Analyse des PeBoW-Komplexes in der Ribosomenbiogenese

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München, 27. März 2008

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Diese Arbeit ist meiner Familie gewidmet.

INHALTSVERZEICHNIS

1 EINLEITUNG

1.1 Vorwort

Die Bildung von Tumoren resultiert aus genetischen Veränderungen, die die Vermehrung, Differenzierung und das Überleben von Zellen kontrollieren. Die betroffenen Gene werden in Proto-Onkogene und Tumor-Suppressor-Gene unterschieden. Onkogene sind abnormal exprimierte oder mutierte Formen von zellulären Genen. Ihre Wirkungsweise ist dominant. Sie regulieren Wachstum, Teilung und Adhäsion von Zellen. Tumor-Suppressor-Gene sind rezessiv und kodieren für Proteine, die Zellteilung unterdrücken. Im weitesten Sinne kontrollieren sie die Aktivität der Onkogene. Fällt diese Kontrolle weg, induzieren Onkogene Zellproliferation und Tumorentwicklung. Viele Krebszellen zeigen chromosomale Instabilität (CIN), was das Auftreten von chromosomalen Translokationen, Duplikationen sowie Deletionen und somit Abnormalitäten in Struktur bzw. der Zahl der Chromosomen begünstigt (Lengauer et al., 1998). Hierdurch wird die Wahrscheinlichkeit der Aktivierung von Onkogenen und Inaktivierung von Tumor-Suppressoren erhöht.
Ein Beispiel für die Aktivierung eines Onkogens durch Chromosomentranslokation ist die transkriptionelle Aktivierung des *c-myc*-Gens in Burkitt-Lymphomen. Das Gen gelangt durch eine Translokation unter die Kontrolle der regulatorischen Einheiten eines der drei Immunglobulingene (Adams et al., 1983; Dalla-Favera et al., 1982; Marcu et al., 1983; Taub et al., 1982) und wird dadurch transkriptionell aktiviert. Die konstitutive Expression des *c-myc*-Gens trägt ursächlich zur Entstehung des Burkitt-Lymphoms sowie vieler anderer Tumoren bei.

1.2 Das Proto-Onkoprotein c-Myc

1.2.1 Deregulierte Expression von *c-myc* führt zur Tumorbildung

Das Proto-Onkoprotein c-Myc spielt eine Schlüsselrolle in der Regulation der Zellproliferation, Differenzierung und Apoptose (Henriksson und Lüscher, 1996). Seine Expression wird durch mitogene Faktoren aktiviert bzw. durch anti-proliferative

Signale sowie durch Induktion von Differenzierung reprimiert. Die Expression von *c-myc* in ruhenden Zellen ist ausreichend, um in Abwesenheit von Wachstumsfaktoren Zellen in den Zellzyklus eintreten zu lassen und Proliferation zu induzieren (Eilers et al., 1991; Littlewood et al., 1995). In Tiermodellen konnte der direkte Zusammenhang zwischen deregulierter *c-myc*-Expression und Tumorentstehung bewiesen werden (Adams et al., 1985; Langdon et al., 1986; Pelengaris et al., 1999). Neuere Arbeiten zeigten, dass die Deregulation von *c-myc* auch zu genomischer Instabilität führen kann (Oster et al., 2002). c-Myc kann in Abwesenheit von Wachstumsfaktoren aber auch Apoptose induzieren (Evan et al., 1992). Dabei scheint es sich um einen Rettungsmechanismus zu handeln, der hilft, die dominante Wirkung von c-Myc auf die Zellproliferation einzuschränken. Tumorzellen mit einer erhöhten Menge an c-Myc weisen daher auch häufig Defekte in Apoptosewegen auf.

1.2.2 Molekulare und biologische Funktion von c-Myc

c-Myc ist ein Transkriptionsfaktor, der zur Klasse der basischen Helix-Loop-Helix/Leucin-Zipper-Proteine (bHLH-LZ) gehört. Das Protein ist Mitglied eines großen Netzwerkes von interagierenden Transkriptionsfaktoren. c-Myc dimerisiert mit dem Helix-Loop-Helix/Leucin-Zipper-Protein Max (Zusammenfassung in Eisenman, 2001). Heterodimere dieser Proteine binden die DNA und modulieren die transkriptionelle Aktivität ihrer Zielgene. c-Myc interagiert über SNF5 mit dem SWI/SNF-Komplex (Cheng et al., 1999). Durch dessen ATPase-Aktivität kommt es zu Veränderungen des dicht gepackten Chromatins am Promotor (Chromatin-Remodellierung), wodurch der Zugang für weitere transkriptionelle Regulatoren und die RNA-Polymerase II möglich wird. Außerdem rekrutiert c-Myc das Coaktivatorprotein TRRAP („transformation/transcription domain-associated protein“) (McMahon et al., 1998) und einen assoziierten Komplex, der die Histon-Acetyltransferase (HAT) GCN5 enthält (McMahon et al., 2000). Durch Erhöhung des Acetylierungsstatus der Histone wird die Interaktion von Nukleosom und umwickelter DNA gelockert (Kingston et al., 1999; Vignali et al., 2000), die Bindung des Präinitiations-Komplexes an den aktiven Promotor ermöglicht und die Transkription gestartet (Bouchard et al., 2001; Frank et al., 2001; Gregory et al., 2001; McMahon et al., 2000). Auf der anderen Seite wurde

kürzlich beschrieben, dass c-Myc die Demethylase (JARID1-Familie) hemmt, die die Aminosäure Lysin an Position 4 des Histons H3 demethyliert, und dadurch die Aktivität von Genen aufrecht erhält (Secombe und Eisenmann, 2007; Secombe et al., 2007).

Als Antwort auf Signale der zellulären Umgebung kann c-Myc eine Vielzahl von zellulären Aktivitäten regulieren. Diese Funktionen übernimmt c-Myc durch Induktion oder Repression einer Reihe von Zielgenen. Dadurch werden separate Signalkaskaden stimuliert, die dann wiederum verschiedene biologische Aktivitäten ausführen. Nachfolgend soll die Wirkung von c-Myc auf Zellzyklus und Zellwachstum beschrieben werden.

Proliferierende Zellen müssen an Masse zunehmen bevor sie sich teilen (Polymenis und Schmidt, 1999; Thomas, 2000). Dadurch wird eine kritische Zellgröße garantiert, bevor es zur Zellteilung kommt. Die Koordination von Zellwachstum und Zellteilung findet während des Zellzyklus statt, den eine proliferierende Zelle durchläuft. Das Zellwachstum findet hauptsächlich während der G1-Phase statt. Der Punkt in der G1-Phase, an dem Zellen entscheiden, in den Zellzyklus einzutreten und sich zu teilen, wird Restriktionspunkt genannt (Pardee, 1989; Zetterberg et al., 1995). Während in ruhenden Zellen eine Expression von *c-myc* kaum stattfindet, wird diese durch Stimulation von Zellen mit Serum oder Mitogenen stark induziert. c-Myc stimuliert die Expression einiger Cycline sowie die Aktivierung der G1-spezifischen Cyclin-abhängigen Kinasen (Cdks) (Bouchard et al., 1999), wodurch der Tumor-Suppressor Rb und seine verwandten Proteine p107 und p130 inaktiviert werden (Sherr, 1994; Weinberg, 1995). Proteine der Rb-Familie (so genannte „Pocket“-Proteine) sind negative Wachstumsregulatoren. Sie binden und inaktivieren die Transkriptionsfaktoren der E2F-Familie, deren Aktivität für den Übergang der Zellen von der G1- in die S-Phase und für die DNA-Replikation essentiell ist, und reprimieren dadurch deren Zielgene (Bandara et al., 1993; Helin et al., 1993; Krek et al., 1993; Weinberg, 1995).

Während die Induktion der Zellzyklusprogression durch c-Myc in der Literatur ausführlich beschrieben wurde, ist über die Regulation des Zellwachstums durch c-Myc noch wenig bekannt. Verschiedene Untersuchungen deuten allerdings darauf hin, dass c-Myc ein von der Aktivierung des Zellzyklus unabhängiges Wachstumsprogramm induzieren kann. So wurde unter anderem gezeigt, dass die Aktivierung eines konditionalen *c-myc*-Gens in einer humanen B-Zelllinie

Zellwachstum auch dann induziert, wenn die Zellzyklusprogression durch den Cdk-Inhibitor Roscovitin blockiert wird (Schuhmacher et al., 1999). Unterstützt wird das Modell durch Untersuchungen in Mäusen mit einer konditionalen Depletion des S6-Gens, das für eine Komponente der kleinen ribosomalen Untereinheit codiert. Dort wurde gezeigt, dass Wachstum von Leberzellen, nicht aber DNA-Synthese und Zellproliferation, auch in Abwesenheit von Ribosomenbiogenese noch stattfindet (Volarevic et al., 2000). c-Myc könnte das Zellwachstum über die Aktivierung von Zielgenen regulieren, die an Stoffwechselprozessen für Zellwachstum beteiligt sind und darüber auch den Zellzyklus steuern. Daher soll an dieser Stelle auf c-Myc-Zielgene eingegangen werden (Abb. 1; Coller et al., 2000; Fernandez et al., 2003; Guo et al., 2000; Oster et al., 2002; Schuhmacher et al., 2001; www.myccancergene.org).

1.2.3 Zielgene von c-Myc

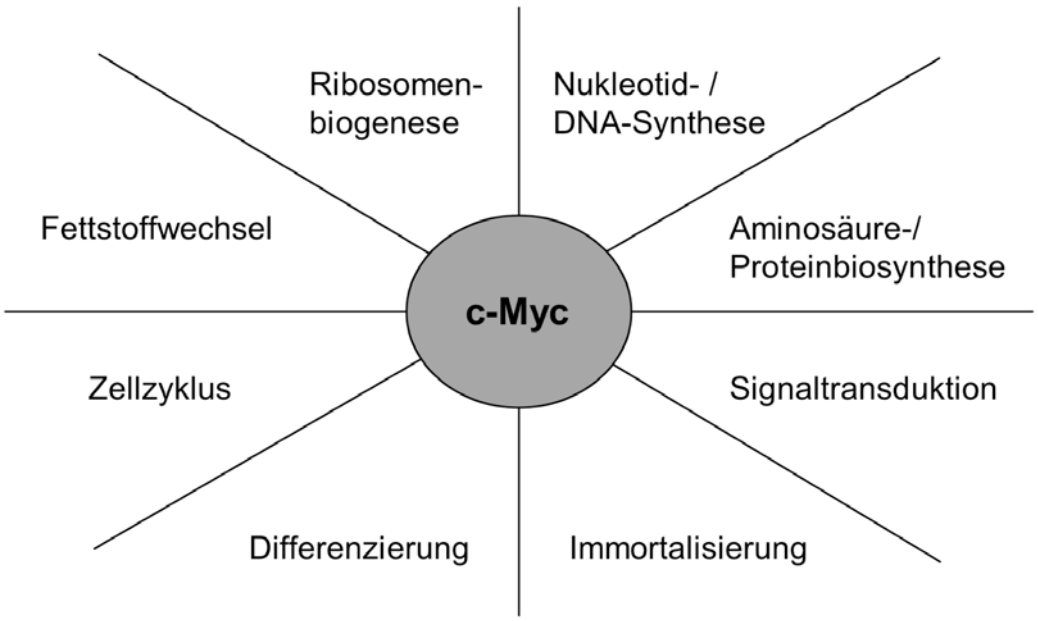

Abbildung 1. Auswahl von verschiedenen zellulären Prozessen, in die c-Myc über die Regulation seiner Zielgene eingreift.

Eines der ersten beschriebenen Zielgene von c-Myc war das *cad*-Gen für die Carbamoylphosphat Synthetase-Aspartat Transcarbamoylase-Dihydroorotase (CAD) (Miltenberger et al., 1995), dessen Produkt am Metabolismus, an der DNA-Reparatur sowie der Nukleotid- und DNA-Synthese beteiligt ist. Zusätzlich reguliert c-Myc die Expression der DNA-Replikationsfaktoren RFC4 und MCM4 sowie der DNA-Polymerase δ (Schumacher et al., 2001). Der Transkriptionsfaktor beeinflusst die DNA-Synthese auch indirekt, indem er an vielen Stellen in der Nukleotid-Synthese involviert ist. c-Myc reguliert u.a. die Expression der Gene für die PRPP Synthetase Untereinheit II, UMP-Kinase, CTP-Synthetase, Adenylat-Kinase, Dihydroorotat-Dehydrogenase und Thioredoxin-Reduktase. Nukleotide sind nötig für die Replikation des Genoms und die Transkription der genetischen Information in RNA. ATP ist aber auch der universelle Energielieferant in der Zelle, während GTP als Energiequelle für ausgewählte biologische Prozesse dient. Des Weiteren dient ATP als Donor für Phosphorylgruppen, welche durch Proteinkinasen übertragen werden. Nukleotidderivate wie UDP-Glucose sind Teil von biosynthetischen Prozessen wie der Bildung von Glykogen. Schließlich sind Nukleotide wie zyklisches AMP und GMP essentielle Komponenten von Signaltransduktionswegen, da sie die Signale innerhalb und zwischen Zellen vermitteln. c-Myc scheint damit sehr global in den Energiehaushalt einer Zelle einzugreifen.

Zahlreiche c-Myc-Zielgene sind an der Regulation der Proteinsynthese beteiligt. c-Myc induziert die Expression der Gene der Translationsfaktoren ElF3 und EEF-2 und einer Untereinheit der ribosomalen S6 Proteinkinase, die für die Initiation der Translation wichtig ist (Schuhmacher et al., 2001; Thomas und Hall, 1997).

Sowohl der Auf- als auch der Abbau von Fettsäuren wird durch c-Myc u.a. durch die Induktion der Bildung von Fettsäure-Synthase, Fettsäure-Coenzym-A-Ligase, Enoyl-Coenzyme-A-Hydratase beeinflusst (Schumacher et al., 2001). Fettsäuren besitzen unterschiedliche Aufgaben. Sie sind Komponenten von Phospholipiden und Glykolipiden, die wichtige Bestandteile biologischer Membranen sind. Durch kovalente Modifikation mit Fettsäuren lokalisieren viele Proteine an/in Membranen. Fettsäuren werden in Form von Triacylglycerol gespeichert. Aus diesen können sie mobilisiert und oxidiert werden, um den Energiebedarf eines Organismus zu decken. Zuletzt können Fettsäuren als Hormone und intrazelluläre Botenstoffe dienen.

Schlosser et al. (2003) zeigten in einem globalen Screen mit Oligonukleotid-Genchips, dass Gene mit nukleolärer Funktion die größte Klasse c-Myc regulierter

Gene darstellen. Viele dieser Gene besitzen Homologe in der Hefe mit bereits bekannter Funktion. Die Mehrzahl dieser Gene spielt in der Hefe eine Rolle in der Ribosomenbiogenese. Diese Beobachtung war Anlass, den Focus unseres Labors auf die Untersuchung der Rolle von c-Myc in der Ribosomenbiogenese zu legen. Daher soll im folgenden Kapitel auf die Ribosomenbiogenese eingegangen werden.

1.3 Ribosomenbiogenese in Eukaryonten

Es ist schon lange bekannt, dass in Tumorzellen Komponenten der Translationsmaschinerie häufig dereguliert exprimiert sind. Obwohl Veränderungen des Nukleolus als verlässlicher Marker von zellulärer Transformation schon sehr früh beschrieben wurde (Gani, 1976), ist diese Beobachtung bisher nicht eingehend untersucht worden. Langsam verändert sich dieses Bild nun, da gezeigt wurde, dass zahlreiche Proto-Onkogene und Tumor-Suppressoren die Ribosomenbiogenese bzw. die Initiation der Proteintranslation direkt regulieren. Daher könnte eine veränderte Regulation der Translation und der Proteinsynthesemaschinerie nicht nur eine Folge sondern auch eine Ursache für Krebsentstehung sein (Ruggero und Pandolfi, 2003).
So führen zum Beispiel Mutationen in dem Gen für das ribosomale Protein S19 zu Diamond-Blackfan Anämie (Draptchinskaia et al., 1999). Dieses Syndrom ist durch eine erhöhte Veranlagung zur Tumorbildung charakterisiert. Auf welcher Ebene Mutationen im S19-Gen für Tumorentstehung prädisponieren ist unklar. Daher sind Untersuchungen zum besseren Verständnis der Ribosomenbiogenese in Eukaryonten dringend notwendig. Diese Untersuchungen können auch dazu beitragen, die Koordination von Zellzyklus und Ribosomenbiogenese besser zu verstehen.

1.3.1 Ribosomenbiogenese in der Hefe und in Säugern

Die Synthese von Ribosomen ist die größte biosynthetische und energieverbrauchende Aktivität schnell wachsender Zellen, da sie einen Hauptteil der zellulären Ressourcen verbraucht (Hannan et al., 1998; Thomas, 2000; Warner, 1999). Ausreichende Mengen an rRNA, die ca. 80% der gesamten RNA einer Zelle

ausmacht, können nur produziert werden, da die rDNA-Gene in ca. 400 Kopien in humanen Zellen vorliegen. rRNA-Transkription, Prozessierung, Zusammenbau der Ribosomen und deren Transport benötigen Hunderte von akzessorischen Proteinen und ca. 200 kleine nukleoläre RNAs (snoRNAs) (Fattica und Tollervey, 2002; Smith und Steitz, 1997; Warner, 2001). Die Ribosomenbiogenese muss daher eng mit dem Zellwachstum und der Proliferation koordiniert werden. Es ist dennoch wenig bekannt über die molekularen Mechanismen, die ein Gleichgewicht zwischen Zellteilung und Ribosomenbiogenese sicherstellen. Während der G1-Phase ist eine erhöhte rRNA-Synthese und Ribosomenbiogenese die Voraussetzung für eine erhöhte Proteinsynthese während der S-Phase. Weiterhin könnte die Abnahme der Ribosomenaktivität bzw. -bildung während der M-Phase verwendet werden, um den Ausstieg aus dem Zellzyklus zu regulieren. Daher existiert eine wichtige Beziehung zwischen Zellzyklus und Ribosomenproduktion. Diese Balance wird durch Schlüssel-Kontrollpunkte gehalten.

In den letzten Jahren hat es wichtige Erkenntnisse für unser Verständnis des hoch dynamischen und evolutionär stark konservierten Prozesses der Ribosomenbiogenese gegeben. Die Biogenese von eukaryontischen Ribosomen findet im Nukleolus statt, einem komplexen nukleären Organell aus drei verschiedenen Komponenten (Abb. 2). Die fibrillären Zentren (FC) enthalten Hunderte von rDNA-Genen in Tandem-Wiederholungen (Scheer und Weisenberger, 1994; Warner, 1989). Diese liegen an zahlreichen chromosomalen Loci, welche als nukleoläre Organisationsregionen (NORs) bezeichnet werden (Thiry und Lafontaine, 2005). Die dichte, fibrilläre Komponente (DFC) enthält aktiv transkribierte rDNA-Gene sowie rRNA-Transkripte. In dieser Region finden frühe Reifungsschritte der pre-rRNA statt, während die granuläre Komponente (GC) die Stelle der späten Prozessierungsschritte in der Reifung der rRNAs sowie des Zusammenbaus der pre-ribosomalen Partikel darstellt.

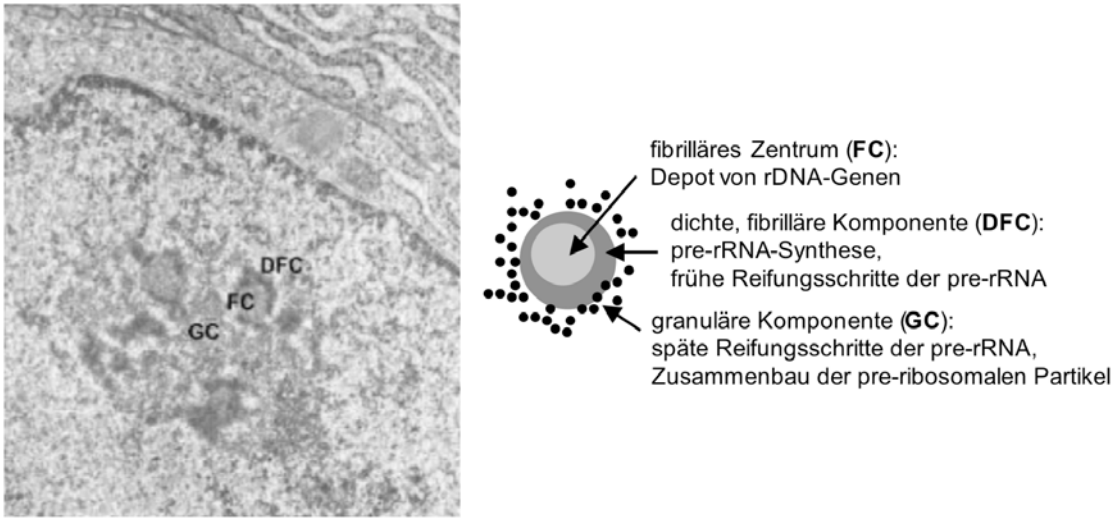

Abbildung 2. Nukleoli von Säugerzellen. Die Nukleoli von Säugerzellen weisen zahlreiche fibrilläre Zentren (FC) auf, welche Hunderte von rDNA-Genen enthalten. Jedes fibrilläre Zentrum ist von der dichten, fibrillären Komponente (DFC) umgeben, an die wiederum die granuläre Komponente (GC) anschließt (Carmo-Fonseca et al., 2000).

Eukaryontische Ribosomen bestehen aus der großen 60S- und der kleinen 40S-Untereinheit. Die ribosomalen 18S, 5.8S und 28S rRNAs (25S rRNA in Hefe) werden als polyzistronisches Transkript, der 47S pre-rRNA (35S pre-rRNA in Hefe), von der RNA-Polymerase I im Nukleolus produziert. Die 5S rRNA wird außerhalb des Nukleolus durch die RNA-Polymerase III gebildet (Geiduschek und Tocchini-Valentini, 1988). Die 47S pre-rRNA wird bereits während der Transkription extensiv modifiziert und mit ribosomalen und nicht-ribosomalen Proteinen zu 90S pre-ribosomalen Partikeln verpackt. Es kommt zur Umwandlung von Uridin- zu Pseudouridinresten in der rRNA, zu Methylierungen an der 2´Hydroxyl-Gruppe von Riboseresten und zur Methylierung von Basen (Eichler und Craig, 1994; Ganot et al., 1997; Kiss-László et al., 1996; Maden, 1990; Rozenski et al., 1999; Tollervey, 1996a; Warner, 1999). Die Funktion einzelner Modifikationen ist noch unbekannt. Allerdings treten sie um das Peptidyltransferasezentrum von Ribosomen auf, was auf eine Rolle während der Peptidsynthese schließen lässt (Bakin et al., 1994).

Nach ihrer Modifikation wird die pre-rRNA an spezifischen Stellen durch Exo- und Endonukleasen gespalten, wodurch eine Serie an charakteristischen Intermediaten (45S, 41S, 36S, 32S, 12S; Abb. 3) und schließlich die reifen 18S, 5.8S und 28S rRNAs entstehen. Die 18S rRNA ist Teil der kleinen 40S, die 5.8S und 28S rRNA

sind Teile der großen 60S ribosomalen Untereinheit. Die pre-40S und pre-60S Untereinheiten werden während der Reifung aus dem Nukleolus über das Nukleoplasma ins Zytoplasma transportiert (Abb. 4). Dort finden die letzten Reifungsschritte dieser Untereinheiten, wie Produktion der reifen 18S rRNA aus der 20S pre-rRNA, statt (Udem und Warner, 1973).

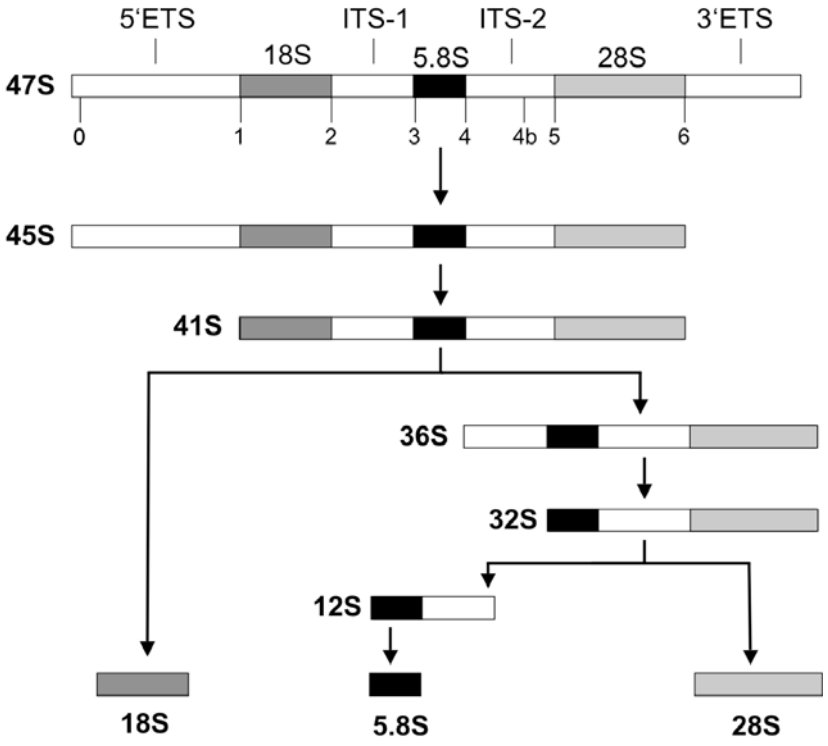

Abbildung 3. Schematischer Überblick über die Prozessierungsschritte der pre-rRNA in Säugerzellen. Das 47S Transkript besteht aus der 5´-externen transkribierten Sequenz (5´ETS), gefolgt von der 18S rRNA, der internen transkribierten Sequenz 1 (ITS-1), der 5.8S rRNA, der internen transkribierten Sequenz 2 (ITS-2), der 28S rRNA und der 3´-externen transkribierten Sequenz (3´ETS). Die Prozessierungsstellen sind angegeben (0 - 6).

1.3.2 Regulation der Ribosomenbiosynthese

Während die Ribosomenbiogenese in der Hefe *Saccharomyces cerevisiae* sehr gut beschrieben wurde (Fatica und Tollervey 2002; Dez und Tollervey, 2004), ist wenig über sie in Säugerzellen bekannt. In der Hefe lassen sich die Gene für die Ribosomenbiogenese in zwei Klassen unterteilen (Wade et al., 2001, 2006). (i) Gene für ribosomale Proteine, die in reifen Ribosomen vorkommen, werden als RP-Gene

bezeichnet. Diese werden durch die RNA-Polymerase II koordiniert transkribiert. Die Mehrheit der RP-Gene enthält Sequenzen, an die die Transkriptionsfaktoren Rap1 und Abf1p binden können (Planta, 1997; Warner, 1999). (ii) Für die Synthese der rRNA und die Reifung der Ribosomen hingegen werden Gene des Ribosom- und rRNA-Biosynthese Wegs (RRB-Regulon) benötigt, die ebenfalls transkriptionell co-reguliert sind. Die Produkte dieser Gene sind, im Gegensatz zu den RP-Genen, selbst aber nicht Teil des reifen Ribosoms. Dazu gehören u.a. Transkriptionsfaktoren für die RNA-Polymerase I und III, RNA-Helikasen und RNA-modifizierende Enzyme. Die Promotoren dieser Gene zeigen häufig PAC (RNA-Polymerase A (I) und C (III)) und RRPE (ribosomales RNA Prozessierungselement) Motive (Dequard-Chablat et al., 1991; Hughes et al., 2000). Es ist allerdings nicht bekannt, welche Faktoren an diese Motive binden. Wade et al. (2006) schätzten, dass das RRB-Regulon mehr als 200 Gene enthält. Wie in der Hefe könnten die Gene für die Ribosomenbiogenese in Säugern ebenfalls co-transkriptionell reguliert sein. Während nur wenige Homologe der RP-Gene durch c-Myc induziert werden, sind viele homologe Gene des RRB-Regulons c-Myc-Zielgene.

Die rDNA-Transkription ist eng mit dem Zellzyklus verbunden und kann durch extrazelluläre Stimuli induziert werden. Die Initiation der Transkription ist abhängig von drei basalen Transkriptionsfaktoren: dem „Selektivitätsfaktor“ (SL1 oder TIP-1B in Maus), dem „HMG1-Box architectural upstream binding“ Faktor (UBF) und der DNA-abhängigen RNA-Polymerase I (Learned et al., 1986). SL1 ist ein Multiprotein-Komplex aus einem TATA-Bindeprotein (TBP) und vier unterschiedlichen TBP-assoziierten Faktoren (TAFs) (Comai et al., 1992, 1994; Gorski et al., 2007). Der Initiationsfaktor TIF-1A sowie der Terminationsfaktor TTF-1 sind ebenfalls Teil des Komplexes. UBF bindet vermutlich zuerst an den Promotor und ermöglicht damit die Rekrutierung von SL1 und RNA-Polymerase I. Der Transkriptionsfaktor UBF ist durch seine Fähigkeit, die Transkriptionsaktivität der RNA-Polymerase I zu modulieren, der Schlüssel-Regulator. UBF wird an Serin- und Threoninresten in C-terminalen und internen Regionen des Proteins phosphoryliert, was die Bindungsaffinität zur DNA oder auch die Interaktionen mit anderen Proteinen verändert (Kihm et al., 1998; Stefanovsky et al., 2001; Tuan et al., 1999; Voit und Grummt, 2001). Die Phosphorylierung von UBF ist direkt abhängig von mitogenen Signalen und der Zellzyklusprogression. In ruhenden Zellen ist UBF hypophosphoryliert und transkriptionell inaktiv (O´Mahony et al., 1992; Voit et al., 1992, 1995). Die Reduktion

der rRNA-Synthese während der Mitose ist die Folge einer Inaktivierung von UBF durch Dephosporylierung. Die UBF-kontrollierenden Kinasen sind in Tumorzellen häufig dereguliert (Bortner und Rosenberg, 1997; Wang et al., 1994).
Die „Pocket"-Proteine können neben der Regulation des Zellzyklus durch Modulation der transkriptionellen Aktivität von E2F auch die rRNA-Synthese über UBF regulieren. Dies geschieht durch direkte Bindung an und Inaktivierung von UBF, wodurch UBF nicht mehr mit SL1 interagieren kann (Hannan et al., 2000; Voit et al., 1997). Der Tumor-Suppressor p53 interagiert mit SL1 und verhindert so die Bindung von UBF und damit die Transkription durch die RNA-Polymerase I (Budde und Grummt, 1999; Zhai und Comai, 2000). Rb und p53 reprimieren durch Bindung an TF-IIIB und TFIIIC2 auch die Transkription der RNA-Polymerase III und damit die Synthese der 5S rRNA (Chesnokov et al., 1996; Cairns und White, 1998; Chu et al., 1997; Sutcliffe et al., 2000; White et al., 1996). Daher sollten Mutationen in Rb und p53 auch zu einer verstärkten Expression von ribosomalen RNAs führen.

1.4 Zielgene von c-Myc in der Ribosomenbiogenese

In der Hefe sind zahlreiche Faktoren beschrieben worden, die an der rRNA-Prozessierung beteiligt sind (Fatica und Tollervey, 2002; Maxwell und Fournier, 1995; Tollervey, 1996b; Dez und Tollervey, 2004). Über die Prozessierung der pre-rRNA in höheren Eukaryonten ist dagegen nur wenig bekannt. Daher sollen hier einzelne Aspekte angesprochen werden, die zur Übersicht in Tabelle 1 sowie Abbildung 4 zusammengefasst sind. Es soll nicht nur auf die Produkte der Zielgene von c-Myc, sondern auch auf die Funktionen ihrer Hefehomologen eingegangen und dadurch deutlich gemacht werden, dass c-Myc vielfältigste Schritte in der Ribosomenbiogenese von Säugern beeinflusst.

Tabelle 1: An der Ribosomenbiogenese beteiligte Produkte von c-Myc-Zielgenen.

Produkte von c-Myc-Zielgenen	homologe Proteine in der Hefe	Funktion der Proteine
Fibrillarin	Nop1p	enzymatische Komponente der C/D-Box snoRNPs; Komponente des SSU Prozessoms
Nop56	Nop56p/Sik1p	Komponente der C/D-Box snoRNPs; Komponente des SSU Prozessoms
Nop58	Nop5p/Nop58p	Komponente der C/D-Box snoRNPs; Komponente des SSU Prozessoms
UTP5 / WDR43	UTP5p	Komponente des SSU Prozessoms
UTP12	Dip2 / UTP12p	Komponente des SSU Prozessoms
UTP18 / WDR50	UTP18p	Komponente des SSU Prozessoms
KIAA0185	Rrp5p	Komponente des SSU Prozessoms
DKFZP564O0463	Sof1p	Komponente des SSU Prozessoms
Dyskerin / NAP57	Cbf5p	enzymatische Komponente der H/ACA-Box snoRNPs
Gar1	Gar1p	Komponente der H/ACA-Box snoRNPs
Dim1p Homolog	Dim1p	Adenin-Dimethyltransferase, Reifung der 18S rRNA
p120	Nop2p	m5C-Methyltransferase, Reifung der 25S/28S rRNA
Nopp140	Srp40p	Chaperon der snoRNPs
Nucleolin / p50	Nsr1p	nukleozytoplasmatischer Transport, Regulation der rDNA Transkription, rRNA-Prozessierung
KIAA0111	Fal1p	DEAD-Box RNA-Helikase, Reifung der 40S Untereinheit
DEAD box Helikase 97 kDa	Dbp10p	DEAD-Box RNA-Helikase, Reifung der 60S Untereinheit
Nog1	Nog1p	GTPase
Nog2	Nog2p	GTPase
Nucleostemin	Nug1p	GTPase
Bop1	Erb1p	Reifung der 60S Untereinheit
Pes1	Nop7p	Reifung der 60S Untereinheit
WDR12	Ytm1p	Reifung der 60S Untereinheit

Tabelle 1: Beispiele für Produkte von c-Myc-Zielgenen mit Funktion in der Ribosomenbiogenese. Die homologen Proteine in Hefe sowie die beschriebenen Funktionen sind angegeben.

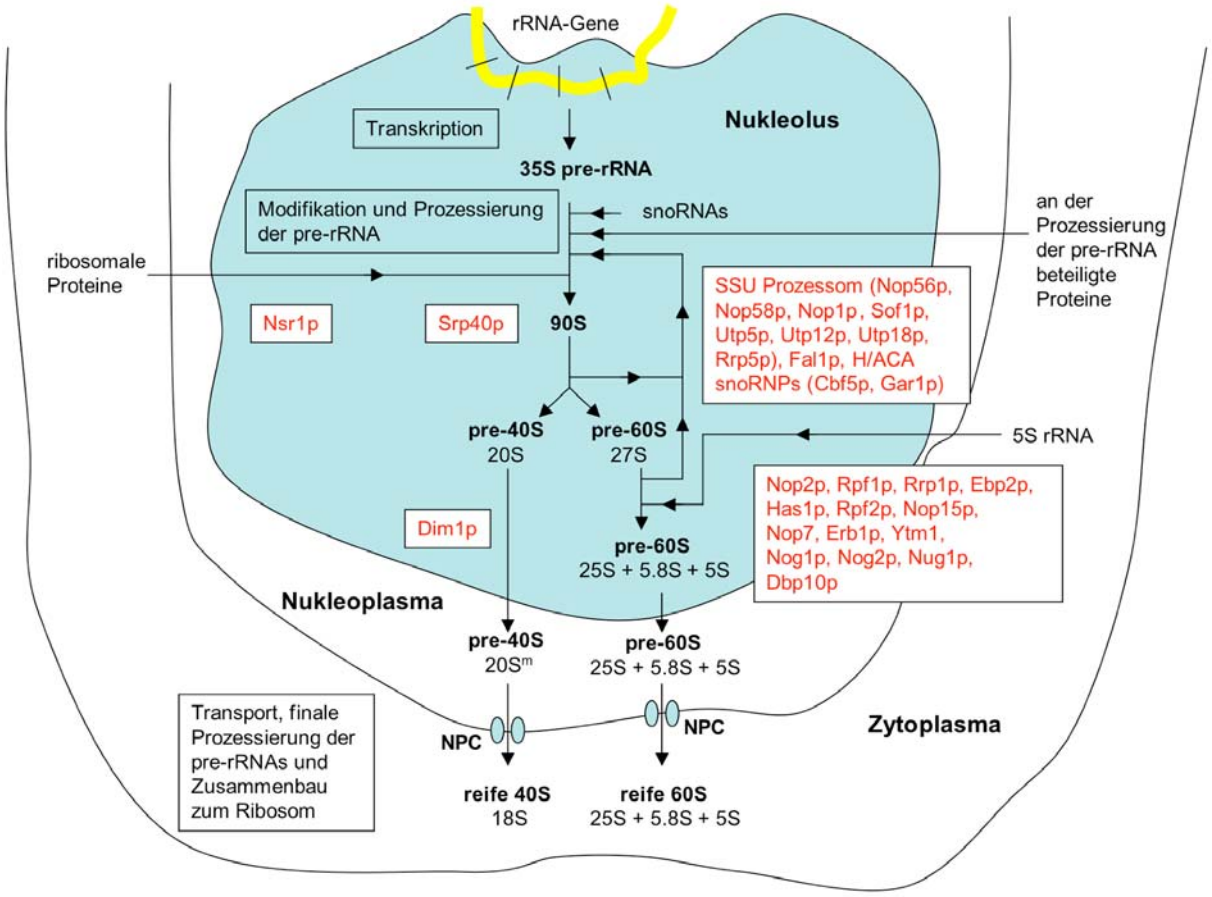

Abbildung 4. Beteiligung verschiedener Proteine an der Ribosomenbiogenese in der Hefe. Die pre-ribosomalen Partikel mit ihren pre-rRNA-Intermediaten und ihre vorhergesagte zelluläre Lokalisation sind angegeben. Rot dargestellte Proteine sind homolog zu den Produkten von c-Myc-Zielgenen (Schlosser et a., 2003). Details sind im Text beschrieben.

Modifikationen der pre-rRNA

Eine der häufigsten Modifikationen der pre-rRNA während der Reifung ist die sequenz-spezifische 2´-O-Methylierung des Riboserestes (Maxwell und Fournier, 1995). Diese wird durch Ribonukleoproteinpartikel (snoRNPs), welche kleine nukleoläre RNAs (snoRNAs) enthalten, durchgeführt (Filipowicz et al., 1999). Die Methylierungsstelle wird durch Basenpaarung der snoRNAs mit der Substrat-RNA festgelegt, während die Methyltransferaseaktivität durch eines der snoRNP-Proteine ausgeführt wird (Bachellerie und Cavaille, 1997; Cavaille und Bachellerie, 1998; Cavaille et al., 1996; Kiss-László et al., 1996; Kiss, 2002). Alle C/D-Box snoRNPs enthalten die evolutionär konservierten Proteine Nop1p (Fibrillarin), Nop56p/Sik1p,

Nop5/58p und Snu13p (Galardi et al., 2002; Gautier et al., 1997; Schimmang et al., 1989; Watkins et al., 2000; Wu et al., 1998). Nop1p ist in der Hefe die enzymatische Komponente dieser snoRNPs und kann direkt an pre-rRNA binden (Tollervey et al., 1993). Seine S-Adenosyl-L-Methionin-Bindungsregion ist verantwortlich für die katalytische Aktivität. S-Adenosylmethionin (SAM) dient dabei als Donor der Methylgruppe (Galardi et al., 2002). Die homologen Säugerproteine von Nop1p, Nop56p und Nop5p/Nop58p sind Zielgene von c-Myc.

Eine weitere wichtige Form der Modifikation von pre-rRNAs ist die Pseudouridylierung (Isomerisierung von Uridin), welche von anderen Ribonukleoproteinen, den so genannten H/ACA-Box snoRNPs, ausgeführt wird (Ganot et al., 1997; Ni et al., 1997). Die Stelle der Pseudouridylierung wird ebenfalls durch RNA-RNA-Interaktion von snoRNA und Ziel-RNA festgelegt. Alle H/ACA snoRNPs teilen die Proteine Cbf5p, Gar1p, Nhp2p und Nop10p. Cbf5p ist in der Hefe die enzymatische Komponente (Lafontaine et al., 1998; Zebarjadian et al., 1999). Das homologe Protein von Cbf5p in humanen Zellen ist Dyskerin (Dkc1), das in Menschen mit Dyskeratosis congenita (DC) mutiert ist. Diese Krankheit ist durch ein vorzeitiges Altern und eine erhöhte Tumorbildungsrate gekennzeichnet (Dokal, 2000; Heiss et al., 1998; Ruggero et al., 2003). Dyskerin und Gar1 sind Zielgene von c-Myc in Säugern.

Die dritte wichtige Modifikation der pre-rRNA ist die Methylierung von spezifischen Basen in der Sequenz. Im Gegensatz zur 2´-O-Methylierung des Riboserestes der pre-rRNA, wird diese durch spezifische Enzyme durchgeführt. Zu diesen Enzymen gehören unter anderem die Adenin-Dimethyltransferase Dim1p, die für die Reifung der 18S rRNA essentiell ist (Lafontaine et al., 1994, 1995) und die m5C-Methyltransferase Nop2p, die für die Synthese der 60S Untereinheit erforderlich ist (Hong et al., 2001). Die einzige bekannte m5C-Modifikationsstelle in der rRNA befindet sich in der 25S bzw. 28S rRNA und ist stark konserviert. Als Donor dient dabei S-Adenosylmethionin (SAM) (Hong et al., 2001). Nop2p zeigt signifikante Homologie zu dem humanen nukleolären Protein p120, einer rRNA 2´-Hydroxy-Methyltransferase, die proliferations-assoziiert exprimiert wird (de Beus et al., 1994; Koonin, 1994). Die Homologen von Dim1p und Nop2p in humanen Zellen sind Zielgene des Transkriptionsfaktors c-Myc.

SSU Prozessom

Die U3 snoRNA gehört zu den C/D-Box snoRNAs und wird für die Reifung der 18S rRNA benötigt (Hughes und Ares, 1991; Maxwell und Fournier, 1995; Venema und Tollervey, 1999). Sie bildet zusammen mit zahlreichen Proteinen, wie den vier C/D-Box snoRNP-Proteinen Nop1p, Nop56p, Nop5p/Nop58p und Snu13p sowie UTP1p-18p („U3 snoRNA associated proteins“), Rrp5 und den U3 snoRNP spezifischen Proteinen Sof1p, Mpp10p, Imp3p, Imp4p, Dhr1p und Rrp9p einen großen RNP-Komplex (Colley et al., 2000; Dragon et al., 2002; Dunbar et al., 1997; Jansen et al., 1993; Lee et al., 1999; Venema et al., 2000; Wiederkehr et al., 1998). Dieser wird als kleine Untereinheit (SSU) Prozessom bezeichnet (Dragon et al., 2002). In Hefe ist die Funktion des SSU Prozessoms wichtig für sehr frühe Schritte in der Prozessierung der pre-rRNA, wie die Abspaltung der 5´ETS Sequenz. Das SSU Prozessom könnte auch als Chaperon der pre-rRNA dienen. Die homologen Proteine von Rrp5p, Nop1p, Nop56p, Nop5p/Nop58p und Sof1p in Säugerzellen sowie UTP5, UTP12 und UTP18 sind bekannte Zielgene von c-Myc.

Chaperon der snoRNPs

Nopp140 lokalisiert sowohl im Zytoplasma, in den Cajal-Körpern und im Nukleolus (Meier und Blobel, 1990, 1992). Cajal-Körper sind kleine nukleäre Organellen, in denen snoRNPs angereichert sind (Bohmann et al., 1995). Nopp140 interagiert mit den C/D-Box und den H/ACA-Box snoRNPs und könnte eine Rolle bei deren Transport zwischen dem Nukleolus und den Cajal-Körpern spielen sowie an der Biogenese bzw. direkt an der Funktion der snoRNPs beteiligt sein (Isaac et al., 1998; Yang et al., 2000). Sowohl die Expression einer dominant-negativen Mutante von Nopp140 in Rattenzellen als auch eine Depletion des homologen Proteins Srp40p in Hefe reduziert die Menge an snoRNP-Proteinen im Nukleolus (Yang et al., 2000; Meier, 1996).

Transport von ribosomalen Proteinen

Ribosomale Proteine werden im Zytoplasma produziert, in den Nukleus transportiert, im Nukleolus mit RNA zu ribosomalen Partikeln zusammengebaut und schließlich wieder ins Zytoplasma exportiert. Der Transport von ribosomalen Proteinen und Partikeln durch die Kernmembran ist daher essentiell für proliferierende Zellen. Das humane Protein Nucleolin (p50) ist an der Regulation zahlreicher Schritte der

Ribosomenbiogenese wie rDNA-Transkription (Bouche et al., 1984; Erard et al., 1988, 1990; Egyhazi et al., 1988; Olson et al., 1983), rRNA-Prozessierung (Abadia-Molina et al., 1998; Ginisty et al., 1998) und nukleozytoplasmatischen Transport (Borer et al., 1989; Bouvet et al., 1998; Schmidt-Zachmann et al., 1993) beteiligt. Nucleolin interagiert sowohl mit ribosomalen Proteinen der großen und kleinen Untereinheit als auch mit pre-rRNA und dem SSU Prozessom. Es ist eines der ersten Proteine, die mit der pre-rRNA interagieren und kann eventuell andere Faktoren wie das SSU Prozessom rekrutieren (Ginisty et al. 1998). In der Hefe bindet das homologe Protein Nsr1p („nuclear signal recognition protein“) an nukleäre Lokalisationssequenzen von Proteinen. Außerdem besitzt es multiple RNA-Bindungsmotive (Lee und Melese, 1989, 1991). Dies impliziert eine Beteiligung des Proteins am Import von ribosomalen Proteinen und anderen zytoplasmatischen Komponenten in den Nukleolus und deren Einbau in pre-ribosomale Partikel (Lee und Melese, 1989; Bouvet et al., 1998).

Strukturelle Veränderungen in ribosomalen Partikeln

Ribosomale Partikel sind höchst dynamische Strukturen, in denen zahlreiche strukturelle Veränderungen für die Reifung der ribosomalen Untereinheiten nötig sind. Dazu ist die Aktivität von RNA-Helikasen, die die RNA-Duplex in einer ATP-abhängigen Art entwinden, notwendig. Während Fal1p eine Rolle in den frühen Prozessierungsschritten der pre-rRNA und damit in der Reifung der 40S Untereinheit (Kressler et al., 1997) spielt, ist Dbp10p (DEAD-Box Protein 10) in der Reifung der 60S Untereinheit involviert (Burger et al., 2000). Die Helikase-Aktivität könnte die pre-rRNA für die endo- und exonukleolytischen Spaltungen zugänglich machen. Außerdem könnte sie für die Interaktion der pre-rRNA mit den snoRNAs, den snoRNPs und ribosomalen Proteinen während der Prozessierung und beim Zusammenbau der Untereinheiten nötig sein (Tollervey, 1996b; Venema und Tollervey, 1995).

GTPasen und Export der ribosomalen Untereinheiten

In eukaryontischen Zellen werden die Ribosomen im Nukleolus gebildet und müssen durch den „nuclear pore complex“ (NPC) ins Zytoplasma transportiert werden, um ihre Funktion in der Translation übernehmen zu können. Es wurde gezeigt, dass zahlreiche GTPasen mit den pre-60S Ribosomen auf deren Weg vom Nukleolus ins

Zytoplasma assoziiert sind (Fromont-Racine et al., 2003; Hedges et al., 2005; Nissan et al., 2002; Tschochner und Hurt 2003). Die genaue Funktion dieser Proteine ist aber noch nicht beschrieben. Nog1p (nukleoläres GTP-Bindungsprotein) wurde als essentielle GTPase charakterisiert (Park et al., 2001), die an der Prozessierung der 35S rRNA (Jensen et al., 2003; Kallstrom et al., 2003; Saveanu et al., 2003) sowie der Translokation der pre-ribosomalen Komplexe vom Nukleolus ins Nukleoplasma und späten Reifungsschritten der 60S-Untereinheit beteiligt ist (Honma et al., 2006). Nug1p (nukleoläre GTPase-1) und Nog2p (nukleäres/nukleoläres GTP-Bindungsprotein-2) sind ebenfalls Teil von pre-60S Partikeln und werden für den Export der 60S Untereinheiten benötigt (Bassler et al., 2001, 2006; Saveanu et al., 2001).

Schnittstelle von Ribosomenbiogenese und DNA-Replikation

Der „Origin Recognition complex“ (ORC) bindet in Hefe an die DNA-Replikationssequenzen (ARS) und bildet dadurch die Plattform für weitere Proteinkomplexe. Als Interaktionspartner des ORC-Komplexes wurde Yph1p (=Nop7p) beschrieben, das in zwei multimeren Proteinkomplexen vorkommt (Du und Stillman, 2002). Der kleinere der beiden Komplexe enthält Yph1p/Nop7p, Erb1p und Ytm1p, während der größere aus vielen zusätzlichen Proteinen aufgebaut ist, zu denen u.a. MCM („minichromosome maintainance“) Proteine (MCM4, 5, 6 und 7) und zellzyklusregulatorische Proteine zählen. Der kleinere trimere Komplex scheint dabei den Kern für den Zusammenbau des größeren zu bilden. Yph1p/Nop7p, Erb1p und Ytm1p sind in der Evolution hoch konserviert und an der Reifung der großen ribosomalen Untereinheit beteiligt (Adams et al., 2002; Harnpicharnchai et al., 2001; Pestov et al., 2001). Du und Stillman (2002) vermuteten, dass der ORC-Komplex und Yph1p eine kritische Rolle in der Koordination von Ribosomenbiogenese und DNA-Replikation spielen könnten. Die rDNA-Gene in eukaryontischen Zellen enthalten jeweils einen Ursprung der DNA-Replikation und damit auch eine Bindungsstelle für den ORC-Komplex. In rasch proliferierenden Zellen könnte die ORC-Yph1p Interaktion die rDNA-Transkription mit der Replikation des Locus koordinieren (Du und Stillman; 2002).

c-Myc reguliert die Expression der homologen Proteine Pes1, Bop1 und WDR12 in humanen Zellen (Schlosser et al., 2003). In Analogie zur Hefe könnten Pes1, Bop1 und WDR12 in Säugerzellen ebenfalls einen Komplex bilden, der eine Funktion an

der Schnittstelle zwischen Ribosomenbiogenese und DNA-Replikation ausübt. Daher sollen im Folgenden Nop7p, Erb1p und Ytm1p sowie ihre Homologen Pes1, Bop1 und WDR12 in Säugern näher beschrieben werden (Abb. 5).

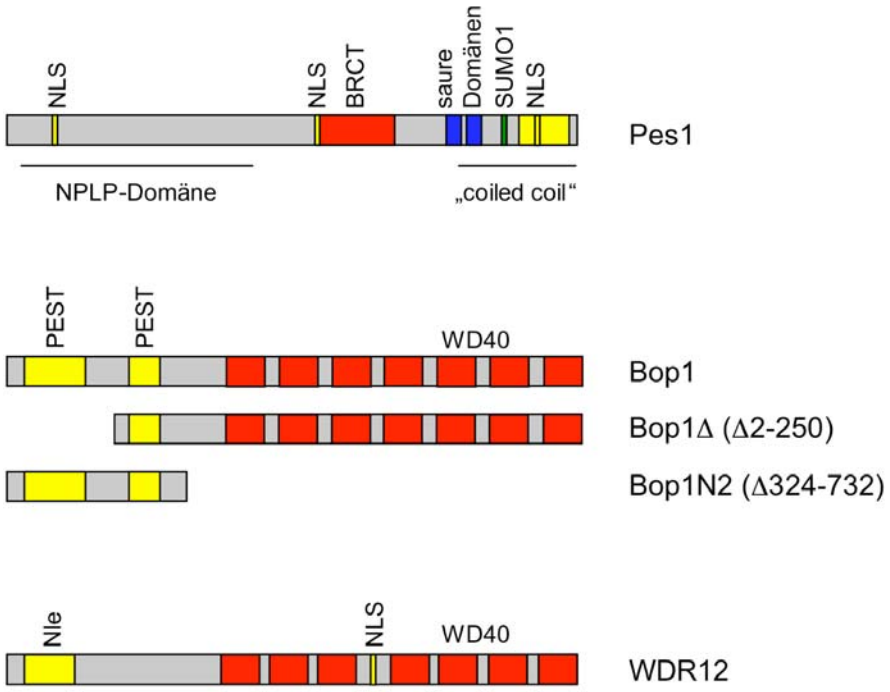

Abbildung 5. Schematische Darstellung von Pes1, Bop1 und WDR12. Pes1 verfügt über eine „N-terminal pescadillo-like protein“ (NPLP) Domäne, drei klassische nukleäre Lokalisationssequenzen (NLS), sowie eine zentral lokalisierte BRCT-Domäne gefolgt von zwei sauren Domänen. Im C-Terminus, der als „coiled coil“ ausgeprägt ist, befinden sich sechs putative NLS und ein Konsensus-SUMOylierungsmotiv. Bop1 weist sieben WD40-Motive sowie zwei PEST-Sequenzen am N-Terminus und in der Mitte des Proteins auf. WDR12 besitzt sieben WD40-Motive, eine nukleäre Lokalisationssequenzen (NLS) und eine N-terminale „Notchless-like“ (Nle) Domäne.

Pes1 und seine Homologen in Zebrafisch *Danio rerio* (pescadillo) und Hefe (Yph1p/Nop7p) sind essentiell (Adams et al., 2002; Allende et al., 1996; Lerch-Gaggl et al., 2002). Die Expression des nukleolären Proteins ist streng assoziiert mit Zellproliferation und daher stark erhöht in Tumorzellen (Charpentier et al., 2000; Kinoshita et al., 2001). Die Proteinsequenz von Pes1 beinhaltet eine zentral lokalisierte BRCT-Domäne, welche als essentiell für die Tumor-Suppressor-Funktion

des BRCA1 Proteins beschrieben wurde. Die BRCT-Domäne besitzt Phosphopeptid-Bindungs-Aktivität, was vermuten lässt, dass sie phosphorylierungsabhängige Protein-Protein-Interaktionen vermittelt (Glover et al., 2004). Das Hefehomolog von Pes1, Yph1p/Nop7p, wurde als Bestandteil der pre-60S Ribosomen identifiziert (Harnpicharnchai et al., 2001). Eine Depletion von Yph1p/Nop7p beeinflusst die Bildung von 60S ribosomalen Untereinheiten, führt zur Akkumulation von pre-Ribosomen im Nukleus und zu einer graduellen Verlängerung der Verdopplungszeit in der Hefe (Adams et al., 2002; Oeffinger et al., 2002). Maus pes1$^{-/-}$ Embryos arretieren in frühen Stadien der Entwicklung (Morulastadium) und weisen abnormale nukleoläre Morphologie sowie eine reduzierte Anzahl an Ribosomen auf (Lerch-Gaggl et al., 2002). Disruption von *pescadillo* im Zebrafisch durch eine Transposoninsertion führt zu einer deutlichen Unterentwicklung verschiedener Organe, welche in einer embryonalen Letalität an Tag 6 resultiert (Allende et al., 1996). Mutationsanalysen von Pes1 belegen eine Beteiligung des Proteins an der Produktion von 28S und 5.8S rRNAs. Je nach Mutante kommt es zur Akkumulation von 36S und 32S pre-rRNAs bzw. durch Nichtabspaltung des 3´-ETS-Bereichs von der 47S pre-rRNA zum Erscheinen von zusätzlichen Formen von 41S*, 36S* und 32S* pre-rRNA. Pes1 ist wichtig für die Reihenfolge und die Effizienz der Schnitte in ITS1, ITS2 und 3´-ETS (siehe Abb. 3). Ein transienter Verlust von Pes1 resultiert in einer erhöhten Anzahl abnormaler Mitosen und Zellen mit multipolaren Spindeln und aberranten Metaphase-Platten (Killian et al., 2004). Eine Folge dieser Ereignisse ist chromosomale Instabilität (CIN).

Bop1 (Block of proliferation) ist ein nukleoläres Protein mit sieben WD40-Motiven. WD40-Motive kommen in verschiedenen Gruppen von Proteinen vor, die in der Regulation von zellulären Prozessen wie Signaltransduktion, Gentranskription und mRNA-Modifikation involviert sind (Neer et al., 1994). Proteine mit WD40-Domänen sind häufig über Protein-Protein-Interaktionen an der Bildung von Multiproteinkomplexen beteiligt. Analysen der Primärsequenz von Bop1 zeigen Cluster von geladenen Aminosäureresten am N-Terminus und in der Mitte des Proteins, bekannt als PEST-Sequenzen, die oft mit regulatorischen und kurzlebigen Proteinen assoziiert sind (Chevaillier, 1993; Rechsteiner und Rogers, 1996). Durch die Deletion des N-Terminus (Bop1Δ) oder des C-Terminus (Bop1N2) wurden dominant-negative Mutanten von Bop1 generiert. Beide Mutanten teilen sich lediglich

ein kurzes Fragment von 72 Aminosäuren. Ihre Expression resultiert in einem starken, aber reversiblen Wachstumsarrest in der G1-Phase und verursacht einen Block in der Prozessierung der pre-rRNA (Pestov et al., 1998; Strezoska et al., 2000, 2002). In beiden Fällen wird die Produktion von 28S rRNA und 5.8S rRNA inhibiert, wohingegen die Mengen an 18S rRNA und des 47S Vorläufers unverändert bleiben. Als Folge kommt es zu einem Defizit an freien 60S ribosomalen Untereinheiten im Zytoplasma. Die Expression von Bop1Δ beeinflusst spezifisch späte Prozessierungsschritte, die zur Reifung der 28S rRNA führen. Gleichzeitig kommt es zur Akkumulation der 36S und 32S pre-rRNAs. Durch die Expression von Bop1N2 entstehen durch Nichtabspaltung des 3´-ETS-Bereichs von der 47S pre-rRNA neue 41S* und 36S* Vorläuferformen. Eine Form der 32S pre-rRNA mit 3´-ETS-Bereich konnte ebenfalls detektiert werden. Diese zusätzlichen Formen konnten auch nach Expression der dominant-negativen Mutanten von Pes1 beobachtet werden (Grimm et al., 2006; Lapik et al., 2004). Die Expression von Bop1N2 blockiert folglich frühe Prozessierungsschritte. Zusätzlich scheint es neben einer Akkumulation der 32S pre-rRNA auch zu einem inhibitorischen Effekt auf den Umsatz der 12S pre-rRNA zu kommen, was eine Abnahme an 5.8S rRNA zur Folge hat (siehe Abb. 3). Eine Reduktion der Menge an endogenem Bop1 durch Antisense-Oligonukleotide führt zur Akkumulation der 36S, der 32S sowie der 12S pre-rRNA. Die Folgen überlappen somit mit denen, die durch Expression von Bop1Δ und Bop1N2 entstehen (Strezoska et al., 2002). Bop1 ist folglich wichtig für die Reihenfolge und die Effizienz der Schnitte in ITS1, ITS2 und 3´-ETS und scheint damit ein multifunktionelles Protein zu sein, das an unterschiedlichen Prozessen der Ribosomenbiogenese beteiligt ist. Neben Pes1 spielt auch Bop1 eine essentielle Rolle in der Mitose. Sowohl die Inaktivierung von Bop1 durch siRNA-Oligonukleotide als auch die Überexpression des Proteins führt zu abnormen Mitosen und einer veränderten chromosomalen Segregation (Killian et al., 2004, 2006).

Das nukleoläre Protein WDR12 ist das putative Homolog von Ytm1p der Hefe. Es besitzt sieben WD40-Motive, die eine Propeller-ähnliche Struktur bilden könnten (Nal et al., 2002). Diese Vermutung basiert auf der Kristallstruktur der Gβ-Untereinheit von heterotrimeren G-Proteinen (Gaudet et al., 1996; Wall et al., 1995). WDR12 verfügt über eine nukleäre Lokalisationssequenzen (NLS) und N-terminale „Notchless-like“ (Nle) Domäne (Nal et al., 2002). Notchless ist an der Modulation des

Notch-Signalweges in *Drosophila melanogaster* beteiligt (Royet et al., 1998). WDR12 mRNA wird während der Embryogenese ubiquitär exprimiert. Eine Interaktion von WDR12 mit Notch1-IC lässt auf eine Rolle in frühen Stadien der primären T-Zell-Differenzierung schließen (Nal et al., 2002). Mutationen in den WD40-Motiven verhindern die Interaktion von Ytm1p mit Erb1p und reduzieren die Assoziation von Ytm1p, Erb1p und Nop7p mit pre-Ribosomen. Als Folge kommt es zu einer gehemmten Reifung der 25S rRNA und einer Reduktion des Exports von pre-Ribosomen aus dem Nukleolus. Die Bildung der 18S rRNA wird jedoch nicht beeinflusst (Miles et al., 2005).

1.5 Verbindungen zwischen Ribosomenbiogenese und Zellzyklusregulation

Proliferierende Zellen können die Zellzyklusprogression als Antwort auf extrazelluläre Signale oder auf Grund von Störungen intrazellulärer Prozesse verlangsamen oder blockieren. Die Schädigung der DNA, Defekte in der DNA-Replikation und der Chromosomensegregation oder auch die Akkumulation von falsch gefalteten Proteinen im Endoplasmatischen Retikulum können Kontrollpunkte des Zellzyklus aktivieren und den Zellzyklus hemmen (Elledge, 1996; Hartwell und Weinert, 1989; Weinert, 1998). Diese Kontrollpunkte sind in Tumorzellen häufig verändert, was darauf schließen lässt, dass ihr Ausfall eine wichtige Rolle in der Entwicklung von Tumoren hat (Hartwell und Kastan, 1994).

Die Störung der strukturellen bzw. funktionellen Integrität des Nukleolus durch zellulären Stress führt zur Induktion des Tumor-Suppressors p53 (Rubbi und Milner, 2003). p53 wiederum aktiviert die Cdk-Inhibitoren p21 und p27, wodurch die Kinaseaktivität von Cdk2 und Cdk4/6 abnimmt (Harper et al., 1995; Vogelstein et al., 2000). Als Folge wird Rb nicht phosphoryliert und die Zellen arretieren in der G1-Phase. Die Korrelation zwischen Funktion des Nukleolus und Stabilisierung von p53 wird auch dadurch bestärkt, dass die Nukleoli während der Mitose aufgelöst werden und eine vollständige nukleoläre Funktionalität (maximale Rate an rRNA-Synthese) nicht vor einer späten G1-Phase wieder erreicht wird (Klein und Grummt, 1999). Diese Zeitspanne entspricht genau dem Fenster, in dem die Menge an p53 während des Zellzyklus erhöht ist (David-Pfeuty, 1999). Rubbi und Milner (2003) vermuteten,

dass dieser Anstieg von p53 das Resultat der mitotischen Auflösung des Nukleolus ist.

Mehrere Ereignisse könnten eine Rolle in der Induktion von p53 nach einer Störung der Ribosomenbiogenese spielen. Zum einen kann p53 kovalent an 5.8S rRNA binden (Fontoura et al., 1992, 1997). Eine intakte Ribosomenbiogenese führt daher zum Transport von p53 zusammen mit Ribosomen ins Zytoplasma und dort zum Abbau von p53. Dieser Mechanismus wurde als p53-Hdm2-Komplex „riding the ribosome“ beschrieben (Sherr und Weber, 2000). Andererseits akkumulieren nicht eingebaute ribosomale Proteine wie L5, L11, L27 und S7 durch eine gestörte Ribosomenbiogenese. Diese können die E3 Ubiquitin-Ligase Mdm2/Hdm2 binden und deren Funktion stören, die normalerweise zum Abbau von p53 führt (Chen et al., 2007; Dai und Lu, 2004; Dai et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). Dadurch kommt es zum p53-abhängigen Zellzyklusstopp und zur Apoptose. Weitere Mechanismen der p53-Aktivierung durch ribosomale Proteine werden diskutiert. Die Produktion von p53 kann beispielsweise durch das ribosomale Protein L26 nach einer DNA-Schädigung gesteigert werden (Takagi et al., 2005). Das mitochondriale ribosomale Protein L41 hingegen bindet nach einer DNA-Schädigung direkt an p53 und verstärkt seine Translokation in die Mitochondrien, was zur p53-abhängigen Apoptose führt (Yoo et al., 2005). Zusätzlich zu ribosomalen Proteinen akkumulieren bei Störung der Ribosomenbiogenese auch andere nukleoläre Komponenten wie Nucleolin, die an der Prozessierung der rRNA beteiligt sind. Nucleolin bindet und inhibiert Mdm2/Hdm2 und führt so zur Stabilisierung von p53 (Saxena et al., 2006). Es scheint folglich mehrere Proteine der Ribosomenbiogenese zu geben, die mit Mdm2/Hdm2 interagieren. Diese könnten parallele oder kollaborierende Signalwege darstellen, die eine effektive p53-Antwort sicherstellen.

2 ZIELSETZUNG DER ARBEIT

Es gibt zunehmend Hinweise für eine Verbindung zwischen Nukleolusfunktion und Zellzykluskontrolle. Die Analyse der Zielgene von c-Myc macht deutlich, dass der Transkriptionsfaktor stark in die Regulation verschiedener Schritte der Ribosomenbiogenese eingreift. c-Myc ist folglich nicht nur ein wichtiger Regulator von Zellzyklus-Genen sondern auch der Ribosomenbiogenese. Die molekularen Mechanismen der Ribosomenbiogenese in Säugern sind allerdings noch nicht gut untersucht.

Daher wurde der Fokus unseres Labors auf die Untersuchung von c-Myc-Zielgenen gelegt, deren Produkte an der Reifung der großen Untereinheit der Ribosomen beteiligt sind. Die Proteine Pes1, Bop1 und WDR12 sollten in ihrer Funktion näher beschrieben werden. c-Myc reguliert die Expression von *pes1*, *bop1* und *wdr12* proliferationsabhängig, wodurch vermutlich die Menge an jeder Komponente an die Rate der Ribosomenbiogenese angepasst wird. Die Homologen von Pes1, Bop1 und WDR12 bilden in Hefe einen Komplex, der über Interaktion mit anderen Proteinen die Ribosomenbiogenese mit der DNA-Replikation und Zellzyklusprogression koppelt.

In dieser Arbeit sollte die Existenz eines Komplexes aus Pes1, Bop1 und WDR12 in Säugern belegt, im Detail charakterisiert und dessen Funktion untersucht werden. Um einen detaillierteren Einblick in den endogenen Komplex von Pes1, Bop1 und WDR12 zu erlangen, war die Herstellung von monoklonalen Antikörpern eines der primären Ziele dieser Arbeit. Außerdem lag der Schwerpunkt aller Untersuchungen bislang auf der Analyse einzelner Proteine durch Mutagenesestudien. In dieser Arbeit sollten Pes1, Bop1 und WDR12 im Kontext des Komplexes näher beschrieben werden. Ein wichtiger Aspekt dabei war, die Auswirkungen einer deregulierten Expression von Komplexkomponenten auf Ribosomenbiogenese und die Zellzyklusprogression zu analysieren.

3 DISKUSSION

In der Hefe *Saccharomyces cerevisiae* wurde ein Komplex der Proteine Nop7p, Erb1p und Ytm1p beschrieben (Du und Stillman, 2002; Harnpicharnchai et al., 2001). Dieser bildet vermutlich den Kern für einen größeren Komplex, der u.a. Proteine des ORC-Komplexes enthält. Der Komplex ist als Teil der pre-ribosomalen 60S-Untereinheit an der Reifung der Ribosomen in der Hefe beteiligt.
In meiner Arbeit habe ich die homologen Proteine von Nop7p, Erb1p und Ytm1p in Säugerzellen näher untersucht und gefragt: Existiert auch in Säugern ein trimerer Komplex aus Pes1, Bop1 und WDR12? Wenn ja, besitzt er eine ähnliche Funktion wie in Hefe? Die Expression der Proteine scheint kritisch zu sein, um den Bedarf einer proliferierenden Zelle an Ribosomen zu decken. Ruhende oder verarmte Zellen zeigen nur geringe Mengen von Pes1, Bop1 und WDR12 auf. Diese steigen in Abhängigkeit von c-Myc nach dem Zellzykluseintritt. Gibt es neben der transkriptionellen Aktivierung durch c-Myc weitere Regulationsmechanismen für Pes1, Bop1 und WDR12? Die Beantwortung dieser Frage erscheint besonders interessant, da eine Überexpression von c-Myc als auch von Bop1 und Pes1 Zellen transformiert (Charpentier et al., 2000; Killian et al., 2004, 2006; Kinoshita et al., 2001; Oster et al., 2002). Neben Überexpression führt auch die Depletion von Pes1 oder Bop1 zu einer Zunahme an abnormen Mitosen und damit zu chromosomaler Instabilität (CIN) (Killian et al., 2004). Die Erklärung hierfür könnte in weiteren Funktionen der Proteine jenseits der Ribosomenbiogenese liegen. So ist beispielsweise die Bedeutung der Interaktion mit den Proteinen des ORC-Komplexes in der Hefe noch nicht aufgeklärt und in Säugerzellen bisher noch nicht bestätigt. Ein Komplex aus Pes1/Bop1 mit den ORC-Komplex-Proteinen könnte in Säugerzellen eine Verbindung zwischen Ribosomenbiogenese und Zellzykluskontrolle herstellen.

3.1 Der PeBoW-Komplex

Mit Hilfe von monoklonalen Antikörpern konnte ich in Co-Immunpräzipitations-Analysen zeigen, dass in Analogie zur Hefe ein stabiler Komplex aus den endogenen Proteinen Pes1, Bop1 und WDR12 auch in Säugern existiert (siehe Hölzel et al., 2005, Abb. 7A). Dieser wurde nach den Komponenten Pes1, Bop1 und WDR12 als

PeBoW-Komplex bezeichnet. Um den Komplex näher zu untersuchen, wurde eine native Gelelektrophorese durchgeführt. Dabei werden Proteinkomplexe unter nicht denaturierenden Bedingungen aufgrund ihrer Größe, Ladung und Gestalt aufgetrennt. Die Analyse ergab, dass alle drei Proteine im nativen Gel gleich schnell in derselben Bande, wahrscheinlich als stabiler Komplex wanderten. Eine anschließende Auftrennung dieser Bande in der zweiten Dimension unter denaturierenden Bedingungen, bestätigte die Anwesenheit von Pes1, Bop1 und WDR12 im PeBoW-Komplex (siehe Hölzel et al., 2005, Abb. 7B). Da stringente Bedingungen gewählt wurden, spricht einiges dafür, dass es sich bei dem im nativen Gel sichtbaren Komplex tatsächlich um den in der Hefe beschriebenen Kernkomplex aus lediglich drei Proteinen handelt (Du und Stillman, 2002). Die Methode der nativen Gelelektrophorese ist gut geeignet für die Analyse von Komplexen. Sie sagt allerdings nichts über die Stöchiometrie der beteiligten Komponenten aus. Da sich die Methode nur für sehr stabile Komplexe eignet, kann über die Interaktion mit anderen Proteinen, die nicht stabil assoziiert sind, keine Aussage gemacht werden. Diese könnten durch Co-Immunpräzipitations-Analysen besser untersucht werden.

Die Darstellung des PeBoW-Komplexes durch Co-Immunpräzipitation und native Gelelektrophorese enthält keinerlei Aussage über die Art der Interaktion der einzelnen Komponenten. Eine direkte Interaktion von Pes1 und Bop1 wurde sowohl in einer „Yeast-Two-Hybrid“-Untersuchung als auch in einem „GST-Pulldown“ beschrieben (Lapik et al., 2004). Um die Interaktion von Pes1, Bop1 und WDR12 genauer zu untersuchen, wurden Co-Immunpräzipitations-Analysen in Bop1-depletierten Zellen durchgeführt (siehe Rohrmoser et al., Abb. 3D). Während in Kontrollzellen Pes1 und WDR12 spezifisch vom jeweils anderen co-immunpräzipitiert wurden, war eine Co-Immunpräzipitation bei einem Fehlen von Bop1 nicht mehr möglich. Es gibt folglich keine direkte Interaktion von Pes1 und WDR12. Die Interaktion der beiden Proteine wird durch Bop1 vermittelt (siehe Abb. 6A). Pes1 und Bop1, als auch Bop1 und WDR12 hingegen interagieren direkt. Diese Ergebnisse entsprechen Beobachtungen aus der Hefe (Miles et al., 2005) und zeigen, dass es einen Komplex aus den homologen Proteinen von Nop7p, Erb1p und Ytm1p auch in Säugerzellen gibt. Dieser scheint in seiner Struktur ähnlich angelegt zu sein. In weiteren Experimenten sollte nun der PeBoW-Komplex näher beleuchtet werden.

3.2 Dominant-negative Mutanten

Die Komponenten des PeBoW-Komplex und damit der Komplex selbst sind essentiell für die Ribosomenbiogenese in Säugern (Grimm et al., 2006; Hölzel et al., 2005; Lapik et al., 2004; Strezoska et al., 2002). Depletion der einzelnen Proteine durch siRNA-Technologie führte zu einer Inhibition der Synthese der 28S rRNA (siehe Grimm et al., 2006, Abb. 5B; Rohrmoser et al., 2007, Abb. 3B). Deletionen der C- bzw. N-terminalen Domänen von Pes1, Bop1 und WDR12 führten zu Mutanten mit einem dominant-negativen Phänotyp (Grimm et al., 2006; Hölzel et al., 2005; Strezoska et al., 2000, 2002). Die konditionelle Expression dieser Mutanten in Tetrazyklin-regulierten Vektoren inhibierte reversibel die Zellproliferation durch einen p53-abhängigen Wachstumsarrest und verhindert die Synthese der 28S rRNA, während die Menge an 18S rRNA nicht beeinflusst wird. Die beeinträchtigte Funktion der Deletionsmutanten wurde in Proliferationsversuchen als auch durch eine *in vivo* Markierung der RNA mit ^{32}P dargestellt. Die Analyse der dominant-negativen Deletionsmutanten von Pes1, Bop1 und WDR12 in Immunfluoreszenzen und Sucrosegradienten zeigte, dass die fehlenden Aminosäuren weder die nukleoläre Lokalisation noch die Komplexbildung der Proteine mit den RNP-Partikeln beeinträchtigen. Die Verteilung der Deletionsmutanten im Sucrosegradient entsprach der der WT-Proteine. Ähnliches wurde für die Transposon-generierten Mutanten von Pes1 gezeigt, in denen einzelne Domänen im Protein durch Insertionen unterbrochen werden (Lapik et al., 2004). Der dominant-negative Phänotyp resultiert folglich aus der Inkorporation der Mutanten in den PeBoW-Komplex und damit in die pre-Ribosomen, wo sie vermutlich zur Ausbildung so genannter „dead-end“ Komplexe führen, die nicht mehr mit anderen, für die Prozessierung wichtigen Faktoren interagieren können. Können Mutanten von Pes1, Bop1 und WDR12 also tatsächlich nur dominant-negativ wirken, wenn sie in den PeBoW-Komplex eingebaut werden? Könnte einer Mutante eine für die Funktion des Proteins essentielle Domäne fehlen, ohne dass bei Überexpression ein dominant-negativer Phänotyp detektierbar ist?
Zur Beantwortung dieser Fragen wurden unterschiedliche Mutanten von Pes1 in der BRCT-Domäne untersucht, deren Überexpression keinen dominant-negativen Phänotyp auf die Prozessierung der pre-rRNA und die Zellproliferation hatten (Grimm et al., 2006; Hölzel et al., 2007a). Die Ausbildung von Komplexen und die Interaktion mit Bop1 und WDR12 wurden durch native Gelelektrophorese und Co-

Immunpräzipitation gezeigt. Nach Deletion oder Punktmutation der BRCT-Domäne wurde Pes1, mit einer Ausnahme, der Mutante Pes1 R380W, nicht mehr in den PeBoW-Komplex eingebaut und die Co-Immunpräzipitation mit WDR12 und Bop1 verlief erfolglos (siehe Hölzel et al., 2007a, Abb. 5C und D). Immunfluoreszenz-Experimente unterstrichen diese Beobachtungen. Sie zeigten, dass die Mutanten im Gegensatz zum nukleolären Pes1 diffus nukleoplasmatisch lokalisierten (siehe Hölzel et al., 2007a, Abb. 3A und C). Die Lokalisation der Mutanten von Pes1 sowie die Interaktion mit Bop1 und WDR12 korrelierten dabei stets mit ihrer Funktionalität in der Reifung der 28S rRNA. In Knockin-Experimenten (Hölzel et al., 2007b) konnte die Expression dieser Mutanten den Knockdown des endogenen Pes1 in Bezug auf Reifung der 28S rRNA nicht rekonstituieren (siehe Hölzel et al., 2007a, Abb. 2B und 4A). Die Mutante Pes1 R380W hingegen verhielt sich sowohl in der Lokalisation, der Inkorporation in den PeBoW-Komplex als auch in der Funktionalität wie das WT-Protein. Daher scheint diese Punktmutation die Funktion der BRCT-Domäne nicht zu zerstören. Aus diesen Ergebnissen ergeben sich folgende Schlussfolgerungen:

1. Die nukleoläre Lokalisation von Pes1, Bop1 und WDR12 und ihrer Mutanten sowie deren Einbau in den PeBoW-Komplex sind wichtig für ihre Funktionalität.
2. Dominant-negative Mutanten beeinflussen nicht die Bildung des Komplexes, sondern ihr Einbau in den Komplex hebt dessen Funktionalität auf. Dies geschieht vermutlich durch Blockade weiterer Interaktionen.
3. Mutanten können nur dominant-negativ wirken, wenn sie in den Komplex eingebaut werden. Zeigt eine Mutante bei Überexpression keinen dominant-negativen Phänotyp, bedeutet dies nicht automatisch, dass die von der Mutation betroffene Region nicht essentiell für die Funktion des Proteins ist.

3.3 Die Integrität des PeBoW-Komplex bedingt seine Funktion

Nicht nur der Einbau von dominant-negativen Mutanten in den PeBoW-Komplex, sondern auch die Depletion einzelner Komponenten durch siRNA-Technologie führt zu einer Inhibition der pre-rRNA-Prozessierung und der Zellproliferation (Grimm et al., 2006; Rohrmoser et al., 2007). Was passiert mit dem PeBoW-Komplex unter diesen Bedingungen? Zur Untersuchung dieser Fragestellung erwies sich erneut die native Gelelektrophorese als geeignete Methode. Der Verlust einer der Komplex-

Komponenten führte zum Verschwinden eines intakten PeBoW-Komplexes (siehe Rohrmoser et al., 2007, Abb. 3C). Durch Depletion von Bop1 kam es zum Erscheinen von monomeren Formen von Pes1 und WDR12, da das integrierende Protein des Komplexes fehlte. Die Depletion von Pes1 führte zur Ausbildung eines Subkomplexes aus Bop1 und WDR12, während die Depletion von WDR12 das Auftauchen von monomerem Pes1 zur Folge hatte. Nach Depletion von Pes1 oder WDR12 konnte aber nie freies Bop1 detektiert werden. Auf diesen interessanten Punkt möchte ich später noch genauer eingehen (Abschnitt 3.5).

Was passiert, wenn eine PeBoW-Komponente überexprimiert wird? Wird dadurch die Integrität des Komplexes ebenfalls gestört? Während die Überexpression von Pes1 und WDR12 keinerlei Auswirkungen auf den Komplex und die Reifung der pre-rRNA zeigten, verursachte die Überexpression von Bop1 eine verminderte Zellproliferation und die Hemmung der 28S rRNA-Reifung (siehe Rohrmoser et al., 2007, Abb. 1). Durch das Überangebot von Bop1 kommt es zur Ausbildung von Pes1/Bop1- als auch Bop1/WDR12-Subkomplexen (Abb. 6). Das Überangebot von Bop1 verhindert letztlich die Bildung eines funktionellen PeBoW-Komplexes. Frühere Experimente zeigten eine Blockade der Proliferation als auch der Prozessierung von pre-rRNA nur durch Expression von dominant-negativen Mutanten von Bop1 (Strezoska et al., 2000, 2002). Auswirkungen einer Überexpression des WT-Proteins wurden bislang nicht beschrieben. Im Vergleich zu den Folgen einer Überexpression der dominant-negativen Mutanten Bop1Δ und Bop1N2 auf die Proliferation und die Biosynthese der 60S-Untereinheit sind die Auswirkungen eines Überangebots von Bop1 in unseren Experimenten in der Tat weitaus weniger ausgeprägt. Dennoch sind sie signifikant. Sie treten außerdem erst bei einer längeren Expression auf (Bornkamm et al., 2005).

Somit gibt es unterschiedliche Bedingungen, die zum Verlust der Funktionalität des PeBoW-Komplexes führen. Zum einen den Einbau von dominant-negativen Mutanten, der weitere Interaktionen des Komplexes verhindert, zum anderen eine Störung der Integrität des Komplexes entweder durch eine Depletion einzelner Komponenten oder durch das Überangebot von Bop1.

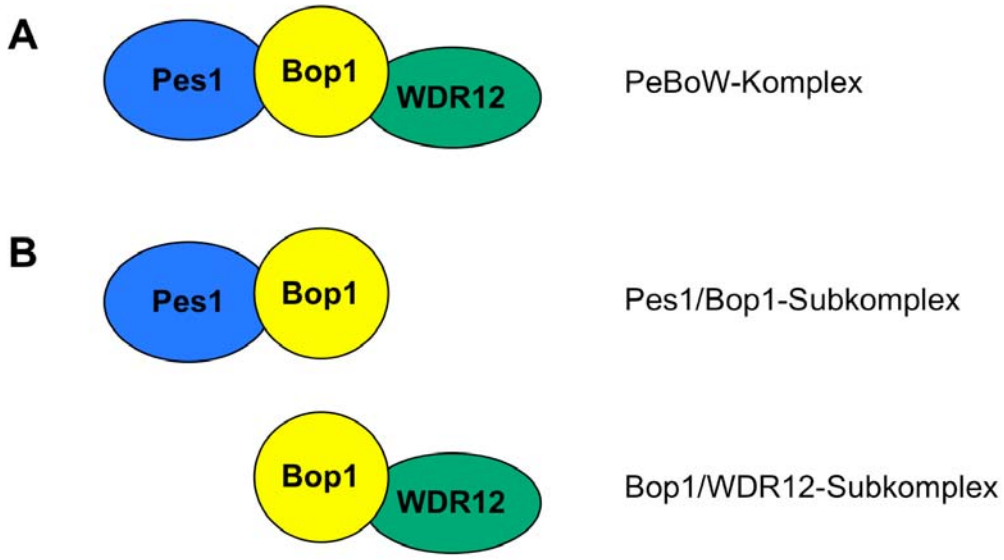

Abbildung 6. Schematische Darstellung des PeBoW-Komplexes und der Auswirkungen einer Bop1-Überexpression. (A) Die Interaktion von Pes1 und WDR12 wird durch Bop1 vermittelt. (B) Durch das Überangebot an Bop1 kommt es zur Ausbildung von Pes1/Bop1- als auch Bop1/WDR12-Subkomplexen. Intakte PeBoW-Komplexe können nicht mehr gebildet werden. Die Überexpression von Pes1 und WDR12 hat dagegen keinerlei Auswirkungen auf die Bildung des PeBoW-Komplexes.

3.4 Lokalisation von Pes1/Bop1- und Bop1/WDR12-Subkomplexen

Die Überexpression von Bop1 führt zur Ausbildung von zwei Subkomplexen, von denen jeder Bop1 enthält. Der dadurch bedingte Verlust eines funktionellen PeBoW-Komplexes scheint den dominant-negativen Phänotyp eines Überangebots von Bop1 zu erklären. In weitergehenden Experimenten konnte ich zeigen, dass die Coexpression von WDR12, nicht aber von Pes1, die negative Wirkung von Bop1 auf rRNA-Reifung und Zellproliferation aufhebt (siehe Rohrmoser et al., 2007, Abb. 1). Zur Analyse dieser Unterschiede wurde die Lokalisation der einzelnen Faktoren und Subkomplexe untersucht. Dazu wurden die Proteine sowohl durch Immunfluoreszenz als auch nach Zellfraktionierung im nativen Gel dargestellt. Während der PeBoW-Komplex sowie überexprimiertes Pes1 und WDR12 sich im Nukleolus befinden, lokalisiert überexprimiertes Bop1 im Zytoplasma (siehe Grimm et al., 2006, Abb. 2; Hölzel et al., 2005, Abb. 2; Rohrmoser et al., 2007, Abb. 8). Diese Beobachtung ist widersprüchlich zu früheren Studien über Bop1 (Strezoska et al., 2000, 2002), die zeigten, dass rekombinantes Bop1 nukleolär lokalisiert und in pre-Ribosomen

eingebaut wird. Auch wir konnten eine nukleoläre Lokalisation von Bop1 feststellen, die allerdings stark von der Expressionsstärke abhängig war. Zellen, die das rekombinante Protein nur schwach exprimierten, zeigten eine nukleoläre Lokalisation von Bop1, wohingegen eine starke Expression zu einer deutlich zytoplasmatischen Lokalisation von Bop1 führte.

Was sind die Folgen einer veränderten Lokalisation von Bop1? Wie bereits beschrieben, zeigten native Gelelektrophorese-Experimente, dass Bop1 monomeres Pes1 und WDR12 in zwei unterschiedliche Subkomplexe zieht. Diese Komplexe verhalten sich unterschiedlich. Während sich der Pes1/Bop1-Subkomplex im Nukleolus befindet, wird der Bop1/WDR12-Subkomplex im Zytoplasma gehalten (siehe Rohrmoser et al., 2007, Abb. 2E und 8). Dies bedeutet, dass im Gegensatz zum Pes1/Bop1-Subkomplex der Komplex aus Bop1 und WDR12 nicht in die pre-Ribosomen eingebaut werden kann. Ein Überangebot an Bop1 hält folglich endogenes WDR12 im Zytoplasma, das dadurch zur Ausbildung eines funktionellen PeBoW-Komplexes im Nukleolus fehlt. Diese Beobachtung erklärt, warum eine Coexpression von WDR12, nicht aber von Pes1, die negativen Folgen einer Bop1-Überexpression ausgleichen kann. Die Daten der Immunfluoreszenzexperimente sowie der Zellfraktionierungen lassen vermuten, dass Pes1 als auch WDR12 selbständig in den Nukleolus gelangen. Bop1 wird dagegen nur in Abhängigkeit von Pes1 in den Nukleolus transportiert.

Wie interagieren die überexprimierten PeBoW-Proteine mit den pre-60S Ribosomen? In der Hefe ist der trimere Komplex aus Nop7p, Erb1p und Ytm1p mit pre-60S Ribosomen assoziiert. Miles et al. (2005) zeigten, dass zunächst Nop7p, gefolgt von Erb1p und schließlich Ytm1p mit dem pre-Ribosom interagieren. Letzteres geschieht vermutlich über Interaktion mit Erb1p. Weiterhin wird vermutet, dass die drei Proteine zusammen als Heterotrimer vom pre-Ribosom dissoziieren können, um eventuell wieder verwendet zu werden. Die Reihenfolge der Assoziation von PeBoW-Komponenten mit den pre-Ribosomen konnte in Säugern bisher nicht beschrieben werden. Die Interaktion von Pes1, Bop1 und WDR12 mit pre-ribosomalen Komplexen wurde von uns und von anderen durch Fraktionierung in Sucrosegradienten dargestellt (siehe Grimm et al., 2006, Abb. 7; Rohrmoser et al., 2007, Abb. 9; Strezoska et al., 2000). Eine Assoziation von überexprimiertem Pes1 als auch WDR12 aber nicht von Bop1 mit den pre-Ribosomen wurde deutlich. Meine Untersuchungen zeigen, dass neben der Lokalisation auch der Einbau von Bop1 in

die pre-ribosomalen Komplexe von der Expressionsstärke abhängt. Schwache Expression führt zu einem teilweisen Einbau des Proteins in pre-ribosomale Partikel. Bei starker Expression bleibt Bop1 zytoplasmatisch.

In Analogie zu den Lokalisationsexperimenten wurde nun die Assoziation der Proteine an pre-Ribosomen bei Coexpression einer anderen PeBoW-Komponente mit Hilfe von Sucrosegradienten untersucht. Der nukleoläre Transport von Bop1 ist von einer Interaktion mit Pes1 abhängig. Verändert eine Coexpression von Pes1 nicht nur die Lokalisation von Bop1, sondern auch seine Assoziation an pre-Ribosomen? Überexprimiertes Bop1 bindet WDR12 im Zytoplasma. Welche Wirkung hat eine Überexpression von Bop1 auf die Assoziation von WDR12 an die pre-Ribosomen? Die Coexpression von Bop1 und WDR12 verminderte die Cofraktionierung von WDR12 mit ribosomalen Partikeln im Sucrosegradienten und hielt WDR12 in Fraktionen mit geringerem molekularem Gewicht (siehe Rohrmoser et al., 2007, Abb.9), das einem Zurückhalten von WDR12 im Zytoplasma entspricht. Durch Bop1-Überexpression befindet sich weniger WDR12 an den pre-Ribosomen. Durch Coexpression von Pes1 und Bop1 hingegen konnte der Anteil von Bop1, der mit pre-Ribosomen assoziiert ist, signifikant erhöht werden. Diese Beobachtung untermauert nochmals die Daten der Immunfluoreszenzexperimente, die zeigen, dass der nukleoläre Transport von Bop1 Pes1-abhängig stattfindet. Ob Pes1 und Bop1 auch als Subkomplex mit den pre-Ribosomen interagieren oder ob sie einzeln mit diesen assoziieren, konnte nicht gezeigt werden. Ebenfalls unklar bleibt, ob WDR12 nach Pes1 und Bop1 an die pre-Ribosomen bindet, wie es in der Hefe beschrieben ist (Miles et al., 2005).

Wie sind nun zusammenfassend die Folgen eines Überangebots an Bop1 auf die Prozessierung der pre-rRNA und die Proliferation zu erklären (Abb. 7)? Bei Überexpression von Bop1 interagiert ein Teil mit endogenem Pes1, wird dadurch in den Nukleolus transportiert und bindet dort an die pre-Ribosomen. Ein anderer Teil von Bop1 hingegen hält endogenes WDR12 im Zytoplasma, welches nun im Nukleolus fehlt. Dadurch kann kein funktioneller PeBoW-Komplex gebildet werden. Durch Coexpression von Pes1 kann dieser Mangel nicht aufgehoben werden. Durch Coexpression von WDR12 wird dagegen wieder genügend WDR12 bereitgestellt und die negative Wirkung einer Bop1-Überexpression auf die Zellproliferation aufgehoben.

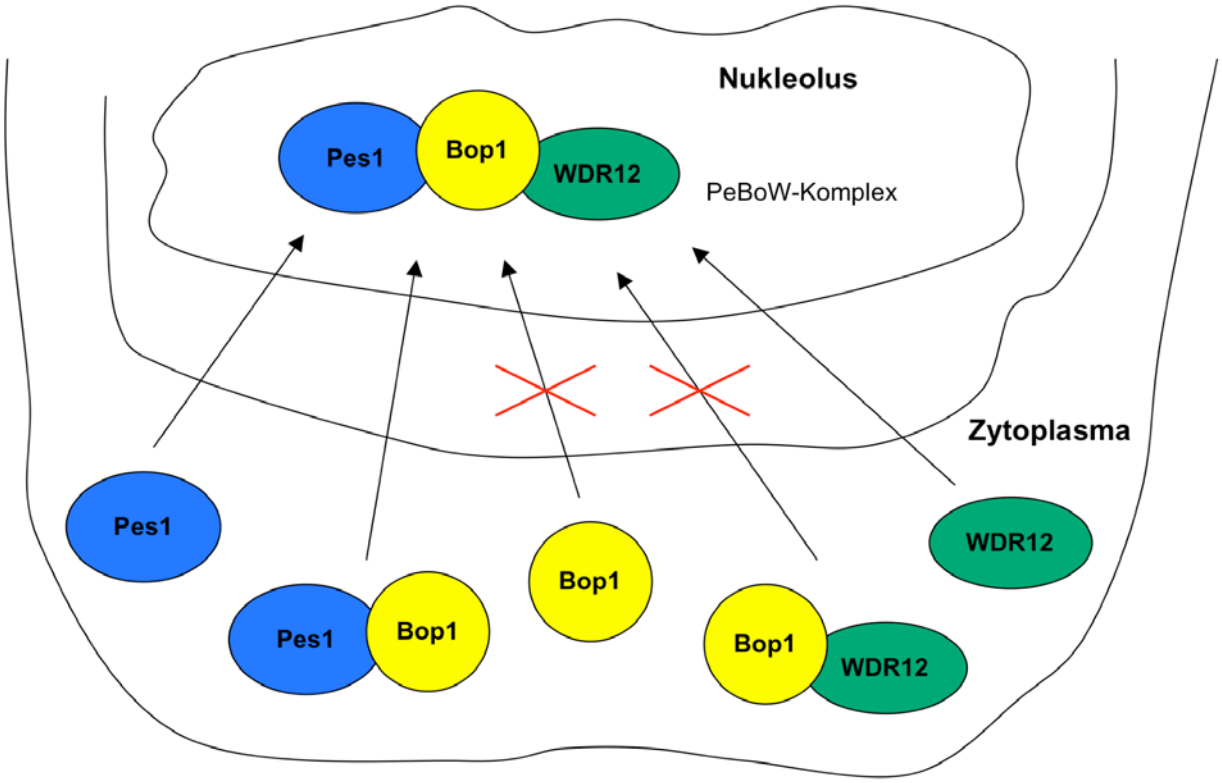

Abbildung 7. Schematische Darstellung der Lokalisation von Pes1, Bop1 und WDR12 sowie deren Komplexe. Während Pes1 und WDR12 selbständig in den Nukleolus gelangen, ist der Transport von Bop1 Pes1-abhängig. WDR12 wird durch erhöhte Mengen an Bop1 in einem Subkomplex mit Bop1 im Zytoplasma zurück gehalten und fehlt im Nukleolus zur Ausbildung eines funktionellen PeBoW-Komplexes.

3.5 Stabilität der Komponenten des PeBoW-Komplexes

Die Folgen einer unbalancierten Expression von Bop1 sind also kritisch für die Zelle. Um ein Ungleichgewicht zu verhindern, werden die Proteine des PeBoW-Komplexes proliferationsassoziiert durch c-Myc induziert und ihre Menge auf der Transkriptionsebene reguliert. Dabei wird sichergestellt, dass die Proteinmenge dem Bedarf an Ribosomen einer wachsenden Zelle angepasst wird. Gibt es zusätzliche Mechanismen, die im Falle einer unbalancierten Expression eines Faktors greifen, um den negativen Auswirkungen eines Bop1-Überangebots entgegen zu wirken?
Mit Hilfe von Cycloheximid-Experimenten konnte ich zeigen, dass Pes1, Bop1 und WDR12 stabile Faktoren sind, sobald sie in den PeBoW-Komplex eingebaut werden

(siehe Rohrmoser et al., 2007, Abb. 7A). Auffällig war allerdings, dass bei Depletion von Pes1 oder WDR12 kein monomeres Bop1 im nativen Gel beobachtet werden konnte (siehe Rohrmoser et al., 2007, Abb. 3C). Langzeitanalysen von Zellen, in denen ein Faktor depletiert wurde zeigten, dass der Verlust einer PeBoW-Komplex-Komponente auch Auswirkungen auf die Stabilität der anderen Bindungspartner hat (siehe Rohrmoser et al., 2007, Abb. 4). Wird die Bildung des Komplexes beispielsweise durch die Depletion eines Faktors verhindert, so reduziert sich auch die Menge der anderen Faktoren dramatisch. Eine gegenseitige Abhängigkeit der Menge der PeBoW-Komponenten konnte somit beschrieben werden.

Wie wird diese gegenseitige Abhängigkeit reguliert? Einerseits kann es sich um eine transkriptionelle Regulation handeln. Analysen der mRNA-Mengen zeigten, dass die Depletion einer Komponente durch siRNA-Oligonukleotide zum Verschwinden der entsprechenden mRNA führt, die Menge der mRNAs der anderen Komponenten wurden allerdings nicht reduziert, sondern stiegen sogar leicht an (siehe Rohrmoser et al., 2007, Abb. 6). Die Ursache für die balancierte Expression der PeBoW-Komponenten scheint ein translationeller oder posttranslationeller Mechanismus zu sein. Überexpression von Pes1, Bop1 oder WDR12 auf dem Hintergrund einer fehlenden Komponente des PeBoW-Komplexes zeigte, dass die gegenseitige Abhängigkeit der Proteinmengen von Pes1, Bop1 und WDR12 durch Proteindestabilisierung verursacht wird (siehe Rohrmoser et al., 2007, Abb. 7B, C und D). Während die Depletion von WDR12 nur geringe Auswirkungen auf die Menge von Pes1 hat, führt die Depletion von Bop1 zu einer starken Abnahme von Pes1. Dieser Unterschied kann durch die Tatsache erklärt werden, dass Pes1 und WDR12 nicht direkt interagieren, sondern dass die Interaktion über Bop1 vermittelt wird. Depletion von Pes1 führt zur starken Abnahme von Bop1. Die gegenseitige Abhängigkeit der PeBoW-Faktoren weist auf die zentrale Rolle von Bop1 hin, die Mengen der anderen Faktoren zu regulieren. Um zu beweisen, dass es sich hierbei um eine spezifische Regulation handelt, wurden Rekonstitutionsexperimente (Knockdown-Knockin) durchgeführt (siehe Rohrmoser et al., 2007, Abb. 5). Tatsächlich konnte eine Destabilisierung der PeBoW-Proteine durch Depletion von endogenem Pes1, Bop1 oder WDR12 nur durch ektopische Expression des entsprechenden Proteins verhindert werden.

Die Proteine des PeBoW-Komplexes bedingen ihre Stabilität gegenseitig, was einem Ungleichgewicht ihrer Mengen entgegenwirkt. Dabei zeigte sich, dass Bop1 das

instabilste Protein ist, wenn es nicht in den Komplex eingebaut wird. Durch die Überexpression von exogenem Bop1 konnte zudem die Menge an endogenem Bop1 stark reduziert werden, ohne die Menge an Pes1 und WDR12 zu beeinträchtigen (siehe Rohrmoser et al., 2007, Abb. 2A). Wahrscheinlich verdrängt das exogen exprimierte Bop1 endogenes Bop1 beim Einbau in den Komplex, was dadurch nicht stabilisiert werden kann.

Wie kann man die Instabilität von freiem Bop1 erklären? Am N-Terminus und in der Mitte von Bop1 befinden sich PEST-Sequenzen (Abb. 5; Strezoska et al., 2000). Regulatorische und kurzlebige Proteine weisen oftmals eine Konsensus-PEST-Sequenz auf, die ein Ziel für den Abbau des Proteins durch das Proteasom darstellt (Chevaillier, 1993; Rechsteiner und Rogers, 1996). Hierbei handelt es sich um eine Anhäufung der Aminosäuren Prolin (P), Glutaminsäure (E), Serin (S) und Threonin (T) sowie zu einem geringeren Anteil von Asparaginsäure (D). Es ist möglich, dass die Instabilität von freiem Bop1 durch die beschriebenen PEST-Sequenzen bedingt wird. Durch Interaktion mit Pes1 und WDR12 und der Inkorporation von Bop1 in den PeBoW-Komplex wird die Funktion der PEST-Motive maskiert und somit das Protein vor einem raschen Abbau geschützt. Im Gegensatz zu Bop1 sind keine PEST-Sequenzen in den Proteinsequenzen von Pes1 und WDR12 beschrieben. Lediglich die Sequenzanalyse mit PESTFind (http://www.at.embnet.org/toolbox/pestfind/) zeigte diese putativen Sequenzen. Inwiefern die PEST-Motive tatsächlich relevant sind, bleibt zu untersuchen.

Die Stabilisierung von Proteinen in Multiproteinkomplexen ist ein wichtiger, regulativer Mechanismus, der verhindert, dass einzelne Komponenten eines Komplexes überproduziert werden. Er könnte damit die Feinabstimmung in der Regulation der Mengen an Komplexkomponenten steuern, wie es für andere Komplexe bereits gezeigt worden ist. Eine gegenseitige Stabilisierung von Proteinen in einem Komplex findet man bei Imp3p, Imp4p und Mpp10p, die Teil des SSU-Prozessoms sind (Wehner et al., 2002). Imp3p reguliert die Aktivität des SSU-Prozessoms, indem es die Interaktionen von Imp4p und Mpp10p mit der U3 snoRNA vermittelt. Mpp10p beeinflusst die Stabilität von Imp3p und Imp4p, seine Depletion führt zur Destabilisierung von Imp3p und Imp4p. Eine gegenseitige Stabilisierung von Komplexkomponenten wurde auch bei Cbf5p, Gar1p, Nhp2p und Nop10p, den Kernkomplexproteinen der H/ACA-Box snoRNPs beobachtet. Cbf5p, Nhp2p und Nop10p sind für die Stabilität von Gar1p und im Allgemeinen der H/ACA-Box

snoRNPs essentiell (Henras et al., 1998; Lafontaine et al., 1998). Die Depletion eines Proteins führt zu einer Abnahme sowohl der Menge an snoRNAs und als auch der Menge an Gar1p. Auch die Stabilität von Nucleolin hängt von der Anwesenheit eines interagierenden Proteins ab. Aprataxin, ein Protein, das in Patienten mit Ataxia-Oculomotor Apraxia 1 (AOA1) mutiert ist, spielt vermutlich eine Rolle in der Reparatur von Einzelstrangbrüchen der DNA (Clements et al., 2004; Gueven et al., 2004; Luo et al., 2004; Mosesso et al., 2005; Sano et al., 2004). AOA1 ist eine seltene degenerative Veränderung des Nervensystems. Bei Depletion von Aprataxin reduziert sich die Stabilität von Nucleolin, während eine Überexpression die Menge an Nucleolin erhöht (Becherel et al., 2006). Bei Abbau von Nucleolin könnten bis zu drei putative PEST-Sequenzen eine Rolle spielen. Diese befinden sich im N-terminalen Bereich des Proteins und wurden mit PESTFind identifiziert. „GST-Pulldown“-Experimente mit einer Serie von Konstrukten von Nucleolin zeigten, dass Aprataxin nur den N-terminalen Bereich von Nucleolin bindet (Becherel et al., 2006). Die Bindung an Aprataxin könnte folglich die PEST-Motive für einen Abbau unzugänglich machen. Der MRE11/RAD50/NBS1-Komplex (MRN-Komplex) bindet an DNA-Doppelstrangbrüche, aktiviert spezifische Zellzyklus-Kontrollpunkte (wie die ATM-Kinase) und bleibt DNA-gebunden, bis der Schaden repariert ist (Carney et al., 1998; Nelms et al., 1998). In Patienten mit dem chromosomalen Instabilitätssyndrom ATLD („Ataxia-Telangiectasia-like disorder“) oder dem Nijmegen Breakage Syndrom (NBS), welche sich durch Hypersensitivität gegen ionisierende Strahlung, Immunschwäche, eine vermehrte Rate an Chromosomentranslokationen und eine erhöhte Prädisposition zur Entwicklung von Tumoren auszeichnen, ist MRE11 (ATLD) bzw. NBS1 (NBS) mutiert (Carney et al., 1998; Stewart et al., 1999; Varon et al., 1998). Fehlt MRE11, zeigt sich auch eine Destabilisierung von NBS1 und RAD50 (Stewart et al., 1999). Die Sequenzanalyse mit PESTFind zeigte putative PEST-Sequenzen in allen drei Proteinen des Komplexes. Diese und unsere Daten zeigen also, dass einzelne Komplexkomponenten nur innerhalb eines Komplexes / Subkomplexes stabilisiert werden können.

3.6 Koordination von Ribosomenbiogenese und DNA-Replikation durch den PeBoW-Komplex

Wir haben gezeigt, dass die Menge an Bop1 für die Funktion des PeBoW-Komplexes entscheidend und daher auch am stärksten reguliert ist. Eine deregulierte Expression von Bop1 führt zu einer erhöhten Tumorprädisposition. Die Inaktivierung von Bop1 in humanen Zellen durch siRNA-Technologie hat eine veränderte chromosomale Segregation und damit abnorme Mitosen zur Folge. Auch eine Überexpression von Bop1 durch eine erhöhte Kopienzahl des Gens führt zu einer Zunahme an multipolaren Spindeln und ist eine der meist häufigen Veränderungen in kolorektalen Tumoren (Killian et al., 2004, 2006). Ob der daraus resultierende Funktionsverlust des PeBoW-Komplexes bzw. fehlende Interaktionen mit anderen zellulären Faktoren, wie der Cdc14 Phosphatase (Ho et al., 2002) oder den ORC-Komplex-Proteinen (Du und Stillman, 2002), die Ursache der erhöhten Tumorprädisposition ist, bleibt zu untersuchen. In der Hefe wird die Cdc14 Phosphatase für den Austritt aus der Mitose und die Koordination der Zytokinese benötigt. Säugerzellen enthalten zwei unabhängige Phosphatasen: Cdc14A und Cdc14B. Die Cdc14A Phosphatase reguliert die Duplikation der Centrosomen. Die Überexpression von Cdc14A hat ein verfrühtes Teilen der Centrosomen und die Ausbildung von multipolaren Spindeln zur Folge. Im Gegensatz dazu führt eine Depletion von Cdc14A durch siRNA-Technologie zu einer verschlechterten Separation der Centrosomen und dadurch zur Bildung von zweikernigen Zellen (Mailand et al., 2002). Die Depletion von Orc6, einer Komponente des ORC-Komplexes, hat die Ausbildung von multipolaren Spindeln, aberrante Mitosen, die Bildung von vielkernigen Zellen und eine verminderte DNA-Replikation zur Folge (Prasanth et al., 2002). Langfristig kommt es zu einer verminderten Zellproliferation und zu einem erhöhten Zelltod. Eine Interaktion des PeBoW-Komplexes mit dem ORC-Komplex in Säugern konnte weder von uns noch von anderen beschrieben werden, da alle Co-Immunpräzipitations-Experimente bislang negativ verliefen (nicht publizierte Daten). Eventuell ist die Interaktion dafür nicht stark genug. Deswegen wurde das Bimolekulare Fluoreszenz-Komplementations-System (BiFC-System) angewendet (Hu et al., 2002), um eine mögliche Interaktion von Pes1 und Orc6 in lebenden Säugerzellen darzustellen. Dabei werden zwei Proteine an das N- bzw. C-terminale Fragment von YFP („yellow fluorescent protein“) fusioniert, wodurch zwei Proteine entstehen, welche

voneinander getrennt keine fluoreszierenden Eigenschaften aufweisen. Bei Interaktion der Proteine kommt es zur Komplementation von YFP und damit zur Fluoreszenz in den Zellen. Der Komplex wird durch die Assoziation der YFP-Fragmente zusätzlich stabilisiert (Hu et al., 2002; Kerppola, 2006). Mit dieser Methode kann nicht nur die Interaktion von Proteinen in der lebenden Zelle bestimmt werden, sondern auch das zelluläre Kompartiment, in dem die Interaktion stattfindet, wie es beispielhaft für Jun und Fos gezeigt worden ist (Abb. 7A). Mit Hilfe der BiFC-Methode konnte ich in Zusammenarbeit mit Andreas Thomae (Helmholtz Zentrum München) zum ersten Mal eine Interaktion von Pes1 mit Orc6 in Säugern nachweisen (Abb. 7B). Das BiFC-Signal weist auf eine nukleoläre Lokalisation der interagierenden Proteine hin.

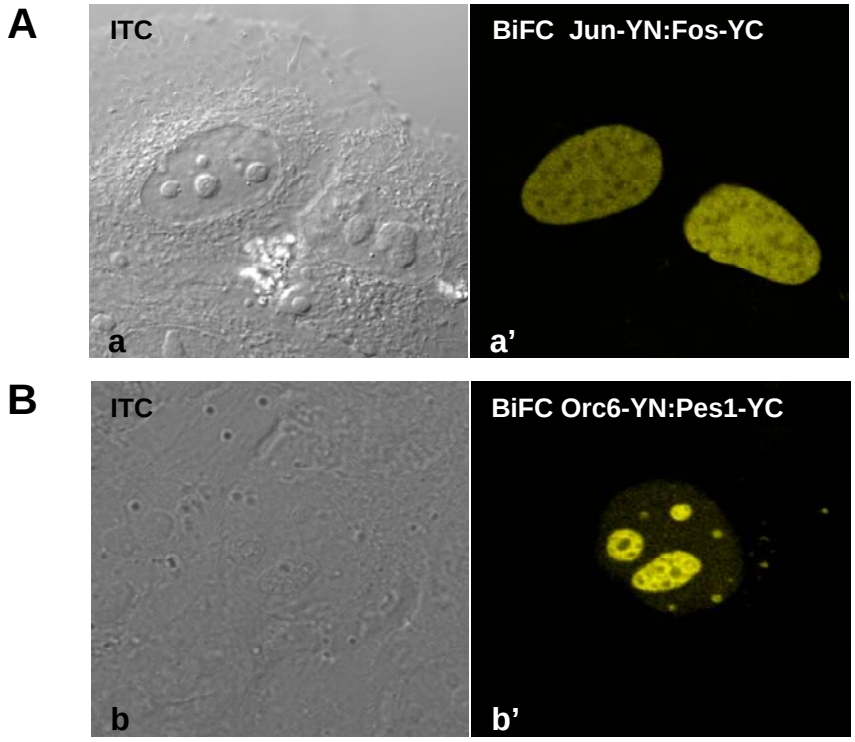

Abbildung 7. Interaktion von Pes1 mit Orc6. (A) Positivkontrolle des BiFC-Systems. Die Konstrukte von Jun und Fos mit den nicht-fluoreszierenden Fragmenthälften von YFP (Jun-YN:Fos-YC) zeigen Fluoreszenz-Komplementation in der lebenden humanen Hepatoblastom-Zelllinie HepG2 (Knowles *et al.*, 1980) (a'). 24-26 Stunden nach Transfektion wurden die Zellen mit einem Leica TCS SP2 Mikroskop untersucht. Die Interaktion kann durch die entstandene Fluoreszenz unter Berücksichtigung der Interferenz-Kontrast-Aufnahme (a) nukleolär lokalisiert werden (Hu et al., 2002). (B) Die Konstrukte von Orc6 und Pes1 mit den nicht-fluoreszierenden Fragmenthälften von YFP (Orc6-YN:Pes1-YC) zeigen Fluoreszenz-Komplementation (b'). Die dazugehörige Interferenz-Kontrast-Aufnahme weist auf eine nukleoläre Lokalisation hin (b).

Das Auftreten von abnormen Mitosen nach Depletion sowohl von Pes1, Bop1 als auch von Orc6 durch siRNA-Technologie (Killian et al., 2004; Prasanth et al., 2002), unterstreicht die Rolle des PeBoW-Komplexes und die Wichtigkeit einer Interaktion mit dem ORC-Komplex für den korrekten Ablauf der DNA-Replikation. Ob eine Störung der Organisation des Zytoskeletts oder eine direkte Störung der DNA-Replikation selbst dabei die Ursache für aberrante Mitosen ist, gilt noch zu klären. Die Interaktion des PeBoW- und des ORC-Komplexes könnte dazu dienen, die rDNA-Transkription mit der rDNA-Replikation zu koordinieren und den erhöhten Bedarf an Ribosomen in proliferierenden Zellen anzupassen.

3.7 Ausblick

Für den homologen Komplex aus Pes1, Bop1 und WDR12 sind in der Hefe neben der Interaktion mit den Proteinen des ORC-Komplexes eine Reihe weiterer Interaktionen beschrieben worden (Krogan et al., 2006), deren Funktion noch ungeklärt ist. Deswegen soll der PeBoW-Komplex aufgereinigt, seine Zusammensetzung massenspektroskopisch bestimmt und Interaktionspartner auf diesem Wege identifiziert werden. Die detaillierte biochemische Charakterisierung des PeBoW-Komplexes stellt einen ersten wichtigen Schritt in der Funktionsanalyse des Komplexes dar. Langfristiges Ziel wird sein, die Verbindung zwischen Ribosomenbiogenese, Zellzykluskontrolle und DNA-Replikation besser zu verstehen.

4 ZUSAMMENFASSUNG

Während der Zellproliferation müssen Zellwachstum und Zellteilung koordiniert werden. Die Kopplung erfolgt in der Hefe durch einen Komplex aus Nop7p, Erb1p und Ytm1p, der sowohl an der Ribosomenbiogenese als auch an der Kontrolle der DNA-Replikation beteiligt ist. Die homologen Proteine Pes1, Bop1 und WDR12 werden in Säugern von Zielgenen des Transkriptionsfaktors c-Myc, einem zellulären Onkoprotein, kodiert.

In dieser Arbeit wurde die Existenz eines evolutionär konservierten Komplexes aus Pes1, Bop1 und WDR12 (PeBoW-Komplex) in Säugern belegt. Dabei wurde gezeigt, dass Bop1 als zentrales Protein des Komplexes agiert und die Interaktion von Pes1 und WDR12 vermittelt. Die Integrität des Komplexes ist wesentlich für seine Funktion. Die Depletion einzelner Komponenten sowie die Überexpression des integrierenden Proteins Bop1 hemmen die Reifung der Vorläufer-rRNA der großen ribosomalen Untereinheit sowie die Proliferation der Zellen. Bop1-Überexpression führt zur Ausbildung von zwei Subkomplexen aus Bop1 und Pes1 bzw. Bop1 und WDR12. Während der Bop1/Pes1-Subkomplex als Teil der pre-Ribosomen im Nukleolus lokalisiert, wird WDR12 durch Bop1-Überexpression im Zytoplasma gehalten und fehlt im Nukleolus zur Ausbildung eines funktionellen PeBoW-Komplexes. Pes1 und WDR12 können unabhängig in den Nukleolus translozieren, während Bop1 dafür die Interaktion mit Pes1 benötigt. Untersuchungen zur Stabilität der einzelnen PeBoW-Komponenten zeigten, dass monomeres Bop1 extrem instabil ist, durch Inkorporation in den PeBoW-Komplex aber vor Abbau geschützt wird. Möglicherweise werden hierdurch interne PEST-Sequenzen in Bop1 maskiert. Die Menge an Bop1 ist somit abhängig von der Anwesenheit von Pes1 und WDR12. Die gegenseitige Abhängigkeit der Stabilität aller drei PeBoW-Komponenten konnte in weitergehenden Experimenten gezeigt werden. Schließlich wurde untersucht, ob der PeBoW-Komplex die Ribosomenbiogenese mit der DNA-Replikation über Interaktion mit dem ORC-Komplex, wie in der Hefe beschrieben, koordiniert. Mit Hilfe der BiFC-Methode konnte eine Interaktion von Pes1 mit Orc6, eines Faktors des ORC-Komplexes, gezeigt werden.

Die koordinierende Funktion des PeBoW-Komplexes für Zellwachstum und Zellproliferation scheint von der Hefe bis zum Menschen stark konserviert zu sein.

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6 ANHANG

6.1 Verzeichnis der verwendeten Abkürzungen

5´/3´-ETS	5´/3´-External Transcribed Spacer – externe transkribierte Sequenz
Abb.	Abbildung
AMP	Adenosinmonophosphat
ATLD	„Ataxia-Telangiectasia-like disorder“, chromosomales Instabilitätssyndrom
ATP	Adenosintriphosphat
bHLH-LZ	basische Helix-Loop-Helix-/Leucin-Zipper Strukturdomäne
BiFC-System	Bimolekulare Fluoreszenz-Komplementations-System
Bop1Δ	Deletion des N-Terminus von Bop1
Bop1N2	Deletion des C-Terminus von Bop1
bzw.	beziehungsweise
C-	Carboxy-
ca.	circa
Cdk	Cyclin-abhängige Kinase
c-Myc	zelluläres Homolog des viralen Onkogens v-myc
CTP	Cytosintriphosphat
DEAD-Box	konserviertes Motiv aus Asparaginsäure (D), Glutaminsäure (E), Alanin (A), Asparaginsäure (D)
DNA	Desoxynukleinsäure
E2F	Transkriptionsfaktor E2F
et al.	et alii (und andere)
G1-Phase	Gap-Phase 1
GMP	Guanosinmonophosphat
GST	Glutathion-S-Transferase
GTP	Guanosintriphosphat
ITS-1/2	Internal Transcribed Spacer 1/2 – interne transkribierte Sequenz 1/2
kDa	kilo-Dalton
M-Phase	Mitose
Max	Myc-assoziierter Faktor X
MCM	„minichromosome maintainance“
mRNA	Boten-RNA

myc	Myeloblastom
N-	Amino-
NBS	Nijmegen Breakage Syndrom
ORC	„Origin Recognition complex“
pes1$^{-/-}$	Knock-out des endogenen *pes1*-Gens
PESTFind	Programm zur Analyse von putativen PEST-Sequenzen, erhältlich unter http://www.at.embnet.org/toolbox/pestfind/
PEST-Sequenz	Anhäufung von Prolin (P), Glutaminsäure (E), Serin (S), Threonin (T); Zielsequenz für den Abbau des Proteins durch das Proteasom
pre-rRNA	Vorläufer rRNA
Rb	Retinoblastom, Tumorsuppressor
PRPP	Phosphoribosyl-pyrophosphat
RNA	Ribonukleinsäure
rRNA	ribosomale RNA
SNF5	Transkriptionsfaktor SNF5
S-Phase	DNA-Synthesephase
siRNA	„small interfering RNA“
SL1	„Selektivitätsfaktor“, TIP-1B in Maus
snoRNA	kleine nukleoläre RNA
snoRNP	Ribonukleoproteinpartikel
SSU	kleine Untereinheit
SWI/SNF	Chromatin-Modellierungskomplex
tRNA	Transfer-RNA
YFP	„yellow fluorescent protein“
u.a.	unter anderem
UBF	„HMG1-Box architectural upstream binding“ Faktor
UDP	Uridindiphosphat
UMP	Uridinmonophosphat
WT	Wildtyp

6.2 Curriculum Vitae

Vor- und Zuname: Michaela Rohrmoser
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Publikationen

Rohrmoser M, Hölzel M, Grimm T, Malamoussi A, Harasim T, Orban M, Pfisterer I, Gruber-Eber A, Kremmer E, Eick D (2007): Interdependence of Pes1, Bop1, and WDR12 controls nucleolar localization and assembly of the PeBoW complex required for maturation of the 60S ribosomal subunit. Mol Cell Biol 27:3682-94.

Hölzel M, Grimm T, Rohrmoser M, Malamoussi A, Harasim T, Gruber-Eber A, Kremmer E, Eick D (2007a): The BRCT domain of mammalian Pes1 is crucial for nucleolar localization and rRNA processing. Nucleic Acids Res 35:789-800.

Hölzel M, Rohrmoser M, Orban M, Hömig C, Harasim T, Malamoussi A, Gruber-Eber A, Heissmeyer V, Bornkamm G, Eick D (2007b): Rapid conditional knock-down-knock-in system for mammalian cells. Nucleic Acids Res 35:e17.

Grimm T, Hölzel M, Rohrmoser M, Harasim T, Malamoussi A, Gruber-Eber A, Kremmer E, Eick D (2006): Dominant-negative Pes1 mutants inhibit ribosomal RNA processing and cell proliferation via incorporation into the PeBoW-complex. Nucleic Acids Res 34:3030-43.

Hölzel M, Rohrmoser M, Schlee M, Grimm T, Harasim T, Malamoussi A, Gruber-Eber A, Kremmer E, Hiddemann W, Bornkamm GW, Eick D (2005): Mammalian WDR12 is a novel member of the Pes1-Bop1 complex and is required for ribosome biogenesis and cell proliferation. J Cell Biol 170:367-78.

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6.3 Danksagung

An dieser Stelle möchte ich mich bei allen Personen bedanken, die mich unterstützen und zum Gelingen dieser Arbeit beigetragen haben.

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6.4 Publikationen als Teil der kumulativen Doktorarbeit

I. Rohrmoser M, Hölzel M, Grimm T, Malamoussi A, Harasim T, Orban M, Pfisterer I, Gruber-Eber A, Kremmer E, Eick D (2007): Interdependence of Pes1, Bop1, and WDR12 controls nucleolar localization and assembly of the PeBoW complex required for maturation of the 60S ribosomal subunit. Mol Cell Biol 27:3682-94.

Die Experimente wurden von mir geplant, durchgeführt und ausgewertet. Die *in vivo* Markierung der RNA mit ^{32}P zur Analyse der Reifung der pre-rRNA übernahm Michael Hölzel. Die Publikation wurde von mir geschrieben, wobei mir Michael Hölzel und Dirk Eick bei der Ausformulierung halfen.

II. Hölzel M, Grimm T, Rohrmoser M, Malamoussi A, Harasim T, Gruber-Eber A, Kremmer E, Eick D (2007a): The BRCT domain of mammalian Pes1 is crucial for nucleolar localization and rRNA processing. Nucleic Acids Res 35:789-800.

Die Fragestellung wurde von Michael Hölzel formuliert. Mein Beitrag beinhaltet die native Gelelektrophorese-Experimente sowie die Co-Immunpräzipitations-Analyse, die jeweils die Inkorporation der Pes1-Mutanten in den PeBoW-Komplex zeigen.

III. Hölzel M, Rohrmoser M, Orban M, Hömig C, Harasim T, Malamoussi A, Gruber-Eber A, Heissmeyer V, Bornkamm G, Eick D (2007b): Rapid conditional knock-down-knock-in system for mammalian cells. Nucleic Acids Res 35:e17.

Die Experimente wurden von Michael Hölzel geplant, durchgeführt und ausgewertet. Ich habe bei der Diskussion der Daten geholfen.

IV. Grimm T, Hölzel M, Rohrmoser M, Harasim T, Malamoussi A, Gruber-Eber A, Kremmer E, Eick D (2006): Dominant-negative Pes1 mutants inhibit ribosomal RNA processing and cell proliferation via incorporation into the PeBoW-complex. Nucleic Acids Res 34:3030-43.

Die Fragestellungen wurden vor allem von Thomas Grimm formuliert und bearbeitet. Ich habe zur Beantwortung der Fragen durch Durchführung und Auswertung der Sucrosegradient-Experimente, in denen die Assoziation der

Pes1-Mutanten mit den pre-RNP-Partikeln untersucht wurde, sowie durch die Etablierung und Diskussion der Co-Immunpräzipitations-Analyse, in denen die Interaktion der Pes1-Mutanten mit Bop1 und WDR12 gezeigt wurden, beigetragen.

V. Hölzel M, Rohrmoser M, Schlee M, Grimm T, Harasim T, Malamoussi A, Gruber-Eber A, Kremmer E, Hiddemann W, Bornkamm GW, Eick D (2005): Mammalian WDR12 is a novel member of the Pes1-Bop1 complex and is required for ribosome biogenesis and cell proliferation. J Cell Biol 170:367-78.

Die Experimente wurden von Michael Hölzel geplant, durchgeführt und ausgewertet. Ich habe den PeBoW-Komplexes in Co-Immunpräzipitations-Experimenten und durch native Gelelektrophorese dargestellt sowie bei der Diskussion und Auswertung der Daten geholfen. Weiterhin war ich verantwortlich für das Testen der Antiseren gegen endogenes Pes1, WDR12 und Bop1.

München, 27. März 2008

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Interdependence of Pes1, Bop1, and WDR12 Controls Nucleolar Localization and Assembly of the PeBoW Complex Required for Maturation of the 60S Ribosomal Subunit▽

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The PeBoW complex is essential for cell proliferation and maturation of the large ribosomal subunit in mammalian cells. Here we examined the role of PeBoW-specific proteins Pes1, Bop1, and WDR12 in complex assembly and stability, nucleolar transport, and preribosome association. Recombinant expression of the three subunits is sufficient for complex formation. The stability of all three subunits strongly increases upon incorporation into the complex. Only overexpression of Bop1 inhibits cell proliferation and rRNA processing, and its negative effects could be rescued by coexpression of WDR12, but not Pes1. Elevated levels of Bop1 induce Bop1/WDR12 and Bop1/Pes1 subcomplexes. Knockdown of Bop1 abolishes the copurification of Pes1 with WDR12, demonstrating Bop1 as the integral component of the complex. Overexpressed Bop1 substitutes for endogenous Bop1 in PeBoW complex assembly, leading to the instability of endogenous Bop1. Finally, indirect immunofluorescence, cell fractionation, and sucrose gradient centrifugation experiments indicate that transport of Bop1 from the cytoplasm to the nucleolus is Pes1 dependent, while Pes1 can migrate to the nucleolus and bind to preribosomal particles independently of Bop1. We conclude that the assembly and integrity of the PeBoW complex are highly sensitive to changes in Bop1 protein levels.

The nucleolus is the site of the highly regulated, evolutionarily conserved processes of rRNA transcription, pre-rRNA processing, and ribosome subunit assembly (30). The mammalian 18S, 5.8S, and 28S rRNAs are derived from a single 47S precursor (pre-rRNA), which is processed to the mature species through a series of endonucleolytic, exonucleolytic, and modification steps (5, 31). Mature rRNAs are assembled into 40S and 60S ribosomal subunits (8). The biogenesis of ribosomes is a major expenditure of cellular resources that needs to be tightly coordinated with cell cycle progression. Doubling of the translational machinery is essential for continuous cell proliferation to sustain the equilibrium between cell growth and cell division (16, 24). The recent years have revealed interesting links between the nucleolus and cell cycle regulation. Ribosome biogenesis is highly sensitive to cellular stresses such as chemotherapeutic agents like actinomycin D. Ribosomal proteins like L11 are no longer incorporated into nascent ribosomes. They accumulate as free proteins and bind and inactivate the E3 ubiquitin ligase Hdm2 that targets the tumor suppressor p53 for degradation (21, 33). Thus, p53 accumulates and elicits cell cycle arrest and apoptosis. Alternatively, it was shown that a subset of ribosomes contained cytoplasmic p53 covalently linked to 5.8S rRNA (7). Therefore, the export of intact ribosomal subunits to the cytoplasm may be important for p53 degradation as well (27). Despite these remarkable connections between ribosome synthesis and other cellular processes, little is known about the mammalian ribosome biogenesis machinery compared to yeast.

We have recently characterized a nucleolar complex of endogenous Pes1, Bop1, and WDR12 in mammalian cells, termed the PeBoW complex (14). Interestingly, expression of N-terminal or C-terminal truncations of Pes1, Bop1, or WDR12 in mammalian cells blocked processing of the 32S pre-rRNA into mature 28S rRNA and triggered p53-dependent cell cycle arrest (10, 14, 20, 23). Apparently, the PeBoW complex is a good target for the generation of dominant-negative mutants and plays a crucial role in rRNA processing and maturation of the large ribosomal subunit.

Coimmunoprecipitation assays showed that dominant-negative mutant forms of Pes1 were indeed incorporated into the PeBoW complex, suggesting that they block its function by building up dead-end complexes that prevent further essential interactions (10, 20). Thus, the amount of PeBoW components needs to be tightly controlled and adjusted to the rate of ribosome synthesis and proliferation. Quiescent or serum-starved cells exhibit low levels of Pes1, Bop1, and WDR12 that are induced by the proto-oncogene c-Myc upon cell cycle entry (14). c-Myc is overexpressed in a variety of human malignancies, suggesting a coordinated upregulation of the PeBoW complex in tumor cells. But interestingly, amplification of the gene for Bop1, but not that for Pes1, was frequently found in colorectal cancers, associated with an increase in Bop1 mRNA (18). The PeBoW complex may play an additional role in mitosis, as transient overexpression of Bop1 increased the percentage of multipolar spindles. Depletion of Pes1 or Bop1 also caused an increase in abnormal mitotic figures (17).

These observations underline the importance of a functional PeBoW complex playing a role in the cross talk between ribo-

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some biogenesis and the cell division cycle. However, it is unknown how the integrity of the PeBoW complex is controlled in mammalian cells.

In this study, we investigated how changes in the abundance of individual PeBoW components affect its functionality. We show that overexpression of Bop1 disturbs cell proliferation and ribosome biogenesis by titrating endogenous WDR12 into a Bop1/WDR12 subcomplex and that its negative effects could be rescued by coexpression of WDR12, but not Pes1. Further, Bop1 was found to be essential for the copurification of Pes1 and WDR12, thus arguing for an indirect interaction mediated by Bop1. Finally, depletion of individual PeBoW components by RNA interference revealed a strong interdependence of their protein levels. Thus, the integrity of the PeBoW complex is tightly controlled by protein-protein interactions and highly sensitive to elevated levels of Bop1.

MATERIALS AND METHODS

Tissue culture. TGR-1 rat fibroblasts, U2OS osteosarcoma cells, and H1299 lung carcinoma cells (non-small-cell lung carcinoma) were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum at 8% CO_2. For generation of polyclonal cell lines, 6×10^5 cells were transfected with the respective pRTS-1 plasmids by using Polyfect (QIAGEN) and stably selected in the presence of 200 μg/ml hygromycin B and/or 1 μg/ml puromycin for 10 to 14 days. Conditional gene expression was induced with 1 μg/ml doxycycline. Pes1 and WDR12 carrying a C-terminal hemagglutinin (HA) tag are of human origin, whereas Bop1 carrying an N-terminal HA tag is mouse specific. Therefore, we analyzed different cell types and species.

RNA analysis and ^{32}P in vivo labeling. Total RNA was isolated with Trifast (PeqLab). Two micrograms of total RNA for detection of ITS-1 and ITS-2 or 10 μg of total RNA for analyzing the endogenous mRNA levels was separated on a 1% agarose-formaldehyde gel and blotted onto Hybond N^+ membranes (GE Healthcare). The following ^{32}P-end-labeled DNA oligonucleotides were used to visualize rRNA precursors: ITS-1 (human specific), 5′-CCTCCGCGCCGGAA CGCGCTAGGTACCTGGACGGCGGGGGGGCGGACG-3′; ITS-2 (human specific), 5′-GCGGCGGCAAGAGGAGGGCGGACGCCGCCGGGTCTGC GCTTAGGGGGA-3′; ITS-1 (rat specific), 5′-GGACCAGACCCGACACCCT GCCACCGCACACCTGTCCCGAAACCCCCT-3′; ITS-2 (rat specific), 5′-GC CCCGGGGAGCGGGCCCTGCGAGCAGACTCCCAGCCGCGCGACGCG A-3′; 18S rRNA (human and rat specific), 5′-CACCCGTGGTCACCATGGTA GGCACGGCGACTACCATCGAAAGTTGATAG-3′.

Metabolic labeling of rRNA has been described elsewhere (14).

Production of antibodies. Monoclonal antibodies (MAbs) against human nucleostemin and human Nog1 were generated as previously described (14). For immunization, we used a glutathione *S*-transferase (GST)–nucleostemin fusion protein and a Nog1-specific peptide coupled to ovalbumin (Peptide Specialty Laboratories GmbH, Heidelberg, Germany). The Nog1 peptide sequence is ESKEKNTQGPRMPRTAKKVQRTVLEKC. The nucleostemin (7H3) MAb belongs to the immunoglobulin G2b subclass, and the Nog1 (1D8) MAb belongs to the immunoglobulin G2a subclass. Polyclonal antibodies against mouse Bop1 were raised by immunization of guinea pigs with a mixture of the peptides GKPHMSPASLPGKRRLEPDQELQIQ and SQEHTQVLLHQVSRRRSQSP FRRSHG.

Immunoblotting, immunofluorescence, and immunoprecipitation. For immunoblotting, cells were directly lysed with 2× sodium dodecyl sulfate (SDS) loading buffer (100 mM Tris/HCl, 200 mM dithioerythritol, 4% SDS, 10 mM EDTA, 0.2% bromophenol blue, 20% glycerol). Whole-cell lysates were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (GE Healthcare). Immunodetection was performed with anti-HA (3F10; Roche), anti-Pes1 (8E9), anti-Bop1 (6H11), anti-WDR12 (1B8), antinucleostemin (7H3), anti-Nog1 (1D8), anti-NPM1 (clone FC82291; Sigma Aldrich), antitubulin (Sigma Aldrich), anti-p53 (PAb240; Dianova), and anti-c-Myc (N-262; Santa Cruz Biotechnology, Inc.). Recombinant mouse Bop1 protein was detected with a 1:10,000 dilution of polyclonal mouse Bop1-specific guinea pig antibodies in methanol-acetone-fixed cells. Immunofluorescence and immunoprecipitation have been described elsewhere (14).

Native gel electrophoresis. Cells (3×10^6) were lysed in 100 μl lysis buffer (50 mM Tris-HCl [pH 8.0], 1% NP-40, 150 mM NaCl, phosphatase inhibitors, protease inhibitors) at 4°C for 20 min. A 7.5-μl volume of 2× sample buffer (125 mM Tris-HCl [pH 6.8], 30% glycerol, 0.02% bromophenol blue) was added to 7.5 μl of total lysate and separated by polyacrylamide gel electrophoresis (6.5%) in the absence of SDS at 4°C. Blotting was performed in the absence of methanol. Immunoblotting was performed as described above.

siRNA transfection. The day before transfection, ~5×10^4 to 10^5 cells were seeded in six-well plates. Five microliters of 20 μM control, Pes1-, Bop1-, or WDR12-specific small interfering RNA (siRNA) was diluted in 150 μl of Opti-MEM (Invitrogen). One hundred fifty microliters of OptiMEM containing 5 μl of Oligofectamine (Invitrogen) was added, and the mixture was incubated for 15 min. Finally, 600 μl of OptiMEM was added and the mixture was applied to cells after aspiration of the culture medium. Cells were incubated for 5 to 6 h. The following sequences (sense) were used: Pes1 UTR, CCAGAGGACCUAAGU GUGAdTdT; Pes1 ORF, AGGUCUUCCUGUCCAUCAAdTDT; Bop1 UTR, UCGUGCUGAAGUCAACAGAdTdT; Bop1 ORF, AUGGCAUGGUGUAC AAUGAdTdT; WDR12 UTR-1, CGUACGUUUCCGUGGGCAAdTdT; WDR12 UTR-2, CGCUUACCUGUGCAGUCUAdTdT; Control (nonspecific siRNA), UUCUCCGAACGUGUCACGUdTdT.

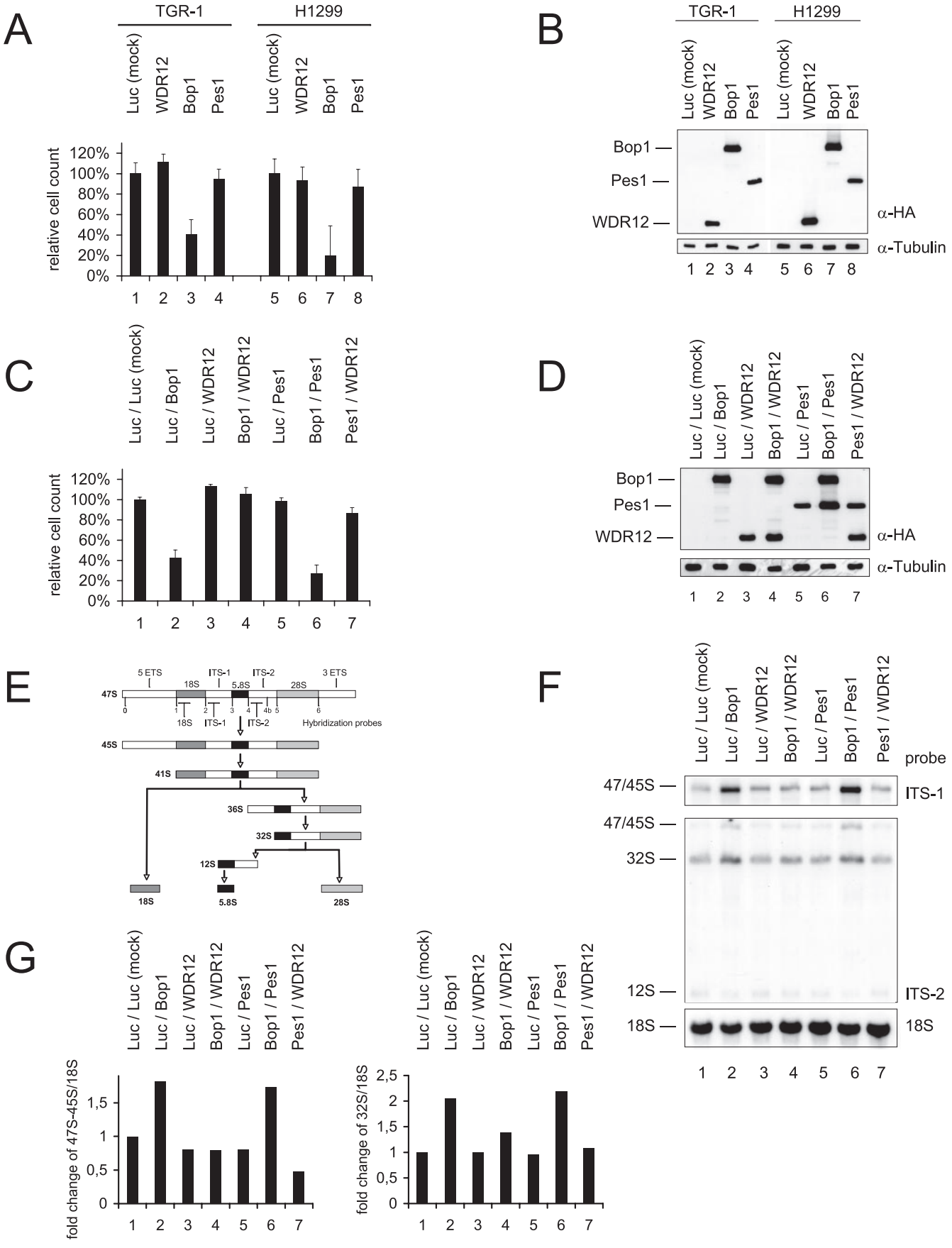
Knockdown–knock-in assay. Exogenous gene expression (Pes1, Bop1, or WDR12) was activated for 3 days by treatment with 1 μg/ml doxycycline and then maintained throughout the subsequent course of two siRNA transfections with siRNAs directed against the 3′ untranslated region (UTR) of the Pes1, Bop1, or WDR12 mRNA.

Cell fractionation and sucrose gradients. Cells were harvested by trypsinization and washed three times with cold phosphate-buffered saline. Cells (3×10^6) were lysed in 100 μl lysis buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5% NP-40, protease inhibitors) at 4°C for 20 min. Cytoplasmic fractions were isolated after centrifugation. The pelleted nuclei were washed three times with cold lysis buffer A and then lysed in 100 μl lysis buffer B (50 mM Tris-HCl [pH 8.0], 1% NP-40, 150 mM NaCl, protease inhibitors) at 4°C for 20 min. Sucrose gradients have been described elsewhere (10).

RESULTS

Overexpression of Bop1 negatively affects proliferation and processing of pre-rRNA. We aimed to investigate the control of PeBoW complex integrity by increasing or decreasing the amount of its individual members. First, we determined the proliferation rates of TGR-1 and H1299 cells overexpressing Pes1, Bop1, or WDR12. Cells were stably transfected with pRTS constructs (1) conditionally expressing the respective HA-tagged wild-type forms or luciferase in a doxycycline-dependent manner. Equal numbers of cells were seeded in the presence of doxycycline, and cell numbers were determined after 6 days. Overexpression of Bop1-HA reduced the cell count up to 41% in TGR-1 and to 20% in H1299 cells (Fig. 1A, lanes 3 and 7) compared to control cells expressing luciferase (lanes 1 and 5). Overexpression of neither WDR12-HA (lanes 2 and 6) nor Pes1-HA (lanes 4 and 8) significantly affected cell proliferation. Expression of the HA-tagged forms of Pes1, Bop1, and WDR12 was verified by Western blot analysis (Fig. 1B).

Next, we tested whether the antiproliferative effect of Bop1 overexpression resulted from an altered stoichiometry of the PeBoW complex and could be alleviated or rescued by coexpression of either Pes1 or WDR12. H1299 cells were stably transfected with two individual pRTS constructs harboring a puromycin or a hygromycin resistance gene. The enhanced green fluorescent protein of the last one was replaced with monomeric red fluorescent protein to better monitor coexpression (data not shown). Cells expressing Pes1, Bop1, or WDR12 or combinations thereof were seeded in equal cell numbers. The number of cells was determined in multiples and compared with the mean cell count of a mock-treated cell line after 6 days (Fig. 1C). The negative effect of Bop1 overexpression on



cell proliferation could be rescued by coexpression of WDR12, but not Pes1 (Fig. 1C, lanes 4 and 6). Expressing WDR12 together with Pes1 did not alter the proliferation rate (Fig. 1C, lane 7). Equal expression levels were determined by Western blot analysis (Fig. 1D).

The PeBoW complex is involved in pre-rRNA processing. Therefore, we studied the maturation of rRNA in cells overexpressing individual PeBoW components. A scheme of mammalian RNA-processing pathways is shown in Fig. 1E. Pes1, Bop1, WDR12, and combinations thereof were stably expressed in H1299 and TGR-1 (data not shown) cells for 24 h. Total RNA was isolated and analyzed by Northern blot analysis with probes specific for internal transcribed spacer 1 (ITS-1) and ITS-2 of the ribosomal pre-rRNA (Fig. 1F and G). Bop1-overexpressing cells accumulated the 47/45S and 32S pre-rRNAs by twofold (Fig. 1F and G, lanes 2) whereas overexpression of WDR12 or Pes1 did not interfere with pre-rRNA processing (Fig. 1F and G, lanes 3 and 5). The Bop1-mediated aberrant accumulation of pre-rRNAs could be completely reversed through coexpression of WDR12, but not Pes1 (Fig. 1F and G, lanes 4 and 6). These results are in line with the proliferation experiments showing that coexpression of WDR12 but not Pes1 alleviates the negative effects of Bop1 overexpression.

Overexpression of Bop1 titrates endogenous WDR12 into a Bop1/WDR12 subcomplex. These results prompted us to investigate the impact of Bop1 overexpression on the PeBoW complex in more detail. We examined whether ectopically expressed Pes1, Bop1, or WDR12 affected the abundance of the endogenous PeBoW proteins. Endogenous Pes1 and WDR12 can be discriminated from the human HA-tagged forms by their lower molecular weights. Recombinant rodent HA-Bop1 is not recognized by the human-specific Bop1 MAb. Overexpression of HA-Bop1, but not WDR12-HA, Pes1-HA, or luciferase, strongly reduced the steady-state levels of endogenous Bop1 in H1299 cells after 6 days (Fig. 2A). However, such a decrease in the endogenous Bop1 protein level was not detected in cells overexpressing Bop1 for only 1 day (data not shown). We did not observe a decrease in the protein levels of endogenous proteins WDR12 and Pes1 (Fig. 2A, lanes 2 and 4) after expression of Pes1-HA and WDR12-HA, respectively.

We have previously shown that the intact PeBoW complex can be visualized by native gel electrophoresis. Therefore, we established stable U2OS cell lines that conditionally overexpressed HA-tagged Pes1, Bop1, WDR12, or luciferase for 1 day and analyzed the PeBoW complex by native gel electrophoresis (14). Immunoblot analysis of all three proteins showed a single band representing the PeBoW complex (Fig. 2B). Overexpression of WDR12 (Fig. 2B, lane 3) and Pes1 (lane 2) resulted in faster-migrating bands indicating free nonincorporated protein. In contrast, overexpression of Bop1 led to the formation of an additional complex consisting of Bop1 and WDR12 but lacking Pes1 (Fig. 2B, lane 6). The immunoblot analysis of WDR12 further indicated that the overexpression of Bop1 titrated free endogenous WDR12 into this subcomplex (Fig. 2B, lane 6). Bop1-overexpressing cells lack monomeric WDR12, in contrast to Pes1-overexpressing cells. The overexpression of another nucleolar protein, nucleostemin or Nog1, did not influence the PeBoW complex (Fig. 2B, lanes 4 and 5).

To verify that recombinant Bop1 is incorporated into the PeBoW complex, we performed native gel electrophoresis after 6 days of Bop1 overexpression (Fig. 2C). Recombinant Bop1, which is only recognized by the HA-specific but not the human-specific Bop1 MAb, replaced the endogenous Bop1 in PeBoW complex assembly (Fig. 2C, lanes 2 and 4).

As overexpression of Bop1 titrates endogenous WDR12 into a Bop1/WDR12 subcomplex without Pes1, we tested whether additional coexpression of Pes1 restores PeBoW complex formation. U2OS cells were stably transfected with two or three individual pRTS constructs. We performed native gel electrophoresis after 6 days of coexpression. The immunoblot of WDR12 shows that monomeric WDR12 (Fig. 2D, lane 1) builds a subcomplex with coexpressed Bop1 (lane 2) but not with coexpressed Pes1 (lane 3). The recombinant expression of all three PeBoW subunits is sufficient for the establishment of the PeBoW complex (Fig. 2D, lane 4). Equal expression levels were determined by Western blot analysis (Fig. 2D).

Overexpression of Bop1 and Pes1 induces a Bop1/Pes1 subcomplex. Overexpression of Bop1 did not reveal a detectable subcomplex with the endogenous Pes1, possibly because of its low stability. To test whether such a complex can generally be formed, we overexpressed Bop1 and Pes1 together. Cell fractionation experiments revealed a Bop1/Pes1 subcomplex in the nuclear fraction but not in the cytoplasmic fraction (Fig. 2E). In contrast, the Bop1/WDR12 subcomplex appeared only in the cytoplasmic fraction and was absent from the nuclear fraction.

Knockdown of Pes1 induces a Bop1/WDR12 subcomplex. Overexpression of Bop1 resulted in the formation of an incomplete PeBoW complex containing Bop1 and WDR12 but not Pes1. Therefore, we aimed to investigate whether this Bop1/WDR12 subcomplex would also appear in cells depleted of

FIG. 1. Overexpression of Bop1 inhibits cell proliferation and pre-rRNA processing. (A) Equal numbers of H1299 and TGR-1 cells stably transfected with the indicated constructs were seeded in the presence of 1 μg/ml doxycycline. After 6 days, cells were trypsinized and counted by trypan blue exclusion. The histogram depicts the cell counts relative to that of the mock-treated cell line expressing luciferase. Error bars indicate standard deviations. (B) Expression levels of HA-tagged proteins were determined with the anti-HA antibody 3F10. Equal loading was verified by immunodetection of α-tubulin. (C) The proliferation of stable polyclonal H1299 cell lines expressing the indicated proteins was analyzed as described for panel A. (D) Expression levels of HA-tagged proteins were determined as described for panel B. (E) Diagram of the primary 47S rRNA transcript and the major rRNA intermediates. Positions of the hybridization probes are depicted. ETS, external transcribed spacer. (F) Northern blot analysis of rRNA precursors. Total RNA was extracted from subconfluent H1299 cells expressing the indicated HA-tagged proteins for 24 h. Equal amounts of total RNA were separated by agarose-formaldehyde gel electrophoresis and hybridized with probes specific for ITS-1 and ITS-2 of the rRNA intermediates. As a loading control, blots were incubated with a probe specific for 18S rRNA. (G) Ratios of 47S-45S/18S and 32S/18S rRNAs.

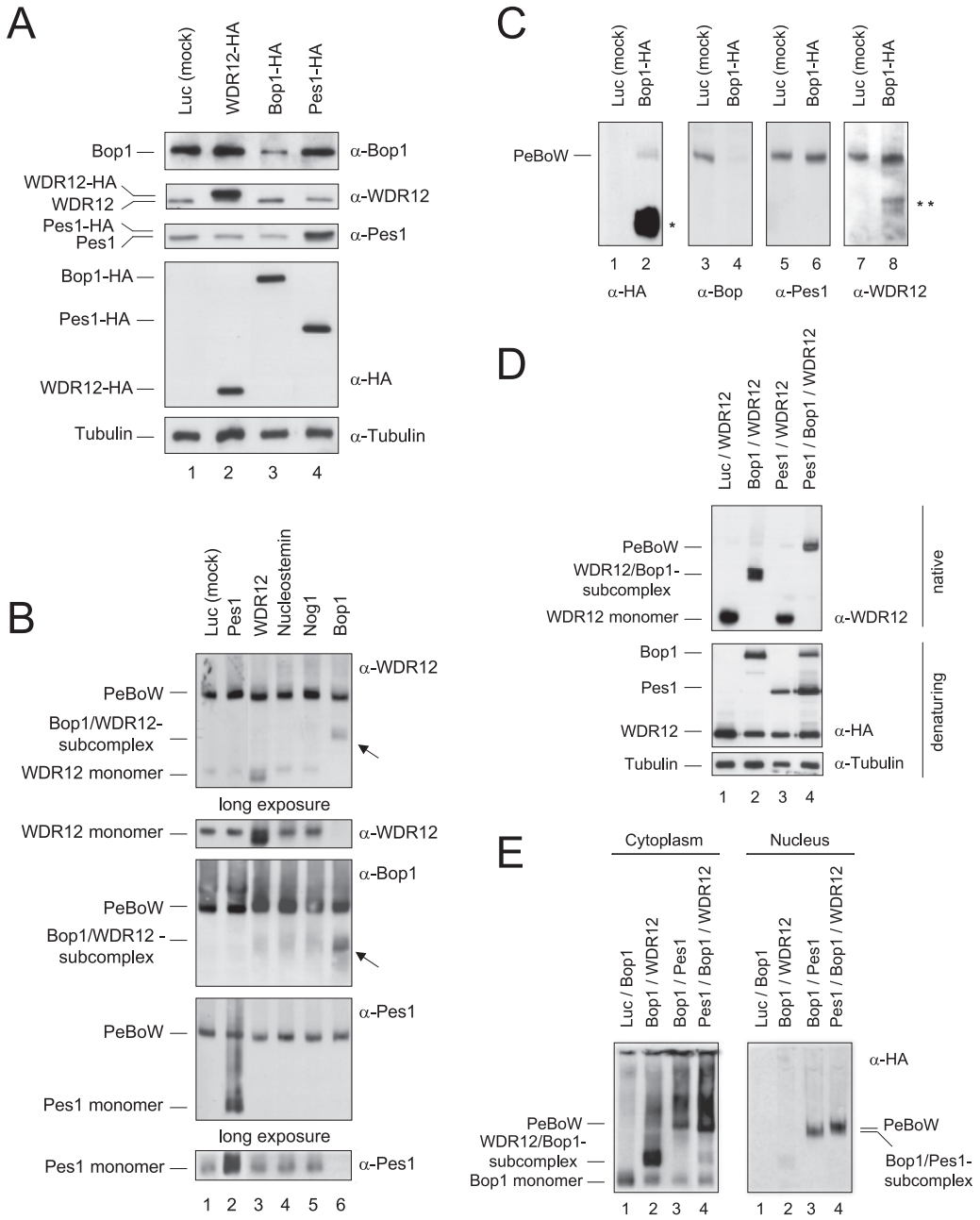


FIG. 2. (A) Overexpression of Bop1 reduces the endogenous Bop1 protein level. H1299 cells were stably transfected with the indicated constructs. After 6 days, endogenous Pes1, Bop1, and WDR12 protein levels were analyzed by Western blotting. Human-specific anti-Bop1 MAb 6H11 does not recognize recombinant mouse Bop1. Expression levels of HA-tagged proteins were determined with anti-HA antibody 3F10. Equal loading was verified by immunodetection of α-tubulin. (B) Overexpression of Bop1 titrates endogenous WDR12 into a Bop1/WDR12 subcomplex. Total cell lysates of U2OS cells expressing the indicated proteins were separated by native gel electrophoresis after 1 day of expression. Pes1, Bop1, and WDR12 were visualized by immunoblotting. Nonincorporated proteins or complexes containing the respective factor are indicated. (C) Overexpression of Bop1-HA replaces endogenous Bop1 in PeBoW complex formation. Total lysates of U2OS cells expressing luciferase or Bop1-HA were separated by native gel electrophoresis after 6 days of expression. Pes1, Bop1, WDR12, and Bop1-HA were visualized by immunoblotting. An asterisk indicates monomeric Bop1-HA. Double asterisks indicate the Bop1/WDR12 subcomplex. (D) Total lysates of U2OS cells expressing the indicated proteins were separated by native gel electrophoresis. WDR12 were visualized by immunoblotting. Nonincorporated proteins or complexes containing the respective factor are indicated. Expression levels of HA-tagged proteins were determined by Western blot analysis with anti-HA antibody 3F10. Equal loading was verified by immunodetection of α-tubulin. (E) Cytoplasmic and nuclear fractions of H1299 cells expressing the indicated genes were analyzed by native gel electrophoresis as described for panel B. Complexes were visualized by immunostaining with HA-specific antibodies.

Pes1. We performed endogenous Pes1, Bop1, and WDR12 siRNA knockdown experiments. U2OS cells were transfected two times with siRNAs directed against the UTR or open reading frame of WDR12, Bop1, or Pes1 mRNA. All siRNAs induced a strong reduction of the respective proteins within 2 days after the last transfection (Fig. 3A). We also tested whether knockdown of Pes1, Bop1, or WDR12 interfered with rRNA processing. Metabolic labeling of nascent rRNA re-

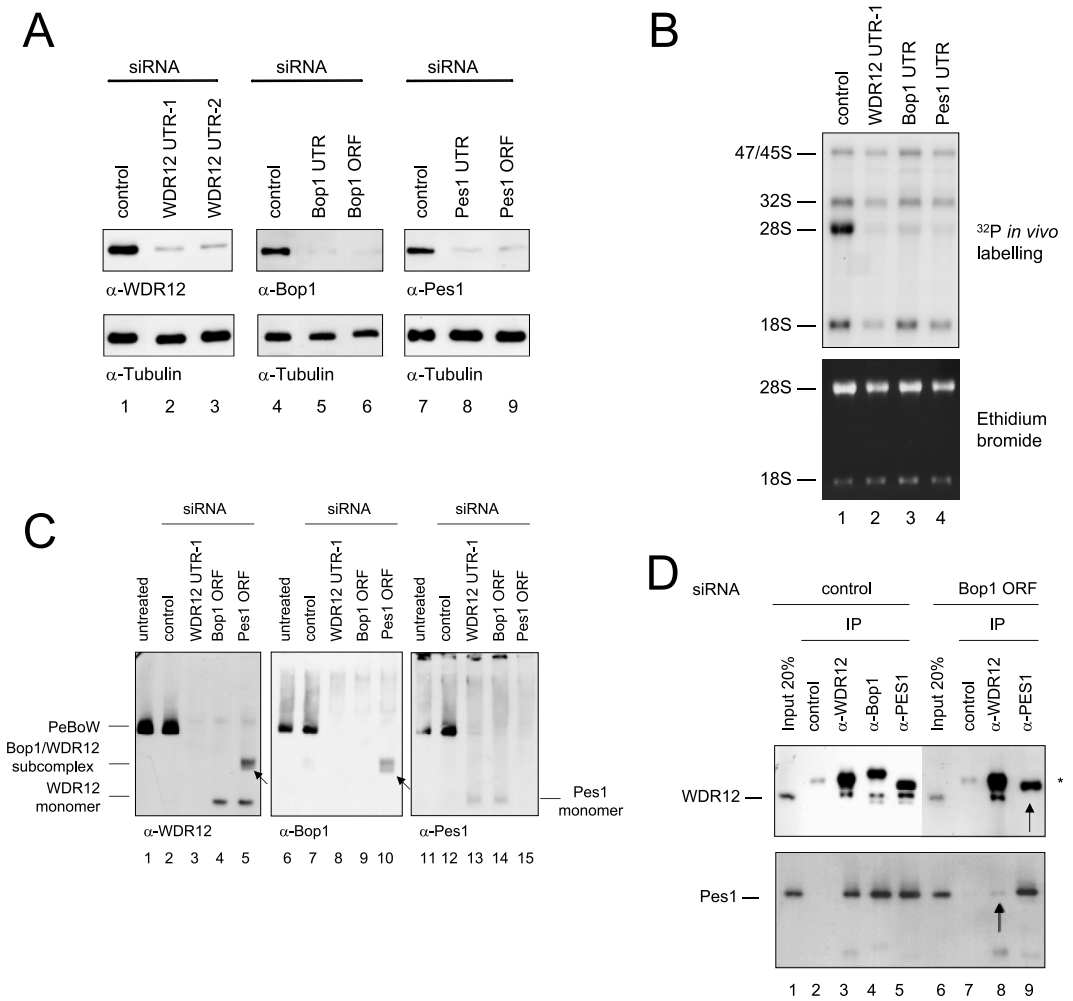


FIG. 3. Bop1 is the core factor of the PeBoW complex. (A) U2OS cells were transfected twice with the indicated siRNAs. Endogenous protein levels were analyzed by Western blotting 2 days after the last transfection. α-Tubulin is shown as a loading control. (B) Cells were treated as described for panel A and metabolically labeled with [^{32}P]orthophosphate for 60 min 1 day after the last transfection. Subsequently, cells were incubated for 2 h in regular culture medium. Labeled rRNAs are indicated. Ethidium bromide staining is shown as a loading control. (C) Total cell lysates of U2OS cells transfected twice with the indicated siRNAs were separated by native gel electrophoresis 1 day after the last transfection. Endogenous Pes1, Bop1, and WDR12 were visualized by immunoblotting. Nonincorporated proteins or complexes containing the respective factor are indicated. (D) U2OS cells were transfected at days 0 and 1 with either control or Bop1-specific siRNA. Total cell lysates were subjected to immunoprecipitation with antibodies either against Pes1 (8E9), Bop1 (6H11), WDR12 (1B8), or the isotype control coupled to protein G-Sepharose beads 2 days after the last transfection. Equivalent amounts of total lysates used for immunoprecipitation (IP) were loaded in each lane or 20% thereof for the input. Immunodetection was performed with the anti-Pes1 (8E9) or anti-WDR12 (1B8) antibody, respectively. An asterisk indicates cross-reactivity to immunoglobulin molecules.

vealed that cells depleted of a single PeBoW component failed to produce mature 28S rRNA (Fig. 3B).

To study the integrity of the PeBoW complex in cells depleted of its individual components, we performed native gel electrophoresis 1 day after the last siRNA transfection (Fig. 3C). Immunoblot analysis of Pes1, Bop1, and WDR12 showed that knockdown of either protein led to the disappearance of an intact PeBoW complex (Fig. 3C, lanes 3 to 5, 8 to 10, and 13 to 15). Bop1- or Pes1-depleted cells accumulated free nonincorporated WDR12 (Fig. 3C, lanes 3 and 4), whereas knockdown of WDR12 and Bop1 led to an accumulation of monomeric Pes1 (lanes 13 and 14). We were not able to detect nonincorporated Bop1 in this assay (Fig. 3B, lanes 6 to 10), possibly because of the low stability of free Bop1 (see below). Interestingly, Pes1-depleted cells exhibited a subcomplex of WDR12 and Bop1 (Fig. 3C, lanes 5, 10, and 15), as observed in Bop1-overexpressing cells (Fig. 2B).

Bop1 mediates the interaction of Pes1 and WDR12. WDR12 and Bop1, as well as Pes1 and Bop1, can form a subcomplexes, but we failed to detect a Pes1/WDR12 subcomplex. Therefore, we studied the interaction of Pes1, Bop1, and WDR12 in more detail. U2OS cells were transfected two times with either control or Bop1-specific siRNA, and coimmunoprecipitation experiments were performed (Fig. 3D). In control cells, WDR12 and Pes1 were specifically immunoprecipitated and also coimmunoprecipitated (Fig. 3D, lanes 3 to 5). In contrast, WDR12 and Pes1 could not be coimmunoprecipitated in Bop1-depleted cells (Fig. 3D, lanes 8 and 9). Therefore, we suppose

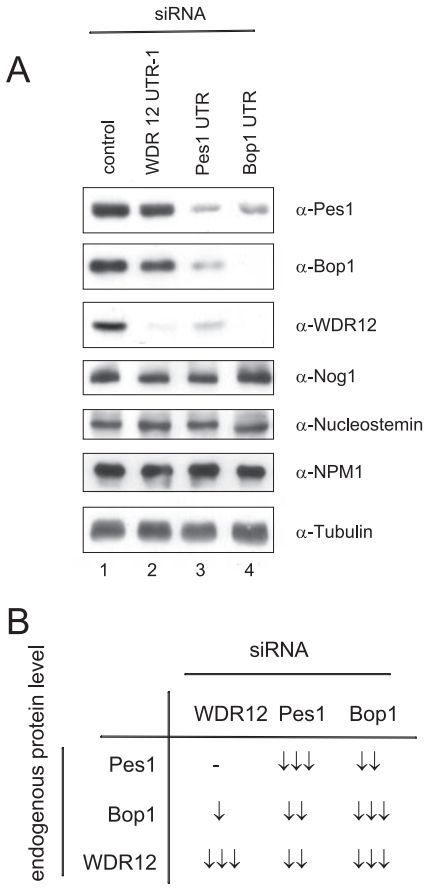


FIG. 4. Specific interdependency of the PeBoW components. (A) H1299 cells were transfected two times with the indicated siRNAs. Effects on the Pes1, Bop1, and WDR12 protein levels were monitored at day 3 after the last transfection by Western blot analysis. (B) A schematic overview of the interdependency of the PeBoW components Pes1, Bop1, and WDR12 is shown. The number of arrows indicates the extent of downregulation.

that the interaction of Pes1 and WDR12 is indirect and mediated by the core factor Bop1.

Interdependent stability of PeBoW components. Native gel electrophoresis revealed that Pes1-, Bop1-, or WDR12-depleted cells lack intact PeBoW complexes. Therefore, we analyzed whether depletion of single PeBoW components affected the abundance of the other components. H1299 cells were transfected twice with siRNA against the 3′ UTR of the Pes1, Bop1, or WDR12 mRNA, and cells were harvested daily. The strongest reduction of the respective proteins was observed 3 days after the last siRNA transfection (Fig. 4A). Knockdown of WDR12 slightly reduced the abundance of Bop1 but not of Pes1. In contrast, depletion of Pes1 resulted in a concomitant loss of Bop1 and WDR12. Furthermore, the knockdown of Bop1 strongly reduced the protein levels of Pes1 and WDR12. The abundances of three other nucleolar proteins, Nog1, nucleostemin, and nucleophosmin (NPM1), were not affected. In addition to their nucleolar localization, Nog1 and NPM1 were found in preribosomal complexes and shown to function in the processing of the 28S rRNA (15, 25, 29). These results indicate that the interdependence of the steady-state levels of individual PeBoW components is specific for and restricted to this subcomplex (Fig. 4B).

Knockdown–knock-in experiments reconstitute steady-state levels of PeBoW proteins. To verify that the mutual reduction of PeBoW proteins after knockdown of a single component is not an off-target effect, we aimed to rescue the depletion phenotype specifically by coexpression of the respective wild-type protein by a knockdown–knock-in approach. H1299 cells were stably transfected with pRTS constructs conditionally expressing the respective HA-tagged form of Pes1, Bop1, or WDR12 in a doxycycline-dependent manner. Exogenous gene expression was activated for 3 days and then maintained throughout the subsequent course of two siRNA transfections. We used siRNAs directed against the 3′ UTRs of the Pes1, Bop1, and WDR12 mRNAs. These siRNAs were used to specifically deplete the endogenous, but not the HA-tagged, recombinant proteins.

The endogenous Pes1, Bop1, and WDR12 protein levels were studied in cells depleted of the endogenous protein and additionally expressing either of the HA-tagged PeBoW components (Fig. 5). The endogenous, as well as the exogenous HA-tagged, Pes1 and WDR12 proteins were detected by the MAbs but could be discriminated by their increased molecular weights and slower migration due to the C-terminal HA tag. The MAb specific for human Bop1 did not recognize recombinant mouse Bop1-HA. First of all, overexpression of Bop1-HA down-regulated the endogenous Bop1, as described above (Fig. 5A to D, lanes 3), consistent with our previous results (Fig. 2A). Knockdown of WDR12 slightly reduced endogenous Bop1 protein levels but not endogenous Pes1 protein levels (Fig. 5B). This reduction of Bop1 was rescued upon coexpression of WDR12-HA (Fig. 5B, lane 1), but not Pes1-HA or Bop1-HA (lanes 2 and 3). Depletion of endogenous Pes1 caused a concomitant loss of Bop1 and WDR12 (Fig. 5C) that was alleviated by expression of Pes1-HA (Fig. 5C, lane 2), but not WDR12-HA or Bop1-HA (lanes 1 and 3).

In addition, the knockdown of Bop1 reduced the Pes1 and WDR12 protein levels (Fig. 5D). The expression of Bop1-HA (Fig. 5D, lane 3) but not WDR12-HA or Pes1-HA (lanes 1 and 2) fully prevented this reduction. Thus, these reconstitution experiments verify that the Pes1, Bop1, and WDR12 protein levels reciprocally depend on each other after experimentally induced knockdown of any one of them.

Interdependence of PeBoW protein levels is transcription independent. In parallel with the Western blot analysis, mRNA levels of Pes1-, Bop1-, or WDR12-depleted cells were investigated by Northern blot analysis (Fig. 6A and B). The knockdown of Pes1, Bop1, or WDR12 strongly reduced the mRNA levels of the respective genes but did not reduce the mRNA levels of other PeBoW components. We even observed a slight increase in mRNA levels. Therefore, the interdependency of the PeBoW proteins is not caused by regulation of the abundance of their mRNA but suggests a translational or posttranslational mechanism.

Interdependence of PeBoW proteins is caused by protein destabilization. These results prompted us to study whether the stability of the PeBoW proteins is reduced in cells depleted of the other PeBoW components. First we analyzed the stability of endogenous PeBoW proteins in untreated cells. Therefore, U2OS cells were incubated with cycloheximide for differ-

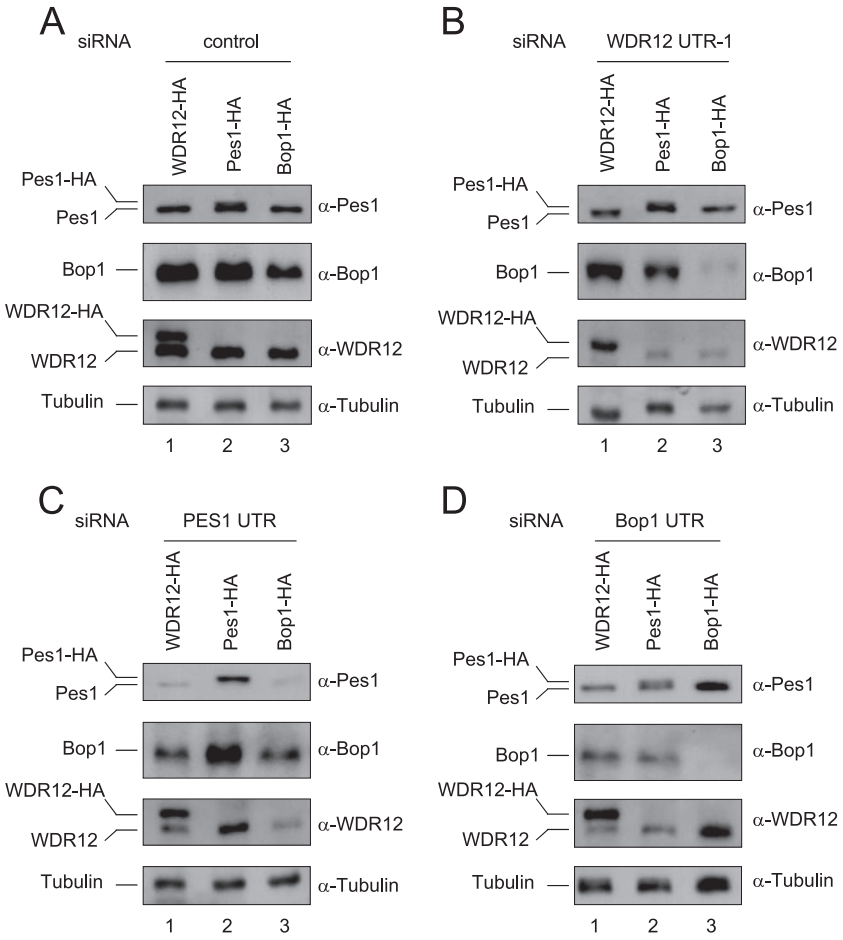


FIG. 5. Knockdown–knock-in assay of stable polyclonal H1299 cell lines expressing Pes1-HA, Bop1-HA, or WDR12-HA. Cells were treated with (A) control or (B) WDR12 (3′ UTR)-, (C) Pes1 (3′ UTR)-, or (D) Bop1 (3′ UTR)-specific siRNA. Endogenous Pes1, Bop1, and WDR12 protein levels were analyzed 2 days after the last transfection by Western blotting. Human-specific anti-Bop1 MAb 6H11 does not recognize recombinant mouse Bop1. α-Tubulin is shown as a loading control.

ent time intervals to block de novo protein synthesis (Fig. 7A). The levels of the Pes1, Bop1, and WDR12 proteins were barely affected over 24 h. In contrast, short-lived proteins such as p53 and c-Myc rapidly decreased within a few hours. Hence, endogenous PeBoW proteins are quite stable.

Next, we aimed to assess whether the turnover of PeBoW proteins is increased in Pes1, Bop1, or WDR12 knockdown cells. Therefore, we monitored the abundance of HA-tagged PeBoW proteins at different time points after a pulse expression in cells depleted of the endogenous PeBoW members. H1299 cells conditionally expressing the respective HA-tagged forms were treated with WDR12 (3′ UTR)-, Pes1 (3′ UTR)-, or Bop1 (3′ UTR)-specific siRNA. Two days after the last siRNA transfection, pulse expression of HA-tagged proteins was performed for 6 h by addition and subsequent removal of doxycycline. The stability of Pes1-HA, Bop1-HA, and WDR12-HA was analyzed by Western blotting with the anti-HA antibody. In control siRNA-treated cells, WDR12-HA and Pes1-HA exhibited only a little turnover within 24 h, consistent with the cycloheximide time course experiments (Fig. 7B and C, lanes 1 to 4). The decline in Pes1 was more pronounced between 24 and 48 h after the pulse expression. The levels of Bop1-HA already decreased substantially within 24 h (Fig. 7D, lanes 1 to 4). This apparent discrepancy between the pulse-chase and the cycloheximide time course experiment suggests that Bop1 is only stable in the context of the PeBoW complex but unstable as a free protein. These results support the observations that the amounts of Bop1 are tightly controlled in mammalian cells (Fig. 2A).

The stability of Bop1-HA was further diminished in Pes1-depleted cells (Fig. 7D, lanes 9 to 12) but not in WDR12-depleted cells (lanes 5 to 8). WDR12-HA protein levels were significantly reduced within 24 h in cells lacking endogenous Bop1 (Fig. 7B, lanes 5 to 8) or Pes1 (lanes 9 to 12), and the stability of Pes1 was dependent on the presence of Bop1 (Fig. 7C, lanes 9 to 12) but not WDR12 (lanes 5 to 8). All of these results are fully in line with the mutual dependency of endogenous PeBoW protein levels observed after siRNA knockdown (Fig. 4). In conclusion, the abundance and stability of Pes1, Bop1, and WDR12 are interdependent.

Nucleolar localization of Bop1 requires Pes1. Bop1 overexpression induces subcomplexes of Bop1/WDR12 and Bop1/Pes1. To study the subcellular distribution of these complexes, we preformed indirect immunofluorescence assays with Bop1-

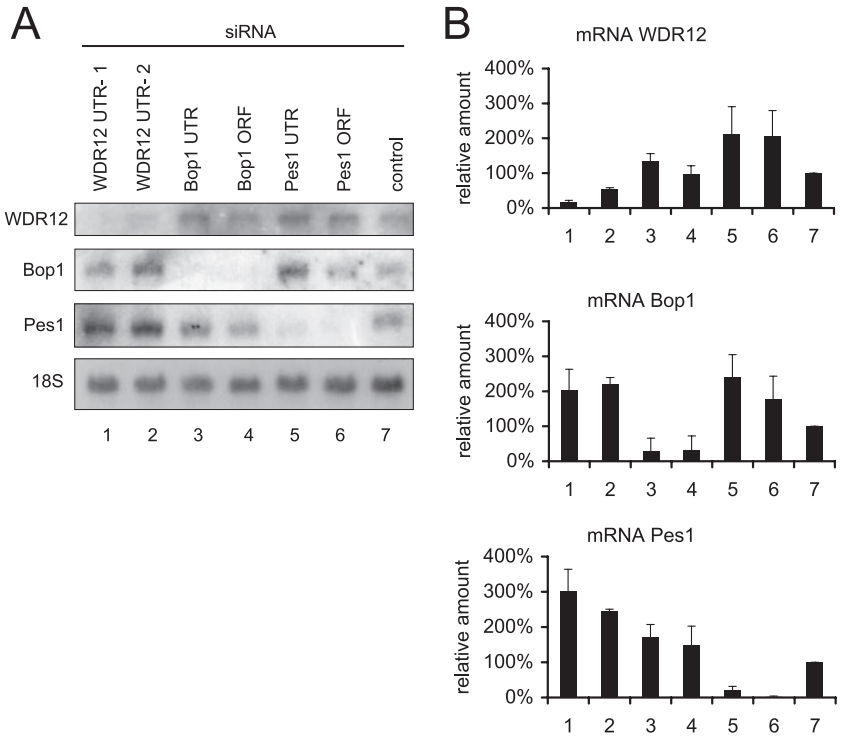


FIG. 6. Knockdown of Pes1, Bop1, or WDR12 does not influence the steady-state mRNA levels of the PeBoW components. (A) U2OS cells were transfected twice with the indicated siRNAs. Levels of the mRNAs of Pes1, Bop1, and WDR12 were analyzed 2 days after the last transfection by Northern blot analysis. As a loading control, blots were incubated with a probe specific for 18S rRNA. (B) The histogram depicts quantification of the mRNA levels of Pes1, Bop1, and WDR12 relative to that of a cell line transfected with control siRNA. Error bars indicate standard deviations.

specific antibodies. Recombinantly expressed Pes1 and WDR12 proteins show predominant nucleolar staining (10, 14). In contrast, recombinant Bop1 localized exclusively to the cytoplasm in H1299 and TGR-1 cells (Fig. 8A and B). Only cells expressing minor levels of Bop1 also revealed nucleolar staining (data not shown). The cytoplasmic localization of Bop1 was not affected by coexpression of WDR12. However, coexpression of Pes1 fostered the nucleolar localization of Bop1. These results are in line with the cell fractionation experiments in Fig. 2E and further suggest that the titration of WDR12 into the Bop1/WDR12 subcomplex retains WDR12 in the cytoplasm.

Do subcomplexes of PeBoW bind to preribosomal particles? The association of Bop1/WDR12 and Bop1/Pes1 with preribosomal complexes was studied by sucrose gradient centrifugation with TGR-1 total cell lysates (Fig. 9). All recombinant proteins accumulated in low-molecular-weight fractions, most likely because of an access of overexpressed free protein in total lysates. However, recombinant Pes1 and WDR12, but not Bop1, exhibited specific enrichment in high-molecular-weight fractions that cofractionated with ribosomal particles. Importantly, coexpression of Bop1 abolished the association of WDR12 with preribosomal particles, while coexpression of Pes1 led to a significant albeit restricted increase in preribosome-associated Bop1. The results are compatible with the model in which overexpression of Bop1 leads to the formation of two subcomplexes, Bop1/WDR12 and Bop1/Pes1. The former is retained in the cytoplasm and inhibits the translocation of WDR12 to the nucleolus, the later enables the transport of Bop1 to the nucleolus, but a functional PeBoW complex cannot assemble in the absence of WDR12.

DISCUSSION

Ribosome biogenesis is an essential and evolutionarily highly conserved process. Mature 60S and 40S subunits contain different species of rRNA and ribosomal proteins (8). Numerous *trans*-acting factors are required along the maturation of ribosomes for processing and modification of rRNA and preribosome assembly (6). So far, it remains elusive how the timely and sterically correct assembly of so many different factors and rRNAs into macromolecular preribosomal complexes is achieved. It is thought that several factors preassemble into subcomplexes that orchestrate the formation and maturation of preribosomes (3). The yeast small-subunit (SSU) processome, the macromolecular correlate of the terminal knob structure of nascent primary rRNA transcripts, was previously shown to consist of distinct subcomplexes (4, 11, 19, 21). Depletion of individual SSU processome components affected ribosome synthesis differently, depending on the complex that they associated with (9). Another subcomplex identified in yeast, containing the factors Nop7p, Erb1p, and Ytm1p, is involved in the maturation of the large ribosomal subunit (11, 19, 21). We have previously characterized the homologous mammalian complex (PeBoW complex), consisting of Pes1, Bop1, and WDR12 (14). The PeBoW complex is critical for mammalian ribosome biogenesis, as it proved to be

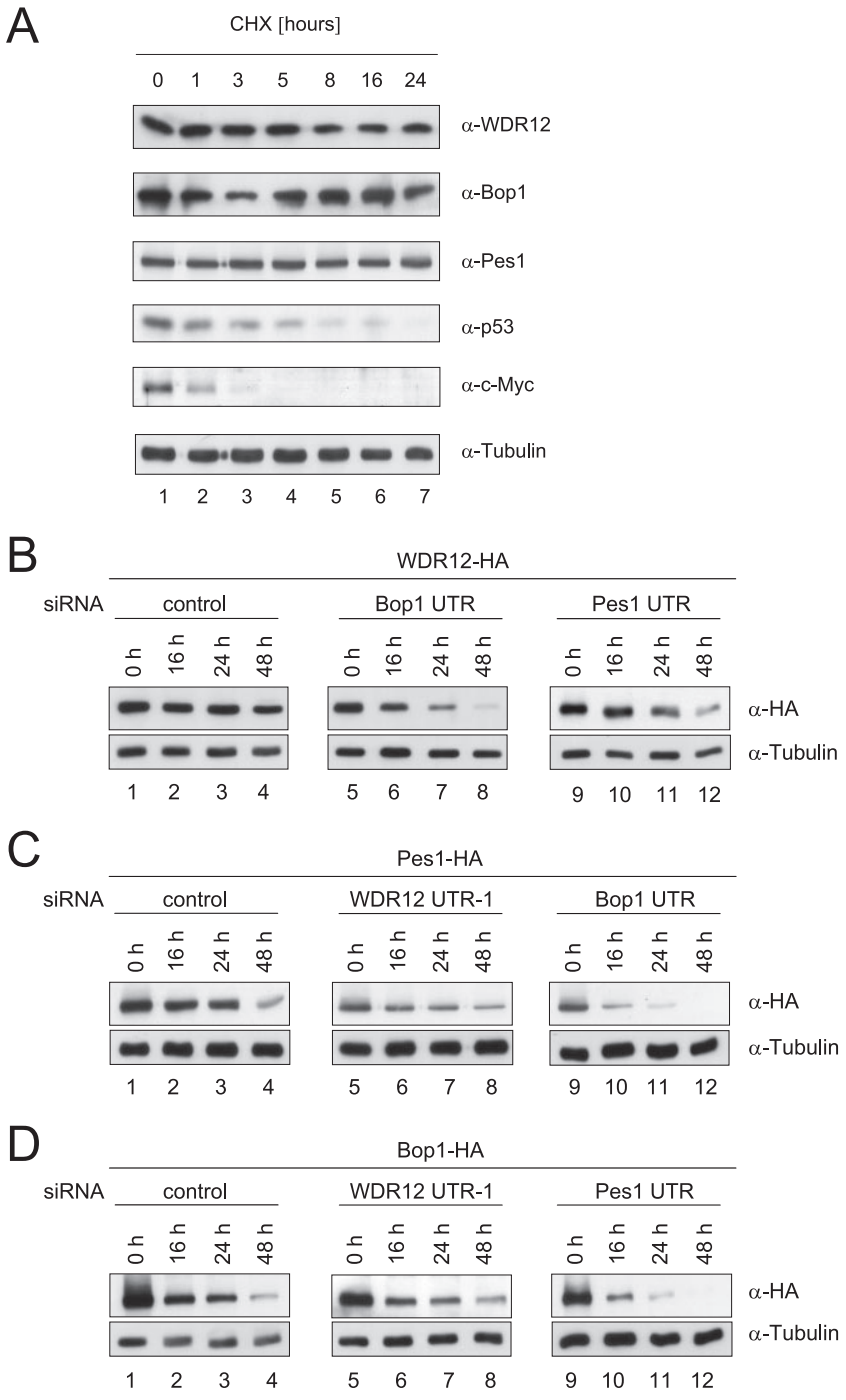


FIG. 7. Knockdown of Pes1, Bop1, or WDR12 influences the stability of the PeBoW components. (A) U2OS cells were treated with 50 μg/ml cycloheximide (CHX) for the indicated times. The stability of the indicated endogenous proteins was monitored by Western blot analysis. Immunodetection was performed with antibodies against Pes1, Bop1, WDR12, p53 (PAb240), and c-Myc (N-262). Equal loading was verified by immunodetection of α-tubulin. (B to D) H1299 cells stably transfected with the indicated constructs were treated two times with control or WDR12 (3′ UTR)-, Pes1 (3′ UTR)-, or Bop1 (3′ UTR)-specific siRNA. Expression of HA-tagged proteins was induced 2 days after the last siRNA transfection for 6 h by addition of 0.1 μg/ml doxycycline and stopped by its removal. The stability of Pes1-HA, Bop1-HA, and WDR12-HA was analyzed by Western blotting with anti-HA antibody 3F10. α-Tubulin is shown as a loading control.

highly susceptible for the generation of dominant-negative mutants that specifically abrogated the synthesis of the 28S rRNA (10, 14, 20, 28). However, it is unknown how the integrity of the PeBoW complex is preserved. First of all, the amount of each component must be adjusted coordinately to the rate of ribosome biogenesis. In mammalian cells that transit from a quiescent into a proliferating state, this concerted action may be accomplished by the oncogenic transcription factor c-Myc,

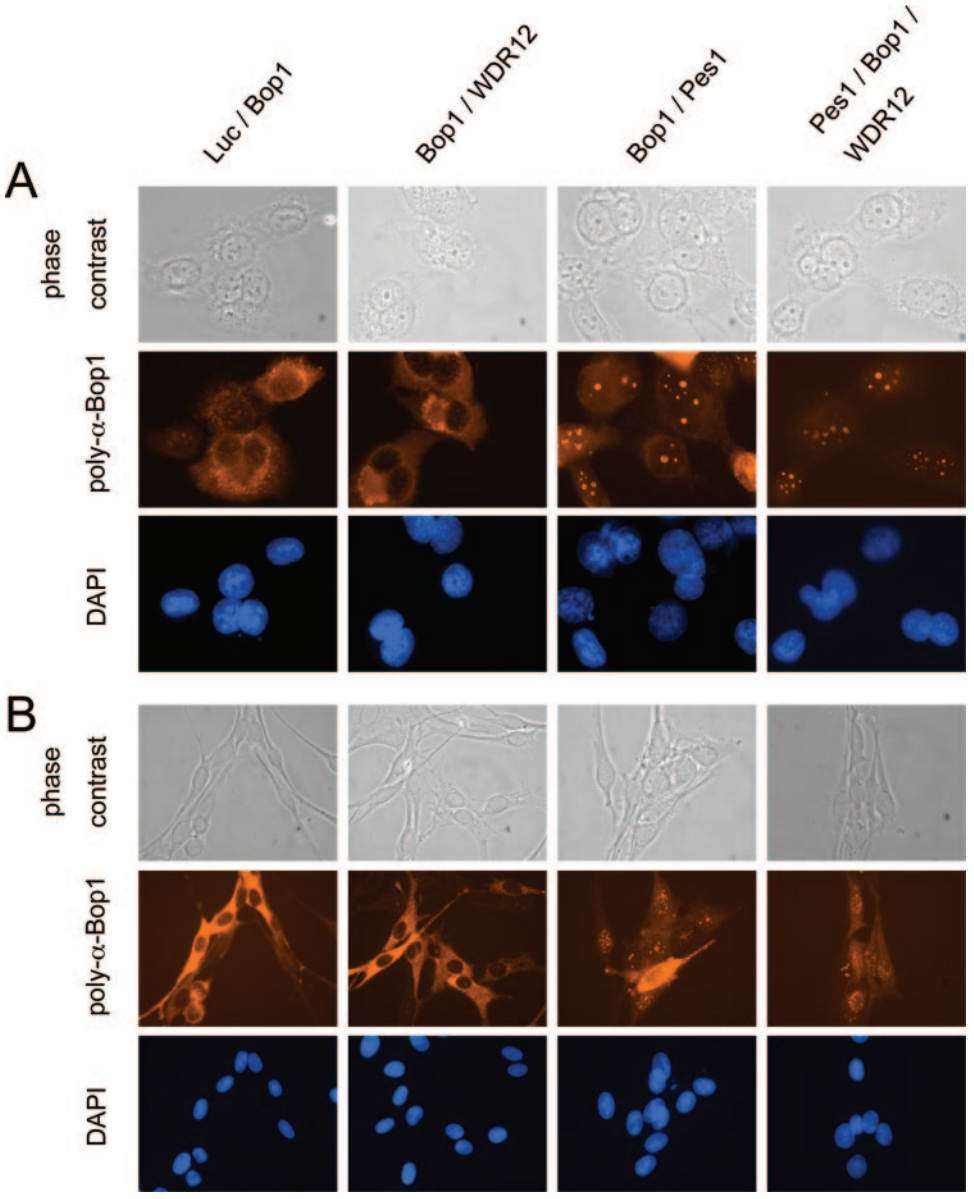


FIG. 8. Pes1 is required for nucleolar localization of Bop1. Bop1-HA expressed in (A) H1299 cells and (B) TGR-1 cells was detected by indirect immunofluorescence assay. Coexpression of luciferase (control), WDR12, and Pes1 is indicated. Recombinant proteins were induced by doxycycline for 30 h, and cells were fixed with methanol-acetone. Bop1 was stained by guinea pig polyclonal antibodies specific for mouse Bop1 (poly-α-Bop1). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI).

which upregulates the mRNAs of Pes1, Bop1, and WDR12 in conjunction with many other genes involved in ribosome biogenesis (26). However, this global transcriptional regulation might be insufficient for fine-tuned control of PeBoW protein levels, as the respective mRNAs are relatively long-lived (unpublished results). Therefore, we aimed to investigate how altered abundances of individual PeBoW proteins affect complex integrity. In proliferating cells, Pes1, Bop1, and WDR12 are relatively stable factors, as revealed by cycloheximide treatment blocking de novo protein synthesis. However, their stability is dramatically reduced in cells depleted of a single PeBoW protein. We identified a specific pattern of their destabilization. For example, pulse-chase expression of tagged wild-type WDR12 or Pes1 in Bop1-depleted cells demonstrated that the half-life of both was strongly reduced compared with that of control siRNA-treated cells, but the half-life of Pes1 was not affected in WDR12-depleted cells. Thus, individual PeBoW factors are only stable in the context of an intact PeBoW complex (Fig. 10). These results also explain the observation that the amounts of Pes1, Bop1, and WDR12 are interdependent in a specific manner. Continuously disturbing the stoichiometry of their expression levels within the cell by RNA interference triggers a cascade of reduced stabilization and thus increased degradation. But it is noteworthy that the extent of the mutual destabilization mediated by knockdown through RNA interference significantly differed, depending on the de-

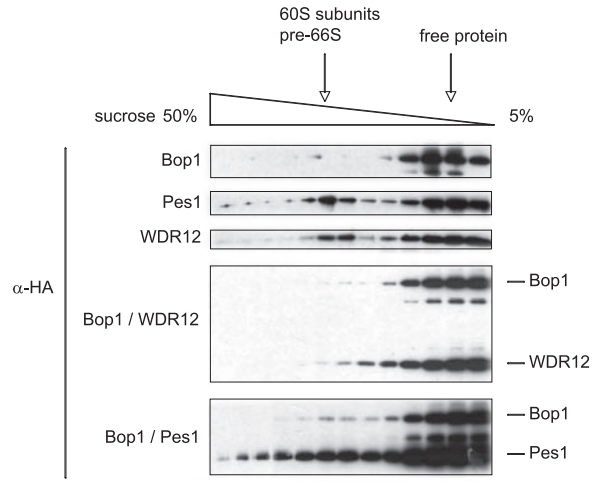


FIG. 9. Association of recombinant PeBoW components with large preribosomal particles after overexpression. Shown is a Western blot analysis of sucrose gradient fractions of TGR-1 cells expressing Bop1-HA, Pes1-HA, and WDR12-HA or coexpressing Bop1-HA/WDR12-HA and Bop1-HA/Pes1-HA. Fractions containing the 60S/pre-66S subunits (28S rRNA) are indicated.

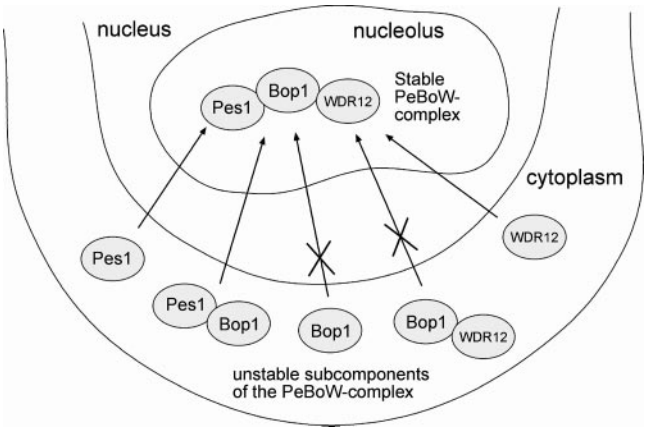


FIG. 10. Model for transport and assembly of Pes1, Bop1, and WDR12 in the nucleolus. Cytoplasmic Pes1, Bop1, and WDR12 are unstable. Bop1 requires Pes1 for nucleolar transport, while nucleolar transport of WDR12 is blocked by high Bop1 levels. PeBoW complex formation occurs in the nucleolus and stabilizes all three proteins.

pleted PeBoW component. Loss of WDR12 had no detectable effect on Pes1 expression levels, whereas loss of Bop1 destabilized Pes1 and caused its concomitant reduction. This observation might be explained by our finding that the interaction of Pes1 and WDR12 is indirect and mediated by Bop1, as revealed by coimmunoprecipitation experiments. Similar results were reported in a recent study that investigated the physical interaction of the corresponding yeast homologues by GST pull-down assays. Nop7p, the homologue of Pes1, interacted with Erb1p but not Ytm1p, the homologues of Bop1 and WDR12, respectively (22). The fact that knockdown of Pes1 very strongly codepletes Bop1 suggests that the Pes1-dependent codepletion of WDR12 is mediated by the reduction of Bop1. As loss of WDR12 only slightly affected the abundance of Bop1, the amount of Bop1 is still sufficient to prevent significant destabilization of Pes1. These results indicated a crucial role for Bop1 in PeBoW complex assembly and integrity. This notion was further supported by our studies of cells overexpressing Bop1. Excess amounts of Bop1 impaired cell proliferation and rRNA processing, and this could be rescued by coexpression of WDR12, but not Pes1. It was previously shown that truncation mutant forms of Bop1, but not wild-type Bop1, impaired cell proliferation and rRNA maturation (23, 28). Indeed, we can fully confirm that dominant-negative Bop1Δ potently blocks rRNA processing and cell cycle progression. Also, in our experiments, Bop1Δ elicited a profound cell cycle arrest, in contrast to wild-type Bop1, as revealed by a bromodeoxyuridine-light assay investigating the efficiency of reversible cell cycle arrests (unpublished results). However, the comparison of mock-treated cells with wild-type Bop1- and Bop1Δ-overexpressing cells in long-term proliferation assays revealed an inhibitory effect of Bop1 (1). Similarly, overexpressed Bop1 disturbs rRNA processing, although less severely than Bop1Δ, but significantly compared to that in control cells.

We also found that the levels of Bop1 within a cell are tightly controlled. Enforced expression of HA-tagged Bop1 was partially compensated for by a decrease in endogenous Bop1. Pulse-chase-expressed, tagged Bop1 was also less stable than Pes1 or WDR12. Thus, any excess of Bop1 is counteracted by its rapid degradation. Therefore, recombinant Bop1 that largely replaces endogenous Bop1, as the tagged form is also incorporated into the PeBoW complex, provokes the degradation of nonincorporated endogenous Bop1. Further, Bop1 contains a PEST motif that is generally thought to mediate destabilization. Our data suggest that the PEST sequence of Bop1 might render the protein unstable in its free form, but incorporation into the PeBoW complex blocks the function of the PEST motif and protects it from rapid degradation.

In addition, depletion of Pes1 triggered the formation of a Bop1/WDR12 subcomplex. Inadequately high levels of Bop1 likewise sequestered endogenous or recombinant WDR12 into this incomplete PeBoW complex. Apparently, the Bop1/WDR12 subcomplex resulted from a relative deficiency of Pes1, as additionally providing sufficient amounts of Pes1 restored PeBoW formation. Alternatively, overexpression of Bop1 and Pes1 induced the formation of a Bop1/Pes1 subcomplex. Thus, overexpression of Bop1 leads to sequestration of Pes1 and WDR12 in two subcomplexes, each containing Bop1 (Fig. 10). In addition, cell fractionation, indirect immunofluorescence, and sucrose gradient centrifugation experiments demonstrated that the subcomplexes behave differently. While the Bop1/WDR12 subcomplex is retained in the cytoplasm, the Bop1/Pes1 subcomplex is located in the nucleolus (Fig. 10). It is further evident that Bop1 requires the help of Pes1 for translocation into the nucleolus. We could recently show that two domains are essential for the nucleolar transport of Pes1, the Bop1 interaction domain and the BRCT domain (10, 13).

Studies of preribosomal complexes in yeast suggest that the Bop1 and Pes1 homologues Erb1p and Nop7p assemble at the preribosome prior to the WDR12 homologue Ytm1p (2, 22). Overexpressed WDR12 is largely located in the nucleolus (14), suggesting that in mammals nucleolar transport of WDR12 also occurs independently of Pes1 and Bop1 and that the PeBoW complex assembles in the nucleolus. Interdependency

of factors has recently also been reported for the activity of the SSU processome in yeast (32).

In the present study, we have identified a crucial role for Bop1 in PeBoW homeostasis. As the gene for Bop1 is frequently amplified in colorectal cancers (18), its deregulation might be involved in carcinogenesis. Overexpression of Bop1, as well as expression of a dominant negative C-terminally truncated Bop protein, affects ribosome biogenesis (28). Whether Bop1 overexpression increases the risk of cancer by impairment of the PeBoW complex or by interaction with other cellular factors like the Cdc14 phosphatase (12), which is important for exit of mitosis, remains unclear and deserves further investigation. We also cannot rule out the possibility that overexpression of Bop1 induces p53 and thereby selects for mutants inactivating p53.

We have conclusively shown that the PeBoW complex is essential for ribosome biogenesis and that its integrity is controlled by its interdependent subunits Pes1, Bop1, and WDR12. The PeBoW complex demonstrates that the stability of free and complex-associated proteins can differ substantially. This complex-specific mutual dependency of protein stability might serve as a control mechanism to accurately adjust expression levels and to ensure correct subcomplex assembly, subsequently required for the proper maturation of a large macromolecule such as the ribosome.

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We have no conflict of interest.

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The BRCT domain of mammalian Pes1 is crucial for nucleolar localization and rRNA processing

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ABSTRACT

The nucleolar protein Pes1 interacts with Bop1 and WDR12 in a stable complex (PeBoW-complex) and its expression is tightly associated with cell proliferation. The yeast homologue Nop7p (Yph1p) functions in both, rRNA processing and cell cycle progression. The presence of a BRCT-domain (BRCA1 C-terminal) within Pes1 is quite unique for an rRNA processing factor, as this domain is normally found in factors involved in DNA-damage or repair pathways. Thus, the function of the BRCT-domain in Pes1 remains elusive. We established a conditional siRNA-based knock-down-knock-in system and analysed a panel of Pes1 truncation mutants for their functionality in ribosome synthesis in the absence of endogenous Pes1. Deletion of the BRCT-domain or single point mutations of highly conserved residues caused diffuse nucleoplasmic distribution and failure to replace endogenous Pes1 in rRNA processing. Further, the BRCT-mutants of Pes1 were less stable and not incorporated into the PeBoW-complex. Hence, the integrity of the BRCT-domain of Pes1 is crucial for nucleolar localization and its function in rRNA processing.

INTRODUCTION

Ribosome biogenesis is the major metabolic challenge of rapidly proliferating cells, in particular tumour cells. However, little is known about the molecular mechanisms that ensure the equilibrium between cell division and ribosome biogenesis required for balanced cell proliferation and thus a successful duplication of the translational machinery (1). Recently it has become evident that ribosome synthesis is cell cycle controlled and sensitive to growth factor and nutrient signalling, and inhibited upon stress signals (2–6). On the other hand, disturbance of the structural and/or functional integrity of the nucleolus is linked to the induction of the tumour suppressor p53 (7–10). Several ribosomal proteins have been identified so far as crucial mediators of the ribosomal stress response. Impaired ribosome synthesis decreases the demand and thereby results in an accumulation of free ribosomal proteins, such as L11, L5 and L23 that have been shown to inhibit the function of Mdm2 (11–14). p53 is targeted by the E3 ubiquitin ligase Mdm2 for proteasomal degradation. Thus, ribosomal stress mediates the accumulation of p53 by blocking its degradation. A profound knowledge of the mammalian ribosome synthesis pathways is required to further unravel the ribosomal stress response, a promising target for non-genotoxic p53 induction in tumour cells. Fortunately, ribosome biogenesis is a highly evolutionary conserved process that has been intensively studied in yeast (15). In addition, the recent characterization of the human nucleolar proteome further facilitates the investigation of mammalian ribosome biogenesis (16–18).

The nucleolar protein Pes1 (Pescadillo) is evolutionarily highly conserved and essential for embryogenesis and nucleologenesis in mice (19). Its expression is tightly associated with cell proliferation and thus highly elevated in tumour cells (20). Two studies have addressed the function of Pes1 in cell proliferation and ribosome biogenesis (21,22). Either transposon mediated insertion mutants or truncations of the N-terminal or C-terminal region resulted in a dominant-negative phenotype, blocking processing of the 32S pre-rRNA and cell proliferation. Ribosomal RNA (rRNA) is synthesized as a large 47S precursor that is subsequently cleaved into the mature 5.8S, 18S and 28S rRNA. Pes1 interacts with two other nucleolar proteins Bop1 and WDR12 in a stable complex, termed PeBoW-complex (8). Both factors are also implicated in the processing of ribosomal RNA (8,23). In yeast, a stable trimeric complex of the respective homologues of Pes1 (Nop7p/Yph1p), Bop1 (Erb1p) and WDR12 (Ytm1p) has been identified (24–26). Further, it was shown that the yeast Pes1 homologue Nop7p (Yph1p) functions in DNA replication independently of ribosome biogenesis (27). In this

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study, Nop7p was co-purified with the origin recognition proteins Orc1p and Orc2p and required for S-phase entry subsequently to the release from a hydroxyurea mediated cell cycle arrest. The presence of a BRCT-domain (BRCA1 C-terminal) within the central region of Pes1 is conserved throughout evolution and remarkable for several reasons. The BRCT-domain has been first identified as target of germline mutations in the BRCA1 protein, that predispose to the early development of breast and ovarian cancer (28). BRCT-domain containing factors, such as BRCA1, XRCC1 and 53BP1 play a role in DNA-damage or repair pathways. Based on recent structural studies, the BRCT-domain is thought to function as phosphopeptide recognition motif (29,30). A bioinformatic analysis of the nucleolar proteome reveals only a few BRCT-domain containing factors, but having all a confirmed role in DNA-damage or replication (http://Lamondlab.com/NOPdb/). Thus, the presence of a BRCT-domain within the Pes1 protein is therefore quite unusual if not unique for an rRNA processing factor. In this study, we established a conditional siRNA mediated knock-down-knock-in system to analyse the function of Pes1 truncation mutants in rRNA processing in the absence of a dominant-negative phenotype. An extended deletion the BRCT-domain resulted in aberrant nucleoplasmic localization, however only in the context of the full-length protein, and failed to reconstitute for the endogenous protein in rRNA processing assays. To study the role of the BRCT-domain more in detail, we inserted single point mutations of highly conserved residues. We found that the BRCT-domain of Pes1 is essential for rRNA processing and nucleolar localization in a context dependent manner. Moreover, point mutations of the BRCT-domain reduce the stability of Pes1 and abolish its interaction with the PeBoW-complex.

MATERIALS AND METHODS

Cloning/plasmids

Conditional Pes1 expression constructs and mutagenesis were described previously (21). The novel Pes1 mutants M9 and M10 were generated by the use of restriction sites. Single point mutations of the BRCT-domain were introduced by site directed mutagenesis. Successful mutagenesis was verified by sequencing. Pes1 mutants were then cloned into the pRTS-1 vector using the SfiI restriction site (31).

Tissue culture

H1299 lung carcinoma cells (non-small cell lung carcinoma) were cultured in DMEM with 10% FBS at 8% CO_2. Polyclonal cell lines were generated by transfection of the respective pRTS-1 plasmids using Polyfect (QIAGEN) and stably selected in the presence of 200 μg/ml hygromycin B for ~10 days. Conditional gene expression was induced with 0.5 μg/ml doxycycline and monitored by determining the percentage of eGFP expressing cells using FACS analysis.

Knock-down-knock-in assay

H1299 cells were treated with 0.5 μg/ml doxycycline to activate expression of Pes1wt and the respective mutants for 3 days. Depletion of endogenous Pes1 was performed by two subsequent transfections of siRNA oligos specific for the 3′-untranslated region (3′-UTR) (5′-CCAGAGGACCUAAGUGUGAtt-3′) of the Pes1 mRNA using oligofectamine (QIAGEN). Cells were harvested for western blot analysis or metabolic labelling of nascent rRNA at 48 h after the last siRNA transfection.

Metabolic labelling of nascent rRNA

H1299 were incubated in phosphate-free DMEM/10% FBS for at least 30 min prior to the *in vivo* labelling. Cells were then incubated for 1 h with 15 μCi/ml ^{32}P-orthophosphate. Subsequently, the metabolic labelling medium was removed and cells were then further cultured in regular DMEM/10% FBS for 4–5 h. Total RNA was then isolated using the RNeasy Mini kit (QIAGEN). A total of 1.5 μg of total RNA was separated on a 1% agarose formaldehyde gel and subsequently dried on a whatman paper using a regular gel drier (BioRad) connected to a vacuum pump. Metabolically labelled RNA was visualized by autoradiography.

Immunoblotting and immunofluorescence

Cells were directly lysed with 2× SDS-loading buffer (100 mM Tris–HCl, 200 mM DTE, 4% SDS, 10 mM EDTA, 0.2% bromphenol blue and 20% glycerol). Cell lysates were separated by SDS–PAGE and blotted on nitrocellulose membranes (Amersham). Immunodetection was performed with monoclonal antibodies directed against the HA-tag (3F10; Roche), Pes1 (8E9) and tubulin (Dianova). The monoclonal antibody against human Pes1 was described previously (8). The antibody against rodent Bop1 was produced by simultaneous immunization of guinea pigs with ovalbumin conjugated peptides specific for 6–30 and 541–566 amino acids of mouse Bop1. The polyclonal serum was only reactive against the N-terminal 6–30 amino acids. For immunofluorescence, cells were grown on cover slides, fixed with ice-cold methanol and air dried. Unspecific binding was blocked with phosphate-buffered saline (PBS)/10% FBS. HA-tagged forms of Pes1 and WDR12 were detected with the anti-HA (3F10) antibody. Primary antibodies were incubated over night at 4°C in a humidified chamber. Cy3 labelled secondary antibodies (Dianova) were incubated for 1 h at room temperature. Nuclei were counterstained with DAPI (Sigma–Aldrich). Digital images were acquired using the Openlab acquisition software (Improvision) and a Zeiss Axiovert 200 M microscope (Carl Zeiss MicroImaging) with a 63 (1.15) plan oil objective connected to a CCD-camera (Hamamatsu, ORCA-479).

Immunoprecipitation

For immunoprecipitations, H1299 cells were seeded at subconfluent density and treated with 0.5 μg/ml doxycycline for 24 h. Cells were harvested by trypsination and washed three times with PBS. Subsequently, cells were resuspended in lysis buffer [50 mM Tris–HCl (pH 8.0), 1% NP-40, 150 mM NaCl, protease inhibitors and phosphatase inhibitors] and incubated on ice for 20 min and sonicated. In the meanwhile protein G beads were incubated either with antibodies against the HA-tag (3F10, Roche), WDR12 (1B8), Bop1 (6H11) or cytohesin for at least 1 h at 4°C. Monoclonal antibodies directed against WDR12, Bop1 and

cytohesin were described previously (8). After centrifugation, cell lysates were incubated with protein G beads at 4°C over night. Finally, the beads were washed three times with lysis buffer and resuspended in SDS-loading buffer/lysis buffer (1:1). The resuspension volume was equal to the volume of the cell lysates initially used for the IP.

Native gel electrophoresis

A total of 3×10^6 cells were lysed in 100 µl lysis buffer [50 mM Tris–HCl (pH 8.0), 1% NP-40, 150 mM NaCl, phosphatase inhibitors and protease inhibitors] at 4°C for 20 min. Total cell lysates were briefly vortexed several times in between. Lysates were then cleared by centrifugation in an Eppendorf FA-24-11 rotor at 24 000*g* for 10 min at 4°C. 7.5 µl of 2× sample buffer [125 mM Tris–HCl (pH 6.8), 30% glycerol and 0.02% bromophenol blue] was added to 7.5 µl of total lysate and separated by PAGE (6.5%) in the absence of SDS at 4°C. Blotting was performed in the absence of methanol. Immunoblotting was performed as described above.

RESULTS

Functional analysis of Pes1 mutants by a knock-down-knock-in assay

Previously, we have analysed a set of truncation mutants of human Pes1 for dominant-negative phenotypes in rRNA processing and cell proliferation assays (Figure 1A). Thereby we identified two mutants, a N-terminal (M1) and a C-terminal (M5) truncation, that impaired rRNA processing and cell cycle progression (21). The respective regions were also found as hotspots for the generation of dominant-negative mutants using transposon mediated mutagenesis (22). In this study we extended the panel of mutants by two novel truncated forms of Pes1, namely M9 and M10. Nomenclature was preserved to facilitate crossreading with our previous study (21).

So far it remained unclear whether all the other deletion mutants were still functional in rRNA processing, in particular the ones that localized to nucleolus as the wild-type protein. Pes1 is composed of several distinct domains and little is known about their role in rRNA processing (Figure 1A). Therefore we established a conditional knock-down-knock-in system for mammalian cells (Figure 1B). Briefly, H1299 human lung carcinoma cells were stably transfected with pRTS-1 constructs, conditionally expressing the respective HA-tagged forms of human Pes1 in a doxycycline dependent manner (21,31). Exogenous gene expression was activated for three days and then maintained throughout the subsequent course of two siRNA transfections using siRNA oligos directed against the 3′-UTR of the Pes1 mRNA to specifically deplete the endogenous Pes1 protein. We used H1299 cells, because stable polyclonal cell cultures could be easily propagated following transfection of the pRTS-1 plasmids and conditional gene expression could be achieved in >95% of the cells (Supplementary Figure 1). A bidirectional promoter drives transcription of eGFP at the same time as the indicated forms of Pes1 and thus allowed easy monitoring by FACS analysis. Efficient knock-down of the endogenous Pes1 protein was verified by western blot analysis 2 days after the last siRNA transfection (Figure 1C). The various Pes1 mutants were detected by antibodies directed against the HA-tag and the C-terminal region of Pes1. The mutants were detected at the expected size, however deletion of the acidic regions in M5, M6 and M10 facilitated faster migration. The Pes1 specific antibody did not recognize the mutants M5, M10 and M7, as they lacked the respective epitope. The enlarged panel at the bottom shows the size-dependent discrimination of the HA-tagged wild-type and the endogenous Pes1 protein. Including the 3 days of pre-activation, the various Pes1 mutants were expressed for 7 days when finally harvested. Thus, the different expression levels of the indicated forms of Pes1 reflect steady-state levels that depend on the respective protein stability. In particular, M2, M4 and M9 are poorly expressed despite identical induction rates as monitored by FACS analysis (Supplementary Figure 1). Interestingly, the steady-state expression of the HA-tagged wild-type Pes1 closely resembled the levels of the endogenous Pes1, thus providing a knock-down-knock-in system at physiological expression conditions.

The acidic domain of Pes1 is dispensable for rRNA processing

In parallel to the western blot analysis, rRNA processing was investigated by *in vivo* labelling of nascent rRNA using ^{32}P-orthophosphate (Figure 2). Ribosomal RNA is transcribed as a large 47S precursor that is subsequently cleaved into the mature rRNA forms. Figure 2A depicts an overview of mammalian rRNA processing pathways. ^{32}P-orthophosphate is incorporated into the nascent rRNA transcripts and thus allows an *in vivo* analysis of rRNA processing. The 45S and the 32S rRNA are the most abundant precursor rRNAs, besides the mature 18S and 28S forms. Expression of Pes1wt-HA but not WDR12wt-HA completely rescued the abrogated production of mature 28S rRNA mediated by depletion of endogenous Pes1 (Figure 2B, lanes 1–4). As the HA-tagged wild-type Pes1 was fully functional in terms of rRNA processing, we now tested the whole panel of Pes1 mutants. The majority of the mutants failed to compensate for depletion of endogenous Pes1. However, M6 and M8 fully restored the synthesis of the mature 28S rRNA (Figure 2B, lanes 17, 18 and 21, 22). As M6 lacks the C-terminal acidic region, this domain is dispensable for rRNA processing. This is intriguing, as the acidic domain of Pes1 is preserved throughout evolution and similar stretches of acidic residues are frequently found in nucleolar proteins, such as nucleolin and nucleophosmin. Several studies provided evidence that these acidic regions might be important for protein–protein interactions (32,33). M8 harbors a point mutation of the potential SUMOylation site of Pes1, however SUMOylation at this site has not been experimentally confirmed yet. Interestingly, all mutants lacking the BRCT-domain were incapable to substitute for endogenous Pes1.

Role of the BRCT-domain for subcellular localization of Pes1

Analysis of the subcellular localization by indirect immunofluorescence revealed that M9, missing the BRCT-domain, was dispersed in the nucleoplasm (Figure 3A), as described previously (21). Further, deletions within the Pes1 protein

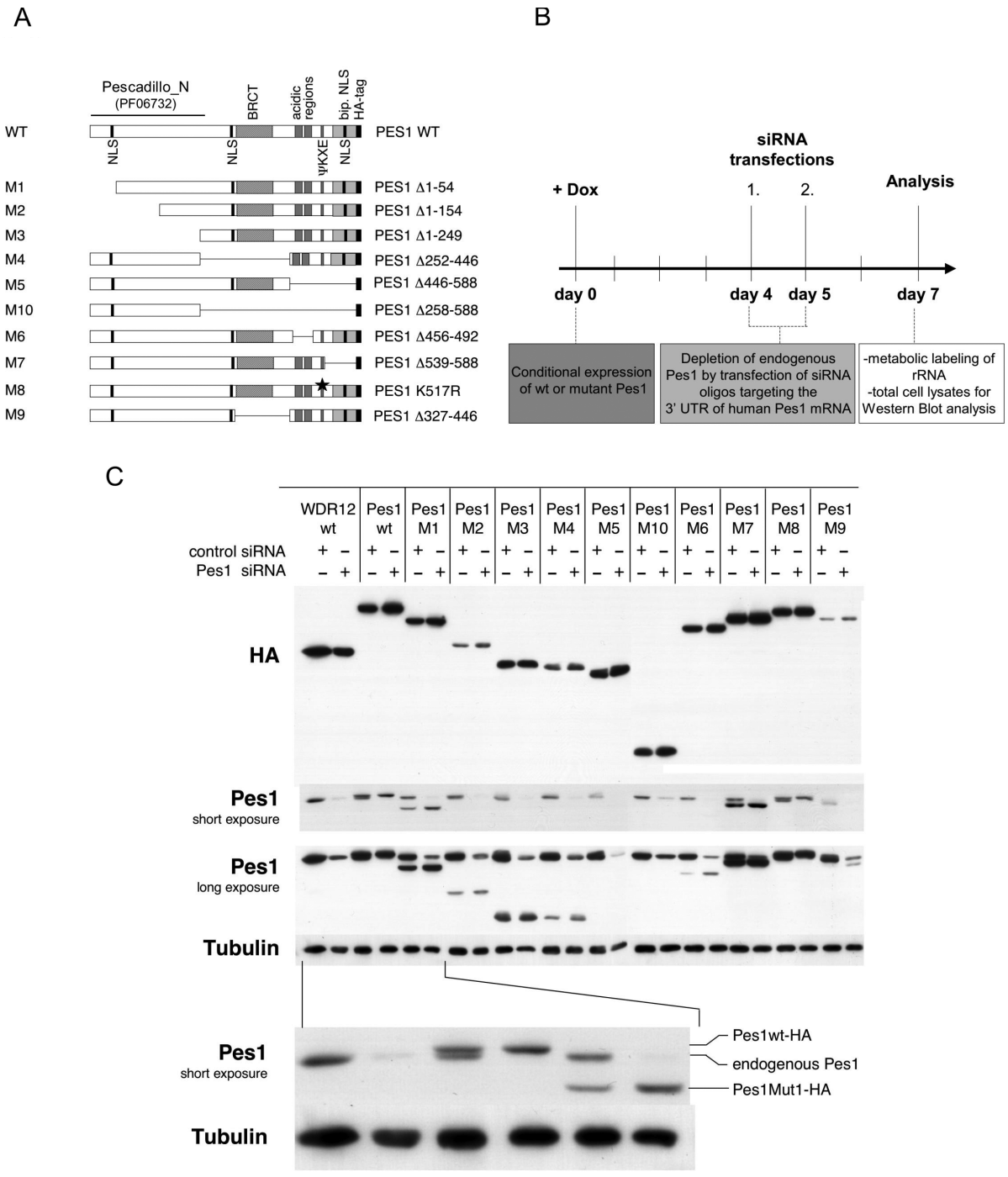


Figure 1. Pes1 mutagenesis, experimental approach of knock-down-knock-in assay and conditional expression of Pes1 mutants. (**A**) Panel of Pes1 deletion mutants harbouring a C-terminal HA-tag, as described previously. M9 and M10 are novel deletion mutants. NLS: classical nuclear localization signal; bip. NLS: bipartite NLS; Pescadillo_N: N-terminal region of Pes1 (Pfam-database: PF06732); BRCT: BRCT-domain; acidic regions: two glutamic acid rich regions; ΨKXE: consensus SUMOylation site; HA: hemagglutinin-tag. (**B**) Experimental approach of knock-down-knock-in assay. (**C**) Western blot analysis of knock-down-knock-in assay as described in (B). Expression of recombinant wt or mutant Pes1 proteins was analysed with the anti-HA antibody (3F10). Knock-down of endogenous Pes1 by siRNA was verified probing with the anti-Pes1 antibody (8E9). All mutants besides M5, M10 and M7 were also detected with the anti-Pes1 antibody. Equal loading was controlled by immunodetection of α-tubulin. Size-dependent discrimination of HA-tagged wt and endogenous Pes1 protein using the anti-Pes1 antibody is shown in the enlarged panel at the bottom.

(M4 and M10) partially or completely restored nucleolar localization [Figure 3A, Supplementary Figure 2 and (21)]. Thus, lack of the BRCT-domain results in a diffuse nucleoplasmic distribution in a context dependent manner. However, the isolated BRCT-domain of Pes1 (Pes1 BRCT) was diffusely distributed in the cytoplasm and therefore insufficient for nuclear/nucleolar localization (Figure 3A). M6, harbouring the deletion of the acidic region, localized to the nucleolus

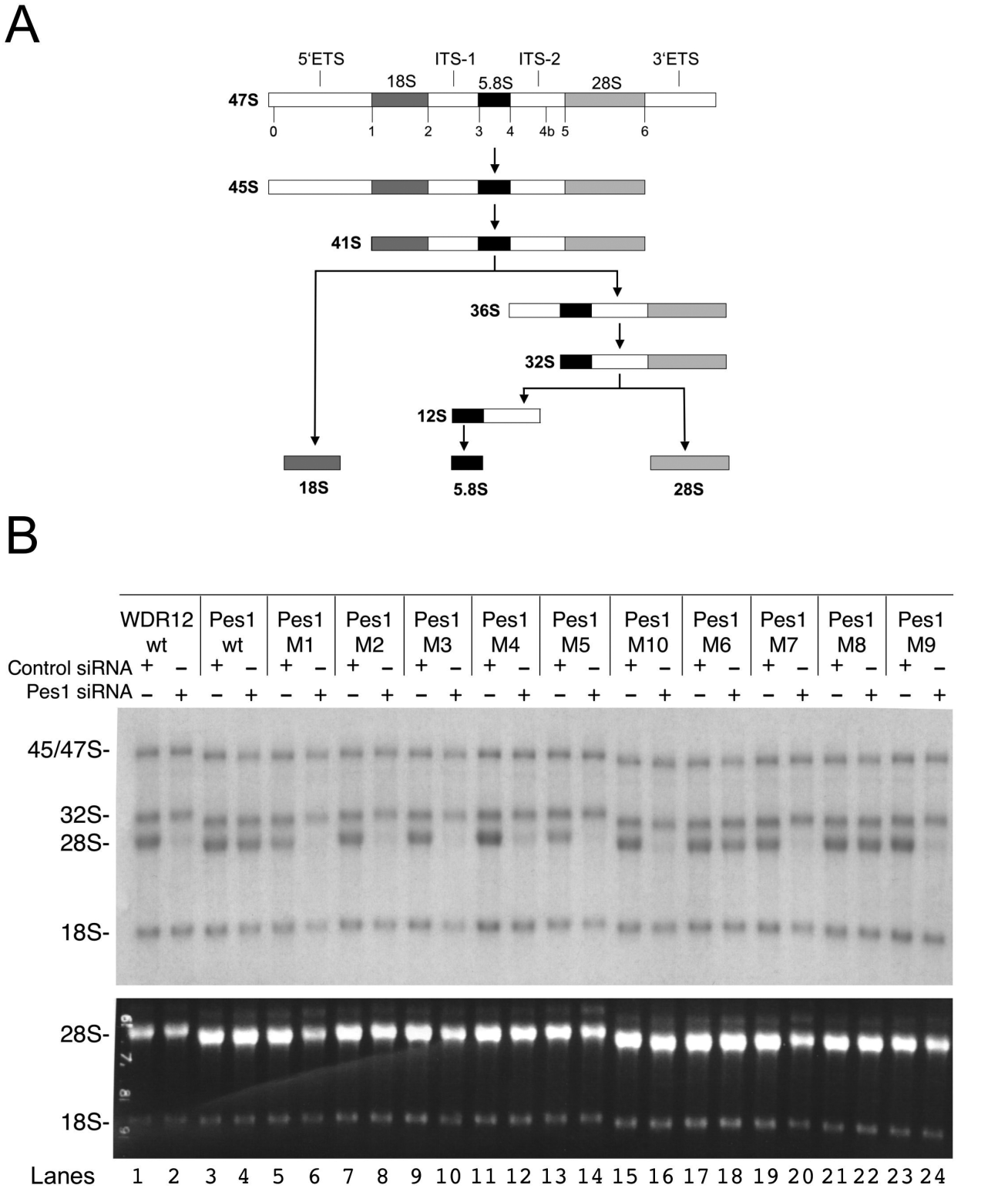
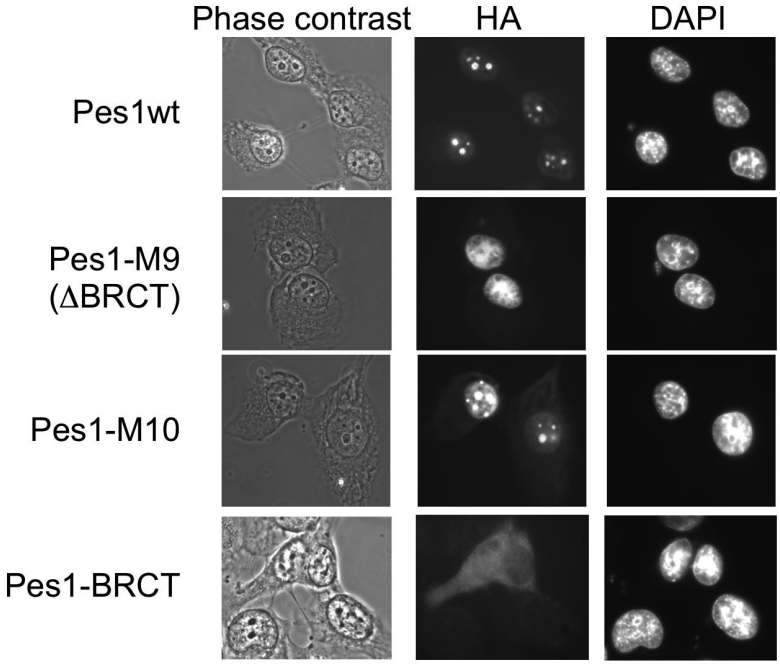
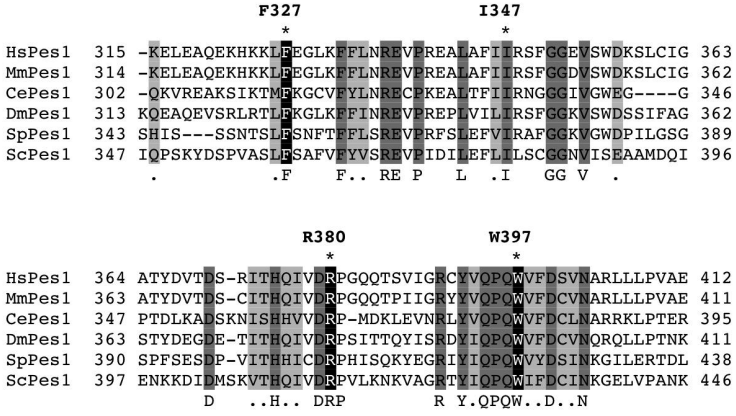


Figure 2. Functionality of Pes1 mutants in rRNA processing in the absence of endogenous Pes1. (**A**) Simplified schematic view of mammalian rRNA processing pathways. The primary 47S transcript is processed into subsequent precursor rRNAs. Cleavage sites are indicated by the corresponding numbers. 5′ETS and 3′ETS (external transcribed spacers), ITS-1 and ITS-2 (internal transcribed spacers) depict the regions that are cleaved off the primary transcript without being used in the mature ribosomes. The 45S and the 32S rRNA are the most abundant rRNA precursors that can be easily detected by the metabolic labelling. (**B**) Autoradiography of metabolic labelling of nascent ribosomal RNA using ^{32}P-orthophosphate. Knock-down-knock-in assay was performed as summarized in Figure 1B. Stable polyclonal H1299 cell lines expressing the indicated HA-tagged proteins were either treated with control or Pes1 (3′-UTR) specific siRNA. Cells were then incubated in phosphate-free medium for 30 min, labelled with ^{32}P-orthophosphate for 1 h and subsequently cultured in regular medium for 4.5 h. Total RNA was separated by formaldehyde-agarose gel electrophoresis. The gel was dried before autoradiography. The ethidium bromide stained gel is shown as loading control.

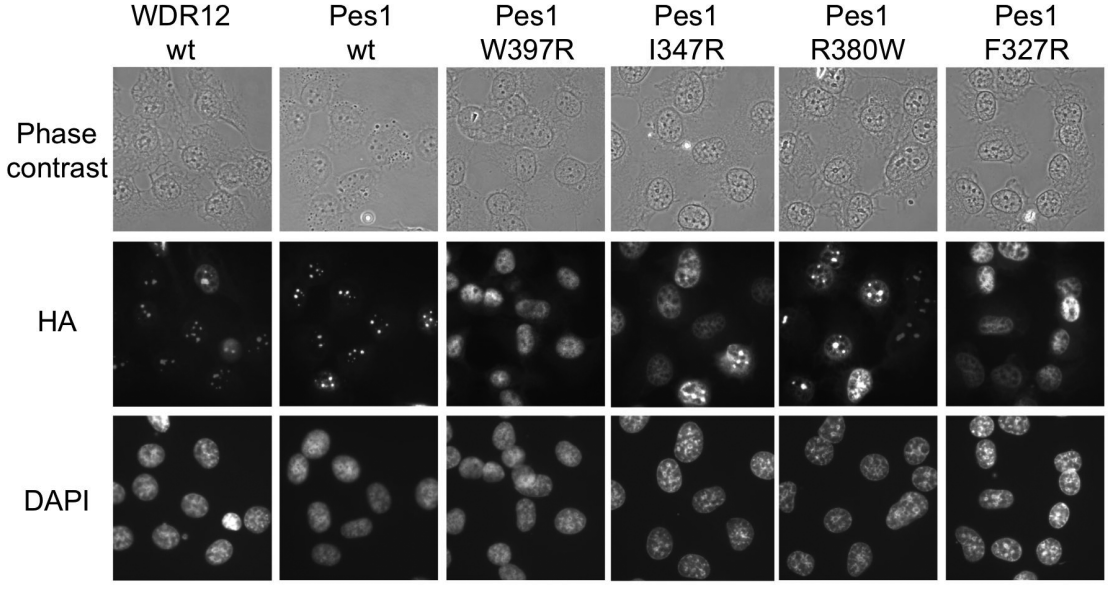
A


Phase contrast
HA
DAPI
Pes1wt
Pes1-M9
(ΔBRCT)
Pes1-M10
Pes1-BRCT

B


F327
I347
HsPes1 315 -KELEAQEKHKKLFEGLKFFLNREVPREALAFIIRSFGGEVSWDKSLCIG 363
MmPes1 314 -KELEAQEKHKKLFEGLKFFLNREVPREALAFIIRSFGGDVSWDKSLCIG 362
CePes1 302 -QKVREAKSIKTMFKGCVFYLNRECPKEALTFIIRNGGGIVGWEG----G 346
DmPes1 313 KQEAQEVSRLRTLFKGLKFFINREVPREPLVILIRSFGGKVSWDSSIFAG 362
SpPes1 343 SHIS---SSNTSLFSNFTFFLSREVPRFSLEFVIRAFGGKVGWDPILGSG 389
ScPes1 347 IQPSKYDSPVASLFSAFVFYVSREVPIDILEFLILSCGGNVISEAAMDQI 396
R380
W397
HsPes1 364 ATYDVTDS-RITHQIVDRPGQQTSVIGRCYVQPQWVFDSVNARLLLPVAE 412
MmPes1 363 ATYDVTDS-CITHQIVDRPGQQTPIIGRYYVQPQWVFDCVNARLLLPVAE 411
CePes1 347 PTDLKADSKNISHHVVDRP-MDKLEVNRLYVQPQWVFDCLNARRKLPTER 395
DmPes1 363 STYDEGDE-TITHQIVDRPSITTQYISRDYIQPQWVFDCVNQRQLLPTNK 411
SpPes1 390 SPFSESDP-VITHHICDRPHISQKYEGRIYIQPQWVYDSINKGILERTDL 438
ScPes1 397 ENKKDIDMSKVTHQIVDRPVLKNKVAGRTYIQPQWIFDCINKGELVPANK 446

C


WDR12 wt
Pes1 wt
Pes1 W397R
Pes1 I347R
Pes1 R380W
Pes1 F327R
Phase contrast
HA
DAPI

as the wild-type protein [Supplementary Figure 2 and (21)]. This observation is in line with its full functionality in the rRNA processing assay. The presence of the BRCT-domain within the Pes1 protein is conserved throughout evolution, from yeast to human. In Figure 3 we have shown that it is crucial for the nucleolar localization of Pes1, but only in the context of the full-length protein, as the highly conserved N-terminal region (M10) was sufficient for nucleolar localization. However, a gross deletion of the BRCT-domain including adjacent residues might affect overall stability and protein folding. Therefore, we generated a set of single point mutations of highly conserved residues within the BRCT-domain (Figure 3B). W397 is one of the most conserved amino acids corresponding to the missense mutation W1837R in the BRCT-domain of BRCA1. Analogous exchanges of W397R, I347R and F327R in the yeast Pes1 homologue Nop7p (Yph1p) resulted in temperature sensitive strains (20,27). So far, no temperature sensitive alleles were isolated in yeast that would correspond to the R380W exchange.

We then investigated the subcellular localization of the respective mutant forms by indirect immunofluorescence (Figure 3C). Pes1-W397R and -F327R exhibited a diffuse nucleoplasmic distribution. Pes1-I347R was also dispersed in the nucleoplasm, however a small percentage of the cells also displayed nucleolar enrichment. Pes1-R380W predominantly localized in the nucleolus, but the intensity of the nucleoplasmic staining was slightly increased in comparison to Pes1wt. Thus, the integrity of the BRCT-domain is crucial for proper nucleolar localization of Pes1, and notably, either deletion or critical point mutations of the BRCT-domain resulted in aberrant nucleoplasmic distribution.

Point mutations within the BRCT-domain of Pes1 abrogate its functionality in rRNA processing

Subsequently, we analysed the functionality of the BRCT-mutants in terms of rRNA processing. We depleted the cells for endogenous Pes1 and performed *in vivo* labelling of nascent ribosomal RNA to test the capacity of the respective mutants to sustain production of the mature 28S rRNA. In analogy to Figure 2, the HA-tagged Pes1wt was fully functional in our assay (Figure 4A, lanes 1–4). In contrast, the predominantly nucleoplasmic BRCT-mutants Pes1-W397R, -I347R and F327R failed to substitute for depletion of endogenous Pes1 (Figure 4A, lanes 5–8 and 11–12). Only the nucleolar Pes1-R380W mutant rescued maturation of the 28S rRNA. For western blot analysis, cells were harvested in parallel to the *in vivo* labelling (Figure 4B). Noteworthy, the non-functional BRCT-domain mutants Pes1-W397R, -I347R, -F327R displayed lower steady-state expression levels than Pes1wt or the functional Pes1-R380W mutant, even though the percentage of ectopic Pes1 expressing cells was equal as determined by detection of eGFP using flowcytometry (Supplementary Figure 3). Successful knock-down of the endogenous Pes1 was verified by using an antibody directed against Pes1 (Figure 4B). As this antibody recognizes the endogenous and exogenous forms of Pes1, the expression of the respective mutants can be analysed in the context of the endogenous Pes1 levels. The non-functional nucleoplasmic BRCT-domain mutants were expressed at significant lower levels than the endogenous Pes1, whereas Pes1wt and Pes1-R380W closely mimicked the physiological expression levels.

Point mutations within the BRCT-domain of Pes1 negatively affect protein stability

Thus, we reasoned that the lower protein expression levels of the non-functional BRCT-domain mutants might result from decreased protein stability. Therefore we inhibited proteasomal degradation by treating the cells with MG-132 for 8 h. Indeed, the non-functional BRCT-domain mutants exhibited a more pronounced accumulation than Pes1wt and Pes1-R380W (Figure 5A). Further, proteasome inhibition resulted in the occurrence of multiple bands with higher molecular weight strongly suggesting that Pes1 and the respective BRCT-domain mutants become polyubiquitinated. We also analysed the panel of Pes1 truncation mutants for the occurrence of polyubiquitination to further map the ubiquitination site. However, all mutants tested so far exhibited signs of polyubiquitination (data not shown). Thus, we concluded that several lysines within different regions of Pes1 might serve for ubiquitination to mediate subsequent proteasomal degradation. Incubation with ALLM, an inhibitor of calpain and cathepsin proteases, had no effect on the protein levels of the BRCT-domain mutants (Figure 5A). Our attempts to analyse whether proteasome inhibition mediated accumulation of the BRCT-domain mutants restored functionality were hampered by the fact that proteasome inhibitors itself potently abrogated rRNA processing (Supplementary Figure 4).

Next, we analysed the stability of the Pes1 BRCT-domain mutants by treating the cells with cyclohexamide to block *de novo* protein synthesis (Figure 5B). Expression levels of Pes1wt and Pes1-R380W remained unchanged over 12 h in the presence of cyclohexamide. However, the non-functional mutants Pes1-W397R, -I347R and -F327R rapidly decreased within several hours in the absence of *de novo* protein synthesis. In conclusion, mutations of highly conserved residues within the BRCT-domain of Pes1 negatively affect protein stability and cause aberrant nucleoplasmic localization. Thus it might be that incorporation of Pes1 mutants into

Figure 3. Mutagenesis of the BRCT-domain of Pes1 and subcellular localization of Pes1 mutants carrying single point mutations within the BRCT-domain. (**A**) Subcellular localization of HA-tagged Pes1wt and mutant M9 (BRCT-domain deletion mutant) and M10 (lacking the BRCT-domain and the C-terminus). Stable polyclonal H1299 cell lines conditionally expressing the indicated Pes1 mutants were grown on cover slips and fixed with ice-cold methanol. Cells expressing HA-tagged wt WDR12 are shown as a control. Nuclei were counterstained with DAPI. Subcellular localization of the full-set of Pes1 mutants is shown in Supplementary Figure 2. Additionally, H1299 cells were transiently transfected with the isolated HA-tagged BRCT-domain of Pes1 (Pes1 BRCT). (**B**) Alignment of the Pes1 BRCT-domain of diverse species: HsPes1: *Homo sapiens* Pes1 (NP_055118); MmPes1: *Mus musculus* Pes1 (NP_075027); CePes1: *Caenorhabditis elegans* Pes1 (NP_498661); DmPes1; *Drosophila melanogaster* Pes1 (NP_609305); SpPes1: *Schizosaccharomyces pombe* Pes1 (NP_596543); ScPes1: *Saccharomyces cervisiae* Pes1 (Nop7p, Yph1p, NP_011617). Highly conserved residues are highlighted in dark grey and similar exchanges in light grey. Conserved residues used for mutagenesis are shown in black and marked by asterisks (*). (**C**) Subcellular localization of Pes1wt and Pes1 BRCT-domain mutants by indirect immunofluorescence using the anti-HA antibody (3F10). Stable polyclonal H1299 cell lines conditionally expressing the indicated proteins were grown on cover slips and fixed with ice-cold methanol. Nuclei were counterstained with DAPI.

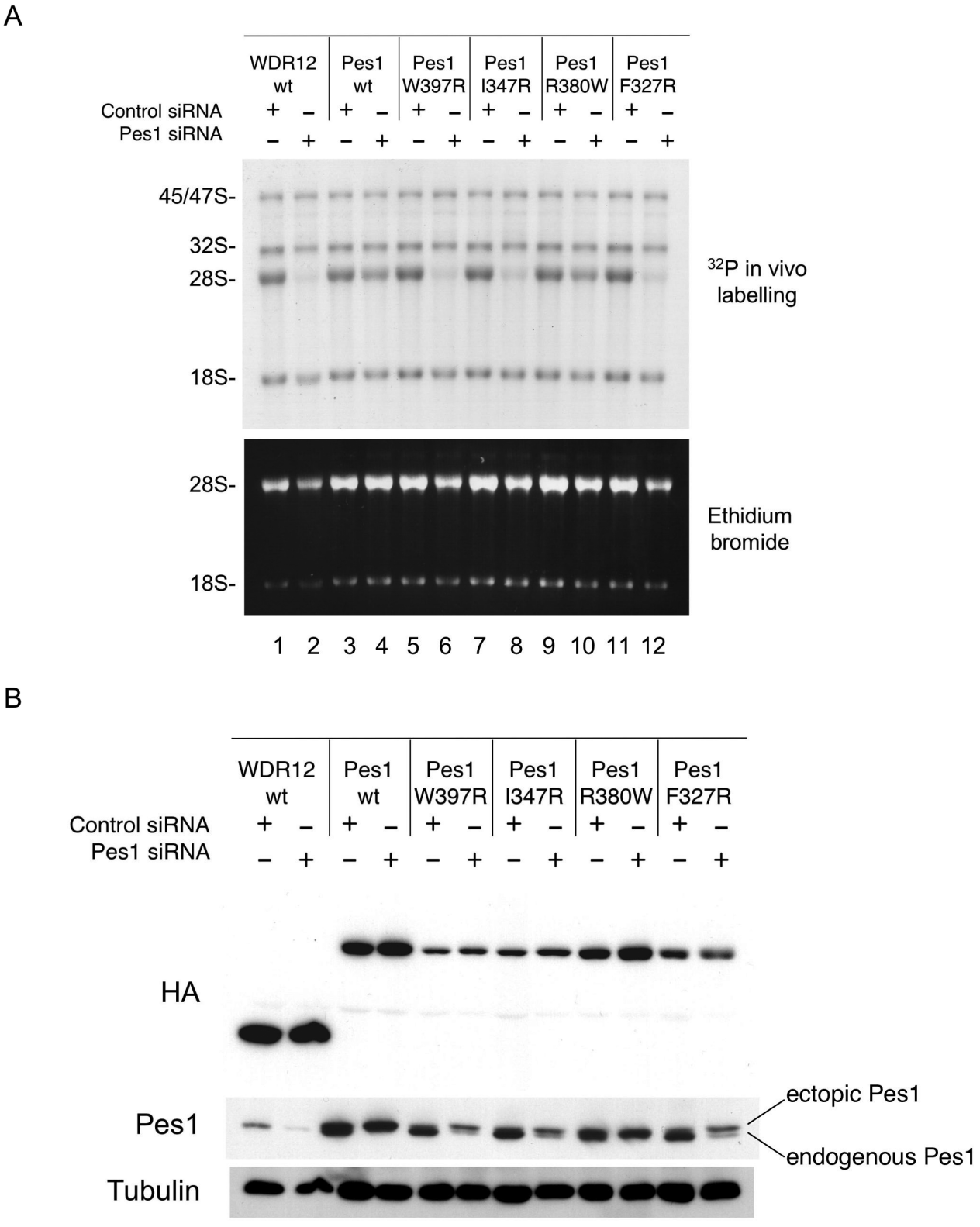


Figure 4. Knock-down-knock-in assay of H1299 cells conditionally expressing wt WDR12, wt Pes1 and the indicated BRCT-domain mutants of Pes1. Experimental procedure was performed as summarized in Figure 1B. (**A**) Metabolic labelling of nascent rRNA. Cells were incubated in phosphate-free medium for 30 min, labelled with ^{32}P-orthophosphate for 1 h and subsequently cultured in regular medium for 4.5 h. Total RNA was separated by formaldehyde-agarose gel electrophoresis. The gel was dried before autoradiography. The ethidium bromide stained gel is shown as loading control. (**B**) Expression of recombinant wt WDR12, wt or mutant Pes1 proteins was analysed using the anti-HA antibody (3F10). Efficient knock-down of endogenous Pes1 by siRNA was verified with the anti-Pes1 antibody (8E9). Equal loading was verified by immunodetection of α-tubulin.

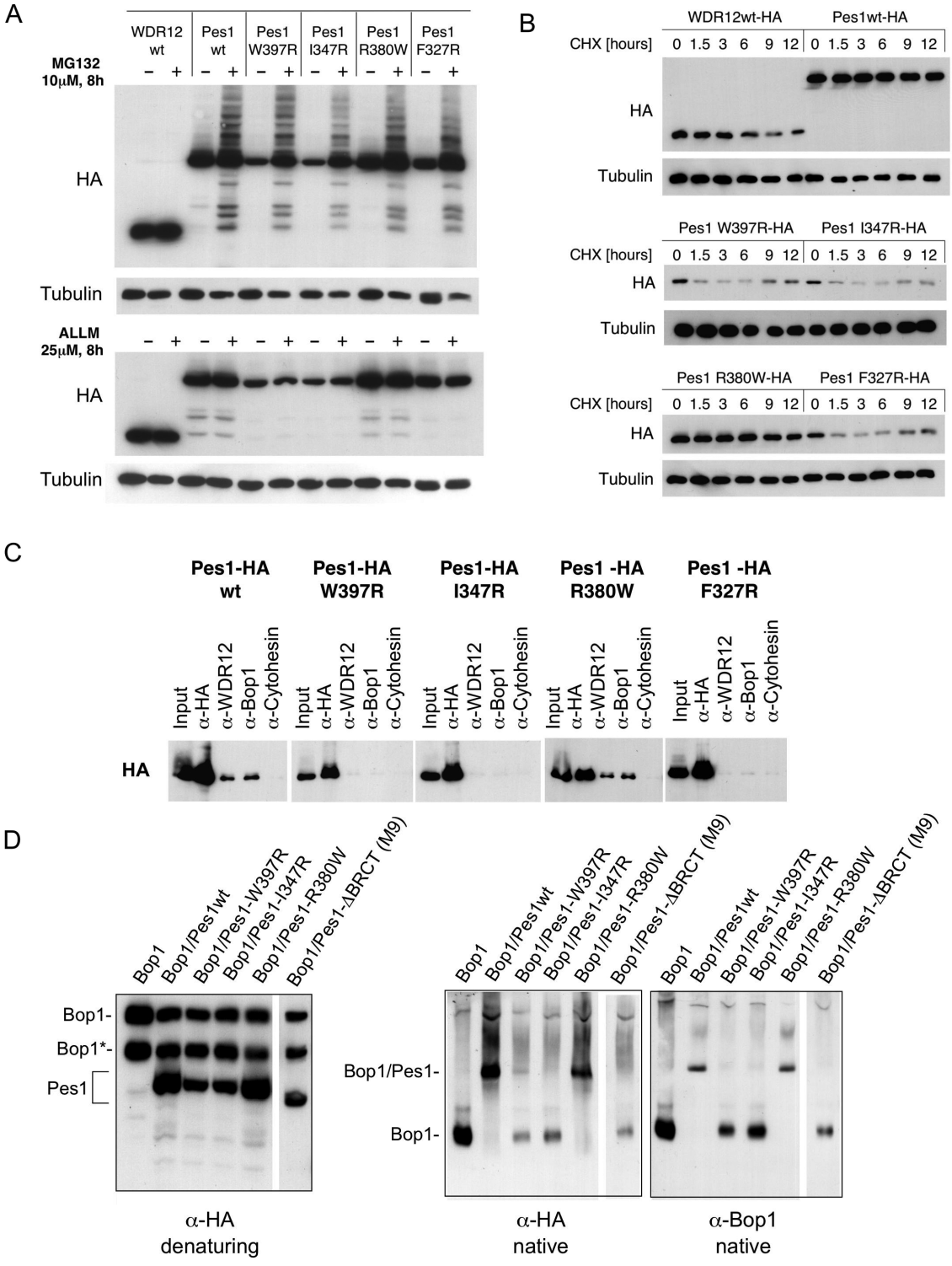


Figure 5. Stability of Pes1 BRCT-domain mutants and incorporation into the PeBoW-complex. (**A**) Stable polyclonal H1299 cells conditionally expressing wt WDR12, wt Pes1 and the indicated Pes1 BRCT-domain mutants were either left untreated or incubated with MG-132 or ALLM for 8 h. Total cell lysates were harvested and analysed by western blot analysis using the anti-HA antibody (3F10). Equal loading was verified by immunodetection for α-tubulin. (**B**) Cells were treated with 50 μg/ml cyclohexamide for the indicated time points to block *de novo* protein synthesis. Western blot analysis of total cell lysates was performed using the anti-HA antibody (3F10). Immunodetection of α-tubulin is shown as loading control. (**C**) Incorporation of Pes1 BRCT-domain mutants into the PeBoW-complex. H1299 cells stably transfected with the indicated constructs were treated with doxycycline for 48 h to induce expression of the respective proteins. Cell lysates were subjected to immunoprecipitations with antibodies either directed against the HA-tag (3F10), WDR12 (1B8), Bop1 (6H11) or the isotype control (cytohesin). Equivalent amounts of total lysates used for immunoprecipitations were loaded in each lane or 20% thereof for the input. Immunodetection was performed using the anti-HA antibody. (**D**) Total cell lysates of stably transfected H1299 cells expressing HA-tagged wild-type Pes1 or the indicated mutant together with HA-tagged rodent Bop1 were separated by native gel electrophoresis. Immunodetection was perfomed using antibodies specific for the HA-tag and rodent Bop1. Denaturing gel electrophoresis and HA-tag immunodetection was performed to control expression levels.

the nucleolar PeBoW-complex (Pes1, Bop1 and WDR12) is crucial for their stability.

Point mutations within the BRCT-domain of Pes1 prevent its incorporation into the PeBoW-complex

Therefore, we performed co-immunoprecipitation studies using antibodies specific for the HA-tag, WDR12, Bop1 and cytohesin, as an isotype control (8,21). Immunodetection was carried out using the HA-tag antibody to test whether the Pes1 BRCT-domain mutants could be co-precipitated with antibodies directed against the two other endogenous PeBoW-components, Bop1 and WDR12 (Figure 5C). Indeed, Pes1wt and Pes1-R380W efficiently co-precipitated with Bop1 and WDR12. However, the nucleoplasmic mutants Pes1-W397R, -I347R and -F327R were not detected in the respective immunoprecipitations. In a previous study, the region of Pes1 required for the interaction with Bop1 was identified by a yeast-two-hybrid assay and localized in the N-terminal region (~200 amino acids) and therefore distant from the BRCT-domain (22). A recent study also demonstrated that the N-terminal region of Pes1 is sufficient and essential for *in vitro* interaction with Bop1 (34). Importantly, a Pes1 mutant harbouring a deletion of the BRCT-domain (corresponding to the nucleoplasmic mutant M9 of our study) precipitated *in vitro* translated Bop1 as efficiently as wild-type Pes1 in glutathione *S*-transferase (GST)-pull down assays. These experiments suggested that deletion or point mutations of the BRCT-domain only play a critical role for the Pes1–Bop1 interaction under *in vivo* conditions. Aberrant nucleoplasmic localization is one possible mechanism that may prevent *in vivo* interaction with endogenous Bop1. However, additional mechanisms may contribute *in vivo* as well. As endogenous Bop1 might be rate limiting for Pes1–Bop1 complex formation we analysed complex assembly by native gel electrophoresis upon enforced expression of Pes1 and Bop1. Wild-type Pes1 and Pes1-R380W efficiently formed a stable complex with Bop1 under these conditions. However, either deletion or the other point mutations of the BRCT-domain abrogated complex formation (Figure 5D). Thus, deletion or critical point mutations of the BRCT-domain of Pes1 prevent its interaction with the endogenous PeBoW-components *in vivo*, whereas its physical interaction with Bop1 under *in vitro* conditions apparently remains unaffected, as recently shown.

DISCUSSION

Several recent studies have underlined the importance of the nucleolus in sensing cellular stress (2,7,11,14). The tremendous metabolic challenge of ongoing ribosome biogenesis is controlled by growth factor and stress signalling cascades, as well as the cell cycle machinery (3). Likewise, disturbed ribosome synthesis impairs cell cycle progression by activating the p53 pathway (8–10). Studying the coordination of cell cycle progression and ribosome biogenesis requires profound knowledge of both processes. The conveniences of yeast genetics, such as the generation of temperature sensitive mutants have provided deep insides into the complex eukaryotic ribosome synthesis machinery (15). However, classical genetic knock-out approaches in mammalian cells are laborious, in particular, if required in a conditional manner for essential genes, such as many ribosome synthesis factors like Pes1 presumably might be (19). The siRNA mediated knock-down technique now provides a rapid and powerful tool for the analysis of factors involved in mammalian ribosome biogenesis. In this study we showed that the combination of conditional gene expression and siRNA mediated knock-down techniques enabled the functional analysis of mutant Pes1 proteins in the absence of a dominant-negative phenotype. First, we noticed that exogenous wt Pes1 closely resembled the endogenous expression levels after the prolonged incubation (7 days) with doxycycline and substituted for endogenous Pes1 in rRNA processing. Interestingly, exogenous wt Pes1 exceeds the endogenous expression levels, when cells were harvested one day after the addition of doxycycline (data not shown). Hence, the Pes1 protein level apparently is tightly controlled in mammalian cells and excess amounts of Pes1 might be subject to proteasomal degradation. Interestingly, polyubiquitination bands were virtually not detected with the endogenous Pes1 upon treatment with proteasome inhibitors (data not shown), however easily with the overexpressed exogenous Pes1 when incubated with doxycycline for one day.

Next, we found that M6, lacking the acidic regions, reconstituted for depletion of endogenous Pes1 in rRNA processing. Acidic domains are frequently found in nucleolar proteins, such as nucleophosmin and nucleostemin, but so far a common function remains elusive. Deletion of the acidic domain of nucleostemin did not interfere with its nucleolar localization (35). In nucleophosmin, the acidic regions were found to be crucial for its interaction with the tumour suppressor p19ARF (32). Hence, the acidic domain of Pes1 might mediate unknown protein–protein interactions, as its deletion does not interfere with incorporation into the PeBoW-complex (21). Interestingly, truncation of the acidic domain of Pes1 in conjunction with the very C-terminal region (M5) results in a nucleolar localized dominant-negative mutant that blocks rRNA processing. However, removal of the C-terminal region only, is insufficient to elicit a dominant-negative phenotype (21). Thus, additional removal of the acidic regions apparently contributes to the inhibitory effects of M5. Nevertheless, further investigations are required to explore the role of the acidic domains of Pes1. We cannot exclude that the acidic regions of Pes1 have any impact on ribosome maturation and may influence overall ribosomal stability, but they are not required for the processing of the 32S rRNA precursor into the mature 28S rRNA. Deletion of the BRCT-domain of Pes1 caused diffuse nucleoplasmic localization and failure to sustain rRNA processing in Pes1 depleted cells. This is remarkable, as the mutant M10, consisting of the Pes1 N-terminal domain only and hence lacking the BRCT-domain, localizes properly to the nucleolus. The Pes1 N-terminal domain (Pfam-domain: PF06732) exhibits the highest degree of evolutionary conservation and apparently is sufficient to direct itself into the nucleolus. Thus, the BRCT-domain is required for nucleolar localization only in the context of the full-length Pes1 protein. These observations are reminiscent of the ones reported for the nucleolar GTP-binding protein nucleostemin. Single residue exchanges within the GTP-binding domain resulted in diffuse nucleoplasmic distribution. Additional removal of an extended region, termed internal-domain (I-domain),

restored nucleolar localization and relieved the nucleoplasmic holdback (35). It will be interesting to explore whether so far unknown regions within the Pes1 protein cooperate with the BRCT-domain in the control of the nucleolar localization.

BRCT-domains are frequently found in factors involved in DNA-damage and repair pathways and are thought to mediate phosphorylation dependent protein–protein interactions (29). Occasionally, the BRCT-repeats specifically contact BRCT-repeats in other proteins, as it has been reported for the interactions of XRCC1 with DNA ligase III (36). Most frequently, however, BRCT-repeats interact with protein domains of different structure. For example, the tandem BRCT-repeats of 53BP1 contact the DNA-binding domain of p53 (37). Only a few BRCT-repeat containing proteins were identified in the nucleolar proteome (http://Lamondlab.com/NOPdb/), such as XRCC1 and TOPBP1 (topoisomerase II binding protein 1). XRCC1 functions as scaffold protein in the base excision response and is therefore directly linked to DNA repair. To our knowledge, Pes1 is the only rRNA processing factor harbouring a BRCT-domain. Yeast strains with point mutations in the BRCT-domain of Nop7p (Yph1p), the respective homologue of Pes1, exhibited a temperature sensitive growth (20,27). However, subcellular localization of Nop7p and rRNA processing were not investigated in these strains. Therefore, we inserted analogous and novel point mutations in highly conserved residues of the BRCT-domain of Pes1 and analysed the subcellular localization and functionality in rRNA processing of the respective mutants. The W397R exchange was performed in analogy to the W1837R cancer predisposing mutation in BRCA1 (27). The BRCT-repeat of Pes1 exhibits a higher homology to the second BRCT-repeat of BRCA1 than to the first repeat. Based on structural analysis W1837 is located within the $\alpha3'$ α-helix of the second BRCT-repeat of BRCA1 and destabilizes the BRCT-repeat (29,38,39). ClustalW-alignment algorithm suggests an identical position for W397 of Pes1 within the $\alpha3$ α-helix of the BRCT-domain (Supplementary Figure 5). Thus, the W397R mutation of Pes1 might also result in specific BRCT folding defects. The I347R substitution is predicted to be located within the a1 a-helix and might affect the secondary structure in a similar way. No point mutation in BRCA1 corresponding to I347R in Pes1 has been described so far, besides an adjacent M1783T exchange. Further, a BIC-database (Breast Cancer Information Core Database) research reveals a F1761S mutation in BRCA1 that correlates with the F327R exchange in Pes1. In conclusion, a comparison of structural elements and missense mutations in BRCA1 with the BRCT-repeat of Pes1 suggests a crucial role for the residues W397, I347 and F327. No such correlations were found for the Pes1-R380W mutant.

Disturbing the integrity of the BRCT-domain by deletion or by point mutations of critical residues resulted in aberrant diffuse nucleoplasmic distribution of Pes1. Further, the nucleoplasmic BRCT-mutants (Pes1-M9, -W397R, -I347R and F327R) exhibited no obvious accumulation in the cytoplasm or formation of precipitates that would be suggestive of general defects in protein folding. Thus, we suppose that the mutations within the BRCT-repeat do not affect the overall structural integrity but rather impose specific conformational changes of the BRCT-domain or defects in protein–protein interactions mediated by the BRCT-domain. Therefore we favour the view of a specific regulatory mechanism that requires an intact BRCT-domain for proper nucleolar localization of Pes1. The nucleoplasmic BRCT-mutants also failed to interact with the endogenous PeBoW-components Bop1 and WDR12. Only Pes1wt and Pes1-R380W were efficiently co-immunoprecipitated. In line with these observations Pes1-W397R, -I347R and F327R could not compensate for depletion of endogenous Pes1 in rRNA processing assays. Bop1 interacts with Pes1 via a region ($\sim$200 amino acids) located in the Pes1 N-terminal domain and thus quite distant from the BRCT-domain (22). Therefore, disabling the nucleolar localization of Pes1 by deletion or point mutations within the BRCT-domain precludes its interaction with endogenous Bop1 and WDR12, even though the interaction site with Bop1 is not affected by the mutagenesis. Importantly, a recent study demonstrated that deletion of the BRCT-domain of Pes1 (corresponding to our ΔBRCT-mutant M9) did not interfere with its capacity to interact with Bop1 *in vitro* (34). Consistent with previous studies, the N-terminal domain of Pes1 was essential and sufficient for *in vitro* interaction with Bop1 (21,22). Thus it is unlikely, that a general folding defect of the ΔBRCT-mutant impairs Bop1 interaction and nucleolar localization *in vivo*, as demonstrated in our study. But nucleoplasmic sequestration may not be the only mechanism that prevents the interaction of the ΔBRCT-mutant (M9) or the respective BRCT point mutants with Bop1 under *in vivo* conditions. Coordinated overexpression of the Pes1 mutants together with Bop1 failed to recapitulate the *in vitro* results, even though we were able to efficiently produce stable complexes of ectopic wild-type Pes1 and Bop1. In principal, providing excess amounts of the Pes1 BRCT-mutant and Bop1 should have at least partially circumvented the regulatory impact of subcellular compartmentalization. It will be interesting to further investigate the role of the BRCT-domain of Pes1 for PeBoW-complex assembly and to explore the mechanism that prevents Pes1 BRCT-mutant interaction with Bop1 *in vivo*. Nevertheless, failure to interact with the endogenous PeBoW-complex might then negatively affect the stability of the BRCT-mutants. Indeed, the stability of PeBoW-proteins is interdependent, and knock-down of Bop1 reduces the level and stability of Pes1 (M. Rohrmoser *et al*., manuscript submitted). This argues for a highly controlled assembly of Pes1 with the remaining PeBoW-members.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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Rapid conditional knock-down–knock-in system for mammalian cells

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ABSTRACT

RNA interference (RNAi) is a powerful tool to analyze gene function in mammalian cells. However, the interpretation of RNAi knock-down phenotypes can be hampered by off-target effects or compound phenotypes, as many proteins combine multiple functions within one molecule and coordinate the assembly of multimolecular complexes. Replacing the endogenous protein with ectopic wild-type or mutant forms can exclude off-target effects, preserve complexes and unravel specific roles of domains or modifications. Therefore, we developed a rapid-knock-down–knock-in system for mammalian cells. Stable polyclonal cell lines were generated within 2 weeks by simultaneous selection of two episomal vectors. Together these vectors mediated reconstitution and knock-down in a doxycycline-dependent manner to allow the analysis of essential genes. Depletion was achieved by an artificial miRNA-embedded siRNA targeting the untranslated region of the endogenous, but not the ectopic mRNA. To prove effectiveness, we tested 17 mutants of WDR12, a factor essential for ribosome biogenesis and cell proliferation. Loss-off function phenotypes were rescued by the wild-type and six mutant forms, but not by the remaining mutants. Thus, our system is suitable to exclude off-target effects and to functionally analyze mutants in cells depleted for the endogenous protein.

INTRODUCTION

The nucleolus is the site of ribosomal RNA (rRNA) transcription, pre-rRNA processing and ribosome subunit assembly (1). For a long time, the nucleolus was considered to be a mere 'ribosome factory' that is solely involved in the production of ribosomes. Now, this view has been challenged, as the recent years have unraveled many essential roles in other processes. In particular, the nucleolus controls cell cycle progression by the sequestration of regulating factors and additionally serves as a site of specific maturation steps of most cellular ribonucleoprotein (RNP) particles such as small nuclear RNAs (snRNAs), transfer RNAs (tRNAs) and telomerase RNA (2–4). Interestingly, recent studies showed that the main nucleolar task, namely ribosome biogenesis itself, is linked to the mammalian cell cycle machinery. Ongoing ribosome synthesis is highly sensitive to stress signals and its disturbance induces the tumor suppressor p53 triggering cell cycle arrest and apoptosis (5–7).

Because of this remarkable connection between ribosome biogenesis and the p53 response, one may speculate that further direct links to other cellular key processes and pathways exist. Unfortunately, our knowledge of the mammalian ribosome synthesis machinery is still incomplete. Ribosome biogenesis factors have been almost exclusively studied in yeast (8). Isolation of pre-ribosomal complexes by mass spectrometry and synthetic lethal screens provided many novel candidates. The convenience of yeast genetics; in particular, the rapid generation of conditional depletion strains then allowed their functional characterization. However, depletion of a protein can interfere with the assembly of a multimolecular complex, if the presence of the factor is required for the formation of a stable complex. Further, many proteins integrate multiple roles through separate domains, and thus depletion of a single protein affects the whole functional repertoire. Therefore, missense mutations can unravel separate specific functions of a protein in cells depleted for the endogenous protein. An elegant recent study identified an essential role for the C-terminal extension of the yeast ribosomal protein rpS14 in 20S pre-rRNA processing by testing the functionality of missense mutations (9). In contrast, depletion of rpS14 affects the pre-rRNA pathway upstream

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of the 20S pre-rRNA intermediate, most likely caused by a defect in the assembly of a multimolecular complex required for the initial pre-rRNA processing steps. Therefore, reconstitution of conditional depletion strains with wild-type or mutant alleles provide a powerful genetic tool for a specific functional analysis of proteins.

Comparable genetic approaches in mammalian cells are laborious, in particular if required in a conditional manner for essential genes. For instance, the rRNA-processing factor Pes1 is essential for embryogenesis in mice which can be expected for many ribosome synthesis factors (10). RNA interference (RNAi) is a powerful tool to circumvent these obstacles and to analyze knock-down phenotypes. However, as mentioned previously, this loss-off function approach might affect multimolecular complex formation and therefore might preclude the investigation of more specific functions. Therefore, in addition to the knock-down approach, a concomitant knock-in of mutant forms is required.

In this study, we present a rapid knock-down–knock-in system for mammalian cells using two episomal constructs. The first one allows the doxycycline-dependent expression of the gene of interest, either as wild-type or mutant form. A second construct encoding a different resistance gene for stable selection in mammalian cells serves for the conditional gene knock-down. We expressed siRNA sequences embedded in an miRNA environment that target the untranslated regions (UTRs) of the endogenous mRNA. Thereby, we specifically deplete the endogenous protein without affecting the ectopic form. We performed a detailed analysis of the essential rRNA processing factor WDR12, a component of a multiprotein complex (PeBoW-complex), to show that our system is a rapid and convenient approach to assess the functionality of mutants by testing their capacity to reconstitute for depletion of the endogenous protein.

MATERIALS AND METHODS

Cloning

The 5′ or 3′ untranslated regions of human WDR12 and Pes1 were targeted by 21mer siRNA sequences designed using the Invitrogen siRNA design algorithm (BLOCK-IT RNAi Designer, https://rnaidesigner.invitrogen.com/rnaiexpress/). Selected siRNA sequences were then further adapted to the miRNA environment of the pcDNA 6.2-GW/EmGFP-miR construct (Invitrogen) that was derived from the endogenous murine miR-155 (11). We generated a subcloning construct (pMIRTOP) to allow initial functional analysis of the selected siRNA sequences, similar to the pcDNA 6.2-GW/EmGFP-miR plasmid (Invitrogen), but providing more convenient subsequent cloning into the pRTS plasmid. pMIRTOP was derived from pEGFP-C1 (Clontech). The oligos were cloned downstream of the eGFP open reading frame (ORF) into the miRNA environment. Functionality of the selected sequences was tested by indirect immunofluorescence to verify knock-down of endogenous WDR12 and Pes1 in H1299 cells. The following DNA oligonucleotides were used in this study. The number indicates the position of the target sequence within the reference mRNA of Pes1 (NM_014303) and WDR12 (NM_018256): miPes1-1903 (3′-UTR; 21mer target sequence: GCCAGAGGACCTAAGTGTGAT; miRNA-oligo: top strand TGCTGATCACACTTAGGTCCTCTGGCGTTTTGGCCACTGACTGACGCCAGAGGCTAAGTGTGAT, bottom strand CCTGATCACACTTAGCCTCTGGCGTCAGTCAGTGGCCAAAACGCCAGAGGACCTAAGTGTGATC), miPes1-308 (ORF; 21mer target sequence: TGTCAACAAGTTCCGTGAATA; miRNA-oligos: top-strand TGCTGTATTCACGGAACTTGTTGACAGTTTTGGCCACTGACTGACTGTCAACATTCCGTGAATA, bottom strand CCTGTATTCACGGAATGTTGACAGTCAGTCAGTGGCCAAAACTGTCAACAAGTTCCGTGAATAC), miWDR12-135 (5′-UTR; 21mer target sequence: TTCGTGTTGTGGGTCTGCTAA; miRNA-oligo: top strand TGCTGTTAGCAGACCCACAACACGAAGTTTTGGCCACTGACTGACTTCGTGTTGGGTCTGCTAA, bottom strand CCTGTTAGCAGACCCAACACGAAGTCAGTCAGTGGCCAAAACTTCGTGTTGTGGGTCTGCTAAC), miWDR12-1663 (3′-UTR; 21mer target sequence: CTGTGGCATACATTCTCTATA; miRNA-oligo: top strand TGCTGTATAGAGAATGTATGCCACAGGTTTTGGCCACTGACTGACCTGTGGCACATTCTCTATA, bottom strand CCTGTATAGAGAATGTGCCACAGGTCAGTCAGTGGCCAAAACCTGTGGCATACATTCTCTATAC), miLuciferase (abbreviated as miLuc; 21mer target sequence: TATTCAGCCCATATCGTTTCA; miRNA-oligo: top strand: TGCTGTATTCAGCCCATATCGTTTCAGTTTTGGCCACTGACTGACT GAAACGATGGGCTGAATA; bottom strand: CCTGTATTCAGCCCATCGTTTCAGTCAGTCAGTGGCCAAAACTGAAACGATATGGGCTGAATAC). An Eco47III/BglII fragment of pMIRTOP containing the eGFP ORF and the miRNA environment was directly cloned into the SwaI/BglII site of the pRTS construct. To obtain the DsRed-miWDR12-1663 construct, the eGFP within pMIRTOP-miWDR12-1663 was replaced by the ORF of DsRed2 (pDsRed2-N1; Clontech). An AgeI/NotI (blunt ended by T4 DNA polymerase) fragment was cloned into the AgeI/PmlI site of pMIRTOP-miWDR12-1663. Then, an Eco47III/BglII fragment containing DsRed2-miWDR12-1663 was cloned into pRTS as described above.

Generation of pRTS constructs expressing HA-tagged Pes1 and WDR12 was described previously. In general, the ORF amplified by PCR using primers that provided a CCACC Kozak sequence before the start codon and lacked the stop codon. Blunt end cloning into the EcoRV site of a subcloning construct (pSfiExpress) then provided a C-terminal HA-tag and two flanking *SfiI* sites for further cloning into pRTS constructs. pSfiExpress was generated by replacing the eGFP of pEGFP-C1 (Clontech) by a linker that was designed as outlined in Supplementary Data. Further, an additional *SfiI* site was also deleted to allow excision of the HA-tagged ORF by a *SfiI* digest. The *SfiI* fragment was then cloned into the *SfiI* sites of pRTS. Detailed information about the cloning strategies is provided in Supplementary Data.

Tissue culture

H1299 lung carcinoma cells (non-small cell lung carcinoma) were cultured in DMEM with 10% FBS at 8% CO_2. H1299 cells were transfected with the respective pRTS constructs using Polyfect (Qiagen) and polyclonal cell lines for knock-down–knock-in assays were generated by stable selection with 200 μg/ml hygromycin B and 1 μg/ml puromycin for ~10 days. Conditional gene and miRNA-embedded siRNA

expression was achieved by the addition of 0.5 μg/ml doxycycline.

Knock-down–knock-in assay

H1299 cells were treated with 0.5 μg/ml doxycycline to activate ectopic gene expression (Luciferase, WDR12 wild-type or mutant forms) together with the respective miRNA-embedded siRNAs (miLuc or miWDR12) for 4 days. Cells were trypsinized, counted and replated for functional assays. After one or two additional days, cells were harvested for metabolic labeling of nascent rRNA, western blot analysis and indirect immunofluorescence.

Metabolic labeling of nascent rRNA

H1299 were incubated in phosphate-free DMEM/10% FBS for at least 30 min and then incubated for 1 h in the presence of 15 μCi/ml [^{32}P]orthophosphate. The metabolic labeling medium was subsequently replaced by regular DMEM/10% FBS and cells were further cultivated for 4–5 h. Total RNA was then isolated using the RNeasy Mini kit (Qiagen). An aliquot of 1.5 μg of metabolically labeled total RNA was separated on a 1% agarose formaldehyde gel. The gel was then dried on a Whatman paper using a regular gel drier (Bio-Rad) connected to a vacuum pump for 2–3 h at 80°C. Metabolically labeled RNA was visualized by autoradiography. Quantification of 28S rRNA signal intensities was performed using a PhosphorImager.

Immunoblotting and immunofluorescence

Cells were directly lysed with 2× SDS loading buffer (100 mM Tris–HCl, 200 mM DTE, 4% SDS, 10 mM EDTA, 0.2% bromophenol blue and 20% glycerol). Whole cell lysates were separated by SDS–PAGE and blotted on to nitrocellulose membranes (Amersham). Immunodetection was performed with monoclonal antibodies directed against the HA-tag (3F10; Roche), WDR12 (1B8), Pes1 (8H11) and anti-tubulin (Sigma–Aldrich). The monoclonal antibodies against human WDR12 and Pes1 were described previously. For indirect immunofluorescence, cells were grown on cover slides, fixed with ice-cold methanol and air dried. Unspecific binding was blocked with PBS/10% FBS. HA-tagged forms of WDR12 were detected with the anti-HA (3F10) antibody. Primary antibodies were incubated overnight at 4°C in a humidified chamber. Cy3-labeled secondary antibodies (Dianova) were incubated for 1 h at room temperature. Nuclei were counterstained with DAPI (Sigma–Aldrich). Digital images were acquired using the Openlab acquisition software (Improvision) and a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging) with a 63 (1.15) plan oil objective connected to a CCD-camera (ORCA-479; Hamamatsu).

RESULTS

Conditional expression of siRNAs embedded in an miRNA environment

It was previously shown that designed siRNA sequences embedded in an miRNA environment are properly processed from ectopic Pol II transcripts and efficiently confer target gene knock-down (12,13). We placed modified miRNA sequences derived from the endogenous murine miR-155 stem–loop downstream of the eGFP ORF that is controlled by a tight doxycycline-dependent bidirectional promoter (Figure 1A) (11). The Epstein–Barr virus (EBV)-derived episomal expression construct pRTS was previously described in detail and contains all features required for conditional gene expression (14). Double-stranded DNA oligonucleotides homologous to the miRNA-embedded siRNAs directed against the rRNA processing factors Pes1 and WDR12 were cloned into a subcloning plasmid (pMIRTOP) following the eGFP ORF. For convenience, we abbreviated the miRNA-embedded siRNAs as miPes1 and miWDR12. They were designed to target the UTRs of the endogenous Pes1 and WDR12 mRNAs to allow future reconstitution experiments without affecting the ectopically expressed genes that only contain the ORF but not the UTRs. Functional sequences were identified by indirect immunofluorescence verifying the knock-down of the endogenous protein in transiently transfected cells (data not shown). Fragments containing the eGFP ORF together with the miRNAs were subsequently transferred into the pRTS construct and transfected into H1299 human lung carcinoma cells by non-viral techniques. Polyclonal cultures were generated within 2 weeks by stable selection with puromycin. As recently reported, GFP expression is reduced by processing of the miRNA structure, but is still easily detected and therefore allows monitoring of conditional miRNA-embedded siRNA expression (Figure 1B). Stable polyclonal H1299 cell cultures exhibit >95% GFP positive cells upon the addition of doxycycline. Similar GFP induction rates (>95%) were also achieved in other cell lines (U2OS, TGR-1). Furthermore, we verified the knock-down of endogenous Pes1 and WDR12 protein by western blot analysis each targeted by two individual miRNA-embedded siRNA sequences (Figure 1C). Depletion of Pes1 or WDR12 resulted in impaired rRNA processing of the 32S pre-rRNA and reduced cell proliferation (data not shown), in line with previous experiments using synthetic siRNA oligos (6,15).

Development of a knock-down–knock-in system by using two individual pRTS constructs

Next, we aimed to rescue the depletion phenotype of Pes1 and WDR12 specifically by co-expression of the respective wild-type proteins. For experimental convenience, we tested whether co-transfection of two individual pRTS constructs is suitable for a knock-down–knock-in approach. One construct encodes a hygromycin resistance gene and expresses HA-tagged Pes1wt, WDR12wt or luciferase as a control (Figure 2A, upper panels, gene of interest). The other contains a puromycin resistance gene and expresses the respective miRNA-embedded siRNA together with DsRed2 and luciferase (Figure 2A, lower panels). The eGFP upstream of the miRNA region was replaced by DsRed2 to better visualize co-expression (Figure 2A). Stable polyclonal H1299 cells were generated by the simultaneous addition of puromycin and hygromycin to the culture medium for 10–14 days. Successful co-selection was verified by monitoring eGFP and DsRed2 double positive cells (Figure 2B). Further, reconstitution of Pes1- or WDR12-depleted cells with the respective HA-tagged wild-type proteins was determined by western

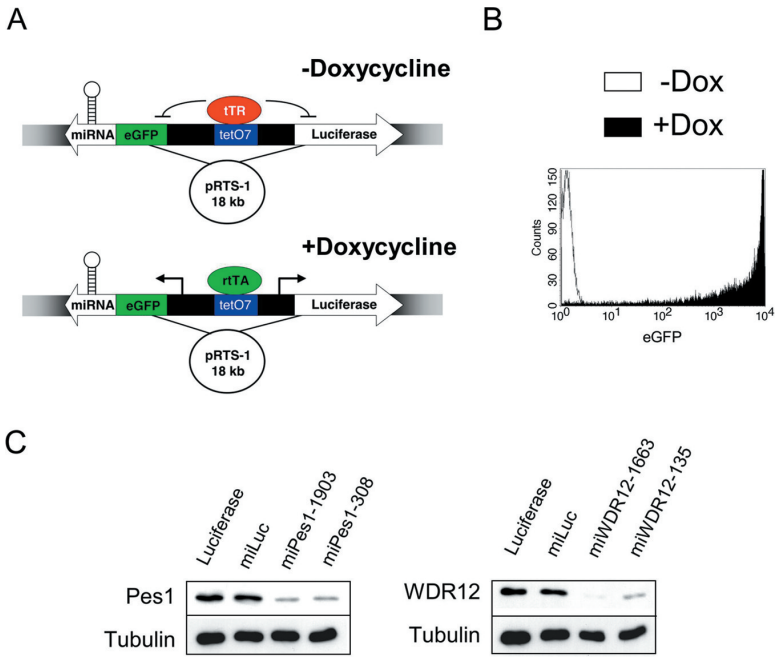

Figure 1. Conditional expression of miRNA-embedded siRNAs. (**A**) Schematic representation of the pRTS construct harboring a target gene-specific siRNA sequence embedded in the modified murine miR-155 structure behind the eGFPORF. Conditional gene activation is achieved in the presence of doxycycline by the tet-activator (rtTA), whereas active repression is mediated by the tet-repressor (rTR) in the absence of doxycycline. (**B**) Detection of eGFP positive cells by flowcytometry upon the addition of doxycycline to the culture medium. (**C**) Western blot analysis of endogenous Pes1 and WDR12 in stably transfected H1299 cells expressing either luciferase or the respective miRNA-embedded siRNAs targeting luciferase (miLuc), Pes1 (miPes1) or WDR12 (miWDR12). The numbers indicate the target site of the 21mer siRNA sequence within the Pes1 and WDR12 reference mRNAs. A tubulin blot is shown as loading control.

blot analysis (Figure 2C). The HA-tagged forms can be discriminated by their increased molecular weight and slower migration due to the C-terminal tag. An miRNA-embedded siRNA targeting luciferase served as knock-down control and likewise a luciferase expression construct as knock-in control. In particular, endogenous WDR12 is strongly depleted and hence replaced by the ectopic form. Then, we investigated whether expression of HA-tagged Pes1wt or WDR12wt rescued 32S pre-rRNA processing, a specific defect observed in Pes1- and WDR12-depleted cells (6,15). A scheme of mammalian rRNA processing pathways is given in Figure 2D. rRNA is transcribed by Pol I as a large 47S transcript that is subsequently cleaved into the mature rRNAs. The 45S and the 32S pre-rRNA are the most abundant precursor rRNAs. To analyze processing of nascent rRNA *in vivo*, we performed metabolic labeling using [^{32}P]orthophosphate. *De novo* synthesis of mature rRNAs can be visualized by autoradiography following gel electrophoresis of metabolically labeled total RNA. In control cells, mature 28S and 18S rRNA are efficiently produced (Figure 2E, lane 1). As expected, cells depleted for Pes1 and WDR12 but co-selected with an additional luciferase expression construct, exhibited a defective maturation of the 28S rRNA, whereas the synthesis of the 18S rRNA is unaffected (Figure 2E, lanes 3 and 5). Co-expression of the HA-tagged wild-type proteins completely restored processing of the 28S rRNA in Pes1- or WDR12-depleted cells (Figure 2E, lanes 2 and 4). In the case of impaired 32S pre-rRNA processing, one would also expect a strong accumulation. However, it is well known that the nuclear exosome potently degrades pre-rRNA that is blocked in its maturation (16,17). In addition, our low-activity [^{32}P]orthophosphate metabolic labeling exhibits a relatively slow uptake and incorporation kinetics. Together, this approach is very suitable to measure the endpoints of rRNA processing pathways, namely production of mature 18S and 28S rRNA, but is not very sensitive to detect accumulation of blocked precursor rRNA. Further, impaired proliferation provoked by depletion of Pes1 and WDR12 was also restored upon co-expression of Pes1 or WDR12 (Figure 2F). Cells were seeded at low density and cultured for 12 days in the presence of hygromycin, puromycin and various doxycycline concentrations. Apparently, strong ectopic gene expression by higher concentrations of doxycycline compromises cell proliferation, even in control cells (Figure 2F, row 1). However, knock-down of Pes1 and WDR12 exhibited a clear proliferation defect that could be fully restored by Pes1wt and WDR12wt co-expression. Thus, our knock-down–knock-in approach is functional, as the depletion phenotypes can be specifically rescued by co-expression of the respective wild-type protein.

Functional analysis of mutants in cells depleted for the endogenous protein

Finally we asked, whether our knock-down–knock-in approach is suitable for the functional analysis of diverse mutants in cells depleted for the endogenous protein. We have previously characterized a panel of WDR12 mutants (Figure 3A; WT, M1, M2, M6–M10). This analysis revealed only one dominant-negative mutant (M1) that blocks rRNA processing (6). We extended this set of mutants by truncation mutants (M2–M5) and various point mutations of putative CKII phosphorylation sites (M13–M17) and of a cysteine

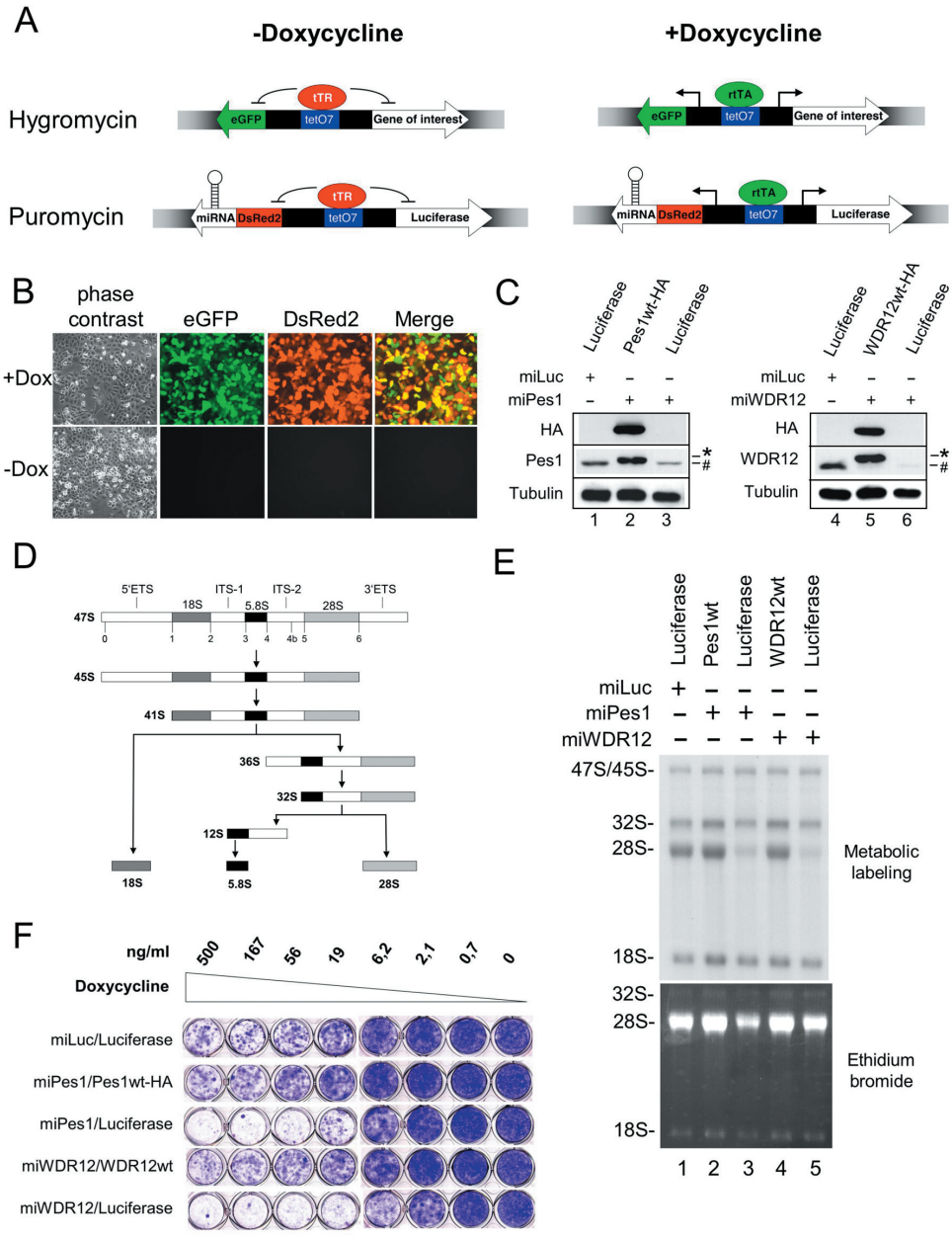

Figure 2. Development of a rapid knock-down–knock-in system for mammalian cells. (**A**) Scheme of the knock-down–knock-in approach by using two pRTS plasmids. (**B**) Verification of successful co-selection with hygromcyin and puromycin by monitoring co-expression of eGFP and DsRed2 in stably selected H1299 cells. (**C**) Western blot analysis of Pes1 and WDR12 in stably transfected H1299 cells after 5 days of doxycycline treatment. Cells co-express one of the indicated miRNA-embedded siRNAs (miLuc, miPes1-1903 or miWDR12-1663) together with either luciferase, Pes1wt-HA or WDR12wt-HA. For convenience, miPes1-1903 and miWDR12-1663 will be designated from now on as miPes1 and miWDR12. Asterisks indicate the ectopic and the hash symbols (#) indicate the endogenous forms that are only discriminated by the increased molecular weight due to the HA-tag. A tubulin blot confirms equal loading. Expression of the ectopic HA-tagged genes was also confirmed by immunodetection of the HA-tag. (**D**) Overview of mammalian rRNA processing pathways starting from the primary 47S transcript. Numbers indicate the cleavage sites within the rRNA. ETS: external transcribed spacer; ITS: internal transcribed spacer. (**E**) Metabolically labeled total rRNA was separated on a 1% agarose formaldehyde gel and visualized by autoradiography. Cell lines as described above (C). The ethidium bromide stained gel is shown as loading control. (**F**) Analysis of cell proliferation. The indicated cell lines were seeded in multiples at low density, fixed after 12 days with ice-cold methanol and stained with Giemsa. Representative wells are shown.

doublet (M12). H1299 cells were stably transfected with the indicated WDR12 expression constructs (WT, M1–M17) together with the WDR12 knock-down plasmid. Expression of the various HA-tagged forms of WDR12 and depletion of endogenous WDR12 were verified by western blot analysis (Supplementary Figure 1A).

To assess the functionality of the mutant forms, we performed metabolic labeling of nascent rRNA (Figure 3B and C). Two control cell lines (Figure 3B and C, lanes 1 and 2) were also included in our analysis to confirm the WDR12 depletion phenotype. HA-tagged WDR12wt successfully sustained the synthesis of the mature 28S rRNA in the

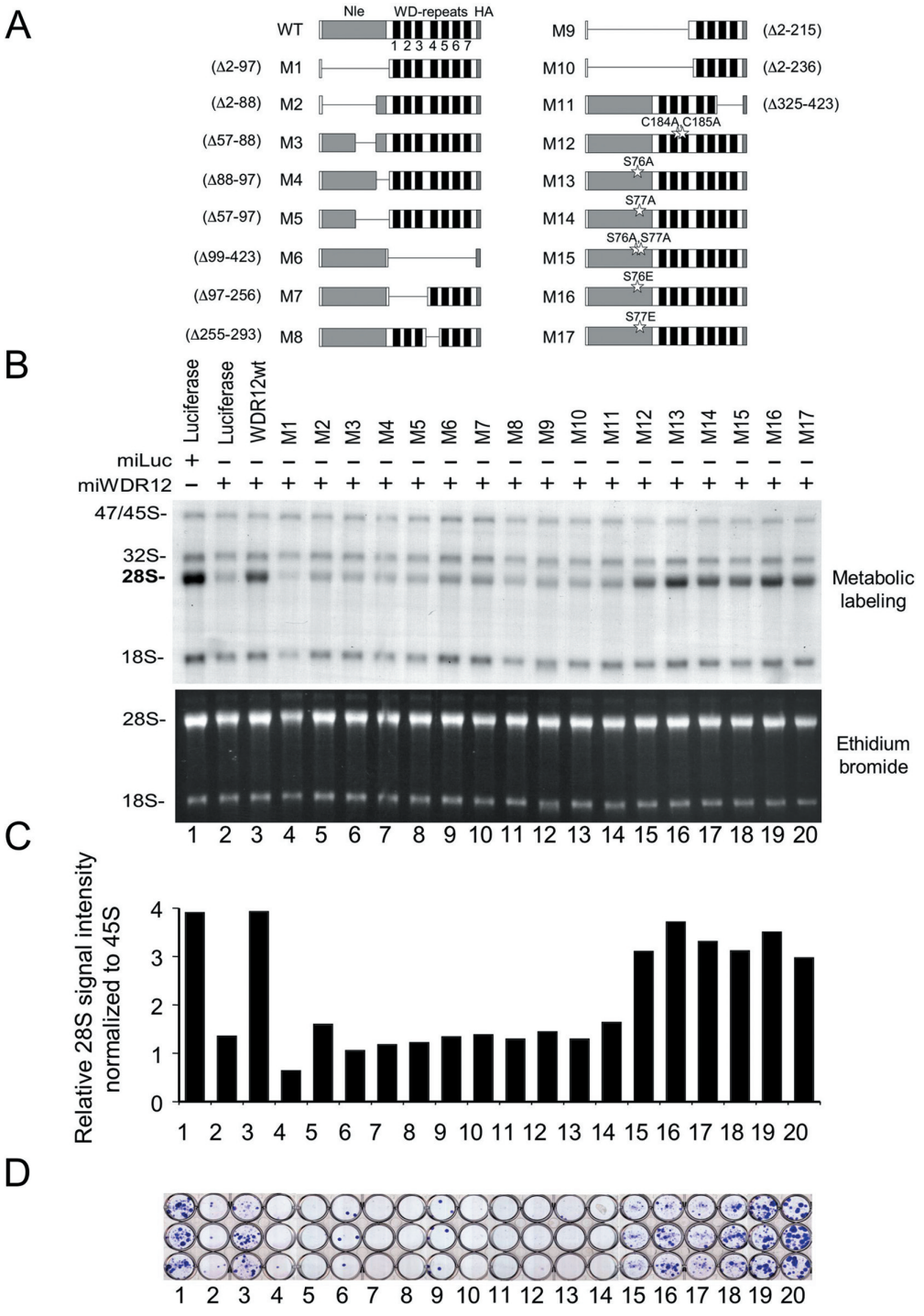

Figure 3. Functional analysis of WDR12 mutants in cells depleted for endogenous WDR12. (**A**) Overview of WDR12 mutants. Deleted amino acids are indicated. Stars depict point mutations at this site. Nle: notchless-like domain; HA: HA-tag. (**B**) Metabolic labeling of nascent rRNA as already described in this study. Stably transfected H1299 cells express either luciferase or the indicated forms of HA-tagged WDR12, in addition to one of the miRNA-embedded siRNAs targeting luciferase (miLuc) or endogenous WDR12 (miWDR12). The ethidium bromide stained gel is shown as loading control. (**C**) Experiment as described in (B). Quantification of the 28S rRNA signal intensity using a PhosphorImager. Histograms reflect arbitrary units of 28S rRNA signal intensity. (**D**) Analysis of cell proliferation. The indicated cell lines were seeded at very low density and cells were fixed and stained with Giemsa after 12 days. Cells were treated with 500 ng/ml doxycycline throughout the course of the experiment in the presence of hygromycin and puromycin.

absence of endogenous WDR12. However, all deletion mutants (M1–M11) failed to rescue rRNA processing (Figure 3B and C, lanes 4–14). Depletion of endogenous WDR12 and co-expression of the dominant-negative mutant M1 synergistically inhibited production of 28S rRNA (Figure 3B and C, lane 4). In contrast, mutants harboring point mutations (M12–M17) were functional in terms of rRNA processing (Figure 3B and C, lanes 15–20). Further,

we seeded the cells at low density to monitor colony outgrowth as a measurement of cell proliferation. Mutants, functional in rRNA processing, also promoted cell proliferation (Figure 3D, lanes 15–20). Interestingly, all functional WDR12 mutants harboring point mutations localized to the nucleolus as the wild-type protein (Supplementary Figure 1B), thus further supporting the previous results. Only the non-functional deletion mutants having deletions within the Notchless-like domain of WDR12 (M1–M5) exhibited nucleolar staining, whereas the remaining mutant forms (M6–M11) were found dispersed in the cytoplasm and nucleoplasm. In conclusion, our analysis demonstrates that the functional importance of specific regions or residues can be readily assessed by our knock-down–knock-in approach.

DISCUSSION

RNAi is a rapid and convenient approach to assess the function of mammalian genes. However, depletion of a protein not only abrogates its specific function but also can compromise the assembly of multimolecular complexes and thus results in a compound phenotype. Therefore, replacing the endogenous protein by mutant forms can preserve associated complexes and thus reveal more specific roles (9). To perform such a mutational analysis, we developed a rapid conditional knock-down–knock-in system for mammalian cells. We investigated the functionality of diverse mutants of WDR12, a factor involved in rRNA processing. Ribosome biogenesis is an essential biological process and therefore served as an appropriate example for the validation of our knock-down–knock-in approach.

Two individual episomal doxycycline-inducible pRTS constructs were used to achieve either the knock-down or the knock-in. In principle, the bidirectional promoter of the pRTS construct would allow a single plasmid strategy; however, in cell lines that are easy to transfect and suitable for double-selection, our approach is fast and convenient, as the combination of a knock-down construct with an established set of mutants does not require further cloning. In addition, knock-down of the endogenous gene was mediated by RNAi targeting the UTR of the mRNA. This strategy avoided laborious target site mutagenesis of the ectopic wild-type and mutant forms, as they only contain ORF sequences and hence lack the UTR. Even though ORF targeting is recommended in general for gene knock-down by RNAi, our UTR directed approach proved to be highly efficient in particular for knock-down–knock-in strategies. We decided to use siRNAs embedded in a modified miRNA environment, as they are processed from Pol II transcripts and could therefore be expressed from the doxycyline-dependent minimal CMV promoter of the pRTS construct. This episomal vector provides all features for doxycycline-dependent gene expression and proved to be suitable for conditional gene expression in several cell lines. Further, stable polyclonal cell lines can be established by non-viral transfection techniques. Depletion of the endogenous protein could also be achieved by synthetic siRNA oligos; however, our plasmid-based approach is more convenient and also cost effective for experiments that require large amounts of cells.

Reconstitution experiments with HA-tagged wild-type proteins demonstrated target gene specificity of the observed knock-down phenotypes induced by the miRNA-embedded siRNAs. We were able to rescue impaired rRNA processing and cell proliferation induced by depletion of endogenous Pes1 and WDR12 with the expression of HA-tagged wild-type forms. Therefore, our knock-down–knock-in approach can experimentally rule out off-target effects. Successful reconstitution experiments further provide a sensitive system to assess the functionality of mutant forms in cells depleted for the endogenous protein. This approach is advantageous over depletion experiments, as mutants can be generated that confer specific defects without affecting other functions or the assembly of associated multimolecular complexes. In respect to the tremendous recent advances in proteomics, our method provides a rapid and convenient tool for the generation of sufficient amounts of mammalian cells that have an endogenous protein replaced by a tagged mutant form. Thus, complexes can be purified and further analyzed by mass spectrometry. Moreover, the impact on a whole organelle, such as the nucleolus, can be investigated. Indeed, the nucleolar proteome was recently characterized in HeLa cells and provided novel insights into its dynamic behavior (18). Interestingly, a large group of unknown proteins was identified that had no respective yeast homologue. Our knock-down–knock-in method allows a rapid and detailed functional analysis of known and novel factors and will substantially support future proteomic approaches, not only in the field of ribosome biogenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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Dominant-negative Pes1 mutants inhibit ribosomal RNA processing and cell proliferation via incorporation into the PeBoW-complex

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ABSTRACT

The nucleolar PeBoW-complex, consisting of Pes1, Bop1 and WDR12, is essential for cell proliferation and processing of ribosomal RNA in mammalian cells. Here we have analysed the physical and functional interactions of Pes1 deletion mutants with the PeBoW-complex. Pes1 mutants M1 and M5, with N- and C-terminal truncations, respectively, displayed a dominant-negative phenotype. Both mutants showed nucleolar localization, blocked processing of the 36S/32S precursors to mature 28S rRNA, inhibited cell proliferation, and induced high p53 levels in proliferating, but not in resting cells. Mutant M1 and M5 proteins associated with large pre-ribosomal complexes and co-immunoprecipitated Bop1 and WDR12 proteins indicating their proper incorporation into the PeBoW-complex. We conclude that the dominant-negative effect of the M1 and M5 mutants is mediated by the impaired function of the PeBoW-complex.

INTRODUCTION

Eukaryotic ribosome biogenesis is a highly regulated, evolutionary conserved process in the nucleolus. A large precursor ribosomal RNA (pre-rRNA) is transcribed by Pol I and rapidly packaged into the 90S ribonucleoprotein particle (90S pre-RNPs) containing ribosomal proteins, non-ribosomal proteins and snoRNA-containing ribonucleoprotein particles (snoRNPs). The 90S pre-RNPs are processed into intermediates, which finally give rise to mature 40S and 60S ribosomal subunits (1).

Ribosome biogenesis is the major metabolic challenge of rapidly proliferating cells, particular in tumour cells, as it consumes up to 80% of the total energy. However, little is known about the molecular mechanism that ensure the equilibrium between cell division and ribosome biogenesis required for balanced cell proliferation (1). Recently it has become evident that ribosome synthesis is cell cycle controlled and sensitive to growth factor and nutrient signalling, and likewise inhibited upon stress signals. Interestingly, several pivotal regulators of cell cycle progression and senescence, such as p19ARF reside within the nucleolus and are also involved in the control of ribosome biogenesis. Moreover, nucleolar proteins like nucleophosmin not only function in the maturation of ribosomes, but are also implicated in the control of the tumour suppressors p53 and p19ARF (2,3). Noteworthy, dysfunction of nucleophosmin is frequently associated with acute myeloid leukaemia and heterozygous mice develop myelodysplastic syndromes (4,5). In conclusion, the recent years have unravelled remarkable links between the nucleolus and cell cycle regulation, thus underlining the importance of the nucleolus far beyond the production of ribosomes.

The nucleolus owns a particular ability in sensitizing cellular stress after ultraviolet (UV) radiation of cells. Using micropore irradiation, Rubbi and Milner (6) demonstrated that large amounts of nuclear DNA damage failed to stabilize p53 unless the nucleolus was affected. In addition, forcing nucleolar disruption by anti-upstream binding factor (UBF) antibody microinjection (in the absence of DNA damage), different chemotherapeutic drugs, or cre-mediated deletion of the Pol I specific transcription factor TIF-IA also caused p53 stabilization and a p53-dependent cell cycle arrest. This suggests that the nucleolus is a stress sensor responsible for the maintenance of low levels of p53, which are automatically elevated as soon as nucleolar function is impaired in response to stress (6,7).

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How does nucleolar stress result in the activation of cell cycle check points? Several mechanisms have been proposed that link ribosome biogenesis to the cell cycle machinery in mammalian cells. A central player in all models is Mdm2, a p53-specific ubiquitin ligase. Disturbance of ribosome biogenesis may decrease the demand for ribosomal proteins and thus lead to an excess of free ribosomal proteins as L5, L11 and L23, which directly bind and inactivate Mdm2 resulting in the accumulation of p53 (8–12). Alternatively, export of ribosomal subunits to the cytoplasm may be a critical step for p53 degradation, which does not take place, if rRNA processing is inhibited (13). However, other models cannot be excluded.

We have recently described the nucleolar complex PeBoW, consisting of Pes1 (Pescadillo), Bop1 (block of proliferation) and WDR12 (WD-repeat protein) in mammalian cells. Knockdown of WDR12 by siRNA technology or expression of a dominant-negative WDR12 mutant blocked processing of the 32S pre-rRNA, evoked stabilization of p53 and induced a strong cell cycle arrest (14). Likewise, expression of dominant-negative mutants of other members of the complex, Pes1 and Bop1, inhibit rRNA processing and cell cycle progression (15,16).

The structure and function of the PeBoW-complex appears to be highly conserved throughout evolution. A homolog complex consisting of Nop7 (Yph1p), Erb1 and Ytm1p has been identified in yeast. As in mammals, mutants of Nop7 and Ytm1 inhibit rRNA processing and cell cycle progression (17). Mutations in Ytm1 disrupt interactions between Ytm1 and Erb1, destabilize the heterotrimer, and significantly reduce association of all three proteins with 66S preribosomes (18).

Even though a function of the PeBoW-complex in processing of the 32S rRNA precursor is established, the structure of the complex and the role of its components Pes1, Bop1 and WDR12 in other cellular processes is largely unresolved e.g. mouse embryos lacking Pes1 arrest at morula stages of development, their nucleoli fail to differentiate and accumulation of ribosomes is inhibited, suggesting an essential role of Pes1 for ribosome biogenesis and nucleologenesis (19). Overexpression of Pes1 can replace the SV40 T antigen in inducing colony formation in soft agar growth but not in inducing cell immortalization (20). Transient depletion of Pes1 resulted in an increase of abnormal mitoses with appearance of binucleate or hyperploid cells, of cells with multipolar spindles and of aberrant metaphase plates (21). Thus, Pes1 appears to be involved in multiple cellular processes, yet it is unclear, whether all these processes require the PeBoW-complex.

Recently, the interaction of Pes1 transposon insertion mutants with Bop1 protein has been studied. Several of the mutants displayed a dominant-negative phenotype for rRNA processing. Interestingly, the dominant-negative phenotype required the interaction of the mutant Pes1 with Bop1, while Pes1 mutants, which did not interact with Bop1, failed to induce a dominant-negative phenotype (15), suggesting that Pes1 mutants might act only in the context of the PeBoW-complex.

Here we report the generation of a set of Pes1 deletion mutants and their interaction with the PeBoW-complex. Two mutants with a N- and C-terminal deletion displayed a dominant-negative effect on rRNA processing and cell growth. Both mutants were incorporated into the PeBoW-complex and induced accumulation of p53. Our data suggests that essential cellular functions of Pes1 are mediated by its incorporation into the PeBoW-complex.

MATERIALS AND METHODS

Cloning/plasmids

Pes1 was cloned from human cDNA using the following primers: HsPescadillo fwd: 5′-GCCACCATGGGAGGCCTTGAGAAGAAG-3′; HsPescadillo bwd: 5′-CTCCGGCCTTGCCTTCTTGGCCTTC-3′. Cloning into the modified pUC18 vector resulted in the addition of a C-terminal HA-tag to the Pes1 open reading frame. The mutant Pes1-HA alleles were created by standard techniques of molecular biology using restriction enzymes and site directed mutagenesis. Pes1-HA wild-type and mutants were cloned into the vector pRTS-1 using the SfiI restriction site (22).

Tissue culture

TGR-1 rat fibroblasts (provided by J. Sedivy, Brown University, Providence, RI) and U2OS osteosarcoma cells were cultured in DMEM with 10% FBS at 8% CO_2. For generation of polyclonal cell lines, 6×10^5 cells were transfected with 7.5 μg pRTS-1 plasmids using Polyfect (QIAGEN) and selected in the presence of 200 μg/ml hygromycin B for 10 to 14 days. Conditional gene expression was induced with 1 μg/ml doxycycline. The percentage of induced cells was monitored by FACS analysis for EGFP expression.

BrdU light assay

BrdU light assays were performed essentially as described previously (23). Briefly, stable polylclonal TGR-1 cells were seeded in the presence of 1 μg/ml doxycycline at a density of 10^5 cells per 100 mm well. After 30 h seeding, the cells were incubated with 100 μM BrdU and doxycycline for 72 h. Culture medium was then removed and replaced by medium containing doxycycline and Hoechst 33 258 at 2 μg/ml for 3 h. Finally, cells were placed on sheet of glass 11 cm above a 30 W fluorescent daylight bulb and irradiated from beneath for 15 min. Cells were washed in phosphate-buffered saline (PBS) two times and regular culture medium without doxycycline was added.

RNA analysis and ^{32}P *in vivo* labelling

Total RNA was isolated from TGR-1 cells using Trifast (PeqLab). A total of 2 μg of RNA were separated on a 1% agarose-formaldehyde gel and blotted on Hybond N^+ membranes (GE Healthcare). The following ^{32}P-labelled oligonucleotides were used to visualize rRNA precursors: ITS-1, 5′-CCGGAGAGATCACGTACCACCCCCGGTGCACACGAGATCACGGAGCCG-3′; ITS-2, 5′-GGAGCGGTCGGCCCCGGTAGAGGGAGCGGGGGAGGAGAGGGACGCGAG-3′; 18S, 5′-CACCCGTGGTCACCATGGTAGGCACGGCGACTACCATCGAAAGTTGATAG-3′.

For metabolic labelling of rRNA, TGR-1 cells were induced with doxycycline for 24 h, followed by preincubation in phosphate-free DMEM (Gibco) with dialyzed

FBS (Gibco) for 30 min. The medium was then replaced by phosphate-free DMEM/10% dialyzed FBS containing 15 μCi/ml ^{32}P-orthophosphate (Amersham). After 60 min the radioactive medium was removed and cells were incubated in regular DMEM/10% FBS for indicated times. A total of 2 μg of RNA were separated on 1% agarose-formaldehyde gels. After electrophoresis, gels were placed on whatman-paper and dried at 80°C under vacuum suction. Dried agarose gels were exposed to regular X-ray films (Kodak) and rRNA was visualized by autoradiography. A PhosphoImager (Fuji) was used for the quantification of signal intensities.

Sucrose gradients

Sucrose solutions were prepared in 50 mM Tris–HCl (pH 8.0), 20 mM NaCl. Sucrose gradients from 50–5% were prepared on top of a 300 μl CsCl (1.4 g/ml) cushion. A total of 1×10^7 cells, without pre-treatment with cycloheximide, were lysed in 1 ml lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40, protease inhibitors] at 4°C for 5 min. A total of 20 μg of glass beads were added each and total cell lysates were sonificated 10 times for 1 s. A total of 100 μl of total cell lysate were loaded on top of the sucrose gradients. Subsequently, sucrose gradients were centrifuged at 30 000 r.p.m. for 2.5 h using a Ti60W rotor. Fractions of 200 μl each were collected. A total of 20 μl of each fraction were used for SDS–PAGE electrophoresis.

siRNA transfection

The day before transfection, 5×10^4 to 10^5 U2OS cells were seeded in 6-well plates. A total of 5 μl of 20 μM control or Pes1-specific siRNA were diluted in 150 μl Optimem (Invitrogen). A total of 150 μl Optimem containing 5 μl Oligofectamine (Invitrogen) was added and incubated for 15 min. Finally, 600 μl Optimem was added and applied to cells after aspiration of the culture medium. Cells were incubated for 6 h. The following sequences (sense) were used: Pes1, CCAGAGGACCUAAGUGUGAdTdT; Control (nonspecific siRNA), UUCUCCGAACGUGUCACGUdTdT.

Immunoblotting and immunofluorescence

Cells were directly lysed with 2× SDS-loading buffer (100 mM Tris–HCl, 200 mM DTE, 4% SDS, 10 mM EDTA, 0.2% bromphenol blue, 20% glycerol). Cell lysates were separated by SDS–PAGE and blotted on nitrocellulose membranes (GE Healthcare). Equal loading of samples was controlled by Ponceau S staining. Immunodetection was performed with anti-HA (3F10; Roche), anti-WDR12 (1B8), anti-Bop1 (6H11), anti-Pes1 (8E9) [Holzel *et al.* (14)], and anti-p53 (Pab-240; Dianova) antibodies.

For immunofluorescence, cells were grown on cover slides, fixed with ice cold methanol or methanol–aceton 1:1 and air dried. Unspecific binding was blocked with PBS/10% FBS. p53 and HA-tagged Pes1 were detected with a 1:100 dilution of anti-p53 antibody (Pab122; Dianova) and a 1:1000 dilution of 3F10 anti-HA antibody, respectively. Primary antibodies were incubated overnight at 4°C in a humidified chamber. Cy3 labelled secondary antibodies (Dianova) were incubated for 1 h at RT. Nuclei were counterstained with DAPI (Sigma–Aldrich). Digital images were acquired using the Openlab acquisition software (Improvision) and a microscope (model Axiovert 200 M; Carl Zeiss MicroImaging, Inc.) with 63 (1.15) and 100 (1.30) plan oil objectives connected to a five charge-coupled device camera (model ORCA- 479; Hamamatsu).

Immunoprecipitation

For immunoprecipitations, U2OS cells were seeded at subconfluent density and treated with 1 μg/ml doxycycline for 20 h. Cells were harvested by trypsination and washing three times with PBS. For lysis, cells were resuspended in lysis buffer [50 mM Tris–HCl (pH 8.0), 1% NP-40, 150 mM NaCl, protease inhibitors, phosphatase inhibitors] and incubated on ice for 20 min. After centrifugation, protein G beads (incubated with antibodies for at least 1 h, washed twice after incubation) were incubated with the lysate over night at 4°C. The beads were washed three times with lysis buffer (lacking phosphatase- and protease inhibitor) and resuspended in SDS-loading buffer/lysis buffer 1:1 with the volume equal to the volume of cell lysate used for the IP.

RESULTS

Cloning and conditional expression of human Pes1 mutants

The Pes1 protein is highly conserved in eukaryotes and several domains and motifs have been predicted based on sequence analysis (Figure 1A). Pes1 contains an evolutionary highly conserved N-terminal pescadillo-like protein domain (NPLP-domain, Pfam-database PF06732) at the N-terminus, a BRCT domain located in the middle of the protein, three classical nuclear localization sequences (NLS) distributed over the protein, six bipartite NLS at the C-terminus and two stretches of acidic amino acids near the C-terminus (24). Pes1 is modified by SUMOylation, but SUMOylation at the consensus site, ψKXE, located at the C-terminus has yet not been experimentally confirmed (25). Human and mouse Pes1 proteins are 89% identical (Figure 1A).

Systematic deletions are an appropriate tool for the investigation of domains involved in subcelluar localization and interactions as well as the generation of dominant-negative mutants. A panel of *pes1* deletion mutants covering the entire open reading frame and a point mutation of the lysine in the consensus SUMOylation site were constructed and tagged with the hemagglutinin (HA) epitope at the C-terminus (Figure 1A). For the conditional expression in mammalian cells, wild-type (wt) and mutant *pes1* alleles were cloned into the pRTS-1 vector (Figure 1B) (14,22).

The expression of the gene of interest is tightly controlled by a doxycycline-regulable trans-activator and trans-repressor. Thus, this tight control prevents any selection against vectors carrying dominant-negative *pes1* alleles during normal tissue culture due to leakiness of the promoter. Moreover, the pRTS-1 vector allows for high expression levels of the gene of interest. In stably transfected polyclonal cell cultures, conditional gene activation is achieved in >95% of cells (Figure 1C). As the bidirectional promoter of the pRTS-1 vector accomplishes simultaneous expression of EGFP in addition to the gene of interest, conditional gene

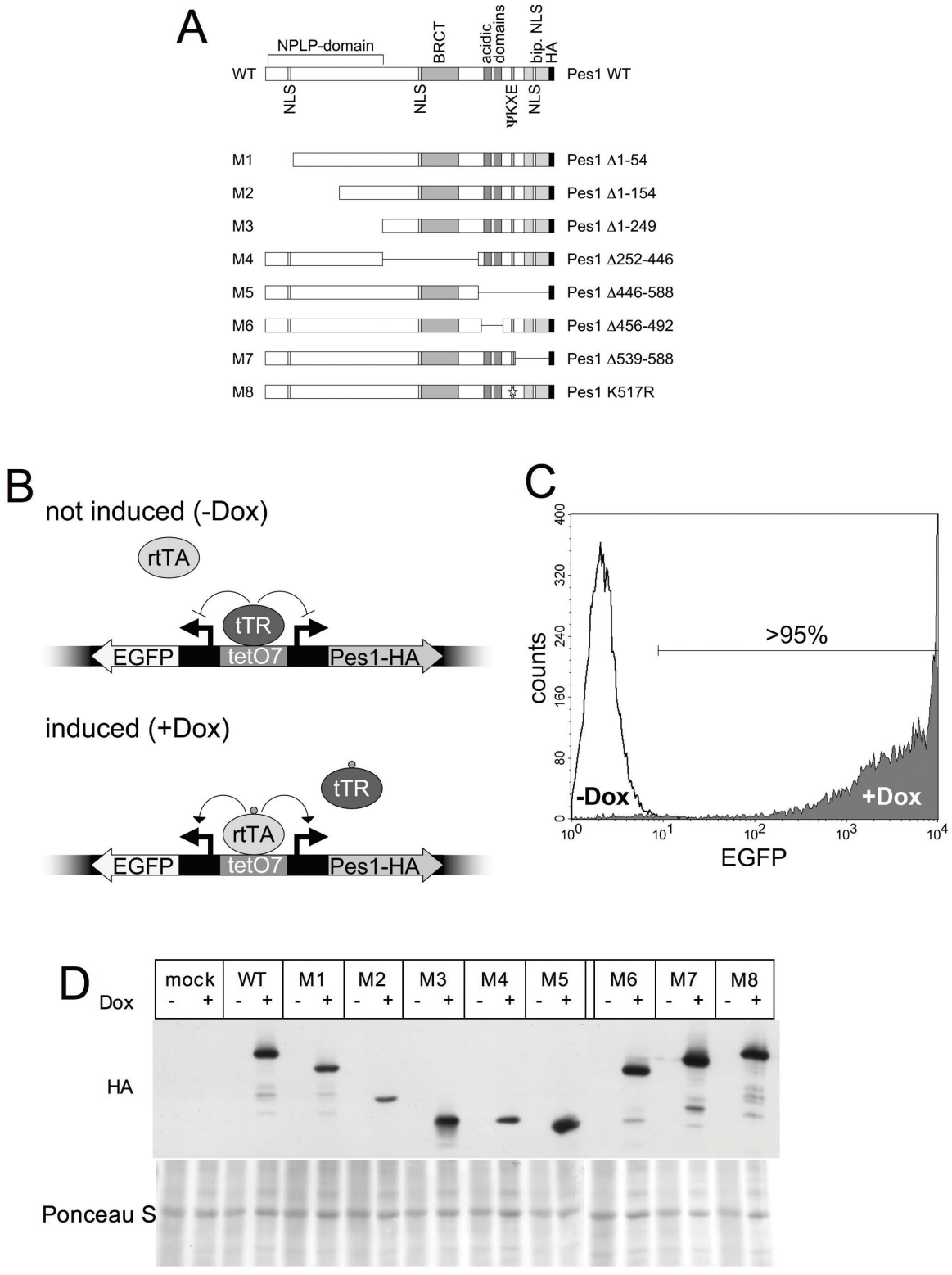

Figure 1. Pes1 mutagenesis and conditional Pes1 expression. (**A**) A panel of Pes1 deletion mutants (M1–M8) were constructed. Recombinant Pes1 wt and mutant proteins carry a C-terminal HA-tag. NLS: classical nuclear localization sequence; the N-terminal NLS consists of three overlapping NLS; bip. NLS: six overlapping bipartite NLS; BRCT: BRCT domain; acidic domains: two glutamic acid rich regions; ΨKXE: consensus SUMOylation site; HA: hemagglutinin-tag. Mutant M8 has replaced Lys517 by Arg. (**B**) Pes1 and its mutants were expressed in rat fibroblasts (TGR-1) stably transfected with the inducible EBV-based vector pRTS-1. pRTS features a bidirectional promoter expressing simultaneously the gene of interest and EGFP. After induction by doxycycline, the vector switches from active silencing to transactivation. (**C**) EGFP expression was induced in >95% of cells 24 h after addition of doxycycline, as determined by FACS analysis. (**D**) Western blot analysis of wt or mutant Pes1 proteins in TGR-1 fibroblasts by anti-HA antibodies (3F10). The mock cell line expresses luciferase instead of Pes1-HA. The indicated cell lines were treated with 1 μg/ml doxycycline for 30 h (+) or left untreated (−). Bottom panel shows Ponceau S staining as a loading control.

activation can be easily monitored by FACS analysis or fluorescence microscopy (Figure 1B and C). Pes wt and mutants M1–M8 were conditionally expressed in the rat fibroblast cell line TGR-1. Expression levels were determined 30 h after addition of doxycycline by western blot analysis. Most of the mutant forms were detected at the expected size, however deletion of the acidic regions in M5 and M6 significantly enhanced migration (Figure 1D).

Subcellular localization of Pes1 mutants

Pes1 protein has been reported to localize predominantly to the nucleolus. To determine the subcellular localization of the Pes1 mutants, we performed indirect immunofluorescence using HA-tag specific antibodies. As expected, the recombinant Pes1 wt protein showed predominantly nucleolar localization and also some nucleoplasmic staining in cells with high expression of Pes1 (Figure 2, wt). The deletion of 54 N-terminal amino acids in mutant M1 did not affect the nucleolar localization whereas the extension of this deletion to amino acid 154 (M2) or 249 (M3) results in a diffuse nuclear staining. Deletion of the central part of Pes1 containing the BRCT domain and adjacent uncharacterized regions in mutant M4 leads to a diffuse nucleoplasmic staining but with nucleolar staining in very few cells (Figure 2, data not shown). The C-terminal deletion mutants M5 to M7, together with mutant M8 harbouring a mutated consensus SUMOylation site, located to the nucleolus as Pes1. Therefore, our mutational analysis suggests that the region between 55 and 154 amino acid plays an important role for the nucleolar localization of the Pes1 protein. A contribution of the region extending from amino acid 155 to 249 remains elusive for

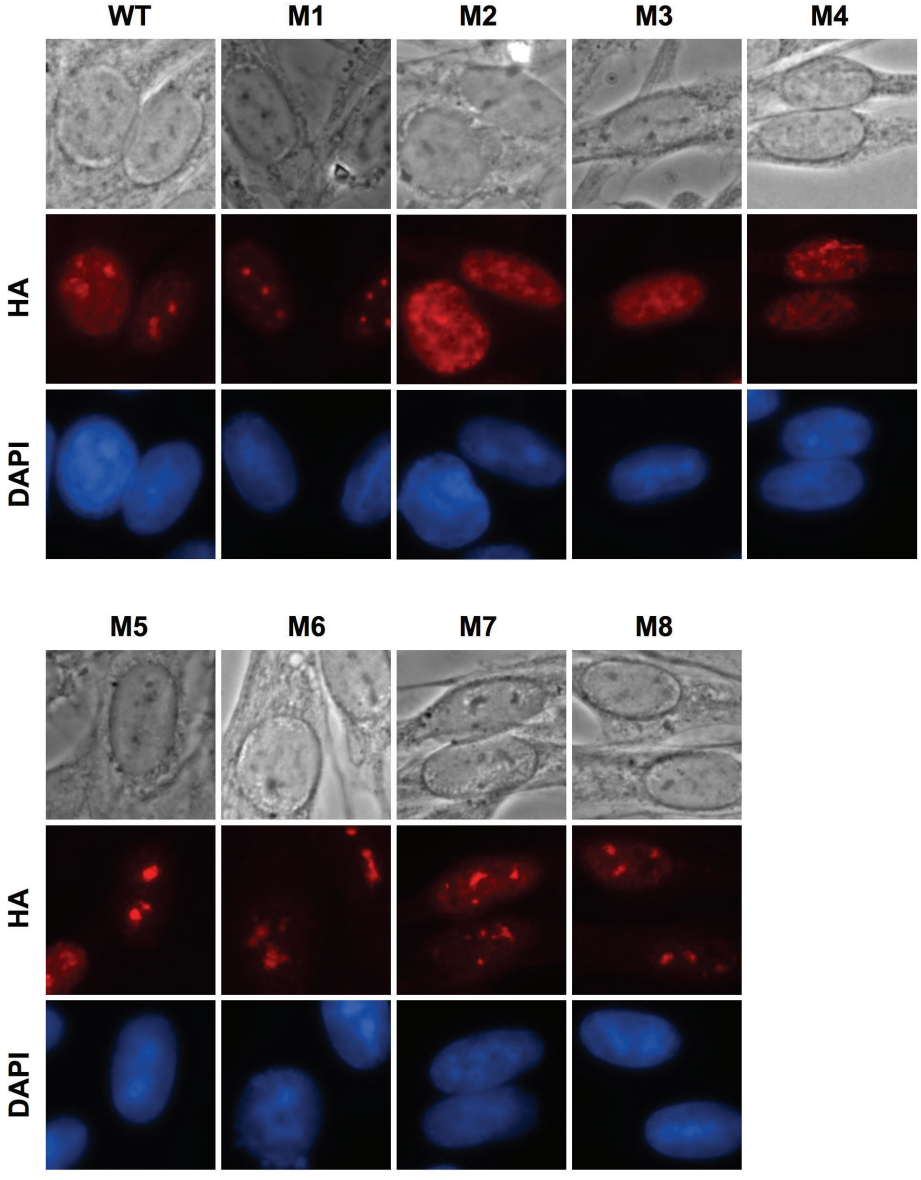

Figure 2. Cellular localization of wt and mutant Pes1 proteins by indirect immunofluorescence. TGR-1 cells transfected with the indicated constructs were fixed with methanol/acetone after induction of the Pes1-HA alleles with doxycycline for 30 h. The HA-tagged proteins were stained by anti-HA antibodies (3F10), the nuclei were counterstained with DAPI.

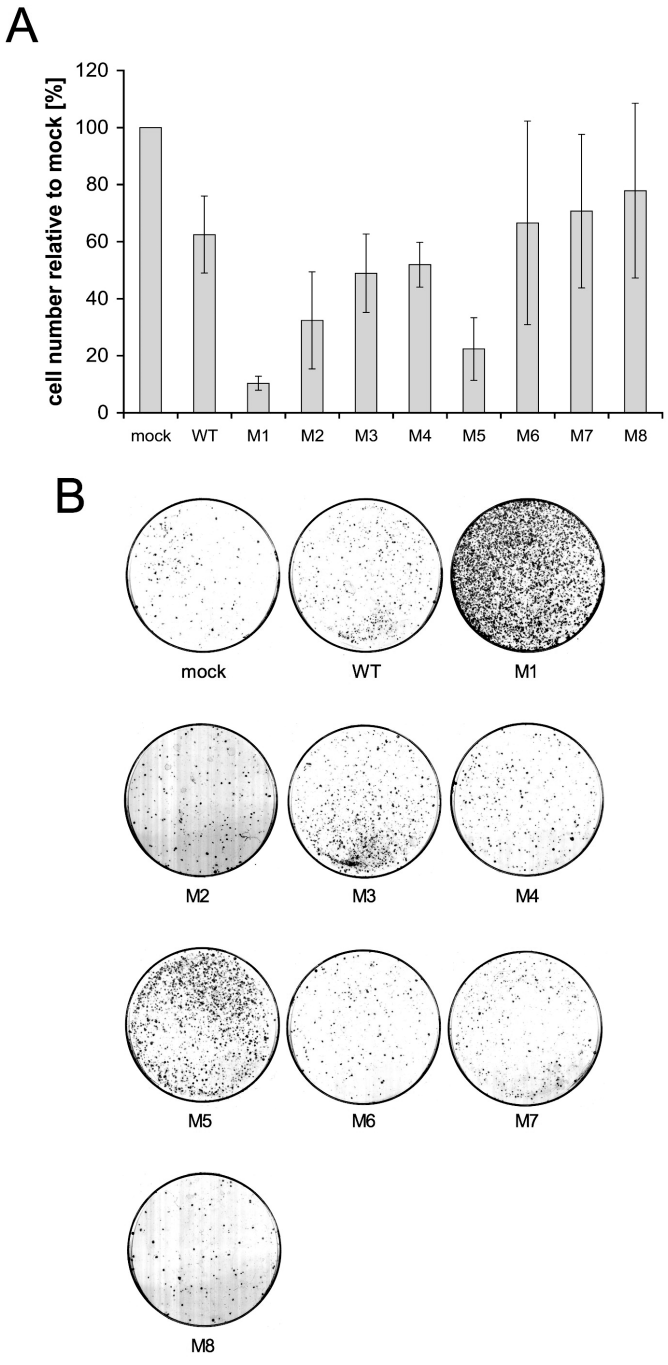

Figure 3. Pes1 mutants M1 and M5 inhibit cell proliferation and elicit a reversible cell cycle arrest. (**A**) Equal numbers of TGR-1 cells stably transfected with the indicated constructs were seeded in multiples in the presence of 1 μg/ml doxycycline. Cells were trypsinized and counted by trypan blue exclusion after 6d. The histogram depicts the cell counts relative to the mock cell line after 6d. Error bars indicate SD. (**B**) Reversible cell cycle arrest by overexpression of mutants M1 and M5. Stably transfected TGR-1 cell lines were seeded at low density and treated with 1 μg/ml doxycycline for 30 h to induce expression of the specified constructs. Subsequently, the cells were incubated with 100 μM BrdU for 72 h to label proliferating cells. Visible light irradiation in the presence of Hoechst 33 258 selectively kills cells that have incorporated BrdU in their DNA. Arrested cells survive BrdU light treatment and give rise to colonies after withdrawal of doxycycline. Images show representative BrdU light assays conducted with the indicated cell lines.

nucleolar localization in this study. Noteworthy, none of the mutants showed cytoplasmic staining suggesting that neither of the deletions affected the nuclear transport of Pes1. Since all mutants retained at least two putative NLS, the functionality of a single NLS could not be addressed by this set of mutants.

N-terminal (M1) and C-terminal (M5) truncation mutants potently inhibit proliferation and trigger a reversible cell cycle arrest

Next, we tested the effect of Pes1 deletion mutants on cell proliferation. Equal numbers of cells were seeded and expression of wt Pes1, mutants M1 to M8 and luciferase (mock) was induced by addition of doxycycline. Cell numbers were determined after 6d (Figure 3A). Overexpression of Pes1 wt reduced the cell count to 62% compared to mock cells. A likewise decrease was observed for the mutants M6, M7 and M8, and to a higher extent for the mutants M2, M3 and M4. Expression of the mutants M1 and M5 resulted in the strongest reduction, 10 and 22% of mock cell count, respectively. FACS analysis revealed a significant increase of cells in the G_0/G_1 phase of the cell cycle 48 h after expression of mutants M1 and M5 (Table 1, Supplementary Figure S1). Hence, the mutants M1 and M5 suppressed cell proliferation in rat fibroblasts most efficiently.

Further, we studied the ability of mutants M1 and M5 to mediate a reversible cell cycle arrest in a BrdU/light assay. In brief, dividing cells that incorporate BrdU into their DNA become highly photosensitive if they are additionally labelled with the Hoechst dye 33 258 and undergo apoptosis upon irradiation with visible light. Cell cycle arrested cells are protected from increased photosensitivity and survive BrdU/light treatment. Thus, cells that were arrested by conditional expression of an anti-proliferative Pes1 mutant, would subsequently give rise to colonies after withdrawal of doxycycline. Mock cells expressing luciferase showed only little colony outgrowth and thus demonstrating the low background of this assay. Expression of Pes1 wt did not increase the number of colonies in comparison to the mock situation. The Pes1 mutant M1 provoked a pronounced rescue effect and subsequent colony outgrowth, consistent with a strong reversible cell cycle inhibition. The effect of mutant M5 was less intense but still significant. Mutant M3 showed a colony number slightly over the background level, while all other Pes1 mutants remained at the background level. The reduced proliferation rates of Pes1 wt (62% in the proliferation assay) and mutants M2–M4 and M6–M8 is not accompanied by an increased apoptosis rate (data not shown) or altered cell cycle distribution (Table 1), thus the reason for the reduced cell count remains currently unclear. Taken together, the mutants M1 and M5 mediated a potent inhibitory effect on cell proliferation and triggered a reversible G_0/G_1-specific cell cycle arrest (Table 1).

The Pes1 mutants M1 and M5 inhibit pre-rRNA processing

Pes1 is involved in ribosome biogenesis and therefore we tested the ability of mutants M1 and M5 to inhibit maturation of ribosomal RNA. A short overview of the major mammalian rRNA processing pathway is depicted in Figure 4A.

Table 1. Cell cycle distribution of Pes1 mutants

Cell line	G_0/G_1 %	S %	G_2 %
Mock	46.6	23.1	30.4
WT	47.7	24.6	27.7
M1	62.3	17.8	19.9
M2	43.7	26.5	29.9
M3	53.5	18.2	28.3
M4	47.6	21.7	30.7
M5	59.1	18.9	21.9
M6	48.6	24.8	26.6
M7	49.5	22.6	27.9
M8	46.7	24.8	28.5

Stably transfected TGR-1 cells were induced at subconfluent density for 48 h with doxycycline and subjected to FACS analysis. The percentage of cells in different cell cycle phases are indicated.

Pes1 wt and mutant forms were expressed in TGR-1 cells for 30 h. Total RNA was isolated, and analysed by northern analysis with hybridization probes specific for the internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) of the ribosomal pre-RNA (Figure 4A). Expression of M1 and M5-induced a significant accumulation of the 36S precursor as visualized by hybridization with the ITS-1 specific probes (Supplementary Figure S2). Inhibition of pre-rRNA processing becomes even more pronounced, if rRNAs were hybridized with the ITS-2 specific probe. Mutants M1 and M5 lead to a strong increase of the amount of 32S rRNA precursor (Figure 4B, upper arrows). While both mutants, M1 and M5, induced accumulation of the 36S/32S rRNA, their effect on processing of the 12S rRNA intermediate differed. The level of 12S pre-rRNA was reduced for the mutant M1, but appeared unaffected for the mutant M5 (Figure 4B, lower arrows). This may indicate separable and common functions of the mutants M1 and M5 in rRNA processing.

In addition we studied the impact of mutants M1 and M5 on rRNA processing in TGR-1 cells by metabolic ^{32}P-labelling (Figure 4C). The production of mature 28S rRNA was severely reduced resulting from an inefficient processing of the 32S rRNA precursor, as concluded from the relative high abundance of metabolically labelled 32S rRNA (Figure 4D). In contrast, synthesis of mature 18S rRNA was almost unaffected. These results are in line with our northern blot analysis. In conclusion, the deletion of the N-terminus or the C-terminus of Pes1 either compromises ribosome biogenesis by blocking rRNA processing at the level of the 32S rRNA precursor.

Endogenous Pes1 is required for rRNA processing and cell proliferation

To confirm the role of Pes1 in ribosome biogenesis and cell proliferation, we performed small interfering RNA (siRNA) knockdown experiments of endogenous Pes1. We used human osteosarcoma U2OS cells, because we had generated a monoclonal antibody directed against human Pes1 (14). Cells were transfected at day 0, 1 and 2 with either control or Pes1-specific siRNA. Expression of endogenous Pes1 was dramatically reduced, as monitored by western blot analysis 2d after the last transfection (Figure 5A). Moreover, proliferation of Pes1-depleted cells was severely impaired (data not shown). Further, we investigated the impact of Pes1 knockdown on ribosome biogenesis after *in vivo* labelling of rRNA. The production of the mature 28S rRNA was strongly compromised (Figure 5B). Notably, synthesis of the large 45/47S precursor was not affected, indicating that Pes1 is not involved in rRNA transcription. In conclusion, expression of dominant-negative Pes1 and depletion of endogenous Pes1 block rRNA processing of the 32S rRNA precursor.

Mutants M1 and M5 induce p53 accumulation in proliferating, but not in starving cells

As mentioned before, p53 is believed to induce cell cycle arrest following nucleolar stress. This raised the question if the observed impact of mutants M1 and M5 on pre-rRNA processing triggers the accumulation of p53. To address this question, we analysed p53 levels in TGR-1 cells 30 h after expression of wt and mutant Pes1 proteins by western blotting (Figure 6A). Pes1 wt and mock cells did not accumulate p53 (Figure 6A, lanes 1–4). Expression of mutant M1 provoked a strong increase of p53 protein (lanes 5 and 6). A less pronounced accumulation was seen for the mutants M3 and M5 (lanes 9, 10 and 13, 14), whereas all other mutants caused no change in p53 levels. If disturbance in ribosome biogenesis is the reason for p53 accumulation, then the accumulation of p53 should be diminished in cells in the absence of active ribosome biogenesis, such as serum-starved cells. To test this assumption, subconfluent TGR-1 cells were cultured with 0.1% FBS for 72 h prior to the addition of doxycycline. The cells were lysed 30 h later. Serum starvation did not affect the induction rate of recombinant proteins (Figure 6B, lower panel, see also Figure 1D), however, neither of the Pes1 mutants was able to trigger the accumulation of p53 (Figure 6B, upper panel).

To further investigate the p53 accumulation induced by the mutant Pes1 proteins on the single cell level, we analysed p53 accumulation by immunofluorescence (Figure 6C). In proliferating cells expressing Pes1 wt, 8.9% of nuclei stained positive for p53. Expression of Pes1 mutants M1 and M5 increased the number of positive cells to 82.0 and 53.4%, respectively (Figure 6D). Serum starvation reduced the number of p53-positive cells for Pes1 wt to 0.71%, and for the mutants M1 and M5 to 8.7 and 3.3%, respectively. Thus neither of the mutants were able to induce p53 significantly in serum-starved cells supporting the notion, that ribosome biogenesis is a prerequisite for the dominant-negative action of Pes1 mutants M1 and M5.

As previously demonstrated, N-terminally deleted WDR12 elicited a p53-dependent cell cycle arrest that was attenuated by the coexpression of the human papillomavirus protein E6 (14), a ubiquitin ligase targeting p53 for degradation. In line with these studies, mutants M1 and M5-induced cell cycle arrest was alleviated in TGR-1 cells stably transfected with HA-tagged E6 (Supplementary Figure S3).

Mutants M1 and M5 associate with the large pre-ribosomal subunit

The association of Pes1 mutants with pre-ribosomal complexes was studied by sucrose gradient fractionation using TGR-1 total cell lysates (Figure 7). All recombinant proteins accumulated in low molecular fractions, most likely due to an access of overexpressed free protein in total cell lysates. However, Pes1,

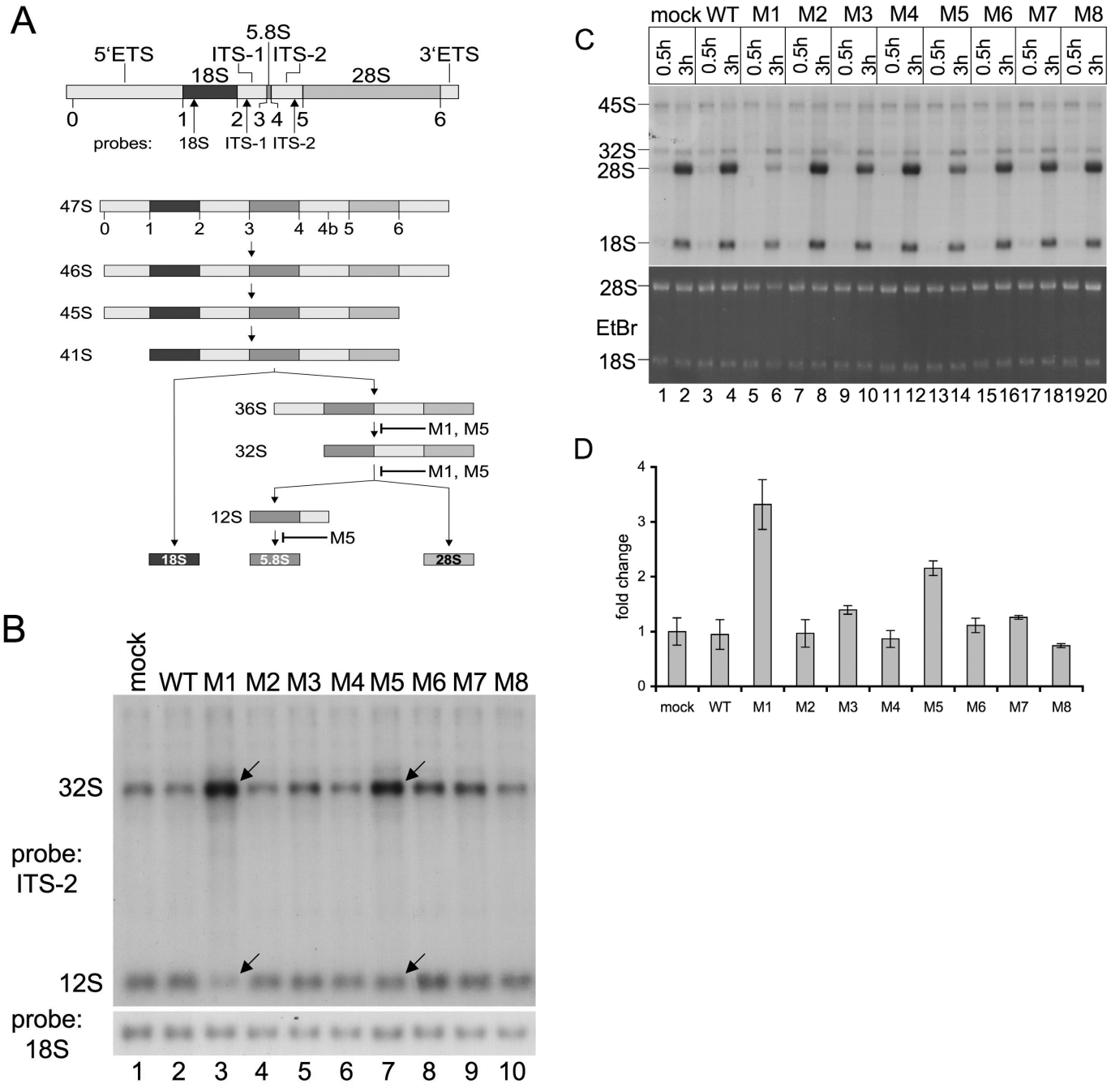

Figure 4. Dominant-negative Pes1 mutants inhibit pre-rRNA processing. (**A**) Schematic of eukaryotic pre-rRNA processing. The top diagram shows the primary 47S transcript at the correct scale and the position of the probes used in the northern blots. The lower diagram shows the processing of the pre-rRNA schematically. The effects of M1 and M5 overexpression are indicated. (**B**) Northern blot for pre-rRNA probed with ITS-2. Total RNA was extracted from subconfluent TGR-1 cells transfected with the indicated constructs after 30 h of induction. Hybridization with a probe against the 18S rRNA confirms equal loading of the blot. (**C**) Expression of dominant-negative Pes1 mutants impairs formation of mature 28S rRNA. Asynchronously growing TGR-1 cells were treated with doxycycline for 24 h. Cells were pulse labelled with ^{32}P-orthophosphate for 1 h and chased in regular medium for 0.5 and 3 h. (**D**) Ratio of 32S/28S rRNA at 3 h after metabolic labelling with ^{32}P-orthophosphate as described in (C).

and mutants M1 and M5, but not the mutant M3, exhibited specific enrichment in high molecular weight fractions that co-fractionated with ribosomal particles. Given the fact that M1 and M5 impair rRNA processing at the level of the 32S pre-rRNA and co-sediment in fractions similar to the 60S peak (see 28S RNA), our data suggests that mutants M1 and M5 accumulate with the 66S pre-ribosomal large subunit.

Incorporation of mutant Pes1 proteins into the PeBoW-complex

Previously we showed that Pes1 forms a stable complex with two other nucleolar proteins, Bop1 and WDR12, respectively (PeBoW-complex). Interestingly, several of the deletion mutants generated in this study localized to the nucleolus

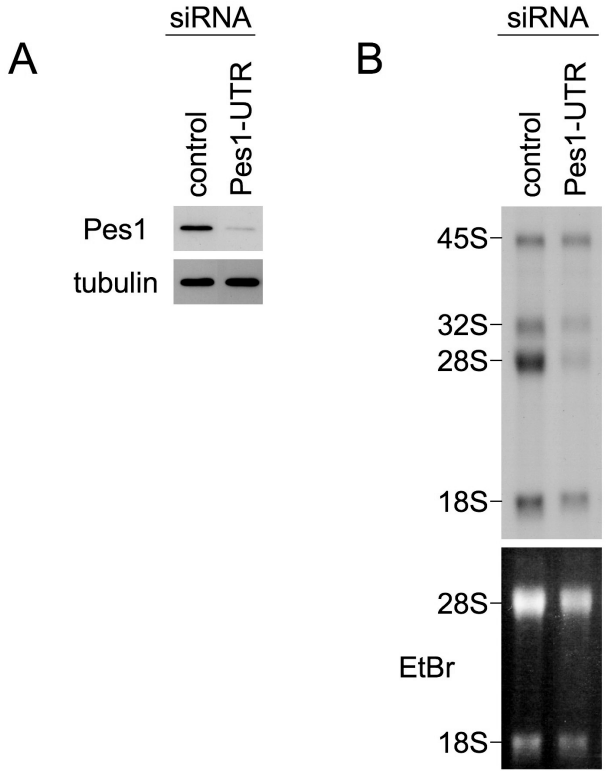

Figure 5. Endogenous Pes1 is required for rRNA processing. (**A**) U2OS cells were transfected at day 0, 1 and 2 with either control or Pes1-specific siRNA. Endogenous Pes1 levels were analysed by western blot analysis 2d after the last transfection. Tubulin is shown as a loading control. (**B**) U2OS cells were transfected only twice at day 0 and 1 and metabolically labelled with ^{32}P-orthophosphate for 60 min at day 3. Subsequently, cells were incubated for 3 h in regular culture medium. Labelled rRNAs are indicated.

with or without a dominant-negative phenotype. Thus we asked, if these mutants are nucleolar upon incorporation into the PeBoW-complex or if nucleolar localization is a feature of Pes1 independent of PeBoW-association. Further, it was unclear if the Pes1 mutants interact with Bop1 or WDR12 in conjunction or independently of each other. Moreover, analysing the incorporation of the dominant-negative mutant proteins M1 and M5 into the PeBoW-complex would help to unravel the mechanism of their inhibitory function. To address these issues, we performed a series of vice-versa immunoprecipitation experiments. As our antibodies against the PeBoW components WDR12 and Bop1 are human specific, we performed the experiments in the human osteosacroma cell line U2OS. This cell line also allowed proper conditional expression of Pes1 mutants by the pRTS-1 vector.

U2OS cells were plated at a subconfluent density and the Pes1 constructs were induced for 20 h with doxycycline. The immunoprecipitation experiments were carried out with antibodies against the HA-tag, WDR12 and Bop1, as well as an isotype control. Western blot analysis was then performed with a HA-tag specific monoclonal antibody (3F10) to detect the recombinant Pes1 protein and monoclonal antibodies against WDR12 and Bop1 to detect the association of the recombinant Pes1 protein with endogenous PeBoW members. In the mock cell line, the lack of the HA-tag, WDR12 and Bop1 specific signals in the IP against the HA-tag revealed no unspecific reactivity of the antibodies (Figure 8A). The endogenous PeBoW-complex was precipitated in IPs against WDR12 and Bop1, as expected (Figure 8A, lanes 3 and 4). HA-tagged Pes1 wt protein co-immunoprecipitated high amounts of WDR12 and Bop1 indicating a proper incorporation into the PeBoW-complex (Figure 8B, lane 2). The IPs against WDR12 and Bop1 could co-precipitate only a small fraction of HA-tagged Pes1 protein (Figure 8B, lanes 3 and 4). This might be due to the fact that 20 h after addition of doxycycline not all PeBoW-complexes have incorporated the HA-tagged Pes1 protein. Hence, the co-immunopreciptation of WDR12 and Bop1 with the anti-HA antibody is the most reliable readout for the incorporation of HA-tagged Pes1 proteins in the BeBoW-complex.

The dominant-negative mutant M1 co-precipitated WDR12 and Bop1 proteins (Figure 8C, lane 2). Mutant M1 was also detectable in IPs against WDR12 and Bop1. This demonstrates that the Pes1 mutant M1 is efficiently incorporated into the PeBoW-complex (Figure 8C, lane 3 and 4). The mutant proteins M2 and M3 are not incorporated into PeBoW. Both proteins did not co-precipitate WDR12 and Bop1 (Figure 8D and E, lane 2) and could not be precipitated in IPs against WDR12 and Bop1 (Figure 8D and E, lane 3 and 4). This finding is compatible with a previous report, that the region in Pes1 needed for nucleolar localization is also needed for binding of Bop1 (15). Pes1 mutant M4 is also not incorporated into the PeBoW-complex according to the failure to co-precipitate significant amounts of WDR12 and Bop1 (Figure 8F, lane 2). The dominant-negative mutant M5, similar to Pes1 wt and mutant M1 protein, co-precipitates WDR12 and Bop1 (Figure 8G, lane 2) and is co-precipitated by WDR12 and Bop1 (Figure 8G, lane 3 and 4). Mutants M6, M7 and M8 are all incorporated into the PeBoW-complex, as they co-precipitate WDR12 and Bop1 (Figure 8H–J, lane 2) and in reciprocal IPs (Figure 8H–J, lanes 3 and 4). Taken together, our immunoprecipitation studies revealed that all mutants are efficiently incorporated in the PeBoW-complex except the mutants M2, M3 and M4. Notably, the mutants M2, M3 and M4 are the only mutants showing no proper nucleolar localization, suggesting that the nucleolar localization of Pes1 is linked to the PeBoW-association. Interestingly, we observed no independent interaction of Pes1 mutants with either Bop1 or WDR12. Further, our results propose that dominant-negative forms of Pes1 require incorporation into the PeBoW-complex for their negative effect on ribosomal rRNA processing and cell cycle progression.

DISCUSSION

In mammals, Pes1, Bop1 and WDR12 proteins are components of the evolutionary highly conserved PeBoW-complex, which is required for maturation of the 60S ribosomal subunit. Although PeBoW is highly abundant in the nucleolus, the way and the place of its assembly is unclear. A homologous complex in yeast, composed of Nop7, Erb1 and Ytm1, associates with four consecutive 66S pre-ribosomal subunits containing the 27SA2, 27SA3, 27SB and the 25.5S plus 7S pre-rRNAs (18,26). Similar as in mammals, the yeast complex is required for proper processing of the 27S pre-rRNA to the mature

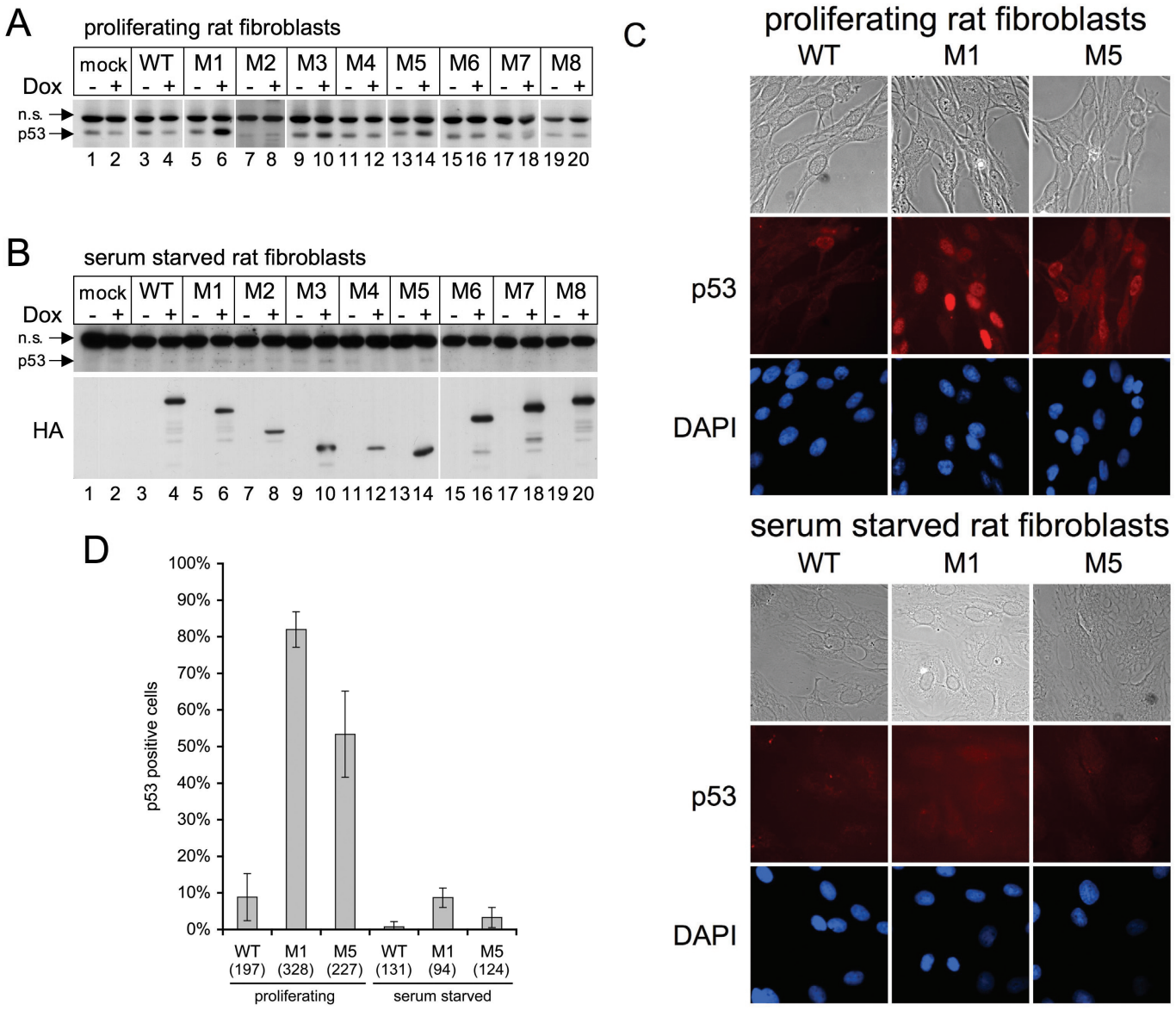

Figure 6. Dominant-negative Pes1 mutants induce p53 accumulation in proliferating cells but not in quiescent cells. (**A**) Western blot for endogenous p53 levels. Stably transfected TGR-1 cells were induced at subconfluent density for 30 h with doxycycline. Cell lysates were prepared and analysed by immunoblotting for p53 accumulation using anti-p53 antibodies (Pab-240). n.s.: non specific. (**B**) Upper panel: western blot as described in (A), but the cells had been serum-starved for 72 h before induction. Lower panel: immunoblot against the HA-tag to confirm the expression of recombinant wt and mutant Pes1. (**C**) Analysis of the endogenous p53 response to expression of Pes1 wt, and mutants M1 and M5 by indirect immunofluorescence. Asynchronously proliferating and serum-starved (72 h) cells were treated with doxycycline for 24 h to induce the Pes1 constructs. Cells were fixed in methanol and stained against p53 with anti-p53 (Pab122). Cell nuclei were counterstained with DAPI. Representative images are shown. (**D**) Percentage of p53-positive nuclei in TGR-1 cells upon induction of Pes1 wt, and the dominant-negative mutants M1 and M5. Proliferating and serum-starved cells were analysed by immunofluorescence as described in (C). Numbers of examined cells are indicated in brackets. Error bars indicate SD of the percentage of p53-positive nuclei cells.

25S rRNA. Recent evidence suggests that the assembly of the trimeric yeast complex occurs in the nucleolus, with assembly of Nop7 and Erb1 into the pre-ribosomes prior to Ytm1 (18). Whether Nop7 and Erb1 assemble before binding to 66S pre-ribosomes is not known. A stable complex of Nop7 and Erb1 has not been demonstrated yet, neither in yeast, nor for the homologues Pes1 and Bop1 in mammalian cells. This is intriguing since the interaction of Pes1 and Bop1 can easily be demonstrated in GST-pull-down and yeast two hybrid experiments (15). This suggests that the interaction of endogenous Pes1 and Bop1 may be inhibited during their passage to the nucleolus and occurs only after association with the pre-ribosome and/or the assembly of WDR12 in the complex.

Nucleolar transport and assembly of Pes1 into the PeBoW-complex

In this study we have constructed a set of deletion mutants to characterize the domains of Pes1 required for the nucleolar localization and assembly into the PeBoW-complex. The Pes1 protein has an evolutionary highly conserved N-terminal domain of pescadillo-like proteins of 250 amino acids

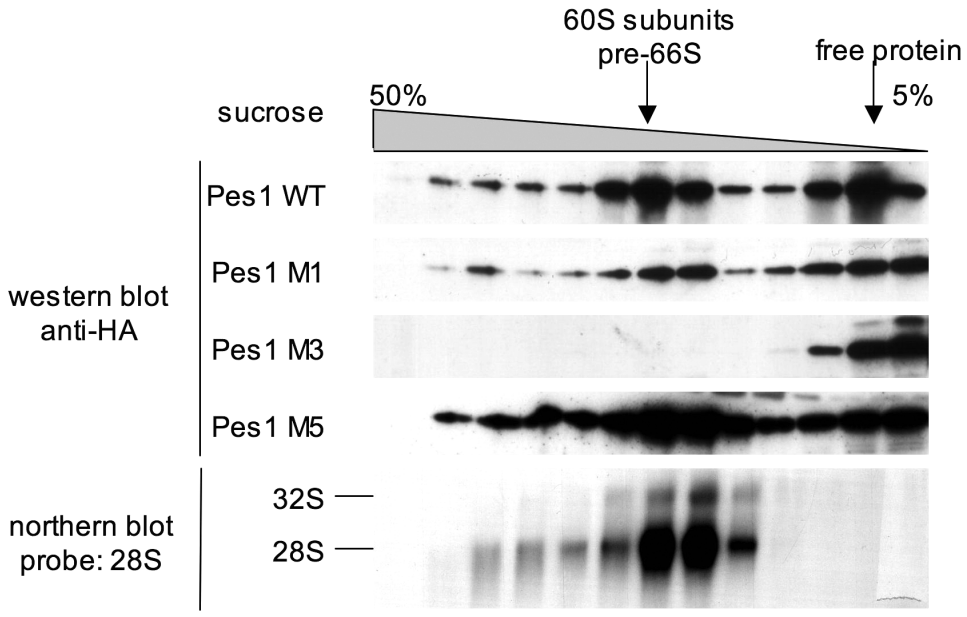

Figure 7. Dominant-negative mutants M1 and M5 associate with large pre-ribosomal particles. Western-blot analysis of sucrose gradient fractions of TGR-1 cells expressing Pes1-HA, and Pes1-HA mutants M1, M3 and M5 with an HA-specific antibody. RNA of each fraction was analysed by northern analysis with 28S rRNA specific probe (lower panel).

followed by a distal BRCT domain (27). While the NPLP-domain is present in all eukaryotes, the BRCT domain is missing in some simple organisms as *Plasmodium falciparum*. Three of our deletions mutants, M1, M2 and M3, successively lacked parts of the NPLP-domain. The removal of 54 N-terminal amino acids of Pes1 in mutant M1 resulted in a strong dominant-negative phenotype. Sucrose gradient centrifugation, co-immunoprecipitation experiments and native gel-electrophoresis experiments (data not shown) revealed proper incorporation of mutant M1 protein into the PeBoW-complex. Hence, the N-terminal 54 amino acids of the NPLP-domain are neither required for the assembly of the PeBoW-complex nor for the transport of Pes1 to the nucleolus. It is more likely that the N-terminal domain of Pes1 fulfils other essential tasks after the assembly of the complex by recruitment of further factors, or by catalysing specific steps in the maturation of the 32S pre-rRNA precursor. However, an enzymatic activity of the complex has not been demonstrated yet, neither in mammals nor in yeast. The mutants M2 and M3 lacked larger parts of the NPLP-domain. Proteins of both mutants were still transported into the nucleus. A transport into the nucleolus, however, was inhibited. This is in line with the observation that mutant M2 and M3 proteins did not co-immunoprecipitate Bop1 and WDR12, because the assembly of the PeBoW-complex in the nucleolus could not take place. The absence of a dominant-negative phenotype of both mutants also supports the model that incorporation of Pes1 mutants into the PeBoW-complex is a prerequisite for a dominant-negative phenotype. The region deleted in M2 and M3 has been reported to interact directly with Bop1 (15). The interaction domain with Bop1 has been identified in murine Pes1 after transposon insertion mutagenesis of 19 extra amino acids downstream of amino acid 198 (Pes1-tn2), amino acid 202 (Pes1-tn11) and amino acid 204 (Pes1-tn12) (Figure 9) in yeast two hybrid experiments (15). The failure of mutants M2 and M3 proteins to immunoprecipitate complexes containing Bop1 and WDR12 supports this notion. Anyway, incorporation of both mutant proteins into the PeBoW-complex cannot take place, since they do not localize to the nucleolus. In conclusion, the NPLP-domain is critical for nucleolar localization and assembly of the PeBoW-complex.

The Pes1 mutant M4 harbours a deletion of the BRCT (BRCA1 C-terminal) domain that was first described as an essential domain of the tumour suppressor function of the BRCA1 protein. Similar repeat sequences have been identified in many proteins that function in DNA damage, repair and replication. Many BRCT-containing proteins have phospho-peptide binding activity suggesting that BRCT repeats might mediate phosphorylation-dependent protein–protein interactions in processes that are central to cell cycle checkpoint and DNA repair functions. It is tempting to speculate that the BRCT domain in Pes1 might fulfil similar tasks and links nucleolar processes to cell cycle control and DNA synthesis. Interestingly, a temperature sensitive mutant of yeast Nop7, a homologue of Pes1, can specifically inhibit S phase entry in yeast cells (17). This points to additional cell cycle control mechanisms by Pes1, which may act independently of ribosome biogenesis. The mutant M4 protein did not co-immunoprecipitate Bop1 and WDR12 from cellular lysates. This lack of interaction suggests that the NPLP-domain in Pes1 is not sufficient for binding of Bop1 in the nucleoplasm, or that the interaction of nucleoplasmic Pes1 and Bop1 is negatively controlled by a yet unknown mechanism. Importantly, in addition to the Bop1 interacting domain, the BRCT domain also plays an important role in nucleolar localization.

The second mutant with a strong dominant-negative phenotype on cell proliferation and rRNA processing was M5. Similar to M1, mutant M5 protein localized to the

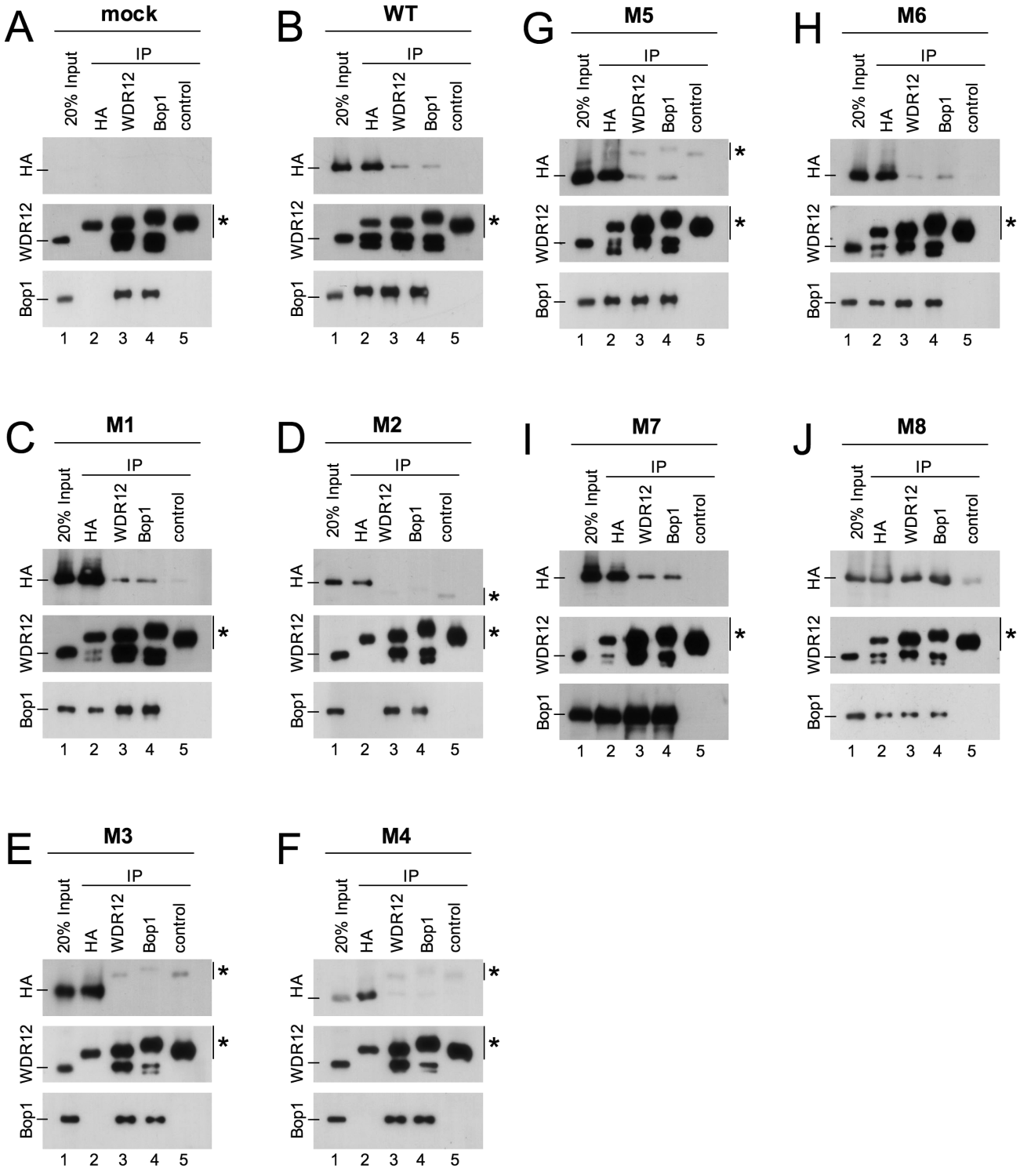

Figure 8. Incorporation of Pes1 mutants into the PeBoW-complex. U2OS cells stably transfected with the indicated constructs **(A–J)** were seeded at subconfluent density and treated with doxycycline for 20 h. The lysates were subjected to immunoprecipitation with antibodies either against the HA-tag (3F10), WDR12 (1B8), Bop1 (6H11) or the isotype control coupled to protein G Sepharose beads. The amount of protein loaded in each lane resembles an equivalent amount of total lysate or 20% thereof for the input. * indicates cross-reactivity to Ig molecules.

nucleolus and co-immunoprecipitated Bop1 and WDR12. However, we noticed a specific difference in the processing of the 5.8S rRNA for mutants M1 and M5. While the amount of 12S pre-rRNA was reduced for mutant M1, the levels of 12S pre-rRNA were unchanged for the mutant M5. This suggests an inhibition of 12S pre-rRNA processing by mutant M5 and an accumulation of this precursor, whereas 12S pre-rRNA processing proceeds in the presence of mutant M1.

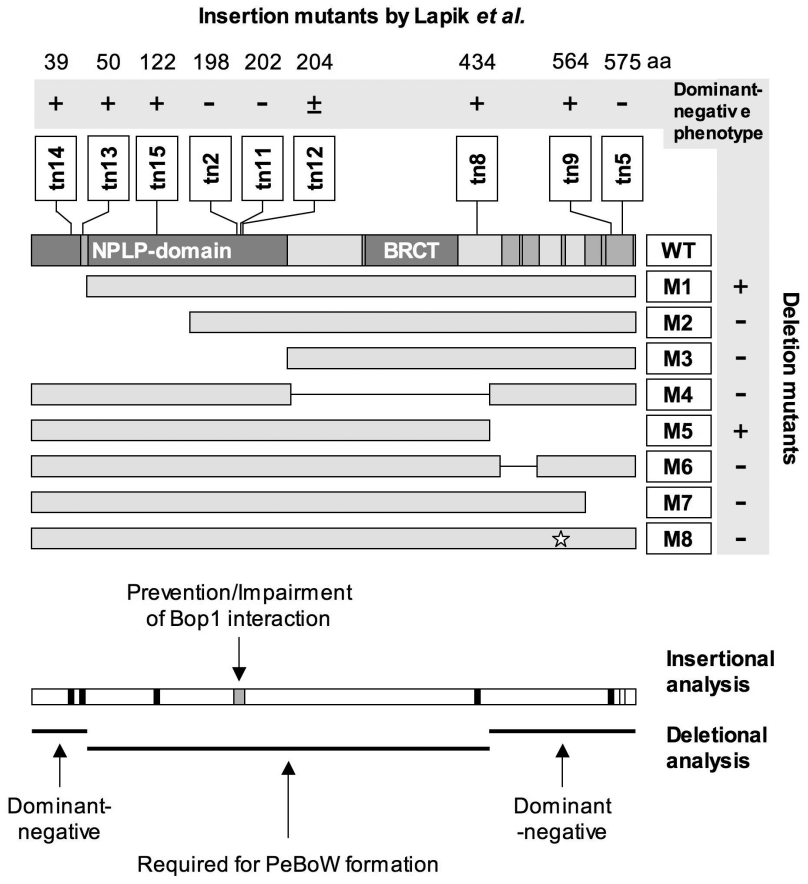

Figure 9. Boundaries and domains important for Pes1 function and PeBoW formation. Pes1 deletions mutants of this work were compared with transposon insertion mutants published by Lapik *et al*. (15). Deletion mutants conferring a dominant-negative phenotype or affecting formation of PeBoW are indicated.

The data suggests that the PeBoW-complex is required for consecutive rRNA processing steps during maturation of the 60S ribosomal subunit and that mutant M5 can block the processing of the 12S pre-rRNA in addition to the 32S pre-rRNA. Accordingly, the C-terminal domain of Pes1 is required for processing of the 32S and 12S pre-rRNA precursors, whereas the N-terminal domain appears to be dispensable for processing of the latter.

Role of the PeBoW-complex in cell cycle control

The impaired function of the PeBoW-complex evokes a p53 response in proliferating but not in serum-starved cells. All three components of the PeBoW-complex are encoded by Myc target genes and usually are not expressed in quiescent cells (14,28,29). This suggests that expression of dominant-negative mutants of Pes1 (this paper), or WDR12 (14) may be not sufficient for the induction of p53, because they cannot assemble into the PeBoW-complex and block its function. However, a recent study showed that human WI-38 fibroblasts accumulated p53 under growth-restricting conditions, such as serum starvation or confluency (30).

Whether the PeBoW-complex is directly involved in the degradation of p53 remains unclear. Since dominant-negative Pes1 mutants act only in the presence of ongoing ribosome biogenesis it is conceivable that signalling of p53 degradation should be linked to this process. Two non-mutually exclusive mechanisms have been proposed for p53 degradation: nucleolar sequestration of Mdm2, and the blockage of a postulated nucleolar route of p53 export for cytoplasmic degradation (13,31). The Mdm2 sequestration model is supported by the recent finding that several ribosomal proteins, L5, L11 and L23, can bind and inhibit the function of Mdm2. Inhibition of ribosome biogenesis leads to accumulation of free L5, L11 and L23 proteins, which bind to and inhibit Mdm2-mediated p53 ubiquitination and degradation. Inhibiton of Mdm2 restores p53-mediated transactivation, accumulation of p21 protein levels, and induction of cell cycle arrest (5–9,27). The second model postulates a nucleolar export route for Mdm2–p53 complexes and subsequent cytoplasmic degradation of p53, termed by Sherr and Weber (13) as Mdm2–p53 complexes 'riding the ribosome'. This model is supported by the observation that p53 can be covalently linked to 5.8S rRNA, and associates with a subset of ribosomes (32–34). Further support comes from the finding that inhibition of nuclear export leads to accumulation of p53 in the nucleus. If p53 has to enter the nucleolus for the second model, or is loaded on ribosomal subunits during the passage through the nucleoplasm, is currently an open question. Evidence for the presence of p53 in the nucleolus has recently been shown following cell permeabilization, where most soluble nucleoplasmic p53 was eliminated, but nuclear-bound p53 remained readily detectable in the nucleoli (6). Accumulation of nucleolar p53 has also been observed

after inhibiton of the proteasome (35). Alternatively, p53 associate with large pre-ribosomal subunits, if they leave the nucleolus. Comparable to processing of the 27S pre-rRNA in yeast (25), processing of the 32S pre-rRNA in mammalian cells probably occurs at the transition of the large pre-ribosomal subunit from the nucleolus to the nucleoplasm. At this moment, the functionality of the PeBoW-complex is required. Successful processing of the 32S rRNA would cause the release of the PeBoW-complex and loading of Mdm2 and p53. A non-functional PeBoW-complex would inhibit processing of the 32S pre-RNA and thereby nuclear export of p53/Mdm2.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Mammalian WDR12 is a novel member of the Pes1–Bop1 complex and is required for ribosome biogenesis and cell proliferation

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Target genes of the protooncogene c-*myc* are implicated in cell cycle and growth control, yet the linkage of both is still unexplored. Here, we show that the products of the nucleolar target genes Pes1 and Bop1 form a stable complex with a novel member, WDR12 (PeBoW complex). Endogenous WDR12, a WD40 repeat protein, is crucial for processing of the 32S precursor ribosomal RNA (rRNA) and cell proliferation. Further, a conditionally expressed dominant-negative mutant of WDR12 also blocks rRNA processing and induces a reversible cell cycle arrest. Mutant WDR12 triggers accumulation of p53 in a p19ARF-independent manner in proliferating cells but not in quiescent cells. Interestingly, a potential homologous complex of Pes1–Bop1–WDR12 in yeast (Nop7p–Erb1p–Ytm1p) is involved in the control of ribosome biogenesis and S phase entry. In conclusion, the integrity of the PeBoW complex is required for ribosome biogenesis and cell proliferation in mammalian cells.

Introduction

Coordination of cell growth and cell division is a fundamental prerequisite for proliferating cells to remain constant in size (Polymenis and Schmidt, 1999; Fingar et al., 2002; Schmidt, 2004). Ribosome biogenesis, the major constituent of cellular growth, accounts for up to 80% of the energy consumption of dividing cells (Thomas, 2000). Disturbances in the ribosome synthesis pathway must be detected and coupled with cell cycle progression to prevent premature cell divisions. The fact that the oncogenic transcription factor c-Myc efficiently promotes proliferation might result from its capacity to positively regulate both ribosome biogenesis and cell cycle progression (Schmidt, 1999, 2004). Yet it remains unclear how c-Myc achieves this concerted action.

The *c-myc* protooncogene is implicated in proliferation, cell growth, differentiation, and apoptosis (Oster et al., 2002; Nilsson and Cleveland, 2003; Pelengaris and Khan, 2003; Schmidt, 2004). Deregulated expression is associated with a variety of human neoplasias. Several high throughput techniques have substantially extended the list of potential c-Myc target genes (Fernandez et al., 2003; Li et al., 2003; Patel et al., 2004). Transcriptional control by c-Myc has been reported on hundreds of genes (Coller et al., 2000; Guo et al., 2000; Schuhmacher et al., 2001). One major subset of target genes is involved in ribosome biogenesis and cell metabolism. Other gene products exert cell cycle control. Indeed, c-Myc is of pivotal importance to promote entry into and to prevent exit from the cell cycle (Eilers et al., 1991; Hölzel et al., 2001; Trumpp et al., 2001). On the other hand, constitutive expression of c-Myc mediates accumulation of cell mass (Schuhmacher et al., 1999; Kim et al., 2000). These findings suggest that c-Myc target genes physiologically act in concert to promote proliferation while ensuring the equilibrium between cell growth and cell cycle progression. However, the mechanisms that survey balanced cell divisions in mammalian cells remain largely unexplored. Nevertheless, several recent studies have considerably enlarged our knowledge. Intriguing links between nucleolar function and cell cycle control have emerged.

Conditional deletion of the ribosomal protein gene S6 in mice impeded cell cycle entry of liver cells after partial hepatectomy (Volarevic et al., 2000). Surprisingly, hepatocytes of starved mice regained their baseline cell size after feeding despite the lack of ribosomal protein S6. Thus, ribosome biogenesis is essential for proliferation but not for accumulation of cell

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Abbreviations used in this paper: rRNA, ribosomal RNA; siRNA, small interfering RNA.

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mass. Several mechanisms have been proposed that couple nucleolar function to cell cycle control. Interestingly, they all imply the tumor suppressor p53.

First, the Mdm2 oncoprotein mediates the proteasomal degradation of p53 (Stommel et al., 1999; Tao and Levine, 1999a; Boyd et al., 2000). The p19ARF tumor suppressor disrupts Mdm2–p53 binding, sequesters the former to the nucleolus, and thus stabilizes p53 (Tao and Levine, 1999b; Weber et al., 1999). Another mechanism proposes nuclear export of p53 via the nucleolus for subsequent degradation in the cytoplasm (Sherr and Weber, 2000). In fact, Mdm2–p53 complexes are found in conjunction with the ribosomal proteins L5 and L11 (Marechal et al., 1994; Lohrum et al., 2003; Zhang et al., 2003). These findings suggest that Mdm2–p53 complexes assemble with ribosomes for their CRM1-dependent nuclear export. Indeed, it was shown that a subset of ribosomes contained cytoplasmic p53 covalently linked to 5.8S ribosomal RNA (rRNA; Fontoura et al., 1992, 1997). Hence, the nucleolar export model directly couples nucleoplasmic p53 levels to the functional state of ribosome biogenesis. This model received substantial support as it was demonstrated that DNA damage by localized UV irradiation of cell nuclei failed to trigger stabilization of the tumor suppressor p53 unless the nucleolus was affected (Rubbi and Milner, 2003). Moreover, the literature confirms that all p53-inducing stresses cause nucleolar disruption besides the ones that act downstream of the nucleolus such as the proteasome and nuclear export inhibitors MG132 and leptomycin B, respectively (Rubbi and Milner, 2003). Recently, it was shown that the ribosomal proteins L5, L11, and L23 negatively regulate Mdm2 activity (Lohrum et al., 2003; Zhang et al., 2003; Dai and Lu, 2004; Jin et al., 2004). Proliferating cells incorporate L5, L11, and L23 into functional ribosomes. Disturbances in the ribosome synthesis pathway, in contrast, result in the accumulation of free ribosomal proteins. The subsequent inhibition of Mdm2 by L5, L11, and L23 causes activation of p53.

Hence, the nucleolus is a common stress sensor of pivotal importance in the p53 response. Therefore, precise knowledge about ribosome biogenesis is required to unravel the signals that transmit nucleolar disturbance to the cell cycle. Several genetic and proteomic studies in yeast provided intriguing details of the complex molecular machinery needed for ribosome synthesis (Fatica and Tollervey, 2002; Fromont-Racine et al., 2003; Saveanu et al., 2003; Takahashi et al., 2003). As an example, yeast Nop7p (Yph1p) has been implicated in the control of ribosome biogenesis and DNA replication via its interaction with origin recognition complex proteins (Du and Stillman, 2002). Two other proteins, Erb1p and Ytm1p, have been copurified with Nop7p in a small core complex. Remarkably, Bop1, the human homologue of Erb1p, plays a role in rRNA processing and cell cycle control (Strezoska et al., 2002). Dominant-negative mutants of Bop1 interfered with proper rRNA processing and provoked a p53-dependent cell cycle arrest (Pestov et al., 2001). More recently, Bop1 was shown to interact with Pes1, the mammalian homologue of Nop7p (Lapik et al., 2004). This indicates that a set of evolutionarily conserved proteins might coordinate ribosome biogenesis with DNA replication in mammalian cells.

Previously, we identified a considerably large number of c-Myc target genes involved in rRNA processing (Schlosser et al., 2003). It received our particular attention that c-Myc up-regulated the expression of *pes1*, *bop1*, and *wdr12*. Mammalian WD repeat protein 12 (WDR12) is the putative homologue of yeast Ytm1p. It is tempting to speculate that in analogy to yeast, Pes1, Bop1, and WDR12 might function in an evolutionarily conserved complex. Thus, c-Myc dependent up-regulation of these three factors might promote the formation of a subcomplex that is important for the assembly of larger preribosomal complexes and the cross talk between ribosome biogenesis and proliferation control.

However, the biological functions of mammalian WDR12 are yet unknown. WDR12 mRNA is ubiquitously expressed during embryogenesis and high levels are found in the thymus and testis of adult mice. In addition, GST pull-down experiments revealed an interaction with Notch1-IC, therefore sug-

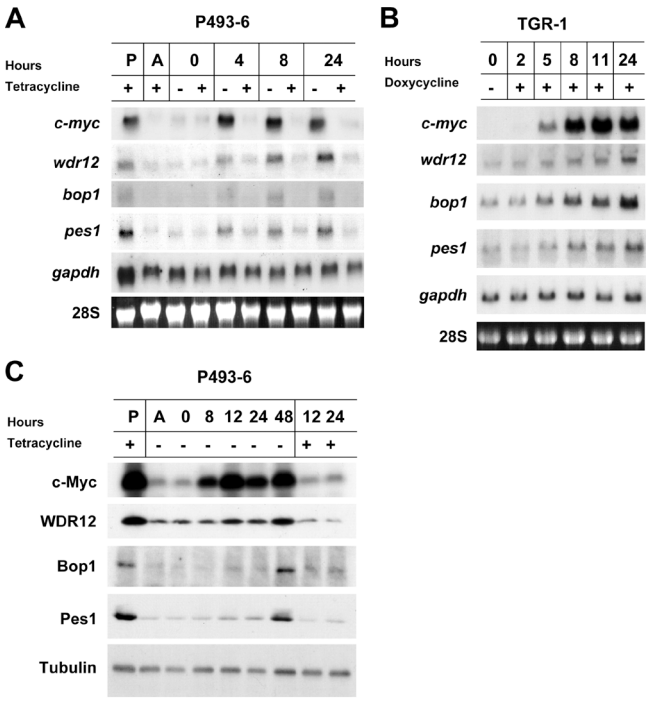

Figure 1. **c-Myc controls endogenous *wdr12* expression.** (A) Northern blot analysis of *wdr12* expression in the human B cell line P493-6 carrying a tetracycline-controllable *c-myc* gene. Removal of tetracycline from the culture medium by several washes with PBS strongly stimulated exogenous *c-myc* expression. As a control, separate cultures were treated equally, however, in the presence of tetracycline. Equal amounts of total RNA were hybridized with radioactively labeled probes specific for human *c-myc*, *wdr12*, *bop1*, and *pes1*. Expression of *gapdh* was not affected by c-Myc. Bottom panel shows ethidium bromide staining as loading control. P, proliferating cells; A, arrested cells. (B) Northern blot analysis of WDR12 expression in TGR-1 rat fibroblasts. TGR-1 cells were stably transfected with a construct encoding a doxycycline-inducible human *c-myc* gene. Cells were arrested by contact inhibition to reduce endogenous *c-myc* levels. Addition of doxycycline to the culture medium strongly activated exogenous *c-myc* expression. Equal amounts of total RNA were hybridized with radioactively labeled probes specific for human *c-myc* and rodent *wdr12*, *bop1*, and *pes1*. Expression of *gapdh* was not affected by c-Myc. Bottom panel shows ethidium bromide staining as loading control. (C) Western blot analysis of WDR12 expression in P493-6 cells. Cells were treated as described in A. Immunodetection was performed with antibodies against c-Myc (N-262), WDR12 (1B8), Bop1, Pes1, and α-tubulin (DM1A).

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gesting a role in early stages of primary T cell differentiation (Nal et al., 2002). The high homology between mammalian WDR12 and yeast Ytm1p prompted us to study the role of WDR12 in ribosome biogenesis and cell proliferation.

Results

c-Myc up-regulates WDR12

First, we analyzed the expression of WDR12 in a human B cell line (P493-6) harboring a *c-myc* tet-off system (Schuhmacher et al., 1999). Proliferation of P493-6 cells is *c-myc* dependent. After the induction of *c-myc*, *wdr12* mRNA was similarly up-regulated as *bop1* and *pes1* (Fig. 1 A). To rule out cell line–specific observations, we established TGR-1 rat fibroblasts carrying a *c-myc* tet-on system. Addition of doxycycline to the culture medium induced rapid and strong *c-myc* expression resulting likewise in up-regulation of endogenous *wdr12*, *bop1*, and *pes1* mRNA levels (Fig. 1 B). Further, we raised monoclonal antibodies specific against human WDR12, Bop1, and Pes1. Proliferating P493-6 cells expressed high levels of all three proteins in contrast to arrested cells (Fig. 1 C). Consistently, endogenous WDR12, Bop1, and Pes1 accumulated subsequent to conditional c-Myc expression.

WDR12 is a nuclear protein with predominant nucleolar localization

To determine the cellular localization of endogenous WDR12, we performed indirect immunofluorescence. WDR12 localized to the nucleolus and nucleoplasm in various cell lines such as U2OS (Fig. 2 A, top), HeLa, and WI-38 (not depicted). Further, human diploid fibroblasts were cotransfected with expression constructs coding for WDR12-eYFP and eCFP-nucleophosmin fusion proteins. WDR12-eYFP exhibited diffuse nucleoplasmic distribution, strong accumulation within the nucleolus, and nucleolar colocalization with nucleophosmin (Fig. 2 A, bottom). Similar results were obtained in rodent fibroblasts (unpublished data). Interestingly, nucleolar localization was also reported for GFP- and HA-tagged forms of yeast Ytm1p, the potential homologue of mammalian WDR12 (Ouspenski et al., 1999; Huh et al., 2003). Hence, our data provide for the first time experimental evidence that mammalian WDR12 and yeast Ytm1p are potential homologues exhibiting similar subcellular distribution.

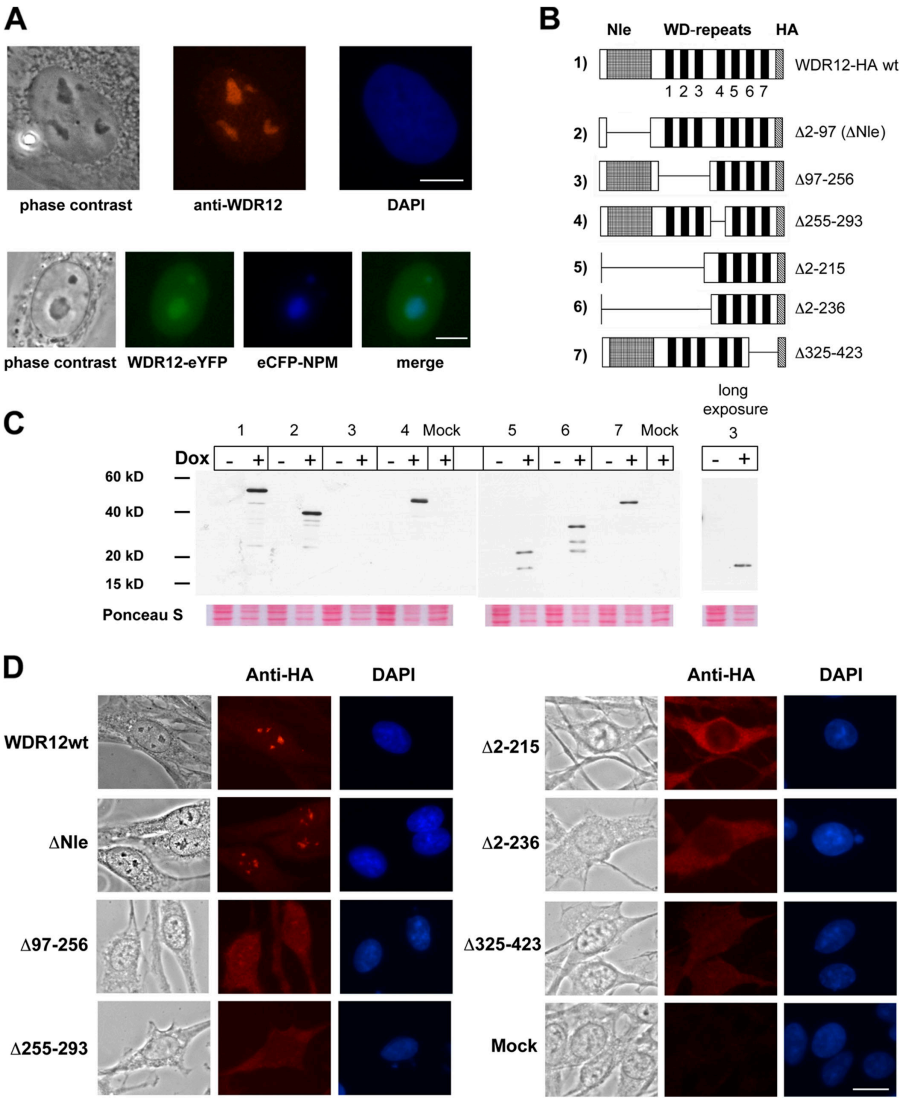

Figure 2. **Subcellular localization and mutagenesis of WDR12.** (A) Endogenous WDR12 was visualized by indirect immunofluorescence in PFA-fixed U2OS cells (top). Nuclei were counterstained with DAPI. Human diploid fibroblasts were transiently cotransfected with constructs encoding the WDR12-eYFP and eCFP-Nucleophosmin fusion proteins. A representative image of a transfected cell is shown (bottom). (B) Outline of domain-specific mutagenesis of WDR12. Several truncation mutants of HA-tagged human WDR12 were generated and cloned into pRTS plasmids allowing doxycycline-dependent gene expression. (C) Western blot analysis of wild-type and mutant WDR12-HA in stably transfected TGR-1 fibroblasts. The indicated polyclonal cell lines were treated with doxycycline for 24 h (+) or left untreated (−). Bottom panel shows Ponceau S staining as loading control. (D) Analysis of subcellular localization of WDR12 mutants. TGR-1 cells were stably transfected with the indicated constructs and treated with doxycycline for 24 h. Methanol-fixed cells were analyzed by indirect immunofluorescence. Representative images are shown in each panel. Nuclei are visualized by DAPI staining. As control, cells expressing luciferase were equally analyzed (Mock). Bars: (A) 10 μm; (D) 20 μm.

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Mutational analysis of WDR12

Next, we aimed to assess the biological functions of WDR12 by mutational analysis. WDR12 belongs to the family of WD repeat proteins that are generally thought to regulate protein–protein interactions (Smith et al., 1999). These factors consist of several copies of a defined amino acid motif, the WD domain. WDR12 contains seven COOH-terminal WD repeats, as predicted by BMERC analysis (Nal et al., 2002). Crystallographic studies of other factors with seven WD repeats revealed that the WD domains form a "propeller-like" structure composed of seven "blades" (Wall et al., 1995). The NH_2 terminus of WDR12 has significant similarity to the NH_2-terminal part of Notchless, a protein implicated in the modulation of Notch signaling in *Drosophila melanogaster* (Royet et al., 1998; Nal et al., 2002). This defined structural organization of WDR12 prompted us to generate a panel of different truncation mutants (Fig. 2 B). We established stable polyclonal cell lines of TGR-1 rat fibroblasts that express WDR12 wild-type or mutant forms together with eGFP from a bidirectional promoter in a doxycycline-dependent manner (unpublished data). After doxycycline treatment, >95% of the cells were eGFP positive, as monitored by flow cytometry in each experiment. Levels of HA-tagged WDR12 mutants were determined by immunoblotting (Fig. 2 C). Some mutant forms of WDR12-HA were detected at the calculated molecular size, whereas others exhibited multiple products at a significantly lower size (Fig. 2 C, lanes 3, 5, and 6). This is most likely due to rapid degradation of unstable mutant forms. Interestingly, all unstable WDR12 mutants lack the first three WD domains.

Further, we examined the cellular localization of WDR12 mutants by immunofluorescence (Fig. 2 D). As expected, the HA-tagged wild-type form of WDR12 localized to the nucleolus. Surprisingly, only WDR12 lacking the NH_2-terminal Notchless-like domain (WDR12ΔNle) also exhibited predominant nucleolar staining. The remaining WDR12 mutants dispersed in the cytoplasm and nucleoplasm.

WDR12ΔNle inhibits proliferation

To test whether wild-type WDR12 or mutants affect proliferation, we seeded equal numbers of stable TGR-1 cell cultures at low density in the presence of doxycycline. After 6 d, the number of cells was determined in multiples and compared with the mean cell count of a mock cell line expressing luciferase (Fig. 3 A). Enforced expression of exogenous wild-type WDR12 neither impaired nor promoted cell proliferation. However, deletion of the NH_2-terminal Notchless-like domain resulted in a strongly reduced cell count. We excluded an increase of apoptosis by Annexin V staining using flow cytometry as a potential explanation for this observation (unpublished data). Hence, the block of proliferation in response to enforced WDR12ΔNle expression is due to cell cycle arrest rather than increased cell death. To support this hypothesis, we performed BrdU light assays (Pestov and Lau, 1994). Thereby we investigated whether enforced WDR12ΔNle expression elicits a reversible cell cycle arrest. In brief, dividing cells that incorporate BrdU into their DNA become highly photosensitive if they are additionally labeled with Hoechst dye and irradiated with light. Hence, cell cycle–arrested cells are protected from increased photosensitivity and survive BrdU light treatment. Conditional WDR12ΔNle expression efficiently rescued TGR-1 cells from BrdU light treatment, whereas wild-type WDR12 failed to do so (Fig. 2 B). As controls, we generated stable cell lines expressing wild-type Bop1, Bop1Δ and Bop1N2, two previously described dominant-negative mutants of Bop1 (Pestov et al., 2001; Strezoska et al., 2002).

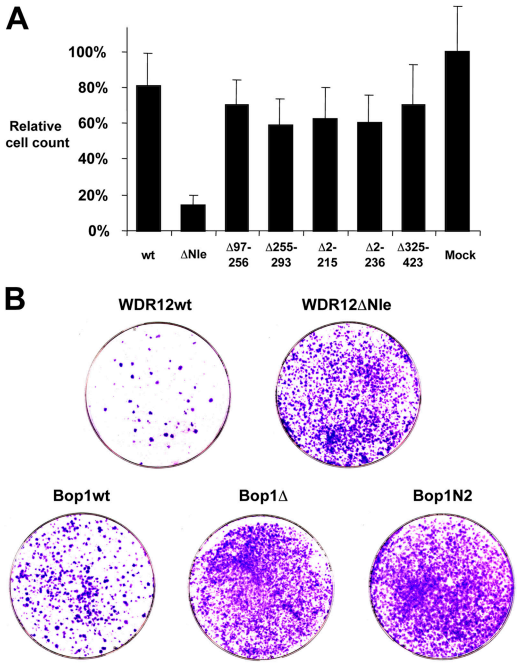

Figure 3. **WDR12ΔNle inhibits cell proliferation and elicits a reversible cell cycle arrest.** (A) Equal numbers of TGR-1 cells stably transfected with the indicated constructs were seeded in multiples in the presence of doxycycline. After 6 d, cells were trypsinized and counted by trypan blue exclusion. Final cell counts of the indicated cell lines were compared with a mock cell line that expresses luciferase. Histograms depict the relative cell counts after 6 d. Error bars indicate SD. (B) Reversible cell cycle arrest by WDR12ΔNle overexpression. Stably transfected TGR-1 cells were seeded at low density and treated with doxycycline for 30 h to induce expression of the specified constructs. Subsequently, cells were incubated with 100 μM BrdU for 48 h to label proliferating cells. Visible light irradiation in the presence of 2 μM Hoechst 33258 selectively kills cells that have incorporated BrdU into their DNA. Arrested cells survive BrdU light treatment and give rise to cell colonies after withdrawal of doxycycline. Images show representative BrdU light assays conducted with the indicated cell lines.

To further examine the impact of WDR12ΔNle expression on cell proliferation, we performed cell cycle analysis using flow cytometry (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200501141/DC1). Overexpression of Bop1Δ, as previously shown, led to accumulation of cells in G1 and reduction in S phase (Pestov et al., 2001). Albeit the strong inhibitory effect of WDR12ΔNle on cell proliferation, we observed only slight alterations of the cell cycle distribution and a less pronounced accumulation in G1 than in Bop1Δ-expressing cells. Hence, our data suggests that in addition to a complete G1 arrest, cells may also exhibit delayed overall cell cycle progression in response to WDR12ΔNle and probably also Bop1Δ.

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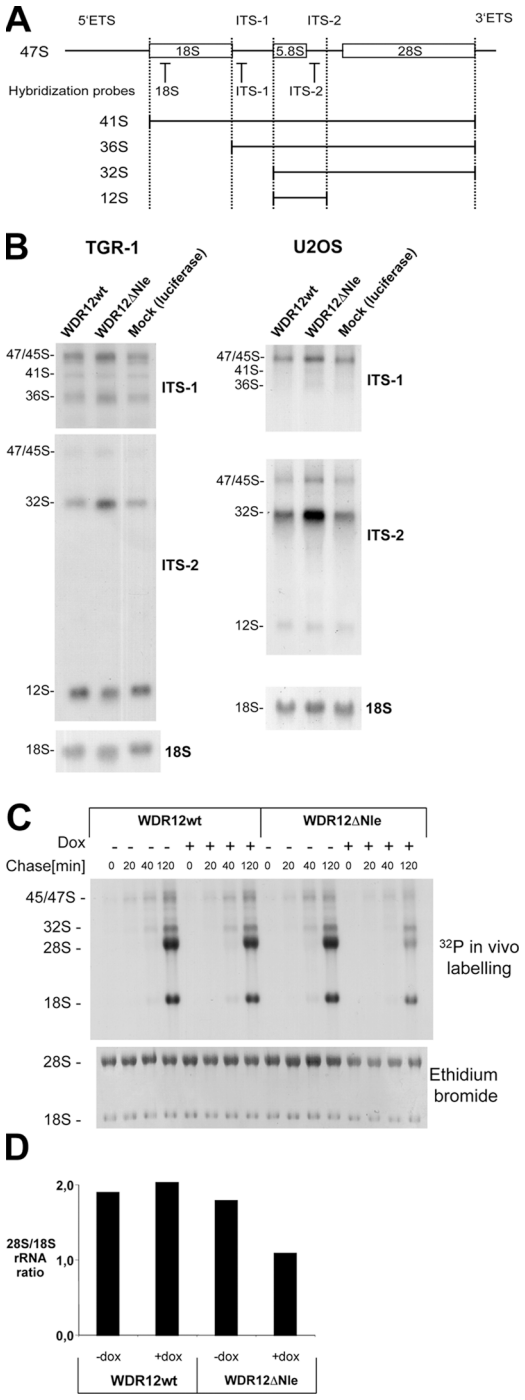

Figure 4. **WDR12ΔNle expression affects rRNA processing.** (A) Diagram of the primary 47S rRNA transcript and the major rRNA intermediates. Positions of the hybridization probes are depicted. ETS, external transcribed spacer; ITS, internal transcribed spacer. (B) Northern blot analysis of rRNA precursors in WDR12ΔNle-expressing TGR-1 and U2OS cells. Indicated cell lines were treated with doxycycline for 30 h. Equal amounts of total RNA were hybridized with probes specific for the ITS1 and ITS2 of rRNA intermediates. As loading control, blots were incubated with a probe specific for 18S rRNA. (C) WDR12ΔNle expression impairs formation of mature 28S rRNA. Asynchronously growing TGR-1 cells were treated with or without doxycycline for 24 h. Cells were pulse labeled with ^{32}P-orthophosphate for 45 min and chased in regular medium as indicated. (D) Ratio of 28S/18S rRNA at 120 min after metabolic labeling with ^{32}P-orthophosphate as described in C.

Mutants lacking the first three WD40 repeats in addition to the deleted Notchless-like domain failed to block proliferation. Thus, disrupted integrity of the WD repeat domains rescued the inhibitory effect of WDR12ΔNle. Noteworthily, these mutants also exhibited aberrant subcellular distribution. Our data suggest that the dominant-negative phenotype of WDR12ΔNle depends on its nucleolar localization.

WDR12ΔNle interferes with rRNA processing

From the aforementioned results we reasoned that WDR12ΔNle might confer its inhibitory effect on cell proliferation via interference with rRNA processing. Besides, there is accumulating evidence in the literature supporting our notion. Yeast Ytm1p, the potential homologue of WDR12, interacts with Erb1p and Nop7p (Yph1p) in a complex that links ribosome biogenesis with DNA replication (Du and Stillman, 2002). Bop1 and Pes1, the candidate mammalian homologues of Erb1p and Nop7p, both play a role in rRNA processing and, moreover, also physically interact (Strezoska et al., 2002; Lapik et al., 2004). Therefore, we investigated the levels of different rRNA species in WDR12ΔNle-expressing TGR-1 and U2OS cells by Northern blot analysis (Fig. 4, A and B). Total RNA was probed with sequences specific for the ITS1 and ITS2 (internal transcribed spacer) of rRNA intermediates (Fig. 4 A). WDR12ΔNle-expressing cells accumulated the 32S rRNA precursor (Fig. 4 B). In another set of experiments, we studied the impact of WDR12ΔNle on rRNA processing in TGR-1 cells by metabolic ^{32}P in vivo labeling (Fig. 4 C). The production of mature 28S rRNA was severely reduced resulting from an inefficient processing of the 32S rRNA precursor, as concluded from the decreased ratio of 28S/18S rRNA (Fig. 4 D). Synthesis of mature 18S rRNA was almost unaffected, as determined by the signal intensity of the 18S rRNA normalized to the total RNA loading. These results are in line with our Northern blot analysis. In conclusion, WDR12ΔNle primarily compromises ribosome biogenesis by blocking processing of the 32S rRNA precursor into mature 28S rRNA.

Endogenous WDR12 is required for rRNA processing and cell proliferation

To confirm the role of WDR12 in ribosome biogenesis and cell proliferation, we performed small interfering RNA (siRNA) knockdown experiments of endogenous WDR12. U2OS cell were transfected at day 0, 1, and 2 with either control or WDR12-specific siRNA. Expression of endogenous WDR12 was dramatically reduced, as monitored by Western blot analysis 2 d after the last transfection (Fig. 5 A). Moreover, proliferation of WDR12-depleted cells was severely impaired (Fig. 5 B). Further, we investigated the impact of WDR12 knockdown on ribosome biogenesis. Levels of precursor and mature rRNA species were determined by Northern blot analysis 2 d after the last siRNA transfection. WDR12-depleted cells exhibited accumulation of the 32S precursor rRNA and a concomitant reduction of the mature 28S rRNA (Fig. 5 C). To study more immediate effects of WDR12 knockdown on rRNA process-

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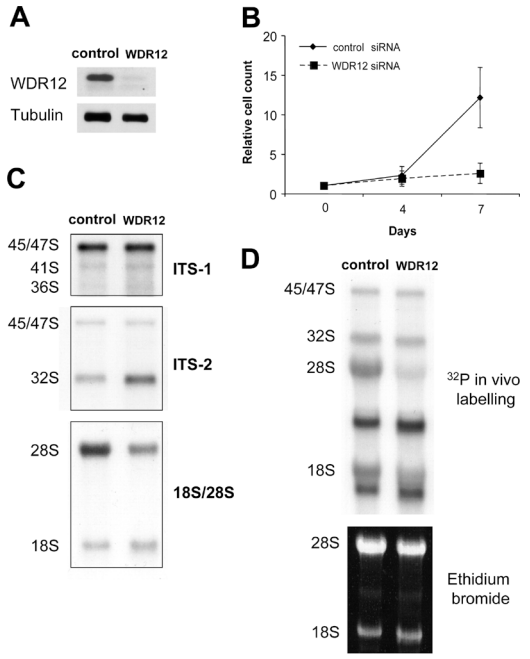

Figure 5. **Endogenous WDR12 is required for rRNA processing and cell proliferation.** (A) U2OS cells were transfected at day 0, 1, and 2 with either control or WDR12-specific siRNA. Endogenous WDR12 levels were analyzed by Western blot analysis 2 d after the last transfection. α-Tubulin is shown as a loading control. (B) U2OS cells were seeded in multiples at low density and treated as described in A. Cells were counted at days 0, 4, and 7. Error bars indicate SD. (C) U2OS cells were treated as in A and total RNA was harvested 2 d after the last transfection. Different rRNA species were visualized by Northern blot analysis using probes specific for human ITS-1, ITS-2, 28S, and 18S rRNA. (D) U2OS cells were transfected only twice at day 0 and 1 and metabolically labeled with ^{32}P-orthophosphate for 60 min at day 3. Subsequently, cells were incubated for 3 h in regular culture medium.

ing, cells were transfected only twice at day 0 and 1 and in vivo labeling of rRNA was performed 2 d later. Again, the production of the mature 28S rRNA was drastically compromised (Fig. 5 D). Notably, synthesis of the large 45/47S precursor was not affected, indicating that WDR12 is not involved in rRNA transcription. In conclusion, expression of dominant-negative WDR12ΔNle and depletion of endogenous WDR12 block rRNA processing of the 32S rRNA precursor and restrain cell proliferation.

WDR12ΔNle triggers accumulation of p53

Disruption of the nucleolus is a common feature of a variety of stresses such as DNA damage. It has been previously postulated that impairment of nucleolar function is the "common denominator" to mediate stabilization of p53 (Rubbi and Milner, 2003). Based on these findings, we explored the p53 response after enforced WDR12ΔNle expression in asynchronously proliferating TGR-1 cells (Fig. 6 A, top). Indeed, WDR12ΔNle in contrast to wild-type WDR12 mediated an accumulation of endogenous p53. The dominant-negative mutants Bop1Δ and Bop1N2 also stabilized p53. Thus, accumulation of p53 apparently is a common feature of compromised rRNA processing. Next, we investigated the conditions under which WDR12ΔNle mediates stabilization of p53. Total cell lysates of proliferating and arrested cells were analyzed by immunoblotting. Interestingly, p53 failed to accumulate in serum-starved cells (Fig. 6 A). Similarly, contact inhibition resulted in an alleviated p53 response. Equal expression of exogenous proteins was verified by immunoblotting (unpublished data). Likewise, we studied the endogenous p53 response by immunofluorescence and examined the percentage of p53-positive cells (Fig. 6 B). In proliferating TGR-1 fibroblasts, WDR12ΔNle provoked an accumulation of p53 in almost 60% of the cells (Fig. 6 C). Intensity and percentage of p53-positive nuclei diminished in the case of contact inhibition and serum starvation. Consistent results were obtained for Bop1Δ and Bop1N2. In summary, p53 accumulation in response to inhibition of rRNA processing is especially pronounced in proliferating cells compared with quiescent cells. We also tested whether the accumulation of p53 was associated with phosphorylation at serine 15. Interestingly, we observed an increase of serine 15–phosphorylated p53 congruently with the overall accumulation of p53 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200501141/DC1). Further, we investigated the WDR12ΔNle-mediated p53 response by analyzing the transcriptional activation of the p53-responsive gene p21 using quantitative real-time PCR (Fig. 6 D). Induction of p21 mRNA resembled the accumulation of p53 in response to WDR12ΔNle expression (Fig. 6 A, top; and Fig. 6 D).

As previously demonstrated, Bop1Δ and Bop1N2 elicited a p53-dependent cell cycle arrest that was attenuated by the coexpression of the human papillomavirus protein E6, a ubiquitin ligase targeting p53 for degradation (Scheffner et al., 1993). In line with these studies, WDR12ΔNle-induced cell cycle arrest was alleviated in TGR-1 cells stably transfected with HA-tagged E6 (Fig. 6 E). Interestingly, overexpression of wild-type Bop1 was accompanied with increased p53 levels. This corresponds to the weak but significant rescue of wild-type Bop1 in the BrdU light assay (Fig. 3 B).

Accumulation of p53 is independent of the tumor suppressor p19ARF

To explore the mechanism of p53 accumulation mediated by interference with rRNA processing, we studied the role of p19ARF in this response. The tumor suppressor p19ARF sequesters Mdm2 to the nucleolus and triggers stabilization of p53 (Sherr and Weber, 2000). In unstressed cells, nucleoplasmic Mdm2 targets p53 for degradation via ubiquitination. Overexpression of p19ARF inhibits rRNA processing in a p53-independent manner and binds to 5.8S rRNA. Therefore, p19ARF might be required for the accumulation of p53 in response to WDR12ΔNle. Stable polyclonal cell lines of p19ARF−/− and p53−/− MEFs were generated that conditionally express wild-type WDR12, WDR12ΔNle, and Bop1Δ. Upon exogenous gene activation, endogenous p53 levels were visualized by immunoblotting (Fig. 6 F). Indeed, overexpression of WDR12ΔNle and Bop1Δ provoked accumulation of p53 in p19ARF−/− cells. Hence, the tumor suppressor p19ARF is dispensable for an appropriate p53 response elicited by inhibition of rRNA processing.

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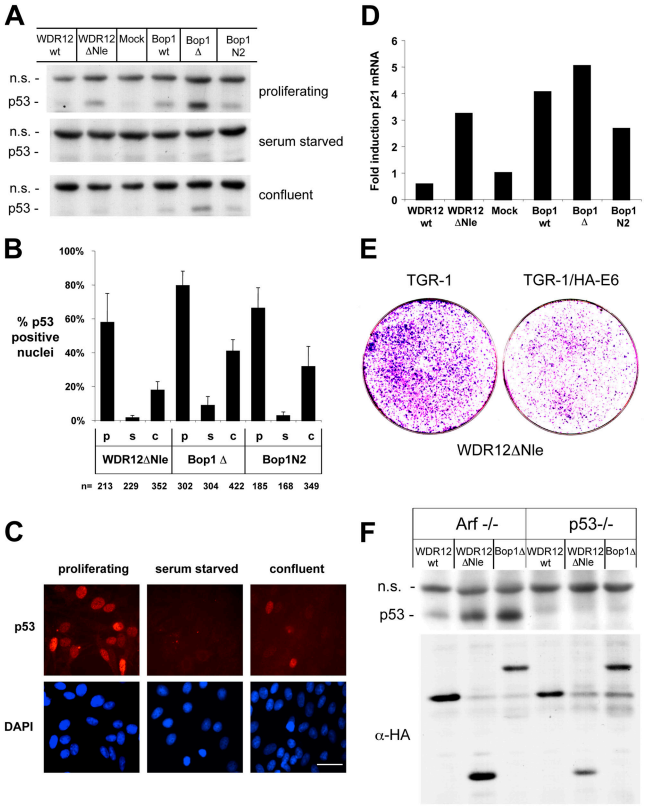

Figure 6. **WDR12ΔNle provokes accumulation of the tumor suppressor p53.** (A) Western blot analysis of endogenous p53 levels. TGR-1 cells were stably transfected with the indicated constructs. Asynchronously proliferating, serum starved (72 h), and contact inhibited cells were treated with doxycycline for 24 h to induce expression of the respective products. Equal amounts of whole cell lysates were analyzed by immunoblotting using anti-p53 (Pab122) antibodies. The top band corresponds to a nonspecific signal, as verified in p53−/− cells (F). The nonspecific band indicates equal loading in addition to Ponceau S staining (not depicted). Identical expression of the HA-tagged constructs was visualized by immunoblotting with anti-HA (3F10) antibody (not depicted). (B) Percentage of p53-positive nuclei in TGR-1 cells upon induction of the dominant-negative mutants WDR12ΔNle, Bop1Δ, and Bop1N2. Asynchronously proliferating (p), serum-starved (s), and confluent (c) cells were analyzed by immunofluorescence as described in C. Numbers of examined cells are indicated at the bottom. Error bars indicate SD of the percentage of p53-positive nuclei cells per high power field. (C) Analysis of the endogenous p53 response to WDR12ΔNle expression by indirect immunofluorescence. Asynchronously proliferating, serum-starved (72 h), and contact-inhibited cells were treated with doxycycline for 24 h to induce WDR12ΔNle. Cells were fixed in methanol and stained with anti-p53 (Pab122). Cell nuclei were counterstained with DAPI. Representative images are shown. Bar, 60 μm. (D) Asynchronously proliferating TGR-1 cells stably transfected with the indicated constructs were treated with doxycycline for 24 h. Levels of p21 mRNA were analyzed by quantitative real-time PCR. Fold induction was normalized to the expression of aldolase. (E) WDR12ΔNle-mediated reversible cell cycle arrest is alleviated by functional impairment of the endogenous p53 response in TGR-1 cells stably transfected with the HA-tagged human papillomavirus protein E6. Identical numbers of TGR-1 and TGR1-HA-E6 cells both conditionally expressing WDR12DNle were subjected to BrdU light treatment as described. Images show representative methanol-fixed and GIEMSA-stained cell cultures. (F) Accumulation of p53 is independent of p19ARF. p19ARF−/− and p53−/− MEFs were stably transfected with the indicated constructs. Asynchronously proliferating cells were treated with doxycycline for 24 h and equal amounts of whole cell lysates were analyzed by immunoblotting with anti-p53 antibody.

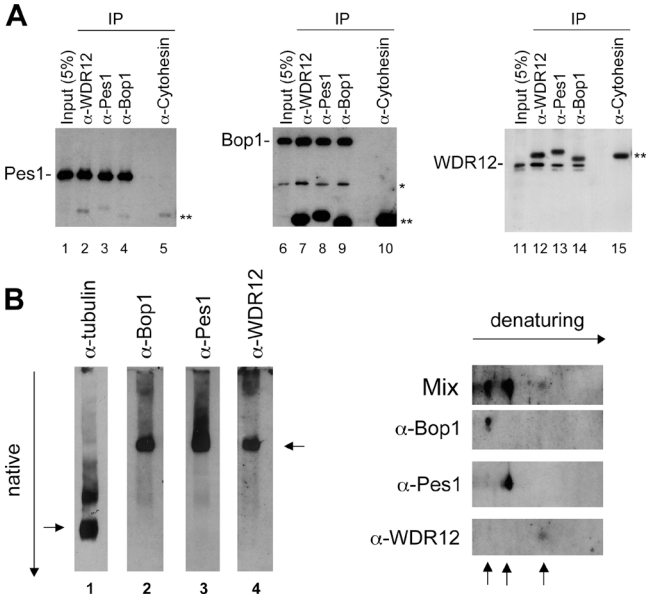

Figure 7. **Endogenous WDR12 forms a stable complex with endogenous Pes1 and Bop1.** (A) Asynchronously proliferating U2OS cells were harvested and immunoprecipitations were performed using antibodies against WDR12, Pes1, Bop1, and Cytohesin, as an IgG1 isotype control. Subsequently, immunoblotting was performed using antibodies against Pes1, Bop1, and WDR12, respectively. *, non specific band; **, IgG heavy chain. (B) Total cell lysates of U2OS cells were separated by native gel electrophoresis. Endogenous Bop1, Pes1, WDR12, and α-tubulin were visualized by immunoblotting. Arrows indicate complexes containing the respective factor. In another set of experiments, native gel electrophoresis was followed by a second denaturing gel electrophoresis. Afterwards, Bop1, Pes1, and WDR12 were detected by immunoblotting using the respective antibodies as mix or alone. Anti-Pes1 usually generates a very strong signal compared with the weaker signal of the anti-WDR12 antibody. (right) Arrows indicate Bop1, Pes1, and WDR12, respectively.

WDR12 forms a stable complex with Bop1 and Pes1

Because the potential yeast homologues Nop7p (Yph1p), Erb1p, and Ytm1p form a stable core complex (Harnpicharnchai et al., 2001; Du and Stillman, 2002), we finally asked whether endogenous Pes1, Bop1, and WDR12 also constitute a stable complex in mammalian cells. Monoclonal antibodies were raised against all three proteins and applied in immunoprecipitation experiments. Each protein was specifically immunoprecipitated and also coimmunoprecipitated the two other proteins (Fig. 7 A). Even though our results strongly suggested that the proteins participate in common complexes, the number and abundance of endogenous complexes containing Pes1, Bop1, and WDR12 remained unknown. Therefore, we performed native gel electrophoresis using stringent cell lysis conditions to unravel stable core complexes. Strikingly, immunoblot analysis of all three proteins exhibited a single major band at the same height of the native gel, indicating the existence of one stable complex. Overexpression of the respective exogenous components of the complex resulted in faster migrating bands indicating free nonincorporated proteins (unpublished data). Moreover, the presence of each protein in this complex was verified by subsequent second dimension gel electrophoresis under denaturing conditions followed by immunoblotting.

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Hence, endogenous Pes1, Bop1, and WDR12, like their potential yeast homologues, form a stable core complex in mammalian cells (PeBoW complex).

Discussion

Using cDNA microarrays, we previously identified numerous c-Myc target genes involved in ribosome biogenesis and demonstrated a global role for c-Myc in the control of efficient rRNA processing (Schlosser et al., 2003). Pes1, Bop1, and WDR12 are the products of three target genes that received our particular attention. Pes1 and Bop1 are directly interacting proteins involved in rRNA processing and proliferation (Strezoska et al., 2002; Lapik et al., 2004). In addition, the respective yeast homologues Nop7p (Yph1p) and Erb1p have been copurified in preribosomal complexes (Harnpicharnchai et al., 2001; Du and Stillman, 2002; Saveanu et al., 2003). Moreover, Nop7p and Erb1p assemble in a small subcomplex together with Ytm1p (Harnpicharnchai et al., 2001; Du and Stillman, 2002). Intriguingly, WDR12 is the most likely homologue of Ytm1p in mammalian cells. Proteomic and genetic studies suggest a role for Ytm1p in the maturation of the 60S ribosomal subunit and chromosomal stability (Ouspenski et al., 1999; Harnpicharnchai et al., 2001). Mammalian WDR12 has been cited as a Notch1-IC interacting protein; however, its biological functions remain unexplored (Nal et al., 2002).

In this study, we demonstrate that WDR12 is a crucial factor in the mammalian ribosome biogenesis pathway that forms a stable complex with Pes1 and Bop1. We were able to show that deletion of the evolutionarily conserved Notchless-like domain of WDR12 results in a dominant-negative phenotype. Interfering with WDR12 function inhibits rRNA processing at specific stages and blocks cell proliferation. Furthermore, we provide evidence that accumulation of the tumor suppressor p53 is a common phenomenon of impaired rRNA processing in a proliferation-dependent manner.

WDR12 and ribosome biogenesis

A genome-wide GFP-tagging approach in yeast revealed nuclear and nucleolar localization of Ytm1p (Huh et al., 2003). We demonstrated that endogenous WDR12, WDR12-HA, and WDR12-eYFP fusion proteins were uniformly distributed in the nucleoplasm and accumulated within the nucleolus. The similar localization of Ytm1p and WDR12 supports the notion that both proteins are indeed homologous proteins. Our subcellular localization experiments using a monoclonal antibody that detects endogenous WDR12 protein appear more reliable than overexpression studies that have reported a more homogeneous nuclear distribution of eGFP-WDR12 in HeLa cells (Nal et al., 2002).

To determine whether WDR12 is a nucleolar factor required for ribosome biogenesis, we developed a series of truncation mutants based on sequence-predicted domains. We aimed to identify dominant-negative phenotypes by screening for proliferation defects. Our results show that the NH_2-terminal Notchless-like domain is not required for nucleolar localization of WDR12. In contrast, disrupting the integrity of the seven WD repeats and therefore the potential propeller-like structure caused aberrant nucleoplasmic and cytoplasmic distribution. Among mutants that were tested, only WDR12ΔNle suppressed cell proliferation and inhibited proper rRNA processing. Prominent accumulation of the 32S prerRNA indicates defective processing in ITS2. Therefore, the NH_2-terminal Notchless-like domain probably mediates pivotal interactions in preribosomal complexes, as its removal severely impairs prerRNA processing. Importantly, our siRNA knockdown experiments confirmed that endogenous WDR12 is required for processing of the 32S precursor rRNA without affecting the synthesis of the 45S/47S primary transcript. Collectively, these results demonstrate that mammalian WDR12 functions in the maturation of the 60S ribosomal subunit.

Inhibition of prerRNA processing and cell cycle control

Overexpression of WDR12ΔNle strongly restrained cell proliferation without significant increase in apoptosis. Conducting BrdU light assays, we demonstrated that WDR12ΔNle imposes a reversible cell cycle arrest on TGR-1 rat fibroblasts. Similar results have been reported on dominant-negative mutants of Bop1 and Pes1, respectively (Pestov et al., 2001; Lapik et al., 2004). Likewise, depletion of endogenous WDR12 severely inhibited cell proliferation. Thus, inhibition of prerRNA processing is coupled with repression of cell proliferation. Noteworthily, changes in cell cycle distributions were less pronounced as expected from the proliferation and BrdU light assays. Hence, our data suggest that in addition to a complete cell cycle arrest, the overall cell cycle progression may also be delayed. The precise nature of this interesting observation needs further investigation.

In a hallmark study, Pestov et al. (2001) demonstrated a link between the tumor suppressor p53 and the response to "nucleolar stress." Impairment of endogenous p53 by the human papillomavirus protein E6 abrogated the Bop1Δ-mediated cell cycle arrest. Congruently, WDR12ΔNle-triggered cell cycle arrest was alleviated by the coexpression of HA-E6. Further, dominant-negative mutants of WDR12 and Bop1 provoked considerable accumulation of nuclear p53 and transcriptional activation of the target gene p21. Our results are in line with the study of Rubbi and Milner (2003), supporting the model that compromised functionality of the nucleolus is the basic principle of p53 stabilization in response to diverse stresses. Several intriguing molecular mechanisms have been unravelled that might contribute concertedly or independently to the overall increase in nuclear p53. First, Mdm2–p53 complexes assembled with ribosomes argue for a nucleolar transit of p53 for subsequent cytoplasmic degradation. On the other hand, the ribosomal proteins L5, L11, and L23 exert negative regulation on Mdm2 if they are not incorporated into functional preribosomal complexes (Lohrum et al., 2003; Zhang et al., 2003; Dai and Lu, 2004; Jin et al., 2004). Inhibition of prerRNA processing might result in an oversupply of free L5, L11, and L23 and may thus facilitate stabilization of p53 by blocking Mdm2 function. Our observations are consistent with either model, because insufficient rRNA processing reduces the production and export of mature ribosomes in the same way as the demand for the ribosomal proteins L5, L11, and L23. From the aforementioned results, we reasoned

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that the impact of WDR12ΔNle or Bop1Δ expression on p53 accumulation should be dependent on the rate of ribosome biosynthesis. Quiescent TGR-1 cells virtually shut down ribosome biogenesis (unpublished data). Hence, WDR12ΔNle and Bop1Δ are not expected to additionally increase nuclear p53 levels in arrested cells. Indeed, we were able to demonstrate that dominant-negative mutants of WDR12 and Bop1 failed to elicit p53 accumulation in serum-starved cells. TGR-1 fibroblasts grown to confluency gradually cease from the active cell division cycle. Again, the p53 response provoked by WDR12ΔNle, Bop1Δ, and Bop1N2 expression was significantly diminished in confluent compared with subconfluent cell cultures. Hence, inhibition of rRNA processing by dominant-negative mutants is a p53-inducing stress that depends on active ribosome biosynthesis. Interestingly, we also found a congruent increase of p53 phosphorylated at serine 15. Further investigation is needed to determine whether it reflects concomitant baseline phosphorylation of accumulated p53 or a specific signaling cascade mediated by disturbed rRNA processing. A recent study showed that human WI-38 fibroblasts accumulated p53 under growth-restricting conditions such as serum starvation and confluency (Bhat et al., 2004). Evidently, TGR-1 rat fibroblasts differ in this aspect from WI-38 cells. The precise explanation remains unclear. TGR-1 cells, however, are immortalized rodent cells that must have acquired genetic aberrations to overcome senescence and therefore might behave differently when challenged by unfavorable growth conditions. Despite this obvious discrepancy, we conclude that WDR12ΔNle, Bop1Δ, and Bop1N2 have no or little effect on the baseline p53 levels under growth-restricting conditions. Conclusively, proliferating cells require functional integrity of the nucleolus to prevent accumulation of p53.

The tumor suppressor p19ARF regulates stability of p53 via sequestration of Mdm2 to the nucleolus (Sherr and Weber, 2000). In addition, overexpression of p19ARF impairs rRNA processing (Sugimoto et al., 2003). Thus, p19ARF is a reasonable candidate to meditate the p53 response triggered by WDR12ΔNle and Bop1Δ. Our results, however, demonstrate that p19ARF is not required for accumulation of p53 provoked by dominant-negative mutants of ribosome synthesis factors.

Finally, we demonstrated that endogenous Pes1, Bop1, and WDR12 interact with each other in coimmunoprecipitation assays. Moreover, native gel electrophoresis revealed that all three proteins constitute only one major complex (PeBoW complex). In yeast, several studies showed that the potential homologous proteins Nop7p (Yph1p), Erb1p, and Ytm1p form a stable trimeric core complex (Harnpicharnchai et al., 2001; Du and Stillman, 2002). Potentially, the mammalian PeBoW complex may also exist as a trimeric core complex; however, we still cannot rule out that other factors may participate in the PeBoW complex. Most likely, the PeBoW complex is a stable subcomponent of larger preribosomal particles. Thus, previous studies in yeast and our data suggest that this core complex is evolutionarily conserved and its integrity is crucial for ribosome biogenesis and cell proliferation. It will be of outstanding interest to unravel whether the mammalian PeBoW complex directly plays a role in processes other than ribosome biogenesis, such as cell cycle control.

Similarly to DNA replication, cells are obliged to duplicate the translational machinery within each cell division cycle. Thus, intact ribosome biogenesis is crucial for normal and malignant cell proliferation. For decades, nucleolar morphology has served pathologists as a reliable indicator of malignancy. Aberrations in the protein synthesis machinery have been frequently implicated in cancer development (Ruggero and Pandolfi, 2003; Holland et al., 2004). Now, intriguing connections between cell cycle control, tumor suppressors, and nucleolar function have emerged. Exploration of individual factors is required to unravel the complex molecular machinery of ribosome synthesis and its role in tumor pathogenesis. Noteworthily, a recent study demonstrated that Pes1 enhances colony formation of SV40 large T-antigen immortalized cells (Maiorana et al., 2004). Mammalian ribosome synthesis factors might be directly implemented in malignant transformation apart from their function in ribosome synthesis. Therefore, it will be exciting to unravel positive and negative regulators of ribosome synthesis and their respective impact on tumor pathogenesis. Comparison of ribosome biogenesis and cell growth control in noncancerous and cancerous cells will be a promising approach to identify novel therapeutic targets. Specific inhibition of ribosome biogenesis might provide a nongenotoxic chemotherapy in addition to the classic chemotherapeutic drugs.

Materials and methods

Plasmids

Human *wdr12* cDNA was cloned into the EcoRV site of pUC18-HA tag using the following primers: 5′-GCCACCATGGCTCAGCTCCAAACACG-3′ and 5′-TGCCCCAACATGGGAAGTGGTAG-3′. A consensus Kozak sequence was added in front of the start codon. pUC18-HA tag was generated by cloning the following linker into the XbaI and HindIII sites of pUC18: 5′-TCTAGAGGCCTCACTGGCCGGCGATATCGGTTACCCTTATGATGTGCCAGATTATGCCTAAGGCCAGTGAGGCCAAGCTT-3′. pUC18-HA tag provides a COOH-terminal HA tag. *Wdr12*-HA was cloned via SfiI sites into pRTS-1, a single episomal plasmid enabling tight doxycycline-dependent gene expression. The bidirectional promoter of pRTS-1 plasmids controls the expression of eGFP and *wdr*12-HA wild type or mutants in a doxycycline-dependent manner. Human *c-myc* cDNA was flanked with SfiI sites and cloned into pRTS-1. Mouse HA-*bop1*, HA-*bop1Δ*, and *bop1N2* cDNAs were provided by D. Pestov (University of Illinois, Chicago, IL) and cloned into pRTS-1 plasmids. Human *wdr12* cDNA was cloned into pGEX-2T coding for a GST-WDR12 fusion protein. HA-E6 cDNA was provided by M. Scheffner (University of Konstanz, Constance, Germany) and cloned into pRTS-1 and pLXSP plasmids. *Wdr12*-eYFP and eCFP-nucleophosmin expression constructs were created by cloning *wdr12* and *nucleophosmin* into pEYFP-N1 and pECFP-C1 plasmids (CLONTECH Laboratories, Inc.), respectively. *Nucleophosmin* cDNA was provided by A. Lamond (University of Dundee, Dundee, UK).

Production of mAbs

Approximately 50 μg of GST-WDR12 fusion protein was injected both i.p. and subcutaneously into Lou/C rats using CPG2006 (TIB MOLBIOL) as adjuvant. After an 8-wk interval, a final boost was given i.p and subcutaneously 3 d before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed according to standard procedures. Hybridoma supernatants were tested in a solid phase immunoassay. A monoclonal mouse mAb specific for the GST part of the fusion protein (M-GST 2C8, IgG1; Forschungzentrum für Umwelt und Gesundheit Research Centre [GSF]) was coated over night at a concentration of 3 μg/ml. After blocking with nonfat milk, the GST-WDR12 fusion protein or an irrelevant GST fusion protein was incubated, followed by the hybridoma supernatants. Bound rat mAbs were detected with a cocktail of biotinylated mouse mAbs against the rat IgG heavy chains, thus avoiding IgM mAbs. The biotinylated mAbs were visualized with peroxidase-labeled Avidin (Alexis) and *o*-phenylenediamine as chromogen in the peroxidase reaction.

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A rat mAb against GST, binding to another epitope of the GST fusion protein (GST-6G9; GSF), served as a positive control.

The hybridoma designated WDR12-1B8 was stably subcloned and used for further analysis. The immunoglobulin isotype was determined using mAbs against the rat IgG heavy chains and light chains. The WDR12 (1B8) mAb has the IgG subclass IgG1. mAbs against human Pes1 (8E9) and Bop1 (6H11) were generated by using the following peptides coupled to ovalbumin (H.R. Rackwitz, Peptide Specialty Laboratories GmbH, Heidelberg, Germany): Pes1, AGSEKEEEARLAALEEQRMEGK; Bop1, SRGAGRTAAPSVRPEK. The Pes1 (8E9) and Bop1 (6H11) mAbs have the IgG subclass IgG1. Besides slight modifications, hybridoma cell lines were generated and supernatants were tested as described in this section. Generation of the anti-cytohesin antibody was described previously (Geiger et al., 2000).

Cell culture, transfections, and stable selection

TGR-1 rat fibroblasts, U2OS osteosarcoma cells, and p53−/− and p19ARF−/− MEFs were cultured in DME with 8% FBS (BioSer) at 8% CO_2. TGR-1 fibroblasts were provided by J. Sedivy (Brown University, Providence, RI), p53−/− MEFs were provided by G. Fingerle-Rowson (University Hospital Cologne, Cologne, Germany), and p19ARF−/− MEFs were provided by C.J. Sherr (Howard Hughes Medical Institute, St. Jude Children's Research Hospital, Memphis, TN). P493-6 cells were cultured in RPMI with 10% FBS at 5% CO_2. TGR-1 and U2OS cells, $\sim 7 \times 10^5$, were transfected with 4 μg pRTS-1 plasmids using polyfect (QIAGEN). Stable polyclonal cell cultures were selected in the presence of 200 μg/ml hygromycin B (TGR-1 and U2OS) or 1 μg/ml puromycin (TGR-1) for 10 to 14 d. Induction of conditional gene expression was performed with 1 μg/ml doxycycline. The percentage of eGFP-positive cells was monitored by FACS analysis. Human diploid fibroblasts were transfected by electroporation with 10 μg of plasmid DNA.

siRNA transfection

The day before transfection, $\sim 5 \times 10^4$ to 10^5 U2OS cells were seeded in 6-well plates. 5 μl of 20 μM control or WDR12-specific siRNA were diluted in 150 μl Optimem (Invitrogen). 150 μl Optimem containing 5 μl Oligofectamine (Invitrogen) was added and incubated for 15 min. Finally, 600 μl Optimem was added and applied to cells after aspiration of the culture medium. Cells were incubated for 5–6 h. The following sequences (sense) were used: WDR12, CGUACGUUUCCGUGGGCAAdTdT; Control (nonspecific siRNA), UUCUCCGAACGUGUCACGUdTdT.

Immunoblotting and immunofluorescence

TGR-1 and P493-6 cells were directly lysed in SDS-loading buffer (50 mM Tris-HCl, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Total cell lysates were separated on SDS-PAGE gels and blotted on nitrocellulose membranes (GE Healthcare). Equal loading was verified by Ponceau S staining. Immunodetection was performed with anti-c-Myc (N-262; Santa Cruz Biotechnology, Inc.), anti-WDR12 (1B8), anti-Pes1, anti-Bop1, anti–α-tubulin (DM1A; Dianova), anti-HA (3F10; Roche), anti-p53 (Pab240; BD Biosciences), and anti–phospho-p53-Ser15 (Cell Signaling Technology Inc.).

For immunofluorescence, cells were grown on coverslips and fixed in ice-cold methanol for 10 min and air dried. Alternatively, cells were fixed in 4% PFA for 10 min and permeabilized with PBS/Tween 0.1% for 15 min at RT. Unspecific binding was blocked with PBS/10% FBS. p53 and HA-tagged WDR12 were detected with a 1:100 dilution of anti-p53 (Pab122; Dianova) and a 1:10 dilution of 3F10 hybridoma supernatant, respectively. Primary antibodies were incubated over night at 4°C in a humidified chamber. Cy3 or FITC-labeled secondary antibodies (Dianova) were incubated for 30 min at RT. Nuclei were counterstained with DAPI (Sigma-Aldrich). Digital images were acquired using the Openlab acquisition software (Improvision) and a microscope (model Axiovert 200M; Carl Zeiss MicroImaging, Inc.) with 63× (1.15) and 100× (1.30) plan oil objectives connected to a 5 charge-coupled device camera (model ORCA-479; Hamamatsu).

BrdU light assay

BrdU lights assays were performed essentially as previously described (Pestov and Lau, 1994). In brief, stable polyclonal TGR-1 cells were seeded in the presence of 1 μg/ml doxycycline at a density of 10^5 cells per 100-mm well. 30 h after seeding, cells were incubated with 100 μM BrdU and doxycycline for 48 h. Culture medium was then removed and replaced by medium containing doxycycline and Hoechst 33258 at 2 μg/ml for 3 h. Finally, cells were placed on glass 11 cm above a 30-W fluorescent daylight bulb and irradiated from beneath for 15 min. Cells were washed in PBS two times and regular culture medium without doxycycline was added.

RNA analysis and ^{32}P in vivo labeling

Total RNA of P493-6 and TGR-1 cells was isolated using Trifast (PeqLab). 10 μg of total RNA was separated on a 1% agarose-formaldehyde gel and blotted on hybond N^+ membranes (GE Healthcare). The following ^{32}P end-labeled DNA oligonucleotides were used to visualize rRNA precursors: ITS-1 (rat specific), 5′-GGACCAGACCCGACACCCTGCCACCGCACACCTGTCCCGAAACCCCCT-3′; ITS-2 (rat specific), 5′-GCCCCGGGGAGCGGGCCCTGCGAGCAGACTCCCAGCCGCGCGACGCGA-3′; ITS-1 (human specific), 5′-CCTCCGCGCCGGAACGCGCTAGGTACCTGGACGGCGGGGGGGCGGACG-3′; ITS-2 (human specific), 5′-GCGGCGGCAAGAGGAGGGCGGACGCCGCCGGGTCTGCGCTTAGGGGGA-3′; 18S rRNA (human and rat specific), 5′-CACCCGTGGTCACCATGGTAGGCACGGCGACTACCATCGAAAGTTGATAG-3′; 28S (human and rat specific), 5′-CCAGCTATCCTGAGGGAAACTTCGGAGGGAACCAGCTACTAGATGGTTCG-3′.

For metabolic labeling of rRNA, TGR-1 and U2OS cells were preincubated in phosphate-free DME (GIBCO BRL) with dialyzed FBS (Sigma-Aldrich) for 30 min. The medium was then replaced by phosphate-free DME/10% dialyzed FBS containing 15 μCi/ml ^{32}P-orthophosphate (GE Healthcare). After 45 min, the radioactive medium was removed and cells were incubated in regular DME/10% FBS for 2 h. 10 μg of total RNA were separated on 1% agarose-formaldehyde gels. After electrophoresis, gels were placed on whatman-paper and dried at 80°C under vacuum suction. Dried agarose gels were exposed to regular x-ray films (Kodak) and rRNA was visualized by autoradiography. A PhosphoImager (Fuji) was used for the quantification of signal intensities.

Immunoprecipitation

3×10^7 cells were lysed in 1 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 1% NP-40, 150 mM NaCl, phosphatase inhibitors, and protease inhibitors) at 4°C for 20 min. 20 μg of glass beads were washed twice with PBS and incubated with 100 μl of anti-WDR12, anti-Bop1, anti-Pes1, and anti-Cytohesin supernatant for 1 h at 4°C. Afterwards, beads were washed once in lysis buffer. Subsequently, beads were incubated with 100 μl of total cell lysate at 4°C for 5 h. Immunoprecipitations were then washed three times with lysis buffer at 4°C and were incubated at 95°C for 10 min in a 1:1 dilution of 2× SDS loading buffer and lysis buffer. Finally, 5 μl of the immunoprecipitations were separated by SDS-PAGE.

Quantitative real-time PCR

Asynchronously proliferating TGR-1 cells were treated with doxycycline for 30 h and total RNA was isolated using Trifast (PeqLab). cDNA was produced using 1 μg of total RNA using oligo(dT)-primers and M-MLV reverse transcriptase (Promega). Subsequently, cDNA was diluted at 1:100 for quantitative real-time PCR using a LightCycler PCR analysis system (Roche) according to the manufacturer's recommendations. The following primers were used for detection of rat p21 mRNA: 5′-TGTTCCACACAGGAGCAAAG-3′ and 5′-CTCTTGCAGAAGACCAATCG-3′. Quantitative real-time PCR of aldolase was performed for normalization using the following primers: 5′-GGTCACAGCACTTCGTCGCACAG-3′ and 5′-TCCTTGACAAGCGAGGCTGTTGGC-3′.

Native gel and two-dimensional gel electrophoresis

3×10^6 cells were lysed in 100 μl lysis buffer at 4°C for 20 min. 7.5 μl of 2× sample buffer (125 mM Tris-HCl, pH 6.8, 30% glycerol, and 0.02% bromphenol blue) was added to 7.5 μl of total cell lysate and separated by PAGE (6.5%) in the absence of SDS at 4°C. The voltage did not exceed 100 V during electrophoresis. Blotting was performed in the absence of methanol. Immunoblotting was performed as described in the Immunoblotting and immunofluorescence section. For two-dimensional gel electrophoresis, subsequently to native gel electrophoresis, the appropriate lanes were cut out from the gel and incubated in SDS-running buffer for 10 min. Afterwards, the strips were applied horizontally to second dimension SDS-PAGE (10%). Immunoblotting was performed as described in the Immunoblotting and immunofluorescence section.

Online supplemental material

Fig. S1 shows cell cycle analysis of WDR12 mutant–expressing cells. Fig. S2 shows analysis of p53 phosphorylation at serine 15 in response to dominant-negative WDR12. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200501141/DC1.

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