DNA polymerase α is essential for the onset of eukaryotic DNA replication. Its correct folding and assembly within the nuclear replication pre-initiation complex is crucial for normal cell cycle progression and genome maintenance. Due to a single point mutation in the largest DNA polymerase α subunit, p180, the temperature-sensitive mouse cell line tsFT20 exhibits heat-labile DNA polymerase α activity and S phase arrest at restrictive temperature. In this study, we show that an aberrant form of endogenous p180 in tsFT20 cells (p180tsFT20) is strictly localized in the cytoplasm while its wild-type counterpart enters the nucleus. Time-lapse fluorescence microscopy with enhanced green fluorescent protein-tagged or photoactivatable green fluorescent protein-tagged p180tsFT20 variants and inhibitor analysis revealed that the exclusion of aberrant p180tsFT20 from the nucleus is due to two distinct mechanisms: first, the inability of newly synthesized (cytoplasmic) p180tsFT20 to enter the nucleus and second, proteasome-dependent degradation of nuclear-localized protein. The nuclear import defect seems to result from an impaired association of aberrant de novo synthesized p180tsFT20 with the second subunit of DNA polymerase α, p68. In accordance, we show that RNA interference of p68 results in a decrease of the overall p180 protein level and in a specific increase of cytoplasmic localized p180 in NIH3T3 cells. Taken together, our data suggest two mechanisms that prevent the nuclear expression of aberrant DNA polymerase α.

The highly conserved DNA polymerase α-primase complex is the only eukaryotic polymerase that can initiate DNA synthesis de novo. Thus, its recruitment is a crucial step in the tightly regulated stepwise assembly of the replication machinery in eukaryotic cells. This complex is required for the synthesis of RNA primers, an essential prerequisite for the initiation of replication, and for the discontinuous synthesis of Okazaki fragments on the lagging strand (1–4). Moreover, DNA polymerase α plays a fundamental role in coordinating DNA replication, DNA repair, and cell cycle progression (1), in telomere capping and length regulation (5–9), and in the epigenetic control of transcriptional silencing and nucleosome reorganization (10, 11).

The DNA polymerase α-primase complex consists of four subunits, each of which is conserved in eukaryotes; in yeast, all four subunits are essential for viability (2). The largest subunit, 180 kDa (p180), harbors the catalytic polymerase α activity. The two smallest subunits, 54 kDa (p54) and 46 kDa (p46), provide primase activity. The p46 protein, which is coupled to p180 by p54, synthesizes RNA primers and is involved in regulating their length; it also functions in cell cycle checkpoints (12). The 68-kDa subunit (p68) plays a crucial regulatory role in the early stage of chromosomal replication in yeast and has been shown to be essential for the nuclear import of p180 in mouse cells (13, 14).

DNA replication takes place during a restricted period in the cell cycle, in the S phase. To avoid errors in DNA duplication that compromise genome integrity in vertebrate cells, the transition from G1 to S phase and the onset of DNA polymerase activity are highly regulated by at least two cyclin-dependent kinases, cyclin E/cdk2 and cyclin A/cdk2 (15, 16). In an SV40 in vitro system, phosphorylation of p68 inhibits replication initiation as well as primer synthesis and elongation (17), and the p180 subunit contains multiple regulatory phosphorylation sites (18, 19). In addition to regulation by phosphorylation, expression of the DNA polymerase α gene is controlled during the cell cycle by E2F, GABP, and Sp1 transcription factors (20, 21). Thus, the activity of DNA polymerase α is tightly controlled at the transcriptional and post-translational levels, whereas less is known about its subcellular distribution, regulation, and behavior under various stress conditions.

The tsFT20 cell line, a temperature-sensitive mutant clone identified in a screen of N-methyl-N’-nitro-N-nitroguanidine-treated mouse mammary carcinoma FM3A cells, has been the subject of several genetic and biochemical studies (22–34). Compared with parental FM3A cells, this cell line grows normally at 33 °C. However, at the restrictive temperature of 39.5 °C, they exhibit a phenotype closely resembling that con-
ferred by arrest at the G1/S boundary. At the restrictive temperature, tsFT20 cells were further characterized as defective in DNA replication, with a highly decreased DNA synthesis rate, reduced frequencies of replicon initiation, and extensive chromosome aberrations. Purified DNA polymerase α from tsFT20 cells was found to be temperature-sensitive although composed of the same hetero-tetrameric complex as that of FM3A cells, and this defect was ascribed to a single point mutation that changes amino acid 1180 of the p180 subunit from serine to phenylalanine (28). The abnormal cessation of DNA replication in tsFT20 cells at the restrictive temperature results in cell death via the induction of DNA double strand breaks, suggesting that aberrant DNA polymerase α is cytotoxic and also that in general a specific quality control mechanism for the pool of aberrant DNA polymerase α in wild-type cells may be crucial to ensure genomic stability and accurate DNA replication. Recently, it was reported that in Saccharomyces cerevisiae, reduced levels of the replicative DNA polymerase α result in greatly elevated frequencies of chromosome translocations and chromosome loss (35). Similarly, when Mcm10 or Ctf4 are greatly elevated frequencies of chromosome translocations and expressed DNA polymerase α protein expression of an aberrant form of the endogenously expressed DNA polymerase α in mammalian cells, DNA polymerase α is cytotoxic and also that in general a specific quality control mechanism for the pool of aberrant DNA polymerase α in wild-type cells may be crucial to ensure genomic stability and accurate DNA replication. Recently, it was reported that in Saccharomyces cerevisiae, reduced levels of the replicative DNA polymerase α result in greatly elevated frequencies of chromosome translocations and chromosome loss (35). Similarly, when Mcm10 or Ctf4 are depleted in mammalian cells, DNA polymerase α levels are markedly reduced, suggesting that DNA polymerase α levels are indeed crucially regulated by as yet unknown cellular mechanisms (36, 37).

Misfolded and non-functional proteins must be degraded to assure the correct functioning of cellular processes. Several mechanisms that ensure the removal of defective proteins have been described, the most prominent being ER5-associated degradation, a process that governs the degradation of unfolded ER proteins in the cytoplasm (38, 39). Degradation systems that function in quality control have also been identified in the secretory pathway and in mitochondria (40). These processes are carried out by proteasomes, organelles consisting of several proteases that are located in the cytoplasm as well as in the nucleus (41, 42, 43). Moreover, most cystic fibrosis patients inherit at least one mutant allele of the ion channel cystic fibrosis transmembrane regulator, which has a temperature-sensitive folding defect causing its detection by the endoplasmic quality control system (44). In contrast, the degradation-dependent protein quality control mechanisms that act in the nucleus are still poorly understood. Recently, Gardner et al. (45) reported that San1-mediated degradation acts as a protein quality control system in S. cerevisiae nuclei. However, it is still unclear how aberrant nuclear proteins are recognized by ubiquitin E3 ligases and whether analogous systems exist in higher eukaryotes.

In this study, we observed the subcellular distribution and protein expression of an aberrant form of the endogenously expressed DNA polymerase α subunit p180 in tsFT20 cells (p180<sup>tsFT20</sup>) at the restricted temperature using a specific p180 antibody. Surprisingly, we found that endogenous p180<sup>tsFT20</sup> was rapidly degraded in the nucleus at the restricted temperature, concomitantly with de novo synthesized protein accumulating in the cytoplasm. Based on these crucial findings on endogenous p180<sup>tsFT20</sup>, we transiently expressed GFP-tagged p180<sup>tsFT20</sup> in NIH3T3 or COS-1 cells and made the very same observation that the aberrant p180 variant is excluded from the nucleus and accumulates in the cytoplasm at the restrictive temperature, whereas the wild-type counterpart normally localizes to the nucleus. Inhibitor studies combined with immunofluorescence analysis suggest two mechanisms that exclude aberrant p180<sup>tsFT20</sup> from the nucleus: defective nuclear import of de novo synthesized protein and proteasome-dependent degradation of nuclear localized protein. Our data further suggests that a single point mutation in p180<sup>tsFT20</sup> presumably causes conformational changes that alter the hydrophobic character of the protein surface, rendering p180<sup>tsFT20</sup> unable to bind the p68 subunit, which leads in turn to its inability to enter the nucleus (cytoplasmic fraction) or its recognition by the proteasome-dependent degradation machinery (nuclear fraction). In accordance with this assumption, we find that the knockdown of p68 by RNA interference results in a decrease of the overall p180 protein level and a specific increase of cytoplasmically expressed p180. Our findings thus offer mechanistic insights into how the aberrant DNA polymerase α subunit p180 (and probably other malformed nuclear proteins) are cleared from the nucleus to avoid its inappropriate involvement in DNA replication.

### Experimental Procedures

**Plasmid Construction**—Expression plasmids encoding p180 or p68 were previously described (14). Using the primers tsFT20-s 5′-GTGATACGGTGTTCTATGTTATTTG-3′ and tsFT20-as 5′-CAAATAACATAGAACCGTATCAC-3′, a specific point mutation that creates the S1180F alteration was inserted in plasmid vectors pSRα-p180, pSRα-p180-GFP, and pSRα-H-core (14, 46) with the PCR-based QuickChange II Site-directed mutagenesis kit (Stratagene, La Jolla, CA). To generate the expression plasmid pSRα-p180<sup>tsFT20</sup>-PA-GFP encoding photoactivatable GFP-tagged p180<sup>tsFT20</sup>, four point mutations (L64F, T65S, V163A, and T204H in EGFP) were introduced (47). The p68-mRFP fusion expression plasmid was constructed by fusing mRFP1 CDNA to the C terminus of p68. The identity of each construct was confirmed by sequencing using an Applied Biosystems 3130 automatic DNA sequencer.

**Cell Culture, Transfection Techniques, and Addition of Inhibitors**—NIH3T3 mouse cells were cultured in a 5% CO2 incubator in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and used for direct immunofluorescence analysis and time-lapse fluorescence microscopy. For Western blot analysis and co-immunoprecipitation, COS-1 cells were grown in the same medium supplemented with 10% fetal bovine serum instead of calf serum. Mouse FM3A cells and the tsFT20 strain were maintained at the permissive temperature (33 °C) in RPMI 1640 medium supplemented with 10% calf serum. NIH3T3 cells (1–3 × 10<sup>5</sup> cells/well) were kept at 37 °C for 16 h and transfected in 8-well chamber slides (Nunc) using Lipofectamine (Invitrogen) or transfectin (Bio-Rad) according to the manufacturer’s instructions. COS-1 cells were transfected using FuGENE 6 reagent (Roche). If not otherwise men-

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The abbreviations used are: ER, endoplasmic reticulum; GFP, green fluorescent protein; LMB, leptomycin B; CHX, cycloheximide; PA-GFP, photoactivatable green fluorescent protein; mRFP, mouse red fluorescent protein; CSK, cytoskeleton buffer; PIPES, 1,4-piperazinediethanesulfonic acid; siRNA, small interfering RNA.
tioned, transfected cells were incubated in appropriate media for 48 h at 33 °C and then kept at 33 °C or shifted to 39.5 °C. The inhibitors leptomycin B (LMB) (2.5 μm, Sigma), MG132 (5 μm, Calbiochem), and cycloheximide (CHX) (0.1 μm, Aldrich) were added 1 h before the temperature shift, if not otherwise mentioned.

Preparation of Cell Extracts—Following transfection, COS-1 cells were washed twice with phosphate-buffered saline, scraped from plates into iced-cold phosphate-buffered saline, centrifuged (3000 × g, 5 min, 4 °C) and frozen as pellets at −80 °C. After thawing, the cells were resuspended in cytoskeleton buffer (CSK) (10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 1 mM EGTA) supplemented with a protease inhibitor mixture (Roche), 0.2 mg/ml phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.1% Triton X-100 for 1 h at 4 °C, and centrifuged (3000 × g, 5 min, 4 °C). The supernatant (S1 fraction) was collected and the insoluble chromatin fraction was further extracted with the same solution as above but supplemented with 0.3 M KCl, incubated for 30 min at 4 °C, and separated by centrifugation (3000 × g, 5 min, 4 °C). Again, the supernatant was collected (S3 fraction) and the remaining insoluble material (pellet fraction) was dissolved in 70 μl of SDS sample buffer. The tsFT20 cells were incubated at permissive (33 °C) or restrictive (39.5 °C) temperatures for the indicated times, harvested by centrifugation, and resuspended in SDS sample buffer.

Western Blot Analysis—For Western blot analysis, the S1 or the pellet fraction was subjected to 8% SDS-PAGE and transferred to a 0.45-μm polyvinylidene difluoride membrane (Millipore). The membranes were washed three times (5 min at room temperature) with TBSSM buffer (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl), blocked with 5% skim milk (30 min at room temperature), incubated with primary antibodies dissolved in TBSSM buffer (16 h at 4 °C), and washed three times with TBSSM buffer containing 0.05% Tween 20. Horseradish peroxidase-coupled secondary antibodies dissolved in TBSSM buffer were applied to the membranes (16 h at 4 °C), and washed three times with TBSSM buffer. The membranes were then washed again three times, incubated in 2 ml of SuperSignal reagent (Pierce), and observed using the LAS system (Fuji). To detect polyubiquitinated p180 in tsFT20 cells, Western blot analysis was carried out using a monoclonal anti-polyubiquitin antibody (FK2, Nippon Biotest Laboratory).

Co-immunoprecipitation Analysis—To detect polyubiquitinated p180, 1 × 10⁷ tsFT20 cells were harvested after incubation for 9 h at 39.5 °C in the presence of 2 nm MG132. Cells were extracted with CSK/Triton X-100 buffer for 20 min at 4 °C and the detergent-resistant fraction was separated by centrifugation (10,000 × g for 5 min). The remaining pellet was mixed with CSK/Triton X-100 buffer and sonicated well by handy sonic UR-20P (TOMY SEIKO Inc. Japan). One mg of extract was mixed with 3 μl of anti-p180 antisera for 16 h at 4 °C. Each sample was mixed with 20 μl of Protein G-Sepharose for 2 h at 4 °C and the unbound fraction was separated by centrifugation (10,000 × g for 2 min). The remaining pellet was washed with CSK/Triton X-100 buffer, dissolved in 30 μl of SDS sample buffer, and subjected to SDS-PAGE and Western blot analysis using polyclonal anti-p180 antibody and monoclonal anti-polyubiquitin antibody.

Immunofluorescence Microscopy—Eight-well chamber slides were pre-treated with poly-L-lysine (1 mg/ml) for 2 h at 37 °C and 3 × 10⁴ cells were seeded per well and incubated for 16 h at 37 °C. The cells were transfected as described above and incubated for 48 h at 33 °C. Inhibitors were applied either separately or in combination as described above and the cultures were incubated for another 1 h at 33 °C. The cells were then either shifted to 39.5 °C or kept at 33 °C as a control. Cell fixing, staining with anti-p180, anti-p68, and anti-Mcm7 (Sigma) antibodies, and subsequent observation by fluorescence microscopy (Olympus, AX70) were done as previously described (14, 46). To increase the detection sensitivity and to eliminate the endogenous p180 signal, monoclonal anti-GFP antibodies (Sigma) were used for staining. In three independent experiments, a total of 600 cells was counted and localization of overexpressed protein was classified as nuclear, nuclear and cytoplasmic, or cytoplasmic. The direct GFP signal and the signal detected with anti-GFP antibodies were visualized as green and red, respectively. DNA was stained with Hoechst 33258 (blue). To detect endogenous p180 in tsFT20 cells, digital images were captured and processed with fluorescence microscopy and deconvolution analysis using a nearest neighbor method with HazeBuster software (VayTek, Inc.) (48). The stack images of 10 optical sections with a step size of 200 nm were then deconvolved in three dimensions. To visualize cytoplasm in FM3A cells, endogenous biotin in the mitochondria was labeled with Alexa Fluor 488 streptavidin (Invitrogen).

Time-lapse Fluorescence Microscopy—To observe living cells, 2.0 × 10⁵ NIH3T3 cells in 2 ml of Dulbecco’s modified Eagle’s medium containing 10% calf serum were seeded in 35-mm dishes with a glass slide on the bottom and incubated for 16 h at 37 °C. The cells were transfected with 2.4 μg of p180(tsFT20)GFP DNA, 1.6 μg of p68 DNA, 6 μl of transfectin, and 500 μl of serum-free media. After incubation for 48 h at 33 °C, the media was changed to CO₂-independent media and the temperature was up-shifted and kept constant at 39.5 °C using a microscope stage heater (Olympus, MI-IBC-I). To monitor p180(tsFT20)GFP, cells were kept for 5 h at 39.5 °C, and the temperature was then reduced and maintained at 33 °C. The GFP signal was observed and pictures were taken every 6 or 10 min. For photoactivation experiments, cells expressing PA-GFP-tagged p180 or p180(tsFT20)GFP were photoactivated by a 10-s exposure to 420 nm light (filter BP400–440, Olympus) from a shuttered 100-watt mercury epi-illuminator with a ×40 UPlanApo objective. Cells were co-transfected with monomeric RFP-p68 to identify those expressing p180tsFT20GFP. Live cell images were acquired with a ×20 UPlanApo objective.

siRNA Depletion—Two rounds of siRNA oligonucleotide transfections were performed to reduce the expression level of p68. siRNA duplexes were transfected into NIH3T3 cells using siLentFect lipid reagent (Bio-Rad) according to the manufacturer’s instructions. 20 pmol duplex RNA was used for each transfection. The procedure was repeated on the second day, and cells were harvested on the fourth day. The following RNA oligonucleotides were used: control, 5′-UUCUCCGAACGUGUCACGUU-3′; si68-1, 5′-CGUAAAGCGAAA-
Aberrant Polymerase α Is Excluded from the Nucleus

FIGURE 1. Degradation of aberrant endogenous p180 in tsFT20 cells. A, FM3A and tsFT20 cells were incubated at the restrictive or permissive temperatures for the indicated time, harvested, and lysed in SDS sample buffer. Twenty μg of whole cell extract was loaded on each lane and Western blot analysis was carried out using anti-p180, anti-p68, anti-Mcm7, and anti-proliferating cell nuclear antigen antibodies. B, tsFT20 cells were incubated in the presence of LMB (1.8 or 3.6 nM) or MG132 (1 or 2 μM) or without treatment at 33 or 39.5 °C for the indicated hours and protein levels of p180 were quantified by Western blot analysis. C, tsFT20 cells were incubated at 33 or 39.5 °C for 9 h in the presence or absence of 2 mM MG132, harvested, and fractionated into a soluble protein fraction (CSK+T solution without sonication (−)), after sonication (+). One-mg extracts were immunoprecipitated with anti-p180 antibody, and precipitates were loaded on a 6% acrylamide gel and Western blot analysis was carried out using anti-p180 antibody, and anti-polyubiquitin monoclonal antibody.

RESULTS

Endogenous p180tsFT20 Is Degraded in the Nucleus at Restrictive Temperature and Expressed in the Cytoplasm—The temperature-sensitive mutant cell line tsFT20 is characterized by severe defects in cell cycle progression and DNA replication. These phenotypes were assigned to a single point mutation in the largest subunit of the mouse DNA polymerase α complex, p180. The properties of the respective protein (designated p180tsFT20) have been studied on a biochemical level, but its nuclear transport properties remained unknown. For the intriguing possibility to use this protein variant as a tool to study protein quality control mechanisms specifically in the nucleus, we decided to look at the endogenous p180tsFT20 expression levels, localization pattern, turnover rate, and its polyubiquitinated form. Interestingly, polyubiquitinated p180 was only observed in the soluble fraction of tsFT20 cell extracts at the restrictive temperature, whereas its degradation of endogenous p180tsFT20 is dependent on the ubiquitin-mediated proteasome pathway and may be specifically in the nucleus. To verify ubiquitin-mediated degradation, proteasome function was inhibited by incubation with MG132 and samples were prepared for immunoprecipitation and Western blot analysis (Fig. 1C). One mg of protein from MG132-treated tsFT20 cells at the restrictive temperature was incubated with anti-p180 antisera and Western blot was performed with a monoclonal anti-polyubiquitin antibody as shown in Fig. 1C. Interestingly, polyubiquitinated p180 was only observed in the soluble fraction of tsFT20 cell extracts at the restrictive temperature, and in the presence of MG132. In sonication-dependent insoluble fractions, p180 is clearly detectable, however, not in its polyubiquitinated form.

Cytoplasmic Localization of the p180tsFT20 Protein in tsFT20 Cells—To further examine whether endogenous p180tsFT20 is degraded in the nucleus or cytoplasm at the restrictive temperature, tsFT20 cells were stained with anti-p180 antibodies. For visualizing the cytoplasm in tsFT20 cells, endogenous biotin in the mitochondria was stained with Alexa Fluor 488-streptavidin and deconvolution imaging performed. We found that the disappearance of endogenous p180tsFT20 is coincident with the tightly associated p68 subunit. To define whether the disappearance of endogenous p180tsFT20 from the nucleus is dependent on nuclear export or the ubiquitin-mediated proteasome pathway, tsFT20 cells were incubated in the presence or absence of the nuclear export inhibitor LMB or the proteasome inhibitor MG132, and protein levels of p180tsFT20 were quantified by Western blot analysis (Fig. 1B). LMB showed no significant effect on the reduction of p180tsFT20, whereas MG132 reduced the degradation of p180tsFT20. This suggests that first, the CRM1-dependent nuclear export pathway is not involved in the degradation of endogenous p180tsFT20 and second, that the degradation of endogenous p180tsFT20 is dependent on the ubiquitin-mediated proteasome pathway and may be specifically in the nucleus.
Aberrant Polymerase α Is Excluded from the Nucleus

FIGURE 2. Immunofluorescent staining of aberrant endogenous p180 in tsFT20 cells. A, tsFT20 cells were cultured on poly-L-lysine-coated slide chambers and incubated at 33 or 39.5 °C for 10 h. Endogenous p180 mutant was detected with anti-p180 antibody, endogenous biotin in mitochondria was visualized by Alexa Fluor 488-conjugated streptavidin and DNA was stained with Hoechst 33258. B, tsFT20 cells were incubated for 5 h at 39.5 °C in the presence of 2 μM cycloheximide or 6.8 μM LMB, or 2 μM MG132. Endogenous p180GFP was detected with anti-p180 antibodies, endogenous biotin in mitochondria was visualized by Alexa Fluor 488-conjugated streptavidin, and DNA was stained with Hoechst 33258.

p180<sup>tsFT20</sup> level (Fig. 1A). To test the dependence of cytoplasmic accumulation of aberrant endogenous p180<sup>tsFT20</sup> on protein synthesis, nuclear export, and proteasome-dependent degradation, tsFT20 cells were incubated in the presence of various inhibitors (Fig. 2B). In the presence of the protein synthesis inhibitor CHX, cytoplasmic expression of p180 was clearly decreased, indicating that the accumulation of cytoplasmic p180<sup>tsFT20</sup> in the absence of CHX is newly synthesized p180. In the presence of the proteasome inhibitor MG132, most of the signal remained in the nucleus p180. Interestingly, aggregates of aberrant proteins that cannot be degraded by the proteasome are only visible in the nucleus. In the presence of leptomycin B, a nuclear export inhibitor, the overall effects concerning protein expression (Fig. 1B) and subcellular staining of p180<sup>tsFT20</sup> (Fig. 2B) are milder, indicating that nuclear export mechanisms are not a major cause of the observed cytoplasmic accumulation. Taken together, these results suggested that clearance of aberrant endogenous p180<sup>tsFT20</sup> from the nucleus at the restricted temperature is mainly due to proteasome-dependent degradation, whereas its cytoplasmic expression is due to de novo protein synthesis.

Transient Expression of p180<sup>tsFT20</sup> in Mammalian Cultured Cells—To confirm and extend our results on endogenous p180<sup>tsFT20</sup> and get a clearer picture on the mechanisms that exclude aberrant p180<sup>tsFT20</sup> from the nucleus, we transiently expressed p180 (WT and p180<sup>tsFT20</sup>) in mammalian cultured cells (supplemental Fig. S1A).

All constructs were expressed at the expected molecular weights and at similar levels (supplemental Fig. S1B). Co-transfection with a plasmid encoding the second largest subunit of the DNA polymerase α complex, the p68 protein, strongly increased the expression of p180 (14) and p180<sup>tsFT20</sup>, independent of the presence of the GFP tag. These results indicate that like wild-type p180, the level of newly synthesized p180<sup>tsFT20</sup> increases in the presence of p68. The more rapidly migrating p180 and p180GFP bands are due to amino-terminal degradation of full-length p180, as described previously (14). Moreover, temperature up-shift decreased the expression level of p180GFP and p180<sup>tsFT20</sup>GFP (data not shown; Fig. 3D), however, NH<sub>2</sub>- and COOH-terminal truncated variants of His-tagged p180, designated H-core and H-core<sup>tsFT20</sup>, showed constant expression in the presence and absence of p68 (supplemental Fig. S1B). Because p68 associates with the COOH-terminal region of p180, the presence of p68 had no effect on H-core expression, as previously described (14). The finding also raises a model in which p180 is degraded via degron motifs in the COOH terminus that can be covered via association with p68.

Nuclear Exclusion of Transiently Expressed Aberrant p180<sup>tsFT20</sup> Protein—To determine the subcellular localization of transiently expressed p180GFP and p180<sup>tsFT20</sup>GFP, NIH3T3 cells were transfected with plasmids in the presence or absence of the p68 expression construct. The cells were seeded in 8-well chamber slides and incubated for 48 h at 33 °C to assure full protein expression. The temperature was then shifted to 39.5 °C or kept at 33 °C as a control, and the cells were fixed and observed by immunofluorescence microscopy. For protein detection, we monitored the direct GFP signal and also used polyclonal anti-p180 antibodies that could be visualized with Alexa 594-labeled secondary antibodies. In cells that were not transfected with the p180GFP or p180<sup>tsFT20</sup>GFP constructs, anti-p180 antibodies did not produce a signal under these conditions (data not shown).

At permissive temperature, both wild-type p180GFP and mutant p180<sup>tsFT20</sup>GFP clearly localized in the nucleus of mouse NIH3T3 cells in the presence of ectopically expressed p68 protein (supplemental Fig. S2, A and C). At restrictive temperature in the presence of p68, the wild-type protein remained in the nucleus, whereas the mutant form was found exclusively in the cytoplasm (supplemental Fig. 2, B and C). We confirmed these results in COS-1 cells (data not shown). In the absence of transfected p68, both p180GFP and p180<sup>tsFT20</sup>GFP exclusively localized in the cytoplasm, thus confirming the essential role of the p68 protein in nuclear entry of the p180 protein (supplemental Fig. S2, A–C) (14). Cell counting experiments revealed that these correlations are very specific. For each condition, more than 150 transfected cells were observed and the staining pattern was categorized as nuclear or cytoplasmic (supplemental Fig. S2C). Notably, in the case of p180<sup>tsFT20</sup>GFP co-expressed with p68, we found that more than 80% of transfected cells exhibited nuclear fluorescence at the permissive temperature, whereas after incubation for 16 h at the restrictive temperature, the protein was expressed in the cytoplasm in more than 95% of transfected cells.

For comparison, we determined the localization pattern of the p180 H-core constructs. As expected, H-core proteins with or without the tsFT20-specific point mutation were expressed exclusively in the cytoplasm at 33 °C as well as at 39.5 °C, independent of the presence of p68 (supplemental Fig. S2D). Thus, these truncated proteins exhibited a localization pattern identical to that of p180<sup>tsFT20</sup>GFP at the restrictive temperature.

To eliminate the possibility that the GFP tag affects the subcellular distribution of p180<sup>tsFT20</sup>GFP, we monitored the exogenous expression of untagged protein in NIH3T3 cells by immunostaining with anti-p180 antibodies. Consistent with the experiments in which the GFP-tagged constructs were used, untagged p180 entered the nucleus at the permissive temperature in the presence of p68, whereas the p180<sup>tsFT20</sup> protein

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was found exclusively in the cytoplasm (data not shown), thus indicating that the GFP tag has no effect on the observed phenotype.  

Accumulation of p180tsFT20GFP in the Cytoplasm Is Rapid, Temperature-dependent, and Reversible—To further investigate the changes in the intracellular localization of p180tsFT20GFP after temperature up-shift, we carried out time-lapse fluorescence microscopy analysis. Therefore, NIH3T3 cells transfected with p180tsFT20GFP and p68 were cultured for 48 h at 33 °C. The temperature was then shifted to 39.5 °C, kept constant using a stage heater apparatus, and the GFP signal was monitored by taking a picture every 10 min. These observations indicated that the transition from nuclear to cytoplasmic expression was complete after less than 5 h (supplemental Fig. S3A and Movie 1). The protein began to accumulate in the cytoplasm a few minutes after the temperature shift, significantly increased during the first 3 h, and then reached a plateau. Instead, wild-type p180GFP showed stable nuclear expression in control experiments (data not shown).

Next, to test whether the mutation-specific accumulation of p180tsFT20GFP in the cytoplasm is reversible, we observed cytoplasmically stained cells after incubation for 5 h at 39.5 °C, lowered the temperature back to 33 °C, and quantified the signal every 6 min. Nuclear entry rapidly occurred, and most of the p180tsFT20GFP protein was found in the nucleus within 1 h (supplemental Fig. S3B and Movie 2).

Alterations in p180tsFT20GFP Localization Involve de Novo Synthesis, Nuclear Degradation, and Export—Next, we wanted to gain further insights into the mechanisms that cause the changes in temperature-dependent localization of aberrant p180tsFT20. Therefore, cells expressing p180tsFT20GFP in the presence of p68 were treated with specific inhibitors such as CHX, LMB, and MG132. For this, NIH3T3 cells were co-transfected with p180tsFT20GFP and p68 and incubated for 48 h at 33 °C (Fig. 3A). The cells were incubated with inhibitors for 1 h, shifted to 39.5 °C for 5 h, fixed, and stained. To overcome the cytotoxicity of LMB and MG132, we seeded NIH3T3 cells at higher concentrations (3 × 10⁴ cells/well) in 8-well chamber slides in which the wells were pre-treated with poly-l-lysine for 2 h at 37 °C. To avoid detecting endogenous p180 and to increase the sensitivity to GFP-tagged proteins, we used monoclonal anti-GFP antibodies in combination with Alexa 594-la-
Indeed, when CHX was included with LMB or MG132, the nucleus (CSK protein fraction containing free proteins from the cytoplasm and aggregates (ppt) were significantly reduced, whereas MG132 treatment results in an increase of this fraction due to the inhibition of proteasome-dependent degradation. LMB treatment also led to a slight decrease in free protein expression when applied in higher concentrations. Regarding the pellet fraction, CHX and LMB had no significant effect, whereas MG132 treatment causes a dramatic increase in p180⁰⁷⁰⁰-GFP, which is presumably complexed with the similarly abundant p68. The smeared band of higher migrating bands may represent ubiquitinated p180⁰⁷⁰⁰ protein variants.

To confirm these inhibitory effects, we tested combinations of the compounds following the same protocol (Fig. 3A). Indeed, when CHX was included with LMB or MG132, the cytoplasmic expression of p180⁰⁷⁰⁰-GFP remained low and the number of cells exhibiting nuclear staining increased dramatically compared with that of untreated cells and increased slightly compared with that of cells treated only with CHX. Thus, CHX again clearly inhibited de novo protein synthesis in the cytoplasm, whereas LMB and MG132 both increased the fraction of cells with nuclear staining. When MG132 and LMB were applied together, the number of treated cells exhibiting nuclear staining was greater than that of untreated cells. However, there was no significant difference in these frequencies with respect to cells treated with either MG132 or LMB alone. Therefore, MG132 and LMB seem to affect the same protein fraction, and the export and degradation processes might be linked. Comparison of CHX-treated cells with those treated with both CHX and LMB reveals that LMB might inhibit the expression of a small fraction of cytoplasmically localized protein that is visible when only CHX but not LMB was applied. This fraction may represent exported protein.

The effect of each inhibitor was further examined by Western blot analysis using COS-1 cells (Fig. 3D). The soluble protein fraction containing free proteins from the cytoplasm and the nucleus (CSK+T) and the pellet protein fraction containing proteins bound to nuclear structures and aggregates (ppt) were analyzed. The results of this experiment confirmed that CHX significantly reduces the level of free protein, whereas MG132 treatment results in an increase of this fraction due to the inhibition of proteasome-dependent degradation.
in Fig. 4C, fluorescence of p180tsFT20PA-GFP was undetectable before photoactivation in NIH3T3 cells but could be observed upon irradiation with 400–440 nm light and time-lapse images were acquired at the restrictive temperature. Photostimulated GFP signals gradually decreased equally in the nucleus and the cytoplasm and can be detected until 200 min after photoactivation under these conditions (Fig. 4C and supplemental Movie 3). As a control, photostimulated p180sFT20PA-GFP was visualized in the nucleus, where the fluorescent intensity also decreased gradually (supplemental Movie 4). Furthermore, the cytoplasmic fraction of p180sFT20 was still observed 200 min after the up-shift to the restrictive temperature, whereas nuclear p180sFT20 is totally removed from the nucleus, indicating degradation of aberrant DNA polymerase α in the nucleus. As the cytoplasmic staining remained rather constant throughout the time course, these results are in accordance with the conclusions that most of the nuclear-localized p180sFT20 is rapidly degraded in the nucleus at the restrictive temperature and that the cytoplasmic p180sFT20 seen at the restrictive temperature is mainly due to de novo protein synthesis.

**Temperature-dependent Interaction between p180sFT20 and p68**—Based on the results obtained from the immunofluorescence experiments in NIH3T3 cells (supplemental Fig. S2, A and B), we considered that the mutation in p180sFT20 leads to conformational changes affecting its COOH-terminal domain. These alterations could result in an inability to bind the smaller p68 subunit and a subsequent defect in the nuclear entry. Indeed, wild-type p180GFP protein in the absence of p68 as well as wild-type H-core protein lacking the p68 binding site were both exclusively localized in the cytoplasm (supplemental Fig. S2, A, B, and D). To test whether failure of newly synthesized p180sFT20GFP protein to bind p68 causes its accumulation in the cytoplasm, we carried out co-immunoprecipitation experiments. Therefore, we co-expressed p180GFP or p180sFT20GFP with p68 and then precipitated the p180-associated p68 from cell lysates using anti-GFP antibodies. This experiment showed that although both p180GFP and p180sFT20GFP interact with p68 at the permissive temperature, the amount of p68 co-precipitating with p180sFT20GFP was significantly reduced at the restrictive temperature (Fig. 5A), suggesting that the binding capacity of p180sFT20GFP to p68 is weakened under these conditions. To further examine the effect of temperature up-shift on the interaction between p180sFT20 and p68, immunoprecipitated complexes containing p180sFT20 and p68, which were expressed at the permissive temperature, were incubated for 1 h at 39.5 or 4 °C, and associated p68 was then detected by Western blot analysis. As shown in Fig. 5B, both p180 and p180sFT20 associated with p68 at 39.5 or 4 °C, suggesting that once p68 and p180sFT20 bind at the permissive temperature, the heterodimer is stable even at the restrictive temperature. We should note here that the amounts of p68 retained at 39.5 °C are significantly lower than those at 4 °C, even for the wild-type p180 subunit, probably because the interaction between p180 and p68 is less strong at 39.5 °C, especially when the p180 subunit is bound to the anti-p180 antibody. Taken together, the reduced level of p68 complexed with p180sFT20GFP at the restrictive temperature as shown in Fig. 5A might be caused by the inability of newly synthesized p180sFT20GFP to bind p68 rather than to the dissociation of pre-existing p180sFT20/p68 heterodimers. We conclude that the p180sFT20 mutation leads to conformational changes that prevent newly synthesized p180sFT20 from binding p68, thus causing the subsequent defect in nuclear entry.

**Depletion of Endogenous p68 by siRNA Causes a Decrease of Overall Endogenous p180 Protein but Specific Accumulation of Cytoplasmic p180**—Previously, we reported that p68 facilitates both production and nuclear translocation of p180 (14). We showed that overexpressed p68 stabilizes p180 expression at the post-transcriptional level and in addition activates the nuclear localization signal of p180 at the COOH-terminal region. As all these data were based on a transiently transfected cDNA expression system, we want to confirm the roles of p68 in p180 expression and localization at the physiological level by depleting p68 with siRNA oligonucleotides. Four different oligonucleotides were designed and transfected into NIH3T3 cells. As shown in Fig. 6, A and B, the expression level of p68 was reduced after two rounds of transfection with three of four siRNAs revealed by Western analysis (si68-2, -3, and -4). Interestingly, the overall protein level of p180 is also decreased by depletion of p68, whereas the expression levels of Mcm7 and proliferating cell nuclear antigen are not altered. Next, to examine whether p180 localization is affected by depletion of p68, immunofluorescence analysis was performed. NIH3T3 cells were transfected with siRNAs, fixed with formaldehyde, and stained with anti-p180 and anti-p68 antibodies. Transfection with the control oligonucleotide exhibited nuclear localization of p180 and nuclear and cytoplasmic localization of p68. When siRNAs were introduced into NIH3T3 cells, the immunofluorescence signal of p68 could be reduced successfully. In conjunction with the depletion of p68, the localization pattern of p180 changed from nuclear to cytoplasmic expression. Taken together, these results indicate that endogenous p68 indeed ensures stable expression and nuclear localization of p180.
Moreover, the subcellular localization of p68 has not been characterized so far. As we previously detected small amounts of monomeric p68 in NIH3T3 cells by glycerol density gradient sedimentation (46), the cytoplasmic expression of p68 observed in this study may represent an excess of its monomeric form.

**DISCUSSION**

**Quality Control of DNA Polymerase α Is Crucial for Maintaining Genome Integrity**—Several lines of evidence suggest that the quality of DNA polymerase α is critical for maintaining genome integrity. First, we previously found that tsFT20 cells transiently cultured at the restrictive temperature showed general and massive chromosomal aberrations, including chromatid gaps and breaks, chromosome pulverizations, and ring chromosomes (23). Second, tsFT20 cells cultured at the semi-permissive temperature exhibited alterations of telomeric DNA and chromatin structure (50). Third, in yeast cells with reduced permissive temperature exhibited alterations of telomeric DNA chromosomes (23). Second, tsFT20 cells cultured at the semi-matid gaps and breaks, chromosome pulverizations, and ring general and massive chromosomal aberrations, including chro-transiently cultured at the restrictive temperature showed genome integrity. First, we previously found that tsFT20 cells at the restrictive temperature and is excluded from the nucleus. We with anti-p180, anti-p68, anti-proliferating cell nuclear antigen (PCNA), and anti-Mcm7 antibodies. B, subcellular distribution of p180 in p68-depleted NIH3T3 cells was observed by immunofluorescence analysis. Cells were transfected with p68 siRNA twice and fixed 48 h after transfection. Endogenous p180 and p68 were detected by anti-p180 polyclonal and anti-p68 monoclonal antibodies (E4 (54)), DNA was stained with Hoechst 33258.

FIGURE 6. Effect of RNA interference-mediated depletion of p68 on endogenous p180 expression and localization. A, NIH3T3 cells were transfected with control or four different oligonucleotides designed to deplete p68. Transfection was repeated once after 24 h. Cells were then grown 48 h after siRNA transfection, and proteins were extracted with CSK buffer containing Triton X-100. An extract with control oligonucleotides was serially diluted to compare the expression levels. Western analysis was carried out with anti-p180, anti-p68, anti-proliferating cell nuclear antigen (PCNA), and anti-Mcm7 antibodies. B, subcellular distribution of p180 in p68-depleted NIH3T3 cells was observed by immunofluorescence analysis. Cells were transfected with p68 siRNA twice and fixed 48 h after transfection. Endogenous p180 and p68 were detected by anti-p180 polyclonal and anti-p68 monoclonal antibodies (E4 (54)), DNA was stained with Hoechst 33258.

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**Quality Control of DNA Polymerase α Is Crucial for Maintaining Genome Integrity**—Several lines of evidence suggest that the quality of DNA polymerase α is critical for maintaining genome integrity. First, we previously found that tsFT20 cells transiently cultured at the restrictive temperature showed general and massive chromosomal aberrations, including chromatid gaps and breaks, chromosome pulverizations, and ring chromosomes (23). Second, tsFT20 cells cultured at the semi-permissive temperature exhibited alterations of telomeric DNA and chromatin structure (50). Third, in yeast cells with reduced protein levels of DNA polymerase α, highly elevated frequencies of chromosome translocations and chromosome loss was observed (35). Thus, the protein level as well as integrity of DNA polymerase α must be accurately controlled to avoid genome instability. In this report, we show that control systems for DNA polymerase α exist to ensure its proper quality in the nucleus.

**Aberrant p180tsFT20 Is Excluded from the Nucleus**—As protein control mechanisms regulating the fate of aberrant proteins are largely unknown in the nucleus, we decided to look at p180 as a central nuclear component of the DNA polymerase α complex. We took advantage of a p180 variant harboring a point mutation that results in phenotypes specifically upon temperature up-shift to the restrictive temperature. Unexpectedly, we found that endogenous as well as transiently over-expressed p180tsFT20 strictly localizes to the cytoplasm at the restrictive temperature and is excluded from the nucleus. We therefore defined p180tsFT20 as a suitable model substrate that is prone to detection by nuclear quality control mechanisms. In the tsFT20 cell line, p180tsFT20 is the only source of p180 at the restrictive temperature and therefore deleterious for the cells. However, it is very likely that in wild-type cells, those p180 molecules that are misfolded and show aberrant structures are cleared from the nucleus by the same mechanisms. Notably, such mechanisms seem to occur in two different compartments, at the nuclear entry step in the cytoplasm as well as via degradation in the nucleus (Fig. 7).

**Control Mechanisms for Nuclear Entry**—Time-lapse observation of living cells and the effects of treatments with different inhibitors suggested that following a temperature up-shift to 39.5 °C, de novo synthesized p180tsFT20GFP protein is sequestered in the cytoplasm. We could show in several experiments in this and previous studies that the smaller DNA polymerase α subunit p68 is crucial for this observation. First, newly synthesized p180tsFT20GFP is significantly impaired in binding p68 and consequently inhibited nuclear localization signal exposure, newly synthesized mutant p180 accumulates in the cytoplasm, whereas nuclear-localized mutant protein is directly degraded, both in the nucleus as well as to a smaller extent in the cytoplasm after export.
bled p180-p68 heterodimers enter the nucleus, thereby excluding aberrant proteins from nuclear import. We speculate that this mechanism is a common concept in cell biology to assure the stoichiometry and correct folding of multiprotein complex subunits in the nucleus.

Proteasome-dependent Degradation of Aberrant Nuclear Proteins—In addition to cytoplasmic sequestration of aberrant DNA polymerase α, the levels of nuclear-localized mutant protein decrease rapidly in a separate process apparently due to proteasome-dependent degradation in the nucleus. This is suggested by the findings that inhibition of proteasome-dependent degradation leads to aggresome formation in the nucleus, that nuclear endogenous p180<sup>SFT20</sup> is degraded independently of nuclear export, and that experiments with PA-GFP tagged p180<sup>SFT20</sup> exhibit degradation of nuclear-localized p180<sup>SFT20</sup> in the nucleus at the restrictive temperature. Although proteasomes are also found in the nucleus (40, 41), degradation-mediated protein quality control in higher eukaryotic nuclei has not been demonstrated. Recently, it was reported that San1p-mediated degradation functions in quality control in yeast nuclei (45). San1 is a ubiquitin ligase that has exquisite specificity for aberrant proteins in the nucleus. The authors propose that San1-mediated degradation acts as the last line of proteolytic defense against the deleterious accumulation of improperly folded nuclear proteins. Thus, it is interesting to speculate that aberrant DNA polymerase α in mammalian nuclei is recognized by a similar protein quality control system finally leading to its clearance from the nucleus.

How Might Such Protein Control Mechanisms Work?—Aberrant nuclear proteins have to be at first recognized as being malformed (e.g. by chaperones due to exposed hydrophobic patches). Afterward, the malformed subunits have to be labeled with ubiquitin for protein degradation (mediated, e.g. by San1 in <i>S. cerevisiae</i> or chromatin immunoprecipitation and yet unknown E3 ligases in higher eukaryotes), extracted from a multisubunit complex (e.g. by Cdc48 in <i>S. cerevisiae</i> or p97 in higher eukaryotes), and finally delivered to nuclear proteasomes for degradation. Apart from this direct nuclear degradation, the exposition of normally hidden domains in aberrant proteins might directly lead to their recognition by the nuclear export machinery as a minor parallel pathway. Additionally, the nuclear entrance step demonstrates a quality control mechanism for DNA polymerase α in such a way that only intact p180-p68 heterodimeric complexes are permitted for nuclear entry, whereas newly synthesized aberrant DNA polymerase α is retained in the cytoplasm. This could indeed be a general mechanism, which holds true for other nuclear protein complexes. In such a model, only those proteins, which are fully expressed and able to form complexes with other subunits are capable for nuclear entry. If this stabilization by complex formation with other subunits does not occur, the protein is rapidly targeted for degradation in the cytoplasm. Especially for essential and accurately regulated proteins like proteins that are involved in DNA replication, such control mechanisms are of high value. Concerning other B-type DNA polymerases, DNA polymerase δ and ε both contain a second largest subunit similar to p68, which might have an analogous function in stabilization and nuclear entry of the largest subunit in the complex.

In this context, it is further interesting to note that in temperature-sensitive yeast Mcm10 mutants, depletion of Mcm10 during S phase results in degradation of DNA polymerase α without affecting other fork components (36). Thus, the nuclear expression level of DNA polymerase α may be tightly controlled at the nuclear entry step as well as by protein degradation in the nucleus, and Mcm10 may be directly involved in the quality control of DNA polymerase α. In addition, several further potential “substrates” (other than DNA polymerase α) of such control mechanisms have been mentioned in recent reports, thus underlining the biological significance of nuclear protein quality control. First, we believe that the phenomenon of nuclear exclusion of aberrant proteins does not only apply for DNA polymerase α, but also for other nuclear multisubunit complexes such as ORC, MCM, GINS, and other replicative or translesion DNA polymerases. In accordance, a recent paper (51) describes the protein stability of 8000 human proteins and found that cell cycle control categories including proteins in DNA replication are classified into a short-medium half-life group. Second, we believe that not only aberrant proteins but also basal proteins are recycled during cell cycle progression. Indeed, DNA polymerase β and α are controlled by ubiquitination and degradation and seem to be very unstable proteins under basal conditions (52, 53). Such protein turnovers in the nucleus might be intimately linked with protein quality control mechanisms.

In summary, our results explain that many phenotypes associated with the original tsFT20 cell line, which are severely defective in DNA replication and cell cycle progression are probably due to the strict exclusion of aberrant p180<sup>SFT20</sup> (the only source of p180 in these cells) from the nucleus. However, more importantly, our study suggests two distinct concepts of how the pool of malformed proteins in wild-type cells might generally be prevented from functioning inappropriately in the nucleus. First, in the cytoplasm, import of correctly folded subunits is assured by only allowing the import of protein complexes (as shown here for p180-p68 heterodimers). Second, proteasome-dependent degradation clears defective proteins in the nucleus. These mechanisms might very well hold true for assuring the functionality of other nuclear protein complexes.

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