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Quality Control of DNA Polymerase α

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1. Introduction

In the human genome, at least 14 DNA polymerases were identified. 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 (Bell and Dutta 2002, Hubscher et al. 2002, Waga and Stillman 1998, Wang 1991). Moreover, DNA polymerase α plays a fundamental role in coordinating DNA replication, DNA repair and cell cycle progression (Hubscher et al. 2002), in telomere capping and length regulation (Adams Martin et al. 2000, Dahlen et al. 2003, Grossi et al. 2004, Qi and Zakian 2000), and in the epigenetic control of transcriptional silencing and nucleosome reorganization (Nakayama et al. 2001, Zhou and Wang 2004).

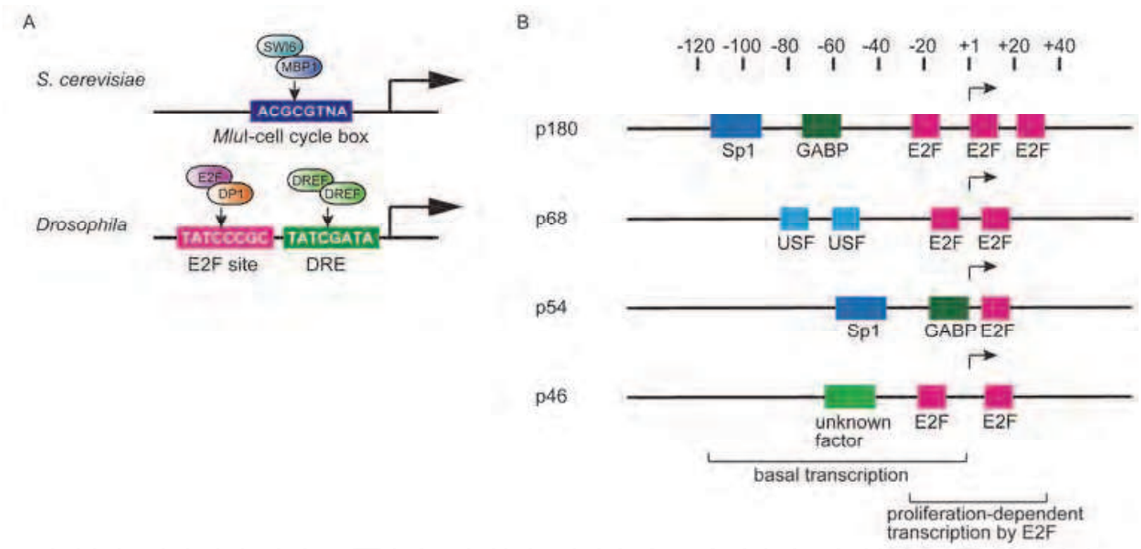
The DNA polymerase α-primase complex consists of four subunits, each of which is conserved in eukaryotes; in yeast, all four subunits are essential for viability (Wang 1991). The largest subunit, 180 kDa (p180), harbors the catalytic polymerase activity. The two smallest subunits, 54 kDa (p54) and 46 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 (Arezi and Kuchta 2000). 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 (Foiani et al. 1994, Mizuno et al. 1998).

In this review, we discuss several layers of control that exist to ensure proper functioning of DNA polymerase α in the cell: regulation of transcription via *cis*- and *trans*-acting elements (part 2), ordered assembly of its four subunits into a functional complex (part 3.1), regulation of nuclear import (part 3.2), co-translational regulation (part 3.3), and quality control mechanisms to exclude aberrant proteins from acting in the nucleus (part 4).

2. Transcriptional regulation of DNA polymerase α

In eukaryotes, the expression of the genes encoding DNA replication proteins is tightly coupled with cell cycle control. In budding yeast, more than 20 genes involved in DNA

replication and nucleotide metabolism (DNA polymerases, thymidylate synthase, ribonucleotide reductase, DNA topoisomerase II, etc.) show periodic fluctuations in mRNA levels during the cell cycle (McIntosh 1993). These genes have a common *cis*-acting element called the *Mlu*I-cell cycle box (MCB) in their 5′ non-transcribed regions (Fig.1A). In late G1 shortly after the execution point, the transcription factor SWI6, which is activated by $p34^{CDC28}$, binds to MCB with MBP1 (*Mlu*I-binding protein 1) to transactivate a set of genes. Also in higher eukaryotes, MCB-like elements control DNA replication-related genes. For instance in *Drosophila*, DNA replication–related elements (DREs) and E2F-binding sites are identified in the promoter regions of DNA replication factors including polymerase α, PCNA, and Orc2 and involved in proliferation-dependent expression (Matsukage et al. 2008). Although the human homolog of DRE-binding factor (DREF) is reported as a putative positive transcriptional factor, it is unknown whether human DREF is involved in the transcriptional regulation of DNA replication factors. On the other hand, E2F-binding sites are generally found in the upstream regions of DNA replication-related genes as well as proliferation-related genes such as c-*myc*, N-*myc*, and c-*myb* protooncogenes in mammalian cells (Muller and Helin 2000). E2F transcription factors are activated by release from retinoblastoma protein (Rb), which is hyperphosphorylated by cyclin-dependent kinase (CDK) at the G1 to S transition.



A. Swi6 interacts with MBP1 at *Mlu*I-cell cycle box sequence and regulates proliferation-dependent transcription in budding yeast, while DREF homodimer as well as E2Fs are involved in *Drosophila*. B. The promoter regions of the four subunits of mouse DNA polymerase α are presented. The E2F-binding sites are localized adjacent to the transcription initiation sites and involved in proliferation-dependent transcription, whereas constitutive transcription is controlled by different factors.

Fig. 1. Transcriptional regulation of DNA replication-related genes.

As for DNA polymerase α, the mRNA and protein levels of all four subunits are coordinately regulated in mammalian cell (Miyazawa et al. 1993). The mRNA levels of quiescent cells (i.e. prior to DNA replication) become 10-fold upregulated when stimulated to proliferate and to enter S phase. We isolated the promoter region of the four subunits of mouse DNA polymerase α and identified *cis*-acting elements and *trans*-activating factors

(Izumi et al. 2000, Nishikawa N. et al. 2001, Nishikawa N. S. et al. 2000). The mechanism of transcriptional regulation of the four subunits have common features as summarized in Fig. 1B. The upstream sequences of all four DNA polymerase α subunits exhibit a high G/C content which is similar to many other housekeeping genes and lack TATA or CAAT boxes. In addition, basal transcription and the proliferation-dependent transcription can be distinguished as a common theme for the regulation of each subunit and are controlled by a different set of transcription factors and *cis*-regulatory elements. A unique factor that stimulates proliferation-dependent transcription of all four DNA polymerase α subunits is E2F. E2F binding sites are located near the transcription initiation sites and 20-90 bp downstream of the *cis*-elements for basal transcription. Other than the common feature of proliferation-dependent transcription of each subunit by E2F, the regulation of basal transcription of each subunit seems to be subunit-specific. Namely, GA-binding protein (GABP), a member of Ets-binding transcription factors, and Sp1 are involved in the transcription of p180 and p54, whereas USF (upstream transcription factor) is involved in the transcription of p68. Interestingly, GABP is shown to be involved in the transcriptional control of Skp2, which is required for degradation of CDK inhibitors including p21 and p27 (Yang et al. 2007). Therefore, GABP may regulate the cell cycle-dependent expression of DNA polymerase α by a pathway that is distinct from E2F.

3. Functional analysis of individual DNA polymerase α subunits

3.1 Complex assembly and domain organization of DNA polymerase α

In order to functionally analyse subunit assembly, cDNAs of each DNA polymerase α subunit were subcloned into mammalian expression plasmids and expressed in cultured mammalian cells. To examine interactions among the four subunits, we transfected four subunits in various combinations, and tested interactions of co-expressed subunits by co-immunoprecipitation analysis (summarized as a cartoon in Fig. 2; Mizuno et al. 1998, 1999). A crucial tool to express different combinations of subunits from a single plasmid was the introduction of IRES (Interal Ribosomal Entry Site) elements, which allowed analysis of subunit interdependency concerning protein expression and subcellular localisation (Fig. 4). Moreover, a set of deletion constructs of p180 revealed that p180 can be divided into three domains (Fig. 2). 1) The amino-terminal (N-terminal) region is not only dispensable for interaction to other subunits but also nonessential for DNA polymerase activity. In addition, it has been reported that the N-terminal domain serves as a binding site for other replication factors (large T antigen, RPA, CDC13, Pol32, Mcm10). 2) The central core-domain is solely responsible for DNA polymerase activity, namely, single-stranded DNA binding activity, and deoxynucleotide tripohosphate binding activity as well as phosphoryl-transfer catalytic reaction. 3) The carboxyl terminal (C-terminal) domain is a binding site for other DNA polymerase α subunits. It contains a conserved cystein rich region, which resembles most closely the iron-binding motif found in rubredoxin (Klinge et al. 2009). These cluster of cysteins are essential for binding to the second-largest subunit. Primase activity is located in the smallest subunit, p46. This subunit does not directly bind to p180 or p68, but is tethered to p180 via a fourth subunit, p54.

DNA polymerase α belongs to the B-type (α-type) DNA polymerase family, and we speculate, that domain arrangement of the largest, catalytical subunit as well as general complex architecture of DNA polymerase α might well be conserved among members of this family like polymerases α, δ, ε and ζ (Fig. 3). All B-type polymerases have a cystein rich

C-terminal region and an accompanying small non-catalytic subunit. In the catalytic subunit, the sequence and order of six short amino acid motifs (I-VI) is also highly conserved (Fig. 3). Molecular insights gained from the analysis of DNA polymerase α might indeed hold true for each member of the B-type polymerase family. In addition, the second-largest subunit p68 and p50 for polymerase δ, and p59 for polymerase ε share significant homology (Mäkiniemi et al. 1999) suggesting that accompanying subunit may as well have common conserved function.

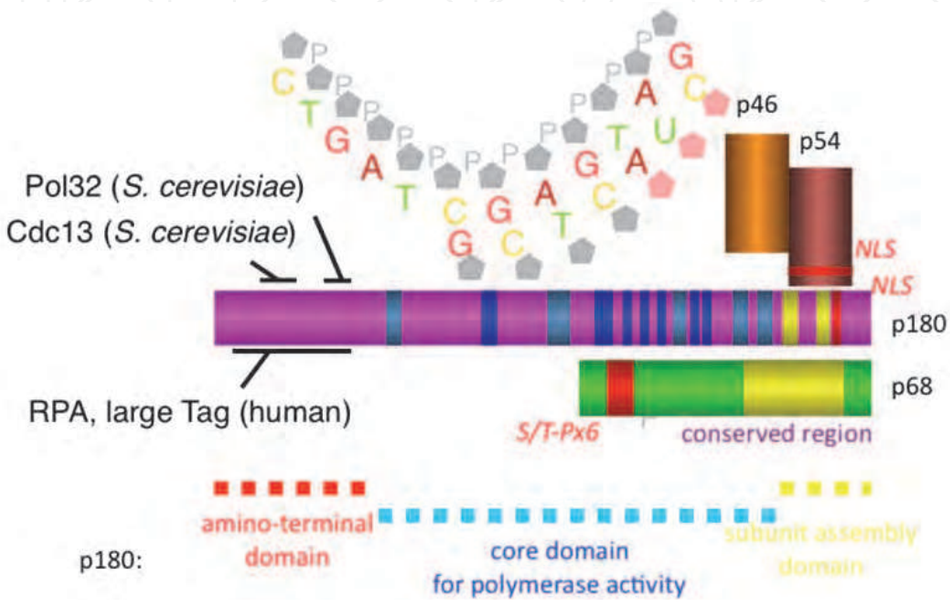


Fig. 2. Organization of four subunits of mouse DNA polymerase α-primase complex. Functional domains of the catalytic subunit p180 are divided into three domains. Other accompanied subunits assemble through the C-terminal domain of p180. The primase catalytic subunit p46 associates with the C-terminal part of p180 through p54. The N-terminus serves as a docking site for various interactors (e.g. Pol32, Cdc13 or RPA), while the core region possesses catalytic polymerase activity. Nuclear localization signal (NLS)s are localized in C-terminus of p180 (Mizuno et al, 1998) and N-terminus of p54 (Mizuno et al. 1996), and depicted as red lines.

3.2 Nuclear translocation of DNA polymerase α depends on its subunit interactions

Multi-protein complex organization seems to be similar among B-type DNA polymerases. We chose DNA polymerase α to carefully analyse subunit organisation, protein expression and sub-cellular organisation of single subunits and all possible combinations to gain insights into common features of complex assembly and function. For this, we co-expressed combinations of subunits and examined sub-cellular distributions by immunofluorescence techniques (Fig. 4). Interestingly, nuclear entry and accumulation of p180 is strictly dependent on co-expression of p68. In the absence of p68, p180 does not translocate into the nucleus (Fig. 4; Mizuno et al. 1999). One hypothetical explanation is the notion that an NLS at the C-terminal part of p180 is only exposed when p68 is bound. A basic amino acid cluster, which might be responsible for nuclear entry, is well conserved among metazoan. However, a potential NLS of p180 in *S. pombe* has been identified at its N-terminal region

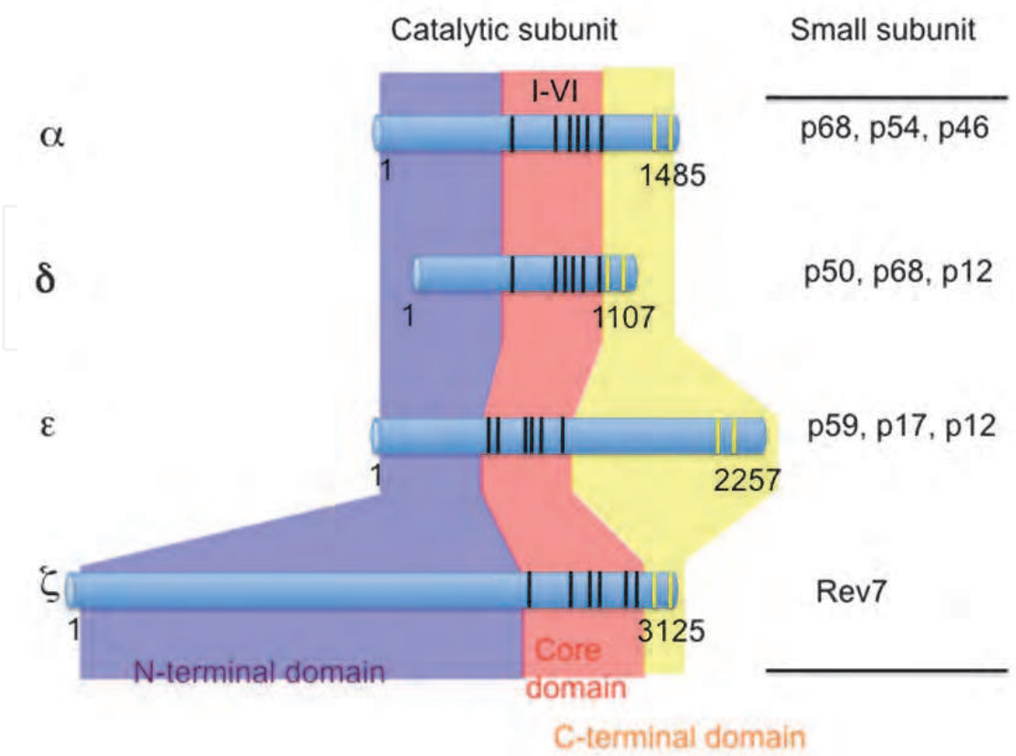


Fig. 3. B-type DNA polymerase family in mammalian cells. Proposed domain organization of catalytic subunits is depicted. Functional domains of catalytic subunit p180 are divided into three domains. Number of amino acids for catalytic subunit and highly conserved polymerase catalytic motifs I-VI (black bars) and cysteine rich motif (yellow bars) are shown.

(Bouvier D and Baldacci, 1995). Also recently, the crystal structure of the C-terminal part of p180 in *S. cerevisiae* suggested that at least in yeast, the C–terminus of yeast p180 is not responsible for nuclear entry (Klinge et al. 2008).

During the course of our studies, we also found a dependency p54 and p46 to enter into the nucleus (Fig. 4; Mizuno et al. 1997). The general idea emerging from these experiments is a necessity of regulated subunit pre-assembly to expose functional NLS and trigger subsequent nuclear translocation. This mechanism might potentially ensure the right protein stoichiometry of nuclear multi-subunit complexes such as DNA polymerase α.

3.3 Co-translational interaction of p68 with p180 enhances p180 protein expression

Our extensive analysis of co-expressing several subunits of polymerase α also revealed that p180 expression is strictly dependent on co-expressed p68. To clarify the effect of p68 on p180 protein expression, we analyzed transcriptional levels as well as the protein turn-over rate in the presence or absence of p68, and found that p68 affects protein expression rate or protein stability at a post-transcriptional level (as mRNA levels are not affected; Mizuno et al. 1998). This finding suggests an important role of p180-p68 interaction at a co-translational step to ensure full expression of each protein (Fig. 5).

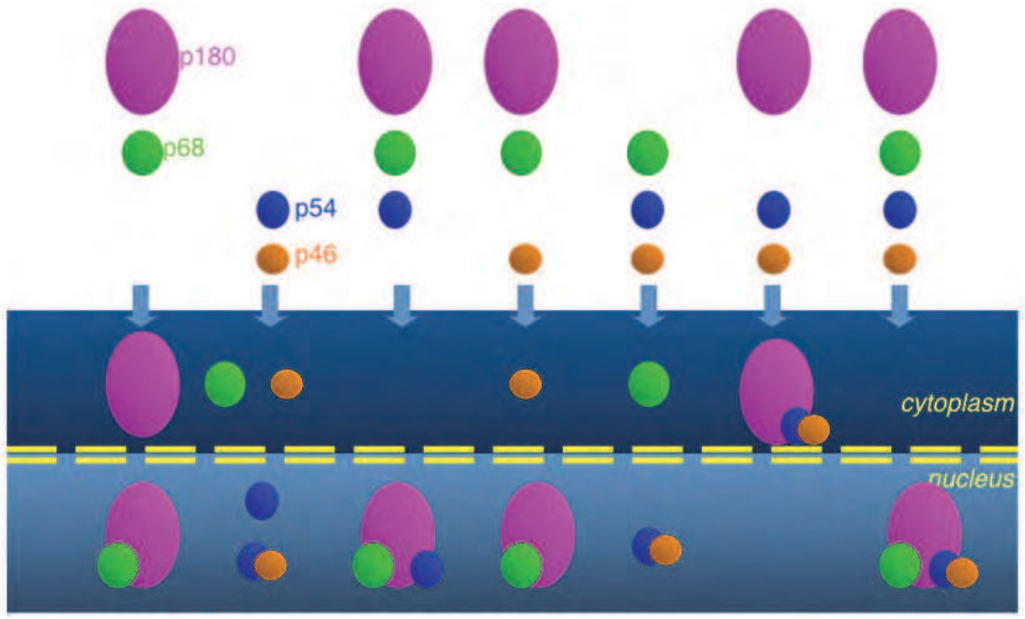


Fig. 4. Nuclear translocation of polymerase α subunits. Various combination of co-expression of four subunits revealed, that nuclear import of the catalytic subunit of polymerase α is dependent on accompanying subunits. In summary, p68 or p54 are essential for p180 or p46 nuclear translocation, respectively.

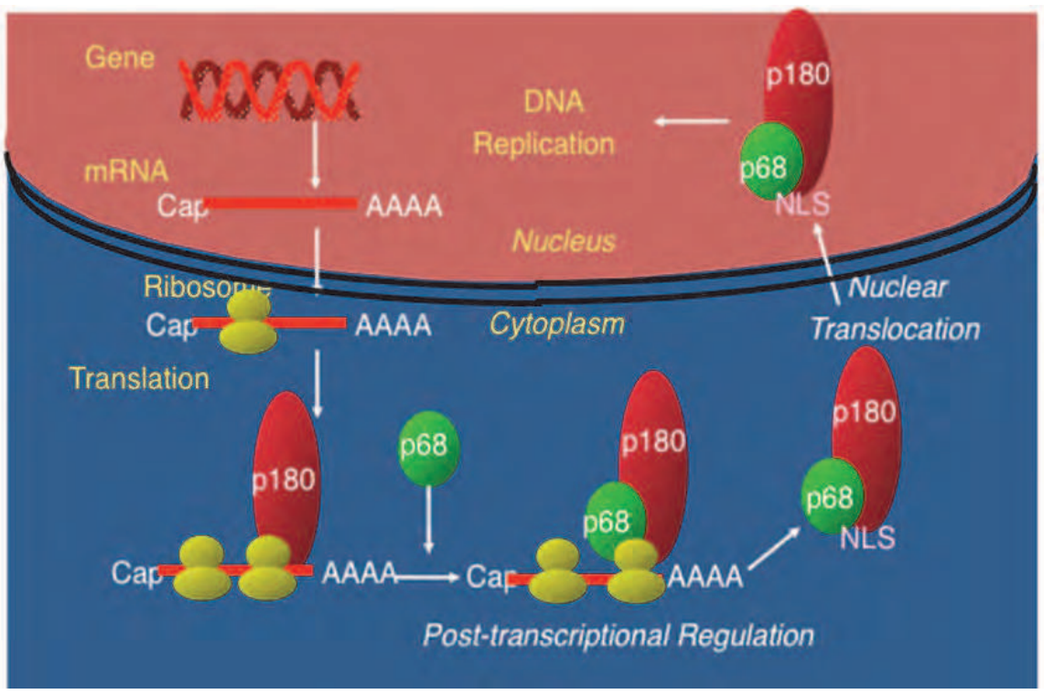


Fig. 5. The second-largest subunit p68 associated with p180 at a post-transcriptional step to enhance translation of p180 and stabilize p180 protein level. In the cytoplasm, free p68 immediately associates with p180 C-terminal region at a co-translational step and it seems association of p68 with p180 may directly stabilize and enhance translation of p180.

4. Quality control of polymerase α in the nucleus

When we found that p68 is important for both p180 protein expression and nuclear translocation, we first could not understand the entire physiological meaning of this observation. Unexpectedly, our investigation using an aberrant mutant version of p180 ($p180^{tsFT20}$) and live cell imaging techniques allowed us to recognize novel protein quality control mechanisms acting on DNA polymerase α which ensure exclusion of aberrant forms of p180 from the nucleus (Eichinger et al. 2009). In general, 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, such as the unfolded protein response and ER-associated degradation (ERAD); the latter process governs the degradation of unfolded ER proteins in the cytoplasm (Richly et al. 2005, Ye et al. 2001). Degradation systems that function in quality control have been identified in the secretory pathway and in mitochondria (Goldberg 2003). These processes are carried out by proteasomes, organelles consisting of several proteases that are located in the cytoplasm as well as in the nucleus (Bennett et al. 2005, Hershko and Ciechanover 1998). Proteasomes function in regulatory processes, allowing cells to balance protein expression and to eliminate misfolded proteins that are useless or even harmful (Hershko and Ciechanover 1998). In contrast, the degradation-dependent protein quality control mechanisms that act in the nucleus are still poorly understood. Although several proteins such as the transcription factors p53 and Myc as well as the replication-associated factors Orc1 and Cdt1 are degraded in the nucleus by a proteasome-dependent pathway over the course of the cell cycle (Nishitani and Lygerous. 2002), the principles underlying the degradation of damaged nuclear proteins have not been uncovered to date. Recently, Gardner et al. reported that San1-mediated degradation acts as a protein quality control system in *Saccharomyces cerevisiae* nuclei (Gardner et al. 2005, Rosenbaum JC, et al. 2011). However, it is still unclear how aberrant nuclear proteins are recognized by E3 proteins and whether analogous systems exist in higher eukaryotes.

4.1 tsFT20 is a model system of aberrant p180 in the cell

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 many studies during the last 30 years (Fig.6; Eki et al. 1986, Eki et al. 1987a, 1988, Eki et al. 1987b, Eki et al. 1990, Ikehata 1994, Ikehata et al. 1997, Izumi et al. 1994, Miyazawa et al. 1986, Murakami et al. 1985, Murakami et al. 1986, Pendergrass et al. 1994, Takada-Takayama et al. 1991, Yamaguchi et al. 1995). Compared to 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 conferred by arrest at the G1/S boundary. At restrictive temperature, tsFT20 cells were further characterized as defective in DNA replication, with a highly decreased DNA synthesis rate and a reduced frequency of replicon initiation. 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 (Izumi et al. 1994). Compared to the wild-type form, the heat-labile DNA polymerase activity of the mutant protein exhibits altered properties with respect to optimal pH and KCl concentration, as well as changes in the dCTP-binding site (Izumi et al. 1994, Takada-Takayama et al. 1991). Furthermore, tsFT20 cells exhibit

extensive chromosomal aberrations after incubation from two hours to 4 hours at restrictive temperature, due to the point mutation. The abnormal cessation of DNA replication in tsFT20 cells at restrictive temperature results in cell death via the induction of DNA double-strand breaks, suggesting that aberrant DNA polymerase α is cytotoxic and that a specific quality control mechanism for DNA polymerase α may be crucial to ensure genomic stability and accurate DNA replication under normal as well as stress conditions in a cell.

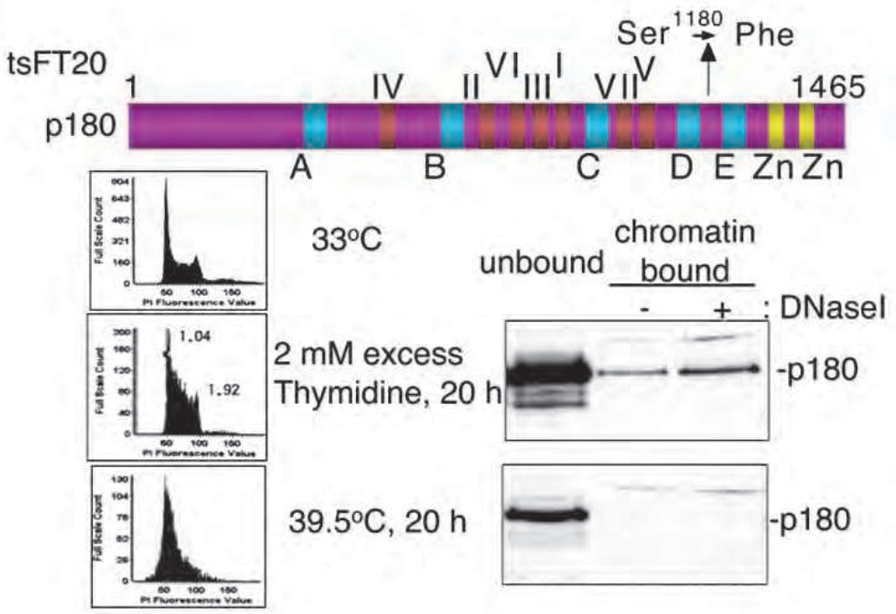


Fig. 6. tsFT20 is a temperature-sensitive mutant cell line derived from mouse FM3A cells. In tsFT20 cells, the DNA polymerase α subunit p180 contains a point mutation altering serine at amino acid position 1180 to phenylalanine. Due to this mutation, tsFT20 cells arrest at the G1/S transition at the restricted temperature (39.5°C) as shown in FACS analysis with total protein levels and chromatin bound p180 gradually decreasing as shown by Western blot analysis.

4.2 Exclusion of aberrant p180 from the nucleus due to defective complex assembly

To test localization of aberrant nuclear proteins and monitor their sub-cellular distribution directly, we constructed p180 or $p180^{tsFT20}$ with GFP. At permissive temperature (33°C), both wild-type p180GFP and mutant $p180^{tsFT20}$GFP clearly localized in the nucleus of mouse NIH3T3 cells in the presence of ectopically over-expressed p68 protein (Fig. 7A). However, at restrictive temperature (39,5°C) in the presence of p68, the wild-type protein remained in the nucleus, whereas the mutant form was found exclusively in the cytoplasm (Fig. 7B). By using co-immunoprecipitation analysis, we found that at restricted temperature, $p180^{tsFT20}$ cannot fully associate with p68, presumably due to a conformational change. Thus, we concluded that cytoplasmic expression of $p180^{tsFT20}$ at restricted temperature is caused by its deficiency to bind p68. Moreover, knock down p68 by siRNA results in expression of endogenous p180 exclusively in the cytoplasm. Taken together, both unbalanced subunit composition and defective interaction of cognate subunits cause nuclear protein sequestration in the cytoplasm.

This finding provides a novel principle of a quality control mechanism to ensure exclusion of misfolded proteins from nucleus. Very recently, Boulon *et al.* reported that RNA polymerase II subunits Rpb1 and Rpb3 accumulate in the cytoplasm when assembly is prevented (Boulon S, et al. 2011). This illustrates a similar example of cytoplasmic sequestration of subunits (of a complex normally acting in the nucleus) that are not properly incorporated into a functional complex or sub-complex. We speculate that subunit-subunit interactions within a complex or pre-assembled sub-complex play a pivotal role to confirm their correct folding state, ability to interact and stoichiometry. Inability of subunits to form pre-assemblies in the right ratio, will mark them as damaged or misfolded proteins and potentially exclude them from the cellular environment (e.g. the nucleus) where they usually act.

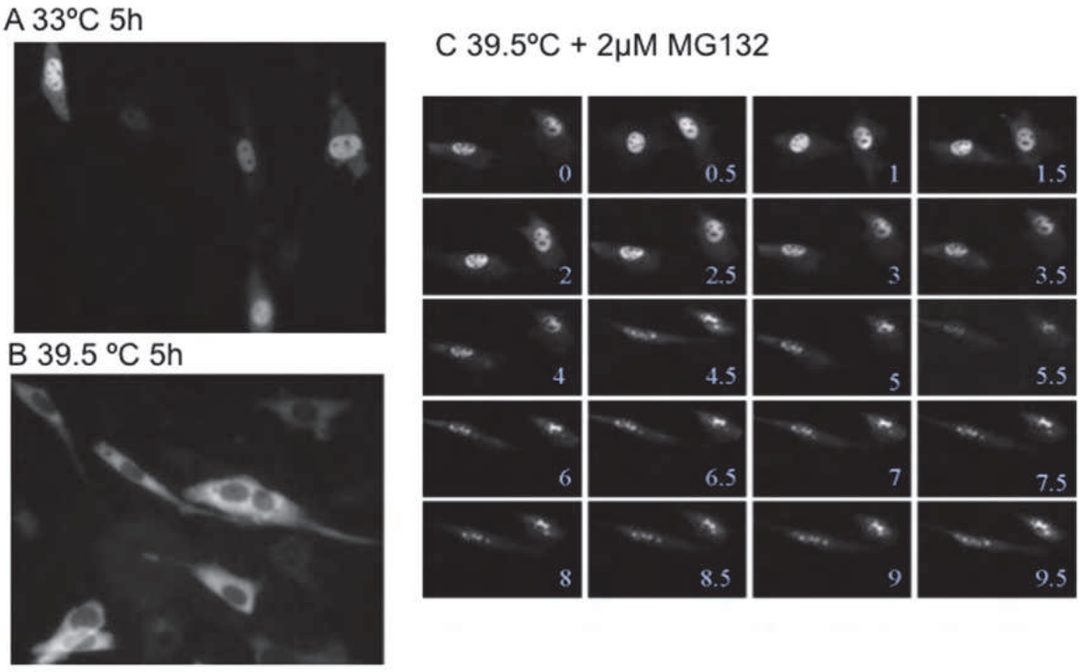


Fig. 7. Subcellular distribution of p180^{tsFT20}GFP . p180^{tsFT20}GFP is expressed in NIH3T3 cells at permissive temperature for 2 days. Afterwards, cells were incubated for further 5 hours either at permissive temperature (A) or at restrictive temperature (B). In the presence MG132, cells transfected with p180^{tsFT20}GFP were incubated at restrictive temperature for the indicated hours in a live cell time-course (C).

4.3 Degradation of aberrant proteins in the nucleus by ubiquitin-mediated proteasome system

In addition to cytoplasmic sequestration, we found that nuclear degradation of misfolded proteins is another way to exclude aberrant proteins from the nucleus. Upon temperature shift to restrictive temperature, nuclear localized p180^{tsFT20}GFP is rapidly removed from the nucleus, a process that can be blocked by addition of MG132, a specific proteasome inhibitor (Fig. 7C; Eichinger, et al. 2009). Thus, we concluded that aberrant p180 is degraded in the nucleus in a proteasome-dependent manner. Taken together, we suggest that the turnover of p180^{tsFT20}GFP after a temperature shift to 39.5˚C may be separated into distinct processes:

first, at restrictive temperature, the mutant protein is rapidly synthesized *de novo* in the cytoplasm but is unable to enter the nucleus due to a mutation-specific, temperature-sensitive effect that affects binding to the second largest subunit p68. Second, nuclear-localized protein is degraded in a proteasome-dependent manner in the nucleus and to a minor extent in the cytoplasm after export from the nucleus (Fig. 8; Eichinger, et al. 2009).
Very recently it has been reported that misfolded nuclear proteins are degraded by a unique E3 ubiquitin ligase in *S. cerevisiae*, San1 (Gardner et al. 2005; Rosenbaum JC, et al. 2011). However, the exact mechanism for such a pathway to control the quality of nuclear proteins is not known in yeast and conserved mechanisms are yet to be identified in higher eukaryotes. Given, the importance of ensuring that only properly folded and assembled multi-subunit complexes can enter and act in the nucleus; this field of biology provides many exciting open questions.

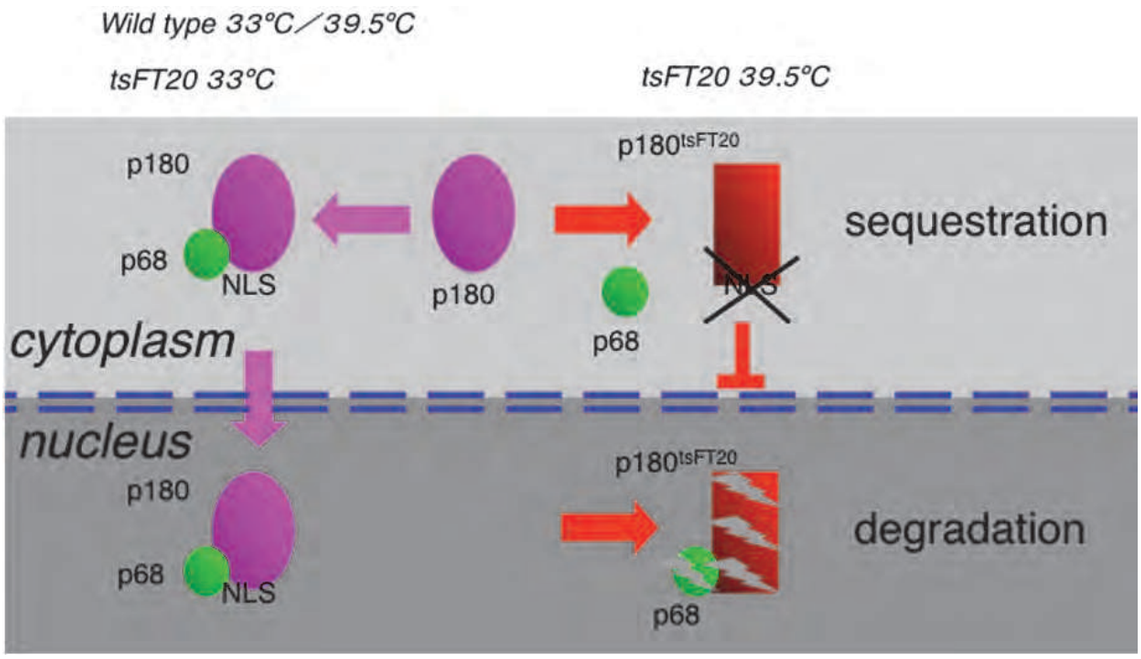


Fig. 8. Two independent quality control mechanisms to exclude aberrant proteins from the nucleus: First, sequestration of aberrant proteins in the cytoplasm, and second, degradation of aberrant proteins through ubiquitin-mediated degradation pathway in the nucleus.

5. Conclusion

DNA polymerase α, a member of the very conserved B-type polymerase family, is a crucial factor to initiate DNA replication and therefore its activity has to be tightly regulated at several stages of its life cycle. Important steps of regulation include basal- and proliferation-dependent control of transcription by *cis*-acting elements and *trans*-activating factors, coordinated complex assembly, regulation of nuclear import, co-translational control of subunit expression as well as quality control mechanisms that ensure exclusion of aberrant proteins acting in the nucleus. These principles to keep a check on such central cellular regulators like DNA polymerase α, might be a common feature shared with other B-type polymerases like DNA polymerases δ, ε and ζ.

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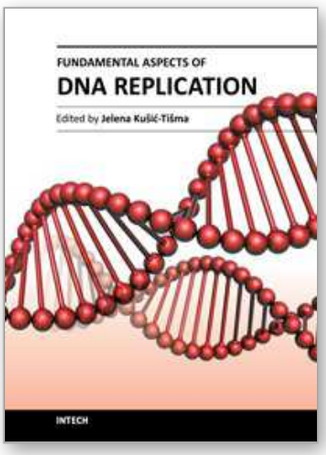

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DNA replication, the process of copying one double stranded DNA molecule to produce two identical copies, is at the heart of cell proliferation. This book highlights new insights into the replication process in eukaryotes, from the assembly of pre-replication complex and features of DNA replication origins, through polymerization mechanisms, to propagation of epigenetic states. It also covers cell cycle control of replication initiation and includes the latest on mechanisms of replication in prokaryotes. The association between genome replication and transcription is also addressed. We hope that readers will find this book interesting, helpful and inspiring.

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