-N1-RECQL4 (Δ84) Clone 5 and GM07532 shRECQL4 and GM07532 shControl have been described earlier (De et al., 2012). Protein levels of RECQL4 in GM07532, GM07532 shRECQL4 and RTS fibroblasts have been reported previously (De et al., 2012). AcGFP-N1 Clone 5 cells were created by lentiviral transduction followed by selection with puromycin (0.5 µg/ml). HCT116 cells (gift from Bert Vogelstein) were maintained in McKoy's 5A medium (Thermo Fisher Scientific).

In vitro import assay

^{35}S-labeled wild-type RECQL4 and RECQL4 (Δ84) were used in import assays as described previously (Tammineni et al., 2013). Labeled proteins were incubated with 200 µg of isolated mitochondria from HEK293T cells. After import, one-half of each sample was treated with trypsin (5 µg/ml, Sigma-Aldrich) on ice for 15 min followed by inhibition of trypsin with soybean trypsin inhibitor (50 µg/µl, Sigma-Aldrich). For mitoplast preparation, mitochondria were re-suspended in hypotonic buffer (20 mM KCl, 10 mM HEPES pH 7.2) or 0.1% digitonin and incubated on ice for 20 min. The obtained mitoplasts were isolated again by centrifugation (14167 ***g*** for 10 min) and processed further.

Immunofluorescence, reverse-transcription PCR, matrigel-invasion assays, transmission electron microscopy

Immunofluorescence for SOD2 and AcLys68 SOD2 was carried out as described (Kharat et al., 2015). Imaging was carried out by using a 63×/1.2 oil immersion objective. Fluorescence intensity was determined by quantitative confocal microscopy (n=200 cells) by using a Zeiss LSM 510 Meta system. The region of interest (ROI) function present in the in-built software was used to keep all acquisition parameters constant. Primers used for reverse-transcription (RT)-PCR are listed in Table S2. Matrigel invasion assays were carried out in six-well BD Biocoat Matrigel Invasion Chambers as described earlier (Chandra et al., 2013). Transmission electron microscopic studies were carried out as published before (Frasca and Parks, 1965; Vijayalakshmi et al., 2015).

Measurements of mitochondrial functions

Mitochondrial copy number was calculated from the ratio of mitochondrial cytochrome b to nuclear β-globin genes by using quantitative PCR (Miller et al., 2003). Mitochondrial mass was determined by using either Mitotracker Red 580 dye (0.25 µM, Thermo Fisher Scientific, measured by quantitative confocal microscopy) or by using NAO (5 µM, Thermo Fisher Scientific, measured in Varioskan Flash at 530 nm). Kits used to measure different mitochondrial parameters are listed in Table S3. Determination of phosphofructokinase activity, lactate, pyruvate concentrations were done in parallel. Membrane potential was measured either by the cationic carbocyanine dye JC-1 (2 µM, Thermo Fisher Scientific) that accumulates in mitochondria or by the cell-permeant, cationic, red-orange fluorescent dye TMRE (1 nM, Thermo Fisher Scientific) that is readily sequestered by active mitochondria. For JC-1, the ratio of the quantitative fluorescence between the Argon and DPSS lasers was taken as the average fluorescence intensity. Mitochondrial ROS was measured by Mitosox™ Red (2.5 µM, Thermo Fisher Scientific) staining. Quantitative imaging was carried out in a Zeiss LSM 510 Meta system by using a 63×/1.2 oil objective and DPSS 561 nm laser lines. The relative amount of proteins modified by oxidation was determined by using an Oxyblot protein oxidation detection kit (Millipore). For glucose uptake, live cells were stained with 2-NBDG (100 µM, Thermo Fisher Scientific) for 30 min at 37°C. The incorporated dye was measured in BD FACS Calibur.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
J.K., M.H., S.D., S.C., P.M., S.T., A.S. and C.S. carried out the experimental work and analyzed data. N.B.S. only analyzed data. S.S. analyzed data and wrote the manuscript.

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Supplementary information
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