RECQL4 is essential for the transport of p53 to mitochondria in normal human cells in the absence of exogenous stress

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Summary

Mutations in RECQL4 helicase are associated with Rothmund–Thomson syndrome (RTS). A subset of RTS patients is predisposed to cancer and is sensitive to DNA damaging agents. The enhanced sensitivity of cells from RTS patients correlates with the accumulation of transcriptionally active nuclear p53. We found that in untreated normal human cells these two nuclear proteins, p53 and RECQL4, instead colocalize in the mitochondrial nucleoids. RECQL4 accumulates in mitochondria in all phases of the cell cycle except S phase and physically interacts with p53 only in the absence of DNA damage. p53–RECQL4 binding leads to the masking of the nuclear localization signal of p53. The N-terminal 84 amino acids of RECQL4 contain a mitochondrial localization signal, which causes the localization of RECQL4–p53 complex to the mitochondria. RECQL4–p53 interaction is disrupted after stress, allowing p53 translocation to the nucleus. In untreated normal cells RECQL4 optimizes de novo replication of mtDNA, which is consequently decreased in fibroblasts from RTS patients. Wild-type RECQL4-complemented RTS cells show relocalization of both RECQL4 and p53 to the mitochondria, loss of p53 activation, restoration of de novo mtDNA replication and resistance to different types of DNA damage. In cells expressing Δ84 RECQL4, which cannot translocate to mitochondria, all the above functions are compromised. The recruitment of p53 to the sites of de novo mtDNA replication is also regulated by RECQL4. Thus these findings elucidate the mechanism by which p53 is regulated by RECQL4 in unstressed normal cells and also delineates the mitochondrial functions of the helicase.

Key words: 53BP1, Bloom helicase, Nuclear localization signal, Tom20 receptor, Mitochondrial localization signal

Introduction

BLM helicase (BLM), Werner helicase (WRN) and RECQL4 helicase are members of the evolutionarily conserved RecQ family of DNA helicases. Mutations in *BLM* and *WRN* genes result in autosomal recessive cancer predisposition disorders namely Bloom syndrome (BS) and Werner syndrome (WS), respectively. Mutations in *RECQL4* cause three autosomal recessive disorders – Rothmund–Thomson syndrome type II (RTS type II), RAPADILINO syndrome and some cases of Baller–Gerold syndrome (BGS) (Bachrati and Hickson, 2008; Ouyang et al., 2008). RTS type II is characterized by congenital skeletal abnormalities, signs of premature aging and cancer predisposition, especially to osteosarcoma (Siitonen et al., 2009). The *RECQL4* gene encodes a protein of 1208 amino acids with a centrally conserved helicase domain. The N-terminal region of RECQL4 has been implicated in the initiation of nuclear DNA replication. RECQL4 functions in prereplication complex formation (Sangrithi et al., 2005) and is required for chromatin binding of DNA polymerase α during initiation of DNA replication (Matsuno et al., 2006). Recently it has been reported that two distinct domains of RECQL4, the conserved helicase motifs and the N-terminal Sld2-like domain, could independently exhibit helicase activity (Xu and Liu, 2009). Using transfection-based experiments, RECQL4 was characterized as a nuclear protein (Kitao et al., 1999a), consisting of a nuclear localization signal (NLS) encompassing amino acids 363–492 in RECQL4 (Burks et al., 2007). However, using antibodies against endogenous RECQL4, the protein was shown to have a cytoplasmic localization (Petkovic et al., 2005; Werner et al., 2006; Yin et al., 2004).

Tumor suppressor p53 is arguably the most studied tumor suppressor because of its role as a sequence-dependent transcription factor in the nucleus, thereby affecting many cellular processes including cell cycle arrest, apoptosis, aging and senescence in response to variety of stress (reviewed by Riley et al., 2008; Vousden and Lane, 2007). However, in recent years the role of p53 in cytoplasm especially during autophagy, energy metabolism and mitochondrial functions (reviewed by Galluzzi et al., 2011; Green and Kroemer, 2009) have also come into sharper focus.

It has been demonstrated that p53 physically and functionally interacts with either BLM or WRN (Blander et al., 1999; Garkavtsev et al., 2001; Guay et al., 2006; Yang et al., 2002) and attenuates their helicase activities in vitro (Sommers et al., 2005; Yang et al., 2002). Reciprocally, BLM and WRN cooperate with p53, and are involved in the modulation of p53 functions

(Blander et al., 2000; Garkavtsev et al., 2001). Endogenous BLM binds with Ser15-phosphorylated p53 in the presence of DNA damage (Restle et al., 2005; Sengupta et al., 2003). BLM is required for the localization of p53 to the sites of damage and the two factors together regulate homologous recombination (Sengupta et al., 2003). Both WS and BS cells show attenuated p53-dependent apoptosis (Spillare et al., 1999; Wang et al., 2001), which can be complemented by microinjection of *WRN* and *BLM* cDNA (Spillare et al., 2006).

We had earlier reported that tumor suppressor p53 attenuates the expression of RECQL4 during DNA damage by repressing its promoter activity (Sengupta et al., 2005). Repression of the RECQL4 promoter was accompanied by an increased accumulation of HDAC1, and simultaneous the loss of SP1 and p53 binding to the promoter. We here provide evidence that the enhanced sensitivity observed in the cells of RTS patients correlates with the accumulation of transcriptionally active p53 in the nucleus. We report, for the first time, that endogenous RECQL4 predominantly localizes to the mitochondria by utilizing a mitochondrial localization signal (MLS) located in its N-terminal 84 amino acids. In wild-type untreated human cells RECQL4 relocates p53 to the mitochondrial nucleoids, thereby preventing the accumulation of active p53 in the nucleus and, consequently, its function as a transcription factor. In the mitochondrial nucleoids, RECQL4 itself regulates mitochondrial DNA (mtDNA) replication and also the localization of p53 to the sites of de novo mtDNA synthesis. Altogether, we hypothesize that the localization of RECQL4 in mitochondria might account for some of the phenotypic features in RTS patients.

Results

Enhanced sensitivity of cells of RTS patients to genotoxic drugs correlates with the accumulation of transcriptionally active nuclear p53

It has been reported that the cells of RTS patients (hereafter referred to as RTS cells) are sensitive to many genotoxic agents (Jin et al., 2008; Werner et al., 2006). To confirm the earlier reports, long-term clonogenic assays were carried out after exposure of RTS cells and normal human fibroblasts (NHFs) to four different inducers of DNA damage, namely: camptothecin (Camp; a topoisomerase I inhibitor), neocarzinostatin (NCS; an inducer of double strand breaks), mitomycin C (Mito; a potent DNA crosslinker) and hydroxyurea (HU; causes replication stress). Indeed, the survival of all tested RTS cells in the long-term clonogenic assays was significantly less than the survival of NHFs following many types DNA damage (supplementary material Fig. S1A). To understand the molecular basis of the enhanced sensitivity of the RTS cells to DNA damage, levels of p53 were analyzed in untreated cells and in cells exposed to the DNA damage inducers. Compared with NHFs, RTS cells, when treated with DNA damage inducers, had enhanced levels of transcriptionally active p53, along with induction of its transcriptional targets p21 (encoded by the gene *CDKN1A*) and Bax (Fig. 1A,B; supplementary material Fig. S2). The presence of transcriptionally active p53 was observed for all the RTS fibroblasts except L9552914-J, which expresses mutant p53 (Fig. 1A, and see Materials and Methods section). Transcriptionally active p53 was also observed in untreated RTS cells (Fig. 1A,B, compare lanes for NHFs and RTS cells without DNA damage; supplementary material Fig. S2). The increase in the basal level of transcriptionally active nuclear p53 [carried out with p53 antibody, sc-126 (DO-1)] was also substantiated by immunofluorescence analysis (at 40× magnification) in untreated cultures of RTS cells and not in NHFs (Fig. 1C).

p21 and Bax are known to be upregulated by both p53-dependent and -independent mechanisms (Michieli et al., 1994; Strobel et al., 1996). Hence, we wanted to verify whether nuclear p53 in RTS fibroblasts could upregulate its other known targets. Apart from p21 and Bax, other targets of p53 (such as MDM2 and PUMA) were also upregulated in all the RTS cell types tested that expressed wild-type p53: i.e. AG03587, AG05013, B1865425K and D8903644-K (Fig. 1D, and data not shown). Transcriptionally active p53 is also characterized by specific phosphorylation events at N-terminal serine residues of p53, including Ser15 and Ser46 (Xu, 2003). The stabilized p53 in the RTS cells was phosphorylated at Ser15 and Ser46 (Fig. 1D, and data not shown). In untreated NHF cells there was complete absence of p53 phosphorylation at Ser15 and Ser46. To further validate that the upregulation of p21 and Bax in RTS cells was a p53-dependent effect, p53 siRNA (or siRNA control) was transfected into RTS cells. The increase in the level of p21 and Bax observed in AG05013 was not observed in cells expressing p53 siRNA (Fig. 1E). To determine whether absence of only RECQL4 was sufficient to cause activation of nuclear p53, we transiently silenced *RECQL4* expression in NHF cells by its cognate short interfering RNA (siRNA; Fig. 1F) or stably depleted its expression by use of *RECQL4* short hairpin RNA (shRNA; Fig. 1G). In both cases upregulation of p53 and its cellular targets (p21, Bax, MDM2 and PUMA) was observed in fibroblasts in which RECQL4 expression was depleted. The stabilized p53 was also phosphorylated at the N-terminal serine residues (Ser15 and Ser46). These phenomena were observed by both western analysis (Fig. 1F,H, and data not shown) and immunofluorescence experiments (data not shown).

Next we wanted to determine the reason for the p53 phosphorylation and stabilization in the nucleus of untreated RTS cells. Untreated NHFs and three different RTS fibroblasts were fixed and stained for 53BP1, a DNA damage sensor protein known to respond to different types of DNA damage (Schultz et al., 2000). Untreated NHFs had very few 53BP1 foci. The number of 53BP1 foci was reproducibly higher in the tested RTS fibroblasts (supplementary material Fig. S3A), indicating the accumulation of spontaneous DNA damage in RTS cells. This was further validated when more 53BP1 foci were observed in untreated NHF shRECQL4 cells compared with NHF shControl cells (supplementary material Fig. S3B). The spontaneous DNA damage in cells not expressing RECQL4 was probably recognized by the sensory kinases ATM and ATR, which in turn phosphorylated p53 at Ser15 and Ser46 leading to activation and stabilization of p53 in these cell lines (supplementary material Fig. S3C). The presence of *N*-acetylcysteine, a potent anti-oxidant and reactive oxygen species (ROS) scavenger, did not lead to any loss of p53 activation, indicating that mitochondrial stress is possibly not the only reason for spontaneous DNA damage in the nucleus of RTS cells.

RECQL4 and p53 colocalize in mitochondria

p53 had exclusively nuclear localization in approximately 5–10% of NHFs and 80–90% of RTS fibroblasts when examined at low (40×) magnification (Fig. 1C) with sc-126 antibody (which recognizes an epitope in the N-terminal region of p53). The same experiment was also done in parallel but stained with SIG-3250-1000 (which recognizes the entire p53 protein without any

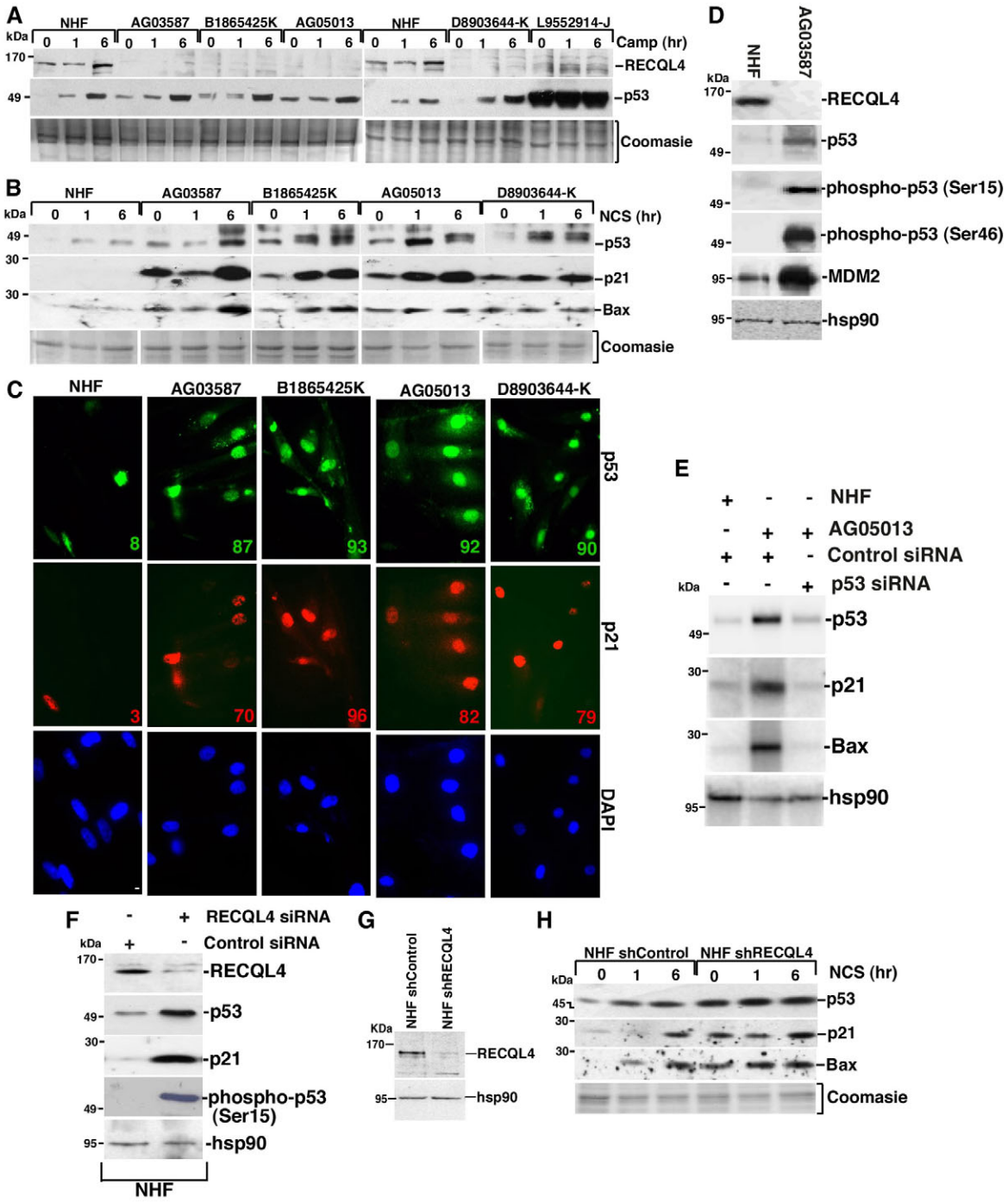

Fig. 1. Lack of RECQL4 leads to the accumulation of transcriptionally active nuclear p53. (**A**,**B**) Basal and drug-induced levels of p53 are elevated in RTS cells. NHFs and RTS cells were left untreated or treated with either camptothecin (Camp; A) or neocarzinostatin (NCS; B) for 1 hour or 6 hours and whole cell lysates prepared. Westerns blots were carried out with RECQL4 (K6312), p53 (sc-126), p21 and Bax antibodies. Coomassie Blue gels (at the bottom) were used for equalization. (**C**) Nuclear p53 is elevated in untreated RTS cells. Immunofluorescence analysis (IFs) on NHFs or RTS cells with p53 (sc-126) and p21 antibodies. Nuclei were stained with DAPI. Cells were examined using epifluorescence microscope at 40× magnification. The percentage of cells with p53–p21 colocalization is indicated at the bottom right of each panel. Scale bar: 5 μm. (**D**) In RTS cells N-terminal serine residues of p53 are phosphorylated. The cells were treated as in A and B except that only untreated NHFs and AG03587 were used to prepare the whole cell lysates. Western blotting was carried out with RECQL4 (K6312), p53 (sc-126), phosphorylated p53 Ser15, phosphorylated p53 Ser46, MDM2 and hsp90 antibodies. (**E**) p53-dependent increase in the level of p21 and Bax in AG05013 cells. NHFs and AG05013 cells were transfected with either control siRNA or RECQL4 siRNA. Whole cell lysates were probed with p53 (sc-126), p21, Bax and hsp90 antibodies. (**F**–**H**) Transcriptionally active nuclear p53 is present in cells with depleted RECQL4. NHFs were transfected with either a control siRNA or RECQL4 siRNA for 48 hours (F). Alternately NHFs were stably transfected with either control shRNA or shRECQL4 (G). NHF shControl or NHF shRECQL4 cells were either left untreated or treated with NCS for 1 hour or 6 hours (H). The protein levels in the whole cell lysates were detected by western blot analysis with RECQL4 (K6312), p53 (sc-126), p21, Bax, phosphorylated p53 Ser15 and hsp90 antibodies. The Coomassie Blue gel at the bottom of H was used for equalization.

detectable cross reactivity in NHFs as observed by both western analysis and immunofluorescence (supplementary material Fig. S4A,B) and examined at a higher magnification with oil immersion (63× or 100×) and epifluorescence microscopy. This process revealed that in the 80–90% of NHFs p53 localization was cytoplasmic, and it colocalized with Mitotracker Red. However, the localization of p53 (as detected by SIG-3250-1000) in RTS cells, was predominantly nuclear (Fig. 2A), though some cytoplasmic p53 was still observed. To examine the subcellular distribution of endogenous p53 and RECQL4 in more detail, untreated NHFs were co-stained with RECQL4 and p53 antibodies and examined by confocal microscopy. Specific punctate cytoplasmic p53 staining was observed (Fig. 2B). This staining pattern was verified using several fixation procedures, in two other primary fibroblast cultures (GM08402 and MRC-5; supplementary material Fig. S5A) and by loss of staining in NHF E6 cells (constitutively expressing HPV E6 oncoprotein, which degrades endogenous p53; data not shown). Incidentally the punctate cytoplasmic staining of p53 was not observed in transformed cell lines, e.g. HCT116 (data not shown).

Two different antibodies (the specificities are shown in supplementary material Fig. S4C,D) were used to determine RECQL4 localization. Similar to p53, RECQL4 staining was also punctate in the cytoplasm of untreated NHFs, and it colocalized with p53 (Fig. 2B). Again this staining pattern was verified in other primary human fibroblasts (GM08402 and MRC-5; supplementary material Fig. S5A) but was not observed in transformed cell lines (HCT116, MCF7) with several fixation processes (data not shown). We then investigated whether the cytoplasmic localization of p53 and RECQL4 was mitochondrial, as indicated by their punctate pattern. Subcellular fractionation identified a substantial amount of RECQL4 and p53 in the mitochondrial fraction (Fig. 2C). Furthermore, we found that both p53 and RECQL4 colocalized extensively with the mitochondrial marker, cytochrome *c* (Fig. 2D,E). Incidentally both p53 and RECQL4 localized predominantly at the mitochondrial nucleoids, the storehouse of mtDNA (enlarged images in Fig. 2D,E). Both p53 and RECQL4 also colocalized at the sites of mtDNA synthesis during BrdU labeling experiments (Fig. 2F; supplementary material Fig. S5B,C).

Interaction between RECQL4 and p53

The localization of both p53 and RECQL4 in the mitochondrial nucleoids suggests that the two proteins physically interact. To determine whether this occurs, we carried out in vitro GST pull-down assays. The minimal region of p53 that interacted with RECQL4 in a nucleic-acid-independent manner encompassed amino acids 293–362 [spanning NLS1 and the tetramerization domain (TD) of p53] (supplementary material Fig. S6). Reciprocally the N-terminal 459 amino acids of RECQL4 interacted with p53 (supplementary material Fig. S7A,D). Using N- and C-terminal mutations within the first 459 amino acids, an internal stretch of 130 amino acids (270–400) of RECQL4 was considered to be the minimal region of interaction (supplementary material Fig. S7A,D,F). This region in RECQL4 partially overlaps with its predicted NLS, which spans amino acids 363–492 (Burks et al., 2007). Together the results suggest that the interaction between p53 and RECQL4 leads to the masking of their respective NLSs (summarized in Fig. 3A). The interaction of RECQL4 (270–400) with p53 was verified either by using a GST-tagged RECQL4 (270–400) fragment in vitro (supplementary material Fig. S7G,H) or by using a FLAG-tagged RECQL4 (1–459) fragment in vivo (supplementary material Fig. S8A). The full-length RECQL4 but not the N-terminal 404-amino-acid-truncated variant interacted with GFP-tagged full-length p53 (supplementary material Fig. S8B). The interaction between p53 and RECQL4 was limited to the untreated NHFs. Exposure of NHFs to different agents that cause DNA damage, such as camptothecin, etoposide and neocarzinostatin led to the loss of p53–RECQL4 interaction (supplementary material Fig. S8C, and data not shown) and instead both proteins accumulated in the nucleus (supplementary material Fig. S8D,E, and data not shown). The extent of p53–RECQL4 colocalization in the mitochondrial nucleoid decreased in a time-dependent manner (supplementary material Fig. S8F). Hence these experiments indicate that in untreated cells localization of both p53 and RECQL4 in the mitochondria could be due to their physical interactions, which in turn could mask their NLS.

Loss of mtDNA inhibits localization of RECQL4 and p53 in mitochondria

Next we investigated the parameters that regulate the localization of RECQL4 and p53 to the mitochondria. Using NHF E6 cells, the localization of RECQL4 with mtDNA was determined to be independent of p53 (supplementary material Fig. S9A, left). The level of RECQL4 was increased in the absence of p53 (supplementary material Fig. S9A, right), possibly because of the lack of p53-mediated repression of RECQL4 transcription (Sengupta et al., 2005).

To determine whether mtDNA was required for the localization of RECQL4 in the mitochondria, we created transient NHF ρ^0 cells, which were completely depleted of mtDNA, as determined by the absence of cytochrome *b* gene expression (supplementary material Fig. S9B). Compared with NHFs, NHF ρ^0 cells had less RECQL4 protein (supplementary material Fig. S9C). The residual RECQL4 and p53 accumulated in the nucleus of NHF ρ^0 cells (Fig. 3B,C). The nuclear p53 in NHF ρ^0 cells was phosphorylated in the N-terminus and transcriptionally active, thereby upregulating its downstream targets, such as p21, Bax and MDM2 (supplementary material Fig. S9C). Spontaneous DNA damage also occurred in the nucleus of NHF ρ^0 cells as indicated by the accumulation of 53BP1 foci (data not shown). These results indicate that the presence of mtDNA is an essential requirement for RECQL4 and p53 to accumulate in the mitochondrial nucleoids in untreated cells.

Next we wanted to determine whether the accumulation of p53 and RECQL4 in the mitochondria was due to the shuttling of the two proteins out of nucleus. Hence we stained RECQL4 and p53 either together (Fig. 3D) or individually with a mitochondrial protein marker PolγA (supplementary material Fig. S9D). Detection of PolγA was carried out with a validated antibody (supplementary material Fig. S4E) in both untreated cultures and in cultures treated with the nuclear export inhibitor, leptomycin B. Although p53 localized to the nucleus after leptomycin B treatment, a substantial fraction of p53 still colocalized with RECQL4 in mitochondrial nucleoids. RECQL4 was not present in the nucleus but was in mitochondria in untreated cells and cell treated with leptomycin B (Fig. 3D; supplementary material Fig. S9D). Hence these experiments suggest the presence of two pools

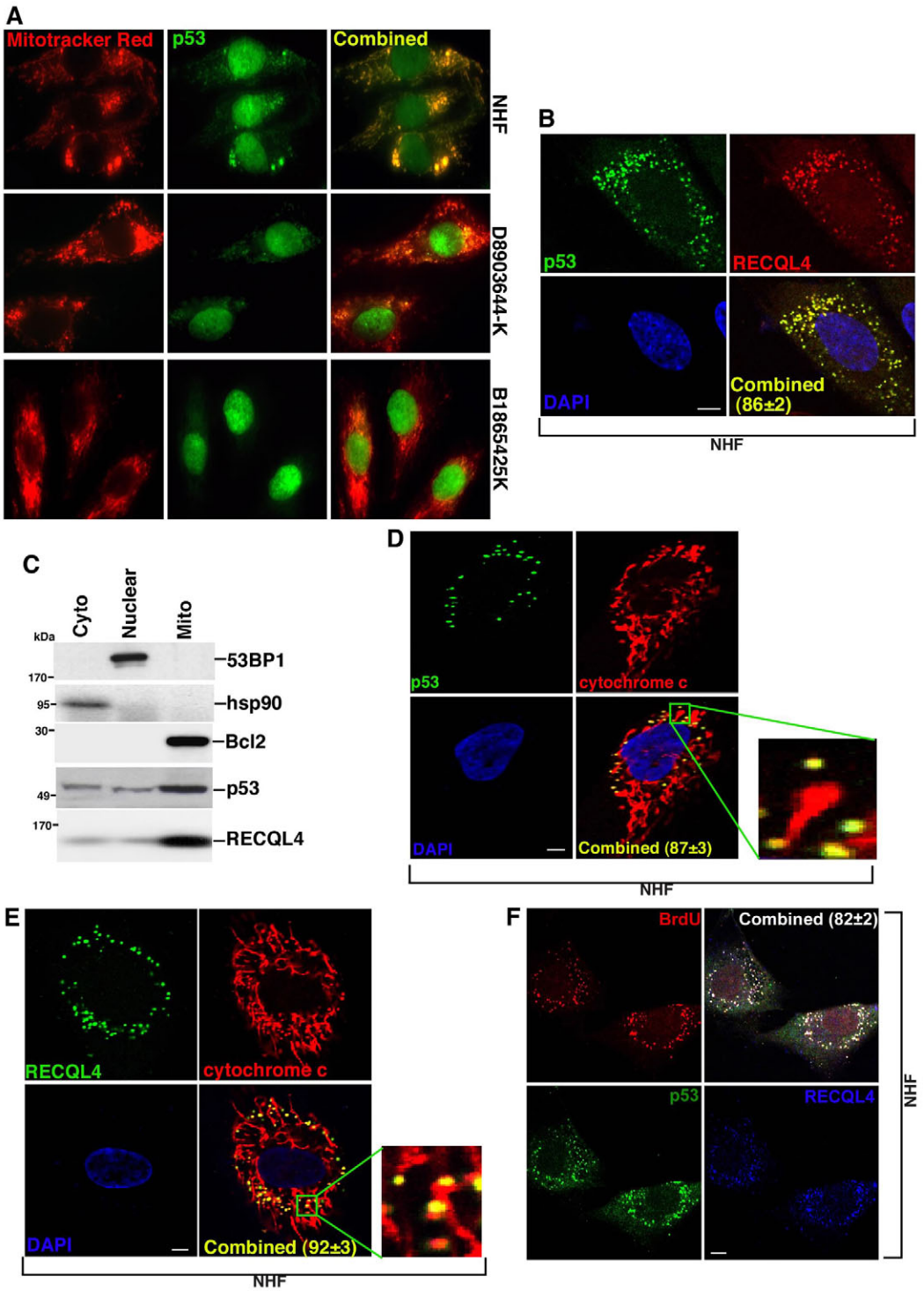

Fig. 2. RECQL4 and p53 localize to the mitochondria. (**A**) Localization of p53 is predominantly nuclear in untreated RTS cell lines. NHFs and the indicated RTS cell lines were stained with Mitotracker Red and for p53 (SIG-3520-1000) and examined using epifluorescence microscopy with a100× oil immersion lens. (**B**,**D**,**E**) p53 and RECQL4 localize to mitochondria. Colocalization of p53 and RECQL4 in NHFs (B). Colocalization of p53 (D) or RECQL4 (E) with cytochrome *c*. RECQL4 and p53 were stained with K6312 and SIG-3520-1000, respectively. Nuclei were stained with DAPI. Percentages of cells showing the colocalizaton of p53 and RECQL4 (in B), p53 and cytochrome *c* (in D), RECQL4 and cytochrome *c* (in E) are indicated in the combined images. Scale bars: 5 μm. (**C**) Subcellular fractionation reveals the localization of RECQL4 in mitochondria. 53BP1, hsp90 and Bcl2 were used as controls for nuclear, cytoplasmic (Cyto) and mitochondrial (Mito) fractions, respectively. Antibodies used were: RECQL4 (K6312), p53 (sc-126), Bcl2, hsp90 and 53BP1. 600 μg of the respective fractions were used. (**F**) p53, RECQL4 and BrdU colocalize with mtDNA in NHFs. NHFs, after incubation with BrdU for 3 hours, were stained with p53 (SIG-3520-1000), RECQL4 (sc-16924) and BrdU (RPN202) antibodies. Percentage of cells having BrdU–p53–RECQL4 triple colocalizaton is indicated. Scale bar: 5 μm.

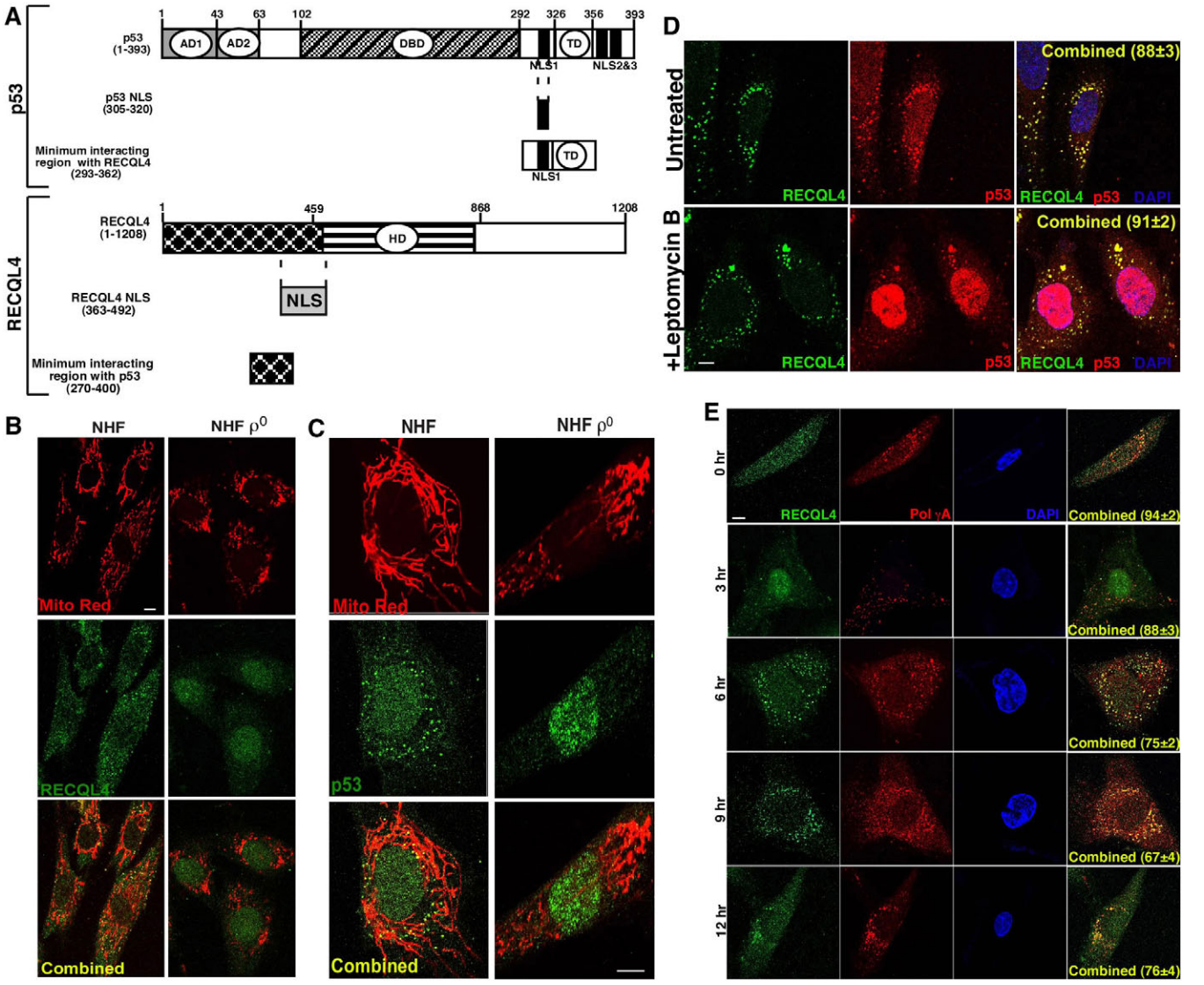

Fig. 3. Parameters regulating the localization of RECQL4 and p53 to the mitochondria. (**A**) Summary of the physical interaction between p53 and RECQL4. (Top) Schematic diagram showing the different domains of p53. The p53 NLS and the minimum region of p53 deduced to interact with RECQL4 from (supplementary material Fig. S6) is shown. (Bottom) Schematic diagram showing the different domains of RECQL4. The RECQL4 NLS and the minimum region of RECQL4 deduced to interact with p53 (supplementary material Fig. S7) is shown. (**B**,**C**). Localization of RECQL4 and p53 in the mitochondria depends on mtDNA. IF in NHF and NHF ρ^0 cells with (B) RECQL4 (sc-16924) or (C) p53 (SIG-3520-1000) antibodies. Mitochondria were visualized using Mitotracker Red CMXRos. Scale bar: 5 μm. (**D**) RECQL4 and a fraction of p53 colocalize in mitochondrial nucleoids in the presence of leptomycin B. NHFs were either left untreated or treated with leptomycin B. Staining was carried out with antibodies against RECQL4 (sc-16924) and p53 (SIG-3520–1000). Nuclei were stained with DAPI. Numbers indicate the percentage of cells showing colocalization of RECQL4 and p53. Scale bar: 5 μm. (**E**) RECQL4 shuttles between the nucleus and the mitochondria during the cell cycle. Cells were stained with RECQL4 (sc-16924) and PolγA (sc-48815) antibodies, immediately after release from double thymidine block (0 hours) and after 3 hours, 6 hours, 9 hours and 12 hours. Nuclei were stained with DAPI. The percentage of cells showing RECQL4–PolγA colocalization is indicated. Scale bar: 5 μm.

of endogenous p53 in untreated cells: one p53 fraction that was targeted to the mitochondria bound to RECQL4, and another fraction that utilized its NLS and was transported to the nucleus, where it was retained as a result of the leptomycin B treatment.

However, it has been reported that RECQL4 is also a nuclear protein (Burks et al., 2007). Hence the possibility exists that the subcellular localization of RECQL4 was cell-cycle dependent. Using synchronized cell populations (after double thymidine block; supplementary material Fig. S10A) we found that RECQL4 colocalized with the catalytic subunit of mitochondrial DNA polymerase, PolγA, in all phases of the cell cycle (Fig. 3E). The punctate mitochondrial staining for RECQL4 was observed 6 hours and 9 hours after the release of the second thymidine block, indicating its accumulation in mitochondrial nucleoids in late S and G2 phases of the cell cycle. During S phase (3 hours after release) RECQL4 was present predominantly in the nucleus, and it colocalized with PCNA (supplementary material Fig. S10B), confirming the role of RECQL4 in the initiation of nuclear replication (Matsuno et al., 2006; Sangrithi et al., 2005).

RECQL4 has a functional mitochondrial localization signal

We next wanted to determine how RECQL4 and p53 were localized to the mitochondria. To date, efforts to identify the mitochondrial targeting signal in p53 have failed. Four web-based tools: Target P, PSORT, MitoProt and iPSORT predicted with high probability an MLS for RECQL4 in the N-terminal domain (supplementary material Table S1). However, no MLS was predicted for p53 (data not shown).

We hypothesized that for RECQL4 to localize to the mitochondria, it has to physically interact with the TOM40 receptor complex located on the outer mitochondrial membrane of human cells. Members of the TOM40 complex (Tom20, Tom22, Tom70), recognize the N-terminal targeting signal sequences of proteins destined for mitochondria (Chacinska et al., 2009). Tom20 is the major import receptor (Pfanner, 2000), which is anchored in the outer mitochondrial membrane, thereby exposing its evolutionarily conserved C-terminal receptor domain to the cytosol. We found that RECQL4 interacted in vitro with the cytosolic part of the Tom20 receptor (Fig. 4A,B). Hence we hypothesized that RECQL4 probably has a specific MLS in the N-terminus that allowed it to interact with Tom20 receptor. A systematic analysis with the disulfide-tethering peptide library had previously revealed the amino acid residues of the substrates involved in the interaction with Tom20 (Obita et al., 2003). The hypothesized motif was $\sigma\ \Phi\ \chi\ \beta\ \Phi\ \Phi\ \chi$ where Φ denotes a hydrophobic, σ, a hydrophilic, β a basic and χ any amino acid. We searched the N-terminal 100 amino acids of RECQL4 using the above motif. Interestingly, one sequence stretch $A^{13}WERAF^{18}$ of RECQL4 matched with the defined motif (Fig. 4C). No sequence with the defined motif was found in p53 (data not shown). Hence, it was hypothesized that the majority of p53 localized to mitochondria when it physically interacted with RECQL4.

To obtain in vivo validation of the predicted MLS of RECQL4 we generated two deletion mutants of FLAG-tagged RECQL4: Δ13–18 and Δ84. The Δ84 RECQL4 mutant was generated to determine whether any other sequence in the extreme N-terminus of RECQL4 apart from amino acids 13–18 was also required for interaction with Tom20 and the subsequent mitochondrial localization of RECQL4. We found that although there was a reproducible 15–20% decrease in the binding between RECQL4 and Tom20 in the Δ13–18 mutant, the binding was almost completely abolished in the Δ84 mutant (Fig. 4D). Hence additional amino acid residues in the N-terminus of RECQL4 apart from residues 13–18 were probably required for Tom20–RECQL4 interaction. To further verify the MLS, wild-type FLAG–RECQL4 (1–1208), FLAG–RECQL4 (Δ13–18) or FLAG–RECQL4 (Δ84) was transiently expressed in the human TERT immortalized RTS patient cell line AG05013 (Kitao et al., 1999b). FLAG-tagged wild-type RECQL4 appeared as punctate staining outside the nucleus and also within the nucleus itself. Both Δ13–18 and Δ84 RECQL4 mutants, however, localized predominantly to the nucleus (Fig. 4E, top). The efficiency of nuclear localization was higher for the Δ84 RECQL4 mutant thereby validating the in vitro results in vivo. However, the nuclear and mitochondria localization of the transfected wild-type RECQL4 was different from the endogenous RECQL4 that localizes predominantly to the mitochondria (Fig. 2B,D,F). This is probably because of the higher expression level of FLAG–RECQL4 (1–1208) during transient transfection than the endogenous level in NHFs (Fig. 4E, bottom right). For further validation, RECQL4 (13–18) was fused in frame with EGFP and expressed transiently in AG05013 cells (Fig. 4F). EGFP alone was homogeneously expressed in both nucleus and cytoplasm, whereas EGFP–RECQL4 (13–18) had a punctate pattern that colocalized with Mitotracker Red in a majority of the transfected cells. Together, the results indicate that RECQL4 has a functional MLS that dictates its own localization and consequently leads to the localization of p53 in the mitochondria.

Rescue of RTS fibroblasts by expression of RECQL4

Having established how RECQL4 and p53 are localized to the mitochondria in untreated normal human cells, we hypothesized that the transcriptionally active nuclear p53 observed in cells of RTS patients (Fig. 1) could be relocated back to the mitochondria by stably overexpressing wild-type RECQL4. Hence we rescued AG05013 cells with AcGFP–RECQL4 (1–1208) to generate AG05013 AcGFP–RECQL4 (1–1208) clone 1. Very few AG05013 AcGFP–RECQL4 (1–1208) clone 1 cells had spontaneous 53BP1 foci (supplementary material Fig. S3A). AcGFP–RECQL4 localized to mitochondria, as it colocalized with Mitotracker Red (Fig. 5A). In contrast to the parental AG05013 cells, p53 colocalized with AcGFP–RECQL4 in the mitochondrial nucleoids of AG05013 AcGFP–RECQL4 clone 1 cells (Fig. 5B), thereby proving that accumulation of p53 on the mitochondria in untreated cells depended on RECQL4. In untreated AG05013 AcGFP–RECQL4 (1–1208) clone 1 cells a decrease in the level of endogenous p53 was observed. The levels of p53 targets (p21, Bax, PUMA and MDM2) and the N-terminal phosphorylation of p53 at Ser15 and Ser46 were also decreased (Fig. 5C, and data not shown). Using immunofluorescence, the high level of transcriptionally active nuclear p53 observed in untreated AG05013 cells was found to be reduced in AG05013 AcGFP–RECQL4 clone 1 cells (Fig. 5D, and data not shown). Clonogenic assays carried out on AG05013 and AG05013 AcGFP–RECQL4 (1–1208) clone 1 cells after exposure to camptothecin and neocarzinostatin indicated that the hypersensitivity of RTS fibroblasts could be rescued by the re-expression of wild-type RECQL4 (supplementary material Fig. S1B). In summary, endogenous RECQL4 relocates p53 to the mitochondria in normal untreated cells, thereby preventing its nuclear activation in the absence of exogenous DNA damage.

RECQL4 is required for optimal de novo mtDNA replication

RECQL4 has been implicated in the regulation of nuclear replication during S phase (Matsuno et al., 2006; Sangrithi et al., 2005; Xu et al., 2009). Hence a probable role of RECQL4 in mitochondria could be to regulate mtDNA replication. To determine whether mtDNA replication in NHFs would only be altered as a result of loss of RECQL4 expression, Southwestern analysis with anti-BrdU antibody was carried out in control cells and in cells that lack RECQL4. There was an approximately 50% decrease in the mtDNA replication in shRECQL4 NHFs compared with NHF shControl cells (Fig. 6A). These observations were further validated by the decreased incorporation of BrdU in all RTS fibroblasts (AG05013, AG03587, B1865425K and D8903644-K) expressing wild-type p53 (Fig. 6B, and data not shown).

To investigate whether RECQL4 affects mtDNA replication, we scored for de novo mtDNA replication in isogenic control ρ cells (NHF shControl ρ) and in RECQL4-depleted ρ cells (NHF shRECQL4 ρ). Cells were grown in ethidium bromide, uridine

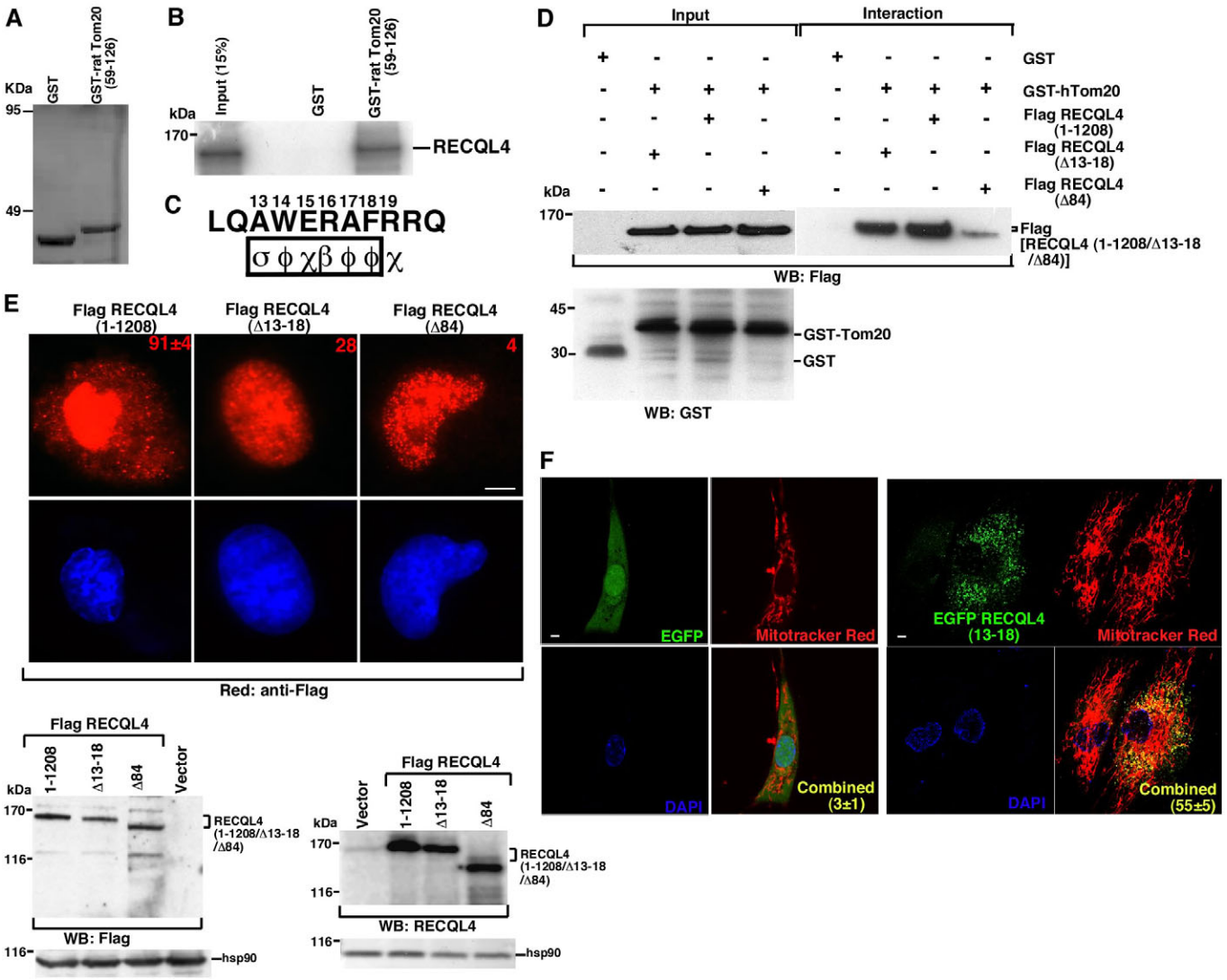

Fig. 4. RECQL4 has a functional MLS in its N-terminus. (**A**) Coomassie Blue gels showing purified GST and GST–rat Tom20 (59–126). (**B**) RECQL4 interacts with Tom20. Interactions carried out between [S^{35}]methionine-labeled in vitro translated RECQL4 and either bound GST or GST-tagged rat Tom20 (59–126). After washing, the bound radioactivity was visualized by autoradiography. (**C**) Predicted pre-sequence peptide in RECQL4 (residues 13–19). The corresponding hexamer consensus motif of Tom20 recognition is shown in the box where Greek letters represent different types of amino acids: Φ, hydrophobic; σ, hydrophilic; β, basic; χ, any amino acid. (**D**) The N-terminal 84 amino acids of RECQL4 are required for its interaction with Tom20. Interactions were carried out with bound GST-tagged Tom20 and whole cell lysates expressing different FLAG-tagged RECQL4 proteins in HEK293T cells. After washing, the bound FLAG-tagged proteins were detected by western blotting with anti-FLAG antibody (top). Levels of GST–Tom20 used for interactions are depicted in the bottom panel. (**E**) Deletion of the first 84 amino acids causes complete nuclear accumulation of RECQL4. FLAG-tagged wild-type RECQL4 or truncated RECQL4 (Δ13–18) or (Δ84) were expressed in NHFs using limiting amounts of transfected DNA, and whole cell lysates were prepared. Western blots were carried out with either anti-FLAG and anti-hsp90 antibodies (bottom, left) or with anti-RECQL4 (K6312) and anti-hsp90 antibodies (bottom, right). The level of endogenous RECQL4 (as seen in the vector lane, bottom right) were compared with the transfected RECQL4 constructs. Post-transfection (24 hours) the cells were washed and IF carried out with anti-FLAG antibody (top). Nuclei were stained with DAPI. The percentage of transfected cells in which RECQL4 was present in both nucleus and as punctate staining in cytoplasm is indicated. Scale bar: 5 μm. (**F**) Amino acids 13–18 of RECQL4 fused to EGFP localized predominantly to the mitochondria. Amino acids 13–18 of RECQL4 were fused in frame to EGFP and transiently transfected into AG05013 cells. In parallel control transfections were done with EGFP alone. Cells were fixed after Mitotracker Red CMXRos staining. The percentage of transfected cells showing EGFP colocalization with Mitotracker Red is indicated. Scale bar: 5 μm.

and pyruvate (see Materials and Methods) to generate the ρ cells. Subsequently ethidium bromide was washed away and the cells were grown in normal medium containing BrdU for specific intervals. Colocalization of BrdU with PolγA was scored at an early (10 minutes) time point to determine the effect on the initiation of mtDNA replication. Subsequently the effect on the elongation stage during mtDNA replication was scored by determining the colocalization after 30 minutes and 60 minutes. Lack of RECQL4 in NHF shRECQL4 cells decreased BrdU–PolγA colocalization at all time points (Fig. 6C,D), indicating that RECQL4 is involved in the regulation of both initiation and elongation steps of mtDNA replication. AG03587 ρ cells also

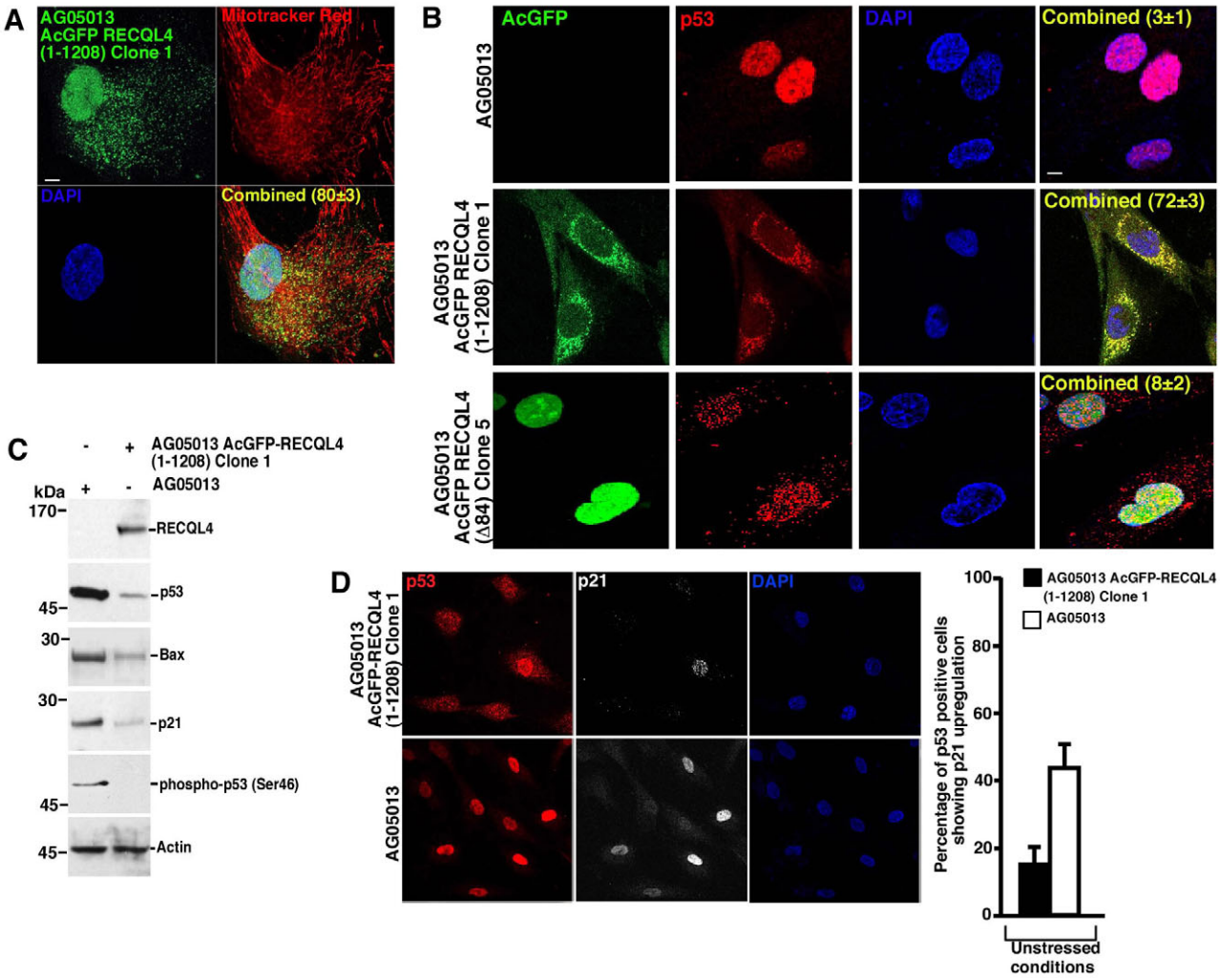

Fig. 5. Rescue of RTS cells with wild-type RECQL4 leads to depletion of p53 and p21. (**A**) Re-expression of AcGFP–RECQL4 (1–1208) in AG05013 cells leads to mitochondrial localization of the helicase. Complementation of AG05013 cells with AcGFP–RECQL4 (1–1208) leads to the generation of AG05013 AcGFP–RECQL4 (1–1208) clone 1. AcGFP–RECQL4 visualization was carried out after staining the cells with Mitotracker Red. Percentage of cells showing colocalization of EGFP and Mitotracker Red is indicated. Scale bar: 5 μm. (**B**) p53 localizes to the mitochondria in AG05013 AcGFP–RECQL4 (1–1208) clone 1 cells. AG05013, AG05013 AcGFP RECQL4 (1–1208) clone 1 and AG05013 AcGFP RECQL4 (Δ84) clone 5 cells were stained for p53 (SIG-3520–1000) and subsequently AcGFP and p53 were visualized. Nuclei were stained with DAPI. Percentage of cells showing EGFP colocalization with p53 in the mitochondria is indicated. Scale bar: 5 μm. (**C**,**D**) Decreased expression levels of nuclear p53 and its downstream targets in AG05013 AcGFP–RECQL4 (1–1208) clone 1 cells. (C) Western blots using whole cell lysates from untreated AG05013 and AG05013 AcGFP RECQL4 (1–1208) clone 1 cells. Antibodies used were: RECQL4 (K6312), p53 (sc-126), Bax, p21, phosphorylated p53 Ser46 and actin. (D) Same as C except that IF was carried out on untreated AG05013 AcGFP–RECQL4 (1–1208) clone 1 cells with p53 (sc-126) and p21 (stained with Cy5-conjugated secondary antibody and represented in monochrome) antibodies and observed using an epifluorescence microscope at 40× magnification. Nuclei were stained with DAPI. Scale bar: 5 μm. Quantification of the IF is represented on the right as a percentage of p53-positive cells with p21 upregulation. Values are means ± s.d.

demonstrated compromised mtDNA replication compared with NHF ρ cells (Fig. 6D).

To elucidate, in more mechanistic detail, the effect of RECQL4 on mtDNA replication, a new cell line was generated using AG05013, namely AG05013 RECQL4 (Δ84) clones 3, 4 and 5 (all of which expressed RECQL4 at the same level without the N-terminal 84 amino acid; supplementary material Fig. S11A). All AG05013 RECQL4 (Δ84) clones were exclusively nuclear (supplementary material Fig. S11B, and data not shown). In RECQL4 (Δ84) cells only background BrdU staining in the cytoplasm was observed after de novo mtDNA replication. The extent of BrdU–PolγA colocalization after the initiation of de novo mtDNA replication was decreased at all the times examined in AG05013 RECQL4 (Δ84) cells (compared with AG05013 AcGFP–RECQL4 clone 1 cells), indicating that the localization of RECQL4 to the mitochondria was essential for the process (Fig. 6E; supplementary material Fig. S11C). Clonogenic assays with AG05013 RECQL4 (Δ84) cells indicated that the nuclear localization of RECQL4 could not rescue the high sensitivity of RTS fibroblasts to camptochecin and neocarzinostatin, especially at higher concentrations of the drug (supplementary material Fig. S1B). The lack of mitochondrial localization of RECQL4 caused the accumulation of p53 in the nucleus in the majority of AG05013 RECQL4 (Δ84) cells (Fig. 5B). However, in a minority of

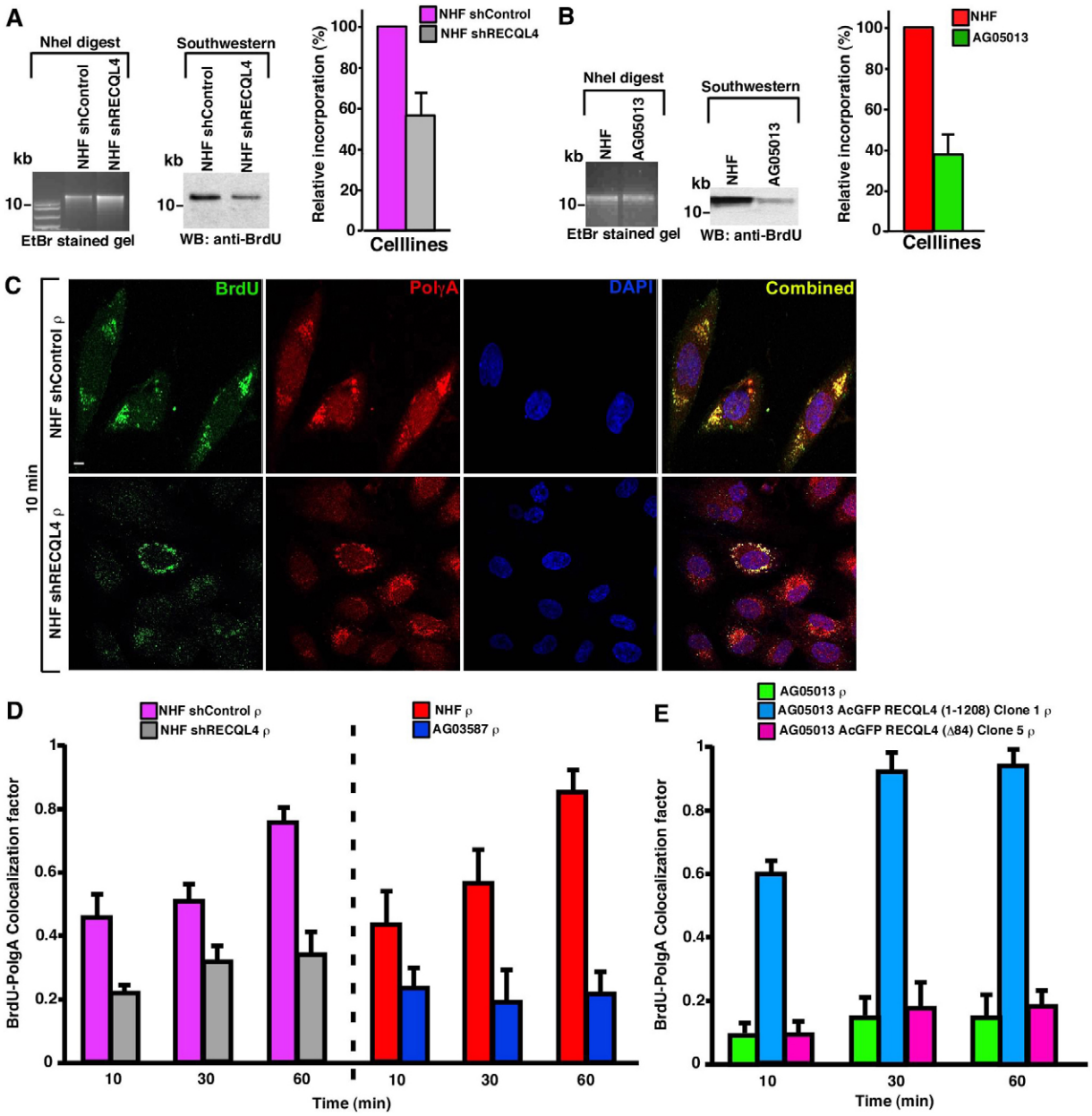

Fig. 6. RECQL4 affects mitochondrial replication in vivo. (**A**,**B**) Absence of RECQL4 reduces the incorporation of BrdU during mitochondrial replication in (A) NHF shControl and NHF shRECQL4 and (B) NHF and AG05013 cells. Cells are grown in presence of BrdU for 24 hours. The isolated mtDNA was digested with *Nhe*I and stained with ethidium bromide (EtBr; left). The extent of BrdU incorporation was determined by southwestern analysis using anti-BrdU (ab1893) antibody (middle) for the western analysis. (Right) Quantification of the BrdU incorporation ($n=3$). Values are means ± s.d. (**C**,**D**) RECQL4 affects initiation and elongation phases of mtDNA replication. After creation of two pairs of cells in ρ state (NHF shRECQL4 ρ and NHF shControl ρ), the cells were washed and grown in BrdU-containing normal growth medium for 10 minutes, 30 minutes and 60 minutes. (C) After fixation immunofluorescence was carried out with antibodies against BrdU (RPN202) and PolγA. The image is a representative BrdU-PolγA staining at 10 minutes. Nuclei were stained with DAPI. Scale bar: 5 mm. (D) Quantification of BrdU–PolγA colocalization in the two pairs of ρ cells (left, NHF shRECQL4 ρ and NHF shControl ρ cells, and right, NHF ρ and AG03587 ρ cells). The extent of colocalization is represented as the colocalization factor (details in Materials and Methods). Values are means ± s.d. (**E**) The extent of BrdU–PolγA colocalization after de novo mtDNA replication in AG05013 ρ, AG05013 AcGFP–RECQL4 (1–1208) clone 1 and AG05013 AcGFP–RECQL4 (Δ84) clone 5 ρ cells. Values are means ± s.d.

RECQL4 (Δ84) cells, p53 was still mitochondrial. This indicated that mechanisms not mediated by RECQL4 could also lead to p53 accumulation in the mitochondria. The extent of activation of p53 in RECQL4 (Δ84) cells (as determined by phosphorylation of p53 Ser46, and p21 accumulation) was equivalent to that in AG05013 cells (supplementary material Fig. S11D).

Mitochondrial p53 is known to be involved in enhancing the accuracy of mtDNA synthesis (Bakhanashvili et al., 2008) and DNA repair (de Souza-Pinto et al., 2004; Wong et al., 2009). p53 in the mitochondria interacts with mtSSB (Wong et al., 2009) and has been postulated to enhance DNA replication function of Polγ (Achanta et al., 2005). Hence we wanted to determine the

inter-regulation, if any, between p53 and RECQL4 during the initiation and elongation phases of mtDNA replication in vivo. For this purpose we carried out the above assay in two cell line pairs: (1) NHF ρ and AG03587 ρ cells; and (2) NHF shControl ρ and NHF shRECQL4 ρ cells. Lack of RECQL4 drastically affected the recruitment and maintenance of p53 at the sites of mtDNA initiation and elongation (Fig. 7A,B, and data not shown). However, the lack of p53 in NHF E6 ρ cells did not affect the localization of RECQL4 to the sites of de novo mtDNA replication (Fig. 7C,D). Therefore, RECQL4 is required to transport p53 to the mitochondria and its (i.e. p53) subsequent recruitment onto the mtDNA.

Discussion

p53 has important functions in the cytoplasm and specifically in mitochondria (reviewed by Galluzzi et al., 2011; Green and Kroemer, 2009). It has been demonstrated that under a variety of apoptotic stimuli, a fraction of p53 translocates rapidly to the mitochondria. Mdm2-mediated monoubiquitylation of p53 promotes its translocation to the mitochondria where it subsequently undergoes deubiquitylation, thereby generating the active mitochondrial p53 capable of acting in the apoptotic pathway (Marchenko et al., 2000). Mitochondrial p53 is known to also be involved in enhancing the accuracy of mtDNA synthesis (Bakhanashvili et al., 2008) and DNA repair (de Souza-Pinto et al., 2004; Wong et al., 2009). p53 in the mitochondria interacts with mtSSB (Wong et al., 2009) and has also been postulated to enhance the DNA replication function of Polγ (Achanta et al., 2005). The cytoplasmic pool of p53 has been shown to function in energy metabolism, act as an anti-oxidant and inhibit autophagy, reticulophagy and mitophagy (Green and Kroemer, 2009; Tasdemir et al., 2008). The above p53 functions in the mitochondria occur in the absence of any external stress, indicating that a basal level of p53 is constitutively present in the mitochondria (Mahyar-Roemer et al., 2004).

However, an unanswered question remains: how is p53 translocated to mitochondria in untreated cells? In this report, we have provided evidence that in untreated cells, a fraction of p53 interacted with RECQL4 (thereby masking the strong NLS of p53 and also its own NLS; summarized in Fig. 3A). Under these conditions RECQL4 utilizes its own MLS in the N-terminal region (Fig. 4) to accumulate in the mitochondria along with the complexed p53 (Fig. 2D–F; supplementary material Fig. S5B,C).

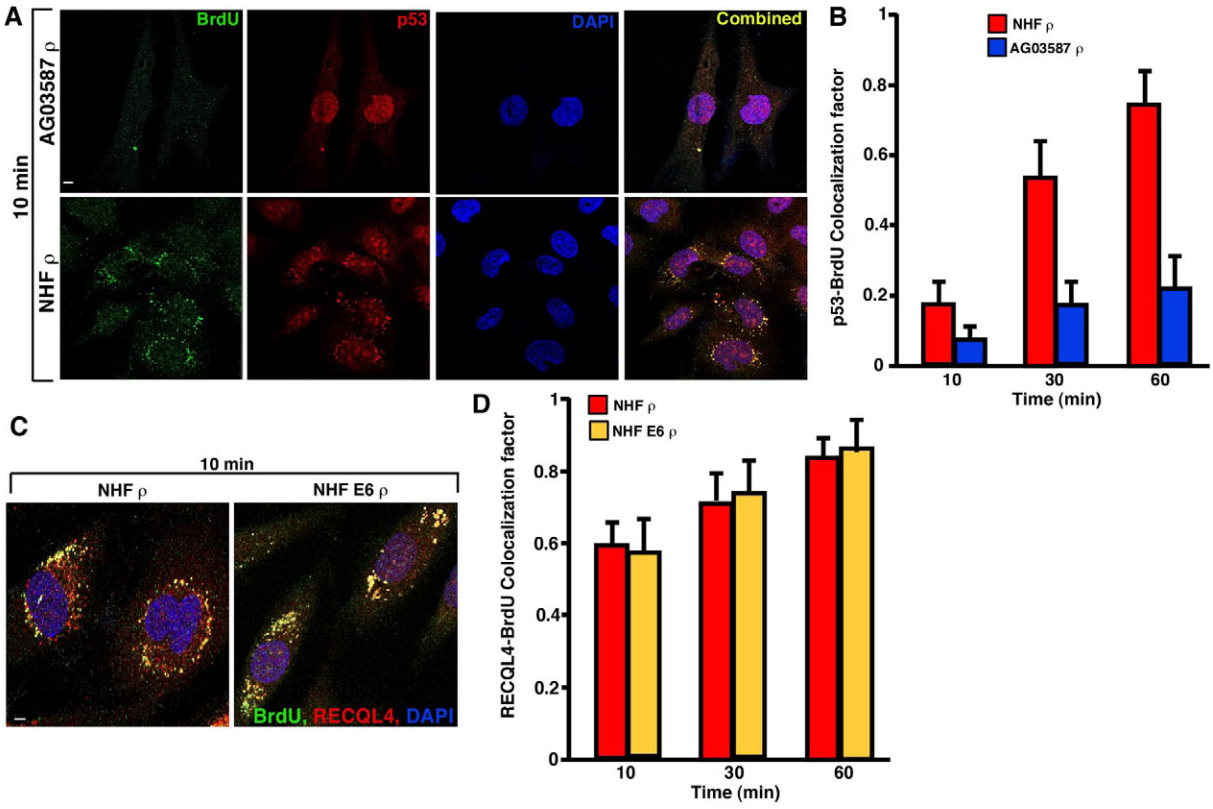

Fig. 7. (A,B) Lack of RECQL4 affects p53 recruitment to the sites of mtDNA replication. (A) After creation of two pairs of cells in ρ state (NHF ρ and AG03587 ρ), the cells were washed and grown in BrdU-containing normal growth medium for 10 minutes, 30 minutes and 60 minutes. After fixation of the cells, immunofluorescence was carried out with antibodies against BrdU (RPN202) and p53 (SIG-3520–1000). The image is a representative BrdU–p53 staining at 10 minutes. Nuclei were stained with DAPI. Scale bar: 5 μm. (B) Quantification of BrdU–p53 colocalization in NHF ρ and AG03587 ρ cells. Extent of colocalization has been represented as the colocalization factor. Values are means ± s.d. (**C**,**D**) Lack of p53 does not affect RECQL4 localization during mtDNA replication. After creation of two pairs of cells in ρ state (NHF ρ and NHF E6 ρ), the cells were washed and grown in BrdU-containing normal growth medium for 10 minutes, 30 minutes and 60 minutes. (C) After fixation, immunofluorescence was carried out with antibodies against BrdU (RPN202) and RECQL4 (sc-16924). The image is a representative BrdU–RECQL4 staining at 10 minutes. Nuclei were stained with DAPI. Scale bar: 5 mm. (D) Quantification of BrdU–RECQL4 colocalization in NHF ρ and NHF E6 ρ cells. Extent of colocalization is represented as the colocalization factor. Values are means ± s.d.

We propose that RECQL4 acts as a regulator of the mitochondrial localization of p53, thereby functioning in a similar manner to mitochondrial hsp70 and hsp90, known to transport proteins to the mitochondria (Deshaies et al., 1988; Young et al., 2003). Lack of RECQL4 (either in RTS cells or in cells with a genetically defined isogenic background) led to accumulation of spontaneous DNA damage in the nucleus (as detected by formation of 53BP1 foci; supplementary material Fig. S3A,B). The spontaneous DNA damage activated ATM and/or ATR, which phosphorylated and stabilized p53 in RTS cells (Fig. 1D; supplementary material Fig. S3C). In NHFs, RECQL4–p53 interaction was disrupted in the presence of DNA damage (supplementary material Fig. S8C–F). Hence, in either case (i.e the lack of RECQL4 and/or after DNA damage) p53 utilizes its own NLS and accumulates directly in the nucleus, thereby carrying out its functions as a transcription factor (Fig. 1; supplementary material Fig. S1A, Fig. S2). Complementation experiments involving re-expression of wild-type RECQL4 in RTS cells (Fig. 5) validated the hypothesis that full-length RECQL4 acts as an important component in the regulatory circuit of p53 in untreated cells. Consequently in cells expressing RECQL4 (Δ84) mutant where RECQL4 expression is nuclear, the majority of the p53 accumulated in the nucleus also, as it was no longer able to accumulate in the mitochondria (Fig. 5B). Interestingly, the nuclear p53 in RECQL4 (Δ84) cells was transcriptionally active (supplementary material Fig. S11D). This also possibly accounts for the high sensitivity of the RECQL4 (Δ84) cells to multiple forms of DNA damage (supplementary material Fig. S1B).

Earlier studies had indicated that a substantial fraction of endogenous RECQL4 was located in the cytoplasm (Petkovic et al., 2005; Werner et al., 2006; Yin et al., 2004). It had been proposed that five lysine residues in RECQL4 could be acetylated by p300. Acetylation of RECQL4 by p300 in vivo led to its cytoplasmic localization (Dietschy et al., 2009). All the predicted lysine residues are within the 270–400 amino acid stretch of RECQL4 that interact with p53 (supplementary material Fig. S7H). Future research might indicate whether acetylation of RECQL4 in vivo regulates its binding to p53 and consequently its accumulation in the mitochondria.

The role of RECQL4 in nuclear DNA replication (Matsuno et al., 2006; Sangrithi et al., 2005; Xu et al., 2009) coupled with the colocalization of RECQL4 and the mtDNA (Fig. 2F; supplementary material Fig. S5B) provided a clue to the function of RECQL4 during mtDNA replication. The colocalization of RECQL4 with Polγ*A* throughout the cell cycle (Fig. 3E) is in agreement with the known fact that mtDNA replication occurs throughout the cell cycle (Magnusson et al., 2003). De novo mtDNA replication indicated that lack of RECQL4 led to a decrease in the extent of colocalization between BrdU (i.e the sites of new DNA synthesis) and PolγA (Fig. 6C,D). This suggests that mtDNA replication is hampered in the absence of RECQL4. Indeed southwestern analysis indicated that mtDNA replication was lower in RTS cells and in isogenic cells in which RECQL4 was genetically ablated (Fig. 6A,B). Hence the role of RECQL4 in de novo mtDNA replication was dependent on its mitochondrial localization (Fig. 6E; supplementary material Fig. S11C). It was also observed that RECQL4 was essential for the accumulation of p53 at the sites of de novo mtDNA replication as stained by BrdU (Fig. 7A,B). p53 has known functions during mtDNA replication (Achanta et al., 2005; Bakhanashvili et al., 2008; Wong et al., 2009). Therefore, by controlling the recruitment of p53 to the sites of de novo mtDNA replication, RECQL4 might also be regulating the mitochondrial functions of p53. This role of RECQL4 is similar to that of another member of the RecQ family of helicases, BLM helicase. BLM is an anti-recombinogenic protein that has been shown to be involved in the transport of p53 to sites of DNA damage in the nucleus (Sengupta et al., 2003). Given the important roles of both p53 and RECQL4 in the nucleus during DNA replication, repair and recombination (Matsuno et al., 2006; Petkovic et al., 2005; Sangrithi et al., 2005; Sengupta and Harris, 2005), it is possible that the mitochondrial and nuclear functions of these two tumor suppressors are altered during neoplastic transformation. This study provides insight into the specific roles of RECQL4 helicase, which are dependent on its localization to the mitochondria. These include the effect of RECQL4 on de novo mtDNA replication (Fig. 6E; supplementary material Fig. S11C), sensitivity to DNA damaging drugs (supplementary material Fig. S1B) and the accumulation of p53 in the mitochondria (Fig. 5B). Future studies to determine the interplay of RECQL4 and p53 between the two subcellular compartments might provide important insight into the progression of cancer and other abnormalities seen in RTS patients.

Materials and methods

Antibodies

Anti-RECQL4: mouse monoclonal K6312 (Kawabe et al., 2000); goat polyclonal sc-16924 (Santa Cruz). Anti-p53: mouse monoclonal sc-126 (Santa Cruz), rabbit polyclonal SIG-3520-1000 (Covance). Anti-FLAG antibody: mouse monoclonal F1804 (Sigma). Anti-cytochrome *c*: rabbit polyclonal sc-7159 (Santa Cruz). Anti-GST: rabbit polyclonal 71-7500 (Invitrogen). Anti-GFP: rabbit polyclonal 632460 (Takara). Anti-hsp90: rabbit polyclonal sc-7947 (Santa Cruz). Anti-p21: mouse monoclonal sc-397 (Santa Cruz). Anti-MDM2: mouse monoclonal ab16895 (Abcam). Phosphorylated p53 Ser15: #9284 rabbit polyclonal (Cell Signaling). Phosphorylated p53 Ser46: rabbit monoclonal ab76242 (Abcam). Anti-Bax: rabbit polyclonal sc-493 (Santa Cruz). Anti-Bcl-2: mouse monoclonal 100 (Santa Cruz). Anti-BrdU: mouse monoclonal RPN202 (GE Healthcare) and sheep polyclonal ab1893 (Abcam). Anti-TBP: mouse monoclonal sc-421 (Santa Cruz). Anti-actin: sc-8432 (Santa Cruz). Anti-53BP1: mouse monoclonal 612523 (BD Pharmingen). Anti-PolγA: rabbit polyclonal sc-48815 (Santa Cruz). Anti-PCNA: mouse monoclonal sc-56 (Santa Cruz). All secondary antibodies were all purchased from Jackson ImmunoResearch.

Recombinants

GST-p53 deletion constructs (Sengupta and Wasylyk, 2001); p53-EGFP-C1 (1–393) (a gift from Tyler Jacks, Koch Institute of Integrative Cancer Research, MA, USA). pcDNA3.1 p53 (1–393) (a gift from Ronald Hay; Welcome Trust Centre for Gene Regulation and Expression, Dundee, UK). pcDNA3-FLAG-RECQL4 (1–1208), pcDNA3-FLAG-RECQL4 (Δ84), pcDNA3-FLAG-RECQL4 (Δ404), pcDNA3-FLAG-RECQL4 (1–459) were obtained by cloning RECQL4 fragments between the *Nhe*I and *Xho*I sites of the FLAG-tagged pcDNA3 construct. pLVX-AcGFP-N1-RECQL4 (1–1208) and pLVX-AcGFP-N1-RECQL4 (Δ84) were obtained by cloning the respective RECQL4 fragments between the *Xho*I and *Eco*RI sites of the pLVX-AcGFP-N1 construct. pcDNA3.1 hygro (+)-EGFP-RECQL4 (13–18) was obtained by cloning the RECQL4 amino acids in frame with EGFP into the *Afl*II–*Bam*HI sites of pcDNA3.1 hygro(+) vector. GST-RECQL4 constructs were obtained by cloning the 1–459 fragment into the *Bam*HI–*Eco*R1 sites and 460–868, 869–1208, 1–150, 151–300, 301–459, 151–459, 180–459, 210–459, 240–459, 270–459, 151–430, 151–400, (151–370), 151–340 and 270–400 fragments into the *Eco*R1–*Xho*I sites of the pGEX4T-1 vector. pcDNA3.1 hygro(+)-FLAG-PolγA obtained by cloning the PCR product into the *Nhe*I–*Not*I sites of the vector, using pBacPAK-PolγA (a gift from Maria Falkenberg, University of Gothenburg, Sweden) as the template. pSilencer2.1-U6 hygro-RECQL4 was obtained by cloning the 19-nucleotide RECQL4 siRNA sequence (5′-GCTTCGAGAGCTACGTGCA-3′) into the *Bam*HI–*Hin*dIII sites of pSilencer2.1-U6 hygro (Ambion). The corresponding siRNA for RECQL4 having the same sequence and p53 siRNA having the sequence (5′-GACTCCAGTGGTAATCTAC-3′) were synthesized from Dharamacon. ON-TARGETplus non-targeting siRNA #1 siRNA (Dharmacon) was used as the control siRNA in all experiments. pGEX-6P-1-ratTom20 (59–126) was a gift from

Daisuke Kohda, Kyushu University, Japan. pcDNA3-FLAG-RECQL4 (Δ13–18) was obtained by site directed mutagenesis.

Cell culture conditions

Immortalized normal human fibroblasts (NHFs, GM07532) and NHF E6 cells were maintained as described previously (Sengupta et al., 2003). GM08402 and MRC-5 were obtained from Coriell Cell Repositories and ViroMED Laboratories, respectively. Primary RTS fibroblasts (Kitao et al., 1999b) were obtained either from Coriell Cell Repositories (AG03587, AG05013 and AG05139) or from the Mayo Clinic (L9552914-J, B1865425K and D8903644-K). All the RTS fibroblasts used (AG03587, AG05013, AG05139, L9552914-J, B1865425K and D8903644-K) were subsequently immortalized by induction of telomerase into the cells by standard procedures. By Genechip PCR analysis and functional assays, p53 was found to be wild-type in the cells from RTS patients (AG03587, AG05013, AG05139, B1865425K and D8903644-K), whereas it was mutated in L9552914-J cells (data not shown). Lentiviruses carrying AcGFP-N1-RECQL4 (1–1208) and AcGFP-N1-RECQL4 (Δ84) were prepared by cotransfecting Lenti-X HT packaging mix with the corresponding plasmid into the Lenti-X^{TM} 293T cell line using the Lenti-X HT packaging mix (Takara). AG05013 cells were transduced with recombinant lentiviruses and cells were selected with puromycin (0.5 μg/ml) for 10 days. The clones obtained were analyzed by western blotting. NHF shRECQL4 and NHF shControl cells were prepared by stably integrating pSilencer2.1-U6 hygro-RECQL4 and pSilencer2.1-U6 hygro, respectively, into NHF cells. Clones were selected with hygromycin (300 μg/ml) for 7 days and analyzed by western blotting.

The ρ^0 cells were created by culturing the parental cells for 14 days in presence of 50 ng/ml ethidium bromide, 50 μg/ml uridine and 2 mmol/l pyruvate. The medium was changed every third day and fresh supplements were added. On the 14th day, the levels of the nuclear genome encoded cytochrome *b* were analyzed by PCR to determine the extent of the depletion of mitochondrial DNA (mtDNA). If complete depletion of mtDNA was observed, the cells were classified as ρ^0 cells. The ρ cells used for de novo replication assays were prepared by following exactly the above process except that the cells were treated with the supplements (ethidium bromide, uridine and pyruvate) for only 10–12 days, when depletion of mtDNA was not complete (as observed by cytochrome *b* amplification). The resulting cells were classified as ρ cells.

For immunofluorescence analysis cells were treated with BrdU (15 μM) for 3 hours or leptomycin B (5 ng/ml) for 4 hours. Cells were treated with *N*-acetylcysteine (20 μM) for 1 hour. For de novo replication assays the ρ cells were treated with BrdU (15 μM) for the indicated time intervals, and then fixed. For double thymidine block, NHFs was treated with thymidine (2.5 mM) for 17 hours, washed, released into thymidine-free medium for 12 hours and then re-treated with thymidine (2.5 mM) for 17 hours. This treatment regime blocked the cells at the G1–S boundary (taken as the 0 hours time point). The final concentrations of drugs used for the DNA damage experiments were: camptothecin (0.5 ng/μl; Sigma), etoposide (2ng/μl; Sigma) and neocarzinostatin (0.2 ng/μl; NCI, NIH). All transient transfections were carried out with Lipofectamine 2000, using Optimem (Invitrogen) as the transfection medium. Mitochondria were stained using the fluorescent dye Mitotracker Red CMXRos (200 nM; Invitrogen). Live cells were incubated with of this dye for 30 minutes. The cells were subsequently washed, fixed and then analyzed. For long-term colony formation assays the cells were exposed for 6 hours with the indicated concentrations of the respective drugs. The cells were extensively washed and the culture continued for the next 14–21 days, after which the clones formed were stained with Giemsa.

Microscopy techniques

Confocal microscopy was done according to published protocols (Sengupta et al., 2003). Cells were fixed initially with either 100% ethanol, 100% methanol, methanol:acetone (1:1), 4% paraformaldehyde or 3.7% formaldehyde. All fixation conditions gave similar results. Subsequently freshly prepared 4% paraformaldehyde fixation was used for all the immunofluorescence staining in which antibodies were used. For fixation of cells treated with Mitotracker Red, 3.7% formaldehyde was used in the medium. Subsequent to fixation, the cells were rinsed three times with 1× PBS, twice treated with 0.1% Triton X-100 for 5 minutes each, washed with 1× PBS with 0.5% Tween 20 and blocked overnight with 10% normal chicken serum. Subsequently the staining was carried out with the indicated antibodies at 37℃, for 1 hour in a humidified chamber. Incubation with secondary antibody was also carried out under similar conditions. Adequate controls, such as use of the corresponding IgG or not using secondary antibody, were always included. At least 150 cells were analyzed for all immunofluorescence experiments. The slides were analyzed using a Zeiss Axio Imager M1 epifluorescence microscope with 100×/1.4 NA oil, 63×/1.25 NA oil or 40×/0.75 NA objective or a Zeiss 510 Meta system with 63×/1.4 NA oil immersion or 40×/0.95 NA Corr objective. The laser lines used were argon 458/477/488/514 nm (for FITC), DPSS 561 nm (for Texas Red and Mitotracker dyes), HeNe 633 nm (for Cy5) and a Chameleon Ultra autotunable femtosecond laser with a tuning range 690–1050 nm (for DAPI). LSM5 software (Carl Zeiss) was used for image acquisition. Details of each quantification are indicated in the respective figure legends. In specific cases quantification has also been defined by colocalization factor. Colocalization factor is defined as [(fraction of cells having colocalization) × (fraction of foci colocalized per cell)].

Southwestern analysis

Cells, cultured in 8×175 cm tissue culture flasks, were grown to 60% confluency and incubated for 24 hours with BrdU (15 μM). Mitochondria were isolated by differential centrifugation. Isolated mitochondria was lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.55 Tween-20) and treated with proteinase K. Proteins were removed by phenol–chloroform–isoamyl alcohol extraction and the isolated mtDNA was digested with *Nhe*I (1 μg was used per sample) for 40 minutes at 37℃. *Nhe*I cuts the mtDNA at one site. The digested DNA was run on a 1% agarose gel, stained with ethidium bromide or transferred onto a nitrocellulose membrane and probed with anti-BrdU antibody.

Genomic PCR

Genomic DNA was isolated by standard processes. The primers were: 5′-CTAC-AATGAGCTGCGTGTGGC-3′ and 5′-CAGGTCCAGACGCAGGATGGC-3′ for actin, and 5′-GGCTTACTTCTCTTCATTCTCTCCT-3′ and 5′-GGTTGTCCT-CCAATTCATGTTA-3′ for cytochrome B.

Expression, purification and interactions of proteins

FLAG-tagged RECQL4 and its mutants were expressed in HEK 293T cells for interaction studies. GST-tagged proteins were expressed according to standard protocols in *Escherichia coli* at 16℃ or 18℃ and subsequently purified by binding to glutathione S-Sepharose (GE Healthcare). pcDNA3-FLAG-RECQL4 and pcDNA3.1-p53 were used for coupled in vitro transcription and translation of RECQL4 and p53, respectively, using the T7 Quick Coupled Transcription/Translation System kit (Promega) in the presence of [S^{35}]methionine. GST-bound target proteins were incubated with the in-vitro-translated interacting partner (p53 or RECQL4) for 4 hour at 4℃ with constant inversion. Interaction was assayed by autoradiography. For interactions involving GST-tagged bound proteins and FLAG-tagged interacting partners, the two components were mixed overnight in an end-to-end rocker. The bound proteins were then centrifuged, washed and analyzed by western blotting. The mitochondrial fraction was isolated as previously described (Achanta et al., 2005). Cytoplasmic and nuclear extracts from cells were made using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce). For preparation of whole cell lysates the cells were lysed in a modified RIPA buffer [1 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitors]. For endogenous interactions, immunoprecipitations (IPs) were carried out with whole cell extracts using standard protocols (1 mg/IP, 2 μg antibody/IP).

Web-based tools

Four web-based tools: Target P (Emanuelsson et al., 2007), PSORT (Nakai and Horton, 1999), MitoProt (Claros and Vincens, 1996) and iPSORT (Bannai et al., 2002) were used to predict the MLS in RECQL4.

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